THE EFFECTS OF DELTA-9-TETRAHYDROCANNABINOL AND CANNABIDIOL IN SYMPTOMATIC INSOMNIA CAUSED BY NEUROPATHIC PAIN

Antonio Farina, Pharm. D.

Integrated Program in Neuroscience

Neurobiological Psychiatry Unit, Department of Psychiatry,

McGill University,

Montreal, QC, Canada

Submitted July, 2020

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

NP	Neuropathic pain
THC	Delta-9-tetrahydrocannabinol
CBD	Cannabidiol
NREM sleep	Non-Rapid Eye Movement sleep
REM sleep	Rapid Eye Movement sleep
TCA	Tricyclic antidepressants
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
5-HT1A	Serotonin 1A receptor
FAAH	Fatty acid amide hydrolase
TRPV1	Transient receptor potential cation channel subfamily V member 1
CCI	Chronic constriction injury
SNI	Sciatic nerve injury
SWS	Slow-wave sleep
PAG	Periaqueductal gray
vlPAG	Ventrolateral Periaqueductal gray
RVM	Rostral ventromedial medulla
EEG	Electroencephalogram
EMG	Electromyography

BF	Basal forebrain
AEA	Anandamide
2-AG	2-arachidonoylglycerol
NAPE-PLD	N-acyl-phosphatidylethanolaminehydrolysing phospholipase D
DGLα	Diacylglycerol lipase-α
DGLβ	Diacylglycerol lipase-α
FAAH	Fatty acid amide hydrolase 1
MAGL	Monoacylglycerol lipase
DRN	Dorsal raphe nucleus
AUC	Area under the curve

ACKNOWLEDGEMENTS

I would like to express my gratitude and appreciation to my supervisor Dr. Gabriella Gobbi for her exceptional guidance, and, foremost, for giving me the opportunity to learn from her.

I will be eternally grateful to Dr. Sabatino Maione, who was the first to believe in my abilities.

I am particularly grateful to Dr. Martha Lopez-Canul, Dr. Danilo De Gregorio, Dr. Luca Posa, and the other members of Gobbi Lab for their friendship, constant guidance, patience and invaluable support.

Additionally, I wish to show my gratitude to Serena Boccella, Livio Luongo, and Francesca Guida for training me in the experimental techniques and for their honest critiques.

I thank all my friends for their love and vital support: Roberta Montanaro, Biagio Mancini, Agnese Ricciardi, Giovanna Cattolico, Pasquale Ferrante, Martina Merola, Hussein Bedeir, Maisha Iqbal, Marie Sato Fitoussi, and Raquel Rousselet Farias.

I wish to express my deepest gratitude to Rita Zakarian and Paulo Sequeiria for their invaluable encouragements, patience and guidance.

I wish to acknowledge the work and support of my advisory committee members, my mentor, and Professor Joseph Rochford.

Finally, I would like to thank my entire family, as well as my counselor Alessandro De Franciscis, who encouraged me to be professional and do the right thing even when the road got tough. They encouraged me to keep going and this experience could not have been possible without their love and support.

CONTRIBUTION OF AUTHORS

Ethics and funding: Gabriella Gobbi

Study design: Antonio Farina, Martha Lopez-Canul, Gabriella Gobbi

Data collection: Antonio Farina, Martha Lopez-Canul, Alexandra Teggin, Luca Posa, Justine Enns, Elena He

Data analysis: Antonio Farina, Martha Lopez-Canul, Alexandra Teggin, Luca Posa, Danilo De Gregorio, Maria-Luisa Vigano, Shelly Yin

Preparation of the thesis: Antonio Farina

ABSTRACT

Neuropathic pain (NP) is a major health problem that results in a high degree of suffering, physical and psychosocial impairments and exorbitant health care costs. Additionally, patients who suffer from NP experience sleep disturbances. Only a restricted number of drugs are available for treating NP associated insomnia, and side effects are common. Preclinical and clinical studies indicate that delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) possess both analgesic and hypnotic effects. However, their mechanisms of action in models of NP are not fully understood yet. In this study, for the first time, we demonstrated that animals with a NP condition also develop sleep perturbations characterized by a decrease in non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep and an increase in wakefulness. Successively, we investigated the effects of CBD and THC in both chronic pain and comorbid insomnia. Acute systemic administration of THC or CBD dose-dependently reduced spared nerve injury-induced mechanical allodynia and was able to restore the normal sleep-wake cycle in NP rats. Antiallodynic and hypnotic effects of THC were fully prevented by the administration of the CB1 receptor selective antagonist rimonabant (1 mg/kg). By contrast, the administration of 5-HT1A selective antagonist WAY 100635 (2 mg/kg) totally prevented the hypnotic effects of CBD, but only partially antagonized its analgesic effects. Employing in vivo single-unit extracellular recordings in NP rats, we observed that both THC and CBD modulated the descending pathway of anti-nociception. Specifically, microinjection of THC into the ventrolateral periaqueductal gray decreased the firing activity of ON cells and activated the firing of OFF cells in the rostroventral medulla. Unlike THC, CBD reduced the ongoing activity of both ON and OFF neurons in anaesthetized NP rats. These findings suggest that THC and CBD have potential in the treatment of neuropathic pain and comorbid insomnia.

RÉSUMÉ

La douleur neuropathique (DN) est un problème majeur de santé qui conduit à un grand niveau de souffrance, troubles physiques et psychosociaux et des coûts exorbitants en termes de santé publique. De plus, les patients affectés par la DN montrent des problèmes de sommeil. Malheureusement, un nombre limité de médicaments pour le traitement de la DN associé à l'insomnie est disponible et souvent ils ont plusieurs effets secondaires. Des études précliniques et cliniques montrent que le delta-9-tetrahydrocannabinol (THC) et cannabidiol (CBD) ont des propriétés analgésiques et hypnotiques, mais le mécanisme d'action dans un modèle de DN n'a jamais été étudié. Dans cette étude, pour la première fois, nous avons démontré que des rats en conditions de DP ont développé de perturbations du sommeil, caractérisées par une diminution du sommeil paradoxal (REM) et du sommeil à mouvements oculaires non-rapides (NREM) et par une augmentation de l'état d'éveil. Nous avons donc étudié les effets du THC et du CBD dans la comorbidité de la douleur chronique et de l'insomnie. L'administration aiguë de THC ou CBD a réduit de manière dose-dépendante l'allodynie mécanique dans un modèle animal de DP, et a rétabli le normal cycle veille-sommeil dans des rats neuropathiques. Les effets antiallodyniques et hypnotiques du THC ont été complètement bloqués par le traitement avec l'antagoniste CB1 Rimonabant (1 mg/kg). Au contraire, l'antagoniste sélectif pour le récepteur 5-HT1A WAY 100635 (2 mg/kg) a totalement bloqué l'effet hypnotique, mais il a partiellement antagonisé l'effet analgésique du CBD. En utilisant l'électrophysiologie en-vivo dans des rats neuropathiques, nous avons observé que tant le THC que le CBD modulent la voie descendante de la nociception. En effet, des micro-injections de THC dans la substance grise périaqueducale diminuent l'activité neuronale des neurones ON et augmentent celle de neurones OFF dans la médulla rostral médiale. Différemment du THC, le CBD réduit l'activité neuronale des neurones ON et OFF dans de rats

anesthésiés. Nos résultats montrent le potentiel thérapeutique du THC et du CBD dans la comorbidité entre la douleur neuropathique et insomnie.

1. INTRODUCTION

1.1. Neuropathic Pain

Neuropathic pain (NP) is a chronic pain disorder resulting from damage to the nervous system, which can be caused by medical conditions such as cancer, diabetes, infection, or traumatic injury [1-4]. NP represents a major economic burden and considerably impairs patients' quality of life [5, 6]. Individuals with NP display distinct sensory symptoms that can coexist in multiple combinations [2, 6, 7]. Between 15 and 50% of NP patients are affected by allodynia (pain caused by normally innocuous stimuli) and hyperalgesia (extreme pain response to a stimulus that normally causes pain) [2]. These cardinal and intractable symptoms result from peripheral sensitization and maladaptive central change [2, 8]. For example, substantial molecular and cellular changes at the level of the primary afferent nociceptor occur following the nerve damage, leading to the development of an aberrant spontaneous activity [5]. Cytokines, nerve growth factors, and other algogenic molecules invade the injured tissue area, leading to changes in expression and trafficking of specific sodium channels, particularly the isoforms NaV1.3, NaV1.7, NaV1.8, and NaV1 [2, 6, 9]; thus eliciting spontaneous firing or inducing alterations in conduction and in neurotransmitter release [10]. Indeed, nociceptors express receptors that are able to interact with proinflammatory agents. Together these events increase the nerve fibers excitability, enhancing their sensitivity to pressure or temperature [10, 11]. Despite tremendous progress, our understanding of the pathological mechanisms underlying allodynia and hyperalgesia is still incomplete, and current treatments are largely ineffective [1, 8, 12]. Psychiatric and medical comorbidities usually co-occur, in fact, patients who suffer from chronic pain often experience depression and insomnia [4, 13, 14].

These symptoms are regulated by shared central nervous system mechanisms and appear to be functionally related. A biochemical theory suggests that these comorbidities are the result of a neurochemical imbalance of the key neurotransmitters serotonin, norepinephrine, and dopamine [3, 4, 13]. These monoaminergic neurotransmitters positively or negatively modulate pain transmission, depending on receptor type and functional area [15]. Evidence suggests that serotonin plays an important role in promoting wakefulness by reducing cortical activation [16], whereas norepinephrine, which also play an important role in waking, is involved in wakefulness associated with stressful situations. Furthermore, both norepinephrine and serotonin play a key role in the suppression of REM sleep [16]. Similarly, clinical and preclinical studies suggest that dopamine is involved in the promotion and maintenance of wakefulness [4, 16]. In fact, amphetamines and modafinil, which mainly act on the dopaminergic system, are the most powerful wake-promoting agents currently known [16]. Additional evidence comes from studies in D2 receptor knockout mice, which display a significant reduction in wakefulness and a concomitant increase in sleep [16]. Functional deficiency of monoamines also relate to specific symptoms of depression. Nutt (2008) stated that norepinephrine could be associated with anxiety, energy, attention, alertness and interest in life; dopamine can be associated with reward, motivation, and attention; serotonin can be associated to anxiety, obsessions, and compulsions. Therefore, increasing the levels of one of these neurotransmitters will probably improve mood [17]. Although serotonin, norepinephrine and dopamine are involved in sleep, pain, and mood, no common pathway has been recognised [3, 4]. Thus, clinicians should consider treating each specific symptom with possible additional beneficial effects on the others [4].

1.2. Sleep and Pain

Sleep is a regulated biological state characterized by a reduction in voluntary motor activities, attenuated response to stimulation, and stereotypic posture. It is conserved across species and essential for survival [18]. The mammalian sleep-wake cycle progresses in three stages distinguished by electroencephalographic (EEG) activity and muscular movements measured by electromyography (EMG). Wakefulness stage is characterized by Alpha and Beta waves, (8-13 and 13-30 Hz. respectively) and sustained EMG signals; following the transition into Non-Rapid Eye Movement (NREM) sleep, the EEG signal increases in voltage and decreases in frequency into Delta waves (0.5-4Hz), and the muscular movement is also decreased; lastly, the REM sleep is characterized by fast, low amplitude EEG oscillation theta waves (t6.0-9.0 Hz) and muscle atonia [19]. Multiple neurotransmitters are involved in the modulation of the sleep-wake cycle. We already mentioned serotonin, dopamine and norepinephrine and their role in the promotion of wakefulness. Sleep homeostasis is regulated by other modulators, defined as homeostatic sleep factors. Among them we should mention the neuromodulator adenosine [16]. Systemic or central administrations of adenosine or other adenosine A1 receptor agonists inhibits wake-active neurons located in the basal forebrain (BF) and other brain areas, inducing sleepiness and affecting vigilance [16, 20-22]. Moreover, in the BF and cortex the endogenous adenosine levels increase proportionally to the time spent awake, demonstrating that adenosine levels monitor sleep need and induce sleep [16, 23-25].

The absence of sleep cycles, or the presence of irregular sleep cycles, are associated with sleep disorders such as parasomnias, sleep disruptive events, insomnia or lack of sleep [26-28]. Symptomatic insomnia is a sleep disorder caused by chronic pain and other medical conditions [4, 29]. Specifically, between 67% and 88% of people with chronic pain report insomnia as a major

source of distress [14, 29, 30]. The pattern of comorbid insomnia in individuals with chronic pain is generally indistinguishable from that of primary insomniacs, in fact, most patients report: longer sleep onset latency, more frequent awakenings, and shorter total sleep time [14, 31]. Additionally, these individuals also show higher level of pain, longer pain duration, greater intensity of anxiety and depression, and worse physical and psychosocial impairments compared to chronic pain patients that did not report poor sleep quality [31].

It is generally believed that chronic pain and sleep disturbances are reciprocally related [4, 31]. Epidemiological studies have demonstrated that patients experiencing chronic pain are more likely to develop sleep disorders [29, 31], and poor sleep quality is considered as a risk factor for chronic pain development [29, 32-34]. Furthermore, the lack of sleep not only has adverse short-term health consequences (emotional distress, mood disorders, impaired cognition, and functional deficits) but also long-term consequences including metabolic syndrome, hypertension, cardiovascular disease, type 2 diabetes mellitus, and an increased risk of developing cancer [35].

1.3. Current pharmacological treatments

The mechanisms underlying NP and pain-induced insomnia still have to be elucidated [4], and only a restricted number of effective drugs are available for treating NP associated with insomnia. However, it is challenging to identify the most adequate treatment, as the response to most drugs remains unpredictable, quite variable among individuals and frequently results in several side effects [36]. Gabapentinoids, tricyclic antidepressants (TCAs), serotonin reuptake inhibitors (SSRIs) are generally recommended, and opioids are commonly used for treating pain with comorbid insomnia [1, 14, 37]. Both TCAs and gabapentinoids have confirmed efficacy in different NP conditions, but only gabapentinoids were found to improve sleep quality and sleep

consolidation. Conversely, TCAs have only weak effects on REM and NREM sleep. Daytime somnolence and sedation are common adverse effects of both TCA and gabapentinoids treatments. SSRIs are effective in treating pain, but they also diminish sleep time and suppress REM sleep [1, 14, 37, 38]. Opioid agonists, are only partially effective, and due to their side effects and abusive/addictive potential, there is concern when prescribing them [1]. Further studies have pointed out that sleep disturbances may be triggered or intensified by opioid treatment, contributing to the development of depression and even enhancing pain [31, 37, 39]. Therefore, developing better therapeutics is of paramount importance to the public health system.

1.4. The Endocannabinoid System

The discovery of cannabinoid receptors and their endogenous ligands [40-42] has generated an exponential increase of studies investigating the functions of the endocannabinoid system and the endocannabinoid-related network. More importantly, it has become clear that modulating the activity of cannabinoid receptors and endogenous cannabinoids involved in restoring homeostasis after endogenous and environmental insults, might represent a possible therapeutic strategy in a wide range of diseases and pathological conditions [40, 43, 44]. Indeed, there is increasing experimental evidence about the therapeutic promise of medicinal cannabis in a plethora of medical conditions raging from cancer, atherosclerosis, mood and anxiety disorders, stroke, glaucoma, metabolic syndromes, insomnia to neuropathic pain, epilepsy and other neurological disorders such as Parkinson's, Alzheimer and Huntington's disease [40, 43, 45]. Thus, despite its unacceptable psychoactive properties and the social controversies surrounding legal and ethical implications associated with use, medicinal cannabis gained increased interest during the last two decades. At present, only two different cannabinoid receptors have been identified: 1) Cannabinoid

receptor type 1 (CB1) cloned by Matsuda and colleagues in 1990 [41], and 2) Cannabinoid receptor type 2 (CB2) which was cloned in 1993 by Munro and colleagues [42]. Cannabinoid receptors CB1 and CB2 share 48% amino acid sequence identity [46] and both are coupled to Gi/o proteins, through which they regulate the activity of adenylate cyclase and mitogen-activated protein kinase [44, 46]. Additionally, CB1 receptors are also coupled through G proteins to different kinds of voltage-activated Ca^{2+} channels and inwardly rectifying K^{+} channels. CB1 is the most abundant G-protein–coupled receptor in the mammalian brain [43, 44, 46]. It is widely expressed in different areas of the central nervous system (substantia nigra, globus pallidus, hippocampus, cerebral cortex, putamen, caudate, cerebellum, spinal cord, hypothalamus and amygdala) and in the peripheral nervous system (for example, sensory nerve fibers) where it inhibits the release of different excitatory or inhibitory neurotransmitter [45]. Functionally significant levels of CB1 have been found also in non-neuronal cells such as adipocytes, hepatocytes, or tissues such as liver tissue, and skeletal muscle [45, 46]. The anatomical localization of CB1 receptors provides further insight into their biological effects [45, 46]. Specifically, activation of CB1 receptors leads to analgesia, nausea attenuation, reduction of intraocular pressure, appetite stimulation, relief from muscle spasms, and decreased intestinal motility as well as hyper-stimulation, sedation, catalepsy, and other depressant effects [46-48]. Notably, it has been demonstrated that the CB1 receptor has a key role in modulating the sleep-wake cycle. Indeed, its expression in the rats' pons is regulated by the light/dark cycle and by sleep [49]. Accordingly, Santucci and colleagues (1996) demonstrated that the administration of the CB1 selective antagonist SR 141716A (rimonabant) dose-dependently increased the time spent in wakefulness, while reducing the time spent in NREM sleep and REM sleep [50]. In contrast, CB2 receptors are mostly expressed in immune cells and tissues, and in the hematopoietic system [45, 46]. However, it has been found in nonparenchymal

cells of the cirrhotic liver, in the endocrine pancreas, in bone, and at lower levels, in neuronal and non-neuronal cells of the brain [43, 45, 51, 52]. After the identification of CB1 and CB2 receptors, two endogenous ligands (endocannabinoids) were isolated and characterized: anandamide (AEA) and 2-arachidonoylglycerol (2-AG), along with the enzymes involved their biosynthesis and inactivation: N-acyl-phosphatidylethanolaminehydrolysing phospholipase D (NAPE-PLD), diacylglycerol lipase-α (DGLα), DGLβ, fatty acid amide hydrolase 1 (FAAH) and monoacylglycerol lipase (MAGL) [40, 43, 44].

AEA behaves as a partial agonist of CB1 receptors and it also binds and activates CB2 receptors. Nevertheless, it has low efficacy and may act as an antagonist CB2 receptor [45, 53, 54]. Murillo-Rodriguez provided experimental evidence about the sleep-promoting proprieties of AEA, which modulates sleep by increasing NREM and REM sleep at expenses of wakefulness, via CB1 receptor activation [55-57]. Preclinical studies pointed out that AEA is also effective in chronic pain of both neuropathic and inflammatory origin, by reducing thermal and mechanical hyperalgesia and mechanical allodynia [58-64].

Similarly to AEA, 2-AG activates the CB1 receptor but it is also a full agonist of the CB2 receptor. Like AEA, 2-AG has a role in pain modulation. Indeed, coordinated release of 2-AG and AEA in the periaqueductal grey mediate stress-induced analgesia [65].

Given that the endocannabinoid system is involved in both analgesia and sleep, this suggests that targeting components of this system and the endocannabinoid-related network with exogenous cannabinoids may represent a valuable therapeutic strategy for treating NP and pain-induced insomnia. Therefore, exogenous cannabinoids may offer a novel approach to chronic pain management and comorbid insomnia, but their efficacy in these two conditions is not fully understood yet.

1.5. Delta-9-tetrahydrocannabinol and Cannabidiol

Among 100 and more cannabinoids, delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are the most abundant components present in the flowers of *Cannabis sativa* (the Cannabis plant) [40, 43, 45, 54]. THC, chemically characterized by Mechoulam and colleagues in the early 60s [66, 67], is the major psychoactive component of cannabis and its effects are mediated by the activation of CB1 and CB2 receptors [40, 54]. THC behaves as a partial agonist at CB1 receptors, and, *in vitro*, shows lower efficacy at CB2 (K_i = 36.4 nM) than at CB1 (K_i = 21 nM) receptors [54, 68]. The biological actions of THC mostly result from activation of CB1 receptors. In particular, in rodent models these responses include locomotor impairments, hypothermia, antinociception, and catalepsy (kwon as 'tetrad' of effects), which are induced with a potency that is consistent with THC affinity for CB1 receptor [46, 54].

Unlike THC, the mechanism of action of CBD is more complex. CBD does not significantly interact with CB1 (K_i = 4350 nM) and CB2 (K_i = 2860 nM) [54] receptors, and it has been hypothesized that its therapeutic actions might be mediated by its antioxidant properties or the interaction with the serotonin 5-HT1A receptor and other molecular targets, such as the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor and the enzyme FAAH [45, 54, 69, 70].

Cannabidiol has been shown to possess several properties of potential therapeutic interest, such as anticonvulsive, antinausea, antinociceptive, hypnotic, anti-inflammatory, and more importantly anxiolytic effects [45, 46, 69, 71]. Recently, it has been proven that the anxiolytic effect of CBD are mediated by 5-HT1A receptor activation [69]. The 5-HT1A receptor is also involved in the sleep-wake cycle modulation. Monti and colleagues (1994) reported that the microinjection into dorsal raphe nucleus (DRN) of 5-HT1A agonist 8-OH-DPAT increases NREM sleep and decreases

wakefulness [72], while Portas (1996) demonstrated that 8-OH-DPAT perfusion in the DRN increases REM sleep without affecting wakefulness [73]. Therefore, it could be that 5-HT1A receptor might also mediate CBD hypnotic proprieties.

1.6. THC and CBD in chronic pain

In preclinical studies, cannabinoids have been found to exert anti-nociceptive effects [74] in animal models of acute, inflammatory and chronic pain [69, 75-78]. Casey et al. (2017) showed that both acute systemic administration of THC and CBD dose-dependently reduced mechanical and cold allodynia in the chronic constriction injury (CCI) model of neuropathic pain. Moreover, through an isobolographic analysis they demonstrated that the co-administration of THC and CBD in a fixed ratio (1:1) induced a synergic reduction in allodynia [76]. Similarly, in a model of chemotherapy-induced neuropathic pain, both CBD and THC alone attenuated mechanical allodynia in mice, while the combination of low ineffective doses of the two phytocannabinoid synergistically attenuated paclitaxel-induced mechanical sensitivity [79].

In our laboratory, we demonstrated that chronic treatment with low-dose CBD (5 mg/k/day, s.c., for 7 days) reduces mechanical allodynia in the sciatic nerve injury (SNI) model of neuropathic pain. This effect can be fully antagonized by administering the TRPV1 antagonist capsazepine (10 mg/kg/day, s.c., for 7 days) and partially prevented by administering the 5-HT1A antagonist WAY 100635 (2 mg/kg/day, s.c., for 7 days) [69].

Additionally, several clinical trials described the ability of THC, dronabinol (the synthetic form of THC) or Sativex (cannabis-based medicines containing a 1:1 combination of THC and CBD) in reducing neuropathic pain symptoms in patients with traumatic nerve injury, multiple sclerosis or with opiate-resistant, intractable pain due to cancer [17, 45, 80, 81].

1.7. Cannabinoids modulate the descending pathway of antinociception

Cannabinoids have been shown to modulate pain transmission in the periaqueductal gray (PAG)-rostral ventromedial medulla (RVM) pathway, involved in the descending modulation of nociception [82, 83]. The PAG indirectly controls nociceptive transmission in the dorsal horn of the spinal cord through its connection with the RVM. In the RVM, two classes of neurons are involved in the pain modulatory circuit, which are characterized by changes in activity evoked by noxious stimuli. ON cells show a burst of activity in response to a noxious stimulus, exerting a pro-nociceptive role. On the contrary, in response to noxious stimuli, OFF cells display a pause in their activity, exerting an anti-nociceptive activity. In nerve-injured animals, both ON and OFF cells in the RVM are sensitized to innocuous and noxious stimuli, and this neuronal hypersensitivity correlates with behavioural hypersensitivity [84]. Moreover, these neurons usually exert opposing modulatory actions in response to pharmacological stimulation with analgesics: systemic or local injections of μ-opioids induce a continuous and increased firing of OFF cells, and inhibits the firing and burst activity of ON cells [85, 86]. Likewise, in healthy animals, cannabinoids depress ON cells firing and burst activity, while increasing the firing of OFF cells, nullifying their pause [82, 83]. Nevertheless, Maione and colleagues demonstrated that CBD microinjection in the ventrolateral periaqueductal gray (vlPAG) paradoxically reduced the ongoing activity of both ON and OFF neurons [70].

1.8. THC and CBD in sleep

The effects of exogenous cannabinoids on sleep have been controversial, and the research on the effects of exogenous cannabinoids on insomnia related to chronic pain conditions is still in its early stages. A preliminary study revealed that CBD behaves as a short-acting hypnotic in healthy rats.

Acute systemic administration of 20 mg/kg of CBD reduced slow-wave sleep (SWS) latency with no significant effect on sleep duration and sleep parameters; whereas, single doses of 40 mg/kg not only decreased SWS latency but also increased SWS without affecting REM sleep [71]. In a recent preclinical study, Chagas and colleagues (2013) confirmed this trend. CBD doses of 10 and 40 mg/kg significantly increased the total percentage of sleep, however, the increase in SWS duration observed with CBD dose of 40 mg/kg was not statistically significant [87]. Conversely, Murillo-Rodriguez and colleagues provided experimental evidence about the wake-inducing proprieties of CBD. Intracerebroventricular injection of CBD (10μg/5μL) in healthy rats induced an increase in wakefulness and a decrease in REM sleep [88]. It is known that the administration of THC increases sleep, conclusive evidence of whether the administration of THC improves or decreases sleep in NP conditions is lacking [89, 90]. Recently, Nicholson and colleagues (2004) investigated the effects of exogenous cannabinoids on nocturnal sleep, early-morning performance, memory, and sleepiness, in eight healthy volunteers. They found that 15 mg of THC had no effect on sleep and the patients reported increased sleepiness 30 minutes after rising, whereas CBD administrated in combination with THC had alerting properties as it increased wakefulness during nocturnal sleep and neutralised the residual sedative activity of THC [91].

Finally, different clinical trials have shown a positive effect of a cannabis-based medicine in patients with sleep disturbances derived from different chronic medical conditions such as multiple sclerosis, rheumatoid arthritis, cancer pain, and chronic pain among others; suggesting that cannabinoids may have a beneficial impact on symptomatic insomnia and chronic pain [39, 92]. However, several limitations were observed in that study, such as the small sample size, the fact that sleep was evaluated as a secondary outcome in the context of chronic pain, and the use of non objective measurement [39, 92].

1.9. General Hypothesis and Specific Aims

Despite the above discussed research findings, there is scarce preclinical and clinical evidence of the effectiveness of synthetic or natural cannabinoids used as sedative-hypnotics, particularly in patients who suffer from NP [39, 92]. Therefore, determining the dose at which THC and CBD can relieve neuropathic pain and restore the sleep/wake cycle remains of primary importance. We hypothesize that an acute systemic administration of THC and CBD may relieve mechanical allodynia and restore the normal sleep/wake cycle in NP rats. Additionally, we think that CB1 and 5-HT1A receptors mediate the hypnotic and analgesic proprieties of THC and CBD, respectively. In the present study the specific aims are:

1) To confirm that animals with a NP condition also develop sleep perturbations characterized by a decrease in NREM and REM sleep and an increase in wakefulness, electroencephalographic (EEG) activities and muscular movements measured by electromyography (EMG) were recorded for 6 hours.
2) To assess THC and CBD analgesic proprieties, different NP animals were treated with increasing doses of CBD or THC and mechanical allodynia was evaluated employing von Frey test.
3) To evaluate the impact of the two compounds on the physiology of sleep, a single effective analgesic dose of THC or CBD was administered in a different cohort of NP rats and EEG/EMG activities were recorded for 6 hours.
4) To test the involvement of the CB1 and 5-HT1A receptors in the sleep-promoting and analgesic effects of THC and CBD, selective antagonists were administered 10 minutes prior to CBD and THC and the same experiments were repeated.

5) Finally, due to the well-documented presence of potential molecular targets of THC and/or CBD in the vlPAG [70, 82, 93-95], we investigated the ability of these two compounds to modulate the electrical activity of ON and OFF neurons of the RVM in anaesthetized NP rats before and after microinjection into the vlPAG.

2. MATERIAL AND METHODS

2.1. Animals

The experiments were performed on male Wistar rats weighing 250 g (six weeks). All animals were housed in standardized animal facilities under a 12 h light/dark cycle (lights on at 7 AM) with *ad libitum* access to food and water. All surgeries and experimental procedures were performed during the light cycle. Experimental protocols were approved by the Animal Ethics Committee of the local institutional committee for animal use and care (McGill University, Qc, Canada). These protocols follow ethical guidelines for investigation of experimental pain in conscious animals of the IASP, the Canadian Institute of Health Research guidelines for animal care and scientific use.

2.2. Drugs

Delta-9-tetrahydrocannabinol (THC; Tocris Bioscience, Ellisville, MO) and the CB1 antagonist Rimonabant (CBD; Tocris Bioscience, Ellisville, MO) were prepared in a vehicle of PEG 400/Tween 80/0.9% Saline (1:1:18).Cannabidiol (Cayman Chemical, Ann Arbor, MI) was prepared in a vehicle of ethanol/Tween 80/0.9% saline (3:1:16). The 5-HT1A antagonist WAY 100635 (Tocris Bioscience, Ellisville, MO) was dissolved in 0.9% saline.

2.3. Spared nerve injury (SNI)

SNI was performed according to the method of Decosterd and Woolf [96]. Rats were deeply anaesthetized with isofluorane (5%), anaesthesia was confirmed by the absence of a nociceptive reflex reaction to a paw pinch. The sciatic nerve was exposed at mid-thigh level distal to the trifurcation and freed of connective tissue; the three peripheral branches (sural, common peroneal,

and tibial nerves) of the sciatic nerve were exposed without stretching nerve structures. Both tibial and common peroneal nerves were ligated and transected together. Carprofen (5 mg/kg) was administrated subcutaneously pre-surgery as well as three days every 24 hours post-surgery as analgesic. After SNI, each rat was housed in its home cage for 15 days until the neuropathy is developed. Naïve rats did not undergo any surgery [69, 97].

2.4. Mechanical allodynia

On day 15 after SNI, mechanical allodynia was assessed via von Frey test [98]. Rats were placed in a test chamber and they were allowed to acclimate for 60 minutes. Tactile allodynia was determined by measuring paw withdrawal thresholds in response to mid-plantar hind paw stimuli with Calibrated von Frey filaments [69]. These filaments are of a logarithmically incremental stiffness corresponding to an applied force ranging from 0.4 to 15 g. Every filament was applied during 10 seconds in the hind paw to measure the withdrawal threshold. The 2.0 g force filament was applied first; in the presence of a response, the next smaller filament was applied. In the absence of a response, the next higher filament was applied. After the first change in response, the test continued until six responses were collected. The paw withdrawal threshold was then converted to the cutaneous nociceptive threshold by using the "up–down" method [99]. The stimulus intensity (filament stiffness) required to produce a response in 50% of the applications for each animal was defined as 50% withdrawal threshold (expressed in g). The 50% withdrawal threshold was determined according to the following equation: 50% Threshold (g) = (10^ [Xf + kδ])/10,000. Where Xf is the value of the last von Frey filament used (in logarithmic units), k is the correction factor based on the response patterns of a calibration table and the tabulated value based on the pattern of positive and negative responses, and δ indicates the average

differences between stimuli in logarithmic units [98, 99]. In non-lesioned animals (naïve) a value 11.98–15 g was considered normal, while the presence of allodynia was considered when the 50% withdrawal threshold of the limb is less than 4 g. All nerve-ligated rats were verified to be allodynic before the experiments. Rats without allodynia were excluded.

2.5. Study design for mechanical allodynia assessment

Rats were randomly assigned to receive a single intraperitoneal (i.p.) injection of THC (1, 1.5, 2, 2.5, 5 mg/kg), CBD (5, 10, 20 mg/kg), rimonabant (1 mg/kg), WAY 100635 (2 mg/kg) or vehicle. To investigate the involvement of CB1 receptors in the antiallodynic effects of THC, some neuropathic rats received a single dose of the selective CB1 antagonist rimonabant (1 mg/kg) 10 minutes before THC (5 mg/kg). Similarly, to evaluate the involvement of 5-HT1A receptors in the analgesic effect of CBD, neuropathic rats received a single dose of the 5-HT1A antagonist WAY (2 mg/kg) 10 min before CBD (20 mg/kg) was given. Following establishment of basal responses, tactile allodynia was assessed again at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5 h post-administration of THC and related treatments, and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5 h post-administration of CBD and related treatments. The area under the curve (AUC) of the paw withdrawal threshold during the 5.5 and 7.5 hours of testing was also analyzed and compared between treatments.

2.6. EEG/EMG Implantation

Neuropathic rats (10 days after surgery) and naïve rats were placed in a stereotaxic frame following isoflurane-induced anesthesia (5%). Anaesthesia was confirmed by the absence of a nociceptive reflex reaction to a paw pinch. For EEG monitoring, three stainless-steel epidural electrodes were

positioned through 1.5 mm burr holes: one over the parietal cortex on each side, and the third (as a reference) in the right prefrontal cortex. In rats, their respective locations relative to bregma were −2 mm anteroposterior (AP) and −3 mm lateral (L), −7 mm AP and −3 mm L, and −4.5 mm AP and +3 mm L, according to Paxinos and Watson [100]. For EMG monitoring, three flexible stainless-steel wire electrodes, silicon insulation removed at the terminal 3-4mm, were implanted into the neck muscles (two bilaterally and one in the middle). Wires were fused with the electrodes and the connectors were fixed to the skull with dental acrylic (Coltene/Whaledent). Carprofen (10mg/kg) was administrated subcutaneously pre-surgery as well as three days every 24 hours post-surgically as analgesic. Each rat was single-caged post-surgery and allowed to recover for the next 5 days [19]. 24 hours after surgery, the rats were placed in the recording chamber and connected to a flexible 6-flat wire (3M scotchflex) in a freely moving manner for several hours during the subsequent 5 days. No recordings were performed, but tolerance to the cable and sleep behavior were observed. All rats from a home cage were also grouped for one hour each day for socialization to circumvent anxious-depressive-like behavior induced by social isolation [19].

2.8. EEG and EMG recording

Six days after surgery (16 days post-SNI), mechanical allodynia was assessed via von Frey test, and EEG/EMG was recorded for a period of 6 h (from 6 AM to 12 PM). The treatments (described below) were performed at 6:00 A.M., right after the recording had started. EEG/EMG signals were amplified at a total gain of 10.000 and filtered locally (EEG, low filter, 1 Hz; high, 1 kHz; EMG, low filter, 30 Hz; high, 3 kHz; Grass, P55), digitized using a CED power 1401 converter and Spike 2 software (CED) [101], stored with a resolution of 128 Hz, and displayed on a PC monitor.

Consecutive 10 s epochs were subjected to a fast Fourier transform (FFT), and EEG power spectra density was computed in the frequency range of 0–64 Hz [19].

2.9. Analysis of EEG and EMG data

The three classical vigilance states as described in the rat were discriminated on the basis of the cortical EEG and neck EMG activities [102]. Wakefulness was identified by a low-amplitude and desynchronized EEG signal (alpha waves 8-13 Hz), with sustained EMG activity (8-13 Hz). NREM sleep was clearly distinguished by high-voltage delta waves (1–4 Hz) and spindles (10-15 Hz) associated with a weak EMG activity. REM sleep was characterized by a low-amplitude EEG, comparable to that of wakefulness, with a pronounced theta rhythm (4-8 Hz) and a complete loss of muscle tone. Thresholds of EEG and EMG signals between NREM and REM sleep were kept consistent within each animal across the recording period. To avoid transitional periods such as drowsiness, only periods of typical stationary EEG and EMG lasting at least 10 s were considered for further analyses of wakefulness, NREMS, and REMS [19].

2.10. Study design for EEG and EMG assessment

For EEG and EMG recordings, THC's vehicle [PEG 400/Tween 80/0.9% Saline (1:1:18)], CBD's vehicle [ethanol/Tween 80/0.9% saline (3:1:16)], THC (5 mg/kg), CBD (20 mg/kg), WAY (2 mg/kg), and rimonabant (1 mg/kg) were injected via intraperitoneal administration at the beginning of the recording period (6 A.M.). To investigate the participation of CB1 and 5-HT1A receptors in the hypnotic effect of THC and CBD, Rimonabant (1 mg/kg) and WAY (2 mg/kg) were injected 10 min before an effective dose of THC (5 mg/kg) and CBD (20 mg/kg) respectively.

2.11. In vivo electrophysiology

In vivo single-unit extracellular recordings of ON and OFF cells into the RVM were performed. NP rats were anaesthetized with urethane (1.2 g/kg, i.p.) and placed in a stereotaxic frame. Anaesthesia was confirmed by the absence of a nociceptive reflex reaction to a paw pinch. Body temperature was maintained throughout the procedure using a thermistor-controlled heated pad. An incision was made on the scalp, from behind the eyes to the back of the head and the skin was held apart using stainless steel clips. A few drops of lidocaine are applied to the open wound to provide pain relief to the area. Hydrogen peroxide was then applied to the exposed periosteum, and using a gauze sponge, the periosteum was scrubbed away thereby exposing the dorsal skull surface. Using a rat brain atlas, the appropriate brain regions can be located, in which the vl-PAG is -7.8 mm caudal to the bregma and 0.5 mm lateral from the midline, and the RVM is 9.16 – 11.6 mm posterior to the bregma, and 1 mm laterally on both sides of the midline according to the atlas of Paxinos and Watson (2006) [100]. A hole was drilled into the skull above the vl-PAG to allow a stainless steel guide cannula to be stereotaxically lowered into the hole until its tip was above the vl-PAG (by coordinates 4 mm below the dura). The cannula was anchored with dental cement to a stainless steel screw in the skull. A 2 mm by 2 mm window was then created above the RVM using the previously mentioned coordinates and the Dura mater revealed was then removed from the RVM site to allow insertion of a recording electrode [70, 97].

2.12. Intra-PAG microinjections

The drugs used for intra-vlPAG microinjections were the following: THC (10 µg) and CBD (1 µg) [70] . Direct intra-PAG administration of drugs, or appropriate vehicle, was conducted with a stainless steel cannula connected by a polyethylene tube to a SGE 1-microlitre syringe, inserted through the guide cannula and extended to reach the PAG. Vehicle and drug solutions were administered into the vl-PAG in a final volume of 2 µl [70, 97].

2.13. RVM Extracellular Recordings

Recordings were carried out using single-barreled glass micropipettes pulled on a Narishige (Tokyo, Japan) PE-21 glass microelectrode puller. The micropipettes were preloaded with fiberglass strands to promote capillary filling with a 2% Pontamine Sky Blue solution in 3 M NaCl and their tips were broken down to allow an impedance ranged from 2-4 MΩ. A hydraulic micro-positioner was used to lower the electrode into the RVM at approximately 2 µm/sec. RVM ON cells were identified by a burst of activity that begins just before nocifensor reflex to the tail-flick whereas OFF cells are identified by the fact that they cease firing at that time [70]. Once an ON and OFF cell were identified, the spontaneous single-spike activity of the neurons was recorded for at least 60 minutes; the first 5 minutes immediately after detecting the neuron was not considered to eliminate mechanical artifacts, and toe pinches were elicited every 5 minutes for 15 minutes before microinjecting the tested substance into the PAG, in which following substance administration toe pinches were elicited every 15 minutes. At the end of each recording session, the recording site was marked by iontophoretic ejection (–530 mA, negative current for 10 min) of Pontamine Sky Blue for later histological verification of recording sites.

Each rat had one neuron recorded before and after vehicle or during drug administration, and these neuronal responses were measured and expressed as spikes/sec (Hz). Spike2 analyses focused on two parameters: the mean number of spikes contained in a burst recorded for 5 seconds and the mean firing rate after toe pinch recorded for 5 minutes [70, 97].

2.14. Statistical Analysis

Data were analyzed with GraphPad Prism version 8.1.1 (Graph-Pad Software). Results are expressed as the mean ± SEM. Two-way mixed-design ANOVA was used to analyze statistical differences in mechanical allodynia, using treatment, and time as factors in the analysis. One-way ANOVA was used to analyze statistical differences in the area under the curve, using treatment as factor in the analysis. Unpaired t-test (one-tailed) was used to compare statistical differences between naïve rats and neuropathic rats in EEG/EMG recordings. One-way ANOVA was used to calculate statistical differences between groups in EEG/EMG recordings, using treatment as factor in the analysis. One-way ANOVA was used to analyze statistical differences in number of awakenings, REM and NREM sleep events, using treatment as factor in the analysis. Two-way mixed-design ANOVA was used to analyze statistical differences between groups in *in vivo* electrophysiological recordings, using treatment, and time as factors in the analysis. Bonferroni post hoc tests were used to calculate statistical differences between groups. Only p values <0.05 were considered significant.

3. RESULTS

3.1. Increasing doses of THC produce analgesic effects on neuropathic rats

Once neuropathy in rats was confirmed by a paw withdrawal threshold below 4 g using the von Frey test, intraperitoneal administration of THC was able to reverse tactile allodynia induced by the SNI, increasing the paw withdrawal threshold in a dose-dependent manner, whereas administration of the vehicle did not change the withdrawal threshold (Fig. 1A). Two-way, mixed-design, ANOVA on the time course of THC at different doses revealed a significant interaction between treatment and time ($F_{[55, 616]} = 4.734$; $p < 0.001$). Bonferroni post hoc comparisons computed on the simple main effect of dose over hours revealed that THC treatment was able to reverse mechanical allodynia at the dose of 5 mg/kg between 1h and 3.5 h ($p < 0.05$), at the dose of 2.5 mg/kg between 1.5 h and 3 h ($p < 0.05$), at the dose of 2 mg/kg between 1.5 and 2.5 h ($p < 0.05$) and at the dose of 1.5 mg/kg between 2h and 2.5h ($p < 0.05$). The maximal anti-allodynic effect was reached by 5 mg/kg of THC and this effect lasted up to 2.5 hours post-administration. Accordingly, a one-way between subject ANOVA computed on the area under the curve (AUC) during 5.5 hours ($F_{[5, 56]} = 31.75$, $p < 0.001$) and subsequent Bonferroni pairwise comparisons confirmed these results (Fig. 1B). The AUC of THC at 1 mg/kg did not show any antiallodynic effect, but the AUC for the majority of the doses (1.5, 2, 2.5, 5 mg/kg) was significantly higher when compared to the vehicle ($p < 0.01$). Moreover, the AUC of the 5 mg/kg dose was significantly higher compared to the 1 mg/kg dose ($p < 0.001$) and the 1.5 mg/kg dose AUC ($p < 0.001$). Even though the AUC of the doses of 5, 2.5 and 2 mg/kg were not statistically different, the 5 mg/kg dose of THC was used for the following experiments.

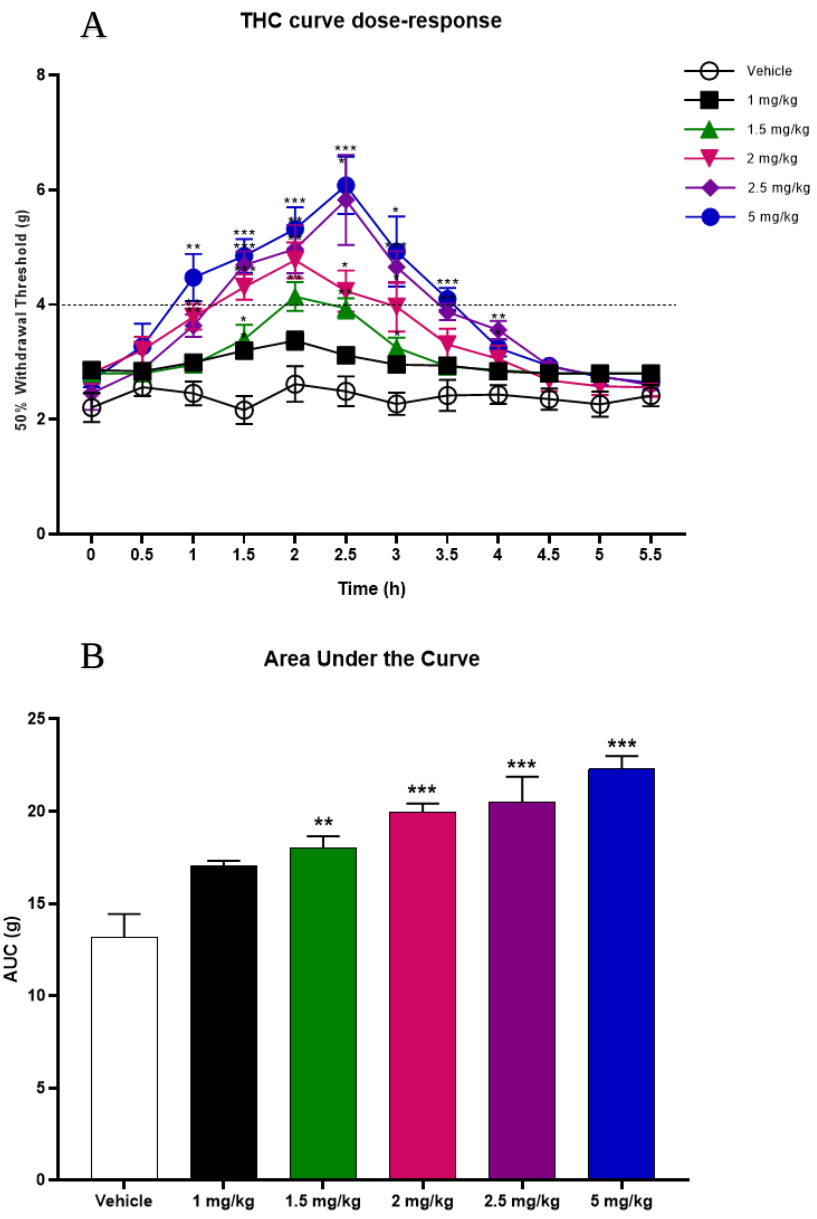


Figure 1: THC reduces mechanical allodynia in a dose-dependent manner and CB1 antagonism blocked this effect. (A) Time course of paw withdrawal threshold after von Frey filament stimulation in rats with SNI before (time 0) and after (0.5-5.5 hours) increasing doses of THC (1, 1.5, 2, 2.5, and 5 mg/kg, intraperitoneally) in comparison with vehicle treated rats. The dashed line through the graph represents the threshold cut off (4g) for allodynia in NP rats, in which values above the line are considered anti-allodynic. Data are expressed as mean ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001 vs. vehicle, by Bonferroni post hoc test. (B) Area under the curve (AUC) of the antiallodynic effect of increasing doses of THC. Data are expressed as mean ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001 vs. vehicle, by Bonferroni post hoc test.

3.1.1. Rimonabant blocks the analgesic effects of THC

To investigate the possible role of CB1 receptor in the analgesic effects of THC, we pretreated NP rats with Rimonabant (1 mg/kg, i.p.) 10 minutes before the administration of THC (5 mg/kg). Two-way, mixed-design, ANOVA revealed a significant interaction between treatment and time ($F_{[33, 330]} = 3.318$; $p < 0.001$). Bonferroni post hoc comparisons computed on main effect of treatments over hours revealed that NP rats treated with Rimonabant + THC (n = 5) were statistically different from NP rats treated with THC (n = 11; $p < 0.001$), but they did not differ from NP animals treated with vehicle (n = 13), suggesting that CB1 antagonism prevented the antiallodynic effects of THC (Fig. 2A). Additionally, treatment with Rimonabant alone (n = 5) did not alter the mechanical threshold when compared with NP rats treated with vehicle (n = 13; $p > 0.05$). Likewise, One-way between subject ANOVA computed on the area under the curve (AUC) during 5.5 hours ($F_{[3, 30]} = 21.36$, $p<0.001$) confirmed these results (Fig. 2B). Subsequent pairwise comparison tests, conducted using Bonferroni post hoc test, indicated that the AUC from the THC (5 mg/kg) group was significantly higher than that from the group receiving 1 mg/kg of Rimonabant ($p < 0.001$), the group receiving 1 mg/kg of Rimonabant + 5 mg/kg of THC ($p < 0.001$), and vehicle ($p < 0.001$). No other pairwise contrast was significant ($p > 0.05$).

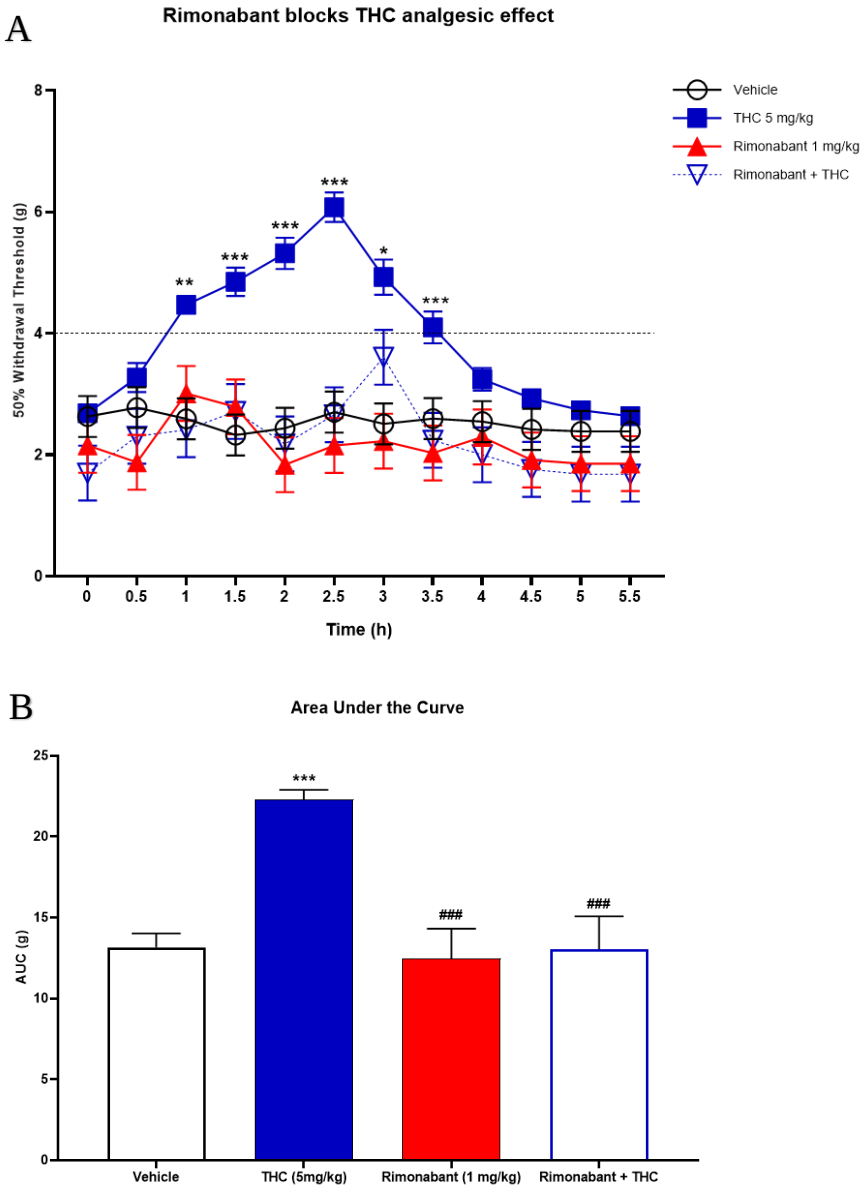


Figure 2: Antiallodynic effect of THC was fully prevented by the administration of the CB1 receptor selective antagonist rimonabant. (A) Time course of paw withdrawal threshold after von Frey filament stimulation in rats with SNI before (time 0) and after (0.5-5.5 hours) rimonabant (1 mg/kg), rimonabant + THC, THC (5mg/kg), and vehicle. Data are expressed as mean ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001 vs. vehicle, by Bonferroni post hoc test. (D) AUC of the treatments with rimonabant alone and rimonabant + THC compared to THC and vehicle. Data are expressed as mean ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001 vs. vehicle, and # p < 0.05, ## p < 0.01, and ### p < 0.001 vs. THC, by Bonferroni post hoc test.

3.2. Increasing doses of CBD produce analgesic effects on neuropathic rats

The intraperitoneal administration of CBD was able to reverse tactile allodynia induced by the SNI, increasing the paw withdrawal threshold in a dose-dependent manner, whereas administration of the vehicle did not change the withdrawal threshold (Fig. 2A). Two-way, mixed-design, ANOVA on the time course of CBD at different doses revealed a significant interaction between treatment and time ($F_{[45, 390]} = 3.545$; $p < 0.001$). Bonferroni post hoc comparisons computed on the simple main effect of dose over hours revealed that CBD treatment was able to reverse mechanical allodynia at the dose of 20 mg/kg between 1h and 5 h ($p < 0.05$), and at the dose of 10 mg/kg between 2 h and 4.5 h ($p < 0.05$). Whereas CBD at 5 mg/kg did not show any antiallodynic effect. These effects lasted up to 4 hours post-administration (Fig. 3A). A one factor between-subject ANOVA performed on the AUC revealed a significant effect for group ($F [3, 26] = 27.22$, $p < 0 .001$; Fig. 3B). Subsequent Bonferroni post hoc analysis indicated that the AUC of the 20 mg/kg group was significantly higher compered to the AUC of the group receiving CBD at 5 mg/kg ($p < 0.001$) and to the group receiving vehicle ($p < 0.001$). Even though the AUC of the doses of 10 and 20 mg/kg were not statistically different, the 20 mg/kg dose of CBD was used for the following experiments.

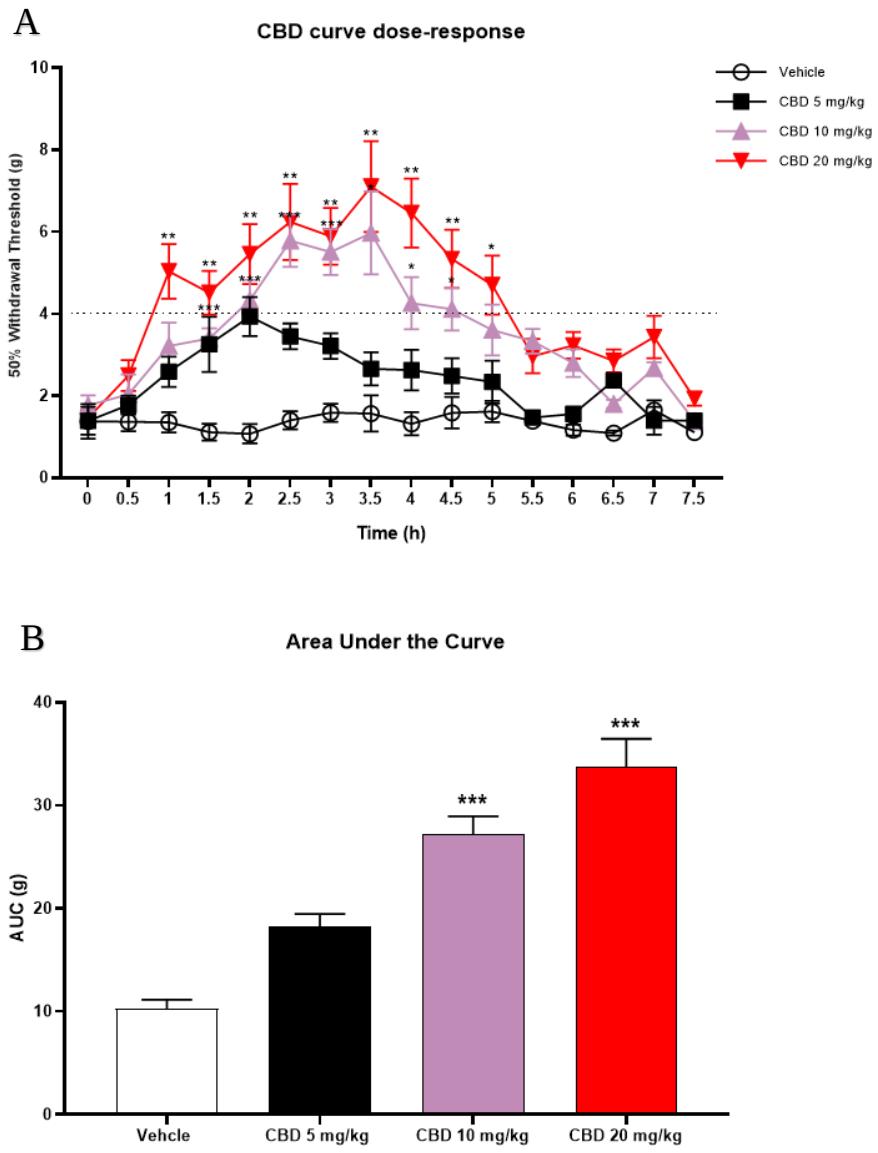
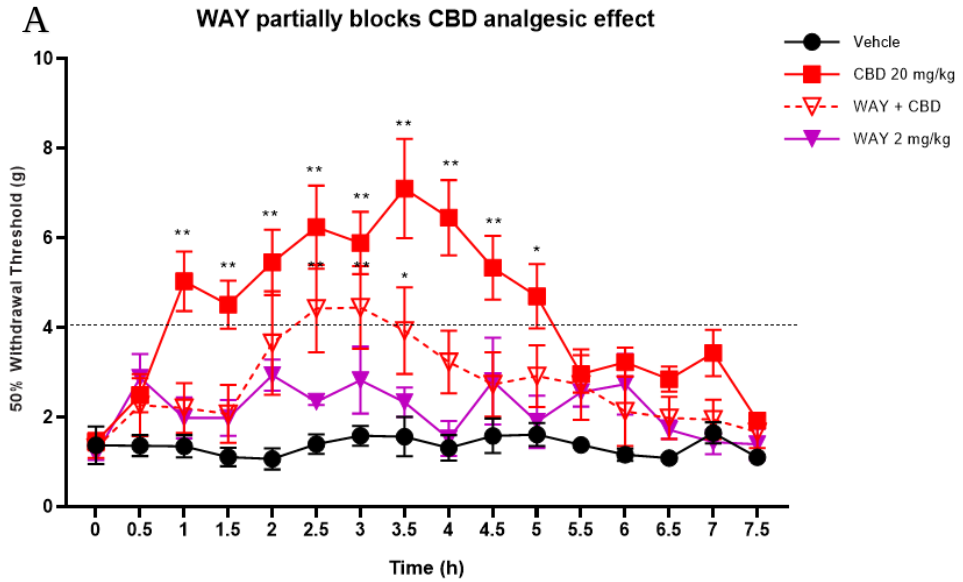


Figure 3: THC reduces mechanical allodynia in a dose-dependent manner and CB1 antagonism blocked this effect. (A) Time course of paw withdrawal threshold after von Frey filament stimulation in rats with SNI before (time 0) and after (0.5-7.5 hours) increasing doses of CBD (5, 10, and 20 mg/kg, intraperitoneally) in comparison with vehicle treated rats. The dashed line through the graph represents the threshold cut off (4g) for allodynia in NP rats, in which values above the line are considered anti-allodynic. Data are expressed as mean ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001 vs. vehicle, by Bonferroni post hoc test. (B) Area under the curve (AUC) of the antiallodynic effect of increasing doses of CBD. Data are expressed as mean ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001 vs. vehicle, by Bonferroni post hoc test.

3.2.1. WAY 100635 partially blocks the analgesic effects of CBD

To investigate the possible role of 5HT1A receptor in the analgesic effects of CBD (20 mg/kg), we pretreated NP rats with WAY 100635 (2 mg/kg, i.p.) 10 minutes before CBD (Fig. 4A). Two-way, mixed-design, ANOVA revealed a significant interaction between treatment and time ($F_{[33, 330]} = 3.318$; $p < 0.001$). Bonferroni post hoc comparisons computed on the simple main effect of treatments over hours revealed that WAY + CBD group (n = 5) was not different from NP rats treated with CBD between 2 and 3.5 hour ($p > 0.05$), suggesting that 5HT1A antagonism only partially prevented the antiallodynic effects of CBD at 20 mg/kg. Additionally, treatment with WAY 100636 alone (n = 5) did not alter the mechanical threshold compared with NP rats treated with vehicle ($p > 0.05$). Likewise, a one-way ANOVA analysis of the AUC ($F_{[3, 20]} = 14.43$; $p < 0.001$) and subsequent Bonferroni post hoc comparison revealed statistical difference between groups (Fig. 4B). The AUC for the 20 mg/kg doses was significantly higher when compared to all the other groups ($p < 0.05$). No other pairwise contrast was significant ($p > 0.05$).



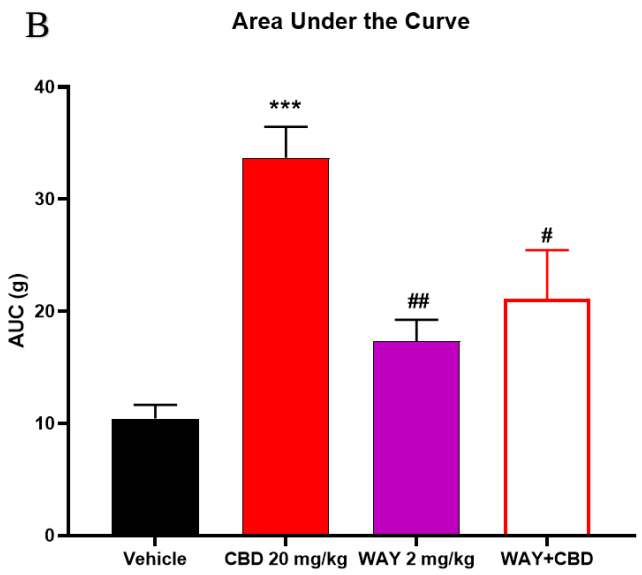


Figure 4: Antiallodynic effect of THC was fully prevented by the administration of the CB1 receptor selective antagonist rimonabant. (A) Time course of paw withdrawal threshold after von Frey filament stimulation in rats with SNI before (time 0) and after (0.5-7.5 hours) way (2 mg/kg), WAY + CBD, CBD (20 mg/kg), and vehicle. Data are expressed as mean ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001 vs. vehicle, by Bonferroni post hoc test. (D) AUC of the treatments with WAY alone and WAY + CBD compared to CBD and vehicle. Data are expressed as mean ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001 vs. vehicle, and # p < 0.05, ## p < 0.01, and ### p < 0.001 vs. CBD, by Bonferroni post hoc test.

3.3. Neuropathic animals develop sleep perturbations

To evaluate whether neuropathy might induce sleep fragmentation also in rats, we measured the EEG/EMG parameters (wakefulness, NREM and REM sleep) for 6 hours in NP and naïve rats. An unpaired t-test (one-tailed) conducted on data revealed that the mean wakefulness for NP rats (n = 11) was significantly higher than the mean for naïve rats (n = 9), $t_{[18]} = 3.007$, $p < 0.01$ (Fig. 5C). An unpaired t-test (one-tailed) revealed the average spent in NREM sleep by NP rats (Fig. 5B) was significantly lower compared to that of naïve rats, $t_{[18]} = 2.912$, $p < 0.01$. In addition, also the average of time spent in REM sleep by NP rats was statistically lower when compared to that of naïve rats, $t_{[18]} = 3.862$, $p < 0.001$ (one-tailed; Fig. 5A).

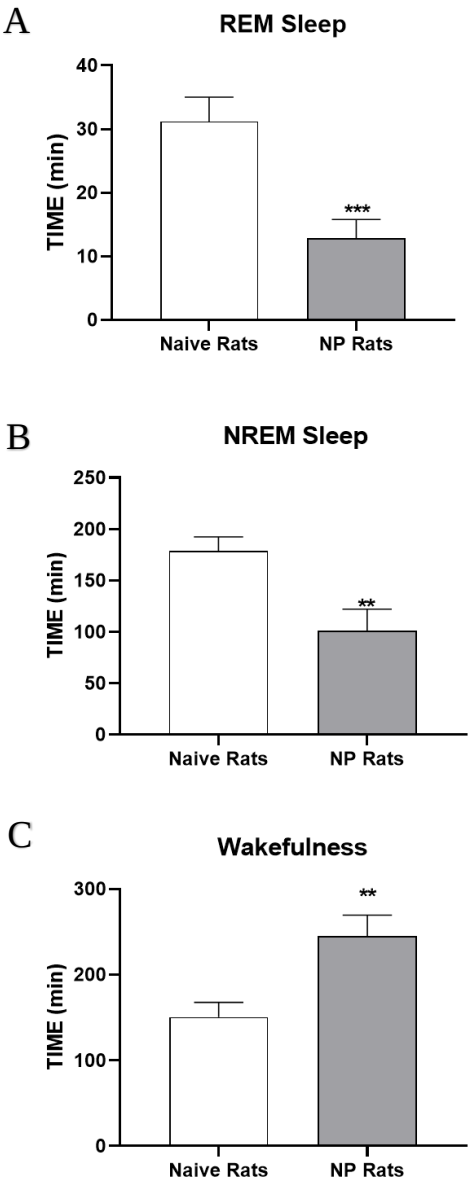


Figure 5: Neuropathic animals develop sleep perturbations. Animals with a NP condition develop sleep perturbations characterized by a decrease in NREM (B) and REM (A) sleep and an increase in wakefulness (C).Data are expressed as mean ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001 vs. Naïve.

3.4. THC reverses the sleep perturbations induced by Neuropathic Pain

To evaluate whether THC (5 mg/kg) might reverse sleep fragmentation associated with NP, we measured the same parameters (wakefulness, NREM and REM sleep) obtained by the EEG/EMG recordings during 6 hours in three groups of rats. The first group were naïve rats treated with THC vehicle (n = 9), the second group were NP rats treated with THC vehicle (n =11), and the third group were NP rats treated with a THC dose of 5 mg/kg (n = 10). One-way ANOVA performed on the total time of wakefulness revealed a statistical difference between groups ($F_{[2, 27]} = 9.28$, $p < 0.001$; Fig. 6C). Bonferroni post-hoc analysis revealed that NP rats treated with THC display a decrease in the amount of time spent in wakefulness, when compared to NP rats treated with vehicle ($p < 0.01$), whereas no difference was detected between naïve animals treated with vehicle and NP rats treated with THC. One-way ANOVA between subjects performed on total NREM sleep revealed a significant main effect for groups ($F_{[2, 27]} = 8.357$, $p < 0.01$; Fig 6B). Bonferroni post hoc comparison revealed that THC (5 mg/kg) was able to reverse NREM sleep impairments in NP rats ($p < 0.01$) when compared to NP rats treated with vehicle. Additionally, One-way ANOVA between subjects performed on REM sleep (total time) revealed a significant main effect for groups ($F_{[2, 27]} = 11.21$, $p < 0.001$). Bonferroni post-hoc analysis showed that THC (5 mg/kg) administration restored the normal REM sleep in neuropathic rats ($p < 0.001$) compared to NP rats treated with vehicle (Fig. 6A). Whereas no difference was detected between naïve animals treated with vehicle and NP rats treated with THC.

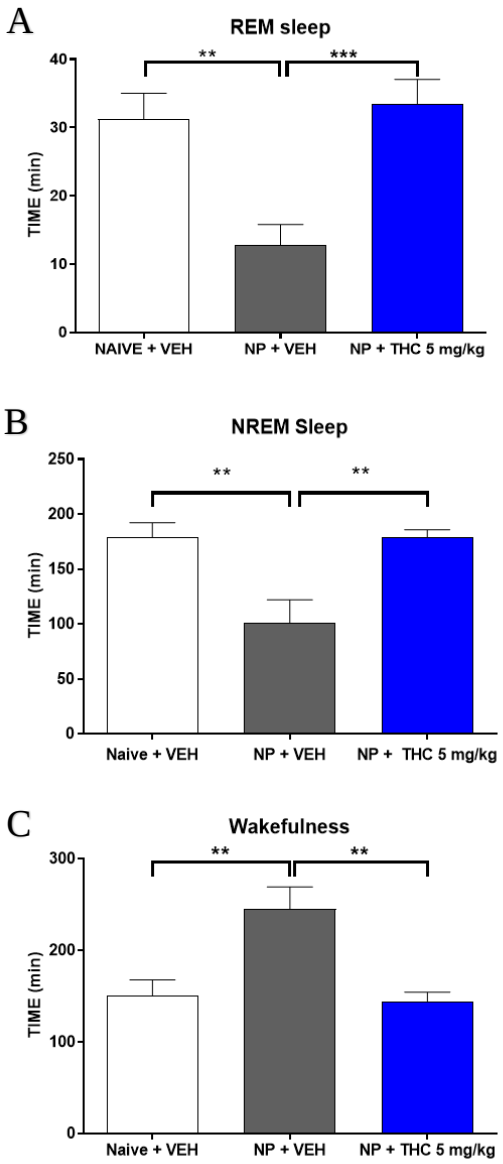


Figure 6: THC (5 mg/kg) restored the normal sleep-wake cycle in NP rats. (A) THC restored REM sleep in Neuropathic animals. Bonferonni post hoc comparison detected no difference between Naïve animals treated with vehicle and Neuropathic rats treated with THC (5 mg/kg). Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. NP + VEH. (B) THC restored NREM sleep in Neuropathic animals. In fact, Bonferonni post hoc comparison detected no difference between Naïve animals treated with vehicle and Neuropathic rats treated with THC (5 mg/kg). Data are expressed as mean ± SEM, (n=9). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. NP + VEH. (C) THC decreased wakefulness in Neuropathic animals. In fact, Bonferoni post hoc comparison revealed no difference between Naïve animals treated with vehicle and Neuropathic rats treated with THC (5 mg/kg). Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. NP + VEH

3.4.1. Rimonabant blocks the hypnotic effects of THC

To evaluate whether Rimonabant (1 mg/kg) might reverse the hypnotic effect of THC (5 mg/kg), we measured the EEG/EMG parameters (wakefulness, NREM and REM sleep) for 6 hours in four groups of rats. The first group were NP rats treated with THC vehicle (n = 11), the second group were NP rats treated with rimonabant (1 mg/kg), the third group (n = 8) were NP rats treated with rimonabant (1 mg/kg) + THC (5 mg/kg). Whereas the fourth group (n = 8) were NP treated with a THC dose of 5 mg/kg (n =10). One-way ANOVA performed on the total time of wakefulness ($F_{[3, 33]} = 12.10$, $p < 0.001$) revealed a statistical difference between groups (Fig. 7C). Bonferroni post-hoc analysis did not detect any difference between NP rats treated with rimonabant (1 mg/kg) + THC (5 mg/kg) and NP rats treated with vehicle ($p > 0.05$); moreover, rimonabant alone did not alter wakefulness compared with NP rats treated with vehicle, ($p > 0.05$). Furthermore, the post-hoc analysis showed that NP rats treated with rimonabant (1 mg/kg) + THC (5 mg/kg) display an increase in wakefulness when compared to NP rats treated with THC at 5 mg/kg ($p < 0.001$). One-way ANOVA between subjects performed on total NREM sleep revealed a significant main effect for groups ($F_{[3, 33]} = 8.763$, $p < 0.001$; Fig. 7B). Bonferroni post-hoc comparison analysis did not reveal any difference between NP rats treated with rimonabant (1 mg/kg) + THC (5 mg/kg) and NP rats treated with vehicle; moreover, rimonabant alone (1 mg/kg) did not alter NREM sleep compared with NP rats treated with vehicle. On the other hand, rimonabant (1 mg/kg) was able to prevent the positive effect of THC on NREM sleep in NP rats ($p < 0.001$). Finally, One-way ANOVA between subjects performed on REM sleep revealed a statistical difference between groups ($F_{[3, 33]} = 31.47$, $p < 0.001$; Fig. 7A). Bonferroni post-hoc analysis showed that rimonabant (1 mg/kg) administration was able to block the positive effect of THC on REM sleep in NP rats ($p < 0.001$). Furthermore, the post-hoc analysis showed that NP rats treated with rimonabant (1

mg/kg) + THC (5 mg/kg) display a decrease in REM sleep when compared to NP rats treated with vehicle ($p < 0.05$), and also NP rats treated with rimonabant alone (1 mg/kg) showed a decrease in REM sleep ($p < 0.05$).

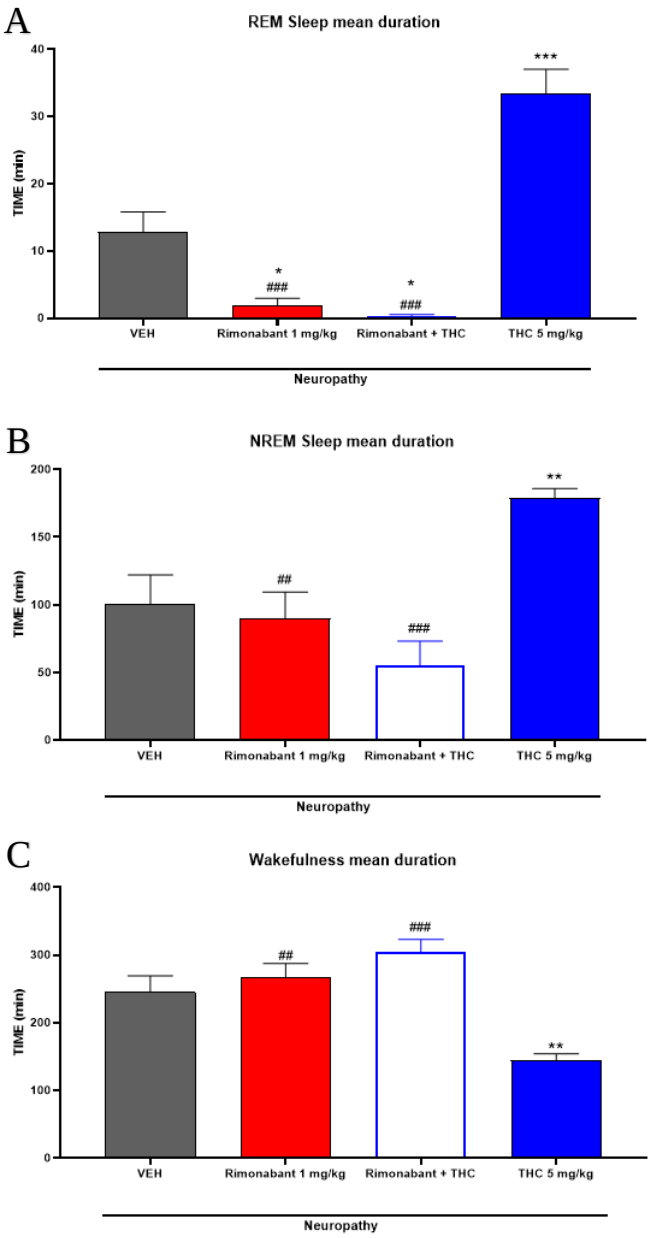


Figure 7: Rimonabant blocks the hypnotic effects of THC: (A-B-C) in Neuropathic rats, the hypnotic effects of THC (5 mg/kg) were fully prevented by the administration of the CB1 receptor selective antagonist rimonabant (1 mg/kg). Moreover, rimonabant impaired REM sleep in Neuropathic rats when compared to NP rats treated with vehicle (A). Data are expressed as mean ± SEM, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. VEH, and ###$P < 0.001$ vs. 5 mg/kg THC by Bonferroni post hoc test

3.5. CBD reverses the sleep perturbations induced by Neuropathic Pain

To evaluate whether CBD (20 mg/kg) might reverse sleep fragmentation associated with NP, we measured the EEG/EMG parameters (wakefulness, NREM and REM sleep) for 6 hours in three groups of rats. The first group were naïve rats treated with CBD vehicle (n = 9), the second group were NP rats treated with CBD vehicle (n =8), and the third group were NP rats treated with a CBD dose of 20 mg/kg (n = 8). One-way ANOVA performed on the total time of wakefulness ($F_{[2, 22]} = 9.888$, $p < 0.001$) revealed a statistically significant difference between groups (Fig. 8C). Bonferroni post-hoc analysis revealed that NP rats treated with CBD display a decrease in the amount of time spent in wakefulness, when compared to NP rats treated with vehicle ($p < 0.01$), whereas no difference was detected between naïve animals treated with vehicle and NP rats treated with CBD. One-way ANOVA between subjects performed on total NREM sleep revealed a significant main effect for groups ($F_{[2, 22]} = 7.553$, $p < 0.01$). Bonferroni post-hoc comparison revealed that CBD (20 mg/kg) was able to reverse NREM sleep impairments in NP rats ($p < 0.05$) when compared to NP rats treated with vehicle, whereas no difference was detected between naïve animals treated with vehicle and NP rats treated with CBD. Additionally, one-way ANOVA between subjects performed on REM sleep (total time) revealed a significant main effect for groups ($F_{[2,22]} = 9.680$, $p < 0.001$). Bonferroni post-hoc analysis showed CBD (20 mg/kg) administration restored the normal REM sleep in neuropathic rats ($p < 0.05$) compared to NP rats treated with vehicle and no difference was detected between naïve animals treated with vehicle and NP rats treated with CBD.

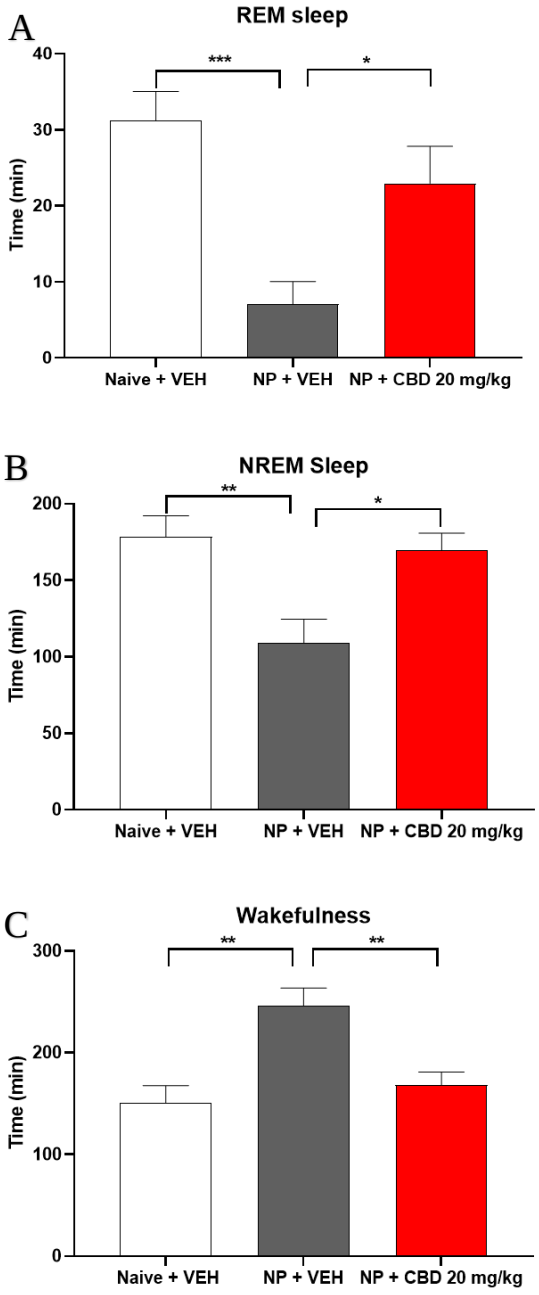


Figure 8: CBD (20 mg/kg) restored the normal sleep-wake cycle in NP rats. (A) CBD restored REM sleep in Neuropathic animals. Bonferonni post hoc comparison detected no difference between Naïve animals treated with vehicle and Neuropathic rats treated with CBD (20 mg/kg). Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. NP + VEH. (B) CBD restored NREM sleep in Neuropathic animals. In fact, Bonferonni post hoc comparison detected no difference between Naïve animals treated with vehicle and Neuropathic rats treated with CBD (20 mg/kg). Data are expressed as mean ± SEM, (n=9). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. NP + VEH. (C) CBD decreased wakefulness in Neuropathic animals. In fact, Bonferoni post hoc comparison revealed no difference between Naïve animals treated with vehicle and Neuropathic rats treated with CBD (20 mg/kg). Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. NP + VEH.

3.5.1. WAY blocks the hypnotic effects of CBD

To evaluate whether the 5-HT1A selective antagonist WAY 100635 (2 mg/kg) might reverse the hypnotic effect of CBD (20 mg/kg), we measured the EEG/EMG parameters (wakefulness, NREM and REM sleep) for 6 hours in four groups of rats. The first group were NP rats treated with CBD vehicle (n = 8), the second group were NP rats treated with WAY 100635 alone at 2 mg/kg (n = 6), the third group were NP rats treated with WAY 100635 (2 mg/kg) + CBD (20mg/kg; n = 6), and the fourth group were NP treated with CBD at 20 mg/kg (n = 8). One-way ANOVA performed on the total time of wakefulness ($F_{[3, 24]} = 19.21$, $p < 0.001$) revealed a statistical difference between groups (Fig. 9C). Bonferroni post-hoc analysis did not detect any difference between NP rats treated with WAY 100635 (2 mg/kg) + CBD (20 mg/kg) and NP rats treated with vehicle, however, they display an increase in wakefulness when compared with NP rats treated with CBD at 20 mg/kg ($p < 0.001$). Moreover, the group treaded with WAY 100635 alone (2 mg/kg) show an increase in wakefulness when compared with NP rats treated with vehicle ($p < 0.001$) and NP rats treated with CBD at 20 mg/kg ($p < 0.001$). One-way ANOVA between subjects performed on total NREM sleep revealed a significant main effect for groups ($F_{[3, 24]} = 18.85$, $p < 0.001$; Fig. 9B). Bonferroni post-hoc comparison analysis did not reveal any difference between NP rats treated with WAY 100635 (2 mg/kg) and CBD (20mg/kg) and NP rats treated with vehicle or with NP rats treated with WAY 100635 alone ($p > 0.05$). However, the WAY 100635 + CBD group display a decrease in time spent in NREM sleep when compared with NP rats treated with CBD at 20 mg/kg ($P<0.001$). Furthermore, WAY 100635 alone (2 mg/kg) statistically reduced the time spent NREM sleep compared with NP rats treated with vehicle ($p > 0.01$). Additionally, One-way ANOVA between subjects performed on REM sleep revealed a statistical difference between groups ($F_{[3, 24]} = 10.27$, $p < 0.001$; Fig. 9A). Bonferroni post-hoc analysis showed that WAY

100635 (1 mg/kg) administration was able to block the positive effect of CBD (20 mg/kg) on REM sleep in NP rats ($p < 0.001$), whereas no statistical difference was detected between NP rats treated with WAY 100635 + CBD and NP treated with WAY 100635 alone. Furthermore, the post-hoc analysis showed that NP rats treated with WAY 100635 alone display a decrease in REM sleep when compared to NP rats treated with CBD at 20 mg/kg ($p < 0.01$), whereas no statistical difference was detected between NP rats treated with vehicle and NP rats treated with WAY 100365 (2 mg/kg).

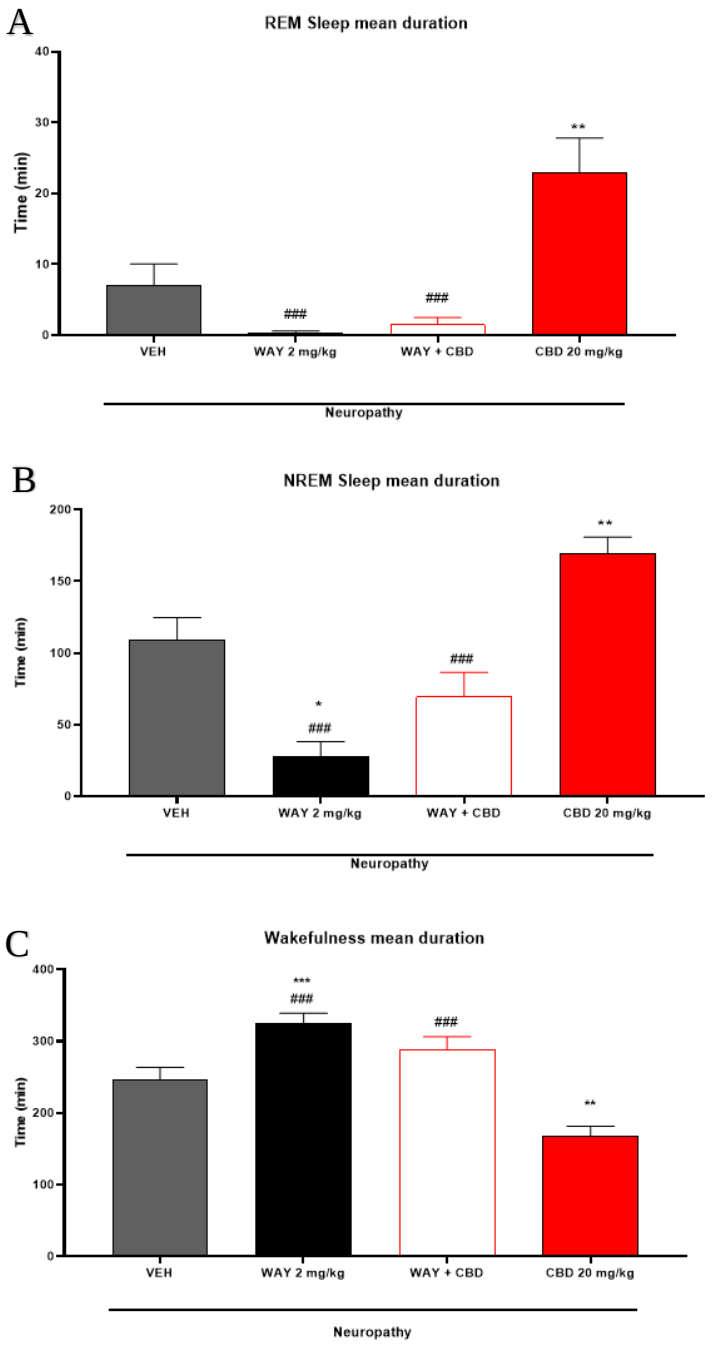


Figure 9: WAY blocks the hypnotic effects of CBD. (A-B-C) in Neuropathic rats, the hypnotic effects of CBD (20 mg/kg) were fully prevented by the administration of the 5-HT1A receptor selective antagonist WAY (2 mg/kg). Moreover, WAY impaired NREM sleep (B) and increased wakefulness (C) in Neuropathic rats when compared to NP rats treated with vehicle. Data are expressed as mean ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001 vs. VEH, and ###P < 0.001 vs. 20 mg/kg CBD by Bonferroni post hoc test.

3.6. Number of awakenings, NREM and REM sleep events

In NP rats, we also evaluated the number of awakenings, NREM and REM sleep events after administration of THC (5 mg/kg), CBD (20 mg/kg), rimonabant (1 mg/kg), WAY 100635 (2 mg/kg) and vehicle. One way between subjects ANOVA ($F_{[3, 27]} = 17.00$, $p < 0.001$) and subsequent Bonferroni post hoc comparisons revealed that rimonabant and rimonabant + THC treatments significantly reduced the number of REM sleep events compared with vehicle ($p < 0.01$: Fig 10A). Whereas THC did not alter the number of REM sleep events in NP rats. Furthermore, rimonabant, rimonabant + THC, and THC had no effect on number of NREM sleep events and awakenings ($p > 0.05$, Fig. 10B-C). One way between subject ANOVA and subsequent Bonferroni post hoc comparisons revealed that the administration of WAY 100635 significantly reduced the number of awakenings ($p < 0.05$; $F_{[3, 24]} = 4.279$, $p < 0.05$; Fig. 11C) and of NREM sleep events ($p < 0.05$; $F_{[3, 24]} = 8.817$, $p < 0.001$; Fig. 11B) compared to vehicle. Conversely, CBD and WAY 100635+ CBD treatments had no effect (Fig. 5B-D). However, CBD statistically increased the number of REM sleep events ($p < 0.05$; $F_{[2, 19]} = 8.250$, $p < 0.01$) compared to vehicle (Fig. 11A), whereas the administration of WAY 100635 had no effect ($p > 0.05$) compared with vehicle but prevented the CBD-induced increase in REM sleep events ($p < 0.01$).

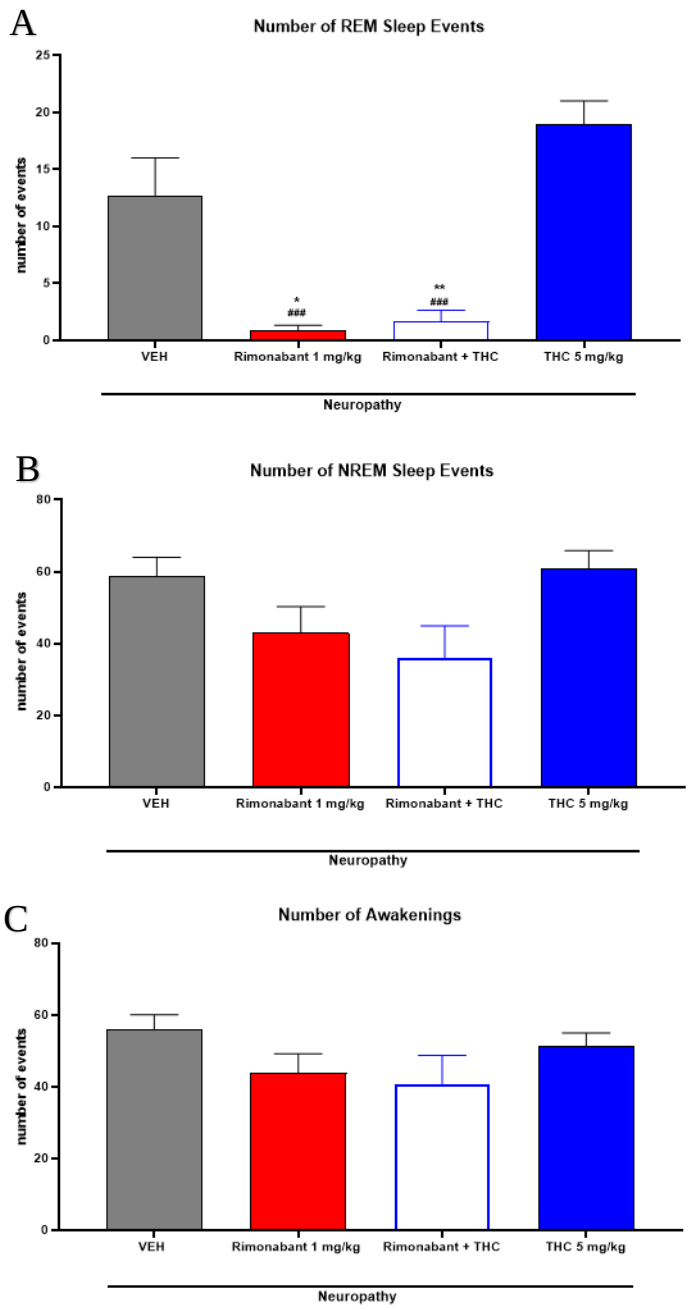


Figure 10: Number of events. (A) Rimonabant and rimonabant + THC treatments significantly reduced the number of REM sleep events compared with vehicle or THC.THC did not alter the number of REM sleep events in NP rats. (B-C) rimonabant and THC had no effect on number of NREM sleep events and awakenings. Data are expressed as mean ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001 vs. VEH, and ###P < 0.001 vs. 5 mg/kg THC by Bonferroni post hoc test.

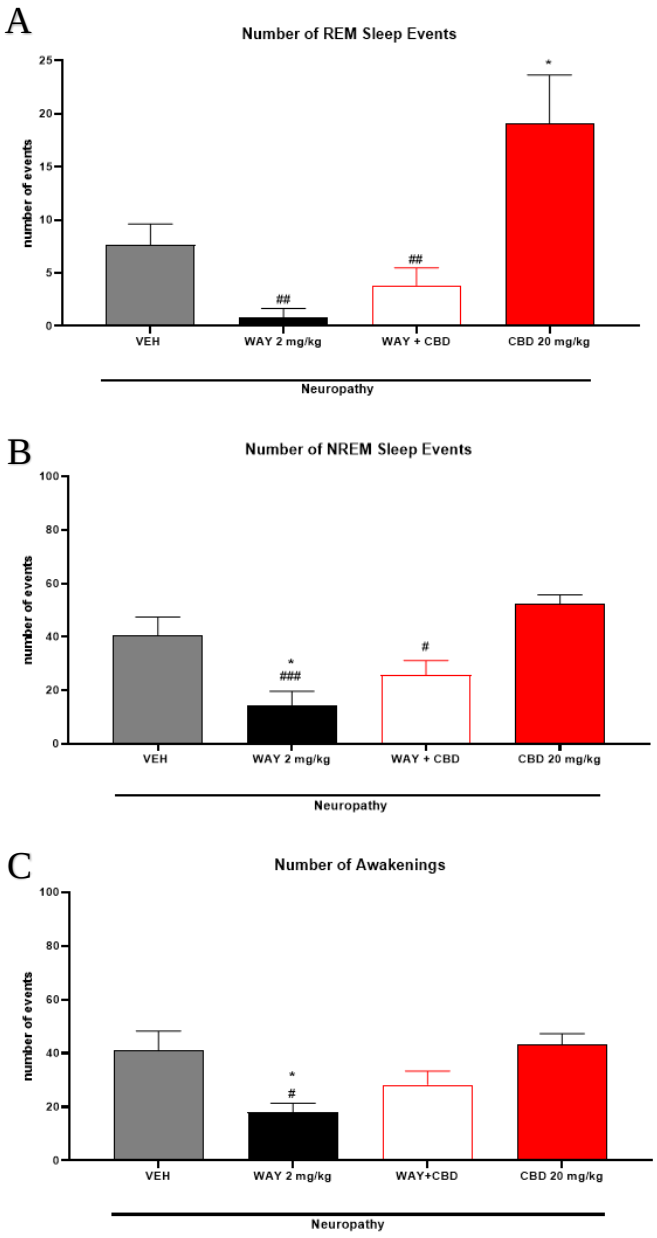


Figure 11: number of events. (A) CBD statistically increased the number of REM sleep events in NP rats when compared with vehicle. Conversely, CBD and WAY 100635+ CBD treatments had no effect on the number REM sleep events when compared to vehicle, however the number of events is reduced when compared to CBD treated rats. (D-F) Administration of WAY significantly reduced the number of awakenings and of NREM sleep events when compared with vehicle. Data are expressed as mean ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001 vs. VEH, and ###P < 0.001 vs. 20 mg/kg CBD by Bonferroni post hoc test.

3.7. Effect of intra-vlPAG injection of THC or CBD on the ongoing activity of RVM ON and OFF cells in NP rats

To investigate the effects of THC (10 μg) and CBD (1 μg) in the modulation of the ON and OFF cells in the PAG-RVM circuit, a new cohort of neuropathic rats underwent *in vivo* electrophysiology, in which ON and OFF cells (within the RVM) were recorded before and after the appropriate drug or vehicle microinjection into the vlPAG. THC microinjection altered the spontaneous activity of ON cells (n = 3), a two way ANOVA (mixed design) computed on the data yielded a significant treatment x time interaction ($F_{[9, 36]} = 26.82$, $p < 0.001$; Fig. 12 A). Bonferroni post hoc comparisons computed on the simple effect of treatments over time indicated that microinjection of vehicle (n = 3) did not alter the spontaneous activity of ON cells whereas microinjection of THC (10 μg) significantly reduced ON cells firing activity between 10 and 45 minutes post-microinjection ($p < 0.001$; Fig. 6A). Similar results were observed with CBD microinjection (Fig. 12 C). A two way ANOVA, mixed design, computed on the data yielded a significant treatment x time interaction ($F_{[9, 36]} = 11.52$, $p < 0.001$). Bonferroni post hoc comparisons computed on the simple effect of treatments over time indicated that microinjection of vehicle (n = 3) did not alter the spontaneous activity of ON cells whereas microinjection of CBD (1 μg; n = 3) significantly reduced ON cells firing activity between 5 and 45 minutes post-microinjection ($p < 0.001$). The population of OFF cells had a mean frequency of spontaneous activity of 4.9 ± 0.45 spikes per second in NP rats. Also in this case, THC microinjection altered the OFF cells (n = 3) spontaneous activity, a two way mixed design ANOVA computed on the data revealed a significant treatment x time interaction ($F_{[9, 36]} = 3.885$, $p < 0.01$). Bonferroni post hoc comparisons computed on treatments at each time indicated that microinjection of THC (10

μg) increased the spontaneous activity of OFF cells between 10 and 30 minutes post-microinjection ($p < 0.05$; Fig. 12B) when compared with vehicle ($n = 3$). Different results were observed after CBD microinjection. A two way ANOVA, mixed design, computed on the data yielded a significant treatment x time interaction ($F_{[9, 36]} = 2.311$, $p < 0.05$). Bonferroni post hoc comparisons computed on treatments at each time indicated that microinjection of CBD (1 μg; $n = 3$) reduced the spontaneous activity of OFF cells between 20 and 30 minutes post-microinjection ($p < 0.05$; Fig. 12D) when compared with vehicle ($n = 3$).

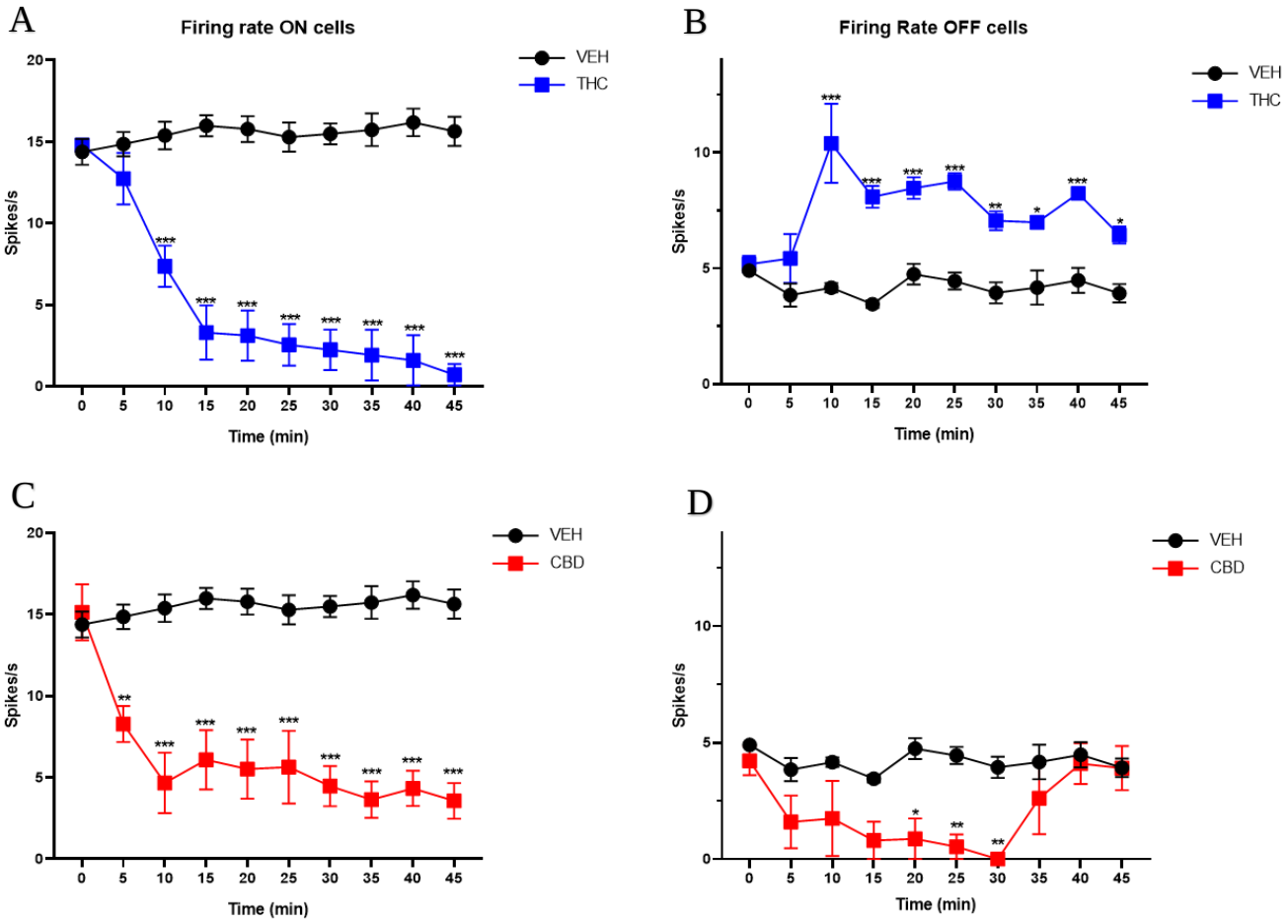


Figure 12: THC and CBD modulate the descending pathway of anti-nociception. THC, when microinjected into the vlPAG, inhibited the ongoing activity of ON cells (A) and the spontaneous activity of OFF cells (B). Conversely, CBD inhibited spontaneous activity of both ON (C) and OFF cells (D), when injected into the vlPAG. Data are expressed as mean ± SEM, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. VEH.

3.7.1. *Effect of intra-vlPAG microinjection of THC or CBD on ON cell burst magnitude and OFF cell pause duration in NP rats*

In NP rats, the population of ON cells had a toe pinch–induced burst of firing of 20.36 $\pm$ 1.37 spikes per second, whereas the population of OFF cells had a pause of 6.53 $\pm$ 1.01 seconds.

Two way mixed design ANOVA (treatment x time interaction: $F_{[3, 12]} = 18.15$, $p<0.001$) and subsequent Bonferroni post hoc comparison computed on treatments at each time revealed that the microinjection of THC (10 μg) significantly reduced ON cells burst activity between 15 and 45 minutes post-microinjection ($p < 0.001$; Fig. 13A) when compared with vehicle. Moreover, THC microinjection significantly reduced OFF cells pause between 30 and 45 minutes post-administration ($p < 0.01$; treatment x time interaction: $F_{[3, 12]} = 58.70$, $p < 0.001$; Fig. 13B).

Similar results were observed after CBD microinjection. Two way mixed design ANOVA (treatment x time interaction: $F_{[3, 12]} = 76.26$, $p < 0.001$) and subsequent Bonferroni post-hoc comparison computed on treatments at each time revealed that the microinjection of CBD (1 μg) significantly reduced ON cells burst activity between 15 and 45 minutes post-microinjection ($p < 0.01$, Fig. 13c) when compared with vehicle. However, CBD microinjection significantly increased OFF cells pause duration between 15 and 30 minutes post administration ($p < 0.05$; treatment x time interaction: $F_{[3, 12]} = 6.264$, $p < 0.01$, Fig. 13D)

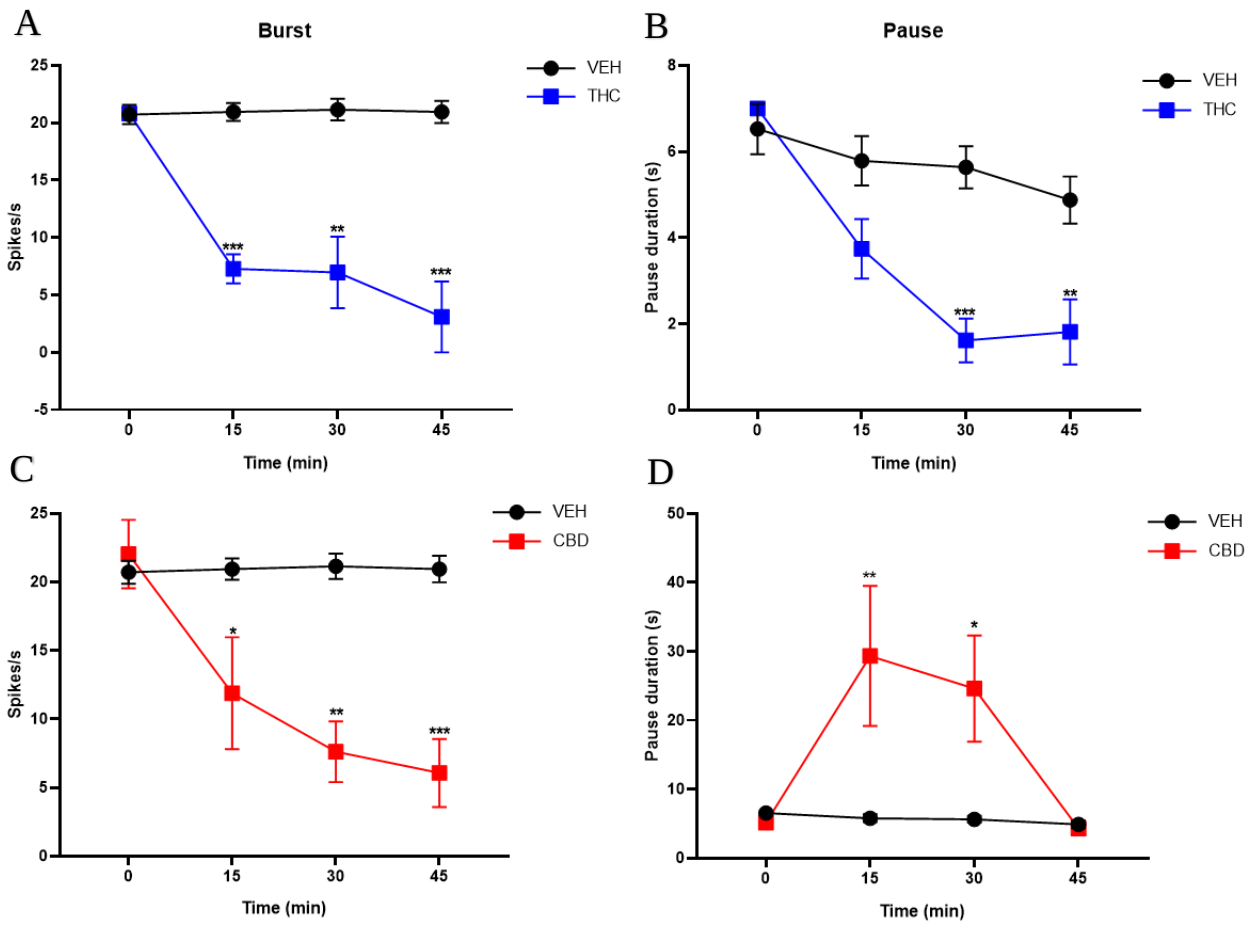


Figure 13: THC and CBD modulate the descending pathway of anti-nociception. THC (A) and CBD (c), when microinjected into the vlPAG, inhibited toe pinch–evoked activity of ON cells. (B) THC, when injected into the vlPAG, and reduced the pause duration in OF cells (B). Conversely, CBD increased the pause duration in OFF cells (D). Data are expressed as mean ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001 vs. VEH.

4. DISCUSSION

4.1. Summary

In this study, we examined the extent to which THC and CBD modulate nociception and sleep in a rodent model of neuropathic pain. Wistar rats underwent the SNI model of induced mechanical allodynia according to the method of Decosterd and Woolf [96]. Neuropathy was confirmed using the von Frey test in which filaments of varying thickness are pressed against the operated paw to determine changes in mechanical thresholds. NP rats received increasing i.p. doses of THC and CBD and compared against a control group receiving vehicle. In line with previous findings, THC and CBD decreased mechanical allodynia in a dose-dependent manner, through different mechanisms. The above results replicate and extend previously reported findings. Casey and colleagues showed that acute systemic administration of THC and CBD dose-dependently reduced mechanical and cold allodynia in the chronic constriction injury (CCI) model of neuropathic pain [76]. De Gregorio and colleagues demonstrated that repeated administration of low doses of CBD (5 mg/kg/day, for 7 days) was able to prevent mechanical allodynia in NP rats [69]. Due to the fact that cannabis is most often administered via inhalation or oral ingestion in humans [103], it is important to consider that the pharmacokinetic profile of inhaled/oral administrated cannabis compounds is different than an equivalent injected dose [104, 105]. Despite these different pharmacokinetic profiles, our results using injected cannabis compounds are consistent with human clinical trials using inhaled or orally administrated cannabis. For example, a clinical study found that dronabinol, the synthetic form of THC, at the dose of 10 mg/day (orally administered) led to a significant reduction in central pain in patients with multiple sclerosis [106]. Similarly, a five-day trial conducted on patients affected by sensory neuropathy due to HIV found that smoked cannabis (3.56% THC) reduced pain scores in 30% of patients compared to those who received

placebo [39, 107]. In our study, we determined that the 1.5, 2, 2.5 and 5 mg/kg doses of THC produced significant analgesic effects, which last up to 2.5 hours post-administration. Moreover, we found that 10 and 20 mg/kg doses of CBD were able to prevent mechanical allodynia, up to 4 hours post-administration. We also confirmed that CB1 receptors are required for the antiallodynic effects of THC, whereas 5-HT1A receptors only partially mediated the analgesic effects of CBD [69, 76]. Indeed, pre-treatment with the CB1 selective antagonist rimonabant totally prevented the analgesic effects of THC, while the 5-HT1A receptor antagonist WAY 100635 partially antagonized the antiallodynic effects of CBD. Importantly, the treatments with the sole selective antagonists (rimonabant and WAY) did not alter pain perception.

We also described the effects of intra-vlPAG microinjections of these two major phytocannabinoids on the activity of the descending antinociceptive pathway in anaesthetized NP rats. The CB1 receptor agonist THC, when microinjected into the vlPAG, inhibited the spontaneous and toe pinch–evoked activity of ON cells, whereas it enhanced the ongoing activity of OFF cells and almost nullified the pause duration. Conversely, CBD not only inhibited spontaneous and toe pinch–evoked activity of ON cells, but also inhibited OFF cells ongoing activity and increased the pause duration, confirming the findings reported by Maione and colleagues [70]. Due to the complexity, and not fully-elucidated mechanism of action of CBD, it is complicated to explain its paradoxical effect on the OFF cells. Maione and colleagues, indeed, demonstrated that numerous antagonists blocked the CBD-mediated inhibition of both ON and OFF cells: the CB1 receptor selective antagonist AM251, the selective adenosine A1 receptor antagonist DPCPX, and the 5-HT1A selective antagonist WAY 100635. Maione hypothesized that the sole inhibition of ON cell activity could be sufficient to deliver an anti-nociceptive effect in specific pathological condition, even with the parallel inhibition of OFF cell activity [70].

Experimental evidence supports this hypothesis. In fact, the selective ablation of RVM ON cells prevents the increase in sensitivity to non-noxious mechanical or noxious thermal stimuli caused by nerve injury [85, 108].

Since the maximal antiallodynic effect was reached by 5 mg/kg of THC and 20 mg/kg, these doses were used for exploring the effect of THC and CBD in comorbid insomnia. The EEG/EMG data analysis in freely moving rats, as expected, revealed that neuropathic rats is associated with sleep fragmentation. Indeed, NP rats show an increase in the total time of wakefulness (+77%, $p < 0.001$) and a decrease in total REM sleep (-67 %, $p < 0.01$) and NREM sleep (-54%, $p < 0.001$) compared with non-NP animals. For the first time, we demonstrated that significant benefits in sleep duration were obtained with acute administration of THC (5 mg/kg) and CBD (20 mg/kg) in a chronic pain paradigm in rodents. NP animals treated with THC showed improvements in NREM (+55%, $p>0.01$) and REM (+66%, $p < 0.001$) sleep when compared with NP animals treated with THC vehicle. In addition, NP animals treated with CBD showed improvements in NREM sleep (+55.57%, $p<0.05$) and REM sleep (+226%, $p<0.05$) when compared to NP animals treated with CBD vehicle. These results corroborate findings from previous clinical trials, in which cannabis-based medicine caused noticeable improvement in sleep parameters in patients with a wide variety of pain conditions [39]. The hypnotic effect of delta-9-tetrahydrocannabinol is mediated by the activation of the CB1 receptor. We demonstrated that administration of rimonabant (CB1 selective antagonist) blocks THC hypnotic effect also in a NP paradigm. We also observed that rimonabant impaired REM sleep in NP animals by reducing the total mean duration and the number of REM events ($p < 0.01$), confirming observations in healthy rats [50].

The localization of the CB1 receptor in sleep-inducing brain areas might explain a role for this receptor in the sleep-wake cycle [109]. In fact, the CB1 receptor is localized in the basal-forebrain,

specifically on cholinergic neurons, which are involved in the promotion of high-frequency oscillations typical of wakefulness and REM sleep [16, 56]. The CB1 receptor is also expressed in the brainstem, an area in which the cholinergic system plays a key role in REM sleep control [16, 56]. Therefore, stimulation or inhibition of CB1 in these areas may explain the THC sleep-promoting proprieties and Rimonabant-induced REM sleep suppression.

Conversely, CBD hypnotic properties are mediated by the 5-HT1A receptors, and we provided experimental evidences that the pre-treatment with the 5-HT1A antagonist WAY 100635 antagonized these effects. In NP rats, the sole administration of WAY increased the total mean duration of wakefulness and reduced the number of awakenings. Furthermore, WAY alone reduced NREM sleep total mean duration and the number of events, confirming the paramount importance of 5-HT1A receptors and serotonin in the sleep-wake cycle [16, 72].

4.2. Limitations of the study and future directions

As previously stated, cannabinoids elicit a wide range of effects, including several depressant proprieties, sedation, and specifically in rodent models locomotor impairments, catalepsy, antinociception, and hypothermia, which are known as the tetrad. Hence, a dose-response analysis of THC and CBD side effects is fundamental to provide further insight into the efficacy and therapeutic window of these phytocannabinoids [76]. However, Casey and colleagues demonstrated that THC dose-dependently induces sedation, catalepsy and motor impairment. Conversely, these side effects were not observed after systemic administration of increasing doses of CBD [76]. Beside central side effects, the clinical usage of cannabinoids is limited, partly because of the development of tolerance after long term or frequent exposure [110]. Future studies should explore if and when THC and CBD tolerance is achieved after chronic administration in

NP animals. The resulting data could be used to provide further insights into the psychopharmacology of THC and CBD analgesic and hypnotic proprieties.

Since CBD antiallodynic effect is likely mediated by TRPV1 receptors [69], it would be interesting to determine if the administration of a selective antagonist also blocks the hypnotic effect elicited by CBD. Additionally, experimental evidences suggests that THC might act as direct agonists to the 5HT1A receptor [111]. Braida and colleagues demonstrated that THC exerted a dose–response anxiolytic effect in the open field test, which was prevented by WAY 100635 administration [111]. Hence, future experiments should address whether or not WAY 100635 attenuates the hypnotic and analgesic effects of THC. Likewise, since CBD indirectly activates CB1 receptor [70], it would be also essential to evaluate whether or not rimonabant attenuates its effects.

Additionally, due to the sex-related differences in the development and recovery from neuropathic pain [112], it would be fundamental to confirm that female rats with a NP condition also develop sleep perturbations, and to determine whether CBD (20 mg/kg) and THC (5 mg/kg) might reverse this condition.

Our results strongly suggest that THC and CBD might be effective in treating chronic pain and comorbid sleep disturbances. However, clinical trials are needed to confirm their hypnotic and analgesic proprieties in humans. Based on FDA conversion guidelines, the estimate human-equivalent doses of those presented in this study are 3.24 mg/kg for CBD, and 0.81 mg/kg for THC [113, 114], which land in their therapeutic window [115, 116]. Nevertheless, more studies are needed to determine an effective clinical dose of THC and CBD in humans.

Despite increasing knowledge, much remains to be learned about chronic pain and comorbid insomnia. Understanding how and where neurobiological pathways of chronic pain and insomnia overlap is critical to help elucidating the underlying pathophysiology of these comorbid disorders.

The combined use of electrophysiological, optogenetics, chemogenetic, pharmacological, and behavioral techniques might help answer these questions. A clearer grasp of events leading to the co-occurrence of these medical conditions could help to identify effective treatments with fewer side effects, which specifically target the underlying molecular dysfunctions leading to chronic pain and comorbid insomnia.

4.3. Conclusion

In conclusion, the analysis demonstrated the benefits of THC and CBD on pain and sleep quality in a rat model of NP. THC and CBD alone have analgesic effects and hypnotic proprieties in a NP paradigm, similar to clinical outcomes reported in humans. Noteworthy, the same doses are able to reverse the sleep fragmentation induced by NP. From a pharmacological point of view, THC and other cannabinoids might be considered in the future as an efficacious alternative to gabapentinoids for treating chronic pain and comorbid sleep disturbances.

References

1. Colloca, L., et al., *Neuropathic pain.* Nature reviews Disease primers, 2017. **3**(1): p. 1-19.
2. Jensen, T.S. and N.B. Finnerup, *Allodynia and hyperalgesia in neuropathic pain: clinical manifestations and mechanisms.* The Lancet Neurology, 2014. **13**(9): p. 924-935.
3. Chung, K.-F. and K.-C. Tso, *Relationship between insomnia and pain in major depressive disorder: A sleep diary and actigraphy study.* Sleep medicine, 2010. **11**(8): p. 752-758.
4. Finan, P.H. and M.T. Smith, *The comorbidity of insomnia, chronic pain, and depression: dopamine as a putative mechanism.* Sleep medicine reviews, 2013. **17**(3): p. 173-183.
5. Baron, R., *Mechanisms of disease: neuropathic pain—a clinical perspective.* Nature clinical practice Neurology, 2006. **2**(2): p. 95-106.
6. von Hehn, C.A., R. Baron, and C.J. Woolf, *Deconstructing the neuropathic pain phenotype to reveal neural mechanisms.* Neuron, 2012. **73**(4): p. 638-652.
7. Cruccu, G., et al., *EFNS guidelines on neuropathic pain assessment.* European journal of neurology, 2004. **11**(3): p. 153-162.
8. Tsuda, M., *New approach for investigating neuropathic allodynia by optogenetics.* Pain, 2019. **160**: p. S53-S58.
9. Devor, M., *Sodium channels and mechanisms of neuropathic pain.* The Journal of Pain, 2006. **7**(1): p. S3-S12.
10. Basbaum, A.I., et al., *Cellular and molecular mechanisms of pain.* Cell, 2009. **139**(2): p. 267-284.
11. Dubin, A.E. and A. Patapoutian, *Nociceptors: the sensors of the pain pathway.* The Journal of clinical investigation, 2010. **120**(11): p. 3760-3772.
12. Petitjean, H., et al., *Dorsal horn parvalbumin neurons are gate-keepers of touch-evoked pain after nerve injury.* Cell reports, 2015. **13**(6): p. 1246-1257.
13. Bair, M.J., et al., *Depression and pain comorbidity: a literature review.* Archives of internal medicine, 2003. **163**(20): p. 2433-2445.
14. Nicholson, B. and S. Verma, *Comorbidities in chronic neuropathic pain.* Pain medicine, 2004. **5**(suppl_1): p. S9-S27.
15. Argoff, C., *Mechanisms of pain transmission and pharmacologic management.* Current medical research and opinion, 2011. **27**(10): p. 2019-2031.
16. Brown, R.E., et al., *Control of sleep and wakefulness.* Physiological reviews, 2012.
17. Nutt, D.J., *Relationship of neurotransmitters to the symptoms of major depressive disorder.* The Journal of clinical psychiatry, 2008. **69**: p. 4-7.
18. Fuller, P.M., J.J. Gooley, and C.B. Saper, *Neurobiology of the sleep-wake cycle: sleep architecture, circadian regulation, and regulatory feedback.* Journal of biological rhythms, 2006. **21**(6): p. 482-493.
19. Ochoa-Sanchez, R., et al., *Promotion of non-rapid eye movement sleep and activation of reticular thalamic neurons by a novel MT2 melatonin receptor ligand.* Journal of Neuroscience, 2011. **31**(50): p. 18439-18452.
20. Christie, M.A., et al., *Microdialysis elevation of adenosine in the basal forebrain produces vigilance impairments in the rat psychomotor vigilance task.* Sleep, 2008. **31**(10): p. 1393-1398.
21. Dunwiddie, T.V. and T. Worth, *Sedative and anticonvulsant effects of adenosine analogs in mouse and rat.* Journal of Pharmacology and Experimental Therapeutics, 1982. **220**(1): p. 70-76.

22. Portas, C., et al., *Role of adenosine in behavioral state modulation: a microdialysis study in the freely moving cat.* Neuroscience, 1997. **79**(1): p. 225-235.
23. Blanco-Centurion, C. and P.J. Shiromani, *Adenosine and sleep homeostasis in the basal forebrain: Final comment*. 2006, Oxford University Press.
24. Kalinchuk, A.V., et al., *The time course of adenosine, nitric oxide (NO) and inducible NO synthase changes in the brain with sleep loss and their role in the non-rapid eye movement sleep homeostatic cascade.* Journal of neurochemistry, 2011. **116**(2): p. 260-272.
25. Porkka-Heiskanen, T., R. Strecker, and R. McCarley, *Brain site-specificity of extracellular adenosine concentration changes during sleep deprivation and spontaneous sleep: an in vivo microdialysis study.* Neuroscience, 2000. **99**(3): p. 507-517.
26. Chaput, J.-P., C. Dutil, and H. Sampasa-Kanyinga, *Sleeping hours: what is the ideal number and how does age impact this?* Nature and science of sleep, 2018. **10**: p. 421.
27. Smith, C. and L. Lapp, *Increases in number of REMS and REM density in humans following an intensive learning period.* Sleep, 1991. **14**(4): p. 325-330.
28. Zepelin, H. and A. Rechtschaffen, *Mammalian Sleep, Longevity, and Energy Metabolism; pp. 447–470.* Brain, behavior and evolution, 1974. **10**(6): p. 447-470.
29. Finan, P.H., B.R. Goodin, and M.T. Smith, *The association of sleep and pain: an update and a path forward.* The Journal of Pain, 2013. **14**(12): p. 1539-1552.
30. Gore, M., et al., *Pain severity in diabetic peripheral neuropathy is associated with patient functioning, symptom levels of anxiety and depression, and sleep.* Journal of pain and symptom management, 2005. **30**(4): p. 374-385.
31. Hester, J. and N.K. Tang, *Insomnia co-occurring with chronic pain: clinical features, interaction, assessments and possible interventions.* Reviews in pain, 2008. **2**(1): p. 2-7.
32. Afolalu, E.F., F. Ramlee, and N.K. Tang, *Effects of sleep changes on pain-related health outcomes in the general population: a systematic review of longitudinal studies with exploratory meta-analysis.* Sleep medicine reviews, 2018. **39**: p. 82-97.
33. Besedovsky, L., T. Lange, and M. Haack, *The sleep-immune crosstalk in health and disease.* Physiological reviews, 2019. **99**(3): p. 1325-1380.
34. Ferini-Strambi, L., *Neuropathic pain and sleep: a review.* Pain and therapy, 2017. **6**(1): p. 19-23.
35. Medic, G., M. Wille, and M.E. Hemels, *Short-and long-term health consequences of sleep disruption.* Nature and science of sleep, 2017. **9**: p. 151.
36. Attal, N., et al., *EFNS guidelines on the pharmacological treatment of neuropathic pain: 2010 revision.* European journal of neurology, 2010. **17**(9): p. 1113-e88.
37. Woo, A. and G. Ratnayake, *Sleep and pain management: a review.* Pain Management, 2020(0).
38. Kapustin, D., et al., *Evaluating the impact of gabapentinoids on sleep health in patients with chronic neuropathic pain: a systematic review and meta-analysis.* Pain, 2020. **161**(3): p. 476-490.
39. Russo, E.B., G.W. Guy, and P.J. Robson, *Cannabis, pain, and sleep: lessons from therapeutic clinical trials of Sativex®, a cannabis-based medicine.* Chemistry & biodiversity, 2007. **4**(8): p. 1729-1743.
40. Cristino, L., T. Bisogno, and V. Di Marzo, *Cannabinoids and the expanded endocannabinoid system in neurological disorders.* Nature Reviews Neurology, 2019: p. 1-21.

41. Matsuda, L.A., et al., *Structure of a cannabinoid receptor and functional expression of the cloned cDNA.* Nature, 1990. **346**(6284): p. 561-564.
42. Munro, S., K.L. Thomas, and M. Abu-Shaar, *Molecular characterization of a peripheral receptor for cannabinoids.* Nature, 1993. **365**(6441): p. 61-65.
43. Di Marzo, V., *New approaches and challenges to targeting the endocannabinoid system.* Nature Reviews Drug Discovery, 2018. **17**(9): p. 623-639.
44. Di Marzo, V., M. Bifulco, and L. De Petrocellis, *The endocannabinoid system and its therapeutic exploitation.* Nature reviews Drug discovery, 2004. **3**(9): p. 771-784.
45. Pacher, P., S. Bátkai, and G. Kunos, *The endocannabinoid system as an emerging target of pharmacotherapy.* Pharmacological reviews, 2006. **58**(3): p. 389-462.
46. Howlett, A., et al., *International Union of Pharmacology. XXVII. Classification of cannabinoid receptors.* Pharmacological reviews, 2002. **54**(2): p. 161-202.
47. Pertwee, R.G., *Cannabinoid receptor ligands: clinical and neuropharmacological considerations, relevant to future drug discovery and development.* Expert opinion on investigational drugs, 2000. **9**(7): p. 1553-1571.
48. Piomelli, D., et al., *The endocannabinoid system as a target for therapeutic drugs.* Trends in pharmacological sciences, 2000. **21**(6): p. 218-224.
49. Martnez-Vargas, M., et al., *Sleep modulates cannabinoid receptor 1 expression in the pons of rats.* Neuroscience, 2003. **117**(1): p. 197-201.
50. Santucci, V., et al., *Arousal-enhancing properties of the CB1 cannabinoid receptor antagonist SR 141716A in rats as assessed by electroencephalographic spectral and sleep-waking cycle analysis.* Life sciences, 1996. **58**(6): p. PL103-PL110.
51. Juan-Picó, P., et al., *Cannabinoid receptors regulate Ca2+ signals and insulin secretion in pancreatic β-cell.* Cell calcium, 2006. **39**(2): p. 155-162.
52. Julien, B., et al., *Antifibrogenic role of the cannabinoid receptor CB2 in the liver.* Gastroenterology, 2005. **128**(3): p. 742-755.
53. Gonsiorek, W., et al., *Endocannabinoid 2-arachidonyl glycerol is a full agonist through human type 2 cannabinoid receptor: antagonism by anandamide.* Molecular pharmacology, 2000. **57**(5): p. 1045-1050.
54. Pertwee, R., *The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: Δ9-tetrahydrocannabinol, cannabidiol and Δ9-tetrahydrocannabivarin.* British journal of pharmacology, 2008. **153**(2): p. 199-215.
55. Murillo-Rodriguez, E., et al., *Anandamide enhances extracellular levels of adenosine and induces sleep: an in vivo microdialysis study.* Sleep, 2003. **26**(8): p. 943-947.
56. Murillo-Rodríguez, E., et al., *Anandamide-induced sleep is blocked by SR141716A, a CB1 receptor antagonist and by U73122, a phospholipase C inhibitor.* Neuroreport, 2001. **12**(10): p. 2131-2136.
57. Murillo-Rodrıguez, E., et al., *Anandamide modulates sleep and memory in rats.* Brain research, 1998. **812**(1-2): p. 270-274.
58. *Neuropathic pain.* Nature Reviews Disease Primers, 2017. **3**(1): p. 17003.
59. Fox, A. and S. Bevan, *Therapeutic potential of cannabinoid receptor agonists as analgesic agents.* Expert opinion on investigational drugs, 2005. **14**(6): p. 695-703.
60. Guindon, J. and P. Beaulieu, *Antihyperalgesic effects of local injections of anandamide, ibuprofen, rofecoxib and their combinations in a model of neuropathic pain.* Neuropharmacology, 2006. **50**(7): p. 814-823.

61. Guindon, J., A. De Léan, and P. Beaulieu, *Local interactions between anandamide, an endocannabinoid, and ibuprofen, a nonsteroidal anti-inflammatory drug, in acute and inflammatory pain.* Pain, 2006. **121**(1-2): p. 85-93.
62. Richardson, J.D., L. Aanonsen, and K.M. Hargreaves, *Antihyperalgesic effects of spinal cannabinoids.* European journal of pharmacology, 1998. **345**(2): p. 145-153.
63. Richardson, J.D., S. Kilo, and K.M. Hargreaves, *Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral CB1 receptors.* Pain, 1998. **75**(1): p. 111-119.
64. Tsou, K., et al., *Suppression of noxious stimulus-evoked expression of Fos protein-like immunoreactivity in rat spinal cord by a selective cannabinoid agonist.* Neuroscience, 1996. **70**(3): p. 791-798.
65. Hohmann, A.G., et al., *An endocannabinoid mechanism for stress-induced analgesia.* Nature, 2005. **435**(7045): p. 1108-1112.
66. Gaoni, Y. and R. Mechoulam, *Isolation, structure, and partial synthesis of an active constituent of hashish.* Journal of the American chemical society, 1964. **86**(8): p. 1646-1647.
67. Mechoulam, R. and Y. Gaoni, *A total synthesis of dl-Δ1-tetrahydrocannabinol, the active constituent of hashish1*. Journal of the American Chemical Society, 1965. **87**(14): p. 3273-3275.
68. Showalter, V.M., et al., *Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB2): identification of cannabinoid receptor subtype selective ligands.* Journal of Pharmacology and Experimental Therapeutics, 1996. **278**(3): p. 989-999.
69. De Gregorio, D., et al., *Cannabidiol modulates serotonergic transmission and reverses both allodynia and anxiety-like behavior in a model of neuropathic pain.* Pain, 2019. **160**(1): p. 136.
70. Maione, S., et al., *Non-psychoactive cannabinoids modulate the descending pathway of antinociception in anaesthetized rats through several mechanisms of action.* British journal of pharmacology, 2011. **162**(3): p. 584-596.
71. Monti, J.M., *Hypnoticlike effects of cannabidiol in the rat.* Psychopharmacology, 1977.
72. Monti, J.M., et al., *Depletion of brain serotonin by 5, 7-DHT: effects on the 8-OH-DPAT-induced changes of sleep and waking in the rat.* Psychopharmacology, 1994. **115**(1-2): p. 273-277.
73. Portas, C.M., et al., *Microdialysis perfusion of 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) in the dorsal raphe nucleus decreases serotonin release and increases rapid eye movement sleep in the freely moving cat.* Journal of Neuroscience, 1996. **16**(8): p. 2820-2828.
74. Donvito, G., et al., *The endogenous cannabinoid system: a budding source of targets for treating inflammatory and neuropathic pain.* Neuropsychopharmacology, 2018. **43**(1): p. 52-79.
75. Bolognini, D., et al., *The plant cannabinoid Δ9-tetrahydrocannabivarin can decrease signs of inflammation and inflammatory pain in mice.* British journal of pharmacology, 2010. **160**(3): p. 677-687.
76. Casey, S.L., N. Atwal, and C.W. Vaughan, *Cannabis constituent synergy in a mouse neuropathic pain model.* Pain, 2017. **158**(12): p. 2452-2460.

77. Costa, B., et al., *The non-psychoactive cannabis constituent cannabidiol is an orally effective therapeutic agent in rat chronic inflammatory and neuropathic pain.* European journal of pharmacology, 2007. **556**(1-3): p. 75-83.
78. Harris, H.M., et al., *Effects of delta-9-tetrahydrocannabinol and cannabidiol on cisplatin-induced neuropathy in mice.* Planta medica, 2016. **82**(13): p. 1169-1172.
79. King, K.M., et al., *Single and combined effects of Δ9-tetrahydrocannabinol and cannabidiol in a mouse model of chemotherapy-induced neuropathic pain.* British journal of pharmacology, 2017. **174**(17): p. 2832-2841.
80. Svendsen, K.B., T.S. Jensen, and F.W. Bach, *Does the cannabinoid dronabinol reduce central pain in multiple sclerosis? Randomised double blind placebo controlled crossover trial.* Bmj, 2004. **329**(7460): p. 253.
81. Wade, D.T., et al., *A preliminary controlled study to determine whether whole-plant cannabis extracts can improve intractable neurogenic symptoms.* Clinical rehabilitation, 2003. **17**(1): p. 21-29.
82. Meng, I.D., et al., *An analgesia circuit activated by cannabinoids.* Nature, 1998. **395**(6700): p. 381-383.
83. Richardson, J.D., *Cannabinoids modulate pain by multiple mechanisms of action.* The Journal of Pain, 2000. **1**(1): p. 2-14.
84. Carlson, J.D., et al., *Sensitization of pain-modulating neurons in the rostral ventromedial medulla after peripheral nerve injury.* Journal of Neuroscience, 2007. **27**(48): p. 13222-13231.
85. Fields, H., *State-dependent opioid control of pain.* Nature Reviews Neuroscience, 2004. **5**(7): p. 565-575.
86. Ossipov, M.H., G.O. Dussor, and F. Porreca, *Central modulation of pain.* The Journal of clinical investigation, 2010. **120**(11): p. 3779-3787.
87. Chagas, M.H.N., et al., *Effects of acute systemic administration of cannabidiol on sleep-wake cycle in rats.* Journal of Psychopharmacology, 2013. **27**(3): p. 312-316.
88. Murillo-Rodríguez, E., et al., *Cannabidiol, a constituent of Cannabis sativa, modulates sleep in rats.* FEBS letters, 2006. **580**(18): p. 4337-4345.
89. Feinberg, I., et al., *Effeets of marijuana extract and tetrahydrocannabinol on electroencephalographic sleep patterns.* Clinical Pharmacology & Therapeutics, 1976. **19**(6): p. 782-794.
90. Mechoulam, R., et al., *Cannabidiol–recent advances.* Chemistry & biodiversity, 2007. **4**(8): p. 1678-1692.
91. Nicholson, A.N., et al., *Effect of Δ-9-tetrahydrocannabinol and cannabidiol on nocturnal sleep and early-morning behavior in young adults.* Journal of clinical psychopharmacology, 2004. **24**(3): p. 305-313.
92. Kuhathasan, N., et al., *The use of cannabinoids for sleep: A critical review on clinical trials.* Experimental and clinical psychopharmacology, 2019.
93. Maione, S., et al., *Elevation of endocannabinoid levels in the ventrolateral periaqueductal grey through inhibition of fatty acid amide hydrolase affects descending nociceptive pathways via both cannabinoid receptor type 1 and transient receptor potential vanilloid type-1 receptors.* Journal of Pharmacology and Experimental Therapeutics, 2006. **316**(3): p. 969-982.
94. Pazos, A. and J. Palacios, *Quantitative autoradiographic mapping of serotonin receptors in the rat brain. I. Serotonin-1 receptors.* Brain research, 1985. **346**(2): p. 205-230.

95. Wilson-Poe, A., et al., *Distribution of CB1 cannabinoid receptors and their relationship with mu-opioid receptors in the rat periaqueductal gray.* Neuroscience, 2012. **213**: p. 191-200.
96. Decosterd, I. and C.J. Woolf, *Spared nerve injury: an animal model of persistent peripheral neuropathic pain.* Pain, 2000. **87**(2): p. 149-158.
97. Lopez-Canul, M., et al., *Selective melatonin MT2 receptor ligands relieve neuropathic pain through modulation of brainstem descending antinociceptive pathways.* Pain, 2015. **156**(2): p. 305-317.
98. Chaplan, S.R., et al., *Quantitative assessment of tactile allodynia in the rat paw.* Journal of neuroscience methods, 1994. **53**(1): p. 55-63.
99. Dixon, W.J., *Efficient analysis of experimental observations.* Annual review of pharmacology and toxicology, 1980. **20**(1): p. 441-462.
100. Paxinos, G. and C. Watson, *The rat brain in stereotaxic coordinates: hard cover edition*. 2006: Elsevier.
101. Urbain, N., K. Creamer, and G. Debonnel, *Electrophysiological diversity of the dorsal raphe cells across the sleep–wake cycle of the rat.* The Journal of physiology, 2006. **573**(3): p. 679-695.
102. Jha, S.K., et al., *Sleep-dependent plasticity requires cortical activity.* Journal of Neuroscience, 2005. **25**(40): p. 9266-9274.
103. Newmeyer, M.N., et al., *Free and glucuronide whole blood cannabinoids' pharmacokinetics after controlled smoked, vaporized, and oral cannabis administration in frequent and occasional cannabis users: identification of recent cannabis intake.* Clinical chemistry, 2016. **62**(12): p. 1579-1592.
104. Huestis, M., *Pharmacokinetics of THC in inhaled and oral preparations*, in *Marihuana and medicine*. 1999, Springer. p. 105-116.
105. Ruiz, C.M., et al., *Pharmacokinetic, behavioral, and brain activity effects of Δ 9-tetrahydrocannabinol in adolescent male and female rats.* Neuropsychopharmacology, 2020: p. 1-12.
106. Lynch, M.E. and F. Campbell, *Cannabinoids for treatment of chronic non-cancer pain; a systematic review of randomized trials.* British journal of clinical pharmacology, 2011. **72**(5): p. 735-744.
107. Abrams, D.I., et al., *Cannabis in painful HIV-associated sensory neuropathy: a randomized placebo-controlled trial.* Neurology, 2007. **68**(7): p. 515-521.
108. Porreca, F., et al., *Inhibition of neuropathic pain by selective ablation of brainstem medullary cells expressing the μ-opioid receptor.* Journal of Neuroscience, 2001. **21**(14): p. 5281-5288.
109. Murillo-Rodríguez, E., *The role of the CB1 receptor in the regulation of sleep.* Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2008. **32**(6): p. 1420-1427.
110. Tappe-Theodor, A., et al., *A molecular basis of analgesic tolerance to cannabinoids.* Journal of Neuroscience, 2007. **27**(15): p. 4165-4177.
111. Braida, D., et al., *5-HT1A receptors are involved in the anxiolytic effect of Δ9-tetrahydrocannabinol and AM 404, the anandamide transport inhibitor, in Sprague–Dawley rats.* European journal of pharmacology, 2007. **555**(2-3): p. 156-163.
112. Vacca, V., et al., *Higher pain perception and lack of recovery from neuropathic pain in females: a behavioural, immunohistochemical, and proteomic investigation on sex-related differences in mice.* PAIN®, 2014. **155**(2): p. 388-402.

113. Food and D. Administration, *Estimating the safe starting dose in clinical trials for therapeutics in adult healthy volunteers.* Rockville, Maryland, USA: US: Food and Drug Administration, 2005.
114. Reagan-Shaw, S., M. Nihal, and N. Ahmad, *Dose translation from animal to human studies revisited.* The FASEB journal, 2008. **22**(3): p. 659-661.
115. Devinsky, O., et al., *Cannabidiol in patients with treatment-resistant epilepsy: an open-label interventional trial.* The Lancet Neurology, 2016. **15**(3): p. 270-278.
116. Whiting, P.F., et al., *Cannabinoids for medical use: a systematic review and meta-analysis.* Jama, 2015. **313**(24): p. 2456-2473.