Anti-allodynic effects of delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) in neuropathic pain with tolerance to morphine

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Abstract

Neuropathic pain (NP) is a persistent pain disorder caused by damage to the nervous system. Current recommended treatments do not always provide sufficient pain relief, so patients are often prescribed opioids, which produce tolerance and dependence. There is therefore a need to identify novel pharmaceutical approaches to the treatment of NP in subjects with tolerance to morphine. We explored the hypothesis that cannabinoids delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) may be effective alternative analgesics to treat NP in morphine-tolerant subjects. Male Wistar rats underwent spared nerve injury and von Frey filaments were used to assess mechanical allodynia, a common symptom of NP. Neuropathic rats were treated with morphine (5 mg/kg) or vehicle twice daily for 7 days. Morphine-naïve and morphine-tolerant rats were then treated with THC (2.5 mg/kg) or CBD (20 mg/kg). THC significantly reduced mechanical allodynia in morphine-naïve and morphine-tolerant rats with NP. Conversely, CBD had no effect on mechanical allodynia in morphine-naïve or morphine-tolerant rats, even at an increased dose (50 mg/kg). In vivo electrophysiological recording of ON and OFF cells in the rostroventral medulla were recorded after microinjection of THC (10 μg) or CBD (1 μg) into the periaqueductal gray of morphine-naïve and morphine-tolerant rats to evaluate the role of descending pain modulation in these effects. Both THC and CBD reduced the spontaneous firing rate and toe-pinch evoked burst rate of pronociceptive ON cells. However, neither cannabinoid had any effect on the firing rate nor pause rate of antinociceptive OFF cells. Overall, these data suggest that THC may be an effective analgesic to manage NP in morphine-tolerant subjects. Additionally, they indicate that CBD, while ineffective at treating mechanical allodynia acutely, may play a more complex role in pain management.

Résumé

La douleur neuropathique (DN) est un trouble neurologique chronique causé par des lésions du système nerveux. Les traitements actuels visent à soulager les symptômes avec plus ou moins de succès, les patients sont donc orientés vers des traitements aux opioïdes qui sont responsables de phénomènes de dépendance et d'accoutumance. Il existe donc un besoin d'identifier de nouvelles approches pharmacologiques pour le traitement de la DN chez les sujets tolérants à la morphine. Nous avons formulé l'hypothèse selon laquelle les dérivés cannabinoïdes delta-9-tétrahydrocannabinol (THC) et cannabidiol (CBD) pourraient être des analgésiques alternatifs efficaces pour traiter la DN chez les sujets tolérants à la morphine. Le test des filaments de von Frey, développé pour évaluer l'allodynie mécanique qui est un symptôme courant de la DN a été utilisé chez des rats mâles Wistar présentant des lésions nerveuses. Les rats développant des symptômes neuropathiques ont été traités avec de la morphine (5 mg/kg) ou un véhicule deux fois par jour pendant 7 jours. Les rats naïfs et tolérants à la morphine ont ensuite été traités avec du THC (2,5 mg/kg) ou du CBD (20 mg/kg). Le THC réduit significativement l'allodynie mécanique chez les rats naïfs et tolérants à la morphine atteints de DN contrairement au CBD y compris à des concentrations accrues de (50 mg/kg). L'enregistrement électrophysiologique des cellules ON et OFF dans la moelle rostroventrale in vivo a été effectuée après micro-injection de THC (10 μg) ou de CBD (1 μg) dans la région périaqueducal grise de rats naïfs et tolérants à la morphine pour évaluer le rôle descendant de la douleur. Le THC et le CBD ont réduit le taux de déclenchement de potentiel d'actions spontanés et le taux de rafale bouffée des cellules ON nociceptives évoqué après pincement des orteils. Cependant, les cannabinoïdes n'ont pas eu d'effet sur le taux de décharge ni sur le taux de pause des cellules OFF anti-nociceptives. Dans l'ensemble, ces données

suggèrent que le THC peut être un analgésique efficace pour gérer la DN chez les sujets tolérants à la morphine. De plus, ils indiquent que le CBD, bien qu'inefficace pour traiter l'allodynie mécanique de manière aiguë, peut jouer un rôle plus complexe dans la gestion de la douleur.

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Contribution of Authors

My supervisor, Dr. Gabriella Gobbi, oversaw all aspects of this project, including conceiving of experiments, experimental design, compliance with ethics protocols, funding, interpretation of results, and the preparation of this thesis. My co-supervisor, Dr. Benoit Gentil, provided advice on the direction of this project, edited this thesis, and translated the abstract into French. I planned and conducted all behavioural experiments involving CBD, tracking development of morphine tolerance, and electrophysiological recordings in morphine-tolerant rats of ON and OFF cells treated with CBD and OFF cells treated with THC. I also analyzed and interpreted all data, created all figures, and wrote this thesis. Alexandra Teggin conducted behavioural experiments involving THC and performed electrophysiological recordings of ON cells with THC. Antonio Farina trained me to perform spared nerve injury. Some electrophysiological experiments in morphine-naïve rats were conducted by Antonio Farina and presented in his own thesis (Farina, 2021) and re-analyzed by me to include in the control groups of this study. Dr. Luca Posa trained me in electrophysiology and assisted me in conducting electrophysiological experiments. Dr. Martha Lopez-Canul trained me to assess pain using von Frey filaments and provided assistance with troubleshooting throughout this project, advice on data analysis, and feedback about this thesis.

Abbreviations

ºC	Degrees Celsius
2-AG	2-arachidonoylglycerol
5-HT	Serotonin (5-hydroxytryptamine)
5-HT1A	Serotonin 1A receptor subtype
ACC	Anterior cingulate cortex
AEA	Arachindonoylethanolamide
ANOVA	Analysis of variance
AP	Antero-posterior
AUC	Area under the curve
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CB1	Cannabinoid receptor 1 subtype
CB2	Cannabinoid receptor 2 subtype
CBD	Cannabidiol
CCI	Chronic constriction injury
CNS	Central nervous system
DOR	Delta-opioid receptor
DRG	Dorsal root ganglia
DRN	Dorsal raphe nucleus
DV	Dorsoventral

EtOH	Ethanol
FAAH	Fatty acid amide hydrolase
g	Gram
GABA	Gamma aminobutyric acid
GIRK	G-protein-coupled inwardly rectifying potassium channel
GPCR	G-protein-coupled receptor
HIV	Human immunodeficiency virus
Hz	Hertz (spikes/second)
IASP	International association for the study of pain
i.p.	Intraperitoneal administration
K^+	Potassium
KOR	Kappa-opioid receptor
kg	Kilogram
LC	Locus coeruleus
M	Molar
MAPK	Mitogen-activated protein kinase
mg	Milligram
MGL	Monaoacylglycerol lipase
$mGlu_5$	Metabotropic glutamate receptor subtype 5
min	Minute
ML	Medial-lateral
MOR	Mu-opioid receptor

Mor	Morphine
mRNA	Messenger ribonucleic acid
MΩ	Mega ohms
NaCl	Sodium chloride
nM	Nanomolar
NP	Neuropathic pain
PAG	Periaqueductal gray
PEA	Palmitoylethanolamide
PEG	Polyethylene glycol
PWT	Paw withdrawal threshold
RM	Repeated measures
rpm	Revolutions per minute
RVM	Rostroventral medulla
s	Second
s.c	Subcutaneous administration
SNI	Spared nerve injury
SNRI	Serotonin norepinephrine reuptake inhibitor
t	Time
TCA	Tricyclic antidepressant
THC	Delta-9-tetrahydrocannabinol
TRPV1	Transient receptor potential vanilloid type 1
VEH	Vehicle

vl	Ventrolateral
µA	Microamp
µg	Microgram
µL	Microlitre
µm	Micrometer

Background

Neuropathic pain

Neuropathic pain (NP) is a persistent pain disorder resulting from damage to the nervous system; causes of NP include surgery, cancer, trauma, diabetes and viral infections such as HIV and shingles (Colloca et al., 2017). The prevalence of chronic neuropathic symptoms in the general population is estimated to range from 7-10% (Colloca et al., 2017; van Hecke, Austin, Khan, Smith, & Torrance, 2014). NP often manifests as a wide range of symptoms, including burning, stabbing, and tingling sensations, and hypersensitivity, including allodynia (pain evoked by normally innocuous stimuli) and hyperalgesia (increased pain evoked by normally painful stimuli) to thermal or mechanical stimuli. The condition often results in reduced productivity and comorbidities such as insomnia, depression, and anxiety, representing a major public health issue in today's society (McCarberg & Billington, 2006).

A plethora of mechanisms have been shown to contribute to the development and maintenance of NP at the peripheral, spinal, and supraspinal level. Briefly, primary afferent fibers transmit sensory signals from the periphery to the dorsal horn of the spinal cord, where they synapse with second- and third-order neurons that project to the brain. There are two types of primary afferents that typically transmit noxious signals (termed nociceptors): Aδ and C fibers (Basbaum, Bautista, Scherrer, & Julius, 2009). Under normal conditions, nociceptors only fire in response to noxious stimulation. However, under neuropathic conditions, a series of physiological changes lead to sensitization and spontaneous firing of these fibers. Local release of inflammatory cytokines and mediators, excitatory neuropeptides, and the insertion of ion channels all contribute

to this phenomenon (Cohen & Mao, 2014; Nickel, Seifert, Lanz, & Maihofner, 2012). Furthermore, following nerve injury, Aβ fibers (low-threshold mechanoreceptors that transmit innocuous touch information) are newly able to activate nociceptive pathways (Finnerup, Kuner, & Jensen, 2021; Latremoliere & Woolf, 2009). Second-order neurons in the spinal cord dorsal horn are also sensitized in NP states (termed central sensitization). Microglial activation, synaptic potentiation, and disinhibition in the spinal cord contribute to this increased transmission of pain signals from the periphery to the brain (Campbell & Meyer, 2006). Additionally, pain transmission at the level of the spinal cord is also influenced by alterations in descending inputs from the brain.

The PAG-RVM descending modulatory pathway

Studies have identified vast networks of brain regions that contribute to pain processing and modulation, including those involved in both sensory and emotional/motivational aspects of pain (Ossipov, Morimura, & Porreca, 2014). One of the most well-characterized endogenous systems for pain modulation involves a descending pathway from the brainstem to the spinal cord. Specifically, the periaqueductal gray area (PAG) receives inputs from higher brain centres and is capable of modulating nociceptive processing through reciprocal connections with the rostroventral medulla (RVM), which projects to the dorsal horn of the spinal cord to modulate ascending inputs from the periphery (Ossipov, Lai, Malan, & Porreca, 2000).

Three distinct types of cells in the RVM have been characterized based on their response to nociceptive simulation. ON cells, which increase their firing rate in response to nociceptive stimulation, facilitate pain transmission. OFF cells, on the other hand, decrease their firing rate in response to nociceptive stimulation and inhibit pain transmission. A third type of “neutral” cell

does not show any response to painful stimulus (Ossipov, Dussor, & Porreca, 2010). It has been suggested that changes in the activity of these cells may play a role in the pathophysiology of NP. Early studies found that microinjection of lidocaine into the PAG or RVM reduced mechanical allodynia, and it was proposed that descending facilitation through tonic activity of ON cells may be responsible for the development of NP (Pertovaara, Wei, & Hamalainen, 1996). However, more recent studies have shown mixed results regarding changes in ON and OFF cell activity in NP states. While one study demonstrated an increase and decrease in the spontaneous activity of ON and OFF cells, respectively, in a rodent model of diabetic neuropathy (Silva et al., 2013), others found lasting changes only in the spontaneous activity of OFF cells (Goncalves, Almeida, & Pertovaara, 2007) in surgical models of NP. On the other hand, Carlson, Maire, Martenson, and Heinricher (2007) found no changes in the basal firing rates of ON or OFF cells. Instead, they demonstrated that the threshold required to elicit an ON cell "burst" or OFF cell "pause" was lower, and the burst rate and pause duration were increased, in the nerve-injured paw. Overall, there is a consensus that ON and OFF cells display some form of sensitization in neuropathic states. In fact, enhanced descending facilitation following nerve damage has even been identified in human subjects with NP, who display increased functional connectivity between the PAG and RVM (Mills et al., 2018).

Current treatments for NP

Current treatments for NP include anticonvulsant and certain antidepressant medications (Mu, Weinberg, Moulin, & Clarke, 2017). Gabapentinoids, including gabapentin and pregabalin, are anticonvulsant medications that act presynaptically in the dorsal horn of the spinal cord to

reduce excitatory transmission of pain signals (Moulin, 2014). Tricyclic antidepressants (TCAs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) have also been shown to reduce NP in clinical studies, in part by increasing synaptically available serotonin and norepinephrine (Sindrup, Otto, Finnerup, & Jensen, 2005). Unfortunately, these first-line treatments do not always provide patients with sufficient pain relief. In fact, a 2017 Cochrane review revealed that only 32-38% of patients with diabetic or post-herpetic neuropathy experienced at least a 50% reduction in pain after taking gabapentin (Wiffen et al., 2017). Therefore, patients may be prescribed stronger, second-line analgesics to manage their pain, including Tramadol and opioid medications (Mu et al., 2017). Although these medications are considered effective analgesics, they are not recommended as a first approach to treatment due to their adverse side effects, risks, and complications from long-term use.

Opioids

Opioids are considered some of the most effective pain relievers available. Morphine, a popular opioid analgesic, was first derived from the opium poppy in the early 1800s, although the plant had been used to treat pain for thousands of years prior (Rosenblum, Marsch, Joseph, & Portenoy, 2008). Opioid medications act on three main G protein-coupled receptors (GPCRs) located throughout the body: μ-, κ-, and δ-receptors. Research has also identified endogenous peptides that bind to each of these receptors. Specifically, β-endorphin mainly acts at the mu-opioid receptor (MOR), Met- and Leu- enkephalins are the primary agonists of the delta-opioid receptor (DOR), and dynorphin A and B agonize the kappa-opioid receptor (KOR) (Pecina et al., 2019). Activation of these receptors by their ligands inhibits adenylyl cyclase, thus reducing

intracellular cyclic adenosine monophosphate (cAMP) levels, and modulates the activity of ion channels, including K^+ and Ca^{2+} channels. In neurons, the net result is cellular inhibition (James & Williams, 2020). Most relevant to the study of pain is the MOR, which is necessary for the antinociceptive actions of most opioid medications (Fields, 2004).

Morphine antinociception

Morphine, a MOR-agonist, exerts a profound analgesic effect by inhibiting pain transmission in the periphery, spinal cord, and brain through signaling at both presynaptic and postsynaptic nerve terminals (Fields, 2004). On presynaptic terminals, MORs activate voltage-gated K^+ channels to inhibit transmitter release (Fyfe, Cleary, Macey, Morgan, & Ingram, 2010). On postsynaptic terminals, MORs inhibit voltage-gated Ca^{2+} channels and activate G-protein-gated inwardly rectifying K^+ channels (GIRKs), causing hyperpolarization and thus blocking excitation of second-order neurons (Bagley, Chieng, Christie, & Connor, 2005; Fyfe et al., 2010; Stein, 2013; Vaughan, Connor, Bagley, & Christie, 2000). Importantly, MORs are strongly expressed throughout both ascending and descending pain pathways, including in the terminals of nociceptors, the spinal cord dorsal horn, the amygdala, and the PAG and RVM (Fields, 2004; Ossipov et al., 2004).

The PAG-RVM descending modulatory pathway is known to play an important role in the antinociceptive actions of morphine. Administration of MOR-agonists either systemically or directly into the PAG or RVM has an antinociceptive effect by decreasing ON cell activity, increasing OFF cell activity, and eliminating nociceptive stimulus-evoked burst/pause (Fields, 2004; Heinricher, Morgan, & Fields, 1992; Tortorici & Morgan, 2002). The mechanism through

which MOR agonists exert these effects has been a topic of great interest. In the PAG, MOR-agonists inhibit tonically active GABAergic neurons, thereby disinhibiting projections to the RVM. It is generally believed that this releases tonic inhibition of glutamatergic projections to OFF cells, thus increasing OFF cell activity and inhibiting pain transmission at the level of the spinal cord (Lueptow, Fakira, & Bobeck, 2018). However, some neurons in the PAG that project directly to the RVM have also been found to express MORs (Commons, Aicher, Kow, & Pfaff, 2000), and evidence suggests that PAG-RVM projections include both glutamatergic and GABAergic neurons (Morgan, Whittier, Hegarty, & Aicher, 2008), indicating that the circuitry involved in morphine antinociception may be more complex. In the RVM, Heinricher et al. (1992) demonstrated that ON cells are directly inhibited by morphine. OFF cells, on the other hand, are believed to be activated via disinhibition (presynaptic inhibition of GABAergic inputs) (Heinricher, Morgan, Tortorici, & Fields, 1994).

Problems with the use of opioids to treat NP

While it is widely recognized that opioids provide a robust analgesic response in the treatment of acute pain, there is significantly less evidence supporting their use in chronic conditions, with few high-quality studies following patients for more than 6 weeks of treatment (Chou et al., 2015). In fact, there are a number of problems that arise when using long-term opioid therapy to treat chronic pain conditions, from side effects including nausea, constipation, drowsiness, and dizziness (Mu et al., 2017), to severe risks such as dependence, withdrawal, and overdose. Taking opioid medication is even associated with increased disability and reduced physical functioning in NP patients (Bostick et al., 2015), which decreases their quality of life.

Yet, opioid prescriptions have still increased substantially over the years, and rates of abuse and overdose have increased along with them (Chou et al., 2015). In patients with chronic non-cancer pain, receiving prescription opioids increases their risk of developing an opioid use disorder (Edlund et al., 2014), and studies have reported aberrant drug behaviors in up to 80% of patients who were prescribed opioids for chronic pain (Sullivan & Howe, 2013). Furthermore, in Ontario, prescribing levels of some opioid analgesics have been correlated with overdose deaths (Fischer, Jones, Urbanoski, Skinner, & Rehm, 2014). This emphasizes the dangers of using opioids to treat NP. Additionally, patients who take opioids chronically may develop tolerance to their antinociceptive effects, experiencing reduced efficacy after repeated exposure.

Opioid tolerance and paradoxical pain

One of the biggest problems with the use of opioids to treat NP is that patients may develop tolerance to their anti-allodynic effects. Tolerance describes the phenomenon where the effect of a drug decreases after repeated exposure, provoking patients to take higher doses to achieve the desired effect. This is means that opioids are unlikely to produce lasting pain relief in chronic pain patients, and these patients may increase their risk of harm if they are led to use higher doses.

In animal studies, opioid tolerance is even evident in the descending modulatory pathway, where repeated morphine treatment leads to alterations in cellular activity. As mentioned previously, acute morphine administration into the PAG alters the baseline firing rate of ON and OFF cells and attenuates their response to nociceptive stimulation (Cheng, Fields, & Heinricher, 1986; Tortorici & Morgan, 2002). However, as tolerance to the effects of morphine develops with repeated administration, this tolerance is reflected by cellular changes in the RVM, where ON and

OFF cells no longer respond to morphine administration (Lane, Tortorici, & Morgan, 2004; Tortorici, Morgan, & Vanegas, 2001). Within this descending system, the development of tolerance is believed to be mainly mediated by the activity of MORs in the PAG (Lueptow et al., 2018). This is clear from experiments showing that blocking MORs in the PAG attenuates the development of tolerance to morphine, whereas blocking RVM output has no effect (Lane, Patel, & Morgan, 2005). Furthermore, antinociceptive tolerance to morphine occurs more readily from repeated microinjection into the PAG compared to the RVM (Morgan, Clayton, & Boyer-Quick, 2005).

Interestingly, it has even been suggested that chronic morphine-induced changes in the descending modulatory system may increase pain facilitation, precipitating the emergence of paradoxical opioid-induce allodynia and hyperalgesia and possibly contributing to behavioural tolerance. For example, administration of lidocaine into the RVM blocked opioid-induced pain and restored the antinociceptive effects of morphine following the development of tolerance (Vanderah et al., 2001). Additionally, one study reported an increase in the number of ON cells in the RVM following chronic morphine administration (Meng & Harasawa, 2007). This led some authors to speculate that opioid-induced abnormal pain was produced by spontaneous activity of ON cells, facilitating pain transmission at the level of the spinal cord (Ossipov et al., 2004). However, this theory was not supported by other studies that found no changes in the spontaneous firing rate of ON or OFF cells in morphine-tolerant rodents (Lane et al., 2004; Tortorici et al., 2001; Viisanen et al., 2020). Interestingly, Viisanen et al. (2020) recently demonstrated that tolerance to morphine increased noxious heat-evoked burst-rate in ON cells and pause duration in

OFF cells, although no changes were seen in noxious mechanical stimulus-evoked cellular responses.

Intracellularly, the mechanisms that underly the development of tolerance are complex and diverse. Briefly, after repeated exposure to their agonist, MORs are desensitized via uncoupling of the receptor from G-protein signaling (Allouche, Noble, & Marie, 2014; P. A. Smith, Selley, Sim-Selley, & Welch, 2007). One study conducted by Bagley et al. (2005) examined changes in MOR signaling in the mouse PAG following chronic morphine administration. They demonstrated a reduction in the ability of opioids to activate GIRK currents and inhibit calcium channel activity in mice that displayed antinociceptive tolerance to morphine. Studies have demonstrated an important role for β-arrestins in the process of receptor uncoupling and the associated development of tolerance. In fact, chronic morphine treatment does not induce tolerance or receptor uncoupling in the PAG of β-arrestin2-KO mice, compared to their wildtype littermates (Bohn, Gainetdinov, Lin, Lefkowitz, & Caron, 2000). Together, the above changes lead to a reduction of MOR-induced disinhibition in the PAG in morphine-tolerant animals (Lueptow et al., 2018).

Overall, there is a need to identify novel approaches to the treatment of chronic NP that avoid the use of strong opioids without compromising pain management. Clinicians and scientists have therefore begun exploring alternative medications, including cannabis.

Cannabinoids

The *Cannabis sativa* plant has been used for medicinal purposes for thousands of years. In fact, physicians in ancient China, Egypt, Greece, Rome, and the Middle East used the plant to treat a variety of ailments, including malaria, rheumatic pain, impotence, kidney stones, depression, and

anxiety (Mechoulam, 1986). In Canada, cannabis was not approved for medical use until 2000 (Canada, 2016). Now, it is widely used to ease the symptoms of various neurological disorders, psychiatric disorders, digestive disorders, and even cancer (Fraguas-Sanchez & Torres-Suarez, 2018). There has therefore been great interest over the years in identifying the components of the cannabis plant responsible for its therapeutic effects and characterizing the endogenous system that they interact with.

The endocannabinoid system includes two main receptors: CB1 and CB2 receptors. Both receptors are $G_{i/o}$-coupled receptors whose activation leads to a decrease in cAMP (via adenylyl cyclase suppression) and an increase in MAPK signalling (Jensen, Chen, Furnish, & Wallace, 2015). CB1 receptors are highly expressed on nerve terminals throughout the central nervous system (CNS), including in the cortex, cerebellum, basal ganglia, spinal cord, and PAG (Maayah, Takahara, Ferdaoussi, & Dyck, 2020; Milligan, Szabo-Pardi, & Burton, 2020; Vuckovic, Srebro, Vujovic, Vucetic, & Prostran, 2018). CB2 receptors, on the other hand, are located primarily on peripheral and immune cells (Vuckovic et al., 2018). The endogenous agonists of the cannabinoid receptors are arachindonoylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG). The enzymes responsible for their hydrolysis, fatty acid amide hydrolase (FAAH) and monaoacylglycerol lipase (MGL), respectively, have also drawn interest as potential therapeutic targets for their ability to modulate endocannabinoid levels (Maayah et al., 2020). Indeed, inhibition of either of these enzymes reduces allodynia in surgical models of NP (de Novellis et al., 2011; Schlosburg et al., 2010), supporting a role for this system in NP and analgesia.

The most abundant pharmacologically active phytochemical in the cannabis plant is delta-9-tetrahydrocannabinol (THC), followed by cannabidiol (CBD) (Jensen et al., 2015). Research

suggests that both cannabinoids are promising candidates for the treatment of NP. In fact, in 2014, the Canadian Pain Society moved cannabinoids from recommended fourth- to third-line analgesics due to increasing clinical evidence of their analgesic efficacy in HIV, diabetic, posttraumatic and postsurgical neuropathy (D. Moulin et al., 2014; D. E. Moulin et al., 2007). Importantly, although THC and CBD are derived from the same plant, their therapeutic effects and mechanisms of action differ.

Delta-9-tetrahydrocannabinol (THC)

THC for the treatment of NP

Preclinical studies have demonstrated that treatment with THC reduces pain in a variety of neuropathic conditions. Both acute and repeated treatment with THC have been shown to reduce mechanical allodynia in surgical models of NP (Abraham et al., 2019; Casey, Atwal, & Vaughan, 2017). The antinociceptive efficacy of THC has also been demonstrated in models of chemotherapy-induced (Harris, Sufka, Gul, & ElSohly, 2016; Henderson-Redmond et al., 2020; King et al., 2017) and diabetic (J. Williams, Haller, Stevens, & Welch, 2008) neuropathy. Furthermore, unpublished results from our lab found that THC at doses of 1.5, 2, 2.5, and 5 mg/kg significantly reduce mechanical allodynia in neuropathic rats in the spared nerve injury (SNI) model of NP (Farina, 2021).

THC antinociceptive mechanism of action

THC is a partial agonist of both CB1 and CB2 receptors, with higher affinity for CB1 (Vuckovic et al., 2018). Indeed, CB1 receptors appear to be necessary for THC's anti-allodynic

effects in rodent models of NP. For example, Casey et al. (2017) demonstrated that the reduction of mechanical allodynia by THC was blocked by the administration of a CB1-antagonist, a finding that was recently replicated by our lab (Farina, 2021). The antinociceptive effects of THC are also reduced or absent in $CB1^{-/-}$ mice (Ledent et al., 1999). As mentioned previously, CB1 receptors are located throughout the CNS, including in regions involved in pain transmission and modulation. In the periphery, CB1 receptors are expressed on terminals of nociceptors, and selective deletion of CB1 on these neurons was found to enhance pain and reduce cannabinoid antinociception in mice (Agarwal et al., 2007). At the spinal level, CB1 receptors are located in the dorsal horn and the dorsal root ganglia (DRG), where they also act to inhibit pain transmission. In the brain, CB1 activation can inhibit ascending pain transmission in the thalamus and limbic areas (Nadal, La Porta, Andreea Bura, & Maldonado, 2013). Agonists of CB1 may also act in the brainstem to influence descending pain control.

CB1 receptors are extensively expressed in the PAG (Wilson-Poe, Morgan, Aicher, & Hegarty, 2012), and microinjection of synthetic cannabinoids into the PAG also induces antinociception (Lichtman, Cook, & Martin, 1996; Martin, Patrick, Coffin, Tsou, & Walker, 1995). Palazzo et al. (2012) even demonstrated that intra-PAG administration of a synthetic cannabinoid, WIN55212-2, decreased and increased ON and OFF cell activity, respectively, and decreased tail-flick related burst and pause in neuropathic rats, prevented by administration of a CB1 antagonist. Furthermore, CB1 mRNA and protein have been identified in the RVM (M. H. Li, Suchland, & Ingram, 2017), and administration of synthetic cannabinoids either systemically or directly into the RVM reduces pain-evoked ON and OFF cell responses and associated pain behaviour in a CB1-dependent manner (Martin, Tsou, & Walker, 1998; Meng & Johansen, 2004; Meng,

Manning, Martin, & Fields, 1998). Intracerebroventricular administration of THC has also been found to produce antinociception (Lichtman et al., 1996), but its direct effects on the descending pain modulatory pathway have not been investigated. It is therefore possible that THC's antinociceptive effects occur through modulation of ON and OFF cell activity via CB1 agonism at the level of the PAG.

CB1-agonists inhibit synaptic transmission, allowing them to influence nociceptive signalling by modulating the release of various neurotransmitters (Maayah et al., 2020). In general, presynaptic CB1 activation can inhibit neurotransmission via activation of voltage-gated K^+ channels, and inhibition of Ca^{2+} channels and vesicle release (Bouchet & Ingram, 2020). In the PAG, CB1 activation has been found to inhibit both GABAergic and glutamatergic neurotransmission *ex vivo* (Vaughan et al., 2000), suggesting that it is capable of modulating output to the RVM. Interestingly, one study found that CB1 receptors in the PAG were mostly expressed on GABAergic rather than glutamatergic cells, and that the antinociceptive effects of intra-PAG cannabinoid administration required $mGlu_5$ receptor activation (Palazzo et al., 2012). This finding supports the hypothesis that, similarly to opioids, cannabinoids acting on CB1 receptors in the PAG inhibit GABAergic neurons, disinhibiting glutamatergic projections to the RVM. In the RVM, CB1-mediated antinociception is also dependent on $mGlu_5$ receptor activation (de Novellis et al., 2005), and patch-clamp recordings indicate that CB1 activation also inhibits GABAergic transmission in RVM slices (Vaughan, McGregor, & Christie, 1999).

Cannabidiol (CBD)

CBD for the treatment of NP

CBD has also been shown to reduce NP in rodent models, including surgical (Abraham et al., 2019; Comelli, Giagnoni, Bettoni, Colleoni, & Costa, 2008; Costa, Trovato, Comelli, Giagnoni, & Colleoni, 2007; De Gregorio et al., 2019; H. Li et al., 2018), chemotherapy-induced (King et al., 2017; Ward et al., 2014; Ward, Ramirez, Neelakantan, & Walker, 2011) and diabetic (Toth, Jedrzejewski, Ellis, & Frey, 2010) neuropathy, when administered repeatedly. However, conflicting results about the acute analgesic effect of CBD have been reported. Some studies have demonstrated an antinociceptive effect of acute CBD (Comelli et al., 2008; Jesus et al., 2019), while others have found that acute CBD is unable to relieve NP (Costa et al., 2007; Toth et al., 2010). Unpublished findings from our lab demonstrated that acute CBD at doses of 10 and 20 mg/kg significantly reduced mechanical allodynia in neuropathic rats in the SNI model of NP (Farina, 2021).

CBD antinociceptive mechanism of action

Unlike THC, CBD is believed to act as an *antagonist* of CB1 and CB2 receptors in the presence of THC, and is capable of negative allosteric modulation of CB1 (Vuckovic et al., 2018). CBD can also influence the endocannabinoid system by inhibiting FAAH, thereby increasing endocannabinoid concentrations by blocking their degradation. Outside of the endocannabinoid system, CBD interacts with a wide variety of molecular targets, including 5-HT-, adenosine-, dopamine-, and opioid-receptors, and calcium-, sodium-, and other ion channels (de Almeida & Devi, 2020).

Considering this range of possible targets, the exact mechanism of CBD's analgesic actions is not completely clear. A number of studies have shown that the antinociceptive effects of CBD

in rodent models of NP are not dependent on CB1 or CB2 receptors (Costa et al., 2007; Jesus et al., 2019; Ward et al., 2014). Instead, there are two receptors that have been the main focus of CBD's antinociceptive mechanism of action in NP: the 5-HT1A receptor and the transient receptor potential vanilloid 1 (TRPV1) receptor. 5-HT1A receptors are GPCRs that exert an inhibitory effect on neurons by opening and closing K^+ and Ca^{2+} channels, respectively. They are expressed on 5-HT neurons and act as negative feedback on further 5-HT release (Haleem, 2018). CBD is an agonist of 5-HT1A receptors with modest affinity (Russo, Burnett, Hall, & Parker, 2005), and CBD's anti-allodynic effects are prevented by administration of a 5-HT1A antagonist in rodents with diabetic (Jesus et al., 2019), surgically induced (De Gregorio et al., 2019), and chemotherapy-induced (Ward et al., 2014) neuropathy. Interestingly, while De Gregorio et al. (2019) found that a 5-HT1A antagonist partially blocked the anti-allodynic effect of CBD in neuropathic rats, a TRPV1 antagonist blocked it completely. TRPV1 is a ligand-gated non-selective cation channel activated by capsaicin, heat, low pH, and endovanilloids (Palazzo, Rossi, & Maione, 2008). *Ex vivo* studies have verified that CBD agonizes and rapidly desensitizes TRPV1 receptors (Bisogno et al., 2001; Iannotti et al., 2014), and Costa et al. (2007) further demonstrated that a TRPV1 antagonist prevented CBD's anti-hyperalgesic effect in neuropathic rats.

Both TRPV1 and 5-HT1A receptors have been implicated in descending pain modulatory circuitry. For example, a 5-HT1A agonist was found to decrease firing activity via hyperpolarization of neurons in the PAG *in vivo* and *in vitro* (Behbehani, Liu, Jiang, Pun, & Shipley, 1993). Furthermore, TRPV1 receptors are expressed in both the PAG and RVM (Palazzo et al., 2008), and microinjection of capsaicin into the vlPAG produces antinociception and decreases and increases the firing rate of ON and OFF cells, respectively (Maione et al., 2006;

Starowicz et al., 2007). Together, these findings indicate that CBD may exert an antinociceptive effect via modulation of the PAG-RVM descending circuitry through agonism of 5-HT1A and/or TRPV1 receptors. In fact, one study, conducted by Maione et al. (2011), evaluated the effects of intra-PAG CBD on pain and ON and OFF cell activity in healthy rats. They found that CBD decreased tail-flick latency and spontaneous activity of both ON and OFF cells. Furthermore, CBD reduced the stimulus induced ON cell burst, but had no effect on the OFF cell pause. Interestingly, these effects were inhibited by microinjection of a 5-HT1A or CB1 antagonist, but a TRPV1 antagonist only slightly prevented CBD's modulation of OFF cells and had no effect on ON cells. Clearly, more research is needed into the anti-allodynic effects and mechanism of action of CBD in the treatment of NP.

Interactions between the opioid and cannabinoid systems

Increasing evidence has suggested the potential use of cannabis as a substitute for opioids in the treatment of chronic pain conditions. In fact, chronic pain patients who are currently using opioids decrease their use by 40-60% when given access to cannabis, citing fewer side effects and increased quality of life as reasons for preferring cannabis (Wiese & Wilson-Poe, 2018). Cannabis has even been reported to enhance the analgesic effect of morphine in patients with chronic pain (Abrams, Couey, Shade, Kelly, & Benowitz, 2011). The apparent synergistic effects and overlapping mechanisms of these compounds have led authors to begin exploring cannabis as an alternative to opioids and a tool to combat the opioid crisis (Abrams et al., 2011; Lucas, 2017).

Morphine and THC interactions

In support of this, several studies have shown that THC and morphine have synergistic antinociceptive effects in animals (Cichewicz & McCarthy, 2003; Cox, Haller, & Welch, 2007; Reche, Fuentes, & Ruiz-Gayo, 1996). THC has also been suggested to attenuate the development of tolerance to morphine (Cichewicz & Welch, 2003; P. A. Smith et al., 2007) and decrease its rewarding properties (Jardinaud, Roques, & Noble, 2006). While chronic administration of either THC or morphine decreases the agonist-stimulated signaling of their respective receptors in the PAG, co-administration appears to circumvent this adaptation, avoiding the problem of tolerance (P. A. Smith et al., 2007). Roberts, Gennings, and Shih (2006) even demonstrated that THC and morphine have a synergistic affective analgesic effect in humans. This synergy may result from the overlap between the endogenous opioid and cannabinoid systems; MORs and CB1 receptors are both GPCRs that are co-localized on cells in the PAG (Wilson-Poe et al., 2012), where agonists of both these receptors inhibit GABAergic transmission (Wilson-Poe, Lau, & Vaughan, 2015).

While these overlapping mechanisms may contribute to acute synergy, it remains unclear whether THC maintains analgesic efficacy following the development of tolerance to morphine. In fact, cross-tolerance to THC has been demonstrated in morphine-tolerant mice (Thorat & Bhargava, 1994) and monkeys (Gerak, Zanettini, Koek, & France, 2015). However, studies demonstrating that MORs are not necessary for the antinociceptive effects of THC indicate that cannabinoid antinociception may actually be independent of morphine tolerance. For example, co-administration of a MOR antagonist does not block THC antinociception (Wakley & Craft, 2011), and THC relieves pain even in $MOR^{-/-}$ mice (Ghozland et al., 2002). In fact, some authors have suggested that morphine-tolerant mice may actually be *more* sensitive to THC antinociception

(Cichewicz & Welch, 2003; Rubino, Tizzoni, Vigano, Massi, & Parolaro, 1997). Still, no studies to date have evaluated the anti-allodynic potential of THC in morphine-tolerant subjects with NP.

Morphine and CBD interactions

Unlike THC, few studies have evaluated possible synergistic effects of CBD and morphine in the treatment of pain. One study demonstrated that pretreatment with CBD enhanced the antinociceptive efficacy of morphine in a thermal pain assay (Rodriguez-Munoz, Onetti, Cortes-Montero, Garzon, & Sanchez-Blazquez, 2018). However, Neelakantan et al. (2015) found that CBD actually reduced the efficacy of morphine in the hot plate test but acted synergistically with morphine to reduce acetic acid-stimulated stretching, suggesting that morphine-CBD interactions may vary depending on the type of pain evaluated. Interestingly, Kishimoto, Koyama, and Akaike (2001) found that MOR and 5-HT1A receptor agonists synergistically inhibit GABA release in the PAG. TPRV1 and MORs are also co-expressed on neurons in the PAG, and co-administration of TRPV1 and MOR agonists into the PAG stimulates antinociception and increases glutamate release into the RVM (Maione et al., 2009). In line with the view of 5-HT1A and TRPV1 agonism as a primary antinociceptive mechanism of CBD in NP, these findings provide a possible mechanism for morphine-CBD antinociceptive synergy. Still, CBD's potential ability to alleviate NP in subjects with tolerance to morphine has not been evaluated.

Hypothesis and Objectives

Anecdotal evidence suggests that cannabinoids may be promising alternative analgesics in the treatment of chronic pain conditions (Lynch & Clark, 2003; Reiman, Welty, & Solomon, 2017). Yet, the potential ability of THC and CBD to alleviate symptoms of NP in subjects with tolerance to morphine has not been directly studied. The main hypothesis of this project is therefore that THC and CBD may be used as alternative analgesics for the treatment of NP in rats that have developed tolerance to the anti-allodynic effects of morphine. Specific objectives include:

1. Determine whether THC and CBD reduce mechanical allodynia in morphine-naïve and morphine-tolerant rats with NP.
2. Evaluate the effects of intra-PAG THC and CBD on the activity of ON and OFF cells in the RVM of morphine-naïve and morphine-tolerant rats with NP.

Materials and Methods

Animals

Male Wistar rats (Charles Rivers, Quebec, Canada) weighing 130g at the beginning of experimental procedures were housed in groups of 2 or 3. All animals were housed in a standard animal facility (12-12-hour light-dark cycle, lights on at 7am), and provided with *ad libitum* access to food and water. All experimental protocols were performed during the light phase – between 7am and 7pm – and approved by the Animal Ethics Committee of McGill University (protocol #2009-5764), following the IASP ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983) and the Canadian Institutes of Health Research guidelines for animal care and experimental use. For all behavioural experiments, the experimenter was blind to drug treatment.

Drugs

Morphine (Sigma Aldrich, St Louis, MO; Cayman Chemical, Ann Arbor, MI; Health Canada Licence 49561.11.19AMDSTK) was dissolved in saline (0.9% NaCl) and injected s.c. at a dose of 5 mg/kg twice daily for 7 days. Cannabidiol (CBD) and gabapentin were purchased from Cayman Chemical (Ann Arbor, MI; Health Canada Licence LIC-UIWBIXH9RW-2019-1) and dissolved in a vehicle consisting of ethanol (EtOH), Tween80, and saline (0.9% NaCl) at a ratio of 3:1:16. CBD was administered i.p. at a dose of 20 or 50 mg/kg for behavioral experiments, and 1 μg intra-PAG (Maione et al., 2011) for electrophysiological experiments. Gabapentin was administered i.p. at a dose of 100 mg/kg. Delta-9-tetrahydrocannabinol (THC) was purchased from Tocris Bioscience (Ellisville, MO; Health Canada Licence LIC-UIWBIXH9RW-2019-1),

prepared in a vehicle consisting of polyethylene glycol (PEG) 400, Tween80, and saline (0.9% NaCl) at a ratio of 1:1:18, and administered i.p. at a dose of 2.5 mg/kg for behavioral experiments and 10 μg intra-PAG for electrophysiological experiments. The doses of THC and CBD chosen were based on dose-response curves previously obtained by our lab (Farina, 2021).

Spared Nerve Injury (SNI)

Rats weighing approximately 130g were used for spared nerve surgery according to the method described by Decosterd and Woolf (2000). Anesthesia was induced using oxygen gas with 5% isoflurane and maintained by 2-3% isoflurane gas once a full anesthetic state was established, confirmed by absence of a toe-pinch reflex. Ophthalmic ointment was administered before beginning the surgery. Hair from the right leg was removed using an electric shaver to expose the skin. An incision was made along the skin followed by blunt dissection of the biceps femoris muscle to reveal the sciatic nerve. The common peroneal and tibial branches of the nerve were ligated with 4.0mm Vicryl and then cut, leaving the sural nerve intact. The muscle was closed using 3.0mm Vicryl and EZclips were used to close the skin. Carpofen at a dose of 5 mg/kg was administered before the surgery and the following 2 days as post-operative care. Wound closures were removed 10 days post-surgery.

Assessment of mechanical allodynia

14 days after the surgery, animals weighing approximately 300g were tested for mechanical allodynia using von Frey filaments through the up-down method described by Chaplan, Bach, Pogrel, Chung, and Yaksh (1994). Briefly, rats were placed in individual plexiglass cubicles on a

wire mesh surface and allowed to habituate for 45 minutes, until still. First, a filament exerting a standard force of 4.31 grams was applied to the plantar surface of the right hind paw, ipsilateral to the nerve injury, for 10 seconds. If the stimulus elicited a paw withdrawal, a filament of lower force was applied next. If the filament did not elicit a withdrawal, a filament of higher force was applied, up to a maximum force of 15g. The 50% Paw Withdrawal Threshold (PWT) (the force that elicited a paw withdrawal 50% of the time) was calculated using the equation proposed by Dixon (1980). A cut-off of 4g of force was used to establish neuropathy in rats, where rats with a baseline threshold below this value were considered allodynic and designated "neuropathic" throughout this thesis. All experiments were performed on neuropathic animals. Animals with motor impairments were also excluded. Allodynic animals were randomly allocated between behavioral or electrophysiological experiments.

In-vivo electrophysiology

Surgical preparation:

Neuropathic rats were anesthetised with an i.p. injection of urethane at a dose of 1.2 g/kg. Full anesthetic state was confirmed by lack of response to toe pinch. Body temperature was maintained throughout the procedure using a heat pad. Rats were placed on a stereotaxic frame and an incision was made from behind the eyes to the base of the skull. Stainless steel clips were used to separate the skin, revealing the skull, and lidocaine was applied for local anesthesia. The coordinates of the RVM and vlPAG were identified using a rat brain atlas (Paxinos & Watson, 2006), in which the PAG (7.8mm antero-posterior (AP) and 0.5mm medial-lateral (ML)) and RVM (9.16-11.6mm AP and 1mm ML) were located according to the intersection of the midline and

bregma. A hole was drilled through the skull over the PAG, and a steel cannula was stereotaxically lowered to a depth of 4mm dorsoventrally (DV) below the dura and anchored, using dental cement, to a stainless-steel screw in the skull. A section of the skull and dura above the RVM was removed to prepare for insertion of a recording electrode.

Intra-PAG microinjections:

A stainless-steel cannula connected by a polyethylene tube to an SGE 1-microlitre syringe was inserted through the guide cannula into the PAG for administration of drugs. Vehicle, followed by THC (10 μg) or CBD (1 μg), were administered a volume of 1 μL each.

Extracellular recordings in the RVM:

A single-barreled glass micropipette was pulled on a Narishige (Tokyo, Japan) PE-21 glass microelectrode puller for recording and filled with a 2% Pontamine Sky Blue solution in 3 M NaCl. The tip was broken down to create an impedance between 2-4 MΩ. A hydraulic micro-positioner was used to lower the electrode into the RVM at approximately 2 μm/s. Recorded cells were located at a depth 7.5-9.5mm below the dura. ON cells were identified by a burst of activity in response to a toe pinch while OFF cells were identified by a decrease in activity. Once a cell was identified, recordings were carried out for 60 minutes. The baseline firing rate was recorded for 5 minutes, followed by injection of vehicle, then either THC or CBD 5 minutes later. A toe pinch was administered every 5 minutes for the first 10 minutes, and then every 15 minutes after drug administration. Only one cell was recorded from each rat. Firing rate was calculated as mean

spikes/s (Hz) over each 5-minute interval. For analysis, the data were converted to a percentage of the baseline firing rate.

After the recording was complete, Pontamine Sky Blue dye was injected iontophoretically by passing a constant positive current of 20 μA for 10 minutes through the recording pipette to mark the location of the recording, after which the rat was euthanized by decapitation and its brain removed and placed in a -80°C freezer for histological verification. A cryostat was used to slice 25 μm thick coronal sections, which were mounted on glass microscope slides to confirm the location of the recording site.

Statistical analysis

All statistical analyses were performed on GraphPad Prism (version 9.0.2), with the exception of Three-Way Analysis of Variance (ANOVA) and Levene's test, which were performed using JASP (version 0.14.1). In order to determine the appropriate test to run, data were tested for normality using Shapiro-Wilk's test or the Q-Q plot, and for homogeneity of variance using Levene's test. Data were tested for sphericity using Mauchly's test, and a Greenhouse Geisser correction was applied where appropriate. For all parametric analyses, post-hoc tests were performed using Bonferroni's post-hoc comparisons. All data are expressed as mean ± SEM. p values < 0.05 were considered significant.

The effect of repeated treatment with morphine on mechanical allodynia was analyzed using Two-Way repeated measures (RM) ANOVA with a Greenhouse Geisser correction, with drug and time as factors. Baseline firing rates of ON and OFF cells were reported as mean spikes/s over a 5-minute period before vehicle or drug injection. The difference in the baseline firing rate

between morphine-naïve and morphine-tolerant rats was compared using unpaired t-tests. The difference between the baseline burst or pause rate in morphine-naïve and morphine-tolerant rats was compared using Mann Whitney tests.

The effect of THC or CBD on mechanical allodynia in morphine-naïve vs. morphine-tolerant rats was compared using Three-Way RM ANOVA with a Greenhouse Geisser correction, with drug, time, and tolerance as factors. The area under the curve (AUC) was analyzed using Two-Way ANOVA with drug and tolerance as factors.

For electrophysiological analysis of the firing rate of ON and OFF cells over time, the average firing rate in spikes/s during each 5-minute interval from 0 to 45 or 50 minutes was converted to a percentage of the baseline firing rate. The effect of THC or CBD on the firing rate and burst rate of ON cells was analyzed using Two-Way RM ANOVA with a Greenhouse Geisser correction, with time and tolerance as factors. The effect of THC or CBD on the firing and pause rate of OFF cells was analyzed using mixed effects analysis, with time and tolerance as factors. The AUC of the firing rate over time was also compared between morphine-naïve and morphine-tolerant rats. In THC-treated animals, the AUC was analyzed using an unpaired t-test. The AUC of CBD in OFF cells was analyzed using Welch's t-test, and the AUC of CBD in ON cells was analyzed using a Mann Whitney test. For the analysis of ON cells, the firing rate following vehicle injection (0 minutes) was also directly compared to the firing rate 45 minutes after THC or CBD injection using Two-Way RM ANOVA, with time and tolerance as factors. The percent decrease in firing rate from vehicle (0 minutes) to 45 minutes after THC or CBD injection was also calculated using the following formula: $((t_0-t_{45})/t_0)x100$. The percent decrease was then compared between morphine-naïve and morphine-tolerant rats using an unpaired t-test.

Finally, the timeline and AUC of the effect of acute CBD (20 mg/kg or 50 mg/kg) and gabapentin (100 mg/kg) on mechanical allodynia in morphine-naïve neuropathic rats was analyzed using individual Kruskal Wallis tests at each time point. Post-hoc tests were then conducted using Mann Whitney tests to compare each group to vehicle.

Results

Repeated treatment with morphine for 7 days induces tolerance to its anti-allodynic effects. First, neuropathic rats were treated with morphine (5 mg/kg) or vehicle (0.9% NaCl) twice daily for 7 days to induce tolerance to morphine's anti-allodynic effect (**Fig. 1**). Mechanical allodynia was assessed using von Frey filaments at baseline (before morphine administration) and 30 minutes after treatment with vehicle or morphine on day 1 and day 7 of treatment. Two-Way RM ANOVA revealed a significant Drug x Day interaction ($F_{2,92} = 187.0$, $p < 0.0001$). Bonferroni post-hoc tests indicated that morphine significantly increased mean PWT compared to vehicle and compared to baseline on day 1 and day 7 ($p < 0.0001$). However, the mean PWT of rats treated with morphine was significantly lower on day 7 compared to day 1, demonstrating over a 50% reduction in morphine's anti-allodynic effects by the 7th day of treatment ($p < 0.0001$). Rats in which no tolerance to morphine developed were removed and excluded from future experiments.

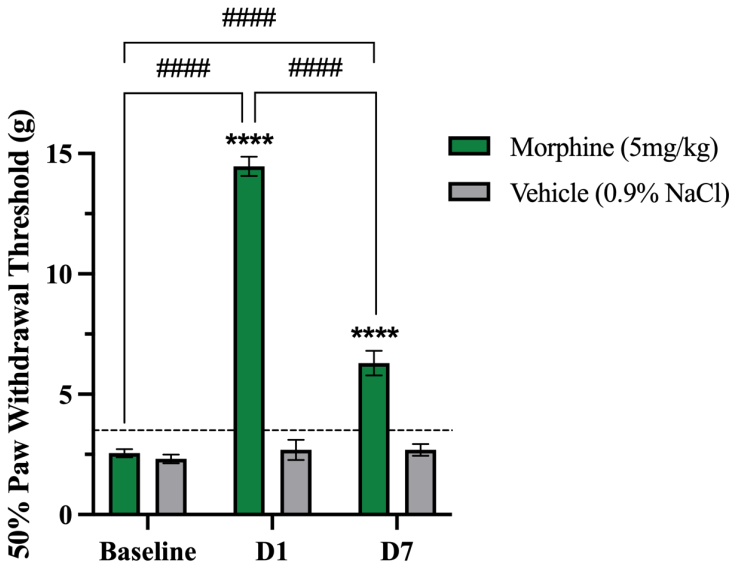

Figure 1. The anti-allodynic effect of morphine is reduced after 7 days of repeated treatment. The dashed line represents the cutoff threshold for neuropathy (4g). Results are expressed as mean ± SEM. N=22-26/group. Two-way RM ANOVA revealed a significant Drug x Day interaction ($p < 0.0001$). **** $p < 0.0001$ vs. vehicle; #### $p < 0.0001$ vs. other time-points by Bonferroni's post-hoc test.

Morphine tolerance does not change the baseline firing characteristics of ON or OFF cells in the RVM. Once tolerance to the anti-allodynic effects of morphine was confirmed, in vivo electrophysiology was used to examine the effects of morphine tolerance on ON and OFF cells in the RVM by analyzing their baseline firing characteristics over a 5-minutes period (**Fig. 2**). Unpaired t-tests revealed no difference between the mean spontaneous firing rate of morphine-naïve and morphine-tolerant rats in ON (**Fig. 2A**; $t_{15} = 1.941$, $p = 0.0713$) or OFF (**Fig. 2C**; $t_{14} = 0.4920$, $p= 0.6303$) cells. Furthermore, Mann Whitney tests indicated that there were no differences in the median pinch-evoked ON cell burst rate (**Fig. 2B**; $U_{8,9} = 17.50$, $p = 0.0789$) or OFF cell pause rate (**Fig. 2D**; $U_{7,9} = 20$, $p = 0.2523$) between morphine-naïve and morphine-tolerant rats.

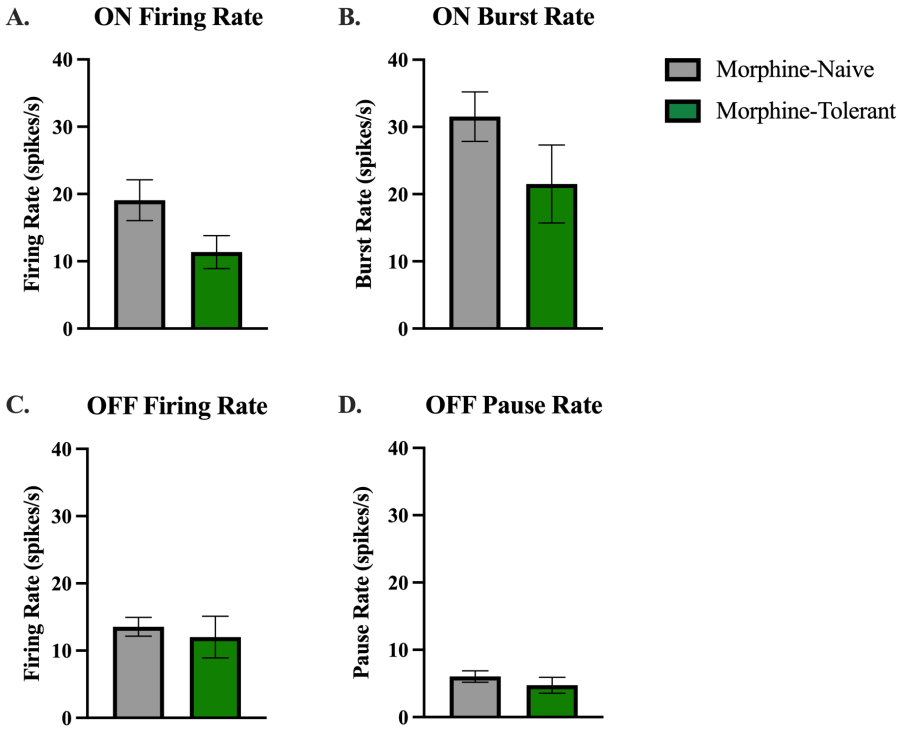

Figure 2. Morphine tolerance has no effect on the baseline firing characteristics of ON or OFF cells in rats with NP. Results are expressed as mean ± SEM. N=7-9/group. **A, C)** Mean firing rate over 5 minutes preceding vehicle injection. The data were analyzed using unpaired t-tests, revealing no significant difference. **B, D)** Burst/pause rate in response to toe-pinch immediately prior to vehicle injection. The data were analyzed using Mann Whitney tests, which revealed no significant differences between morphine-naive and morphine-tolerant rats.

THC (2.5 mg/kg) reduces mechanical allodynia in morphine-naïve and morphine-tolerant rats with NP. The ability of THC (2.5 mg/kg) to alleviate mechanical allodynia in morphine-naïve and morphine-tolerant rats with NP was tested over a 5-hour period on the day after the last dose of morphine (**Fig. 3**). Three-Way RM ANOVA with a Greenhouse Geisser correction revealed significant Drug x Time ($F_{4.270,123.822} = 29.761$, $p < 0.001$) and Tolerance x Time ($F_{4.270,123.822} = 4.187$, $p = 0.003$) interactions (**Fig. 3A**). Bonferroni post-hoc tests indicated that THC increased the mean PWT compared to vehicle 1-3 hours after administration (1h: $p < 0.001$, 2h: $p < 0.001$, 3h: $p < 0.001$). Importantly, Bonferroni tests revealed no significant difference between morphine-tolerant and morphine-naïve rats at any time point. Drug x Tolerance x Time ($F_{4.270,123.822} = 1.622$, $p = 0.169$) and Drug x Tolerance $F_{1,29} = 6.817e\text{-}4$, $p = 0.979$) interactions were not significant. Analysis of the area under the curve (AUC; **Fig. 3B**) by Two-Way ANOVA revealed a significant difference between the mean AUC of rats treated with THC compared to morphine (Drug: $F_{1,29} = 57.93$, $p < 0.0001$), but no effect of tolerance (Tolerance: $F_{1,29} = 1.051$, $p = 0.3138$), or interaction effect (Drug x Tolerance: $F_{1,29} = 0.1401$, $p = 0.7109$).

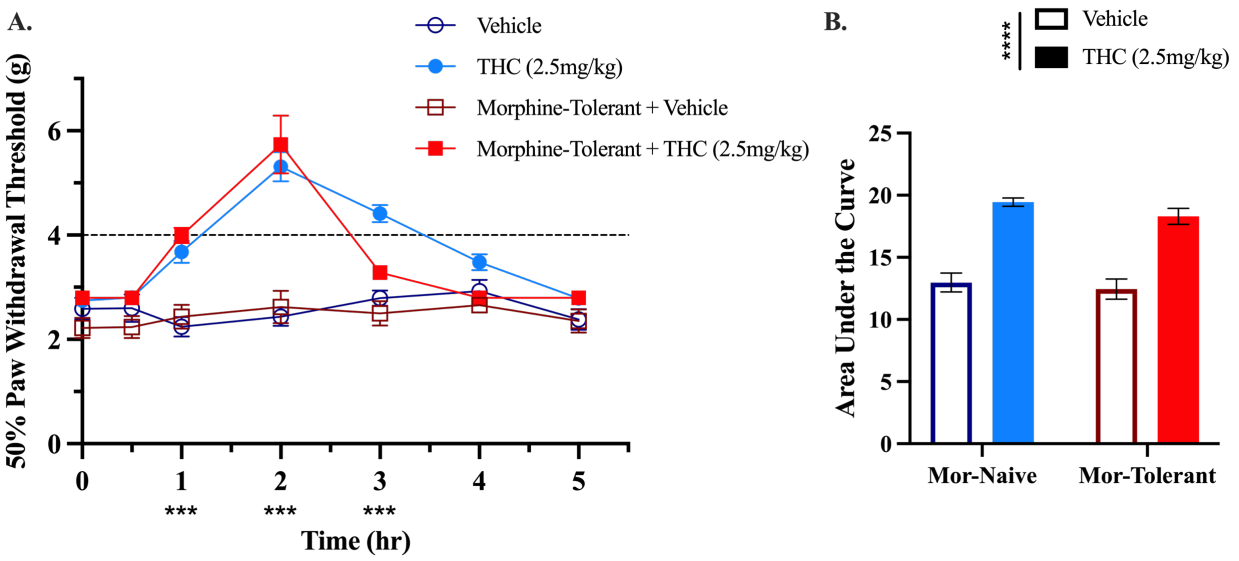

Figure 3. THC (2.5 mg/kg) reduces mechanical allodynia in morphine-naïve and morphine-tolerant rats with NP. The dashed line represents the cutoff threshold for neuropathy (4g). Results are expressed as mean ± SEM. N=4-11/group. **A)** Mechanical allodynia over time. Three-Way RM ANOVA revealed significant Drug x Time ($p < 0.001$) and Tolerance x Time ($p < 0.001$) interactions, where THC is significantly different from vehicle 1, 2, and 3 hours after administration, *** $p < 0.001$ by Bonferroni post-hoc test. **B)** Area under the curve of mechanical allodynia over time. Two-Way ANOVA revealed a main effect of Drug ($p < 0.0001$).

Microinjection of THC into the PAG decreases the firing rate and burst rate of ON cells in the RVM of morphine-naïve and morphine-tolerant rats with NP. The effect of THC (10 µg intra-PAG) on the firing rate and pinch-evoked burst rate of ON cells in the RVM was evaluated in morphine-naïve and morphine-tolerant rats with NP using in vivo electrophysiology (**Fig. 4**). Each cell was recorded for 50 minutes. The data were analyzed by converting the mean firing rate (spikes/s) in each 5-minute interval to a percentage (%) of the baseline firing rate obtained during the first 5 minutes of the recording. The response of each cell to a nociceptive stimulus was also evaluated by administering a toe-pinch to assess baseline response, response to vehicle, and every 15 minutes following injection of THC. In this study, the first 5 minutes of baseline vehicle recording (VEH) were used as a control. However, previous experiments from our lab have demonstrated that the firing rate of ON and OFF cells is stable over 1 hour in

neuropathic rats (Lopez-Canul et al., 2015). Two-Way RM ANOVA with a Greenhouse-Geisser correction revealed a significant main effect of Time on the mean firing rate of ON cells treated with THC (Time: $F_{1.490,10.43} = 9.693$, $p = 0.0063$; Tolerance: $F_{1,7} = 0.8918$, $p = 0.3764$; Time x Tolerance: $F_{9,63} = 0.2717$, $p = 0.9800$) (**Fig. 4A**), indicating that THC decreased the firing rate of ON cells over time. THC significantly reduced the firing rate of ON cells compared to vehicle ($t = 0$) at 25-, 30-, and 45-minutes post-administration (25min: $p = 0.0153$, 30min: $p = 0.0171$, 45min: $p = 0.0404$). An unpaired t-test of the AUC of the firing rate over time revealed no significant differences between the mean AUC in morphine-naïve compared to morphine-tolerant rats ($t_7 = 0.9347$, $p = 0.3811$) (**Fig. 4B**). The effect of THC was further assessed by comparing the firing rate 40 minutes after THC injection to the firing rate after vehicle injection (0 min). Again, Two-Way RM ANOVA revealed a main effect of time (Time: $F_{1,7} = 25.95$, $p = 0.0014$; Tolerance: $F_{1,7} = 0.7378$, $p = 0.4188$; Time x Tolerance: $F_{1,7} = 0.7016$, $p = 0.4299$) on the mean firing rate of ON cells treated with THC (**Fig. 4D**), indicating that THC decreased the spontaneous firing rate of ON cells compared to vehicle. Furthermore, the percent (%) decrease in firing rate from vehicle to 40 minutes after THC injection was compared between morphine-naïve and morphine-tolerant rats (**Fig. 4E**). An unpaired t-test of the mean percent decrease revealed no significant difference in the change in firing rate of ON cells between morphine-naïve and morphine-tolerant rats ($t_7 = 0.8338$, $p = 0.4319$). Finally, the effect of THC on the pinch-evoked burst rate of ON cells was assessed in morphine-naïve and morphine-tolerant rats using Two-Way RM ANVOA with a Greenhouse-Geisser correction, which revealed that THC decreased the mean burst rate of ON cells compared to vehicle ($t=0$) at all time points (Time: $F_{1.806,12.64} = 14.07$, $p = 0.0008$; 20min: $p = 0.0371$, 35min:

p = 0.0090, 50min: p = 0.0045 by Bonferroni post-hoc tests), independent of tolerance (Tolerance: $F_{1,7} = 0.2525$, p = 0.6307; Time x Tolerance: $F_{3,21} = 0.1257$, p = 0.9439) (**Fig. 4F**).

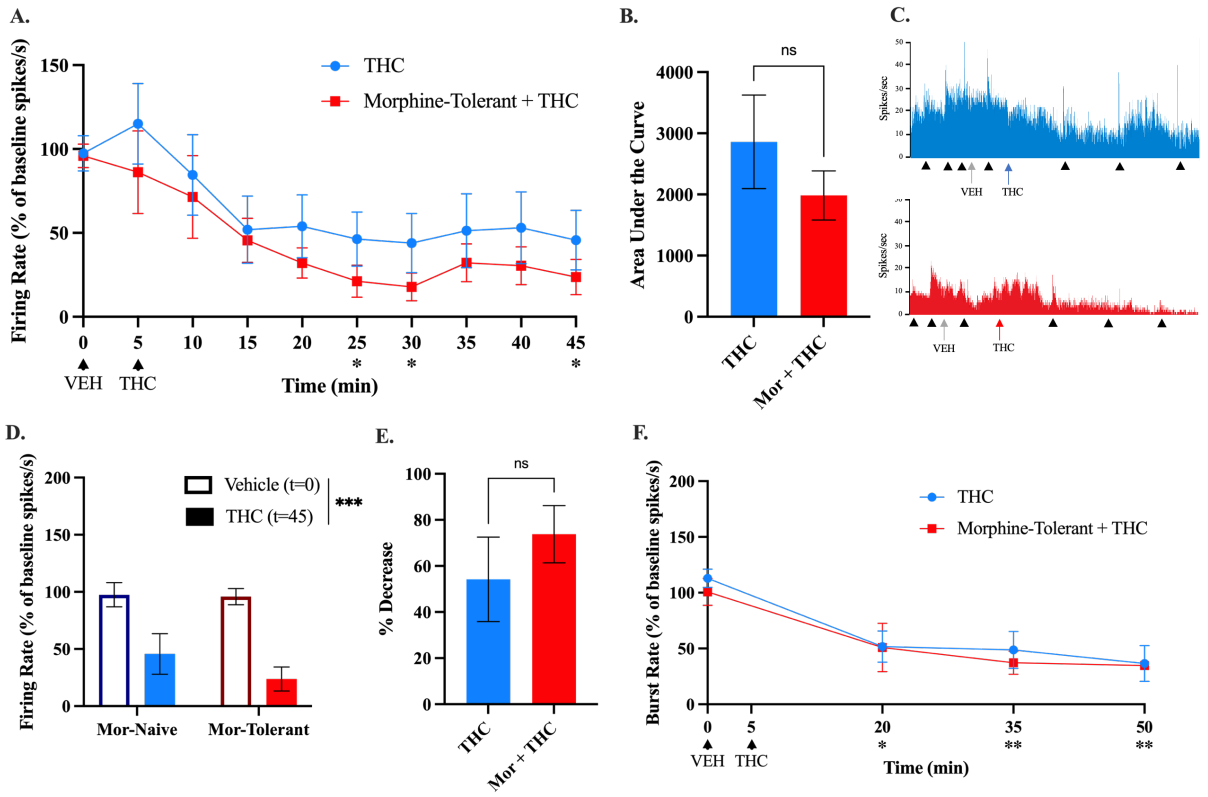

Figure 4. Microinjection of THC into the PAG reduces the firing rate and burst rate of ON cells over time in rats with NP. Results are expressed as mean ± SEM. N=4-5/group. **A)** Firing rate over time. The data are expressed as a % of baseline firing rate. Two-Way RM ANOVA with a Greenhouse-Geisser correction revealed a significant main effect of Time ($p < 0.01$), * $p < 0.05$ vs. t=0 by Bonferroni post-hoc tests. **B)** The area under the curve of firing rate over time. An unpaired t-test revealed no significant difference between morphine-naive and morphine-tolerant rats. **C)** Sample histograms displaying the activity of ON cells in morphine-naïve (top) and morphine-tolerant (bottom) rats. Triangles represent a toe-pinch. **D)** Firing rate after vehicle injection (t = 0) compared to 40 minutes after THC injection (t = 45). The data are expressed as a % of baseline firing rate. Two-Way RM ANOVA revealed a main effect of Time ($p < 0.001$). **E)** Percent decrease in firing rate from 0 to 45 minutes. An unpaired t-test revealed no significant difference between morphine-naive and morphine-tolerant rats. **F)** Pinch-evoked burst rate. The data are expressed as a % of baseline burst rate. Two-Way RM ANOVA with a Greenhouse-Geisser correction revealed a significant main effect of Time (*** $p < 0.001$), * $p < 0.05$, ** $p < 0.01$ vs. t=0 by Bonferroni post-hoc test.

THC has no effect on the firing rate or burst rate of OFF cells in the RVM of morphine-naïve or morphine-tolerant rats with NP. The effect of THC (10 µg intra-PAG) on the firing rate and pinch-evoked pause rate of OFF cells in the RVM was also evaluated in

morphine-naïve and morphine-tolerant rats with NP using in vivo electrophysiology (**Fig. 5**). Mixed effects analysis with a Greenhouse Geisser correction revealed no significant effect of THC on the firing rate of OFF cells over time (Time x Tolerance: $F_{10,45} = 1.521$, $p = 0.1632$; Time: $F_{1.889,8.502} = 1.511$, $p = 0.2733$; Tolerance: $F_{1,5} = 2.925$, $p = 0.1479$) (**Fig 5A**). Likewise, an unpaired t-test revealed no significant difference between the mean AUC of firing rate over time in morphine-naïve compared to morphine-tolerant rats ($t_4 = 1.039$, $p = 0.3576$) (**Fig. 5B**). Finally, mixed-effects analysis revealed no significant effects of THC on pinch-evoked pause rate (Time x Tolerance: $F_{3,12} = 0.9443$, $p = 0.4498$; Time: $F_{2.218,8.870} = 1.285$, $p = 0.3278$; Tolerance: $F_{1,5} = 0.3786$, $p = 0.5653$) (**Fig. 5D**).

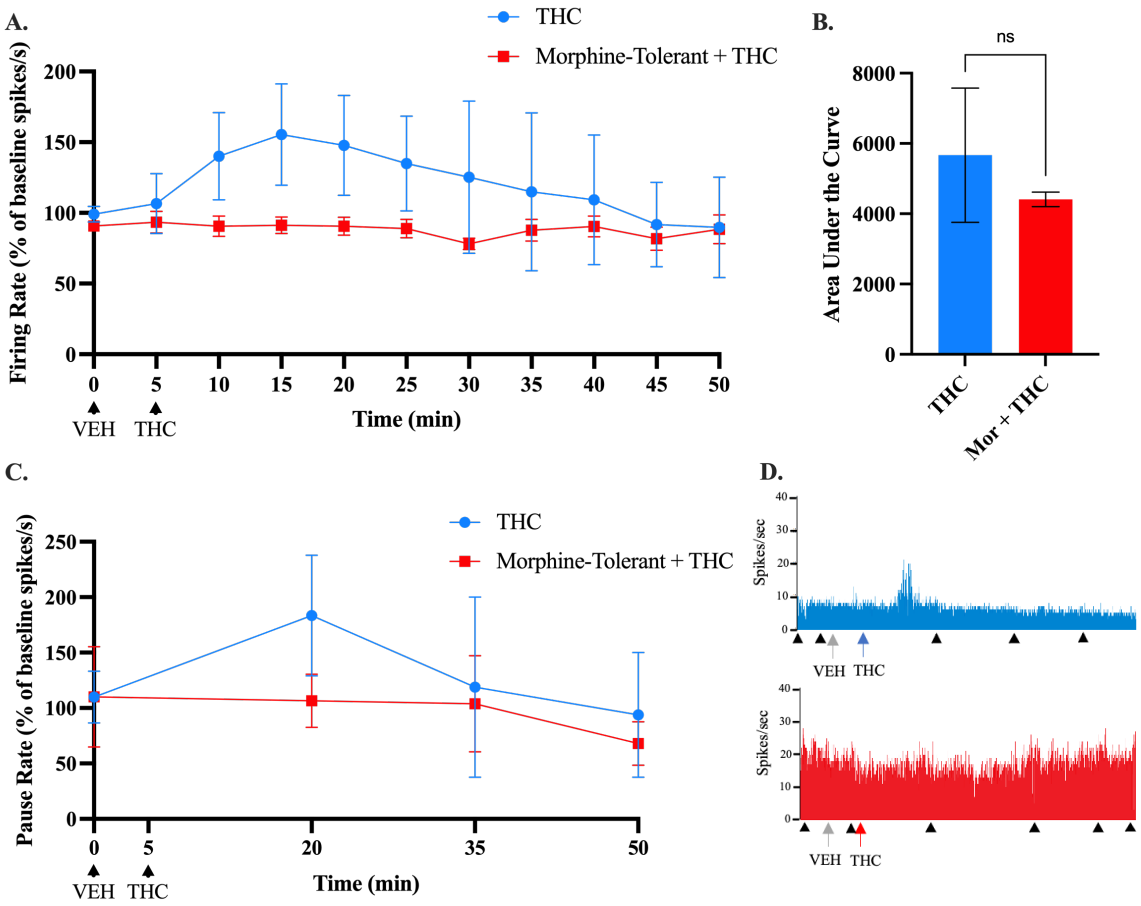

Figure 5. THC has no effect on the firing or pause rate of OFF cells in rats with NP. Results are expressed as mean ± SEM. N=2-4/group. **A)** Firing rate over time. The data are expressed as a % of baseline firing rate. Mixed-effects analysis revealed no significant effects. **B)** The area under the curve of firing rate over time. An unpaired t-test revealed no significant difference. **C)** Pinch-evoked pause rate. The data are expressed as a % of baseline pause rate. Mixed-effects analysis revealed no significant effects. **D)** Sample histogram displaying the activity of OFF cells in morphine-naïve (top) and morphine-tolerant (bottom) rats. Triangles represent a toe-pinch.

CBD (20 mg/kg) does not reduce mechanical allodynia in morphine-naïve or morphine-tolerant rats with NP. The ability of CBD (20 mg/kg) to alleviate mechanical allodynia in morphine-naïve and morphine-tolerant neuropathic rats was tested over a 5-hour period on the day after the last dose of morphine (**Fig. 6**). Three-Way RM ANOVA with a Greenhouse Geisser correction revealed no significant interactions (Time x Tolerance x Drug: $F_{3.817,114.504} = 1.136$, $p = 0.343$; Tolerance x Drug: $F_{1,30} = 1.062$, $p = 0.311$; Time x Tolerance:

$F_{3.817,114.504}$ = 1.744, p = 0.148; Time x Drug: $F_{3.817,114.504}$ = 0.867, p = 0.482) or main effects (Tolerance: $F_{1,30}$ = 0.067, p = 0.798; Drug: $F_{1,30}$ = 1.451, p = 0.238; Time: $F_{3.817,114.504}$ = 1.977, p = 0.106) of CBD on mechanical allodynia (**Fig. 6A**). Furthermore, Two-Way ANOVA of the AUC revealed no significant effects (Tolerance x Drug: $F_{1,30}$ = 1.127, p = 0.2969; Drug: $F_{1,30}$ = 1.232, p = 0.2758; Tolerance: $F_{1,30}$ = 0.2417, p = 0.6266) (**Fig. 6B**).

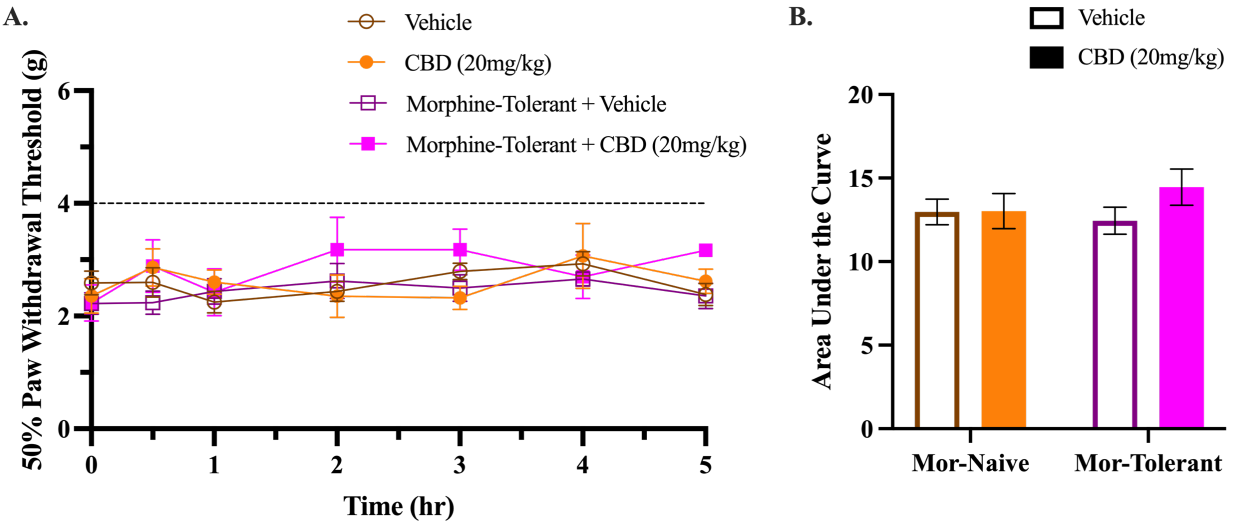

Figure 6. Effect of CBD (20 mg/kg) on mechanical allodynia in morphine-naïve and morphine-tolerant rats with NP. The dashed line represents the cutoff threshold for neuropathy (4g). Results are expressed as mean ± SEM. N=6-11/group. **A)** Mechanical allodynia over time. Three-Way RM ANOVA revealed no significant interactions or main effects. **B)** The area under the curve of mechanical allodynia over time. Two-Way ANOVA revealed no significant interactions or main effects.

Microinjection of CBD into the PAG decreases the firing and burst rate of ON cells in the RVM of morphine-naïve and morphine-tolerant rats with NP. The effect of CBD (1 µg intra-PAG) on the firing rate and pinch-evoked burst rate of ON cells in the RVM was also evaluated in morphine-naïve and morphine-tolerant rats with NP using in vivo electrophysiology (**Fig. 7**). Two-Way RM ANOVA with a Greenhouse-Geisser correction revealed no statistically significant effects of CBD on the firing rate of ON cells over time. However, the main effect of Time approached statistical significance (Time: $F_{1.559,9.351}$ = 3.928, p = 0.0652; Tolerance: $F_{1,6}$ =

0.4733, p = 0.5172; Time x Tolerance: $F_{9,54}$ = 1.016, p =0.4396). (**Fig. 7A**). A Mann Whitney test revealed no significant differences in the median AUC between morphine-naïve and morphine-tolerant rats ($U_{4,4}$ = 6, p = 0.6857) (**Fig. 7B**). The effect of CBD over time was further assessed by comparing the firing rate 40 minutes after CBD injection to the firing rate following vehicle injection (0 min) (**Fig. 7D**). Two-Way RM ANOVA indicated that CBD significantly reduced the mean firing rate of ON cells 40 minutes after administration compared to vehicle (0 min) (Time: $F_{1,6}$ = 8.585, p = 0.0263). There was no significant interaction or main effect of tolerance, indicating that the effect of CBD did not differ between morphine-naïve and morphine-tolerant rats (Time x Tolerance: $F_{1,6}$ = 3.289, p = 0.1197; Tolerance: $F_{1,6}$ = 0.01516, p = 0.9060). An unpaired t-test of the mean % decrease from vehicle (0 min) to 40 minutes after CBD injection also revealed no significant difference between morphine-naïve and morphine-tolerant rats (t_6 = 1.611, p = 0.1584) (**Fig. 7E**). Finally, Two-Way RM ANVOA with a Greenhouse-Geisser correction revealed that CBD significantly decreased the mean pinch-evoked burst rate of ON cells compared to vehicle (t=0) (Time: $F_{1.994,11.97}$ = 10.38, p = 0.0024; 20min: p = 0.0168, 35min: p = 0.0181, 50min: p = 0.0329), independent of tolerance (Tolerance: $F_{1,6}$ = 0.1563, p = 0.7063; Time x Tolerance: $F_{3,18}$ = 0.8154, p = 0.5020) (**Fig. 7F**).

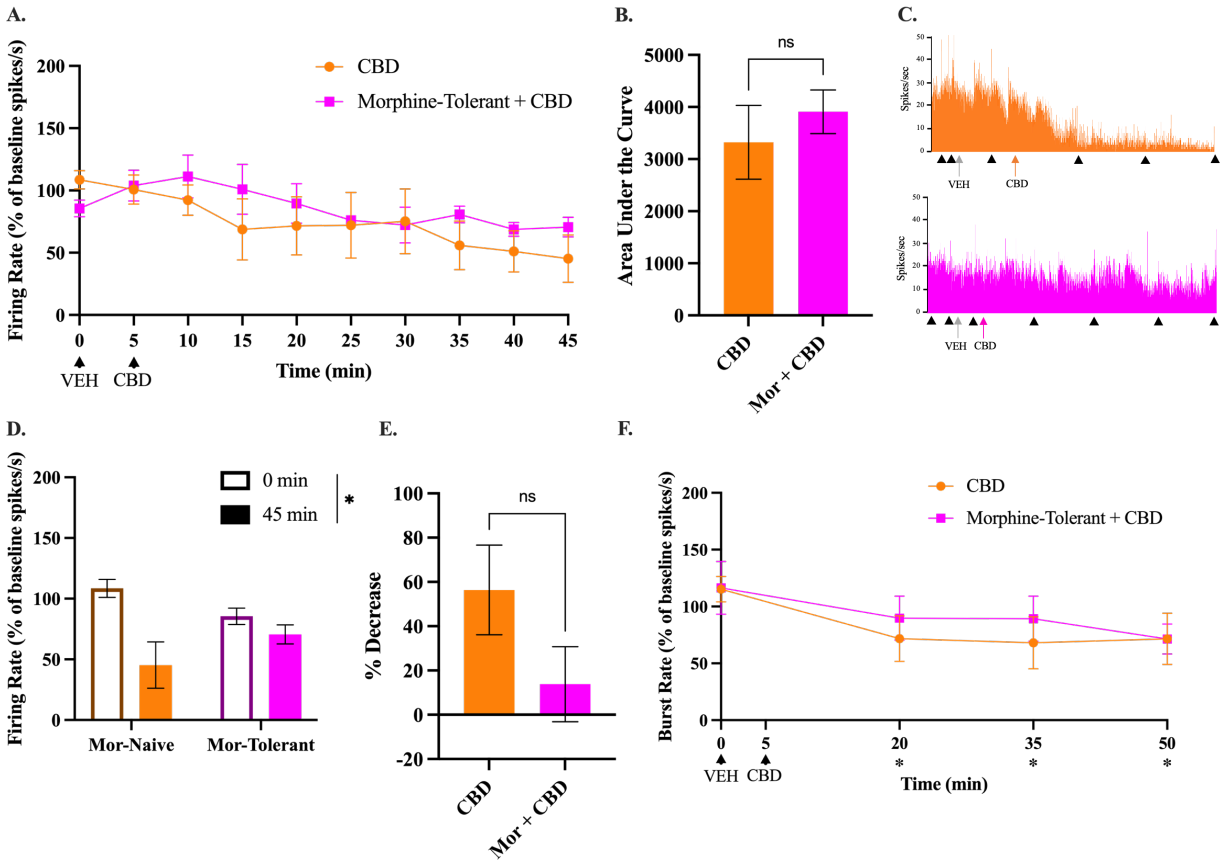

Figure 7. Microinjection of CBD into the PAG reduces the firing rate and burst rate of ON cells in rats with NP. Results are expressed as mean ± SEM. N=4/group. Triangles represent a toe-pinch. **A)** Firing rate over time. The data are expressed as a % of baseline firing rate. Two-Way RM ANOVA with a Greenhouse Geisser correction revealed a trend toward a main effect of Time ($p < 0.07$). **B)** The area under the curve of firing rate over time. A Mann Whitney test revealed no significant difference between morphine-naive and morphine-tolerant rats. **C)** Sample histogram displaying the activity of ON cells in morphine-naïve (top) and morphine-tolerant (bottom) rats. Triangles represent a toe-pinch. **D)** Firing rate after vehicle injection (0 min) compared to 45 minutes after CBD injection. The data are expressed as a % of baseline firing rate. Two-Way RM ANOVA with a Greenhouse Geisser correction revealed a significant main effect of Time, * $p < 0.05$. **E)** % decrease in firing rate from 0 to 45 minutes. An unpaired t-test revealed no significant difference between morphine-naive and morphine-tolerant rats. **F)** Pinch-evoked burst rate. The data are expressed as a % of baseline burst rate. Two-Way RM ANOVA with a Greenhouse Geisser correction revealed a significant main effect of Time ($p < 0.01$), * $p < 0.05$ vs. $t = 0$ by Bonferroni post-hoc tests.

CBD has no effect on the firing rate or burst rate of OFF cells in the RVM of morphine-naïve or morphine-tolerant rats with NP. The effect of CBD (1 µg intra-PAG) on the firing rate and pinch-evoked pause rate of OFF cells in the RVM was evaluated in morphine-

naïve and morphine-tolerant rats with NP using in vivo electrophysiology (**Fig. 8**). Mixed-effects analysis with a Greenhouse Geisser correction revealed no significant effect of CBD on the mean firing rate of OFF cells over time (Time x Tolerance: $F_{10, 65} = 0.1796$, $p = 0.9838$; Time: $F_{1.603,10.42} = 0.3595$, $p = 0.6612$; Tolerance: $F_{1,7} = 0.3718$, $p = 0.5613$) (**Fig 8A**). Welch's t test was used to compare the mean AUC of firing rate over time in morphine-naïve and morphine-tolerant rats, revealing no significant difference ($t_{5.124} = 0.7179$, $p = 0.5042$) (**Fig. 8B**). Finally, Mixed-effects analysis with a Greenhouse Geisser correction revealed no significant effects of CBD on mean pinch-evoked pause rate (Time x Tolerance: $F_{3,19} = 1.810$, $p = 0.2159$; Time: $F_{1.991,12.61} = 0.4910$, $p = 0.6225$; Tolerance: $F_{1,7} = 0.1803$, $p = 0.6839$) (**Fig. 8C**).

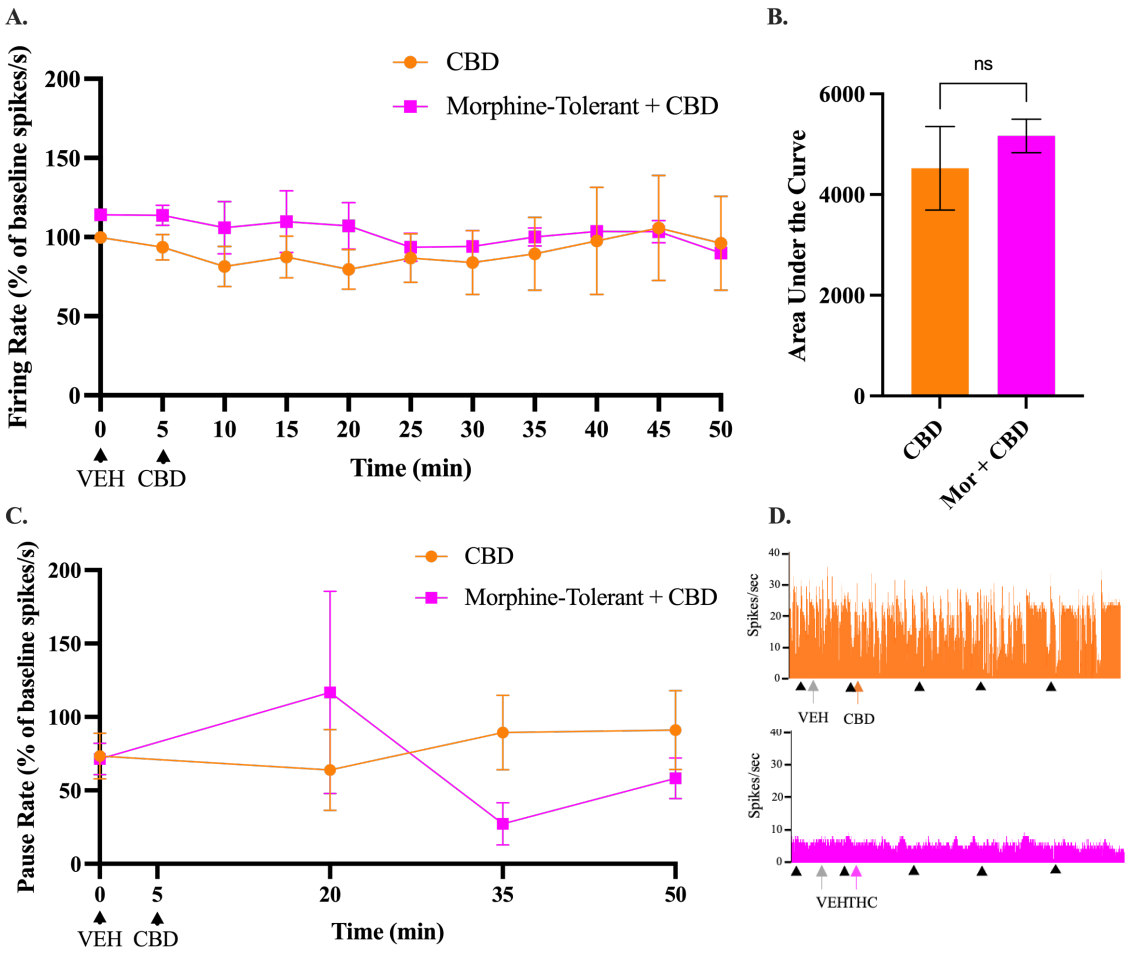

Figure 8. CBD has no effect on the firing or pause rate of OFF cells in rats with NP. Results are expressed as mean ± SEM. N=3-6/group. Triangles represent a toe-pinch. **A)** Firing rate over time. The data are expressed as a % of baseline firing rate. Mixed effects analysis revealed no significant effects. **B)** The area under the curve of firing rate over time. A Welch's t-test was revealed no significant difference between morphine-naive and morphine-tolerant rats. **C)** Pinch-evoked pause rate. The data are expressed as a % of baseline pause rate. Mixed effects analysis revealed no significant effects. **D)** Sample histogram displaying the activity of ON cells in morphine-naïve (top) and morphine-tolerant (bottom) rats. Triangles represent a toe-pinch.

High-dose CBD (50 mg/kg) does not reduce mechanical allodynia in rats with NP. In order to determine whether a higher dose of CBD was needed to elicit an anti-allodynic effect, neuropathic rats were treated with 50 mg/kg of CBD, or 100 mg/kg of gabapentin as a positive control, and mechanical allodynia was assessed periodically for 5 hours (**Fig. 9**). Kruskal Wallis tests at each time point in the curve of mechanical allodynia over time (**Fig. 9A**) revealed a

significant effect 1-5 hours after administration ($H_3 \geq 9.536$, $p < 0.0229$), and Mann Whitney tests were used to compare each treatment group to vehicle every hour during that time. As expected, gabapentin significantly increased the median PWT compared to vehicle 1-5 hours after administration (1h: $p < 0.0001$, 2h: $p = 0.0001$, 3h: $p = 0.0015$, 4h: $p = 0.0012$, 5h: $p = 0.0150$). However, neither a dose of 20 nor 50 mg/kg of CBD produced a significant increase in median PWT compared to vehicle at any time point. Kruskal Wallis analysis of the AUC (**Fig. 9B**) revealed a significant difference between groups ($H_3 = 16.99$, $p = 0.0007$). However, only gabapentin was found to increase the median AUC compared to vehicle by Mann-Whitney test ($p < 0.0001$). Together, these results demonstrate that CBD does not reduce mechanical allodynia compared to vehicle at any dose tested.

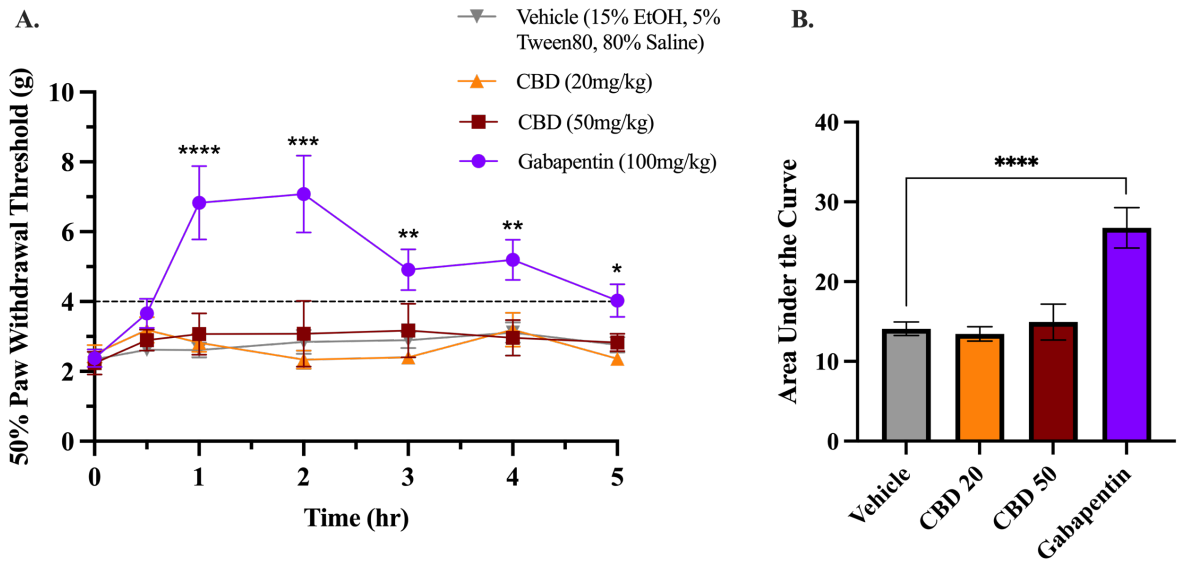

Figure 9. Effect of CBD (20 mg/kg or 50 mg/kg) on mechanical allodynia in rats with NP. Results are expressed as mean ± SEM. N=8-27/group. The dashed line represents the cutoff threshold for neuropathy (4g). Post-hoc tests only compared each group to vehicle. **A)** Mechanical allodynia over time. Individual Kruskal Wallis tests were used to compare the groups at each time point. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Vehicle by Mann-Whitney test. **B)** Area under the curve of mechanical allodynia over time. Kruskal Wallis test indicated a significant difference ($p < 0.001$). **** $p < 0.0001$ by Mann-Whitney test.

Discussion

Neuropathic pain (NP) is a persistent pain disorder that is often difficult to treat. First-line treatment recommendations do not always provide adequate pain relief, so patients may be prescribed opioids. However, opioids also present significant problems, including severe side effects and safety risks, which contribute to a reduced quality of life. Furthermore, patients who take opioids chronically are likely to develop tolerance to their analgesic effects; not only does this reduce the level of pain relief that patients experience, but may lead them to use higher doses, increasing their risk of harm. Research has therefore sought to identify novel approaches to the management of NP, especially in the context of morphine-tolerance. We explored the hypothesis that THC and/or CBD may be effective alternative analgesics to treat NP in subjects that have developed tolerance to the anti-allodynic effects of morphine.

Morphine tolerance

First, we showed that our protocol induces tolerance to morphine's anti-allodynic effects. It is well established that morphine exerts its antinociceptive effects via modulation of descending pain circuitry in the PAG-RVM pathway (Cheng et al., 1986; Heinricher et al., 1992; Tortorici & Morgan, 2002). Acutely, morphine decreases and increases ON and OFF cell firing, respectively, and reduces the response of these cells to noxious stimulation. While it is known that these cellular responses to morphine are absent in morphine-tolerant rodents (Lane et al., 2004; Tortorici et al., 2001), to our knowledge, the effects of morphine tolerance on the basal firing characteristics of these cells in rodents with NP has not been studied. Understanding the cellular adaptations that underly morphine tolerance in subjects with NP may guide future approaches to attenuate or

alleviate tolerance. Therefore, we used in vivo electrophysiology to record the spontaneous firing rate and pinch-evoked burst and pause rate of ON and OFF cells in the RVM of morphine-naïve and morphine-tolerant rats with NP. We demonstrated that tolerance to the anti-allodynic effect of morphine does not change the spontaneous firing rate or pinch-evoked burst/pause rate of ON or OFF cells in the RVM of neuropathic rats. This is in line with previous studies that have observed no changes in the spontaneous activity of these cells in healthy, morphine-tolerant rodents (Lane et al., 2004; Tortorici et al., 2001; Viisanen et al., 2020). However, it is also true that we observed a trend toward a decrease in the spontaneous firing rate and pinch-evoked burst rate of ON cells induced by morphine-tolerance that approached, but did not reach, statistical significance ($p < 0.08$). In contrast, an increase in facilitatory influence from the RVM has been proposed as a possible mechanism that may counteract opioid antinociception and contribute to tolerance (Ossipov et al., 2004). Indeed, Viisanen et al. (2020) recently showed an increase in noxious heat-evoked ON and OFF cell responses in morphine-tolerant rats. On the other hand, they observed no changes in mechanical stimulus-evoked cellular response. It is therefore possible that there is a modality-specific role of the descending modulatory system in the development of morphine tolerance. Furthermore, it is worth considering the possibility of opioid-induced allodynia and hyperalgesia (Vanderah et al., 2001); using our protocol, morphine tolerant rats did not display increased allodynia compared to saline-treated rats, indicating that they did not develop opioid-induced paradoxical pain. Therefore, we cannot rule out that descending facilitation from the brainstem may contribute to this phenomenon at higher doses or durations of morphine treatment.

Delta-9-tetrahydrocannabinol (THC)

Anti-allodynic effects of THC

The antinociceptive efficacy of THC is quite well established in rodent models of NP (Abraham et al., 2019; Casey et al., 2017; Harris et al., 2016; Henderson-Redmond et al., 2020; King et al., 2017; J. Williams et al., 2008). Furthermore, a number of studies have demonstrated the ability of THC pre-treatment (Cichewicz, Martin, Smith, & Welch, 1999; F. L. Smith, Cichewicz, Martin, & Welch, 1998; Wilson-Poe, Pocius, Herschbach, & Morgan, 2013) or co-administration (Cichewicz & McCarthy, 2003; Cox et al., 2007) to potentiate morphine antinociception and attenuate tolerance (Cichewicz & Welch, 2003; P. A. Smith et al., 2007). However, until now, it remained unclear whether THC produces antinociception in neuropathic subjects with tolerance to morphine.

In this thesis, we demonstrated that THC at a dose of 2.5 mg/kg reduces mechanical allodynia in morphine-tolerant rats with NP. Previous studies evaluating whether THC maintains its efficacy in morphine-tolerant animals have shown mixed results (Cichewicz & Welch, 2003; Gerak et al., 2015; Rubino et al., 1997; Thorat & Bhargava, 1994). This variability may be due to differences in species, dose, duration of treatment, pain assay, or pathological condition. We showed for the first time that THC reduces mechanical allodynia in neuropathic rats with tolerance to morphine, with the same overall effect as in morphine-naïve rats. This finding supports the main hypothesis posed in this thesis: that THC may be an effective alternative analgesic to treat NP in subjects that have developed tolerance to the anti-allodynic effects of morphine.

Effects of THC on descending pain modulation

Both opioids (Fields, 2004) and synthetic cannabinoids (Martin et al., 1998; Meng & Johansen, 2004; Meng et al., 1998) have been shown to modulate the activity of brainstem descending pain circuitry by decreasing and increasing the firing rate of ON and OFF cells, respectively, and reducing their response to nociceptive stimulation. However, to our knowledge, the effects of THC on the activity of RVM ON and OFF cells has not been explored. We therefore evaluated the effects of intra-PAG administration of THC (10 μg) on the firing rate and pinch-evoked burst/pause rate of ON and OFF cells in the RVM of neuropathic rats. We demonstrated that THC decreases the spontaneous firing rate and pinch-evoked burst rate of ON cells in rats with NP. On the other hand, THC has no effect on the spontaneous firing rate or pinch-evoked pause rate of OFF cells. Our findings are in contrast to the results reported by Meng and Johansen (2004) demonstrating an increase in spontaneous activity of OFF cells, but not ON cells, induced by the synthetic cannabinoid WIN55,212-2. However, it is worth noting that their experiments were performed in healthy, not neuropathic, rats. Indeed, Palazzo et al. (2012) demonstrated that a higher dose of WIN55,212-2 (intra-PAG) was needed to induce cellular responses in neuropathic compared to sham rats, suggesting that the effect of cannabinoids may vary in different pathological conditions. Furthermore, in support of our findings, Palazzo et al. (2012) reported a decrease in ON cell spontaneous activity and stimulus evoked bursts by WIN55,212-2. However, they also reported that WIN55,212-2 increased the spontaneous firing rate and decreased the pause duration of OFF cells in neuropathic rats. Still, it is important to consider that WIN55,212-2 is a full agonist, whereas THC is a partial agonist, of CB1 receptors (Kirschmann, McCalley, Edwards, & Torregrossa, 2017), which may explain the differences between our results.

We also showed for the first time that there is no effect of morphine-tolerance on the THC-induced decrease in spontaneous firing rate and pinch-evoked burst rate of ON cells in neuropathic rats. This is supported by findings that administration of a MOR antagonist does not block the effect of WIN55,212-2 on ON or OFF cells (Meng et al., 1998), and antinociception induced by intra-PAG administration of a CB1 receptor agonist is enhanced in morphine-tolerant rats (Wilson-Poe et al., 2013). The overlapping but distinct signaling mechanisms of opioids and cannabinoids may account for the lack of cross-tolerance that we observed. While both opioids and cannabinoids reduce GABAergic transmission via presynaptic inhibition in the PAG, only opioids also act at postsynaptic terminals (Vaughan et al., 2000). Research suggests that, on a cellular level, morphine tolerance may manifest mainly as changes in postsynaptic, rather than presynaptic, signaling. Specifically, morphine tolerance reduces MOR-stimulated activation of post-synaptic currents in the PAG (Bagley et al., 2005; Wilson-Poe et al., 2015), but has no effect on MOR-agonist- or synthetic cannabinoid-induced presynaptic inhibition of GABAergic transmission (Wilson-Poe et al., 2015). It is therefore possible that the diminished efficacy of intra-PAG morphine observed in morphine-tolerant animals (Morgan et al., 2005) occurs via reduced postsynaptic MOR-stimulated GIRK and Ca^{2+} channel activation (Bagley et al., 2005), without influencing THC's ability to modulate PAG neurons via presynaptic inhibition. However, more experiments are needed to fully understand the relationship between morphine-tolerance and THC-antinociception on a cellular level.

Cannabidiol (CBD)

Anti-allodynic effects of CBD

We also evaluated the anti-allodynic efficacy of CBD in neuropathic rats. We found that CBD does not reduce mechanical allodynia in morphine-naïve or morphine-tolerant rats with NP, even at a high dose. Although the current literature generally supports the use of CBD as an analgesic, most studies evaluating its efficacy in rodent models of NP have used repeated instead of acute treatment regimens (Abraham et al., 2019; De Gregorio et al., 2019; Ward et al., 2014), and those that have assessed its acute effects have shown mixed results (Costa et al., 2007; Jesus et al., 2019; Toth et al., 2010). For example, unpublished results from our lab demonstrated that 20 mg/kg of CBD decreased mechanical allodynia in the SNI model of NP (Farina, 2021). However, Costa et al. (2007) found that the same dose did not reduce thermal or mechanical hyperalgesia in rats following chronic constriction injury (CCI). Similarly, Jesus et al. (2019) showed that acute CBD was able to reduce mechanical allodynia in diabetic animals, whereas Toth et al. (2010) found no acute effect, even at a higher dose. It is therefore possible that CBD only exerts an anti-allodynic effect when administered repeatedly. With repeated administration, CBD may alter the response properties of the receptors that it interacts with, altering pain transmission and modulation. For example, De Gregorio et al. (2019) demonstrated that CBD at a dose of 5 mg/kg did not alleviate pain acutely in rats that underwent SNI; however, after 7 days of repeated treatment, there was a reduction in mechanical allodynia. This was paralleled by neuronal activity in the dorsal raphe nucleus (DRN), where acute CBD decreased 5-HT firing, and repeated CBD increased it through desensitization of 5-HT1A receptors.

Another possible explanation for the results that we observed is that CBD modulates the affective component of pain, and that the assessment of mechanical allodynia is not necessarily representative of CBD's full analgesic potential. Importantly, pain is defined as both a sensory and emotional experience (Raja et al., 2020), and it is possible that these two components are differently modulated by analgesics. Previous researchers have actually suggested that the pain-relieving effects of cannabis may be largely mediated by a reduction in the affective component of pain (Lee et al., 2013; Weizman et al., 2018). In fact, Genaro et al. (2017) demonstrated that CBD administered systemically or into the rostral anterior cingulate cortex (ACC) relieves pain aversiveness at doses that do not reduce mechanical allodynia. Indeed, the ACC, along with other cortical and limbic structures, is known to play a role in the emotional and motivational components of pain processing (Genaro et al., 2017; Ossipov et al., 2014). For example, placebo analgesia, which involves the modulation of pain by emotional/motivational factors, is associated with functional connectivity between the ACC and PAG (Bingel, Lorenz, Schoell, Weiller, & Buchel, 2006; Eippert et al., 2009). This provides a clear pathway through which emotional factors may be capable of modulating pain transmission via "top-down" control of the brainstem descending modulatory system. Overall, it is possible that acute CBD may in fact produce an analgesic effect if a different paradigm were used to assess pain, especially since we found that CBD, like THC, modulated the activity of ON cells in the RVM, supporting an antinociceptive effect.

Effects of CBD on descending pain modulation

We showed that intra-PAG administration of CBD (1 μg) decreases the spontaneous firing rate and pinch-evoked burst rate of ON cells in the RVM of morphine-naïve and morphine-tolerant rats with NP. Conversely, CBD produces no change in the activity of OFF cells in morphine-naïve or morphine-tolerant rats. Only one previous study has directly evaluated the effects of intra-PAG CBD on the activity of pain responsive cells in the RVM. Maione et al. (2011) demonstrated that CBD (3 nmol) produced antinociception, decreased the firing rate and tail-flick related burst rate of ON cells, and paradoxically decreased the firing rate of OFF cells, in healthy rats. It is possible that the effects that Maione et al. (2011) observed on OFF cells were absent in our results because our experiments were performed on neuropathic rats. A number of changes in the endocannabinoid system have been identified in neuropathic animals, supporting the notion that CBD's effects on the descending modulatory system may differ based on pathological condition. For example, in neuropathic rats, CB1 receptors appear to be downregulated or desensitized in the PAG (Knerlich-Lukoschus et al., 2011; Palazzo et al., 2012) and ACC (Hoot et al., 2010; Knerlich-Lukoschus et al., 2011). AEA and 2-AG levels are also elevated in the PAG, RVM, and spinal cord of neuropathic rats (Petrosino et al., 2007). These changes could alter the indirect effects of CBD on CB1 receptors via inhibition of FAAH (outlined below).

There are a number of possible mechanisms through which CBD may modulate cellular activity in the PAG. As outlined previously, CBD is known to agonize 5-HT1A (Russo et al., 2005) and TRVP1 (Bisogno et al., 2001; Iannotti et al., 2014), but not CB1 (Vuckovic et al., 2018), receptors. Still, CBD is capable of affecting the activation of CB1 receptors through negative allosteric modulation (Vuckovic et al., 2018) and elevation of endocannabinoid levels via

inhibition of FAAH (de Almeida & Devi, 2020). Maione et al. (2011) showed that the effects of intra-PAG CBD on ON and OFF cells were blocked by administration of a CB1, adenosine A1, TRPA1, or 5-HT1A antagonist, emphasizing the complexity of CBD's mechanism of action. Similar to morphine, 5-HT1A receptors presynaptically inhibit GABA release in the PAG, where populations of neurons have been identified that respond to 5-HT, MOR agonists, or both (Kishimoto et al., 2001). CBD may therefore act on overlapping and separate cells from morphine to exert its effects on ON cells, even in morphine-tolerant rats. TRPV1 receptors, which play a role in CBD antinociception (Costa et al., 2007; De Gregorio et al., 2019), are also located in the PAG and have been implicated in descending modulation of pain (Palazzo et al., 2008). Although intra-PAG administration of a TRPV1 agonist does produce antinociception and decrease ON cell activity (Starowicz et al., 2007), Maione et al. (2011) reported that CBD's modulation of ON cells is independent of TRPV1. Instead, they found that the reduction of OFF cell activity by CBD was blocked by administration of a TRPV1 antagonist, but only for the first 10 minutes. Finally, it is possible that CBD's effects in the PAG occur via indirect activation of CB1 receptors, since Maione et al. (2011) demonstrated that CB1 antagonists blocked CBD's modulation of ON and OFF cell activity. CBD inhibits FAAH, an enzyme that inactivates endocannabinoids AEA and 2-AG, which are both CB1 agonists (Maione et al., 2006). Administration of an FAAH inhibitor into the PAG produces antinociception and reduces spontaneous and tail flick related ON cell firing via endocannabinoid elevation and CB1 receptor activation (Maione et al., 2006). Interestingly, morphine-tolerance increases endocannabinoid inhibition of GABAergic activity in the PAG (Wilson-Poe et al., 2015). Therefore, if CBD indirectly increases CB1-mediated signalling by

blocking endocannabinoid hydrolysis, its effects should remain in rats with tolerance to morphine, as we demonstrated here.

Summary of cannabinoid effects on descending pain modulation

The ability of analgesic substances to modulate the activity of ON and OFF cells has become a hallmark of their antinociceptive actions. In line with this, we showed that THC and CBD both decrease the spontaneous activity and pinch-evoked burst rate of ON cells in the RVM of neuropathic rats, independent of morphine tolerance. However, we also demonstrated that these cannabinoids have no effect on the activity of OFF cells.

There are a number of possible mechanisms that could explain the unexpected results that we observed of cannabinoids on OFF cell activity. 1) A reduction in ON cell activity may be sufficient to produce antinociception. THC, like morphine (Fields, 2004), decreases the firing rate of ON cells and produces an antinociceptive response, despite its lack of effect on OFF cells. Furthermore, findings from Sebatino Maione and colleagues demonstrating a paradoxical decrease in the activity of OFF cells by antinociceptive doses of a dual FAAH inhibitor/TRPV1 antagonist (de Novellis et al., 2008), the endocannabinoid palmitoylethanolamide (PEA) (de Novellis et al., 2012), and CBD (Maione et al., 2011), have led the authors to suggest that a reduction in ON cell activity is sufficient to produce antinociception. However, previous research has contradicted this proposal. For example, Heinricher and McGaraughty (1998) reported that the elimination of tail flick-related ON cell burst (but not OFF cell pause) by administration of a glutamate antagonist into the RVM had no effect on tail flick latency, indicating that modulation of ON cell activity does not play a significant role in antinociception. Furthermore, Maione et al. (2011) reported that

their highest dose of CBD reduced ON cell firing without producing antinociception. Since we also demonstrated that ON cell activity was reduced by CBD, which did not reduce allodynia, our results seem to suggest that ON cell modulation is not sufficient for antinociception. Therefore, it is possible that 2) antinociception may occur through a different pathway from the PAG. The PAG projects to the noradrenergic locus coeruleus (LC) and serotonergic DRN, both of which play a role in pain and analgesia (Mendiguren, Aostri, & Pineda, 2018; Ossipov et al., 2010). These regions also express CB1 receptors, and cannabinoids have been shown to modulate their neuronal activity (De Gregorio et al., 2019; Mendiguren et al., 2018; Mendiguren & Pineda, 2009). It is therefore possible that administration of cannabinoids into the PAG produces antinociception through one of these pathways, without necessarily modulating the activity of the RVM. 3) THC and CBD administration into the PAG may not be sufficient to produce antinociception. It is important to recognize that we did not verify that antinociception was produced by intra-PAG administration of THC and CBD in this study. Because we were working with deeply anesthetized rats, we were not able to correlate cellular response with a behavioural measure. It is therefore possible that the anti-allodynic effects of THC that we observed may have occurred via activation of receptors elsewhere in the body, and that intra-PAG administration of THC did not in fact produce antinociception. While findings from Palazzo et al. (2012) demonstrating an antinociceptive effect of intra-PAG WIN55,212-2 contradict this theory, we must consider the difference in potency between WIN55,212-2 and THC at CB1 receptors (Howlett et al., 2002), suggesting that their effects could differ. Therefore, we cannot exclude the possibility that THC antinociception is partially mediated by peripheral actions. After all, CB1 receptors are expressed

on most peripheral nociceptors (Mitrirattanakul et al., 2007), and selective activation of peripheral CB1 receptors reduces NP (Milligan et al., 2020; Seltzman et al., 2016).

Implications, limitations, and future directions

We showed for the first time that THC reduces mechanical allodynia and pronociceptive ON cell activity in rats with NP and tolerance to morphine. These findings represent early proof of concept supporting further research into the use of THC as an analgesic in patients with NP who have been previously treated with opioids. The inefficacy, side effects, and risks of abuse and overdose that are associated with long-term opioid use emphasize the need to identify novel approaches to pain management that avoid the use of strong opioids. Researchers and physicians have suggested cannabis as a potential alternative to opioids in the management of pain and as a tool to combat the opioid crisis (Lucas, 2017; Reiman et al., 2017). Still, there is not enough efficacy and safety data available for all physicians to confidently prescribe cannabinoids to their patients with NP. In particular, there is a lack of controlled clinical studies evaluating the analgesic potential of CBD (Svensson, 2020), and more preclinical findings are needed in order to inform clinical studies. The results presented here should therefore guide and encourage future research, both preclinical and clinical, into the potential use of cannabinoids in the treatment of NP. In Canada, easy access to cannabis products enables patients to self-medicate with cannabinoids, even when alternative treatments are available, making it increasingly important to produce research that can inform physicians and users alike of the potential risks and benefits of cannabis use in the context of chronic pain.

While the cannabis plant contains both THC and CBD at varying ratios, we chose to evaluate the effects of these cannabinoids separately. It is important to develop an in-depth understanding of both THC and CBD since they act through separate mechanisms (outlined throughout this thesis) and often exert different effects. Indeed, the findings presented here indicate that we are at different stages of understanding the role of THC compared to CBD in pain treatment. Specifically, we have learned that THC reduces NP in morphine-tolerant rats, suggesting that we are in need of controlled, clinical studies evaluating its analgesic potential in humans that have developed tolerance to opioids. On the other hand, it appears that the analgesic capabilities of CBD may be much more complex. Our results indicate that CBD does not reduce mechanical allodynia, despite its characteristically antinociceptive effects on ON cells. It is true that the evaluation of mechanical allodynia in isolation may not appreciate the complexity pain and analgesia. Thus, future research into CBD as a potential treatment for NP should aim to understand which components of pain (i.e., allodynia, hyperalgesia, spontaneous pain, or affective/motivational pain), if any, are modulated by CBD. More preclinical research that includes multiple measures of pain and focuses on mechanism of action is therefore needed in order to inform future clinical studies into the use of CBD for the treatment of NP.

Although a clear understanding of the physiological effects exerted by THC and CBD in isolation is beneficial in allowing more precise approaches to treatment, it is also true that THC and CBD appear to exert a synergistic effect when administered in combination (Casey et al., 2017; Comelli et al., 2008; King et al., 2017). On top of this, it has been suggested that plant cannabis produces an "entourage effect"; that is, a unique therapeutic effect produced by some combination of THC, CBD, and the other active constituents contained in the cannabis plant (terpenes,

flavonoids, amino acids, and fatty acids, to name a few) (Bonn-Miller, ElSohly, Loflin, Chandra, & Vandrey, 2018). It will therefore be worthwhile to better understand the therapeutic effects of THC/CBD combinations and plant cannabis, especially given its widespread availability.

Despite mounting evidence of its therapeutic benefits, the potential risks and harms of cannabis use should not be understated. In fact, it has been suggested that the risks of cannabinoids may outweigh their potential benefits in the treatment of NP. Possible adverse effects associated with cannabis use include "feeling high", sedation, confusion, and psychosis (Allan et al., 2018; Mucke, Phillips, Radbruch, Petzke, & Hauser, 2018). Indeed, the sedative effects of THC and CBD were not assessed in this study, and previous studies have confirmed that anti-allodynic doses of THC (but not CBD) can produce sedation in rats (Casey et al., 2017). Although sedative effects may interfere with behavioural testing of mechanical allodynia, rats used in this study that appeared sedated were woken up by the experimenter as needed. It is also true that we did not evaluate the effects of chronic cannabinoid treatment, even though most patients with NP require long-term medication to manage their symptoms. It has previously been shown that repeated THC administration induces tolerance to its anti-nociceptive effects in neuropathic animals (Abraham et al., 2019; Henderson-Redmond et al., 2020) via desensitization and downregulation of CB1 receptors (Sim-Selley, 2003). Thus, it will be important to determine whether the efficacy that we observed persists through repeated administration. Of note, the involvement of β-arrestin has been implicated in the development of tolerance at both MOR and CB1 receptors (Raehal & Bohn, 2014). That is, β-arrestin binding to these receptors contributes to desensitization by facilitating uncoupling from the G protein in multiple CNS regions, including the PAG (Bohn et al., 2000;

Nguyen et al., 2012). In mice genetically lacking β-arrestin2, tolerance to both morphine (Bohn et al., 2000) and THC (Nguyen et al., 2012) is attenuated. Despite this common mechanism of tolerance, some studies have reported that THC antinociception is actually enhanced in morphine-tolerant rodents (Cichewicz et al., 1999; Rubino et al., 1997; I. J. Williams et al., 2006). As mentioned previously, THC pre-treatment also appears to enhance morphine antinociception (Cichewicz et al., 1999; F. L. Smith et al., 1998; Wilson-Poe et al., 2013). This bidirectional relationship between opioids and cannabinoids has led some authors to propose that alternating administration of these substances could provide long-term, effective analgesia, while bypassing antinociceptive tolerance (Wilson, Maher, & Morgan, 2008; Wilson-Poe et al., 2013). The results reported here provide support for this theory by demonstrating that THC anti-allodynia and modulation of descending pain circuitry is unaffected by morphine tolerance. While opioid rotations have long been used to avoid the problem of tolerance, opioid-cannabinoid rotations are a fairly new concept that merit more exploration. Furthermore, even if THC is a valid alternative to opioids to avoid tolerance, this study did not evaluate the addiction liability of THC compared to opioids, presenting another possible limitation in its use. However, CBD is believed to attenuate the rewarding properties of opioids (Wiese & Wilson-Poe, 2018), and cannabis use decreases opioid dose in patients with chronic pain (Lynch & Clark, 2003), suggesting that cannabis-based adjuvants could actually minimize opioid abuse liability.

Finally, it should be mentioned that these experiments were selectively performed on male rats, which presents some limitations to the potential applications of our findings. Clinically, female patients are more likely than males to experience a chronic pain condition (Mogil, 2012).

Instead, research suggests that morphine antinociception is stronger in male rats (Holtman, Sloan, & Wala, 2004), who also display higher MOR expression in the PAG (Loyd, Wang, & Murphy, 2008). On the other hand, female rats have been reported to have more PAG-RVM projections than males, but morphine administration activates a higher proportion of these projections in males than females (Loyd & Murphy, 2009). Finally, a recent study reported that THC, CBD, and their combined administration, prevented the development of NP in male, but not female, mice (Linher-Melville et al., 2020), revealing a profound sex difference in cannabinoid analgesia. It will therefore be of great importance to replicate our results in female animals before applying these findings to human patients.

Summary and Conclusions

Neuropathic pain (NP) represents a major public health problem, with limited treatment options that provide effective pain relief without imposing severe side effects and safety risks. Since the legalization of cannabis in Canada, it is increasingly easy for patients to self-medicate with THC and CBD for chronic pain and other conditions. Therefore, it is of great clinical importance to understand the contexts in which cannabinoids can be effectively used to treat NP, as well as their mechanism of action. This thesis explored the anti-allodynic potential of THC and CBD to treat NP in morphine-naïve and morphine-tolerant subjects. We showed that THC (2.5 mg/kg) significantly reduces mechanical allodynia in both morphine-naïve and morphine-tolerant rats in the SNI model of NP. Conversely, CBD does not reduce mechanical allodynia in neuropathic rats at either dose of 20 mg/kg or 50 mg/kg. We also examined the effects of these cannabinoids on the activity of descending pain modulatory circuitry. We showed that tolerance to morphine does not alter the spontaneous firing rate or pinch-evoked burst/pause rate of ON or OFF cells in the RVM of neuropathic rats. Furthermore, intra-PAG administration of THC (10 μg) and CBD (1 μg) both decrease the spontaneous firing rate and pinch-evoked burst rate of ON cells in morphine-naïve and morphine-tolerant neuropathic rats. However, neither THC nor CBD modulate the spontaneous firing rate or pinch-evoked pause rate of OFF cells in morphine-naïve or morphine-tolerant neuropathic rats. Together, the results reported here support a potential use for THC in the treatment of NP in subjects with tolerance to morphine. Additionally, our data suggest that, although CBD influences descending modulation of pain, its acute administration does not reduce mechanical allodynia in neuropathic rats. These findings highlight the complicated nature of pain processing and emphasize the need for more research into cannabinoid safety and

efficacy in the treatment of sensory and affective components of pain, in both preclinical and clinical settings.

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