

UNIVERSITY OF CALGARY

Non-Psychotropic cannabinoids attenuate visceral pain in colitis

by

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## Abstract

The inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis, are complex chronic diseases that affect an increasing proportion of the population. Abdominal pain is a major clinical symptom, but current treatments are limited and a source of frustration for patients, many of whom seek alternatives such as cannabis. Cannabis contains many compounds with therapeutic potential that do not have the prohibitive psychotropic effects of tetrahydrocannabinol (THC). These non-psychotropic cannabinoids (npCBs) have a variety of effects including analgesia and anti-inflammatory actions and show potentiating effects when administered in combination. This project explored the analgesic effects of cannabichromene (CBC), cannabidiol (CBD), cannabidivarin (CBDV), and cannabigerol (CBG), individually and in combination, in the treatment of colitis-evoked visceral hypersensitivity using the acute dextran sulfate sodium model. Abdominal pain was quantified by electromyographic recordings of the reflexive contraction of the external oblique muscle in response to colorectal distension using an animal of experimental colitis. Activation of the spinal cord was assessed using immunohistochemistry to the neuronal activity marker c-Fos in neurons of the lumbosacral dorsal horn. A single injection intraperitoneal injection of 10 mg/kg of either CBD or CBG was found to reduce both nocifensive behaviors in the functional assay and c-Fos activity in spinal cord in animals with colitis. Similarly, a combination of npCBs consisting of 5 m/kg CBD with 1 m/kg each CBC, CBDV, and CBG—all sub-therapeutic dosages—reduced both measures to the level of untreated control animals. Investigations of mechanism of actions via whole-cell patch-clamp electrophysiology of primary dorsal root ganglia neurons revealed CBD to act via a voltage-gated calcium channel with preliminary evidence indicating a high-voltage activated isoform. Preliminary data also suggest that the mixture of npCBs may act through a similar

mechanism. These results suggest CBD, CBG, and a mixture of npCBs may be beneficial in managing pain associated with IBD.

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## List of Abbreviations

2-AG	2-arachidonylglycerol
AC	Adenylyl Cyclase
AEA	Anandamide
AP	Action Potential
ATP	Adenosine Triphosphate
BDS	Biological Drug Substance
BK	Bradykinins
Cav	Voltage-Gated Calcium Channel
CB <sub>1</sub>	Cannabinoid Receptor Type 1
CB <sub>2</sub>	Cannabinoid Receptor Type 2
CBC	Cannabichromene
CBD	Cannabidiol
CBDV	Cannabdivarin
CBG	Cannabigerol
CBN	Cannabinol
CD	Crohn's Disease
CGRP	Calcitonin Gene-Related Peptide
CNS	Central Nervous System
COX-2	Cyclooxygenase-2
CRD	Colorectal Distension
DAGL	Diacylglycerol Lipase
DAI	Disease Activity Index
DHP	Dihydropyridines
DNBS	Dinitrobenzene Sulfonic Acid
DRG	Dorsal Root Ganglia
ECS	Endocannabinoid System
ENS	Enteric Nervous System
EPSC	Excitatory Presynaptic Current
ExPANs	Extrinsic Primary Afferents
FAAH	Fatty Acid Amide Hydrolase
FO	Fish Oil
GDNF	Glial Cell Derived Neurotrophic Factor
GI	Gastrointestinal
GIRKs	G-Protein Coupled Inwardly Rectifying Potassium Channels
GPCR	G-Protein Coupled Receptor
HBSS	Hank's Balanced Salt Solution
HVA	High-Voltage Activated
IBD	Inflammatory Bowel Diseases
LPS	Lipopolysaccharides
LVA	Low-Voltage Activated
MAGL	Monoacylglycerol Lipase
NAAA	N-Acylethanolamine Acid Amide Hydrolase
NAPE-PLD	N-acyl phosphatidylethanolamine-phospholipase D
Nav	Voltage-Gated Sodium Channel

NBA	Neurobasal A
NF- $\kappa$ B	Nuclear Factor-Kappa B
NGF	Nerve Growth Factor
npCBs	Non-Psychotropic Cannabinoids
ODN	Oligodeoxynucleotides
PAG	Periaqueductal Gray
PEPD	Paroxysmal Extreme Pain Disorder
PGE2	Prostaglandin E2
PKC	Phosphokinase C
PPAR	Peroxisome Proliferator Receptor
RVM	Rostroventral Medulla
SOD	Super Oxide Dismutase
SP	Substance P
THC	Tetrahydrocannabinol
THCV	$\Delta^9$ -tetrahydrocannabidiol
TNF	Tumor Necrosis Factor
TrkA	Tropomyosin Receptors Kinase A
TRP	Transient Receptor Potential
TRPM8	Transient Receptor Potential Melastatin 8
TRPV1	Transient Receptor Potential Vanilloid 1
TRPV2	Transient Receptor Potential Vanilloid 2
TRPV4	Transient Receptor Potential Vanilloid 4
TTX	Tetrodotoxin
Tukey HSD	Tukey Honestly Significant Difference
UC	Ulcerative Colitis
VMR	Visceral Motor Response

## **CHAPTER ONE: INTRODUCTION.**

This chapter will provide the background for the project. Beginning with an explanation of the inflammatory bowel diseases (IBD) and the need for alternative treatment options for the associated visceral pain. Next, how painful stimuli are transduced and transmitted to the CNS is discussed as well as key ion channels related to this process. Finally, the four non-psychotropic cannabinoids addressed in this project—cannabichromene (CBC), cannabidiol (CBD), cannabidivarin (CBDV), cannabigerol (CBG), and their combinations—are discussed within this framework.

### **1.1 Inflammatory bowel diseases and cannabis**

The inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are characterized by chronic relapsing inflammation of the gastrointestinal (GI) tract accompanied with weight loss, diarrhea, bloody stools, and abdominal pain. A key difference between the two diseases is the localization of inflammation within the GI tract. UC primarily affects the mucosal layer of the colon and rectum in a continuous fashion, while CD affects all layers of the bowel wall and can affect any part of the GI tract in a discontinuous fashion (Kobayashi et al., 2020).

In Canada, typical onset of IBD occurs between the ages of 20 and 30; however, IBD can be diagnosed at any age. CD shows a slight sex bias with a female to male diagnosis ratio between 1.2 and 1.3. UC however, shows no sex bias (Kaplan et al., 2019). The incidence rate within Canada is currently (2019) estimated at 0.7% of the population but this rate is expected to rise to 1% by 2030 (Kaplan et al., 2019)

Pain is a highly prevalent symptom in IBD patient populations. It is estimated that approximately 70% of IBD patients experience pain during active inflammation and 30 – 50% of patients develop chronic pain, regardless of whether the disease has resolved or relapsed (Hurtado-Lorenzo et al., 2021; Weaver & Szigethy, 2020). IBD pain impacts more than the lower GI tract. For example, cross-organ hypersensitization has been reported to include the skin, muscles, bladder, and uterus (Qiao & Tiwari, 2020). Additionally, the transition from acute to chronic pain is accompanied by an increased risk of comorbid psychiatric disorders and other psychological issues such as sleep disturbances and anxiety that can exacerbate pain perception (Weaver & Szigethy, 2020).

IBD has a profound impact on the quality of life of patients. A 2018 study on the impact of IBD in Canada reported an overall decrease in patient's quality of life for at least some time of the disease and a chronic decrease for many patients. Disease severity was reported as the main predictor of quality of life with pain and diarrhea as key symptoms (Jones et al., 2019). IBD also affects patients psychologically with many suffering from fear and anxiety during remission, a lack of need fulfilment and achieving personal goals such as career or family building, social isolation, and fear of stigmatization and dependency. This psychological distress extends to caregivers and family members who also report decreased quality of life (Jones et al., 2019).

The pathogenesis of IBD is not completely understood but appears to be related to a complex interaction of genetics, immune, environmental, and microbial factors (Ananthkrishnan et al., 2018; Kobayashi et al., 2020; Lavelle & Sokol, 2020; Levine et al., 2018). Treatment options for IBD include, steroids, immunomodulator, anti-tumor necrosis factor drugs (TNF) and other so-called biologicals, most of which have undesirable side-effects (Kobayashi et al., 2020; Roda et al., 2020). Pain management in IBD is complicated as non-steroidal anti-inflammatory drugs and

narcotics, the mainstays of chronic pain management, may worsen IBD symptoms (Lamb et al., 2019).

Many IBD patients are dissatisfied with their current treatment options and have sought alternative measures such as cannabis. A survey of 313 IBD patients completed at the University of Calgary between 2008 and 2009 exploring their cannabis usage reported 17.6% of patients to use cannabis for symptom management. This is notable because this survey was completed prior to recreational cannabis legalization in Canada. Of those 17.6%, 91.1% reported some improvement with their IBD symptoms while only 3.6% reported no improvement with cannabis. Additionally, 76.8% reported reduced abdominal cramping, 48.2% reduced joint pain, 83.9% reduced abdominal pain, and 28.6% reported reduced diarrhea (Storr et al., 2014). Regardless of usage, pain and diarrhea were reported to be the most problematic symptoms. When asked about their motivation for trying cannabis, 26.8% reported that their current medication did not help with their symptoms, 43% reported fewer symptoms with cannabis than steroids, and 82% stated that they preferred cannabis to steroids (Storr et al., 2014).

Similarly, a 2019 study of IBD patients with existing medical cannabis prescriptions in Israel showed similar results. Of 127 patients, 56.7% smoked dried cannabis, 16.5% consumed a cannabis oil, and 12.6% consumed through other forms of inhalation. Patients consumed an average of 21mg tetrahydrocannabinol (THC) and 170mg cannabidiol (CBD) per day or about 30g of dried cannabis per month. Cannabis consumption was associated with improved quality of life and reduction in the usage of other medications. Common side-effects reported included dry-mouth (63%) and memory issues (34%) (Naftali et al., 2019).

Finally, two recent randomized control studies examined cannabis in IBD patients in Israel. CD patients were given an oral CBD/THC oil at a dose of 80mg CBD and 20mg THC twice per

day. UC patients smoked a 0.5g cannabis cigarette (containing 16% THC and <0.5% CBD and other cannabinoids) twice per day. Both studies showed similar results: No effect was observed on the inflammatory markers c-reactive protein and calprotectin nor on the endoscopic score in either study. However, significant improvements in quality of life and abdominal pain were reported in both studies with memory issues reported as the most common side-effect (Naftali, Bar-Lev Schleider, Almog, et al., 2021; Naftali, Bar-Lev Schleider, Scklerovsky Benjaminov, et al., 2021). Based on these studies it appears that cannabis provides some relief for IBD patients; however, an important consideration is that a major barrier to cannabis usage was the psychotropic effects.

Cannabis has been used medicinally for centuries and the psychotropic effects are largely attributed to a single constituent, THC, among >100 cannabinoids identified in the plant. Cannabis (*Cannabis sativa*) has been cultivated to produce fibers, food, oil, and for recreational, religious, and medicinal purposes for many centuries (Bonini et al., 2018). One of the earliest recorded medical usages was in 2700 BCE China where it was used as an appetite stimulant. In the 2<sup>nd</sup> century cannabis was used to manage chronic pain in Greece. The earliest record of its use to manage visceral pain comes from England in the 11<sup>th</sup> century. In 1888, cannabis was given to Parkinson's patients to aid with pain and spasticity. More recently, cannabis derivatives Marinol (first approved in the United States in 1985) and Sativex (first approved in the United Kingdom in 2003) have been used as anti-nauseants in cancer and for pain and spasticity management in multiple sclerosis respectively (Russo, 2015). Sativex is an oromucosal spray containing equal parts THC and CBD. While this medication has shown great therapeutic utility in many domains such as sleep and pain in patients with multiple sclerosis, the accompanying psychotropic side-effects severely limit its applications and usage (MacCallum & Russo, 2018;

Russo et al., 2007). Importantly, these psychotropic effects can be largely avoided. Presently, over 100 biologically active compounds have been identified in cannabis. The overwhelming majority of these compounds show little to no psychotropic effects and are termed non-psychotropic cannabinoids (npCBs).

## **1.2 Gastrointestinal innervation**

The GI tract is innervated both intrinsically and extrinsically. The intrinsic component comprises the enteric nervous system (ENS) which is part of the autonomic nervous system and, within the large and small intestine, can generate its own responses independent of the central nervous system (CNS). The ENS is composed of many continuous nerve networks and ganglia that occur throughout the GI tract and are grouped into the myenteric and submucosal plexuses. The myenteric plexuses are located within the muscle layers of the GI tract and are responsible for generating the rhythmic proximal contraction and distal relaxation required for peristaltic propulsion of a bolus through the large and small intestine. The submucosal plexus is located within the submucosa of the GI tract and is involved in secretion and absorption (Abdullah et al., 2020; Furness, 2012; Spencer & Hu, 2020).

Extrinsic innervation of the GI tract connects the gut to the CNS. Of greatest relevance to visceral pain is the extrinsic primary afferent (ExPANs) innervation of the gut which has two major anatomical pathways: the vagal afferents and the spinal afferents. The vagal afferents have their cell bodies within the nodose and jugular ganglia and project to the nucleus tractus solitarius in the brain stem. The functions of this population include the monitoring of stomach volume, intestinal nutrients and transmitting nonpainful sensations such as nausea and satiety.

Interestingly, vagal afferents may be involved in the descending modulation of nociceptive signaling at the level of the dorsal horn via the periaqueductal gray (PAG) and rostroventral medulla (RVM) (Abdullah et al., 2020; Drewes et al., 2020).

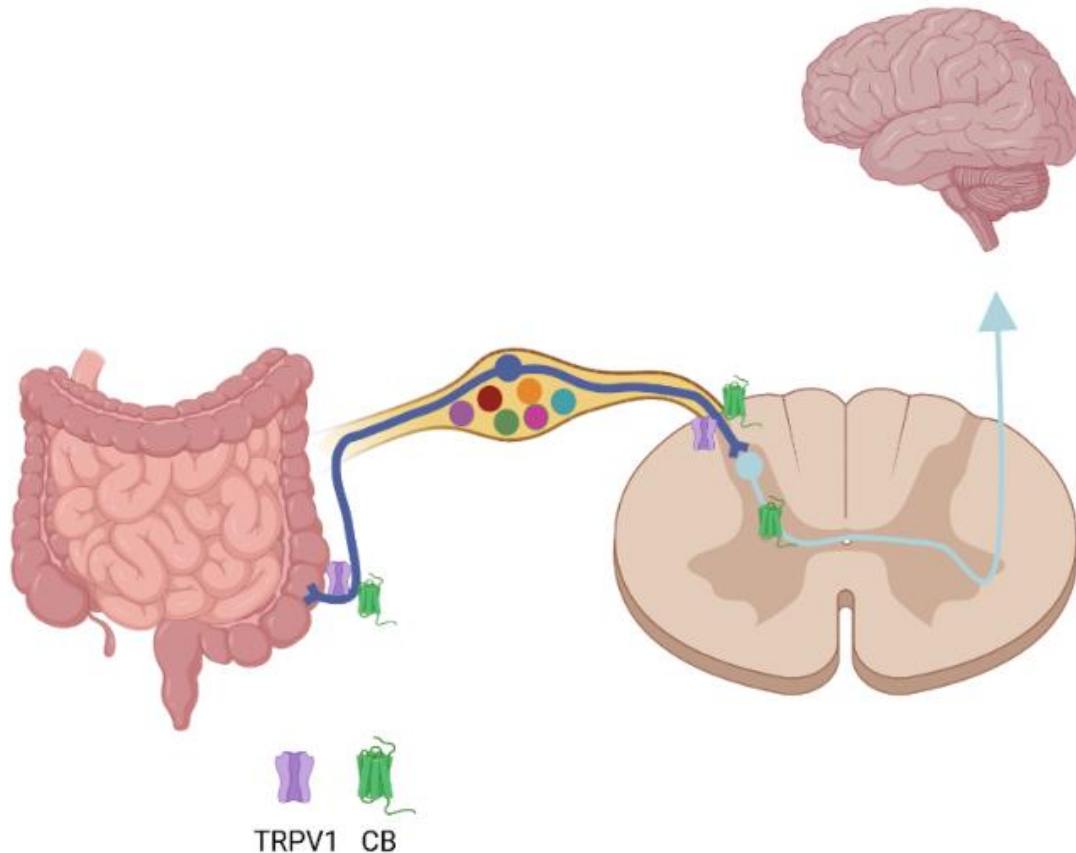
Spinal afferents are the predominant transmission pathway for visceral pain. Their cell bodies are located within the dorsal root ganglia (DRG) and possess a bifurcated axon, which projects from the upper GI tract and small intestine to the thoracolumbar level of the spinal cord via the splanchnic nerve while those innervating the distal colon and rectum project to the lumbosacral level of the spinal cord via the pelvic nerve. Additionally, so-called silent afferents which are inactive under normal physiological conditions will transduce nociceptive signals once they become sensitized under inflammatory conditions (Abdullah et al., 2020; Drewes et al., 2020).

There are four major subtypes within these DRG fibers. Proprioceptors have large diameter axons and are heavily myelinated resulting in a fast action potential conduction velocity (~30-70 m/s) and respond to muscle twitch. Similarly, A $\beta$  fibres have large diameter axons and are heavily myelinated with a fast conduction velocity (~30-70 m/s) and respond to low threshold mechanical stimuli. A $\delta$  fibres have medium diameter axons, are lightly myelinated with a medium conduction velocity (~30 m/s) and are responsible for the fast sharp pain associated with injury that activates protective reflexes. Finally, C-fibers have narrow unmyelinated axons resulting in a slow conduction velocity of ~2 m/s. These fibers are polymodal and sense temperature, tissue damage, chemical irritants, and mechanical stress and provide the slow, dull pain sensation (Abdullah et al., 2020).

C-fibers are further divided into peptidergic and non-peptidergic fibres. The defining difference is that peptidergic C-fibres can release neuropeptides such as of substance P (SP) and

calcitonin gene-related peptide (CGRP) both of which have pronociceptive and proinflammatory roles. Non-peptidergic C-fibers terminate in inner laminae II of the dorsal horn while peptidergic C-fibers terminate in laminae I and outer laminae II. Approximately 65-95% (species dependent) of these peptidergic C-fibers express the non-selective cation channel TRPV1 (Abdullah et al., 2020).

Once the nociceptive signal is transduced at the nerve endings, it travels along the axon to the spinal cord where the primary afferents synapse onto secondary afferents within lamina I and II of the dorsal horn (Figure 1). At this level, the signal can be amplified or diminished by descending modulation driven by the PAG and RVM. Interestingly, the transient receptor potential vanilloid 1 (TRPV1) and the endocannabinoid receptor cannabinoid receptor type 1 (CB<sub>1</sub>) are highly expressed in these controlling regions and studies show this mechanism to potentially underlie some analgesic effects observed in cannabis administration (Lau & Vaughan, 2014; Maione et al., 2011; Starowicz & Finn, 2017).



**Figure 1.** A simplified schematic of the spinal ascending pain pathway. npCBs may act to desensitize TRPV1 (purple) reducing the amount of signal transduced. Additionally, through the ability of some npCBs to modify endocannabinoid tone, CB<sub>1</sub> receptors (green) may be activated to decrease the amount of nociceptive signal transduced. Created with BioRender.com.

The primary afferents synapse on to second order neurons within the dorsal horn and ascend through several pathways. The primary pathway is the spinothalamic tract which terminates in the thalamus. Nociceptive signals also ascend via the spinoparabrachial pathways which project to the brain via the PAG and RVM and thus may be involved in descending modulation of nociception. Additionally, these pathways project to the insula cortex which is involved in the integration of visceral sensory and motor information as well as the anterior

cingulate cortex and prefrontal cortex which are involved in the emotional and cognitive experience of pain. Finally, some signals are transmitted along the spinoreticular tract which projects to the reticular formation to mediate arousal and autonomic responses to nociception (Drewes et al., 2020). One of the key regulatory systems of pain control is the endocannabinoid system.

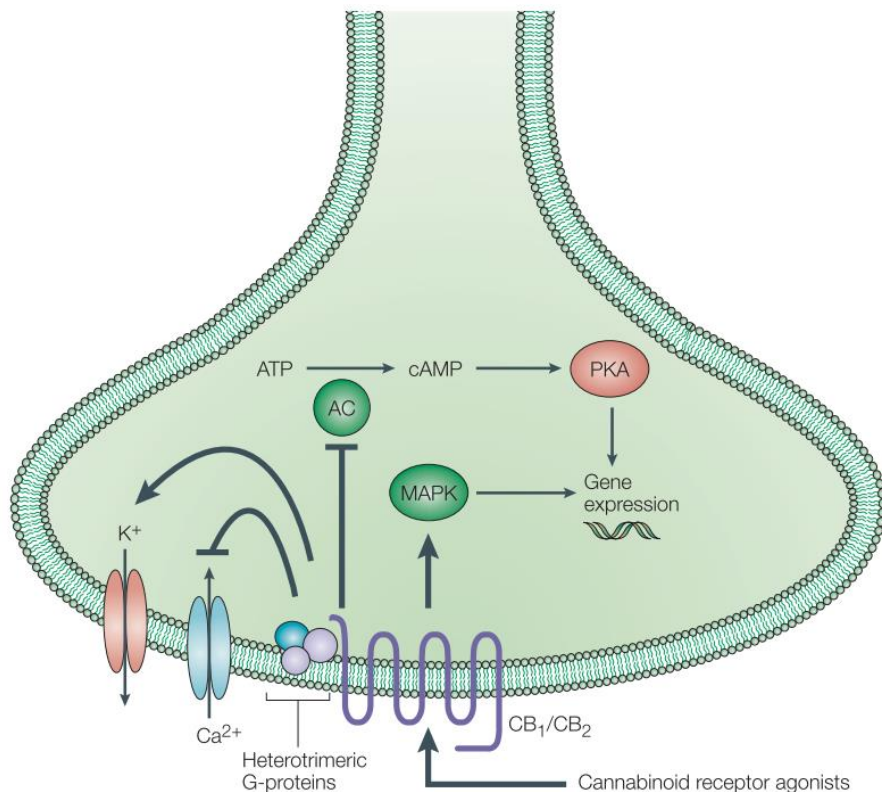
### **1.3 The endocannabinoid system and pain**

The discovery of the endocannabinoid system (ECS) followed from the isolation of THC in 1964 (Gaoni & Mechoulam, 1964). While behavioral experiments showed that this compound was key to the psychotropic effects of cannabis, an endogenous receptor for this compound would not be discovered until 24 years later and termed the cannabinoid receptor 1 (CB<sub>1</sub>) (Devane et al., 1988). Four years later, an endogenous ligand for this receptor termed N-arachidonylethanolamine or anandamide (AEA) was discovered (Devane et al., 1992), followed quickly by the cannabinoid receptor 2 (CB<sub>2</sub>) (Munro et al., 1993) and the second classical ECS ligand 2-arachidonylglycerol (2-AG) (Mechoulam et al., 1995). These two ligands and receptors along with their biosynthetic and degradation enzymes (N-acyl phosphatidylethanolamine-phospholipase D or NAPE-PLD and fatty acid amide hydrolase or FAAH for AEA and diacylglycerol lipase or DAGL and monoacylglycerol lipase or MAGL for 2-AG, respectively) comprise the classical constituents of the ECS (Di Marzo & Piscitelli, 2015).

Stemming from the discovery of the ECS through the actions of THC, the classical ECS does not encompass the sites of actions of most of the >100 cannabinoids present in cannabis. In fact, only THC and  $\Delta^9$ -tetrahydrocannabinol (THC) show significant affinity for the CB

receptors (Pertwee, 2005). In the last 20 years the ECS has been expanded to include several transient receptor potential (TRP) channels, orphan G-protein coupled receptors (GPCRs), peroxisome proliferator receptor (PPARs), additional ligands, as well as multiple enzymatic pathways for the synthesis and degradation of the various endocannabinoids (Cristino et al., 2020; Di Marzo & Piscitelli, 2015).

The two cannabinoid receptors are GPCRs and primarily coupled to  $G_{i/o}$  proteins. Subsequently, they inhibit the actions of adenylyl cyclase (AC), activate G-protein coupled inwardly rectifying potassium channels (GIRKs) and close calcium channels (Figure 2). Interestingly,  $CB_2$  appears to only inhibit AC without affecting ion channels.  $CB_1$  is most commonly found on nervous tissue while  $CB_2$  is predominantly expressed in immune cells such as microglia and mast cells (Alexander, 2015). Importantly, under inflammatory conditions, both CB receptors are upregulated and  $CB_2$  is expressed in neurons of the dorsal horn (Davis, 2014). Thus, activation of these channels inhibits neurotransmission and dampens the immune response making the ECS a prime target for the modulation of pain and inflammation.



**Figure 2.** Canonical cannabinoid receptors signaling pathways. Both CB<sub>1</sub> and CB<sub>2</sub> receptors are GPCRs coupled to Gi/o proteins. The activation of these receptors results in the opening of G-protein coupled inwardly rectifying potassium channels and closing of calcium channels resulting in decreased excitability. From Di Marzo et al., 2004 with permission.

The ligands of the ECS have many targets and degradation pathways. Both AEA and 2-AG will bind the CB receptors, with 2-AG showing greater affinity. Additionally, both AEA and 2-AG inhibit the purinoceptors P2X<sub>2</sub> and P2X<sub>2/3</sub>, producing analgesic effects. AEA will also bind PPAR- $\alpha$  decreasing nuclear factor-kappa B (NF- $\kappa$ B) signaling subsequently reducing cytokine production. Also, AEA was the first endogenous ligand of TRPV1 discovered and acts to desensitize the channel at high concentrations and activate the channel at lower concentrations. This suggests that FAAH inhibitors would be good analgesic targets; however,

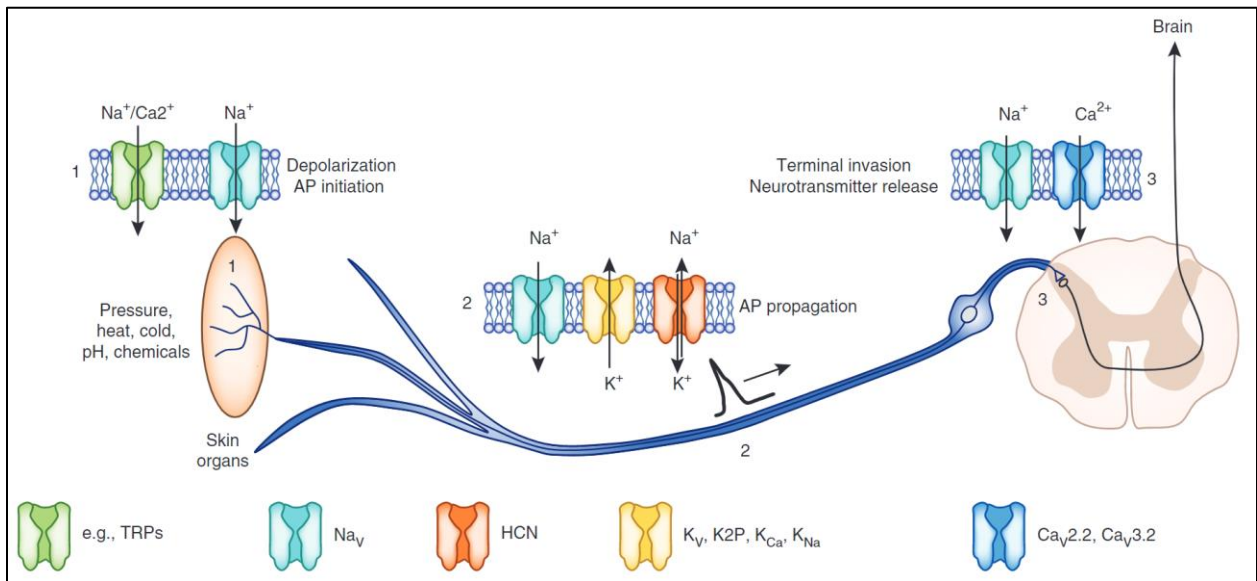
they are frequently accompanied by undesirable side-effects. CBD acts as FAAH inhibitor (De Petrocellis et al., 2011) but despite being well tolerated, FAAH inhibition can shunt AEA degradation towards alternative pathways with both beneficial and deleterious effects (Cristino et al., 2020; Davis, 2014).

Degradation of AEA is primarily through FAAH yielding rather benign metabolites; however, alternative degradation pathways exist that result in much more active compounds. AEA as well as 2-AG can be degraded by cyclooxygenase-2 (COX-2) to yield endoperoxides which can be acted on by prostaglandin synthases to produce the proinflammatory and pronociceptive compounds prostamide  $F_{2\alpha}$  (Ligresti et al., 2014) and prostaglandin E2 glycerol (Hu et al., 2008) respectively. Interestingly, CBD is also able to inhibit COX-2 via the activation of PPAR $\gamma$  (Sunda & Arowolo, 2020). AEA can also be degraded by cytochrome P450s to 5,6-epoxyeicosatrienoic acid ethanolamide a potent TRP vanilloid 4 (TRPV4) and CB $_2$  agonist resulting in dampening of the inflammatory response and decreased nociceptive signaling (Cristino et al., 2020; Davis, 2014; Watanabe et al., 2003). While the ECS is a clear target to modulate nociception, there are many channels along the visceral pain pathway that are also targets for modulation.

#### **1.4 Key ion channels in visceral pain**

There are numerous ion channels involved in the transduction, propagation, and transmission of noxious stimuli to the brain. As shown in Figure 3 noxious stimuli can be transduced by TRP channels and propagated by sodium and potassium channels. Finally, opening of voltage-gated calcium channels in the primary afferent terminal can stimulate

calcium-dependent vesicle fusion resulting in the transmission of the nociceptive signal to the second order neurons in the dorsal horn (Waxman & Zamponi, 2014). This section will outline several key ion channels involved in the visceral pain pathway to provide a framework for how npCBs may interact at these sites to modify nociceptive signaling.



**Figure 3.** Key ion channels in the primary afferent. Nociceptive signals are initially transduced by noxious signal detectors such as TRP channels. If sufficient to initiate an action potential, this signal is transduced along the axon by coordinated opening and closing of  $\text{Na}_v$  and  $\text{K}_v$  channels. Finally, activation of channels such as  $\text{Ca}_v2.2$  results in calcium-dependent vesicle fusion at the primary-afferent and secondary-afferent junction in the dorsal horn transmitting the signal to the spinal cord and subsequently the brain. Adapted from (Waxman & Zamponi, 2014) with permission.

### 1.4.1 Transient receptor vanilloid 1 (TRPV1)

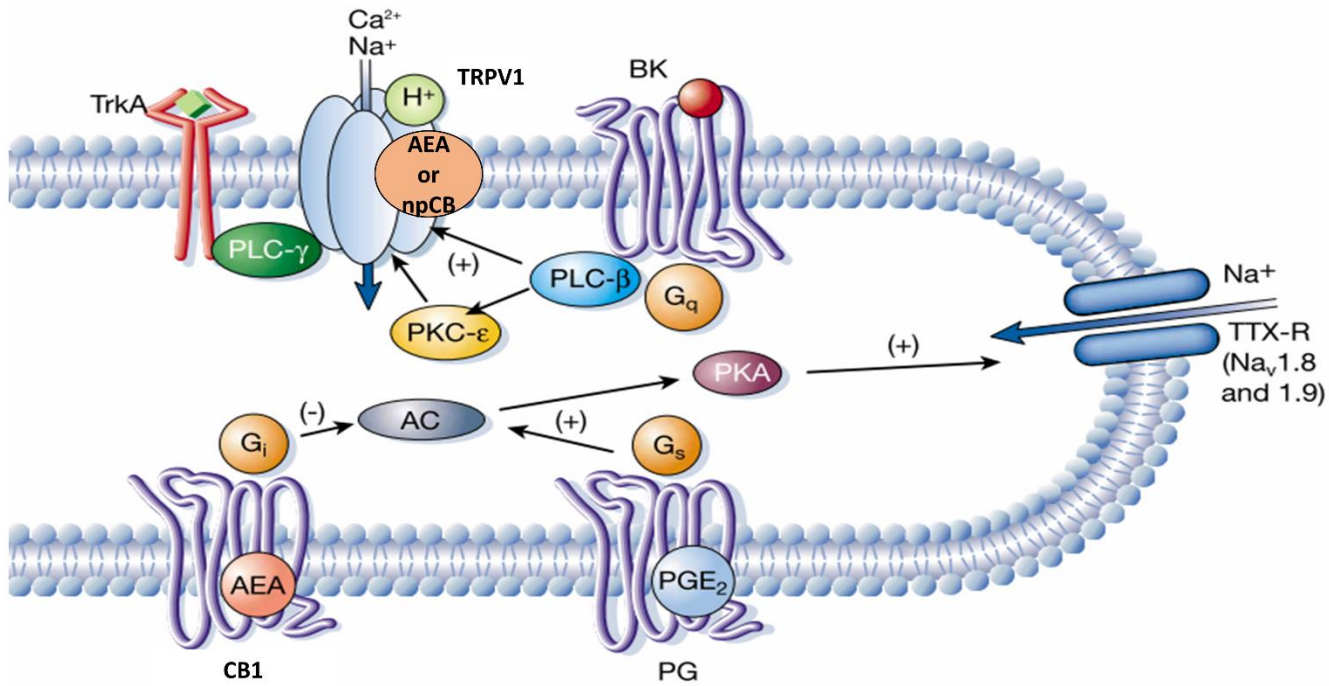
TRPV1 is of keen interest in pain research. This polymodal channel responds to numerous stimuli including heat ( $>43^{\circ}\text{C}$ ), low extracellular pH ( $\text{pH} \leq 5.9$ ), mechanical, and chemicals including capsaicin, the endocannabinoid AEA, and many npCBs. Activation of this channel can result in  $\text{Ca}^{2+}$  influx and depolarization of gut-innervating C fibers, which will directly stimulate CGRP or SP release in the ascending pain pathway (Abbas, 2020; Bourinet et al., 2014).

TRPV1 can become upregulated and sensitized under inflammatory conditions. Nerve growth factor (NGF) stimulates the externalization of TRPV1 via binding of tropomyosin receptor kinase A (TrkA) receptors. Inflammatory molecules such as serotonin, prostaglandins, bradykinins, and adenosine triphosphate activate GPCR signaling pathways to phosphorylate the channel on its intracellular carboxy terminus via phosphokinase A & C (Figure 4). This is a key feature of the peripheral sensitization which drives the visceral hypersensitivity experienced by IBD patients. Paradoxically, TRPV1 demonstrates the counterintuitive property of agonists acting as analgesics. This is achieved through the rapid desensitization of the channel following agonist binding (Abbas, 2020; Bourinet et al., 2014). A common application of this feature can be observed in capsaicin based topical creams for the treatment of muscle soreness and arthritis. Notably, many npCBs bind TRPV1 as an agonist (see Table 1) and have been shown to desensitize the channel (De Petrocellis et al., 2011).

**Table 1.** EC50s of the npCBs of interest as well as capsaicin at TRPV1

Compound	EC50 ( $\mu\text{M}$ )	System	Reference
CBC	$24.2 \pm 3.1$	Human TRPV1 in HEK293	(De Petrocellis et al., 2011)
CBD	$1.0 \pm 0.1$	Human TRPV1 in HEK293	(De Petrocellis et al., 2011)
	30	HEK TRexTRPV1	(Starkus et al., 2019)
CBDV	$3.6 \pm 0.7$	Human TRPV1 in HEK293	(De Petrocellis et al., 2011)
	56	HEK TRexTRPV1	(Starkus et al., 2019)
CBG	$1.3 \pm 0.5$	Human TRPV1 in HEK293	(De Petrocellis et al., 2011)
	19	HEK TRexTRPV1	(Starkus et al., 2019)
Capsaicin	$0.1 \pm 0.003$	Murine TRPV1 in HEK293	(Yin et al., 2019)

TRPV1 most commonly exists as a homotetramer and each monomer consists of 6 transmembrane domains with a hydrophobic pore loop between S5 and S6 (Flynn et al., 2014). Opening of the channel results from conformational changes of an upper and lower gate. In the closed (apo) state, both gates are closed. Different agonists appear to be able to open the two gates to different degrees. For example, a capsaicin crystal structure shows the lower gate and part of the upper gate expanded. While a resiniferatoxin (an ultrapotent TRPV1 agonist) crystal structure shows both gates fully open (Abbas, 2020). Interestingly, recent electrophysiological evidence shows CBD, CBDV, and CBG to activate TRPV1 to a less permeable state than the classic agonist capsaicin (Starkus et al., 2019).



**Figure 4.** Regulation of TRPV1 by npCBs. npCBs directly bind TRPV1 to desensitize the channel. Inflammatory molecules such as bradykinins (BK) bind GPCRs, resulting in phosphorylation of TRPV1 via phosphokinase C (PKC). Additionally, certain npCBs can increase the levels of endocannabinoids such as anandamide (AEA) which can similarly bind and desensitize TRPV1 as well as bind cannabinoid (CB) receptors to decrease the excitability of the cell. TrkA receptor binding of NGF results in trafficking of TRPV1 to the plasma membrane. Modified from Julius & Basbaum (2001) with permission.

#### 1.4.2 Transient receptor ankyrin 1 (TRPA1)

Another important channel in visceral pain is the transient receptor ankyrin 1 (TRPA1). TRPA1 is a calcium ion permeable non-selective cation channel expressed broadly in sensory neurons including colocalization with TRPV1 on peptidergic nodose and spinal ExPANs

innervating the colon and in myenteric motor neurons (Talavera et al., 2020; Vellani et al., 2010). They respond to chemical irritants such as mustard oil (allyl isothiocyanate), mechanical stimulation, and temperature changes and, like TRPV1, can be sensitized by proinflammatory mediators and are subsequently implicated in visceral pain. Also, like TRPV1, agonists of this channel can prove analgesic. For example, activation of this channel by CB agonists WIN 55,212-2 and AM1241 decreases responses to capsaicin and mustard oil. Additionally, many cannabinoids have been shown to activate this channel with similar  $\text{Ca}^{2+}$  responses to mustard oil (Talavera et al., 2020).

### **1.4.3 Voltage-gated sodium channels**

Voltage-gated sodium channels (Navs) are a key family of channels in pain signaling. Navs are composed of a single alpha-subunit which forms the pore as well as beta-subunits which modify the functional properties of the alpha subunits. There are 9 voltage-gated alpha subunits termed Nav1.1 – Nav1.9 as well as non-voltage gated subunit termed Nax which is involved in sensing salts (Bennett et al., 2019).

Each Nav isoform has different properties and thus plays a different role in action potential generation and propagation. Three key isoforms involved in pain signaling are Nav1.7, Nav1.8, and Nav1.9. Nav1.7 is encoded by the SCN9A gene, it is inhibited by tetrodotoxin (TTX), and shows a low activation threshold with slow inactivation kinetics. As a result of these properties, this channel functions to amplify sub-threshold stimuli and is conceptually the gain control on a neuron (Bennett et al., 2019). Under inflammatory conditions, Nav1.7 KO mice show decreased nocifensive behaviors (Nassar et al., 2004). Interestingly, it appears that the role

of Nav1.7 in inflammatory pain is due to an upregulation of the channel (Liang et al., 2013; Strickland et al., 2008; Wada, 2004) and not its phosphorylation which actually decreases its activity (Nassar et al., 2004; Vijayaragavan et al., 2004). Importantly, in the context of visceral pain, Nav1.8 conditional Nav1.7 KO mice showed no differences from controls both in vivo in response to intracolonic application of either capsaicin or mustard oil as well as ex vivo in response to colonic distension (Hockley et al., 2017). This result is even more intriguing when the Nav1.7 channelopathies paroxysmal extreme pain disorder (PEPD) and congenital insensitivity to pain are considered. PEPD is a gain of function mutation of the SCN9A gene which results in an inhibition of fast-inactivation and rapid recovery from inactivation of the channel resulting in a persistent and resurgent sodium current. One of the symptoms of this disorder is severe anal pain. Congenital insensitivity to pain is a loss of function mutation of the SCN9A gene and results in a loss of painful sensation (Bennett et al., 2019). Together, this suggests that in visceral pain overactivity of this channel is deleterious, but another mechanism can compensate for its inhibition.

Another important Nav is Nav1.8. While Nav1.7 helps to amplify the small excitatory post-synaptic potentials to drive the neuron to the firing threshold, Nav1.8 drives the main action potential contributing 58-90% of the inward current during the rising phase of an action potential (AP) in nociceptors. Nav1.8 is encoded by the SCN10A gene and primarily expressed in sensory neurons. It is TTX-resistant, inactivates from open-state slowly and recovers from inactivation rapidly. Nav1.8 shows both fast and slow inactivation kinetics. The slow-inactivating state occurs when a depolarization is applied for more than 10s and is enhanced in nonpeptidergic nociceptors compared to peptidergic nociceptors suggesting that nonpeptidergic fibers show greater adaptation to a sustained stimulus. Fast-inactivation is incomplete which results in small

persistent currents which contribute to increased AP duration, broadening and to current spikes following an AP resulting from the rapid repriming of the channel. Interestingly, human DRG neurons show double the persistent current of rat DRG neurons as well as more spike firings (Bennett et al., 2019; Waxman & Zamponi, 2014). Subsequently, any compound decreasing the firing of this channel has great analgesic potential.

Nav1.8 has a clear role in pain. Nav1.8 KO mice show decreased nocifensive behaviors in response to intra-colonic application of either capsaicin or mustard oil (Laird et al., 2002). Additionally, antisense oligodeoxynucleotides (ODN) targeting Nav1.8 (Joshi et al., 2006) as well as selective pharmacological blockade (Jarvis et al., 2007) of Nav1.8 attenuates mechanical allodynia and thermal hyperalgesia in a CFA model. Inflammation can increase the inward currents through upregulation and phosphorylation of the channel (Figure 4) (Bennett et al., 2019). Numerous studies have examined Nav1.8 in visceral pain (Beyak et al., 2004; K. Bielefeldt et al., 2002; Klaus Bielefeldt et al., 2002). For example, (Yoshimura et al., 2001) used an acetic acid induced model of bladder pain and Intrathecally injected Nav1.8 antisense ODN. Results showed antisense ODN to prevent an increase in bladder contractions, decrease cFos expression in the L6 dorsal horn, and to decrease TTX-R currents in Fast Blue retrogradely-labeled L6-S1 DRG neurons.

Nav1.9 is encoded by the SCN11A gene and is comparatively poorly characterized. Its expression is largely restricted to DRG neurons and the C-fiber population. Within somatosensory populations its expression is biased towards the nonpeptidergic fibers; however, in colonic afferents, its expression is biased towards the peptidergic fibers. It is expressed along the entire length of primary afferents, shows ultra-kinetics, and is activated at hyperpolarized potentials very close to resting membrane potentials. As a result, this channel produces a

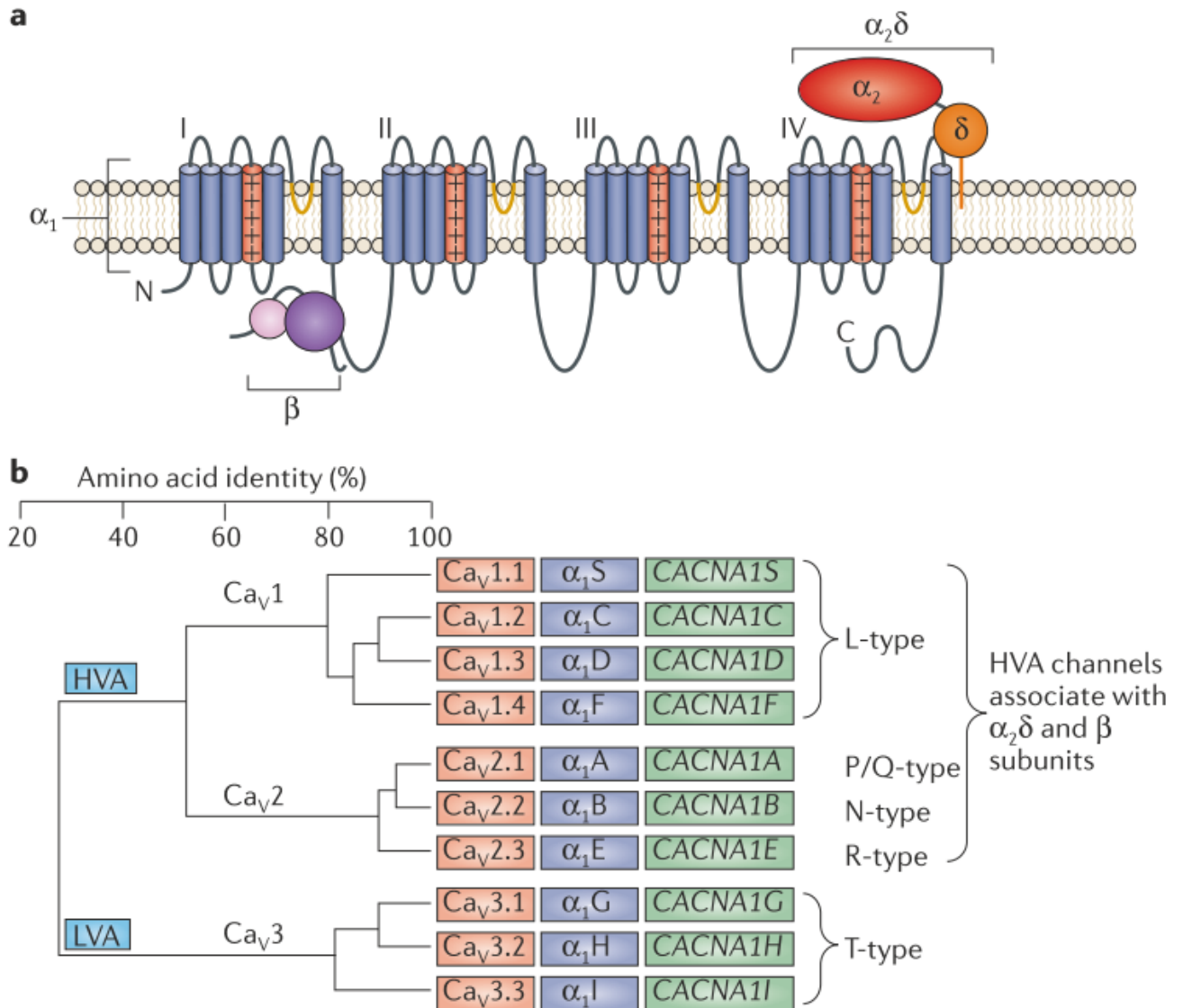
persistent current that serves a similar role as Nav1.7: to amplify subthreshold stimuli. Additionally, increased expression or activity of this channel (such as by inflammatory mediators, see Figure 4) results in an overall increase of the excitability of the cell: decreased rheobase, increased repetitive firing, increased spontaneous activity (Bennett et al., 2019; Waxman & Zamponi, 2014).

Nav1.9 appears to play an important role in visceral pain. Hockley et al., (2014), applied adenosine triphosphate (ATP), prostaglandin E2 (PGE2), an “inflammatory soup”, and supernatants from CD and UC patient biopsies to multiple ex-vivo colon preparations from Nav1.9 KO mice. Multi-unit recordings from splanchnic nerves showed ATP, PGE2, and IBD supernatant induced firing to be significantly reduced in KO mice. In another experiment, inflammatory hyperexcitability was induced with the application of an inflammatory soup mixture. The colon was then stimulated with a 2g von Frey filament. KO mice showed no sign of hyperexcitability. Finally, distension stimuli were applied in a ramping fashion and KO mice showed significantly increased activation threshold when recording from the colonic afferents.

#### **1.4.4 Voltage-Gated calcium channels**

Another important class of channels in pain transmission is the voltage-gated calcium channels (Cav). These channels have a similar structure to Navs: The alpha-1 is the pore-forming subunit that determines the channel subtype. It is composed of 4 homologous domains each with 6 transmembrane segments, and the S4 of each domain serves as the voltage sensor for the channel (Figure 5a). The high voltage activated (HVA) channels also include an  $\alpha_2\delta$  subunit.

Finally, Cavs also associate with beta subunits which function to alter the biophysical properties of the channel (Dolphin, 2012).



**Figure 5.** An overview of voltage-gated calcium channels. **A)** The structure of the Cavs. These channels consist of a single  $\alpha_1$  subunit which is composed of 4 domains each with 6 transmembrane segments, a  $\beta$  subunit, and the high voltage family of Cavs also possess an  $\alpha_2\delta$  subunit. **B)** Subtypes of Cavs (orange) and their associated  $\alpha$  (purple) and genes (green). From (Dolphin, 2012) with permission.

These channels can be broadly categorized as high voltage activated (HVA) and low voltage activated (LVA) (Figure 5b). The HVA channels can be further categorized based on their sensitivity to different toxins. L-type Caves (Cav1.1 – Cav1.4) were the first identified. They are sensitive to dihydropyridines (DHP) and show long-lasting (“L”) currents. L-type channels are involved in the contraction of muscles including the heart but are also found on the soma of neurons and may play a postsynaptic role in pain signaling. Next, N-type Caves (Cav2.2) were identified based on their sensitivity to  $\omega$ -conotoxins GVIA. These non-L type channels are highly expressed in nerve terminals where they are involved in  $\text{Ca}^{2+}$ -dependent neurotransmitter release. Subsequently, inhibition of these channels can decrease transmission of nociceptive stimuli at various junctions along the pain pathway to produce analgesia. Additionally, these channels are regulated by numerous GPCRs including opioid and cannabinoid receptors. P-type Caves (Cav2.1) were identified in Purkinje cells of the cerebellum based on their sensitivity to  $\omega$ -agatoxin IVA. Another  $\omega$ -agatoxin IVA sensitive current was identified in cerebellar granule cells which showed more rapid inactivation than the P-type currents. Initially, this Q-type current was attributed to a separate Cav channel, however, these two currents arise from either splice variants or beta-subunit modification of the same channel and are now typically grouped as P/Q-type Cav and are involved in migraines. Finally, the residual current, can be attributed to the R-type Cav (Cav2.3) which is inhibited by SNX-482 and has a role in regulating neuronal excitability and inflammatory pain (Bourinet et al., 2014; Dolphin, 2006, 2012).

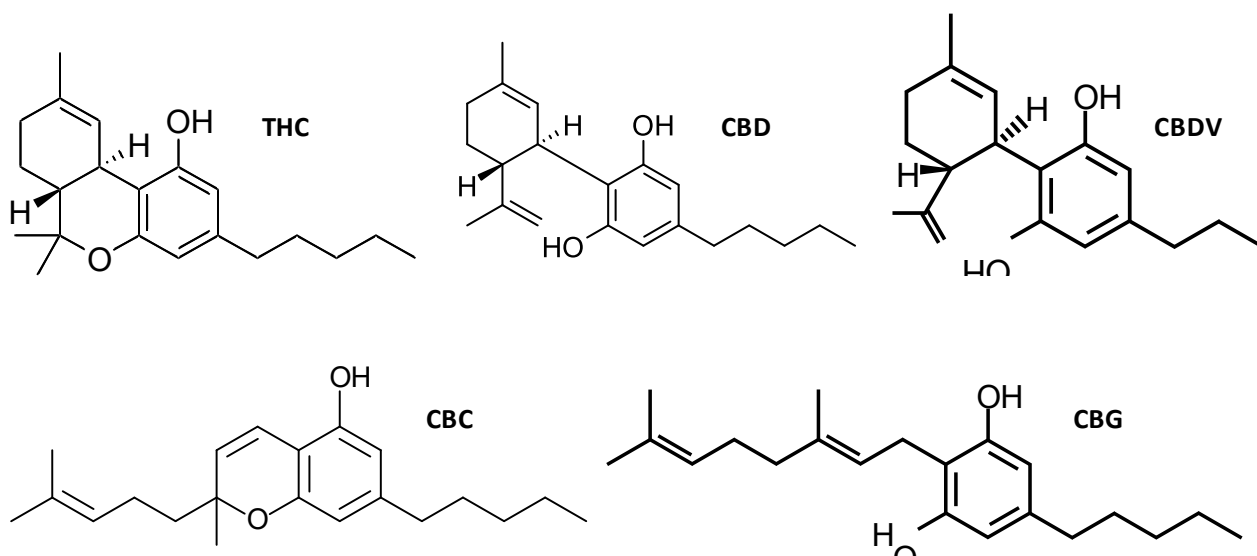
The LVA Caves all belong to the same T-type family (Cav3.1 – Cav3.3). Similar to Nav1.7 and Nav1.9, these channels are activated at hyperpolarized potentials and subsequently play a role in regulating neuronal excitability. However, the exact mechanism for this action is unclear. Some potential mechanisms include T-type channels acting as mechanoreceptors,

facilitating burst firing of colocalized Nav channels, activation of the ERK pathway, lowering firing thresholds, and increasing NT release in the dorsal horn. Interestingly, T-type channels can be directly inhibited by the endocannabinoid AEA (Bourinet et al., 2014; Waxman & Zamponi, 2014). In terms of visceral pain, Marger et al., (2011) demonstrated the importance of Cav3.2. The authors induced IBS-related colonic hypersensitivity by intracolonic administration of butyrate. The rats underwent ramp colorectal distension up to 80mmHg: animals treated with antisense ODN for Cav3.1 and Cav3.3 showed no attenuation of the hypersensitivity; however, knockdown of Cav3.2 prevented hypersensitivity in this model. The authors go on to show that butyrate upregulates Cav3.2 in retrogradely-labeled colonic afferents likely through increased trafficking of the channel to the cell membrane. Importantly, this study also showed Cav3.2 to be the most relevant channel in the butyrate model of visceral hypersensitivity.

With the relevant innervation, systems, and channels outlined, we can now consider how the npCBs may operate within this framework to potentially produce anti-inflammatory and analgesic effects.

### **1.5 Non-Psychotropic cannabinoids**

As previously stated, cannabis contains over 100 biologically active compounds which, except for THC, show little to no psychotropic effects. Generally, the npCBs show little affinity for the classical CB receptors (Pertwee, 2005) and demonstrate many potentially beneficial effects for IBD patients on inflammation, motility, and pain, as will be described in the following sections. Cannabichromene (CBC), cannabidiol (CBD), cannabidivarin (CBDV), and cannabigerol (CBG) are the npCBs of interest for this project (Figure 6).



**Figure 6.** Chemical structures of various cannabinoids. The psychotropic cannabinoid tetrahydrocannabinol (THC) and the non-psychotropic cannabinoids cannabidiol (CBD), Cannabidivarin (CBDV), Cannabichromene (CBC), and Cannabigerol (CBG).

### 1.5.1 Cannabichromene (CBC)

CBC, like other npCBs, shows low affinity for the CB<sub>1</sub> receptor (Pertwee, 2005) and was unable to modify excitatory presynaptic current (EPSC) amplitudes or CB<sub>1</sub> receptor signaling in hippocampal neurons (Straiker et al., 2021). Interestingly, it does show greater affinity for the CB<sub>2</sub> receptor than THC *in vitro*; however, as will be discussed further, *in vivo* it appears to act through a non-CB mechanism (Booker et al., 2009; DeLong et al., 2010a; Ligresti et al., 2016; Udoh et al., 2019). *In vitro* work has shown CBC to be a TRPA1, TRPV1, and TRP vanilloid 2 (TRPV2) agonist as well as an antagonist of TRP melastatin 8 (TRPM8) (de Petrocellis et al.,

2011; Ligresti et al., 2016) and T-type Caves (Mirlohi et al., 2022). Despite its affinity for TRP channels reported in expression systems, CBC was unable to produce significant  $\text{Ca}^{2+}$  responses in cultured rat DRG neurons further suggesting that CBC may act at alternative sites in more complex systems (Straiker et al., 2021).

CBC was tested in a croton oil model of intestinal inflammation, which induces GI hypermotility. Results showed reduced hypermotility within the small intestine. The effect was unaffected by  $\text{CB}_1$ ,  $\text{CB}_2$ , or TRPA1 antagonists. Interestingly, CBC had no effects on transit of control mice suggesting that CBC may be able to reduce hypermotility without inducing hypomotility. However, *ex vivo*, CBC affected the contractions of both inflamed and uninflamed ileum preparations. Interestingly, the effects of CBC were attenuated by  $\omega$ -conotoxin and by verapamil suggesting that CBC may reduce smooth muscle contraction by acting on L-type  $\text{Ca}^{2+}$  channels (Izzo et al., 2012).

(Romano et al., 2013) examined CBC in a dinitrobenzene sulfonic acid (DNBS) model of colitis in male mice. CBC was administered *i.p.* at 1 mg/kg for 2 days following DNBS administration. Results showed CBC to be primarily anti-inflammatory and to reduced intestinal permeability potentially through a TRPA1 mechanism. CBC was also shown to be anti-inflammatory in lipopolysaccharides (LPS) challenged macrophages.

CBC has also been tested in the classic tetrad assay commonly used to evaluate marijuana-like effects of molecules. The tetrad examines locomotor suppression, antinociception, hypothermia, and catalepsy. A tail-flick test showed a small analgesic effect at 100 mg/kg *i.v.* that was not reproduced in a later experiment in the same paper. Overall, results showed weak evidence of analgesic effects not mediated by the  $\text{CB}_1$  receptor and an anti-inflammatory effect not mediated by either the  $\text{CB}_1$  or  $\text{CB}_2$  receptors. Zagzoog et al. (2020) also showed weak

evidence of analgesic effects of CBC in a tail-flick test where 3 mg/kg showed very small analgesic effects, but 10 mg/kg did not. Finally, CBC failed to produce anti-nociceptive effects in an acetic acid model (Booker et al., 2009).

Overall, CBC appears to be anti-inflammatory with weak evidence of analgesic effects and to be able to rectify hypermotility without inducing hypomotility. Unfortunately, no clear mechanism for these effects has been reported with in vitro and in vivo work often contradicting each other. Despite this lack of mechanism, CBC is a promising target for research as it shows many effects which could benefit IBD patients.

### **1.5.2 Cannabidiol (CBD)**

CBD is by far the most studied of the npCBs. Its usage has been examined in the context of psychosis, epilepsy, anxiety, sleep, neurodegenerative diseases, cancer, pain, inflammation, and others. CBD has shown anxiolytic, antidepressant, anti-nausea, antioxidant, anti-inflammatory, and analgesic properties (Iannotti et al., 2014). Like most npCBs, CBD shows low affinity for the orthostatic site of CB receptors but instead acts as a negative allosteric modulator of CB<sub>1</sub> receptors (Straiker et al., 2018). Interestingly, the artificial (+)-enantiomer of CBD shows greater CB<sub>1</sub> mediated inhibition in autaptic hippocampal neurons than the naturally produced (-)-enantiomer (Bosquez et al., 2022). Additionally, CBD interacts with many targets relevant for abdominal pain (Figure 7) including transient receptor potential (TRP) channels (TRPV1, TRPV2, TRPV4, and TRPA1) which it activates and rapidly desensitizes (Iannotti et al., 2014), Nav1.8 (Zhang & Bean, 2021), T-type voltage activated calcium channels (Cavs) (Harding et al., 2023; Mirlohi et al., 2022; Ross et al., 2008a), and Kv7 channels (Zhang et al., 2022). These

effects and sites of action suggest CBD is an excellent candidate for the treatment of abdominal pain in IBD. A summary of the effects of CBD in IBD models is provided in Table 2.

**Table 2.** A summary of the effects of CBD in various IBD and abdominal pain models.

Dose (mg/kg)	Administration Route	Model	Results	Reference
10	I.p. daily for 4 days beginning 1 day prior to TNBS	TNBS	Reduced inflammation: <ul style="list-style-type: none"> <li>• Reduced DAI</li> <li>• Decreased MPO activity</li> <li>• Histology showed greater epithelial integrity, reduced colon thickness, less immunocytes infiltration</li> </ul>	Schicho and Storr, 2012
20	Gavage daily for 4 days beginning 1 day prior to TNBS		n.s. on DAI, MPO activity, or histology	
20	Intrarectal daily for 4 days beginning 1 day prior to TNBS		Reduced inflammation: <ul style="list-style-type: none"> <li>• Reduced DAI</li> <li>• Decreased MPO activity</li> <li>• Histology showed reduced leukocyte infiltration</li> </ul>	
1, 2.5	I.p. daily for 6 days beginning 3 days before DNBS	DNBS	n.s. on colon weight to length ratio and macroscopic damage score	Borrelli et al., 2009
5			Greatest effect of doses tested to reduce macroscopic damage score and colon weight to length ratio  No change in COX-2 expression Reduced overexpression of iNOS  Reduced Nitrite levels	

			Decreased IL-1b Increased IL-10	
10			Reduced macroscopic damage score and colon weight to length ratio	
5, 10, 30	i.p.	DNBS	n.s. on body weight n.s. on colon weight to length ratio	Pagano et al., 2016
1, 2.5, 5, 10		Croton Oil	n.s. on motility in healthy 5-10 mg/kg reduced motility in croton	
10, 30, 60	Gavage (CBD dissolved in canola oil)	DNBS	n.s. on body weight n.s. on colon weight to length ratio	
5, 10, 30, 60		Croton Oil	n.s. on motility in healthy controls 5 mg/kg was only dose to reduce motility in croton oil treated mice	
1, 3, 10	Gavage (CBD dissolved sesame oil)	Acute model: 5 days 4% DSS, 3 days water. CBD days 2-8	n.s. on MPO activity, DAI, body weight, or colon weight to length ratio	Silvestri et al., 2020
0.3, 3, 10, 30				
1		4% remission DSS (5 days DSS, 9 days water)		
1	i.p. 30 mins prior to TNBS daily for 5 days	TNBS (125 mg/kg) daily for 5 days	Reduced inflammation: <ul style="list-style-type: none"> <li>• Decreased MPO activity</li> <li>• Decreased IL-6 levels</li> </ul>	(Wei et al., 2020)
20, 50	s.c. 60 mins prior to i.p. 0.6% acetic acid	0.6% Acetic Acid Stretch Test	n.s. on nociceptive behaviors	(Booker et al., 2009)
10, 30, 90	i.p. 30 mins prior to i.p. 0.8% acetic acid	0.8% Acetic Acid Stretch Test	Reduced writhing with 30 and 90 mg/kg	(Silva et al., 2017)

As shown in Table 2, CBD can reduce inflammation in a TNBS model in both rats and mice (Schicho & Storr, 2012; Wei et al., 2020). There is mixed evidence of its efficacy in a dinitrobenzene sulphonic acid (DNBS) model of colitis (Borrelli et al., 2009)(Pagano et al., 2016). No effect was observed in a DSS model (Silvestri et al., 2020). Finally, CBD rectified motility in a croton oil model of hypermotility (Capasso et al., 2008; Pagano et al., 2016). CBD showed mixed results in the acetic acid test (Booker et al., 2009; Silva et al., 2017) and anti-nociceptive effects have not been directly examined in an IBD model despite showing anti-nociceptive effects in other pain modalities (Silva-Cardoso & Leite-Panissi, 2022).

### **1.5.3 Cannabidivarin (CBDV)**

CBDV is the propyl analog of CBD. It is a TRPV1, TRPV2, and TRPA1 agonist as well as a TRPM8 antagonist (Iannotti et al., 2014; Ligresti et al., 2016). Few studies have examined CBDV. A 2014 study by Iannotti et al., showed CBDV to activate and rapidly desensitize TRPV1, TRPV2, and TRPA1 in a dose dependent manner in HEK293 cells. The study also showed hippocampal slices treated with CBDV to reduce epileptic spike generation and to dephosphorylate TRPV1. Recently, CBDV has been shown to inhibit T-type Caves (Mirlohi et al., 2022) and to potentially act post-synaptically to antagonize CB<sub>1</sub> signaling in autaptic hippocampus neurons (Straiker et al., 2021). Interestingly, despite reports of activating TRP channels in an expression system (Iannotti et al., 2014; Ligresti et al., 2016), CBDV showed no significant Ca<sup>2+</sup> signal in cultured DRG neurons (Straiker et al., 2021) suggesting that other pathways or affinities may overwhelm the TRP channel dependent effects. Finally, CBDV showed no significant effects in a tail-flick test for thermal nociception (Zagzoog et al., 2020).

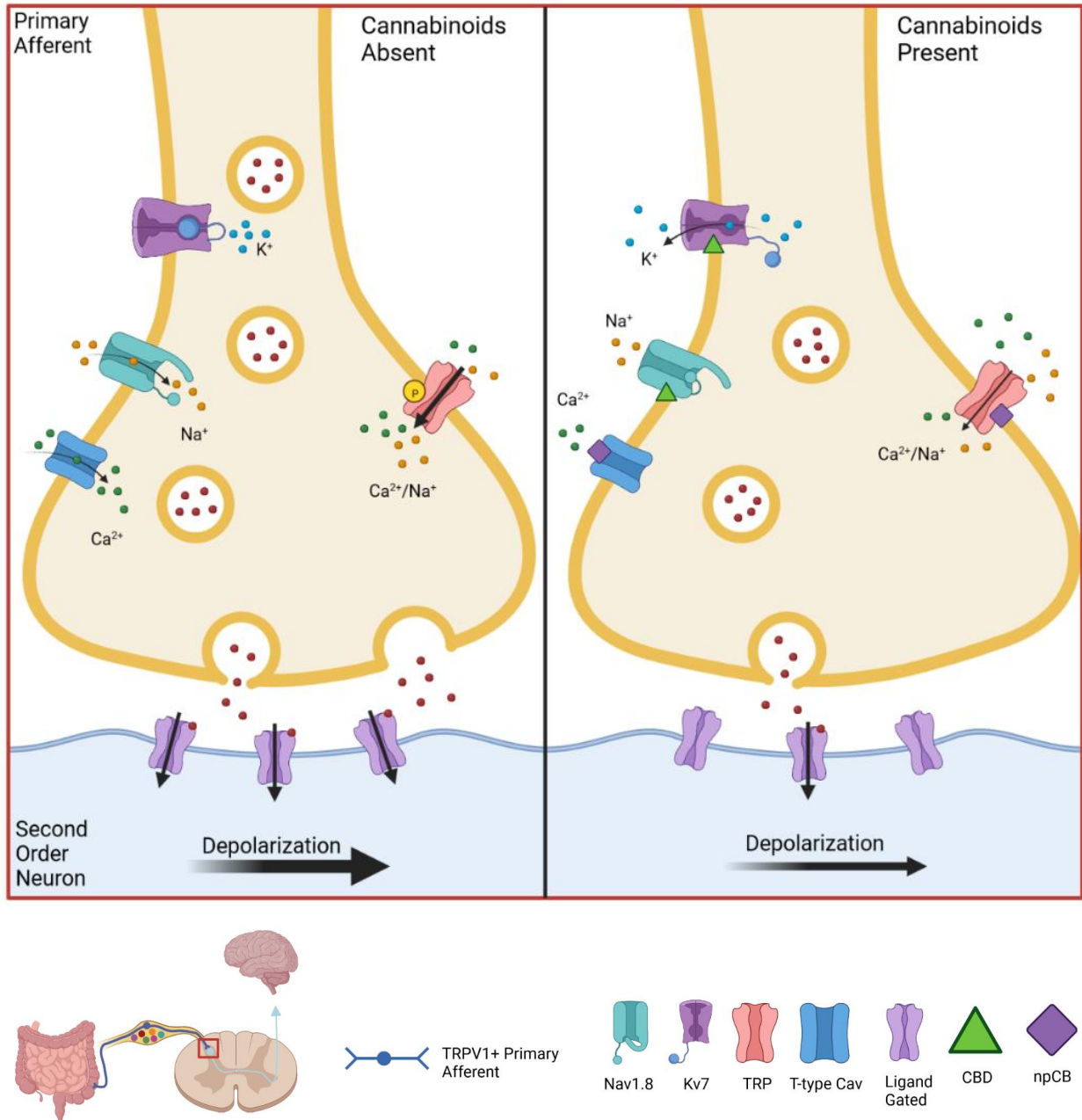
A study by (Pagano et al., 2019) examined CBDV in both DNBS and DSS models of colitis as well as in human pediatric UC colon biopsies. In the DNBS colitis model, CBDV reduced inflammation with stronger effects shown via oral gavage (o.g.) than i.p. suggesting that a metabolite may be exerting effects as well. Additionally, CBDV was shown to rectify the upregulation of TRPA1 associated with DNBS. No effect on TRPV1 or TRPV2 expression was observed. Finally, the TRPA1 antagonist HC030031 was able to partially reduce the anti-inflammatory effects of CBDV suggesting that part of the effects observed can be attributed to activity at TRPA1. In the DSS colitis model, CBDV reduced inflammation. Inflamed pediatric UC patient biopsies were shown to have upregulated TRPA1 compared to uninflamed samples. Additionally, 10 $\mu$ M CBDV was able to reduce IL-1 $\beta$  in inflamed biopsies.

This study clearly shows CBDV to have positive effects on inflammation in UC with the potential to rectify the upregulation of TRPA1 associated with UC. While no studies have directly examined CBDV in the context of IBD pain, its ability to rectify TRPA1 expression and to desensitize TRPA1, TRPV1 and TRPV2 shows that there is potential for CBDV to prove analgesic by acting to decreasing the peripheral sensitization that occurs in IBD.

#### **1.5.4 Cannabigerol (CBG)**

CBG is an antagonist of TRPM8, 5-HT<sub>1A</sub>, T-type Cavs(de Petrocellis et al., 2011; Ligresti et al., 2016; Mirlohi et al., 2021) as well as COX-1 and COX-2(Ruhaak et al., 2011). It is an agonist of TRPV1, TRPV2, TRPA1, and  $\alpha$ 2-adrenoreceptors (de Petrocellis et al., 2011; Ligresti et al., 2016). Recently, CBG was also shown to inhibit Nav1.7 in DRG neurons suggesting an analgesic mechanism (Ghovanloo et al., 2022). However, Nav1.7 KO experiments

have shown this channel to be a major player in somatosensory pain but not in abdominal pain (Hockley et al., 2017).



**Figure 7.** Mechanisms of action of non-psychotropic cannabinoids (npCB) at the primary afferent -second order neuron junction in the dorsal horn of the spinal cord. Cannabidiol (CBD) opens Kv7 channels and stabilizes the inactivated state of Nav1.8 channels. Generally, npCBs

(including CBD) inhibit T-type Cav channels and dephosphorylate TRP channels. Created with BioRender.com.

Few studies have examined CBG in the context of abdominal pain. The first study examined daily i.p. administration in a DNBS model of IBD and showed anti-inflammatory effects. This study also investigated CBG at 0.001 to 1  $\mu$ M concentrations in LPS treated macrophages. Treatment with 0.01, 0.1, and 1  $\mu$ M CBG resulted in significantly decreased nitrite levels. At the 1  $\mu$ M concentration, the nitrite production was found to be unaffected by the CB<sub>1</sub> antagonist rimonabant and enhanced by the CB<sub>2</sub> antagonist SR144528 suggesting a modulatory role of this receptor (Borrelli et al., 2013). This study showed clear anti-inflammatory effects of CBG in a DNBS model and these effects were not dependent upon CB<sub>1</sub> although CB<sub>2</sub> may play an unclear role. A more recent study confirmed the anti-inflammatory of CBG in the DNBS model of IBD but this time administration was by oral gavage. (Pagano et al., 2021).

Non-visceral pain models show mixed evidence of analgesic effects of CBG. This npCB attenuated mechanosensitivity in cisplatin-induced peripheral neuropathy. Effects were enhanced when combined with CBD, reduced with alpha2-adrenergic, CB<sub>1</sub>, and CB<sub>2</sub> antagonists, and unaffected by a TRPV1 antagonist. CBG was ineffective in either a tail-flick or formalin assay (Sepulveda et al., 2022). Finally, CBG produced small analgesic effects in a tail-flick test at 3mg/kg but no effect at 10mg/kg (Zagzoog et al., 2020).

Overall, CBG appears to be anti-inflammatory in IBD models but there is a lack of research in IBD pain; however, mixed results on non-visceral pain studies suggest that CBG warrants investigation in a visceral pain context.

### 1.5.5 The entourage effect

The entourage effect is an interesting feature of cannabinoids to potentiate the actions of each other (Anand et al., 2021). It allows for small amounts of cannabinoids to be administered together to produce greater results than a single cannabinoid administered alone. It was first described in 1998 when the actions of 2-AG in the tetrad assay, and in its inhibitory actions on AC were enhanced by 2-linoleoyl-glycerol and 2-palmitoyl-glycerol despite both compounds showing no ability to inhibit AC activity or bind CB<sub>1</sub> or CB<sub>2</sub>. The effect could only partially be explained by inhibition of MAGL (Ben-Shabat et al., 1998). Many studies have examined the entourage effect produced by different combinations of cannabinoids, but its mechanisms have never been sufficiently explained.

A common way to examine the entourage effect is the comparison of a pure compound with an extract from a strain of cannabis bred to produce large amounts of the compound of interest—frequently termed a botanical drug substance (BDS). In vitro work showed CBC-BDS to have a lower EC<sub>50</sub> than pure CBC at TRPV1. CBC-BDS and CBG-BDS show inhibition of N-acyl ethanolamine acid amide hydrolase (NAAA; the main degradation enzyme of PEA) at IC<sub>50</sub>s of  $14.2 \pm 6.2 \mu\text{M}$  and  $18.3 \pm 9.4 \mu\text{M}$  respectively, while both of their pure counterparts show no significant effect (IC<sub>50</sub> >100 $\mu\text{M}$ ) on the activity of this enzyme. In vivo work showed pure CBD to be entirely ineffective by both i.p. (5 - 30 mg/kg) and gavage (5 - 60 mg/kg) in DNBS model of colitis while CBD-BDS effectively reduced inflammatory markers at 30 mg/kg i.p. and 60 mg/kg o.g.. Additionally, in a croton oil model of hypermotility, pure CBD reduced motility at 5 - 10 mg/kg i.p. and at 5 mg/kg o.g. while CBD-BDS reduced motility at 1 - 10 mg/kg i.p. and 5 - 60 mg/kg o.g. (Pagano et al., 2016).

The entourage effect has also been examined by combining individual pure cannabinoids. CBD, THC, THCV, cannabinol (CBN), and CBC were administered subcutaneously to male mice in an acetic acid model of abdominal pain. While CBD, CBC, and THCV failed to produce any analgesic effects, CBN and THC did produce analgesic effects that were potentiated when co-administered. Interestingly, THCV antagonized the analgesic effects of THC (Booker et al., 2009). Also, CBC and THC when given in combination showed additive effects in the tetrad assay and in an LPS-induced paw edema model. The administration of 0.3 mg/kg i.v. THC 10 minutes following CBC administration (3, 10, 30, 100 mg/kg i.v.) enhanced responses for catalepsy, antinociception, hypothermia, but not hypomotility. THC was co-administered in a 1:10 ratio with CBC in an LPS-induced paw edema model of inflammation and showed greater effects than either compound alone at the 40mg/kg CBC with 4 mg/kg THC dosage. Additionally, the authors examined the blood and brain levels of CBC and THC when administered alone and together. CBC was found to increase brain THC levels at 30 and 100 mg/kg i.v. doses (DeLong et al., 2010). This finding is of great practical importance when considering the administration route of cannabinoids. The heating of CBD to the temperatures of e-cigarettes or combustion has been shown to convert 25-52% of the CBD to other cannabinoids predominantly THC, CBN, and CBC (Czégény et al., 2021). Combined, these results suggest that e-cigarettes or smoking would be an inappropriate administration route to avoid psychotropic effects.

At least two studies have examined the combination of cannabinoids and fish oil with the rationale that a synergistic effect may be achieved through CBD and CBG inhibition of superoxide dismutase (SOD) preventing oxidative stress associated with fish oil consumption in IBD. (Borrelli et al., 2013; Pagano et al., 2021; Silvestri et al., 2020; Sunda & Arowolo, 2020).

The first study examined the combination of CBD and fish oil in a DSS model. Results showed no significant effects of CBD alone (0.3 – 10 mg/kg o.g.). However, when a subtherapeutic dosage of fish oil (20 mg/kg) was combined with previously inactive doses of CBD, significant anti-inflammatory effects were observed (Silvestri et al., 2020). Similarly, a second study using the DNBS model showed CBD to be ineffectual alone but show significant anti-inflammatory effects when co-administered with fish oil as well as with both fish oil and CBG (Pagano et al., 2021). It must be noted that not all combinations are beneficial. For example, while in this study the combination of CBD, CBG, and fish oil was shown to be anti-inflammatory, CBG has actually shown to block the anti-emetic and anti-nausea effects of CBD in rats and shrews likely owing to its antagonism of 5-HT<sub>1A</sub> (Ligresti et al., 2016; Rock et al., 2011). Similarly, THCV has been shown to attenuate the analgesic effects of THC (Booker et al., 2009). Interactions can be more complex, for example, the inclusion of CBD with CBN in equal proportion potentiated the duration of effects of CBN; however, increasing the proportion of CBD attenuated this potentiation (Wong & Cairns, 2019).

The described studies show evidence for an entourage effect of npCBs for both anti-inflammatory and analgesic effects. This effect provides an opportunity to combine the therapeutic attributes of individual npCBs—with additional effects potentially emerging from their combination—and to increase the potency of any therapeutics developed from npCBs to create new pharmacological tools for the management of IBD symptoms.

## 1.6 Hypothesis and Aims

### Hypothesis

Non-psychotropic cannabinoids attenuate DSS-induced visceral hypersensitivity

### Specific Aims

1. Examine the ability of npCBs to attenuate visceral hypersensitivity in the DSS model of experimental colitis
  - 1.1. Hypothesis: One or more npCB will attenuate visceral hypersensitivity in the DSS model of experimental colitis
    - 1.1.1. Determine the efficacy of CBD in this model.
    - 1.1.2. Determine the efficacy of CBG in this model.
    - 1.1.3. Determine the efficacy of CBC in this model.
    - 1.1.4. Determine the efficacy of CBDV in this model.
    - 1.1.5. Develop a combination of npCBs to increase anti-nociceptive potency.
2. Determine mechanisms of action of npCBs effective in vivo using electrophysiology
  - 2.1. Hypothesis: One or more npCB will interact with an ion channel to decrease nociceptor excitability
    - 2.1.1. Examine effects on Na<sup>+</sup> channels
    - 2.1.2. Examine effects on Ca<sup>2+</sup> channels

## CHAPTER TWO: METHODS

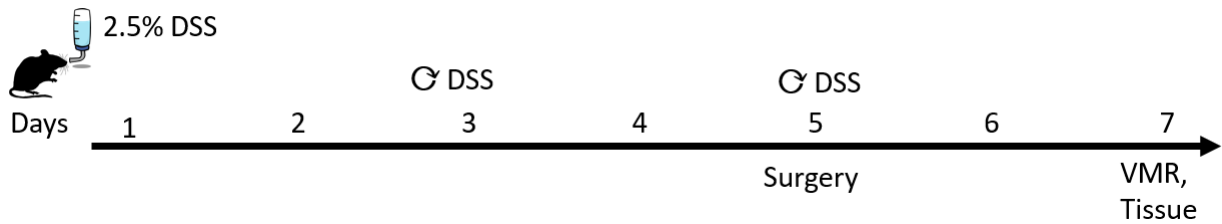
### 2.1 Animals

All procedures were approved by the University of Calgary Animal Care Committee (protocol: AC19-0124) and performed in accordance with guidelines established by the Canadian Council on Animal Care. Unless otherwise stated, male C57BL/6J (Jackson Laboratory, Bar Harbor, Maine, USA) aged 6 weeks were used. Mice were housed, up to 4 mice per cage, in plastic cages with sawdust bedding and food and water available *ad libitum*. Male mice were chosen for the acute DSS model because they typically show a more pronounced and rapid phenotype (Chassaing et al., 2015) and visceral pain response have been shown in female mice to be affected by the estrous cycle (Ji et al., 2008). Additionally, for some electrophysiological experiments, TRPV1-GFP mice were used. These transgenic mice express a pH-sensitive GFP on the TRPV1 channel which will fluoresce when the channel is inserted in the membrane (Agosti & Altier, 2019). These animals were bred in-house and kept under same conditions listed above.

### 2.2 Dextran Sulfate Sodium (DSS) colitis

DSS is a synthetic sulfated polysaccharide that is water soluble and highly negatively charged due to the presence of a sulfate group. The compound is toxic to epithelial cells and increases the permeability of the colonic epithelial layer allowing for the infiltration of commensal bacteria into the underlying mucosa resulting in the initiation of the inflammatory response. DSS is also an anticoagulant and subsequently increases intestinal bleeding. The net results of DSS administration includes weight loss, bloody stool, diarrhea, inflammation, and

visceral hypersensitivity. Thus, this model recapitulates many of the key symptoms of IBD and can be easily administered in drinking water (Chassaing et al., 2015). The acute DSS protocol used was 7 days of 2.5% DSS dissolved in tap water available *ad libitum* with the DSS-water replaced on days 3 and 5 (Figure 8).



**Figure 8.** Acute DSS protocol timeline. Mice received 2.5% DSS in their drinking water for 7 days which was refreshed on days 3 and 5. Additionally on day 5, mice underwent surgery for the implantation of two electrodes into the external oblique muscles. Finally, on day 7, the visceral motor response was measured and tissue collected.

### 2.3 The visceral motor response (VMR)

The visceral motor response (VMR) served as a measure of evoked pain (pain behaviors in response to a mechanical stimulus). Mice were housed together based on treatment group until two days prior to VMR when they were housed in individual cages following surgery and remained separated for the remainder of the time course. For the surgery, mice were anesthetized with a mixture of ketamine and xylazine and two electrodes were implanted in the oblique muscles and the electrodes externalized at the back of the neck and protected with a plastic tube sutured to the skin. Two days later, these electrodes were used to measure the contraction of the oblique muscles via an electromyogram acquisition system through a Bio Amplifier (ADInstruments, Colorado Springs, CO) and a VMR was computed using LabChart7 in response

to colorectal distension (CRD) (Lapointe et al., 2015). The CRD stimulus was applied via a 10.5-mm-diameter balloon catheter (Edwards Life-Sciences, Irvine, CA) inserted approximately 5mm into the rectum. The balloon was subsequently inflated to pressures of 15, 30, 45, and 60 mmHg for 10s. The oblique contraction measured by the VMR is largely reflexive and thus provides an objective measure of visceral pain and hypersensitivity (Lapointe et al., 2015). Following the VMR the mice were euthanized, and tissue was collected.

## **2.4 Disease activity index (DAI)**

A daily disease activity index (DAI) was calculated for each mouse. To determine this measure, mice were weighed daily, and the percent weight decrease calculated as follows: no change or weight gain = 0, 1-5% decrease = 1, 5-10% decrease = 2, 10-20% decrease = 3 (Defaye et al., 2022; Lapointe et al., 2015). A stool sample was also obtained, scored for consistency and blood content as follows: normal consistency = 0, moist and sticky = 1, soft = 2, diarrhea = 3; normal color = 0, red-brown color = 1, visible blood = 2, rectal bleeding = 3. This yields a daily score out of 9. The stool sample was then stored at -80°C for later testing. Importantly, any mouse showing rectal bleeding was not subjected to the visceral motor response (VMR) as this increases the risk of perforating the bowel and any mouse in visible pain or showing excessive weight loss (>20%) was euthanized to minimize suffering. Later experiments examined only changes in visceral hypersensitivity and subsequently used only percent weight change from day 0 to monitor DSS progress.

## **2.5 Macroscopic damage score**

Following VMR, tissue was collected. Mice were euthanized via isoflurane overdose and the colon isolated and removed. The colon was rinsed in PBS and its length recorded and scored for macroscopic damage as follows: adhesion, absent = 0, moderate = 1, severe = 2; edema, absent = 0, moderate = 1, severe = 2; restrictions, absent = 0, 1 present = 1, 2 present = 2, more than 2 = 3; blood, absent = 0, present = 1; ulcer, absent = 0, present = 1; mucus, absent = 0, present = 1. This yields a total score out of 10 and provides a quick measure of any protective or anti-inflammatory effects at a macroscopic scale (Defaye et al., 2022). Next, the most distal 5 mm were removed and stored in 4% PFA for histological analysis, and the next most distal 5 mm removed and placed on dry ice and later stored at -80°C for biochemical analysis. Once the colon was removed, mice were perfused first with PBS and then fixed with 4% PFA. Finally, the spinal cord was collected and placed in 4% PFA before transfer to a 30% sucrose in PBS solution for cryoprotection.

## **2.6 Immunohistochemistry**

To complement the VMR measure of pain, the lumbosacral region (L6 to S1) of collected spinal cords was sectioned at 10 µm and immunohistochemically stained for the proto-oncogene cFos. This gene is rapidly expressed in activated neurons and is commonly used as a marker of neuronal activity (Harris, 1998). Lamina I, II and V of this region receive nociceptive input from the colon (Abdullah et al., 2020) and subsequently mice experiencing greater pain from the CRD should show greater cFos expression in this region. cFos counts were quantified by imaging all processed dorsal horn sections and then randomly selecting 8 – 10 images per mouse, counting

the number of cFos positive cells within the entire dorsal horn and taking the average of these images. Additionally, to account for the same neuron being present in multiple slices, slices were collected onto 4 separate slides alternating between slices. All counts were completed blindly using coded slides.

## **2.7 Primary Dorsal Root Ganglia (DRG) culture**

DRGs were isolated and cultured on glass coverslips for electrophysiological recordings. Six- to eight-week-old, Male C57Bl/6 mice or transgenic TRPV1-GFP were euthanized by isoflurane overdose, the spinal cord removed via hydraulic extrusion, the spinal column bifurcated, and the individual DRGs dissected out and collected on ice in a sucrose solution containing kynurenic acid which decreases neuronal activity (Stone, 2021) (Table 3). DRGs were then washed in 0mM Ca<sup>2+</sup> Hank's Balanced Salt Solution (HBSS) 3 times and enzymatically digested with 2mg/mL collagenase type 1 and 4 mg/mL dispase in HBSS with calcium for 39 minutes at 37°C with 5% CO<sub>2</sub> in 96% humidity. After digestion, enzymes were inactivated with neural basal A (NBA) and washed twice in 0mM Ca<sup>2+</sup> HBSS. Next, cells were triturated with fire-polished Pasteur pipettes in NBA containing 2% B27, 100 µg/mL streptomycin, 100 units/mL penicillin, 10% fetal bovine serum, 0.25 µg/mL NGF, and 0.25 µg/mL glial cell derived neurotrophic factor (GDNF). The dissociated cells were then placed on laminin and polyornithine-coated glass coverslips and maintained for up to two days at 37°C with 5% CO<sub>2</sub> in 96% humidity (Lapointe et al., 2015).

Table 3. Contents of sucrose solution used for DRG collection

Sucrose Solution	
Compound	Molarity (mM)
Sucrose	248
NaHCO <sub>3</sub>	26
Glucose Monohydrate	11
KCl	2
MgSO <sub>4</sub>	2
NaH <sub>2</sub> PO <sub>4</sub> H <sub>2</sub> O	1.25
Kynurenic Acid	1

## 2.8 Electrophysiology

The receptors affected by npCBs were examined using whole cell patch-clamp electrophysiology of DRG neurons isolated from either C57Bl/6 mice or transgenic TRPV1-GFP mice. Cells were recorded using borosilicate glass (Harvard Apparatus, Holliston, MA) pipettes of  $\sim 3\text{M}\Omega$  pulled on a DMZ Universal puller (Zeitz, Martinsried, Germany). Recordings were completed using the pClamp 10.7 (Molecular Devices) software and a CV203BU headstage connected to an Axopatch 200b amplifier (both Axon Instruments) and digitized at 10 kHz with a Digidata 1440A digitizer. External and internal solution contents are listed in Table 4 and Table 5 respectively.

Table 4. Contents of the external solutions used for electrophysiology recordings.

	<b>Normal External Solution</b>	<b>Low Ca<sup>2+</sup> External Solution</b>	<b>Low Na<sup>+</sup> External Solution</b>	<b>Low Na<sup>+</sup> with Ba<sup>2+</sup> External Solution</b>
<b>Compound</b>	<b>Molarity (mM)</b>	<b>Molarity (mM)</b>	<b>Molarity (mM)</b>	<b>Molarity (mM)</b>
<b>NaCl</b>	60	60	2	
<b>Sucrose</b>	110	168	168	95
<b>KCl</b>	3	3	3	
<b>MgCl<sub>2</sub></b>	1	1	1	
<b>Hepes</b>	10	10	10	10
<b>CaCl<sub>2</sub></b>	2.5	0.1	2.5	
<b>Glucose</b>	10	10	10	10
<b>TEA-Cl</b>	10	10	10	30
<b>NMDG</b>			58	110
<b>BaCl<sub>2</sub></b>				10
	pH 7.4 with HCl	pH 7.4 with HCl	pH 7.4 with HCl	pH 7.4 with HCl

Table 5. Contents of the internal solution used for electrophysiological recordings

<b>Internal Solution</b>	
<b>Compound</b>	<b>Molarity (mM)</b>
CsCl <sub>2</sub>	120
MgCl <sub>2</sub>	3
EGTA	10
HEPES	10
ATP	2
GTP	0.5

## **2.9 LABORAS**

Non-evoked pain behaviors were evaluated using the laboratory animal behavior observation registration and analysis system (LABORAS) (Metris, Hoofddorp, Holland). This is an automated behavior acquisition system which monitors the vibrations generated by mice placed on the apparatus and correlates these vibrations to typified behaviors. It is important to also quantify non-evoked pain behaviors because these behaviors can be more representative of the pain experienced by IBD patients. The behaviors of interest were locomotion and climbing which are pain-related behaviors that require stretching of the abdomen (Lapointe et al., 2015).

## **2.10 Statistics**

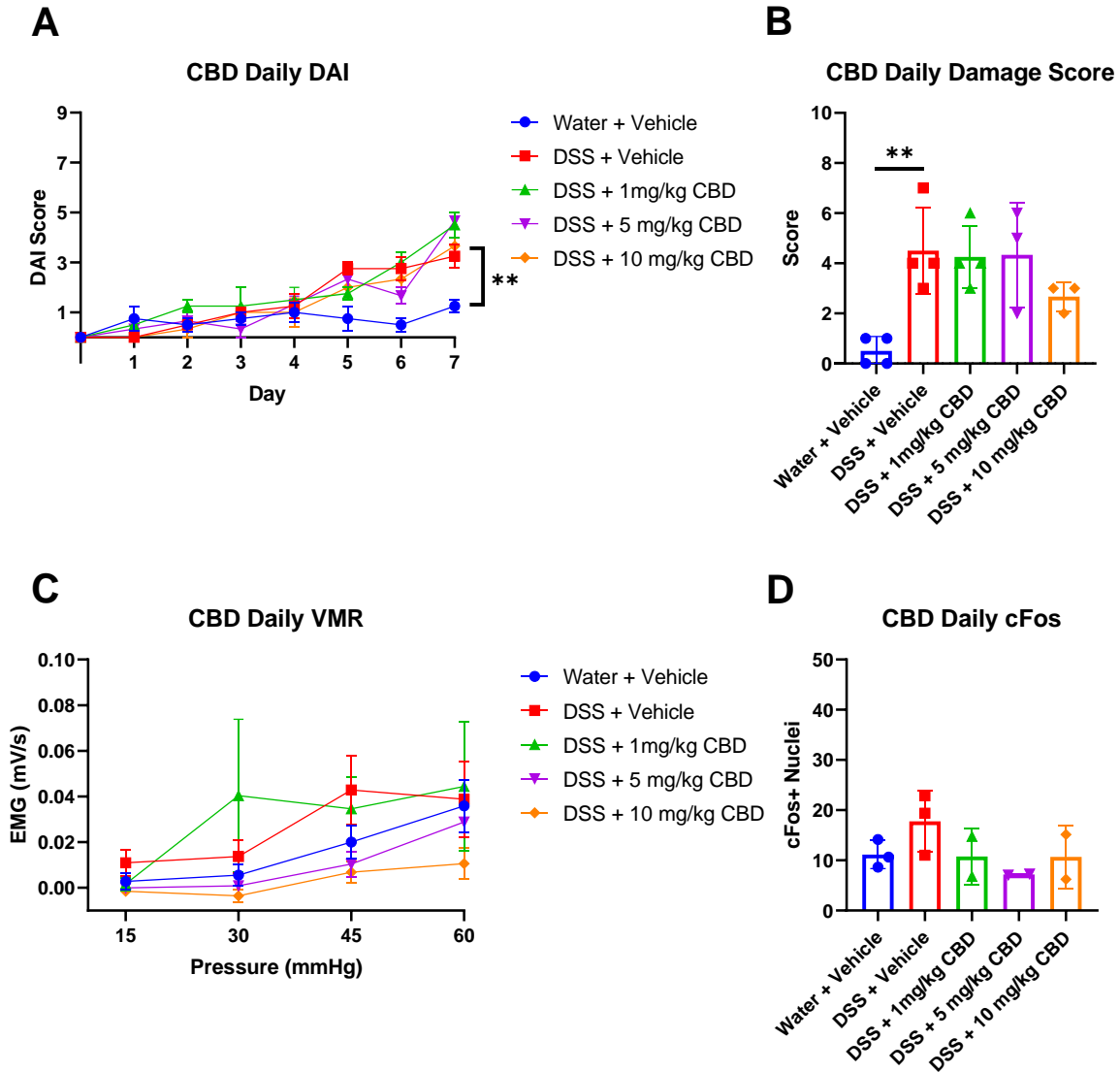
VMR, DAI, and body weight data were subjected to two-way ANOVA (mean type II) followed by post-hoc analysis with a Tukey honestly significant difference (Tukey HSD). Similarly, macroscopic damage score, and cFos counts will be subjected to a one-way ANOVA (mean type II) followed by post-hoc analysis with a Tukey HSD. These statistics and their accompanying figures were computed using RStudio 1.4.1106 with the tidyverse, ggplot2, and RColorBrewer packages (Erich Neuwirth 2014, R Core Team, 2013; Wickham, 2016; Wickham et al., 2019). Comparison of current densities from distinct peaks in electrophysiological recordings were examined using a paired t-test computed using GraphPad Prism 8.0.2 (GraphPad Software, San Diego, California USA, [www.graphpad.com](http://www.graphpad.com)).

## **CHAPTER THREE: RESULTS**

### **3.1 Effects of single npCBs in the acute DSS model**

#### **3.1.1 Effects of daily administration of CBD in the acute DSS model**

Preliminary experiments sought to determine the effect of CBD on inflammation and pain using the acute DSS model and a daily i.p. injection (Figure 8). Based on literature summarized in the introduction (Table 2), doses of 1, 5, and 10 mg/kg were used, daily. No strong evidence of anti-inflammatory effects were observed in either the DAI or the macroscopic damage score (Figure 9A and 9B). However, VMR data showed no significant difference but a clear trend of reduced response in the 5 and 10 mg/kg conditions (Figure 9C). Similarly, cFos staining showed the same trend (Figure 9D).

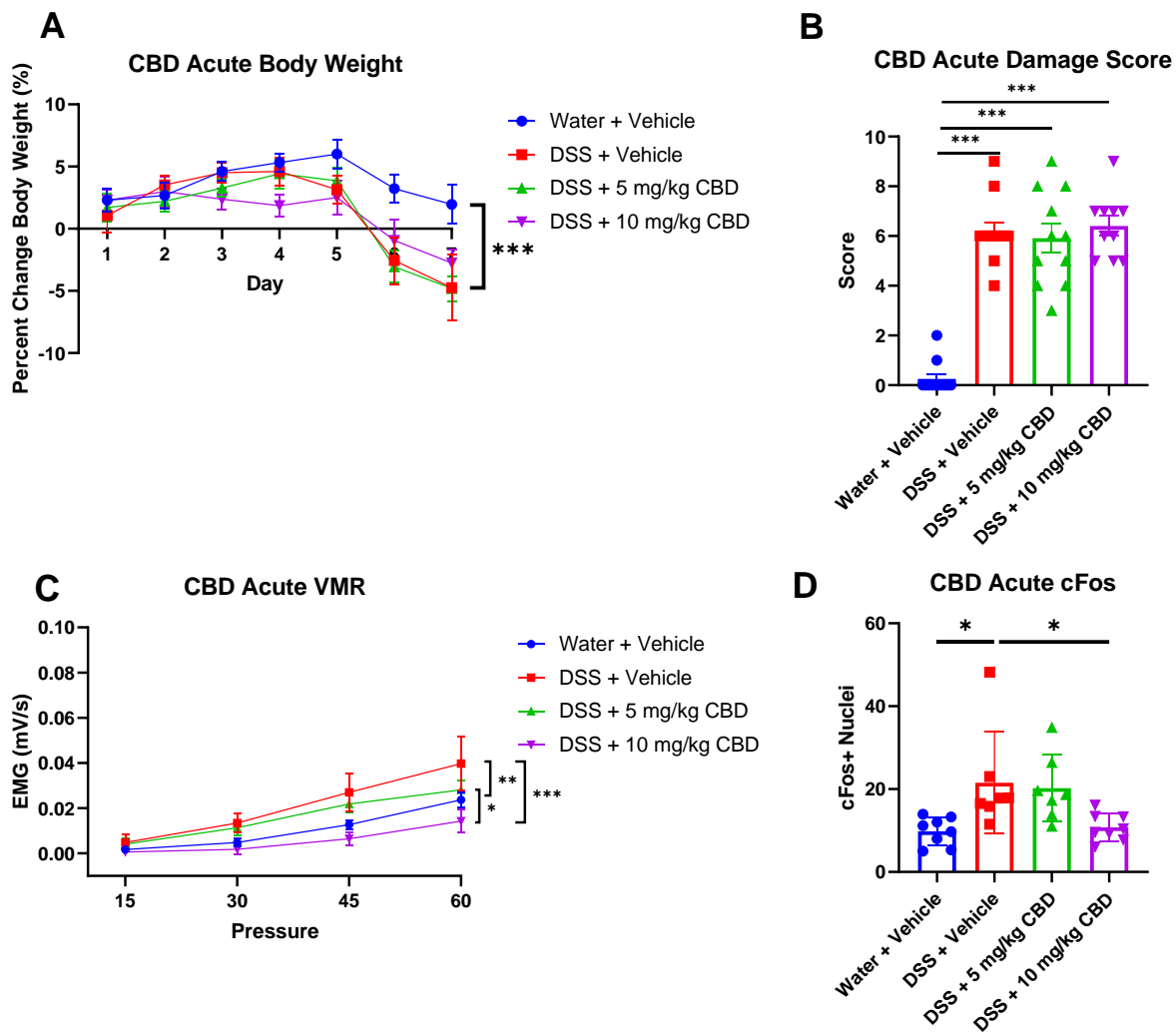


**Figure 9.** Daily injections (i.p.) of CBD at 1 mg/kg, 5 mg/kg, and 10 mg/kg does not reduce inflammation or visceral sensitivity in mice treated with 2.5% DSS. Positive control animals received the drug vehicle and 2.5% DSS. Negative control animals received the drug vehicle and normal drinking water. Each treatment condition contains 3 – 4 mice except for cFos staining where conditions contain 2 – 3 mice. Error bars represent standard error of the mean (SEM). **A)** DAI score. A two-way ANOVA showed a significant effect of treatment condition ( $F(4) = 10.278$ ,  $p < 0.001$ ); however, post-hoc analysis (Tukey Honestly Significant Difference (HSD))

showed significant differences only for the negative control compared to all DSS treated conditions (all  $p < 0.01$ ) with no significant effects of any CBD dosage. **B)** Macroscopic damage score. A one-way ANOVA showed a significant effect of treatment condition ( $F(4) = 6.076$ ,  $p = 0.005$ ); however, post-hoc analysis (Tukey HSD) again showed significant differences only for the negative control compared to the positive control ( $p = 0.008$ ), 1mg/kg CBD ( $p = 0.0131$ ), and 5 mg/kg CBD ( $p = 0.0191$ ) conditions with no significant effects for any CBD dosage compared to the positive controls. **C)** VMR data. A two-way ANOVA no significant effect of treatment condition ( $F(4) = 2.548$ ,  $p = 0.0501$ ). **D)** cFos count of the dorsal from the lumbosacral region presented as the average of all images counted per mouse (2 – 3 mice per group).

### **3.1.2 Effects of acute administration of CBD in the acute DSS model**

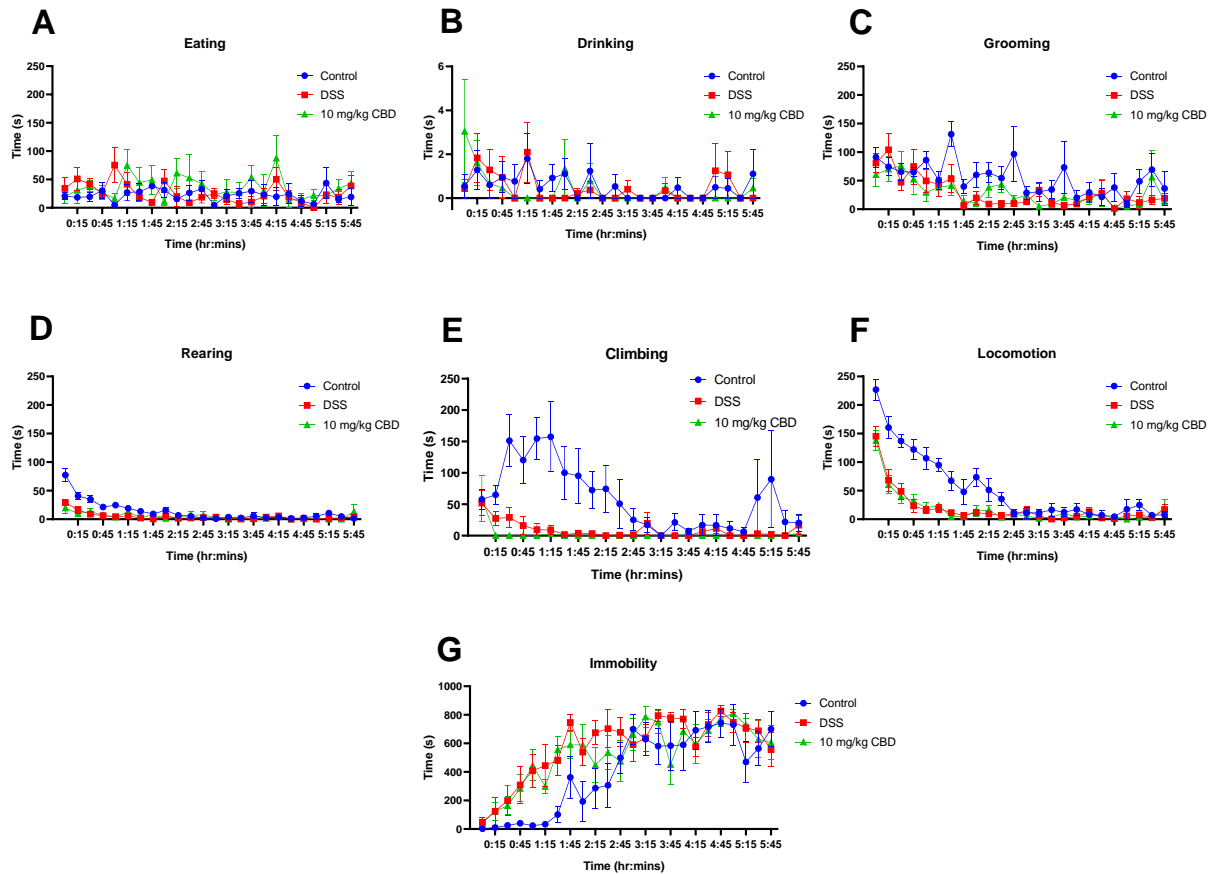
The preliminary study with daily injections of CBD showed potential analgesic effects. However, to remove any anti-inflammatory or protective effects that CBD may have contributed through daily administration, a single injection 30 minutes prior to undergoing the VMR measure was used. The same acute DSS model (Figure 8) was used. Additionally, instead of a full DAI daily body weight measurements in conjunction with the macroscopic damage score were used to confirm DSS activity. Results showed a significant differences in body weight and damage scores between all DSS treated groups and water controls (Figure 10A & B) as well as a reduction in VMR response (Figure 10C) as well as in the number of cfos positive neurons (Figure 10D) and



**Figure 10.** A single injection (i.p.) of CBD at 10 mg/kg attenuates visceral hypersensitivity in mice treated with 2.5% DSS. Animals treated with 2.5% DSS received a single injection (i.p.) of CBD at either 5 mg/kg or 10 mg/kg. Positive controls consisted of DSS treated mice which received the drug vehicle (DSS) and negative controls consisted of mice with normal drinking water which also received the drug vehicle (Veh). Each treatment contains 10 – 12 mice except for cFos data which contains 7 – 9 mice per condition. Error bars represent SEM. **A)** Percent change in bodyweight. A two-way ANOVA showed a main effect of treatment condition ( $F(3) = 10.2074$ ,  $p < 0.0001$ ). Post-hoc analysis (Tukey HSD) showed no significant differences between

any DSS treated group and all DSS treated groups showed significant differences from Veh (all  $p < 0.001$ ). **B)** Macroscopic damage score. A one-way ANOVA showed a significant effect of treatment condition ( $F(3) = 52.272$ ,  $p < 0.0001$ ). Post-hoc analysis (Tukey HSD) showed no significant differences between any DSS treated group and all DSS treated groups showed significant differences from Veh (all  $p < 0.0001$ ). **C)** VMR data. A two-way ANOVA showed a significant effect of condition ( $F(3) = 7.9749$ ,  $p < 0.0001$ ). Post-hoc analysis (Tukey HSD) showed significant differences between Veh and DSS ( $p = 0.0015$ ), 10 mg/kg CBD and DSS ( $p = 0.0003$ ), and 10 mg/kg CBD and 5mg/kg CBD ( $p = 0.0177$ ). **D)** cFos data. A one-way ANOVA showed a significant effect of condition ( $F(3) = 5.5605$ ,  $p = 0.0042$ ). Post-hoc analysis (Tukey HSD) showed significant differences between Veh and DSS ( $p = 0.0231$ ), 10 mg/kg CBD and DSS ( $p = 0.0263$ ), as well as 5mg/kg CBD and Veh ( $p = 0.0496$ ).

Next, to explore potential effects of CBD on non-evoked behaviors, sickness behaviors were examined using the LABORAS system for 6 hours following a single i.p. injection of either drug vehicle or 10 mg/kg CBD (Figure 11). Results showed no significant effect of 10 mg/kg CBD on any parameter examined except for an increase in eating behaviors compared to negative control mice ( $p = 0.0104$ ; Figure 11A). Suggesting CBD attenuates visceral hypersensitivity without inducing major motor side-effects or relieving sickness behaviors.



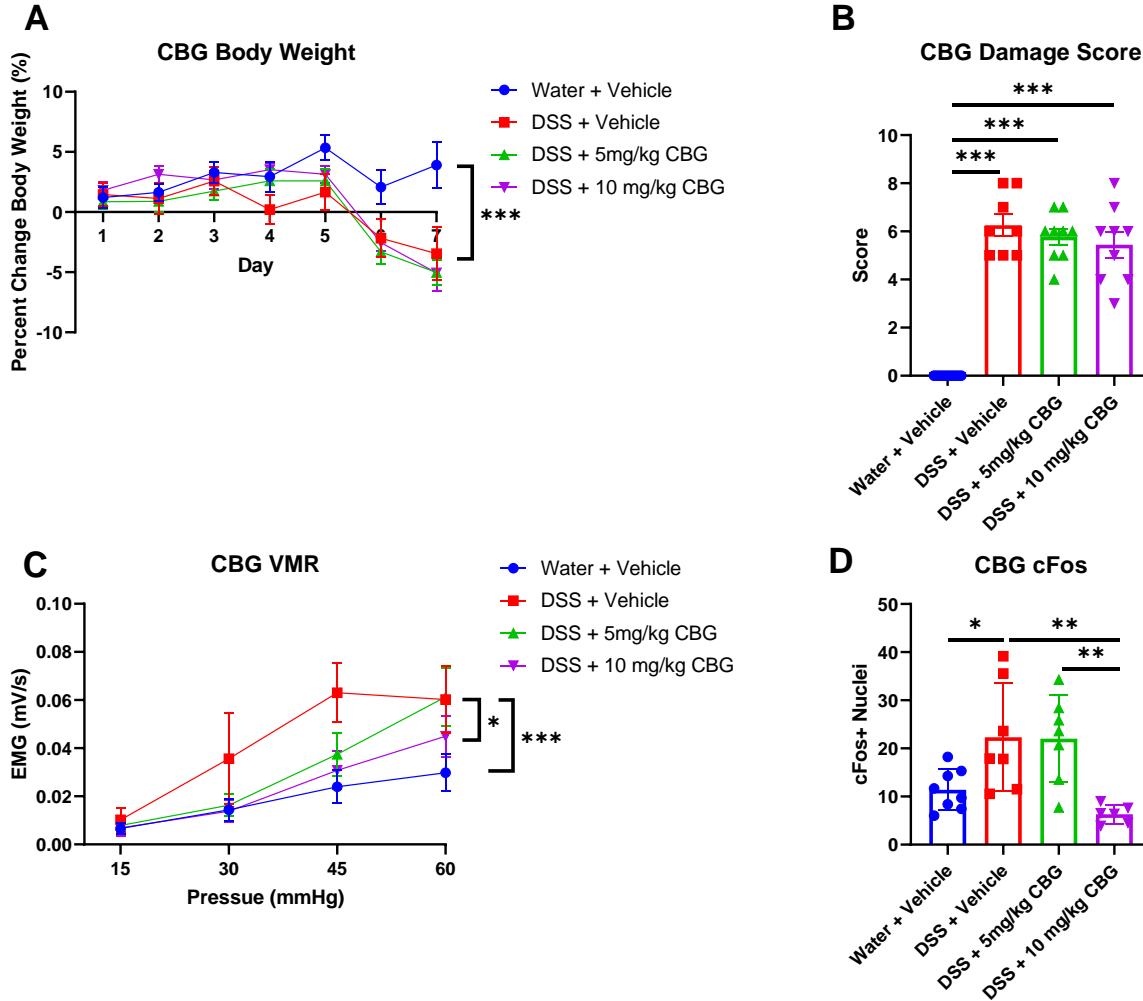
**Figure 11.** CBD increases eating behaviors but does not affect sickness behaviors. LABORAS results for 6 hours following a single injection (i.p.) of 10mg/kg CBD in mice treated with 2.5% DSS. Control groups are vehicle in DSS mice (DSS) and vehicle in untreated water mice (Veh). All groups contain 6 mice. Error bars represent SEM **A)** Eating data. A two-way ANOVA showed a main effect of treatment ( $F(2) = 4.464$ ,  $p < 0.0122$ ). Pair-wise comparison (Tukey HSD) showed a significant difference between Veh and 10 mg/kg CBD groups ( $p = 0.0104$ ) and a near significant difference between DSS and 10mg/kg CBD groups ( $p = 0.0871$ ). **B)** Drinking data. A two-way ANOVA showed no main effect of treatment ( $F(2) = 0.4247$ ,  $p = 0.6543$ ). **C)** Grooming data. A two-way ANOVA showed a main effect of treatment ( $F(2) = 16.01$ ,  $p < 0.0001$ ). Pair-wise comparison (Tukey HSD) showed a significant difference between Veh and

10 mg/kg CBD groups ( $p < 0.0001$ ) as well as Veh and DSS groups ( $p = 0.0001$ ). No significant was observed between DSS and 10 mg/kg CBD groups ( $p > 0.9999$ ). **D)** Rearing data. A two-way ANOVA showed a main effect of treatment ( $F(2) = 36.06$ ,  $p < 0.0001$ ). Pair-wise comparison (Tukey HSD) showed a significant difference between Veh and 10 mg/kg CBD groups ( $p < 0.0001$ ) as well as Veh and DSS groups ( $p = 0.0001$ ). No significant was observed between DSS and 10 mg/kg CBD groups ( $p = 0.8198$ ). **E)** Climbing data. A two-way ANOVA showed a main effect of treatment ( $F(2) = 49.74$ ,  $p < 0.0001$ ). Pair-wise comparison (Tukey HSD) showed a significant difference between Veh and 10 mg/kg CBD groups ( $p < 0.0001$ ) as well as Veh and DSS groups ( $p = 0.0001$ ). No significant was observed between DSS and 10 mg/kg CBD groups ( $p = 0.5970$ ). **F)** Locomotion data. A two-way ANOVA showed a main effect of treatment ( $F(2) = 93.64$ ,  $p < 0.0001$ ). Pair-wise comparison (Tukey HSD) showed a significant difference between Veh and 10 mg/kg CBD groups ( $p < 0.0001$ ) as well as Veh and DSS groups ( $p = 0.0001$ ). No significant was observed between DSS and 10 mg/kg CBD groups ( $p = 0.9433$ ). **G)** Immobility data. A two-way ANOVA showed a main effect of treatment ( $F(2) = 19.27$ ,  $p < 0.0001$ ). Pair-wise comparison (Tukey HSD) showed a significant difference between Veh and 10 mg/kg CBD groups ( $p < 0.0001$ ) as well as Veh and DSS groups ( $p = 0.0001$ ). No significant was observed between DSS and 10 mg/kg CBD groups ( $p = 0.3224$ ).

### **3.1.3 Effects of acute administration of CBG in the acute DSS model**

CBG was also investigated using the same acute DSS and single injection protocol. Body weight and macroscopic damage score both showed significant differences between all DSS treated groups and water controls (Figure 12A & B). VMR data showed significant differences between positive and negative controls as well as between 10 mg/kg CBG and positive controls

(Figure 12C). This pattern of results was also found by cFos counts in the dorsal horn of the spinal cord (Figure 12D). Suggesting 10 mg/kg of CBG attenuates visceral hypersensitivity.



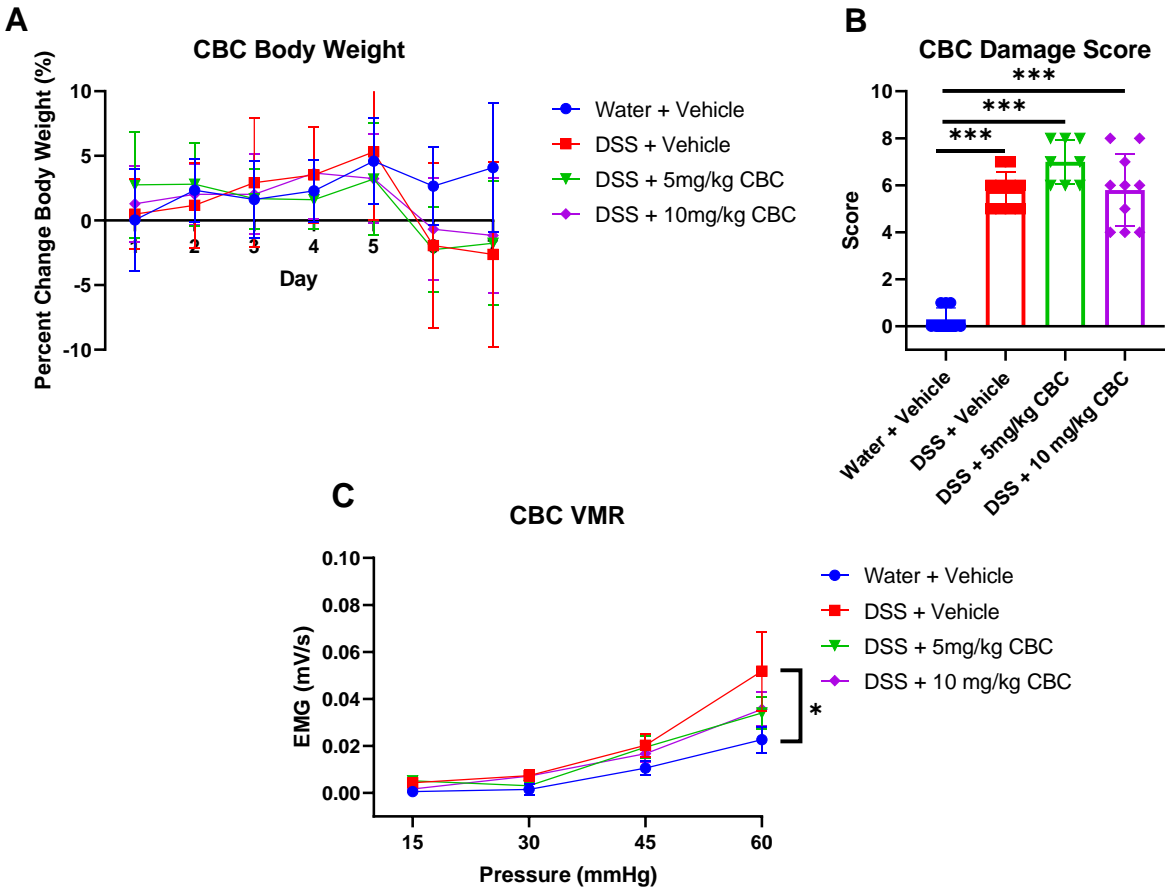
**Figure 12** A single injection (i.p.) of CBG at 10 mg/kg attenuates visceral hypersensitivity in mice treated with 2.5% DSS. Animals treated with 2.5% DSS received a single injection (i.p.) of CBG at either 5 mg/kg or 10 mg/kg 30 minutes prior to VMR. Positive controls consisted of DSS treated mice which received the drug vehicle (DSS) and negative controls consisted of mice with normal drinking water which also received the drug vehicle (Veh). Each treatment contains 8 – 10 mice except for cFos data which contains 6 – 8 mice per condition. Error bars represent SEM

**A)** Percent change in bodyweight. A two-way ANOVA showed a main effect of treatment condition ( $F(3) = 10.1779$ ,  $p < 0.0001$ ). Post-hoc analysis (Tukey HSD) showed no significant differences between any DSS treated group and all DSS treated groups showed significant differences from Veh (all  $p < 0.001$ ). **B)** Macroscopic damage score. A one-way ANOVA showed a significant effect of treatment condition ( $F(3) = 39.106$ ,  $p < 0.0001$ ). Post-hoc analysis (Tukey HSD) showed no significant differences between any DSS treated group and all DSS treated groups showed significant differences from Veh (all  $p < 0.0001$ ). **C)** VMR data. A two-way ANOVA showed a significant effect of condition ( $F(3) = 5.5371$ ,  $p = 0.0013$ ). Post-hoc analysis (Tukey HSD) showed significant differences between Veh and DSS ( $p = 0.0008$ ), and 10 mg/kg CBG and DSS ( $p = 0.0202$ ). **D)** cFos data. A one-way ANOVA showed a significant effect of condition ( $F(3) = 7.2751$ ,  $p = 0.0012$ ). Post-hoc analysis (Tukey HSD) showed significant differences between Veh and DSS ( $p = 0.0483$ ), 10 mg/kg CBG and DSS ( $p = 0.0048$ ), as well as 10 mg/kg CBG and 5mg/kg CBG ( $p = 0.0056$ ).

### **3.1.4 Effects of acute administration of CBC in the acute DSS model**

CBC was also tested in this same acute DSS protocol. Again, mice treated with 2.5% DSS received 5 or 10 mg/kg i.p. 30 minutes prior to undergoing the VMR measure. Positive controls consisted of DSS treated mice which received the drug vehicle (DSS) and negative controls consisted of mice with normal drinking water which also received the drug vehicle (Veh). All groups contain 9 – 10 mice. Results showed no significant difference between any condition in terms of percent body weight change (Figure 13A). However, the macroscopic damage score showed significant differences between Veh and all conditions receiving DSS (Figure 13B). VMR results showed a significant difference between Veh and DSS conditions but

no effect of either concentration of CBC (Figure 13C). Suggesting that CBC did not attenuate visceral hypersensitivity.

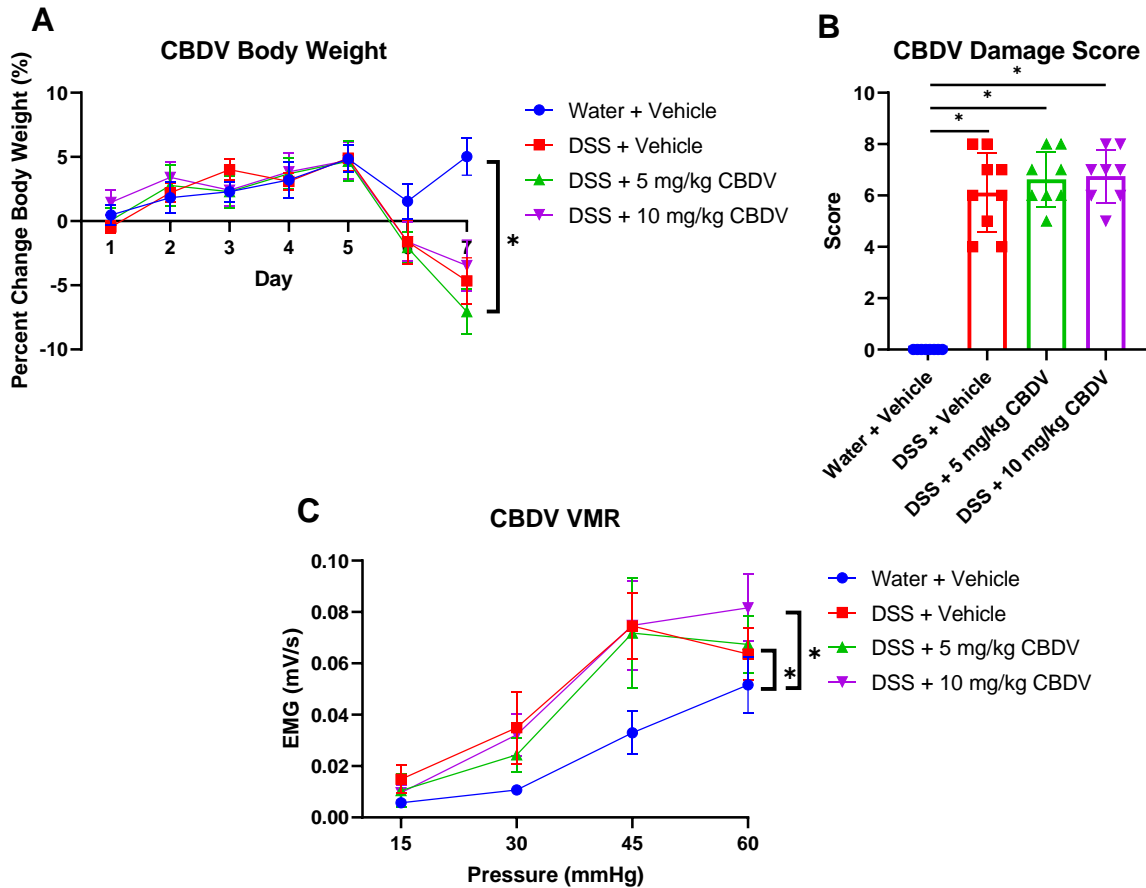


**Figure 13.** A single injection (i.p.) of CBC at 5 or 10 mg/kg does not affect visceral hypersensitivity in mice treated with 2.5% DSS. Control groups are vehicle in mice receiving 2.5% DSS (DSS) and vehicle in mice receiving normal water (Veh). All groups contain 9 - 10 mice. Error bars represent SEM **A**) Percent change in bodyweight. A two-way ANOVA showed no significant effect of treatment condition ( $F(3) = 1.9680$ ,  $p = 0.1194$ ). **B**) Macroscopic damage score. A one-way ANOVA showed a significant effect of treatment condition ( $F(3) = 83.404$ ,  $p <$

0.0001). Post-hoc analysis (Tukey HSD) showed no significant differences between any DSS treated group and all DSS treated groups showed significant differences from Veh (all  $p < 0.0001$ ). C) VMR data. A two-way ANOVA showed a significant effect of condition ( $F(3) = 3.0554$ ,  $p = 0.03061$ ). Post-hoc analysis (Tukey HSD) showed significant differences for Veh-DSS ( $p = 0.01564$ ).

### **3.1.5 Effects of acute administration of CBDV in the acute DSS model**

CBDV was also tested in the same acute DSS protocol. Again, mice treated with 2.5% DSS received 5 or 10 mg/kg CBDV i.p. 30 minutes prior to undergoing the VMR measure. Positive controls consisted of DSS treated mice which received the drug vehicle (DSS) and negative controls consisted of mice with normal drinking water which also received the drug vehicle (Veh). Both body weight and macroscopic damage score showed significant differences between all conditions receiving DSS and water controls (Figure 14A & B). VMR results showed a significant increase in the VMR measure for both the positive control and 10 mg/kg CBDV conditions compared to water controls (Figure 14C). Suggesting that CBDV did not attenuate visceral hypersensitivity.



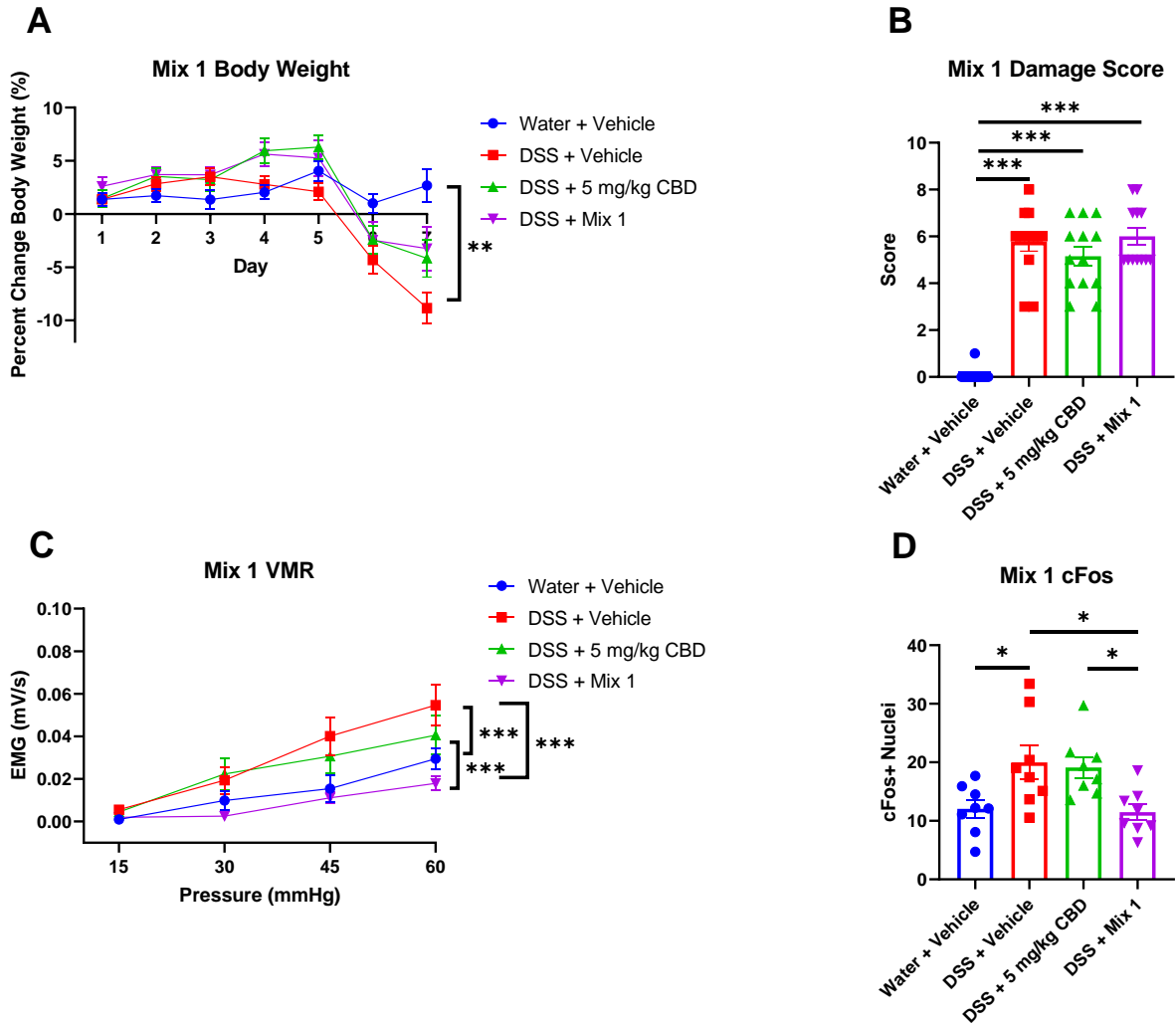
**Figure 14.** A single injection (i.p.) of CBDV at 5 or 10 mg/kg does not affect visceral hypersensitivity in mice treated with 2.5% DSS. Control groups are vehicle in DSS mice (DSS) and vehicle in untreated water mice (Veh). All groups contain 8 – 9 mice. Error bars represent SEM **A**) Percent change in bodyweight. A two-way ANOVA showed a significant effect of treatment condition ( $F(3) = 3.5082$ ,  $p = 0.0159$ ). Post-hoc analysis (Tukey HSD) showed a significant difference between Veh and 5 mg/kg CBDV groups only ( $p = 0.0125$ ). **B**) Macroscopic damage score. A one-way ANOVA showed a main effect of treatment condition ( $F(3) = 29.84$ ,  $p < 0.0001$ ). Post-hoc analysis (Tukey HSD) showed no significant differences between any DSS treated group and all DSS treated groups showed significant differences from Veh (all  $p < 0.0001$ ). **C**) VMR data. A two-way ANOVA showed a significant effect of

condition ( $F(3) = 3.8961$ ,  $p = 0.0107$ ). Post-hoc analysis (Tukey HSD) showed significant differences between Veh and DSS conditions ( $p = 0.0306$ ) as well as Veh and 10mg/kg CBDV ( $p = 0.0114$ ).

### **3.2 The effects of mixing npCBs in the acute DSS model**

#### **3.2.1 Effects of acute administration of Mix 1 in the acute DSS model**

A mixture of npCBs was tested to investigate any potentiation effects of combinations of npCBs. This mixture was composed 5mg/kg CBD as well as 1mg/kg each of CBC, CBDV, and CBG—all shown to be sub-therapeutic dosages. Results showed a significant reduction in body weight for all DSS treated animals and a significant increase the macroscopic damage score for all DSS treated animals compared to water controls (Figure 15A & B). Both VMR and cFos data showed a significant difference between positive and negative controls, Mix 1 and positive controls, as well as Mix 1 and 5 mg/kg CBD (Figure 15C & D). Suggesting that Mix 1 attenuated visceral hypersensitivity.

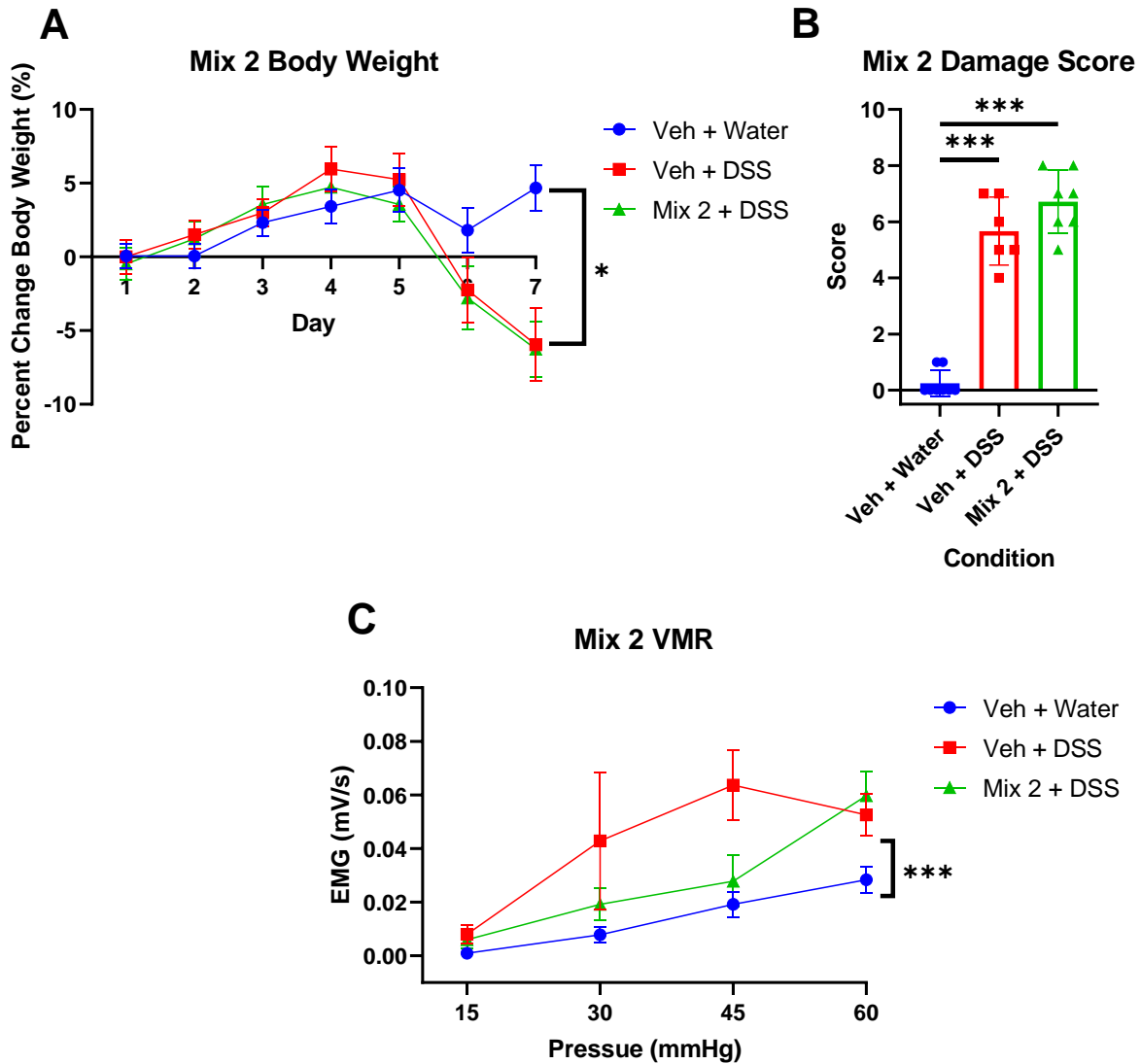


**Figure 15.** A single injection (i.p.) of Mix 1 (5 mg/kg CBD, 1mg/kg each of CBC, CBDV, and CBG) attenuates visceral hypersensitivity in mice treated with 2.5% DSS. Animals treated with 2.5% DSS received a single injection (i.p.) of 5 mg/kg CBD or mix 1 (5 mg/kg CBG + 1mg/kg each of CBC, CBDV, and CBG) 30 minutes prior to VMR. Positive controls consisted of DSS treated mice which received the drug vehicle (DSS) and negative controls consisted of mice with normal drinking water which also received the drug vehicle (Veh). Each treatment contains 12 – 13 mice except for cFos data which contains 8 mice per treatment condition. Error bars represent

SEM **A**) Percent change in bodyweight. A two-way ANOVA showed a main effect of treatment condition ( $F(3) = 6.5178$ ,  $p = 0.0002$ ). Post-hoc analysis (Tukey HSD) showed no significant differences between any DSS treated group and all DSS treated groups showed significant differences from Veh (all  $p < 0.01$ ). **B**) Macroscopic damage score. A one-way ANOVA showed a significant effect of treatment condition ( $F(3) = 69.034$ ,  $p < 0.0001$ ). Post-hoc analysis (Tukey HSD) showed no significant differences between any DSS treated group and all DSS treated groups showed significant differences from Veh (all  $p < 0.0001$ ). **C**) VMR data. A two-way ANOVA showed a significant effect of condition ( $F(3) = 11.0480$ ,  $p < 0.0001$ ). Post-hoc analysis (Tukey HSD) showed significant differences between Veh and DSS ( $p = 0.0007$ ), Mix 1 and DSS ( $p < 0.0001$ ), as well as Mix 1 and 5mg/kg CBD ( $p = 0.0009$ ). **D**) cFos data. A one-way ANOVA showed a main effect of condition ( $F(3) = 5.3037$ ,  $p = 0.0050$ ). Post-hoc analysis (Tukey HSD) showed significant differences between Veh and DSS ( $p = 0.0366$ ), Mix 1 and DSS ( $p = 0.0232$ ), as well as Mix 1 and 5mg/kg CBD ( $p = 0.0481$ ).

### **3.2.2 Effects of acute administration of Mix 2 in the acute DSS model**

To check for a threshold of effect of Mix 1, a second mixture was also attempted in the same protocol. Mixture 2 was composed of 1 mg/kg of each of the 4 npCBs. Body weight data showed a significant reduction in Mix 2 compared to water controls (Figure 16A), and macroscopic damage scores showed significant differences between all DSS treated conditions and water controls (Figure 16B). VMR results showed a significant difference between positive and negative controls but no significant effect of mixture 2 (Figure 16C). Suggesting Mix 2 did not attenuate visceral hypersensitivity.

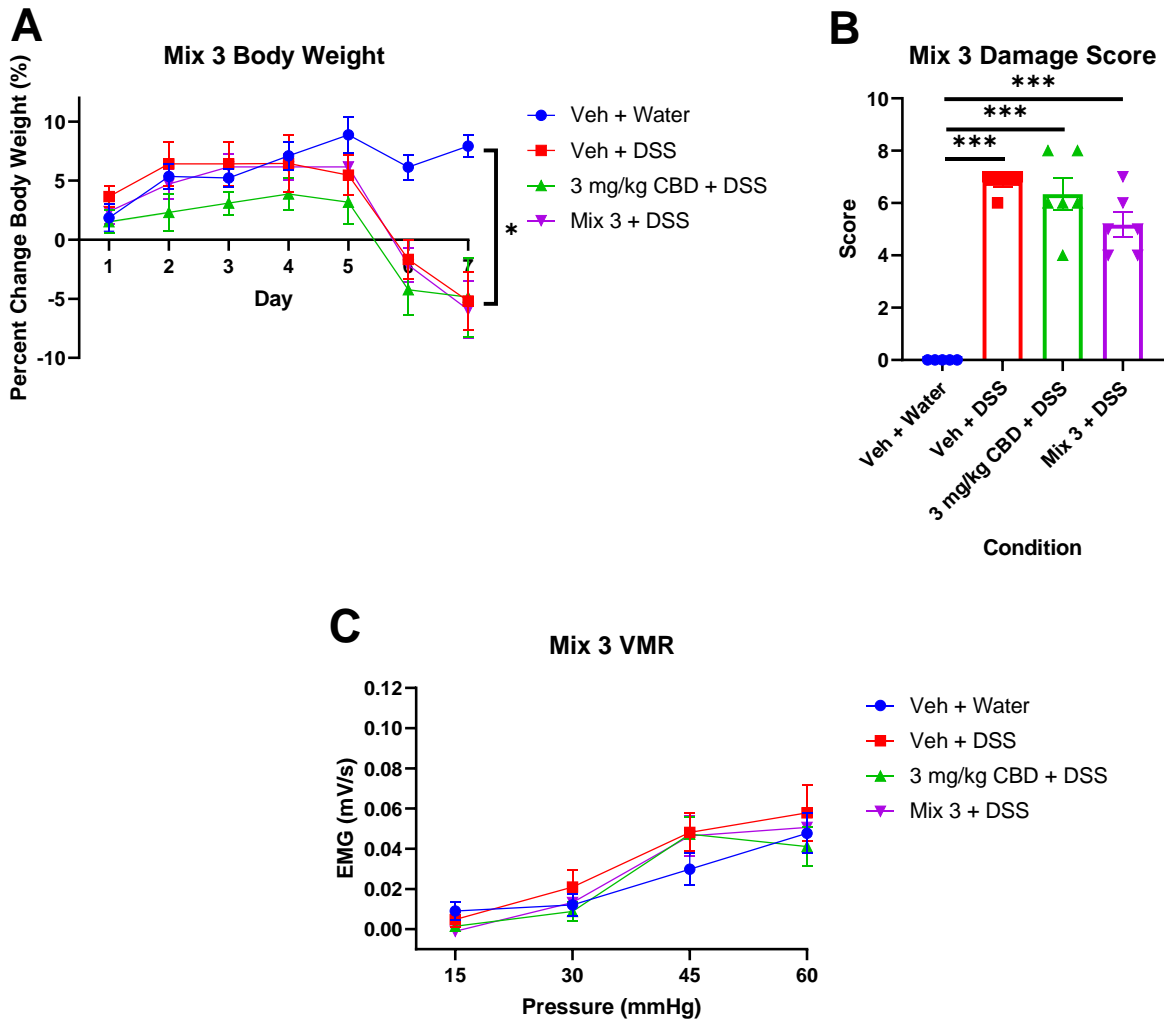


**Figure 16.** A single injection (i.p.) of Mix 2 (1 mg/kg each of CBD, CBDV, CBC, and CBG) does not affect visceral hypersensitivity in mice treated with 2.5% DSS. Control groups are vehicle in DSS mice (DSS) and vehicle in untreated water mice (Veh). All groups contain 6 – 8 mice. Error bars represent SEM **A**) Percent change in bodyweight. A two-way ANOVA showed a significant effect of treatment condition ( $F(2) = 3.4734$ ,  $p < 0.0338$ ). Post-hoc analysis (Tukey HSD) showed significant differences between Mix2-Veh ( $p = 0.0294$ ). **B**) Macroscopic damage score. A one-way ANOVA showed a significant effect of treatment condition ( $F(3) = 100$ ,  $p <$

0.0001). Post-hoc analysis (Tukey HSD) showed no significant differences between any DSS treated group and all DSS treated groups showed significant differences from Veh (all  $p < 0.0001$ ). C) VMR data. A two-way ANOVA showed a significant effect of condition ( $F(2) = 9.5987$ ,  $p = 0.0002$ ). Post-hoc analysis (Tukey HSD) showed significant differences for Veh-DSS ( $p = 0.0001$ ).

### **3.2.3 Effects of acute administration of Mix 3 in the acute DSS model**

Following-up on the results of Mix 2, a third mixture was also attempted to investigate a threshold of effects observed in Mix 1. Mixture 3 was composed of 3 mg/kg CBD and 1 mg/kg each of CBDV, CBC, and CBG. Both body weight (Figure 17A) and macroscopic damage score (Figure 17B) data showed significant differences between all DSS treated conditions and water controls. VMR results were inconclusive and showed a trend of no-effect of the mix or 3 mg/kg CBD; however, positive and negative controls were not significantly different and thus no real conclusions can be drawn from this experiment (Figure 17C).



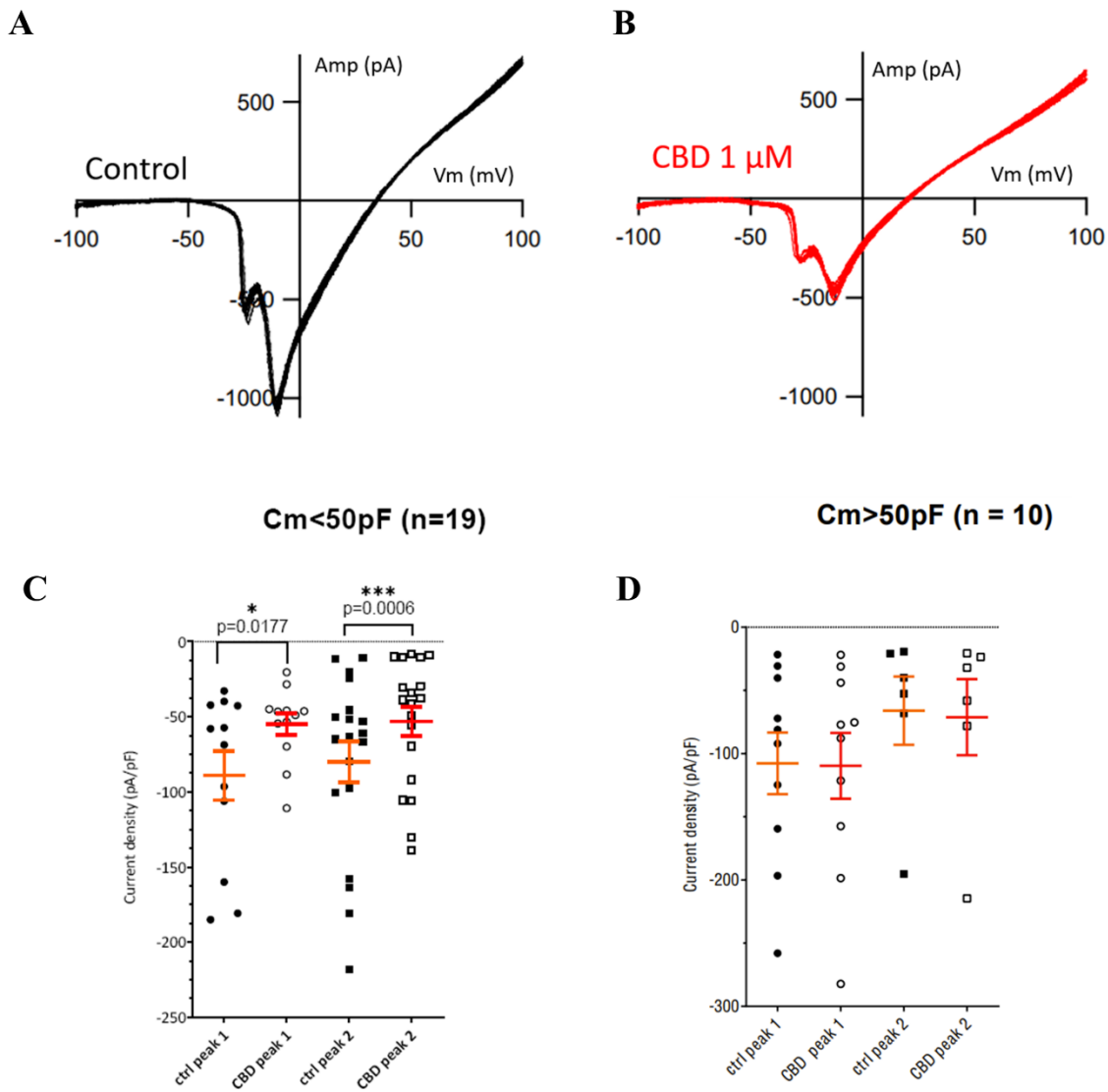
**Figure 17** The effect of a single injection (i.p.) of Mix 3 (3 mg/kg CBD + 1mg/kg each of CBC, CBDV, and CBG) on visceral hypersensitivity in mice treated with 2.5% was inconclusive. DSS Control groups are vehicle in DSS mice (DSS) and vehicle in untreated water mice (Veh). All groups contain 5 – 6 mice. Error bars represent SEM **A**) Percent change in bodyweight. A two-way ANOVA showed a significant effect of treatment condition ( $F(3) = 12.573$ ,  $p < 0.0001$ ). Post-hoc analysis (Tukey HSD) showed significant differences between Veh mice and all DSS treated mice (at least,  $p < 0.01$ ), as well as between CBD3 and DSS conditions ( $p = 0.0398$ ). **B**) Macroscopic damage score. A one-way ANOVA showed a significant effect of treatment

condition ( $F(3) = 47.39$ ,  $p < 0.0001$ ). Post-hoc analysis (Tukey HSD) showed no significant differences between any DSS treated group and all DSS treated groups showed significant differences from Veh (all  $p < 0.0001$ ). C) VMR data. A two-way ANOVA showed no significant effect of condition ( $F(3) = 0.9611$ ,  $p = 0.4159$ ).

### **3.3 Investigations of mechanisms of npCBs by electrophysiology**

#### **3.3.1 The effect of CBD on evoked inward-currents in primary DRG neurons**

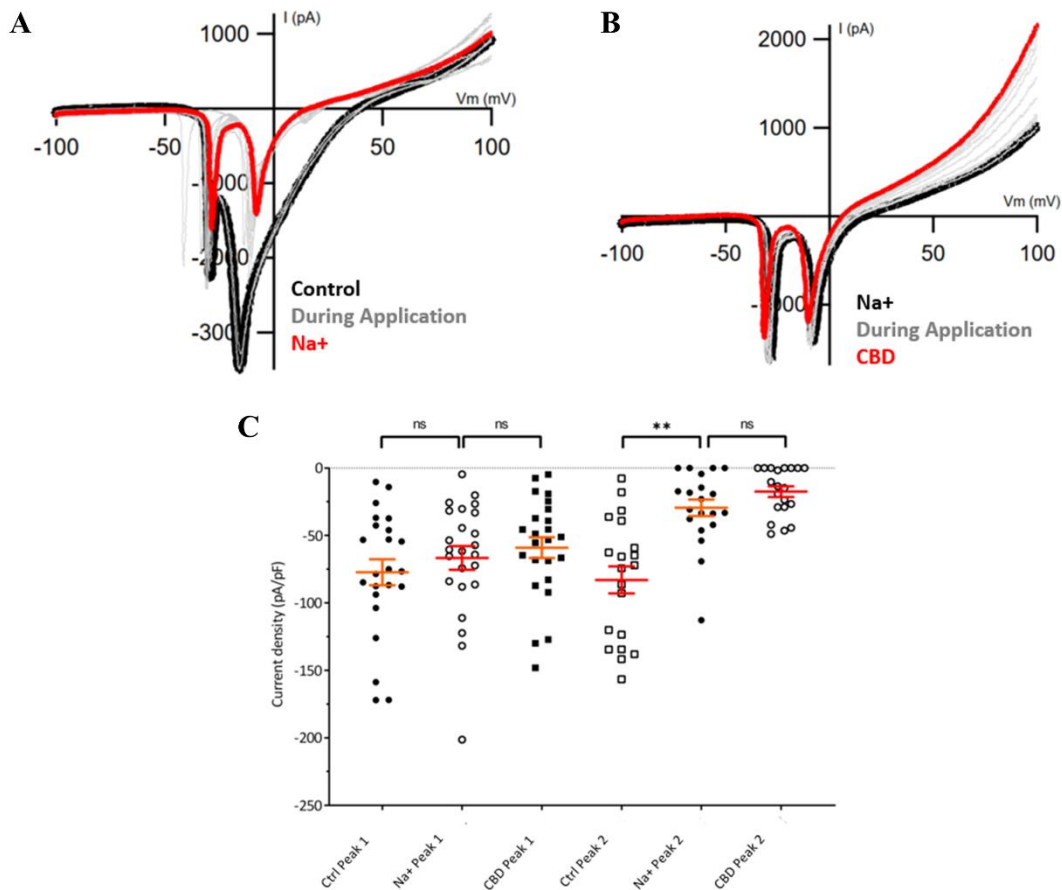
The mechanisms of CBD were investigated by whole-cell voltage-clamp electrophysiology on primary DRG neurons in culture. Initially, CBD was applied at  $1 \mu\text{M}$  concentration in normal external bath solution (Table 4) which contained TEA-Cl to block potassium channels and a voltage-ramp protocol from  $-100\text{mV}$  to  $+100\text{mV}$  applied (Figure 18A & B). This stimulation protocol will yield an inward-current trace with two peaks. The first peak will be primarily due to  $\text{Na}^+$  ion flux and the second peak  $\text{Ca}^{2+}$  ion flux due differences in their kinetics and activation thresholds (Bennett et al., 2019; Cummins et al., 2020; Harding & Zamponi, 2022). Results showed a significant reduction of both inward-current peaks for small ( $C_m < 50 \text{ pF}$ ) DRG neurons (Figure 18C) while no effect was observed for large ( $C_m > 50 \text{ pF}$ ) DRG neurons (Figure 18D). Suggesting that CBD inhibited either  $\text{Na}^+$ , or  $\text{Ca}^{2+}$ , or both currents.



**Figure 18.** CBD (1  $\mu$ M) reduces inward-currents evoked by a -100 mV to +100mV voltage-ramp on primary DRG neurons. **A)** Control example trace showing two distinct current peaks. **B)** CBD (1  $\mu$ M) example trace showing a reduction in both current peaks. **C)** Quantification of current density at peaks 1 and 2 for small ( $C_m < 50$  pF;  $n = 19$ ) DRG neurons. A significant reduction of current density was observed for both peaks (peak 1,  $p = 0.0177$ ; peak 2,  $p = 0.0006$ ). **D)** Quantification of currents density at peaks 1 and 2 for large ( $C_m > 50$  pF;  $n = 10$ ) DRG neurons. No significant change was observed at either peak. Courtesy of Dr. Amyaouch Bradaia.

### 3.3.2 The effect of CBD on isolated Na<sup>+</sup> currents in primary DRG neurons

Following the initial result that 1  $\mu\text{M}$  CBD can affect inward-currents in small DRG neurons, the same voltage-ramp protocol was repeated using the same external bath solution with the addition of cadmium to block voltage-gated Ca<sup>2+</sup> channels (isolating the Na<sup>+</sup> current) and patching only small DRG neurons. Results showed application of cadmium to significantly reduce peak 2. The subsequent application of 1  $\mu\text{M}$  CBD in the bath solution showed no significant effect on either peak (Figure 19C) suggesting no effect of CBD on Na<sup>+</sup> currents.

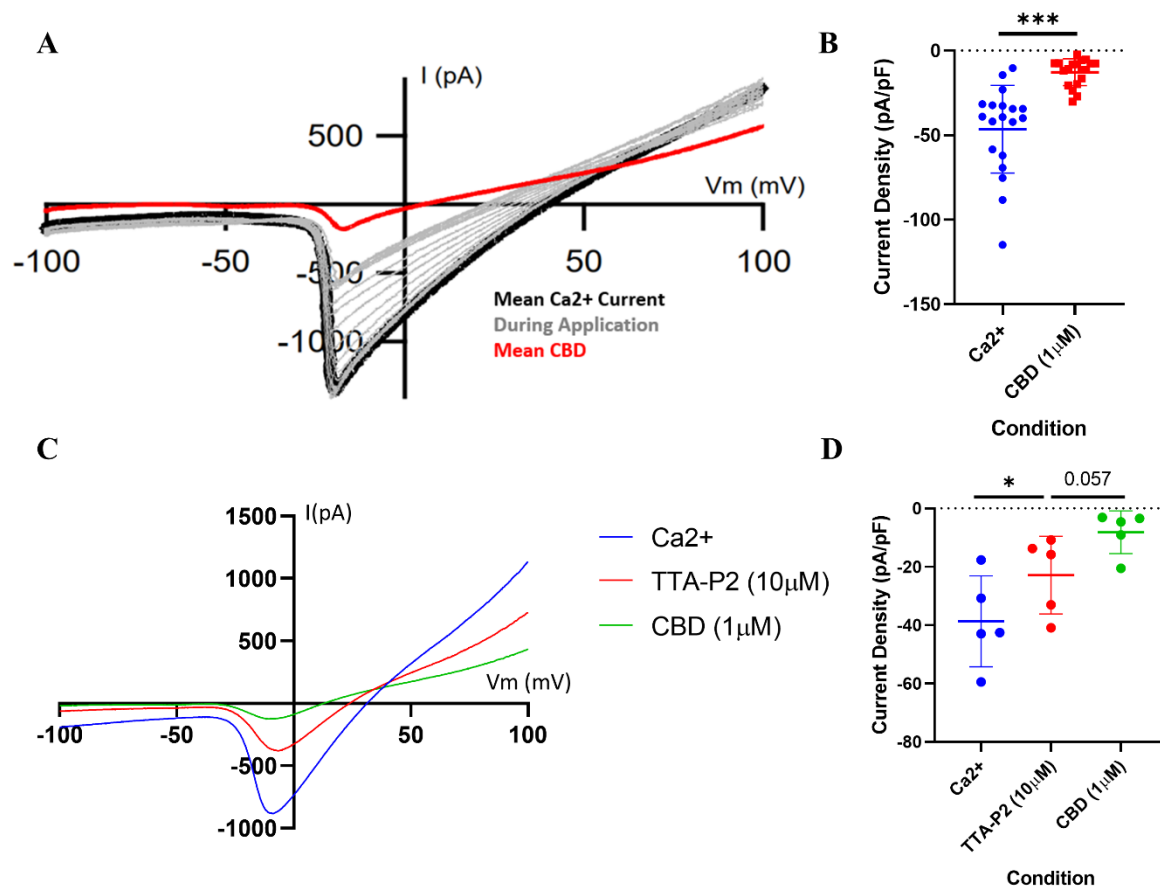


**Figure 19.** CBD (1  $\mu\text{M}$ ) showed no effect on isolated Na<sup>+</sup> currents evoked by a -100 mV to +100 mV voltage-ramp in small ( $C_m < 50$  pF) primary DRG neurons. **A)** Example trace showing

the control current (black), the change in current during application of cadmium (gray), and the isolated Na<sup>+</sup> current (red). **B**) Example trace showing the isolated Na<sup>+</sup> current (black), the application of 1 μM CBD (grey), and the stable current in the presence of CBD (red). **C**) Quantification of the current densities at peaks 1 and 2 for control, isolated Na<sup>+</sup>, and CBD condition. The addition of cadmium to isolate the Na<sup>+</sup> showed a significant reduction in peak 2 current density compared to control ( $p < 0.01$ ); however, CBD showed further reduction of this isolated Na<sup>+</sup> current. Courtesy of Dr. Amyaouch Bradaia.

### **3.3.3 The effect of CBD on isolated Ca<sup>2+</sup> currents in primary DRG neurons**

After observing no effect of CBD on Na<sup>+</sup> currents, the Ca<sup>2+</sup> current was isolated by using a low Na<sup>+</sup> solution (Table 4) and the same -100 mV to +100 mV voltage-ramp applied (Figure 20A). Results showed a significant reduction of this isolated Ca<sup>2+</sup> current with the application of 1 μM CBD (Figure 20B). Next, to begin to uncover which Cav isoform may be affected by CBD, the T-type Cav blocker TTA-P2 was applied to isolate non-T-type Cav currents (Figure 20C). Results showed a significant reduction in peak current density following TTA-P2 application to the isolated Ca<sup>2+</sup> current and a trend of reduction in peak current density following CBD application to the isolated non-T-type Ca<sup>2+</sup> current (Figure 20D).

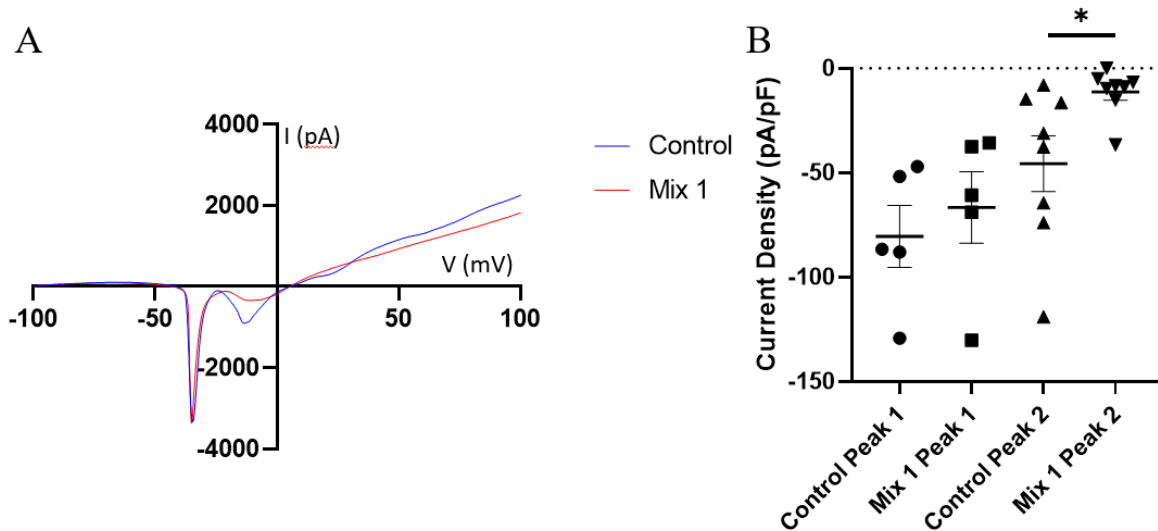


**Figure 20.** CBD (1  $\mu\text{M}$ ) reduces isolated  $\text{Ca}^{2+}$  currents evoked by a -100 mV to +100 mV voltage-ramp in small ( $C_m < 50$  pF) primary DRG neurons. **A)** Example traces showing the average isolated calcium current (black), individual traces during the application of CBD, and the average stable trace following CBD application (red). **B)** Quantification of the peak current density of the isolated  $\text{Ca}^{2+}$  before and after CBD application. The application of CBD significantly reduced peak current density ( $p < 0.001$ ). **C)** Example traces showing the isolated  $\text{Ca}^{2+}$  current (blue), the non-T-type  $\text{Ca}^{2+}$  current (red), and the remaining current following CBD application. **D)** Quantification of peak current density. Results showed a significant reduction with  $\text{Ca}^{2+}$  current with application of TTA-P2 ( $p = 0.0233$ ; Sidak's multiple

comparison test) and trend of further reduction with CBD application ( $p = 0.0568$ ; Sidak's multiple comparison test). A and B are courtesy of Dr. Amyaouch Bradaia.

### 3.3.4 The effect of Mix 1 on evoked inward-currents in primary DRG neurons

The Mix 1 used in vivo (5 mg/kg + 1 mg/kg each CBC, CBDV, CBG) was recapitulated in vitro by combining 1  $\mu\text{M}$  CBD with 200 nM each of CBC, CBDV, CBG thus maintaining the same proportion of npCBs. Mix 1 was applied in normal external bath solution (Table 4) and the same voltage-ramp protocol from -100mV to +100mV applied (Figure 21A). Results showed no significant effect on the first peak evoked (peak 1) but a significant reduction of the second peak evoked (peak 2; Figure 21B) suggesting an effect either  $\text{Na}^+$ , or  $\text{Ca}^{2+}$ , or both currents.

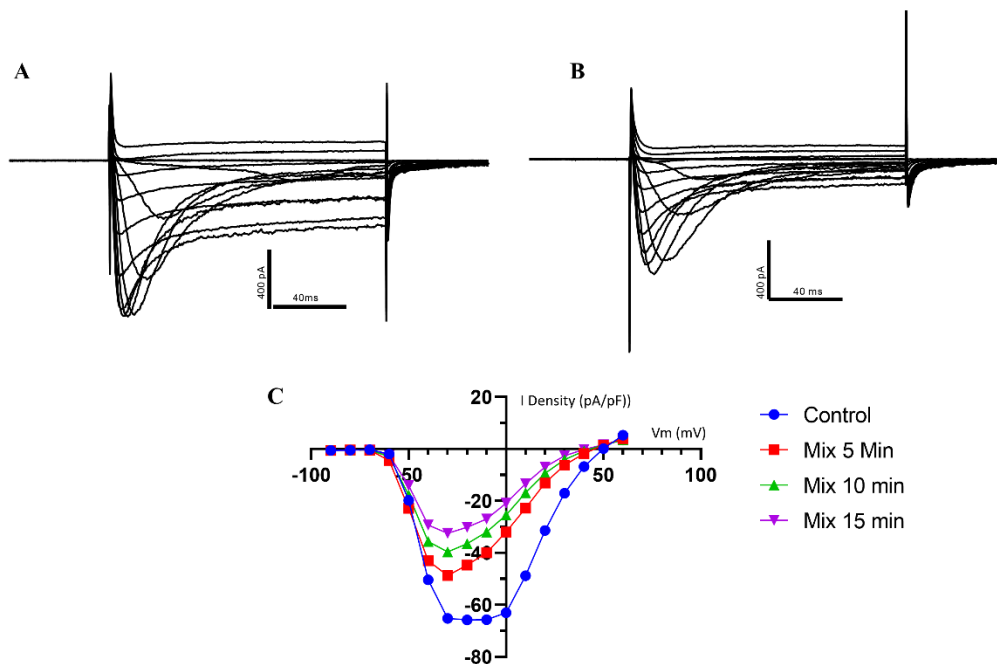


**Figure 21.** Mix 1 (1  $\mu\text{M}$  CBD + 200 nM each of CBC, CBDV, CBG) inhibits inward-currents evoked by a -100 mV to +100mV voltage-ramp on primary DRG neurons. Error bars represent SEM. A) Example traces showing the effect of Mix 1 on evoked inward-currents. B)

Quantification of peak current density. Results showed no significant effect on peak 1 ( $p = 0.3015$ ,  $n = 5$ ) and a significant reduction of peak 2 ( $p = 0.0251$ ,  $n = 8$ ).

### 3.3.5 The effect of Mix 1 on isolated $\text{Ca}^{2+}$ currents in primary DRG neurons

Following-up on the result that Mix 1 can affect the second peak of evoked inward-currents, the calcium current was isolated by using a low  $\text{Na}^+$  solution with  $\text{Ba}^{2+}$  used as the charge carrier to mitigate run-down effects (Harding et al., 2023) (Table 4). Additionally, a step protocol was used: initially, cells were held at  $-90$  mV and stepped to  $+60$  mV in  $10$  mV increments every  $10$  seconds. Results suggest a reduction in peak current density that is greater around  $0$  mV; however, an  $n$  of  $1$  precludes any statistical analysis (Figure 22C).



**Figure 22.** Mix 1 ( $1 \mu\text{M}$  CBD +  $200$  nM each of CBC, CBDV, CBG) may inhibit high-voltage activated calcium channels. Effect in isolated  $\text{Ca}^{2+}$  currents evoked by a  $-90$  mV to  $+60$  mV ( $\Delta V$

= 10mV) on primary DRG neurons. **A)** Example control trace. **B)** Example trace 15 minutes after Mix 1 application. **C)** I-V curve showing peak current density at each step. A trend of reduction is shown, and is more pronounced around 0 mV; however, no statistical analysis could be completed due to insufficient n.

## CHAPTER FOUR: DISCUSSION

### Summary of main findings

This project has shown CBD, CBG, and a blend of npCBs to attenuate visceral hypersensitivity in the acute DSS model of ulcerative colitis. CBD and CBG both significantly reduced nocifensive behaviors using the VMR as a surrogate measure of visceral pain and second-order neuron activation in the lumbosacral dorsal horn at doses of 10 mg/kg. Conversely, CBDV and CBC did not affect VMR responses at either 5 mg/kg or 10 mg/kg doses. Interestingly, potentiation effects were observed by combining sub-therapeutic doses of npCBs. Namely, 5 mg/kg CBD combined with 1 mg/kg each of CBC, CBDV, and CBG (Mix 1) showed a significant reduction in VMR responses and second-order neuron activation. Mechanistic experiments using whole-cell patch-clamp electrophysiology demonstrated CBD to inhibit voltage-gated calcium channels in DRG neurons, with preliminary evidence suggesting a non-T-type site of action. Initial evidence suggests that Mix 1 may act through a similar mechanism. Together, these data demonstrate CBD, CBG, and Mix 1 to be potential therapeutic options for the management of IBD-associated pain.

### CBD attenuates visceral hypersensitivity

The goal of this project was to explore the therapeutic potential of 4 npCBs in ulcerative colitis. The acute DSS model of experimental colitis was chosen due to its ease of administration through the drinking water and recapitulation of key symptoms of ulcerative colitis such as weight loss, inflammation of the colon, and visceral hypersensitivity (Chassaing et al., 2015).

Additionally, male mice (C57Bl/6J) were chosen because males show a stronger phenotype with this model (Chassaing et al., 2015).

Four npCBs were studied in this project and CBD was chosen for the initial experiments as it is best studied npCB in the literature and is found at much higher levels in some strains of cannabis. For the first experiment, CBD was administered daily (i.p.) at 1 mg/kg, 5 mg/kg, and 10 mg/kg doses. Daily administration allowed for the screening of any anti-inflammatory or protective effects which had been previously reported in the literature (Borrelli et al., 2009; Schicho & Storr, 2012; Wei et al., 2020); although, other studies had also reported no such effects of CBD (Pagano et al., 2016; Silvestri et al., 2020). DAI results suggested no effect of CBD while the macroscopic damage score showed a trend of reduction with the 10 mg/kg condition. Additionally, 10 mg/kg also showed a trend of reduction in the VMR measure which was paralleled in immunohistochemical staining for the neuronal activity marker cFos in the lumbosacral dorsal horn. To tease apart any protective effects brought on by the daily administration of CBD, the next experiment used a single injection 30 minutes prior to the VMR measure. This administration protocol precludes the majority of anti-inflammatory effects from confounding acute analgesic actions of the compound. These experiments showed significant analgesic effects of 10 mg/kg CBD in both the VMR and cFos measures. This is the first evidence of CBD analgesia in an IBD model. Two other studies have examined CBD in visceral pain using the acetic acid writhing test. One showed no effect of CBD at 20 or 50 mg/kg (s.c.) (Booker et al., 2009) while the other showed an effect only at the high doses of 30 and 90 mg/kg (i.p.) (Silva et al., 2017). This difference in efficacy may be related to differences in dosage administered or to differences in bioavailability between sub-cutaneous and intraperitoneal administration route. Observing 10 mg/kg CBD to effectively reduce nociception in a model IBD

is a novel finding, but not therefore entirely unexpected when considering the success of CBD as an analgesic in other pain models such as spared nerve injury or diabetic neuropathy (Silva-Cardoso & Leite-Panissi, 2022).

An important caveat to the translation of this finding is the administration of route of CBD. Administration by i.p. injection was chosen for this study as it provides simpler pharmacology compared to oral gavage or inhalation. Oral gavage would subject CBD to hepatic-metabolism which converts approximately 97.5% of CBD to 7-COOH-CBD which may or may not be bioactive (Stella, 2023). The heating of CBD to the temperatures required for inhalation either by e-cigarette or combustion converts 25-52% to other cannabinoids predominantly the psychoactive constituent THC as well as CBN and CBC (Czégény et al., 2021).

The impact of the analgesic dose of CBD on sickness behaviors was investigated using the LABORAS system for 6 hours following administration. The LABORAS system is an automatic behavior acquisition system which quantifies various behaviors including eating, drinking, grooming, rearing, climbing, and locomotion. CBD was shown to increase eating behaviors in DSS treated mice and no other behavioral effects were observed. Importantly, while CBD did not alleviate the reduced locomotion, climbing, and rearing behaviors observed in positive controls, it also did not further depress these behaviors. An effect which has been reported some studies of CBD (Moore & Weerts, 2022). Overall, these results suggest that CBD may be an effective treatment option for visceral hypersensitivity with at least minimal movement related side-effect, but administration route remains a major barrier.

### **CBG attenuates visceral hypersensitivity**

Considering the novelty of the analgesic effects of CBD, the project focused on the single injection paradigm to screen the remaining npCBs for similar effects. Both VMR and cFos measures showed 10 mg/kg of CBG to also attenuate visceral hypersensitivity. This is a highly novel finding as no studies have examined CBG in the context of visceral pain. Non-visceral pain models have shown CBG effective in cisplatin-induced peripheral neuropathy but ineffective in either the tail flick or formalin tests (Sepulveda et al., 2022; Zagzoog et al., 2020). CBG has also shown strong anti-inflammatory effects both by i.p. and o.g. in a DNBS model (Borrelli et al., 2013; Pagano et al., 2021). Finally, CBG shows greater bioavailability by o.g. than i.p. (Deiana et al., 2012; Jastrzab et al., 2022) overcoming a key limitation to CBD based therapeutics. Together, these results suggest CBG to be a very promising potential therapeutic for IBD.

### **CBDV and CBC have no effect on visceral hypersensitivity**

The remaining two npCBs, CBC and CBDV, showed no significant attenuation of visceral hypersensitivity in the VMR measure. Previous studies have shown weak evidence of anti-nociceptive effects of CBC. Mixed results have been observed on the tail-flick test: One study showed a significant effect of 100 mg/kg (i.v.) that was not reproduced later in the study (DeLong et al., 2010a). Another study showed a significant effect at 3mg/kg (i.p.) but not at the higher dose of 10 mg/kg (Zagzoog et al., 2020). Finally, no effect in the acetic acid writhing test (Booker et al., 2009). While CBC may not show anti-nociceptive effects, numerous studies have shown effects which may be beneficial in IBD such as anti-inflammatory, reduction of intestinal

permeability (Romano et al., 2013), and motility regulating effects (Izzo et al., 2012). CBDV has not been examined directly in pain models; however, in vitro evidence of TRP channel desensitization (Iannotti et al., 2014; Ligresti et al., 2016) suggested CBDV might show anti-nociceptive properties in a model of visceral hypersensitivity. Like CBC, CBDV may not be anti-nociceptive, but has other promising features that may be relevant in IBD treatment such as anti-inflammatory effects (Pagano et al., 2019). Interestingly, CBDV has been shown to prevent the upregulation of TRPA1 associated with UC and to exert stronger anti-inflammatory effects by o.g. than i.p. (Pagano et al., 2019) suggesting that CBDV may prove beneficial through either the daily administration paradigm or through administration by oral gavage. Finally, CBDV was recently shown to enhance morphine anti-nociception and attenuate morphine analgesic tolerance (Wang et al., 2022) suggesting CBDV may be best used in combination with other drugs.

### **Mix 1 attenuates visceral hypersensitivity**

This project also sought to examine a combination of npCBs. Numerous studies have reported interactions from combining multiple cannabinoids together. Some studies show positive interactions such as increasing anti-nociceptive or anti-inflammatory effects (DeLong et al., 2010b; Pagano et al., 2016), while others have shown negative interactions such as decreasing anti-nociceptive effects (Booker et al., 2009) or blocking anti-emetic effects (Rock et al., 2011). One motivation for combining cannabinoids is to increase their potency which is a common issue in cannabinoid-based therapeutics. For example, effective doses of CBD have been reported as high as 300-600 mg/day for anti-anxiety effects (Bergamaschi et al., 2011) which has motivated some researchers to attempt to increase the bioavailability of CBD through chemical modifications such as fluorination (Silva et al., 2017). Additionally increasing potency

may aid to achieve a desired effect without also inducing side-effects that may occur at higher doses, such as memory issues which have been reported in mice at 10 mg/kg (i.p.) CBD (Stella, 2023) the same dose that was found to be analgesic in this project.

To show strong evidence of a potentiation or “entourage” effect, subtherapeutic doses of npCBs were selected based on their results individually. The first combination attempted consisted of 5 mg/kg CBD with 1mg/kg each of CBC, CBDV, and CBG (Mix 1). Mix 1 showed significant attenuation of visceral hypersensitivity in the VMR measure and was confirmed by cFos staining. Other studies have shown enhanced analgesia by combining cannabinoids but have focused on enhancing the effects of THC (Booker et al., 2009; DeLong et al., 2010a). This result is the first to show a potentiation effect with only npCBs.

Two other combinations were also tested. Mix 2 consisted of 1 mg/kg each of CBD, CBC, CBDV, and CBG. This mix proved ineffective at reducing visceral hypersensitivity in the VMR measure. Suggesting that there may be a threshold for effect and serving as a control for the 1 mg/kg doses used in Mix 1. To further probe this threshold, a third combination was attempted consisting of 3 mg/kg CBD with 1 mg/kg each of CBC, CBDV, and CBG. Unfortunately, due to variance inherent in the VMR measure and DSS model, positive and negative controls were not different and thus no conclusions could be drawn from this experiment.

### **CBD and Mix 1 may act through a non-T-type mechanism**

Potential mechanisms were explored using whole-cell patch-clamp electrophysiology in primary DRG neurons. The voltage ramp protocol that was used evokes a current with two

peaks. The first peak will be primarily due to  $\text{Na}^+$  ion flux and the second peak  $\text{Ca}^{2+}$  ion flux due differences in their kinetics and activation thresholds (Bennett et al., 2019; Cummins et al., 2020; Harding & Zamponi, 2022). Importantly, some overlap exists in ions contributing to these two peaks. Initial experiments showed a decrease in current density for both peaks with 1  $\mu\text{M}$  CBD; however, this was only found in small DRG neurons not the larger neurons as measured by cell membrane capacitance. DRG neurons are an extremely heterogenous population of neurons with unique cell populations serving diverse functions. Nociceptors have small cells bodies while other populations such as proprioceptors have larger cell bodies (Abdullah et al., 2020). Thus, these results suggest CBD to act primarily on nociceptors while leaving other populations like proprioceptors unaffected; subsequently, any motor affects attributed to CBD likely emerge from sites other than the DRG neurons.

Both  $\text{Na}^+$  and  $\text{Ca}^{2+}$  currents were isolated to identify which ion channel populations were affected by CBD in the previous experiment.  $\text{Na}^+$  currents were isolated by blocking  $\text{Ca}^{2+}$  channels with cadmium and no effect of CBD was found on this current. This result is surprising considering a recent paper has shown CBD to stabilize the inactivated-state of Nav1.8(H.-X. B. Zhang & Bean, 2021), the main driver of action potentials in nociceptors (Waxman & Zamponi, 2014). However, the disparate findings may be explained by a difference in concentration of CBD: 1  $\mu\text{M}$  in this project compared to 2 and 5  $\mu\text{M}$  in Zhang & Bean (2021). Next,  $\text{Ca}^{2+}$  currents were isolated by reducing the  $\text{Na}^+$  concentration from 60 mM to 2 mM and balancing the osmolarity with N-Methyl-D-glucamine. CBD significantly reduced the peak current density of this isolated  $\text{Ca}^{2+}$  current. There are 10 isoforms of Caves (Dolphin, 2006) which are broadly categorized into LVA or T-type and HVA. Transcriptomic data has shown Cav2.2 (N-type) and Cav3.2 (a T-type) to be the most highly expressed in the nociceptor population (Zheng et al.,

2019), and functional experiments have shown these two isoforms to be key analgesic targets in visceral pain (Marger et al., 2011; Waxman & Zamponi, 2014). Additionally, three studies have shown CBD to inhibit T-type Cav channels (Harding et al., 2023; Mirlohi et al., 2022; Ross et al., 2008b). Thus, the effect of CBD on an isolated Ca<sup>2+</sup> current was investigated in the presence of a T-type channel blocker (TTA-P2) and showed a near-significant reduction of the peak current density of this non-T-type Ca<sup>2+</sup> current suggesting activity of CBD at an HVA Cav most likely Cav2.2. This would be a novel site of action for CBD and future experiments should seek to confirm the modulation of ion flux through this channel. A recent study in an expression system showed CBD to directly inhibit Cav3.2 by occluding the pore but to not affect Cav2.2 ion flux directly or when co-expressed with the CB1 receptor (Harding et al., 2023). Thus, if Cav2.2 is affected by CBD, it is either interacting with a different splice variant of Cav2.2 (Lipscombe & Lopez Soto, 2019) or acting indirectly through modulation by a GPCR (Bourinet et al., 2014) other than CB1 which is present in primary DRGs but not in the expression system used in the previously mentioned study.

Mix 1 was recapitulated in vitro by combining 1  $\mu$ M CBD with 200 nM each of CBC, CBDV, and CBG maintaining the same 5:1:1:1 ratio used in vivo. Similar to CBD, Mix 1 was initially tested on combined Na<sup>+</sup> and Ca<sup>2+</sup> currents and was found to inhibit only peak two suggesting an effect on Ca<sup>2+</sup> currents. The final follow-up experiments isolated Ca<sup>2+</sup> currents as before, but additionally replaced Ca<sup>2+</sup> with Ba<sup>2+</sup>. Ba<sup>2+</sup> is commonly used when studying Caves because it provides a slightly larger current—allowing for easier study of small Cav currents—and can mitigate the phenomenon of run down wherein evoked Ca<sup>2+</sup> currents decrease in magnitude due to Ca<sup>2+</sup> being sequestered and interacting with other components of the cellular machinery (Harding et al., 2023). The final change was in the stimulation protocol. Instead of a

linear ramp of voltage from -100 mV to +100 mV, a step protocol was used. Neurons were held at -90mV and stepped sequentially to voltages increasing by 10 mV with each step up to +60 mV. This allows for the generation of an I-V curve showing the current density at each potential. Because T-type Cavs are activated at lower potentials while HVA Cavs such as Cav2.2 are activated at higher potentials, this I-V curve can inform on which population (LVA or HVA) is primarily affected by Mix 1. Results showed a trend of reduction at low voltage but a greater reduction at higher voltages, suggesting that Mix 1 may similarly be acting on HVA Cavs.

Preliminary data suggest a common mechanism of action for CBD and Mix 1 through non-T-type currents. This is not surprising considering that the same concentration of CBD is present in Mix 1. An important follow-up experiment will be to directly compare CBD alone and Mix 1 on the percent inhibition of inward-currents or isolated Ca<sup>2+</sup> currents. The results of this experiment would be informative on mechanistic actions of Mix 1. If Mix 1 shows greater inhibition than CBD alone, this would suggest that the entourage effects can at least partially be attributed to interactions at the level of the DRG. One possible mechanism could be converging pathways resulting from activation of non-CB GPCRs, such as GPR18 and GPR55 (Morales & Reggio, 2017) combined with direct interactions with ion channels. If no difference is found between the conditions, this would suggest that other sites must be involved such as the CNS as npCBs are able to pass the blood-brain barrier (Pertwee, 2005) or the involvement of K<sup>+</sup> channels such as GIRKs. A possible mechanism with this outcome could include the recruitment of top-down inhibition via the periaqueductal grey and rostroventral medulla coupled with direct effects in the DRG such as inhibition of Cav2.2 via GPCR modulation (Bourinet et al., 2014), Cav3.2 (Harding et al., 2023), Nav1.8 (Zhang & Bean, 2021), Nav1.7 (Ghovanloo et al., 2022) or activation of Kv7 channels (Zhang et al., 2022).

## Limitations and future experiments

A major limitation of this project is the use of only male mice *in vivo*. As stated previously, male mice were chosen due to showing a more pronounced phenotype with the DSS model (Chassaing et al., 2015). Additionally, studies have shown responses on the VMR measure to vary with the estrous cycle (Ji et al., 2008) adding a layer of logistical complexity to these experiments. It seems reasonable then, to perform high-throughput screenings with male mice; however, a key step would be to repeat the three significant findings—10m/kg CBD, 10mg/kg CBG, and Mix 1—in female mice to increase the translatability of the study and to address the sex bias in preclinical research (Karp & Reavey, 2019). Future studies could also repeat these significant findings in a post-inflammatory model of colitis (Lapointe et al., 2015). In this model, mice receive DSS for 5 days then are allowed to recover for 5 weeks. This model recapitulates the visceral hypersensitivity experienced by some IBD patients during remission in the absence of active inflammation (Hurtado-Lorenzo et al., 2021; Weaver & Szigethy, 2020). Results of this experiment would further increase the translatability of the project.

There are technical limitations sources of variance with each technique used in this study. The DSS model is easy to administer it is also variable and thus difficult to compare across experiments. Sources of variance include interbatch variability of the compound. Additionally, administration through drinking water means that the exact dose an animal receives is dependent upon how much the animal drinks and any changes in motility that a treatment may induce. Finally, disease severity has been shown to be affected by the microbiome of the animal (Katsandegwaza et al., 2022). Performing the VMR measure requires the implantation of electrodes into the oblique muscles of the animal. This action may result in initiation of host-tissue response to the foreign body and responses to anesthesia. Additionally, anti-biotic usage

has been shown to impact VMR response (Larauche et al., 2011). Thus, rigorous use of control conditions is required when using this measure. The gene c-Fos is commonly used as a marker of neuronal activity in nociceptors (Harris, 1998). Importantly, this technique has several limitations including the sensitivity and consistency of the measure. Expression of this gene requires strong and prolonged stimulation and it is unclear if there is a consistent threshold for stimulation between neurons (Harris, 1998). Additionally, there is very low event-measure specificity with this approach i.e. it is difficult to know exactly what stimulus was responsible for change in c-Fos expression. Finally, the timing between noxious stimulus application and tissue fixation may impact relative c-Fos expression. Electrophysiology is a very powerful technique for examining pharmacological actions and functional outcomes of compounds. Importantly, it is an *in vitro* model and thus not a facsimile of the *in vivo* system and will be missing many components of the true *in vivo* system. This study used primary DRGs in place of an expression system to examine the effects of npCBs in a more intact neuronal system; however, these were DRGs from all levels of the spinal cord not just from the levels innervating the colon. DRGs are extremely heterogenous, and differences exist across levels of the spinal as well (Harrington et al., 2019). Thus, any effects observed may not be representative of what is happening in the intact system.

This study leaves many mechanistic questions unanswered. A previous study has shown CBD to preferentially block Cav3.2 T-type over Cav2.2 N-type channels in a heterologous expression system (Harding et al., 2023), our work suggests Cav2.2 as a putative target in sensory DRG neurons. Moreover, it is possible that the site of direct interaction is a non-CB GPCR receptor i.e GPR18 or 55 (Guerrero-Alba et al., 2019). Additionally, it is unclear if the potentiation effects of Mix 1 occur at the level of the DRG or elsewhere such as the CNS due to

the absence of a direct comparison of the inhibitory effects of Mix 1 and CBD in vitro. Finally, CBG was not examined in vitro. Previous work suggests CBG to inhibit T-type CavS (Mirlohi et al., 2022) as well as Nav1.7 (Ghovanloo et al., 2022). It is unlikely that inhibition of Nav1.7 will play a role in the effect of CBG on visceral pain as Nav1.8 conditional knock-out of this channel does not change responses to noxious visceral stimuli (Hockley et al., 2017). Any novel mechanisms of action found would also help inform actions of Mix 1. Finally, any key mechanisms identified in vitro would best be replicated in vivo by attempting to abolish the effects observed through pharmacological and genetic manipulation of pathway identified. Although, considering the multivarious nature of npCB pharmacology, it is unlikely that a complete blockade of effect would be observed as other interactions may contribute to the overall effects observed.

### **Summary and significance**

This study reported multiple novel findings related to npCBs in visceral pain. Both CBD and CBG were shown to attenuate visceral hypersensitivity while CBC and CBDV showed no attenuation. This is the first study to show a positive impact on visceral pain in an IBD model for CBD or any model for CBG. Additionally, this study reports the first potentiation or entourage effect in any pain model using only non-psychotropic cannabinoids and not augmenting the effects of THC. Preliminary mechanistic studies suggest a non-T-type mechanism of action for CBD. These results show rapid translational value when considering the easy availability of npCBs with the legalization of recreational cannabis in Canada. This is especially notable for CBG which shows excellent bioavailability when consumed orally. Hopefully, npCB-based

therapeutics can be developed to address the needs of IBD patients and remove the psychoactive barrier found in other cannabis-based therapeutics.

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