Cannabinoid CB1 receptor expression and localization in the dorsal horn of male and female human and rat spinal cord tissue

by

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Abstract

Preclinical and clinical evidence suggests that cannabis, a potent cannabinoid, has potential analgesic properties. However, there is a gap in the literature with respect to cannabinoid receptor expression and localization in the spinal cord across both sex and species, with almost nothing known in humans. We aimed to investigate the differential expression of the cannabinoid type 1 receptor (CB1R) across dorsal horn laminae and cell populations in male and female adult rats and humans. Human spinal cord samples were collected from organ donors 1-3 hours post-aortic cross-clamping. To investigate and quantify CB1R expression in the spinal dorsal horn, we used an immunohistochemistry approach along with confocal imaging. We successfully refined and applied staining procedures from rat to human fixed tissue. Qualitatively, we observed increased neuropil immunostaining in the superficial dorsal horn (SDH) of rats and humans, and somatic staining in the deeper laminae. Quantitative results indicated a significant increase in CB1R immunoreactivity in the SDH when compared to the deeper dorsal horn laminae of both rat and humans. This significant difference in receptor expression across dorsal horn laminae was conserved across sex in both species. The preferential expression of CB1Rs in the SDH across both sex and species has significant implications for both the understanding and treatment of pain.

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2-AG	2-arachidonoylglycerol
Δ^9-THC	Δ– 9 – tetrahydrocannabinol
ABHD6	αβ Hydrolase domain 6
ABHD12	αβ Hydrolase domain 12
AEA	Anandamide
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoaxazolepropionic acid
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
CBD	Cannabidiol
CGRP	Calcitonin gene related peptide
DAG	Diacylglycerol
DDH	Deep dorsal horn
DGL	Diacylglycerol
DRG	Dorsal root ganglia
eCB-LTD	Endocannabinoid long term depression

FAAH	Fatty acid amide hydrolase
GABA	γ-Aminobutyric acid
GFAP	Glial fibrillary acidic protein
IB4	Isolectin B4
Iba1	Ionized calcium binding adaptor molecule 1
MAGL	Monoacylglycerol lipase
NAPE	N-arachidonoyl phosphatidyethanolamine
NeuN	Neuronal nuclear protein
NKA	Neurokinin A
OD	Optical density
PB	Phosphate buffer
PBS	Phosphate buffered saline
PET	Positron emission tomography
PFA	Paraformaldehyde
PKC	Protein kinase C
PLCβ	Phospholipase C β

SP	Substance P
SDH	Superficial dorsal horn
TRPV1	Transient receptor potential cation channel subfamily V member 1
TSA	Tyramide signal amplification

Introduction

General Overview

Acute pain is a healthy, essential process, informing the organism of a dangerous or harmful situation. When acute pain persists longer than the natural healing process, it is referred to as chronic pain, a maladaptive process. The prevalence of chronic pain in Canada has been on the rise with an increase from 15.3% to 19.5% between 1996 and 2006 respectively (Reitsma et al., 2012). Current pharmacological treatments for chronic pain include opioids, anti-inflammatory medication, antidepressants, and anticonvulsant analgesics. These treatments are not effective in all individuals, by not providing sufficient pain relief, inducing adverse side effects that are unbearable, or having highly addictive properties (Yekkirala et al., 2017). For these reasons, it is critical that other therapies and treatments for chronic pain be explored.

Cannabis sativa has been used to treat pain for hundreds of years, however research in this area has only begun to advance in the last sixty years (Aviram et al., 2017). Cannabis belongs to a chemical class of compounds called cannabinoids, which act on cannabinoid receptors. There are several rodent and human studies reviewing the efficacy of cannabinoids in treating chronic pain conditions, including neuropathic pain with a loose consensus that cannabis has analgesic effects while producing minimal adverse side effects (Andreae et al., 2015; Deshpande et al., 2015; Ware et al., 2010). While there are several studies providing evidence for the efficacy of cannabis in treating pain, there is a lack of consensus with regards to its efficacy between sexes, and specific pain conditions. It also remains unclear as to whether cannabis is acting at the level of the spinal cord to influence pain signalling, acting at the level of the brain to suppress the psychological response or perception of pain, or acting at both levels to suppress

pain signalling and perception. The underlying mechanisms of the endocannabinoid system, how it may be altered during pain conditions, and how it can be used therapeutically, remains poorly understood. For these reasons, it is imperative that these mechanisms be further explored so we can have a better understanding on how cannabis may be used in the treatment of pain.

History of Cannabis Use

The first documented use of cannabis dates to 4000 B.C. where the Chinese used cannabis fruits as food and obtained fibers from the plant to create textiles, ropes, and strings (Li, 1973). Around 2700 B.C. the Chinese began to use cannabis as a medicine for the treatment of rheumatic pain, intestinal constipation, and malaria (Touw, 1981). The use of cannabis for medicinal purposes became popular in India around 1000 B.C, where it was used for various purposes such as an analgesic, anticonvulsant, tranquilizer for anxiety, anti-inflammatory, and a diuretic (Mikuriya & Francisco, 1969). The use of cannabis for medicinal purposes became increasingly popular around the world, however, was not recognized by western medicine until the early 19th century (Zuardi, 2006). In 1924, a book was published by an endocrinologist outlining the primary medicinal uses of cannabis discovered thus far, which included: as a sedative, analgesic, and to promote digestion (Sajous & Sajous, 1924).

Cannabis was first prohibited in Canada in 1923 when it was added to the *Confidential Restricted List* under the *Narcotics Drug Act Amendment* Bill, likely due to influences from international partners such as the United States. Interestingly, cannabis use in Canada did not begin to gain significant attention until the 1930s. In 2001, Canada's government legalized access to medical cannabis through the Medical Marijuana Access Regulation, and this initiated

the public's significant lobbying efforts for the legalization of cannabis for all Canadian adults (Owusu-Bempah & Luscombe, 2021). The Canadian government developed the Cannabis Act in 2018, in hopes of protecting the health and safety of adults in the Canadian population who wish to access cannabis for medicinal or recreational purposes. This act allows those of provincially-determined legal age to grow, possess, and purchase cannabis or cannabis products, therefore increasing accessibility to cannabis.

Cannabis and Pain in the Clinical Population

Chronic pain is described as maladaptive pain lasting longer than the regular healing time. Due to the high heterogeneity amongst chronic pain conditions, in order to study the effect of cannabis on chronic pain, each specific chronic pain syndrome must be investigated individually. In this section we will focus on fibromyalgia, arthritis, and neuropathic pain.

Fibromyalgia is a condition affecting approximately 1.78% of the population, characterized by chronic widespread pain all over the body and increased sensitivity to painful stimuli (Heidari et al., 2017). A 2019 study in Canada found that out of 5452 patients prescribed cannabinoid medication, 34% had fibromyalgia (Berger et al., 2020). A randomized, double-blind, placebo-controlled clinical trial of cannabinoids in 2008 investigated the use of nabilone, a synthetic cannabinoid, in 40 patients with fibromyalgia (Skrabek et al., 2008). When compared to the placebo, patients treated with nabilone had improvements in their pain after four weeks of treatment. While the treatment group experienced mild side effects after 2 and 4 weeks of taking nabilone, the drug was overall well-tolerated and produced significant pain relief and improved quality of life compared to placebo group. The participants in this study continued to use their

regular therapies for the treatment of fibromyalgia (including pharmacological aids), suggesting that a combined approach of cannabinoids and other therapies may be required in order to see significant improvements in pain. These studies provide evidence for the acute efficacy of cannabis as a treatment for fibromyalgia, however the long-term effects of cannabis use for the treatment of fibromyalgia have yet to be evaluated.

Osteoarthritis is the most common form of arthritis and is characterized by degeneration of the joints due to 'wear and tear' (A. D. Woolf & Pfleger, 2003). Rheumatoid arthritis, on the other hand, is an autoimmune and inflammatory disease leading to severe joint inflammation and pain (D. M. Lee & Weinblatt, 2001). Both forms of arthritis, while highly prevalent, have low efficacy in response to available treatments such as non-steroidal anti-inflammatory drugs and disease-modifying antirheumatic drugs. When researchers take samples of joint fluid from patients with arthritis, an increase of CB1 and CB2 receptor proteins and endocannabinoids 2-arachidonoylglycerol (2-AG) and anadamide (AEA) were observed compared to healthy controls (D. Richardson et al., 2008). The increased presence of cannabinoid receptors and endocannabinoids could suggest that cannabinoid signalling is an important therapeutic target for the treatment of pain in those with arthritis.

Neuropathic pain is characterized by an increase in spontaneous activity of afferent nerves, and an increased or abnormal response of the somatosensory nervous system to incoming stimuli (G. Lee et al., 2018). Numerous randomized, double-blind, placebo-controlled clinical trials have shown that cannabis, regardless of route of administration, provides significant acute pain relief for individuals with neuropathic pain, when compared to the placebo (Ellis et al., 2008; Nurmikko et al., 2007; Wilsey et al., 2013). While there is some evidence that supports the efficacy of cannabis for the treatment of neuropathic pain, there is also significant evidence

stating the contrary. A meta-analysis analysing evidence of inhaled cannabis for the treatment of chronic neuropathic pain found that less than 1 in 5 patients with neuropathic pain experienced moderate benefit from inhaled cannabis, with an odds ratio of 3.2 (Andreae et al., 2015). For this reason, more research is needed to look at the underlying mechanisms of these systems to gain a better understanding of the effects of the cannabinoid system in neuropathic pain conditions.

Together, these findings provide evidence for the mild to moderate effective use of cannabis in some patients for the treatment of various chronic pain conditions, such as fibromyalgia, arthritis, and neuropathic pain, while having little adverse effects. The most common side effects reported from medicinal cannabis use were gastrointestinal issues, dizziness, and intoxication; however, these were resolved upon cessation of the medication. The primary focus of the literature has been on acute effects of cannabis use, however the mechanisms by which this occurs, along with dose and long-term effects, are poorly understood.

Nociceptive Signalling in the Spinal Cord

Signalling along the nociceptive pathway is induced when noxious stimuli activate nociceptors in the periphery, which send signals along the afferents of first order sensory neurons to the dorsal horn of the spinal cord, where the signals are integrated. Projection neurons within the dorsal horn are then activated and the neuronal signals travel through the ascending pathway where the information is processed as painful by the cortical areas of the brain pain matrix (Todd, 2010, 2017; C. J. Woolf & Salter, 2000). Descending projections are sent from the brain down the spinal cord to synapse in the dorsal horn of the spinal cord, modulating these nociceptive pain responses. Therefore, the spinal dorsal horn is a key region involved in pain processing and modulation.

The spinal cord is a delicate part of the central nervous system, protected by the vertebral column. The spinal cord can be divided into four main segments based on location, listed here from rostral to caudal: cervical, thoracic, lumbar, and sacral. Within each of these segments, there are separate nerves which carry sensory and motor information from distinct body regions dermatomes, each having different functions. Ventral and dorsal roots emerge from either side of the spinal cord to form the spinal nerves, carrying motor and sensory information respectively. The grey matter of the spinal cord can be divided into ten layers, referred to as laminae, first described in the cat by Rexed in 1952 (Rexed, 1952). The first six laminae contain groups of neurons responsible for sensory information, making up the dorsal horn, and laminae VII-IX make up the ventral horn and are responsible for motor functions. Lastly laminae X is found around the central canal of the spinal cord, and is where axons decussate from one side of the spinal cord to the other.

Nociceptive afferents terminate primarily in laminae I and II, which form the superficial dorsal horn, a key region in the nociceptive pathway. Lamina I contains a high concentration of projection neurons which have axons that terminate in the brain, while other projection neurons are dispersed through deeper laminae of the dorsal horn (Al-Khater et al., 2008). Myelinated A-δ afferents terminate in lamina I while unmyelinated C-fibers that contain neuropeptides substance P and calcitonin gene-related peptide (CGRP) are primarily found in laminae I and laminae II outer (Light & Perl, 1979; Todd, 2017). CGRP is a peptide that acts on G protein-coupled receptors to activate adenylyl cyclase and protein kinase A (Wimalawansa, 1996), and plays a critical role in the modulation of spinal nociceptive processing (Bird et al., 2006). Noxious stimulation and peripheral inflammation can lead to release of CGRP in the spinal dorsal horn (Galeazza et al., 1995; Morion & Hutchison, 1989; Schaible et al., 1994). Since CGRP is found

in laminae I and II of the spinal cord, it can be used as an anatomical marker of the superficial dorsal horn in immunohistochemical studies in both rodents and humans (Ribeiro-da-Silva, 1995; Shiers et al., 2021). Signal integration occurs in the superficial dorsal horn, where the first synapse of the nociceptive pathway is located. Most of the neurons in laminae I and II are interneurons, many of these being either inhibitory (i.e. GABA) or excitatory (i.e. glutamate) neurons (Todd, 2017). Inhibitory neurons (GABA and glycine) in mice make up 26% of the neurons in the superficial dorsal horn, and it is thought that the remaining neurons are glutamatergic (Polgár et al., 2013; Yasaka et al., 2010). The deep dorsal horn consists of laminae III-VI, where processing of other sensory modalities such as touch occurs (Light & Perl, 1979). For example, neurons in laminae III-V of the spinal dorsal horn receive inputs from myelinated Aβ primary afferents, where they function as low-threshold mechanoreceptors (Todd, 2002). Therefore, the superficial dorsal horn (laminae I and II) is the primary region for nociceptive processing.

The Endocannabinoid System

The endocannabinoid system is a neuromodulatory system existing in the central nervous system and the periphery, and consists of endogenous cannabinoid compounds (endocannabinoids), cannabinoid receptors, and related enzymes. The endocannabinoid system regulates the excitability of neurons through G-protein coupled cannabinoid receptor type 1 and 2 (CB1 and CB2 receptors respectively). CB1 receptors are highly expressed in the brain, with high expression in the neurons of the hippocampus, striatum, substantia nigra, and cerebral cortex (Moldrich & Wenger, 2000; Tsou et al., 1998). Low to moderate density of CB1 receptors

has also been detected in the thalamus, hypothalamus, amygdala, and spinal cord. CB2 receptors are primarily expressed in the periphery, however under normal physiological conditions, CB2 receptors can be found in the brainstem and hippocampal neurons (Bie et al., 2018). CB2 receptors are highly expressed in circulating immune cells, the spleen, and macrophages, with their primary role being to suppress the release of inflammatory mediators (Galiègue et al., 1995; Munro et al., 1993) . Following injury or inflammation, CB2 receptor expression is upregulated, and can be found on the membranes of microglia, both in the brain and in the spinal cord (Benito et al., 2003, 2005; Zhang et al., 2003).

Cannabinoid receptors are categorized as a class A G protein-coupled receptor, where they activate inhibitory $G\alpha_{i/o}$ heterotrimeric G proteins, and interact with β-arrestins (Howlett, 1985; Jin et al., 1999). The activation of CB1 receptors in neurons leads to the inhibition of adenylyl cyclase, decreasing cyclic AMP levels and protein kinase A activity (Mackie & Hille, 1992; Wilson & Nicoll, 2002). Voltage-gated Ca^{2+} channels are inhibited, leading to the activation of A-type potassium channels and the hyperpolarization of the cellular membrane, decreasing the release of neurotransmitters (Henry & Chavkin, 1995; Mackie & Hille, 1992; Wilson & Nicoll, 2002).

Endogenous and Exogenous Cannabinoids

The endocannabinoid system has two primary ligands (endocannabinoids) that bind to the CB1 and CB2 receptors: anandamide and 2-arachidonoylglycerol (2-AG). These endocannabinoids are synthesized on demand from membrane phospholipid precursors, generally following extreme stimulation of the cell leading to prolonged increase of calcium

intracellularly. 2-AG is synthesized from the production of diacylglycerol (DAG). First, DAG species are produced from phospholipids in the membrane by PLCβ, and then they are broken down by the enzymes diacylglycerol lipase-α and β, to form 2-AG (Bisogno et al., 2003). DAG on its own can activate protein kinase C (PKC), which is increased in pain states. When 2-AG is synthesized, this in turn will terminate DAG signalling, and decrease PKC activity. To date, the synthesis of AEA in nociceptive pathways has not been determined, however there are currently three known pathways from which anandamide can be generated (Liu et al., 2008; Woodhams et al., 2015). The first pathway is through the membrane phospholipid N-arachidonoyl phosphatidylethanolamine (NAPE), which is hydrolyzed by phospholipase D to become anandamide. A second pathway that has been identified begins with α,β-hydrolase 4 causing the deacylation of NAPE and the cleavage of glycerophosphate to create anandamide. The third identified pathway for anandamide synthesis begins with phospholipase-C causing the hydrolysis of NAPE which creates phosphoanandamide, which is then dephosphorylated by phosphatases to become anandamide.

The synaptic action of these endocannabinoids is unique in the sense that they are released from the post-synaptic cell and travel in retrograde fashion to bind to cannabinoid receptors on the presynaptic membrane and decrease the release of neurotransmitters from the presynaptic cell. 2-AG was found to act as a full agonist to the CB1 and CB2 receptors, while anandamide acts as a partial agonist (Sugiura et al., 2000). Following synaptic action, AEA and 2-AG are promptly metabolized by their respective enzymes. 2-AG is primarily metabolized by monoacylglycerol lipase (MAGL) with approximately 15% of its metabolism by αβ hydrolase domains 6 and 12 (ABHD6 and ABHD12) (Blankman et al., 2007; Dinh et al., 2002; Savinainen et al., 2012). ABHD6 is found post-synaptically and may play a role in the regulation of the

release of 2-AG, and ABHD12 is highly expressed in microglia. AEA is primarily metabolized by the enzyme fatty acid amide hydrolase (FAAH), where it is broken down into arachidonic acid and ethanolamine (Cravatt et al., 1996).

While endocannabinoids are cannabinoids that are naturally produced in the body, there also exists exogenous cannabinoids that can bind to CB1 and CB2 receptors, which can be categorized as either phytocannabinoids (originating from the cannabis plant) or synthetic cannabinoids (originating from a lab). The cannabis plant includes various cannabinoids, Δ9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) being two of the most common cannabinoids found in the cannabis plant (Radwan et al., 2009). Δ^9-THC is a full agonist for the CB1 receptor, and a partial antagonist for the CB2 receptor, and is known to produce psychoactive symptoms (Bayewitch et al., 1996).

Endocannabinoid Signalling and Long-Term Synaptic Plasticity

Endocannabinoids regulate synaptic transmission in the central nervous system by acting on cannabinoid receptors to inhibit the release of neurotransmitters in both a short and long-term fashion (Devane et al., 1988, 1992; Freund et al., 2003; Lovinger, 2008; Stella et al., 1997). The activation of CB1 receptors can lead to a prolonged reduction of neurotransmitter release at the synapse, provoking endocannabinoid-mediated long-term depression (eCB-LTD) (Heifets & Castillo, 2009).

One mechanism by which eCB-LTD can be induced in the brain begins with repetitive excitatory signalling causing the activation of postsynaptic glutamate receptors (Heifets & Castillo, 2009). Through G protein-coupled signalling, 2-AG is synthesized and released from

the post-synaptic neuron, binding to presynaptic CB1 receptors. 2-AG release can also be upregulated through increase in Ca^{2+} influx (Adermark & Lovinger, 2007; Nevian & Sakmann, 2006; Ohno-shosaku et al., 2007). When these CB1 receptors are activated, there is subsequent inhibition of adenylyl cyclase which reduces PKA activity (Heifets & Castillo, 2009). When Ca^{2+}-sensitive phosphatase calcineurin is activated, along with the reduction in PKA activity, this shifts the balance of the kinase and phosphatase activity, promoting dephosphorylation of the presynaptic neuron, mediating a long-lasting reduction in neurotransmitter release. These findings however, are limited to the brain and are primarily defined in rodent models, highlighting a gap in the literature with respect to how endocannabinoid-mediated plasticity may occur in the spinal cord, and how it may translate to humans.

Endocannabinoid System and Pain

CB1 receptors are a primary target for cannabinoid-related therapies for acute and chronic pain. When cannabinoids are administered in mice intrathecally, an antinociceptive effect has been seen (Welch & Stevens, 1992). In contrast, an increase in pain responses is seen in mice when CB1 receptors are blocked by a CB1 receptor antagonist (J. D. Richardson et al., 1997). These data suggest an important role of the endocannabinoid system, and especially the CB1 receptor in the modulation of pain. There is a large amount of evidence for the activation of peripheral CB1 receptors in peripheral sensory neurons leading to analgesia, however, little literature exists regarding the role of activated CB1 receptors in nociception. For example, an immunohistochemistry study found that approximately 1/3 of dorsal root ganglia (DRG) neurons express the CB1 receptor, in which most expressed CGRP (Veress et al., 2013).

Not only are CB1 receptors a promising target for cannabinoid therapies for pain conditions, but there is evidence that the endocannabinoid 2-AG may also play an important role in pain processing, and therefore may be a target of interest. When there are inputs from noxious stimuli, this initiates 2-AG signalling, which inhibits the release of pronociceptive neurotransmitters from the primary afferent terminals (Woodhams et al., 2015). Interestingly, when the enzyme that metabolizes 2-AG is blocked in the spinal cord, nociceptive transmission is inhibited (Woodhams et al., 2012). When levels of the endocannabinoid AEA are increased in the spinal cord – by administration of a FAAH inhibitor, an antinociceptive effect is also seen, however not as strong as inhibiting MAGL (Jhaveri et al., 2006). These results suggest that endocannabinoids 2-AG and AEA, and specifically the enzymes that break them down, may be a favourable target at the level of the spinal cord for reduction in pain signalling.

Electrophysiological and neurochemical studies in rodents have shown evidence of antinociception following the administration of cannabinoids after a thermal, mechanical, or chemical noxious stimulus was applied (Walker et al., 2001). In 1995, Hohmann and colleagues tested the effects of the synthetic cannabinoid WIN 55,212-2 on the nociceptive responses of neurons in the lumbar spinal cord of rats (Hohmann et al., 1995). When the synthetic cannabinoid was administered, but not the vehicle control, inhibition of noxious stimulus-evoked activity was found in the lumbar dorsal horn nociceptive neurons. This inhibition was reversed following the cessation of cannabinoid use, suggesting that cannabinoids modulate the activity of nociceptive neurons in the spinal cord of rats.

Researchers Kelly and Chapman administered a CB1 receptor agonist in the spine of rats and tested the evoked responses of nociceptive fibers (Kelly & Chapman, 2001). The CB1

agonist inhibited Aδ- and C-fiber-mediated responses of dorsal horn neurons when administered in healthy and inflamed rats. This effect was reversed when a CB1 receptor antagonist was administered, demonstrating that the activation of CB1 receptors in the spine of rats reduces nociceptive activity.

Pain and Cannabinoids: Sex differences

In animal studies testing the effects of antinociception in healthy male and female rats, the administration of either cannabinoids or cannabinoid receptor agonists in females produces a stronger analgesic response at baseline compared to male rats (Craft et al., 2012; Romero et al., 2002; Tseng & Craft, 2001). This effect was seen in both adolescent and adult animals and was validated in various behavioural tests, such as warm water tail withdrawal and paw pressure tests. Female rats also appear to be more sensitive to the effects that cannabinoids have on locomotor activity, compared to male rats, which may contribute to their performance on behavioural tasks. For example, several studies have shown an increase in locomotor activity of female rats at low doses of THC administration, but a decrease in locomotor function at higher doses, suggesting both a dose and sex dependent response to cannabinoids (Craft et al., 2012; Tseng & Craft, 2001; Wiley, 2003).

Similar to rodent studies, several studies in humans have revealed that women tend to have increased sensitivity to the psychological and physiological effects of cannabis compared to men (Blanton et al., 2021). Males and females achieved the same acute cognitive effects from smoked cannabis at different doses, where males required a higher dose of smoked cannabis to see the same effects as females (Matheson et al., 2020). Notably, the female subjects had a lower

body mass compared to males but there was no statistical difference in BMI between sexes. It is unclear if the results regarding cannabis dosage were corrected for body weight and composition, which could play a role in the absorption of cannabis in the bloodstream. Similarly, women have also been found to be more sensitive to the psychological and physiological effects of cannabis, however this effect seems to be dose dependent. For example, women experienced a greater response to 5mg dose of THC compared to men, however with a 15mg dose of THC, men showed an enhanced response (Fogel et al., 2017). While few studies exist examining the sex differences in antinociceptive effects of cannabis products, one study in 2008 found that women, and not men experienced anti-hyperalgesia when given Nabilone (Redmond et al., 2008) . In this double-blind, placebo-controlled, crossover study, heat pain was experimentally-induced and temporal summation was assessed in healthy adult male (n=7) and female (n=10) participants. While Nabilone at either 0.5mg or 1.0mg dose did not affect heat pain in neither males nor females, there was a significant difference between sex with regards to temporal summation. Women, but not men showed reduced temporal summation of heat pain following administration of 1.0 mg dose of Nabilone. These results suggest that cannabinoids, specifically Nabilone, may act as an anti-hyperalgesic tool in women but not men. Whether this effect is due to Nabilone's action on the CB1 receptor remains unknown.

Sex Differences and CB1 Receptor Expression

Sex differences in CB1 receptor density and affinity have been observed in various brain regions, however these differences are not consistent across studies. For example, some studies have found an increase in CB1 receptor expression in the anterior pituitary, mesencephalon,

prefrontal cortex, and amygdala of male rats when compared to females, and no difference in the hypothalamus (Castelli et al., 2014; González et al., 2000; Rodríguez de Fonseca et al., 1994). The prefrontal cortex and amygdala play important roles in the emotional processing of pain, and therefore this sex difference may have implications for the perception of pain between sexes. In contrast, other studies have found increased CB1 receptor density in the amygdala and decreased expression in the hypothalamus of female rats compared to males (Riebe et al., 2010). The reason for these contrasting results remains unclear, given that similar species and CB1 receptor agonists were used, suggesting that further investigations are required. There are limited studies examining CB1 receptor expression in the spinal cord, such that to our knowledge no studies make quantitative comparisons between sexes, hence the current literature focuses primarily on the brain.

The literature for sex differences in CB1 receptor expression in humans is limited. Positron emission tomography (PET) radiotracers have been used in humans to study CB1 receptor density. One study found greater CB1 receptor density in the brains of women during the follicular phase (when estrogen levels are high), when compared to men (Normandin et al., 2015). Understanding how the menstrual cycle impacts cannabinoid receptor expression in women remains to be fully understood and should be considered an important target for future studies.

A similar PET scan study, which used a different radioligand to assess CB1 receptor expression in the brain, found region-specific differences in CB1 receptor density between sexes (van Laere et al., 2008). Women had increased CB1 receptor density in the basal ganglia, lateral temporal cortex, and limbic system, while men had increased density in the cortico-striato-thalamic-cortical circuit. Together, these results suggest that not only are there sex differences in

cannabinoid receptor expression, but that these differences are also region-specific. While these differences have been briefly examined in the brains of human male and female participants, this has yet to be assessed in the human spinal cord. Examining sex differences in cannabinoid signalling is crucial, but in order to achieve strong translational potential, we need further evidence as to how rodent studies may translate to the human clinical population.

The majority of studies investigating the effects of CB1 receptor expression and signalling have been done in rodents, with little known on how these rodent studies will translate into the human population. An electrophysiological study found that human CB1 receptors have reduced activity compared to rat CB1 receptor activity, where the human CB1 receptors are less effective at inhibiting synaptic transmission (Straiker et al., 2012). These results suggest that careful consideration should be taken when translating a treatment/drug in rodents to the human population, as there is evidence that there are differences in the signalling patterns of the endocannabinoid system between species.

CB1 Receptor Localization in the Spinal Cord

There is mixed evidence that CB1 receptors are found pre- and post-synaptically in the spinal cord. Rodent studies have shown strong immunostaining for CB1 receptors in the spinal dorsal horn as well as in dorsal root ganglia, and electrophysiological studies have confirmed CB1 signalling in the lumbar dorsal horn (Hohmann, 2002). Intracellular recordings in juvenile rat spinal cord tissue revealed evidence for presynaptic CB1 receptor localization in the spinal dorsal horn (Morisset & Urban, 2001). When a CB1 receptor agonist is administered, neuronal activity of afferent C fibers following capsaicin administration was significantly reduced. These

results suggest that when presynaptic CB1 receptors in the dorsal horn of the spinal cord are activated, they inhibit the release of glutamate, decreasing the excitatory signals being sent to the brain and therefore producing an analgesic effect. Additionally, when the same CB1 receptor agonist was administered in capsaicin-induced animals, there was no change in the AMPA/kainite receptor-mediated postsynaptic current. These results suggest that there are likely few CB1 receptors localized postsynaptically. Furthermore, immunohistochemical studies reveal CB1 receptor localization to GABAergic interneurons in the spinal dorsal horn (Jennings et al., 2001). This suggests that cannabinoids may also act through disinhibition of GABAergic neurons in lamina II of the dorsal horn.

Many primary nociceptive afferents express the peptide CGRP, making it a strong target for immune staining of afferent axon terminals. When co-staining for CB1 receptors and CGRP in the spinal cord of adult rats, approximately 50% of CGRP staining co-expresses CB1 receptors (Hegyi et al., 2009). On the other hand, approximately 20% of non-peptidergic nociceptive primary afferent axon terminals (labelled with isolectin B4) express CB1 receptors (Hegyi et al., 2009). Transient receptor potential vanilloid type 1 (TRPV1), a nociceptive receptor, is another example of an afferent marker, as it is predominantly expressed on primary afferent fibres. There has been evidence of colocalization of the CB1 receptor and TRPV1 in some regions of the mouse and rat brain, one of these regions being the periaqueductal gray, a key structure in the propagation and modulation of pain (Cristino et al., 2006; Maione et al., 2006). In the dorsal root ganglia of wild-type mice, immunostaining revealed presence of CB1 receptors in approximately 40% of nociceptors, including, IB4, substance P-expressing peptidergic nociceptors, and Nav1.8-expressing nociceptors (Agarwal et al., 2007). When the same immunostaining procedures are performed in SNS-CB1 knockout mice (lacking CB1

receptor specifically in primary nociceptors), there is almost an entire loss of CB1 staining in these nociceptor populations, however CB1 immunoreactivity is still present in large DRG neurons. Furthermore, when CB1 immunoreactivity was measured in the brain and spinal cord of SNS-CB1 knockout mice, levels remained normal. Comparably, when the same immunostaining techniques were applied to global CB1 knockout mice (knockout of all CB1 receptors), there was a complete loss of signal in all regions (brain, spinal cord, DRG). This co-expression of primary afferent markers with CB1 receptors indicates that CB1 immunostaining may be pre-synaptic, however this has not yet been studied in the spinal cord, especially in the spinal dorsal horn of humans.

Aims:

Aim 1: Investigation of CB1 receptor expression across dorsal horn laminae between male and female adult humans

We aimed to investigate CB1 receptor expression in male versus female adult humans. Using immunohistochemistry, human spinal cord tissue from organ donors was analyzed to compare relative CB1 receptor expression in the superficial dorsal horn compared to the deep dorsal horn as well as across the mediolateral axis of the superficial dorsal horn. Given that most studies staining for CB1 receptors have been done in male rodent models, and with there being a higher clinical population of women who experience chronic pain, it is imperative that both sexes are considered.

Aim 2: Comparison of CB1 receptor expression across superficial dorsal horn between species from rodents to humans

Using immunohistochemistry methods, we aimed to characterize CB1 receptor expression across the dorsal horn laminae, between adult rodents and humans. Relative expression will be analyzed between the SDH and the DDH in rats compared to humans. The comparison across species from rats to humans, will help bridge past findings using rodent models to potential targets for humans.

Methods:

Animals

Spinal cord tissue was used from male and female adult Sprague-Dawley rats purchased from Charles River. Experimental procedures using these animals were approved by the Carleton University Animal Care Committee and performed in accordance with the guidelines set by the Canadian Council for Animal Care.

Rat spinal cord isolation and preparation

Twelve adult Sprague-Dawley rats (six females and six males) were used for the purposes of this study. First, the rats were anesthetized with intraperitoneal injection of 3g/kg urethane (Sigma Aldrich). Next, the spinal cord was isolated and removed via posterior laminectomy, then the meninges were removed, and the nerve roots trimmed. The cord was then immediately placed in ice-cold sucrose solution (50nM sucrose, 92nM NaCl, 17nM D-glucose,

26nM NaHCO3, 5mM KCl, 1.25 mM NaH2PO4, 0.5 mM CaCl2, 7 mM MgSO4) bubbled with carbogen (95% O_2, 5% CO_2) to preserve the tissue prior to fixation. The tissue was then fixed in a 4% PFA solution in PB for 24-36 hours at 4℃, washed in 10% sucrose solution in PB for 24 hours, washed again in 10% sucrose solution in PB for 6-24 hours, and then finally placed in 30% sucrose solution in PB for at least 72 hours, all at 4℃.

For spinal cord freezing, tissue sections were embedded in cryomatrix, and frozen in isopentane that was chilled with liquid nitrogen and stored at -80℃ prior to sectioning. The rat spinal cord tissue was sectioned at 25μm on a microtome cryostat (ThermoScientific) at -20℃ and mounted immediately onto microscope slides in serial fashion and stored at -80℃ in preparation for immunohistochemical experiments.

Human donors and tissue collection

Adult (18-70 years old) male and female human spinal cord tissue was collected from organ donors identified by the Trillium Gift of Life Network. Consent was obtained from the donor's family prior to collection. Ethics approval was obtained to collect and conduct experiments with human tissue by the Ottawa Health Science Network Research Ethics Board and the Carleton University Research Ethics Board A.

Donors were pre-screened to exclude any blood-borne illnesses such as HIV, Hepatitis, and syphilis. Donors with serious chronic illnesses such as (cancer, chronic pain, etc.) or with damage to the spinal cord were excluded from the study to prevent any confounding variables from interfering with our results. The most common cause of death was hemorrhagic stroke.

During the donor surgery, a cooling bed was used to induce hypothermia and the body was perfused with a preservation solution designed to prolong the viability of the organs while without blood and oxygen (ex. Custodiol® HTK Solution or Perfadex® Plus). Once the organs were removed for transplant donation, the vertebral column was opened to isolate and remove the spinal cord within 1-3 hours of aortic cross-clamping or flushing the body with protective solutions. The dura mater was removed, and thoracic or lumbar sections of the spinal cord were cut into 6-10mm sections and placed in ice-cold sucrose cutting solution (50mM sucrose, 92mM NaCl, 15mM glucose, 26mM $NaHCO_3$, 5mM KCl, 1.25mM NaH_2PO_4, 0.5mM $CaCl_2$ and 7mM $MgSO_4$) to preserve the tissue in preparation for immunohistochemical experiments. The spinal cord was then fixed in a 4% PFA solution in PB for 24-36 hours at 4°C, washed in 10% sucrose solution in PB for 24 hours, re-washed in 10% sucrose solution in PB for 6-24 hours, and then finally placed in 30% sucrose solution in PB for at a minimum of 72 hours, all at 4°C.

For spinal cord tissue freezing, the fixed human tissue sections were embedded in cryomatrix and then frozen in isopentane that was chilled with liquid nitrogen and stored at -80°C prior to sectioning. The human spinal cord tissue was sectioned at 25-35μm on a microtome cryostat (ThermoScientific) at -20°C and immediately mounted onto microscope slides in serial fashion and stored at -80°C in preparation for immunohistochemical experiments.

Free-floating tissue preparation

A subset of human spinal cord tissue was prepared as free-floating slices, as opposed to slide mounted. For these samples, the extraction technique in the operating room was identical to

the procedure described above, however instead of immediately fixing the tissue in 4% PFA, the tissue was bubbled in saline for 70 minutes prior to being fixed in 4% PFA for 24-36 hours. The tissue was then washed in 10% sucrose solution in PB for 24 hours and washed for a second time in 10% sucrose solution for 6-24 hours, and finally placed in 30% sucrose solution for 72 hours. The tissue sections were then placed in antifreeze solution (28.7 mL of sodium phosphate monobasic dihydrate 31.2 g/L (pH 7.3), 96.3ml of sodium phosphate dibasic anhydrous 28.4 g/L (pH 7.3) 375 mL DEPC water, 300 mL of Ethylene glycol and 200 mL glycerol) at -20℃ until sectioning. Transverse sections of the spinal cord were sliced at 25 μm using a Leica SM2000R microtome and stored again in antifreeze solution at -20℃ in preparation for immunohistochemical experiments.

Immunohistochemistry

Immunohistochemical methods were used to investigate the distribution pattern of CB1 receptors in the spinal dorsal horn of adult rats and humans. After 3 x 5 min washes in PBS, the tissue was incubated in a peroxidase blocking solution (50% methanol, 48.2% PBS, and 1.8% hydrogen peroxide) for 30 minutes at room temperature. Following the peroxidase block, the tissue was washed 3 x 5 min in PBS and blocked for one hour in a PBS solution containing 5% NGS, 0.3% 10M Triton-X, and 0.3% BSA. The blocker was then pipetted out and the tissue was incubated in a solution containing the primary antibodies against the CB1 receptor: rabbit anti-CB1 (1:1000, Immunogenes) and antibodies against CGRP: mouse anti-CGRP (1:5000, Sigma, C7113), diluted in the blocking solution (described above) for 48 hours at 4℃. Following the incubation in the primary antibody, the tissue sections were washed 3 x 10 min with PBS and

incubated with secondary antibodies: goat anti-mouse AlexaFluor 647 (1:1000, Invitrogen, A21235) and goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (1:500; Jackson ImmunoResearch Laboratories) for 2 hours at room temperature and protected from light. Next, the slides were washed 3 x 10 min PBS and incubated for 10 minutes at room temperature in Tyramide Signal Amplification (TSA) diluted 1:50 in amplification diluent (TSA plus cyanine 3 system, Akoya Biosciences) to amplify the CB1 signal. The slides were washed 3 x 10 min in PBS and incubated in Hoechst 33258 (1:1000, Abcam, ab228550) diluted in PBS for 5 minutes to stain for nuclei. The tissue was then washed for one last round of 3 x 10 min wash in PBS before being mounted with fluoromount and covered with a coverslip. The slides were sealed with clear nail polish within 24-48 hours of placing the coverslip to prevent the tissue from drying out.

Image Acquisition

All images were acquired using a Zeiss LSM 800 AxioObserverZ1 confocal microscope, and further processed using Zen 2.6 software. Laser intensity was appropriately chosen to limit photobleaching of the sample, and identical microscope settings were used for all images to maintain continuity. Tiling was used to obtain an image of the entire dorsal horn, and appropriate z-stacks were obtained to create a hyperstack to account for multiple layers of tissue. The tiled images were stitched in Zen 2.6 software and then further analyzed in Fiji ImageJ.

Analysis

Quantification of CB1 receptor expression was obtained and analyzed using FIJI ImageJ. Two main regions are of interest in this study: the superficial dorsal horn (SDH), and the deep

dorsal horn (DDH). Using CGRP as a marker for the SDH (Eftekhari & Edvinsson, 2011), a contour line was drawn to outline the SDH where there was CGRP-positive staining. Using the contour tool, a second selection was made to encompass the DDH.

We used Fiji ImageJ to measure the optical density (O.D.) of the cy3 stained areas (representing CB1 receptors), in the SDH, and then the DDH. All values were normalized to the background which was determined by a square selection of the white matter, medial to the dorsal horn, where there should be minimal cy3 fluorescence. Comparisons in receptor expression were made between the SDH and DDH regions in each animal and human, and then further compared between sex and species.

Statistical Analysis

All statistical tests were completed in OriginLab and verified using IBM SPSS Statistics Software. Data is presented as mean ± standard error of the mean (SE). For all rodent data, each animal has been immunostained in triplicates, and a mean of the three slides was obtained to create a mean for each animal. For all human data, each donor was immunostained in duplicates, and a mean of the two slides was obtained to create a mean for each human. To test for statistical difference of sex differences, unpaired t-test was conducted. A paired t-test was used to test for statistical difference between the O.D. of the SDH against the DDH, where $p < 0.05$ was considered statistically significant.

Results

CB1 immunostaining patterns in the rat dorsal horn

To investigate the baseline expression of cannabinoid CB1 receptors in pain processing areas of the spinal cord, we compared the relative expression in the SDH and DDH of the lumbar spinal cord of adult rats. To achieve this, we selected the CGRP-positive region representing the SDH (Eftekhari & Edvinsson, 2011) and the CGRP-negative region of the dorsal horn representing the DDH, and measured the optical density (O.D.) of CB1 immunoreactivity in each region (Figure 1). The O.D. per area of each region of interest was normalized to the O.D. per area of the background by selecting a square region located within the white matter of the spinal cord dorsal column, near the medial region of the dorsal horn. When we investigated the patterns of immunoreactivity for the CB1 receptor in the dorsal horn of rats, we qualitatively observed primarily punctate neuropil staining in the SDH, with more localization to lamina I in male and females (Figures 2,3). We also observed mild localization of CB1 receptors to CGRP in the SDH of male and female rats (Figures 2A and 3A). Qualitatively, the O.D. of CB1 receptors within the SDH region is consistent across the mediolateral axis of the dorsal horn, unlike that found for synaptic NMDA receptors (Armstrong et al., 2021). Cellular CB1 immunolabelling can be seen primarily in DDH regions (laminae III-V), suggesting that CB1 receptors are localized to both neuropil and cell bodies within the dorsal horn of the spinal cord. These qualitative observations are conserved across sex.

Next, we quantified the relative expression of CB1 receptors in the SDH versus the DDH. An average CB1 O.D. per area within the SDH and DDH was obtained for each animal, by staining and imaging triplicate spinal sections from the same animal and taking an average

amongst the three samples. We found that the CB1 O.D. in the SDH was significantly higher than the O.D. in the DDH, suggesting significantly increased expression of CB1 receptors in the SDH compared to the DDH (Figure 4 C,D) for both female rats ($p= 0.0068$) and male rats ($p= 0.015$). To effectively compare across sex, we took the normalized CB1 O.D. in the SDH and divided it by the normalized O.D. in the DDH to create a ratio of SDH/DDH (Figure 4 E). In female rats, the average SDH/DDH ratio was 1.78 ± 0.10 (SEM), which was not significantly different from the SDH/DDH ratio of 1.84 ± 0.09 (SEM) found in male rats ($p=0.34$). Together, these results suggest that CB1 receptors are primarily localized to the SDH in rats, with a preference for lamina I, and that this CB1 localization is conserved across sex.

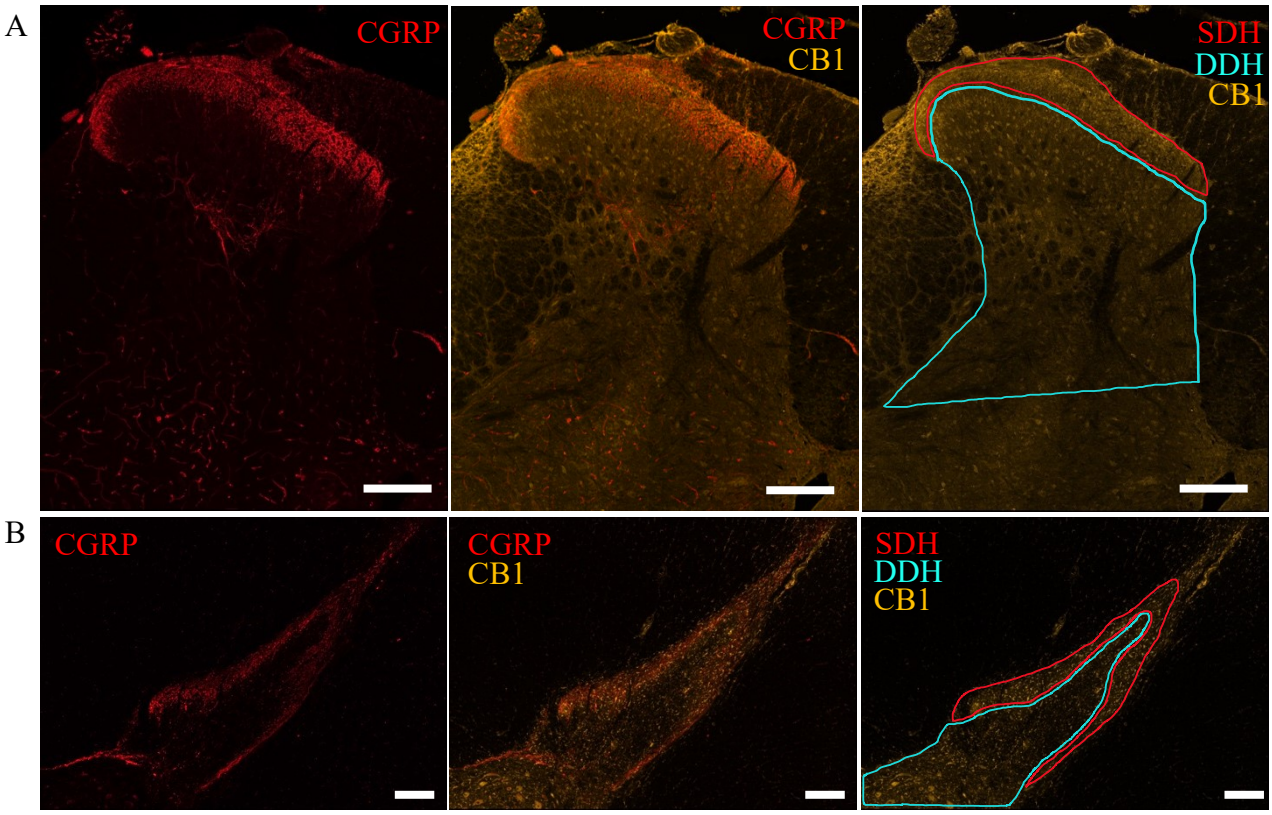

Figure 1. Immunohistochemistry staining of CB1 receptors in the CGRP-positive SDH region and CGRP-negative DDH region. Left: CGRP immunostaining (red) identifies the SDH. Middle: CGRP (red) and CB1 (yellow) immunostaining overlay in the dorsal horn. Right: CB1 immunostaining, red outlines the SDH and blue represents the DDH. A: Female rat dorsal horn. B Male human dorsal horn. Scalebar 200 µm.

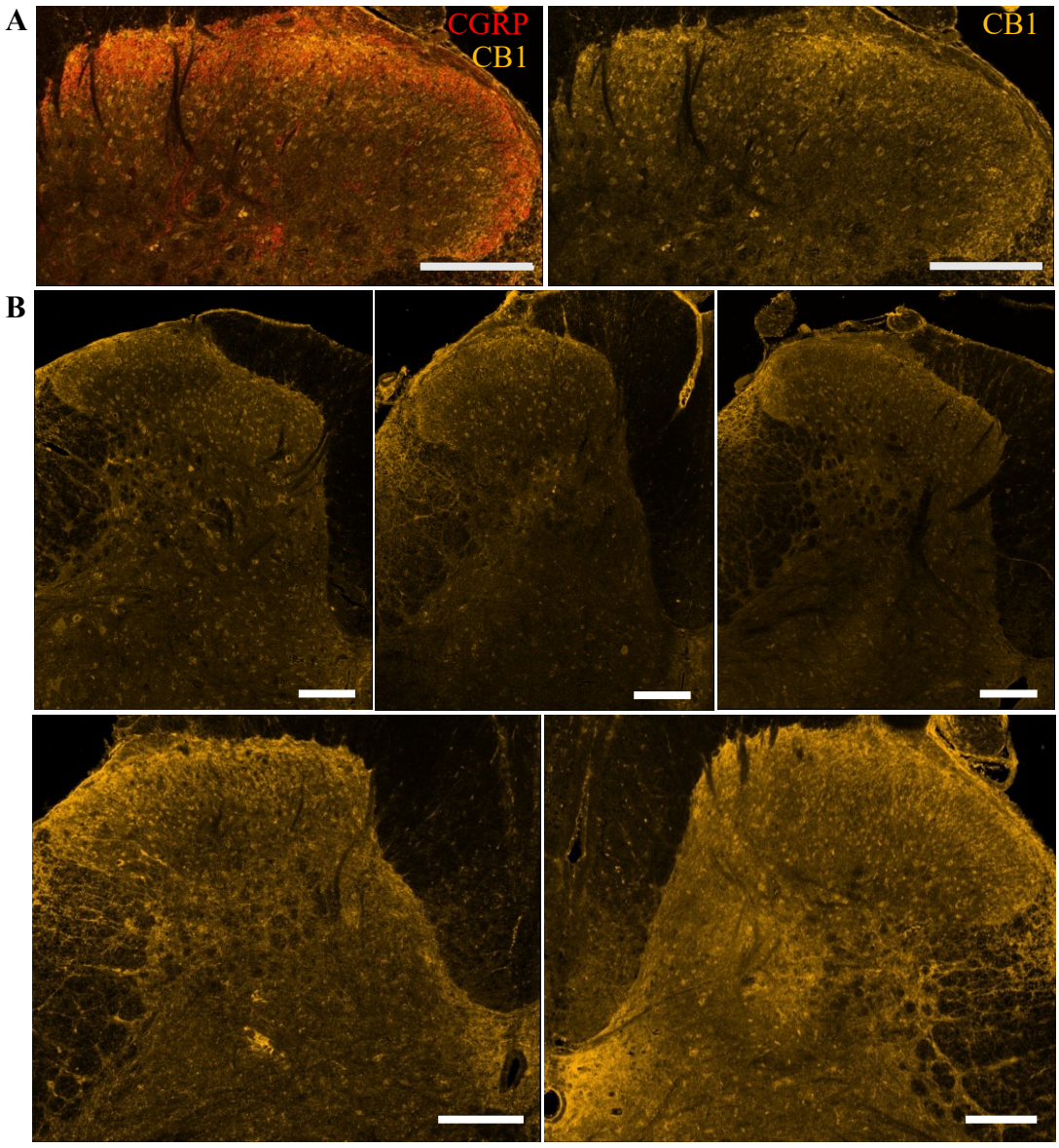

Figure 2: Representative images demonstrating consistent CB1 immunostaining patterns across female animals. Representative confocal images of CB1 immunostaining in the dorsal horn of female rats. **(A), left:** CGRP representing the SDH shown in red overlayed with CB1(yellow) immunostaining in the dorsal horn; **(A), right:** CB1 immunostaining in the dorsal horn showcasing primarily neuropil staining in the SDH. **(B):** Representative images of CB1 immunostaining from each female animal showing neuropil staining across the SDH and more cellular staining in the DDH (laminae III-VI). Scalebar 200 μm.

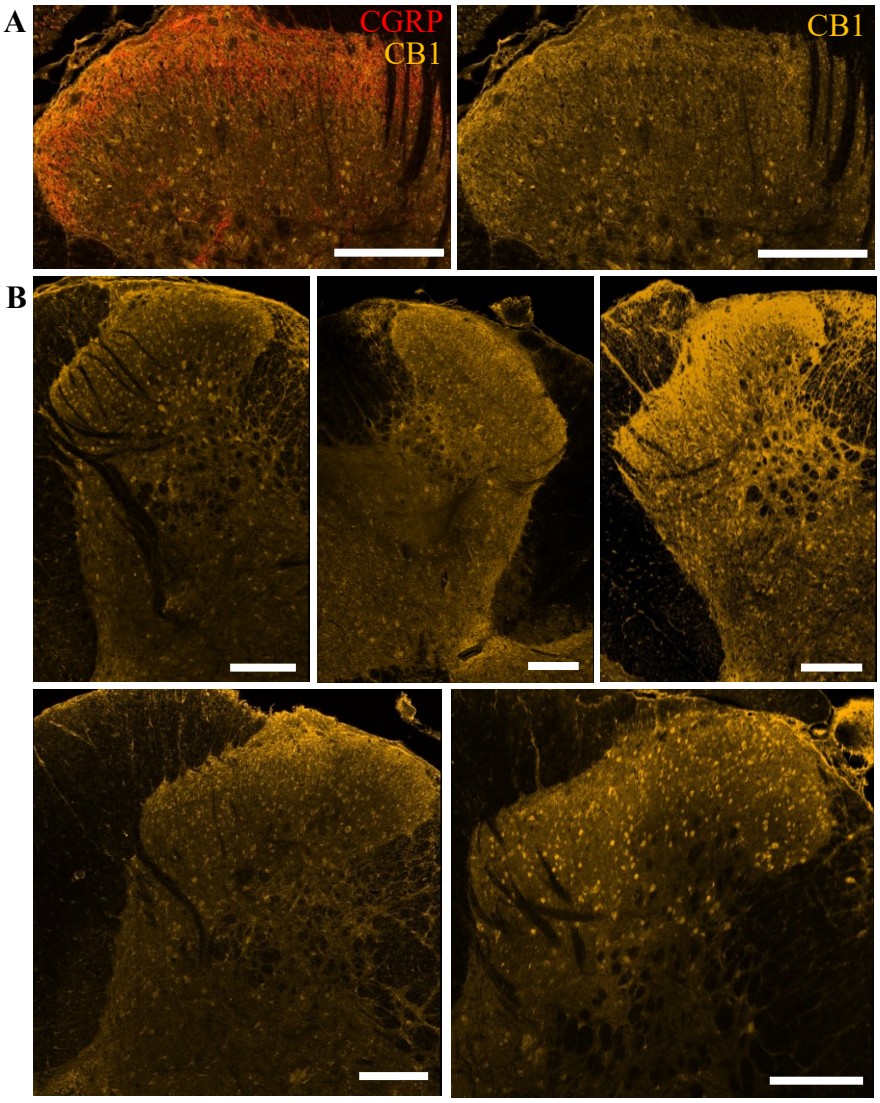

Figure 3: Representative images demonstrating consistent CB1 immunostaining patterns across male animals. Representative confocal images of CB1 immunostaining in the dorsal horn of male rats. **(A), left:** CGRP+ (red), representing the SDH overlayed with CB1 (yellow); **(A), right:** CB1 immunostaining in the SDH indicates increased neuropil staining in the SDH compared to more somatic staining in laminae III and deeper laminae. **(B):** Representative images of CB1 immunostaining from each male animal showing neuropil staining across the SDH and more cellular staining in the DDH (laminae III-VI). Scalebar 200 μm.

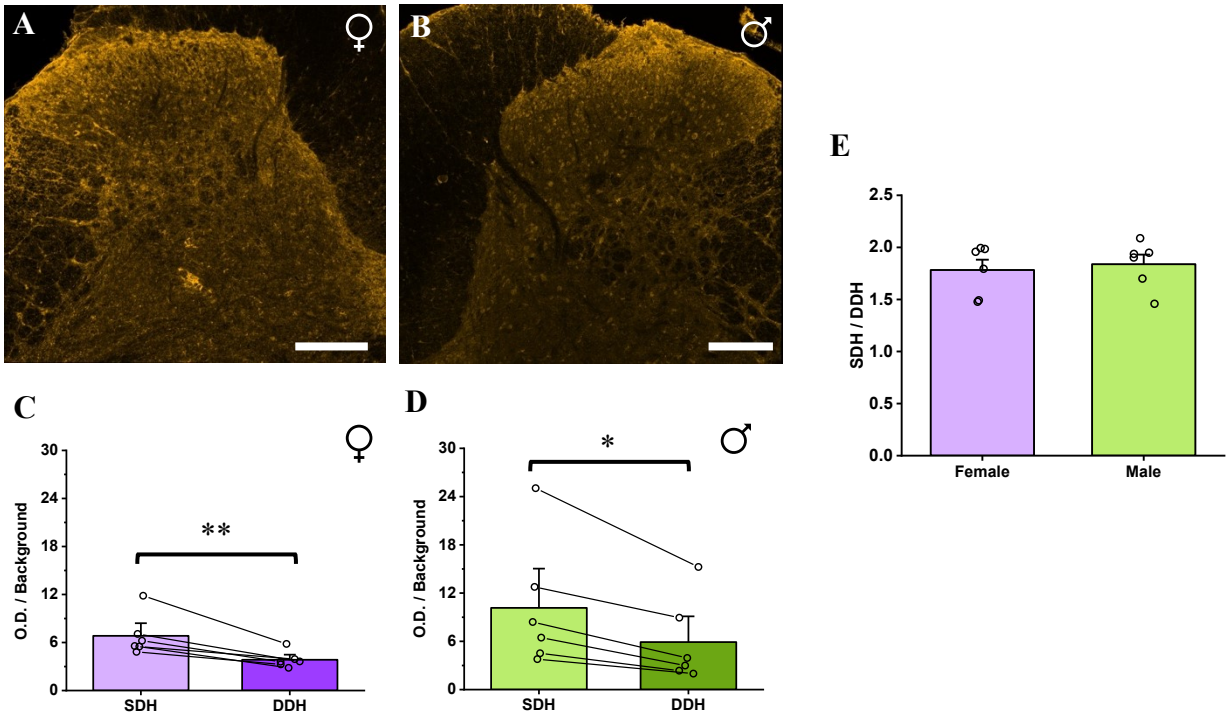

Figure 4: CB1 receptor expression increased in SDH compared to DDH in rats. **A,B:** representative images of CB1 receptor expression in rat dorsal horn in female **(A)**, and male **(B)**. **C,D:** The normalized optical density in the SDH of rats is statistically higher than the normalized optical density in the DDH for female **(C)** and male **(D)** rats. **p <0.01 for females and *p <0.02 for males. **E:** A ratio comparing the normalized optical density of the SDH to the normalized optical density of the DDH reveals that the increased optical density of CB1 receptors in the SDH of rats is conserved across sex. n=6. Scalebar 200 μm.

CB1 immunostaining patterns in the human spinal dorsal horn

We successfully investigated CB1 immunostaining patterns across the dorsal horn of rats, so next we explored whether these same trends were observed in humans. Similar to the rodent model, we compared the relative expression in the SDH and DDH of the thoracic and lumbar regions of the spinal cord of adult human organ donors (Figure 1). Punctate neuropil staining was observed in the SDH and DDH, with some somatic staining in deeper laminae (IV-VI) of the dorsal horn, and these observations are conserved across sex (Figures 5,6). Similar to what was seen in rats, the immunoreactivity of CB1 receptors across the SDH is fairly consistent across the mediolateral axis. Qualitatively, we also investigated the colocalization of CB1 receptors to CGRP, and found mild colocalization (<50%) in the SDH of male and female humans (Figure 5A and 6A), a trend which was conserved across sex and species.

We then quantified the immunoreactivity of CB1 receptors in the SDH compared to the DDH of fixed human spinal cord sections. Each donor's experiments were done in duplicate, and an average O.D. of CB1 expression in the SDH and DDH was obtained. The O.D. of the SDH is significantly higher than the O.D. of the DDH, suggesting increased expression of CB1 receptors in the SDH compared to the DDH (Figure 7C, D). This statistically significant difference of expression between regions is seen in female humans ($p= 0.0018$) and in male humans ($p= 0.017$). To effectively compare across sex, created a ratio of SDH:DDH was created for both males and females (Figure 7E). In female humans, the average SDH:DDH ratio was 1.45 ± 0.079 (SEM), and 1.54 ± 0.13 (SEM) for males, with no statistical differences between sexes ($p= 0.32$). Interestingly, when we qualitatively compare the ratio of expression of CB1 receptors in SDH:DDH, it is observed that humans have a decreased ratio when compared to rats.

Together, these results suggest that CB1 receptors are primarily localized to the SDH in humans and this observation is conserved across sex.

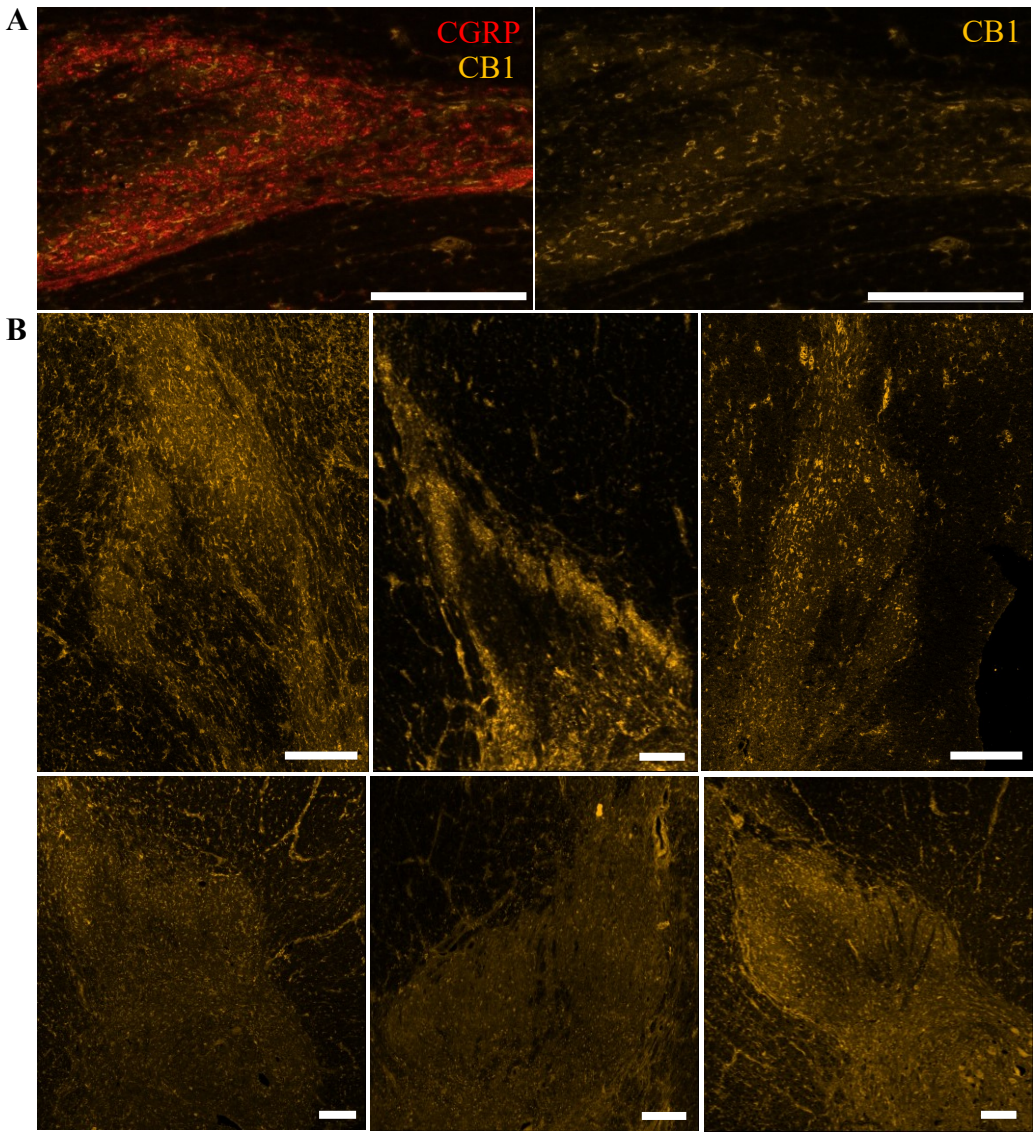

Figure 5: Representative confocal images demonstrating consistent CB1 immunostaining patterns across female humans. **(A), left:** close-up image of the SDH, CGRP-positive immunostaining (red) representing the SDH overlayed with CB1 (yellow), representing the SDH; **(A), right**: CB1 immunostaining showing neuropil staining in the SDH. **(B):** Representative images of CB1 immunostaining from each female human donor showing neuropil staining throughout the dorsal horn, and somatic staining primarily localized to the DDH, in this case laminae IV-V. Scalebar 200 μm.

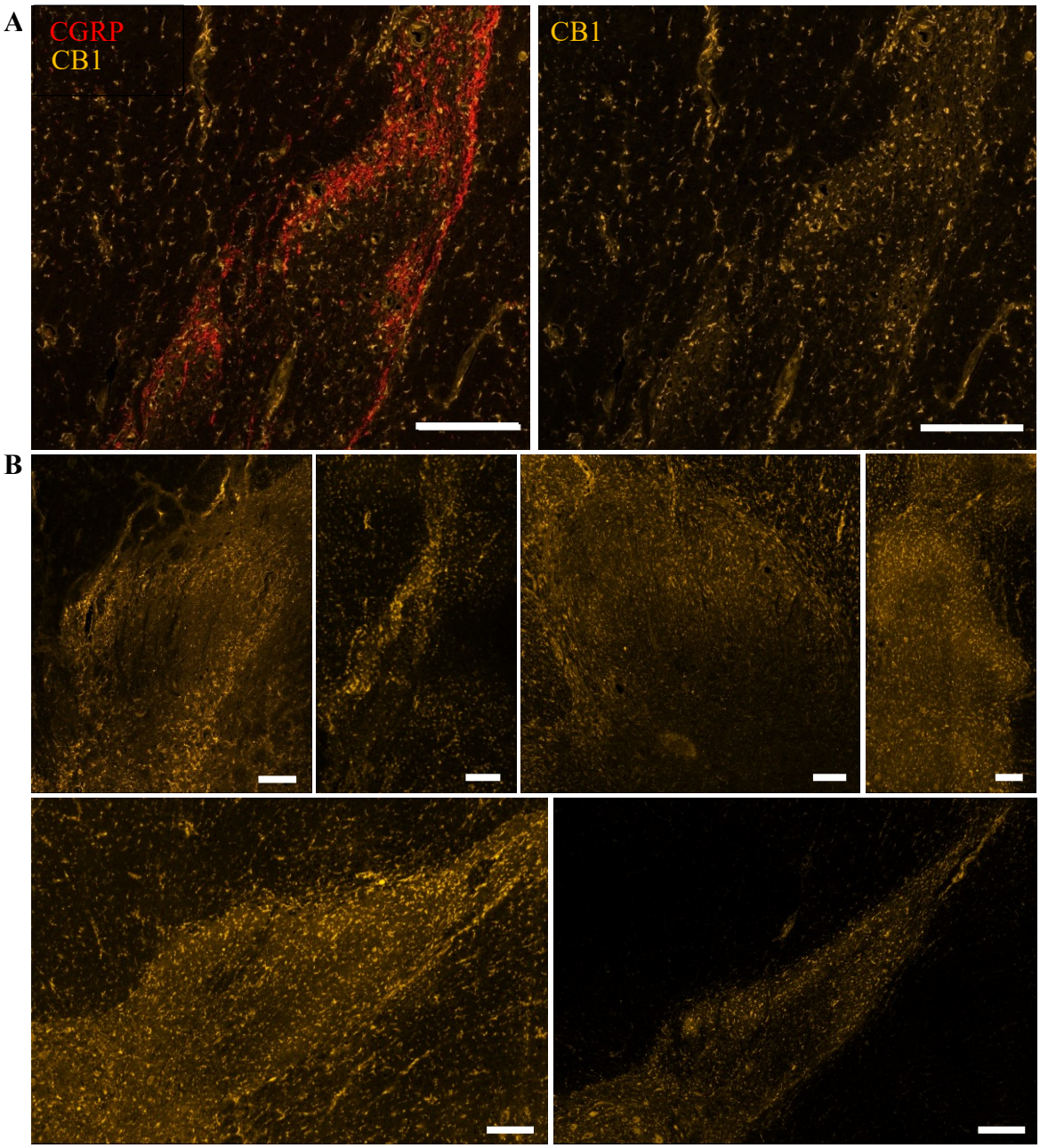

Figure 6: Representative images demonstrating consistent CB1 immunostaining patterns across male humans. **(A), left:** close-up of the SDH, CGRP-positive region (red) representing the SDH overlayed with CB1 (yellow); **(A), right:** CB1 immunostaining showing neuropil staining in the SDH **(B):** Representative images of CB1 immunostaining from each male human donor showing

neuropil staining throughout the dorsal horn, and somatic staining primarily localized to the DDH, in this case laminae IV-V. Scalebar 200µm.

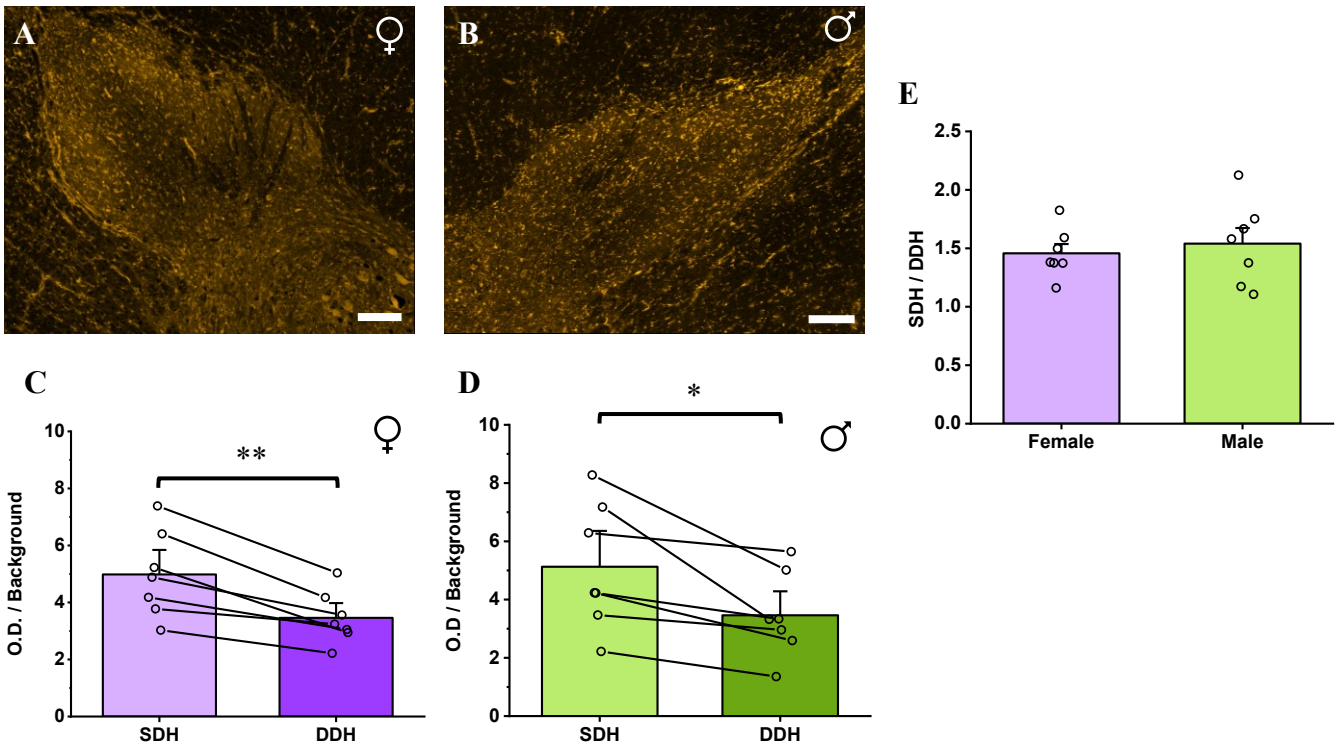

Figure 7: CB1 receptor expression increased in SDH versus DDH in humans. **A,B:** representative images of CB1 receptor expression in human dorsal horn in female **(A)**, and male **(B)**. **C,D:** The normalized optical density in the SDH of humans is statistically higher than the normalized optical density in the DDH for female **(C)** and male **(D)** humans. **p <0.002 for females and *p <0.02 for males. **E:** A ratio comparing the normalized optical density of the SDH to the normalized optical density of the DDH reveals that the increased optical density of CB1 receptors in the SDH of humans is conserved across sex. n=7. Scalebar 200 µm.

Discussion

The present study aimed to investigate the CB1 receptor expression in spinal pain processing areas of male and female rats and humans. This was accomplished by developing an appropriate protocol for immunostaining for CB1 receptors in human spinal cord tissue and acquiring high-resolution confocal images which were further analysed and quantified. Previous studies have shown increased expression of CB1 receptor immunoreactivity in the superficial dorsal horn of the rat spinal cord (Farquhar-Smith et al., 2000). The aim of the present study was to quantify those expression patterns and determine if similar patterns of expression are observed in the human spinal cord.

CB1 immunostaining in the dorsal horn

The present study explored the pattern of CB1 immunostaining present in the dorsal horn of the spinal cord. Previous studies have found predominantly CB1 immunopositive puncta in the superficial dorsal horn, which are hypothesized to be dorsal horn neurons which either express CB1 receptors themselves or receive inputs from fibres which express CB1 receptors (Hegyi et al., 2009; Veress et al., 2013). Some studies have found evidence for somatic staining in the spinal cord, particularly in the ependymal region (lamina X), with little evidence for cellular staining in the dorsal horn or other laminae of the spinal cord (Farquhar-Smith et al., 2000). Results regarding punctate versus cellular staining in the rodent spinal cord seem to differ based on the type of antibody used. In our study we used a knockout-validated polyclonal antibody, which has the affinity for multiple epitopes of the same antigen, compared to monoclonal antibodies which only have specificity for one epitope. Our results indicated the

presence of both punctate (i.e. neuropil) staining and cellular staining in the dorsal horn of the spinal cord. As previously reported (Farquhar-Smith et al., 2000; Hegyi et al., 2009; Veress et al., 2013), we see primarily punctate staining in the SDH in rodent and human spinal cord tissue. However, in contrast to previous studies, we identified strong somatic staining and some punctate staining in the deeper dorsal horn laminae. These findings were consistent across sex; however, there are some notable differences between species. In our rat samples, we observed somatic staining beginning in the DDH as high as laminae III and going into deeper laminae (ex. V-VI). In contrast, we found that somatic staining was restricted to deeper laminae (IV-VI) in our human samples. Together, these results provide evidence that CB1 receptors are primarily localized to neuropil (ex. nerve fibre endings) in the SDH, and have increased cellular localization in the DDH, which contributes more insight into its possible mechanisms of action in the context of pain and other modalities of somatosensory processing. For example, one hypothesis could be that the activation of CB1 receptors localized on the terminals of primary afferent fibres in the SDH lead to decreased nociceptive signalling through the ascending spinothalamic tract and potentially decreased perception of pain. While CB1 receptors are most commonly reported to be localized pre-synaptically, there is evidence from brain studies showing localization of CB1 receptors post-synaptically in regions such as the hippocampus (Busquets-Garcia et al., 2017). In the hippocampus, it is suggested that the post-synaptic CB1 receptors causes an increase in hyperpolarization-activated K^+ currents, which in turn decreases excitatory signalling and inhibiting long term potentiation (Maroso et al., 2016). Using similar rationale, it could be hypothesized that activation of somatic (i.e. post-synaptic) CB1 receptors in the DDH may increase hyperpolarization via K^+ currents, impacting signal transmission and integration for other somatosensory modalities.

CB1 localization to SDH vs. DDH

One primary aim of this study was to examine the level of localization of CB1 receptors to the SDH in comparison to the DDH in rodent and human male and female spinal cord tissue. Previous studies have shown evidence for increased CB1 receptor expression in the SDH compared to the DDH in all four levels of the spinal cord (Farquhar-Smith et al., 2000; Salio, Fischer, et al., 2002). Given that no significant differences in expression patterns between spinal cord levels were observed in the Farquhar-Smith study in 2000, we used both thoracic and lumbar samples for our human studies. Consistent with previous findings, our results show a significant increase of CB1 receptor expression in the SDH compared to the DDH in rodent and human spinal cord samples. Most early studies looking at cannabinoid receptor expression in the central nervous system failed to compare across sex, and typically focus on the male rat population (Farquhar-Smith et al., 2000; Salio, Fischer, et al., 2002). In our study, we used male and females in both our rodent and human experiments and found that expression patterns were conserved across sex in both species.

The SDH of the spinal cord is a critical region involved in pain processing, where the first synapse of the pain pathway occurs, before the signal travels up the ascending pathway to the brain where we perceive pain, whereas the DDH is responsible for integrating non-painful somatosensory information from the periphery. The preferred localization of CB1 receptors to the SDH could indicate an important role that CB1 receptors play in the regulation of pain. We can hypothesize that the CB1 receptors in the SDH are localized to either primary afferent fibres or the excitatory axon terminals of interneurons, which when activated would lead to a decrease in pain signaling to the brain. Moreover, in rats, but not humans, we qualitatively observed an increase in CB1 receptor expression localized to lamina I in comparison to lamina II. Lamina I

contains a higher proportion of excitatory interneurons as well as ascending projection neurons for pain signals, while lamina II has a notable population of inhibitory interneurons (30%) (Iwagaki et al., 2012; Maxwell et al., 2007; Wang et al., 2013). Given the difference in neuronal subtypes and function between these laminae, future investigations should use distinct lamina- and cell-specific histochemical markers to quantitively investigate this putative differential CB1 expression between subpopulations of SDH neurons.

Colocalization of CB1 receptors to CGRP

CGRP is a peptide found on peptidergic primary afferents, which acts on G protein-coupled receptors to activate adenylyl cyclase and PKA (Wimalawansa, 1996) and is important for the modulation of nociceptive processing in the spinal cord (Bird et al., 2006). Given the mixed evidence of CB1 receptors being localized pre- and post-synaptically in the spinal cord, we co-stained for CB1 receptors and CGRP, a peptide expressed on many nociceptive afferents. There is contrasting evidence for the degree of colocalization between CB1 and CGRP in the rodent spinal cord and DRG, with some studies indicating limited co-expression (Farquhar-Smith et al., 2000; Khasabova et al., 2004) and others showing considerable colocalization (>60%) (Veress et al., 2013). Given that different CB1 receptor antibodies were used in these studies, the conflicting results across various studies could be attributed to differences in the antibody recognizing different epitopes of the CB1 receptor. To our knowledge, there have been no studies examining the colocalization of CGRP and CB1 receptors in human spinal cord samples, particularly in the SDH. Using a knockout validated antibody, we demonstrated moderate colocalization (<50%) between CB1 receptors and CGRP in the SDH of the spinal cord of rats

and humans. When CB1 receptors are localized to CGRP containing peptidergic sensory neurons, it is thought that the activation of CB1 receptors by CB1 receptor agonists lead to a decrease in that sensory neuron's activity, and therefore a decrease in pain (Agarwal et al., 2007; Hohmann, 2002; Morisset & Urban, 2001; Veress et al., 2013). Our results indicate that some CB1 receptors may be acting to decrease nociceptive signalling via inhibition of peptidergic neuron terminals in the SDH of the spinal cord. However, given that the majority of CB1 receptors in the SDH were not localized to CGRP, this suggests that they may also be acting on non-peptidergic neuron terminals and/or acting post-synaptically. It is important to note that CGRP is but one marker of peptidergic afferents in the spinal cord, and testing for the localization to other peptidergic afferents was beyond the scope of this study. Future experiments should target colocalization of CB1 receptors with other peptidergic afferents such as neurokinin A (NKA) and substance P (SP), along with non-peptidergic afferents such as the purinergic receptor P2X3 and isolectin B4 (IB4), which could provide further insight into the workings of CB1 receptors in the spinal cord. Additionally, it has been established in rodents that CB1 receptors are localized to excitatory and inhibitory interneurons in the SDH, which is another strong target for pain research and should be investigated in human spinal cord samples (Farquhar-Smith et al., 2000; Salio, Fischer, et al., 2002). Furthermore, emerging single-cell and single-nucleus sequencing datasets from rodent and human DRG and spinal cord samples could be used to examine what specific subpopulations of presynaptic peripheral sensory neurons as well as dorsal horn neurons express the CB1 gene, CNR1 (Alkaslasi et al., 2021; Nguyen et al., 2021; Russ et al., 2021; Tansley et al., 2022; Tavares-Ferreira et al., 2022).

Sex differences in CB1 receptor expression patterns

Previous literature remains inconclusive with regards to sex differences in CB1 receptor expression in the central nervous system. While there is some evidence suggesting region-specific sex differences in expression patterns in the brain, there is currently no evidence for sex differences in CB1 receptor expression in the spinal cord (Castelli et al., 2014; González et al., 2000; Riebe et al., 2010; Rodríguez de Fonseca et al., 1994). The present study found that CB1 receptor expression patterns in the dorsal horn of the spinal cord were conserved across sex in both rats and humans. While no sex differences were observed with regards to CB1 receptor expression at baseline, future studies should examine whether expression patterns in the spinal cord change across sex in pain conditions and with prolonged cannabis use. In addition, it is important that research in the field of cannabis and pain, regardless of species, continue to include sex as a factor, to enable further investigation of sex differences and pain.

Future Directions

CB1 Receptors and Glial Cells in the Central Nervous System

In the brain there are low, but significant levels of cannabinoid receptors on astrocytes, which are thought to play a role in metabolic functions and inflammation (Bosier et al., 2013). For example, in the brains of rats, astrocytic CB1 activation increases the rate of glucose oxidation and ketogenesis, which are involved in supplying energy to the brain (Blázquez et al., 1999; Sánchez et al., 1998). The activation of astrocytic CB1 receptors can also hinder the production of inflammatory mediators (ex. nitric oxide) by astrocytes (Molina-Holgado et al.,

2002; Sheng et al., 2005). Lastly, there is evidence that CB1 receptors located on astrocytes in the mouse brain may mediate neuron-astrocyte interactions (Navarrete & Araque, 2008).

Immunohistochemical studies in rodents have demonstrated colocalization of astrocytes with CB1 receptors, and microglia with CB1 receptors in the superficial dorsal horn of the spinal cord (Hegyi et al., 2018; Salio, Doly, et al., 2002). Approximately 50% of astrocytes had CB1-positive staining, and approximately 80% of microglia had positive CB1 receptor staining (Hegyi et al., 2009). Astrocytes can produce 2-AG, which is a full agonist for the CB1 receptor (Walter et al., 2004; Walter & Stella, 2003). Immunohistochemical analyses reveal close proximity between astrocytes, CB1 receptors and DGLα, the precursor for 2-AG, suggesting that endocannabinoids, and particularly 2-AG play an important role in the communication between neurons and astrocytes (Hegyi et al., 2018).

Approximately 80% of stained microglia in the superficial dorsal horn express CB1 receptors (Hegyi et al., 2009). Co-localization of CB1 receptors on microglia has also been identified in the brain, particularly in the cerebral cortex, where they are involved in the modulation of chemokine and cytokine expression (Cabral & Marciano-Cabral, 2005). It is proposed that CB1 receptors on microglia in the spinal dorsal horn may have the same effect, however this mechanism needs to be further explored in the spinal cord. The understanding of the relationship between the cannabinoid system and glial cells has significant implications for the treatment of chronic pain and should be studied in humans both at baseline and in pain conditions.

Pain models and CB2

Our study investigated the CB1 receptor expression in the dorsal horn of rats and humans at baseline (ie. with no chronic pain conditions), which limits how much we can translate to rodent pain models and to the clinical chronic pain population. Future studies should continue to use baseline models, but also provide comparisons to pain conditions as well as determine how mechanisms of pathological pain may affect CB1 expression and function at the level of the spinal cord.

Given the lack of evidence of CB2 receptors being present at baseline in the spinal cord (Bie et al., 2018; Lu & MacKie, 2016; Zhang et al., 2003), the focus of the present study was to analyze the expression of CB1 receptors in healthy animals and human donors. There is a large amount of literature supporting the evidence that CB2 receptors are upregulated in inflammatory states such as pain (Bie et al., 2018; Garcia-Ovejero et al., 2009; Lu & MacKie, 2016; Zhang et al., 2003). Given this, future expansion of human donor tissue collection programs may enable collection of human spinal cord tissue from donors with specified chronic pain conditions, where expression of CB2 receptors could be investigated.

Conclusion

In conclusion, the results from the present study depict the first successful immunostaining of CB1 receptors in the dorsal horn of the human spinal cord. CB1 receptors have higher expression in the SDH compared to the DDH, and this finding is conserved across both sex and species. Within the dorsal horn, CB1 receptors are localized to both neuropil and

cell bodies, however neuropil staining is more pronounced in the SDH, and cell body staining in the DDH. Lastly, there is slightly less pronounced localization of CB1 to the SDH in humans compared to rats. CB1 receptors in the spinal cord remain a key target for therapeutics in the context of pain.

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