Persistent Inflammation Desensitizes the Presynaptic Cannabinoid CB1 Receptor

By

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A DISSERTATION

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List of Abbreviations

2-AG	arachidonolyglyerol
AA	arachidonic acid
AEA	anandamide
BLA	basolateral amygdala
CB1	cannabinoid receptor subtype 1
CB2	cannabinoid receptor subtype 2
CFA	Complete Freund’s adjuvant
DAGL-α	diacylglycerol lipase alpha
DAMGO	[D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin
DSE	depolarization-induced suppression of excitation
DSI	depolarization-induced suppression of inhibition
eCB	endocannabinoid
FAAH	fatty acid amide hydrolase
FABP	fatty acid binding protein
GIRK	G protein-coupled inwardly rectifying potassium channel
GPCR	G protein-coupled receptor
GR	Glucocorticoid Receptor
GRK	G protein coupled receptor kinase
Het	Heterozygous
MAGL	monoacylglycerol lipase
ME	met-enkephalin
mGluR	metabotropic glutamate receptor
MOR	mu opioid receptor
NADA	N-arachidonoyl dopamine

NAPE-PLD	N-acylphosphatidylethanolamine phospholipase D
OEA	Oleoylethanolamine
PAG	periaqueductal gray
PPARα	perixome proliferator-activated receptor alpha
RGS	Regulator of G-protein Signaling
RIM	Rimonabant (SR144528)
RVM	rostral ventromedial medulla
THC	tetrahydrocannabinol
TRPV1	transient receptor potential cation channel subfamily V member 1
vlPAG	Ventrolateral periaqueductal gray
WIN	WIN-55,212-2 (CBR agonist)
WT	Wildtype

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In loving memory of Thurmond A. Williamson, PhD
Dec. 11, 1922 – Dec 13, 2021

The man who inspired my curiosity and passion for life-long learning.

// Abstract

The majority of pharmaceutical drugs on the market target G protein coupled receptor (GPCRs); however, GPCR signaling and regulation adapt in response to environmental stimuli. One GPCR gaining attention due to the increasing legalization of cannabis is the cannabinoid 1 receptor (CB1R). Cannabis legalization is leading to increased use to treat pain and other ailments, including pain caused by inflammation. Studies presented throughout this dissertation investigate adaptations in synaptic CB1R suppression of GABA release after persistent inflammation induced by Complete Freund's Adjuvant (CFA).

In Chapter 2, we investigate adaptations in the cannabinoid system after persistent inflammation in the ventrolateral periaqueductal gray (vlPAG). We find that persistent inflammation induces GRK2/3-dependent desensitization of the presynaptic cannabinoid 1 receptor (CB1R). This finding is important because presynaptic GPCRS do not readily desensitize under normal conditions. Additionally, CB1Rs respond differently to exogenous versus endogenous agonists after persistent inflammation. While CB1R-dependent suppression of GABA release in response to exogenous agonists is significantly reduced after persistent inflammation, endocannabinoid-induced suppression of GABA release is prolonged. This prolonged effect appears to be due to an increase in the endocannabinoid, 2-arachadonylglycerol (2-AG), via reduced breakdown by MAGL. Together, these data highlight a mechanism of altered cannabinoid regulation after persistent hindpaw inflammation.

In Chapter 3, we investigate a potential mediator of the impacts of inflammation on the cannabinoid system: circulating corticosterone. Corticosterone has been implicated in CB1R adaptations after stress; however, it is not known whether corticosterone mediates the impacts of inflammation on the cannabinoid system within the vlPAG. Corticosterone measurements from trunk blood reveal increases in corticosterone at multiple timepoints after CFA-induced inflammation and overall elevated corticosterone levels in females compared to males. After persistent inflammation, CB1R suppression of GABA release is equally reduced in both males and females; however, a further examination of the timecourse of inflammation revealed a stark sex difference in CB1R suppression of inhibition 24 hours after CFA injection. At this timepoint, activation of CB1R by exogenous cannabinoid agonists suppresses GABA release to a similar level as observed in naïve animals, but in females yields significantly reduced suppression of GABA release- comparable to persistent inflammation. By washing corticosterone over vlPAG slices, we found that acute corticosterone suppresses GABA release through glucocorticoid receptor-mediated eCB release that acts through CB1Rs. These data suggest that corticosterone is increased by inflammation and can stimulate eCB synthesis within the vlPAG. Further, the glucocorticoid receptor (GR) antagonist RU486 reverses CB1R suppression of GABA release in a subset of neurons after persistent inflammation. Together these data indicate that corticosterone may play a role in the reduction of CB1R function after persistent inflammation but is likely not the only factor. Together, data presented in this dissertation highlight adaptations to presynaptic CB1R regulation after persistent inflammation and identify a circulating factor involved in that regulation.

Chapter 1 : Introduction

Intense interest has recently focused on the use of cannabinoids for novel pain therapeutics. Ironically, *Cannabis sativa* (colloquially known as marijuana or cannabis) has been used for millennia as a medicine for pain and other ailments, but its mechanisms of action are not completely understood. With the emerging legalization of cannabis throughout the United States and the world, there is an urgent need for a deeper understanding of the mechanisms underlying cannabinoid effects. Studies of endogenous cannabinoids (endocannabinoids) have begun to elucidate the far-reaching roles of these lipid signaling molecules in modulating neuronal function.

1.1 Endocannabinoid system

Endocannabinoids

The endogenous cannabinoid system is comprised of the best characterized cannabinoid receptor subtypes 1 (CB1) and 2 (CB2), as well as their natural ligands; termed endocannabinoids (eCBs; Cristino et al 2019, Lu & Mackie 2016). Anandamide (AEA; Devane et al 1992) and 2-Arachidonoylglycerol (2-AG; Mechoulam et al 1995, Stella et al 1997, Sugiura et al 1995)) are the most well-studied endocannabinoids. These ligands are synthesized “on demand” from membrane lipids in response to cellular signals including activation of the postsynaptic metabotropic glutamate receptors (mGluRs; Maejima et al 2001a, Maejima et al 2001b, Ohno-Shosaku et al 2001) and depolarization-induced calcium mobilization (Brenowitz & Regehr 2003). AEA and 2-AG are synthesized primarily by N-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) and diacyglyerol lipase alpha (DAGLα), respectively in calcium

dependent processes. Termination of signaling occurs rapidly through enzymatic degradation by specific lipases. The most thoroughly characterized lipases are monoacyglycerol lipase (MAGL; Bisogno et al 2003, Dinh et al 2004), which degrades 2-AG, and fatty acid amide hydrolase (FAAH; Maccarrone et al 1998, Natarajan et al 1984, Schmid et al 1985) which predominantly degrades AEA (For review see Hillard 2000). Each of these components is necessary for maintaining tight control over endocannabinoid levels and their actions on brain circuits.

eCBs are unique messengers that are distinct from other neurotransmitters or neuromodulators. They are synthesized “on demand” in the postsynaptic neuron (Kreitzer & Regehr 2001, Ohno-Shosaku et al 2001, Wilson & Nicoll 2001) for review see (Ahn et al 2008), primarily in response to intense stimulation of afferents impinging on the postsynaptic neurons that result in activation of postsynaptic mGluRs or robust increases in internal Ca^{2+}. The eCBs then travel in a retrograde manner across the synapse, this retrograde signaling was first described in hippocampal synapses (Maejima et al 2001a, Wilson & Nicoll 2001). eCBs travel retrogradely and inhibit the release of neurotransmitters from the presynaptic terminal by binding to and activation presynaptic cannabinoid receptors (Maejima et al 2001a, Maejima et al 2001b, Ohno-Shosaku et al 2001, Wilson & Nicoll 2001). In addition to the classic cannabinoid receptors, endocannabinoids bind directly to channels to modulate their gating and/or ion flux, including A-type potassium channels, TRPV1, $GABA_A$, nicotinic acetylcholine, glycine and HCN channels (Gantz & Bean 2017, Maroso et al 2016, Oz et al 2003, Sigel et al 2011, Xiong et al 2012, Zygmunt et al 1999). The lipid composition of eCBs begs the question of how they travel across the synapse, a question which is still not well

understood. Transport of endocannabinoids across the membrane into the synapse has been proposed to use facilitated transport (Adermark & Lovinger 2007) or passive diffusion (Glaser et al 2003, Kaczocha et al 2006) via binding to fatty acid binding proteins (FABPs) (Kaczocha et al 2009), or possibly as constituents of extracellular vesicles (Gabrielli et al 2015).

In addition to these well-characterized endocannabinoids, there are other members that have been shown to modulate pain (Walker et al 2002). Noladin ether, an analog of 2-AG that binds to the CB1 receptor, has similar characteristics to 2-AG but is not found in appreciable quantities in the brain (Oka et al 2003), distinguishing it from AEA and 2-AG. N-arachidonoyl dopamine (NADA) is an agonist at both the CB1 receptor and transient receptor potential channel (TRPV1) (Grabiec & Dehghani 2017) and increases firing of spinal nociceptive neurons in a TRPV1-dependent manner (Huang & Walker 2006). Oleoylethanolamine (OEA) is an endocannabinoid-like compound that stimulates the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARa; Laleh et al 2019)) has been implicated in pain modulation. However, the role of OEA in pain is complex and its pain modulatory effects may be indirect through PPAR-α interacting pathways (Donvito et al 2017, Suardiaz et al 2007). Further understanding of the biosynthesis, regulation and functions of these molecules is no doubt an important step in defining novel targets for pain therapies.

Cannabinoid receptors

The cannabinoid receptors (CB1R and CB2R), as well as a putative cannabinoid receptor GPR55, are seven transmembrane G protein-coupled receptors (GPCRs) that

signal predominately through inhibitory $G\alpha_{i/o}$ G proteins. The CB1 receptor was cloned in 1990 (Matsuda et al 1990) based on its binding affinity for the natural ligand (delta9-tetrahydrocannabinol, THC) and a synthetic analogue with potent analgesic properties (CP-55,940). CB1R inhibits forskolin-stimulated adenylyl cyclase activity in a G protein-dependent manner. Discovery of CB1R structure opened the door for development of synthetic compounds, both agonists and antagonists, that bind CB1Rs (Herkenham et al 1990). Cannabinoid receptors can be activated by phytocannabinoids, synthetic cannabinoids, and endocannabinoids (**Figure 1.1**). CB1Rs are the most abundant GPCRs in the central nervous system (Herkenham et al 1990, Howlett et al 2002) and are expressed in neurons throughout the central nervous system (Busquets-Garcia et al 2018, Herkenham et al 1990, Stella 2010, Turcotte et al 2016), where they are primarily expressed in presynaptic terminals and act to inhibit neurotransmitter release (Chevaleyre et al 2006, Freund & Hajos 2003, Freund et al 2003, Hajos et al 2000, Huang et al 2001b, Kano et al 2009, Katona et al 2001, Katona et al 1999, Katona et al 2006, Mackie 2005, Morisset & Urban 2001). CB1Rs are also found postsynaptically, where their activation increases outward K+ conductance (Maroso et al 2016, Schweitzer 2000), although postsynaptic CB1Rs seem to play a relatively minor role compared to the ubiquitous expression and activity of presynaptic CB1R (for review see Busquets-Garcia et al 2018).

Historically, CB2Rs were thought to be expressed exclusively in the periphery, primarily on immune cells, but functional and anatomical evidence now indicates that these receptors are also expressed in the central nervous system (Atwood & Mackie 2010). CB2Rs are expressed at significantly lower levels than CB1Rs in the midbrain

and brainstem (Gong et al 2006), although localization studies using putative CB2R antibodies should be interpreted with caution due to issues with specificity (Brownjohn & Ashton 2012, Cecyre et al 2014, Marchalant et al 2014). Nonetheless, functional studies using multiple CB2-selective agonists and antagonists provide convincing evidence for CB2-dependent effects in the rostral ventromedial medulla (RVM) (Deng et al 2015, Li et al 2017) and spinal cord (Beltramo et al 2006, Burston et al 2013, Guindon & Hohmann 2008a, Guindon & Hohmann). Interestingly, CB2R expression appears to be highly dynamic and dependent on the environment (Hsieh et al 2011, Li et al 2017) as CB2R expression is induced by inflammation and neuropathic pain. CB2R expression has also been observed on microglia (Stella 2010) and is upregulated in inflammation (Maresz et al 2005).

While CB1R and CB2R are the best studied receptors in the cannabinoid system, both endocannabinoids and exogenous cannabinoids can act on other receptors. GPR55 is an orphan GPCR that is stimulated by AEA and some lipophilic derivatives of endocannabinoids, as well as the CB1R antagonist, AM251, and inverse agonist SR141716A (rimonabant) (Kapur et al 2009, Yang et al 2016). GPR55 is expressed on neurons in the dorsal root ganglion (Lauckner et al 2008), on adipose tissue (Tuduri et al 2017) and microvascular endothelial cells (Leo et al 2019) suggesting myriad functions of the endocannabinoid system that are largely unexplored.

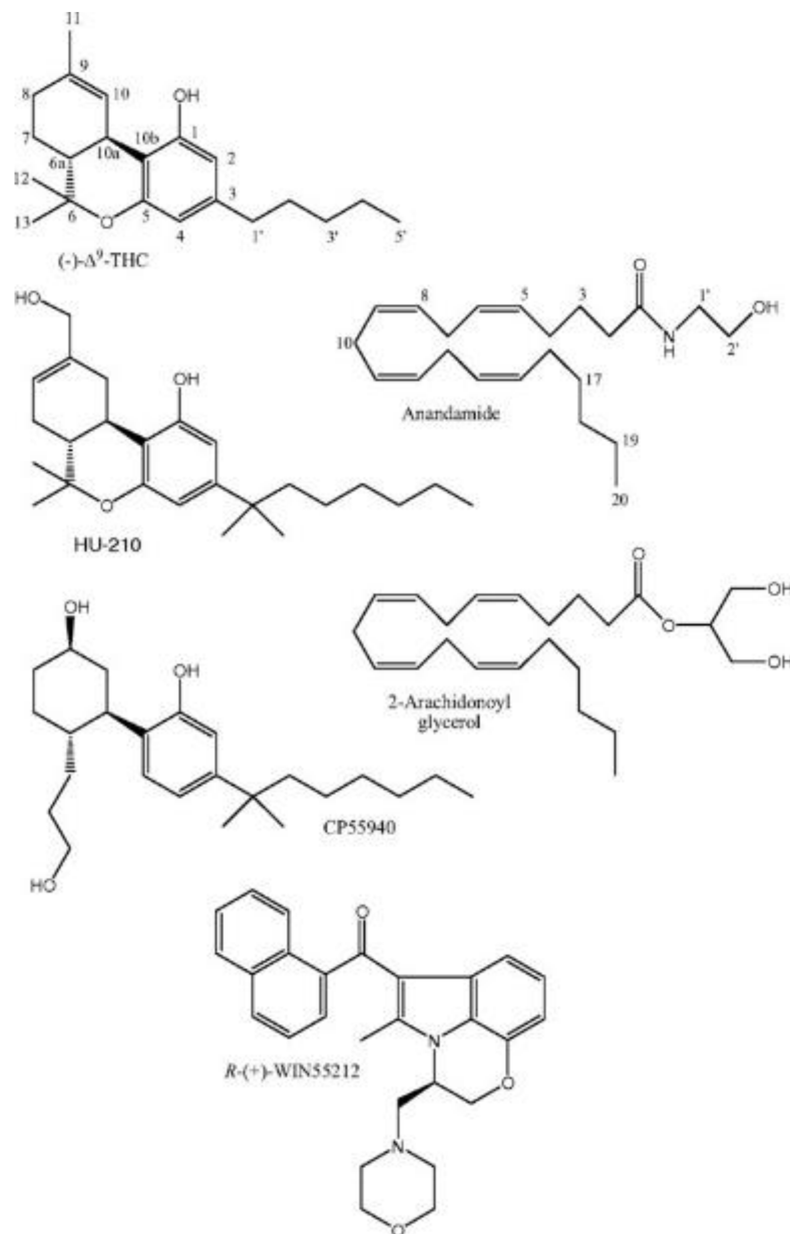

Figure 1.1 Structure of synthetic and endogenous cannabinoid ligands

The structure of phytocannabinoid Δ^9-THC, synthetic cannabinoids: WIN55,212-2, CP-55940, and endocannabinoids: anandamide and 2-arachadidonyl glycerol (2-AG).

[Provided with permission from Curr Med Chem; Adapted by C. Bouchet]

Adapted from: Pertwee, R. G. (2010). "Receptors and channels targeted by synthetic cannabinoid receptor agonists and antagonists." Curr Med Chem **17**(14): 1360-1381.

AEA also binds to the transient receptor potential channel TRPV1 (Di Marzo & De Petrocellis 2010, Di Marzo et al 2002). AEA is a full agonist at TRPV1 channels expressed on nociceptive primary afferents, as well as on many central neurons comprising ascending pain circuits. TRPV1 channels are non-selective cation channels gated by capsaicin, protons and heat that promote neuronal excitability. AEA is pro-nociceptive in some situations, promoting responses to painful stimuli (Dinis et al 2004) but AEA activation of TRPV1 channels is also antinociceptive, especially in the presence of inflammation and neuropathic pain (Guindon et al 2013, Horvath et al 2008). Taken together, the actions of endocannabinoids depend both on expression of the target receptors on specific cells and adaptations within specific brain areas that are induced in different pain states.

It should also be noted that there are documented variations in cannabinoid receptor sequence across species. The CB1R appears to be well conserved with 98.7% amino acid sequence homology between guinea-pig and human, 99.2% homology between guinea pig and rat or mouse (Kurz et al 2008), and even 70% homology between the amino acid sequence between pufferfish and human CB1R (Yamaguchi et al 1996). In contrast, the CB2 receptor is not as well conserved across species. CB2 receptor mRNA splicing and expression vary between mice and rats, which impacts CB2 receptor-dependent effects on cocaine self-administration between the species (Zhang et al 2015). Rat and human CB2 receptors share 81% amino acid homology (Mukherjee et al 2004) and the profound sequence divergence in the carboxy terminus of mammalian CB2 receptors could differentially impact receptor regulation including

desensitization and internalization (Brown et al 2002). Appropriate caution should be employed when comparing CB2 receptor function across species.

CB1 receptor desensitization

CB1R regulation has been extensively studied in cell culture and similar systems. In their systems, acute agonist exposure induces desensitization and internalization. In CHO cells, one-hour incubation in the synthetic agonist, CP-55940, induces dose-dependent desensitization of forskolin-stimulated cAMP (Rinaldi-Carmona et al 1998). In AtT20 cell culture, CB1Rs internalize with as little as 5 minutes of exposure to synthetic agonists WIN55,212-2, CP55,940, or HU-210. Almost all CB1 receptors are internalized after 15 minutes of agonist exposure and internalization requires C-terminal residues between 460 and 463 on the CB1R (Hsieh et al 1999). The eCB analog methandamide also induce CB1R desensitization; however, a much higher concentration is required compared to exogenous agonists (Hsieh et al 1999), highlighting potentially important differences between exogenous and endogenous cannabinoid agonists in CB1R desensitization. In *Xenopus* oocytes, CB1R desensitization requires GRK3 phosphorylation of the C-terminal tail (Jin et al 1999). These events are similar to what is known about mu opioid receptor (MOR) regulation within the postsynaptic environment. Briefly, acute MOR activation by an agonist rapidly induces G-protein coupled receptor kinase (GRK) phosphorylation of the C-terminal tail and recruitment of β-arrestin (**Figure 1.2**). This process leads to MOR desensitization and internalization (for review see Gurevich & Gurevich 2019, Williams et al 2013). However, the presynaptic localization of the CB1R within the central nervous system

(Chan & Yung 1998, Irving et al 2000, Katona et al 1999, Szabo et al 1998, Vaughan et al 2000) but see (Wilson-Poe et al 2012) suggests that regulation of the CB1R within the CNS differs from what has been observed in cell culture.

Presynaptic and postsynaptic GPCRs are differentially regulated, a process that has been thoroughly studied with the MOR. While postsynaptic GPCRs rapidly desensitize in response to agonist-induced activation (Alvarez et al 2002, Williams et al 2013), similar to the regulation of CB1R observed in cell culture, presynaptic MORs do not desensitize with up to 30 minutes of agonist-induced activation (Fyfe et al 2010). This finding has been expanded to multiple brain regions and multiple GPCRs: while the postsynaptic response to activation desensitizes within the course of minutes, the presynaptic response is resistant to desensitization (Pennock et al 2012, Pennock & Hentges 2011). These data force us to think critically about regulatory features of presynaptic GPCRs and how they differ from regulation of GPCRs in postsynaptic membranes or regulatory events established in cell culture models.

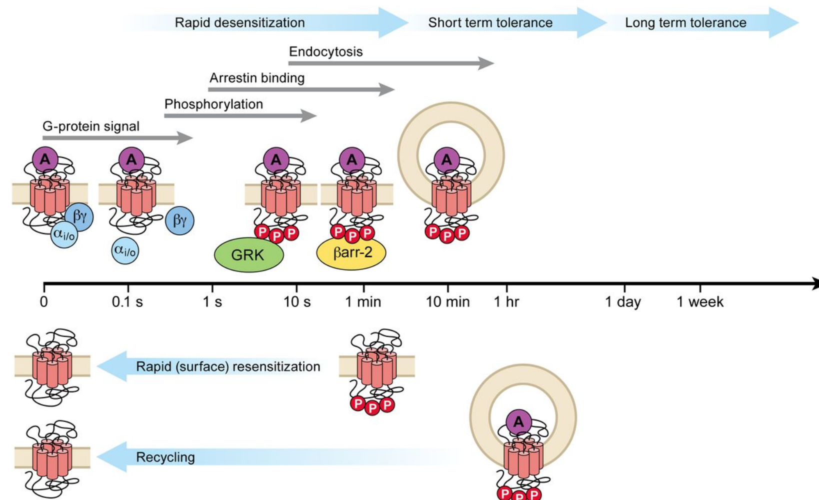

Figure 1.2 Canonical GPCR desensitization

Cartoon depiction of postsynaptic MOR regulation following binding of an efficacious agonist. Time scales for each process are shown (log scale). Agonist (A) binding to the extracellular binding sites of the GPCR initiates intracellular G-protein signaling. Gα dissociates from Gβ/γ subunits. (3) C-terminal tail of GPCR is phosphorylated by G protein coupled receptor kinase (GRK) very rapidly, saturating in less than 20 seconds. (4) β–arrestin is recruited to the C-terminal tail and saturates in several minutes, desensitization reaches stead state around 5 minutes. (5) The GPCR undergoes endocytosis and is removed from the extracellular surface, endocytosis reaches steady state in approximately 30 minutes. This process can lead to short- and long-term tolerance, presumably through additional mechanisms. Throughout this process, the GPCR can become active again through phosphate removal with phosphatases or recycling of endocytosed receptors back to the membrane.

[Provided with permission from Pharmacological reviews; Adapted by C. Bouchet]

Adapted from: Williams JT, Ingram SL, Henderson G, Chavkin C, von Zastrow M, et al. 2013. Regulation of mu-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. *Pharmacol Rev* 65: 223-54

Cannabinoid signaling pathways

Activation of cannabinoid receptors suppresses neurotransmitter release from the presynaptic termial. CB1R and CB2R are both inhibitory GPCRs that signal primarily via $G\alpha_{i/o}$ subunits (Huang et al 2001a, Lichtman et al 1996), although there is evidence that some cannabinoid receptor agonists stimulate $G\alpha_q$ signaling (Lauckner et al 2005). Cannabinoid agonists inhibit glutamate release in many synapses in the central nervous system, including the prefrontal cortex (Melis et al 2004), hippocampus (Misner & Sullivan 1999), cerebellum (Levenes et al 1998, Takahashi & Linden 2000), striatum (Huang et al 2001a, Robbe et al 2001), and spinal cord (Morisset et al 2001, Morisset & Urban 2001). Cannabinoid agonists also inhibit GABA release in many synapses throughout the brain, including the amygdala (Katona et al 2001), cerebellum (Diana et al 2002, Yamasaki et al 2006), nucleus accumbens (Mato et al 2004), and brainstem (Li et al 2017, Vaughan & Christie 1997a, Vaughan et al 1999). These lists are by no means exhaustive but illustrate the widespread modulation of neurotransmitter release by the cannabinoid receptors.

CB1 receptors effectively couple to several G protein subtypes but efficacy and potency for activation varies widely throughout the brain (Prather et al 2000) and is dependent on the ligand (Diez-Alarcia et al 2016), as well as on the complement of signaling proteins expressed in each synapse. **Figure 1.3** depicts several of these signaling pathways. CB1 receptors inhibit presynaptic neurotransmitter release through inhibition of presynaptic Ca^{2+} channels (Brown et al 2004, Huang et al 2001a, Kushmerick et al 2004, Mackie & Hille 1992) and activate K^+ channels (Daniel et al 2004, Robbe et al 2001). CB1 receptors also modulate local translation in GABAergic

presynaptic terminals through a mTOR signaling pathway (Younts et al 2016). The multiple cellular mechanisms underlying CB1 receptor inhibition of neurotransmitter release may contribute to the difficulty in targeting CB1 receptors for novel therapeutics because the signaling pathways are different in individual synapses, even within specific brain areas.

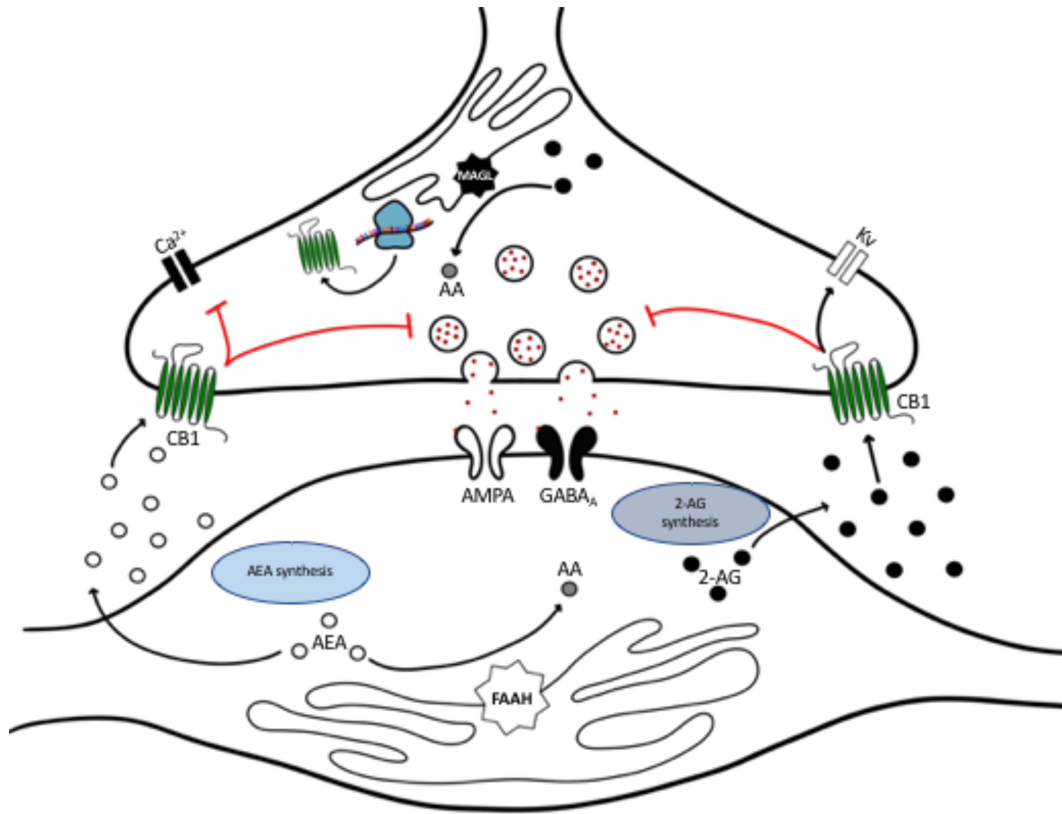

Figure 1.3 CB1R signaling pathways

Schematic of the CB1 receptor-mediated inhibition of presynaptic neurotransmitter (glutamate and GABA) release. The endocannabinoids anandamide (AEA) and 2-Arachidonoylglycerol (2-AG) are synthesized in response to stimulation of postsynaptic cells. They diffuse through the membrane or are transported across the synapse to act at cannabinoid subtype 1 (CB1) receptors expressed on presynaptic terminals. CB1 receptors are translated in the presynaptic terminals in a mTOR-dependent manner in some synapses. Activation of CB1 receptors inhibit release via several signaling cascades, including activation of voltage-gated potassium channels (Kv), inhibition of Ca^{2+} channels and direct inhibition of vesicle release machinery. Termination of signaling occurs through enzymatic breakdown. Monoacyglycerol lipase (MAGL) is expressed in the presynaptic terminal and degrades 2-AG while fatty acid amide hydrolase (FAAH), which predominantly degrades AEA, is localized to postsynaptic cells.

Regulation of synaptic plasticity

Endocannabinoids can produce both short-term, transient inhibition of neurotransmitter release (Diana & Marty 2004, Kreitzer & Regehr 2002, Wilson & Nicoll 2002) and longer, more sustained inhibition (Chevaleyre & Castillo 2003, Chevaleyre et al 2006, Gerdeman & Lovinger 2003, Gerdeman et al 2002, Robbe et al 2002, Ronesi et al 2004, Sjostrom et al 2003, Yin et al 2006). Depolarization-induced suppression of excitatory (DSE) or inhibitory (DSI) transmission are examples of short-term plasticity that last during stimulation or shortly after stimulation is terminated. In these experiments, the postsynaptic cell is depolarized, increasing intracellular calcium and synthesis of endocannabinoid ligands. Interestingly, not all synapses display DSE/DSI (Hentges et al 2005, Kreitzer & Malenka 2005). From these studies, it is not clear whether these synapses lack eCB signaling (potentially through insufficient CB1R expression or eCB synthesis enzymes) or if these synapses use a mode other than depolarization to initiate eCB signaling. eCB synthesis can be initiated by means other than depolarization, such as activation of GPCRs coupled to $G\alpha_q$, for example Group I mGluRs and muscarinic receptors (Kreitzer & Malenka 2005, Martin & Alger 1999, Morishita et al 1998). These studies indicate that there are multiple modes of endocannabinoid production, with some cells potentially requiring activation of $G\alpha_q$, depolarization, or a combination of the two. Endocannabinoids have been implicated in short-term depression (STD) via mGluR activation of phospholipase C (Sternweis et al 1992), as well as long-term depression (LTD) at both glutamatergic (Robbe et al 2002) and GABAergic (Friend et al 2017, Younts et al 2016) synapses. These forms of endocannabinoid-induced plasticity are carefully reviewed in (Lovinger 2008).

Regulation of the endocannabinoid system by corticosterone

CB1R expression and function, as well as eCB production, are affected by circulating factors including glucocorticoids. Glucocorticoids stimulate eCB synthesis through activation of glucocorticoid receptor (GR) that has actions through both genomic and non-genomic mechanisms. Canonically, corticosterone activates the GR by crossing the cell membrane, binding to and activating cytosolic GRs which induces their dimerization and translocation to the nucleus where they influence transcription (Zanchi et al 2010). However, corticosterone conjugated to BSA, rendering it unable to cross the plasma membrane, can exert effects through the GR indicating the presence of a membrane-bound GR (mGR; Strehl et al 2011, Vernocchi et al 2013). The rapid effects of the glucocorticoid, corticosterone, on eCB levels within the amygdala and hippocampus (Hill et al 2010a, Tasker et al 2006) support the theory that elevations in circulating corticosterone act through non-genomic mbGRs. In one example, eCB levels were elevated in several limbic regions 10 minutes after systemic corticosterone injection and returned to baseline 1 hour later (Hill et al 2010a). This rapid time course of eCB synthesis indicates actions through mbGRs. It is not fully understood how GR activation stimulates eCB synthesis but pharmacological evidence indicates the necessity of postsynaptic kinases including PKC and PKA, as well as calcium mobilization (Harris et al 2019). Together, these data provide strong evidence that eCB synthesis can be stimulated through activation of mbGRs.

There is evidence that some, although potentially not all, of the mbGRs are GPCRs (Tasker et al 2006). The first evidence was through a series of experiments showing that [^{3}H]corticosterone binding to membranes is negatively regulated by GTPγS

or the addition of G-proteins and is enhanced by the addition of Mg^{2+} (Orchinik et al 1992). The change in affinity of [^{3}H]corticosterone for the “corticosterone receptor” and enhancement by Mg^{2+} is consistent with a G protein signaling. This was notable because it was the first time that a steroid hormone was found to act through a GPCR. Another group examined corticosterone inhibition of nicotine-induced calcium influx in PC-12 cells. Briefly, their main findings were that corticosterone conjugated to BSA had the same effects as free corticosterone and this effect was blocked by pertussis toxin (Qiu et al 1998). These data suggest that, in PC-12 cells, corticosterone acts through membrane-bound $G\alpha_{i/o}$ GPCR receptor signaling. In the hypothalamic paraventricular nucleus (PVN), glucocorticoids dexamethasone and corticosterone rapidly suppress excitatory neurotransmission. This suppression is maintained when corticosterone is conjugated BSA, making it membrane-impermeable, but not when administered intracellularly and completely blocked by CB1R antagonists (Di et al 2003), supporting the hypothesis that glucocorticoids stimulate eCB synthesis through activation of mbGRs. Additional data indicate that glucocorticoid-induced synthesis of eCBs is mediated by the $G\alpha_s$-cAMP-PKA signaling pathway within the hypothalamus following the binding of glucocorticoids to a membrane-associated GPCR (mbGR; Di et al 2009, Malcher-Lopes et al 2006). Although mbGRs can have a multitude of downstream effects and even act through either $G\alpha_{i/o}$ or $G\alpha_s$ subtypes, evidence indicates that corticosterone activates mbGRs which then stimulate the release of intracellular Ca^{2+}. Together, these actions lead to increased eCB synthesis (Fig. 1.4). Corticosterone-induced synthesis of eCBs within the brain presents a mechanism that connects the endocrine system with neural function and neurotransmitter release.

The role of corticosterone on the central cannabinoid system has been primarily studied in the context of stress. Chronic stress alters CB1R expression and eCB levels within multiple brain regions (for an excellent review see Morena et al 2016). Stress can induce CB1R upregulation or downregulation depending on the brain region of interest, although it should be noted that most of the studies cited here are conducted in male rats and mice, so it is less clear how these processes work in females. Chronic unpredictable stress reduces cannabinoid receptor expression in the hippocampus, hypothalamus, and ventral striatum, increases binding in the PFC, and does not impact binding in the amygdala or midbrain in male rats (Hill et al 2008b). In other brain regions, such as the amygdala, chronic stress does not reduce expression (Lee & Hill 2013) but changes eCB levels and CB1R suppression of inhibition. In male mice, 10 days of repeated restraint stress increases the synthesis 2-AG and prolongs DSI while reducing the effect of WIN on suppression of GABA release (Patel et al 2009). This set of studies elucidates stress-induced changes in the cannabinoid system that differentially impact eCB and synthetic cannabinoid agonist actions at the CB1R. Overall, chronic stressors alter CB1R regulation while endocannabinoid levels rapidly adjust to acute stressors. These adaptations often persist through the duration of chronic stressors and reverse after the cessation of the stressor (Wamsteeker et al 2010).

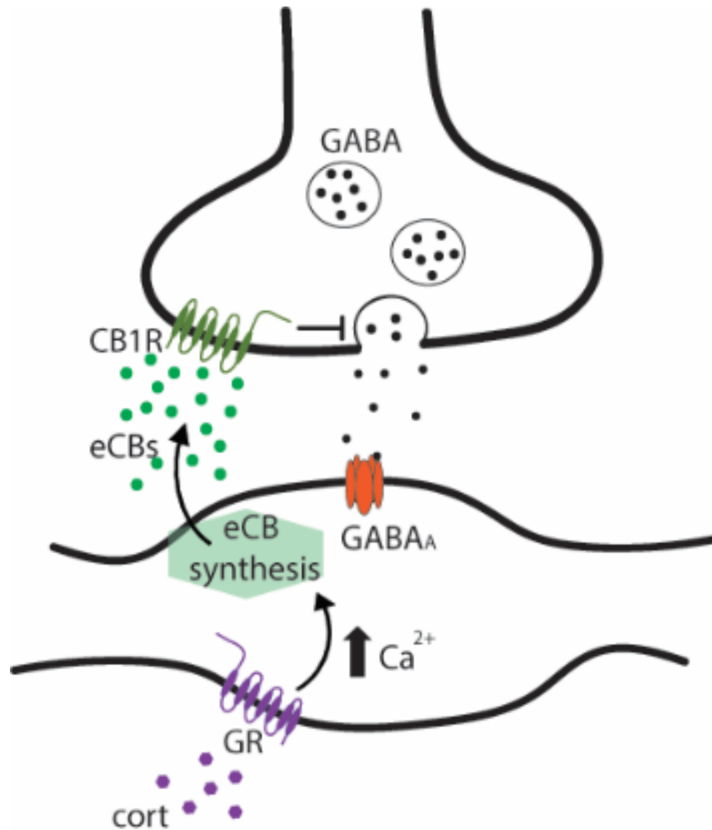

Figure 1.4 Corticosterone-induced eCB synthesis

Corticosterone (cort) binding to the membrane-bound glucocorticoid receptor (mbGR) initiates intracellular pathways that increase Ca^{2+} levels within the postsynaptic neuron. The rate-limiting enzymes for eCB synthesis are Ca^{2+} sensitive, so increased Ca^{2+} is required for eCB synthesis. These enzymes are thought to be coincidence detectors that integrate incoming signals from excitatory GPCRs and the influx of intracellular Ca^{2+} (Baggelaar et al 2018). The eCBs travel in a retrograde manner across the synapse to act on presynaptic CB1Rs, causing suppression of neurotransmitter release (GABA in this example).

The impacts of stress on the endocannabinoid system are thought to occur primarily through increased circulating levels of corticosterone acting at membrane-bound GRs (mbGRs). Acutely, a 30-minute stressor enhances hippocampal DSI via a corticosterone-dependent mechanism, as it is blocked by application of the GR and progesterone receptor (PR) antagonist, RU-486, on hippocampal slices and recapitulated by treating the slice with corticosterone (Wang et al 2012). Repeated stressors lead to persistently increased basal corticosterone levels and, in the amygdala, reduced AEA and increased 2-AG (Hill et al 2010b). Corticosterone treatment alone also recapitulates the effect of chronic stress on CB1R expression with no change in the amygdala, downregulation in the hippocampus (Bowles et al 2012, Hill et al 2008a), striatum (Rossi et al 2008), and dorsal root ganglion (Hong et al 2011). Within the dorsal striatum, activating CB1R in the dorsal striatum enhances learning retention, a phenomenon that is mimicked by stress. Co-injection of the CB1R antagonist AM251 blocks corticosterone-induced learning and memory retention (Siller-Perez et al 2019) indicating that the effects of corticosterone act indirectly through CB1Rs. Importantly, these effects are not due to the stress of injection, as non-invasive corticosterone administered via drinking water changes CB1R expression and eCB levels, paralleling the effects of corticosterone injections (Bowles et al 2012). Moreover, systemic administration of the mixed GR/PR antagonist, RU486, during chronic stress reverses stress-induced changes in CB1R expression in the dorsal root ganglion (Hong et al 2011), striatum (Rossi et al 2008) and hypothalamus (Wamsteeker et al 2010). Together, these studies provide strong evidence that stress-induced changes in the endogenous cannabinoid system occur through corticosterone actions at the mbGR.

Cannabinoids in pain and inflammation

Although cannabis has been used for centuries to relieve pain, the cloning of cannabinoid receptors and identification of an endogenous cannabinoid lagged behind characterization of the opiate system. The effects of opioids in the descending pain modulatory system have been studied in detail and reviewed previously (Heinricher & Ingram 2008a, Heinricher & Ingram 2020, Lau & Vaughan 2014). In contrast to opioids, much less is known about cannabinoid regulation of this circuit. Early studies from the Hargreaves laboratory discovered that intrathecal administration of a CB1 inverse agonist SR 141716A produced hyperalgesia (increased pain sensitivity) when measuring thermal hot plate latencies in mice (Richardson et al 1997, Richardson et al 1998). These studies documented tonic release of endocannabinoids, at least in some areas of the CNS, that regulate thermal nociceptive thresholds. The tonic release of endocannabinoids under normal conditions is in stark contrast to the opioid system which is engaged following stress or threatening situations (Walker et al 1999). Although it is clear that cannabinoids are not as efficacious as opioids in reducing acute pain when administered directly into the PAG or RVM (Martin et al 1998), they appear to have similar or increased efficacy in chronic pain states (Donvito et al 2018, Woodhams et al 2017). Clinically, the use of chronic opioids lacks efficacy for the treatment of chronic pain (Krebs et al 2018) and while there is evidence that cannabinoids are more efficacious than opioids for the treatment of chronic pain (Cousijn et al 2018, Whiting et al 2015), a more thorough analysis of clinical impacts of cannabinoids on the multiple aspects of pain and pain syndromes must be conducted to determine efficacy and safety of targeting the cannabinoid system for analgesia.

Cannabinoids have an important role in regulating inflammatory processes in the periphery. Many studies, both *in vitro* and *in vivo*, have shown that CB1R and CB2R agonists, as well as FAAH and MAGL inhibitors, inhibit the development and maintenance of inflammation (for reviews, see (Donvito et al 2018, Guindon & Hohmann 2009). Agonist activation of either cannabinoid receptor inhibits edema associated with carrageenan and Complete Freund's Adjuvant (CFA) injections into rodent paws, and regulate the release of pro-inflammatory and anti-inflammatory cytokines (Cabral & Griffin-Thomas 2009). Although these peripheral effects of cannabinoids are important in the overall response to systemic administration of cannabinoid receptor agonists and other drugs that modulate the endocannabinoid system in inflammation, this thesis focuses on the role of cannabinoids in the descending pain modulatory circuit in the brain.

1.2 Descending pain modulatory system

The descending pain modulatory circuit is comprised of the ventrolateral periaqueductal gray (vlPAG) projections to the rostral ventromedial medulla (RVM) and their reciprocal connections with upstream cortical and subcortical brain areas and downstream spinal cord neurons, respectively. Activation of this system typically results in analgesia (Barbaro 1988, Fardin et al 1984, Heinricher & Ingram 2008b, Hosobuchi 1980, Mayer 1984). However, this circuit is subject to plasticity during pain states, and prolonged pain results in a switch in the output from the RVM from inhibition of pain to facilitation of pain (Burgess et al 2002, Carlson et al 2007, Cleary & Heinricher 2013, Kincaid et al 2006, Roberts et al 2009, Zhang et al 2009) indicating that the circuit can modulate pain bi-directionally (Carlson et al 2007, Cleary & Heinricher 2013).

Cannabinoid receptors within the descending pain modulatory pathway have been reviewed previously (Palazzo et al 2010).

Inputs and outputs of the descending pain modulatory pathway

The vlPAG is an integration center for the descending pain modulatory pathway, receiving inputs from a variety of cortical and subcortical brain regions (Heinricher & Ingram 2008b, Keay & Bandler 2001, Silva & McNaughton 2019a). The vlPAG receives inputs from regions that are targets of the ascending nociceptive fibers (Mantyh 1982), as well as regions associated with affective aspects of pain including the ventral tegmental area (VTA) (Breton et al 2019), prefrontal cortex (Floyd et al 2000), hypothalamus (Keay & Bandler 2001) and amygdala (Hopkins & Holstege 1978, Li & Sheets 2018).

Efferents of the vlPAG project to many brain regions including the VTA and substantia nigra (**Figure 1.5)** (Silva & McNaughton 2019a, Suckow et al 2013), but pertinent to the descending pain modulatory pathway is the dense projection to the RVM. The RVM also receives nociceptive transmission from the parabrachial complex (Chen et al 2017). The RVM sends a dense projection to the dorsal horn of the spinal cord (Francois et al 2017) and the trigeminal nucleus (Aicher et al 2012). While the PAG and RVM are involved in multiple brainstem circuits, their role in descending modulation of pain is well documented (Heinricher 2016).

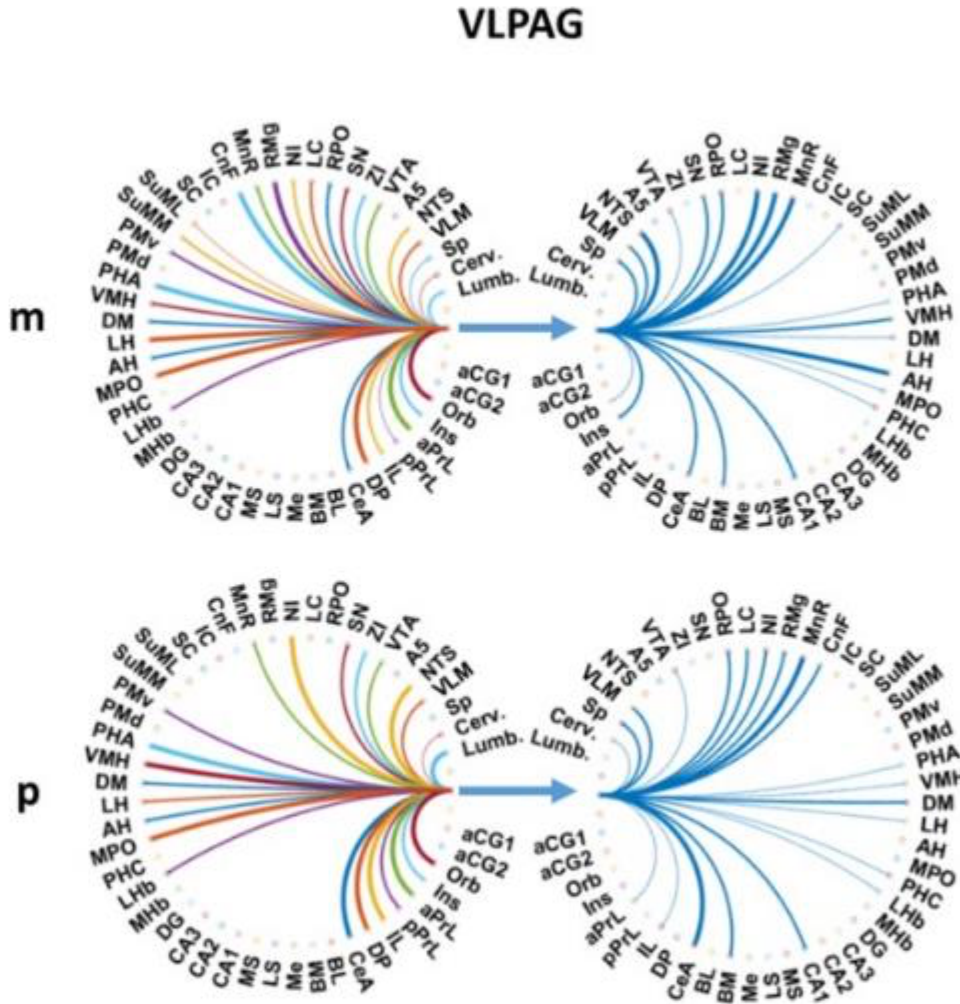

Figure 1.5 Inputs and outputs of the vlPAG

Illustrartion of the vast number of inputs and outputs of the vlPAG at the middle and posterior levels.

[Provided with permission from Elsevier]

Adapted from: Silva, C. and N. McNaughton (2019). "Are periaqueductal grey and dorsal raphe the foundation of appetitive and aversive control? A comprehensive review." Prog Neurobiol.

Periaqueductal Gray (PAG)

The PAG is a heterogeneous cell-dense structure that contains circuits that respond to threatening and stressful stimuli, as well as being involved in homeostatic control of feeding, lactation and respiration (Keay & Bandler 2001, Silva & McNaughton 2019a). The PAG surrounds the cerebral aqueduct and is organized in columns running in the rostral-caudal axis that serve different functions (Figure 1.6). Stimulation of the ventrolateral column produces opioid-mediated analgesia, as well as freezing and quiescent behaviors, whereas stimulation of the lateral column and more dorsal columns produce escape behaviors such as jumping and flight responses (Keay & Bandler 2001).

The disinhibition hypothesis of analgesia claims that PAG output neurons to the RVM are inhibited by GABA under normal conditions (Lau & Vaughan 2014). Removal of this inhibition, termed *disinhibition*, results in activation of the descending pain modulatory circuit and analgesia (reviewed in Heinricher & Ingram 2008b, Lau & Vaughan 2014). Microinjections of either glutamate receptor agonists or $GABA_A$ receptor antagonists into the PAG produce antinociception and support this hypothesis (Bobeck et al 2009, Moreau & Fields 1986, Morgan et al 2009, Tortorici & Morgan 2002). Opioids inhibit GABA release to disinhibit PAG output neurons to the RVM (Stiller et al 1996, Tortorici & Morgan 2002). Based on these results, a simple circuit where opioid inhibition of GABAergic interneurons in the PAG result in disinhibition of excitatory PAG output neurons has been proposed in many models of the descending pain modulatory circuit. However, there is substantial data that suggests that the PAG-RVM circuit is more complex. For example, the PAG receives GABAergic afferents from

several brain areas, including the amygdala (Li & Sheets 2018) and ventral tegmental area (VTA; Breton et al 2019) and we find that the VTA-PAG GABAergic projections are sensitive to opioid inhibition (Ingram lab, unpublished observations). In addition, opioid receptors are expressed on glutamatergic terminals in the PAG (Vaughan & Christie 1997b) and the physiological relevance of these actions are not clearly understood. Finally, both GABAergic and glutamatergic neurons project to RVM (Morgan et al 2008) and some of the GABAergic projection neurons are directly inhibited by opioids (Commons et al 2000, Osborne et al 1996). Thus, PAG to RVM circuitry is more complicated than simply disinhibition of excitatory descending projections and probably reflect the existence of parallel circuits that contribute to the bidirectional control of pain mediated by the RVM.

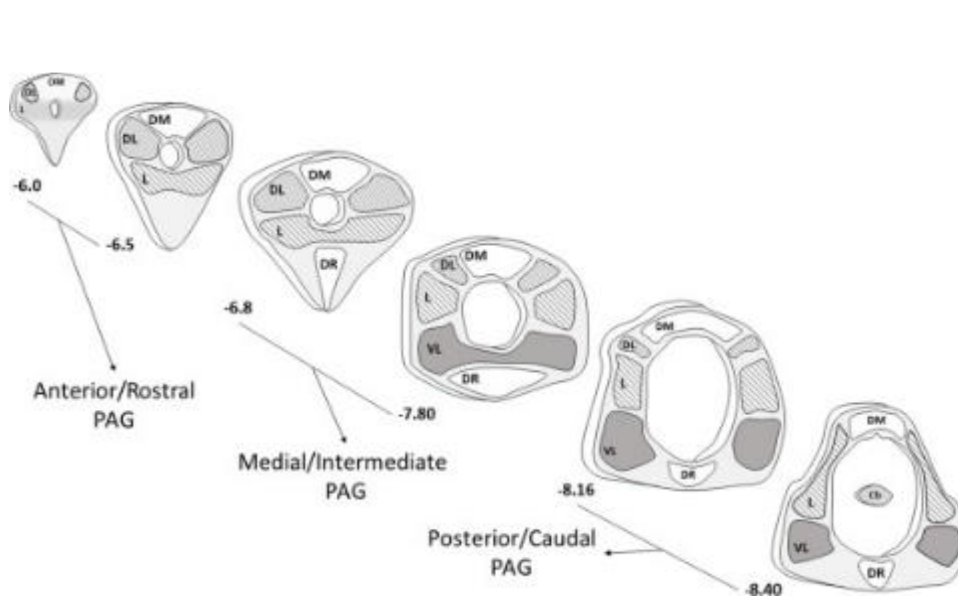

Figure 1.6 Columnar organization of the PAG

The PAG surrounds the aqueduct and is organized into distinct columns through the rostral-caudal axis. The vlPAG (VL) is located in the caudal portion, from -7.8 to -8.4 bregma.

[Provided with permission from Elsevier]

Adapted from: Silva, C. and N. McNaughton (2019). "Are periaqueductal grey and dorsal raphe the foundation of appetitive and aversive control? A comprehensive review." Prog Neurobiol.

The descending pain modulatory circuit is sexually dimorphic (Loyd et al 2007, Loyd & Murphy 2006, Loyd et al 2008b) whereby activation of the PAG results in differential behavioral responses to pain in males and females. The reasons for these differences probably reflect environmental, as well as biological factors. Additionally, GRs are expressed within the vlPAG (Mor & Keay 2013) and can be activated by chronic stress or systemic administration of glucocorticoids (Ho et al 2018). Stressors also impact eCB levels within the PAG. Chronic, homotypic stressors reliably increase 2-AG but reduce anandamide (AEA) levels (Dubreucq et al 2012) while foot shock increases both 2-AG and AEA (Hohmann et al 2005).

Rostral Ventromedial Medulla (RVM)

While the focus of this thesis is the vlPAG, a brief introduction to the RVM is important for understanding the descending pain modulatory pathway. The RVM receives a dense input from the PAG, as well as afferents from the hypothalamus, parabrachial nucleus, and a variety of other cortical and subcortical areas (Heinricher & Ingram 2008b). The RVM provides the main output from the descending pain modulatory circuit to the spinal cord (Fields et al 1995, Heinricher et al 2009). The RVM contains two types of neurons that respond to noxious stimuli; OFF-cells stop firing and ON-cells fire just prior to the behavioral response to a noxious stimulus. ON-cell firing promotes hyperalgesia (Heinricher & Neubert 2004, Neubert et al 2004). Opioids, but not cannabinoids, directly hyperpolarize RVM ON-cells (Vaughan et al 1999). ON-cells in the RVM express the mu opioid receptor (Barbaro et al 1986, Heinricher et al 1992) and it has become widely accepted that mu opioid receptor agonist sensitivity defines

this cell population (Phillips et al 2012, Porreca et al 2001). RVM OFF-cells pause firing in response to a nociceptive stimulus and, just prior to the behavioral, withdrawal from the stimulus (Fields et al 1983). Opioids and cannabinoids reduce the pause response from these cells and prolong the latency to withdraw from the stimulus (i.e., antinociception, (Heinricher et al 1994, Meng & Johansen 2004, Meng et al 1998)). The drugs elicit firing of OFF-cells by reducing GABAergic inputs to the cells. Interestingly, if OFF-cells are firing and do not pause, the behavioral output is analgesia, regardless of the activity of ON-cells. This fairly simple classification of neurons in the RVM is an interesting comparison to the heterogeneous cell populations in the PAG. While 'ON' and 'OFF' cells have been identified in the PAG *in vivo* (Heinricher et al 1987b), PAG neurons are fairly "quiet" with few basally active neurons and a low percentage of neurons that respond to noxious stimuli (Heinricher et al 1987a, Tryon et al 2016).

1.3 Mechanisms of cannabinoid action in the descending pain modulatory pathway

Cannabinoid signaling in the PAG

The PAG is an important region for the antinociceptive effects of cannabinoids (Lichtman et al 1996). Behavioral studies indicate that direct injection of cannabinoid agonists into the PAG produces mild hypoalgesia that is approximately 1/3 of an equi-potent dose of morphine (Palazzo et al 2001, Wilson-Poe et al 2013). PAG microinjections of cannabinoids also reduce hyperalgesia induced by formalin (de Novellis et al 2005, Finn et al 2003). These studies provide evidence that CB1 receptors

in the PAG can activate the descending pain modulatory circuit and reduce inflammatory hyperalgesia.

The CB1 receptor is highly expressed throughout both the dorsolateral and ventrolateral periaqueductal gray (Wilson-Poe et al 2012) and expression extends throughout the rostral and caudal PAG (Tsou et al 1998). Activation of CB1 receptors in the vlPAG produces antinociception and anti-hyperalgesia (Palazzo et al 2012) and have actions similar to opioids. CB1 receptors in the dorsolateral PAG mediate opioid-independent stress-induced analgesia (Hohmann et al 2005). Immunohistochemical evidence indicates that CB1 receptors may be expressed abundantly (50%) on the postsynaptic membrane within the PAG (Wilson-Poe et al 2012), although electrophysiological studies have not found evidence of postsynaptic function of CB1 receptors (Vaughan et al 2000). The function of these putative postsynaptic CB1 receptors is not known so they either couple to signaling pathways that do not alter electrophysiological properties of the neurons or they represent an artifact of antibody staining in the rat. Thus far, there is no evidence of CB2 receptor activity within the PAG under normal conditions (Ingram lab, unpublished observations).

Similar to the mu opioid receptor, cannabinoid receptors are expressed on both GABAergic and glutamatergic terminals within the lateral and ventrolateral PAG (Vaughan et al 2000) and inhibit the release of both neurotransmitters (Vaughan et al 2000). The signaling pathways involved in CB1-mediated inhibition of neurotransmitter release in the PAG are not currently known. **Figure 1.7** depicts a few of the known signaling pathways involved in inhibition of neurotransmitter release in other central synapses. However, CB1 receptor-mediated inhibition of GABA release in the vlPAG is

still occurs in the absence of Ca^{2+} and cannabinoid agonists do not block voltage-gated Ca^{2+} channels in isolated PAG neurons (Vaughan et al 2000). In addition, db-cAMP microinjections into the PAG do not reverse the analgesia produced by a CB1 receptor agonist (Lichtman, et al., 1996) indicating that CB1 receptors are not eliciting analgesia via inhibition of adenylyl cyclase. Another possibility is that CB1 receptors couple to phospholipase A2 and voltage-gated potassium channels in presynaptic terminals, since mu opioid receptors act via this pathway in vlPAG GABAergic terminals. Preliminary experiments show that alpha-dendrotoxin, an inhibitor of voltage-gated potassium channels diminishes, but does not abolish, the effects of the cannabinoid agonist WIN55212 (Ingram lab, unpublished observations) indicating that Kv channels may be a target of CB1 receptor activation but also hinting that there is a pathway that has not been identified in PAG terminals. This would be consistent with the lack of cross-tolerance between cannabinoids and opioids (Vigano et al 2005, Wilson et al 2008) so these two receptors likely use non-overlapping signaling pathway.

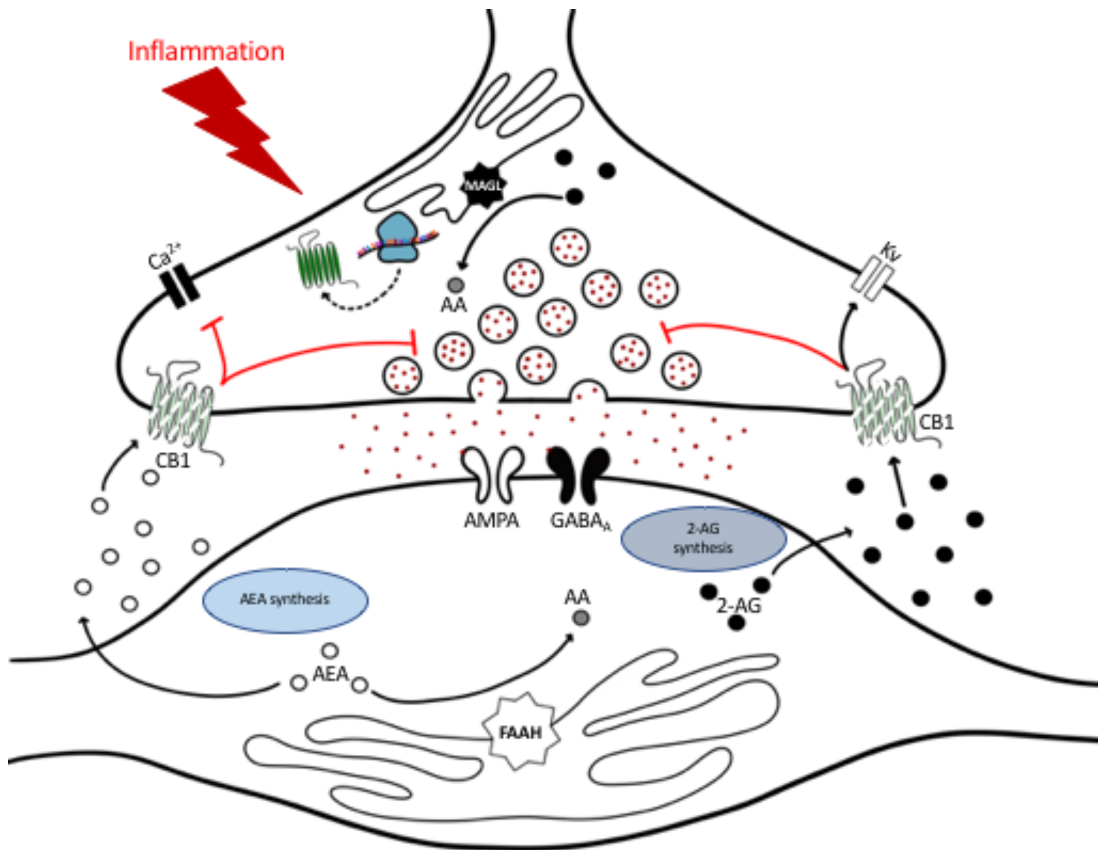

Figure 1.7 Inflammation induced adaptations in presynaptic terminals

Persistent inflammation increases the release of glutamate and GABA from presynaptic terminals. This is partially due to a loss of CB1 receptor-mediated inhibition of release. At 5-7 days post-CFA, there is decreased CB1 receptor protein without a change in CB1 or CB2 receptor mRNA, AEA or 2-AG levels. However, it is not known if changes occur at earlier time points after induction of inflammation.

CB1 receptor signaling regulates different modes of release from presynaptic terminals within the PAG. First, they reduce the probability of release, similar to the effects of opioids (Drew et al 2009, Drew et al 2008, Vaughan et al 2000). Second, cannabinoids shift the mode of GABA vesicle release from multivesicular to univesicular (Aubrey et al 2017). It is thought that the multivesicular release from GABAergic terminals in the vlPAG is critical for normal suppression of the descending analgesic circuit so tight regulation of release mode by endocannabinoids would allow for rapid responses to pain or stress.

Under stress or pain stimuli, enhanced release of glutamate activates mGluRs that stimulate production of endocannabinoids in the PAG (Drew et al 2009, Drew et al 2008, Drew & Vaughan 2004). Endocannabinoid synthesis is also stimulated by muscarinic M1 receptors (Lau & Vaughan 2008), as well as several neuropeptides (Substance P, neurotensin, CCK) that induce glutamate release in the PAG (Drew et al 2009, Drew et al 2005, Mitchell et al 2011, Mitchell et al 2009). Activation of these GPCRs could lead to retrograde inhibition of neurotransmitter release in the PAG. Increased endocannabinoid release has also been detected after chronic constriction injury (Petrosino et al 2007) and formalin injections (Walker et al 1999). Further, blockade of endocannabinoid hydrolysis by FAAH and MAGL in the PAG produces antinociception (Adamson Barnes et al 2016, Anderson et al 2014, Maione et al 2006) and enhances stress-induced analgesia (Hohmann et al 2005, Suplita et al 2005). Thus, endocannabinoid actions in the PAG are stimulated by multiple neurotransmitters and neuropeptides that are linked to modulation of nociceptive thresholds.

GPR55, the orphan cannabinoid receptor, has a controversial role in antinociception. GPR55 agonists reduce nociceptive thresholds when microinjected into the PAG (Deliu et al 2015) suggesting that GPR55 may have pro-nociceptive actions in the PAG. GPR55 knock-out mice display decreased mechanical hyperalgesia in inflammatory and neuropathic pain models (Staton et al 2008). However, in a different set of studies, there were no detectable differences in the development of pathological pain in chemical and neuropathic pain models (Carey et al 2017). These results indicate that studies of cannabinoid regulation of pain need to be interpreted with caution and use appropriate controls to rule out potential actions at GPR55.

Cannabinoid signaling in the RVM

In the RVM, acute inflammation or injury is associated with sustained activation of ON-cells and suppression of OFF-cell firing, leading to hyperalgesia (Cleary & Heinricher 2013, Kincaid et al 2006, Xu et al 2007). Lidocaine injections into the RVM inhibit ON-cell firing and hyperalgesia (Kincaid et al 2006) indicating that the RVM provides a pro-nociceptive output to the spinal cord under acute conditions. In contrast, in chronic pain models, ON- and OFF-cells in the RVM exhibit profoundly lowered thresholds, responding to innocuous as well as noxious peripheral stimulation (Carlson et al 2007, Cleary & Heinricher 2013). Interestingly, lidocaine block within the RVM under these conditions worsen hyperalgesia (Cleary & Heinricher 2013) indicating that output from the RVM is antinociceptive. This plasticity in the properties of RVM pain-modulating neurons are probably important in the transition from acute to chronic pain.

Cannabinoids microinjected into RVM, similar to results in the PAG, also produce a modest analgesic effect (Martin et al 1998) and potentiate the analgesic response to a

low dose of opioid (Wilson-Poe et al 2013). CB1 receptor agonists activate RVM OFF-cells during *in vivo* recordings (Meng & Johansen 2004, Meng et al 1998), consistent with their effects on behavior. In *ex vivo* slice recordings, CB1 receptor agonists inhibit GABA release in the RVM (Li et al 2015, Vaughan et al 1999) but it is not known if glutamate release in the RVM is inhibited by cannabinoids. It is also not currently known what role endocannabinoids play in the transition from antinociception to hyperalgesia after prolonged pain, although inflammation down-regulates CB1 receptors in the RVM (Li et al 2017) which will be discussed in more detail below. Given that this area is the key output node of the descending pain modulatory system, continued study of cannabinoid signaling in the RVM is important.

An interesting side note is that tonic endocannabinoid signaling is observed in adult RVM slices but not in early postnatal rats, indicating that the endocannabinoid system is developmentally regulated in this area (Kwok et al 2017, Li et al 2015). Thus, the cannabinoid system is developing, at least in some brain areas, during adolescence which potentially has important clinical implications considering the increasing prevalence of cannabis use in adolescents.

Phytocannabinoids in the descending pain modulatory pathway

Phytocannabinoids, or the cannabinoids derived from the plant *Cannabis sativa*, have different properties than synthetic cannabinoids such as WIN 55,212-2 or CP 55,940 that are used in the majority of the animal studies cited throughout this review. Tetrahydrocannabinol (THC) is a partial agonist (Felder et al 1995, Showalter et al

1996), whereas WIN 55,212-2 and CP 55,940 are both full agonists at the CB1 receptor (Felder et al 1995, Ross et al 1999, Shire et al 1996, Showalter et al 1996). Cannabidiol (CBD), a phytocannabinoid and constituent of *Cannabis sativa* that does not appreciably bind to CB1 receptors (Thomas et al 1998), appears to have antinociceptive properties (De Gregorio et al 2019). The effects of CBD on the descending pain modulatory pathway are largely unknown. CBD microinjections into the vlPAG decreased both RVM ON- and OFF-cell activity (Maione et al 2011) and produced antinociceptive effects. However, these results are not consistent with known actions of antinociceptive drugs on RVM neurons (Heinricher et al 1994). In addition, CBD produces anti-anxiogenic actions in the PAG that are probably mediated via 5-HT_{1A} receptors (Campos & Guimaraes 2008, Moreira et al 2009) so it is possible that CBD targets different brainstem descending circuits.

1.4 Synaptic plasticity with inflammation

Studies examining the analgesic actions of both opioids and cannabinoids in the descending pain modulatory circuit have largely focused on regulation of GABA release and $GABA_A$ receptors in the PAG and RVM. Blocking $GABA_A$ receptors in either the PAG or RVM elicits antinociception (Bobeck et al 2009, Gilbert & Franklin 2001, Moreau & Fields 1986, Tortorici & Vanegas 1994). In addition, chronic inflammation and neuropathic pain modulate GABA release in these areas, although both increases and decreases in GABA release have been reported (Chen et al 2013, Hahm et al 2011, Li et al 2017, Zhang et al 2011). The Ingram lab recently observed that GABA release was increased selectively in females in the presence of inflammation (Tonsfeldt et al 2016).

Because modulation of GABA release is important for activation of the descending pain modulatory circuit, sex-dependent adaptations contributing to increases in GABA release could elucidate cellular mechanisms leading to sex differences in chronic pain. These studies indicate that GABAergic transmission within the descending pain modulatory circuit can undergo compensatory changes in response to inflammation and neuropathic pain but highlight the complexity of these changes and the gaps in our knowledge.

There is plasticity in the cannabinoid system within the descending pain modulatory pathway, particularly with cannabinoid receptors whose expression and function change in response to various manipulations including persistent inflammation. The Ingram lab recently documented this plasticity in the RVM following CFA injections into the hind paw of rats and the development of persistent inflammation lasting 5-7 days. We observed a decrease in CB1 receptor-mediated inhibition of GABA release in the RVM of CFA-treated rats (Li et al 2017). Although there is evidence that CB1 receptors desensitize in response to exogenous cannabinoid administration (Lazenka et al 2014a, Mikasova et al 2008, Morgan et al 2014, Selley et al 2004) and upregulation of endocannabinoids (Imperatore et al 2015, Navia-Paldanius et al 2015), no changes in AEA or 2-AG levels at 5-7 days post-CFA were observed. However, inflammation led to down-regulation of CB1 receptor protein without a change in CB1 mRNA in the RVM (Li et al 2017) indicating that CB1 receptor translation or degradation are modulated by inflammation. These results are consistent with observed down-regulation of CB1 receptor protein (but not mRNA) in the PAG following the chronic constriction injury model of neuropathic pain (Palazzo et al 2012). CB1 receptor down-regulation has also

been documented following repeated exposure to cannabinoids (Breivogel et al 1999, Dudok et al 2015, Sim et al 1996).

Consistent with previous findings (Beltramo et al 2006, Burston et al 2013), CB2 receptors in the RVM are upregulated in persistent inflammation and CB2 receptor agonists inhibit presynaptic GABA release in the RVM of CFA-treated but not naïve rats (Li et al 2017). It is interesting that these receptors are upregulated and appear to function in a manner comparable to that of CB1 receptors in the region; however, there are many aspects of the CB2 receptor actions in the RVM that are not understood. For instance, it is not known what signals trigger CB2 receptor expression nor it is known where the CB2 receptors are localized. An intriguing possibility is that CB2 receptors are upregulated on microglia (Maresz et al 2005, Stella 2010) that are known to be activated in the descending pain modulatory pathway after inflammation (Doyle et al 2017). Low levels of mRNA and inadequate CB2 receptor antibodies have not allowed visualization of these receptors and we have been reliant on pharmacological tools to examine the functions of CB2R. There are also studies that support the idea that CB2 receptors are relevant targets for chronic pain therapeutics for inflammatory (Beltramo et al 2006, Burston et al 2013, Deng et al 2015, Guindon & Hohmann 2008a) and neuropathic (Guindon & Hohmann 2008a, Ibrahim et al 2003, Sagar et al 2005, Zhang et al 2003) pain. Taken together, these data indicate that drugs that selectively target CB1 receptors may not be clinically useful in some types of inflammatory and neuropathic pain. Conversely, cannabinoid agonists that bind to CB2 receptors may be beneficial for the treatment of inflammatory and neuropathic pain.

1.5 Clinical implications

Endocannabinoids and cannabinoid receptors are well-situated to regulate pain processing and modulation. Unfortunately, the ubiquitous expression of CB1 receptors and the enzymes that regulate endocannabinoid synthesis and degradation throughout the brain probably limits the therapeutic potential of pharmacological drugs that target these proteins exclusively. Another potential issue is that the endocannabinoids inhibit release of both glutamate and GABA, so it is difficult to predict how the drugs would alter any specific circuit. While there is little evidence of clinically-relevant acute or experimental analgesia in humans, there an ever-growing literature documenting evidence of pain management with cannabis and cannabinoids (Cousijn et al 2018, Hill 2015). The clinical data has been elegantly reviewed recently (Lotsch et al 2018, Woodhams et al 2017) and will not be discussed in detail here. Interestingly, studies predominately observe weak to no analgesic effects of cannabinoid agonists even though cannabinoids decrease functional connectivity of the "pain matrix" in functional magnetic resonance (fMRI) studies (Walter et al 2016). However, it should be noted that many of the fMRI studies have examined pain responses in healthy subjects to date, not subjects in chronic pain. The incongruency between pain relief in clinical studies and lack of reliable antinociception produced by cannabinoids emphasizes the multifaceted aspects of pain and that analgesia is only one aspect of clinical pain relief. Meta-analyses of clinical chronic pain studies show that the modest effects of cannabinoids may be a result of effects of cannabinoids on sleep and mood (Andreae et al 2015, Sharon & Brill 2019, Walitt et al 2016, Yanes et al 2019). Most studies conclude that

more double-blind, placebo-controlled research is needed to understand the utility of cannabinoid therapies for pain (Savage et al 2016).

One interesting strategy is to use cannabinoid therapies in conjunction with other analgesics, such as NSAIDs or opioids. There is a substantial preclinical literature on synergistic analgesia produced by FAAH inhibitors with morphine or other opioids for neuropathic pain (Casey et al 2017, Christie et al 1999, Kazantzis et al 2016). A clinical trial using a FAAH inhibitor for osteoarthritis in the knee found no significant benefit (Huggins et al 2012) but a recent study found that a patient with a FAAH mutation presented with higher AEA levels and insensitivity to pain (Habib et al 2019). CB2 receptor agonists are also synergistic with morphine in rodent models of acute and chronic inflammatory, post-operative, and neuropathic pain (Grenald et al 2017). These studies suggest that lower doses of opioids, when used in combination with cannabinoid agonists, can be used to effectively treat pain, decreasing frequency of opioid-induced side-effects. Indeed, chronic pain patients reliably reduce their opioid consumption by 40-50% when using adjunct cannabis (Boehnke et al 2016, Gruber et al 2016, Haroutounian et al 2016, Reiman et al 2017). The opioid-sparing effects alone may support the use of cannabinoid-based therapies. However, long-term clinical use of cannabinoid therapies is at the early stage of investigation and more clinical trials are necessary to fully evaluate the efficacy of this class of drugs.

This introduction was adapted from Bouchet CA, Ingram SL. 2020. Cannabinoids in the descending pain modulatory circuit: Role in inflammation. Pharmacol Ther: 107495

Chapter 2

Persistent inflammation promotes endocannabinoid release and presynaptic cannabinoid 1 receptor desensitization

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Summary

Pain therapies targeting the cannabinoid system are increasing with expansion of cannabis legalization but adaptations in the endogenous cannabinoid system during inflammatory pain could limit their efficacy. Presynaptic inhibition of GABA release mediated by cannabinoid 1 receptor (CB1R) agonists in the ventrolateral periaqueductal gray (vlPAG) is markedly reduced in male and female Sprague Dawley rats after persistent inflammation induced by Complete Freund's Adjuvant (CFA). Inflammation results in increased endocannabinoid (eCB) synthesis and desensitization of presynaptic CB1Rs that is reversed by a GRK2/3 inhibitor, Compound 101. Despite CB1R desensitization, eCB activation of CB1Rs is maintained after inflammation. Depolarization-induced suppression of inhibition (DSI) in naïve animals is rapid and transient, but is prolonged in recordings after inflammation. Prolonged DSI is mediated by 2-arachidonoylglycerol (2-AG) indicating reduced monoacylglycerol lipase (MAGL) activity. These adaptations within the endogenous cannabinoid system have important implications for the development of future pain therapies targeting CB1Rs or MAGL.

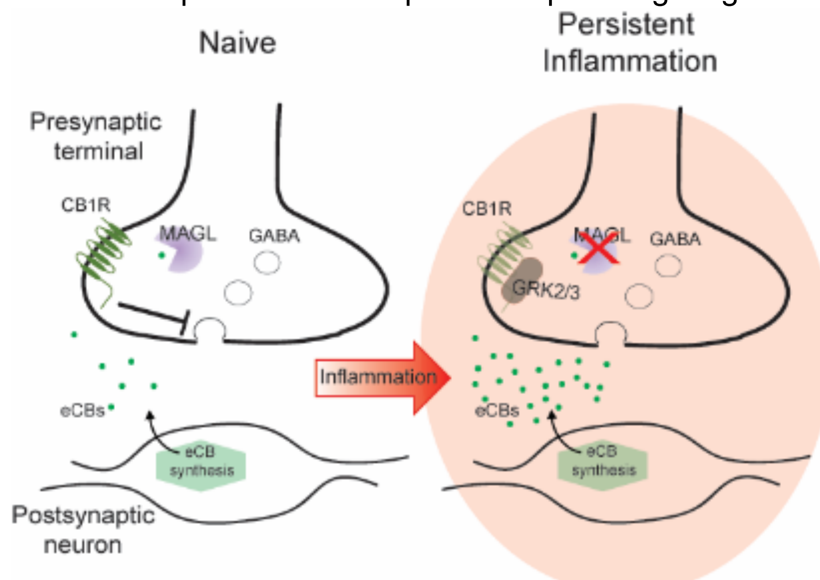

Figure 2.1: Graphical Abstract

Introduction

The cannabinoid 1 receptor (CB1R) is one of the most highly expressed GPCRs in the brain (Herkenham et al 1990) and is primarily localized to presynaptic terminals, where its activation inhibits neurotransmitter release (Katona et al 1999, Mikasova et al 2008, Vaughan & Christie 2005, Vaughan et al 2000). CB1Rs are activated by endogenous cannabinoid ligands, endocannabinoids (eCBs) that negatively regulate synaptic transmission through on-demand synthesis, retrograde transport and activation of CB1Rs. eCB activation of CB1Rs is tightly controlled by enzymes responsible for eCB on-demand synthesis and rapid degradation (for review see Ahn et al 2008). The two most well studied eCBs are 2-arachidonylglycerol (2-AG) and anandamide (AEA). In the brain, levels of 2-AG are more than 100 times higher than AEA (Stella et al 1997). 2-AG is regulated by the synthesis enzyme, diacylglycerol (DAGL; Bisogno et al 2003) and the catabolism enzyme, monoacylglycerol lipase (MAGL; Dinh et al 2002, Dinh et al 2004). Through this endogenous cannabinoid system, eCBs and the CB1R regulate neurotransmitter release from the presynaptic terminal.

Expression of eCBs and their degradation enzymes are altered by inflammation in several brain areas (Vecchiarelli et al 2021). Our prior study demonstrated a reduction in CB1R suppression of GABA release that was the result of reduced protein expression in the rostral ventromedial medulla (RVM) with persistent inflammation (Li et al 2017). The RVM is integral to descending pain modulation and, along with the ventrolateral periaqueductal gray (vlPAG), constitutes the descending pain modulatory pathway. Within the vlPAG, CB1Rs are densely expressed (Wilson-Poe et al 2012) and their activation modulates neurotransmitter release in naïve animals (Aubrey et al 2017,

Drew et al 2009, Lau et al 2014, Vaughan et al 2000, Wilson-Poe et al 2015), but adaptations in the cannabinoid system after persistent inflammation in the vlPAG are not understood. Therefore, we sought to investigate how persistent hindpaw inflammation impacts cannabinoid regulation of GABA release within the vlPAG.

The present results describe an inflammation-induced increase in eCB levels in the vlPAG, leading to desensitization of CB1Rs by a GRK2/3-dependent mechanism. While this desensitization is clearly observed with exogenous agonists, endogenous release of 2-AG continues to induce CB1R-dependent suppression of inhibition after inflammation. Compared to naïve, the eCB-induced suppression is prolonged after persistent inflammation. Together, results show a distinction between CB1R activation by exogenous and endogenous cannabinoid ligands, as well as alteration in the endogenous cannabinoid system in the vlPAG after persistent inflammation. These adaptations have important implications for future therapeutic drug development.

Results

Persistent inflammation reduces CB1R inhibition induced by exogenous agonists

Plasticity within the cannabinoid system induced by persistent inflammation in the vlPAG was examined following Complete Freund's Adjuvant (CFA) injection into the hindpaw of male and female Sprague Dawley rats. All experiments were conducted 5-7d after CFA injection. Whole-cell patch clamp recordings of electrically evoked inhibitory postsynaptic currents (eIPSCs) were used to measure GABA IPSCs and the inhibition of GABA release by the non-selective cannabinoid receptor agonist, WIN-55,212-2 (WIN; 3 μM). In tissue from naïve animals, WIN reduced eIPSC amplitudes by 57 ± 5% compared to baseline (Fig. 2.2 A,B). CFA-induced inflammation significantly

reduced WIN-mediated inhibition of eIPSCs to 18 ± 4% (Fig. 2.2 A,B). WIN inhibition was reversed by the CB1R selective antagonist, SR141716A (rimonabant, RIM; 3 μM). No sex differences were observed in WIN-mediated suppression of GABA release in recordings from either naïve or CFA-treated rats (Fig. 2.8), so data from male and female rats were combined for all analyses. There were no differences in baseline eIPSC paired pulse ratios (unpaired t-test, t_{13}=0.59; p=0.6) or decay kinetics (unpaired *t*-test, t_{11} = 1.0; p=0.3) between recordings from naïve and CFA-treated animals.

To determine whether inflammation also affects spontaneous GABA release and the inhibition of spontaneous release by CB1Rs, we measured miniature IPSCs (mIPSCs) in the presence of tetrodotoxin (TTX; 500 nM). WIN suppressed mIPSC frequency by 56 ± 5% in tissue from naïve animals and this effect was significantly reduced (14 ± 6%) after persistent inflammation (Fig. 2.2 C-F). Activating CB1Rs had no effect on mIPSC amplitude (One-way ANOVA: F(1.1, 5.5)=0.43; *p*=0.56), consistent with a presynaptic effect of CB1R activation.

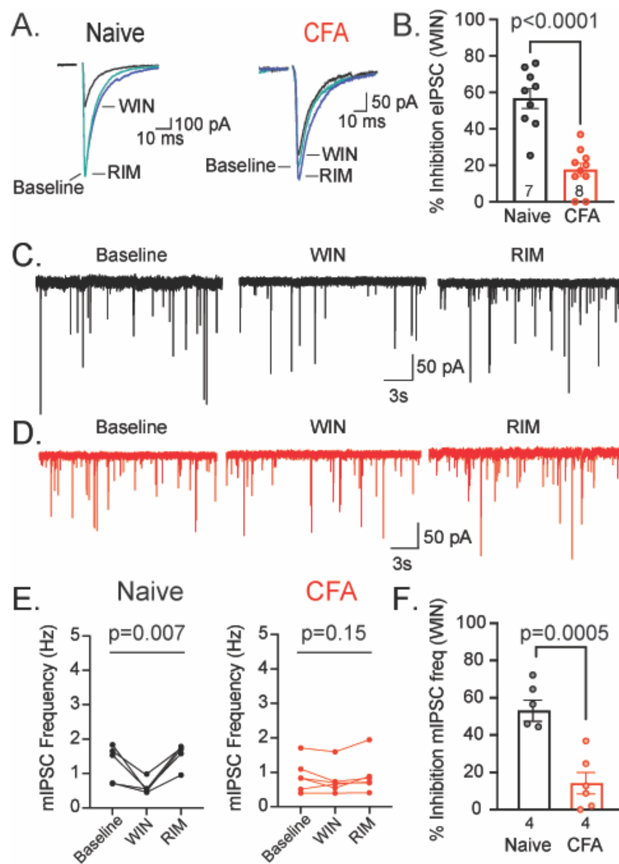

Figure 2.2: Persistent inflammation reduces WIN-induced inhibition of GABA release.

(A) Representative traces of eIPSCs isolated in NBQX recorded from vlPAG neurons in baseline (5µM; teal), the cannabinoid receptor agonist WIN,55212 (WIN 3 µM; black) and the CB1R selective antagonist rimonabant (RIM; 3 µM; blue) from naïve (left) and CFA-treated (right) animals. (B) Percent inhibition of eIPSC amplitude by WIN in slices from naïve (black bar) and CFA-treated rats (red bar) (unpaired t-test, t_{14}=5.34; p=0.0002). (C,D) Representative trace of mIPSCs recorded from vlPAG neurons in baseline containing TTX (500 nM) and NBQX (5 µM), WIN (3 µM), and RIM (3 µM) from slices of naïve (black, C) or CFA-treated rats (red, D). (E) mIPSC frequency at baseline, WIN, and RIM from slices of naïve (black) and CFA-treated (red) rats. (F) WIN percent inhibition of mIPSC frequency from naïve (black) and CFA-treated (red) rats (unpaired t-test, $t_{(10)}$=4.65; p=0.0009).

To determine whether the reduction in CB1R suppression of GABA release is due to a general change in presynaptic GPCR signaling or downstream signaling pathways, we investigated the effects of persistent inflammation on the cannabinoid 2 receptor (CB2R) and presynaptic μ-opioid receptor (MOR) inhibition of GABA release. The CB2R agonist, AM1241 (3 μM) did not affect mIPSC frequency in vlPAG slices from naïve animals (14 ± 4% inhibition; Fig. 2.3 A) and this was not changed after persistent inflammation (17 ± 10% inhibition; Figure 2.3 A,B; unpaired t-test, t_{15}=0.71; p=0.5). While CB2R activation does not affect GABA release within the vlPAG, MOR activation suppresses GABA release to a similar extent in both naïve and CFA-treated slices. The MOR selective agonist DAMGO (1 μM) inhibited eIPSCs to the same extent in slices from naïve and CFA-treated rats (Naïve: 69 ± 16%; CFA: 66 ± 23%; Fig. 2.3 C,D). DAMGO-induced suppression of mIPSC frequency was also unaffected by persistent inflammation (naïve: 64 ± 12%; CFA: 53 ± 18%; Fig. 2.3 E,F). Together, these data indicate that the effects of persistent inflammation are selective to the CB1R within the vlPAG.

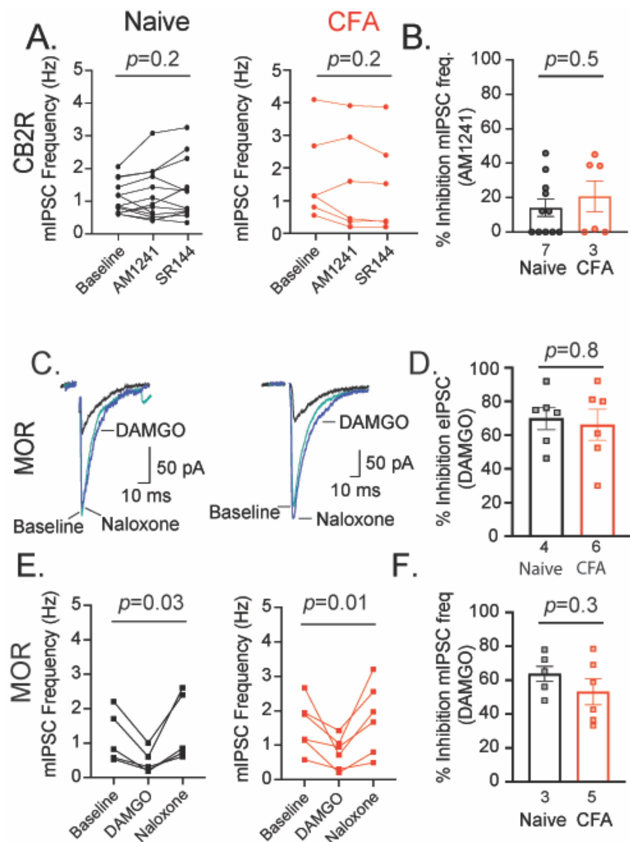

Figure 2.3: Persistent inflammation does not affect CB2R or MOR suppression of GABA release.

(A) spontaneous mIPSC frequency in slices from naïve (black) or CFA-treated (red) animals during baseline, CB2R agonist AM1241 (3 µM) and CB2R antagonist SR144528 (3 µM). (B) mIPSC frequency inhibition by AM1241 (unpaired t-test; t_{15}=0.71; p=0.5). (C) Representative eIPSC traces at baseline (5 µM; teal), DAMGO (1 µM; black) and naloxone (1µM; blue). (D) Percent inhibition of eIPSCs by DAMGO in naïve (black bar) and CFA-treated (red bar) conditions (unpaired t-test, t_{10}= 0.32; p=0.8). (E) spontaneous mIPSC frequency in slices from naïve (black) or CFA-treated (red) animals during baseline, DAMGO (1µM), and naloxone (1µM). (F) mIPSC frequency inhibition by DAMGO (unpaired t-test, t_9=1.11; p=0.3). Error bars represent SEM, dots indicate individual recordings and numbers represent the number of rats represented per bar.

Cannabinoid receptor expression is unchanged following persistent inflammation

Persistent inflammation downregulates CB1R protein in the RVM (Li et al 2017), so we hypothesized that persistent inflammation downregulates CB1R expression in the vlPAG as well. Expression levels were measured using radioligand binding with [^{3}H]CP-55,940. Since this is a different ligand than previously used, we first replicated our findings from Fig. 1 and found that CP-55,940 suppression of GABA release is significantly reduced after persistent inflammation (Naïve: 50 ± 5%, CFA: 4 ± 4%; Fig. 2.4 A,B). Radioligand binding was then carried out using [^{3}H]CP-55,940 in vlPAG dissected from naïve and CFA-treated. Surprisingly, there was no difference in total cannabinoid receptor binding (Fig. 2.4 C; Naïve B_{max}: 785 ± 61 fmol/mg; CFA $B_{max:}$ 708 ± 126 fmol/mg) or the dissociation constant (Naïve K_d = 1.8 ± 0.3 nmol; CFA K_d = 1.7 ± 0.4 nmol) in vlPAG tissue from naïve and CFA-treated animals. Similarly, persistent inflammation did not impact cannabinoid receptor binding in the dorsolateral striatum or hypothalamus (Fig. 2.9).

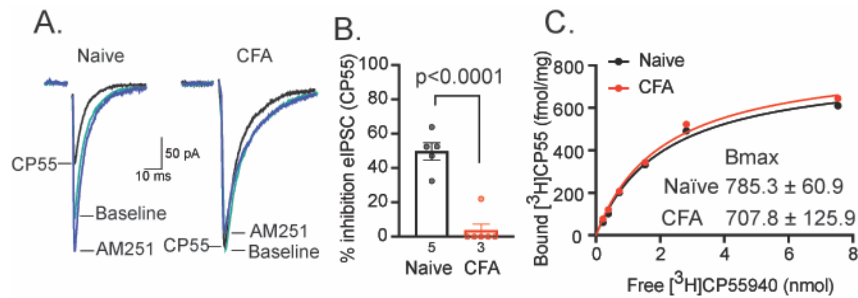

Figure 2.4: Cannabinoid receptor binding is unaffected by persistent inflammation.

(A) Representative traces of eIPSC recorded from vlPAG neurons in baseline containing NBQX (5μM; teal; Baseline), cannabinoid agoinst CP-55,940 (3μM; black; CP55), and CB1-selective antagonist AM251 (3μM; blue) from naïve and CFA-treated rats. (B) Percent inhibition of eIPSC amplitude by CP-55,940 in vlPAG slices from naïve (black bar) or CFA-treated (red bar) rats (unpaired t-test, t_9=7.8; p<0.0001). (C) Representative radioligand binding saturation curve with [^{3}H]CP-55,940 and vlPAG tissue from naïve (black) and CFA-treated (red) rats (vlPAG from 8 rats pooled per curve, statistics run on average of 3 curves). Error bars represent SEM, dots indicate individual recordings and numbers represent the number of rats per bar.

CB1Rs do not display acute desensitization to exogenous agonist

The observation that total cannabinoid receptor binding was unchanged in slices from CFA-treated rats suggested that CB1Rs might be desensitized with persistent inflammation. Similar to other presynaptic GPCRs, CB1Rs do not desensitize during 30 minutes of WIN (3 μM; Fig. 4A). To test whether CB1Rs in the vlPAG desensitize with multiple hours of agonist exposure, slices containing vlPAG were incubated in WIN (3 μM) for 90 minutes up to 5.5 hours and RIM was used to determine the extent of inhibition by WIN over time. RIM increased eIPSC amplitudes similarly after 15 minutes of WIN (275 ± 48%) or >1 hour of WIN (274 ± 92%; Fig. 4B,C). These results indicate that CB1Rs are resistant to desensitization, even after several hours of WIN exposure.

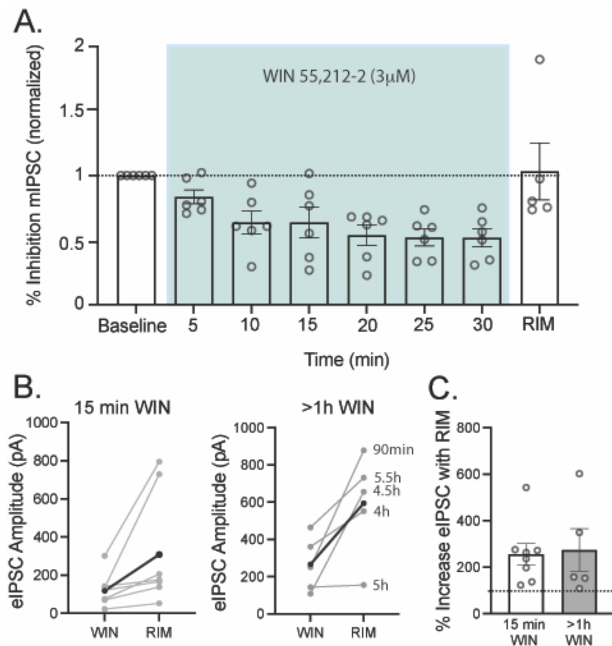

Figure 2.5: CB1R function is sustained throughout 5h WIN-induced activation

(A) Percent inhibition of mIPSC frequency in vlPAG neurons during 30 min of WIN exposure (3 μM; n=8). Data are normalized to mIPSC frequency during baseline in TTX (500 nM) and NBQX (5 μM). WIN (3 μM) reduced mIPSC frequency over the first 10 minutes of drug application. Frequency remained reduced for the entirety of the 30 min drug application and was reversed by RIM (3 μM; two-tailed paired t-test, t_7= 7; p=0.016). (B) eIPSC amplitude with bath application of CB1R selective antagonist RIM (3μM) after 15 minutes in WIN (3 μM; paired t-test, t_7=2.42; p=0.046; data from 6 animals) or >1h WIN incubation (3 μM; paired t-test, t_5=3.53; p=0.02; 5 cells from 3 animals). Average is shown in thick black. (C) Bar graph depicting RIM percent increase from WIN after 15 minutes in WIN (white bar) or >1 hour in WIN (gray bar; unpaired t-test, t_{11}=0.2; p=0.8). Error bars represent SEM, dots indicate individual neurons.

Persistent inflammation induces phosphorlylation dependent CB1R desensitization

Although CB1Rs are resistant to desensitization with acute agonist application over multiple hours (Fig. 2.5), it is possible that CB1R desensitization is induced by endogenous agonist(s) over the course of 5-7 days. A key step in canonical postsynaptic GPCR desensitization is G protein-coupled receptor kinase (GRK) phosphorylation of the GPCR C-terminal tail (Kovoor et al 1998, Lefkowitz 1993, Wang 2000, Zhang et al 1998). To block this step, we incubated slices in Compound 101 (Cmp101, 1 μM, ≥1 h), a potent and membrane permeable inhibitor of GRK 2/3 (Ikeda et al 2007, Thal et al 2011). Incubating slices in Cmp101 recovered CB1R suppression of GABA release after persistent inflammation (Fig. 2.6 A; 41 ± 5% inhibition compared to CFA vehicle: 9 ± 2% inhibition). This result indicates that persistent inflammation induces GRK2/3-dependent desensitization of the presynaptic CB1R. We also tested Cmp101 (30 μM) incubation on CB1R function (Adhikary et al 2022b, Leff et al 2020, Lowe et al 2015) and found that Cmp101 increased CB1R function in a concentration-dependent manner (30 μM incubation >1h, WIN inhibition 62 ± 10%). Interestingly, GRK2/3 desensitization after persistent inflammation appears to be selective to the CB1R as presynaptic MOR suppression of GABA release after Cmp101 incubation is not different between slices from either naïve or CFA-treated rats (30 μM; Fig. 2.6 B).

The next experiments examined the role of eCB levels on the desensitization of CB1Rs after inflammation. GABAergic IPSCs were evoked and RIM (3 μM) was applied to evaluate tonic activation of CB1Rs by eCBs. Consistent with previous findings in the vlPAG (Aubrey et al 2017), RIM did not consistently increase eIPSC amplitude in recordings from slices from naïve rats (paired t-test: baseline vs. RIM: t_{13}=1.54; p=0.15),

nor was there consistent eCB tone in slices from CFA-treated rats (paired t-test: baseline vs. RIM: t_{11}=1.13; p=0.28). Since inflammation induces CB1R desensitization, we hypothesized that eCB tone is masked by CB1R desensitization in rats treated with CFA. This was tested by incubating slices in Cmp101 prior to RIM superfusion. Cmp101 incubation did not reveal eCB tone in slices from naïve animals (Fig. 2.6 C; naïve drug free: 13 ± 5% inhibition; naïve Cmp101: 12 ± 6% inhibition) but revealed significant eCB tone in slices from CFA-treated animals, (Fig. 2.6 C; CFA drug free: 16 ± 6% inhibition; CFA Cmp101: 37 ± 6% inhibition). RIM is an inverse agonist, so we also tested eCB tone with the CB1R neutral antagonist, NESS 0327 (NESS; 0.5μM) to determine if the increased eCB tone resulted from constitutive activity of the CB1R (Ruiu et al 2003). After Cmp101 incubation, NESS revealed eCB inhibition (40 ± 8%; n=9) which was similar to that produced by RIM (30 ± 5%; n=5). Thus, constitutive activity of CB1Rs does not account for the effect of the inverse agonist, RIM. A closer analysis of eIPSC kinetics revealed a decrease in eIPSC decay in recordings from CFA-treated rats, consistent with eCB modulation of vesicle release mode, changing multi-vesicular release to univesicular release in the vlPAG (Aubrey et al 2017). Even in the absence of Cmp101, RIM significantly increased eIPSC decay time in vlPAG slices from CFA-treated rats while it has no impact on decay in slices from naïve rats (Fig. 2.6 D).

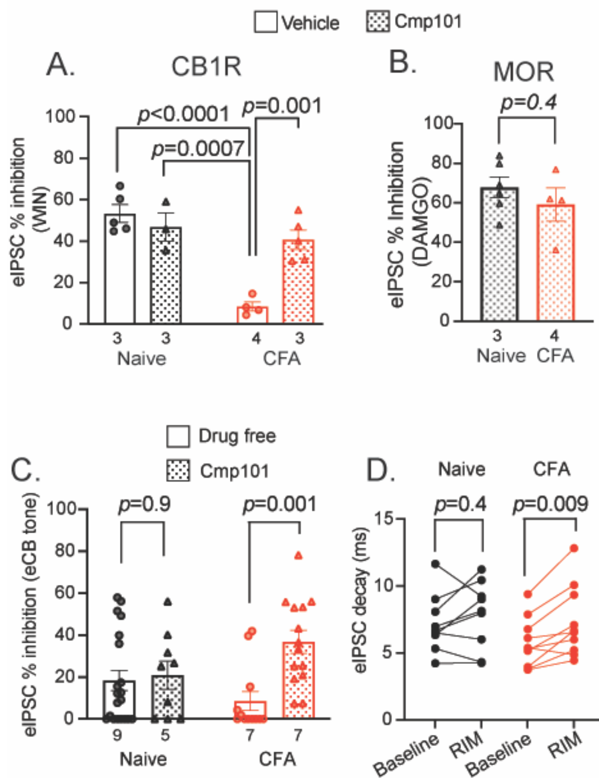

Figure 2.6: Compound 101 (Cmp101) incubation recovers CB1R inhibition of GABA release after persistent inflammation

(A) WIN (3 μM) inhibition of eIPSC amplitudes from naïve (black) or CFA-treated (red) rats. vlPAG slices were incubated in vehicle (no fill) or Cmp101 (filled bar) for >1h. Cmp101 incubation fully recovered CB1R signaling in slices from CFA-treated rats (2-way ANOVA: main effect of Cmp101: $F(1,13)=7.6$; $p=0.016$; main effect of CFA: $F(1,13)=29.9$; $p=0.0001$; CFA x Cmp101 interaction: $F(1,13)=17.29$, $p=0.001$). Post-hoc analysis (Tukey test) reveals the effect of WIN in CFA-treated slices incubated in vehicle was significantly reduced compared to all other conditions. (B) DAMGO (1 μM) inhibition of eIPSC amplitude after Cmp101 (30 μM) incubation from naïve (black bar) or CFA-treated (red bar) rats. (C) Cmp101 incubation reveals eCB tone in recordings from CFA-treated rats (2-way ANOVA: main effect of Cmp101: $F(1,46)=6.06$; $p=0.02$). Post-hoc analysis (Šidák's multiple comparisons test) reveals a significant effect of Cmp101 in CFA-treated rats but not naïve. RIM and NESS are combined. (D) eIPSC decay at baseline and after addition of RIM in slices from naïve (black) and CFA-treated (red)

rats (2-way ANOVA: main effect of RIM $F(1,17)=9.98$; $p=0.006$; Šídák post-hoc test). Error bars represent SEM, dots indicate individual neurons and numbers represent the number of animals per bar.

Persistent inflammation prolongs 2-AG signaling in the vlPAG

With evidence that eCBs change eIPSC decay kinetics in the absence of Cmp101 (Fig. 2.6 D), it appeared that eCBs activate CB1Rs even though the majority are desensitized after persistent inflammation (Fig. 2.6 A). We used depolarization-induced suppression of inhibition (DSI) to directly examine eCB activation of CB1Rs. The DSI protocol (+20mV for 5 seconds; Wamsteeker et al 2010) induced a rapid and transient suppression of presynaptic GABA release in a subset of PAG neurons (Fig. 2.7 A). This suppression was blocked by the CB1R antagonist NESS (0.5 μM; Fig. 2.7 A), indicating that DSI induces CB1R activation in the vlPAG. In slices from CFA-treated rats, we observed prolonged DSI (Fig. 2.7 B) that was also blocked by NESS (Fig. 2.7 B). The prolonged time course was analyzed by measuring the maximal percent inhibition immediately following depolarization (max DSI) and 30 s later (late DSI; Fig. 2.7 C). Max DSI in recordings from naïve rats is 41 ± 6% is similar to 37 ± 4% in recordings from CFA-treated rats. The eIPSCs from naïve slices return close to baseline (14 ± 5% inhibition) but did not recover in 30 s (37 ± 5%) in recordings from CFA treated rats (Fig. 2.7 C). In addition to prolonged DSI, the proportion of experiments that yield DSI after depolarization is significantly increased (Fig. 2.7 D) in recordings from CFA treated rats (16 out of 19 cells exhibited DSI) compared to recordings from naïve rats (10 out of 24 cells exhibited DSI). In the remaining neurons, the DSI protocol had no effect on eIPSC amplitude (Fig. 2.10 A).

DSI is dependent on 2-AG signaling in other brain regions and can be prolonged by inhibiting 2-AG degradation (Hashimotodani et al 2007, Straiker & Mackie 2005). To determine the effects of prolonging 2-AG levels in vlPAG, slices from naïve rats were

incubated in the 2-AG degradation inhibitor, JZL184 (1μM) for at least one hour (Lau et al 2014, Long et al 2009). Incubation with JZL184 prolonged DSI and recapitulated the DSI time course observed in recordings from CFA-treated rats (Fig. 2.7 E,F). Interestingly, JZL184 incubation did not change the proportion of cells that exhibit DSI (Fig. 2.10 B). DSI after CFA is completely blocked by inhibiting 2-AG synthesis with incubation in the DAGLα inhibitor, DO34 (Fig. 2.7 G; 1μM; >1h). Together, these data indicate that CFA-induced inflammation increases 2-AG levels in the vlPAG.

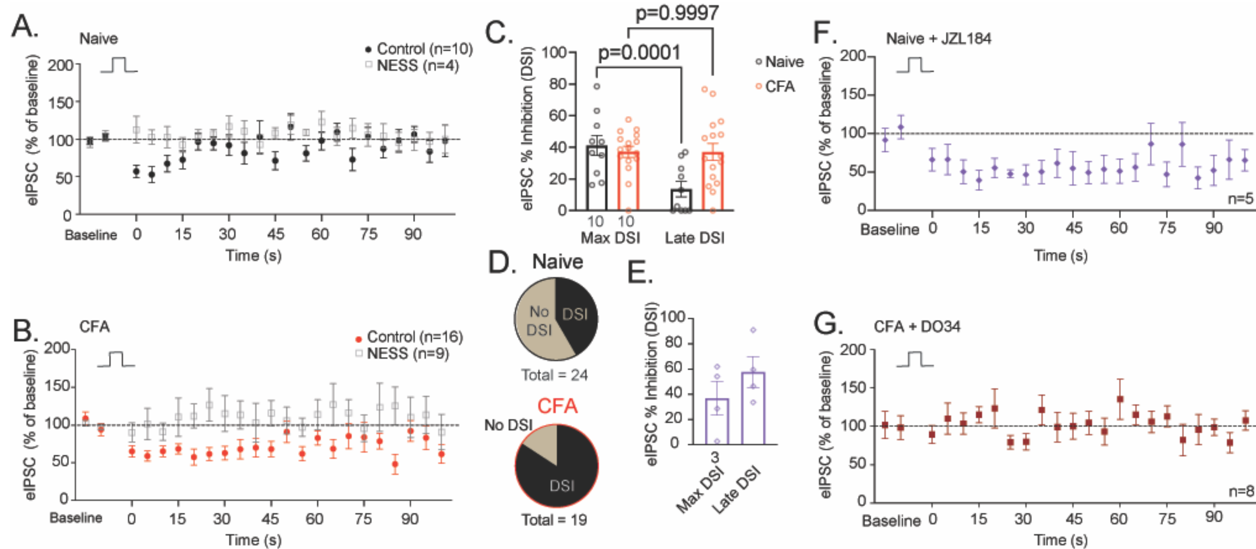

Figure 2.7: Persistent inflammation increases 2-AG activity at the CB1R.

(A) Summary of DSI (5s; +20 mV) in tissue from naïve rats (black circles; n=10 recordings from 10 rats). DSI is blocked by NESS (0.5μM; gray open boxes; n=4 recordings from 3 rats) (B) Summary of DSI in tissue from CFA-treated rats (red dots; n=16 recordings from 10 rats). DSI is blocked by NESS (0.5μM; gray open boxes; n=9 recordings from 5 rats). (C) Quantification of eIPSC % inhibition at max DSI and late DSI in vlPAG tissue from naïve (black) and CFA-treated (red) animals (2-way repeated-measures ANOVA: main effect DSI length: $F(1,24)=14.5$; $p=0.0009$; interaction DSI length x CFA: $F(1,24)=14.3$; $p=0.0009$; Šídák post-hoc test). (D) Proportion of patched neurons that respond to DSI. In slices from naïve rats, after depolarization 10 neurons exhibited DSI and 14 did not. In slices from CFA treated rats, 16 exhibited DSI and 3 did not. The proportion of neurons that produced DSI was significantly higher in slices from CFA-treated slices (Fishers exact test: $p=0.006$). (E) Quantification of eIPSC % inhibition at max DSI and late DSI in vlPAG tissue from naïve animals incubated in MAGL inhibitor JZL184 (1 μM, >1h). (F) Summary of DSI in tissue from naïve rats after incubation in JZL184 (1 μM, >1h). (G) Summary of DSI in tissue from CFA-treated rats incubated in DAGLα inhibitor, DO34 (1 μM incubation; >1h). Dots represent individual recordings, numbers below the bar represent number of animals; error bars represent SEM.

Discussion

Here, we describe a mechanism by which persistent inflammation induces adaptations in the endogenous cannabinoid system. Inflammation promotes desensitization of presynaptic CB1Rs that suppress GABA release in the vlPAG. This desensitization is dependent on CB1R and GRK2/3 activity and is recovered in the presence of the GRK2/3 inhibitor, Cmp101. Cmp101 also reveals an underlying increase in tonic activation of CB1Rs by eCBs 5-7 days after CFA injection. Despite these adaptations, desensitization does not affect maximal CB1R activation by eCBs, but actually prolongs CB1R activation by depolarization-induced eCB release in CFA-treated rats. These data have important implications for the development of pharmaceuticals targeting the cannabinoid system for inflammatory diseases.

Our data show direct evidence of GRK2/3-dependent desensitization of presynaptic CB1Rs. While postsynaptic GPCRs readily desensitize and internalize in response to agonist exposure (Williams et al 2013), it is well established that presynaptic GPCRs are resistant to desensitization (Fyfe et al 2010, Pennock et al 2012, Pennock & Hentges 2011, Pennock & Hentges 2016, Wetherington & Lambert 2002). Sustained signaling from presynaptic receptors during prolonged agonist exposure may be due to multiple mechanisms. One such mechanism involves protein-protein interactions with presynaptic scaffold proteins that immobilize the receptors close to the plasma membrane, as observed for presynaptic $GABA_B$ receptors (Boudin et al 2000, Laviv et al 2011, Vargas et al 2008, Vigot et al 2006). An alternative mechanism, observed for presynaptic MORs, describes presynaptic GPCRs internalizing into endosomes in response to agonist stimulation, but maintaining

signaling through rapid receptor replacement by a pool of receptors that diffuse laterally through axon membranes (Jullie et al 2020). Both mechanisms result in sustained GPCR signaling from presynaptic terminals. CB1Rs exhibit rapid mobility through the synapse under basal conditions; however, in contrast to MOR regulation, prolonged agonist exposure significantly reduces CB1R mobility and expression of CB1Rs in the synapse (Mikasova et al 2008). We demonstrated that CB1Rs are also resistant to desensitization under normal conditions but are desensitized after persistent inflammation, in contrast to presynaptic MORs which were unaffected by inflammation. Differences in CB1R and MOR regulation and mobility could underly their differential desensitization after persistent inflammation.

CB1R desensitization in response to prolonged administration of exogenous agonists, such as tetrahydrocannabinol (Δ^9-THC) or WIN, has been reported by many groups (Breivogel et al 1999, Kouznetsova et al 2002, Lazenka et al 2014b, Rubino et al 2000, Sim et al 1996). Long-term increases in eCBs also induce CB1R desensitization (Imperatore et al 2015, Kinsey et al 2013, Long et al 2009, Navia-Paldanius et al 2015, Schlosburg et al 2010). eCB levels in the PAG are increased almost immediately after acute inflammation induced by formalin injection into the hindpaw (Walker et al 1999) as well as after 7 days of chronic constriction injury, a model of neuropathic pain (Petrosino et al 2007). The observed CB1R desensitization in our study is likely a result of increased CB1R-induced G protein signaling within the vlPAG early in inflammation (Wilson-Poe et al 2021).

We observed prolonged DSI after persistent inflammation, which is consistent with the time course in other studies pharmacologically or genetically inhibiting MAGL

(Chen et al 2016, Pan et al 2009, Schlosburg et al 2010, Straiker & Mackie 2005). We show that the prolonged inhibition of GABAergic IPSCs following DSI in slices from CFA-treated rats is blocked by inhibiting DAGLα, the enzyme responsible for 2-AG synthesis, implicating 2-AG in the adaptations induced by CFA in the vlPAG. The prolonged time course could be the result of increased synthesis or decreased activity or levels of the degradation enzyme, MAGL. Since we observe a comparable maximal effect of DSI in recordings from both naïve and CFA-treated rats, suggesting comparable levels of 2-AG synthesis, we hypothesize that MAGL activity is diminished following persistent inflammation. Under normal conditions in the vlPAG, MAGL catabolizes 2-AG quickly enough that washing 2-AG over the slice does not suppress GABA release unless MAGL is blocked (Lau et al 2014). Consistent with this interpretation, experiments using MAGL knockout mice or pharmacological inhibition of MAGL show increases in 2-AG signaling that lead to CB1R desensitization (Imperatore et al 2015, Kinsey et al 2013, Long et al 2009, Navia-Paldanius et al 2015, Schlosburg et al 2010). However, if alterations in MAGL degradation of 2-AG are the sole mechanism underlying these adaptations in CFA-treated rats, we expected inhibiting MAGL activity with JZL184 would also increase the proportion of neurons in naïve rats that displayed DSI. This was not the case suggesting that CFA treatment may also affect synthesis in neurons that do not readily display DSI or diffusion of eCBs within the vlPAG. Therefore, reductions in MAGL degradation of 2-AG play a role but other mechanisms may be also involved in inflammation-induced adaptations in the cannabinoid system.

These results also highlight differences in signaling between exogenous and endogenous cannabinoids following persistent inflammation. Desensitization of CB1Rs clearly diminishes effects of exogenous cannabinoid agonists but eCBs continue to activate CB1Rs and induce prolonged suppression of GABA release, even though the majority of CB1Rs are desensitized. Similar reductions in exogenous but not endogenous ligand-mediated CB1R suppression of GABA release have been observed after chronic stress paradigms (Patel et al 2009). Importantly, this indicates that eCBs synthesized through DSI protocols are coupled more effectively to effectors and may indicate spare receptors in synapses. Alternatively, eCBs target different signaling pathways. Further studies are necessary to understand the consequences of long-term alterations in eCB synthesis and CB1R desensitization.

Physiological Relevance

Direct microinjections of cannabinoid agonists into the PAG induce antinociception (Lichtman et al 1996, Martin et al 1995, Wilson et al 2008, Wilson-Poe et al 2013) through activation of CB1Rs that inhibit GABA release in the PAG (Vaughan et al 2000). Recent work has highlighted MAGL inhibitors as analgesic therapeutic options (Anderson et al 2014, Curry et al 2018, Della Pietra et al 2021, Diester et al 2021, Ignatowska-Jankowska et al 2015) but the data presented here suggest that MAGL inhibition may not be a viable strategy if inflammation impairs MAGL function and desensitizes CB1Rs. However, systemic administration of MAGL inhibitors, as well as fatty acid hydrolase (FAAH) inhibitors and combinations of the two, increase levels of the eCBs 2-AG and anadamide and result in anti-hyperalgesia in both neuropathic and inflammatory pain models (Anderson et al 2014, Jayamanne et al 2006, Mitchell et al

2005). In addition, the anti-hyperalgesic effects of systemic cannabinoid agonist, Δ^9-THC (Craft et al 2013, Smith et al 1998, Sofia et al 1973), and WIN (Bridges et al 2001, Herzberg et al 1997, Li et al 1999) are not altered in similar inflammatory or neuropathic pain models, suggesting either that the reduced functional CB1Rs in the vlPAG are sufficient for cannabinoid-induced analgesia or that CB1Rs in the vlPAG are not required. One intriguing possibility is that inflammation-induced increases in 2-AG contribute to hyperalgesia and CB1R desensitization is a compensatory response that protects synapses. Indeed, there is precedent for cannabinoids to contribute to hyperalgesia (Dunford et al 2021, Khasabova et al 2022). Understanding the behavioral consequences of this altered cannabinoid signaling within the vlPAG after persistent inflammation, the generalizability to other brain areas, and the reversibility of this process have important implications for future drug development.

Materials and Methods

Animals

Adult male and female Sprague Dawley rats (Harlan Laboratories and bred in-house; postnatal day 30-90) were used for all experiments. All procedures were performed in strict accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted by the Institutional Animal Care and Use Committee of Oregon Health & Science University. Care was taken to minimize discomfort.

Inflammation

Complete Freund's Adjuvant (CFA: heat-killed *Mycobacterium tuberculosis* in mineral oil, 1 mg/ml, 0.1 ml volume injected, Sigma-Aldrich) was injected subcutaneously into the plantar surface of the right hindpaw. The CFA injection produces an intense tissue inflammation of the hindpaw characterized by erythema, edema, and hyperalgesia (Iadarola et al 1988). Electrophysiological recordings and tissue dissections were performed 5-7d following CFA injection.

vlPAG slice preparation

vlPAG slices were prepared as previously described (Tonsfeldt et al 2016). Rats were deeply anesthetized with isofluorane and the brain was rapidly removed and placed in ice-cold sucrose-based cutting buffer containing the following (in mM): 75 NaCl, 2.5 KCl, 0.1 $CaCl_2$, 6 $MgSO_4$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 dextrose, 80 sucrose. Ventrolateral PAG (vlPAG) slices were cut to a thickness of 220 µm on a vibrotome (Leica Microsystems) in sucrose-based cutting buffer and transferred to a holding chamber with aCSF containing the following (in mM): 126 NaCl, 21.4 $NaHCO_3$, 22 dextrose, 2.5 KCl, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4 and the osmolarity was adjusted to 300-310 mOsm. Slices were maintained with 95% O_2- and 5% CO_2-oxygenated until transfer to a recording chamber on an Olympus BX51WI upright microscope and superfused with aCSF maintained at 32°C.

Whole-cell patch-clamp recordings

Voltage-clamp recordings (holding potential -70 mV) were made in whole-cell configuration using an Axopatch 200B amplifier (Molecular Devices). Patch-clamp

electrodes were pulled from boroxilicate glass (1.5 mm diameter; WPI) on a two-stage puller (PP83, Narishige). Pipettes had a resistance of 2.5-5 MΩ. IPSCs were recorded in an intracellular pipette solution containing the following (in mM): 140 CsCl, 10 HEPES, 4 MgATP, 3 NaGTP, 1 EGTA, 1 $MgCl_2$, 0.3 $CaCl_2$, pH adjusted to 7.3 with CsOH, 290-300 mOsm. QX314 (100μM) was added to the internal solution for eIPSC experiments to reduce action potentials in the recording cell. Access resistance was continuously monitored. Recordings in which access resistance changed by >20% during the experiment were excluded from data analysis. A junction potential of -5mV was corrected during recording. GABAergic events were isolated in the presence of glutamate receptor antagonist NBQX (5 μM). Spontaneous miniature IPSCs (mIPSCs) were recorded in the presence of TTX (500 nM). Events were low-pass filtered at 2 kHz and sampled at 10-20 kHz for off-line analysis (Axograph 1.7.6) and individual events were visually confirmed. In experiments using exogenous cannabinoid agnoists, one neuron was recorded per slice due to the lipophilic nature of cannabinoid receptor drugs. After each experiment with exogenous cannabinoid agonists or antagonists, lines were washed with 50% EtOH. Each set of experiments was repeated using at least 3 distinct rats with no more than 2 cells from a single rat included in a specific dataset.

Drugs

WIN55,212-2 (Caymen Chemicals), SR141716A (rimonabant; RIM; Caymen Chemical), and NESS (Tocris) were dissolved in DMSO, aliquoted, and stored at -20°C. CP55,940 and AM251 (Caymen Chemical Company) were dissolved in methanol and stored at -20°C. DMSO and methanol at appropriate concentrations were used as vehicle

controls. 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline (NBQX; (Sheardown et al 1990)), [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO), Naloxone and tetrodotoxin (TTX) were purchased from Abcam, dissolved in distilled water, and stored at 4°C. CMP101 (3- [(4-methyl-5- pyridin-4-yl-1,2,4-triazol-3-yl)methylamino]-N-[[2-(trifluoromethyl) phenyl]methyl]benzamide hydrochloride) was purchased from Hello Bio and prepared as described previously (Leff et al 2020). Briefly, Cmp101 (made fresh daily) was first dissolved in a small amount of DMSO (10% of final volume), sonicated, then brought to its final volume with 20% (2-Hydroxypropyl)-b-cyclo-dextrin (HPCD; Sigma-Aldrich) and sonicated again to create a 10mM solution. For experiments using a higher concentration of Cmp101, Cmp101 was applied to the slice as follows: 30μM incubation for >1h, 1μM maintenance while patching, 10μM in drug tubes (Adhikary et al 2022b, Leff et al 2020, Lowe et al 2015). For experiments using a lower concentration of Cmp101, [1μM] was used for incubation (>1h), maintenance while patching, and in drug tubes. DMSO and 20% HPCD were used as the vehicle control.

Radioligand Binding Assay – tissue dissection

Rats were deeply anesthetized with isoflurane, brains were removed and submerged in ice cold Tris-HCl buffer (pH=7.4 at 4°C). Over ice, the brain was sectioned into 1mm slices from which the vlPAG, DLS and hypothalamus were dissected and immediately flash frozen on dry ice. Tissue samples were stored at -80°C.

Radioligand Binding Assay- total particulate tissue preparation

Tissue preparation was adapted from (Eastwood et al 2018). Since brain regions sampled are so small, tissue from each brain region from multiple animals (8 vlPAG, 2

DLS, 2 hypothalamus) was pooled to ensure ample protein levels for saturation binding. Tissue was removed from -80°C and transferred to 2 ml tube containing 0.5 ml Tris-HCl (pH 7.4 at 4C) with protease inhibitor (EMD Millipore; protease inhibitor cocktail set #539134). Tissue was homogenized with a polytron PT1200E 4 x 6s, placing sample on ice for 20s between homogenizations. The polytron was washed with water between each sample. The volume was increased to 1.5ml, then the sample was transferred to a mini-centrifuge and spun at 13,000 x g for 20 min at 4°C. The supernatant was discarded and pellet was resuspended in 0.5ml Tris-HCl with protease inhibitor. Tissue was homogenized for 7s and spun as described above once more. After the final spin, the supernatant was discarded, the pellet was resuspended in TME Binding Buffer (200 mM Tris Base, 50mM $MgCl_2$, 10mM EDTA, pH=8.0) with protease inhibitor and homogenized for 10s. TME with protease inhibitor was added for a final volume of 1.5ml. Samples were kept on ice throughout the preparation. Protein levels were determined with the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA).

Radioligand Binding Assay- Saturation Curve

Binding assays were conducted in the absence of Na^+. [^{3}H]CP-55,940 was used to measure cannabinoid receptor binding (Catani & Gasperi 2016, Chanda et al 2010, Freels et al 2020, Hill et al 2008a, McLaughlin et al 2013, Romero et al 1995). Binding assays were conducted using 5 concentrations [^{3}H]CP-55,940 (0.1-7nM) in a final volume of 1 ml. Assays were performed in duplicate in a 96-well plate with 50 mM TME binding buffer with bovine serum albumin (BSA; 1mg/ml; pH 7.4 at 30°C). Nonspecific binding was subtracted from total binding to yield specific binding. Nonspecific binding was determined with 1μM WIN55,212-2 and was 59%, 19%, or 55% in naïve and 53%,

17%, or 55% in CFA in vlPAG, DLS, and hypothalamus, respectively. Prepared membranes were incubated with [^{3}H]CP-55,940 at 30°C for 60 min. The incubation was terminated using a Tomtec cell harvester (Hamden, CT) by rapid filtration through Perkin Elmer Filtermat A filters presoaked in 0.2% polyethylenimine. The filters were dried, spotted with scintillation cocktail, and radioactivity was determined using a Perkin Elmer microBetaplate 1405 scintillation counter.

Data Analysis

In all electrophysiological experiments, each dataset included recordings from at least 3 rats. For DSI experiments, “Max DSI” averaged the first 4 eIPSCs after depolarization and “Late DSI” averaged eIPSCs 30-45 seconds after depolarization. In radioligand binding experiments, 3 replicates per group were run. All analysis were conducted in Graphpad Prism 9 (Prism version 9.2; San Diego, CA). Values are presented as mean ± SE and all data points are shown in bar graphs to illustrate variability. Statistical comparisons were made using two-tailed paired or unpaired T-test, one-way ANOVA, or two-way ANOVA when appropriate. In all summary bar graphs, each dot represents an individual cell while the numbers in the bars represent the animal number. When post-hoc analysis was appropriate Tukey test and Šidák’s multiple comparisons tests were used. $P < 0.05$ was used.

Acknowledgments

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Author Contributions

C.A.B. performed experiments and C.A.B. and S.L.I. conceived of the experiments, analyzed the data and wrote the manuscript. A.J. provided essential reagents, equipment, and helped with analysis of radioligand binding assays.

Declaration of interests

The authors declare no competing interests.

Supplemental Data

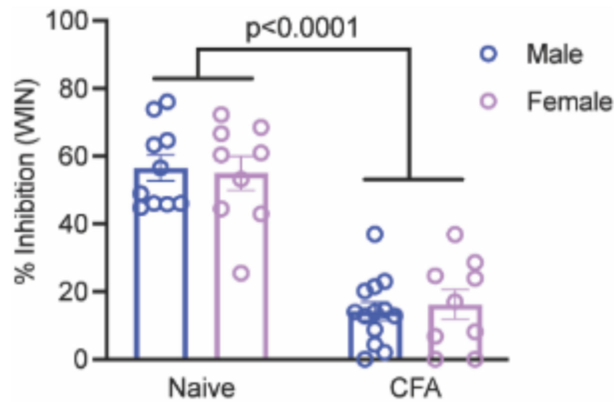

Figure 2.8 (Supplement) No sex differences in WIN suppression of GABA release

WIN inhibition of all eIPSCs (Fig. 1), mIPSCs (Fig. 2), and vehicle eIPSCs (Fig. 5) were pooled and separated by sex. 2-way ANOVA reveals a significant main effect of CFA ($F_{(1,37)}$=107; p<0.0001) and no sex difference (p=0.96). Dots represent individual recordings, error bars represent SEM.

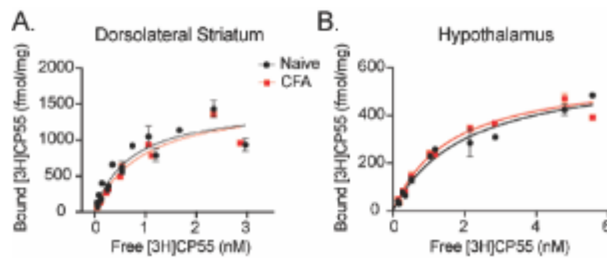

Figure 2.9 (Supplement) Cannabinoid receptor binding in DLS and hypothalamus

[^{3}H]CP-55,940 binding in the (A) dorsolateral striatum and (B) hypothalamus dissected from brains of naïve (black) and CFA-treated (red) rats.

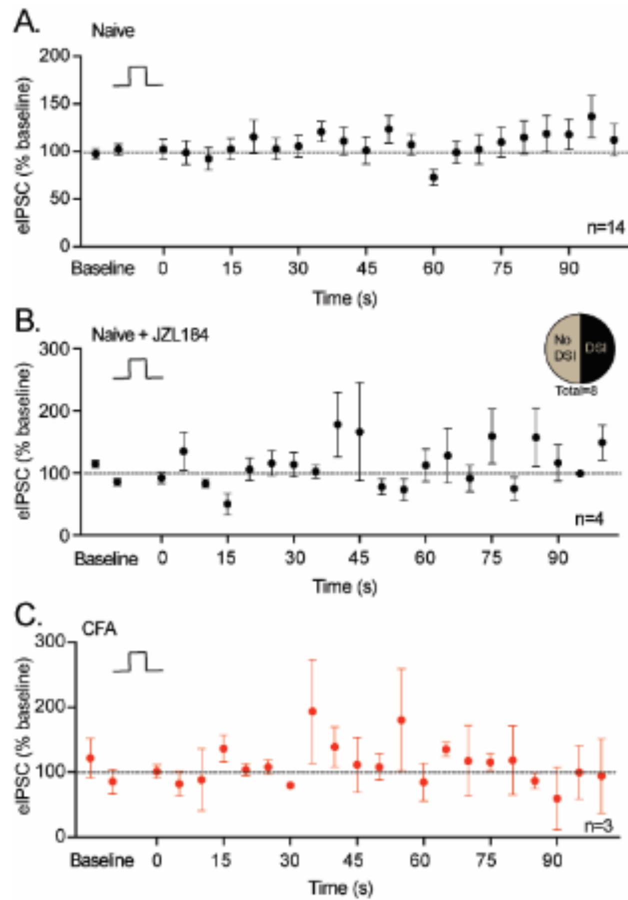

Figure 2.10 (Supplement) Subset of neurons do not exhibit DSI

Summary of traces from naïve and CFA-treated slices where depolarization (5s; +25mV) did not induce suppression of inhibition. (A) Naïve; (B) Naïve incubated in the 2-AG degradation inhibition, JZL184 (1μM; >1h); out of 8 recordings 4 had DSI and 4 did not (inset). (C) CFA-treated animal. Error bars represent

Chapter 3

The role of corticosterone in cannabinoid 1 receptor adaptations to inflammation

Abstract

Corticosterone has been identified as a critical signaling molecule that translates stressful experiences to cannabinoid signaling within the brain. Our previous study determined that persistent inflammation induces desensitization of CB1Rs, but the underlying mechanism is not understood. Here, we examine the possibility that inflammation-induced release of corticosterone and glucocorticoid receptor (GR)-mediated potentiation of 2-Arachidonoylglycerol (2-AG) synthesis desensitizes CB1Rs. We find sex differences in CB1R suppression of GABA release 24 hours after inflammation induced by Complete Freund's Adjuvant (CFA) injection into the hindpaw. Trunk blood corticosterone measurements show that circulating corticosterone levels are elevated in females and increased by CFA-induced inflammation. Corticosterone activation of GRs inhibit GABA release through a CB1R-dependent mechanism in the vlPAG. The mixed GR/PR antagonist, RU486, induces partial recovery of CB1R suppression of GABA release in slices from CFA-treated rats. Together, these data identify corticosterone release as a mechanism involved CFA-induced CB1R adaptations, especially in females.

Introduction

Cannabinoids are ubiquitous signaling molecules that suppress neurotransmitter release from the presynaptic terminal. Endocannabinoids (eCBs) are synthesized in the postsynaptic neuron in response to strong stimulation, resulting in increased intracellular Ca^{2+}, and travel in a retrograde manner across the synapse (Kreitzer & Regehr 2001, Ohno-Shosaku et al 2001, Wilson & Nicoll 2001). The presynaptic cannabinoid 1 receptor (CB1R) is a G-protein coupled receptor (GPCR) whose activation suppresses neurotransmitter release from the presynaptic terminal (Vaughan et al 2000). eCB-induced CB1R activation provides the central nervous system with an endogenous negative feedback regulatory system. This system is differentially regulate after persistent inflammation (Li et al 2017, Vecchiarelli et al 2021) but the mechanisms underlying inflammation-induced changes in the cannabinoid system are not clear.

While persistent inflammation induces a plethora of alterations in circulating mediators and neural activity, an interesting candidate for CB1R adaptations to inflammation is circulating corticosterone. Corticosterone activation of the membrane-bound glucocorticoid receptor (mbGR) increases synthesis of eCBs that activate the presynaptic CB1R (Di et al 2003), likely through increases in postsynaptic Ca^{2+} levels (Malcher-Lopes et al 2006). Further, systemic corticosterone administration results in brain region-dependent changes in eCB levels (Hill et al 2010a). Thus, in the following set of experiments we test the role of corticosterone in CB1R adaptations to inflammation in male and female Sprague Dawley rats.

Cannabinoid signaling in the periaqueductal gray (PAG) is critical for stress-induced analgesia (Hohmann et al 2005), indicating that cannabinoids in this region are

sensitive to adaptations involving stress. The cannabinoid system within the ventrolateral region PAG (vlPAG) is also sensitive to persistent inflammation, as 5-7 days of hind paw inflammation increase eCB tone and desensitize the presynaptic CB1R. The GR is expressed within the vlPAG (Mor & Keay 2013); therefore, we hypothesize that corticosterone activation of mbGR plays an important role in inflammation-induced adaptations in the cannabinoid system within the vlPAG.

Materials and Methods

Animals

Adult male and female Sprague Dawley rats (Harlan Laboratories and bred in-house; postnatal day 30-90) were used for all experiments. All procedures were performed in strict accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted by the Institutional Animal Care and Use Committee of Oregon Health & Science University. Care was taken to minimize discomfort.

Inflammation

Complete Freund's Adjuvant (CFA: heat-killed *Mycobacterium tuberculosis* in mineral oil, 1 mg/ml, 0.1 ml volume injected, Sigma-Aldrich) was injected subcutaneously into the plantar surface of the right hindpaw. The CFA injection produces an intense tissue inflammation of the hindpaw characterized by erythema, edema, and hyperalgesia (Iadarola et al 1988). Electrophysiological recordings and tissue dissections were performed 24h or 5-7d following CFA injection.

vlPAG slice preparation

Slices containing vlPAG were prepared as previously described (Bouchet et al 2021, Tonsfeldt et al 2016). Rats were deeply anesthetized with isofluorane and the brain was rapidly removed and placed in ice-cold sucrose-based cutting buffer containing the following (in mM): 75 NaCl, 2.5 KCl, 0.1 $CaCl_2$, 6 $MgSO_4$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 dextrose, 80 sucrose. Ventrolateral PAG (vlPAG) slices were cut to a thickness of 220 μm on a vibrotome (Leica Microsystems) in sucrose-based cutting buffer and transferred to a holding chamber with aCSF containing the following (in mM): 126 NaCl, 21.4 $NaHCO_3$, 22 dextrose, 2.5 KCl, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4 and the osmolarity was adjusted to 300-310 mOsm. Slices were maintained with 95% O_2- and 5% CO_2-oxygenated until transfer to a recording chamber on an Olympus BX51WI upright microscope and superfused with aCSF maintained at 32°C.

Whole-cell patch-clamp recordings

Voltage-clamp recordings (holding potential -65 mV) were made in whole-cell configuration using an Axopatch 200B amplifier (Molecular Devices). Patch-clamp electrodes were pulled from boroxilicate glass (1.5 mm diameter; WPI) on a two-stage puller (PP83, Narishige). Pipettes had a resistance of 2.5-5 MΩ. IPSCs were recorded in an intracellular pipette solution containing the following (in mM): 140 CsCl, 10 HEPES, 4 MgATP, 3 NaGTP, 1 EGTA, 1 $MgCl_2$, 0.3 $CaCl_2$, pH adjusted to 7.3 with CsOH, 290-300 mOsm. QX314 (100μM) was added to the internal solution for eIPSC experiments to reduce action potentials in the recording cell. Access resistance was continuously monitored. Recordings in which access resistance changed by >20%

during the experiment were excluded from data analysis. A junction potential of -5mV was corrected during recording. GABAergic events were isolated in the presence of glutamate receptor antagonist NBQX (5 μM). Spontaneous miniature IPSCs (mIPSCs) were recorded in the presence of TTX (500 nM). Events were low-pass filtered at 2 kHz and sampled at 10-20 kHz for off-line analysis (Axograph 1.4.3) and individual events were visually confirmed. When using cannabinoid agonists or antagonists, one neuron was recorded per slice due to their lipophilic nature. After each experiment with cannabinoid agonists or antagonists, lines were washed with 50% EtOH. Each set of experiments was repeated using at least 3 rats.

Drugs

WIN55,212-2 (Cayman Chemicals), SR141716A (rimonabant; RIM; Caymen Chemical), corticosterone (Abcam), and 11b-(4-dimethyl-amino)-phenyl-17bhydroxyl-17-(1-propynyl)-estra-4,9-dien-3-one (RU486; Abcam) were dissolved in DMSO, aliquoted, and stored at -20°C. DMSO was dissolved to <1:10,000 DMSO:aCSF in all cases and added to the appropriate baseline measurements. NBQX was purchased from Abcam, dissolved in distilled water, and stored at 4°C. All drugs except for those dissolved in DMSO were kept on ice throughout experimental days.

Corticosterone measurements

Rats were deeply anesthetized with isoflurane and trunk blood was collected in EDTA blood collection tubes (BD Vacutainer) at time of slicing for electrophysiological recordings. Blood was centrifuged for 20 min at 914 x g to isolate plasma and stored at -80°C until assayed. Steroid concentrations were measured using a commercially

available ^{125}I radioimmunoassay kit (ImmuChem Double Antibody Corticosterone for rodents; MP Biomedicals, Orangeburg, NY). The manufacturer supplied protocol was slightly modified so that corticosterone concentration in plasma samples (5μL) was single-determined via interpolation from a standard curve derived from six standards (ranging from 25 to 1000 ng/mL; i.e. 2.5 – 100 μg/dL).

Data Analysis

In all electrophysiological experiments, enough data was collected to get at least 5 replicates per group. All analysis were conducted in Graphpad Prism 9 (Prism version 9.2; San Diego, CA). Values are presented as mean ± SE and all data points are shown in bar graphs to illustrate variability. Statistical comparisons were made using two-tailed paired or unpaired T-test, one-way ANOVA, two-way ANOVA, or repeated measures ANOVA when appropriate. In all summary bar graphs, each dot represents an individual cell while the numbers in the bars represent the animal number. The vlPAG is a heterogenous brain region therefore each cell is considered an individual sample and all data sets contain data from at least 3 animals. When post-hoc analysis was appropriate, Tukey test and Šidák's multiple comparisons tests were used. $P < 0.05$ was used as a marker of significance for all experiments.

Results

The time course of CB1R desensitization is sex dependent

CB1R suppression of GABA release is significantly reduced after 5-7 days of inflammation (Bouchet et al in preparation; Dissertation Chapter 2) but it is not clear when in the 5-7 days the reduction occurs. To better understand the time course of CB1R adaptations to CFA-induced inflammation, CB1R-induced suppression of inhibition with the exogenous CB agonist, WIN-55,212-2 (WIN; 3μM; (Li et al 2017)) and CB1R-selective antagonist/inverse agonist, rimonabant (RIM; 3μM; (Li et al 2017)) was tested 24 hours after CFA injection (Fig. 3.1A). WIN suppressed GABA release 73 ± 8% in males (Fig. 3.1B) and only 22 ± 7% in females (Fig. 3.1C), resulting in a significant difference between males and females (Fig. 3.1D). This indicates a sex-dependent sensitivity of CB1R to inflammation 24h after CFA injection.

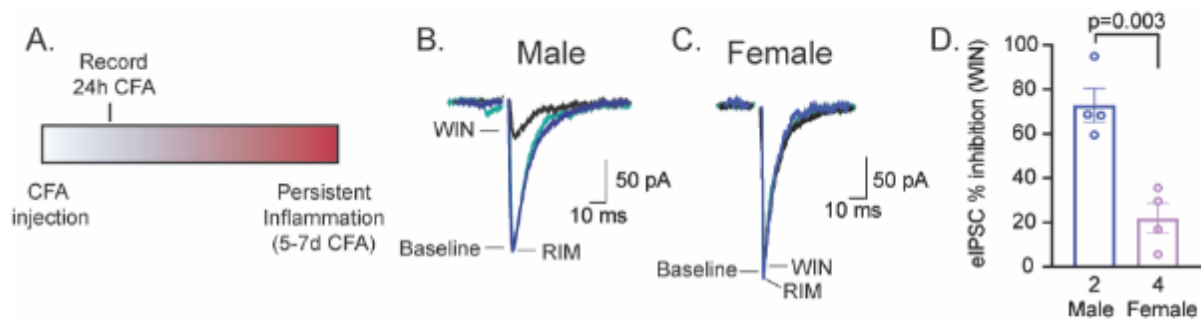

Figure 3.1 CB1R suppression of GABA release 24 hours after hindpaw CFA injection

(A) Experimental design. CFA was injected and recordings were conducted 24 hours later. The time course for persistent inflammation is 5-7 days; (B) Example trace from male rat 24h after CFA injection. Baseline in NBQX (5 μM, teal), WIN (3 μM, black), RIM (3 μM, blue). (C) Example trace of CB1R-induced eIPSC suppression in female rat 24h after CFA injection. (D) Summary of eIPSC percent inhibition by WIN in male (blue, 4 recordings from 2 rats) and female (pink, 4 recordings from 4 rats) 24h after CFA-induced inflammation. Percent inhibition is significantly reduced in females compared to males (unpaired t-test: $t_{(6)}=5$; $p=0.003$). Dots represent separate neurons, numbers under each bar represent the number of animals, error bars represent mean ± SEM.

Circulating corticosterone is elevated in females compared to males

Females are more sensitive to changes in corticosterone, so we hypothesized that the difference in CB1R suppression of GABA release after 24 hours of inflammation (Fig. 1) could be due to changes in corticosterone. To test circulating corticosterone levels, we collected trunk blood from male and female rats at different times CFA post-injection. CFA significantly increased circulating corticosterone levels in males and females (2-way ANOVA main effect of CFA: $F_{(4,76)}=5$, p=0.001). Consistent with the literature, there is a main effect of sex with higher corticosterone levels in females compared to males.

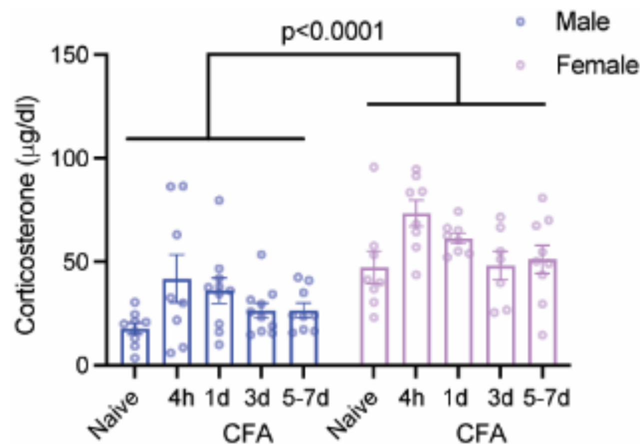

Figure 3.2 Circulating corticosterone levels

Corticosterone levels measured from trunk blood of male and female Sprague Dawley rats at timepoints throughout persistent inflammation. Inflammation increases circulating corticosterone levels in a sex-dependent manner (2-way ANOVA: main effect of CFA: $F_{(4,76)}=5$, p=0.001; main effect of sex: $F_{(1,76)}=45$; p<0.0001). Dots represent individual animals, n= 7-10 per group. Bars represent mean ± SEM.

Corticosterone suppresses GABA release in vlPAG through a CB1R-dependent mechanism

Within multiple brain regions, corticosterone acts through the mbGR to increase eCB synthesis and, thereby, suppress neurotransmitter release through the CB1R (Di et al 2003, Ragozzino et al 2020, Wamsteeker et al 2010). While GRs are expressed in the vlPAG (Mor & Keay 2013), corticosterone-induced suppression of GABA has not been investigated within this region. Therefore, we tested the effects of acute corticosterone on GABA release within the vlPAG. Consistent with its actions in other brain regions, corticosterone superfusion over vlPAG slices suppressed eIPSC amplitudes and was fully reversed by the mixed GR/PR antagonist, 11b-(4-dimethyl-amino)-phenyl-17bhydroxyl-17-(1-propynyl)-estra-4,9-dien-3-one (RU486, 10 μM). Corticosterone suppressed GABA release to the same extent as WIN-induced activation of the CB1R (Fig. 3.3B; WIN: N=3; 56 ± 18% inhibition; cort: N=5; 55 ± 15% inhibition) and was reversed by the CB1R antagonist RIM (Figure 3.3A). Reversal by RIM indicates that corticosterone-induced suppression of inhibition is dependent on CB1R

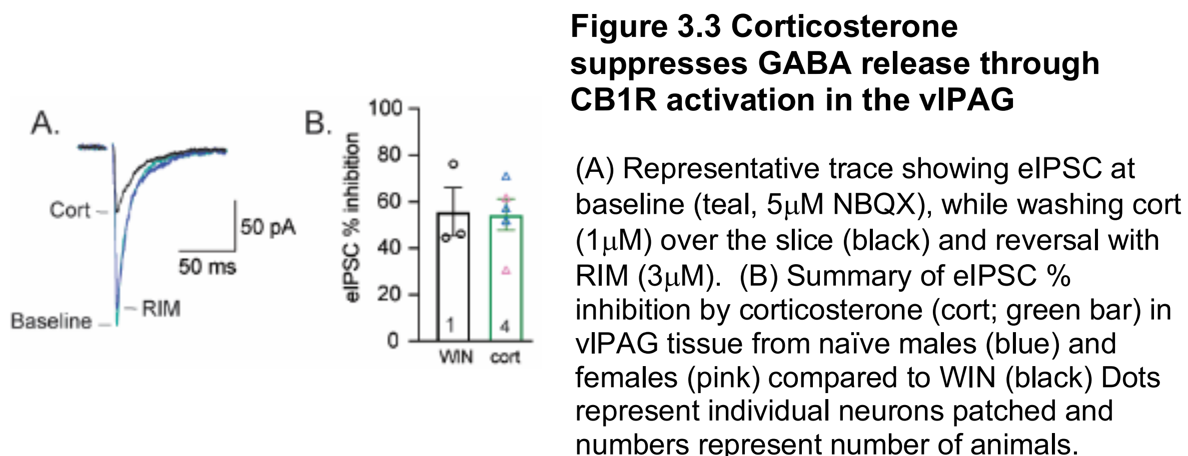

Figure 3.3 Corticosterone suppresses GABA release through CB1R activation in the vlPAG

(A) Representative trace showing eIPSC at baseline (teal, 5μM NBQX), while washing cort (1μM) over the slice (black) and reversal with RIM (3μM). (B) Summary of eIPSC % inhibition by corticosterone (cort; green bar) in vlPAG tissue from naïve males (blue) and females (pink) compared to WIN (black) Dots represent individual neurons patched and numbers represent number of animals.

activation. Corticosterone reversal by RU486 and WIN were not different, so they are pooled (Fig. 3.3B).

Pharmacologically blocking GR after persistent inflammation rescues CB1R function in a subset of neurons

To determine the role of increased GR activation during persistent inflammation, we blocked GR signaling with the GR and progesterone antagonist, RU486 (>1h incubation), in vlPAG slices from rats treated with CFA 5-7 prior (persistent inflammation). In a subset of vlPAG neurons, RU486 incubation recovered CB1R suppression of GABA release (Fig. 3.4). In both males and females, RU486 incubation after persistent inflammation partially recovers CB1R suppression of GABA release. This partial recovery indicates that corticosterone acting at mbGRs is involved, but not the sole mechanism driving reduced CB1R suppression of GABA release after persistent inflammation.

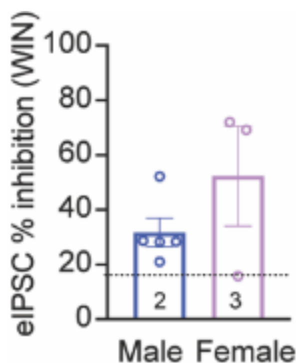

Figure 3.4 RU486 incubation partially recovers CB1R suppression of GABA release after persistent inflammation

Slices containing vlPAG were incubated in the GR antagonist, RU486 (1μM), for at least one hour before recordings. RU486 incubation recovers a CB1R suppression of GABA release in a subset of recordings from males (blue) and females (pink). Dotted line represents average eIPSC percent inhibition by WIN after 5-7d inflammation (from Chapter 2). Dots represent individual neurons; numbers represent the number of animals used for each experiment.

Discussion

This study examined the role of corticosterone in adaptations to CB1R suppression of GABA release after inflammation. Our previous study determined that persistent CFA-induced inflammation significantly reduces CB1R suppression of GABA release in both male and female Sprague Dawley rats. Here we show that there are sex differences in the time course of CFA-induced effects at CB1Rs, as well as circulating corticosterone levels throughout persistent inflammation. Together, the data presented here suggest that corticosterone may be part of the mechanism by which peripheral, hindpaw inflammation alters the cannabinoid system within the vlPAG.

The effects of persistent inflammation on the cannabinoid system are strikingly similar to those induced by chronic stress (for review see Morena et al 2016). These changes include brain region-dependent CB1R downregulation (Hill et al 2008b, Li et al 2017), increased 2-AG throughout the brain (Patel et al 2009, Patel et al 2005, Rademacher et al 2008), and reduced expression of membrane MAGL (Sumislawski et al 2011). Interestingly, the impacts of chronic stress in the basolateral amygdala (Patel et al 2009) are nearly identical to the impacts of persistent inflammation in the ventrolateral periaqueductal gray: reduced CB1R suppression of GABA release by exogenous agonist, WIN, and prolonged depolarization-induced suppression of inhibition. Therefore, it is plausible that persistent inflammation and chronic stress act through similar mechanisms. Corticosterone has been identified as a critical component of stress-induced adaptations to cannabinoid signaling. For example, stress reduces CB1R sensitivity to exogenous cannabinoid, HU210, at inhibitory synapses within the striatum of male mice (Rossi et al 2008). The authors show that this effect is

recapitulated with systemic corticosterone injections in the absence of stress and blocked by blocking GRs with RU486 just prior to stressor exposure (Rossi et al 2008). Recovering CB1R expression and function by blocking GR during stressor exposure has also been demonstrated in the hypothalamus (Wamsteeker et al 2010) and dorsal root ganglion (Hong et al 2011). Together, these studies indicate that corticosterone actions at GRs during chronic stress induce adaptations in CB1R expression and suppression of GABA release.

In our study, we did not find a robust recovery of CB1R suppression of GABA release after incubation in RU486. Importantly, the studies that report CB1R recovery after stress administer RU486 *in vivo* prior to each stressor to block GR signaling *during* the stressor (Hong et al 2011, Rossi et al 2008, Wamsteeker et al 2010). This indicates a potential critical window of GR activation during the stressor that is important for adaptations in cannabinoid signaling. Since persistent inflammation produces prolonged increases in corticosterone that are not time-locked to a specific repeated stressor, this would be a difficult experiment to replicate. However, these studies suggest that corticosterone is important for the induction of cannabinoid adaptations to stress; therefore, blocking GR signaling earlier in the inflammatory time course may result in a more consistent CB1R recovery. It is important to note, however, that the effects of activating mbGRs is not always reversed by RU486 (Di et al 2003). Additionally, since RU486 is an antagonist for both GRs and PRs, there are potentially different modes of action in males and females within our preparation.

Data presented here indicate that CB1R signaling in females is already reduced 24 hours after CFA injection. Our study did not determine the time point where male

CB1R suppression of GABA release is first reduced, but recent evidence using GTPγS indicates that CB1R function in males is not reduced 48h after CFA injection. Knowing the time point at which male CB1R suppression of GABA release is reduced could enable more temporally specific manipulations. Perhaps corticosterone plays a critical role in the initial reduction in CB1R suppression of GABA release, so blocking GR signaling at that timepoint would be more efficacious.

The majority of work investigating the effects of stress on cannabinoid regulation within the brain was done on males, but there is vast evidence that the stress response differs between males and females (Dearing et al 2021, Jones et al 1998, Kendler et al 2000, Reich et al 2009, Solomon et al 2015, Wallace et al 2021). We found sex differences in CB1R suppression of GABA 24 hours after CFA injection (Fig. 3.1) and circulating corticosterone levels (Fig. 3.2). There are well established sex differences in HPA axis responsivity (for review see Kudielka & Kirschbaum 2005) with overall higher responsivity in females. Increased HPA axis responsivity in females compared to males may underlie the difference in CB1R sensitivity to inflammation 24 hours after CFA administration.

Together, these data identify sex differences in adaptations to CB1R suppression of GABA release after inflammation. Further we provide evidence for corticosterone as a partial mechanism for reduced CB1R suppression of GABA release in the vlPAG after persistent inflammation. These data highlight the importance of investigating inflammation-induced adaptations in both males and females and have implications for the treatment of inflammatory diseases.

Acknowledgments We would like to thank Melinda L. Helms, Michelle A. Nipper, and Dr. Doborah A. Finn for help with the corticosterone measurements (Fig. 3.2)

Chapter 4 : Additional Unpublished Data

4.1 Ventral Tegmental Area (VTA) projections to vlPAG are predominantly GABAergic and sensitive to both opioids and cannabinoids

4.1.1 Introduction

The vlPAG is an integration center that receives information from a vast array of brain regions (Silva & McNaughton 2019b). A dense GABAergic projection from the ventral tegmental area (VTA) projects to the vlPAG (Breton et al 2019). VTA GABA plays a role in pain modulation (Franklin 1989), in addition to the important roles in drug addiction (Wang et al 2002) and mood-related behaviors (Bolanos et al 2003). Chronic neuropathic pain leads to increased putative GABAergic neuronal activity within the VTA (Ko et al 2018), indicating a role for VTA GABA in chronic pain. While these studies focus on GABA within the VTA, my studies focused on the GABAergic projection from the VTA to the vlPAG, as increased GABA release within the vlPAG leads to hyperalgesia (Takasu et al 2015). This unexplored GABAergic projection from the VTA to the vlPAG could have important functions in pain-related signaling within the vlPAG.

4.1.2 Materials and Methods

Naïve male Sprague Dawley rats were used for these experiments. Brains were sliced at 220μM in ice-cold cutting buffer (in mM): 75 NaCl, 2.5 KCl, 0.1 $CaCl_2$, 6 $MgSO_4$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 dextrose, 50 sucrose and placed in a holding chamber with artificial cerebral spinal fluid (aCSF) containing the following (in mM): 126 NaCl, 21.4 NaHCO3, 11.1 dextrose, 2.5 KCl, 2.4 CaCl2, 1.2 MgCl2, and 1.2 NaH2PO4, pH 7.35, equilibrated with 95% O2/5% CO2 at 34°C until moved into a recording chamber. Recording solutions included NBQX (5μM) to block glutamate transmission. CB1R function was measured by calculating % inhibition by WIN55,212-2 (3μM) and reversal by Rimonabant (3μM). MOR function was assessed with DAMGO (1μM) with reversal by Naloxone (1μM).

Rats (p25-p35) were deeply anesthetized for surgery with a Ketamine/Xylazine/acepromazine cocktail (250, 50, 10 mg/kg, respectively injected IP). ChR2 AAV (AAV9.hSyn.hChR2(H134R)-EYFP.WPRE.hGH, Penn Vector Core; 50nL; bilateral injection) was injected bilaterally into the VTA (from bregma: AP: -4.35mm, ML: 0.8mm; DV: -7.5mm from skull) (Matsui et al 2014). Recordings were conducted 2-4 weeks after injection to allow for optimal viral expression. Light stimulation was delivered using high-powered LED systems (Thorlabs) to deliver blue (M470F1 LED, 470nm, ChR2, 0.3-10mW/mm^2) light to vlPAG slices containing VTA terminals. Brain slices were fixed to map recording site, VTA microinjection site, and distribution of terminals all experiments.

4.1.3 Results

Projections from the VTA to the vlPAG are sensitive to both opioids and cannabinoids (Figure 4.1). Figure 4.1A shows experimental design: ChR2 was injected into the VTA and neurons were patched in the vlPAG, measuring VTA afferents. Bilateral VTA ChR2 injection was confirmed for each experiment (Fig. 4.1B). In some experiments, inhibition by both WIN and DAMGO were tested while patched onto the same neuron (Fig. 4.1C). The order of opioid and cannabinoid drugs was

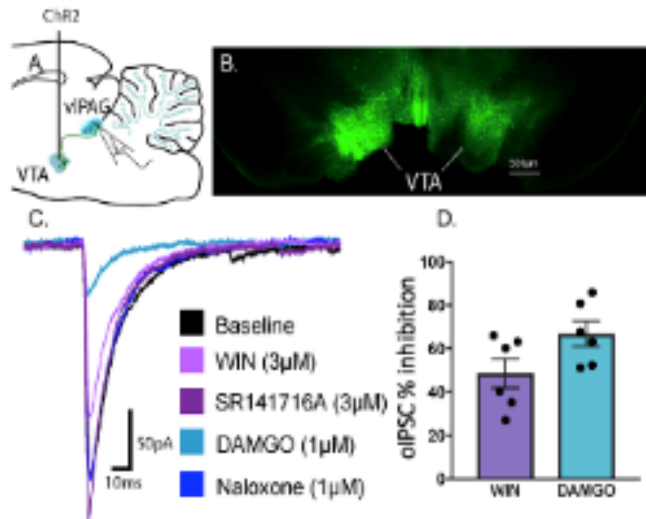

Figure 4.1 VTA-vlPAG projections are sensitive to opioids and cannabinoids

(A) Channelrhodopsin (AAV9-hSyn-ChrR2-EYFP) is injected into the VTA and neurons are patched in the vlPAG. (B) Representative image of injection site. (C) Light stimulation of vlPAG elicits optically-induced IPSCs (oIPSCs) that are reduced by either CB receptor agonist WIN 55,212-2 (3μM) or MOR agonist DAMGO (1μM) and reversed by antagonists SR141716A (3μM) and Naloxone (1μM), respectively. (D) Quantification of % inhibition. Dots in the bar graph are individual cells and error bars represent mean ± SEM.

counterbalanced. Overall, results indicate that GABAergic inhibition by WIN is slightly lower than that of DAMGO at VTA afferents within the vlPAG (Fig. 4.1D).

4.1.4 Discussion

Data from this project illuminate a specific projection from the VTA to the vlPAG that is sensitive to both cannabinoid- and opioid-suppression of GABA release. Using optogenetics we investigated CB1R and MOR suppression of GABA release from targeted afferents within the vlPAG. We found that these terminals were sensitive to both opioids and cannabinoids. Maximal concentrations of both WIN (Wilson-Poe et al 2015) and DAMGO (Vaughan et al 1997a) were used for these experiments. These data could be useful in the future to target cellular processes in presynaptic terminals using pathway-specific projections to the vlPAG.

4.2 Regulation of cannabinoid receptors by protein translation

4.2.1 Introduction

Protein synthesis is a key component of synaptic remodeling and memory processes; however, relatively little is known about presynaptic synaptic plasticity in comparison to postsynaptic. Synaptic plasticity involves rapid protein translation that enables dendritic remodeling and long-term changes in synaptic strength (Huber et al 2000, Steward & Schuman 2001, Waung & Huber 2009). The presynaptic terminal also undergoes structural changes that result in long lasting changes in neurotransmitter release (for review see Castillo 2012, Monday & Castillo 2017, Yang & Calakos 2013) indicating that local machinery required for structural changes is present. Since protein synthesis is such a major component of postsynaptic plasticity processes, whether protein synthesis machinery existed in the presynaptic compartment was a major question. The first evidence of local protein synthesis machinery in axons came from surgically dissociated growth cones, where protein synthesis inhibitors blocked chemotropic responses and both protein synthesis machinery and protein degradation machinery were identified (Campbell & Holt 2001). This discovery was particularly interesting because it showed that the external cues can alter presynaptic compartment function. A long-standing belief prior to this was that the axon and presynaptic compartment were purely there to send along a message generated in the dendrites and soma, but these data collected from a dissociated growth cone changed that view. Since then, it has become well established that the presynaptic compartment and axon contain protein translation machinery, such as the presence of ribosomes within the presynaptic terminal (Younts et al 2016), and can foster local protein synthesis.

CB1Rs are highly expressed throughout the brain (Herkenham et al 1990, Howlett et al 2002) and retrograde endocannabinoid (eCB) transmission induces presynaptic long-term depression (Gerdeman et al 2002, Robbe et al 2002); (for review see Heifets & Castillo 2009). Indeed, CB1R signaling induces mammalian target of rapamycin (mTOR)-dependent protein synthesis in the hippocampus (Monday et al 2020, Younts et al 2016). Not only does CB1R promote protein synthesis, but protein synthesis is required for CB1R-dependent long-term plasticity within the hippocampus (Younts et al 2016). In addition to protein synthesis involvement in long term synaptic remodeling, protein synthesis is required for surface expression of certain receptors. For example, cell surface expression of calcium-permeable (CP)-AMPA receptors is regulated by protein translation (Loweth et al 2019). Multiple groups have found that changes in CB1R protein levels often do not correspond with changes in CB1R mRNA (Li et al 2017, Wilson-Poe et al 2021) suggesting that protein translation may be a key regulatory step in CB1R expression and function. Here, we investigate the role of protein translation in CB1R suppression of GABA release.

4.2.2 Materials and methods

Naïve male Sprague Dawley rats were used for these experiments. Brains were sliced at 220μM in ice-cold cutting buffer (in mM): 75 NaCl, 2.5 KCl, 0.1 $CaCl_2$, 6 $MgSO_4$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 dextrose, 50 sucrose and placed in a holding chamber with artificial cerebral spinal fluid (aCSF) containing the following (in mM): 126 NaCl, 21.4 NaHCO3, 11.1 dextrose, 2.5 KCl, 2.4 CaCl2, 1.2 MgCl2, and 1.2 NaH2PO4,

pH 7.35, equilibrated with 95% O2/5% CO2 at 34°C until moved into a recording chamber. Slices were incubated in aCSF or aCSF containing the protein synthesis inhibitors anisomyocin (20μM) or cycloheximide (80μM) for 30 minutes before being put onto the microscope and washed with warm aCSF. Time course (Figure 4.2.2) separates data from figure 4.2.1 into groups depending on the amount of time in aCSF after 30-minute anisomyocin incubation. Recording solutions included TTX (500nM) and NBQX (5μM) to measure mIPSCs. CB1R function was measured by calculating % inhibition by WIN55,212-2 (3μM) and reversal by Rimonabant (3μM). MOR function was assessed with DAMGO (1μM) with reversal by Naloxone (1μM).

4.2.3 Results

Blocking protein translation with just 30 minutes of anisomycin exposure reduces CB1R-mediated suppression of GABA release (Figure 4.2). Preliminary data suggests that the same effect can be produced by 30-minute incubation in cycloheximide (Figure 4.2.1). This sensitivity to protein translation is selective to the CB1R as neither anisomyocin nor cycloheximide impacted presynaptic MOR-induced suppression of GABA release (Figure 4.2).

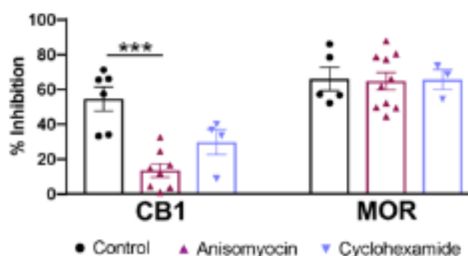

Figure 4.2 CB1R suppression of GABA release is sensitive to protein synthesis inhibitors

Blocking protein synthesis with Anisomyocin (20μM) or Cyclohexamide (80μM) for 30 min reduces CB1-mediated inhibition of GABA release but has no effect on MOR inhibition of GABA release. *** $p<0.001$.

The effect of blocking protein translation occurs surprisingly quickly, as assessed by a time course (Figure 4.3). All slices were incubated in anisomyocin for 30 minutes and the aCSF wash following incubation varied and is documented in figure 4.3. Neurons patched within an hour of anisomyocin incubation show a slight reduction in CB1R-induced GABA suppression from 58 ± 5% inhibition at baseline to 36 ± 10%, but 1-2h following anisomyocin incubation CB1R suppression of GABA release is nearly abolished completely (4 ± 3%). Anisomyocin did not have an impact on MOR suppression of GABA release throughout this time course.

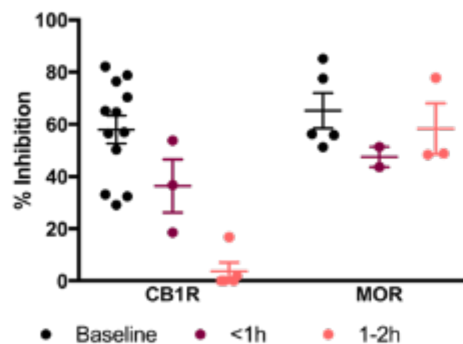

Figure 4.3 Anisomyocin time course

CB1R and MOR inhibition of GABA release was measured with mIPSCs following slice incubation in anisomyocin (20μM, 30 min) and then wash in aCSF for <1h or 1-2h. CB1R function is reduced over the course of aCSF wash. There is no effect on MOR function.

4.2.4 Discussion

Data presented here indicate that protein translation is a novel regulatory mechanism of the presynaptic CB1R. This mechanism indicates that CB1Rs are tightly regulated and their signaling can be rapidly abolished. Protein translation inhibitors are notoriously non-selective, but the observation of reduced CB1R suppression of GABA release with both anisomyocin and cycloheximide (Fig. 4.2) suggest that the reduced CB1R activity is indeed occurring through inhibition of protein synthesis. The rapid time course of reduced CB1R suppression of GABA release after inhibition of protein synthesis suggests that this process is targeting local protein synthesis. Due to the time required for axonal transport, it would take on the course of hours for proteins translated in the soma to get to the presynaptic terminal; therefore, inhibition of translation impacting proteins translated in the soma would likely not be visible in the time course that we see anisomyocin-induced reductions in CB1R suppression of GABA release. Indeed, local presynaptic regulation of protein degradation and translation have been implicated in presynaptic function (Monday et al 2020). Local translational regulation of the CB1R could help explain the CB1R dichotomy: it is expressed at such high levels; how does it exert any sort of specificity? Perhaps extremely tight regulation over CB1R expression and function play an important role in CB1R signaling specificity.

Another interesting finding from this experiment was the stark difference between protein translation inhibitors on CB1R and MOR suppression of GABA release. While CB1R suppression of GABA release was significantly reduced after inhibition of protein translation, MOR suppression of GABA release was unaffected. This suggests different basal regulatory mechanisms in the vlPAG of naïve rats between the two GPCRs. A

recent paper elucidated a mechanism of MOR regulation that focuses on mobility: MORs exhibit rapid lateral mobility and when a synaptic MOR gets internalized, an axonal MOR replaces it to maintain signaling (Jullie et al 2020). This rapid lateral movement could explain the continued signaling through MOR during protein synthesis inhibitors. Cannabinoid receptors also exhibit rapid lateral movement in the absence of agonist (Mikasova et al 2008) and there is a large pool of intracellular CB1Rs (Wilson-Poe et al 2012) that, presumably, could be transported to the synapse and sustain signaling. The loss of CB1R suppression of GABA release so quickly suggests rapid CB1R turnover, synthesis and degradation. Importantly, we do not know if the molecule modulated by protein synthesis inhibitors is the CB1R itself, a scaffolding protein, or a protein important for trafficking CB1R to the membrane. However, these data do indicate that CB1R is tightly regulated, and continuous protein synthesis is required for continual signaling.

Future Directions

1. *Experimental question*: How quickly does the CB1R recycle through the synapse? *Experiment*: Incubate in a CB1R irreversible antagonist, test CB1R suppression of GABA release through aCSF wash.
2. *Experimental question:* Is the CB1R translated locally? *Experiment:* Combine optogenetic stimulation with protein synthesis inhibitors. Using optogenetics to target a region, such as the VTA, that is not in the same slice as the vlPAG would sever axons from their respective cell bodies. If protein synthesis inhibitors block CB1R suppression of inhibition from axons dissociated from

their cell bodies, that would provide compelling evidence that CB1R is locally translated.

Chapter 5 : Discussion & Future Directions

Using electrophysiology and pharmacology, we investigated regulatory mechanisms governing presynaptic CB1R suppression of GABA release in the vlPAG and adaptations in this regulation induced by persistent inflammation in the hindpaw. Data presented within this dissertation reveal unique CB1R adaptations to persistent inflammation in male and female Sprague Dawley rats. In Chapter 2, we show that 5-7 days of hindpaw inflammation prolongs eCB signaling and desensitizes the CB1R in the vlPAG. In Chapter 3, we demonstrate sex differences in the time course of changes in CB1R inhibition of GABA release and show that corticosterone engages CB1R signaling in the vlPAG- highlighting a link between inflammation and CB1R function. This new understanding of inflammation-induced adaptations of presynaptic CB1R regulation invite a host of questions involving presynaptic GPCR regulation, regional differences in the impacts of inflammation, and mechanistic questions underlying GRK2/3-induced desensitization. In this section, I outline key findings from my dissertation, limitations, and future directions.

5.1 Presynaptic CB1R and MOR are differentially regulated

Activation of either presynaptic CB1R or MOR suppresses GABA release from the presynaptic terminal. Through my studies, I found that persistent inflammation and inhibition of protein synthesis significantly reduced CB1R suppression of GABA release but had no impact on MOR. This indicates that these presynaptic GPCRs are

differentially regulated within the vlPAG. Additionally, the rapid loss of CB1R suppression of GABA release after treatment with protein synthesis inhibitors, as discussed in Chapter 4.2, indicate rapid CB1R turnover in tissue from naïve animals. In the following paragraphs, I will highlight key differences in CB1R and MOR regulation within the presynaptic terminal.

CB1R and MOR both exhibit rapid lateral movement but respond differently to agonist exposure. Single quantum dot imaging confirms rapid lateral movement of native CB1Rs in cortical neuronal culture (Mikasova et al 2008). In this study, the authors found CB1R localized primarily to the axon, as opposed to somatodendritic localization, of GABAergic neurons. Basally, more than 80% of the CB1Rs are mobile and move laterally through the synapse. This movement through the synapse is very rapid, CB1R dwell time is 6 times shorter than that of postsynaptic AMPA receptors. Importantly, CB1R mobility is impacted by prolonged agonist exposure. While CB1R mobility was not affected by 30 minutes of WIN, more than 2 hours of WIN exposure reduced the expression of surface CB1Rs and reduced the mobility of the receptors that remained on the membrane, which were predominantly located at the axon and not in the synapse. These data show that CB1R lateral mobility, moving rapidly through the synaptic compartment in basal conditions, and surface expression are greatly reduced by prolonged agonist exposure.

In contrast to reduced CB1R mobility during prolonged agonist, presynaptic MOR dynamic mobility is maintained during agonist exposure. In an elegant study, Jullié et al. use GFP-labeled MORs to investigate MOR dynamics at the presynaptic terminal of medium spiny neurons (Jullie et al 2020). They fund that MORs are localized along the

axon with a slight enrichment within the synapse and rapidly diffuse throughout the axon surface in the absence of ligand. Application of the MOR agonist, DAMGO, induces accumulation within terminals. While MORs undergo ligand-induced internalization within 30 minutes of agonist application, surface MORs maintain their dynamic mobilization throughout agonist exposure and move laterally into the synapse to replace internalized MORs. Thus, MORs also exhibit lateral mobility but maintain their rapid mobility during agonist exposure.

A clear similarity between CB1R and MOR is their rapid diffusion through the presynaptic terminal. While rapid lateral mobility is a consistent feature of these two presynaptic GPCRs, their response to prolonged agonist is starkly different. Agonists induce receptor internalization in both systems, but the time course is vastly different, as is the impact on receptor mobility. While 2 hours of agonist exposure is required for partial desensitization and internalization of the CB1R, MORs are internalized within the course of 30 minutes. This difference in the timing of desensitization strongly suggests that there is a different regulatory mechanism involved in desensitization of the two presynaptic GRCRs. Another important difference is that CB1Rs immobilize after prolonged agonist exposure, rendering very few CB1Rs in the synapse and an inability of extrasynaptic CB1Rs to move laterally to permeate the synapse. MORs, in comparison, maintain their dynamic movement throughout the course of agonist exposure and, therefore, when synaptic MORs are internalized, extrasynaptic MORs move laterally into the synapse and maintain signaling. Because of these differences, presynaptic MORs maintain signaling throughout agonist exposure while CB1R signaling is vastly reduced. MOR lateral movement in the presence of agonist

corroborates the lack of MOR desensitization in response to prolonged agonist exposure in vlPAG and hypothalamus slices (Fyfe et al 2010, Pennock et al 2012, Pennock & Hentges 2011), although CB1Rs are also resistant to desensitization (Chapter 2) so further research is needed to better understand lateral mobility and presynaptic GPCRs.

Overall, presynaptic CB1R and MOR both move rapidly through the synaptic compartment under basal conditions but respond differently to agonist exposure. These differences in response to agonist, reductions in CB1R mobility and surface expression with consistent MOR mobility, could underlie the differences in CB1R and MOR suppression of GABA release after persistent inflammation and protein synthesis inhibitors.

Future Directions

Here, we tested CB1R and MOR suppression of GABA release with a near-maximal concentrations of WIN (Wilson-Poe et al 2015) and DAMGO (Vaughan et al 1997a), respectively. While adaptations at CB1R have been shown to reduce the maximal effect of WIN (Wamsteeker et al 2010), adaptations in presynaptic MOR suppression of GABA release have been observed at sub-maximal concentrations of DAMGO, such as MOR sensitization after repeated morphine treatment (Fyfe et al 2010, Ingram et al 1998). Since our experiments tested a near maximal concentration of DAMGO, we may have missed a shift in the concentration-response curve. To better understand the impacts of persistent inflammation on MOR sensitivity, we could run a

concentration-response curve after persistent inflammation to see if there is a shift in DAMGO efficacy. This experiment would elucidate whether presynaptic MORs in the vlPAG are insensitive to persistent inflammation, as our studies with maximal concentration of DAMGO suggest.

5.2 Persistent inflammation desensitizes the presynaptic CB1R

We find that persistent inflammation significantly reduces CB1R suppression of GABA release in response to exogenous agonist. This could be the result of multiple regulatory mechanisms, including CB1R downregulation or desensitization. Previous work from the Ingram lab demonstrates that persistent inflammation induces CB1R downregulation in the RVM (Li et al 2017). In the vlPAG, however, radioligand binding and Western blot indicate that the CB1R is not downregulated. We found, instead, that inflammation induces GRK2/3-dependent desensitization. This finding is interesting for two reasons: (1) this is the first evidence that GRK2/3-dependent desensitization reduces GPCR suppression of GABA release; and (2) GRK2/3-dependent desensitization of the presynaptic CB1R is induced by persistent inflammation.

GRK2/3 phosphorylation of the GPCR C-terminal tail is a critical step in canonical / postsynaptic GPCR desensitization (Kovoor et al 1998, Zhang et al 1998). Similarly, there is evidence that GRK2/3 is important for CB1R regulation. GRK2/3 phosphorylation of the CB1R C-terminal tail is required for CB1R internalization in cell culture where CB1Rs internalize within 15 minutes of agonist exposure (Daigle et al 2008a, Daigle et al 2008b, Hsieh et al 1999, Jin et al 1999). In cultured hippocampal neurons, where CB1Rs are maintained in the presynaptic environment, 2 hours of WIN

exposure induces partial desensitization and 18-24 hours of WIN is required for full desensitization (Kouznetsova et al 2002). In this system, CB1R desensitization is reduced by expressing presynaptic dominant-negative GRK2, supporting a requirement for GRK2 in CB1R desensitization. *In vivo* studies using a transgenic mouse with mutations in the putative GRK2/3 phosphorylation sites on the CB1R C-terminal tail (S426A/S430A) provide evidence for phosphorylation of these sites in behavioral tolerance to THC. These transgenic mice exhibit delayed tolerance and increased sensitivity to Δ^9THC, increased analgesic effects of Δ^9THC, and increased sensitivity to DSE at the synaptic level (Morgan et al 2014). Together, these studies provide ample evidence that GRK2/3 phosphorylation of the CB1R C-terminal tail is important for CB1R regulation in response to prolonged agonist in cell culture, slice, and *in vivo*. Adding to this literature of CB1R regulation by GRK2/3, we found that the reduction in CB1R function after persistent inflammation is fully recovered by blocking GRK2/3 in the slice (Fig. 2.5). Blocking GRK2/3 activity in slices from naïve animals had no impact on CB1R suppression of GABA release, indicating that persistent inflammation alters GRK2/3 interactions with the CB1R. These data indicate that that persistent inflammation recruits GRK2/3 and induces desensitization of presynaptic CB1Rs.

Methodological Limitations

While Cmp101 is commonly used to block GRK2/3 activity (Adhikary et al 2022b, Leff et al 2020, Lowe et al 2015), it also inhibits other kinases. Among the kinases that Cmp101 targets with over 50% efficacy are protein kinase C-related protein kinase

(PRK2) and serum and glucocorticoid-regulated kinase (SGK1) (Lowe et al 2015). Either of these kinases could be impacted by persistent inflammation. SGK1 is particularly interesting given the connection between glucocorticoids and the cannabinoid system (Chapter 3). SGK1 is a serine-threonine kinase that is transcriptionally regulated by glucocorticoids (Webster et al 1993) and known for its role in learning and memory and synaptic plasticity (Ma et al 2006, Tsai et al 2002). SGK1 protein expression is increased in the superficial dorsal horn of the spinal cord 6h after CFA-induced joint inflammation and appears to mediate the *initial* inflammatory pain state, but local knockdown does not impact *persistent* pain states (>48h after CFA injection) (Geranton et al 2007). Currently, there is only one published study investigating interactions between SGK1 and CB1R (Finn et al 2003) and the authors did not find a connection between formalin-induced increases in *Sgk1* mRNA expression in the dorsal horn and cannabinoid signaling. Therefore, although SGK1 expression is increased by inflammation and would be interesting to study in the future, we do not have ample evidence to believe that Cmp101 recovery of CB1R signaling after persistent inflammation involves inhibition of SGK1. In addition, we used lower concentrations of Cmp101 that are more selective to GRK2/3 (Lowe et al 2015) and still observe full recovery of CB1R suppression of GABA release after persistent inflammation.

Future Directions

(1) It is not clear what leads to this increase in GRK2/3-dependent desensitization of the CB1R after persistent inflammation. Our primary hypothesis is that inflammation

upregulates GRK2/3 within the vlPAG. We could test this by analyzing GRK2/3 protein expression in a Western blot or by assessing co-localization of CB1R and GRK2/3 (Blasic et al 2012). One problem with the co-localization assay is CB1R antibodies, which are notoriously non-specific. Additionally, since the CB1R-GRK2/3 interaction that we are interested in is within the presynaptic terminal it would be extremely difficult to visualize and quantify co-localization. That said, techniques to visualize terminals exist (Dudok et al 2015, Jullie et al 2020, Mikasova et al 2008, O'Neil et al 2021) and could be used to visualize CB1R and GRK2/3 proximity.

(2) An important question that is not addressed within this dissertation is how long inflammation-induced adaptations last. Δ^9THC treatment induces desensitization, as measured by GTPγS, that reverses days after the cessation of administration (Sim-Selley et al 2006). Additionally, stress-induced reductions in CB1R signaling in the hypothalamus recover 3 days after stress cessation (Wamsteeker et al 2010). Therefore, we hypothesize that inflammation-induced adaptations described here will reverse after recovery from inflammation. Further evidence supporting this hypothesis is the rapid recovery of CB1R signaling after blocking GRK2/3 for just 1 hour after 5-7 days of inflammation, indicating that the adaptations we observe at the receptor level are rapidly reversible.

5.3 Persistent inflammation prolongs eCB signaling

Data presented within this dissertation provide evidence that somatic / dendritic depolarization within the vlPAG induces the mobilization of eCBs that suppress

presynaptic GABA release through CB1R activation. This adds the vlPAG to the list of other brain regions that exhibit DSI/DSE including the hippocampus (Wilson & Nicoll 2001), hippocampal cultures (Ohno-Shosaku et al 2001), hippocampal autaptic cultures (Straiker & Mackie 2005), Purkinje cells of the cerebellum (Kreitzer & Regehr 2001), paraventricular nucleus of the hypothalamus (Wamsteeker et al 2010), cortical interneuron-pyramidal neuron synapses (Trettel & Levine 2003) and substantia nigra (Yanovsky et al 2003).

We found that depolarizing vlPAG neurons for 5s to +25mV induced approximately 20s of CB1R-dependent suppression of GABA release (Figure 2.6). This time course is similar to what is observed in the hypothalamus (Wamsteeker et al 2010) and hippocampus (Wilson & Nicoll 2001), suggesting transient suppression of inhibition is an important feature of eCB signaling. Endocannabinoids are rapidly degraded by specific degradation enzymes: MAGL and FAAH degrade the majority of 2-AG and AEA, respectively. Blocking MAGL genetically with knock-out mice (Hashimotodani et al 2007) or pharmacologically prolongs DSI while blocking FAAH has no impact (Pan et al 2009, Straiker & Mackie 2005), indicating that 2-AG degradation is a critical component of the time course of DSI. In the brain, 2-AG is rapidly degraded, consistent with the transient nature of DSI. Within the vlPAG itself, 2-AG degradation is so rapid that washing 2-AG over the slice doesn’t suppress GABA release unless MAGL is blocked (Lau et al 2014). The transient time course of DSI in our experiments that is prolonged by blocking MAGL with JZL184 supports rapid degradation of 2-AG in tissue from naïve animals. MAGL inhibition in tissue from naïve animals recapitulates the time course of DSI after persistent inflammation, suggesting that MAGL activity is reduced by

inflammation. This conclusion also explains the observed increase in eCB tone within the vlPAG after persistent inflammation. Together, these data indicate that reduced MAGL activity is at the core of cannabinoid adaptations induced by persistent inflammation; however, the mechanism underlying reduced MAGL activity is not clear.

One potential mechanism is transcriptional repression. Evidence indicates that MAGL is transcriptionally regulated after peripheral injury. PRDM5, a transcriptional repressor for *mgll*, the gene that encodes MAGL (Galli et al 2014) is elevated in the spinal cord after spinal cord injury (Liu et al 2016). If PRDM5 is increased in the vlPAG after persistent inflammation, that would provide a mechanism by which persistent inflammation reduces MAGL activity. This transcriptional repressor is implicated in effects of ketamine. Within the striatum, ketamine increases 2-AG, reduces MAGL, and increases PRDM5. Genetic overexpression of MAGL or silencing of PRDM5 robustly reduces 2-AG levels (Xu et al 2020). These experiments provide evidence that a physiological stimulus alters PRDM5 levels that can suppress MAGL and, therefore, increase 2-AG levels within brain regions. It is not known whether CFA-induced inflammation impacts PRDM5 expression but would be interesting to assess PRDM5 within the vlPAG in naïve and CFA-treated animals. PRDM5 presents a potential mechanism by which persistent inflammation reduces MAGL activity within the vlPAG after persistent inflammation.

Methodological Limitations

DSI in the vlPAG is incredibly variable. Less than half of the neurons in slices from naïve rats that were tested exhibited DSI (Fig. 2.6) and when DSI was produced, the maximal inhibition was not consistent between neurons. Presumably, this is the case in other brain regions as well given that experiments providing evidence for DSI from multiple brain regions average more than 15 cells for an experiment (Patel et al 2009, Wamsteeker et al 2010, Wilson & Nicoll 2001) and even average multiple DSI experiments *per cell* (Wilson & Nicoll 2001). There are multiple potential reasons for this variability of DSI:

(1) Location of the Ca^{2+} release in different cells or the proximity of the recording electrode to the dendrites. The amount of Ca^{2+} needed to elicit eCB-induced suppression of inhibition is not clear. Investigating the concentration of Ca^{2+} sufficient for 50% DSI in cerebellar purkinje cells, one group concluded 200 nM and 40 nM dendritic and somatic Ca^{2+}, respectively, was sufficient (Glitsch et al 2000) while another group found 15 μM dendritic Ca^{2+} was necessary (Brenowitz & Regehr 2003). These concentrations are substantially different and have vastly different implications for eCB synthesis *in vivo.* A third group found that 4 μM Ca^{2+} is required for half-maximal DSI in the hippocampus (Wang & Zucker 2001). This discrepancy between studies could be due to the difference in Ca^{2+} detectors or exactly where the Ca^{2+} was being measured within the cell (For review see (Diana & Marty 2004)). Nonetheless, differences in Ca^{2+} handling between cells could account for variability in DSI.

(2) CB1R expression on impinging presynaptic terminals. In the hippocampus, where CB1R expression is well established, DSI at sites of high CB1R expression yield ~90% inhibition (Wilson & Nicoll 2001) and presynaptic sensitivity to cannabinoids determines DSI magnitude (Ohno-Shosaku et al 2002). Presumably, then, DSI magnitude in the vlPAG (~40% inhibition) and variability between cells is dependent on CB1R expression on impinging presynaptic terminals and indicate that some vlPAG inputs are insensitive to cannabinoids.

(3) Multivesicular release. Under basal conditions, GABAergic transmission within the vlPAG is multivesicular (Aubrey et al 2017) which could account for the large mIPSCs observed within this region (Llano et al 2000). Multivesicular release stabilizes unstable, stochastic nature of synaptic transmission and is found throughout the brain (Rudolph et al 2015). This release mode in the vlPAG highlights the importance of GABAergic transmission, which saturates $GABA_A$ receptors under basal conditions (Aubrey et al 2017). In my experience this multivesicular release is often desynchronized, leading to multiple peaks in an eIPSC or mIPSC which can be very difficult to quantify and increase variability.

(4) Isofluorane anasthesia. The Ingram lab uses isofluorane anasthesia; however, isofluorane reduces circulating levels of AEA (Weis et al 2010). Further, WIN-55 treatment increases the minimum alveolar concentration of anasthesia (Chavez-Monteagudo et al 2022). This means that our anasthetic could be differentially impacting naïve and CFA-treated animals, as data

presented in this dissertation shows higher levels of endocannabinoids after CFA treatment. This interaction between isofluorane and the cannabinoid system highlights an important limitation to our experiments.

Future Directions

Blocking 2-AG degradation by MAGL prolongs DSI (Pan et al 2009, Schlosburg et al 2010, Straiker & Mackie 2005). Another explanation for prolonged DSI after persistent inflammation is changes in Ca^{2+} handling in the postsynaptic neuron. To further understand this process, we could conduct DSI experiments with varying concentrations of internal Ca^{2+} or Ca^{2+} chelators in naïve tissue. It would also be interesting to do Ca^{2+} imaging DSI studies with tissue from naïve and CFA-treated animals. If there is a change in Ca^{2+} handling after persistent inflammation, the same DSI protocol would result in increased intracellular Ca^{2+}. This mechanism could also explain the increase in the proportion of cells that exhibit DSI after persistent inflammation.

5.4 Overall Methodological Limitations

Slice Electrophysiology

If I have learned anything throughout the course of this dissertation, it is that slice electrophysiology is an incredibly useful tool to understand synaptic physiology. That said, there are important limitations to this technique that must be acknowledged when considering slice electrophysiological data.

The first methodical consideration is the preparation of brain tissue. When preparing the slices, we cut right through the axons that are traveling into our region of interest from regions outside of the slice. This is considered when determining the optimal slicing protocol. In the Ingram lab, we slice the vlPAG coronally which keeps somas and intact axons of any neurons that are within 220μm of our recording cell but severs many others. This is especially important when thinking about the mechanisms determining presynaptic function. It is never clear whether the soma of the presynaptic terminals releasing GABA that we are measuring are in our slice or not. This could have important functional consequences as far as protein trafficking.

Another consideration is slicing conditions. In the Ingram lab we slice our brains in ice-cold cutting buffer with high levels of sucrose. In our hands this yields healthy cells. Throughout the course of my PhD, I have tested multiple slicing conditions, as other labs find slicing warm more fruitful than slicing cold. I tested CB1R- and MOR-suppression of optically evoked GABA release from VTA afferents after slicing in cold

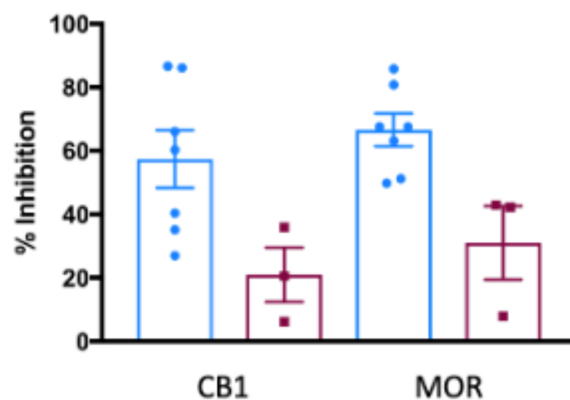

Figure 5.1 Slicing warm vs. cold

Brains from male SD rats injected with ChR2 virus (Chapter 4.1) sliced in cold sucrose solution (blue) or warm Krebs solution (red). Warm Krebs solution was tested over the course of 2 days with 2 animals. Slicing warm resulted in a reduced CB1R- and MOR-oIPSC percent inhibition. (2-way ANOVA: Main effect of slicing temperature $p = 0.007$).

sucrose cutting solution or warm Krebs solution. These experiments indicated that both CB1R and MOR have significantly reduced function in vlPAG tissue after slicing in warm Krebs solution (Fig. 5.1). A possible explanation is that sucrose, which inhibits vesicle release and was included in the cold cutting solution but not the warm, that may maintain a vesicle pool that allows us to measure changes in presynaptic release. These data were sufficient to convince me to continue slicing in cold sucrose solution, but it would be interesting to understand these differences more thoroughly. Additionally, I would like to test this in other brain regions to determine if the vlPAG is particularly sensitive to slicing conditions. It's location immediately adjacent to the aqueduct could render it more sensitive than other brain regions. Other groups slice in warm Krebs solution and do not encounter problems with GPCR function in the locus coeruleus (Adhikary et al 2022b) or dorsal striatum (Adhikary et al 2022a).

A third consideration of experimental conditions is bath temperature while recording. Many cellular functions are temperature sensitive and recording at different temperatures can induce unexpected experimental variables. GPCR activation, signaling, and trafficking are likely temperature dependent, so different recording temperatures could alter a variety of important measurements. A critical example of this regarding experiments within this dissertation is DSI. DSI lasts longer (Ohno-Shosaku et al 2002) and depolarization-induced eCBs diffuse farther (Kreitzer et al 2002) at room temperature than physiological temperature. I accidentally tested this one day when I forgot to turn on the heating mechanism that heats the external Krebs solution as it washes to my bath. Indeed, DSI lasted noticeably longer! Alas, this is only an N of 1

experiment because it was so obvious that I looked for the culprit and realized I had forgotten to turn on my heater.

Radioligand Binding Assays

Another technique that I used to study cannabinoid pharmacology is radioligand binding, a classic method used to assess receptor expression and binding kinetics. This technique is particularly useful when specific antibodies are difficult to find. A consideration, though, is the ligand used to test binding. I used [^{3}H]CP55,940, which is a non-selective cannabinoid receptor agonist and, while it is used in many studies to assess CB1R expression (Basavarajappa et al 1998, Hill et al 2008b, Hill et al 2005, Tao & Abood 1998), these results cannot determine CB1R binding levels specifically. For example, in the RVM CB1R was downregulated while the CB2 receptor was upregulated after persistent inflammation (Li et al 2017). If these changes occurred at similar levels, this alteration would not be detectable by radioligand binding with a non-specific cannabinoid receptor agonist, like CP-55,940. Since we do not see changes in CB2R function after persistent inflammation and eIPSC inhibition by CP-55,940 is reduced after persistent inflammation (Fig. 2.3), we feel confident that the radioligand binding represents lack of CB1R downregulation in the vlPAG after persistent inflammation. An alternative ligand to more accurately measure CB1R binding in brain tissue, as opposed to overall cannabinoid binding, is [^{3}H]SR141716A (RIM; Hirst et al 1996).

Another limitation of radioligand binding is the dissection and lack of regional specificity. The vlPAG is a very small brain region and was dissected out by hand. As careful as I tried to be, it is very likely that the dissections captured some nearby brain regions and missed parts of the vlPAG. Additionally, the vlPAG is so small that it required pooling tissue from 8 animals / curve. This is a lot of animals per experiment and there are likely more efficient ways to test receptor expression. Lack of regional analysis pertains to (1) pre- vs. post-synaptic receptor localization and (2) located in the synapse or internalized in vesicles. While CB1Rs are thought to be located exclusively on terminals and not on soma / dendrites, immunohistochemistry analysis suggests that CB1Rs can be located on the postsynaptic membrane in the PAG as well (Wilson-Poe et al 2012). Radioligand binding indiscriminately measures pre- and post-synaptic cannabinoid receptor binding. CB1Rs could also be in the soma if they are transcribed there and transported along the axon. Additionally, this technique would also detect internalized CB1Rs. With this technique, it is not possible to differentiate receptors at the synapse / along the axon versus internalized receptors.

5.5 Physiological Relevance

Pain

Pain is a complex sensation, encompassing emotional, nociceptive, and motivational facets. Even with the complex emotions and motivations associated with pain, similar behaviors and stages of pain behaviors can be observed from wild animals, such as a deer wounded by a hunter's bullet, to a dog injured in a fight, to a human

surprisingly hit in the head with a rock (Wall 1979). The complexity of pain makes it difficult to study in animal models; however, there are many different animal models to study the nociception that can underlie “pain” (Chapman et al 1985, Tappe-Theodor et al 2019). Nociception is the process of sensing potentially damaging stimuli, which can be chemical, mechanical or thermal. A single animal model will not perfectly encompass all nociception, but the combination of these models can start to help us understand the neural processing underlying nociceptive behaviors and, potentially, pharmacological manipulations that can help to alleviate that nociception. It is important to remember that pain itself is *adaptive* and helps animals learn to avoid danger in the future, tend to their injury, and recover. The goal of an analgesic should not be to block an animal’s ability to acknowledge a nociceptive input – as this could impair the animal’s ability to adapt and avoid further injury. The goal for future pain treatments is to reduce hyperalgesia, the increased sensitivity to nociceptive inputs, or allodynia, increased sensitivity to a stimulus that does not normally cause pain, back to baseline levels (Tappe-Theodor et al 2019).

Anecdotal evidence suggests that activation of the cannabinoid system is effective for pain relief in humans. With the increasing legalization of cannabis throughout America and the world, it is increasingly important to understand the mechanisms underlying cannabinoids in pain. Data presented throughout this dissertation indicate that inflammation *reduces* the effect of exogenous cannabinoid agonists at the CB1R within the vlPAG. If CB1R function is reduced in the descending pain modulatory pathway after persistent inflammation (Chapter 2,3, (Li et al 2017)), do systemic cannabinoids still induce anti-hyperalgesia?

Multiple exogenous cannabinoid agonists via different routes of administration induce antinociception, even after inflammation. Unlike the increased efficacy of morphine-induced antinociception after CFA treatment (Stein et al 1988, Wang et al 2006), systemic THC and anandamide administration produce equal levels of anti-nociception in CFA- and saline-treated male rats 19 days after CFA injection (Smith et al 1998). THC administered orally also induces anti-hyperalgesia at low doses and antinociception at high doses, in both rats and mice (Sofia et al 1973). Systemic or local THC induces dose-dependent anti-hyperalgesia and anti-allodynia in CFA-treated male and female rats 1, 3, and 7 days after CFA injection (Craft et al 2013). Similar levels of anti-hyperalgesia induced by local and systemic THC indicate that THC may be acting locally at the level of the inflammation as opposed to supraspinal (Craft et al 2013). WIN administered intrathecally 24 hours after CFA hindpaw injection induces anti-allodynia in male rats (Martin et al 1999). The anti-hyperalgesia effects of cannabinoids generalize beyond CFA-induced inflammation, as capsaicin-induced hyperalgesia can also be reversed by intravenous administration of the synthetic agonist, WIN (Li et al 1999). Additionally, WIN reduces hyperalgesia when administered intraperitoneally, decreasing neuropathic hyperalgesia to thermal and mechanical stimuli (Herzberg et al 1997)). Collectively, these data highlight cannabinoids efficacy for reducing hyperalgesia and allodynia when delivered systemically. Further investigation into how cannabinoids specifically target the pain modulatory circuit illustrates a more complicated picture.

Systemic cannabinoids can exert actions through supraspinal pain modulatory networks. Transection of the lumbar spinal cord blocks WIN suppression of heat-evoked firing to systemic cannabinoid administration, as measured *in vivo* in the thoracic spinal

cord, indicating that systemic cannabinoid-induced analgesia is dependent on supraspinal mechanisms (Hohmann et al 1999). The PAG itself has also been implicated in cannabinoid-induced antinociception. Activating cannabinoid receptors in the dorsal PAG reduces acute formalin--induced pain behaviors in adult male Sprague Dawley rats and is blocked by co-injection of rimonabant (Finn et al 2003). Furthermore, activation of cannabinoid receptors with the non-selective cannabinoid receptor agonist, CP-55,940, in the vlPAG induces antinociception (Lichtman et al 1996). However, consistent THC-induced anti-allodynia and anti-hyperalgesia 1, 3, and 7 days after CFA (Craft et al 2013) is incongruent with CB1R function in the vlPAG after CFA (Chapter 2). This suggests that systemic cannabinoid agonists do not require activation of CB1Rs in the vlPAG or RVM to exert anti-hyperalgesia after CFA injection, because CB1R response to the exogenous agonists that we tested in these regions is significantly reduced (Chapter 2, 3, Li et al 2017). Alternatively, reduced CB1R suppression of GABA release after persistent inflammation (~30% of naive) could be sufficient to induce antinociception with systemic cannabinoids. Most likely, cannabinoids administered systemically act in a variety of tissues on a variety of receptors (Hempel & Xi 2022) to exert anti-nociception and anti-hyperalgesia.

While cannabis has been used for years for pain relief and anecdotal evidence indicates antinociceptive properties, the mechanisms of action are not well understood. Emerging evidence indicates that cannabinoids only produce modest antinociception (Johnson et al 2010, Lichtman et al 2018), especially when compared to opioids (Maguma & Taylor 2011). This is surprising, given the similarities between MOR and CB1R within the vlPAG. MORs and CB1Rs both suppress presynaptic GABA release

(Chieng & Christie 1994b, Vaughan et al 2000, Vaughan et al 1997a), a process that is thought to be critical to opioid-induced analgesia (Lamberts et al 2013, Lau & Vaughan 2014, McPherson et al 2018). Activation of MOR in the vlPAG are necessary (Bernal et al 2007, Lane & Morgan 2005) and sufficient (Bodnar et al 1990, Jacquet & Lajtha 1974, Jensen & Yaksh 1986, Mehalick et al 2013, Morgan et al 2009, Morgan et al 1998, Zambotti et al 1982) to induce analgesia; therefore, it is surprising that cannabinoids in the vlPAG would not be important for analgesia. The fact that the overwhelming public perception is that cannabinoids are effective for pain, in addition to the recent rapid changes in legalization of cannabis and its constituents throughout the United States and other countries, highlights the need for further study of endocannabinoids and their receptors, and plasticity within the endocannabinoid system in different chronic pain states.

Functional Relevance

The robust adaptations in CB1R signaling after persistent inflammation do not appear to impact cannabinoid-induced analgesia, so what is the functional relevance of this adaptation? As discussed earlier, the PAG is an integration center that processes physiological threats including pain, stress, and fear and prompts appropriate escape behavior. The vlPAG, in particular, is responsible for passive coping behavior in response to these physiological threats (Keay & Bandler 2001). Therefore, reduced CB1R function in the vlPAG could be involved in a multitude of behavioral outcomes. Since we do not observe consistent eCB tone in naïve conditions, reduced CB1R function does not impact basal GABAergic tone in the vlPAG. Therefore, the impact of

functionally reduced CB1Rs after inflammation may not be apparent until the system is tested. In other words, this adaptation may shift an animal's escape behavior in response to a stressor. Importantly, eCBs still induce suppression of inhibition after inflammation so actions involving eCBs, such as stress-induced analgesia, could still occur but behavioral outcomes to exogenous cannabinoids is likely different. Additionally, it is not clear if the increased eCB tone and CB1R desensitization within the vlPAG after persistent inflammation are adaptive or maladaptive.

5.6 Conclusions

The data presented throughout this dissertation highlight adaptations in the endogenous cannabinoid system after persistent inflammation. These adaptations alter the regulation of multiple components of the otherwise tightly controlled system and appear to selectively effect cannabinoids. This begs the question: what exactly are cannabinoids doing in the vlPAG and how does this altered regulation change that function? While we don't know the answer to that question, hopefully the data presented in this dissertation provides a piece of the puzzle. The cannabinoid system is vast, unique, and mysterious-- there is so much left to uncover.

Appendix A: RGS-insensitive mice define roles of μ-opioid receptor (MOR)-Gαo and Gαi subunit coupling in inhibition of presynaptic GABA release

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A.1 Background

In addition to investigating regulation of the presynaptic CB1R, I helped on a project investigating Regulators of G protein Signaling (RGS) proteins on presynaptic MOR suppression of GABA release within the vlPAG. Agonist activation of MOR initiates G protein signaling with the exchange of GTP for GDP on the intracellular Gα subunit. Gα then dissociates from G$\beta\gamma$, both of which activate downstream effectors. RGS proteins act as GTPase-accelerating proteins (GAPs), hydrolyzing GTP on active Gα subunits. This yields Gα bound to GDP, which then re-associates with G$\beta\gamma$ and terminates signaling (for review see (Traynor 2010)). Consistent with the theme running through this dissertation, this project sought to understand RGS regulation of MOR signaling in the presynaptic terminal. These studies investigate RGS actions at MOR using transgenic animals with a genetic knock-in mutation in Gα_o that renders it insensitive to RGS hydrolysis. Previous research using this transgenic mouse line show increased MOR-induced suppression of GABA release in the vlPAG (Lamberts et al 2013), consistent with the role of RGS proteins as negative regulators of GPCR signaling. Interestingly, a recent publication out of the Ingram lab showed an *opposite* effect on postsynaptic MORs: MOR-induced signaling to GIRK channels was reduced in the transgenic mouse line (McPherson et al 2018). In the following study, we further probed presynaptic MOR signaling using the RGS-insensitive transgenic mouse line as well as peptide inhibitors that block Gα_o or Gα_i signaling. We investigated MOR signaling with different MOR agonists and two different modes of GABA release using both evoked- and spontaneous, miniature inhibitory postsynaptic currents.

A.2 Abstract

Regulators of G protein signaling (RGS) proteins modulate signaling by G protein-coupled receptors (GPCRs). Using a knock-in transgenic mouse model with a mutation in Gαo that does not bind RGS proteins (RGS-insensitive), we determined the effect of RGS proteins on presynaptic mu opioid receptor (MOR)-mediated inhibition of GABA release in the ventrolateral periaqueductal gray (vlPAG). The MOR agonists [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO) and met-enkephalin (ME) inhibited evoked inhibitory postsynaptic currents (eIPSCs) in the RGS-insensitive mice compared to wildtype (WT) littermates, respectively. Fentanyl inhibited eIPSCs similarly in both WT and RGS-insensitive mice. There were no differences in opioid agonist inhibition of spontaneous GABA release between the genotypes. To further probe the mechanism underlying these differences between opioid inhibition of evoked and spontaneous GABA release, specific myristoylated Gα peptide inhibitors for Gαo_1 and Gαi_{1-3} that block receptor-G protein interactions were used to test the preference of agonists for MOR-Gα complexes. The Gαo_1 inhibitor reduced DAMGO inhibition of eIPSCs but Gαi_{1-3} inhibitors had no effect. Both Gαo_1 and Gαi_{1-3} inhibitors separately reduced fentanyl inhibition of eIPSCs, but had no effects on ME inhibition. Gαi_{1-3} inhibitors blocked the inhibitory effects of ME and fentanyl on mIPSC frequency, but both Gαo_1 and Gαi_{1-3} inhibitors were needed to block the effects of DAMGO. Finally, baclofen-mediated inhibition of GABA release is unaffected in the RGS-insensitive mice and in the presence of Gαo_1 and Gαi_{1-3} inhibitor peptides suggesting that $GABA_B$ receptor coupling to G proteins in vlPAG presynaptic terminals is different than MOR coupling.

A.3 Significance statement

Presynaptic mu opioid receptors (MORs) in the ventrolateral periaqueductal gray (vlPAG) are critical for opioid analgesia and are negatively regulated by RGS proteins. These data in RGS-insensitive mice provide evidence that MOR agonists differ in preference for Gαo versus Gαi and regulation by RGS proteins in presynaptic terminals, providing a mechanism for functional selectivity between agonists. The results further define important differences in MOR and $GABA_B$ receptor coupling to G proteins that could be exploited for new pain therapies.

A.4 Introduction

Regulators of G protein signaling (RGS) proteins accelerate the hydrolysis of GTP to GDP, terminating G protein signaling. These proteins comprise a large family of proteins that differ in structure and function and are expressed in various tissues with overlapping distributions (Traynor & Neubig 2005). RGS proteins bind to active G proteins to regulate both temporal and spatial signaling to downstream effectors (Hollinger & Hepler 2002, Neubig 2015). In addition, RGS proteins recognize specific Gα proteins (Masuho et al 2020) highlighting the importance of understanding RGS-Gα interactions within discrete neural circuits. Specific RGS protein knockout mouse models have been generated to probe regulation of GPCR signaling by RGS proteins but there is evidence of strong compensation by redundant RGS proteins in various knockout lines (Grillet et al 2005). To circumvent this issue, we use a mutant mouse line that has a knock-in mutation in the Gαo subunit (G184S) that does not bind to any RGS proteins (RGS-insensitive) (Goldenstein et al 2009).

Mu opioid receptors (MORs) are GPCRs that typically couple to inhibitory G proteins, including Gαo and Gαi subunits (Gaibelet et al 1999). However, opioid analgesia is dependent on MOR coupling to Gαo, but not Gαi (Lamberts et al 2011, Lamberts et al 2013). In addition, different MOR agonists preferentially bind MORs coupled to specific G protein subunits (Clark et al 2006, Massotte et al 2002). This differential coupling constitutes one determinant of functional selectivity of opioid agonists and differential activation of G proteins by MOR agonists could have important impacts in understanding opioid-mediated behaviors. The ventrolateral periaqueductal gray (vlPAG) is a supraspinal site important for opioid-induced analgesia. MORs expressed postsynaptically on a subpopulation of vlPAG neurons are coupled to GIRK channels that hyperpolarize the cells (Chieng & Christie 1994a, Ingram et al 2007, Ingram et al 2008). We observed in our prior studies that MOR coupling to GIRK channels was reduced in the RGS-insensitive mice indicating that RGS proteins support signaling to some effectors (McPherson et al 2018), in addition to their well-known negative regulation via GTPase accelerating activity (Clark et al 2003, Clark et al 2008, Lamberts et al 2013). High efficacy synthetic agonists DAMGO and fentanyl were less effective in the RGS-insensitive mice but the GIRK currents induced by the peptide agonist met-enkephalin (ME) were unaffected (McPherson et al 2018). These effects were further confirmed using selective peptide inhibitors of Gαo and Gαi subunits showing that ME-induced GIRK currents could be inhibited only with the Gαi peptide inhibitor. Taken together, these results support the idea that different opioid agonists recruit or prefer receptors bound to specific G proteins, similar to observations in cell lines (Clark & Traynor 2006, Milligan et al 1990a, Moon et al 2001). However, the loss in

MOR coupling to GIRK channels in the RGS-insensitive mice does not explain the enhanced analgesia observed in these mice (Lamberts et al 2013) so we have continued to examine presynaptic MOR signaling in the vlPAG.

MORs expressed on presynaptic terminals are coupled to phospholipase A2 resulting in inhibition of neurotransmitter release (Ingram et al 1998, Vaughan et al 1997b). RGS proteins negatively regulate presynaptic MORs that inhibit GABA release (Lamberts et al 2013) but the G proteins that are involved in presynaptic MOR signaling have not been identified previously. In these studies, we have examined several MOR agonists for their ability to activate MOR signaling via $G\alpha o$ or $G\alpha i$, using the RGS-insensitive mice to further define MOR signaling in GABAergic terminals within the vlPAG. Based on observations that RGS-insensitive mice display enhanced antinociception, we hypothesized that MOR inhibition of presynaptic GABA release is enhanced in these mice. Further, we expected to find differences between agonists in the presence of the selective $G\alpha o$ and $G\alpha i$ peptide inhibitors.

A.5 Materials and Methods

These studies used male and female heterozygous (RGS-insensitive Het) mice for a mutation in the $G\alpha o$ protein (G184S) that is insensitive to RGS protein binding (Goldenstein et al 2009) and wildtype (WT) 129S1/SvImJ littermates. Homozygous knock-in mice die *in utero*, so WT mice were compared with Het mice. WT mice were used in the studies assessing the effect of G protein peptide inhibitors. Mice were group housed with unlimited access to food and water. Lights were maintained on a 12 h light/dark cycle (lights on at 7:00 A.M.). Mice were sacrificed and cellular recordings were conducted during the light phase of this cycle. The Institutional Animal Care and

Use Committee at Oregon Health & Science University approved all experimental procedures. Experiments were conducted in accordance with the United States National Research Council *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

Electrophysiological recordings. Mice (postnatal day >25) were anesthetized with isoflurane, brains were removed, and brain slices containing the vlPAG were cut with a vibratome (180–220 μm thick) in sucrose cutting buffer containing the following (in mM): 75 NaCl, 2.5 KCl, 0.1 $CaCl_2$, 6 $MgSO_4$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 dextrose, 50 sucrose and placed in a holding chamber with artificial cerebral spinal fluid (ACSF) containing the following (in mM): 126 NaCl, 21.4 NaHCO3, 11.1 dextrose, 2.5 KCl, 2.4 CaCl2, 1.2 MgCl2, and 1.2 NaH2PO4, pH 7.35, equilibrated with 95% O2/5% CO2 until moved into a recording chamber. In experiments using myristoylated Gαo and Gαi peptide inhibitors, slices were incubated for at least 30 min in ACSF plus inhibitors (1-10 μM) before recording. Recordings were made with electrodes pulled to 2–4 MOhm resistance with an internal solution consisting of the following (in mM): 140 CsCl, 10 HEPES, 10 KCl, 1 MgCl2, 1 EGTA, 0.3 CaCl2, 4 MgATP, and 3 NaGTP, pH 7.4. Neurons were voltage-clamped at -70 mV. Junction potentials of 5 mV were corrected at the beginning of the experiments. Access resistance was monitored throughout the experiments. Data were collected with Axopatch 200B microelectrode amplifier (Molecular Devices) at 5 kHz and low-pass filtered at 2 kHz. Currents were digitized with InstruTECH ITC-18 (HEKA), collected via AxoGraph data acquisition software and analyzed using AxoGraph (Axograph Scientific). The Het mice tend to be smaller, so experimenters were not blind to genotype; however, data analysis was done blind to

genotype. In experiments using Gαo and Gαi inhibitor peptides, all mice were WT, but the analyses of peak drug effects were measured blind to slice treatment.

Reagents. [D-Ala(2),N-Me-Phe(4),Gly(5)-ol]-enkephalin (DAMGO), met-enkephalin acetate salt hydrate (ME) and fentanyl citrate salt (fentanyl) were obtained from Sigma-Aldrich, and (*R*,*S*)-baclofen and CGP 55845 hydrochloride were purchased from Abcam. Myristoylated Gα peptide inhibitors were synthesized by GenScript (Piscataway, NJ), as follows: Gαo1 (MGIANNLRGCGLY), Gαi1/2 (MGIKNNLKDCGLP), and Gαi3 (MGIKNNLKECGLT) according to sequences for mini-gene vectors designed by the Hamm laboratory (Vanderbilt University Medical Center, Nashville TN; (Gilchrist et al 2002). We were unable to obtain the peptide for $G\alpha o_2$ at sufficient purity (<60%) to use in slice experiments. The Gαi inhibitors were combined as a cocktail.

Statistical analyses. All data are expressed as mean & standard deviation (SD). Data were analyzed with Prism 9 (GraphPad Software). Each electrophysiological recording from a single neuron is treated as an individual observation because the vlPAG contains heterogenous cell populations; however, all datasets contain recordings from at least three separate animals. Drug effects were reversed by specific antagonists, and peak drug effects were measured as an increase in current from the average of baseline and washout or the presence of antagonists. Differences between groups were assessed using Student's t-test or ANOVA when appropriate (significance is denoted as *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$).

A.6 Results

Opioid inhibition of evoked GABA release

In order to test the hypothesis that RGS proteins affect opioid signaling in presynaptic terminals, we compared the ability of several opioid agonists to inhibit evoked GABAergic inhibitory postsynaptic currents (eIPSCs) in vlPAG neurons of wildtype (WT) and RGS-insensitive Het mice. The studies used concentrations of opioid agonists that were consistent with our previous study examining opioid activation of GIRK channels in the vlPAG (McPherson et al 2018). For comparison, we also tested maximal concentrations of each agonist so we could assess the efficacy of each agonist at inhibiting presynaptic GABA release (Fig. A. 1). The maximal % inhibition was the same for all three opioid drugs. Het mice had similar effects to WT mice at the maximal concentration (mean ± SD; DAMGO: 54 ± 5%, n = 6; ME: 67 ± 28%, n = 5; fentanyl: 55 ± 10%, n = 4). Interestingly, differences between WT and RGS-insensitive Het mice appeared at sub-maximal concentrations of these MOR agonists. The selective MOR agonist DAMGO inhibited eIPSCs 37% more in neurons from the RGS-insensitive Het mice (95% CI = 54 to 73; Fig A. 1A,B). The nonselective agonist ME inhibited the eIPSCs 88% more in cells from the RGS-insensitive Het mice (95% CI = 38 to 52; Fig. A.1C). These results are consistent with our prior report showing an increase in morphine and ME inhibition of eIPSCs in RGS-insensitive Het mice (Lamberts et al 2013). In contrast, fentanyl inhibited GABAergic eIPSCs similarly in neurons from both WT and RGS-insensitive Het mice (Fig. A.1D).

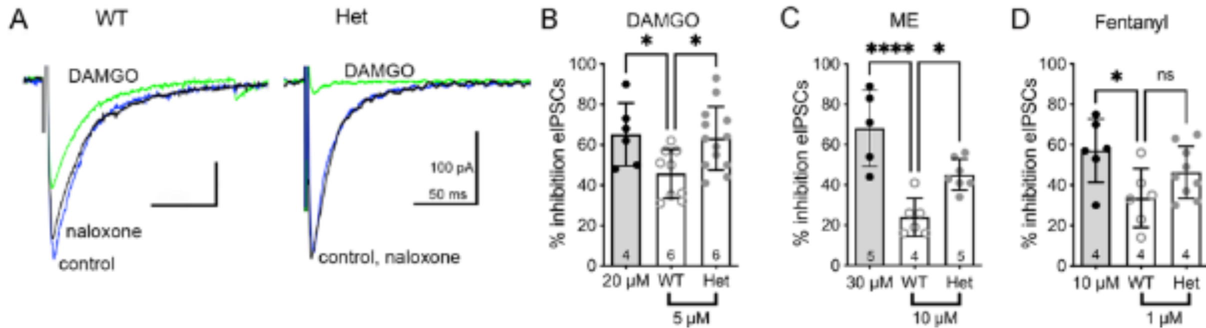

Figure A.0.1 Opioid inhibition of evoked IPSCs are differentially affected in RGS-insensitive mice

A. Representative traces depicting inhibition of eIPSCs by DAMGO (5 µM) in wildtype (WT) and RGS-insensitive (Het) mice. The inhibition is reversed by naloxone. B. Combined experiments of % inhibition (± SD) by a maximal DAMGO concentration (20 µM; gray bar) and a sub-maximal concentration (5 µM) in WT compared to Het mice (One way ANOVA; $F_{(2, 25)} = 4.9$, $p = 0.02$; Dunnett's, *$p < 0.05$). C. Combined experiments of % inhibition (± SD) by a maximal met-enkephalin concentration (30 µM; gray bar) and a sub-maximal concentration (10 µM) in WT compared to Het mice (One way ANOVA; $F_{(2, 15)} = 17.7$, $p = 0.0001$; Dunnett's, *$p < 0.05$; ****$p < 0.0001$). D. Combined experiments of % inhibition (± SD) by a maximal fentanyl concentration (10 µM; gray bar) and a sub-maximal concentration (1 µM) in WT compared to Het mice (One way ANOVA; $F_{(2, 18)} = 4.1$, $p = 0.03$; Dunnett's, *$p < 0.05$). Symbols in bars denote recordings and numbers denote number of animals used in each group.

In a subset of experiments, a paired pulse protocol (2 stimuli, 50-100 ms apart) was used to examine the probability of GABA release from presynaptic terminals in WT and RGS-insensitive Het mice. Paired-pulse ratios (PP ratio = P2/P1) for eIPSCs in slices from Het mice (0.6 ± 0.2; n = 8) were lower than WT mice (1.1 ± 0.1; n = 12; $t_{(18)}$ = 2.2, *p = 0.04). A lower PP ratio indicates a higher release probability in the RGS-insensitive Het mice. This change in release probability could be due to changes in endogenous opioid tone, so we tested whether an endogenous opioid tone could be measured in slices from either the WT or RGS-insensitive Het mice using naloxone (5 – 10 µM). Spontaneous IPSCs (in the absence of tetrodotoxin) were similar in the absence and presence of naloxone for both genotypes (% change ± SD in naloxone for WT: 103 ± 20%; One sample t-test, t_5 = 0.4, p = 0.7 and Het: 95 ± 19%; One-sample t-test, t_6 = 0.8, p = 0.5) indicating a lack of endogenous opioid tone in either genotype.

In order to determine whether the difference between the agonists in the RGS-insensitive mice was due to a preference for Gαo versus Gαi subunits coupling to MORs in presynaptic terminals, selective peptide inhibitors of each subunit binding sites were tested. Inhibitor peptides corresponding to the carboxy terminal amino-acids of the Gα subunit compete for binding to the receptor, inhibiting activation of the G proteins (Gilchrist et al 2002). The effect of DAMGO was reduced in the presence of the Gαo inhibitor but the Gαi inhibitor had no effect (Fig. 2A). Adding all of the peptide inhibitors together essentially abolished DAMGO-mediated inhibition of eIPSCs. Neither the Gαo or Gαi peptide inhibitors significantly reduced ME inhibition (Fig. 2B); however the combined inhibitors also significantly reduced ME inhibition of eIPSCs. Finally, both Gαo and Gαi inhibitor peptides superfused alone reduced fentanyl inhibition (Fig. 2C).

These data suggest that DAMGO preferentially activates MOR-Gαo in presynaptic terminals in the vlPAG, but ME and fentanyl are less selective.

Male and female mice were used throughout the studies. DAMGO recordings from WT mice had approximately equal numbers of recordings from male and female mice with similar inhibition in both sexes (males: 49 ± 10% (SD); n = 8 versus females: 41 ± 10% (SD), n = 7; $t_{(13)} = 1.4$, $p = 0.19$). In addition, no noted differences were observed with the other agonists. The lack of sex differences is consistent with our prior study (McPherson et al 2018).

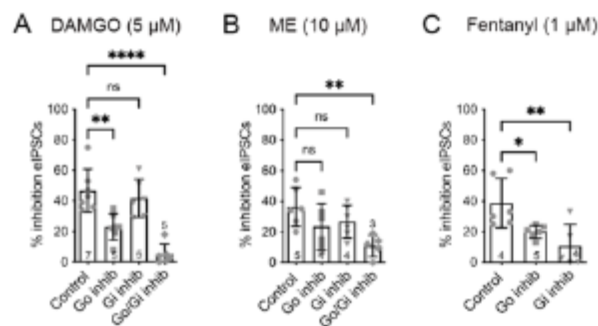

Figure A.0.2: MOR agonists differentially activate Gα subunits to inhibit evoked GABA release

A. DAMGO (5 µM)-mediated inhibition of eIPSCs in the absence (control) and presence of Gαo peptide inhibitor and Gαi peptide inhibitors (One way ANOVA, $F_{(3, 22)} = 19.1$, $p = 0.0001$; Dunnett's, **$p < 0.001$, ****$p < 0.0001$). B. ME (10 µM)-mediated inhibition of eIPSCs in absence and presence of inhibitors (One way ANOVA, $F_{(3, 19)} = 4.2$; $p = 0.02$; Dunnett's, **$p < 0.001$). C. Fentanyl (1 µM)-mediated inhibition of eIPSCs in absence and presence of inhibitors (One way ANOVA, $F_{(2, 14)} = 7.3$, $p = 0.007$, Dunnett's, *$p < 0.05$, ** $p < 0.001$). Symbols in bars denote recordings and numbers denote number of animals used in each bar.

Opioid inhibition of spontaneous GABA release

The change in PP ratio indicated that the RGS-insensitive Het mice have altered evoked GABA release so we were interested in whether spontaneous release (in the presence of TTX) was altered in the knock-in mouse line. Inter-event intervals of mIPSCs measured in the presence of NBQX (5 μM) and TTX (500 nM) were similar in WT (0.33 ± 0.22 s (SD), n = 27) and Het mice (0.24 ± 0.15 s (SD), n = 27; $t_{(52)} = 1.6$, $p = 0.1$).

All three opioid agonists inhibited spontaneous mIPSC frequency to similar degrees in both the WT and Het mice (Fig. 3). The mIPSC amplitude, as well as rise and decay kinetics (data not shown) were also not different between agonists indicating that the opioid modulation of mIPSC frequency was due to presynaptic modulation of release. There were no sex differences in the amount of inhibition induced by any of the 3 opioid agonists (data not shown). These results suggest that either RGS proteins have little impact on opioid modulation of spontaneous release in presynaptic terminals or that inhibition of spontaneous release is not dependent on Gαo since the RGS-insensitive knock-in mutation is specific for Gαo. In order to test whether MOR-Gαi coupling is involved in opioid inhibition of spontaneous GABA release in WT mice, we examined the effects of the specific Gαo and Gαi peptide inhibitors. Neither of the inhibitors superfused alone reduced inhibition of mIPSC frequency by DAMGO (Fig. 4A), but the inhibitors applied to slices together reduced DAMGO-mediated inhibition by 83% (95% CI = -5 to 27) compared to control. In contrast, inhibition by ME and fentanyl was reduced in the presence of the Gαi inhibitors but unaffected in the presence of the Gαo inhibitor peptide (Fig. 4B,C). These results are consistent with results in the RGS-

insensitive Het mice which have a mutation specifically in Gαo that perturbs RGS protein binding and subsequent GTP-hydrolysis and indicate that MOR-Gαi coupling is important for the inhibition of spontaneous GABA release.

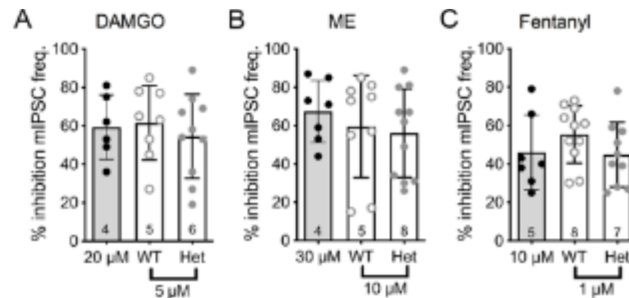

Figure A.0.3 Opioid inhibition of GABAergic mIPSCs is not altered in RGS-insensitive mice

A. Combined experiments of % inhibition (± SD) by a maximal DAMGO concentration (20 μM; gray bar) and a sub-maximal concentration (5 μM) in WT compared to Het mice (One way ANOVA; $F_{(2, 21)} = 0.3$, $p = 0.8$). B. Combined experiments of % inhibition (± SD) by a maximal met-enkephalin concentration (ME 30 μM; gray bar) and a sub-maximal concentration (10 μM) in WT compared to Het mice (One way ANOVA, $F_{(2, 24)} = 0.5$, $p = 0.6$). C. Combined experiments of % inhibition (± SD) by by a maximal fentanyl concentration (10 μM; gray bar) and a sub-maximal concentration (1 μM) in WT compared to Het mice (One way ANOVA; $F_{(2, 25)}= 1.2$, $p = 0.3$). Symbols in bars denote recordings and numbers denote number of animals used in each bar.

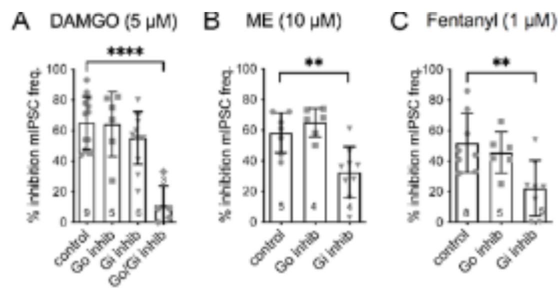

Figure A.0.4 MOR-Gαi coupling is more important for inhibition of spontaneous GABA release

A. Inhibition of mIPSCs by DAMGO (5 μM) is unaffected by Gαo and Gαi inhibitors (One way ANOVA; $F_{(3, 32)} = 12.6$, $p = 0.0001$; Dunnett's, ****$p = 0.0001$). B. Inhibition by ME is reduced in the presence of Gαi inhibitors, but not by the Gαo inhibitor ($F_{(2, 19)} = 11.8$, $p = 0.001$, Dunnett's, **$p < 0.01$). C. Inhibition by fentanyl is reduced in the presence of Gαi inhibitors, but not by the Gαo inhibitor ($F_{(2, 19)} = 6.2$, $p = 0.01$, Dunnett's, **$p < 0.01$). Symbols in bars denote recordings and numbers denote number of animals used in each bar.

$GABA_B$-mediated inhibition of GABA release is unaffected by Gαo or GαI peptide inhibitors

Our previous study found no difference in the amount of inhibition of evoked GABA release induced by a maximal concentration of the $GABA_B$ agonist baclofen (20 μM) between slices from WT and RGS-insensitive Het mice (McPherson et al 2018). Because RGS proteins have less influence on high efficacy agonists, especially at maximal concentrations (Clark et al 2008), we repeated the studies using a lower concentration of baclofen (5 μM). This concentration of baclofen also inhibited evoked GABA release to a similar extent in slices from the two genotypes (Fig. 5A). Consistent with these results, incubation of slices in the Gαo and Gαi inhibitors did not alter the responses to either concentration of baclofen (5 μM or 20 μM; Fig. 5B and 5C,

respectively). Baclofen was typically tested on the same cells before or after an opioid response that was affected by either the Gαo or Gαi inhibitor indicating that these peptide inhibitors were effective in blocking binding of the Gα subunits in a given experiment and providing positive controls.

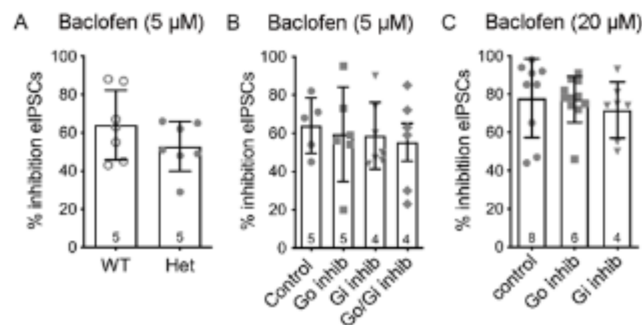

Figure A.0.5 Baclofen-mediated inhibition of evoked GABA release is not affected in slices from RGS-insensitive mice or by Gαo/i peptide inhibitors

A. Baclofen (5 μM) inhibition is similar in wildtype (WT) and RGS-insensitive (Het) mice ($t_{(12)}$ = 1.3, p = 0.2). B. Bacofen (5 μM)-mediated inhibition is not altered in the presence of peptide inhibitors ($F_{(3, 20)}$ = 0.2, p = 0.9). C. Baclofen (20 μM)-mediated inhibition is not altered by the peptide inhibitors ($F_{(2, 24)}$ = 0.3, p = 0.7). Symbols in bars denote recordings and numbers denote number of animals used in each bar.

Both concentrations of baclofen were also tested for inhibition of spontaneous release of GABA (Fig. 6). The data show that baclofen inhibition of mIPSC frequency is similar in both the WT and RGS-insensitive Het mice and the inhibition is unaffected by the Gαo and Gαi peptide inhibitors. Similar results were obtained at both 5 and 20 μM concentrations of baclofen.

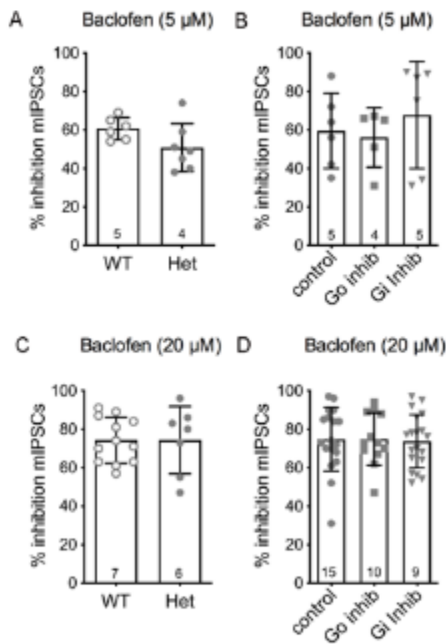

Figure A.6 Baclofen-mediated inhibition of spontaneous GABA release in slices is not affected in RGS-insensitive mice or in the presence of Gαo/i peptide inhibitors

A. Baclofen (5 µM) inhibition is similar in wildtype (WT) and RGS-insensitive (Het) mice ($t_{(11)} = 1.8$, $p = 0.1$). B. Bacofen (5 µM)-mediated inhibition is not altered in the peptide inhibitors ($F_{(2, 14)} = 0.4$, $p = 0.7$). C. Baclofen (20 µM) inhibition is similar in wildtype (WT) and RGS-insensitive (Het) mice ($t_{(17)} = 0.005$, $p = 1.0$). B. Bacofen (20 µM)-mediated inhibition is not altered in the peptide inhibitors ($F_{(2, 46)} = 0.03$, $p = 1.0$). Symbols in bars denote recordings and numbers denote number of animals used in each bar.

A.7 Discussion

These studies used a transgenic knock-in mutant mouse model with a mutation in Gαo (G148S) that blocks RGS protein binding (Goldenstein et al 2009). The advantage of this model is that it is unbiased with regard to RGS protein subtypes because compensatory expression of RGS proteins can obscure RGS regulation in knockout mice (Grillet et al 2005). Opioid analgesia is reduced in Gαo knockout mice (Lamberts et al 2011) providing evidence that MOR couples to Gαo in analgesia pathways. Consistent with the knockout data, RGS-insensitive Het mice display enhanced supraspinal morphine analgesia (Lamberts et al 2013). Since MOR inhibition of GABA release in the vlPAG is important for opioid analgesia (Bobeck et al 2014, Budai & Fields 1998, Moreau & Fields 1986, Vaughan & Christie 1997b), we expected

that inhibition of GABA release by opioid agonists would be increased in the RGS-insensitive Het mice. Importantly, we observed differences between MOR agonists in the RGS-insensitive Het mice suggesting that RGS regulation plays a role in functional selectivity of MOR agonists. In addition, MORs activate different $G\alpha$ subunits to inhibit evoked and spontaneous GABA release. Finally, $GABA_B$-mediated inhibition of GABA release is not altered in the Het mice and is unaffected by peptide inhibitors of either $G\alpha o$ or $G\alpha i$ subunits.

We first examined opioid inhibition of evoked GABA release in the vlPAG. DAMGO and ME, but not fentanyl, inhibited GABA release more in the RGS-insensitive Het mice. Since sub-maximal concentrations were used for each of the agonists, the lack of increase with fentanyl in the recordings from RGS-insensitive Het mice was not attributed to a ceiling effect. These data are consistent with recent data showing that inhibition of RGS4 in the PAG enhanced morphine, but not fentanyl, antinociception (Morgan et al 2020). There is evidence that RGS protein GTPase accelerating activity is more evident with low compared to high efficacy MOR agonists (Clark et al 2008); however, the maximal inhibition by all agonists was comparable. Thus, the differences between agonists in inhibiting GABA release in the two genotypes are likely due to a different mechanism, such as the ability of fentanyl-bound MORs to couple to $G\alpha i$.

MORs activate pertussis-toxin (PTX)-sensitive $G\alpha o$ and $G\alpha i$ subunits (Williams et al 2013). Analgesia induced by morphine (Lutfy et al 1991, Parenti et al 1986, Shah et al 1994) and DAMGO (Sanchez-Blazquez & Garzon 1988) is reduced in the presence of PTX. In order to probe the signaling of specific G proteins in vlPAG presynaptic terminals further, we used myristoylated peptide inhibitors of $G\alpha o$ and $G\alpha i$ subunits.

Incubation of slices with the Gαo peptide inhibitor reduced the inhibition by DAMGO and fentanyl, but not ME. Gαo inhibition of Ca^{2+} channels is more potent than Gαi (Hescheler et al 1987), and there are differences in coupling between Gα subunits and effectors (McKenzie & Milligan 1990, Milligan et al 1990a, Milligan et al 1990b, Moon et al 2001). Thus, it is reasonable that inhibition by DAMGO and fentanyl was reduced by the Gαo peptide inhibitor since evoked release is dependent on voltage-gated Ca^{2+} channels (Hubbard et al 1968). Incubation of slices with Gαi peptide inhibitors reduced fentanyl, but not DAMGO or ME, inhibition of eIPSCs. These results indicate that coupling to Gαi subunits is equally effective at inhibiting eIPSCs in the vlPAG, and that DAMGO and fentanyl form different MOR complexes in presynaptic terminals. Combining the peptide inhibitors reduced the effects of both DAMGO and ME compared to incubating slices in either inhibitor alone. Together with the knowledge that only small differences exist in the potency of DAMGO to stimulate different Gαi versus Gαo subtypes (Clark et al 2006), these results indicate there is redundancy of Gi/o proteins for activation by MOR. It is interesting to note that the endogenous peptide ME is less sensitive to both peptide inhibitors given alone compared to DAMGO and fentanyl suggesting that ME-bound MORs couple equally well to $G\alpha o_1$ and $G\alpha i_{1-3}$ subunits. The data highlight the importance of G protein subunit expression and levels as a factor in MOR coupling to effectors (Connor & Christie 1999).

A surprising finding in these studies was the difference in G protein subunits involved in MOR inhibition of spontaneous GABA release. Inhibition by all three opioid agonists was similar in both the WT and RGS-insensitive Het mice, and the Gαo peptide inhibitor did not affect opioid inhibition of mIPSC frequency. Instead, the $G\alpha i_{1-3}$

peptide inhibitors applied alone decreased inhibition by ME and fentanyl without affecting DAMGO-mediated inhibition. However, DAMGO inhibition was reduced in the presence of all inhibitors. This pattern supports the results with DAMGO on evoked release and further suggests that DAMGO preferentially couples to MOR-Gαo subunits (Chakrabarti et al 1995, Clark et al 2006, Laugwitz et al 1993). The data presented here indicate that opioid inhibition of spontaneous release is mediated by Gαi subunits, explaining why opioid inhibition of spontaneous GABA release was unaffected in the RGS-insensitive Het mice. Thus, these studies are not able to determine if RGS proteins regulate MOR inhibition of spontaneous release. The molecular mechanisms involved in MOR regulation of spontaneous release are not completely understood but there is data to support direct G protein $\beta\gamma$ subunit regulation of release machinery (Zurawski et al 2016, Zurawski et al 2019).

GABA$_B$ receptors also readily inhibit evoked and spontaneous GABA release in the vlPAG (Vaughan et al 1997b). In the RGS-insensitive Het mice, baclofen inhibited both evoked and spontaneous GABA release similarly to WT mice. Since the Gαo and Gαi peptide inhibitors were ineffective at blocking baclofen inhibition, even when applied together, we are not able to make a statement regarding the ability of RGS proteins to modulate GABA$_B$ signaling. The results are interesting considering data that GABA$_B$ coupling to voltage-gated Ca^{2+} channels is abolished by PTX (Connor & Christie 1998). However, GABA$_B$-Gi protein coupling has different structural features compared to other GPCR classes. Agonists at this receptor do not induce outward movement of transmembrane domain 6 to provide a cavity for the binding of the C-terminus of the G proteins (Shen et al 2021). Consequently, the peptide inhibitors used in this study,

designed to mimic the $G\alpha$-C-terminal interaction with the receptor core, may not bind to the $GABA_B$ receptor to block G protein binding. Alternatively, $G\alpha z$ is a G protein with 60% sequence homology to the Gi family (Tsu et al 1997), is densely expressed in the vlPAG and couples to MOR (Garzon et al 1998, Garzon et al 2005, Gaspari et al 2018). The $G\alpha z$ residues that bind to MOR have not been identified so it is possible the peptide inhibitors would not block $G\alpha z$ coupling to MOR or $GABA_B$ receptors, especially given substitution of tyrosine in the place of the PTX-sensitive cysteine in the $G\alpha z$ C-terminus.

The descending pain modulatory pathway is sexually dimorphic (Loyd et al 2008a, Loyd & Murphy 2006, Loyd & Murphy 2014) and MOR agonists are more efficacious in males than females (Fullerton et al 2018). There were no sex differences in either genotype in opioid agonist inhibition of evoked and spontaneous GABA release. Thus, sex differences in opioid signaling are not explained by RGS-mediated regulation of signaling, at least via $G\alpha o$ subunits. This is consistent with the lack of sex differences in MOR coupling to GIRK channels in the WT and RGS-insensitive Het mice (McPherson,et al., 2018).

Our results showing enhanced MOR inhibition of presynaptic GABA release by several opioid agonists in the RGS-insensitive mice, in addition to morphine which we examined in our previous paper (Lamberts, et al., 2013), provide a mechanism for the increase in opioid antinociception on the supraspinal hot plate test observed in RGS-insensitive mice (Lamberts, et al., 2013). There is substantial evidence that opioid inhibition of GABA release in the vlPAG activates descending pain modulatory circuits that produce analgesia (Cheng et al 1986, Moreau & Fields 1986). We previously reported that postsynaptic MOR coupling to GIRK channels is reduced in RGS-

insensitive mice (McPherson, et al., 2018), possibly through loss of a scaffolding function of RGS proteins (Zhong et al 2003). Although it is tempting to argue that postsynaptic MORs in the vlPAG do not play a role in opioid-induced antinociception, an equally valid interpretation is that MOR coupling to GIRK channels opposes supraspinal antinociceptive circuits and removal of this MOR signaling supports opioid analgesia in the RGS-insensitive Het mice (Lamberts et al 2013). Indeed, blocking both GIRK channels and presynaptic MOR signaling decreases morphine antinociception (Morgan et al 2020). Inhibition of RGS4 in the vlPAG enhances opioid-induced antinociception suggesting that RGS4 may play an important role in regulating presynaptic MOR signaling through Gαo. However, RGS gene expression in the PAG includes RGS4, RGS7, RGS8, RGS10, RGS17 and RGS20 (https://alleninstitute.org/legal/citation-policy/ Allen Brain Atlas), and these RGS proteins bind preferentially to different G proteins (Masuho et al 2020). Thus, additional RGS proteins may also regulate opioid analgesia through MOR coupling in the PAG.

A.8 Acknowledgments

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Appendix B: Recipes

Sucrose-based Cutting Buffer

1. Add the following to 400mL MilliQ H_2O for a final volume 500mL Cutting Buffer:
    a. 14 g sucrose (80 mM)
    b. 1 g $NaHCO_3$ (25 mM)
    c. 0.25 g D-Glucose (2.5 mM)
    d. 50 mL 10 Cutting solution (1 L recipe below)
        i. 43.8 g NaCl (75 mM)
        ii. 1.9 g KCl (2.5 mM)
        iii. 7.3 g $MgSO_4$ (6 mM)
        iv. 0.15 g $CaCl_2$*$2H_2O$ (0.1 mM)
        v. 1.7 g NaH_2PO_4*H_2O (1.2 mM)
2. Bring final volume to 500mL with MilliQ H_2O.
3. Check osmolarity and adjust to 290 $\pm$ 5 mOsm.
4. Split into 2 x 250mL containers. Store at 4°C overnight. Chill in -20°C freezer for ~30 minutes before use.

Krebs artificial cerebrospinal fluid (aCSF; External solution)

1. Add the following to 1L MilliQ H2O to obtain 2L modified Krebs buffer:
    a. 8.0 g D-Glucose (22mM)
    b. 3.6 g $NaHCO_3$ (25 mM)
    c. 200 mL 10x stock solution (4 L 10x stock recipe below):

        i. 294 g NaCl (126mM)
        ii. 7.4 g KCl (2.5 mM)
        iii. 5.8 g MgSO4 (1.2 mM)
        iv. 14.12 g CaCl2 (2.6 mM)
        v. 6.64 g NaH_2PO_4 * H_2O (11.1 mM)

2. Fill to 2 L with MilliQ H2O
3. Check osmolarity and adjust to 300-305 mOsm
4. Incubate in 34°C water bath while oxygenating with 95%/5% O2/CO2 gas

CsCl Internal Solution (Stock)

1. Add the following to 50 mL MilliQ H_2O to obtain 100 mL CsCl internal stock
    a. 2.51 g CsCl (150 mM)
    b. 0.238 g HEPES (10 mM)
    c. 0.042 g EGTA (1.1 mM)
    d. 200 μL 1M $MgCl_2$ (2 mM)
    e. 10 μL 1M $CaCl_2$ (0.1 mM)
2. Fill to 100 mL with MilliQ H_2O
3. Separate into 10 mL aliquots and store at -20°C.

CsCl Internal Aliquots

1. To a thawed 10 mL CsCl aliquot add:
    a. 0.02 g ATP
    b. 0.005 g GTP

c. 0.003 g Biocytin (not required, use this if you need to visualize patched neurons post-hoc)

2. Adjust osmolarity to ~290 mOsm
3. Adjust pH to 7.4
    a. This a K^+ free internal, so **<u>use CsOH to adjust pH</u> (do not use KOH).**
4. Filter through 0.2 μm filters into 1 mL aliquots, store at -20°C and thaw a fresh aliquot for each day of recording. Keep internal on ice throughout recording day.

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