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Niu, KY., Noyes, N., Abrams, TW (2012). Novel Approach for Generation of Low Calcium Reagents for Investigations of Heavy Metal Effects on Calcium Signaling. *Journal of Pharmacological and Toxicological Methods*. May-Jun;65(3):122-5.

Niu, KY., Saloman, JL., Zhang, Y and Ro, JY (2011) Sex differences in the contribution of ATP-sensitive K^+ channels in trigeminal ganglia under an acute muscle pain condition. *Neuroscience.* 180:344-52.

Saloman, JL., **Niu, KY.,** Ro, JY (2011) Activation of peripheral delta-opioid receptors leads to anti-hyperalgesic responses in the masseter muscle of male and female rats. *Neuroscience*. 190:379-85

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Abstract

Title of dissertation: Sex differences in cannabinoid and opioid mediated analgesia

Katelyn Niu, Doctor of Philosophy, 2013

Dissertation Directed by: Dr. Jin Y. Ro, Associate Professor, Department of Neural and Pain Sciences

Orofacial musculoskeletal pain conditions, such as temporomandibular disorder (TMD), are debilitating and often difficult to treat. As with many chronic pain conditions, TMD occurs more frequently in women. Thus, understanding mechanisms underlying sex differences in pain and analgesia is essential for effective pain management in both sexes. This study introduces the potential therapeutic advantage of targeting cannabinoid 1 receptor (CB1) localized in primary afferent neurons under myositis conditions. Although sex differences in CB1 responses are recognized in the CNS, it is not known whether such sex differences exist in the periphery. Therefore, I investigated whether peripheral cannabinoid treatment leads to sex differences in anti-hyperalgesic effects, and whether the effects are mediated by sex differences in CB1 level in trigeminal ganglia (TG) under a rodent orofacial myositis model. Peripherally administered ACPA, a specific CB1 agonist, significantly attenuated inflammation-induced mechanical hypersensitivity in the masseter of male rats. In female rats, a 30-fold higher dose of ACPA was required to produce a reduction in mechanical hypersensitivity. CFA injected in masseter muscle significantly up-regulated CB1 mRNA expression in TG from males, but not from females, and CB1 mRNA levels in TG were positively correlated with the anti-hyperalgesic effect of ACPA. IL-1β and IL-6, elevated in the muscle tissue following inflammation, induced a significant up-regulation of CB1 mRNA expression in TG cultures from male rats. The up-regulation of CB1 was prevented in TG cultures from

orchidectomized males, and was restored by testosterone treatment. The cytokines did not alter the CB1 mRNA level in TG from intact or ovariectomized female rats. Neither estradiol nor estrogen receptor blockade had any effect on CB1 expression. Similar results were obtained regarding cytokine-induced regulation of μ-opioid receptor (MOR) in TG, another important peripheral target for pain management. These data indicate that testosterone, but not estradiol, is required for the regulation of CB1 and MOR in TG under inflammatory conditions, which explains the sex differences in the anti-hyperalgesic effects of peripherally administered agonists. These data offer important new insights for the development of mechanism-based sex-specific pharmacological treatment alternatives that can be directed at the peripheral anti-nociceptive systems to ameliorate persistent pain.

Sex differences in cannabinoid and opioid mediated analgesia

By
Katelyn Y. Niu

Dissertation submitted to the faculty of the Graduate School
of the University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2013

Acknowledgements

I would like to thank my mentor Dr. Jin Y. Ro for the support and guidance he has provided throughout my program. He kept me focused on my project, and if it wasn't for Dr. Ro, I wouldn't be a clinician/scientist.

I would like to express my appreciation for my committee members, particularly Drs. Norman Capra and Richard Traub who served as readers. Dr. Norman Capra brought me into the DDS/PhD program and I'm grateful for both his scientific and administrative support. Dr. Frank Margolis has been there since the beginning of my acceptance into the program and was very helpful troubleshooting biochemical experiments. I thank Drs. Richard Traub and Bradley Alger for the wonderful scientific discussion that shaped my dissertation. I also would like to acknowledge Dr. Hiroaki Misono for technical advice.

I would like to thank all current and former members of the Ro lab for training me as well as for their technical support. Gregory Haynes and Youping Zhang were instrumental in the collection of behavioral data. Youping Zhang also prepared cultures for my experiments. Xia Zhang and Youping Zhang contributed to the MOR Real-time PCR experiments. Thank you to Dr. Jami Saloman for editing my dissertation and presentation. I thank Fran Addison, Judy Pennington, Yulvonnda Brown in the Department of Neural and Pain Sciences and Jennifer Aumiller in the Program in Neuroscience. I would like to thank my family and friends for their support throughout my education. I thank you all!

Funding: DE020988 to KYN; DE019448 to JYR; T32 DE007309

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$\Delta\Delta C_T$:	comparative C_T
2-AG:	2-arachidonoylglycerol
ACEA:	arachidonyl-2'-chloroethylamide
ACPA:	arachidonylcyclopropylamide
AMPA:	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid
AP-1:	activator protein 1
AR:	androgen receptor
CB1:	cannabinoid receptor type 1
CB2:	cannabinoid receptor type 2
CBR:	cannabinoid receptors
cDNA:	complementary DNA
CGRP:	Calcitonin Gene-Related Peptide
CNS:	central nervous system
DAMGO:	D-Ala2, N-Me-Phe4, Gly5-ol]-Enkephalin acetate salt
DMSO:	dimethyl sulfoxide
DRG:	dorsal root ganglia
EDTA:	Ethylenediaminetetraacetic acid
ES:	estradiol benzoate
FAAH:	fatty acid amide hydrolase
GAPDH:	glyceraldehyde 3-phosphate dehydrogenase
GDX:	gonadectomized
gp130:	glycoprotein 130
GPR55:	G protein-coupled receptor 55
GPR119:	G protein-coupled receptor 119
HBSS:	Hank's balanced salt solution
IFN-γ:	interferon-gamma
IL-1β:	interleukin-1 beta
IL-1RA:	interleukin-1 receptor antagonist
IL-4:	interleukin-4
IL-6:	interleukin-6
IL-6R:	interleukin-6 and interleukin-6 receptor complex
JAK-STAT:	Janus kinase and signal transducer and activator of transcription
KOR:	kappa opioid receptors
MAGL:	monoacylglycerol lipase
MOR:	mu opioid receptors
NF-IL6:	nuclear factor for IL-6 expression
NMDA:	N-Methyl-D-aspartate
NSAIDs:	non-steroidal anti-inflammatory drugs
OR:	opioid receptors
OVX:	ovariectomized
PBS:	phosphate buffered saline
PPARs:	peroxisome-proliferator-activated receptors
RIPA buffer:	radioimmunoprecipitation assay buffer

RT-PCR:	reverse transcription polymerase chain reaction
SE:	standard error
TG:	trigeminal ganglia
THC:	tetrahydrocannabinol
TMD:	temporomandibular disorders
TMJ:	temporomandibular joint
TNF-α:	tumor necrosis factor-alpha
TRPV1:	transient receptor potential cation channel V1
TS:	testosterone
VPM:	ventral posterior medial nucleus of the thalamus.
xMAP:	Luminex multi-analyte profiling

Chapter 1: General Introduction

1.1 Orofacial muscle pain and temporomandibular disorders (TMD)

Orofacial muscle pain related to TMD is one of the most common persistent orofacial pain conditions. In the 2002 National Health Interview Survey, five percent of US adults reported TMD-type pain (6% women and 3% men; 2:1 ratio) (Isong et al., 2008; Janal et al., 2008). Based on the "Orofacial Pain Prospective Evaluation and Risk Assessment" (OPPERA) studies, the odds of TMD increased successively in older age groups and drops off after reproductive age. The prevalence of TMD-like symptoms reached 7.1% among women aged 35-44 years (Slade et al., 2011). The pathophysiology of TMD-related pain conditions is not well understood; it is multi-factorial in nature characterized by multiple symptoms involving structural as well as inflammatory components (Kopp, 2001; Kaneyama et al., 2002; Kaneyama et al., 2010). The signs and symptoms of TMD vary in their presentation and often involve muscles, temporomandibular joint (TMJ), nerves, tendons, ligaments, disks, bones and connective tissue (Okeson, 2003). TMD patients suffer from dull and aching pain in the face, neck, shoulder and the jaw. Earache and headache are also prevalent along with masticatory difficulty (Okeson, 2003). The focus of this study will be on the primary afferent neurons innervating the masseter muscle because the masseter is the predominant muscle associated with TMD related orofacial pain (Ohrbach et al., 2011).

Another important feature of TMD is that it occurs two times more frequently in women than in men (Von Korff et al., 1990) and the female-to-male ratio of patients seeking care varies between 3:1 and 9:1 (Huber and Hall, 1990). Sex differences also play an important role in individual responses to treatments. For example, women

consume fewer opioids and experience greater pain relief from opioids for post-operative pain (Fillingim and Gear, 2004; Miaskowski et al., 2004; Chia et al., 2002; Gear et al., 1999). The mechanisms underlying sex-related differences in analgesic responses have yet to be fully investigated. With etiology, pathophysiology and sex-related responses not fully understood, mechanism-based diagnosis, prognosis as well as treatment strategies for this painful condition are still inadequate.

Current therapy for orofacial pain related to TMD involves both non-pharmacological and pharmacological options. Non-pharmacological treatments include physical therapy, stress management, dental interventions such as occlusion adjustments and fitting for a night guard, surgeries and other alternative treatments such as acupuncture. These non-pharmacological approaches are palliative, and do not offer resolution of the cause of the TMD. The major classes of drugs used to treat orofacial pain are non-steroidal anti-inflammatory drugs (NSAIDs), anti-depressants, anti-convulsants and narcotics such as opioids. Unfortunately, many of these drugs do not offer reliable analgesia in patients. Despite the strong analgesic effects provided by opioids, systemic use of opioids are associated with unwanted side effects, such as nausea, vomiting, constipation, dependence, and respiratory depression, which often lead to poor compliance and rejection of therapy.

1.2 Trigeminal nociceptive and anti-nociceptive processing

The TMJ is innervated by the fifth cranial nerve (trigeminal nerve) whose cell bodies are found in the trigeminal ganglia (TG). The mechanisms underlying trigeminal pain can be studied at multiple levels of the nervous system, from the periphery (e.g.TG)

to the cerebral cortex. Nociceptors are a specialized class of first order sensory neurons (called primary afferent neurons) that detect tissue damaging stimuli. In the trigeminal system, the cell bodies of these primary afferent nociceptors are located in TG with their bifurcating axons projecting to the peripheral tissue and centrally to the spinal nucleus of the trigeminal nerve in the brainstem. The spinal nucleus of the trigeminal nerve is further divided rostrocaudally into the subnuclei oralis (Vo), interpolaris (Vi) and caudalis (Vc). It is widely accepted that nociceptive input from the orofacial region is initially processed in the Vc. The trigeminal Vi/Vc transition zone also plays an important role in deep tissue pain processing, integrating nociceptive orofacial input and the development of persistent orofacial pain (Ren and Dubner, 2011). The major synaptic transmission between nociceptors and second order neurons is mediated by the amino acid glutamate, which activates 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA) receptors and N-methyl-D-aspartate (NMDA) receptors. The nociceptors also elicit slow excitatory postsynaptic potentials by releasing neuropeptides such as substance P. Substance P is released from C fibers in response to tissue injury such as inflammation (Basbaum,A.I., Jessel, T.M., 2000). The second order neurons from the spinal nucleus of the trigeminal nerve then ascend to synapse onto the ventral posterior medial nucleus of the thalamus and the third order neurons terminate at various structures in the brain (Fig. 1). The sensory and emotional components of pain are processed in parallel by distinct brain structures. Lateral thalamic nuclei and the somatosensory cortex are important in sensory-discriminative aspects of pain, such as quality, location and intensity processing, whereas mesial thalamic nuclei, the prefrontal cortex and limbic system have been proposed to process the affective-motivational dimension of pain

(Coghill et al., 1999). The endogenous descending pain modulatory systems also exist to control pain transmission, both inhibitory and facilitatory. The descending pain circuit includes the periaqueductal gray (PAG), the rostral ventromedial medulla (RVM) and the spinal cord (Ren and Dubner, 2002).

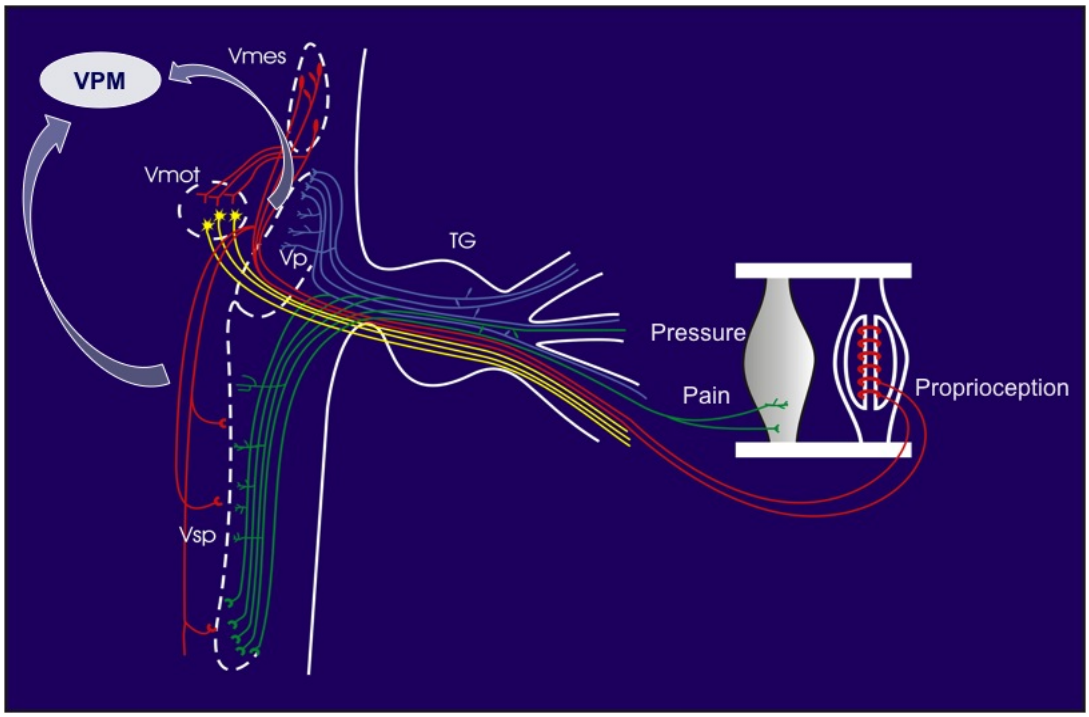

Fig. 1. The anatomy of the trigeminal pain mechanism. Primary afferent neurons in trigeminal ganglia (TG) detect pain and pressure from muscle fibers and proprioceptive information from muscle spindles in the periphery. This information is transmitted to the second order neurons in the spinal nucleus of the trigeminal nerve in the brainstem. The second order neurons then ascend to synapse onto the ventral posterior medial nucleus of the thalamus (VPM) and the third order neurons terminate at various locations in the brain.

While the central nervous system (CNS) is required for the realization of discriminative, affective and motivational components of pain, in most cases the peripheral tissue is where the sensory nociceptive processing is initiated. Furthermore, injury or an inflammatory condition leads to profound changes in nociceptor properties

that underlie the development and maintenance of pathological pain conditions. Since my project focuses on peripheral mechanisms of trigeminal pain and analgesia I will introduce only the literature pertinent to pain processing at the level of primary afferent nociceptors.

During prolonged tissue injury or inflammation, there is a reduction in receptor threshold and an increase in the transmission of nociceptive information from primary afferents, termed nociceptor sensitization (Gold and Flake, 2005). Nociceptor sensitization results from the release of a variety of chemicals including bradykinin, histamine, prostaglandins, and substance P in the injured tissue, which then activate second messenger pathways that lead to the increase in nociceptor excitability (Gold and Flake, 2005; Levine et al., 1993). Nociceptor sensitization, characterized by a decrease in threshold, an augmented response to suprathreshold stimuli and ongoing spontaneous activity, has been suggested as the neural substrate for hyperalgesia (enhanced pain responses to normally noxious stimuli) and allodynia (abnormal pain sensitivity to normally non-painful stimuli). For example, inflammatory mediators such as substance P and Calcitonin Gene-Related Peptide (CGRP) released from afferent neurons can result in neurogenic inflammation of the dura which leads to dural afferent sensitization and is fundamentally important for initiating migraine pain (Sarchielli et al., 1999; Burstein, 2001). Specifically relevant to this study, trigeminal nociceptor sensitization has been demonstrated to underlie orofacial hyperalgesia (Saloman et al., 2012; Cady et al., 2011; Sessle, 2011).

Since primary afferent nociceptors are usually the first structures to be involved in the development of pathological pain conditions, targeting peripheral nociceptors

effectively attenuates hyperalgesia in many pain models. In addition, primary afferent nociceptors are an especially attractive target for the development of pharmacological agents since they are located outside of the CNS. Drugs targeting peripheral nociceptors obviate many centrally-mediated side effects that are associated with systemically administered drugs. For example, recent studies demonstrated that a local injection of keterolac, an NSAID, attenuates jaw muscle pain by inhibiting peripherally localized NMDA receptors (Bendixen et al., 2010; Cairns et al., 2012). The anti-nociceptive and anti-hyperalgesic effects of pharmacological agents that block transient receptor potential (TRP) V1 receptors, which are preferentially expressed in nociceptors, have been extensively studied in the last decade (Eid, 2011; Pal et al., 2009). Similarly, there has been an ongoing effort to target peripheral opioid receptors in primary afferent neurons to reduce or eliminate pain and hyperalgesia (Chung et al., 2012; Spetea and Schmidhammer, 2012; Cunha et al., 2012). Peripheral opioid-receptor mediated analgesia will be investigated in this study as a side project.

1.3 Cannabinoids and pain

Cannabis sativa contains approximately 60 types of cannabinoids. The main psychoactive compound is tetrahydrocannabinol (THC). Cannabis was used as the primary pain reliever until the invention of aspirin, but it was also used to treat migraine, painful rheumatological conditions, neuropathic pain and even dysmenorrhea in the case of Queen Victoria (Geller, 2007). The use of cannabinoids is associated with side effects such as nausea, euphoria and depression. In the early 20th century, cannabis became

federally regulated (Geller, 2007). Today, cannabis for recreational use is illegal in most parts of the world, but its use as a medicine is legal in some countries worldwide.

In recent years, cannabinoids have emerged as attractive alternatives or adjuncts to treatment with opioids. Cannabinoids produce a variety of responses including anti-inflammatory, anti-nociceptive, anti-ulcer, and anti-tumor effects (Saxena, 2009). Peripheral administration of cannabinoids effectively relieves various types of pain without producing centrally mediated side effects (Richardson et al., 1998; Calignano et al., 1998; Agarwal et al., 2007; Brusberg et al., 2009). A better understanding of the mechanisms involved in cannabinoid-mediated analgesia, especially for use as the treatment for orofacial muscle pain related to TMD, is warranted.

Cannabinoid receptors (CBRs) are a family of metabotropic G-protein coupled receptors, which includes CB1, CB2, GPR55, GPR119, and PPARs (peroxisome-proliferator-activated receptors) (Akopian et al., 2009; Matsuda et al., 1990; Ryberg et al., 2007; Sun and Bennett, 2007). Among these, CB1 and CB2 are the most studied. CB1 are expressed throughout the CNS, heart, GI tract, kidney, spleen, liver, lung, testis, uterus and muscle, while CB2 are primarily expressed in immune cells and some neurons (Mackie, 2007; Mackie, 2008). The orphan receptor, GPR55 is characterized as a cannabinoid receptor based on the sequence homology (Ryberg et al., 2007). GPR55 can be activated by a variety of both endogenous and exogenous cannabinoid ligands (Ryberg et al., 2007), and it exhibits the blood pressure lowering properties of cannabinoids (Johns et al., 2007). Another CBR, GPR119, which was initially discovered in the hippocampus, is predominantly expressed in the pancreas and GI tract (Fredriksson et al.,

2003). GPR119 regulates incretin and insulin secretion, which might be important for the treatment of obesity and diabetes (Brown, 2007).

CBRs signal primarily via $G_{i/o}$ coupled pathways, although the involvement of G_s and G_q has also been indicated (Hiley and Kaup, 2007). CB1 activation inhibits adenylyl cyclase (Howlett and Fleming, 1984), decreases N- and P/Q-type Ca^{2+} channel conductance (Caulfield and Brown, 1992; Mackie and Hille, 1992), and activates inward rectifying and A-type potassium channels (Mackie et al., 1995; Deadwyler et al., 1995; Wacnik et al., 2008). Similar to CB1, CB2 inhibits the activity of adenylyl cyclase. Both CB1 and CB2 are also known to be coupled to the MAPK-ERK pathway. However, in contrast to CB1, CB2 stimulation is believed not to modulate ion channel functions (Demuth and Molleman, 2006). For nociception, the intracellular signaling mechanisms and downstream effectors of opioid receptors (ORs) closely resemble those of CB1 (Jordan and Devi, 1998).

1.4 Peripheral cannabinoid receptor-mediated analgesia

Of the two most extensively studied CBRs, CB1 is expressed in primary afferent neurons (Sanudo-Pena et al., 1999; Ahluwalia et al., 2000). Amaya and colleagues (2006) reported that approximately 28% of dorsal root ganglia (DRG) neurons expressed CB1 mRNA in rats. Nociceptor-specific loss of CB1 in the peripheral nervous system in mice substantially reduces the analgesic effect produced by local and systemic delivery of cannabinoids (Agarwal et al., 2007). Intraplantar application of the CB1 agonist, ACEA, attenuates CFA-induced thermal hyperalgesia (Amaya et al., 2006). The peripherally acting CB1-selective agonist SAB-378 inhibits pain-related responses to colorectal

distension in a visceral pain model (Brusberg et al., 2009). These and other pertinent findings suggest that activation of peripheral CB1 leads to effective pain relief without producing centrally mediated side effects in visceral and cutaneous tissue under neuropathic as well as inflammatory conditions (Brusberg et al., 2009; Amaya et al., 2006; Dani et al., 2007; Johanek and Simone, 2004; Grotenhermen, 2005). However, the participation of peripheral CB1 in reduction of orofacial muscle pain conditions, especially under injury or inflammatory conditions has not been documented.

1.5 Modulation of CBR and OR expression

Inflammation or injury has been shown to alter the expression of CB1. Inflammation of the intestine induces up-regulation of the CB1 expression in the jejunum (Izzo et al., 2001). Neuroinflammatory processes lead to a dramatic increase in CB2 expression in the CNS (Benito et al., 2008). Acute and chronic bladder inflammation induces an increase in the expression of CB2 but not CB1 in the bladder detrusor (Merriam et al., 2008). Of direct relevance to this project, peripheral inflammation has been shown to significantly increase the percentage of CB1 mRNA-positive neurons in DRG and CB1 immunoreactivity in DRG neurons and in nerve fibers of the hindpaw dermis in male rats (Amaya et al., 2006). This increase in CB1 immunoreactivity was suggested to contribute to the increased anti-hyperalgesic efficacy of locally administered CB1 agonists. The inflammatory components of TMD conditions could alter the CB1 expression in TG as well, which could contribute to a change in our responses to pain and analgesia.

Of the many factors that are released during inflammation, cytokines have been implicated as potential modulators of CBRs. Cytokines are a large group of polypeptides that are constitutively expressed and can be released during inflammation. For example, tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine that plays an important role in innate immunity that provides early defense against pathogens by activating macrophages to further secrete pro-inflammatory cytokines such as IL-1 and IL-6 (Popa et al., 2007). TNF-α increases mu OR (MOR) expression in various human immune effector cells (Kraus et al., 2003). The Interleukin-1 (IL-1) family of pro-inflammatory cytokines is produced by a wide variety of cells such as macrophages, monocytes, fibroblasts, dendritic cells, glia and neurons (Vidal et al., 1998; Dinarello, 2000). IL-1β up-regulates kappa OR (KOR) expression in dorsal root ganglia in response to peripheral inflammation and induces the expression of MOR in endothelial cells (Vidal et al., 1998; Puehler et al., 2006).

IL-6 is expressed by activated monocytes, macrophages and endothelial cells in inflamed tissue (Moller and Villiger, 2006). IL-6 binds to its receptor IL-6R, which is a cell surface cytokine receptor composed of a ligand binding chain and a signal transducing component glycoprotein 130 (gp130). When IL-6 binds to IL-6R, dimerization of gp130 and IL-6R leads to the activation of the Janus kinase and signal transducer and activator of transcription (JAK-STAT) signaling pathway, which induces the expression of inflammatory genes (Heinrich et al., 2003; Barton, 1997). IL-6 strongly induces MOR mRNA expression in the human neuroblastoma cell line SH SY5Y (Borner et al., 2004). IL-4 is an anti-inflammatory cytokine that induces the differentiation of naïve helper T cells to T helper 2 cells. IL-4 has also been shown to reduce the

production of pro-inflammatory cytokines such as IL-6 and TNF-α (Ledeboer et al., 2000; Ehrlich et al., 1998). Stimulation of IL-4 induces MOR transcripts in human primary blood cells, immune cell lines and dendritic cells (Kraus et al., 2001).

Although the effects of cytokines on the expression of CB1 have not been systematically investigated, cytokine-treated whole blood elevates CB1 and CB2 mRNA and protein levels when compared to non-treated blood (Jean-Gilles and Constantinescu, 2007). The expression of CB2 in spinal cord is up-regulated with a time course consistent with the production of pro-inflammatory cytokines after the onset of multiple sclerosis (Loria et al., 2008). The potential contribution of inflammatory cytokines on the expression of CB1 in sensory neurons has not been studied and is an important topic to be addressed in this dissertation.

1.6 Sex differences in CB1- and MOR-mediated analgesia

Sex differences in opioid analgesia have been extensively documented over the past decade. Animal studies generally report greater mu opioid mediated analgesia in males, whereas the human data are inconclusive (Fillingim and Gear, 2004). For example, activation of peripheral MOR in a visceral pain model produces more potent analgesia in male than female rats, possibly due to the effect of estrogen in female rats (Ji et al., 2006; Ji et al., 2007). Local morphine injected in the TMJ significantly reduces glutamate-evoked jaw muscle activity in male but not female rats (Cai et al., 2001). It is well established that endocannabinoids produce sex specific cognitive and emotional responses (Rubino and Parolaro, 2011). Several studies report cannabinoids produce greater motoric effects in females (Cohn et al., 1972), and more potent analgesia in females than in males in response to noxious mechanical and heat stimuli, mediated by

centrally located CB1 (Tseng and Craft, 2001; Tseng and Craft, 2004). It is proposed that sexually dimorphic responses in the cannabinoid system can be explained by sex differences in the pharmacokinetics of cannabinoids (Tseng et al., 2004), endocannabinoid levels (Bradshaw et al., 2006), and CBR expression levels (Burston et al., 2010; Mateos et al., 2011). There is no information on whether selective activation of peripheral CBRs produces sex-dependent effects, and the potential mechanisms that might lead to sex differences in the cannabinoid effects are unknown.

It has been shown that estrogen inhibits cannabinoid-induced changes in eating behavior and core body temperature (Kellert et al., 2009). Other studies demonstrated that cannabinoids are more potent or more efficacious in females than males in producing anti-nociception and locomotor suppression (Craft and Leitl, 2008; Wiley, 2003). Estradiol has also been shown to enhance THC-induced anti-nociception in ovariectomized female rats (Craft and Leitl, 2008). In addition, testosterone has been shown to attenuate the motoric effects of THC in gonadectomized male rats (Craft and Leitl, 2008). However the mechanisms underlying the effect of sex hormones on the peripheral cannabinoid system is unclear.

Many studies report that CBR expression is sex dependent and it is likely modulated by sex hormones. It has been shown that brain CBR density fluctuates over the estrous cycle in females (Rodriguez de Fonseca et al., 1994). Some studies reported an estrogen mediated decrease in CB1 mRNA expression (Gonzalez et al., 2000). Bonnin et al. reported that ovariectomy decreased brain CBR density in female rats and estradiol treatment of OVX rats increased CBR density (Bonnin et al., 1993). In addition, CB1 density decreases in parotid glands after castration in male rats, and is restored after

testosterone replacement (Busch et al., 2006). CB1 expression in the anterior pituitary gland is higher in ovariectomized (OVX) animals than in animals with estrogen replacement (Gonzalez et al., 2000). However, 17β-estradiol treatment induces CB1 expression in human colon cancer cells (Notarnicola et al., 2008). Collectively, these studies suggest that sex hormones directly or indirectly regulate CB1 expression and the hormonal influences on CB1 regulation depend on the tissue types and experimental models.

Based on these observations I proposed to study how sex hormones modulate the expression of CB1 by cytokines in trigeminal primary afferent neurons under an experimental myositis (masseter inflammation). I hypothesized that sex differences in peripheral cannabinoid effects are mediated by sex differences in CB1 expression in trigeminal primary afferent neurons. Specifically, I predicted that there will be 1) sex differences in local cytokine levels under masseter inflammation; 2) sex differences in peripheral CB1-mediated anti-hyperalgesic responses under masseter inflammation; 3) sex differences in inflammation-induced changes in CB1 expression in TG; 4) sex differences in cytokine-induced CB1 expression in TG, and that 5) sex hormone(s) modulate cytokine-induced CB1 up-regulation in TG.

Because of the well documented similarities in cytokine-induced modulation of receptor expression and intracellular signaling mechanisms between opioids and cannabinoids, I decided to investigate these issues with peripheral opioid. Specifically, a side project investigated whether 1) there are sex differences in peripheral MOR-mediated anti-hyperalgesic responses under masseter inflammation; 2) inflammation and

inflammatory cytokines induce sex-specific MOR expression in TG; and 3) sex hormone(s) modulate cytokine-induced MOR up-regulation in TG.

Chapter 2: Materials and Methods

2.1 Animals

Age matched adult male, female, orchidectomized (GDX) male and ovariectomized (OVX) female Sprague Dawley rats (8 weeks old; 250–300 g for males and 225–260 g for females, Harlan, Indianapolis) were used in the present study. Orchidectomy and ovariectomy were performed by Harlan. All animals were housed in a temperature-controlled room under a 12:12 light-dark cycle with access to food and water *ad libitum*. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and under a University of Maryland approved Institutional Animal Care and Use Committee protocol.

2.2 Experimental myositis (Masseter inflammation)

Inflammation was induced by injecting 50 μl of 50% complete Freund's adjuvant (CFA) in isotonic saline (Sigma, St. Louis, MO) into the mid-region of the masseter muscle via a 27 gauge needle. Rats were briefly anesthetized with 3% isoflurane for the injection procedure. In order to ensure the injection was consistently placed within the same target region of the muscle, the injection site was determined by palpating the masseter muscle between the zygomatic bone and the angle of the mandible. Upon contacting the mandible, the needle was slowly withdrawn into the mid-region of the masseter and the injections were made for 5-10 seconds. Injection of CFA in the rat masseter induces a time-dependent and significant decrease in mechanical thresholds detectible as early as 30 min, which is well developed by three days and that lasts over 12 days (Ambalavanar et al., 2006; Shimizu et al., 2009). Other characteristics of

inflammation and behavioral responses observed following CFA injections in the rat masseter have been described previously (Ambalavanar et al., 2006; Imbe et al., 1999).

2.3 Assessment of local cytokine levels in masseter muscle

2.3a Experimental groups

The levels of cytokines in the masseter muscle were analyzed in naïve rats and rats treated with CFA at 6 hrs, 1, 3 and 7 days following the CFA treatment (n=4-6/group). In addition, serum samples were collected from groups of naïve and CFA-inflamed rats (1 day post CFA) from tail vein with a 21 gauge precision glide IV needle (BD Biosciences). Animals were anesthetized with 3% isoflurane anesthesia described above. Approximately 200 µl to 500 µl of blood was collected with a 1ml syringe. The blood was kept at room temperature for 30 min and centrifuged at 3,000 rpm for 10 min at 4 °C. The serum was collected and stored at -80 °C. Serum cytokine levels were analyzed in order to determine whether changes in cytokine levels in the muscle result from local inflammatory responses or systemic reactions.

2.3b Tissue preparation

All animals were euthanized by decapitation under pentobarbital anesthesia (100 mg/kg). Masseter muscle surrounding the injection site (approximately 100 mg of muscle tissue) was extracted and homogenized in RIPA buffer containing protease inhibitor cocktail on ice. The amount of RIPA buffer added was nine times the weight of the tissue sample. The total protein concentration in lysate was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) and the amount of protein was normalized to

that of the control sample. A total of 2 mg of protein (250 µl, 8 µg/µl) was used in the cytokine assay for each sample.

2.3c Luminex multi-analyte profiling (xMAP) technology

The Luminex multi-analyte profiling (xMAP) technology (Luminex corp.) was used to measure cytokine levels. Bead sets were coated with capture antibody for various cytokines (TNF-α, IL-1β, IL-4 and IL-6). Fluorescence detection antibodies were then applied to bind the cytokine-capture antibody complex on the bead set. Multiple cytokines in the samples were then recognized by the differences in bead sets with fluorogenic emission detection using flow cytometric analysis (Puehler et al., 2006). Each sample was measured in triplicate.

2.3d Data analysis

Two-Way ANOVA was used to compare the cytokine levels between naïve and CFA groups and between male and female rats. All comparisons between multiple groups were followed by a *post hoc* test (Bonferroni). Differences were considered significant at $p<0.05$. All data are presented as the mean ± standard error.

2.4 Behavioral studies

2.4a Behavioral paradigm

For the following experiments, I utilized a behavioral model specifically developed for testing masseter sensitivity in rats (Ren, 1999; Shimizu et al., 2009). A series of calibrated von Frey filaments was applied to the region over the masseter muscle

to determine the force required to produce an aversive response (head withdrawal). An active withdrawal of the head from the filament application was defined as a positive response. Each von Frey filament was applied five times and the response frequencies [(number of responses/number of stimuli) x 100] to a range of filament forces were determined. After a non-linear regression analysis on the response frequencies, an EF_{50} value which is defined as the filament force (g) necessary to produce a 50% response frequency was determined. The EF_{50} value was used as a measure of mechanical threshold. Mechanical sensitivity of the masseter muscle was determined before and 1, 2, 3, 5, 7, 10 and 14 days after the CFA injection. A reduction of EF_{50} suggested the presence of mechanical hypersensitivity.

2.4b Experimental and control groups – CB1 study

The effect of a CB1 agonist on mechanical sensitivity was examined on the third day post CFA injection, a time point at which mechanical hypersensitivity was pronounced. Four doses of ACPA, a specific CB1 agonist (10 μg, 30 μg, 100 μg and 300 μg/20 μl) were administered in the masseter muscle to evaluate the dose-dependent effects on CFA-induced mechanical hypersensitivity. The doses of ACPA were adapted from the literature (Hayase et al., 2001). Control groups received the vehicle injection in the same manner. To rule out the possibility that local administration of ACPA produced systemic effects by activating CB1 in the CNS, another group of rats received the highest doses of ACPA injections in the masseter muscle contralateral to the mechanical stimulation.

To test the receptor specificity of the agonist, a selective antagonist for CB1, AM251 (30 μg/10 μl), or for CB2, AM630 (30 μg/10 μl), was administered 5 min before ACPA treatment in another group of animals. The selected doses of the antagonists have been shown to block the receptor-specific agonist effects (Gibson et al., 2008; Potenzieri et al., 2008). In order to maintain the consistency in assessing behavioral responses, all behavioral experiments were conducted by an experimenter who was blinded to treatment conditions.

2.4c Experimental and control groups – MOR study

For this study, mechanical sensitivity of the masseter muscle was determined before and 1, 2 and 3 days after the CFA injection in the masseter muscle. The effect of a MOR agonist, D-Ala2, N-Me-Phe4, Gly5-ol]-Enkephalin acetate salt (DAMGO; Tocris, MS, USA) on mechanical sensitivity was examined on the third day post CFA injection. On day 3, DAMGO (10 μg/20 μl) or the same volume of vehicle was administered directly in the masseter muscle. The post DAMGO or vehicle effect was measured 30 minutes after the drug injection. The dose of DAMGO was adapted from a previous study published in the lab, which confirmed that DAMGO at this dose specifically targets peripheral MOR without producing central effects (Nunez et al., 2007). In order to maintain the consistency in assessing behavioral responses an experimenter who was blinded to treatment conditions conducted all behavioral experiments.

2.4d Experimental and control groups – cytokine antagonist study

To confirm that the blockade of CB1 up-regulation by the cytokine antagonists is functionally relevant, the same behavioral experiments were performed in another group of male rats. Animals received cytokine antagonists 5 min before CFA injections at the same muscle site and the effect of CB1 agonist on mechanical sensitivity was examined 3 days post CFA injection. The dose of ACPA selected for this experiment was 10 μg. Control groups received the vehicle injection in the same manner.

2.4e Drug preparation and administration

ACPA in Tocrisolve^{TM} 100 (Tocris, MS, USA), was dissolved in phosphate buffered saline (PBS; American Bioanalytical, MA, USA). AM251 (Tocris) and AM630 (Tocris) were dissolved in dimethyl sulfoxide (DMSO; American Bioanalytical, MA, USA). DAMGO was dissolved in PBS. IL-1β (PeproTech, NJ, USA), IL-6 (PeproTech), soluble recombinant mouse IL-6 receptor (R&D Systems, USA), recombinant human IL-1 receptor antagonist (IL-1RA; PeproTech) and recombinant human gp130 (R&D Systems) were dissolved in PBS. Cytokines and IL-6 receptor were prepared to a final concentration of 200 ng/20 μl. Cytokine antagonists were prepared to a final concentration of 2 μg/20 μl.

2.4f Testosterone replacement for MOR experiments

For RT-PCR experiments, testosterone (Sigma-Aldrich, T-1500) was dissolved in safflower oil (16 mg/kg/100 μl) and administered subcutaneously in the abdomen of GDX rats for seven days in the morning between 9:00-11:00 AM. TG from testosterone

replaced GDX rats were subjected to tissue culture four hours after the last testosterone injection.

For behavioral experiments, GDX rats received the same testosterone replacement protocol (16 mg/kg/100 μl) subcutaneously for seven days in the morning between 9:00-11:00 AM. In addition, CFA was injected in the masseter muscle 3 days prior to behavioral studies.

2.4g Data analysis

The time-dependent changes in mechanical thresholds (EF_{50}) before and after CFA treatment were analyzed with a Two-Way ANOVA with repeated measures (within subject variable: time, between subject variable: sex). In order to assess the effects of ACPA or vehicle, the post-CFA behavioral data was represented as a normalized EF_{50} value to the animals' own baseline threshold. Mean percent change of EF_{50} was then calculated by EF_{50} (drug) / EF_{50} (baseline) X 100 – 100. Mechanical hypersensitivity is presented as a negative value on the mean percent change graph. Zero represents the absence of hypersensitivity. A One-Way ANOVA was then used to analyze the mean percent changes in EF_{50} compared to baseline between experimental and control groups.

2.5 TG cultures

2.5a TG primary cultures for CB1 studies

All animals were euthanized by decapitation under pentobarbital anesthesia (100 mg/kg, i.p.). Both TG from each animal were extracted and dissociated by sequential digestion with collagenase D (0.1%) in DMEM-F12 medium (with L-

glutamine) at 37 °C for 30 min, followed by collagenase D (0.1%), trypsin (0.25%), DNase (50 μg) and EDTA (0.02%) in DMEM-F12 medium at 37 °C for 15 min. After trituration, cells were plated on laminin pre-coated 12-well plates and kept in a 37 °C incubator at 5% CO_2 for 3 days.

In culture studies, IL-1β, IL-6, TNF-α (PeproTech) and soluble recombinant mouse IL-6 receptor were dissolved in PBS. Estradiol benzoate and testosterone (Sigma, MO, USA) were dissolved in DMSO at a stock concentration of 2.66 mM. IL-1β, IL-6, TNF-α and soluble recombinant mouse IL-6 receptor or vehicle (PBS) were applied to culture medium to a final concentration of 100 ng/ml. Estradiol benzoate, testosterone or DMSO were applied to culture medium to a final concentration of 100 nM. Medium containing cytokine(s) and/or sex hormone were warmed to 37 ˚C before application to cultured TG cells.

2.5b TG tissue cultures for MOR studies

The rats were euthanized with pentobarbital (100 mg/Kg, i.p.) and decapitated. Both TG from each rat were quickly dissected and kept in ice-cold HBSS solution. Dura and other connective tissues were carefully removed under a microscope. The three branches of trigeminal nerve (V1, V2 and V3) were preserved with about 2 mm of axon fibers on both ends. The TG was cut into two equal sections in the horizontal plane, and each section was split into three pieces in the rostro-caudal direction. The tissue samples were then transferred to a 12-well culture plate with 1 ml of culture media (Dulbecco's Modified Eagle Medium, DMEM-F12 supplemented with penicillin 100 U/ml, streptomycin 100 μl/ml, and 10% serum) and incubated in a 37 °C incubator at 5% CO_2

for 48 hours. A similar protocol for TG tissue culture has recently been published (Lei et al., 2012).

2.6 Real-time RT-PCR

2.6a Real-time RT-PCR for CB1

Total RNA was extracted from TG of naïve rats and CFA inflamed rats at the following time points, 6 hours, 1, 3, 7, 14 days. RNA was also extracted from cytokine inflamed rats 6 hours post injections. In culture experiments, RNA was collected 30 min after cytokine and/or sex hormone application. RNA was extracted using Trizol (Sigma) and purified with an RNeasy kit (Qiagen Sciences, MD, USA) that included a DNase treatment to remove genomic DNA. Reverse transcription was carried out using the Superscript First Stand synthesis kit (Invitrogen, CA, USA). Superscript II (Invitrogen) was used to generate cDNA from 1 μg of RNA along with 2.5 ng of random primer per reaction. Real-time PCR analysis of cDNA equal to 25 ng RNA was performed on the Eppendorf Mastercycler ep realplex 2.0. The amount of a given CB1 mRNA was normalized to the GAPDH mRNA in the same sample. The primer pairs are described in table 1. The cycling protocol used was 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 50 °C for 15 s and 72 °C for 20 s. Relative quantification of the CB1 mRNA was calculated by the comparative C_T method ($\Delta\Delta C_T$ method) between control and experimental groups (Schmittgen and Livak, 2008).

2.6b Real-time RT-PCR for MOR

Total RNAs were extracted from TG of naïve male and female rats, inflamed male and female rats 1, 3, and 7 days following CFA injection in the masseter muscle, and 4 hours after cytokine treatments from TG tissue cultures. RNA was extracted similarly as in the CB1 experiments. SuperScript II (Invitrogen) was used to generate cDNA from 500 ng of RNA along with 2.5 ng of random primer per reaction. Real-time PCR analysis of cDNA equal to 15 ng of RNA was then performed using Maxima SYBR Green/ROX qPCR Master Mix in an Eppendorf Mastercycler Ep Realplex 2.0 (Fermentas, Forest City, CA, USA). The ratio between MOR and β-actin was calculated to obtain the relative abundance of mRNA levels in each sample. The primer pairs are listed in table 1. Relative quantification of the CB1 mRNA was calculated by the comparative CT method (ΔΔCT method) between control and experimental groups.

Table 1: Real-time RT-PCR primers

	Forward primers	Reverse primers
CB1	5′-CTACTGGTGCTGTGTGTCATC-3′	5′-GCTGTCTTTACGGTGGAATAC-3′ (Mitrirattanakul et al., 2006)
GAPDH	5’-TCACCACCATGGAGAAGGC-3’	5’- GCTAAGCAGTTGGTGGTGCA-3’
MOR	5′-GCCCTCTACTCTATCGTGTGTGTA-3′	5′-GTTCCCATCAGGTAGTTGACACTC-3′(Bannister et al., 2011)
β-actin	5′-GGTCCACACCCGCCACCAG-3′	5′-CAGGTCCAGACGCAGGATGG-3′

All data obtained from RT-PCR experiments were analyzed with a One-Way ANOVA on means or Kruskal-Wallis One-Way ANOVA on ranks depending on the outcome of a normality test with the exception of the following experiments. A student t-test was used to compare baseline MOR mRNA levels between intact male and female TG samples. A Two-Way ANOVA was used to compare CB1 mRNA levels between control and experimental groups and between male and female intact TG. All comparisons between multiple groups were followed by a *post hoc* test (Dunnett’s or

Bonferroni's). Data are presented as mean ± SE and differences were considered significant at $p < 0.05$.

Chapter 3: Intramuscular cytokine levels under myositis condition in male and female rats

3.1 Abstract

This study was conducted to examine cytokine profiles in the masseter muscle with and without complete Freund's adjuvant (CFA)-induced inflammation in male and female rats. Age and weight matched male and female Sprague Dawley rats were injected with CFA in the mid-region of the masseter muscle. Muscle tissue surrounding the injection site was extracted 6 hrs, 1, 3 and 7 days after the injection to measure TNF-α, IL-1β, IL-6 and IL-4 levels with Luminex multi-analyte profiling (xMAP) technology. The cytokine levels were compared to those obtained from naïve rats. CFA injection into the masseter muscle led to significant time dependent changes in the level of TNF-α compared to that of naïve rats. The pattern of changes in TNF-α levels after CFA injection was significantly different between the male and female rats. CFA injection induced significant time-dependent increases in the levels of IL-1β and IL-6 in the masseter muscle in both male and female rats. The level of IL-4 was slightly, but significantly, reduced in both sexes at 6 hrs and 3 days after CFA-induced inflammation. No significant sex differences were observed in the levels of IL-1β, IL-6 or IL-4. The results provided novel information about distinct cytokine profiles during CFA-induced muscle inflammation, and provide the basis for further pursuing contributions of each cytokine in pain processing and analgesic responses in both sexes.

3.2 Introduction

Musculoskeletal pain conditions including neck pain, back pain, orofacial muscle pain and other chronic widespread pain conditions such as fibromyalgia are prevalent and severe pain conditions. While most of our knowledge about chronic types of pain is based on cutaneous pain models, muscle pain conditions, including exercise-induced muscle pain and orofacial muscle pain related to temporomandibular disorders, appear to utilize different mechanisms in both the development and maintenance phases (Sharma et al., 2009; Mense, 2008).

Many chronic musculoskeletal pain conditions have been shown to originate from muscle inflammation (Loram et al., 2007; Armstrong, 1990; Inanici and Yunus, 2004; Murata et al., 2005). Cytokines released locally during inflammation modulate nociceptive processing via multiple mechanisms. Cytokines can directly sensitize nociceptors and increase neuronal sensitivity to heat, mechanical and chemical stimuli (Nicol et al., 1997; Sorkin et al., 1997; Junger and Sorkin, 2000; Jin and Gereau, 2006; Obreja et al., 2002). Inflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, contribute to pain and hyperalgesia indirectly by inducing the production of inflammatory agents that sensitize nociceptors (Bernheim et al., 1980; Zucali et al., 1986; Poole et al., 1995). In addition, inflammatory cytokines are powerful modulators of the expression of many receptors involved in pain and analgesia. For example, TNF-α significantly up-regulates TRPV1 in trigeminal sensory neurons (Khan et al., 2008). In the opioid system, IL-1β, IL-6, TNF-α and IL-4 induce mu-opioid receptor expression in neuronal and non-neuronal cells (Vidal et al., 1998; Borner et al., 2004; Kraus et al., 2001; Borner et al., 2002; Ruzicka et al., 1996). Similarly, in the

cannabinoid system, cytokine-treated whole blood elevates CB1 and CB2 mRNA and protein levels when compared to non-treated blood (Jean-Gilles and Constantinescu, 2007). Thus, changes in cytokine profiles in the local tissue during injury or inflammation can provide potent modulation of nociceptive signaling processes.

Studies based on cutaneous tissue have demonstrated distinct changes in cytokine profiles after inflammation and tissue injury: e.g., tissue injury induces bradykinin release leading to the release of TNF-α, which results in the release of IL-6 and IL-1β (Poole et al., 1995). There are, however, distinct differences in inflammation-induced changes in cytokine profiles when cutaneous hindpaw tissue is compared to gastrocnemius muscle in female Sprague Dawley rats (Loram et al., 2007). TNF-α is elevated significantly in the hindpaw but not in the muscle. Both IL-1β and IL-6 are elevated at an earlier time point in the cutaneous hindpaw tissue compared to the muscle tissue. These data confirm that there is a specific cytokine profile associated with muscle inflammation, at least in female rats.

However, since pain responses from males and females vary a great deal in many chronic musculoskeletal pain conditions, and the two sexes also respond differently to inflammation (Flake et al., 2006), it is important to examine whether there are sex differences in the cytokine expression profiles in muscle under inflammatory conditions. At present, there is no study that has directly assessed potential sex differences in the local cytokine levels under inflammatory conditions. Thus, the objective of this study was to establish the cytokine profiles in the masseter muscle before and after complete Freund's adjuvant (CFA)-induced inflammation in both male and female rats. I chose to analyze the levels of three pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and one anti-

inflammatory cytokine IL-4 because of their known roles in inflammation and as modulators of receptor expression and nociceptive signaling at the primary afferent level.

3.3 Results

In male rats, the mean level of TNF-α in the masseter muscle was at its highest (90.1±46.7 pg/ml) under the naïve condition (Fig. 2). The injection of CFA into the masseter muscle induced a gradual decrease in the TNF-α level. The mean TNF-α level was reduced to 86.9±23.3, 40.4±15.4, 24.7±7.6, and 13.3±3.4 pg/ml after 6 hrs, 1, 3 and 7 days, respectively. In female rats, the mean level of TNF-α in the masseter muscle was 6.4±0.65 pg/ml under the naïve condition, about 14 fold less than the level in naïve male rats. However, after CFA injection, the level of TNF-α in female rats increased to 36.6±10.2 pg/ml after 6 hours and 49.7±13.5 pg/ml after one day before returning to the level under the naïve condition after three days. A significant sex difference between male and female rats was observed ($F=6.417$, $p=0.015$). There was also a significant time effect on inflammation-induced TNF-α levels ($F=2.767$, $p=0.038$), but post hoc analysis did not detect significant group differences. A significant group difference was found only between naïve and CFA 7 day male rats. No other individual group comparisons were significant.

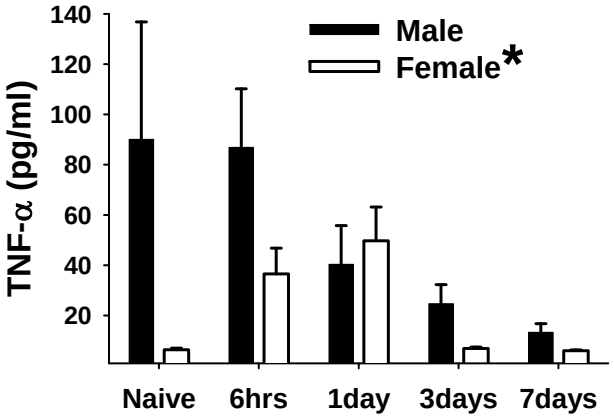

Fig. 2. Mean levels of TNF-α in masseter muscle in naïve and CFA treated rats. Significant sex differences were observed between male and female rats. There was also a significant time effect, but the post hoc test did not reveal any significant group differences within time. * denote significant sex effects with $p<0.05$, n=4–6/group.

The mean level of IL-4 decreased slightly but significantly after the CFA injection (F=4.065, *p=0.007*, Fig. 3). Under the naïve condition, the mean levels of IL-4 were 64.7±11.5 and 61.8±14.7 pg/ml for male and female, respectively. Six hours after the CFA injection, the mean levels of IL-4 decreased significantly to 27.0±4.5 and 26.5±4.4 pg/ml in male and female respectively. A second phase of slight decrease in the IL-4 level was observed three days after CFA injection. No significant sex difference was detected (F=0.138, *p=0.712*).

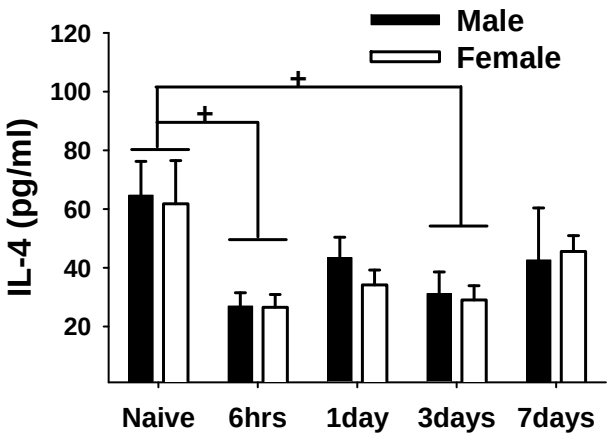

Fig. 3. Mean levels of IL-4 in masseter muscle in naïve and CFA treated rats. The levels of IL-4 decreased slightly but significantly at the 6 hr and 3 day time points in both sexes. No sex difference was observed. + denote significant time effects, respectively with $p<0.05$, n=4–6/group.

The mean levels of IL-1β in the masseter muscle were low in both male (9.3±4.9 pg/ml) and female rats (1.0±0.5 pg/ml) under the naïve condition (Fig. 4). Injection of CFA into the masseter muscle induced a substantial increase in the IL-1β levels in both sexes (~1000 pg/ml) as early as 6 hrs and the levels remained significantly increased for one day ($F=43.258$, $p<0.001$). Seven days after CFA injection, the levels of IL-1β in both male and female rats decreased to approximately 100 pg/ml. No significant difference in the IL-1β level was detected between naïve and CFA 3 or 7 day groups. There appeared to be a slight delay in the increase and decrease in the level of IL-1β in females compared to males; however no significant sex difference was observed ($F=0.548$, $p=0.463$).

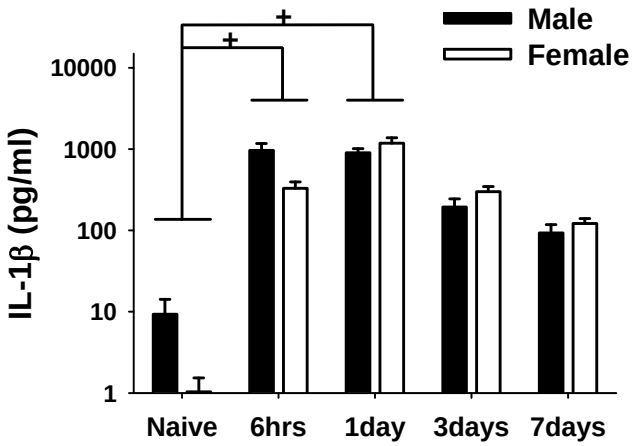

Fig. 4. Mean levels of IL-1β in masseter muscle in naïve and CFA treated rats. The levels of IL-1β increased significantly after 6 hrs and 1 day after CFA injection in both sexes. No significant sex difference was observed. + denotes significant time effect with $p<0.05$, n=4–6/group. Y axis is in a log scale.

The pattern of inflammation-induced changes in the level of IL-6 was similar to that of IL-1β. The mean level of IL-6 similar between male and female rats under naïve conditions (Male: 29.6±13.3 pg/ml, female: 27.1±1.8 pg/ml) (Fig. 5). Injection of CFA into the masseter muscle induced a significant and substantial increase in IL-6 levels in both sexes (F=79.190, *p<0.001*). Six hours after the CFA injection, the level of IL-6 rose to approximately 10,000 pg/ml, more than a 300 fold increase from the naïve condition. The mean level of IL-6 remained significantly increased after one day, which then gradually decreased to approximately 200 pg/ml after 7 days. There were no significant differences in the IL-6 level between naïve and CFA 3 or CFA 7 day groups. No significant sex difference was detected (F=0.940, *p=0.337*). The plasma levels of all four cytokines in male and female rats collected one day after CFA inflammation were not significantly different from those of naïve rats.

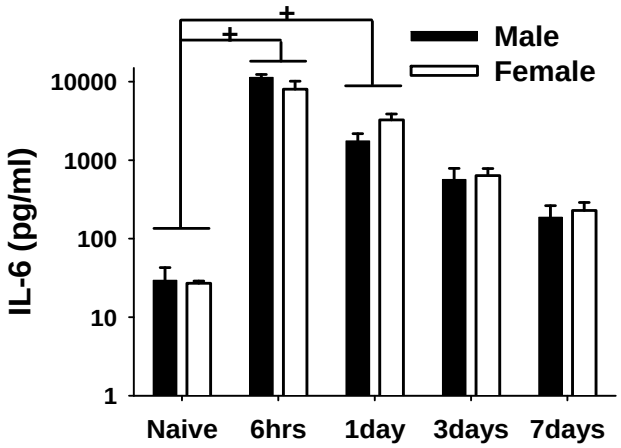

Fig. 5. Mean levels of IL-6 in masseter muscle in naïve and CFA treated rats. The levels of IL- 6 increased significantly after 6 hrs and 1 day following CFA injection in both male and female rats. No significant sex difference was observed. + denotes significant time effect with $p<0.05$, n=4–6/group. Y axis is in a log scale.

3.4 Discussion

The present study examined the cytokine profiles in the masseter muscle under complete Freund's adjuvant (CFA)-induced inflammation and possible sex differences in the cytokine levels. The pattern of changes in TNF-α level after CFA injection was significantly different between the male and female rats. CFA injection induced significant time-dependent increases in the levels of IL-1β and IL-6 in the masseter muscle in both male and female rats. The level of IL-4 was slightly, but significantly, reduced in both sexes at 6 hrs and 3 days after CFA-induced inflammation. No significant sex differences were observed in the levels of IL-1β, IL-6 or IL-4.

Few studies have directly compared cytokine levels between males and females under pathological conditions. A significantly higher plasma IL-1β level in male patients compared to females was reported following abdominal trauma (Ertan et al., 2007). A greater concentration of serum IL-6 in men has been implicated in hepatocellular carcinoma, the most common liver cancer in men, while estrogen-mediated inhibition of

IL-6 reduces the risk of liver cancer in women (Naugler et al., 2007). The present study provides the first report regarding sex-based observations on local cytokine profiles following muscle inflammation. The data showed that CFA-induced inflammation produced similar changes in IL-1β, IL-4 and IL-6 levels between the male and female rats. Inflammation differentially impacted the levels of TNF-α between the sexes.

In contrast to my results and the effects found in cutaneous tissue, the level of TNF-α in gastrocnemius muscle does not increase in female rats after carrageenan-induced inflammation (Loram et al., 2007). Our data showed that the level of TNF-α in the masseter muscle decreased significantly only at a later stage of CFA-induced inflammation in male rats. However, the level of TNF-α in the masseter muscle in female rats rose slightly at 6 hrs and 1 day. The differences in the basal levels and the pattern of inflammation-induced changes in TNF-α levels in female rats between this study and that of Loram et al (2007) could be attributed to the types of muscle (limb vs. craniofacial) or the inflammatory agent (carrageenan vs. CFA).

The increase in the level of TNF-α during CFA-induced inflammation in female rats was only modest compared to that of IL-1β or IL-6. Also, the level of TNF-α during CFA-induced inflammation was comparable between male and female rats. These observations suggest that muscle inflammation does not significantly impact the overall level of TNF-α in either sex. Direct intramuscular injection of a high dose of TNF-α produces mechanical hyperalgesia (Schafers et al., 2003), and sensitizes trigeminal muscle afferents in male rats (Hakim et al., 2009). However, the same concentration of exogenous TNF-α could produce different responses in female rats since the basal level is substantially lower in the masseter muscle of female rats. Thus, the contribution of TNF-

α at a physiological concentration in muscle pain processing and potential sex specific responses merits further study.

IL-1β mediates inflammatory pain and hyperalgesia by inducing a widespread expression of prostanoids both in the periphery and in the CNS (Samad et al., 2001; Ferreira et al., 1988). In the muscle tissue, IL-1β regulates IL-6 production, which may be involved in muscle injury, degeneration and repair of the muscle (Hoheisel et al., 2005). The level of IL-1β increases in human skeletal muscle after eccentric exercise for a prolonged period of time and has also been shown to be a potent hyperalgesic agent (Ferreira et al., 1988; Cannon et al., 1989; Fielding et al., 1993). Similarly, the concentration of IL-1β is elevated in the trapezius muscle of human patients with active myofascial trigger points (Shah et al., 2008). In an animal model of limb muscular hyperalgesia, the surge of IL-1β in the gastrocnemius muscle during carrageenan-induced inflammation occurs at 24 hrs. Since this change followed the initiation of primary muscle hyperalgesia that occurs as early as 3 hrs post injection the role of IL-1β in the maintenance phase of hyperalgesia in muscle tissue has been suggested (Loram et al., 2007). However, carrageenan injected in the masseter muscle causes an immediate increase (i.e. 1 hr post injection) in the level of IL-1β (Ono et al., 2007). The present study also showed a prominent increase in masseteric IL-1β levels following CFA-induced inflammation. The increase in the levels of IL-1β could be observed from 6 hrs and were maintained until the 3 day time point. Again, the differences could be due to the more potent and prolonged effects of CFA compared to carrageenan as well as tissue specific responses.

CFA injected in the rat masseter muscle and the hindpaw have been shown to significantly up-regulate μ and κ opioid receptors, respectively, in sensory neurons (Puehler et al., 2006; Nunez et al., 2007). Interestingly, a local injection of IL-1β also significantly up-regulates κ opioid receptors, thereby increasing κ mediated analgesia in the periphery; treatment with an IL-1 receptor antagonist blocks the CFA-induced up-regulation of κ opioid receptor expression in sensory neurons (Puehler et al., 2006). Taken together, these data suggest that the surge of IL-1β in local tissue following inflammation contributes to pain and hyperalgesia, as well as analgesia. However, a potential role of IL-1β in mediating sex differences in pain and analgesia cannot be explained solely by the local level of IL-1β, since the inflammation changed the cytokine levels similarly between male and female rats.

While there is no clear consensus on the role of IL-6 in inflammatory muscle pain, it is involved in multiple aspects of skeletal muscle physiology and pathophysiology. IL-6 is markedly increased during eccentric and concentric contraction of the muscle and is involved in protein degradation during muscle damage as well as during muscle regeneration (Tomiya et al., 2004; Tsujinaka et al., 1995; Tsujinaka et al., 1996; Cantini et al., 1995; Steensberg et al., 2000). Acute injections with IL-6 in the gastrocnemius muscle do not excite or sensitize muscle nociceptors in naïve rats (Hoheisel et al., 2005). However, pre-treatment with local IL-6 injections primed the muscle and produced muscular hyperalgesia following injection of prostaglandin (E2) that lasts for several hours (Dina et al., 2008). An immediate rise of intramuscular IL-6 mRNA expression following electrically-induced muscle contraction and carrageenan–induced inflammation in the masseter muscle suggests a multifunctional role for IL-6

across different types of muscle tissue (Ono et al., 2007). In addition, my data showing that IL-6 in the masseter muscle increased significantly and dramatically (more than 300 fold) in both male and female rats after CFA-induced inflammation, further support the contribution of IL-6 in pain and metabolism of craniofacial muscles in both sexes.

Both anti-inflammatory and anti-hyperalgesic effects of IL-4 have been demonstrated (Vale et al., 2003; Hao et al., 2006). However, information on inflammation-induced changes in IL-4 in the muscle tissue and potential sex differences in its level is not available. In this study, I showed that the levels of IL-4 in the masseter muscle were reduced slightly but significantly in both sexes at the 6 hr and 3 day time points, indicating a net pro-inflammatory effect at those time points. It is possible that the reduced level of IL-4 contributed, at least partly, to the increase in IL-1β and IL-6 (Üçeyler and Sommer, 2007). IL-4 also contributes to anti-hyperalgesic effects by up-regulating peripheral opioid receptors under inflammatory conditions (Borner et al., 2004; Kraus et al., 2001). Because similar changes were observed in the levels of IL-4 in both sexes, IL-4 mediated anti-hyperalgesic effects are not expected to exhibit sex differences.

In summary, this study established the cytokine profiles in the masseter muscle before and after CFA-induced inflammation. These observations provided the basis for the potential contribution of each cytokine in inflammation-induced pain and analgesic responses in the muscle tissue in both sexes. The following chapters will continue to address the roles of cytokines in regulating inflammatory pain and cannabinoid/opioid receptors mediated analgesia.

Chapter 4: Sex differences in cannabinoid-mediated analgesia and modulation of CB1 receptor expression

4.1 Abstract

I studied the effects of peripherally administered cannabinoids and assessed the role of sex hormones in cannabinoid receptor (CBR) expression in trigeminal ganglia (TG) in an orofacial myositis model. Peripherally administered ACPA, a specific CB1 agonist, significantly attenuated complete Freund's adjuvant (CFA)-induced mechanical hypersensitivity in the masseter muscle in male rats. The ACPA effect was blocked by a local administration of AM251, a specific CB1 antagonist, but not by AM630, a specific CB2 antagonist. In female rats, a 30-fold higher dose of ACPA was required to produce a moderate reduction in mechanical hypersensitivity. CFA injected in masseter muscle significantly up-regulated CB1 mRNA expression in TG in male, but not in female, rats. There was a close correlation between the CB1 mRNA levels in TG and the anti-hyperalgesic effect of ACPA. IL-1β and IL-6, which are elevated in the muscle tissue following CFA treatment, induced a significant up-regulation of CB1 mRNA expression in TG from male rats. The up-regulation of CB1 was prevented in TG cultures from orchidectomized (GDX) male rats, which was restored by the application of testosterone. The cytokines did not alter the CB1 mRNA level in TG from intact or ovariectomized (OVX) female rats. Neither estradiol supplement nor estrogen receptor blockade had any effects on CB1 expression. These data indicate that testosterone, but not estrogen, is required for the regulation of CB1 in TG under inflammatory conditions, which provides an explanation for the sex differences in the anti-hyperalgesic effects of peripherally administered cannabinoids.

4.2 Introduction

In recent years, cannabinoids have emerged as attractive alternatives or adjuncts for treatment of many types of diseases, including pain and inflammation (Hazekamp and Grotenhermen, 2010). However, psychotropic effects mediated by CBRs in the CNS limit the therapeutic use of cannabinoids as analgesics. Activation of peripheral CBRs, particularly CB1, which are primarily expressed in primary sensory neurons, produces anti-nociceptive and anti-hyperalgesic effects in visceral, cutaneous and muscle tissue (Brusberg et al., 2009; Amaya et al., 2006; Johanek and Simone, 2004; Gutierrez et al., 2007), and nociceptor-specific loss of CB1 in mice substantially reduces the analgesic effect produced by local and systemic delivery of cannabinoids (Agarwal et al., 2007). These pertinent findings suggest the potential value of targeting peripheral CB1 to provide effective pain relief without producing centrally mediated side effects.

It is well established that endocannabinoids produce sex differences in cognitive and emotional responses (Rubino and Parolaro, 2011). Furthermore, several cannabinoids produce greater motoric effects in females (Cohn et al., 1972), and more potent analgesia in females than in males in response to noxious mechanical and heat stimuli; the effects that are mediated by centrally located CB1 (Tseng and Craft, 2001; Tseng and Craft, 2004). Sex specific responses in the cannabinoid system can be explained by sex differences in pharmacokinetics of cannabinoids (Tseng et al., 2004), endocannabinoid levels (Bradshaw et al., 2006), and CBR expression levels (Burston et al., 2010; Mateos et al., 2011). There is no information on whether peripheral activation of CBRs produces sex-dependent effects.

While factors that modulate CBR expression are not well understood generally, sex steroids have been implicated as potential modulators of CBR expression in the periphery. Males exhibit higher levels of CB1 mRNA than females in the rat pituitary gland, and orchidectomy in males reduces CB1 transcripts, whereas chronic exposure to estradiol in ovariectomized females decreases CB1 transcripts (Gonzalez et al., 2000). Similarly, CB1 density decreases in parotid glands after castration in male rats, and is restored following testosterone replacement (Busch et al., 2006). Inflammatory cytokines also modulate CBR expression. Cytokine-stimulated whole blood expresses greater CB1 and CB2 mRNA levels than does non-stimulated blood (Jean-Gilles and Constantinescu, 2007). CB2 expression in the spinal cord is up-regulated along a time course consistent with the production of pro-inflammatory cytokines following the onset of multiple sclerosis (Loria et al., 2008). These studies suggest that sex hormones and inflammation can influence the effect of endogenous cannabinoids as well as responses to cannabinoid treatments by modulating CBR expression.

In this study, I used a rat model of orofacial myositis to evaluate the anti-hyperalgesic effects of peripheral CB1 activation in both sexes, and investigated the role of inflammatory cytokines, specifically IL-1β and IL-6 that are up-regulated in the muscle under inflammatory conditions (Niu and Ro, 2010), on CB1 expression in primary sensory neurons in trigeminal ganglia (TG). I then examined whether cytokine-induced CB1 expression is modulated by sex hormones: estrogen and testosterone.

4.3 Results

4.3a Sex differences in the effect of peripheral CB1 activation on masseter hypersensitivity

Masseter injection of CFA in the rat induces a time-dependent and significant decrease in mechanical thresholds as early as 30 min that lasts over 12 days (Ambalavanar et al., 2006; Shimizu et al., 2009). I have confirmed the development of mechanical hypersensitivity following CFA injection in the masseter with a significant decrease in EF_{50} during the 14 days post CFA injection (Fig. 6A). There were no sex differences in the baseline thresholds, the time course, or the extent of mechanical hypersensitivity. In order to examine the effect of CB1 activation in CFA-induced masseter mechanical hypersensitivity, I injected the muscle with ACPA (20 μl) or vehicle 45 min prior to behavioral testing on the third day following CFA treatment. In males, 10 μg of ACPA significantly attenuated the CFA-induced mechanical hypersensitivity (Fig. 6B). ACPA at 30 μg not only reversed the hypersensitivity, but also produced a significant analgesic effect. To rule out possible systemic effects, the high dose of ACPA (30 μg) was injected into the masseter muscle contralateral to the testing site in a separate group of male rats. The contralateral ACPA treatment failed to attenuate the mechanical hypersensitivity, suggesting that ACPA at 30 μg was a systemically low dose and did not produce centrally-mediated effects.

In contrast, the same doses of ACPA were ineffective in female rats. A 30-fold higher dose of ACPA (300 μg vs. 10 μg in males) was required to significantly attenuate the mechanical hypersensitivity in females (Fig. 6C). In a separate group of female rats, the highest dose of ACPA (300 μg) was also injected into the masseter muscle

contralateral to the testing site. Similar to male rats, the contralateral ACPA treatment failed to attenuate the mechanical hypersensitivity. The dose of ACPA (10 μg) that significantly attenuated masseter mechanical hypersensitivity under the inflammatory condition in male rats did not affect the mechanical sensitivity in naïve male or female rats (Fig. 6D). These data demonstrated sex differences in the potency and efficacy of the local CB1 agonist in attenuating mechanical hypersensitivity under inflammatory conditions.

ACPA is a specific CB1 agonist with a 325-fold selectivity over CB2 (Hillard et al., 1999). However, to further rule out the possible involvement of CB2, additional behavioral experiments were conducted with specific antagonists for CB1 and CB2. The effect of ACPA was completely blocked by the pretreatment of the muscle with a specific CB1 antagonist, AM251 (Fig. 6E). In contrast to AM251, pretreatment of the muscle with AM630, a specific CB2 antagonist, did not block the effect of ACPA (Fig. 6F). These data confirmed that the effect of ACPA was mediated by CB1, and not by CB2. AM251 alone, without ACPA, did not augment the masseter hypersensitivity, suggesting that endogenous cannabinoids do not play a major role in this model (Fig. 6E). Since AM630 was ineffective on the CFA effects, I did not examine the effects of AM630 alone.

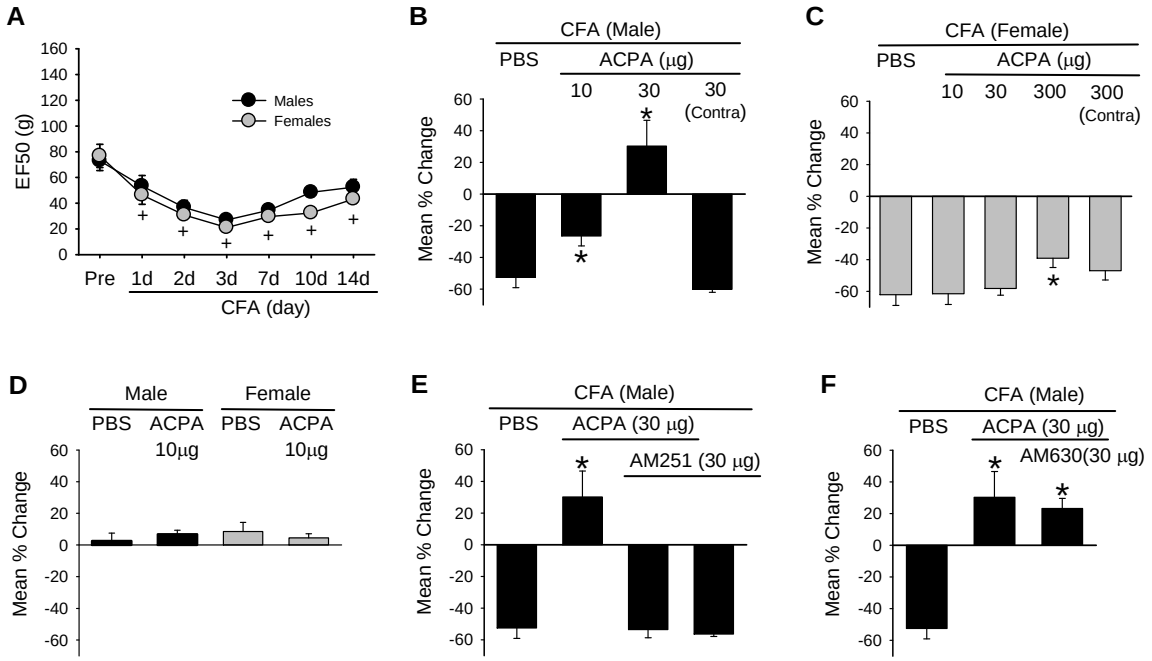

Fig. 6. Effects of ACPA on CFA-induced mechanical hypersensitivity. (A) CFA-induced mechanical hypersensitivity measured in the masseter muscle of male and normally cycling female rats. Mechanical force (g) that produced the head withdrawal responses 50% of the trials is plotted for pre- and 1, 2, 3, 7, 10, and 14 days post-CFA injection. $^{+}$denotes significant time effects at $p < 0.05$ compared to the pre-CFA values. (B, C) Effects of intramuscular ACPA on mechanical sensitivity 3 days after CFA treatment in male and female rats. Bar graphs show mean % change in EF_{50} values in vehicle (phosphate-buffered saline [PBS])- and ACPA-treated rats. *denotes significant differences at $p < 0.05$ compared to the vehicle group. ACPA was also injected in the masseter muscle contralateral to the mechanical sensitivity testing (Contra). (D). Effects of ACPA on mechanical sensitivity of naïve male and female rats. (E, F). Effects of a CB1 antagonist (AM251) and a CB2 antagonist (AM630) on ACPA-mediated anti-hyperalgesic responses 3 days after CFA treatment in male rats. *denotes significant differences at $p < 0.05$ compared to the vehicle group. All data are shown as mean ± SE and each group consisted of 6–7 animals.

4.3b Sex differences in inflammation-induced CB1 expression in TG

In order to examine the impact of inflammation on CB1 expression I measured the changes in CB1 mRNA in TG over a time course similar to the behavioral observations following CFA injection in the masseter. No sex differences in the level of CB1 mRNA in TG were observed under naïve conditions. CFA-induced masseter inflammation resulted in a time-dependent increase in the level of CB1 mRNA in TG of male, but not female rats (Fig. 7). In male rats, the level of CB1 mRNA was significantly greater in CFA treated groups on day 3 and day 7 than in the naïve group. These data

suggested that inflammation-induced changes in CB1 mRNA levels in TG could underlie the sex differences observed in the behavioral studies.

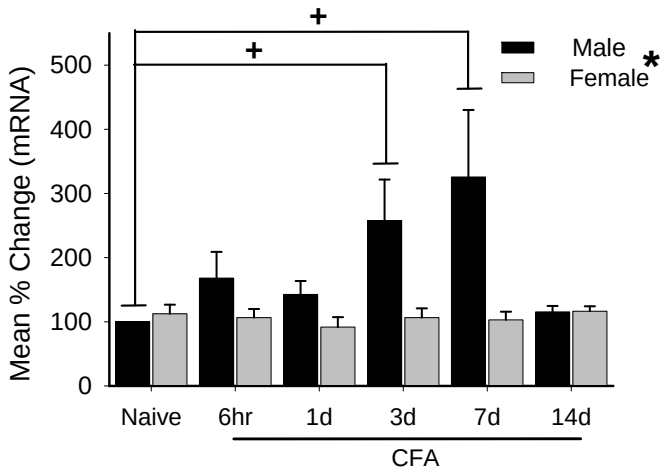

Fig. 7. Real-time PCR showing CFA-induced changes in CB1 mRNA levels in intact TG of male and female rats**.** All data are normalized to naïve male rats and presented as mean percent change. $^{+}$denotes significant effects with respect to naïve condition at $p < 0.05$. Data are shown as mean ± SE and each group consisted of 6 animals.

ACPA (10 μg), a dose that was ineffective in attenuating masseter hypersensitivity under naïve conditions became efficacious on day 3 following CFA treatment, a time point when CB1 mRNA was significantly increased in male rats (cf. Figs. 6B, D). The level of CB1 mRNA in male TG was not significantly increased from the baseline on day 1 or 14 after CFA inflammation. To determine if the effect of ACPA is correlated with the expression of CB1 in TG, I tested whether the same 10 μg dose that significantly attenuated the masseter hypersensitivity 3 days after inflammation also attenuated masseter hypersensitivity 1 or 14 days after CFA-induced inflammation. ACPA, had no effect at day 1 or 14 (Fig. 8A, B). These data suggested that the anti-hyperalgesic effect of ACPA was largely due to an increased level of CB1 expression.

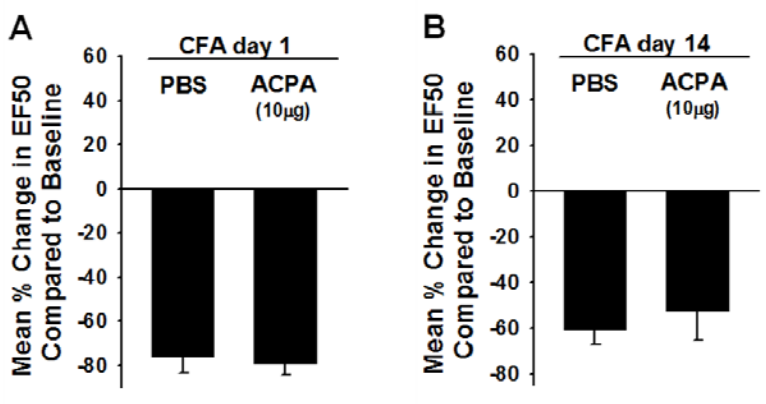

Fig. 8. Effects of ACPA on masseter hypersensitivity. Hypersensitivity was measured 1 day (A) and 14 days (B) after CFA treatment in male rats. Bar graphs show mean % change in EF_{50} values in vehicle (phosphate-buffered saline [PBS])- and ACPA-treated rats. Data are shown as mean ± SE and each group consisted of 6 animals.

4.3c Inflammatory cytokines induce CB1 up-regulation in TG cultures in a sex-dependent manner

CFA-induced masseter inflammation leads to significant increases in the levels of IL-1β and IL-6, but not TNF-α or IL-4, in the masseter muscle of either male or female rats (Niu and Ro, 2010). Since the changes in IL-6 and IL-1β in the masseter muscle precede that of CB1 up-regulation in TG, it is possible that local inflammatory cytokines are involved in the regulation of CB1 expression. Interestingly, CFA increases the levels of IL-1β and IL-6 similarly in the two sexes (Niu and Ro, 2010), but modulates CB1 expression in TG in a sex specific manner (Fig. 7). These observations suggested that cellular mechanisms involved in transcriptional regulation of CB1 following inflammation are different between males and females. In this experiment, I investigated whether inflammatory cytokines induce differential up-regulation of CB1 expression in male and female rats using primary cultures of TG.

In TG cultures from male rats, the level of CB1 mRNA increased significantly with 30 min application of IL-1β or IL-6 along with its soluble α-receptor subunit, IL-6R, but not with application TNF-α (Fig. 9A). Since TNF-α has been shown to produce synergistic effects when combined with other cytokines (Poli et al., 1990; Kuhweide et al., 1990), I also administered TNF-α in combination with IL-1β or IL-6R. The combination of IL-6R and TNF-α, but not the combination of IL-1β and TNF-α, also induced a significant up-regulation of CB1 mRNA compared to controls (Fig. 9B). However, the same concentrations of IL-1β, IL-6R, or TNF-α alone, or as combinations of cytokines did not induce significant changes of CB1 mRNA in TG cultures prepared from female rats (Fig. 9C, D).

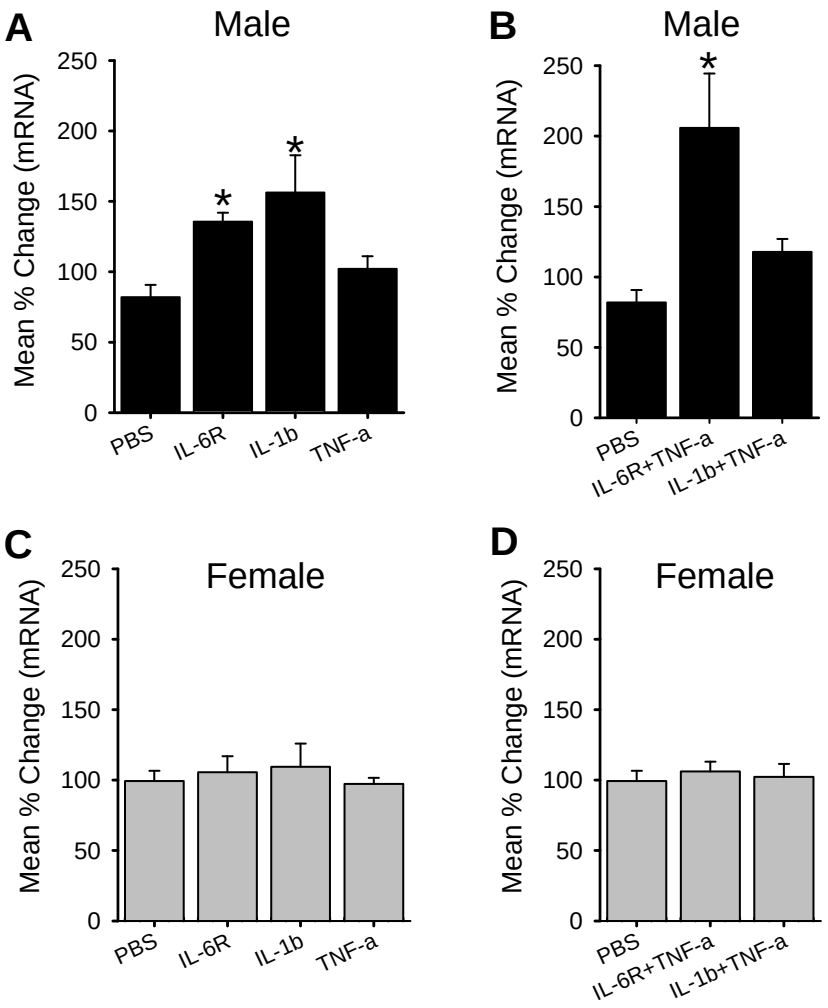

Fig. 9. Effects of inflammatory cytokines on CB1 mRNA expression in TG primary cultures. Real-time polymerase chain reaction data following the applications of individual or combinations of cytokines were compared to those of phosphate-buffered saline (PBS) treatment in cultures prepared from male (A,B) and female (C,D) rats. All data are normalized to untreated samples and presented as mean percent change in CB1 mRNA transcripts. Data are shown as mean ± SE and each group consisted of 5–6 replicates. *denotes significant differences compared to PBS group at $p < 0.05$. IL, interleukin; TNF, tumor necrosis factor.

4.3d IL-1β and IL-6 mediate CFA-induced CB1 up-regulation and ACPA effects *in vivo*

I then investigated whether IL-1β and IL-6 are involved in inflammation-induced up-regulation of CB1 mRNA in intact TG. IL-1 receptor antagonist (IL-1RA) or soluble gp130 (an inhibitor of IL-6 signaling complex) were directly administered into the masseter muscle 5 min prior to the induction of inflammation by CFA in male rats. TG

from these rats were extracted 3 days later and the expression of CB1 mRNA was assessed. IL-1RA, at a dose that has been shown to effectively attenuate the IL-1β effect (2 μg) (Hook et al., 2011), completely prevented the up-regulation of CB1 mRNA. Similarly, soluble gp130 at a dose that effectively antagonizes the IL-6 effect (2 μg) (Obreja et al., 2002) also prevented the CFA-induced up-regulation of CB1 mRNA (Fig. 10A).

In order to confirm that the blockade of CB1 up-regulation by the cytokine antagonists is functionally relevant, I performed the same experiments and tested the effect of ACPA with the behavioral assay in additional groups of male rats. Consistent with the mRNA data, the dose of ACPA (10 μg) that significantly attenuated the CFA-induced masseter hypersensitivity was no longer effective in animals that were pretreated with either IL-1RA or gp130 (Fig. 10B).

Finally, I tested whether direct injections of these cytokines can induce CB1 mRNA up-regulation *in vivo*. IL-1β (200 ng) or IL-6 (200 ng) and its soluble receptor (IL-6R) were injected into the mid-region of the masseter muscle of male rats and CB1 mRNA was measured in intact TG 6 hours after the injection. IL-1β, but not its vehicle control, induced a significant increase in CB1 mRNA compared to that of CB1 mRNA levels in TG from naïve rats (Fig. 10C). However, under the same experimental condition, IL-6R failed to induce a significant up-regulation of CB1 mRNA.

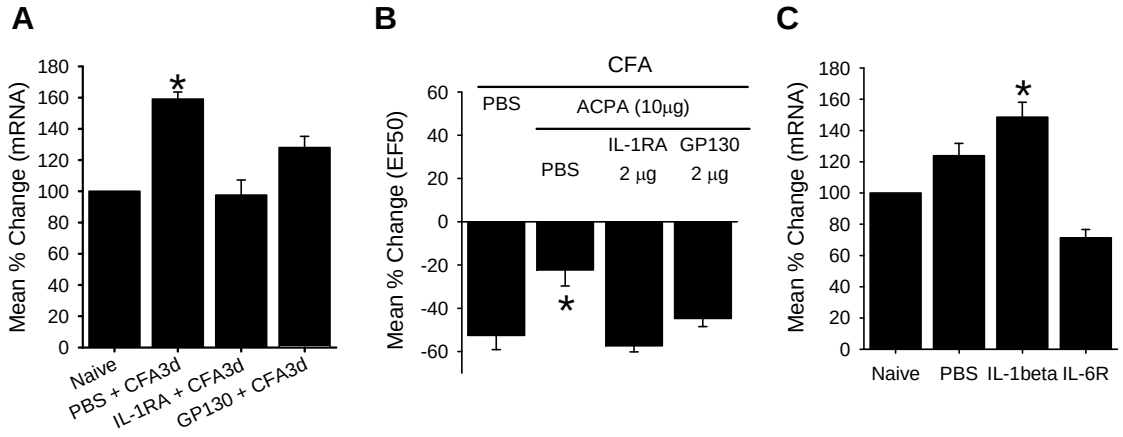

Fig. 10. The role of inflammatory cytokines in CFA-induced up-regulation of CB1 mRNA and the mechanical hypersensitivity in male rats. (A) CFA-induced changes in CB1 mRNA levels were assessed in intact TG of CFA- inflamed male rats treated with interleukin (IL)-1 receptor antagonist (IL-1RA), IL-6 inhibitor, gp130, and vehicle. Data are normalized to that of TG obtained from naïve rats. Each group consisted of 6 rats. (B) Effects of arachidonylcyclopropylamide (ACPA) on CFA-induced masseter hypersensitivity were assessed under the same experimental conditions as in A. Each group consisted of 6–8 rats. (C) CB1 mRNA in intact TG from male rats treated with direct masseteric injections of IL-1β, IL-6 with its receptor (IL-6R), and vehicle control. Data are normalized to that of naïve male rats and each group consisted of 6 rats *denotes significant group effects at $p < 0.05$. PBS, phosphate-buffered saline.

4.3e Testosterone, but not estrogen, modulates cytokine-induced CB1 up-regulation

Since cytokine-induced up-regulation of CB1 mRNA was observed in TG from male rats, I investigated whether sex hormones play a role in modulating cytokine-induced CB1 expression. First, since it is possible that circulating estrogen could have persistent inhibitory effects on cytokine-induced CB1 expression, I applied the same concentrations of inflammatory cytokines to TG cultures prepared from OVX female rats. None of the cytokines exerted a significant effect on CB1 expression (Fig. 11A). I then tested whether additional estrogen is required for cytokine-induced up-regulation of CB1 mRNA. TG cultures from intact female rats were supplemented with estradiol benzoate (100 nM). The estradiol treatment alone or in combination with cytokines did not induce a significant up-regulation of CB1 mRNA (Fig. 11B). Finally, I tested whether estrogen exerts residual inhibitory effects on TG cultures from intact female rats. The application

of estrogen receptor inhibitor (ICI 182,780, 100 nM; Tocris) along with the cytokines in TG cultures from intact females also failed to increase the CB1 mRNA (Fig. 11C). Collectively, these data suggested that estradiol is not involved in the modulation of CB1 expression by inflammatory cytokines in TG.

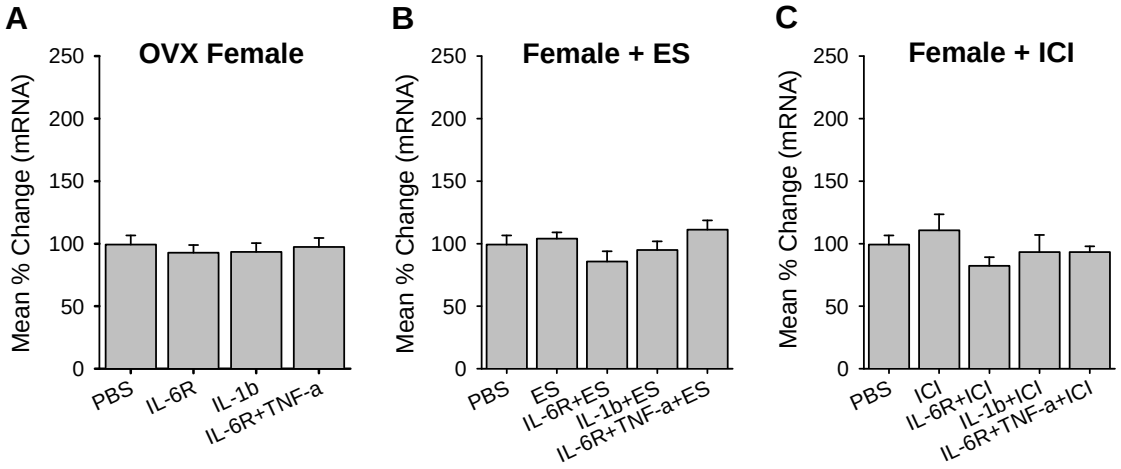

Fig. 11. Role of estrogen in CB1 expression in TG from female rats. (A) CB1 mRNA levels were assessed from TG of ovariectomized (OVX) rats treated with cytokines. (B) Estradiol benzoate (ES) alone or ES combined with cytokines were applied to TG cultures from intact female rats and CB1 mRNA levels assessed. (C) An estrogen receptor antagonist, ICI-182,780 (ICI) alone or combined with cytokines was applied to TG cultures from intact female rats and CB1 mRNA assessed. All data are normalized to untreated samples and presented as mean percent change in CB1 mRNA transcripts. Data are shown as mean ± SE and each group consisted of 5–6 replicates. No significant differences were detected. PBS, phosphate-buffered saline; IL, interleukin; TNF, tumor necrosis factor.

If estrogen is not preventing the CB1 mRNA up-regulation in females, it is possible that testosterone is playing a role in cytokine-induced modulation in males. Therefore, I applied the same cytokine treatments that induced CB1 mRNA up-regulation in TG cultures from intact males to those prepared from GDX rats. Neither IL-1β nor IL-6R was effective in up-regulating CB1 mRNA (Fig. 12A). IL-6R applied in combination with TNF-α was also ineffective. Testosterone treatment in cultures from GDX rats significantly restored the cytokine-induced up-regulation of CB1 mRNA (Fig. 12B). IL-

6R in combination with TNF-α also induced a significant up-regulation of CB1 mRNA in testosterone treated cultures of GDX rats.

Since the cytokine treatment no longer induced CB1 mRNA up-regulation in TG cultures prepared from GDX rats, ACPA should not be efficacious in attenuating CFA-induced mechanical hypersensitivity in GDX animals. Our behavioral data confirmed that the dose of ACPA (10 μg) that significantly attenuate the CFA-induced mechanical hypersensitivity in intact male rats was no longer effective in GDX rats (Fig. 12C). Taken together, these data suggested that testosterone is required for cytokine-induced CB1 up-regulation in male rats, and further corroborate the correlation between the efficacy of peripheral CB1 and CB1 expression in trigeminal ganglia.

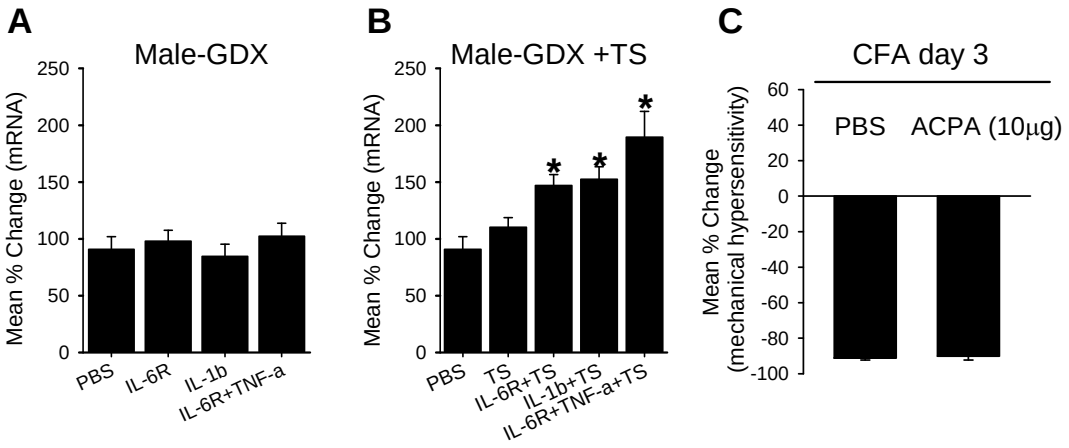

Fig. 12. Role of testosterone in CB1 mRNA expression in TG from male rats. (A) Real-time polymerase chain reaction data following individual or combination of cytokines applications were compared to those of PBS treatment in orchidectomized (GDX) rats. (B) Testosterone (TS) alone or TS combined with cytokines were applied to TG cultures from GDX rats and CB1 mRNA levels assessed. All data are normalized to untreated samples and presented as mean percent change in CB1 mRNA transcripts. Data are shown as mean ± SE and each group consisted of 5–6 replicates. *denotes significant differences compared to PBS group at $p < 0.05$. (C) Effects of ACPA on masseter hypersensitivity 3 days after complete Freund's adjuvant treatment in GDX rats. Bar graphs show mean % change in EF_{50} values in vehicle (phosphate-buffered saline [PBS])- and ACPA-treated rats. Data are shown as mean ± SE and each group consisted of 6 animals.

Finally, I examined whether testosterone modulates cytokine-induced CB1 expression in female rats. IL-1β, IL-6R or the combination of IL-6R and TNF-α was applied to cultures from female rats in the presence of testosterone. The testosterone treatment did not result in cytokine-induced CB1 up-regulation in cultures prepared from female rats (Fig. 13).

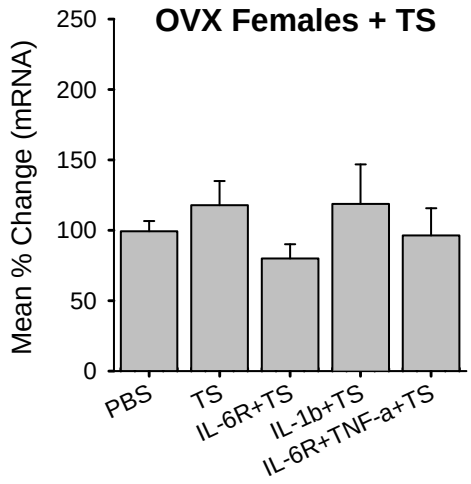

Fig. 13. Role of testosterone in CB1 mRNA expression in female TG cultures. TS alone or TS combined with cytokines were applied to TG cultures from intact female rats and CB1 mRNA levels assessed. All data are normalized to untreated samples and presented as mean percent change in CB1 mRNA transcripts. Data are shown as mean ± SE and each group consisted of 5–6 replicates. OVX, ovariectomized; PBS, phosphate-buffered saline; IL, interleukin; TNF, tumor necrosis factor.

4.4 Discussion

The results from the present study provide additional evidence that activation of CB1 in TG afferents effectively attenuates inflammation-induced mechanical hyperalgesia in the masseter muscle in males. Systemically inactive doses of ACPA reversed the mechanical hyperalgesia in inflamed muscle, but did not alter the mechanical sensitivity in non-inflamed muscle. Although I did not perform complete dose response studies to compare the efficacy of ACPA between normal and inflamed

conditions our data is consistent with previous studies that showed enhanced effects of cannabinoids at the peripheral CB1 under inflammatory conditions (Amaya et al., 2006; Kelly et al., 2003).

Cannabinoids produce greater anti-nociception in female rats compared to male rats, effects predominantly mediated by CB1 in the brain (Tseng and Craft, 2001; Tseng and Craft, 2004). Based on this animal study, the analgesic activity of Cannador, a whole plant extract, was tested in female subjects in human experimental pain models (Kraft et al., 2008), but no analgesic or anti-hyperalgesic activities were found with this cannabis extract. However, Nabilone, a synthetic analogue of THC, significantly attenuates hyperalgesia only in women (Redmond et al., 2008).

As far as we know, there is no study that showed sex differences in behavioral responses mediated by peripheral CB1 under either normal or pathological conditions. Many studies that showed a potent peripheral CB1 effect under inflammatory or neuropathic pain conditions used male subjects (Richardson et al., 1998; Agarwal et al., 2007; Gutierrez et al., 2007; Nackley et al., 2003; Yu et al., 2010) and the few studies that utilized female subjects did not directly assess sex differences (Guerrero et al., 2008; Walczak and Cervero, 2011). Thus, we provide a novel and interesting finding that locally administered CB1 agonist produces more potent anti-hyperalgesic effects in males than females in a rat myositis model.

The sex differences in ACPA effects can be explained by multiple factors such as differences in cannabinoid metabolism in local tissue, CB1 expression level, G-protein coupling efficiency and endocannabinoid signaling between males and females. It is possible that inflammation or injury can have sex-dependent impacts on these factors. For

example, CFA in the hindpaw induced a significant up-regulation of CB1 expression in DRG, which resulted in enhanced anti-hyperalgesic effects of a peripheral CB1 agonist in male rats, but female rats were not studied (Amaya et al., 2006). In the present study, the anti-hyperalgesic effects of ACPA were observable only in male rats, and only during times when CB1 mRNA was significantly increased. In female rats, masseter inflammation did not alter the CB1 expression level in TG, and ACPA was ineffective in attenuating mechanical sensitivity in either inflamed or non-inflamed muscle. Therefore, our data suggest that sex differences in local ACPA effects are a result of a differential impact of inflammation on CB1 expression between male and female rats. Although I did not systematically investigate this in the present study, CB2 expressed in primary sensory neurons also mediates analgesic activity in chronic inflammatory and neuropathic pain conditions (Hsieh et al., 2011). It is possible that the limited effect observed in females at a high dose of ACPA may be due to CB2 activation. Identification of sex differences in peripheral CB2 requires further study.

Of the multitude of inflammatory mediators that are released in the local tissue, cytokines have been frequently implicated in the modulation of both ORs and CBRs. In the OR system, pro-inflammatory and anti-inflammatory cytokines, IL-1β, IL-6, TNF-α and IL-4 are powerful modulators of OR expression in neuronal and non-neuronal cells (Kraus et al., 2003; Vidal et al., 1998; Borner et al., 2004; Borner et al., 2002; Ruzicka et al., 1996). Direct injection of IL-1β into the hindpaw mimics CFA-induced up-regulation of kappa OR in DRG (Puehler et al., 2006). Similarly, in the cannabinoid system, CB1 mRNA and protein levels increase significantly in IL-1β, TNF-α or IL-6-stimulated whole blood compared to non-stimulated blood (Jean-Gilles and Constantinescu, 2007).

In this study, I showed that inflammatory cytokines such as IL-1β and IL-6 induce CB1 expression in TG. Since I only measured CB1 mRNA from intact TG or TG cultures we cannot claim that the cytokines elevated neuronal CB1. In our hands, several commercially available CB1 antibodies did not yield reliable and quantifiable immunohistochemical staining in TG neurons. However, CB1 is expressed primarily in neurons in DRG (Amaya et al., 2006), and there is a high level of co-localization of CB1 and TRPV1 in small diameter DRG neurons (Ahluwalia et al., 2000). These studies strongly suggest that the cytokine-induced up-regulation involves neuronal CB1.

While both IL-1β and IL-6 produced moderate but significant up-regulation of CB1 mRNA, IL-6 and TNF-α combined treatment resulted in a more pronounced increase in CB1 expression. This was not surprising since TNF-α has been shown to potentiate IL-6 effect in other systems (Poli et al., 1990; Kuhweide et al., 1990). An interesting observation was that the IL-1β effect is diminished when combined with TNF-α. Therefore, unlike the synergistic interactions between IL-6 and TNF-α, a high level of TNF-α could inhibit IL-1β in inducing CB1. CFA treatment did not result in CB1 up-regulation in intact animals when IL-1β and IL-6 were blocked, confirming the involvement of IL-6 and IL-1β in CB1 expression in TG. Furthermore, direct injection of IL-1β mimicked the CFA effect of CB1 expression in TG. Thus, the involvement of IL-1β in CB1 regulation is shown at three different levels.

Direct intramuscular injection of IL-6, however, failed to mimic the CFA effect. I collected evidence that IL-1β treatment in the masseter muscle evokes a significant release of IL-6 (data not shown), suggesting that IL-1β treatment accompanies the IL-6 effect. In fact, IL-1β leads to IL-6 production in various systems such as human orbital

fibroblasts, placental mesenchymal cells and Caco-2 cells (Cahill and Rogers, 2008; Chen et al., 2005; Kauma et al., 1994). These observations suggest that under *in vivo* conditions both IL-6 and IL-1β are required for CB1 induction. This interpretation is consistent with the data in Fig. 4 that showed a significant but only partial effect following the blockade of IL-6 compared to IL-1β. Taken together, our data provided compelling evidence that IL-1β and IL-6 are critically involved in the regulation of CB1 in TG under inflammatory conditions.

Gonadal hormones do not seem to modulate basal CB1 level in TG as there were no significant sex differences in the CB1 mRNA levels between the two sexes. A significant role of estrogen in CB1 expression is not supported in our data. We could not determine the estrous stage of female rats since CFA-induced inflammation disrupts estrous cycling (Wang et al., 2006). Therefore, the impact of fluctuations in estrogen levels in normally cycling female rats on CB1 is yet to be determined. In our hands, the cytokine-induced CB1 up-regulation was restored following a brief exposure to testosterone in GDX animals. This data has to be confirmed in GDX animals with testosterone replacement that more closely restore the serum testosterone level. Nevertheless, the present observations suggest that testosterone, but not estrogen, is required in the up-regulation of peripheral CB1 under inflammatory conditions.

Chapter 5: Sex differences in μ-opioid-mediated analgesia and modulation of MOR receptor expression

5.1 Abstract

Peripheral opioid receptor expression is up-regulated under inflammatory conditions, which leads to the increased efficacy of peripherally administered opioids. Sex differences in the effects of inflammation, cytokine levels and gonadal hormones on mu opioid receptor (MOR) expression in trigeminal ganglia (TG) are not well understood. MOR mRNA levels from TG of male and female Sprague Dawley rats following complete Freund's adjuvant (CFA)-induced muscle inflammation were assessed. Cytokine-induced changes in MOR expression from TG cultures prepared from intact and gonadectomized male and female, and gonadectomized male rats with testosterone replacement were examined. Behavioral experiments were then performed to examine the efficacy of a peripherally administered MOR agonist in male, female and gonadectomized male rats under a myositis condition. CFA and cytokine treatments induced significant up-regulation of MOR mRNA in TG of male, but not of female, rats. The cytokine-induced up-regulation of MOR expression did not occur in TG from orchidectomized (GDX) male rats, but this was restored with testosterone replacement. Peripherally administered DAMGO, a specific MOR agonist, significantly attenuated CFA-induced masseter mechanical hypersensitivity only in intact male rats. These data indicate that testosterone plays a key role in the regulation of MOR in TG under inflammatory conditions, and that sex differences in the anti-hyperalgesic effects of peripherally administered opioids are, in part, mediated by peripheral opioid receptor expression levels.

5.2 Introduction

Peripheral inflammation modulates μ-opioid receptor (MOR) expression in dorsal root ganglia (DRG) and trigeminal ganglia (TG). There is a significant increase in the MOR mRNA content and the percentage of MOR protein positive neurons in DRG 4 days following hindpaw inflammation induced by complete Freund's adjuvant (CFA) (Mousa, 2003; Puehler et al., 2004). A marked up-regulation of MOR protein occurs in DRG days after carrageenan-induced hindpaw inflammation (Ji et al., 1995). Inflammation of the deep tissues, such as visceral or muscle tissue, also results in the increase of MOR expression in sensory ganglia (Nunez et al., 2007; Pol and Puig, 2004).

The increase in MOR density in the peripheral tissue has been proposed as one of the major mechanisms underlying pronounced anti-hyperalgesic effects of peripheral opioids under inflammatory conditions (Zollner et al., 2003). However, molecular mechanisms underlying the modulation of MOR expression in sensory ganglia as well as potential mediators that induce such changes under inflammatory conditions have not been systematically investigated. Available data show cytokines such as IL-1β, IL-4, IL-6, and TNFα induce MOR expression in neuronal as well as in non-neuronal cell lines (Vidal et al., 1998; Borner et al., 2004; Kraus et al., 2001; Borner et al., 2002; Ruzicka et al., 1996). There are no data on whether cytokines also modulate MOR expression in sensory ganglia.

Humans and animals show sex differences in analgesia to systemic opioid treatments (Kest et al., 2000; Craft et al., 2004; Fillingim and Gear 2004). Multiple mechanisms underlying sex differences in opioid analgesia may exist, including cellular signaling mechanisms and hormonal and genetic effects (Kest et al., 1999; Cicero et al.,

2002; Mitrovic et al., 2003; Selley et al., 2003). Numerous studies also provide evidence for a sexual dimorphism in the opioid receptor density in various regions of the CNS, rendering opioid receptor expression level as the basis for sex differences in opioid mediated behaviors (Ostrowski et al., 1987; Wilson et al., 2002; Flores et al., 2003; Carretero et al., 2004; Harris et al., 2004). In the periphery, a local morphine administration in the jaw joint of male, but not female rats, significantly reduces nociceptive jaw muscle activity (Cai et al., 2001), and activation of peripheral MOR produces more potent analgesia in male than in female rats in a visceral pain model (Ji et al., 2006). However, the mechanisms underlying sex differences in peripheral MOR mediated analgesia have not been fully explored.

These observations led me to propose that inflammatory cytokines modulate MOR expression in sensory ganglia in a sex dependent manner, which lead to sex-specific in peripheral opioid anti-hyperalgesia. In this study, I used a rat model of orofacial myositis to evaluate: (1) whether inflammation differentially modulates MOR expression in TG between the sexes; (2) the role of inflammatory cytokines, specifically IL-1β, IL-6 and TNF-α, on MOR expression; (3) whether cytokine-induced MOR expression is modulated by sex hormones, and (4) whether sex differences in MOR expression is related to anti-hyperalgesic effects of a peripherally administered MOR agonist under the inflammatory condition.

5.3 Results

5.3a Sex differences in inflammation-induced MOR expression in TG

In order to compare the impact of inflammation on MOR expression between the sexes we measured the changes in the MOR mRNA content in TG following intramuscular injection with CFA. No sex differences in the level of MOR mRNA in TG were observed in naïve rats (Fig. 14A; t=1.03, p=0.34). CFA-induced masseter inflammation resulted in a time-dependent increase in the level of MOR mRNA in TG of male, but not of female rats (Fig. 14B,C). In male rats, the level of MOR mRNA was significantly greater in CFA treated groups on days 1 and 3 compared to the naïve group (H=13.84, p=0.003). The CFA treatment did not alter MOR expression in TG contralateral to the injected side in either male or female rats (data not shown). These data confirmed that masseter inflammation induces changes in MOR mRNA levels in TG of male rats (Nunez et al., 2007), and provided new evidence that similar changes do not occur in female rats.

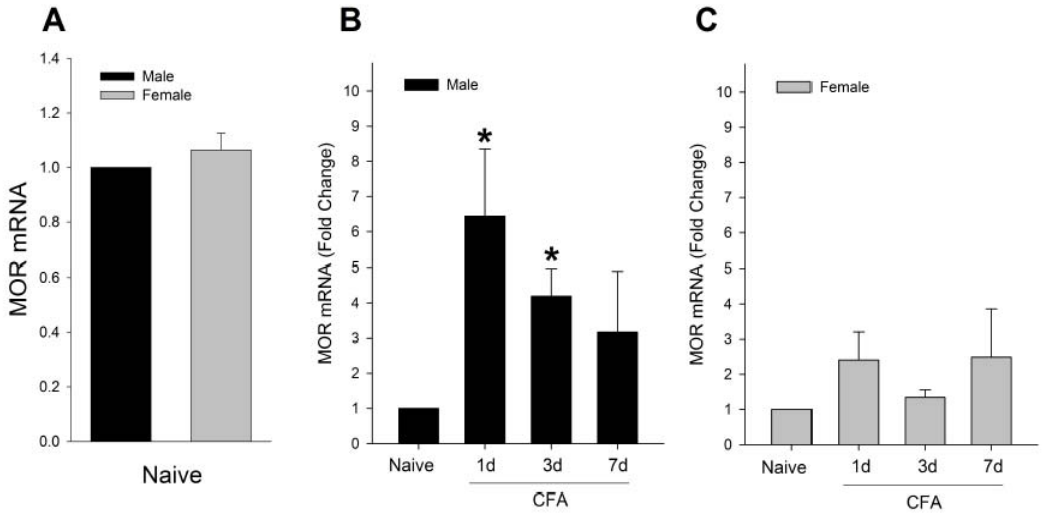

Fig. 14. Sex differences CFA-induced MOR mRNA expression in TG. (A) Real time RT-PCR data showing MOR mRNA levels in TG of age-matched naïve male and female rats. The MOR mRNA level in female was normalized to that of male rats. (B,C) Real time RT-PCR data showing CFA-induced changes in MOR mRNA levels in TG of male and female rats, respectively. Data from CFA inflamed rats were normalized to naïve rats and presented as a fold change. * denotes significant effects with respect to naïve condition at $p < 0.05$. Each group consisted of 6 animals.

5.3b Effects of inflammatory cytokines on MOR expression

In this experiment, we investigated whether inflammatory cytokines induce differential up-regulation of MOR expression in male and female rats using TG tissue cultures. In TG cultures from male rats, the level of MOR mRNA was significantly higher following the application of TNF-α, IL-1β, or IL-6 (along with its soluble α-receptor subunit IL-6R) compared to the vehicle condition (Fig. 15A; F=5.6, p=0.004). The vehicle treatment itself, in this case PBS, did not significantly alter the basal level of MOR mRNA. The same concentration of IL-1β, IL-6R, or TNF-α did not induce significant changes of MOR mRNA in TG cultures prepared from female rats (Fig. 15B; F=0.73, p=0.55).

We then investigated whether these inflammatory cytokines are involved in CFA-induced up-regulation of MOR mRNA in intact TG. IL-1 receptor antagonist (IL-1RA) or soluble gp130 (an inhibitor of IL-6 signaling complex) was directly administered into the masseter muscle 5 min prior to the induction of inflammation by CFA in male rats. TG from these rats were extracted 3 days later and the level of MOR mRNA was assessed. We did not examine the effects of TNF-α receptor antagonist since CFA does not increase the level of TNF-α in the masseter muscle. The pretreatment of the muscle with PBS failed to block the CFA-induced up-regulation of MOR mRNA (Fig. 15C; H=14.2, p=0.03). However, IL-1RA, at a dose that has been shown to effectively attenuate the IL-1β effect (2 μg) (Hook et al., 2011), prevented the up-regulation of MOR mRNA in TG. Similarly, soluble gp130 at a dose that effectively antagonizes the IL-6 effect (2 μg) (Obreja et al., 2002) prevented the CFA-induced up-regulation of MOR mRNA in intact TG (Fig. 15C).

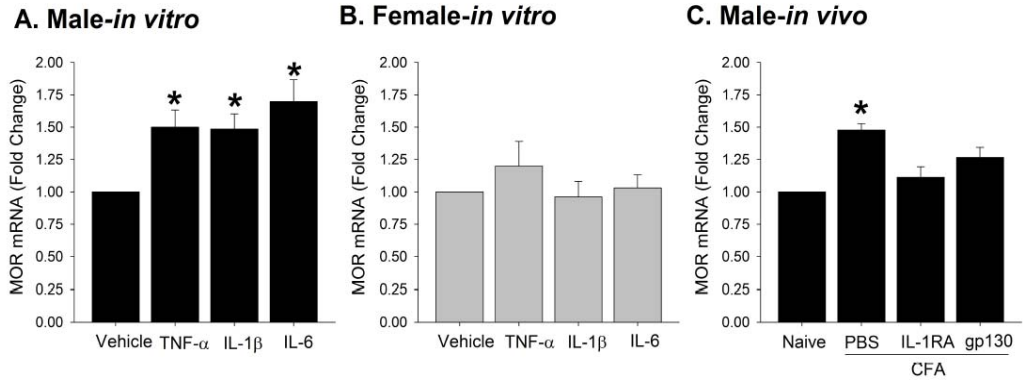

Fig. 15. Sex differences in cytokines' effect on MOR mRNA expression. (A,B) Effects of cytokines on MOR mRNA expression in TG tissue cultures. Real time RT-PCR data following individual cytokine applications were compared to those of PBS treatment in cultures prepared from male and female rats. (C) Role of inflammatory cytokines in CFA-induced up-regulation of MOR mRNA in intact TG of male rats. CFA-induced changes in MOR mRNA in intact TG in the presence of IL-1β or IL-6 receptor antagonist or PBS were compared to that of untreated rats. Data are shown as mean ± SE and * denotes significant differences compared to control group at $p < 0.05$. Each group consisted of 6-9 animals.

5.3c Testosterone is required for cytokine-induced MOR mRNA up-regulation

Since cytokine-induced up-regulation of MOR mRNA was only observed in TG from male rats, we investigated whether sex hormones play a role in modulating cytokine-induced MOR expression. In order to examine the role of testosterone, we applied the same cytokine treatments to TG cultures prepared from GDX rats. Under this condition, none of the cytokines was effective in up-regulating MOR mRNA (Fig. 16A). Instead, IL-1β application resulted in a slight, but significant down-regulation of MOR mRNA expression ($F=4.9$, $p=0.01$). We then applied the same set of cytokines to TG cultures prepared from GDX rats that received testosterone replacement for 7 days. In TG cultures from these rats, all three cytokines significantly increased the MOR mRNA level (Fig. 16B; $H=13.1$, $p=0.04$).

Since it is possible that estrogen could have persistent inhibitory effects on cytokine-induced MOR expression, we applied the same concentrations of the three cytokines to TG cultures prepared from OVX female rats. None of the cytokines exerted a significant effect on MOR mRNA expression (Fig. 16C; $F=0.63$, $p=0.6$).

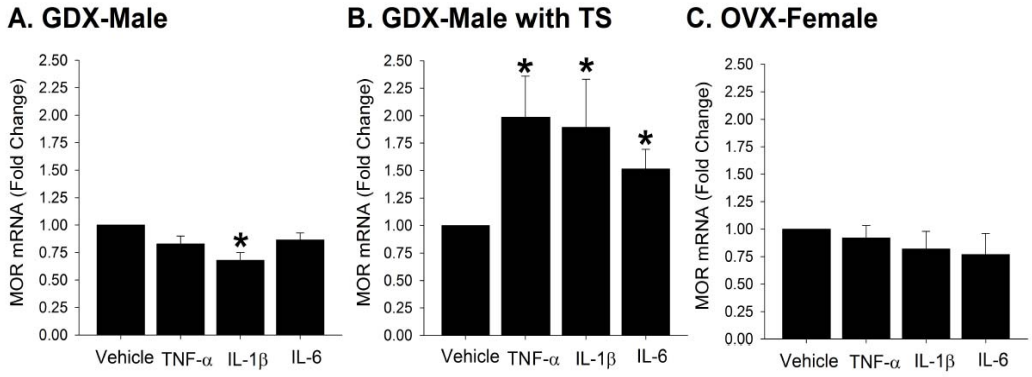

Fig. 16. Role of gonadal hormones in MOR mRNA expression in TG. (A, B) Real time RT-PCR data following individual cytokine applications were compared to that of PBS treatment in GDX rats and in GDX rats that received testosterone (TS). (C) MOR mRNA levels were assessed from TG of OVX rats treated with the same cytokines. All data were normalized to vehicle treated samples and presented as fold changes in MOR mRNA transcripts. Data are shown as mean ± SE and * denotes significant differences compared to PBS group at $p < 0.05$. Each group consisted of 6-8 animals.

5.3d Sex differences in the effect of peripheral MOR activation on masseter hypersensitivity

Masseteric injection of CFA in the rat induces a time-dependent and significant decrease in mechanical thresholds as early as 30 min that lasts over 12 days (Ambalavanar et al., 2006; Shimizu et al., 2009). In chapter four, I confirmed the development of mechanical hypersensitivity following CFA injection in the masseter with a peak decrease in EF_{50} on 1-3 days post CFA injection. In order to examine the effect of MOR activation in CFA-induced masseter mechanical hypersensitivity, we injected the muscle with DAMGO (10μg) or vehicle 45 min prior to behavioral testing on the third day following CFA treatment. At this dose, DAMGO effectively blocks masseter nocifensive responses without producing systemic effects by specifically targeting peripheral MOR (Nunez et al., 2007).

The EF_{50} of baseline mechanical thresholds were comparable between male and female rats (59.9±2 g and 55.1±3.98 g, respectively). Intramuscular CFA injection resulted in a significant mechanical hypersensitivity for the three days we monitored in both male and female rats. In those rats, the vehicle injection into the masseter muscle on day 3 did not alter the mechanical thresholds in either male of female rats (Fig. 17A; Time: F=76.5, p<0.001; Sex: F=2.4, p=0.162). In contrast, DAMGO treatment in the masseter muscle significantly reversed the CFA-induced mechanical hypersensitivity in male, but not in female, rats (Fig. 17B; Time: F=95.3, p<0.001; Sex: F=20.3, p<0.001). Finally, neither DAMGO nor vehicle given 3 days post CFA treatment significantly altered the mechanical hypersensitivity in GDX male rats (Fig. 17C; Time: 75.2, p<0.001; Drug: F=1.2, p=0.29).

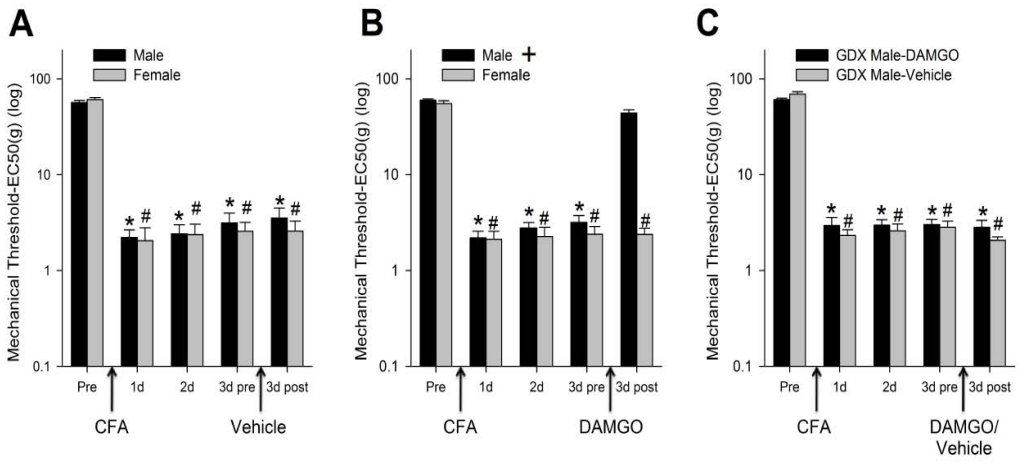

Fig. 17. The role of testosterone in MOR mediated anti-hypersensitivity. (A, B) Effects of intramuscular DAMGO or vehicle on mechanical sensitivity 3 days after CFA treatment in intact male and female rats. Bar graphs show mean EF_{50} values in vehicle and DAMGO treated rats. (C) Effects of DAMGO on mechanical sensitivity 3 days after CFA treatment of GDX rats. + denotes significant sex differences, and * and # significant time dependent changes for each group at $p < 0.05$ compared to the pretreatment condition. All data are shown as mean ± SE and each group consisted of 6-8 animals.

5.4 Discussion

Sex differences in MOR expression have been reported in many regions of the brain. In the rat anterior pituitary gland, MOR is more abundantly expressed in males than in females (Carretero et al., 2004). Similarly, the MOR protein levels in the midbrain and the spinal cord are higher in male rats than in female rats, and CFA-induced arthritis treatment significantly increases MOR expression in the midbrain only in male, but not in female rats (Kren et al., 2008). In the rat periaquaductal gray, males exhibit significantly higher expression of MOR compared with cycling females (Loyd et al., 2008).

Although changes in MOR expression levels following CFA-induced inflammation were not assessed in Loyd et al (2008), a microinjection of morphine into the PAG more effectively relieves CFA-induced thermal hyperalgesia in males than in females, suggesting a more potent morphine analgesic effect that correlates with a higher level of MOR expression in males.

In the periphery, opioid receptor efficacy is augmented under inflammatory or injury conditions due to the increase in opioid receptor density in primary afferent neurons and the recruitment of immune cells that express opioid peptides to the inflamed site (Pol and Puig, 2004; Zollner et al., 2003; Hassan et al., 1993; Mousa et al., 2010; Hackel et al., 2011; Schreiter et al., 2012). Consistent with these observations, it has been shown previously in our lab that masseter inflammation up-regulates MOR mRNA in TG (Nunez et al., 2007), which I confirmed in this study. Here I report that similar changes do not occur in female rats. To the best of my knowledge, it is unknown whether such profound sex differences in MOR expression in sensory neurons also exist in other inflammatory models since only males were used in these studies.

In a visceral pain model, the peripherally restricted MOR agonist, loperamide exhibits greater potency in males compared with females and the peripherally restricted MOR antagonist naloxone methiodide dose-dependently attenuates the systemic morphine effects, which could be mediated by a higher level of peripheral MOR expression in male rats (Ji et al., 2006). Loperamide also exerts greater anti-hyperalgesic effects in CFA treated arthritic male than female rats (Cook and Nickerson, 2005), and direct morphine injection into the TMJ significantly suppresses glutamate-evoked jaw muscle activity only in male rats (Cai et al., 2001). These studies clearly implicate sex differences in peripheral MOR-mediated analgesia, but MOR expression levels were not directly assessed. Our data provided important clues that sex differences in MOR function are, in part, mediated by the MOR expression levels in sensory neurons. While full dose-response functions of DAMGO for male and female rats are required to assess the extent of sex differences, our data revealed novel information on the impact of inflammation on peripheral MOR expression that could explain greater anti-hyperalgesic effects in males by peripheral opioids.

Since masseter inflammation led to the up-regulation of MOR mRNA in TG in a sex dependent manner in this study and inflammatory cytokines are potent modulators of opioid receptors, it is reasonable to propose that inflammation produces different cytokine profiles in local tissue that might be correlated with MOR expression between the sexes. However based on my data, CFA-induced inflammation produces a similar rise in IL-1β and IL-6 in both male and female rat muscle tissue. Muscle inflammation does not significantly increase local TNFα levels (Loram et al., 2007). Despite the similarities in local cytokine levels, direct application of cytokines resulted in MOR mRNA increase

only in TG of male rats. These observations indicate that MOR up-regulation in TG cannot be explained solely by local cytokine effects. While it is possible that functional transcriptional factors or cytokine receptors are differentially expressed in TG of the two sexes, my data showed that testosterone is required for cytokine-induced up-regulation of MOR mRNA. In the previous chapter, I have shown that cytokine-induced up-regulation of CB1 is also dependent on the presence of testosterone. The up-regulation of CB1 is prevented in TG cultures from GDX male rats, only to be restored by testosterone replacement. The cytokines do not alter the CB1 mRNA level in TG either from intact or OVX female rats, and neither estradiol supplement nor estrogen receptor blockade produces any effects on CB1 expression. Together, these data provide compelling evidence that testosterone plays a key role in modulating peripheral anti-nociceptive systems under inflammatory conditions.

Chapter 6: General discussion.

6.1 Summary of main findings

This study demonstrated that masseter inflammation induces significant up-regulation of CB1 and MOR mRNA levels in TG in male, but not in female, rats, which could underlie the sex differences in anti-hyperalgesic responses to peripherally administered agonists. Since inflammatory cytokines are potent modulators of opioid and cannabinoid receptors, I initially proposed that inflammation produces different cytokine profiles in local tissue between the sexes, which might lead to sex differences in CB1 and MOR expression. The data showed that masseter inflammation induces significant and time-dependent changes in the levels of IL-1β, IL-6 and IL-4 in the muscle tissue in both male and female rats. However, no sex differences in the levels of these cytokines are observed. Muscle inflammation induced significant sex differences in the levels of TNF-α. Despite the similarities in local cytokine levels between male and female rats, direct application of individual cytokines or combinations of cytokines increases MOR and CB1 mRNA levels only in male TG. These observations indicate that the regulation of MOR and CB1 mRNA expression in TG cannot simply be explained by local cytokine levels. I also demonstrated that the cytokine-induced up-regulation of CB1 and MOR mRNA is absent in TG cultures from GDX male rats, but can be restored by testosterone replacement. Cytokine treatments do not alter CB1 or MOR mRNA levels in TG from either intact or OVX female rats. Neither estradiol supplement nor estrogen receptor blockade produces any alterations in CB1 expression. Together, these data provide compelling evidence that testosterone plays a key role in modulating peripheral anti-nociceptive systems in males under inflammatory conditions.

6.2 Peripheral CB1 and MOR in chronic pain conditions

Non-steroidal anti-inflammatory analgesics (NSAIDS) and central opioids will currently remain as initial treatments for acute or postoperative pain. However, the analgesic effects of systemic cannabinoids and opioids are accompanied by centrally-mediated side effects. Peripheral cannabinoid and opioid analgesics may become a solution for the treatment of various chronic pain conditions when NSAIDS or opioids are contraindicated or are ineffective. Recent studies amply demonstrate that peripherally localized CBRs are attractive targets which can mediate cannabinoids' therapeutic effects in many types of pain conditions without producing unwanted side effects (Guerrero et al., 2008; Walczak and Cervero, 2011; Reis et al., 2011; Sanchez Robles et al., 2012). Selective knockout of CB1 in nociceptors in mice substantially reduces the analgesic effect produced by systemic delivery of cannabinoids suggesting peripheral CB1 as the major site for cannabinoid-mediated analgesia (Agarwal et al., 2007). Consistent with this finding, systemic delivery of peripherally restricted cannabinoid compounds produces robust and sufficient analgesic and anti-hyperalgesic effects in animal models of inflammatory and neuropathic pain (Walczak and Cervero, 2011; Dziadulewicz et al., 2007). My project provides novel data that locally administered low doses of a CB1 agonist can also effectively alleviate craniofacial muscle pain under inflammatory conditions in males.

Studies implicating peripheral CB1 in the blockade of not only the development, but also the maintenance, of pathological pain conditions lend further support for therapeutic interventions targeting CBRs at the site of injury (Gutierrez et al., 2007; Khasabova et al., 2008). In clinical trials, Sativex, a cannabis extract, provides significant

improvement in movement-induced pain in rheumatoid arthritis patients (Blake et al., 2006), and effectively relieves peripheral neuropathic pain without causing significant psychotropic effects, suggesting a peripheral action of the drug (Spigelman, 2010). These observations provide a strong rationale for the development of pharmacological interventions that selectively target peripherally localized CBRs. Another clinically relevant option is to augment the peripheral cannabinoid system to increase endocannabinoid levels at the sites of injury or inflammation. Specific enzymes have been identified that metabolize endocannabinoids. For example anandamide is metabolized by fatty acid amide hydrolase (FAAH) and 2-arachidonoylglycerol (2-AG) is broken down by monoacylglycerol lipase (MAGL). Selective FAAH and MAGL inhibitors have already been developed (Kathuria et al., 2003; Ghafouri et al., 2004; Quistad et al., 2006). It has been demonstrated that FAAH inhibitors are effective in alleviating neuropathic pain symptoms (Lichtman et al., 2004). These FAAH and MAGL inhibitors permeate the blood brain barrier, however, despite CNS permeability, these inhibitors do not exhibit the same adverse effects produced by activating central cannabinoid receptors (Comelli et al., 2007; Esfandyari et al., 2007). Although further studies are needed to understand the effects of peripherally restricted FAAH and MAGL inhibitors, the development of peripherally restricted FAAH and MAGL inhibitors could be important for cannabinoid-based therapeutics.

Considerable effort was made in the past two decades to characterize peripheral opioid receptors and the effectiveness of peripherally administered opioids under chronic pain conditions. The analgesic efficacy of peripheral opioids in a clinical setting has been most extensively studied after knee arthroscopy. While the majority of studies on intra-

articular application of opioids support the role for peripheral opioid receptors, some do not (Rosseland, 2005). Similarly, injections of intra-articular morphine in the temporomandibular joint (TMJ) effectively relieves pain in temporomandibular disorder patients (Ziegler et al., 2010), but is ineffective in patients following TMJ arthroscopy (Bryant et al., 1999). While the discrepant findings seem to undermine the clinical importance of targeting peripheral opioid receptors, methodological factors such as study sensitivity, tissue inflammation, or superimposition of general or local anesthetics, which can mask the local opioid effects need to be carefully considered when interpreting the existing data (Stein, 2006; Stein and Kuchler, 2012).

In other inflammatory or injury conditions, such as chronic rheumatoid osteoarthritis, chronic tooth inflammation and postoperative bladder surgery, application of opioids at the local site effectively relieves pain and hyperalgesia (Duckett et al., 1997; Likar et al., 2001; Rorarius et al., 1999; Dionne et al., 2001). Tegeder et al (2003) demonstrated that local infusion of morphine-6-â-glucuronide, a major active metabolite of morphine, significantly reduced muscle hyperalgesia induced by a series of concentric and eccentric muscle contractions in human subjects (Tegeder et al., 2003). To the best of my knowledge, there is no clinical study addressing peripheral opioid effects in chronic muscle pain conditions. It is important to note that pre-clinical studies demonstrating the effectiveness of peripheral opioid receptors are accumulating, including those under neuropathic pain conditions (Shinoda et al., 2007; Guan et al., 2008; Sanchez et al., 2010; Saloman et al., 2011; Auh and Ro, 2012; Martins et al., 2012; Ni et al., 2012). Therefore, peripheral opioid receptors remain as viable target for pain management, especially under

inflammatory conditions, and additional human experimental as well as clinical studies are definitely warranted.

My studies provide novel evidence supporting sex differences in both peripheral cannabinoid and opioid treatments. Both cannabinoids and opioids are more effective in reducing inflammatory hypersensitivity in male rats than in female rats. These findings suggest that when treating inflammatory orofacial pain a higher dosage may be required in female patients. This brings out an important issue of whether the sex differences in orofacial pain treatment are quantitative or qualitative in nature. If the sex differences reflect a quantitative difference, the same level of anti-hyperalgesia can be achieved by prescribing of higher doses to females. I have also shown that testosterone is important in modulating both MOR and CB1 expression. Based on my preliminary experiment, testosterone did not induce CB1 up-regulation in cultures from female rats. However, it is possible, and even likely that other hormones or mechanisms could be involved in anti-nociception in females. Thus, there could be both qualitative and quantitative mechanisms involved in MOR and CB1 anti-nociceptive processing in females. Further study is required to address these questions.

6.3 Cytokines and their roles in regulation of CB1 and MOR expression in TG

Cannabinoids and the immune systems interact bi-directionally. Cannabinoids suppress the production of cytokines in innate and adaptive immune responses (Klein et al., 1998; Berdyshev, 2000; Shohami et al., 1997). For example, anandamide suppresses proliferation and the release of cytokines IL-2, TNF-α and INF-γ from primary human T-cells via CB2 (Cencioni et al., 2010). The active psychotropic substance in cannabis, Δ^9-

Tetrahydrocannabinol (THC) treatment suppresses the serum levels of IL-12 and IFN-γ (Klein et al., 2000). Cannabinoids also suppress the migration of inflammatory cells (Ni et al., 2004). Thus, cannabinoids have strong immunosuppressive and anti-inflammatory effects. On the other hand, cytokines have been indicated to modulate cannabinoid receptor expression in various cell types. For example, cytokine-stimulated whole blood elevates CB1 and CB2 mRNA and protein levels when compared to non-stimulated blood (Jean-Gilles and Constantinescu, 2007). The expression of CB2 in spinal cord is up-regulated with a time course consistent with the production of pro-inflammatory cytokines after the onset of multiple sclerosis (Loria et al., 2008). It has also been shown that IL-4 induces CB1 gene transcription in a STAT6-dependent manner in T cells (Borner et al., 2007).

It is relatively well established that opioid and immune systems also interact bi-directionally. Opioid treatments modulate inflammatory responses by altering cytokine production and cytokine receptor expression (Peng et al., 2000; Raghavendra et al., 2004; Hutchinson et al., 2008). Then again, cytokines are the most widely studied factor that modulates opioid receptor expression in various cell types (Kraus, 2009). The promoter regions of the MOR gene contain binding sites for many transcription factors. IL-1α and IL-1β treatment induces MOR expression in neural microvascular endothelial cells, possibly by activating the cytokine response element, NF-IL6 in the opioid receptor gene (Vidal et al., 1998). However, NF-IL6 in the opioid receptor gene is not functional in immune cells (Im et al., 1999). Using transcription factor decoy oligonucleotides, Börner et al. (2004) showed that STAT1 and STAT3, but not other transcriptional factors such as nuclear factor for IL-6 expression (NF-IL6) and activator protein 1 (AP1), are involved in

MOR transcription in the human neuroblastoma cell line (Borner et al., 2004). In the same cell line, AP1 is required for MOR gene expression following the activation of protein kinase C signaling pathways (Borner et al., 2002). TNFα also induces MOR gene expression in immune cells in a nuclear factor-kappa B, but not AP1, dependent manner (Kraus et al., 2003). Thus, specific cytokine-mediated MOR gene expression is determined by transcriptional factors and cell types.

To my knowledge, these studies are the first direct demonstration of cytokine-induced up-regulation of CB1 and MOR in TG, and confirmed in both *in vivo* and *in vitro* conditions. Further experiments are necessary to examine the molecular mechanisms underlying the transcriptional up-regulation of CB1 and MOR expression. I have attempted to examine the protein expression of CB1 and MOR in addition to the mRNA expression. However, due to the quality of the antibodies available, the data were not conclusive. It is possible that the protein levels could be examined by expressing a tagged receptor in culture experiments. Immunohistochemistry studies could be useful as well when high quality antibodies become available.

Another important observation to come from these studies is that pro-inflammatory cytokines may also produce anti-nociceptive effects. Pro-inflammatory cytokines are accountable for initiating inflammation in response to tissue injury. Prolonged inflammation can be detrimental because unregulated increases in inflammatory factors can induce host toxicity and tissue damage. Incidentally, pro-inflammatory cytokines are involved in modulating anti-nociceptive systems such as the CB1 and MOR receptor expression which increase the efficacy of cannabinoid and opioid treatment of chronic pain conditions. Thus, it is important to understand the mechanism

underlying the cannabinoid/opioid and immune interactions as well as anti-inflammatory treatments targeting pro-inflammatory cytokines which may also compromise anti-nociceptive responses.

6.4 Role of testosterone in Cytokine-induced regulation of CB1 and MOR in TG

Currently, the effect of sex hormones on CBR expression is not well understood. 17β-estradiol treatment induces CB1 expression in human colon cancer cells and the co-administration of an estrogen antagonist blocks this effect (Notarnicola et al., 2008). In the hippocampus and the hypothalamus, however, OVX females have higher amounts of cannabinoid receptor binding relative to both cycling females and estradiol-replaced OVX females (Riebe et al., 2010). Similarly, in the anterior pituitary gland, the CB1 mRNA transcript is at the lowest level during the estrus stage in normally cycling female rats, and estradiol-replaced OVX rats exhibit significantly lower CB1 mRNA compared to OVX animals that had not received estradiol replacement (Gonzalez et al., 2000). Interestingly, the same study also showed that males maintain higher levels of CB1 mRNA than female rats and that orchidectomy in males reduced CB1 mRNA levels. In the parotid gland, castration in male rats significantly reduces CB1 expression, which is restored after testosterone treatment (Busch et al., 2006). Given that a higher density of CB1 levels is observed in male animals in many brain regions (Rubino and Parolaro, 2011), it seems that CB1 expression is influenced by gonadal hormones and that testosterone plays a key role in the regulation of CB1 in the CNS as well as in the periphery.

These observations are consistent with my data that testosterone is required for cytokine-induced up-regulation of MOR and CB1 mRNA in primary afferent neurons. While many factors such as sex specific transcriptional factors or the expression level of cytokine receptors in TG of the two sexes could potentially contribute to sex differences in cytokine modulation of CB1 and MOR, we speculate that cytokines may promote a testosterone-androgen receptor complex that directly modulates peripheral MOR and CB1 in sensory ganglia. Recently, it was determined in the lab that the promoter regions of both CB1 and MOR genes contain putative androgen receptor binding sites. Further studies examining such molecular mechanisms should yield important knowledge on immune-hormone-neural interactions in peripheral pain modulatory systems.

Testosterone provides many health benefits, including increased bone mineral density, improved muscle mass and strength, increased sexual desire, increased energy, decreased irritability and depression and improved cognition (Gruenewald and Matsumoto, 2003). Recently, reports have begun to establish the role of testosterone in pain and anti-nociceptive mechanisms. Long term use of morphine induces a dramatic long-lasting decrease in testosterone level in both males and females by inhibiting GnRH secretion in the hypothalamus (Aloisi et al., 2005). The effect of opioids can occur after a few hours of drug use and the level of testosterone can reach castration levels. Usually, once the opioid treatment is interrupted, testosterone levels do recover in a few days (Aloisi et al., 2009).

Testosterone deficiency has also been reported in chronic pain patients and testosterone replacement therapy is necessary for satisfactory pain control (Aloisi et al., 2011). A substantial population of men with spinal cord injury experiences testosterone

deficiency and there is a significant association between testosterone level and the severity of spinal cord injury (Durga et al., 2011). Schopp et al. (2006) demonstrated that there is a significant decline in serum testosterone level immediately after acute spinal cord injury and 83% of men with acute spinal cord injury have testosterone deficiency within four months of injury (Schopp et al., 2006).

My data suggest that testosterone is involved in maintaining endogenous anti-nociceptive systems such as CBRs and ORs in chronic pain conditions. Effective treatment strategies targeting peripheral CB1/MOR should thus consider the hormonal status of patients.

6.5 Conclusion/Future directions

This study investigated the mechanisms involved in modulating CB1 and MOR mRNA expression which in turn contributed to the effect of CB1 and MOR agonists in attenuating mechanical hyperalgesia following inflammation. This project revealed that testosterone is important in the regulation of peripheral cannabinoid and opioid systems in males. Further studies are needed to investigate the exact mechanisms through which testosterone modulates the cannabinoids and opioids. First, it is important to understand the cellular pathways underlying cytokine induced CB1 and MOR expression and how testosterone plays a role in up-regulating CB1 and MOR expression. It is possible that cytokines induce translocation of androgen receptor (AR) from the cytoplasm to the nucleus in sensory neurons in the presence of testosterone. Direct binding of AR to CB1/MOR genes functions as a transcriptional factor to induce the up-regulation of CB1/MOR. Second, it is also imperative to understand the nature of the sex differences in

the cannabinoid and opioid system. If females were treated with testosterone, would this mimic the conditions observed in male rats? Would testosterone replacement alone in females be adequate for achieving effective cannabinoid and opioid analgesia? Does a distinct mechanism for transcriptional regulation of CB1/MOR exist in females? My data offer important clues to further investigate cellular mechanisms that link cytokines and sex hormones in various inflammatory conditions, which should offer important new insights for the development of mechanism-based sex-specific pharmacological treatment alternatives that can be directed at the peripheral anti-nociceptive system to ameliorate persistent pain.

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