The Effects of Estrogen on Carrageenan-Induced Nociceptive Behaviors

and Inflammatory Mediators in Ovariectomized Female Mice

by

Lisa C. Abrams

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy
The City University of New York
2010

This manuscript has been read and accepted for the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

Vanya Quiñones-Jenab, Ph.D.

_____ _____

Date Chair of Examining Committee

Maureen O'Connor, Ph.D.

_____ _____

Date Executive Officer

Vanya Quiñones-Jenab, Ph.D_____

Shirzad Jenab, Ph.D._____

James Gordon, Ph.D._____

Victoria Luine, Ph.D._____

Ann Ho, Ph.D._____

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

ABSTRACT

The Effects of Estrogen on Carrageenan-induced Nociceptive Behaviors and Inflammatory Mediators in Ovariectomized Female Mice

by

Lisa C. Abrams

Advisor: Dr. Vanya Quiñones-Jenab

Epidemiological studies have shown that pain perception is sexually dimorphic; females tend to experience greater sensitivity to painful stimuli and more chronic pain compared to males. Researchers believe that this dichotomy is caused by the distinct endocrinological profile of females. 17β-estradiol has been shown to attenuate inflammatory behaviors in both the formalin and carrageenan (Cg) models of inflammation. Research also shows that estrogen affects many inflammatory mediators, including proinflammatory cytokines and prostaglandins (PG). Estrogen plays an important, yet complicated role, in inflammation, and little is known about the specific biochemical mechanisms involved. The objective of this study is to determine if, similar to rats, estrogen attenuates Cg-induced thermal hyperalgesia by altering cytokine or PG release. To that end, female OVX mice were pretreated with various doses of estradiol and injected with 1% Cg. Paw withdrawal latency was recorded prior to, 1 hour, and 5 hours after Cg-treatment in response to a low, medium, and high heat stimuli. Additional animals were treated with indomethacin, a COX blocker. High doses of estradiol caused an increase in nociceptive responses prior to and subsequent to Cg administration. This increase in these pain behaviors was not directly caused by an increase in proinflammatory cytokine levels or a decrease in anti-inflammatory cytokines levels. However, estradiol caused increases in cytokine levels in the

untreated paw. Furthermore, treatment with indomethacin caused an attenuation of hyperalgesia. Additionally, indomethacin negated the difference between estradiol- and vehicle-treated mice, indicating that estrogen may interact with prostanoid synthesis. This effect, however, was not seen in the Cg-treated paw, suggesting that estradiol may be increasing hyperalgesia via another pathway as well. Taken together, our results suggest that estrogen's hyperalgesic effects are partly mediated through cytokine up-regulation and prostanoid synthesis, but the main mechanism of action still needs further investigation.

ACKNOWLEDGEMENTS

I have been extremely fortunate to have many people in my life who have helped me throughout my graduate career. I sincerely thank you all for giving me the support that I needed to get to this point in my life.

To my mentor, Dr. Vanya Quiñones-Jenab, I thank you for your guidance throughout my career as a graduate student. I appreciate all the advice you gave me on each aspect of my project. I would also like to thank my dissertation committee for taking part in my thesis. To Drs. Shirzad Jenab, Jim Gordon, and Victoria Luine, thank you for helping me with the design of my project. To Dr. Ann Ho, thank you for your input on the statistical analysis of my data.

To the members of the Quinones' lab, thank you so much for the help you have given me on my various projects throughout the years! Thanks go to Drs. Deirtra Hunter and Nicole Amador, along with Tina Mathew and Stephanie Nygard for all of their help with running my experiments. Thanks also goes to Ruhal Hazim for helping me with all of my complicated statistics. A special thank you goes to Kai-Yvonne Shivers, the person who trained me from my first day and came in on holidays to run experiments with me! Without her help as a great colleague and friend, I would not have reached this point in my life. Thank you also to all of the undergraduate students who helped me with my bench work. This project would not be possible without all of your help.

I would also like to thank my friends for their support and understanding. I would especially like to thank Michelle and Karen, who have been the most understanding throughout the last 5 years and who always have an ear to listen if I need it.

I want to thank my family for all of the love and support they have shown me throughout this process. Thanks goes to my parents, for putting up with me when I was stressed out.

Special thanks goes to my sisters, Lyn and Stephanie, who always know how to be there for me, who make me laugh even when I don't want to. The amount of love from you guys has been an extremely important part of my journey, and for that I will be forever thankful. Thanks also goes to my neice, Lia, and nephew, Caden, for they always know how to lift my mood no matter what else is going on. I love you guys so much!

Last of all, I'd like to thank my boyfriend, Dwayne. Without his support over the last 4 years, I would not have been able to make it to this point. It takes a special person to be unalterably supportive to someone going through graduate school, and he has made my journey easier and so much more enjoyable. Thank you for everything that you've done for me!

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

2-AG	2-arachidonoyl-glycerol
AA	Arachidonic acid
AEA	Arachidonol-ethanolamide
AMPA	α-amino-3-hydroxy-5-methylisoxazole
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
βc	β-chain receptor
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CCI	Chronic constriction injury
CFA	Complete Freund's adjuvant
Cg	Carrageenan
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
COX	Cyclooxygenase
CSF	Colony stimulating factor
DRG	Dorsal root ganglia
EP	E prostanoid receptor
ERK	Extracellular signal-regulated kinase
FAAH	Fatty acid amide hydrolase
GABA	γ-aminobutyric acid
GDX	Gonadectomy
GI	Gastrointenstinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HPA	Hypothalamic-pituitary-adrenal
IBS	Irritable bowel syndrome
IFN	Interferon
IL	Interleukin
IL-1R	IL-1 receptor
IL-1ra	IL-1 receptor antagonist
IP	Prostacyclin
JAK	Janus Kinase
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
Mg^{2+}	Magnesium
mRNA	Messenger ribonucleic acid
Na^{+}	Sodium
NF-κB	Nuclear transcription factor kappaB
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAID	Nonsteroidal anti-inflammatory drug
OVX	Ovariectomy
PG	Prostaglandin
PGE_2	Prostaglandin E_2
PGI_2	Prostagladin I_2
PKA	Protein kinase A
PKC	Protein kinase C
PNS	Peripheral nervous system
PWL	Paw withdrawal latency
RA	Rheumatoid arthritis
SP	Substance P
STAT	Signal transducer and activator of transcription
TH1	T-helper 1cell
TH2	T-helper 2 cell
TMD	Temporomandibular disorder
TNF	Tumor necrosis factor
TNFR1	TNF receptor type 1
TNFR2	TNF receptor type 2
TrkA	Tyrosine Kinase A
TRPV1	Vanilloid receptor 1
TTXr	Tetrodotoxin-resistant

Chapter 1: Introduction

Pain, although unpleasant, plays an essential role in the survival of an organism. It serves a physiologically protective function by immediately alerting an individual to the presence of a potentially damaging stimulus in the environment (Riedel & Neeck, 2001; Costigan & Woolf, 2000; Basbaum & Woolf, 1999), while allowing the individual to react appropriately to the dangerous stimulus and minimize its pain (Costigan and Woolf, 2000). Pain also warns an organism of an injury that should be treated or avoided (Basbaum & Jessell, 2000) and serves as a conduit through which an organism learns to avoid harmful stimuli in the future (Weiseler-Frank, Maier, & Watkins, 2004). Finally, after tissue damage, pain promotes injury repair by creating a region of localized sensitivity around the injury, referred to as inflammation. This inflammation reduces movement or contact with the injury while it heals (Basbaum & Woolf, 1999). While pain has evolved with an obvious evolutionary value (Bennett, 2001), it can also be maladaptive. Clinical pain can arise from damage to the nervous system or chronic inflammation which occurs in diseases like arthritis. This type of pain is pathological and has no benefit to the individual (Costigan & Woolf, 2000).

Chronic pain studies are often used to enhance knowledge about maladaptive pain states that can be applied to the management of acute and chronic pain in both humans and animals (Ren & Dubner, 1999). From such studies, a gender disparity has been found in pain perception and discrimination (Gaumond, Arsenault, & Marchand, 2005; Terner, Lomas, & Picker, 2005). In order to understand this dimporphic response to pain, current studies need to investigate the role gonadal hormones play in modulating pain and inflammation. Study of the regulatory actions of hormones may elucidate which biochemical markers involved in the inflammatory response, if any, are affected by hormonal differences in males and females.

Pain and Nociception

Pain, a submodality of somatic sensation, refers to an "unpleasant sensory and emotional experience associated with actual or potential tissue damage" (Basbaum & Jessell, 2000). Pain is a multidimensional experience that not only consists of a physiological response, but also includes motivational, affective, cognitive, and behavioral components (Riedel & Neeck, 2001). This multidimensionality can be broken up into two distinct dimensions of pain perception: physiological and psychological pain. Physiological pain is associated with the sensory discrimination and perception of a noxious stimulus, while psychological pain includes the affective and cognitive components of the painful event. Physiological pain serves an adaptive function because it is linked to motor withdrawal brought about in response to acute exposure to a noxious event which discontinues the exposure to the damaging stimulus (Millan, 1999) and results in the pain subsiding quickly (Weiseler-Frank, Maier & Watkins, 2004). Psychological pain perception includes a cognitive analysis of the event as well as an emotional response to it (Weiseler-Frank, Maier, & Watkins, 2004). This cognitive-emotional analysis includes such aspects of pain perception as the relationship between pain and mood, the attention to pain and its memory, as well as the ability to cope with and tolerate pain (Riedel & Neeck, 2001).

Pain can be separated into chronic and acute pain. Acute pain tends to be short in duration, lasting less than a few weeks (Milligan & Watkins, 2009; Millan, 1999), and often resolves quickly with the termination of the noxious stimulus (Saab, Waxman & Hains, 2008). The resolution of pain is usually due to the induction of antinociceptive mechanisms initiated by the stimulus (Riedel & Neeck, 2001). Normal acute pain accurately reflects the intensity, localization, and/or timing of a stimulus (Kidd & Urban, 2001).

In contrast to the acute type, chronic pain, also known as pathological or clinical pain, contributes no beneficial functions (Costigan & Woolf, 2000) and, in fact, may be life-threatening (Saab, Waxman & Hains, 2008). Chronic pain occurs in response to damage to the nervous system (neuropathic pain) or from chronic inflammatory conditions, as in arthritis (Costigan & Woolf, 2000). It can last up to 6 months and is itself considered a disease condition (Milligan & Watkins, 2009). Chronic pain is often associated with degenerative tissue diseases like rheumatoid arthritis (RA) and does not involve spontaneous recovery (Riedel & Neeck, 2001). While acute pain accurately reflects specifics of the noxious stimulus (e.g. stimulus duration and intensity), chronic pain is exaggerated and may even arise spontaneously in the absence of an external trigger (Kidd & Urban, 2001). Besides the perception of pain, chronic pain is reflected behaviorally and may lead to a long-term state of withdrawal, anhedonia (lack of pleasure in previously pleasurable activities) or an indifference to environmental changes. These behavioral manifestations mimic depressive states that are triggered by constant and inescapable stress and thus, is not adaptive (Millan, 1999).

The terms "pain" and "nociception" (from the Latin "nocere" which means "to hurt"; Coutaux, Adam, Willer & LeBars, 2005) are sometimes used interchangeably but there is an important difference between them. As stated before, pain includes the physiological and psychological components, but nociception is the neural mechanism associated with pain perception. It is defined as the physiological response to actual or perceived tissue damage (Basbaum & Jessell, 2000). Nociception does not include any aspect of psychological response to pain such as analysis or emotional evaluation of the painful event. Nociception does not imply any sort of unpleasantness, like the term pain does, since it is simply the neural detection of a noxious stimulus (Weiseler-Frank, Maier & Watkins, 2004). Further clarifying this point,

pain may be experienced in excess of any actual tissue insult, and at times, pain is absent in the presence of actual tissue damage (McHugh & McHugh, 2000). These phenomena show that "nociception" is indeed different from "pain". One way in which nociception arises is from local inflammation (Basbaum & Jessell, 2000), which will be discussed in more detail later.

In the nervous system, both cutaneous and deep somatic tissues are innervated by primary afferent neurons that convey both noxious and innocuous information (Milligan & Watkins, 2009). The sensory fibers that transmit pain information to the central nervous system (CNS) are called nociceptive afferent neurons, or nociceptors. These afferents have three main functions: to detect toxic or damaging stimuli (transduction), to pass on that sensory message from the peripheral nerve terminals to the spinal cord (conduction), and to transfer that information to specific neurons within specific laminae of the dorsal horn of the spinal cord (transmission; Kidd & Urban, 2001). Nociceptors have terminals located in peripheral tissues and are positioned in such a way that makes them ideal for transmitting pain information (Costigan & Woolf, 2000) and providing the CNS with information about the environment and the state of the organism (Meyer, Ringkamp, Campbell, & Raja, 2005). These fibers have been implicated in pain since the loss of these cell types results in a pain-free phenotype (Abrahamsen et. al, 2008).

More specifically, nociceptors are highly specialized sensory receptors that respond most strongly to noxious stimuli applied to peripheral tissues (Basbaum & Jessell, 2000). Most nociceptors are free nerve endings that form arborizations in peripheral tissue and harbor proteins in their membrane that can convert various types of noxious stimuli (thermal, mechanical, and chemical) into an electrical potential which is needed in order to convey pain information to the spinal cord (Coutaux, et. al, 2005; Basbaum & Jessell, 2000). They have relatively long distal projections and small diameter cell bodies located in the dorsal root ganglia

(DRG) located proximate to the spinal cord (McHugh & McHugh, 2000; Meyer, et. al, 2005). They can be distinguished from other types of sensory receptors by their sensitivity to capsaicin, the ingredient in hot peppers (Kidd & Urban, 2001). Because of their high-threshold nature, nociceptors require a high stimulus intensity to activate them, which poses a risk for tissue damage (Coutaux, et. al, 2005; Basbaum & Woolf, 1999). Another way in which nociceptors are different than other primary sensory afferents is that they do not exhibit the characteristic of adaptation to a constant stimulus. Nociceptors instead sensitize to constant stimulation, resulting in an increase in responding over time (Meyer, et. al, 2005).

Most nociceptors are polymodal in that they respond to different types of stimuli (e.g. thermal, mechanical, chemical), but some respond preferentially to certain stimuli (Meyer, et. al, 2005; Costigan & Woolf, 2000). This quality in nociceptors may play a part in creating the different types of pain sensations, such as burning, aching, and pricking pain (Meyer, et. al, 2005).

There are various kinds of primary afferent fibers in the nervous system. These fibers can be grouped into three types of fibers based on their diameter and thickness of myelination/conduction velocity. Aα and Aβ fibers are large in diameter (>10μM) and thickly myelinated, allowing for fast conduction speeds (30+ m/s). Aδ fibers have axons that are medium-sized in diameter (2-6μM), thinly myelinated, and therefore have an intermediate conduction velocity (4-30 m/s). C fibers, on the other hand, are very thin (0.4-1.2μM in diameter), unmyelinated, and transmits signals more slowly (0.4-2 m/s) (Milligan & Watkins, 2009; Basbaum & Jessell, 2000; Millan, 1999). Each class of afferent fibers encodes sensory information, but they differ in their sensitivity to noxious and innocuous stimuli. Aβ and Aα fibers transmit only innocuous signals, like those from vibration and light touch, during normal

conditions (Milligan & Watkins, 2009; Millan, 1999) due to their low-threshold nature (Costigan & Woolf, 2000). Both C and Aδ fibers, unlike Aα and Aβ fibers, have free nerve endings and, under normal circumstances, only respond to and conduct information arising from noxious stimuli (Kidd & Urban, 2001). Because of their small diameter and lack of thick myelination, both types of these fibers conduct information slowly at the rate of about 10% of that of motor fibers (McHugh & McHugh, 2000).

C fibers are mostly polymodal nociceptors located deeply along vein walls (Millan, 1999) that respond to thermal, mechanical, and chemical stimuli (Coutaux et. al, 2005; Millan, 1999; Meyer, et. al, 2005). Some C fibers, however, respond exclusively to chemical stimuli and are insensitive to mechanical and thermal stimulation (Kidd & Urban, 2001). C fiber activity of a magnitude above threshold will evoke a diffuse, slow, painful burning sensation (McHugh & McHugh, 2000; Meyer, et. al, 2005) after the initial rapid phase of pain carried by Aδ fibers (Millan, 1999). In glabrous skin, C fibers respond to heat simulation of short duration (less than 5s) at a temperature near the pain threshold in humans (~45ºC; Meyer, et. al, 2005), indicating that these fibers are responsible for at least some thermal nociceptive sensation. The fact that many patients with congenital insensitivity to pain lack C fibers can be seen as further evidence that supports their nociceptive role (Abrahamsen, et al., 2008).

A subset of C-fiber afferents have been identified in human skin and are commonly referred to as silent nociceptors that, under normal circumstances, are inactive and unresponsive to typical nociceptive stimuli (Coutaux, et. al, 2005; Meyer, et. al, 2005; Millan, 1999). These fibers have extremely high mechanical thresholds, higher than a typical C fiber nociceptor (Meyer, et. al, 2005). These afferents are recruited and activated gradually during the

inflammatory response and are thought to contribute to the development of post-inflammation hypersensitivity (Coutaux, et. al, 2005)

Aδ nociceptive fibers are thought to evoke pricking, sharp pain and possibly an aching pain (Meyer, et. al, 2005). They peform all of functions of C fibers but are thought to do it more robustly since they have a higher activation threshold (Coutaux, et. al, 2005). Aδ fibers have a more frequent discharge rate, and the information that they relay to the CNS is more discriminable (Meyer, et. al, 2005). Their exact role in inflammation has yet to be elucidated (Coutaux, et. al, 2005).

Studies have found that two different types of Aδ fibers exist. The first type of fiber, Type I, responds weakly to heat stimuli due to a high thermal threshold but respond robustly to high-intensity mechanical stimuli like pinching (Millan, 1999). Type I Aδ fibers may also respond to chemical stimulation due to their polymodal nature. Type I fibers are found in both hairy and glabrous skin (Meyer, et. al, 2005).

Type II Aδ fibers, the second class of these nociceptors, are insensitive to mechanical stimulation unlike their Type I counterparts. These fibers respond intensely to stimulation by heat sources and are thought to send the first signals to the CNS in response to heat or capsaicin. This type of stimulation evokes a double pain response in Type II fibers. There is a quick sharp pricking sensation followed by a slower burning sensation. Type II fibers are found mainly in hairy skin (Meyer, et. al, 2005).

Heat sensitivity in Aδ fibers is most likely mediated by the vanilloid receptor 1 (TRPV1), a non-selective channel with a preference for calcium (Ca^{2+}; Meyer, et. al, 2005; Coutaux, et al.,2005) since it has a similar high activation threshold for heat and is expressed mainly in small neurons with myelinated axons, like the Aδ fibers (Meyer, et. al, 2005). Vanilloids are naturally

occurring chemicals that are responsible for the spicy taste in foods; they include capsaicin, piperine, and zingerone (Coutaux, et al., 2005). TRPV1 receptors are activated by vanilloids, heat, and protons, indicating that their activation may be enhanced by the drop in pH of the environment in inflamed tissue (Kidd & Urban, 2001; Meyer, et. al, 2005). Furthermore, research shows that a substantial loss of cells that express the TRPV1 receptor correlates with the loss of thermal hyperalgesia (Abrahamsen, et al., 2008). Some inflammatory mediators like bradykinin have been shown to decrease the threshold of these receptors in the DRG so that the ambient temperature becomes nociceptive causing spontaneous firing of pain-processing cells (Coutaux, et al., 2005; Meyer, et. al, 2005). To further show the importance of the TRPV1 receptor in pain perception, studies have shown that TRPV1 receptor knockout mice show a significantly inhibited thermal hyperalgesic response to inflammatory stimuli (Kidd & Urban, 2001).

The conscious experience of pain is ultimately produced in the limbic and cortical centers of the brain. As shown in Figure 1.1, interceding between an exposure to a noxious stimulus and the sensation of pain, however, is a complex series of interacting mechanisms whereby the algogenic stimulus is encoded into a nociceptive signal that is perpetuated through the nervous system in a series of progressively higher nervous centers (Millan, 1999). Pain processing is not, however a simple relay from periphery to brain. The nociceptive system is dynamic and consists of multiple, redundant pathways. Because of this, suppression or enhancement of pain can occur at any, and possibly all, level of synaptic connection (Milligan & Watkins, 2009). In order to understand how hormones may affect pain perception, it is important, therefore, to understand the nature of the connections nociceptive neurons make at all levels of the nervous

system. The research to be described will focus mainly on the portion of the nociceptive pathway from the peripheral afferents to the spinal cord.

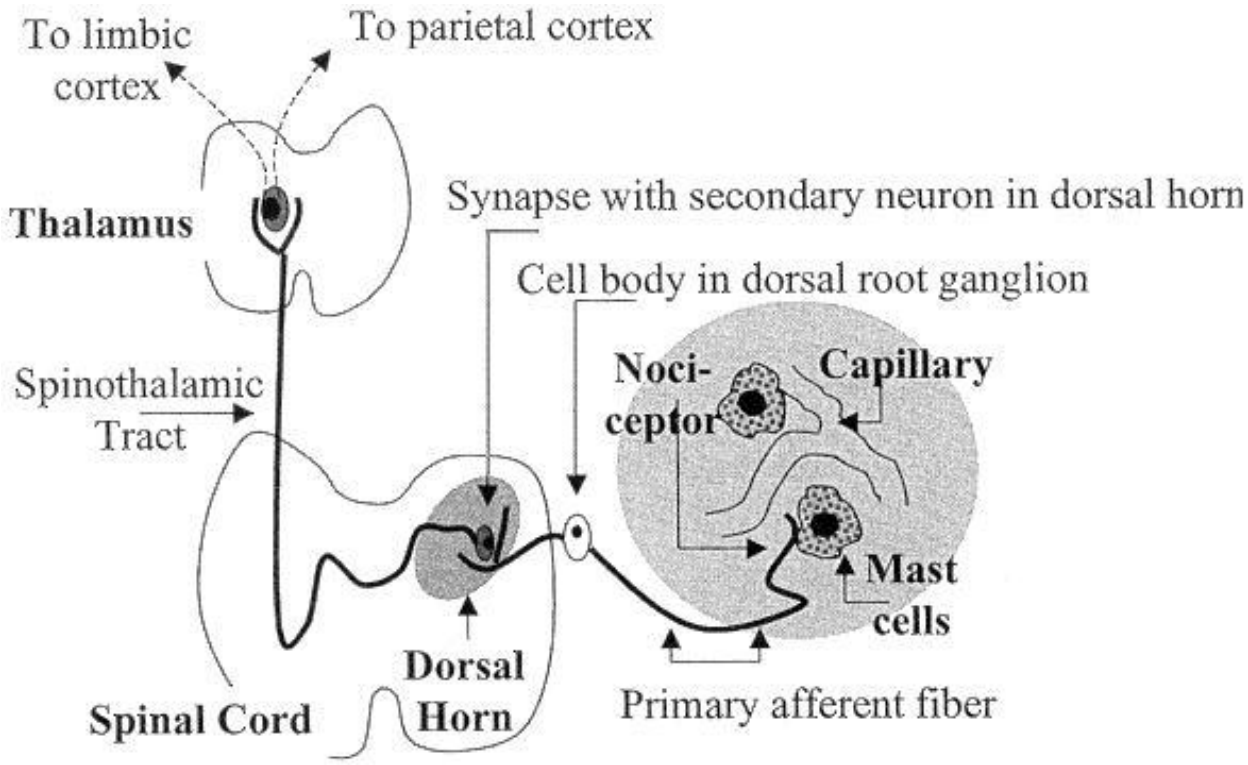

Figure 1.1. Organization of pain responses from the periphery to the brain. Primary nociceptive fibers are found adjacent to capillaries and mast cells. Each of these respond to tissue injury in a coordination way: cell injury, mast cell degranulation, plasma exudation, and capillary dilation all cause chemical and/or mechanical stimulation of the nociceptor. Impulses along the nociceptor pass through the dorsal root ganglion and proceed to the dorsal horn in the spinal cord. These impulses cross to the spinal thalamic tracts on the contralateral side before reaching the thalamic centers in the brain. Once the signal reaches the thalamus and higher brain centers like the limbic and parietal cortices, pain becomes a conscious experience. Meaning and emotions are attributed to the event once it reaches the highest levels of the brain.
From: McHugh & McHugh (2000), AACN CLIN ISSUES ADV PRACT ACUTE CRIT CARE, VOL 11(2), 168-178.

A pain signal is induced when a stimulus that signals potential or actual tissue damage is detected by the primary nociceptive afferents (Milligan & Watkins, 2009). The axons of these primary sensory neurons converge on the area of the spinal cord called the dorsal horn in a topographical fashion, as shown in Figure 1.2 (Milligan & Watkins, 2009; Weiseler-Frank, et al., 2004). The majority of nociceptors synapse specifically on the superficial areas of the dorsal horn, in Laminae I and II, but some nociceptive fibers have been found to terminate in other laminae, such as Lamina V, VI, and X (Riedel & Neeck, 2001). These areas of the spinal cord are the first place of synaptic contact between the primary nociceptive afferents and the CNS. Here, the pain signal is subject to both local and descending modulation, both excitatory and inhibitory (Riedel & Neeck, 2001; Costigan & Woolf, 2000). Because of this, the dorsal horn may be an extremely important place to investigate the amplification or attenuation of nociceptive information. Local modulation of pain signals in the spinal cord will be discussed in detail later.

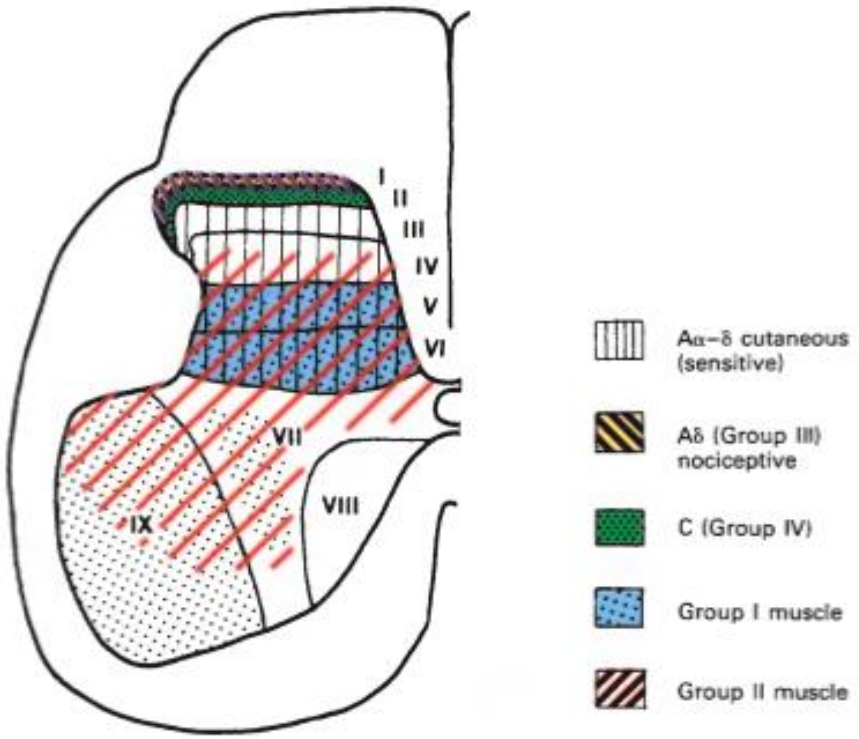

Figure 1.2. Distribution of afferent fibers in the spinal cord grey matter. Fibers associated with nociception synapse at the superficial levels of the dorsal horn in the spinal cord.
From: Riedel & Neeck, 2001, Z RHEUMATOL, VOL. 60, 404-415.

After receiving nociceptive information from the primary afferents, the neurons in the dorsal horn relay pain-related information to higher-order areas in the nervous system (McMahon, Cafferty, & Marchand, 2005). Second-order pain-responsive neurons decussate at the level of the spinal cord (Milligan & Watkins, 2009; McHugh & McHugh, 2000), synapse on neurons in various supraspinal structures like the thalamus and brainstem (Riedel & Neeck, 2001; Kidd & Urban, 2001) and ultimately synapse with cortical neurons responsible for conscious pain experience (Milligan & Watkins, 2009; Riedel & Neeck, 2001). All parts of the pain pathway have to act in concert to produce a protective or adaptive response to a noxious stimulus (Milligan & Watkins, 2009). When one aspect of the pathway is disrupted, pain perception is altered and may become pathological, as is the case with inflammation.

Inflammation

Pain is a classic symptom of inflammation, and many people experience chronic inflammatory pain as part of a disease, like RA (McMahon, et al., & Marchand, 2005). This type of pain is pathological and can occur spontaneously without an external painful stimulus (Coutaux, et. al, 2005; Basbaum & Jessell, 2000), and therefore, serves no adaptive function because it no longer aids in the healing of a wound (Milligan & Watkins, 2009; Weiseler- Frank, et. al, 2004). Inflammation occurs at the site of tissue damage (Costigan & Woolf, 2000) and is associated with heat, rubor (redness) caused by the dilation of peripheral blood vessels, and tumor (swelling) caused by plasma extraversion (Coutaux et. al, 2005; Basbaum & Jessell, 2000). Inflammation is initiated by the activation of the nociceptive unit which is comprised of capillaries, mast cells, and nociceptors. Noxious stimuli can cause injury to cells, plasma exudation, dilation of capillaries, and mast cell degranulation (McHugh & McHugh, 2000).

During inflammation, nociceptive messages are amplified, which results in an exaggerated pain response (Weiseler-Frank, et. al, 2004). Tissue damage triggers a cascade of events that can prolong activation of nociceptive afferents and enhance their sensitivity. This cascade is linked to the inflammatory process (Coutaux et. al, 2005). The nervous system has a characteristic of plasticity, which is the property that allows it to change its functions according to its surrounding conditions. It is this property that allows the nociceptive system to become hypersensitive in response to inflammation (Kidd & Urban, 2001).

After inflammation, nociceptors can be activated by normally innocuous stimuli, which is referred to as allodynia (Riedel & Neeck, 2001; Ren & Dubner, 1999). The hallmark feature of the inflammatory response, however, is an excessive response to a noxious stimulus, a phenomenon known as hyperalgesia (Coutaux, et. al, 2005; Riedel & Neeck, 2001). This is the classic symptom of hypersensitivity that accompanies inflammation (Kidd & Urban, 2001). Essentially, the threshold for pain is lowered and thus pain in response to a suprathreshold stimulus is increased (Meyer, et. al, 2005). Hyperalgesia occurs at both the site of injury (primary hyperalgesia) and in surrounding uninjured areas (secondary hyperalgesia; Coutaux, et. al, 2005).

Hyperalgesia is believed to be due to multiple mechanisms including sensitization of peripheral nociceptors, central sensitization, or both (Ren & Dubner, 1999). Both chemical and neural changes occur both at the site of injury (at the nociceptive nerve endings or in their axon projections) and at the first-order synapses in the dorsal horn of the spinal cord (Milligan & Watkins, 2009). It is thought that primary hyperalgesia is due to the sensitization of peripheral nociceptive afferents since nociceptors show an increase in response to a test stimulus after hyperalgesia is induced (Riedel & Neeck, 2001; Meyer, et. al, 2005). This sensitization

effectively decreases the depolarization threshold and increases the rate of response to a constant stimulus (Coutaux, et. al, 2005; Sweitzer, Arruda, & DeLeo, 2001; Gold, et al., 1996). Furthermore, it is thought that secondary hyperalgesia is due to central sensitization (Meyer, et. al, 2005). Central sensitization occurs at the level of the dorsal horn in the spinal cord and is well-characterized by changes in both neuronal and biochemical processing of pain information (Milligan & Watkins, 2009). This phenomenon plays a major role in the development of chronic pain in absence of an external cause (Coutaux, et. al, 2005).

Injury to peripheral tissues by noxious mechanical, thermal, or chemical stimulation results in the discharge of an intricate mixture of chemical substances such as cytokines, prostanoids [including prostaglandins (PGs)], growth factors [specifically nerve growth factor (NGF)], histamine, nitric oxide (NO), leukotrienes, adenosine, thromboxanes, bradykinin, and serotonin (McMahon, et. al, 2005; Kidd & Urban, 2001; McHugh & McHugh, 2000; Costigan & Woolf, 2000; Basbaum & Woolf, 1999; Meyer, et. al, 2005) which are released during the interaction of the nervous and immune systems with alien noxious material (Riedel & Neeck, 2001). Certain of these mediators serve to facilitate the recovery of injured tissues (Millan, 1999). Others, however, increase the likelihood that nociceptors will activate and transmit pain information to the spinal cord (Milligan & Watkins, 2009; McHugh & McHugh, 2000). These chemical mediators have a synergistic effect by acting both directly via activation of chemosensitive nociceptors and indirectly by initiating the release of more mediators from nociceptors and immune cells (Saab, et. al, 2008; Coutaux, et. al, 2005). This cascade also leads to the phenomena of hyperalgesia and allodynia (Meyer, et. al, 2005), as well as the visible peripheral reactions of edema and rubor (Coutaux et. al, 2005).

Contact with a noxious agent causes the perturbation and damage of peripheral afferent neurons (McHugh & McHugh, 2000). When this occurs, the damaged cells release both adenosine triphosphate (ATP) and protons, creating an acidic environment. Mast cells degranulate in response to NGF (McMahon, et. al, 2005), causing the release of amines like serotonin and histamine, and arachidonic acid (AA) metabolites including PGs, thromboxanes, and leukotrienes (Kidd & Urban, 2001; Costigan & Woolf, 2000). Macrophages at the site of injury release cytokines and growth factors, and bradykinin is released from multiple cell types. Concurrently, primary nociceptors release neuropeptides like substance P (SP), resulting in a neurogenic response, which further enhances the release of cytokines (both inflammatory and anti-inflammatory proteins) from immune cells. This neurochemical reaction increases bloodflow to the area of injury via vasodilation which results in redness, and allows the movement of proteins into the extracellular space via plasma extraversion, resulting in swelling (Costigan & Woolf, 2000). The accumulation of fluid (edema) in the extracellular space produces additional mechanical pressure on peripheral neurons (McHugh & McHugh, 2000). All of these steps in the pain cascade result in the depolarization of nociceptors and transmission of pain information from the periphery to the spinal cord.

Peripheral Sensitization

Researchers have proposed many mechanisms to account for hyperalgesia. These mechanisms include both the direct activation of nociceptors and the sensitization of nociceptors through more indirect means such as the production and release of various mediators (Riedel & Neeck, 2001). The inflammatory mediators mentioned above can interact with peripheral nerve terminals and result in a lowering of their depolarization thresholds and subsequent sensitization

(Kidd & Urban, 2001; Meyer, et. al, 2005). For example, PGs have been found to sensitize nociceptors to physical stimuli and certain algogenic substances (Coutaux, et. al, 2005; McHugh & McHugh, 2000).

One mechanism that has been proposed to account for this change is the induction of post-translational changes in voltage-gated ion channels (Kidd & Urban, 2001; Costigan & Woolf, 2000), specifically sodium (Na^+) channels. It has been proposed that the tetrodotoxin-resistant (TTXr) Na^+ channel plays a modulatory role in peripheral nociceptive afferents (Meyer, et. al, 2005). TTXr Na^+ channels are expressed specifically on small-diameter sensory neurons, indicating a specialized role in nociception (Kidd & Urban, 2001; Gold, et. al, 1996). The biochemical substances released at the location of tissue injury sensitize nociceptors by catalyzing a cascade of events that may cause a change in the ionic conductances in the cells' peripheral terminals (Gold, et. al, 1996).

Because the phosphorylation of TTXr Na^+ channels is driven by PGs and serotonin (Basbaum & Woolf, 1999), Gold and colleagues (1996) conducted a study to investigate the role hyperalgesic agents like prostaglandin E_2 (PGE_2) play in changing TTXr voltage-gated Na^+ currents that are selectively expressed in nociceptive afferents. Using recordings from cultured rat DRG neurons, they found that PGE_2, serotonin, and adenosine, all hyperalgeisc substances, dose-dependently modify the TTXr Na^+ current in a way consistent with nociceptor sensitization. These changes may account for both the decrease in the threshold of the nociceptors as well as an increase in the number of action potentials seen in response to a constant stimulus. These observations, along with the fact that TTXr Na^+ channels are selectively distributed in DRG neurons, are consistent with the hypothesis that modulation of these currents is the underlying mechanism of nociceptor sensitization (Gold, et. al, 1996).

Another mechanism that may account for the increase in nociceptive firing is the recruitment of non-nociceptive, large-diameter afferents. As stated above, insult to peripheral tissues results in the release of many substances, including NGF. This growth factor has been found to sensitize nociceptors and may possibly alter the distribution of previously innocuous Aβ fibers such that some have nociceptive activity (Meyer, et. al, 2005). During inflammation, both large- and medium-sized A fiber DRG neurons can begin to produce and release neuropeptides typically associated with C fiber activity. This switch in cell phenotype coverts both non-nociceptive Aβ fibers and nociceptive Aδ fibers into fibers that begin to act more like C fibers (Milligan & Watkins, 2009; Costigan & Woolf, 2000). To investigate this phenotypic switch in Aβ fibers, Neumann and colleagues (1996) studied whether experimental inflammation alters the chemical phenotype of these fibers and whether this changes their actions in the spinal cord. They found that there is an increase in the number of Aβ fibers expressing SP after inflammation, and that this change is associated with a qualitative change in function. These "new" C fibers can both increase and generate central excitability in vivo (Neumann, et. al, 1996). Transcriptional changes in peripheral cells can also occur, resulting in more long-term changes in the nociceptor responsivity (Costigan & Woolf, 2000). These events are mediated by cytokines and growth factors and result in the production of more receptors, ion channels, as well production of central transmitters and neuromodulators (Kidd & Urban, 2001).

Central Sensitization

In addition to the neuromodulators released in the periphery, there are others that play an important role at the synapse between the primary nociceptor and the second-order neurons in the dorsal horn (Milligan & Watkins, 2009). Allodynia and hyperalgesia reflect an enhanced

excitability of spinal cord nociceptive neurons (McMahon, et. al, 2005; Kidd & Urban, 2001). Long-lasting and repetitive firing of nociceptive afferents produce distinct changes in both the function and activity of the central nociceptive cells (Kidd & Urban, 2001).

Several mechanisms for central sensitization have been described in recent literature. One of the best characterized involves a change in the function of N-methyl-D-aspartate (NMDA) receptors in dorsal horn neurons. It is well-established that NMDA receptors are highly represented in the dorsal horn of the spinal cord and play a role in nociceptive transmission (Riedel & Neeck, 2001; Kidd & Urban, 2001). Under non-nociceptive conditions, the spinal cord responds to non-damaging noxious stimuli with the excitatory amino acid glutamate, which acts via the α-amino-3-hydroxy-5-methylisoxazole (AMPA) receptors (Kidd & Urban, 2001). The repeated activation of peripheral nociceptors accompanying inflammation results in the expression and activation of the NMDA receptors instead (Kidd & Urban, 2001) and the subsequent release of SP and glutamate, depolarizing the pain-projection neurons in the spinal cord. This causes the magnesium (Mg^{2+}) ion that normally blocks the NMDA channel to be removed which results in increased Ca^{2+} influx and facilitated signal transmission. It further increases the production and release of NO via Ca^{2+}-activated nitric oxide synthase (NOS) (Milligan & Watkins, 2009). NO increases neuronal excitability in the spinal cord which results in an inflated release of neuromodulators from the presynaptic terminals of the sensory neurons. These biochemical changes in the neurons of the spinal cord may also lead to the phenotypic changes in non-nociceptive fibers discussed above (Kidd & Urban, 2001). Ultimately, these reactions to the enhanced nociceptive input from the periphery result in the amplification of the pain messages that are being relayed through the spinal cord (Milligan & Watkins, 2009; Kidd & Urban, 2001).

Along with short-term changes in the function of the dorsal horn neurons, if the chain of nociceptive input is consistent, activity-dependent changes in transcription arise in these cells. This provides the cellular basis for more long-lasting changes in neuronal functioning which may underlie chronic pain in some patients (Costigan & Woolf, 2000). Glutamate, neuropeptides, and trophic factors released from the terminals of primary nociceptors during inflammation act as co-transmitters that can induce long-lasting changes in the excitability of spinal cells, leading to central sensitization (Kidd & Urban, 2001). These changes ultimately make these spinal neurons more sensitive to subsequent nociceptive inputs (Costigan & Woolf, 2000).

As stated before, secondary hyperalgesia is attributed to spinal mechanisms. Some researchers believe that secondary hyperalgesia is a consequence of an increase in the size of dorsal horn receptive fields. An increase of this nature would mimic an increase in peripheral output, and it would account for at least part of the hypersensitivity in tissue surrounding the site of injury (Riedel & Neeck, 2001; Kidd & Urban, 2001).

Along with neurons, glial cells have also been found to play a role in sensitization. SP released from primary nociceptive terminals during inflammation is capable of activating glial cells in the spinal cord, which leads to an increase in expression of cyclooxygenase-2 (COX-2) and the production of PGE_2, both of which are known constituents in sensitization (McMahon, et. al, 2005). Further, microglia, a type of glial cell, are thought to release proinflammatory cytokines which also contribute to the increased neural responding in the spinal cord during prolonged inflammation (Saab, et. al, 2008).

Carrageenan Model of Inflammation

Carrageenan (Cg) is a glycoprotein derived from seaweed that is commonly used to induce nonspecific inflammation in animals (Rittner, Machelska, & Stein, 2005; Bennet, 2001). Injection of Cg can induce both muscle and hindpaw inflammation depending on the location of the injection (Loram, Fuller, Fick, Cartmell, Poole, & Mitchell, 2006). Injection of this substance into a rodent's footpad produces more persistent pain and hyperalgesia compared to formalin (Ren & Dubner, 1999). Previous studies have found that hindpaw injection of Cg causes an acute inflammation that peaks from 2 to 4 hours after injection which is associated with mechanical hyperalgesia; both inflammation and hyperalgesia associated with Cg resolves within 24 hours and are dose dependent (Loram, et al., 2006). Studies have also shown that intraplantar injection of Cg into wild-type C57BL//6 mice evoked mechanical hyper-nociception in a time- and dose-dependent manner (Cunha, et al., 2005; Hargreaves, et al., 1988). The same researchers also found that Cg injection stimulates the production of both TNF-α and IL-1β 30 minutes and 1 hour after injection, respectively (Cunha, et al., 2005). The Cg model is representative of the time course of post operative pain and other chronic pain states (Ren & Dubner, 1999).

Inflammatory mediators

When inflammation arises, the nervous system does not act alone. Following tissue injury, inflammatory responses are generated by local macrophages, mast cells, and neutrophils, and these responses are exacerbated by the subsequent migration of similar types of cells and leukocytes to the site of injury (Moalem & Tracey, 2006; Sommer & Kress, 2004). These damage-sensing immune cells become activated upon insult and secrete neuro-excitatory

substances like cytokines, PGs, bradykinin, and SP (Saab, et al., 2008; Angst, et al., 2008; Chichorro, et al., 2004). While these mediators may contribute to an organism's ability to offset infection or hinder the destruction of tissue, they also cause pain by activating and sensitizing nociceptive primary afferents (Hopkins, 2007; Rittner & Stein, 2005). This sensitization can be caused by posttranslational changes in both the periphery and dorsal horn of the spinal cord leading to the alteration of basal transduction levels (Woolf & Costigan, 1999). Of particular interest in the current study are cytokines and PGs.

Cytokines

Cytokines are small polypeptides that are produced and released by a variety of cell types such as white blood cells, mast cells, macrophages, and neutrophils in the periphery and glia in the CNS (Angst, et al., 2008; Moalem & Tracey, 2006; Cunha, et al., 2005). Most cytokines have either proinflammatory or anti-inflammatory actions, but some exhibit both as a function of their concentrations and their cellular and molecular environment (Uceyler & Sommer, 2008). Cytokines are secreted in response to inflammatory stimuli like viruses and bacteria, tissue injury, and other cytokines (Verri, Jr., et al., 2006; Sommer & Kress, 2004). Cytokines act locally on the receptors of the cells that produce them (autocrine effects), as well on receptors of other cells (paracrine effects). These proteins also have hormonal effects in that they affect distant tissues (Verri, Jr., et al., 2006). The great majority, however, act locally as neuromodulators in inflamed tissue (Hopkins, 2007). Over 100 cytokines have been identified, many of which are involved with the regulation of immune responses or act as growth and repair molecules, serving cellular functions such as differentiation, survival, and metabolism (Hopkins, 2007; Verri, Jr., et al., 2006).

It is now well-accepted that cytokines represent the link between tissue injury or immunological responses and resulting nociception (Verri, Jr., et al., 2006; Coutaux, et al., 2005). Much of the relationship between the nervous and immune systems remains imprecise (Coutaux, et. al, 2005). Immune cells, including those that release both pro- and anti-inflammatory cytokines, are implicated in the hyperalgesic and allodynic states that are associated with chronic inflammation. The relationship between these two entities is bi-directional in that inflammation activates immune cells and that these immune cells feed back on neurons and alter their activity (Watkins & Maier, 1999).

Cytokines act directly on nociceptors to sensitize them, or indirectly via a resulting cascade (Uceyler & Sommer, 2008). In response to peripheral injury, mast cells degranulate and recruit neutrophils and macrophages to the injury site (Moalem & Tracey, 2006), from which cytokines are released in a controlled sequence. These cytokines are then responsible for stimulating the production of final inflammatory mediators, like PGs, which are involved in the creation of inflammation and its associated signs and symptoms (Verri, Jr., et al., 2006). Following tissue injury, inflammatory mediators are transported to the site of trauma and local dendritic cells and macrophages are activated. Inflammatory responses are amplified by the migration of leukocytes into the inflamed tissue via the production of inflammatory mediators (Sommer & Kress, 2004; Rittner, Machelska, & Stein, 2005). Triggers like trauma or infection stimulate cells of the monocyte/macrophage lineage to produce cytokines, such as tumor necrosis factor-α (TNF-α) [Hopkins, 2007; Wieseler-Frank, Maier, & Watkins, 2005]. The production of TNF-α is the catalyst for a cascade of cytokine production: TNF-α causes the release of interleukin-1β (IL-1β) and then interleukin-6 (IL-6) and interleukin-8 (IL-8). As seen in Figure 1.3**,** the resulting cascade activates two distinct pathways: the prostanoid pathway and the

sympathetic amine pathway (Loram, et al., 2006). Release of IL-1β and IL-6 leads to the activation of COX-2 and the subsequent prostanoid release via activation of p38 MAP kinase (McMahon, Cafferty, & Marchand, 2005). IL-8 mediates pain by stimulating the release of catecholamines from the sympathetic nervous system (Loram, et al., 2006; Sommer & Kress, 2004). The various mediators that are released during inflammation and the tissue acidification associated with inflammation act in a synergistic fashion to produce increased pain responses like hyperalgesia and allodynia (Sommer & Kress, 2004).

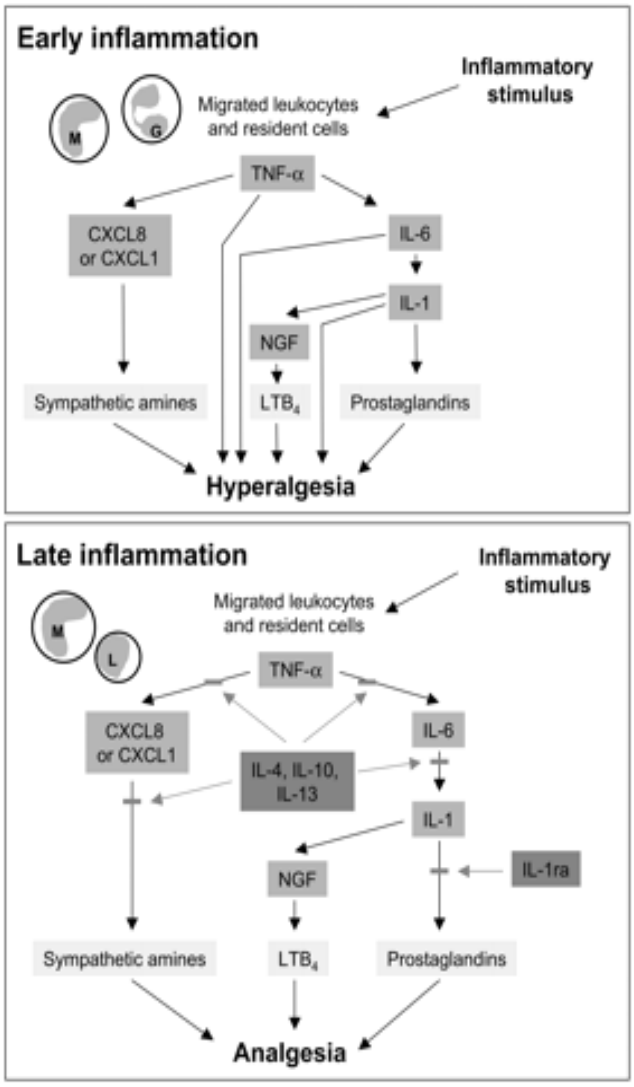

Figure 1.3. Pro- and anti-inflammatory mechanisms in early and late inflammation. During early inflammation, a noxious stimulus causes the migration of leukocytes like monocytes (M) and granulocytes (G) to the inflamed tissue. Once there, these cells initiate a cascade of pro-inflammatory cytokine such as TNF-α and IL-1 and -6, as well as chemokines (e.g. CXC chemokine ligand 8 [CXCL8] and CXCL1), and NGF. Following that, secondary mediators such as sympathetic amines, prostaglandins, and leukotriene B4 (LTB_4) are produced/activated which results in hyperalgesia. During ongoing, late inflammation, lymphocytes (L) begin to produce anti-inflammatory cytokines such as IL-4, -10, -13, and -1ra which inhibit the production of the proinflammatory cytokines. This prevents further hyperalgesia from developing.
From: Rittner, Machelska, & Stein (2005): JOURNAL OF LEUKOCYTE BIOLOGY, VOL 78(6), 1215-1222.

Leukocytes are not only the source of proinflammatory mediators but of anti-inflammatory ones as well (Rittner & Stein, 2005; Verri, Jr., et al., 2006). Because cytokine activation or dysregulation is linked to hyperalgesic and chronic pain states, the release of proinflammatory cytokines is highly regulated by various negative feedback mechanisms (Wieseler-Frank, et al., 2005). Cells that produce inflammatory cytokines also produce endogenous cytokine antagonists like the IL-1 receptor antagonist (IL-1ra; Hopkins, 2007) as well as soluble forms of the TNF and IL-1 receptors which can bind to and neutralize TNF-α and IL-1 (Hopkins, 2007). Further, these cells can release cytokines that have anti-inflammatory actions such as IL-4 and IL-10 (Angst, et al., 2008). The net effect of these analgesic responses is the inhibition of the actions of the inflammatory mediators when they are no longer needed (Wieseler-Frank, et al., 2005).

In order to study the effects of cytokines on inflammation, experimenters use a variety of methods. They apply exogenous cytokines to non-injured tissue, treat a subject with cytokine antagonists, or measure cytokine levels in both the PNS and CNS after inflammation. In this study, we examined the changes in both proinflammatory and anti-inflammatory cytokines to determine if estrogen modulates pain via changes in one or both types of mediators. The ones discussed herein are the proinflammatory cytokines TNF-α, IL-1β, IL-2, IL-5, IFN-γ, and GM-CSF and the anti-inflammatory cytokines IL-10 and IL-4.

Pro-Inflammatory Cytokines

Many cytokines are considered proinflammatory due to their ability to act either indirectly via the production of PGs and sympathetic amines or directly via sensitization of the

primary nociceptive afferent (Verri, Jr., et al., 2006; Uceyler & Sommer, 2008). Six such cytokines are TNF-α, IL-1β, IL-2, IL-5, IFN-γ, and GM-CSF.

Tumor Necrosis Factor-Alpha (TNF-α)

TNF-α is largely considered the prototypic proinflammatory cytokine due to its major role in initiating the cascade of activation and release of cytokines and other inflammatory mediators after the onset of inflammation (Schafers & Sorkin, 2008; Rittner, Machelska, & Stein, 2005). TNF-α is constitutively expressed in mast cells, but during tissue injury or inflammation, this cytokine may be released by other cells including macrophages, neutrophils, and Schwann cells (Moalem & Tracey, 2006; Sommer & Kress, 2004) in response to a variety of stimuli including viral, bacterial, and parasitic products, ischemia, trauma, and other cytokines (Rittner, et al., 2005). Besides being involved in the cytokine cascade subsequent to inflammation, one of major roles of TNF-α is to kill certain types of tumor cells (DeLeo, Colburn, & Rickman, 1997).

Many studies show that TNF-α is associated with or causes hyperalgesia and inflammation. In C57BL/6N mice, intraplantar injection of TNF-α induced hypernociception in a dose- and time-dependent manner (Cunha, et al., 2005). In vivo injection of TNF-α into DRG causes allodynia. Furthermore, in vitro perfusion of TNF into DRG cells has been shown to cause discharges in the neurons that are associated with pain, the Aδ and C fibers (Sommer & Kress, 2004). Additionally, treatment of animals with neuropathic pain with TNF-α antibodies significantly reduces signs of pain (Rittner, et al., 2005) and treatment with etanercept, a substance that acts as a TNF antagonist, reverses hyperalgesia that has already been established in mice with chronic constriction injury (CCI) of the sciatic nerve (Moalem & Tracey, 2006).

Several studies provide correlative data between TNF expression and the development of hyperalgesia and allodynia in multiple pain models (Schaers & Sorkin, 2008). Taking this into account, it seems apparent that TNF-α plays a large role in the development of the most prominent signs of inflammation, hyperalgesia and allodynia.

TNF-α exerts its effects through two types of receptors: the constitutively expressed tumor necrosis factor receptor-1 (TNFR1) and the inducible TNFR2 (Moalem & Tracey, 2006; Rittner, et al., 2005). Unlike TNFR2, TNFR1 is linked to pathways for cell death (Moalem & Tracey, 2006). Both types of receptor are expressed on inflammatory cells and are up-regulated in the DRG during inflammation (Rittner, et al., 2005). The effects that are associated with experimentally-induced hyperalgesia, however, have been shown to be more associated with TNFR1 (Sommer & Kress, 2004). Activation of either receptor results in p38 mitogen-activated protein kinase (MAPK) activation, translocation of nuclear transcription factor-kappaB (NF-κB) to the nucleus and activation of COX-2-dependent prostanoid release (Moalem & Tracey, 2006).

Additionally, TNF-α has been shown to up-regulate NGF subsequent to inflammation. McMahon and colleagues (2005) established that blocking TNF-α, IL-1β or NGF blocks the pain produced by the cytokines, indicating that the pain is produced by a cytokine-dependent release of NGF. Like cytokines, NGF has both direct and indirect effects. It can directly sensitize nociceptors by acting on tyrosine kinase A (trkA) receptors. The intracellular mechanism of this effect is unclear. Indirectly, when NGF binds to trkA receptors, it is internalized and transported to the sensory neuron soma in the DRG, where it acts as a strong regulator of gene expression. This results in the up-regulation of inflammatory mediators such as calcitonin gene-related peptide (CGRP), SP, and brain-derived neurotrophic factor (BDNF; McMahon, Cafferty, & Marchand, 2005).

Interleukin (IL)-1β

IL-1β is a potent hyperalgesic substance (Loram, et al., 2006) that has been shown to have multiple roles in both the body's immune and inflammatory responses (DeLeo, et al., 1997). It is produced and secreted under pathological conditions, like RA, that are associated with increased pain and hyperalgesia (Sommer & Kress, 2004). This cytokine can be released by myocytes and resident macrophages in the periphery (Loram, et al., 2006) as well as fibroblasts, Schwann cells, and endothelial cells (Sommer & Kress, 2004). Its release can be induced by both TNF-α-dependent and -independent pathways (Loram, et al., 2006). IL-1β has both direct sensitization effects on primary nociceptors and indirect effects by instigating the production of PGs (Moalem & Tracey, 2006). In fact, it has been shown to be the major cytokine stimulus for COX-2 expression in the CNS during inflammation. The receptor for IL-1 is the IL-1 receptor (IL-1R) and is located on various immune cells (Rittner & Stein, 2005) as well as DRG neurons (Sommer & Kress, 2004), indicating possible peripheral and central roles. Once this cytokine binds to IL-1R, it inhibits voltage-dependent Na^+ currents in trigeminal neurons. This effect seems to be dependent on protein kinase C (PKC) and G-protein-coupled signaling pathways since inhibitors of each inhibits IL-1β's effects. IL-1β has also been shown to sensitize the TRPV1 currents after brief applications (Schafers & Sorkin, 2008).

Like TNF-α, many experimental studies have shown that IL-1β can cause or increase signs of inflammation. Injection of IL-1β into the rat paw, rat sciatic nerve, and various regions of the brain elicit hyperalgesia (Moalem & Tracey, 2006). In C57BL/6N mice, administration of IL-1β induced mechanical hypernociception in a time- and dose-dependent manner, peaking between 3 and 5 hours post-injection (Cunha, et al., 2005). Inflammatory hyperalgesia caused by injection of Cg can be at least partially prevented by exogenous IL-1ra administration, while

antibodies that neutralize IL-1R reduce pain behavior in mice with neuropathy (Rittner, et al., 2005; Sommer & Kress, 2004). Studies on animals with deletion of the IL-1R type 1 and with transgenic overexpression of the IL-1ra showed a reduction of spontaneous ectopic activity subsequent to a spinal nerve injury (Schafers & Sorkin, 2008). IL-1R has been found to be up-regulated in inflamed tissue (Rittner & Stein, 2005). Furthermore, IL-1β production is up-regulated in injured peripheral nerves (Moalem & Tracey, 2006; DeLeo, et al., 1997) and in the CNS after glial injury (DeLeo, et al., 1997), indicating a direct correlation between inflammation and nerve injury and IL-1β expression in both the PNS and CNS. Not only has it been shown that IL-1 can induce hyperalgesia and allodynia, it has been implicated in the reversal of opioid analgesia, opioid tolerance; endogenous IL-1 has been shown to reduce morphine analgesia (Hutchinson, et al., 2008).

IL-2

IL-2, also known as T cell growth factor, is one of the cytokines that has been shown to have both anti- and proinflammtory effects, depending on the context in which it is secreted (Uceyler & Sommer, 2008). IL-2 activation stimulates the production of B, T, and Natural Killer cells (Hanisch & Quirion, 1996; Waldmann, 2006). IL-2 receptors are found in both the periphery in the DRG as well as the CNS in various regions of the brain (Song, Zhao, & Liu, 2000; Lapchak, et al., 1991).

Many studies show both the anti- and proinflammatory effects of IL-2. For instance, both intraperitoneal and intrathecal injection of IL-2 have analgesic effects during the radiant heat test for thermal nociception (Song, et al., 2002; Yao, et al., 2003). Additionally, IL-2 inhibits nociceptive responses in spinal dorsal horn neurons. Research indicates that the anti-nociceptive

effects of IL-2 are due to IL-2 binding to opiod receptors (Yao, et al., 2002). On the other hand, elevated IL-2 plasma levels were correlated with an increase in the intensity of pain in chronic pain patients (Koch, et al., 2007). In a more recent study, Cata and colleagues (2008) studied the effect of both high and low doses of IL-2 on pain processing by treating rats with IL-2 and analyzing their responses to mechanical and thermal hyperalgesia. These researchers found that following the low dose, animals exhibited less thermal hyperalgesia, while at the high dose, animals experienced more thermal and mechanical hyperalgesia (Cata, Weng, & Dougherty, 2008). Taken together, these studies show the multiple effects IL-2 has in pain processing.

IL-5

IL-5 is produced by Th2 cells, mast cells, and eosinophils. Stimulation of IL-5 via the IL-5α and the common cytokine β-chain receptor (βc) results in activation of eosinophils (Kouro & Takatsu, 2009; Martinez-Moczygemba & Huston, 2003). This cytokine has the ability to enhance survival, differentiation, activation, and mobilization of eosinophils (Chen, et al., 2009; Lei & Martinez-Moczygemba, 2008). It is important in the pathophysiology of asthma and allergic inflammation. As such, eosinophil blood counts in the periphery increase within 24 hours of insult with an allergen, and eosinophil infiltration in the lungs is a key component in asthma. Studies have found that mice lacking IL-5 do not develop airway eosinophilia or bronchial hyperreactivity after challenge with a known allergen (Martinez-Moczygemba & Huston, 2003). Furthermore, IL-5 mRNA is up-regulated in bronchial tissue in asthma patients and transgenic mice that express IL-5 in the lung epithelium show an increase in eosinophil infiltration and airway hyper-responsiveness (Kouro & Takatsu, 2009). Chen and colleagues (2009) show that a small interfering RNA against IL-5 significantly reduces airway hyper-

reactivity in an ovalbumin-induced murine model of allergic asthma. Additionally, Lai and colleagues (2009) display that IL-5 is up-regulated in bronchoalveolar fluid in a rat model of airway inflammation caused by gastroesophageal reflux. Studies have also shown that in βc knockout mice, allergen-induced accumulation of eosinophils in the lungs is non-existent (Asquith, et al., 2008). Taken together, these findings clearly show that IL-5 plays an important role in inflammatory responses in the lung.

Like most other cytokines, IL-5 exerts its effects via different intracellular pathways. One is the JAK/STAT pathway. Subsequent to IL-5 binding to either of its receptors, the JAK1 and JAK2 kinases, key players in causing cytokine-induced proliferation and differentiation of immune cells, are activated. IL-5 also activates the MAPK pathways, which are also important in the growth, proliferation and survival of cells. Activation of the extracellular signal-regulated kinase (ERK) pathway by IL-5 leads to downstream activation of c-Fos and c-Jun, important contributors of cytokine-induced proliferation. It has been shown that the ERK pathway is important in preventing apoptosis in response to cytokines, resulting in an increase in cytokine-producing cells. Additionally, IL-5 stimulates the c-Jun N-terminal kinase (JNK) cascade where JNK phosphorylates the c-Jun transcription factor. It also stimulates the p38 MAPK cascade, where p38 MAPK activates an activating transcription factor (ATF). All three MAPK cascades work in parallel to enhance cell growth (Martinez-Moczygemba & Huston, 2003).

*Interferon gamma (*IFN-γ)

IFN-γ is a cytokine that is produced by a variety of cells types such as T cells, lymphocytes, and macrophages and can be found in elevated levels in the CNS after inflammatory insult (Vikman, Duggan, & Siddal, 2007). IFN-γ production can be stimulated by

other proinflammatory cytokines such as IL-2 and IL-12. This cytokine has been shown to induce monocyte/macrophage differentiation and gene expression and thus plays a crucial role in modulating both immune and inflammatory responses (Young & Hardy, 1995). Receptors responsive to IFN-γ are located in various regions of the nervous system, including the pain-processing superficial region of the dorsal horn in the spinal cord and the DRG (Vikman, Duggan, & Siddell, 2007; Robertson, et al.,1997).

Both clinical and preclinical studies have shown the pronociceptive properties of IFN-γ. In clinical studies, cancer patients that received IFN-γ therapy reported spontaneous pain after treatment (Mahmoud, et al., 1992). Preclinically, rats treated with IFN-γ exhibited a reduction in PWL to mechanical hindpaw stimulation (Vikman, Siddall, & Duggan, 2005). Furthermore, chronic IFN-γ treatment led to an increase in spontaneous activity in dorsal horn neurons and an increase in the responsiveness of DRG axons (Vikman, et al., 2003). In vivo, intrathecal IFN-γ administration in rodents has been shown to evoke pain-related behaviors like biting and scratching as well as activation of the spinal nociceptive flexor reflex (Robertson, et al., 1997; Xu, et al., 1994). Additionally, IFN-γ receptor knockout mice failed to develop the behavioral signs to neuropathic pain after nerve injury in the periphery (Vikman, Duggan, & Siddall, 2007).

Although the spinal mechanisms that increase responsiveness in dorsal horn neurons are largely unknown, recent studies have shown that IFN-γ may act via inhibition of γ-aminobutyric acid (GABA) receptors, the receptors that responds to GABA, the main inhibitory neurotransmitter in the CNS. A reduction in the inhibitory effects of GABA in the CNS has been linked to central sensitization. IFN-γ reduces the efficacy of picrotoxin, a GABA antagonist, indicating that IFN-γ is reducing GABA in the nervous system prior to antagonist treatment (Vikman, et al., 2003). Researchers suggest that this disinhibition is caused activation of glial

cells in the spinal cord by IFN-γ (Vikman, Duggan, & Siddall, 2007). Vikman and colleagues (2007) conducted a study investigating the effect of bicuculline, a $GABA_a$ receptor antagonist, on altering spinal hypersensitivity caused by IFN-γ. Researchers showed that bicuculline is not effective at reducing spontaneous firing of dorsal horn neurons treated with IFN-γ, indicating that this cytokine is decreasing GABA function itself (Vikman, Duggan, & Siddall, 2007). Additionally, Vikman and colleagues (2003) had found that chronic treatment of cultured neurons from the dorsal horn with IFN-γ resulted in a significant decrease in glutamate receptor-1 on neurons, which was accompanied by an increase in spontaneous activity at the synapse.

Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF)

GM-CSF is a haematopoietic factor and inflammatory cytokine which is expressed in many cell types including T-cells, monocytes, mitogen-stimulated lymphocytes, macrophages, fibroblasts, and endothelial cells (Frazen, et al., 2004; Burgess & Metcalf, 1980). It is a part of a group of colony stimulating factors that also includes granulocyte CSF, macrophage CSF, and IL-3 (Campbell, et al., 1997). GM-CSF stimulates the maturation and proliferation of neutrophil, monocyte, macrophage, and eosinophil precursors and thus, is thought to be vital for the survival, proliferation, and differentiation of these progenitor cells into monocyte/macrophages and granulocytes (Campbell, et al., 1997; Burgess & Metcalf, 1980). Receptors for GM-CSF are found in tissues that are derived from haematopoietic cells but also various sites in the nervous system, including microglia, astocytes, oligodendrocytes, and even some neurons. During times of rest, astrocytes are the only source of GM-CSF in the CNS where they regulate microglial functioning. This cytokine can cross both the blood-brain and blood-spinal cord barriers, which indicates a prominent role for GM-CSF in the CNS. In response to peripheral nerve injury, TNF-

α and IL-1α induce GM-CSF production in fibroblasts and subsequent activation of macrophages. In the CNS, GM-CSF affects microglia in many ways, including induction of proliferation and morphological changes. Because of this, GM-CSF may be important for successful repair of injured axons. On the other hand, it is considered a critical mediator in the development of chronic inflammation. GM-CSF has been identified as an anti-apoptotic factor that hinders the cell death program of neutrophils, allowing them to prolong their action and contribute to the inflammatory process for longer (Frazen, et al., 2004).

In humans, GM-CSF has been found in the joint effusions in patients with RA, indicating a possible role in RA disease pathology (Feldmann, Brennan, & Maini, 1996). Furthermore, when patients with Felty's syndrome are treated with GM-CSF, RA symptoms flare up in the affected joints (Campbell, et al., 1997). Experimentally, Campbell and colleagues (1997) found that GM-CSF exacerbated collagen-induced arthritis in mice; animals treated with the cytokine not only had more incidence of disease, but also a more rapid onset of disease as compared to control animals. The GM-CSF-treated animals had more pronounced pathology in general, including more paw edema. GM-CSF levels have been shown to increase following injection of LPS as well (Burgess & Metcalf, 1980). The anti-inflammatory cytokine, IL-4, has been shown to inhibit GM-CSF's effects in an LPS-treated human monocytes (Hamilton, et al., 1992).

Anti-inflammatory cytokines

The cells that produce proinflammatory mediators also produce analgesic ones (Rittner, et al., 2005). Both IL-4 and IL-10 have been identified as anti-inflammatory cytokines. Occurring late in inflammation, these proteins limit the production of proinflammatory cytokines by suppressing the genes that code for the production of IL-1, IL-6, and TNF (Angst, et al., 2008;

Moalem & Tracey, 2006; Rittner, et al., 2005) and therefore counteract the hyperalgesia that is associated with these proinflammatory cytokines (Rittner & Stein, 2005). The analgesic effects of these cytokines have been shown in different models of inflammation (i.e. Cg, TNF-α, bradykinin, experimental peritonitis) and occur independently of endogenous opioid production (Rittner & Stein, 2005). There is evidence that the release of anti-inflammatory mediators parallels the cascade of proinflammatory cytokines that precedes the release of PGs and sympathetic amines in response to inflammation (Verri, Jr., et al., 2006).

IL-4

IL-4 is the protoypic anti-inflammatory cytokine (Hao, et al., 2006) and is a member of the lymphokine family consisting of IL-4, -5, -3, and GM-CSF (Paul, 1991). IL-4 is mainly produced by T cells (te Velde, et al., 1990), but is also manufactured by circulating monocytes, macrophages and mast cells (Cunha, et al., 1999; Hamilton, et al., 1992). Cells of the monocyte/macrophage lineage have been shown to have receptors for IL-4 (Hart, et al., 1989). IL-4 is predominantly responsible for the production of immunoglobulin E in mice in response to a variety of stimuli (Paul, 1991).

IL-4 has been shown to inhibit the secretion of the proinflammatory cytokines TNF-α, IL-1α, IL-1β, and IL-6 in response to LPS- or IFN-γ-induced inflammation (te Velde, et al., 1990). te Velde and colleagues (1990) found that the reduction of TNF-α and IL-1β secretion in response to LPS- and IFN-γ-induced inflammation was directly attributed to IL-4 since anti-IL-4 antiserum completely restored normal cytokine production. TNF-α production by LPS-stimulated macrophages is also inhibited by IL-4 (Mijatovic, et al., 1997). Pretreatment with IL-4 inhibits writhing responses induced by intraperitoneal administration of acetic acid or zymosan

in mice as well as the knee joint incapacitation elicited by an intra-articular injection of zymosan in rats, but not the pain responses created during the hot-plate test. This decrease in pain responses was shown to be correlated with a decrease in both TNF-α and IL-1β (Vale, et al., 2003). In vitro, Hart and colleagues (1989) showed that co-treatment of human monocytes with LPS or IFN-γ with IL-4 significantly blocked the production of the proinflammatory cytokines TNF-α and IL-1, and the AA metabolite PGE_2. Cunha and colleagues (1999) showed that IL-4 administration 30 minutes prior to intraplantar injection of Cg, bradykinin, or TNF-α inhibited subsequent behavioral pain responses. Administration of this cytokine 12-14 hours before IL-1β injection inhibited its subsequent hyperalgesia (Cunha, et al., 1999). Taken together, this data suggests that IL-4 works in a time-dependent manner, inhibiting TNF-α during the early phase of inflammation and IL-1β in the later stages. Hamilton and colleagues (1992) found that IL-4 also inhibited GM-CSF production in LPS-stimulated human monocytes, indicating yet another IL-4 mechanism that works to counteract inflammation.

Not only does IL-4 inhibit the production of proinflammatory cytokines, but it has also been shown to increase the production of IL-1ra, the IL-1 inhibitor. In LPS-stimulated human peripheral blood mononuclear cells, IL-4 acted synergistically with LPS to increase IL-1ra production. When the same cell type was stimulated with IL-1β, IL-4 reduced IL-1β production and increased IL-1ra synthesis (Vannier, Miller, & Dinarello, 1992).

IL-10

IL-10 is a well-characterized endogenous immunoregulatory anti-inflammatory cytokine that is produced and released by many immune cells including T-lymphocytes, macrophages/monocytes, B cells, and Natural Killer Cells (Wagner, Janjigian, & Myers, 1998;

Marshall, et al., 1996; Mertz, et al., 1994). IL-10 decreases proinflammatory cytokine production by macrophages and mast cells activated by tissue injury (Wagner, et al., 1998) and has been shown to enhance mast cell development, especially in conjunction of IL-3 and IL-4, other anti-inflammatory cytokines (Marshall, et al., 1996). This cytokine also modulates B cell function and has numerous effects on monocytes/macrophages, mainly by inhibiting the majority of monocyte activity (Mertz, et al., 1994).

Like IL-4, pretreatment with IL-10 has been shown to inhibit both the writhing response elicited by intraperitoneal injection of acetic acid or zymosan and the knee joint incapacitation created by an injection of zymosan in the knee joint in rats. This decrease in pain response was correlated with IL-10-dependent decreases in TNF-α and IL-1β. Furthermore, antiserum against IL-10 potentiated both the writhing response and incapacitation of the knee joint (Vale, et al., 2003). IL-10 also decreases the thermal hyperalgesia associated with CCI lesions up to 9 days after injury. Two days after injury, IL-10 decreases the number of cells that are immunoreactive for TNF-α (Wagner, et al., 1998). IL-10 inhibits the production of IFN-γ, TNF-α, IL-6, and GM-CSF (Marshall, et al., 1996). Further, Marshall and colleagues (1996) discovered that recombinant murine IL-10 inhibits the production of both IL-6 and TNF-α in response to a variety of stimuli in rat peritoneal mast cells. IL-10 also inhibits IL-6 production in LPS-, PGE_1- and PGE_2-activated cells (Marshall, et al., 1996). Poole and colleagues (1995) found that IL-10 inhibited hyperalgesic responses to bradykinin, TNF-α, IL-1β, IL-6, and Cg. To further support this notion, the same experimenters found that a mouse IL-10 antibody potentiated the hypernociceptive responses to Cg. In vitro analyses show that in IL-1β- and LPS-stimulated human peripheral blood mononuclear cells IL-10 diminishes PGE_2 production but only results in a minor increase in IL-1ra levels (Poole, et al., 1995).

Prostanoids and Cyclooxygenases

Prostanoid synthesis is an important part in the development of inflammatory pain. PGs, thromboxanes and leukotrienes constitute a family of AA metabolites known as the eicosanoids (Meyer, et al., 2005). The eicosanoids are thought to play a role in increased sensitivity to pain not by activating nociceptors directly, but by sensitizing nociceptors in the skin to natural stimuli and various endogenous inflammatory mediators (Meyer, et al., 2005; Kidd & Urban, 2001). They sensitize nociceptors by increasing levels of cyclic adenosine monophosphate (cAMP) and by decreasing the primary afferents' activation thresholds via TTX-R Na^+ channels and the protein kinase A (PKA) pathway (Kidd & Urban, 2001; Basbaum & Jessell, 2000). Prostanoids have been recently recognized as having important effects on both peripheral and central pain processing (McMahon, et al., 2005). PGs exert their effects by acting on G-protein-coupled receptors that include $EP_{1\text{-}4}$ for PGE_2 and the IP receptor for prostaglandin I_2 (PGI_2; Tober, et al., 2007; Moalem & Tracey, 2006).

The cascade starts with AA, a polyunsaturated fatty acid that is found in cell membranes. It is the predominant precursor to the eicosanoids and once freed from the cell membrane by phospholipase A2, it is converted into the various eicosanoids. AA is converted into PGs via the COX enzymes (Moalem & Tracey, 1996). There are 2 COX isoforms: the constitutive enzyme COX-1 and the inducible enzyme COX-2 which is produced in peripheral tissues after tissue injury (Meyer, et al., 2005). COX-1 mainly plays a housekeeping role in most tissues, while basal COX-2 expression is usually low. COX-2 expression can be elicited by inflammatory cytokine in mast cells, macrophages, and spinal neurons. On the other hand, COX-1 may also be induced in some inflammatory conditions such as spinal cord injury (Moalem & Tracey, 2006). Both COX-1 and -2 are constitutively expressed in the brian and spinal cord in humans and rats

(Kidd & Urban, 2001). Both COX isoforms have similar enzyme activity and substrate affinity but are produced in different locations (Coutaux, et al., 2005). Both isoforms stimulate the production of PGs, including the much-studied PGE_2. COX-1 converts AA into the PG precursors PGG_2 and PGH_2. Membrane-associated PGE synthase (mPGES)-1, mPGES-2, and cytosolic prostaglandin E_2 synthase (cPGES), all produce PGE_2 from these precursors (Figure 1.4). Both COX and PGE_2 have been shown to be up-regulated in skin cells in response to ultraviolet B (UVB) light exposure (Tober, et al., 2007).

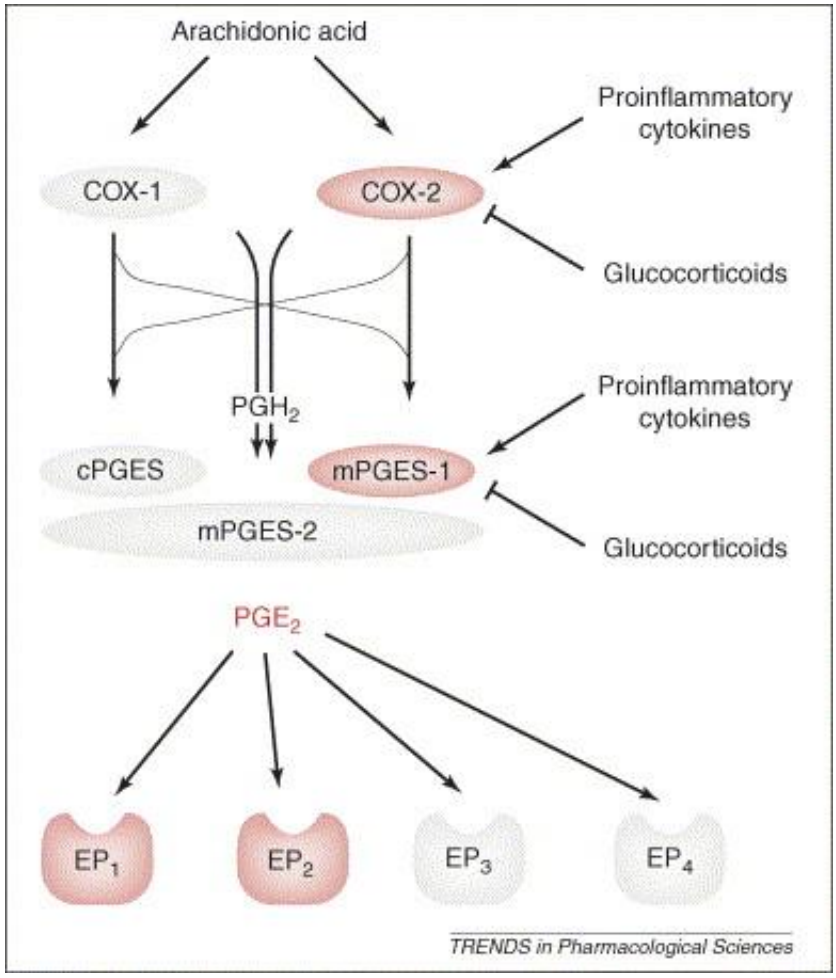

Figure 1.4. Biosynthesis of PGE_2. Arachidonic acid, which is released from the cell membrane by phospholipase A_2 in response to tissue damage and inflammatory stimuli, is the starting point of several biosynthetic pathways that give rise to different lipid messengers including prostaglandins, leukotrienes and endocannabinoids. Constitutively expressed COX-1 and inducible COX-2 metabolize arachidonic acid into the two prostaglandin precursors PGG_2 and PGH_2. At least three PGE synthases, mPGES-1 mPGES-2 and cPGES, produce PGE_2 from these precursors. mPGES-1 and COX-2 are coupled functionally, spatially and temporally: they colocalize at cellular and subcellular levels, and are induced by proinflammatory cytokines and suppressed by glucocorticoids. PGE_2 exerts its cellular effects through four subtypes of GPCRs called EP_1–EP_4. Enzymes and receptors highlighted in pink indicate those that contribute the most to pain sensitization.

From: Zeilhofer & Brune (2006). TRENDS IN PHARM. SCI. VOL. 27(9), 467-474.

Studies using COX inhibitors have demonstrated the importance of COX in the development of inflammation. Moalem and Tracey (2006) showed that injection of indomethacin, a classic COX inhibitor, into an inflamed hindpaw alleviated mechanical hyperalgesia in nerve-injured rats for 10 days. COX inhibitors also mediate the febrile response (Hopkins, 2007). Blocking spinal PGE2 production using COX inhibitors reduces or reverses the enhanced pain associated with peripheral tissue damage (McMahon, et al., 2005). The COX inhibitors indomethacin and piroxicam have been shown to inhibit the effects that IL-1β has on pain behavior (Hopkins, 2007). Additionally, multiple selective COX-2 inhibitors have been effective at reducing Cg-induced paw edema and vascular permeability (Süleyman, et al., 2004). Because of the known analgesic actions of nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit COX enzymes and thus decrease the production of PGs (Kidd & Urban, 2001), it is widely accepted that PGs are the primary inducers of inflammatory pain (Verri, et al., 2006). Some researchers claim that NSAIDs have limited efficacy in relieving inflammation, and thus suggest that other inflammatory mediators are also important in the inflammatory process (McMahon, et al., 2005).

Using a genetic model, experimenters have found that COX-deficient mice display decreased nociceptor activity in response to a variety of stimuli. When comparing female and male COX-2-deficient mice, researchers found a sex difference in the role COX-2 plays in nociception: nociceptive activity only decreased in diffuse pain models in female, but not male, mice (Kidd & Urban, 2001).

Blocking the PG receptors also is another way to investigate the role of PGs in inflammation and pain. Kidd and Urban (2001) report that selective blockade of both the EP and IP receptors has an antihyperalgesic effect in most animal models. Further, mice that lack the

prostanoid receptor have normal acute nociception, indicating that prostanoids are not responsible for acute pain perception but may be linked, instead, to the more long-term processes like allodynia and hyperalgesia that are associated with inflammation (Zeilhofer & Brune, 2006). Additionally, Tober and colleagues (2007) show that blocking EP1 receptors with an antagonist drug reduces acute inflammation associated with UVB exposure. This was shown to be via cross-regulation of the EP3 receptor (Tober, et al., 2007). Research also shows that COX-2 inhibition and treatment with anti-PGE_2 antibodies reverses peroxynitrate-induced hyperalgesia (Ndengele, et al., 2008).

Indomethacin: A COX inhibitor

Indomethacin is an NSAID, a class of drugs primarily used to treat illnesses like inflammation, fever, and cardiovascular disease (Zhou, Hancock, & Lichtenberger, 2010; Okamura, et al., 2008). Although these drugs can treat many diseases due to a wide variety of biochemical effects, the anti-inflammatory effect of NSAIDs, including indomethacin, come from their inhibition of the cyclooxygenase (COX) enzymes, a key component involved in eicosanoid synthesis (Zhou, Hancock, & Lichtenberger, 2010; Gordon, et al., 2007). NSAIDs are connected to side effects such as gastrointestinal (GI) and renal toxicity and GI bleeding (Sabina, Chandel, & Rasool, 2008) but are attractive due to their relatively low toxicity and their availability as over-the-counter medications (Blaha & Leon, 2008).

Indomethacin has been shown to reduce edema in a murine model of gouty arthritis, a disease that manifests in inflammation and extreme pain around the affected joints. In the same model, this drug decreased TNF-α production in the plasma, liver, and spleen (Sabina, Chandel, & Rasool, 2008). Along the same thread, Kamachi and colleagues (2007) showed that

indomethacin decreased vascular permeability in a model of LPS-induced air-pouch-type inflammation, leading to a decrease in overall edema. Furthermore, topical applications of indomethacin at the site of inflammation decreases edema by 65% in both the treated and un-treated ears, indicating that indomethacin may work systemically to reduce inflammation (Bralley, et al., 2008; Gordon, et al., 2007). This drug was also shown to inhibit neutrophil infiltration into the injured area (Gordon, et al., 2007). Additionally, both Lavich and colleagues (2005) and Holt and colleagues (2005) showed that pretreatment of indomethacin abolished Cg-induced hyperalgesia. It is argued that the anti-edema effect of NSAIDs are due to the reduction of the COX-1 enzyme more than the COX-2 enzyme. This is supported by the fact that Cg-induced edema is inhibited by indomethacin by the same amount in wild-type and COX-2-deficient mice (Holt, et al., 2005).

Prusakiewicz and colleagues (2009) showed that indomethacin, but not other NSAIDs, inhibit oxygenation of both AA and 2-arachidonoylglycerol (2-AG), acid and ester precursors to PG synthesis, respectively. Another studied showed that indomethacin inhibits fatty acid amide hydrolase (FAAH), which metabolizes arachidonolethanolamide (AEA), a endocannabinoid synthesized on demand, into AA and ethanol amine (Holt, et al., 2005). As discussed in the previous section, AA is a precursor to the sensitizing agents, PGs, so an inhibition of FAAH would prevent AEA from being converted to AA and stop the sensitizing PGs from being formed. Some NSAIDs show fast, reversible inhibition, while others, such as indomethacin, display slow, tight-binding inhibition. These NSAIDs are harder to reverse the effects of and, therefore, are more potent (Prusakiewicz, et al., 2009).

Sex differences in pain

It is well documented that pain perception differs between males and females. Epidemiological studies show that many chronic pain disorders are more prevalent in females (Gupta, et al., 2007; Evans, 2006; Kuba, et al., 2006). For example, females are 2-3 times more likely to experience migraine headaches (Riley III, et al., 1998), 10 times more likely to have fibromyalgia (Macfarlane, et al., 2002), and roughly 2 times more likely to report temporomandibular disorders (TMD; LaResche, et al., 2003; Warren & Fried, 2001). In addition, women are reported to experience the majority of cases of RA and irritable bowel syndrome (IBS) [Berkley 1997].

Psychophysical studies in humans show that women have lower thresholds for pain, exhibit less tolerance for painful stimuli, and rate similar stimuli as more painful than men (Gaumond, Arsenault, & Marchand, 2005; LaCroix-Fralish, Tawfik, & DeLeo, 2005). Women also report more recurrent pain in multiple parts of their bodies compared to males (Tall, et al., 2001; Riley III, et al., 1998) and tend to show less analgesia to morphine than men (Mannino, et al., 2005; Clemente, et al., 2004; Berkley, 1997).

Differences in pain perception between the sexes could be due to physiological or psychosocial factors, or both. Physiological differences include differences in anatomy and hormonal profile. Psychosocial factors include differing attitudes toward pain (Gaumond, et al., 2005; Berkley, 1997) and gender-role expectations in reporting pain (Wiesenfeld-Hallin, 2005). However, in preclinical studies, male and female rodents exhibited sex differences in basal nociceptive thresholds, which indicates that the differences seen between men and women are mainly due to physiological factors (Berkley,1997). Mounting attention is being paid to the presence and fluctuation of gonadal hormones in pain studies (Gaumond, et al., 2005).

Estrogen and pain

Estrogens are gonadal hormones that are present in both males and females (Ceccarelli, et al, 2003). Receptors for sex hormones are found throughout the nervous system, both in the PNS and CNS (Gaumond, et al., 2005). These receptors develop differently in males and females in certain regions of the nervous system (Berkley, 1997). Among other places, estrogen receptors are located on small-diameter DRG neurons in the periphery (Kuba, et al., 2005) and in neurons in the lumbar section of the spinal cord's dorsal horn (Kuba, et al., 2006).

Females have complex endogenous hormonal fluctuations across the estrous/menstrual cycle where estrogen fluctuates cyclically. Plasma levels of estrogen and progesterone vary throughout the menstrual and estrous cycles (Martin & Behbehani, 2006; Kuba & Quinones-Jenab, 2005). In the human menstrual cycle, estrogen levels are low during early to mid-follicular phase, plateau during mid-luteal phase, and fall quickly just before menstruation, a cycle lasting from 14 to 35 days (Martin & Behbehani, 2006; Kuba & Quinones-Jenab, 2005). The rodent estrous cycle in contrast lasts approximately 4 days, with estrogen levels peaking in proestrus and declining through the estrus phase (Kuba & Quinones-Jenab, 2005).

Menstrual phase and reproductive status (pre-, peri-, and post-menopausal) have been shown to affect pain ratings (Macfarlane, et al., 2002) as well as intensity and prevalence of chronic pain conditions (Gaumond, et al., 2005). The decline of estrogen during the menstrual cycle has been shown to cause an increase in migraine attacks (Gupta, et al., 2007). Using the natural variations of sex hormones in the estrous cycle, Tall and Crisp (2004) showed that female rats in proestrus, a stage that is marked with high levels of estrogen, nociceptive responses decreased in the Cg model of inflammation. It has also been shown that vocalization to paw pressure in rats is lowest during proestrus and estrus, the stages highest in estrogen (Kayser, et

al., 1996). On the other hand, researchers have found that rats are more sensitive to electric foot shocks during the proestrus and estrus stages than in diestrus (Kayser, et al., 1996). Finally, preclinical studies have shown that there are temporal variations in the activity of various opioid receptors across the estrous cycle (Berkley, 1997), indicating that pain sensitivity fluctuates during the cycle as well.

In addition to the study of endogenous hormones and their fluctuations, many studies have examined the effects of exogenous estrogens on the response to noxious stimuli in animals. In humans, oral contraceptive use in women who experience migraines increased the frequency of attacks (Martin & Behbehani, 2006). The use of exogenous estrogen has also been shown to increase the odds of experiencing TMD, and this risk increases with higher doses of estrogen (Warren & Fried, 2001). Postmenopausal women undergoing estrogen replacement therapy show an increased incidence of temporomandibular joint pain (Ceccarelli, et al., 2003b). In the formalin model of inflammation, experimental results indicate that estradiol administration increases male rats' reactivity to a nociceptive stimulus. Specifically, intracerebroventricular injection of estradiol causes an increase in formalin-induced licking (Ceccarelli, et al., 2003b). On the other hand, Kuba and colleagues (2006) found that after 1% formalin administration, estradiol reduced flinching responses in OVX rats.

Gonadectomy

Gonadectomized (GDX) animals are often used to investigate the role of hormones in pain. For instance, GDX abolishes the sex differences in nociceptive responses seen in rats (Gaumond, et al., 2005), including differences in paw withdrawal latency (PWL) in the Cg model and thermal hyperalgesia after CCI (Kuba & Quinones-Jenab, 2005). Bradshaw and

Berkley (2002) showed that removal of the female reproductive glands via ovariectomy (OVX) induces vaginal hyperalgesia which is reversible with estradiol treatment. Further, after OVX, estradiol increased tail-flick latencies in rats (Mannino, et al., 2006). OVX animals have been shown to be more sensitive to subcutaneous injection of formalin compared to intact rats as shown by increased licking of the injection site. Tail withdrawal latency was also found to be lower in the OVX animals (Ceccarelli, et al., 2003b). Additionally, Aloisi and colleagues (2003) show that GDX in male rats causes increases in formalin-induced inflammatory behaviors. Although this result may be due to a decrease in testosterone, it may also be linked to the increase in estrogen that is observed after male GDX (Aloisi, Ceccarelli, & Fiorenzani, 2003). Taking into account the findings from estradiol, estrous and menstrual cycle, and GDX studies, it seems likely that female hormones and their fluctuations may account for the sexually dimorphic nature of pain perception.

Estrogen's Effect on Indomethacin Efficacy

Research has suggested that estrogen can improve circulation by acting as a vasodilator (Ba, et al., 2006). To determine the mechanism of this effect, experimenters designed studies using indomethacin to see if estrogen works by increasing constrictor prostanoid synthesis in vascular tissues (Li & Stallone, 2005). White and colleagues (2005) found that indomethacin reversed the contractile effect of estrogen in a porcine heart. Similarly, Li & Stallone (2005) showed that by blocking the prostanoid pathway pharmacologically using indomethacin in rats, they also blocked the contractile responses to estrogen. Although this research is not specifically aimed at the effects estrogen has on inflammation and pain, it indicates that some of estradiol's physiological effects are due to their alteration of the prostanoid pathway.

Estrogen's Effect on Cytokine Action

Estrogen is thought to play a critical role in immune responses. Estrogen receptors are found on a variety of immunocompetent cells including T cells and macrophages. There is clinical evidence that indicates that the severity of some autoimmune disorders including RA and multiple sclerosis fluctuate based on a woman's hormonal fluctuations during pregnancy and the postpartum period; symptoms decrease during pregnancy, a time of high levels of estrogen, and are exacerbated after pregnancy when estrogen levels drop. Pharmacological doses of estrogen also seem to improve certain autoimmune disorders. Recent findings indicate that estrogen inhibits the production of T-helper 1 cell (TH1) proinflammatory cytokines like IL-12, TNF-α, and IFN-γ, while stimulating the production of TH2 anti-inflammatory cytokines such as IL-10, and IL-4 (Salem, 2004). Ito and colleagues (2001) found that estradiol significantly suppressed experimental autoimmune encephalomyelitis in IL-4, -10, and IFN-γ knockout mice. The decrease in the severity of the disease was associated with a concomitant reduction in the number of cells in the CNS that produce proinflammatory cytokines and chemokines, especially those cells producing TNF-α. Because these experimenters did not see a reduction in TH2 cytokine production, they proposed that that the reduction of TNF-α production may be a main mechanism through which estrogen may be working to decrease inflammation in various diseases (Ito, et al., 2001). Thus, estrogen may be working via two different mechanisms to mediate autoimmune disorders, including those associated with inflammation, such as RA.

Schaefer and colleagues (2005) show that estradiol inhibits the IL-1β-mediated response in a dose-dependent fashion in human uterine epithelial cells stimulated with IL-1β. Estradiol's effect is due to actions mediated by estrogen receptors since the addition of pure estrogen receptor antagonist reversed estradiol's effect. Estradiol also downregulates the IL-1R type 1,

which reduces the uterine epithelial cell's ability to respond to IL-1β. Further studies show that both high and low doses of ethinyl estradiol are effective at suppressing both clinical and histological signs of collagen-induced arthritis in DBA/1LacJ mice via decreased secretion of TNF-α, IFN-γ, IL-1β, and IL-6 as well as mRNA for cytokines and chemokines in the joint tissue (Subramanian, et al., 2005). Taken together, these data suggest a link between the endocrine and immune systems during the inflammatory process (Schaefer, et al., 2005).

Proposed Model

We propose that estrogen's antihyperalgesic effects at both the peripheral and central levels occur via down-regulation of pro-inflammatory cytokines, up-regulation of anti-inflammatory cytokines, or both. Further, we postulate that estrogen may act to decrease COX activation and subsequent PG release (Figure 1.5). Specifically, estrogen may decrease the activation threshold in nociceptors via modulation of these mediators at the site of injury or in the DRG. Further, estrogen may inhibit central sensitization via the modulation of cytokines or prostanoids at the level of the spinal cord in the dorsal horn.

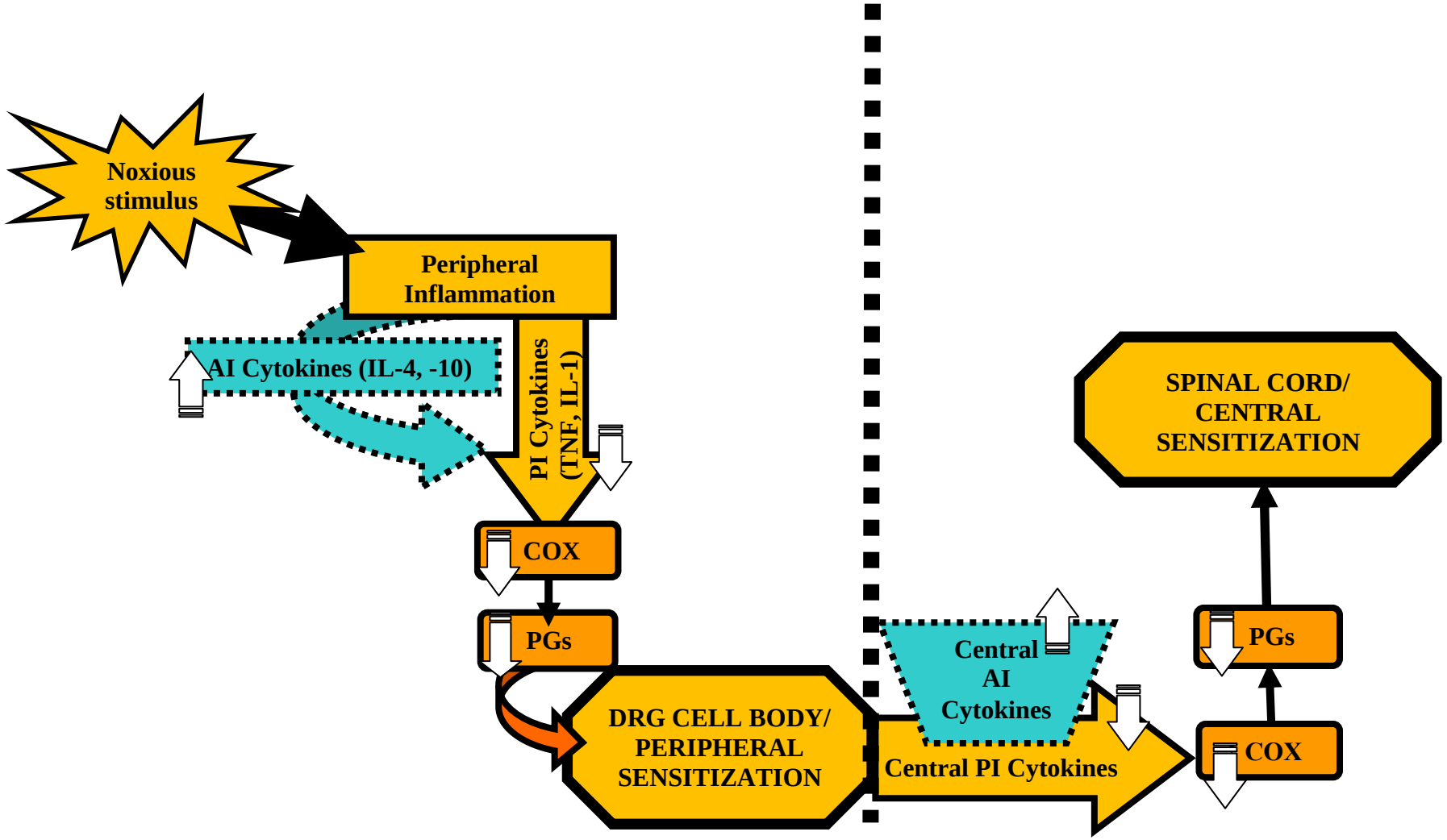

Figure 1.5. Proposed mechanisms for estrogen's attenuation of inflammation in the peripheral and central nervous systems. Estrogen may act to increase anti-inflammator y (AI) mediators such as AI cytokines or decrease proinflammatory (PI) mediators such as PI cytokines, COX, and PG. Each of these mechanisms would result in analgesia, and estrogen may be affecting both in the periphery and the central nervous system. White arrows indicate estrogen's proposed effects.

Specific Aims

Epidemiological studies have found that some chronic pain conditions such as migraine, arthritis, fibromyalgia, and neuropathic pain are significantly more common in females than in males (Fillingin & Ness, 2000). Further, it has been found that females show a greater behavioral response to inflammatory pain than males (Kuba et al., 2005a). It has been suggested that the probable cause of this dimorphic response to pain is due to gonadal hormones. Supporting that notion, research has demonstrated that estradiol attenuates behavior in the chronic phase of inflammation-induced pain (Kuba et al., 2005b). Inflammatory pain is characterized by an increase in sensitivity to both mechanical and heat stimuli post-injury (Sommer & Kress, 2004). Injury to tissue results in the activation of local macrophages, which amplifies the inflammatory response by increasing the migration of leukocytes to the injured tissue. Leukocytes produce cytokines, small regulatory proteins that act to facilitate or attenuate inflammatory responses (Sommer & Kress, 2004). The cytokine cascade results in the activation of cyclooxygenase (COX) and COX-dependent PG release (Sommer & Kress, 2004). PGs sensitize primary afferent fibers by acting on G-protein-coupled receptors that include $EP_{1\text{-}4}$ for PGE_2 and the prostacyclin (IP) receptor for PGI_2 (Moalem & Tracey, 2006). Additionally, estrogen has been shown to reduce cytokine release (Schaefer, et al., 2005; Ito, et al., 2001) which leads to a decrease in subsequent PG expression.

To date, it is not known if estrogen attenuates inflammatory pain responses by regulating prostaglandin production via activation of COX. Furthermore, it is unclear if estrogen is acting at the cytokine level in CNS and PNS tissue by increasing anti-inflammatory cytokine levels, decreasing pro-inflammatory cytokine levels, or both. The objective of my dissertation work is to determine at what levels of the pain pathway estrogen acts to alter inflammatory pain. We

hypothesize that *estradiol acts to decrease inflammatory pain responses by reducing levels of pro-inflammatory cytokines (i.e. IL-1β, TNF-α) while also increasing levels of anti-inflammatory cytokines (i.e. IL-10, IL-4) at both the site of injury and the central nervous system. We also expect that estradiol's effects on these cytokines will lead to a decrease in COX-dependent prostanoid synthesis*. To test these hypotheses, the following aims were proposed:

Aim 1: To determine if, similar to rats, estrogen produces anti-hyperalgesic responses in mice. To this end, 2 experiments were designed to test if estrogen dose-dependently decreases thermal hyperalgesia in C57BL/6N and 129S6 mice and whether this effect is strain-dependent. In each strain, we measured PWL in Cg-treated mice that were pretreated with capsules of cholesterol (0% estradiol), or capsules containing 10%, 20%, 30%, or 40% estradiol. We predict that estrogen will increase PWL to a transient heat stimulus applied to the plantar region of the hindpaw before and after intraplantar injection of 1% Cg.

Aim 2: To determine if estrogen's effects on nocicepetive responses occur via changes in pro-inflammatory and anti-inflammatory cytokines. Tissue from the PNS and CNS extracted 5 hours after Cg administration were measured for IL-1β, IL-2, IL-4, IL-5, IL-10, GM-CSF, IFN-γ, and TNF-α concentrations. Ipsilateral and contralateral paw, ipsilateral and contralateral DRG, and spinal cord tissue were analyzed. Concordant with Aim 1, we hypothesize that pro-inflammatory cytokine levels will decrease after estradiol-treatment, anti-inflammatory cytokine levels will increase after estradiol-treatment, or both.

Aim 3: To determine if blocking COX enzyme activity blocks estrogen's anti-hyperalgesic effects. The effect of high and low doses of indomethacin, a drug that blocks both COX enzymes, on thermal hyperalgesia in animals pretreated with estradiol will be determined. We anticipate that blockade of the COX enzymes will reverse the attenuation of nociceptive behavior caused by estradiol in Aim 1. Therefore, responses to the transient thermal stimulus should be the same in both cholesterol- and estradiol-treated animals after indomethacin administration.

Chapter 2: Methods and Materials

2.1 Animals

Ten-week-old ovariectomized (OVX) C57BL/6N/6N mice and ten-week-old OVX 129S6 mice were purchased from Taconic (Germantown, NY). Mice weighed between 20 and 29g at the start of the experiments. All mice were 7 weeks of age prior to all OVX surgeries. Mice were double-housed with a 12-hour light/dark photoperiod (lights on at 7 A.M. EST). Food and water were available *ad libitum*. All NIH guidelines for the Care and Use of Laboratory Animals (NIH publication 85-23, Bethesda, MD) were followed and approved by the Institutional Animal Care and Use Committee at Hunter College of The City University of New York (CUNY).

2.2 Hormone Replacement Paradigm

Two weeks after ovariectomy, a SILASTIC capsule (1 cm, 0.058in. ID x 0.077in. OD, Dow Corning) was inserted under the skin at the nape of the neck in all animals. For the two dose response studies, the capsules contained either cholesterol (5-Cholestin-3Beta-ol; Sigma Aldrich, St. Louis, MO) for the vehicle group, or 10, 20, 30, or 40% β-Estradiol (1, 3, 5 [10]-Estratriene-3, 17 Beta-diol; Sigma Aldrich) mixed with 90, 80, 70, or 60% cholesterol, respectively, for the experimental groups. The capsules for the indomethacin study contained either cholesterol for the vehicle group, or 40% β-Estradiol mixed in 60% of cholesterol for the experimental groups. Adequate care was taken to minimize pain or discomfort during this procedure. Animals were randomly assigned to either vehicle or experimental groups with the assurance that mice in the same cage received the same treatment.

2.3 Behavioral Apparatus

The Paw Thermal Stimulator system (Department of Anesthesiology, University of California, San Diego, CA) is an apparatus that can be used to determine thermal nociceptive responses in the hind paw of rodents. It consists of 10 plexiglass enclosures (3 x 3 inches) positioned on a heated glass surface that maintains a temperature of 30C ±1C. A mobile infrared heat lamp is used to focus different heat intensities ranging from 4.0-6.0mV. To determine paw withdrawal latencies (PWL), the focused light from the heat lamp is applied to the plantar surface of both hindpaws. The time it takes for the animal to withdraw its paw from the heat source (PWL) is automatically recorded. Using this apparatus, animals are free to remove their paw at the point of discomfort. The light stimulus automatically shuts off after 20 seconds in order to avoid tissue damage. No tissue injury resulted during behavioral testing.

2.4 Behavioral Assay

Behavioral testing was conducted 1 week after hormone replacement. PWL to a low (4.5mV), medium (4.8mV), and high (5.2mV) heat stimuli were determined. To minimize effects of the novel environment, animals were placed in the testing chambers for 1 hour to acclimate prior to baseline readings. After the acclimation period, the animals that received drug treatments injected intraperitoneally with either 0.2mL DMSO for the vehicle group, or 50mL/kg body weight or 25mg/kg body weight injections of indomethacin suspended in DMSO for the experimental groups. For those animals that received a drug treatment, baseline PWL was taken 30 minutes after drug injection. For those animals not treated with indomethacin, baseline PWL were determined directly after the acclimation period. Immediately after baseline assessments (approximately 30 minutes), animals received a 50µl intraplantar injection of 0.9% saline, or 1%

λ-Carrageenan in saline in their right (ipsilateral) hindpaws. Following injection, PWL to all heat intensities were measured in both the ispilateral and contralateral hind paws. All animals were tested 1 hour and 5 hours after the intraplantar injection. Behavioral testing was conducted between the hours of 8:00A.M. and 4:00P.M. EST.

2.5 Tissue Collection and Homogenization

After mice were sacrificed by rapid decapitation following brief exposure to CO_2 (5-10s), trunk blood was collected and placed into sterile tubes containing K_2 EDTA and paw measurements were taken using a dial caliper (General Tools, New York, NY), measuring the width of paws in millimeters. Paws were removed post mortem, weighed, and rapidly frozen on dry ice. Spinal cord and dorsal root ganglia (DRG) dissected from the lumbar region were collected and immediately frozen on dry ice as well. All tissue samples were stored at -80°C until tissue homogenization. Blood samples were stored on ice until they were centrifuged at 2,600RPM for 30 minutes at 4°C. Serum was extracted and stored at -80°C until assays were performed.

Spinal cord, DRG, and paw samples were homogenized in a lysing solution containing cell lysis buffer (from Cell Lysis Kit #171-304012, Bio-Rad, Hercules, CA), a protease inhibitor cocktail provided by the cell lysis kit, and 500mM phenylmethysulfonyl (PMSF, Sigma Aldrich) diluted in DMSO. Spinal cord and paw tissue were homogenized in 100µL lysing solution and DRG tissue was homogenized in 50µL lysing solution. Tissue samples were ground on ice for approximately 10 strokes and immediately frozen on dry ice. Homogenates were then thawed and centrifuged at 5000RPM for 15 minutes at 4°C. Supernatants were collected and stored at -80°C. Total protein concentration was determined in each sample using Bradford analysis. In

order to reduce variation between samples, all samples from the same region were homogenized at the same time.

2.6 Cytokine Multiplex Assay

The Bio-Plex Suspension Array (Bio-Rad) is a system that allows for simultaneous quantification of multiple cytokines. Its array reader combines fluidics, 2 lasers, and real-time digital signaling to distinguish cytokines within each sample. Cytokine analysis is determined using the Bio-Plex cytokine assay (#171F11241, Bio-Rad), a multiplexed (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α) immunoassay consisting of premixed beads coated with target capture antibody that allows for cytokine detection by using the sandwich immune assay technique.

Tissue homogenates were analyzed using the Bio-Plex assay along with the Cytokine Reagent Kit (#171-304001, Bio-Rad). Simultaneous cytokine quantification was analyzed according to the manufacturer's protocol (for details, see Hulse et al., 2004). Standard curves were calculated for each run and sample concentrations were calculated with the Bio-Plex Manager 4.0 software. Standards ranged from .078pg/mL to 3,200pg/mL. The limit of detection was <10ng/mL for each cytokine, and intra-assay coefficients of variance were below 20%. Cytokine concentrations obtained from the Bio-Plex software were divided by the protein concentrations measured for each sample. Cytokine values obtained from tissue samples are expressed as pg/µg of total tissue protein.

2.7 Statistical Analyses

All data are expressed as mean ±SEM. Behavioral Data: Repeated Measures Analysis of Variance (ANOVA) was used to analyze: 1.) baseline PWL following vehicle and estradiol administration; 2.) effects of estradiol when comparing baseline PWL to PWL after Cg or saline injection; 3.) effects of estradiol on PWL post-Cg or –saline injection over low, medium, and high heat intensities; 4.) effects of indomethacin, Cg, and estradiol on baseline PWL; 5.) effects of indomethacin, Cg, and estradiol when comparing baseline PWL to post-injection PWL. Paw Size Data: Paw size was determined by multiplying the width and depth of each hindpaw and was expressed in mm^2. Repeated Measures ANOVA was used to compare ipsilateral and contralateral paw size post-Cg or –saline administration. Cytokine Data: One-Way ANOVA was used to analyze cytokine values in different hormone groups. For all analyses, post hoc tests included One-Way ANOVA (on interactions only), Fisher's least significant difference tests, and planned comparisons when applicable. Expoloratory correlations were also conducted to find relationships between cytokine levels and PWL. No corrections were made for running multiple correlations. For these correlations, group size may differ due to out of range data obtained from the cytokine assay. For all analyses, significance was at the level of $p<0.05$.

Chapter 3: Effects of estradiol administration on Cg-induced nociceptive behavior and paw size in C57BL/6N OVX mice.

3.1. Introduction

Epidemiological studies show that many chronic pain disorders are more prevalent in females (Gupta, et al., 2007). Additionally, preclinical studies show that males and female rodents exhibited sex differences in basal nociceptive thresholds, indicating that the differences seen between men and women are mainly due to physiological factors (Berkley,1997). Supporting this notion, Tall and Crisp (2004) showed that female rats in proestrus, a stage in the rodent estrous cycle marked with high levels of estrogen, nociceptive responses decreased in the Cg model of inflammation. On the other hand, researchers have found that rats are more sensitive to electric foot shocks during the proestrus and estrus stages than in diestrus, indicating that high levels of estrogen can make animals more sensitive to pain (Kayser, et al., 1996). Through the use of exogenous estrogens, various researchers have found that hormone replacement can cause either an increase in rates of inflammatory disorders in humans (Ceccarelli, et al., 2003b) or a decrease in nociceptive behaviors in rats (Kuba, et al., 2006; Mannino, et al., 2006; Gaumond, Arsenault, & Marchand, 2005). Alternatively, Calippe and colleagues (2010) show that chronic estradiol administration causes significant increases in the expression of pro-inflammatory mediators, including IL-1β, IL-6, and TNF-α. Taken together, these findings indicate that estrogen plays an important, but complicated, role in the inflammatory process. Previous studies show that there are strain differences in mice in regards to morphine tolerance (Kest, et al., 2002), and in behavioral responses to thermal, chemical, and neuropathic pain assays (Mogil, et al., 1999). Taking this into consideration, in this experiment, C57BL/6N and 129S6 mice were used in order to establish this model in mouse strains suitable

for future genetic studies. Hormone replacement occurs over 7 days, and we hypothesize that exogenous estrogen will cause a dose-dependent response in inflammatory behaviors such that estradiol will cause a decrease in nociceptive responses. Additionally, we expect that any change in behavior will be accompanied by a corresponding change in the level of edema observed in the injected paw.

3.2. Results

Effects of estradiol administration on Cg-induced nociceptive behavior and paw size in C57BL/6N OVX mice.

Effect of estradiol administration on baseline PWL. Figure 3.1 shows PWL for estradiol- and vehicle-treated OVX mice at low, medium, and high heat intensities in both paws prior to Cg administration. Main effects for hormone treatment and heat intensity were observed [Hormone Treatment: $F(4,51)=5.85$, $p<0.001$; Heat Intensity: $F(2,102)=63.09$, $p<0.001$]. PWL was significantly shorter in the medium ($M=7.15$) and high ($M=5.21$) heat intensities compared to the lowest heat intensity ($M=11.92$; $p<0.001$). Further, the highest heat intensity also showed significantly shorter PWL compared to PWL at the medium heat intensity ($p<0.01$; Figure 3.1A). In addition, the group that received the vehicle treatment ($M=9.1$) showed significantly longer PWL compared to the 30% ($M=6.63$) and 40% ($M=6.37$) estradiol-treated groups ($p<0.05$). Further, treatment with 20% estradiol produced significantly longer PWL ($M=10.25$) compared with all other concentrations of estradiol-treated groups ($p<0.05$; Figure 3.1B).

Effects of estradiol administration on ipsilateral PWL at baseline, 1 and 5 hours after Cg administration. Figures 3.2 shows PWL for both estradiol- and vehicle-treated C57BL/6N

OVX mice at low (Figure 3.2A), medium (Figure 3.2C), and high (Figure 3.2E) heat intensities prior to, 1 hour and 5 hours after Cg administration in the ipsilateral (injected) paw. Figure F also indicates PWL for each hormone treatment group over time (Figure 3.2B, 3.2D, & 3.2F)

Low Heat Intensity. At the lowest heat intensity (Figures 3.2A and 3.2B), significant main effects for hormone treatment and time were observed [Hormone Treatment: $F(4,47)=5.05$, $p<0.01$; Time: $F(2, 94)=25.04$, $p<0.001$]. Specifically, the 10% ($M=6.5$) and 40% ($M=5.8$) estradiol treatments showed significantly decreased PWL compared to vehicle- and 20%-treated groups ($M=10.4$ and 10.8, respectively; $p<0.05$). Furthermore, 30% estradiol treatment ($M=7.5$) produced significantly shorter PWL when compared to the 20% estradiol-treated group ($p<0.01$; Figure 3.2B). In addition, PWL was significantly shorter both 1 and 5 hours after Cg injection ($M=8.1$ and 4.8, respectively) compared to baseline ($M=12.2$; $p<0.001$); PWL was also significantly shorter 5 hours post-Cg administration compared to 1 hour post-injection ($p<0.01$; Figure 3.2A).

Medium Heat Intensity. At the medium heat intensity (Figure 3.2C and 3.2D), main effects for both hormone treatment and time were observed [Hormone Treatment: $F(4,44)=5.9$, $p<0.001$; Time: $F(2,88)=26.05$, $p<0.001$]; PWL was significantly shorter in the 20% ($M=3.9$), 30% ($M=3.5$), and 40% ($M=3.2$) estradiol-treatment groups compared with the vehicle- and 10% estradiol-treated groups ($M=5.5$ and 5.2, respectively; $p<0.05$; Figure 3.2D). Further, PWL was significantly shorter at both 1 hour ($M=3.3$) and 5 hours ($M=2.9$) after Cg administration compared to baseline readings ($M=6.8$; $p<0.001$; Figure 3.2C).

High Heat Intensity. At the highest heat intensity tested (Figure 3.2E and 3.2F), significant main effects for hormone treatment and time as were obtained [Hormone main effect: $F(4,49)=4.07$, $p<0.01$; Time main effect: $F(2,98)=29.61$, $p<0.001$]. PWL was significantly

shorter in the 30% (M=2.6) and 40% (M= 2.8) estradiol-treated groups compared to the vehicle-treated (M=4.1), 10% (M=3.8), and 20% (M=4.0) estradiol-treated groups ($p<0.05$; Figure 3.2F). Further, post-injection PWL was significantly shorter (1 Hour: M=2.8; 5 Hours: M=2.4) compared to PWL prior to Cg injection (M=5.2; $p<0.001$; Figure 3.2E).

A significant hormone by time interaction was also observed [$F(8,98)=2.25$, $p<0.05$]--significant differences in PWL in hormone treatment groups prior to Cg injection [$F(4,51)=2.76$, $p<0.05$] and 5 hours after Cg injection [$F(4,52)=2.84$, $p<0.05$] were obtained. Baseline PWL in the 30% estradiol-treated group (M=3.53) was significantly shorter compared to both the vehicle (M=6.65) and 20% groups (M=6.54; $p<0.05$). In addition, 5 hours after injection of Cg, PWL in both the 30% (M=1.72) and 40% (M=1.92) estradiol-treated groups were significantly shorter compared to the vehicle group (M=3.20; $p<0.05$). Additionally, the 30% estradiol-treated group showed significantly shorter PWL compared to the 10% estradiol-treated group (M=2.81; $p<0.05$; Figure 3.2E).

Effects of 17β-estradiol on contralateral PWL at baseline, 1 and 5 hours after Cg-administration. Figure 3.3 shows PWL for both estradiol- and vehicle-treated C57BL/6N OVX mice at low (Figure 3.3A), medium (Figure 3.3C), and high (Figure 3.3E) heat intensities prior to, 1 hour and 5 hours after Cg administration in the contralateral (uninjected) paw. This figure also shows average PWL for each hormone treatment group over time (Figures 3.3B, 3.3D, and 3.3F).

Low Heat Intensity. Figures 3.3A and 3.3B show contralateral PWL for estradiol- and vehicle-treated OVX mice at the lowest heat intensity tested prior to and 1 and 5 hours after Cg administration. A significant main effect for hormone treatment was observed [$F(4,51)=4.21$,

$p<0.01$]. At this heat intensity, both 30% (M=8.24) and 40% (M=7.83) estradiol-treatment groups had significantly shorter PWL compared to the vehicle-treated (M=11.75) and 20% estradiol-treated (M=13.28) groups ($p<0.05$; Figure 3.3B).

Medium Heat Intensity. Figure 3.3C and 3.3D show contralateral PWL in estradiol- and vehicle-treated groups at medium heat intensity prior to, 1 hour and 5 hours post-Cg injection. A significant main effect for hormone treatment was seen [$F(4,45)=13.67$]; all estradiol-treated groups had significantly shorter PWL (10%: M=5.97; 20%: M= 7.18; 30%: M= 4.66; 40%: M=5.14) compared to the vehicle-treated group (M=9.71; $p<0.01$). Further, the 30% and 40% estradiol-treated groups showed significantly shorter PWL compared to the 20% group ($p<0.05$; Figure 3.3D).

High Heat Intensity. Figure 3.3E and 3.3F show contralateral PWL in vehicle- and estradiol-treated groups at the highest heat intensity prior to, 1 hour and 5 hours after Cg administration. A significant main effect for hormone treatment and a significant interaction of hormone and time were observed [Hormone Treatment: $F(4,48)=4.72$, $p<0.01$; Interaction: $F(8,96)=3.63$, $p<0.001$]. Specifically, 30% (M=3.56) and 40% (M=3.92) estradiol-treated groups had significantly shorter PWL compared to the vehicle-treated (M=5.52) or 10% estradiol-treated (M=5.73) groups ($p<0.05$; Figure 3.3F). In addition, at both 1 and 5 hours after Cg injection, significant differences were seen between hormone treatments [1 Hour: $F(4,51)=5.87$, $p<0.001$; 5 Hours: $F(4,52)=4.94$, $p<0.01$]. One hour after Cg administration, PWL after 30% (M=2.66) and 40% (M=3.33) hormone treatment were significantly shorter compared to vehicle (M=5.26), 10% (M=6.33), and 20% (M=5.41) estradiol-treatment ($p<0.05$). Five hours after Cg injection, all estradiol-treated groups (10%: M=4.70; 20%: M=3.98; 30%: 3.68;

40%: 3.72) displayed significantly shorter PWL compared to the vehicle-treated group (M=7.88; p<0,01; Figure 3.3E).

Effects of 17β-estradiol on PWL across all heat intensities after 1% Cg administration.

Ipsilateral Paw. Figure 3.4A and 3.4B show ipsilateral PWL to low, medium, and high heat intensities in vehicle- and estradiol-treated C57BL/6N OVX mice 1 and 5 hours after Cg administration. One hour after Cg injection, a significant main effect for heat intensity was seen [F(2,94)=30.19, p<0.001]--PWL was significantly shorter in response to medium (M=3.32) and high (M=2.91) heat intensities compared to the lowest heat intensity (M=7.83; p<0.001; Figure 3.4A).

Five hours after Cg administration significant main effects for both hormone treatment and heat intensity were observed [Hormone Treatment: F(4,44)=4.06, p<0.01; Heat Intensity: F(2,88)=12.06, p<0.001]. Specifically, the 30% (M=2.18) and 40% (M=2.22) estradiol-treated groups displayed significantly shorter PWL compared to both the vehicle-treated (M=4.79) and 10% estradiol-treated (M=3.78) groups (p<0.05). In addition, PWL was significantly shorter at medium (M=3.07) and high (M=2.35) heat intensities compared to the lowest heat intensity (M=4.59; p<0.01).

Additionally, a significant interaction between hormone treatment and heat intensity was seen [F(8,88)=2.80, p<0.01]. This significant interaction was fueled by diverging patterns of differences between the hormone-treated groups at each heat intensity. A significant difference between groups was seen at all heat intensities [Low: F(4,49)=3.80, p<0.01; Medium: F(4,49)=5.27, p<0.01; High: F(4,52)=2.84, p<0.05]. At low heat intensity, all estradiol-treated groups (10%: M=3.64; 20%: M=5.01; 30%: 3.01; 40%: 3.20) showed significantly shorter PWL compared to the cholesterol-treated group (M=8.09; p<0.05). In response to medium heat, PWL

in the 10% estradiol group (M=5.17) was significantly longer compared to the vehicle group (M=3.10; $p<0.05$). Further, 20% (M=2.86), 30% (M=1.93), and 40% (M=1.86) estradiol treatments significantly shortened PWL compared to the 10% treatment group ($p<0.01$), but not the vehicle group. At the highest heat intensity, 30% estradiol treatment (M=1.72) showed significantly shorter PWL compared to vehicle (M=3.20) or 10% estradiol treatment (M=2.81; $p<0.05$). The 40% estradiol-treated group (M=1.92) also showed significantly shorter PWL compared to the vehicle-treated group ($p<0.05$; Figure 3.4B).

Contralateral Paw. Figure 3.4C and 3.4D show contralateral PWL to low, medium, and high heat intensities in vehicle- and estradiol-treated C57BL/6N OVX mice 1 and 5 hours after Cg administration. One hour post Cg injection, significant main effects for both hormone treatment and heat intensity were seen [Hormone Treatment: $F(4,48)=3.92$, $p<0.01$; Heat Intensity: $F(2,96)=31.51$, $p<0.001$]. PWL in the 30% (M=4.66) and 40% (M=5.59) estradiol-treated groups was significantly shorter compared to the vehicle-treated (M=8.38) and the 10% (M=7.91) and 20% (M=7.87) estradiol-treated groups ($p<0.05$). Further, PWL was significantly shorter at the medium (M=6.04) and high (M=4.65) heat intensities compared with the lowest heat intensity tested (M=10.20; $p<0.001$).

In addition, a significant interaction between hormone and heat intensity was seen [$F(8,96)=2.33$, $p<0.05$]. The significant interaction at this time point can be taken into account by the differences in hormone-treated groups at each heat intensity. All heat intensities showed a significant difference in hormone groups [Low: $F(4,52)=2.82$, $p<00.05$; Medium: $F(4,50)=3.29$, $p<0.05$; High: $F(4, 51)=5.87$, $p<0.001$]. In response to low heat, PWL was significantly shorter in the 30% estradiol-treated group (M=5.98) compared with the vehicle-treated (M=12.26) and 20% estradiol-treated (M=13.75) groups ($p<0.05$). At medium heat, the vehicle group (M=7.60)

showed significantly longer PWL compared to the 20% (M=4.92) and 40% (M=4.42) estradiol-treated groups ($p<0.05$). Further, PWL in the 10% group (M=8.32) was significantly longer compared to the 20%, 30% (M=5.37), and 40% estradiol groups ($p<0.05$). PWL in response to the highest heat intensity was significantly shorter in the 30% (M=2.66) and 40% (M=3.33) groups compared to all other hormone groups (Vehicle: M=5.26; 10%: M=6.33; 20%: M=5.41; $p<0.05$; Figure 3.4C).

Behavior taken five hours after Cg treatment also showed significant main effects for heat intensity and hormone treatment [Heat Intensity: $F(2,98)=12.34$, $p<0.001$; Hormone Treatment: $F(4,49)=6.3$, $p<0.01$]. Specifically, between all heat intensities, all groups were significantly different from each other; PWL at the lowest heat intensity (M=9.01) was significantly longer compared to the medium (M=6.89) and high (M=4.8) heat intensities ($p<0.05$), and PWL at the medium heat intensity was significantly longer compared to the highest heat intensity ($p<0.05$). PWL was also significantly longer in the vehicle-treated group (M=10.05) compared to the 10% (M=6.32), 20% (M=7.25), 30% (M=5), or 40% (M=5.41) estradiol-treated groups ($p<0.05$; Figure 3.4D).

Effect of estradiol on paw size in C57BL/6N OVX mice. Figure 3.5 shows mean paw size in C57BL/6N OVX mice 5 hours after Cg administration. Pretreatment with estradiol did not significantly affect paw size. However, a significant main effect for injection site was observed [$F(1,35)=213.93$, $p<0.001$] such that ipsilateral paw size (M=12.46) was larger compared to contralateral paw size (M=6.47) across hormone treatment groups.

Effects of estradiol administration on Cg-induced nociceptive behavior and paw size in 129S6 OVX mice

Effects of 17β-estradiol administration on baseline PWL. Figure 3.6 shows PWL for estradiol- and vehicle-treated OVX mice at low, medium, and high heat intensities prior to Cg administration. A significant main effect for heat intensity was observed [$F(2,64)=59.32$, $p<0.001$]; PWL was significantly shorter in response to high ($M=3.28$) and medium ($M=4.41$) heat intensities when compared to the lowest heat intensity ($M=8.23$; $p<0.001$). Further, PWL at the highest heat intensity was also significantly shorter compared to PWL at medium heat intensity ($p<0.05$; Figure 3.6).

Effects of 17β-estradiol on ipsilateral PWL at baseline, 1 and 5 hours after Cg-administration.

Low Heat Intensity. Figure 3.7A shows ipsilateral PWL in response to the low heat stimulus at baseline, 1 and 5 hours post-injection in vehicle- and estradiol-treated 129S6 OVX mice. A significant main effect for time was obtained [$F(2,58)=16.84$, $p<0.001$]; PWL was significantly shorter 1 hour ($M=5.29$) and 5 hours ($M=3.94$) post-injection compared to baseline readings ($M=7.39$, $p<0.001$). In addition, PWL at 5 hours post-injection was significantly shorter compared to 1-hour PWL ($p<0.05$; Figure 3.7A).

Medium Heat Intensity. Figure 3.7B shows ipsilateral PWL in response to the medium heat stimulus at baseline, 1 and 5 hours post Cg administration. A significant main effect for time was observed [$F(2,62)=20.05$, $p<0.001$]. PWL was significantly shorter 1 hour ($M=3.53$) and 5 hours ($M=2.88$) post-injection compared to PWL prior to Cg administration ($M=4.78$; $p<0.001$; Figure 3.7B).

High Heat Intensity. Figure 3.7C shows ipsilateral PWL in response to a high heat stimulus at baseline, 1 and 5 hours after Cg administration. A significant main effect for time was observed [Time: ($F(2,66)=11.92$, $p<0.001$]. PWL 5 hours post-injection ($M=2.52$) was significantly shorter compared to both baseline ($M=3.38$) and 1-hour ($M=2.94$) PWL ($p<0.01$). Further, PWL was significantly shorter at 5 hours compared to 1 hour post-injection ($p<0.05$). A significant interaction between time and and hormone treatment was observed [$F(8,64)=2.67$, $p<0.05$]. The significant time by hormone interaction is explained by the significant differences in treatment groups 1 hour post-Cg injection [$F(4,33)=3.08$, $p<0.05$]. Specifically, PWL in the 30% estradiol-treated group ($M=3.63$) was significantly longer compared to the vehicle-treated ($M=2.56$), 10% ($M=2.58$) and 40% ($M=2.67$) estradiol-treated groups ($p<0.05$; Figure 3.7C).

Effects of 17β-estradiol on contralateral PWL at baseline, 1 hour and 5 hours after Cg-administration. Figure 3.8 shows PWL for estradiol- and vehicle-treated OVX mice at low (Figure 3.8A), medium (Figure 3.8B), and high heat (Figure 3.8C) intensities prior to, 1 and 5 hours after Cg administration in the contralateral (uninjected) paw.

Low Heat Intensity. Figure 3.8A shows contralateral PWL in vehicle- and estradiol-treated OVX 129S6 mice in response to the lowest heat intensity. Although no significant main effects for time or hormone treatment were seen, a significant interaction between estrogen and time was obtained [$F(8,60)=2.25$, $p<0.05$]. This interaction can be taken into account by significant differences in treatment groups at the 1-hour time point [$F(4,35)=2.72$, $p<0.05$]. Specifically, PWL in the vehicle group ($M=11.34$) was significantly longer compared to both the 20% ($M=6.33$) and 40% ($M=6.12$) estradiol-treated groups ($p<0.05$; Figure 3.8A).

Medium Heat Intensity. Figure 3.8B displayed contralateral PWL in vehicle- and estradiol-treated OVX 129S6 mice in response to medium heat intensity. Data did not show any significant main effects and no significant hormone by time interaction.

High Heat Intensity. Figure 3.8C shows contralateral PWL in vehicle- and estradiol-treated OVX 129S6 mice in response to the highest heat intensity tested. A significant main effect for time and a significant time by hormone interaction was observed [Time main effect: $F(2,62)=9.36$, $p<0.001$; Interaction: $F(8, 62)=2.22$, $p<0.05$]. Specifically, PWL was significantly shorter prior to Cg administration (M=2.98) compared to 1 and 5 hours post-injection (M=3.61 and 3.62, respectively; $p<0.001$). The significant interaction can be accounted for by significant differences among hormone-treated groups after Cg administration [1 Hour: $F(4,32)=3.67$, $p<0.05$; 5 Hours: $F(4,34)=3.66$, $p<0.05$]. One hour post-injection, PWL was significantly longer in the 30% treatment group (M=4.33) compared to the vehicle group (M=3.21; $p<0.01$). Further, PWL in the 40% estradiol-treated group (M=2.98) was significantly shorter compared to all other estradiol-treated groups (10%: M=3.99; 20%: M=3.88; 30%: 4.33; $p<0.05$). Five hours after Cg injection, PWL in the 40% group (M=2.81) was significantly shorter compared to all other groups (Vehicle: M=3.88; 10%: M=3.53; 20%: M=4.02; 30%: 3.57; $p<0.05$).

Effect of 17β-estradiol on PWL over heat intensities after 1% Cg administration

Ipsilateral Paw. Figure 3.9A and 3.9B show ipsilateral PWL to low, medium, and high heat intensities in vehicle- and estradiol-treated 129S6 OVX mice 1 hour and 5 hours after Cg administration. In this paw, a significant main effect for heat intensity was seen at 1 and 5 hours after Cg treatment [1Hour: $F(2,62)=17.89$, $p<0.001$; 5 Hours: $F(2,62)=21.89$, $p<0.001$]. Specifically, PWL in response to the lowest heat intensity was significantly longer compared to

the medium and high heat intensities at both one hour (Low: M=5.23; Medium: M=3.66; High: M=2.96; Figure 3.9A) and five hours (Low: M=4.03; Medium: M=2.87; High: M=2.48; Figure 3.9B) after injection ($p<0.001$). Further, a significant interaction of hormone treatment and heat intensity was seen 5 hours after Cg treatment [$F(8, 62)=2.69$, $p<0.05$]. Post hoc tests showed no significant difference between treatment groups at each time point (Figure 3.9B).

Contralateral Paw. Figures 3.9C and 3.9D show contralateral PWL to low, medium, and high heat intensities in vehicle- and estradiol-treated mice 1 hour and 5 hours after Cg administration. At 1 hour post-Cg injection, significant main effects of heat intensity and hormone treatment were seen [Heat Intensity: $F(2,62)=40.02$, $p<0.001$; Hormone Treatment: $F(4,31)=3.27$, $p<0.05$]. A significant interaction between hormone and heat intensity was also observed [$F(8,62)=2.37$, $p<0.05$]. At the lowest heat intensity (M=8.62) was significantly longer compared with the medium (M=4.72) and high (M=3.69) heat intensities ($p<0.001$). PWL was significantly shorter in the 40% estradiol-treated group (M=4.01) compared to the vehicle-treated (M=6.46), 10% (M=6.25), and 30% (M=6.38) estradiol-treated groups ($p<0.05$). Post-hoc analyses of the significant interaction show that were significant differences between hormone treatment groups in response to the lowest and highest heat intensities [Low Heat: $F(4,35)=2.72$, $p<0.05$; High Heat: $F(4,34)=3.67$, $p<0.05$]. Specifically, at the lowest heat intensity, PWL in the vehicle group (M=11.34) was significantly longer compared to the 20% (M=6.33) and 40% (M=6.12) groups ($p<0.05$). In response to the highest heat intensity, PWL in the 30% group (M=4.33) was significantly longer compared to the vehicle group (M=3.21; $p<0.01$). In addition, PWL in the 40% estradiol-treated group (M=2.98) was significantly shorter compared to all other estradiol-treated groups (10%: M=3.99; 20%: M=3.88; 30%: 4.33; $p<0.05$; Figure 3.8C).

Data also show that in the contralateral paw, 5 hours after Cg administration, significant main effects for both heat intensity and hormone treatment were observed [Heat Intensity: $F(2,62)=23.15$, $p<0.001$; Hormone Treatment: $F(4,31)=2.93$, $p<0.05$]. Specifically, at the lowest heat intensity, PWL was significantly longer (M=6.94) compared to both the medium (M=4.5) and high (M=3.51) heat intensities ($p<0.001$). Also, PWL in the 40% estradiol-treated group (M=3.65) was significantly shorter compared to PWL in the vehicle (M=6.1) and 10% estradiol-treated (M=5.52) groups ($p<0.05$; Figure 3.9D).

Effect of 17β-estradiol on paw size in 129S6 OVX mice. Figure 3.10 shows mean paw size 5 hours after Cg administration. A main effect of injection site was observed [$F(1,35)=213.93$, $p<0.001$] such that ipsilateral paw size (M=12.46) was significantly larger compared to contralateral paw size (M=6.47) across all treatment groups ($p<0.001$).

3.3. Discussion

These experiments have compared the effects of low (10 and 20%) and high (30 and 40%) doses of estradiol on nociceptive responses in C57BL/6N and 129S6 OVX mice. Our results show that unlike in rats, in C57BL/6N mice high doses of estradiol increase baseline inflammatory responses, as well as increases responses in both the treated and untreated paw after insult with Cg. Additionally, administration of exogenous estrogen interacts with time and heat intensity in both the injected and uninjected paws. Estradiol had no significant effect on paw edema. Furthermore, estradiol did not have strong effects in 129S6 mice—effects were only seen after Cg injection in the uninjected paw. These findings suggest that in mice, estradiol increases sensitivity to pain, consistent with physiological results in studies conducted by

Calippe and colleagues (2008). Other findings support the notion that there are fundamental differences between mouse and rat species. Researchers show that there are species differences in drug metabolism (Kramer & Testa, 2008), learning and memory (Jonasson, 2005), and in copulatory behavior (Hull & Dominguez, 2009). Our results indicate that estradiol's influence on inflammatory responses also differ between rats and mice.

Our findings indicate that prior to inflammatory insult, estrogen affects pain sensitivity. Pretreatment with high doses of exogenous estrogen caused an increase in nociceptive behaviors. This supports the notion that hormone replacement therapy in humans results in an increase in pain sensitivity and an increase in inflammatory syndromes (Ceccarelli, et al., 2003b; Warren & Fried, 2001).

Additionally, estradiol was shown to have a sensitizing effect in both ipsilateral and contralateral paws. In both paws, high doses of estradiol caused an increase in nociceptive behaviors in response to all heat intensities. Additionally, in response to the high intensity stimulus, estradiol had an effect 5 hours, but not 1 hour, after Cg administration. Because effects were seen on the contralateral side to Cg treatment, estradiol's effects on inflammatory behaviors seem to be happening systemically. Estradiol seems to be causing an increase in inflammatory mediators at the site of injury, causing a cascade of inflammatory responses in the primary sensory neuron and subsequently in the dorsal horn of the spinal cord, leading to the phenomenon of "windup" and subsequent central sensitization. Windup causes changes in neuronal excitability, which allows the cells in the dorsal horn to respond to low-intensity stimulation (Woolf & Costigan, 1999). Sensitization of the spinal cord could cause an increase in behavioral responses to the alternate side of the body as well.

Post-Cg administration, estradiol increased nociceptive behaviors in all but one case. In the treated paw, in response to medium heat, the 10% dose of estradiol caused a decrease in

nociceptive behaviors 5 hours post-treatment. This supports the literature that shows that lower doses of estradiol, usually 20%, attenuates behavioral responses to inflammation (Kuba, et al., 2006; Kuba, Kemen, & Quinones-Jeanb, 2005). It is possible that in a murine model, low doses of estrogen may act to decrease inflammatory responses at longer periods of time while high doses cause an increase in pain responses shortly after inflammation. Experiments with longer time courses would be needed to determine if this is the case.

Exogenous estrogen did not affect the edema observed in the paw after inflammatory insult. This suggests that estrogen's sensitizing effects on nociceptive responses is not due to an increase in neutrophil migration or an increase in plasma exudation, key components in creating edema.

Finally, estrogen did not have an effect on most nociceptive behaviors in the alternative strain of mouse tested (129S6). When estrogen did have an effect, it mimicked the effects seen in the C57BL/6N strain—high doses caused a decrease in pain behaviors in the contralateral paw post-Cg administration. This indicates that estrogen's effects are strain-dependent. This strain can be utilized in the future to compare physiological differences that may affect estrogen's effectiveness at increase pain behaviors in mice.

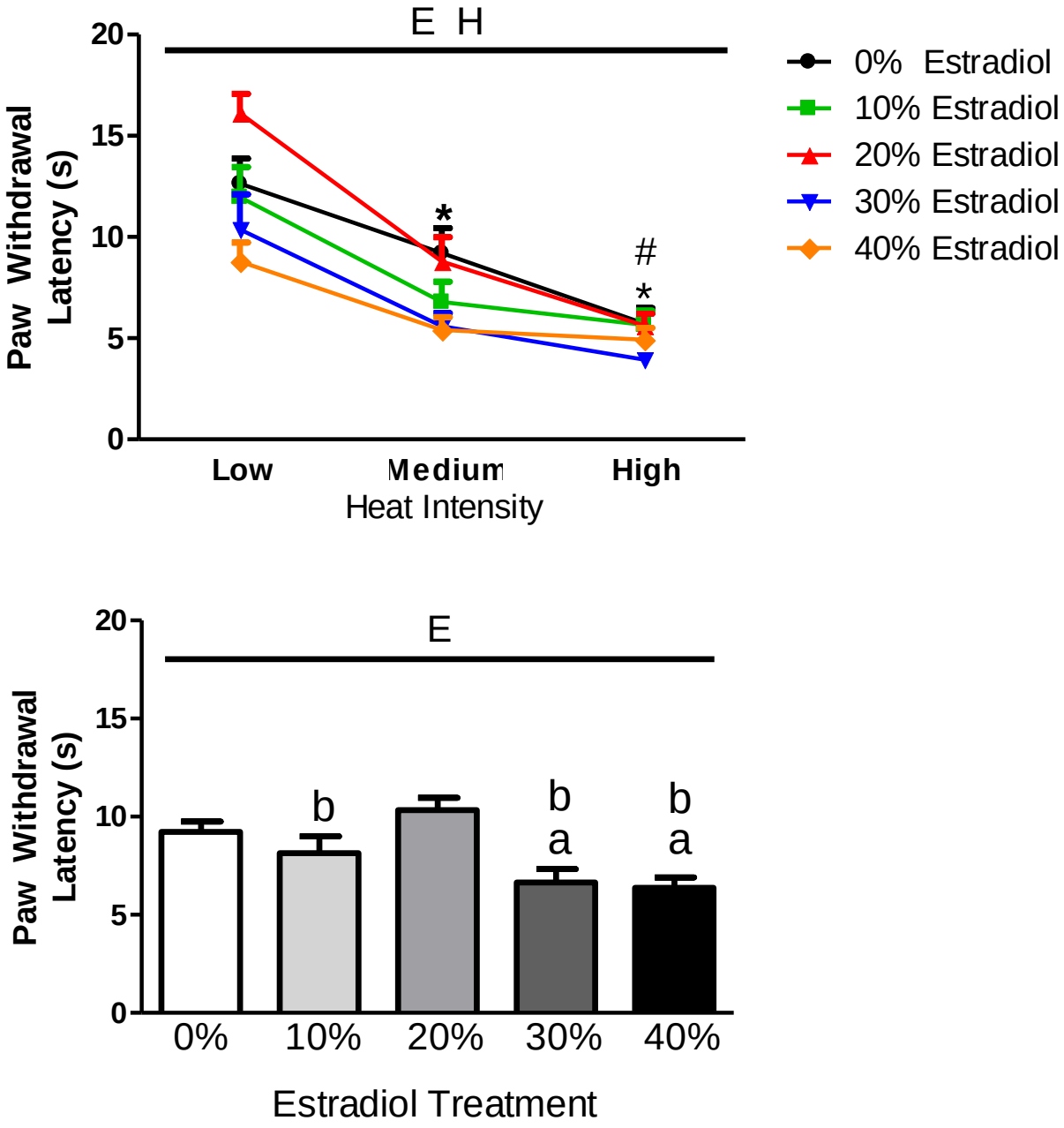

Figure 3.1. Effects of 17β-estradiol on baseline PWL at all heat intensities in C57BL/6N OVX mice. Data represents mean PWL (±SEM) in left and right paws before Cg administration at low (4.5mV), medium (4.9mV), and high (5.2mV) heat intensities. (A). PWL is also shown in all hormone treatment groups across heat intensities (B). (E) denotes a significant main effect of estradiol treatment ($p<0.001$). (H) denotes a significant main effect of heat intensity ($p<0.001$). (*) signifies a significant difference to the lowest heat intensity ($p<0.001$). (#) denotes a significant difference to the medium heat intensity ($p<0.01$). (a) denotes a significant difference to the vehicle-treated group ($p<0.05$). (b) shows a significant difference to the 20% estradiol-treated group ($p<0.05$). *n*=9-12 per group.

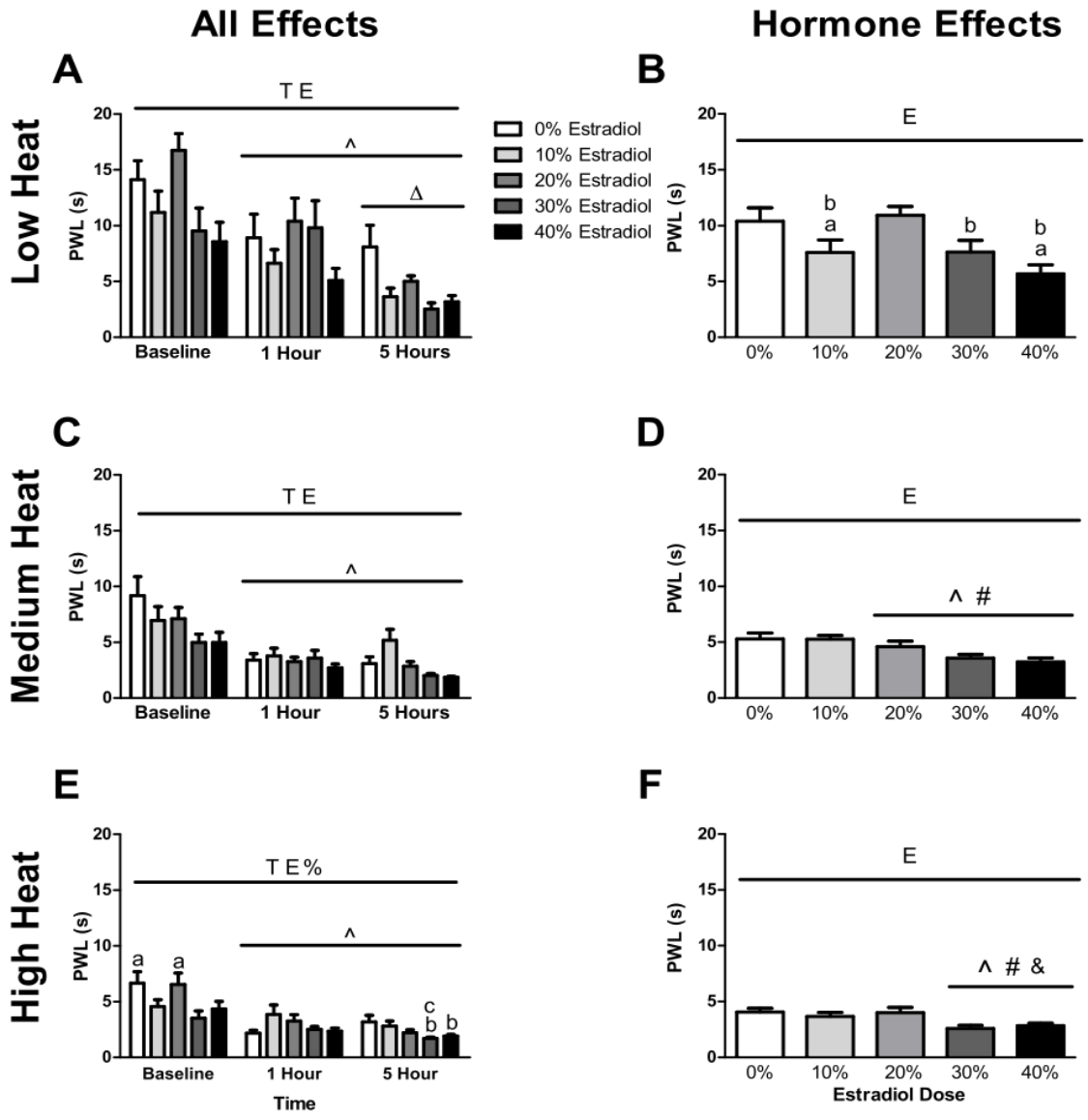

Figure 3.2. Effects of 17β-estradiol on ipsilateral PWL prior to and after Cg administration in C57BL/6N OVX mice. Data represent mean PWL (±SEM) prior to, 1 and 5 hours after Cg injection at low (A-B), medium (C-D), and high (E-F) heat intensities. Figures A, C, and E represent all effects, while Figures B, D, and F represent the significant hormone main effects. (T) represents a significant main effect of time (^) signifies a significant difference to baseline PWL (Δ) denotes a significant difference to PWL 1 hour after Cg administration. (a) denotes a significant difference to the 30% group during baseline readings ($p<0.01$). (b) denotes a significant difference to the vehicle-treated group 5 hours after Cg injection ($p<0.05$). (c) denotes a significant difference to the 10% group 5 hours after Cg injection ($p<0.05$). *n*=10-12 per group.

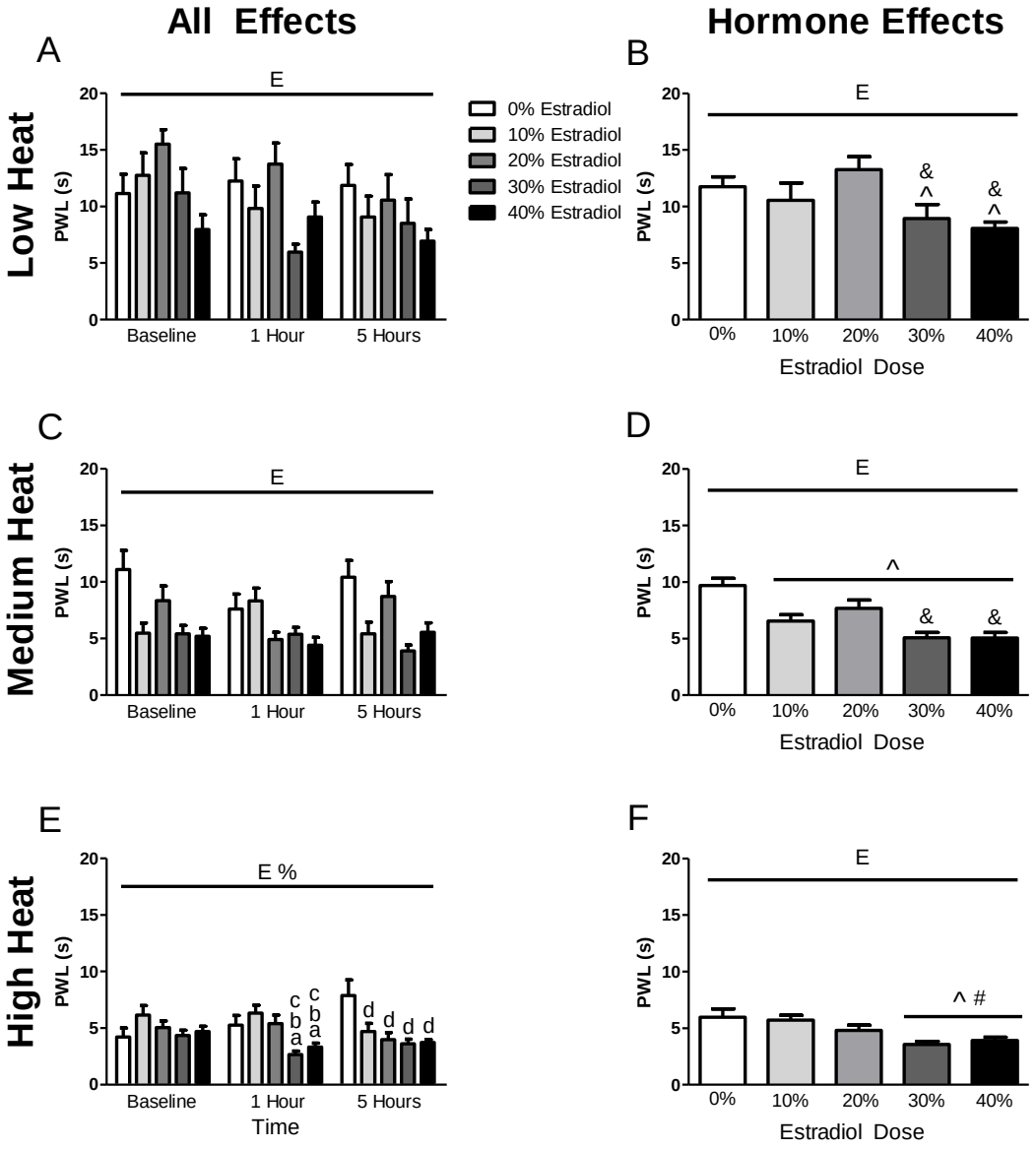

Figure 3.3. Effects of 17β-estradiol on contralateral PWL prior to, 1 and 5 hours after Cg administration in C57BL/6N OVX mice. Data represents mean contralateral PWL (± SEM) at low (A-B), medium (C-D), and high (E-F) heat intensities. Figures A, C, and E represent all effects, while Figures B, D, and F represent the significant hormone main effects. (E) represents a significant main effect of hormone treatment ($p<0.01$). (%) denotes a significant hormone by time interaction effect ($p<0.001$). (^) denotes a significant difference to the vehicle group. (#) indicates a significant difference to the 10%-treated group. (&) denotes a significant difference to the 20%-treated group. (a) indicates a significant difference to the vehicle-treated group 1 hour after Cg administration ($p<0.05$). (b) represents a significant difference to the 10% group 1 hour post-Cg injection ($p<0.05$). (c) denotes a significant difference from the 20% group 1 hour post-injection ($p<0.01$). (d) represents a significant difference to the vehicle-treated group 5 hours post-Cg administration. n=10-12 per group.

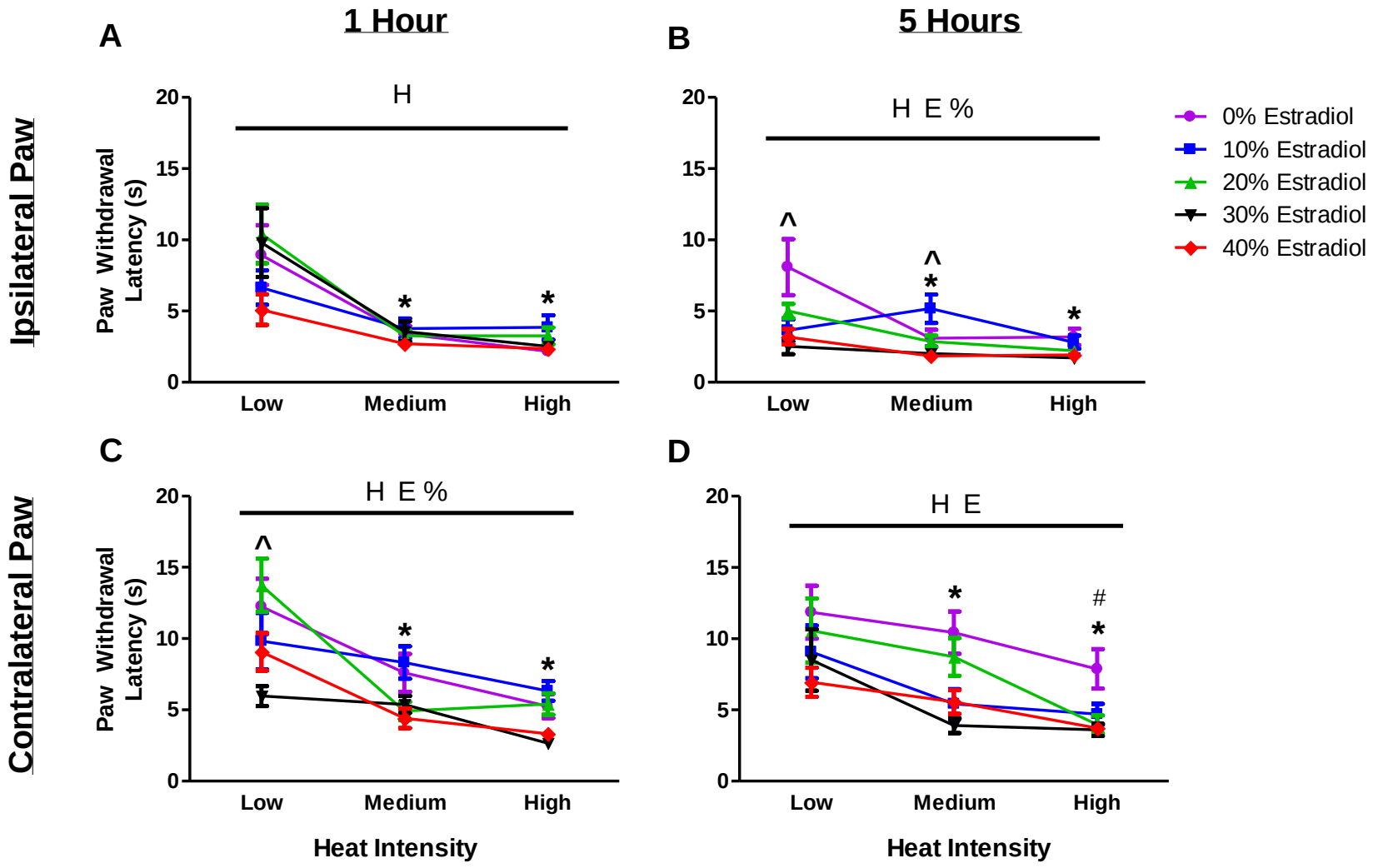

Figure 3.4. Effects of 17β-estradiol on PWL in C57BL/6N OVX mice across low, medium, and high heat intensities after Cg administration. Data represents mean ipsilateral (A-B) and contralateral (C-D) PWL (±SEM) across heat intensities 1 and 5 hours after Cg injection. (H) denotes a significant main effect for heat intensity ($p<0.001$). (E) signifies a significant main effect for hormone treatment ($p<0.01$). (%) signifies a significant hormone treatment by heat intensity interaction ($p<0.05$). (*) shows a significant difference to the lowest heat intensity ($p<0.05$). (#) signifies a significant difference to the medium heat intensity ($p<0.05$). (^) denotes significant differences between the hormone treatments at that heat intensity ($p<0.05$).

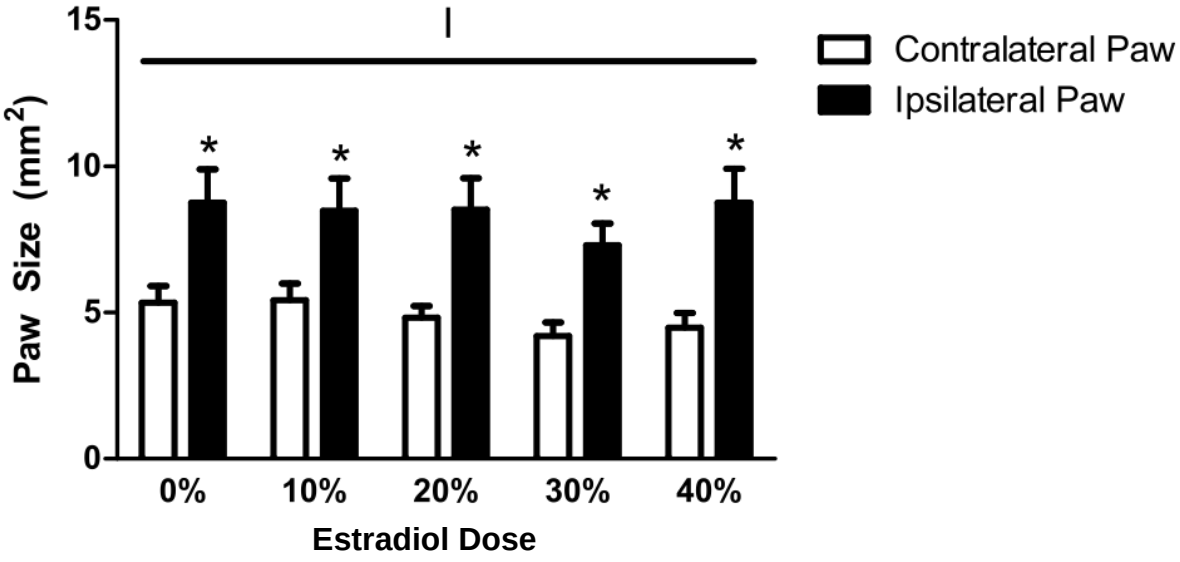

Figure 3.5. Paw size after hormone treatment and Cg administration in C57BL/6N OVX mice. Data represents mean paw size (±SEM) in vehicle- and estradiol-treated groups 5 hours after Cg injection. (I) represents a significant main effect for injection site ($p<0.001$). (*) signifies a significant difference from the contralateral paw ($p<0.001$). n=10-12 per group.

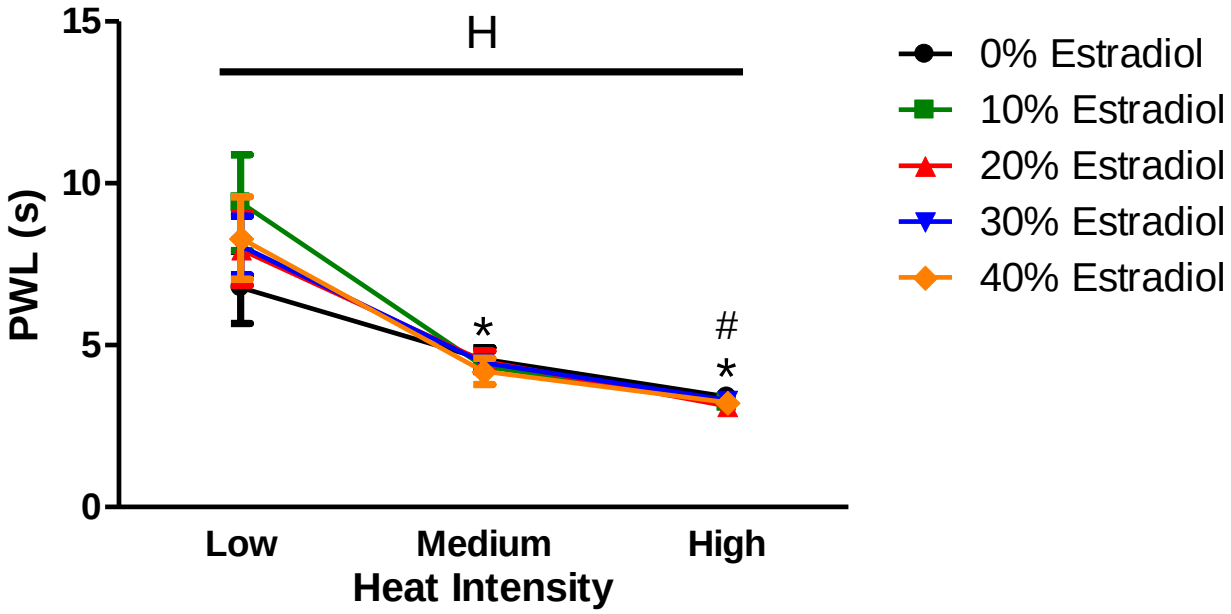

Figure 3.6. Effects of 17β-estradiol on baseline PWL in 129S6 OVX mice. Data represents mean PWL (±SEM) in ipsilateral and contralateral paws prior to Cg administration at low (4.5mV), medium (4.9mV), and high (5.2mV) heat intensities. (H) denotes a significant main effect of heat intensity ($p<0.001$). (*) represents a significant difference to the low heat intensity ($p<0.001$). (#) denotes a significant difference to the medium heat intensity ($p<0.05$). *n*=15-16 per group.

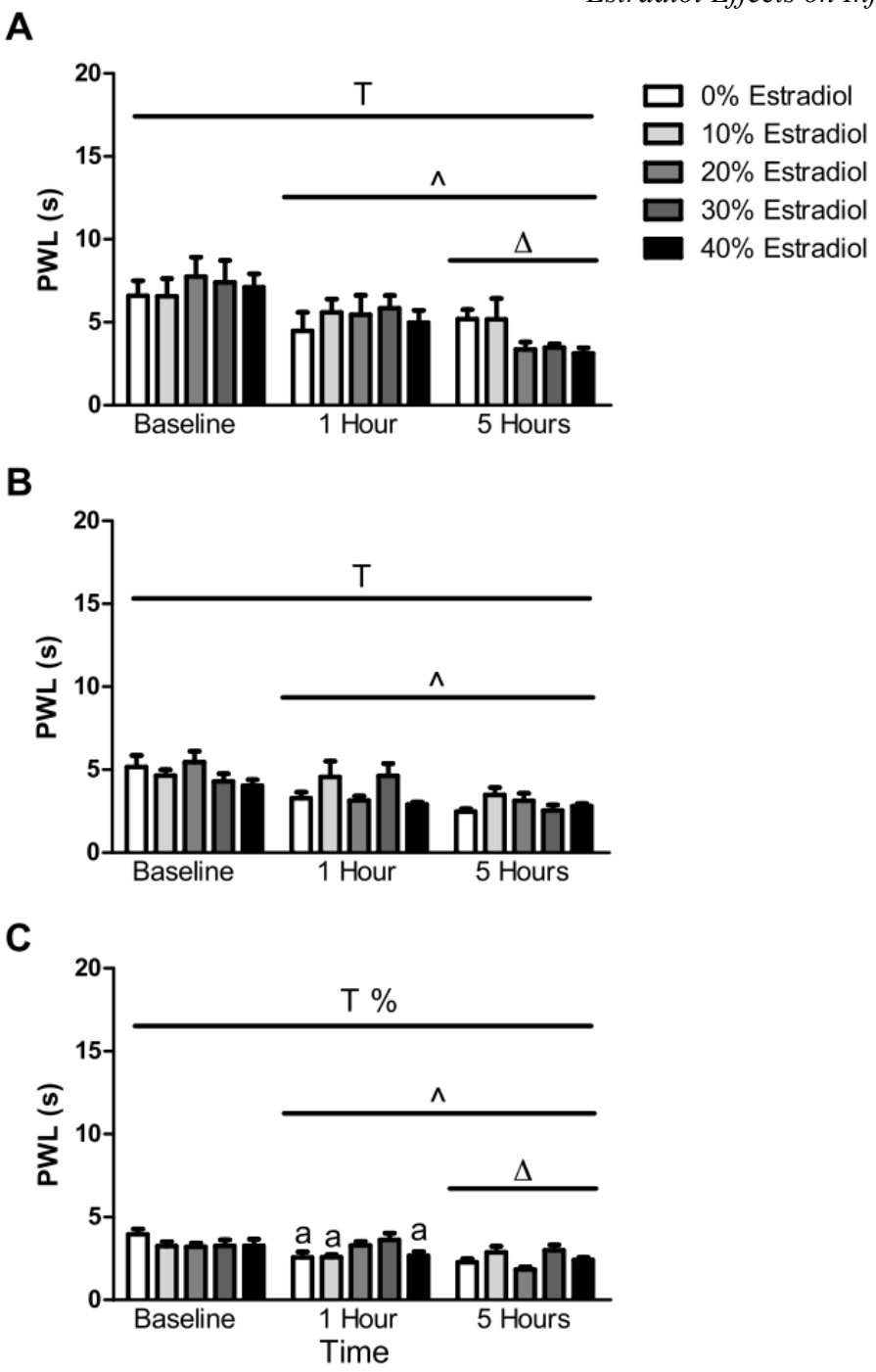

Figure 3.7. Effects of 17β-estradiol on ipsilateral PWL at baseline, 1 and 5 hours after Cg administration. Data represents mean PWL (±SEM) in response to low (A), medium (B), and high (C) heat intensities in 129S6 OVX mice. (T) represents a significant main effect for time ($p<0.001$). (%) represents a significant time by hormone interaction ($p<0.01$). (^) denotes a significant difference to baseline PWL ($p<0.01$). (Δ) signifies a significant difference to PWL at 1 hour post-injection ($p<0.05$). (a) represents a significant difference to the 30% estradiol-treated group 1 hour post-injection ($p<0.05$). n=7-8 per group.

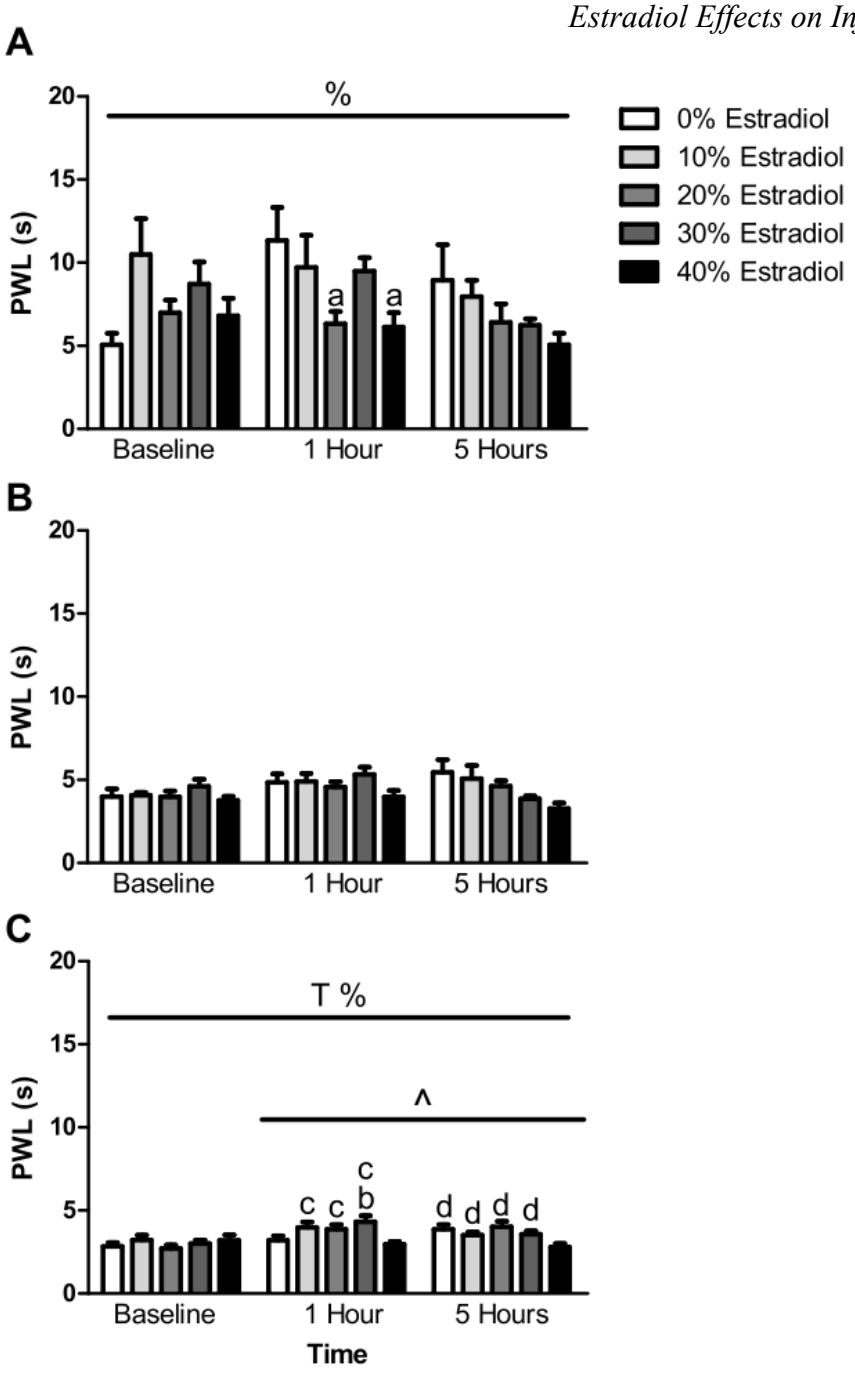

Figure 3.8. Effects of 17β-estradiol on contralateral PWL at baseline, 1 hour and 5 hours after Cg administration. Data represents mean contralateral PWL (±SEM) in response to low (A), medium (B), and high (C) heat intensities in 129S6 OVX mice at baseline, 1 and 5 hours after Cg administration. (T) represents a main effect for time ($p<0.01$). (%) represents a significant time by hormone interaction ($p<0.05$). (^) denotes a significant difference to baseline PWL ($p<0.01$). (a) denotes a significant difference to the vehicle-treated group 1 hour after Cg administration ($p<0.05$). (b) represents a significant difference to the vehicle-treated group 1 hour post-injection ($p<0.05$). (c) denotes a significant difference to the 40% group at 1 hour post-injection ($p<0.05$). (d) signifies a significant difference to the 40% group 5 hours after Cg administration ($p<0.05$). n=7-8 per group.

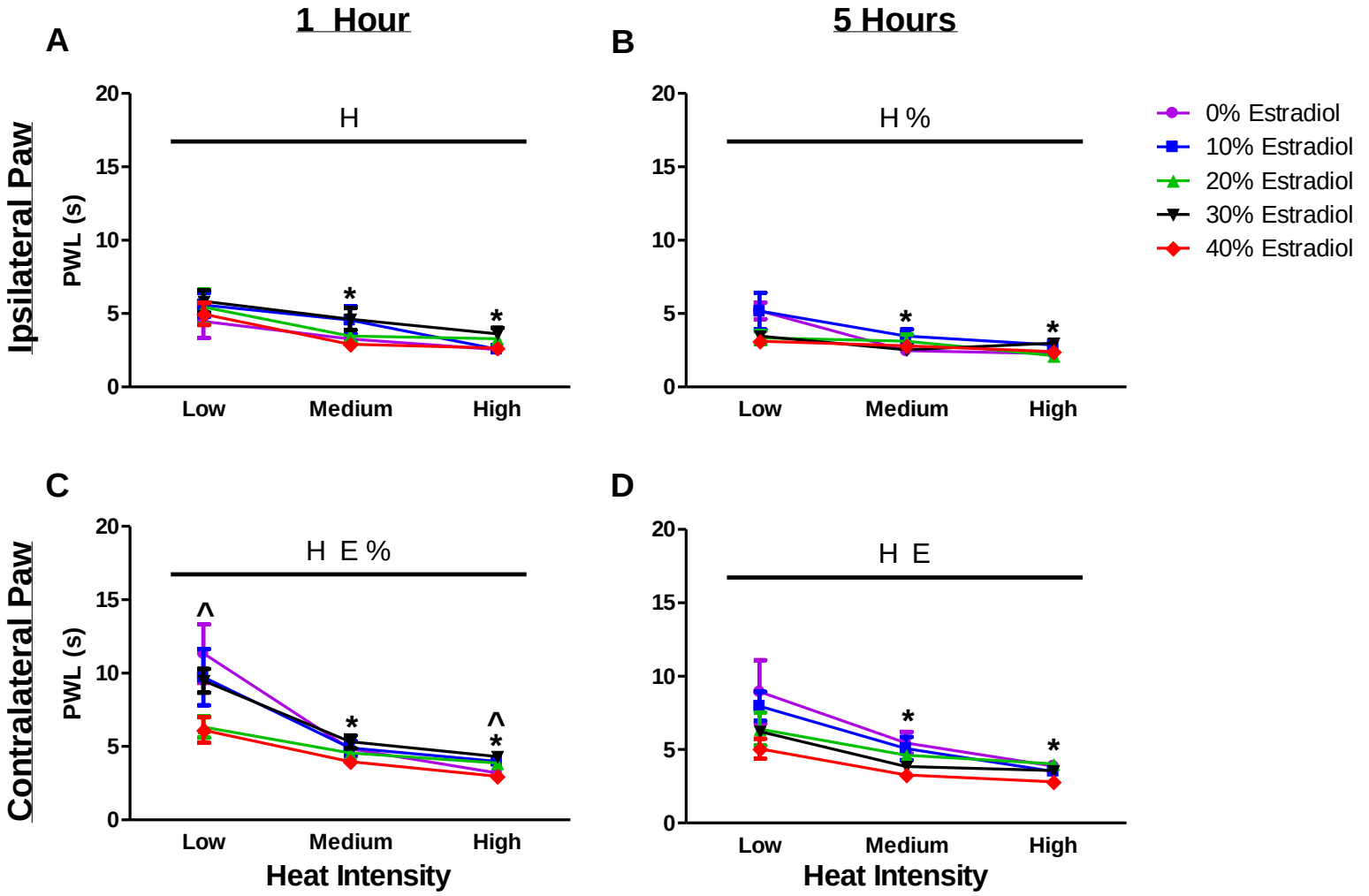

Figure 3.9. Effects of 17β-estradiol on PWL in 129S6 OVX mice across low, medium, and high heat intensities after Cg administration. Data represents mean ipsilateral (A-B) and contralateral (C-D) PWL (±SEM) across heat intensities 1 and 5 hours after Cg injection. (H) represents a significant main effect for heat intensities ($p<0.001$). (E) denotes a significant main effect for hormone treatment ($p<0.05$). (%) represents a significant hormone by heat interaction ($p<0.05$). (^) signifies significant differences in hormone treatment at the specified heat intensity ($p<0.05$). (*) denotes a significant difference to the low heat intensity ($p<0.001$).

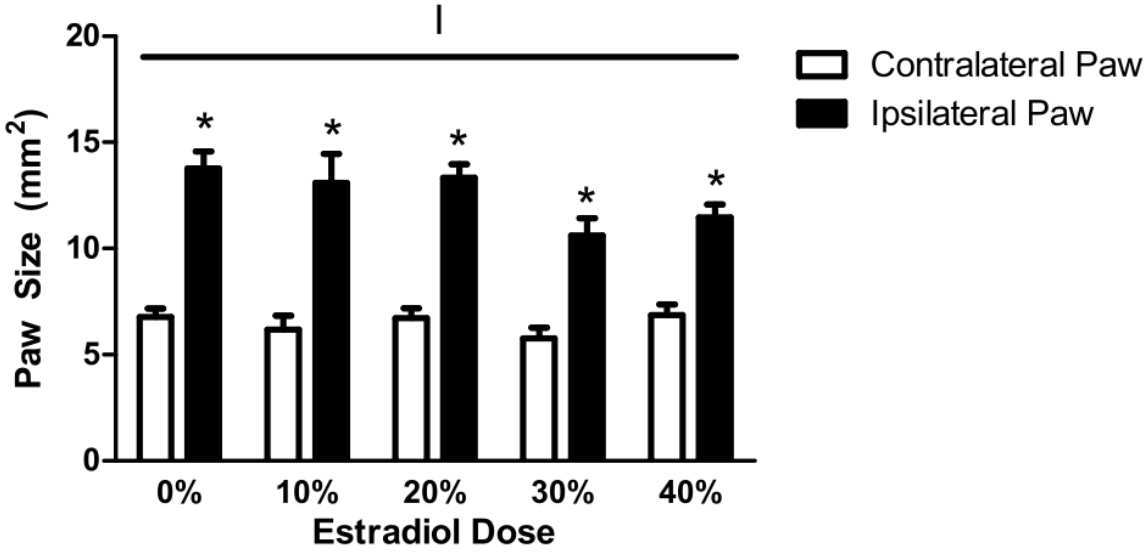

Figure 3.10. Paw size after hormone treatment and Cg administration in 129S6 OVX mice. Data represents mean paw size (±SEM) in vehicle- and estradiol-treated mice. (I) represents a significant main effect for injection site ($p<0.001$). (*) signifies a significant difference from the contralateral paw ($p<0.001$). n=8 per group.

Chapter 4: Effects of 17β-Estradiol and 1% Cg administration on cytokine expression in spinal cord, paw, and DRG tissue in C57BL/6N OVX mice.

4.1. Introduction

Cytokines, small polypeptides produced and released by a variety of cell types, have been shown to have both pro- and anti-inflammatory effects (Angst, et al., 2008). These proteins are the link between tissue injury and nociception, and they are secreted in response to inflammatory stimuli, like Cg (Verri, Jr., et al., 2006). Recent findings show that estrogen modulates cytokine release which results in a decrease of inflammation and hyperalgesia (Salem, 2004; Bradshaw & Berkley, 2002; Ito, et al., 2001). Specifically, studies suggest that estrogen may cause a decrease in TNF-α production, which consequently causes a decrease in nociception and pain behavior (Ito, et al., 2001). Because estrogen has been shown to influence cytokine production, and because cytokines are released from immune cells that are also implicated in the hyperalgesic and allodynic states associated with chronic inflammation (Watkins & Maier, 1999), this experiment was designed to address the possibility that exogenous estrogen alters cytokine production following tissue injury and inflammation. Based on our results that showed that high doses of estradiol increase inflammatory behaviors in OVX mice (see Chapter 3), we hypothesize that we will see a concordant increase in pro-inflammatory cytokine levels, decrease in anti-inflammatory cytokines, or both in animals that received high doses of estradiol.

4.2. Results

Dose-response effects of hormone treatment on cytokine expression in spinal cord, paw, and DRG tissue. To determine if estradiol alters cytokine levels in the peripheral or central nervous systems, estradiol dose-response curves were done in Cg-treated mice. No significant

hormone treatment main effect was observed for IL-2 (Figure 4.1), IL-4 (Figure 4.2), IL-10 (Figure 4.3), or IFN-γ (Figure 4.4) concentrations in any of the tissues tested. However, in contralateral paw tissue, a significant hormone main effect was seen for the following: IL-1β [$F(4,10)=4.49$, $p<0.05$; Figure 4.5], IL-5 [$F(4,10)=4.94$, $p<0.05$; Figure 4.6], TNF-α [$F(4,11)=3.96$, $p<0.05$; Figure 4.7], and GM-CSF [$F(4,10)=4.94$. $p<0.05$; Figure 4.8]. For each of these cytokines, pretreatment with 20% estradiol significantly increased cytokine concentrations compared to all hormone treatment groups ($p<0.05$).

The correlation between cytokine levels in the ipsilateral paw, ipsilateral DRG, and spinal cord and ipsilateral behavioral responses 5 hours after Cg administration. To determine if estradiol effects on PWL 5 hours after Cg administration are mediated by regulation of cytokine levels, in the PNS and CNS PWL in the ipsilateral paw and cytokine levels in the ipsilateral paw, ipsilateral DRG, and spinal cord were measured.

Correlation between PWL in the ipsilateral paw and ipsilateral paw cytokine concentrations. Although estradiol did not significantly alter cytokine concentrations in the ipsilateral paw at the medium heat intensity, significant correlations were observed between IL-1β and GM-CSF concentrations and PWL in the cholesterol-treated group [IL-1β: $r=-.98$, $p<0.05$; GM-CSF: $r=-.98$, $p<0.05$]. In addition, at the medium heat intensity, after 10% estradiol treatment, positive correlations were observed between PWL and GM-CSF and TNF-α levels [GM-CSF: $r=.98$, $p<0.05$; TNF-α: $r=.96$, $p<0.05$]. Furthermore, at the lowest heat intensity, in rats pretreated with 40% estradiol, a significant correlation was found between IL-2 levels and PWL [$r=.96$, $p<0.05$; Table 4.1].

Correlation between PWL in the ipsilateral paw and cytokine levels in the ipsilateral DRG. In response to the highest heat intensity, in the 10% estradiol-treated group, PWL was significantly correlated with IFN-γ levels in the ipsilateral DRG [r=-.95, $p<0.05$]. Furthermore, at medium heat intensity, in the 10% estradiol-treated group, TNF-α levels were negatively correlated with PWL [r=-.96, $p<0.05$] and at the lowest heat intensity, IL-4 levels were positively correlated with PWL [r=.99, $p<0.05$]. In addition, in response to low heat intensity, after treatment with 20% estradiol, TNF-α was negatively correlated with PWL to [r=-.98; $p<0.05$]. At the high heat intensity, in the 40% estradiol-treated group, significant positive correlations were observed between IL-1β [r=.96, $p<0.05$], IL-2 [r=.99, $p<0.05$], IL-5 [r=.96, $p<0.05$], IL-10 [r=.99, $p<0.05$], GM-CSF [r=.99, $p<0.05$], IFN-γ [r=.98, $p<0.05$], and TNF-α [r=.98, $p<0.05$] levels and PWL (Table 4.2).

Correlations between PWL in the ipsilateral paw and spinal cord cytokine levels. In the spinal cord of vehicle-treated mice, after high heat stimulation, a significant positive correlation was seen between spinal IL-1β levels and PWL [r=.98, $p<0.05$]. Further, in the 10% estradiol-treated mice at the same heat intensity, spinal IL-4 concentrations were also positively correlated with PWL [r=.97, $p<0.05$] At this heat intensity, in the 20% estradiol-treated mice, significant negative correlations were also observed between spinal IL-2 [r=-.98, $p<0.05$], IL-4 [r=-.98, $p<0.05$], GM-CSF [r=-.99, $p<0.05$], IFN-γ [r=-.99, $p<0.05$], and TNF-α [r=-1.00, $p<0.05$] levels and PWL. In addition, at the medium heat intensity, in the animals pretreated with 20% estradiol, spinal IL-1β levels were significantly correlated with PWL [r=-.97; $p<0.05$]. Furthermore, in response to medium heat stimulation, in the group that received 30% estradiol, significant negative relationships were seen between IL-5 [r=-1.00, $p<0.05$], IFN-γ [r=-1.00, $p<0.05$], and TNF-α [r=-1.00, $p<0.05$] levels and PWL (Table 4.3).

Correlations between cytokine levels in the PNS and CNS after Cg-induced inflammation. In order to elucidate the nature of cytokine activation in response to an inflammatory stimulus, differences between cytokine expression in the peripheral and central nervous systems, cytokine concentrations were compared in ipsilateral paw and DRG tissues, contralateral paw and DRG tissues, and spinal cord tissue.

Cytokine expression in the ipsilateral PNS. In the ipsilateral paw, significant positive correlations were observed between different cytokines. Specifically, IL-2 was significantly correlated with IL-4 [r=.66, p<0.05] and IFN-γ [r=.71, p<0.05]. IFN-γ was also significantly correlated with IL-4 [r=.77, p<0.05], and GM-CSF was significantly correlated with TNF-α [r=.83, p<0.05; Table 4.4].

In the ipsilateral DRG, with the exception of IL-2, all cytokine levels were significantly correlated with each other. Specifically, IL-1β was significantly correlated with IL-5 [r=.89, p<0.05], IL-10 [r=.81, p<0.05], and GM-CSF [r=.97, p<0.05]. IL-4 was positively correlated with IL-5 [r=.74, p<0.05], IL-10 [r=.86, p<0.05], GM-CSF [r=.67, p<0.05], IFN-γ [r=.94, p<0.05], and TNF-α [r=.95, p<0.05]. IL-5 was significantly correlated with IL-10 [r=.97, p<0.05], GM-CSF [r=.95, p<0.05], IFN-γ [r=.77, p<0.05], and TNF-α [r=.84, p<0.05]. IL-10 was significantly correlated with GM-CSF [r=.92, p<0.05], IFN-γ [r=.88, p<0.05], and TNF-α [r=.93, p<0.05]. GM-CSF was positively correlated with IFN-γ [r=.63, p<0.05] and TNF-α [r=.73, p<0.05]. IFN-γ was significantly correlated with TNF-α [r=.99, p<0.05; Table 4.4].

To determine if a relationship between cytokine levels in the ipsilateral paw and DRG occur, we did correlation analyses of cytokine levels in the paw versus levels in the DRG. IL-1β concentrations in the paw were significantly correlated with levels of IL-4 [r=.88, p<0.05], IFN-γ [r=.82, p<0.05], and TNF-α [r=.80, p<0.05] in the ipsilateral DRG. IL-2 concentrations in the

paw correlated significantly with IL-2 concentrations in the DRG [r=.70, p<0.05]. Furthermore, TNF-α levels in the paw were negatively correlated with IL-10 [r=-.67, p<0.05], IFN-γ [r=-.75, p<0.05], and TNF-α [r=-.72, p<0.05] levels in the DRG (Table 4.4).

Cytokine levels in the contralateral PNS. In the contralateral paw, significant positive correlations were observed between different cytokines in the contralateral paw tissue. IL-1β concentrations were significantly correlated with IFN-γ [r=.66, p<0.05] and TNF-α [r=.99, p<0.05] concentrations in the contralateral paw. In addition, IFN-γ levels were significantly correlated with IL-10 [r=.92, p<0.05] and TNF-α levels [r=.73, p<0.05; Table 4.5].

In the contralateral DRG, significant positive correlations were also found between the different cytokines measured. IL-1β was significantly correlated with IL-2 [r=.74, p<0.05], IL-4 [r=.74, p<0.05], IL-5 [r=.97, p<0.05], IL-10 [r=.91, p<0.05], GM-CSF [r=.95, p<0.05], IFN-γ [r=.83, p<0.05], and TNF-α [r=.87, p<0.05]. IL-2 correlated significantly with IL-4 [r=.86, p<0.05], IL-5 [r=.80, p<0.05], IL-10 [r=.87, p<0.05], GM-CSF [r=.80, p<0.05], IFN-γ [r=.85, p<0.05], and TNF-α [r=.87, p<0.05]. IL-4 was significantly correlated with IL-5 [r=.83, p<0.05], IL-10 [r=.94, p<0.05], GM-CSF [r=.88, p<0.05], IFN-γ [r=.95, p<0.05], and TNF-α [r=.95, p<0.05]. Significant correlations were observed between IL-5 and IL-10 [r=.96, p<0.05], GM-CSF [r=.97, p<0.05], IFN-γ [r=.92, p<0.05], and TNF-α [r=.95, p<0.05]. Furthermore, IL-10 was significantly correlated with GM-CSF [r=.98, p<0.05], IFN-γ [r=.95, p<0.05], and TNF-α [r=.95, p<0.05]. GM-CSF was positively correlated with both IFN-γ [r=.91, p<0.05] and TNF-α [r=.92, p<0.05], and IFN-γ was significantly correlated with TNF-α [r=.99, p<0.05; Table 4.5].

To determine if a relationship exists between cytokine levels in the contralateral paw and DRG, we conducted correlation analyses of cytokine levels in the paw versus that in the DRG.

Cytokine levels in the contralateral paw and DRG tissues were significantly correlated with each other. IL-2 concentrations in the contralateral paw were positively correlated with IFN-γ [r=.75, $p<0.05$] and TNF-α [r=.74, $p<0.05$] levels in the contralateral DRG. In addition, IL-10 levels in the paw correlated negatively with IL-2 levels in the DRG [r=-.67, $p<0.05$; Table 4.5].

Similarly, significant correlations between ipsilateral and contralateral DRG were found. Cytokine concentrations in the ipsilateral DRG were significantly correlated with cytokine concentrations in the contralateral DRG. Levels of IL-2 in the ipsilateral DRG were positively correlated with levels of IL-2 [r=.70, $p<0.05$], IL-1β [r=.72, $p<0.05$], IL-5 [r=.73, $p<0.05$], IFN-γ [r=.68, $p<0.05$], and TNF-α [r=.75, $p<0.05$] in contralateral DRG tissue. Ipsilateral IL-4 levels were positively correlated with contralateral IL-4 [r=.72, $p<0.05$] and IL-10 [r=.68, $p<0.05$] levels. Ipsilateral IL-5 concentrations correlated with contralateral IL-4 levels [r=.71, $p<0.05$]. IL-10 levels in the ipsilateral DRG were positively correlated with IL-10 [r=.67, $p<0.05$], IL-4 [r=.74, $p<0.05$], and GM-CSF [r=.64, $p<0.05$] levels in the contralateral DRG. Ipsilateral IFN-γ concentrations positively correlated with contralateral IFN-γ [r=.74, $p<0.05$], IL-2 [r=.75, $p<0.05$], IL-4 [r=.88, $p<0.05$], IL-10 [r=.81, $p<0.05$], GM-CSF [r=.72, $p<0.05$], and TNF-α [r=.69, $p<0.05$] concentrations. Furthermore, TNF-α levels in the ipsilateral DRG correlated positively with levels of IL-2 [r=.71, $p<0.05$], IL-4 [r=.83, $p<0.05$], IL-10 [r=.76, $p<0.05$], GM-CSF [r=.69, $p<0.05$], and IFN-γ [r=.68, $p<0.05$] in the contralateral DRG (Table 4.6).

Cytokine levels in the CNS. Spinal concentrations of different cytokines correlated significantly with each other. Specifically, IL-1β levels were significantly correlated with IL-4 [r=.97, $p<0.05$], IL-5 [r=.96, $p<0.05$], IL-10 [r=.94, $p<0.05$], GM-CSF [r=.91, $p<0.05$], IFN-γ [r=.83, $p<0.05$], and TNF-α [r=.81, $p<0.05$] levels in the spinal cord. Spinal IL-2 concentrations

correlated positively with spinal GM-CSF [r=.64, p<0.05] and TNF-α [r=.71, p<0.05] concentrations. IL-4 levels were significantly correlated with IL-5 [r=.96, p<0.05], IL-10 [r=.97, p<0.05], GM-CSF [r=.95, p<0.05], IFN-γ [r=.88, p<0.05], and TNF-α [r=.90, p<0.05] levels. Concentrations of IL-5 correlated significantly with spinal IL-10 [r=.99, p<0.05], GM-CSF [r=.97, p<0.05], IFN-γ [r=.92, p<0.05], and TNF-α [r=.90, p<0.05]. IL-10 concentrations were correlated with concentrations of GM-CSF [r=.99, p<0.05], IFN-γ [r=.95, p<0.05], and TNF-α [r=.94, p<0.05]. GM-CSF levels were also significantly correlated with IFN-γ [r=.96, p<0.05], and TNF-α [r=.97, p<0.05] levels, and concentrations of spinal IFN-γ were positively correlated with spinal TNF-α concentrations [r=.98, p<0.05; Table 4.7].

To determine if inflammatory responses after injury in the ipsilateral paw correlate with changes in cytokine concentrations in the spinal cord, we did a series of correlation analyses. Overall, peripheral levels of cytokines in the ipsilateral paw correlated significantly with spinal concentrations of cytokines in the CNS. IL-1β levels in the ipsilateral paw were negatively correlated with both spinal IL-4 [r=-.64, p<0.05] and TNF-α [r=-.63, p<0.05] concentrations. In addition, peripheral IL-4 levels were negatively correlated with spinal IL-5 levels [r=-.64, p<0.05]. Peripheral IL-10 and spinal IFN-γ concentrations were also negatively correlated [r=-.65, p<0.05; Table 4.8].

In the case of contralateral paw and spinal cord cytokine levels after inflammatory stimulation, cytokine concentrations in the contralateral paw correlated significantly with spinal concentrations of cytokines in the CNS. For example, peripheral IL-5 concentrations were negatively correlated with both spinal IL-4 [r=-.64, p<0.05] and spinal IL-1β [r=-.65, p<0.05] concentrations (Table 4.9).

4.3. Discussion

We have compared the effect of estradiol and Cg treatments on PNS and CNS cytokine production. Our findings suggest that cytokine release is related to inflammation and inflammatory responses but failed to fully support the theory that estrogen significantly affects cytokine production post-inflammation in a way that would substantially alter nociceptive responses. However, estradiol affected cytokine levels in the uninjected paw tissue at a moderate dose. Second, significant relationships were found between PWL and cytokine concentrations in the injected paw, ipsilateral DRG, and spinal cord tissues at certain levels of estrogen treatment. Lastly, significant correlations were found in cytokine concentrations in tissues from both the peripheral and central nervous systems.

Our data suggest that estradiol may play a role in mediating cytokine levels. For example, pre-treating mice with 20% estradiol resulted in an increase in cytokine levels in contralateral paw tissue, indicating that estradiol's effects in the up-regulation of cytokines throughout the PNS is dose-dependent. These effects may be seen in the contralateral paw due to central sensitization and a subsequent sensitizing of the contralateral sensory neurons. Previous studies have shown that peripheral inflammatory insult causes both TNF-α and IL-1β up-regulation (Rittner, Machelska, & Stein, 2005). Furthermore, peripheral cytokine release can cause axonal changes in the peripheral nerve, which can lead to glial activation in the spinal cord (Wieseler-Frank, Maier, & Watkins, 2005). It is possible that because we analyzed cytokine levels approximately 5-6 hours after Cg injection, that cytokine levels on the ipsilateral side and spinal cord have already returned back to baseline levels, while there was still an effect seen on the contralateral side.

The behavioral responses discussed in the previous chapter were accompanied by corresponding changes in cytokine levels in both the PNS and CNS. These results indicate that cytokine expression is linked to behavioral responses resulting from persistent inflammation caused by Cg. The inflammatory responses, however, varied based on level of hormone pre-treatment. For instance, a significant negative relationship was found between cytokines in paw and DRG tissue and PWL in the vehicle and low dose (10% and 20%) groups. This supports the notion that cytokine activation is responsible for increased levels of nociceptive behavior (Sommer & Kress, 2004); an increase in PWL indicates a lessening of pain behavior and was accompanied by a decrease in cytokine levels. When pretreated with higher doses (30% and 40%) of estradiol, however, cytokine concentrations in the paw were positively correlated with PWL, which may indicate that high doses of estradiol cause physiological changes divergent from changes observed at lower doses. Taking into account that the higher doses of estradiol caused increases in overall pain behavior (see Chapter 3), this indicates that these sensitizing effects may be working through a pathway divergent from the cytokine pathways.

In conjunction with effects seen at the periphery, a positive relationship was found between spinal cytokines and PWL in the vehicle group, but negative relationships were seen in the higher estradiol groups. This indicates that estradiol may have diverging effects in the peripheral and central nervous systems. In addition, IL-2 and IL-4 tend to have positive correlations with PWL, which supports the anti-inflammatory roles of these cytokines (Verri, Jr., et al., 2006). Since significant relationships were found between PWL and cytokine expression in various nervous system tissues, it is clear that cytokine production in both the CNS and PNS play important roles in the inflammatory response.

We also compared cytokine concentrations in both ipsilateral and contralateral peripheral tissues and in the spinal cord. Results indicate that multiple cytokines are correlated in each tissue type. For example, both pro- and anti-inflammatory cytokines were positively correlated with each other. This supports recent literature that states that proinflammatory cytokines act synergistically to create inflammatory hyperalgesia and allodynia followed by a release of anti-inflammatory cytokines to counteract effects of inflammatory cytokines (Rittner, Machelska, & Stein, 2005; Cunha, et al., 2005). It is unclear, however, whether or not the cytokines are being released concomitantly or sequentially. Most research indicates that cytokines are released as a cascade in response to inflammatory stimuli (Cunha, et al., 2005), but time-course experiments would be needed to further elucidate this matter in this specific model since tissues were extracted 5 hours after inflammatory insult.

In addition to the synergistic role of cytokines, we also found significant correlations between cytokine concentrations in peripheral and central sites and in the ipsilateral and contralateral DRGs. These point to the possibility that the inflammatory stimulus in the ipsilateral paw caused subsequent molecular changes in the ipsilateral DRG, which may have caused changes in the spinal cord and the contralateral DRG. This suggests that treatment with Cg can cause central sensitization in murine models that, in turn, affect inflammatory mediators on the contralateral side.

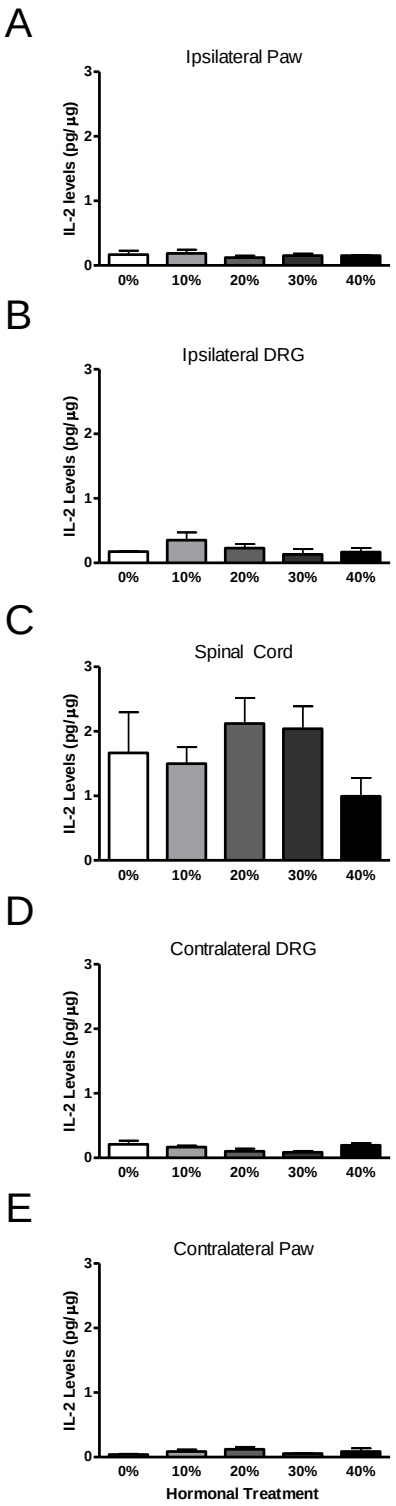

Figure 4.1. Effects of hormone treatment on IL-2 levels in C57BL/6N OVX mice. Data represent mean (±SEM) IL-2 levels (pg/µg) in ipsilateral paw (A), ipsilateral DRG (B), spinal cord (C), contralateral DRG (D), and contralateral paw (E) tissues in vehicle- and estradiol-treated mice. *n*=4 per group.

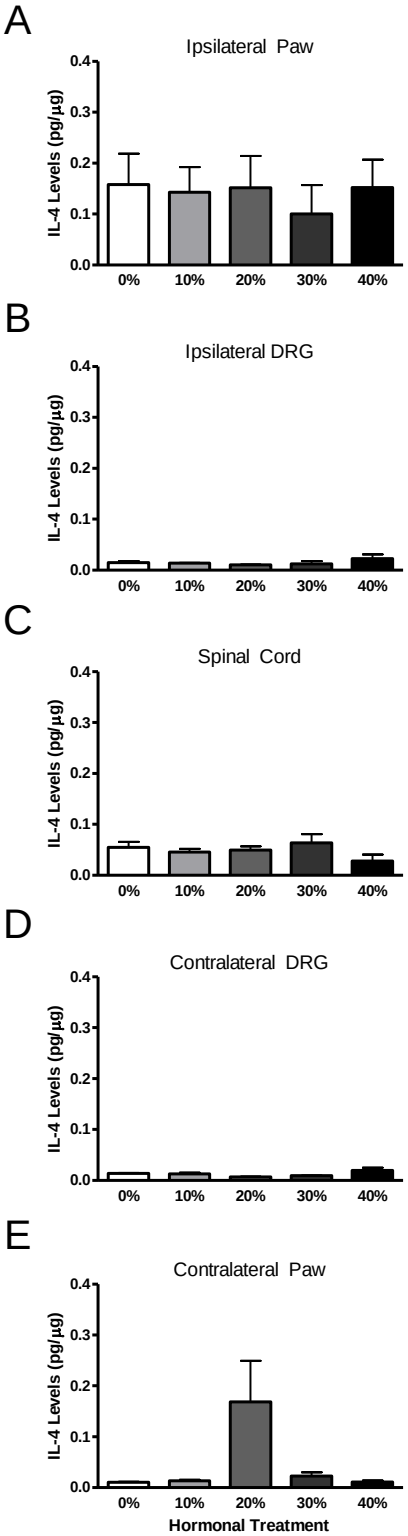

Figure 4.2. Effects of hormone treatment on IL-4 levels in C57BL/6N OVX mice. Data represent mean (±SEM) IL-4 levels (pg/µg) in ipsilateral paw (A), ipsilateral DRG (B), spinal cord (C), contralateral DRG (D), and contralateral paw (E) tissues in vehicle- and estradiol-treated mice. *n*=4 per group.

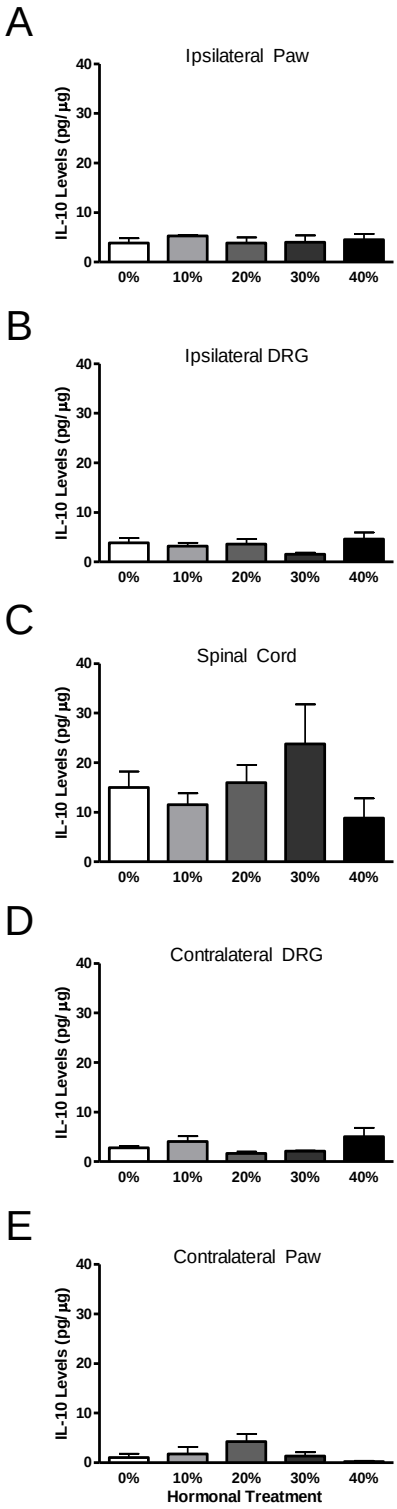

Figure 4.3. Effects of hormone treatment on IL-10 levels in C57BL/6N OVX mice. Data represent mean (±SEM) IL-10 levels (pg/µg) in ipsilateral paw (A), ipsilateral DRG (B), spinal cord (C), contralateral DRG (D), and contralateral paw (E) tissues in vehicle- and estradiol-treated mice. *n*=4 per group.

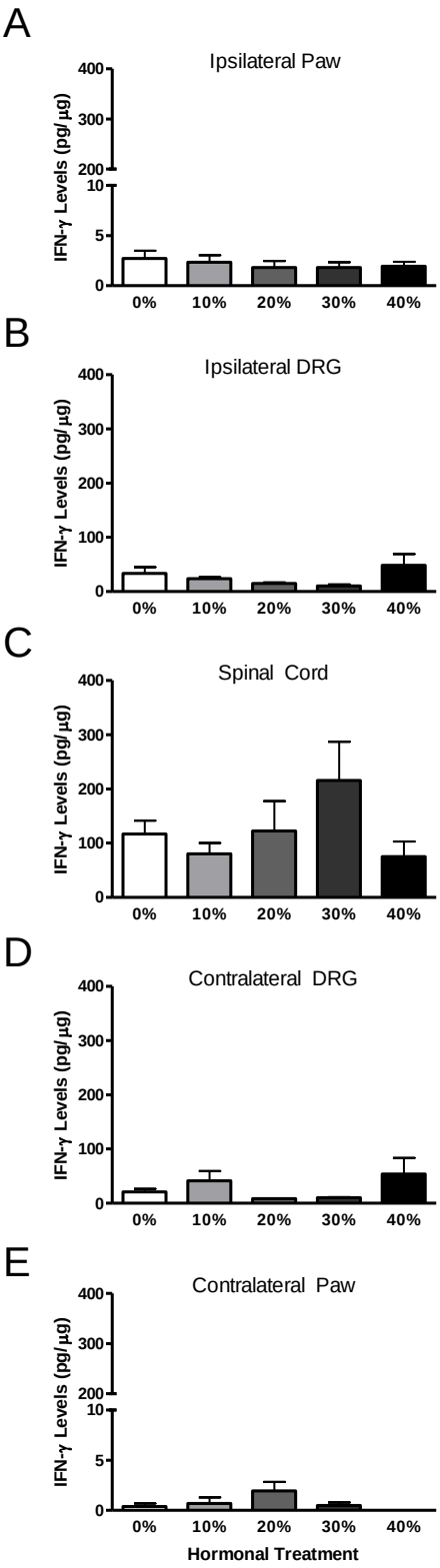

Figure 4.4. Effects of hormone treatment on IFN-γ levels in C57BL/6N OVX mice. Data represent mean (±SEM) IFN-γ levels (pg/µg) in ipsilateral paw (A), ipsilateral DRG (B), spinal cord (C), contralateral DRG (D), and contralateral paw (E) tissues in vehicle- and estradiol-treated mice. *n*=4 per group.

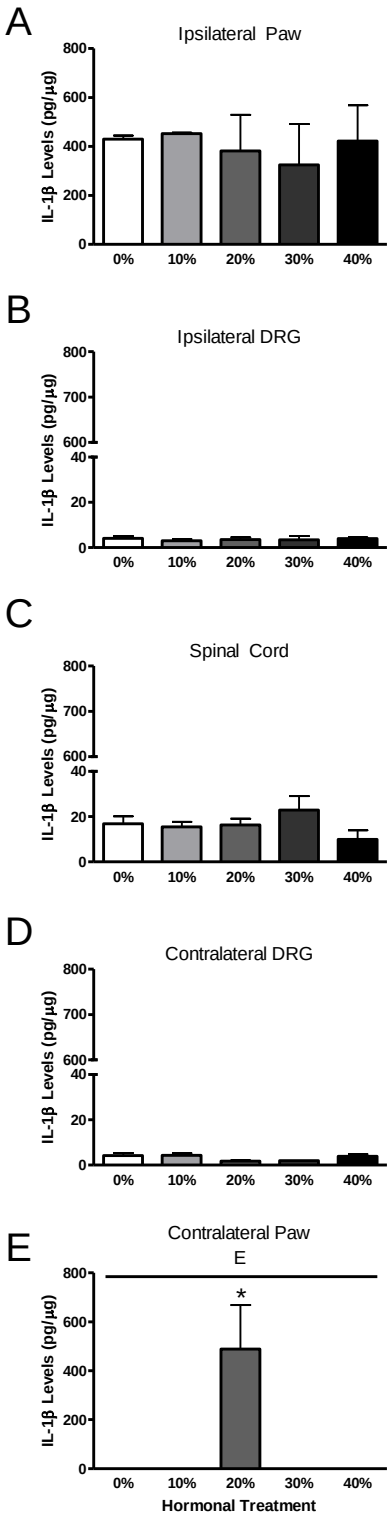

Figure 4.5. Effects of hormone treatment on IL-1β levels in C57BL/6N OVX mice. Data represent mean (±SEM) IL-1β levels (pg/µg) in ipsilateral paw (A), ipsilateral DRG (B), spinal cord (C), contralateral DRG (D), and contralateral paw (E) tissues in vehicle- and estradiol-treated mice. (*) signifies a significant difference to all other treatment groups ($p<0.05$). *n*=4 per group.

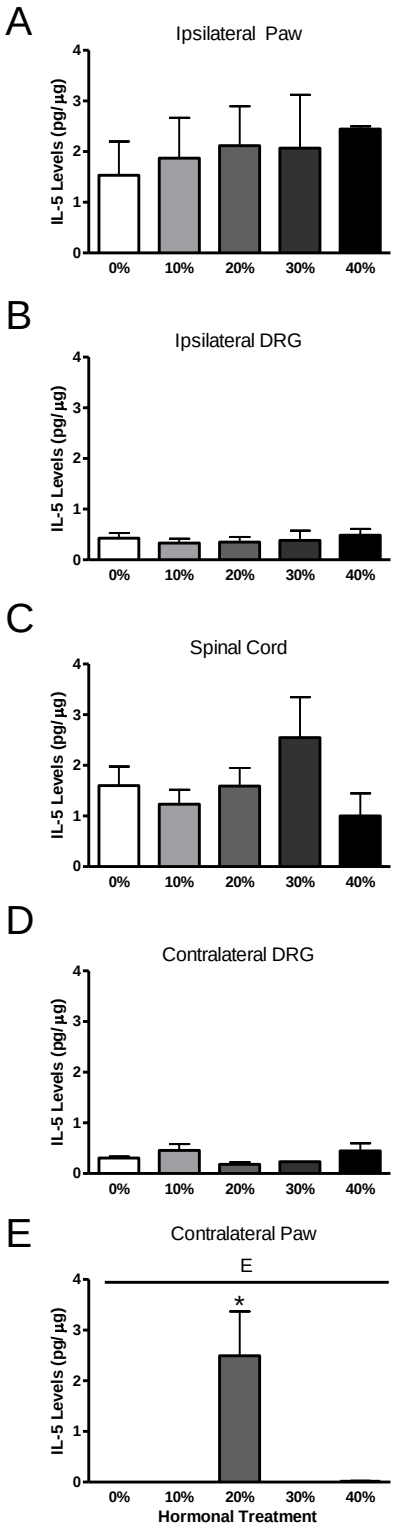

Figure 4.6. Effects of hormone treatment on IL-5 levels in C57BL/6N OVX mice. Data represent mean (±SEM) IL-5 levels (pg/µg) in ipsilateral paw (A), ipsilateral DRG (B), spinal cord (C), contralateral DRG (D), and contralateral paw (E) tissues in vehicle- and estradiol-treated mice. (E) denotes a significant main effect of hormone treatment. (#) signifies a significant difference to the 40% estradiol-treated group. ($p<0.01$). *n*=4-6 per group.

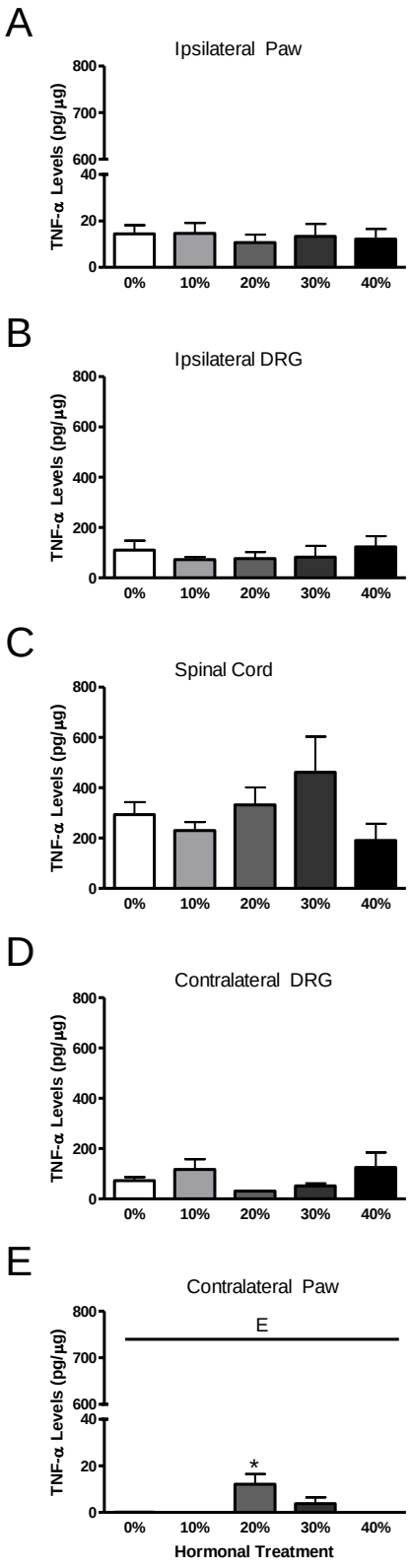

Figure 4.7. Effects of hormone treatment on TNF-α levels in C57BL/6N OVX mice. Data represent mean (±SEM) TNF-α levels (pg/µg) in ipsilateral paw (A), ipsilateral DRG (B), spinal cord (C), contralateral DRG (D), and contralateral paw (E) tissues in vehicle- and estradiol-treated mice. *n*=4-6 per group.

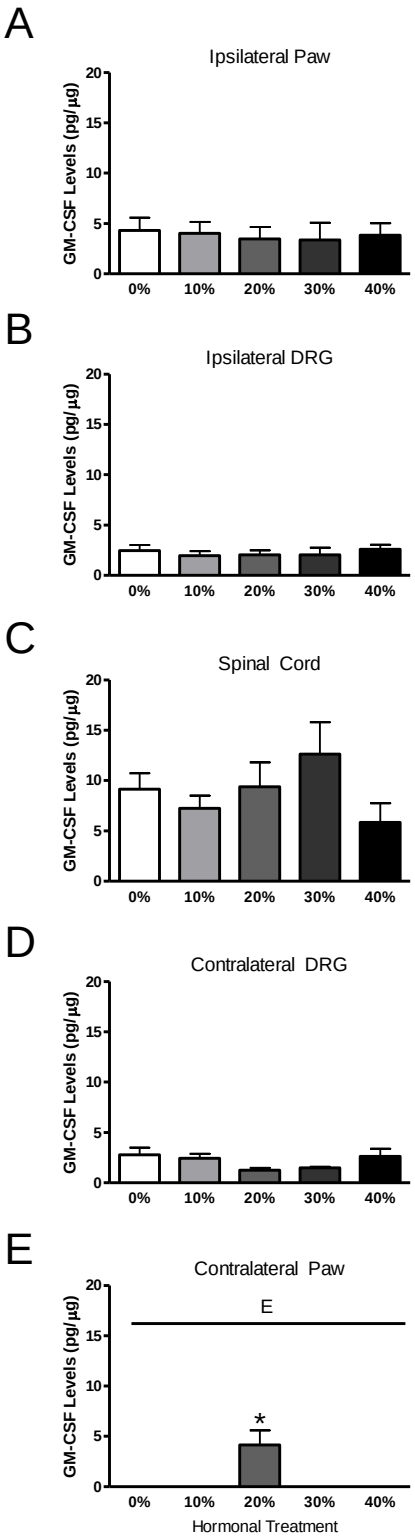

Figure 4.8. Effects of hormone treatment on GM-CSF levels in C57BL/6N OVX mice. Data represent mean (±SEM) GM-CSF levels (pg/µg) in ipsilateral paw (A), ipsilateral DRG (B), spinal cord (C), contralateral DRG (D), and contralateral paw (E) tissues in vehicle- and estradiol-treated mice. *n*=4-6 per group.

Table 4.1
Summary of correlations between ipsilateral PWL and ipsilateral paw cytokine levels in C57BL/6N OVX mice

		Cytokine							
Group		**IL-1β**	**IL-2**	**IL-4**	**IL-5**	**IL-10**	**GM-CSF**	**IFN-γ**	**TNF-α**
Low Heat	**Overall**	-.03	.27	.08	-.10	-.15	.14	.18	-.02
	0% E2	.53	.62	.42	.57	.28	.75	.47	.41
	10% E2	-.90	-.89	-.83	-.90	-.93	-.79	-.83	-.73
	20% E2	-.75	-.25	-.70	-.10	-.78	-.66	-.93	-.72
	30% E2	-.96	-.59	-.75	-.99	-.99	-.99	-.89	-.96
	40% E2	.33	**.96***	.18	-.13	-.16	.01	-.18	-.34
Medium Heat	**Overall**	.04	-.23	-.03	.15	.02	.02	-.14	.02
	0% E2	**-.98***	-.80	-.78	-.74	-.89	**-.98***	-.88	-.94
	10% E2	.79	.31	.31	.76	.82	**.98***	.58	**.96***
	20% E2	.30	.77	.28	.86	.24	.37	-.03	.25
	30% E2	.21	-.43	-.23	.59	.32	.61	.02	.69
	40% E2	.44	-.56	.71	-.09	.50	.24	.69	.20
High Heat	**Overall**	-.04	-.31	-.17	.07	-.07	.08	-.11	.03
	0% E2	-.27	-.74	-.79	-.47	-.21	-.06	-.67	-.10
	10% E2	.07	-.53	-.40	-.21	.04	.36	-.03	.43
	20% E2	.48	.59	.63	.64	.26	.30	.17	.18
	30% E2	-.71	-.99	-.94	-.36	-.63	-.34	-.83	-.23
	40% E2	.80	.61	.81	-.06	.35	.34	.46	-.06

Legend. E2 signifies estradiol treatment. Data represents correlation coefficients for specified analysis. (*****) denotes a significant correlation coefficient ($p<0.05$). *n*=3-4 per group.

Table 4.2
Summary of correlations between ipsilateral PWL and ipsilateral DRG cytokine levels in C57BL/6N OVX mice

		Cytokine							
Group		**IL-1β**	**IL-2**	**IL-4**	**IL-5**	**IL-10**	**GM-CSF**	**IFN-γ**	**TNF-α**
Low Heat	**Overall**	.03	-.12	-.09	.02	.03	.02	.07	-.00
	0% E2	-.10	.98	-.37	-.16	-.18	-.22	-.14	-.25
	10% E2	.82	-.62	**.99***	.87	.89	.84	-.12	.72
	20% E2	-.66	-.33	-.09	-.81	-.89	-.70	-.92	**-.98***
	30% E2	-.32	-.37	-.27	-.34	----	-.28	----	-.38
	40% E2	.86	.78	.33	.87	.73	.76	.60	.58
Medium Heat	**Overall**	-.41	-.19	-.14	-.41	-.39	-.37	-.21	-.26
	0% E2	-.48	-.88	-.44	-.56	-.58	-.39	-.59	-.54
	10% E2	-.42	-.15	-.58	-.52	-.68	-.45	-.64	**-.96***
	20% E2	-.92	-.45	-.98	-.85	-.76	-.86	-.28	-.56
	30% E2	-.69	-.65	-.73	-.67	----	-.72	----	-.64
	40% E2	.03	.20	.69	.03	.28	.21	.44	.46
High Heat	**Overall**	.20	-.05	-.02	.07	.07	.21	-.07	-.06
	0% E2	.43	-.59	-.25	.14	-.00	.42	-.57	-.43
	10% E2	.23	-.85	.38	.18	.03	.23	**-.95***	-.46
	20% E2	-.78	.89	-.94	-.61	-.52	-.78	-.74	-.22
	30% E2	-.98	-.99	-.97	-.99	----	-.97	----	-.99
	40% E2	**.96***	**.99***	.90	**.96***	**.99***	**.99***	**.98***	**.98***

Legend. E2 denotes estradiol treatment. Data represents correlation coefficients for specified analysis. (*****) denotes a significant correlation coefficient ($p<0.05$). (----) signifies an insufficient amount of data to complete the analysis. *n*=3-6 per group.

Table 4.3
Summary of correlations between ipsilateral PWL and spinal cord cytokine levels in C57BL/6N OVX mice

Group		Cytokine							
		IL-1β	**IL-2**	**IL-4**	**IL-5**	**IL-10**	**GM-CSF**	**IFN-γ**	**TNF-α**
Low Heat	**Overall**	.06	-.26	.06	.06	.06	.07	.11	.08
	0% E2	-.14	-.89	-.41	-.39	-.33	-.37	-.35	-.44
	10% E2	.62	-.28	.41	.65	.66	.62	.63	.61
	20% E2	-.16	-.21	.05	.08	.12	-.09	-.12	-.19
	30% E2	.11	.60	.19	.42	.27	.31	.50	.42
	40% E2	.03	.14	-.05	-.10	-.07	.05	.89	.15
Medium Heat	**Overall**	-.12	.00	.02	-.01	-.02	-.01	-.03	-.02
	0% E2	.18	.29	.62	.60	.53	.64	.72	.77
	10% E2	.15	.26	.38	.12	.10	.13	.14	.13
	20% E2	**-.97***	-.88	-.76	-.86	-.81	-.84	-.85	-.78
	30% E2	-.93	-.99	-.96	**-1.00***	-.98	-.99	**-1.00***	**-1.00***
	40% E2	-.37	-.50	-.40	-.32	-.32	-.35	-.56	-.49
High Heat	**Overall**	.18	-.16	.24	.12	.10	.12	.03	.04
	0% E2	**.98***	-.20	.91	.91	.95	.90	.84	.81
	10% E2	.88	.32	**.97***	.86	.89	.93	.90	.94
	20% E2	-.80	**-.98***	**-.98***	-.90	-.93	**-.99***	**-.99***	**-1.00***
	30% E2	-.81	-.40	-.76	-.58	-.70	-.67	-.60	-.58
	40% E2	-.40	-.37	-.50	-.49	-.46	-.36	-.26	-.35

Legend. E2 signifies estradiol treatment. Data represents correlation coefficients for specified analysis. (*****) denotes a significant correlation coefficient ($p<0.05$). *n*=3-4 per group.

Table 4.4.
Summary of correlations between cytokine levels in the ipsilateral paw and DRG in C57BL/6N OVX mice.

Cytokine		IL-1β		IL-2		IL-4		IL-5		IL-10		GM-CSF		IFN-γ		TNF-α	
Tissue		**IP**	**ID**	**IP**	**ID**	**IP**	**ID**	**IP**	**ID**	**IP**	**ID**	**IP**	**ID**	**IP**	**ID**	**IP**	**ID**
IL-1β	**IP**	1.00	.21	-.07	.22	.62	**.88***	-.17	.51	.43	.62	-.21	.40	.15	**.82***	-.29	**.80***
	ID	.21	1.00	-.52	-.18	-.22	.51	.05	**.89***	-.17	**.81***	-.03	**.97***	-.45	.46	-.31	.58
IL-2	**IP**	-.07	-.52	1.00	**.70***	**.66***	-.18	-.35	-.35	-.26	-.28	-.26	-.53	**.71***	.02	-.31	-.07
	ID	.22	-.18	**.70***	1.00	.58	.17	-.31	-.04	-.01	.07	-.58	-.15	.41	.29	-.44	.23
IL-4	**IP**	.62	-.22	**.66***	.58	1.00	.52	-.47	.05	.14	.21	-.29	-.11	**.77***	.58	-.41	.52
	ID	**.88***	.51	-.18	.17	.52	1.00	-.36	**.74***	.17	**.86***	-.39	**.67***	-.01	**.94***	-.55	**.95***
IL-5	**IP**	-.17	.05	-.35	-.31	-.47	-.36	1.00	-.15	.53	-.27	.48	-.02	-.40	-.42	.56	-.411
	ID	.51	**.89***	-.35	-.04	.05	**.74***	-.15	1.00	-.24	**.97***	-.27	**.95***	-.40	**.77***	-.57	**.84***
IL-10	**IP**	.43	-.17	-.26	-.01	.14	.17	.53	-.24	1.00	-.17	.30	-.13	.12	-.06	.55	-.08
	ID	.62	**.81***	-.28	.07	.21	**.86***	-.27	**.97***	-.17	1.00	-.42	**.92***	-30	**.88***	**-.67***	**.93***
GM-CSF	**IP**	-.21	-.03	-.26	-.58	-.29	-.39	.48	-.27	.30	-.42	1.00	-.14	.14	-.53	**.83***	-.47
	ID	.40	**.97***	-.53	-.15	-.11	**.67***	-.02	**.95***	-.13	**.92***	-.14	1.00	-.47	**.63***	-.41	**.73***
IFN-γ	**IP**	.15	-.45	**.71***	.41	**.77***	-.01	-.40	-.40	.12	-.30	.14	-.47	1.00	-.01	.08	-.04
	ID	**.82***	.46	.02	.29	.58	**.94***	-.42	**.77***	-.06	**.88***	-.53	**.63***	-.01	1.00	**-.75***	**.99***
TNF-α	**IP**	-.29	-.31	-.31	-.44	-.41	-.55	.56	-.57	.55	**-.67***	**.83***	-.41	.08	**-.75***	1.00	**-.72***
	ID	**.80***	.58	-.07	.23	.52	**.95***	-.41	**.84***	-.08	**.93***	-.47	**.73***	-.04	**.99***	**-.72***	1.00

Legend. IP signifies ipsilateral paw. ID signifies ipsilateral DRG. (*) denotes a significant correlation coefficient for specified analysis ($p<0.05$). *n*=19-20 per group.

Table 4.5.
Summary of correlations between cytokine levels in the contralateral paw and DRG in C57BL/6N OVX mice.

Cytokine		IL-1β		IL-2		IL-4		IL-5		IL-10		GM-CSF		IFN-γ		TNF-α	
Tissue		CP	CD	CP	CD	CP	CD	CP	CD	CP	CD	CP	CD	CP	CD	CP	CD
IL-1β	CP	1.00	-.34	-.01	-.37	.04	-.31	-.16	-.28	.55	-.30	------	-.24	**.66***	-.27	**.99***	-.28
	CD	-.34	1.00	.51	**.74***	.04	**.74***	-.35	**.97***	-.31	**.91***	------	**.95***	-.13	**.83***	-.34	**.87***
IL-2	CP	-.01	.51	1.00	.59	-.45	.62	-.34	.59	-.38	.62	------	.62	-.19	**.75***	-.01	**.74***
	CD	-.37	**.74***	.59	1.00	-.07	**.86***	-.12	**.80***	**-.67***	**.87***	------	**.80***	-.44	**.85***	-.39	**.87***
IL-4	CP	.04	.04	-.45	-.07	1.00	-.19	.38	-.09	.51	-.12	------	-.08	.43	-.34	.05	-.29
	CD	-.31	**.74***	.62	**.86***	-.19	1.00	.03	**.83***	-.56	**.94***	------	**.88***	-.42	**.93***	-.34	**.90***
IL-5	CP	-.16	-.35	-.34	-.12	.38	.03	1.00	-.30	-.02	-.17	------	-.28	-.30	-.20	-.18	-.26
	CD	-.28	**.97***	.59	**.80***	-.09	**.83***	-.30	1.00	-.44	**.96***	------	**.97***	-.24	**.92***	-.30	**.95***
IL-10	CP	.55	-.31	-.38	**-.67***	.51	-.56	-.02	-.44	1.00	-.50	------	-.35	**.92***	-.63	.60	-.62
	CD	-.30	**.91***	.62	**.87***	-.12	**.94***	-.17	**.96***	-.50	1.00	------	**.97***	-.32	**.95***	-.32	**.95***
GM-CSF	CP	------	------	------	------	------	------	------	------	------	------	1.00	------	------	------	------	------
	CD	-.24	**.95***	.62	**.80***	-.08	**.88***	-.28	**.97***	-.35	**.97***	------	1.00	-.15	**.91***	-.24	**.92***
IFN-γ	CP	**.66***	-.13	-.19	-.44	.43	-.42	-.30	-.24	**.92***	-.32	------	-.15	1.00	-.45	**.73***	-.41
	CD	-.27	**.83***	**.75***	**.85***	-.34	**.93***	-.20	**.92***	-.63	**.95***	------	**.91***	-.45	1.00	-.30	**.99***
TNF-α	CP	**.99***	-.34	-.01	-.39	.05	-.34	-.18	-.30	.60	-.32	------	-.24	**.73***	-.30	1.00	-.31
	CD	-.28	**.87***	**.74***	**.87***	-.29	**.90***	-.26	**.95***	-.62	**.95***	------	**.92***	-.41	**.99***	-.31	1.00

Legend. CP signifies contralateral paw. CD signifies contralateral DRG. (----) signifies an insufficient amount of data to complete the analysis. (*) denotes a significant correlation coefficient for specified analysis ($p<0.05$). *n*=19-20 per group.

Table 4.6.
Summary of correlations between cytokine levels in the ipsilateral and contralateral DRG in C57BL/6N OVX mice.

	Ipsilateral DRG							
Contralateral DRG	**IL-1β**	**IL-2**	**IL-4**	**IL-5**	**IL-10**	**GM-CSF**	**IFN-γ**	**TNF-α**
IL-1β	.24	**.72***	.44	.44	.46	.31	.53	.50
IL-2	.12	**.70***	.61	.44	.51	.24	**.75***	**.71***
IL-4	.34	.45	**.72***	**.71***	**.74***	.49	**.88***	**.83***
IL-5	.20	**.73***	.49	.47	.51	.30	.63	.58
IL-10	.28	.63	**.68***	.61	**.67***	.43	**.81***	**.76***
GM-CSF	.34	.58	.60	.62	**.64***	.46	**.72***	**.69***
IFN-γ	.18	**.68***	.54	.53	.58	.31	**.74***	**.68***
TNF-α	.14	**.75***	.49	.47	.52	.26	**.69***	.63

Legend. (*) denotes a significant correlation coefficient for specified analysis ($p<0.05$). *n*=19-20 per group.

Table 4.7.
Summary of correlations between cytokine levels in the spinal cord in C57BL/6N OVX mice.

Spinal Cord	IL-1β	IL-2	IL-4	IL-5	IL-10	GM-CSF	IFN-γ	TNF-α
IL-1β	1.00	.45	**.97***	**.96***	**.94***	**.91***	**.83***	**.81***
IL-2	.45	1.00	.59	.51	.58	**.64***	.57	**.71***
IL-4	**.97***	.59	1.00	**.96***	**.97***	**.95***	**.88***	**.90***
IL-5	**.96***	.51	**.96***	1.00	**.99***	**.97***	**.92***	**.90***
IL-10	**.94***	.58	**.97***	**.99***	1.00	**.99***	**.95***	**.94***
GM-CSF	**.91***	**.64***	**.95***	**.97***	**.99***	1.00	**.96***	**.97***
IFN-γ	**.83***	.57	**.88***	**.92***	**.95***	**.96***	1.00	**.98***
TNF-α	**.81***	**.71***	**.90***	**.90***	**.94***	**.97***	**.98***	1.00

Legend. (*) denotes a significant correlation coefficient for specified analysis ($p<0.05$). *n*=19-20 per group.

Table 4.8.
Summary of correlations between cytokine levels in the spinal cord and ipsilateral paw in C57BL/6N OVX mice.

	Ipsilateral Paw							
Spinal Cord	**IL-1β**	**IL-2**	**IL-4**	**IL-5**	**IL-10**	**GM-CSF**	**IFN-γ**	**TNF-α**
IL-1β	-.51	-.27	-.54	-.22	-.44	.21	-.19	.15
IL-2	-.51	-.09	-.42	-.29	-.27	-.48	-.28	-.13
IL-4	**-.64***	-.21	-.55	-.22	-.46	.14	-.17	.14
IL-5	-.54	-.44	**-.64***	-.17	-.43	.19	-.34	.14
IL-10	-.59	-.37	-.61	-.22	-.48	.10	-.30	.06
GM-CSF	-.59	-.33	-.58	-.30	-.55	.02	-.30	-.02
IFN-γ	-.57	-.33	-.59	-.25	**-.65***	-.04	-.41	-.15
TNF-α	**-.63***	-.25	-.56	-.29	-.60	-.11	-.33	-.14

Legend. (*) denotes a significant correlation coefficient for specified analysis ($p<0.05$). *n*=19-20 per group.

Table 4.9.
Summary of correlations between cytokine levels in the spinal cord and contralateral paw in C57BL/6N OVX mice.

	Contralateral Paw							
Spinal Cord	**IL-1β**	**IL-2**	**IL-4**	**IL-5**	**IL-10**	**GM-CSF**	**IFN-γ**	**TNF-α**
IL-1β	-.08	.00	-.02	**-.65***	.44	------	.54	.03
IL-2	-.05	.22	-.38	-.47	.02	------	-.01	-.06
IL-4	-.14	-.06	-.11	**-.64***	.37	------	.42	-.04
IL-5	-.07	-.11	-.11	-.54	.48	------	.51	.04
IL-10	-.11	-.10	-.22	-.57	.38	------	.40	-.01
GM-CSF	-.12	-.04	-.31	-.61	.31	------	.34	-.03
IFN-γ	-.19	.03	-.44	-.56	.23	------	.23	-.11
TNF-α	-.21	-.02	-.45	-.58	.17	------	.16	-.14

Legend. (*) denotes a significant correlation coefficient for specified analysis ($p<0.05$). (----) signifies an insufficient amount of data to complete the analysis. *n*=19-20 per group.

Chapter 5: Effects of indomethacin and estradiol administration on Cg-induced nociceptive behaviors and paw size in C57BL/6N OVX mice.

5.1. Introduction

Indomethacin is a nonsteroidal anti-inflammatory drug (NSAID), a type of drug primarily used to treat illnesses like inflammation, fever, and cardiovascular disease (Zhou, Hancock, & Lichtenberger, 2010; Okamura, et al., 2008). Although these drugs can treat many diseases due to a wide variety of biochemical effects, the anti-inflammatory effect of NSAIDs, come from their inhibition of the cyclooxygenase (COX) enzymes, a key component involved in eicosanoid synthesis (Zhou, Hancock, & Lichtenberger, 2010; Gordon, et al., 2007). Both Lavich and colleagues (2005) and Holt and colleagues (2005) showed that pretreatment with indomethacin abolished the Cg-induced hyperalgesic response. Furthermore, topical application of indomethacin at the site of inflammation is shown to decrease edema by 65% and inhibit neutrophil infiltration into the injured area (Bralley, et al., 2008; Gordon, et al., 2007). Additionally, White and colleagues (2005) found that indomethacin reversed the contractile effect of estrogen in a porcine heart. Similarly, Li & Stallone (2005) showed that by blocking the prostanoid pathway pharmacologically using indomethacin in rats, they also blocked the aortic contractile responses to estrogen. Although this research is not specifically aimed at the effects estrogen has on inflammation and pain, it indicates that some of estradiol's physiological effects are due to their alteration of the prostanoid pathway. This experiment was designed to determine if estradiol's hyperalgesic effects (see Chapter 3) are due to a COX-dependent mechanism. Researchers hypothesize that both low and high doses of indomethacin will reverse the hyperalgesic effect high doses of estradiol had on PWL in OVX mice.

5.2. Results

Effect of DMSO and indomethacin on baseline PWL in cholesterol- and estrogen-treated OVX mice. Figure 5.1 shows mean PWL in hormone- and drug-treated groups prior to Cg and saline treatment. Significant main effects for drug treatment and heat intensity were observed [Drug Treatment: $F(2,82)=26.96$, $p<0.001$; Heat Intensity: ($F(2,164)=126.09$, $p<0.001$]. The groups that received the highest dose of indomethacin showed significantly longer baseline PWL ($M=6.76$) when compared to both the vehicle-treated group ($M=3.99$) and the low dose group ($M=4.19$; $p<0.001$). Further, PWL at the lowest heat intensity ($M=7.45$) was significantly longer compared to both the medium ($M=4.67$) and highest ($M=3.09$) heat intensities ($p<0.001$). Additionally, baseline PWL was significantly longer in response to medium heat intensity compared to the high heat intensity ($p<0.001$).

A significant interaction between heat intensity and drug treatment was also observed [$F(4, 164)=11.9$, $p<0.001$]. This significant interaction was fueled the magnitude of differences between the drug-treatment groups at low [$F(2,90)=27.15$, $p<0.001$], medium [$F(2,90)=14.03$, $p<0.001$], and high heat intensities [$F(2,90)=8.29$, $p<0.001$]. In response to each heat intensity PWL in the high-dose group [Low Heat: $M=11.02$; Medium Heat: $M=6.02$; High Heat: $M=3.77$] was significant longer when compared to PWL in the low-dose group [Low Heat: $M=5.93$; Medium Heat: $M=3.84$; High Heat: $M=2.77$] and the control group [Low Heat: $M=5.70$; Medium Heat: $M=3.68$; High Heat: $M=2.70$]. Although the data displays the same pattern of differences in PWL to the different drug treatments at each heat intensity, the effect was strongest in response to the low heat.

Effects of estradiol and indomethacin treatment on ipsilateral PWL prior to, 1 hour, and 5 hours after saline or Cg administration.

Low Heat. Figure 5.2 shows mean ipsilateral PWL in response to low heat in estradiol- and indomethacin-treated animals. Significant main effects for drug treatment, pain condition, and time were observed [Drug Treatment: $F(2,73)=15.04$, $p<0.001$; Pain Treatment: $F(1,73)=5.3$, $p<0.05$; Time: $F(2,146)=20.79$, $p<0.001$]. Treatment with high doses of indomethacin (M=6.68) caused significantly longer PWL in the ipsilateral paw compared to both the low dose (M=5.11) and the control group (M=4.24; $p<0.001$; Figure 5.2d). Furthermore, subjects treated with saline (M=5.77) displayed significantly longer PWL compared to those treated with Cg (4.91; $p<0.05$; Figure 5.2e). PWL prior to Cg or saline injection (M=6.72) were significantly longer compared to PWL at both 1 hour (M=5.18) and 5 hours (M=4.18) post-administratoin ($p<0.001$). Additionally, PWL 1 hour post-injection was significantly longer compared with PWL 5 hours after injection ($p<0.05$; Figure 5.2f).

Significant interactions were observed between time and drug treatment [$F(4,146)=8.26$, $p<0.001$] and between time and pain treatment [$F(2,146)=10.2$, $p<0.001$]. The significant time by drug treatment interaction can be clarified by significant differences between drug treatment groups prior to Cg or saline injection and 1 hour post-injection [Baseline: $F(2,89)=18.73$, $p<0.001$; 1 Hour: $F(2,89)=5.33$, $p<0.05$]. During baseline readings, PWL in the control group (M=4.93) and low dose group (M=5.46) was significantly shorter compared to PWL in the high drug dose group (M=9.67; $p<0.001$). One hour after Cg or saline injection, PWL in the control group (M=4.01) was significantly shorter when compared to the high-dose group (M=6.28; $p<0.01$). The significant time by pain treatment interaction can be understood best by comparing PWL in the saline and Cg groups prior to, 1 hour, and 5 hours after administration; a

significant difference between saline (M=5.59) and Cg (M=2.66) groups were observed only 5 hours after injection [$F(1,73)=61.67$, $p<0.001$; Figure 5.2].

A significant 4-way interaction between time, hormone treatment, pain condition, and drug treatment was also observed [$F(4,146)=4.34$, $p<0.01$]. In order to elucidate this interaction further, data was analyzed at each time point—prior to, 1 hour, and 5 hours after Cg or saline administration. Prior to administration, a main effect for drug treatment was seen [$F(2,80)=21.47$, $p<0.001$]. At this time point, PWL was significantly longer in the high-drug-dose group (M=10.08) when compared to the PWL in the control-dose (M=5.06) and low-dose (M=5.48) groups ($p<0.001$). In addition, a significant interaction was observed between hormone treatment, drug treatment, and pain condition [$F(2,80)=4.70$, $p<0.05$]. In the cholesterol group, a significant drug treatment by pain condition interaction was observed [$F(2,38)=6.66$,m $p<0.01$]. In those animals that received treatment with Cg, PWL was significantly longer in the high-dose group (M=13.91) when compared to PWL in the control-dose (M=6.28) and the low-dose (M=3.51) groups ($p<0.001$). One hour post-Cg or –saline administration, a significant main effect for drug treatment [$F(2,80)=5.66$, $p<0.01$] as well as a significant interaction between drug treatment and pain condition [$F(2,80)=3.32$, $p<0.05$] was observed--PWL was significantly longer in the high-dose group (M=6.41) when compared to the control-dose group (M=4.05; $p<0.01$). In the group that received treatment with Cg, however, PWL was significantly shorter in the control-dose group (M=2.97) when compared with the low-(M=5.20; $p<0.05$) and high-dose (M=7.16; $p<0.001$) groups, accounting for the significant drug by pain interaction. Five hours post-Cg or –saline treatment, a significant main effect for pain condition was observed [$F(1,79)=56.00$, $p<0.001$] such that PWL was significantly longer in the saline group (M=5.58) compared to the Cg group (M=2.71; $p<0.001$; Figure 5.2).

Medium Heat. Figure 5.3 represents ipsilateral PWL in response to medium heat for hormone- and drug-treated animals. Significant main effects were observed for hormone treatment, drug treatment, pain condition, and time [Hormone Treatment: $F(1,73)=9.98$, $p<0.01$; Drug Treatment: $F(2,73)=3.48$, $p<0.05$; Pain Condition: $F(1,73)=10.73$, $p<0.01$; Time: $F(2,146)=20.15$, $p<0.001$]. High doses of estradiol caused significantly shorter PWL ($M=3.17$) when compared with the cholesterol group ($M=3.87$, $p<0.01$; Figure 5.3d). Additionally, PWL in the high-drug-dose group ($M=3.97$) was significantly longer compared to the control group ($M=3.26$; $p<0.01$; Figure 5.3e). Animals that received saline treatment displayed significantly longer PWL ($M=3.89$) compared to those that received Cg treatment ($M=3.14$; $p<0.01$; Figure 5.3f). PWL prior to Cg or saline administration ($M=4.32$) was significantly longer compared with PWL 1 hour ($M=3.4$) and 5 hours ($M=2.85$) post-administration ($p<0.001$). Furthermore, PWL 5 hours after Cg or saline injection was significantly shorter compared with PWL 1 hour post-injection ($p<0.05$; Figure 5.3g).

In response to medium heat intensity, significant interactions between drug treatment and pain [$F(2,73)=6.18$, $p<0.001$], between drug treatment and time [$F(4,146)=4.23$, $p<0.01$], and between pain condition and time [$F(2,146)=3.98$, $p<0.05$] were also observed. Significant differences between drug treatment groups in the response to Cg treatment [$F(1,3)=19.74$, $p<0.05$], but not saline treatment, elucidates the significant drug treatment by pain interaction. In the Cg-treated animals, the high-dose of indomethacin caused significantly longer PWL ($M=4.15$) when compared to both the low-dose ($M=2.75$) and the control-dose groups ($M=2.60$; $p<0.05$). Additionally, to clarify the time by drug treatment interaction, different patterns of differences were found between time points in each drug-treatment group. In the control-dose group, baseline PWL ($M=3.88$) was significantly longer compared to PWL 5 hours ($M=2.77$)

after Cg or saline injection [$F(1,73)=6.34$, $p<0.05$]. In the low-dose group, baseline PWL ($M=3.93$) was significantly longer compared with PWL 1 hour ($M=3.02$) after injection [$F(1,73)=4.02$, $p<0.05$]. In the high-dose drug group, PWL prior to Cg or saline administration ($M=5.27$) was significantly longer when compared with PWL 1 hour [$M=4.24$; $F(1,73)=5.07$, $p<0.05$] and 5 hours [$M=2.40$; $F(1,73)=32.74$, $p<0.001$] post-injection. Additionally, PWL 1 hour post-Cg or saline administration was significantly longer than PWL 5 hours post-administration [$F(1,73)=25.36$, $p<0.001$]. To support the significant pain condition by time interaction, PWL was significantly longer in the saline-treated group 5 hours post-Cg or –saline injection ($M=3.61$) when compared to the Cg-treated group [$M=2.03$; $t(89)=6.59$, $p<0.001$; Figure 5.3].

High Heat. Figure 5.4 represents ipsilateral PWL in response to high heat intensity for hormone- and drug-treated animals. Significant main effects for hormone treatment, drug treatment, and time were observed [Hormone Treatment: $F(1,75)=4.14$, $p<0.05$; Drug Treatment: $F(2,75)=11.36$, $p<0.001$; Time: $F(2,150)=32.62$, $p<0.001$]. Although mean PWL was higher in animals that received vehicle treatment when compared to those that received estradiol treatment, this difference did not reach significance during post hoc analysis (Figure 5.4d). PWL in the high-drug-dose group ($M=2.87$) was significantly longer when compared to PWL in the low-dose ($M=2.22$) and control-dose ($M=2.32$) groups ($p<0.001$; Figure 5.4e). Prior to Cg or saline administration, PWL was significantly longer ($M=2.97$) when compared to PWL 1 hour ($M=2.56$) and 5 hours ($M=1.87$) post-administration ($p<0.01$). Additionally, PWL 1 hour after Cg or saline treatment was significantly longer compared to PWL 5 hours post-treatment ($p<0.001$; Figure 5.4f).

Significant interactions between time and hormone treatment [$F(2,150)=4.12$, $p<0.05$], between time and drug treatment [$F(4,150)=5.83$, $p<0.001$] were observed. To elucidate the nature of the significant time by hormone treatment interaction, PWL was significantly shorter in the estradiol group ($M=2.26$) when compared to the cholesterol group ($M=2.92$) 1 hour after saline or Cg administration [$F(1,75)=13.19$, $p<0.001$], but not prior to administration or 5 hours post-administration. Furthermore, post hoc analyses following up the significant time by drug treatment interaction show that PWL prior to Cg or saline treatment and 1 hour post-treatment differed in the drug-treatment groups, but did not 5 hours after treatment. At baseline readings, PWL in the high-dose group ($M=3.72$) was significantly longer when compared with the control group [$M=2.74$; $F(1,75)=7.44$, $p<0.05$] and the low-dose group [$M=2.47$; $F(1,75)=12.95$, $p<0.01$]. The same pattern was observed 1 hour after Cg or saline administration: PWL in the high-dose group ($M=3.22$) was significantly longer than PWL in the control group [$M=2.20$; $F(1,75)=19.43$, $p<0.001$] and the low-dose group [$M=2.37$; $F(1,75)=14.49$, $p<0.001$; Figure 5.4].

Significant interactions were also observed between drug treatment and pain condition [$F(2,75)=5.96$, $p<0.01$] and between time and pain condition [$F(2,150)=6.02$, $p<0.01$] in response to high heat intensity. To elucidate the significant drug treatment by pain condition interaction, significant differences between drug treatment groups were only observed in the animals treated with Cg [$F(2,45)=9.90$, $p<0.001$], but not saline ($p>0.05$). In those animals treated with Cg, PWL was significantly longer over time in the high-drug-dose group ($M=3.09$) when compared with the control group ($M=2.10$) and the low-dose group ($M=2.04$; $p<0.001$). The time by pain condition interaction can be explained by significant differences between Cg- and saline-treated animals 5 hours after administration [$t(90)=6.17$, $p<0.001$]. Five hours after Cg or saline treatment, PWL was significantly longer in the saline-treated animals ($M=2.19$)

when compared with those animals that received Cg treatment (M=1.51). No differences were found between groups prior to Cg or saline administration or 1 hour post-administration ($p>0.05$; Figure 5.4).

In response to high heat intensity, a three-way interaction between time, drug treatment, and pain condition was also observed [$F(4,150)=2.49$, $p<0.05$]. When looking at the pain condition groups separately, a significant interaction between time and drug treatment was seen in the Cg-treated group [$F(2,78)=22.54$, $p<0.001$] but not the saline-treated group ($p>0.05$). This interaction is explained by diverging patterns of differences in PWL prior to, and 1 and 5 hours after Cg administration in the drug treatment groups. In the control group, PWL was significantly longer prior to Cg administration (M=2.77) when compared to 5 hours post-Cg administration [$F(1,39)=10.02$, $p<0.01$]. In the high-dose group, PWL was significantly shorter 5 hours post-Cg administration (M=9.09) when compared to baseline PWL [M=11.65; $F(1,39)=32.92$, $p<0.001$] and PWL 1 hour post-administration [M=11.35; $F(1,39)=46.27$, $p<0.001$; Figure 5.4].

Effects of estradiol and indomethacin treatment on contralateral PWL prior to, 1 hour, and 5 hours after saline or Cg administration.

Low Heat. Figure 5.5 represents contralateral PWL in response to low heat intensity for hormone- and drug-treated animals. Significant main effects for drug treatment and time were observed [Drug Treatment: $F(2,76)=9.79$, $p<0.001$; Time: $F(2,152)=20.03$, $p<0.001$]. PWL was significantly longer in the high-drug-dose group (M=7.79) when compared with both the control-dose (M=5.32) and the low-drug-dose (M=5.98) groups ($p<0.01$; Figure 5.5d). Furthermore, PWL prior to Cg or saline treatment was significantly longer (M=7.98) when compared with

PWL 1 hour (M=6.07) and 5 hours (M=5.04) post-treatment ($p<0.001$). Additionally, PWL was significantly longer 1 hour post-Cg or –saline administration when compared to PWL 5 hours post-administration ($p<0.05$; Figure 5.5e).

In addition to significant main effects, significant interaction between hormone and drug treatments [$F(2,76)=3.41$, $p<0.05$], and between time and drug treatment [$F(4,152)=10.42$, $p<0.001$] were observed. To elucidate the significant hormone by drug interaction, PWL in the control-drug-dose group was significantly longer in the cholesterol-treated group (M=6.06) when compared with the estradiol-treated group [M=4.91; $t(29)=2.10$, $p<0.05$]. This effect was not seen in either of the indomethacin-treated groups ($p>0.05$). To further explain the significant time by drug interaction, significant differences between baseline, 1 hour, and 5 hour readings were observed only in the high-drug-dose group. In the high-dose group, PWL prior to Cg or saline treatment (M=11.40) was significantly longer than PWL 1 hour [M=7.80; $F(1,76)=18.67$] and 5 hours [M=4.16; $F(1,76)=65.56$] post-treatment ($p<0.001$). In addition, PWL 5 hours post-treatment was significantly shorter compared to PWL 1 hour post-treatment [$F(1,76)=24.43$, $p<0.001$; Figure 5.5].

Medium Heat. Figure 5.6 represents contralateral PWL in response to medium heat intensity for hormone- and drug-treated animals. Significant main effects for drug treatment and time were observed [Drug Treatment: $F(2,77)=5.58$, $p<0.01$; Time: $F(2,154)=4.82$, $p<0.01$]. PWL was significantly longer in the high-drug-dose group (M=4.78) when compared to both the control group (M=3.59) and the low-dose group (M=3.82; $p<0.05$; Figure 5.6d). Additionally, PWL was significantly shorter 5 hours after Cg or saline administration (M=3.58) when compared to baseline readings (M=4.49; $p<0.01$; Figure 5.6e).

Significant interactions between hormone and drug treatments [$F(2,77)=7.32$, $p<0.01$] and between time and drug treatment [$F(4,154)=6.55$, $p<0.001$] were also observed. In the high-drug-dose group, PWL was significantly longer in those animals that received pretreatment with cholesterol ($M=5.59$) than those that received estradiol [$M=3.83$; $t(30)=2.36$, $p<0.05$]. The cholesterol and estradiol groups did not significantly differ in the control or low-dose groups. To elucidate the nature of the significant time by drug interaction, significant differences were observed in the high-drug-dose group between PWL prior to, 1 hour, and 5 hours after Cg or saline administration. In the high-dose group, PWL was significantly longer prior to administration ($M=6.17$) when compared to PWL at both 1 hour [$M=4.91$; $F(1,77)=7.64$, $p<0.01$] and 5 hours [$M=3.27$; $F(1,77)=28.97$, $p<0.001$] post-administration. Furthermore, PWL was significantly shorter 5 hours after Cg or saline treatment than PWL 1 hour post-treatment [$F(1,77)=11.98$, $p<0.001$; Figure 5.6].

In addition to the significant main effects and two-way interactions, a significant three-way interaction was observed between time, hormone treatment, and drug treatment [$F(4,154)=3.38$, $p<0.05$]. This significant interaction can be explained by different patterns of differences in PWL in the cholesterol and estradiol groups before and after treatment with Cg or saline. Before treatment and 1 hour post-treatment, significant interactions between drug and hormone treatments was observed [Baseline: $F(2,87)=3.84$, $p<0.05$; 1 Hour: $F(2,88)=8.69$, $p<0.001$]. In both cases, significant differences in PWL in the different drug treatment groups were observed only in the cholesterol-treated group [Baseline: $F(2,43)=8.31$, $p<0.001$; 1 Hour: $F(2,43)=6.77$, $p<0.01$]. Furthermore, prior to Cg or saline injection, PWL was significantly longer in the high-dose group ($M=7.30$) when compared with the control group ($M=3.27$) and the low-dose group (3.32; $p<0.01$). The same pattern was observed 1 hour post-injection; PWL

was significantly longer in the high-dose group (M=6.43) when compared with the control group (M=3.36) and the low-dose group (3.74; $p<0.01$). No significant interactions were observed 5 hours post-Cg or –saline treatment ($p>0.05$; Figure 5.6).

High Heat. Figure 5.7 represents contralateral PWL in response to high heat intensity for hormone- and drug-treated animals. Significant main effects for hormone treatment, drug treatment, and time were observed [Hormone Treatment: $F(1,77)=8.56$, $p<0.01$; Drug Treatment: $F(2,77)=4.97$, $p<0.01$; Time: $F(2,154)=7.67$, $p<0.001$]. PWL was significantly longer in the cholesterol-treated group (M=3.06) when compared to the estradiol-treated group (M=2.70; $p<0.01$; Figure 5.7d). Furthermore, PWL was significantly longer in the high-drug-dose group (M=3.13) than in the control (M=2.66) and the low-dose (M=2.83) groups ($p<0.05$; Figure 5.7e). PWL was also significantly shorter 5 hours after Cg or saline treatment (M=2.57) when compared to PWL prior to treatment (M=3.12) and 1 hour post-treatment (M=2.94; $p<0.01$; Figure 5.7f).

A significant interaction between hormone and drug treatments was also observed [$F(2,77)=4.44$, $p<0.05$]. Only in the high-drug-dose group, PWL in the cholesterol group (M=3.53) was significantly longer than PWL in the estradiol-treated group [M=2.75; $t(30)=4.07$, $p<0.001$; Figure 5.7].

Furthermore, a significant interaction was observed between time, hormone treatment, and drug treatment [$F(4,154)=2.86$, $p<0.05$]. One hour after Cg or saline administration, a significant interaction between hormone and drug treatments was observed [$F(2,86)=8.73$, $p<0.001$]. PWL was significantly longer in the high-drug-dose group (M=4.12) when compared to the control (M=2.39) and low-dose (M=2.85) groups ($p<0.01$). A significant hormone by drug

interaction was neither observed prior to Cg or saline treatment nor 5 hours after treatment (Figure 5.7).

Effect of indomethacin and estradiol treatment on paw size in Cg- and saline-treated OVX mice. Figure 5.8 shows mean paw size in estradiol- and indomethacin-treated groups as well as their respective controls. Significant main effects for drug treatment, pain treatment, and injection site (ipsilateral versus contralateral) were seen [Drug Treatment: $F(2,83)=6.14$, $p<0.01$; Pain Treatment: $F(1,83)=13.03$, $p<0.001$; Injection Site: $F(1,83)=286.79$, $p<0.001$]. Paw size was significantly smaller in the high-drug-dose group ($M=8.11$) when compared to the control-dose ($M=9.27$) and low dose groups ($M=8.67$; $p<0.001$). Additionally, paw size was significantly larger in the Cg-treated group ($M=9.16$) when compared with the saline-treated group ($M=8.18$; $p<0.001$). Paw size was also larger in the ipsilateral paw ($M=10.35$) when compared with the contralateral paw ($M=7.01$; $p<0.001$).

A significant injection site by pain treatment interaction was also observed [$F(1,83)=52.5$, $p<0.001$]. To clarify this significance, in saline-treated animals, paw size was significantly larger in the contralateral paw ($M=9.14$) when compared to the ipsilateral paw [$M=7.22$; $t(46)=7.43$, $p<0.001$]. In Cg-treated animals, however, paw size was significantly larger in the ipsilateral paw ($M=11.53$) when compared to the contralateral paw [$M=6.79$; $t(47)=15.52$, $p<0.001$]. Additionally, ipsilateral paw size was significantly larger in Cg-treated animals ($M=11.53$) when compared to saline-treated animals [$M=9.14$; $t(47)=5.67$, $p<0.001$]. Paw size did not differ between groups in the contralateral paw.

5.3. Discussion

We have compared the effect that estrogen exerts on PWL in animals treated with high and low doses of indomethacin. First, we found that indomethacin significantly decreased paw edema subsequent to Cg administration. Second, our findings show that high, but not low, doses of indomethacin significantly decrease baseline nociceptive behaviors. We also show that in the injected paw, indomethacin only attenuates pain behaviors in those animals treated with the inflammatory stimulus. In the untreated paw, indomethacin abolished the hyperalgesic effect estradiol had on PWL in response to the low heat stimulus. On the other hand, in response to a more intense heat stimulus, no differences were seen between the drug treatment groups in estradiol-treated mice in the untreated paw.

These results support the idea that indomethacin's anti-nociceptive effects are due to a blockage of COX-1, but not COX-2. Treatment with indomethacin significantly decreased nociceptive behavior in OVX mice pretreated with estradiol prior to inflammatory insult. Since COX-1 is a constituent part of nervous system tissues and COX-2 is synthesized in response to inflammation or injury (Meyer, et al., 2005), the fact that indomethacin decreases pain behaviors prior to inflammation indicates that indomethacin is decreasing inflammation by depleting COX-1 production in the nervous system. Although less studied than COX-2, the COX-1 enzyme has been linked to some inflammatory conditions, including spinal cord injury (Moalem & Tracey, 2006).

Our findings also suggest that there may be some interaction between estradiol and the prostanoid system subsequent to inflammation. Not only did pretreatment with estradiol attenuate the interaction between inflammation and drug treatment, in the uninjured paw, indomethacin reverses estrogen's effect in response to a mild stimulus, indicating that estrogen's

hyperalgesic effects may be due to a COX-dependent mechanism. This is supported by a study conducted by Li & Stallone (2005) which shows an interaction of estradiol and COX in the circulatory system. In addition, in response to a medium or high heat stimulus, treatment with estradiol eliminated the effect that indomethacin had on nociceptive behaviors, indicating that insult with a stronger stimulus may be mediated through a COX-independent mechanism, such as regulation of the sympathetic amines. Additionally, since indomethacin had an effect on both Cg-treated and untreated paws, our results indicate that indomethacin may be acting systemically. Research has shown that topical applications of indomethacin at the site of inflammation decrease edema by 65% and inhibit neutrophil infiltration into the injured area in both the indomethacin-treated ear and the un-treated ear (Bralley, et al., 2008; Gordon, et al., 2007).

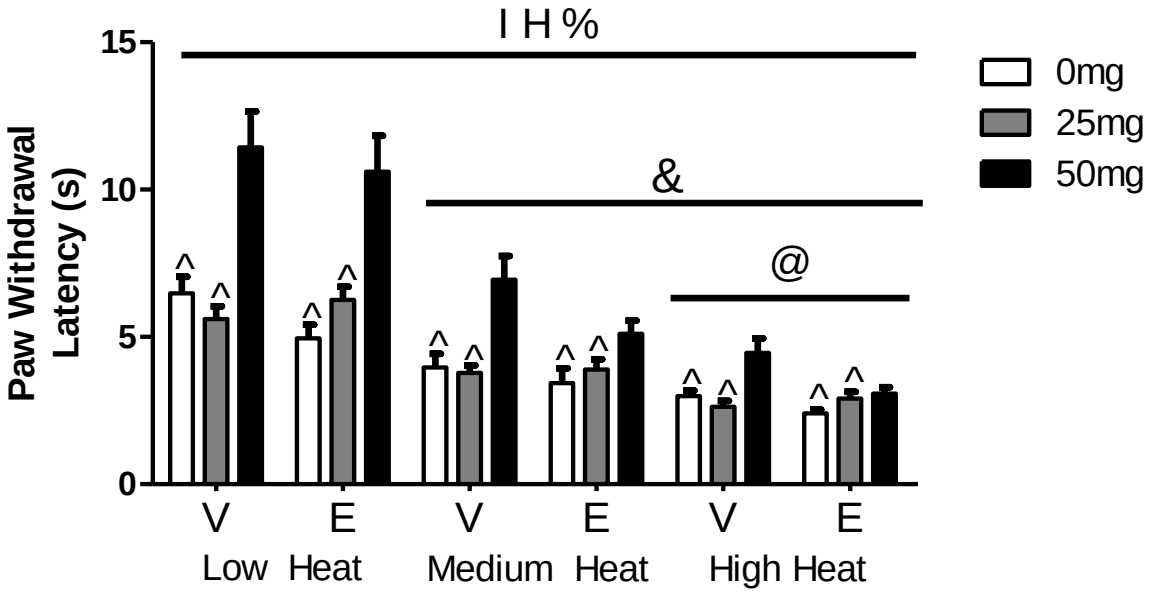

Figure 5.1. Effects of hormone and indomethacin treatment on baseline PWL at low, medium, and high heat intensities. Data represent mean PWL (±SEM) after pretreatment with either vehicle (V) or estradiol (E) averaged across both paws immediately after DMSO or indomethacin treatment and prior to Cg or saline administration. (I) denotes a significant main effect for drug treatment. (H) indicates a significant main effect for heat intensity. (%) denotes a significant heat intensity by drug treatment interaction. (&) indicates a significant difference to low heat intensity (@) denotes a significant difference in PWL to the medium heat intensity. (^) signifies a significant difference to the high-drug-dose group. All p values were <0.001. *n*=14-16 per group.

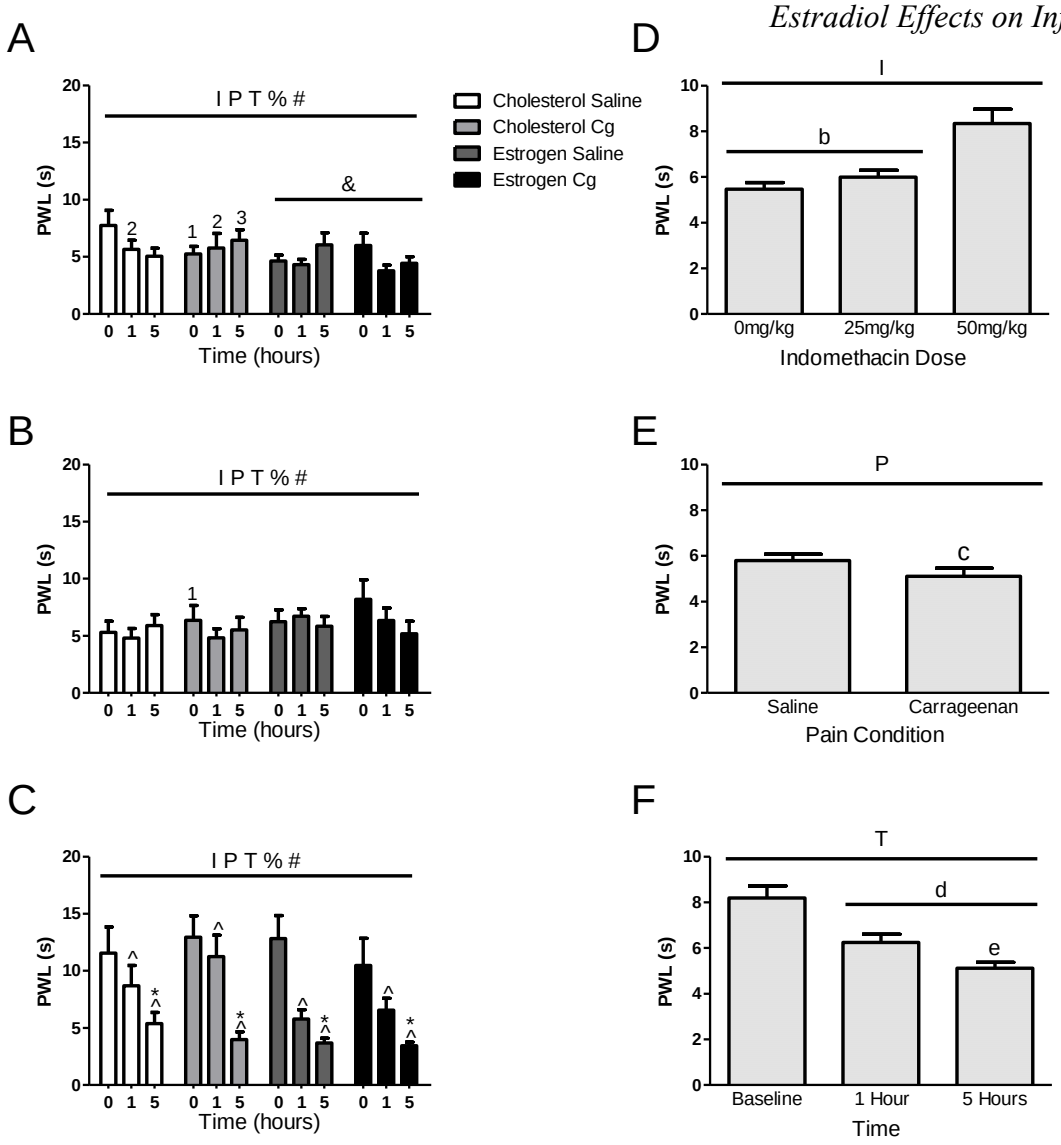

Figure 5.2. Effects of estradiol and indomethacin administration on PWL to low heat intensity prior to, 1 hour, and 5 hours after Cg or saline administration in OVX C57BL/6N mice. Data represent mean PWL (±SEM) in control dose (A), low dose (B), and high dose (C) drug treatment groups. Figures D-F represent main effects of drug treatment (D), pain condition (E), and time (F). (I) denotes a significant main effect for drug treatment. (P) denotes a significant main effect for pain treatment. (T) signifies a significant time main effect. (%) signifies a significant time by indomethacin interaction. (#) denotes a significant time by pain interaction. (@) represents a significant 4-way interaction. (b) denotes a significant difference to the high-drug-dose group. (c) signifies a significant difference to the Cg-treated group. (d) denotes a significant difference to baseline PWL. (e) represents a significant difference to PWL 1 hour post-injection. (1) denotes a significant difference to the high-dose group prior to Cg or saline administration in the animals that received both Cg and cholesterol. (2) signifies a significant difference to the high-dose group 1 hour post-administration in the cholesterol group. (3) indicates a significant difference to the saline-treated animals in the cholesterol group. All p values <0.05. *n*=7-8 per group.

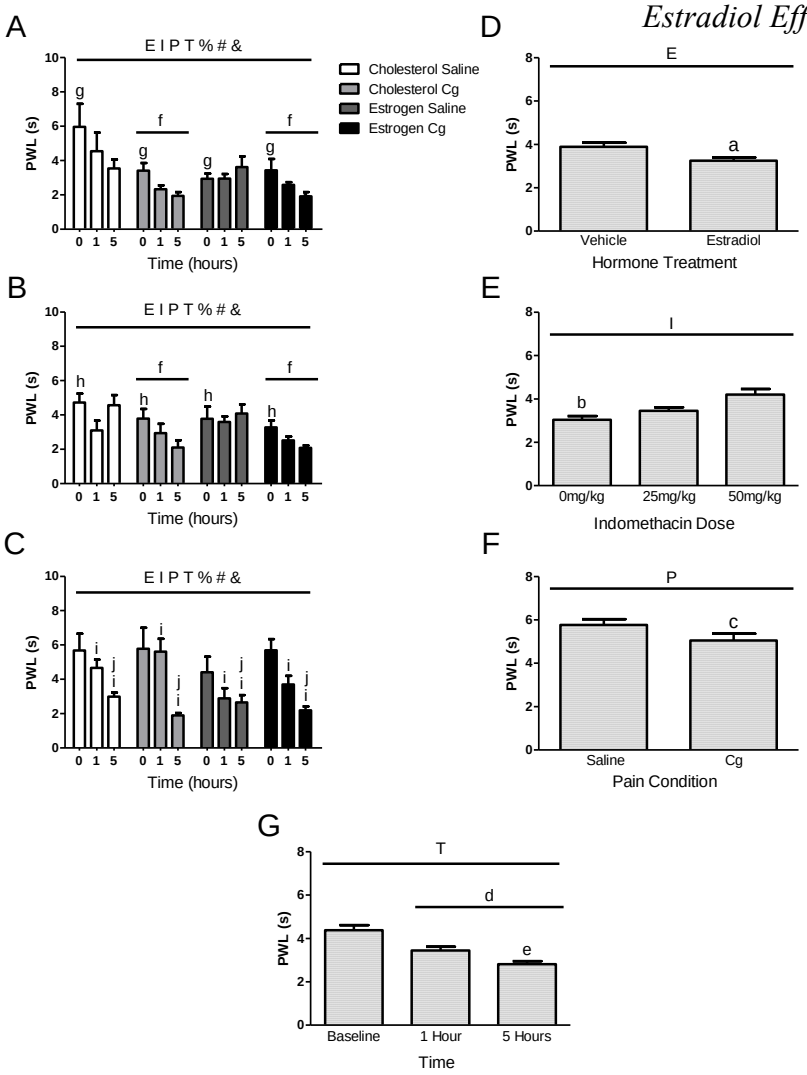

Figure 5.3. Effects of estradiol and indomethacin administration on ipsilateral PWL to medium heat prior to, 1 hour, and 5 hours after Cg or saline administration. Data represent mean (±SEM) PWL in OVX C57BL/6N mice that received the control-dose (A), low-dose (B), and high dose (C) of indomethacin. Figures D-G represent significant main effects of hormone treatment (D), drug treatment (E), pain condition (F), and time (G). (E) signifies a significant hormone main effect. (I) denotes a significant drug treatment main effect. (P) denotes a significant pain condition main effect. (T) represents a significant time main effect. (%) denotes a significant time by pain treatment interaction. (#) signifies a significant time by pain condition interaction. (&) represents a significant drug treatment by pain condition interaction. (a) denotes a significant difference to the cholesterol group. (b) represents a significant difference to the high-dose drug treatment group. (c) signifies a significant difference to the saline-treated group. (d) denotes a significant difference to baseline PWL. (e) represents a significant difference to PWL 1 hour after Cg or saline treatment. (f) signifies a significant difference to the high-dose drug group in Cg-treated animals. (g) denotes a significant difference in PWL 5 hours after Cg or saline treatment in the control-dose drug group. (h) represents a significant difference in PWL 1 hour post-treatment in the low-drug-dose group. (i) denotes a significant difference to baseline PWL in the high-drug-dose group. (j) signifies a significant difference to in PWL 1 hour post-treatment in the high-dose group. All p values < 0.05. *n*=7-8.

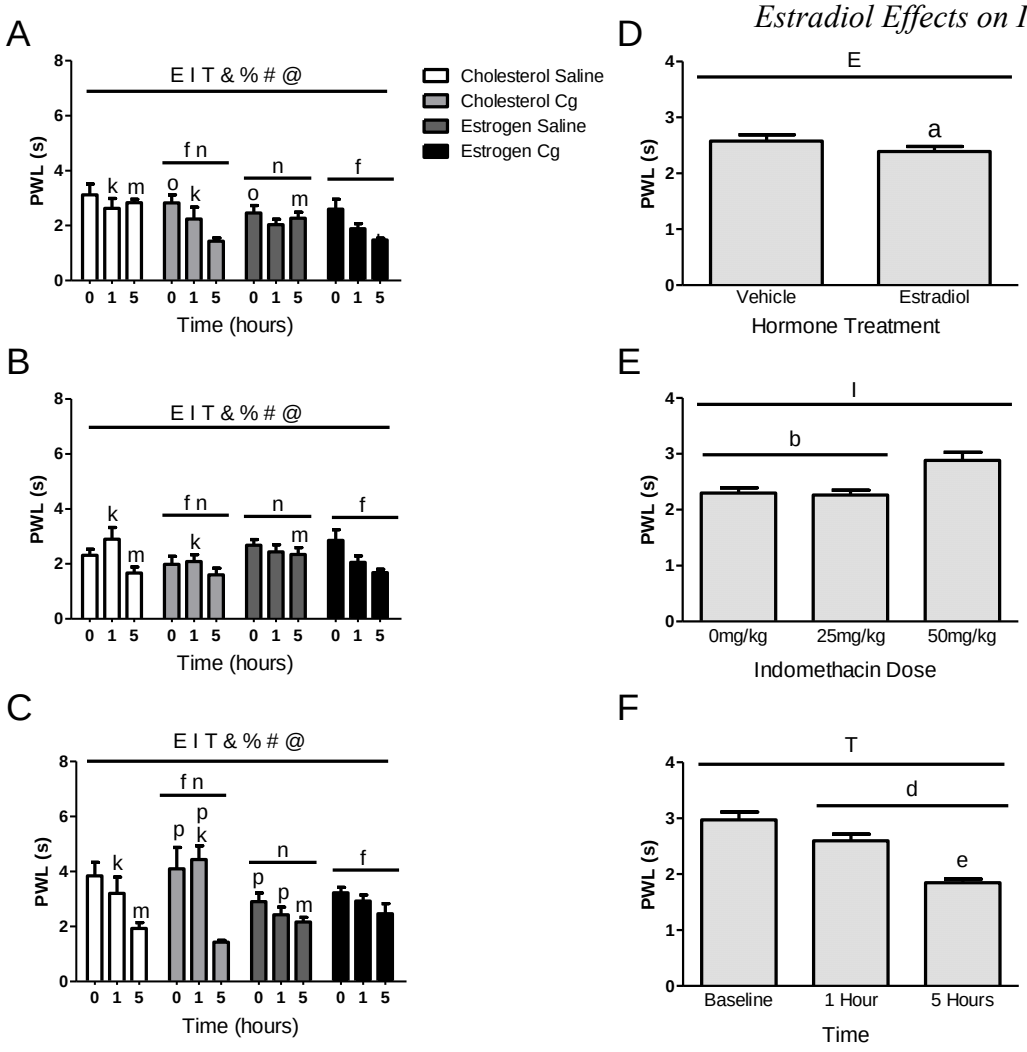

Figure 5.4. Effects of estradiol and indomethacin administration on ipsilateral PWL to high heat prior to, 1 hour, and 5 hours after Cg or saline administration. Data represent mean (±SEM) PWL in OVX C57BL/6N mice that received the control-dose (A), low-dose (B), and high dose (C) of indomethacin. Figures D-F represent significant main effects of hormone treatment (D), drug treatment, (E) and time (F). (E) signifies a significant hormone main effect. (I) denotes a significant drug treatment main effect. (T) represents a significant time main effect. (%) denotes a significant time by pain treatment interaction. (#) signifies a significant time by pain condition interaction. (&) represents a significant drug treatment by pain condition interaction. (@) denotes a significant time by hormone treatment interaction. (a) denotes a significant difference to the cholesterol group. (b) represents a significant difference to the high-dose drug treatment group. (d) denotes a significant difference to baseline PWL. (e) represents a significant difference to PWL 1 hour after Cg or saline treatment. (f) signifies a significant difference to the high-dose drug group in Cg-treated animals. (k) denotes a significant difference from the estradiol group 1 hour post Cg or saline treatment. (m) signifies a significant difference in PWL to the Cg group 5 hours post-treatment. (n) represents a significant time by drug treatment interaction. (o) signifies a significant to PWL 5 hours after treatment in Cg-treated animals in the control-drug-dose group. (p) denotes a significant difference compared to PWL 5 hours post-administration in the high-drug-dose group. All p values < 0.05. *n*=7-8.

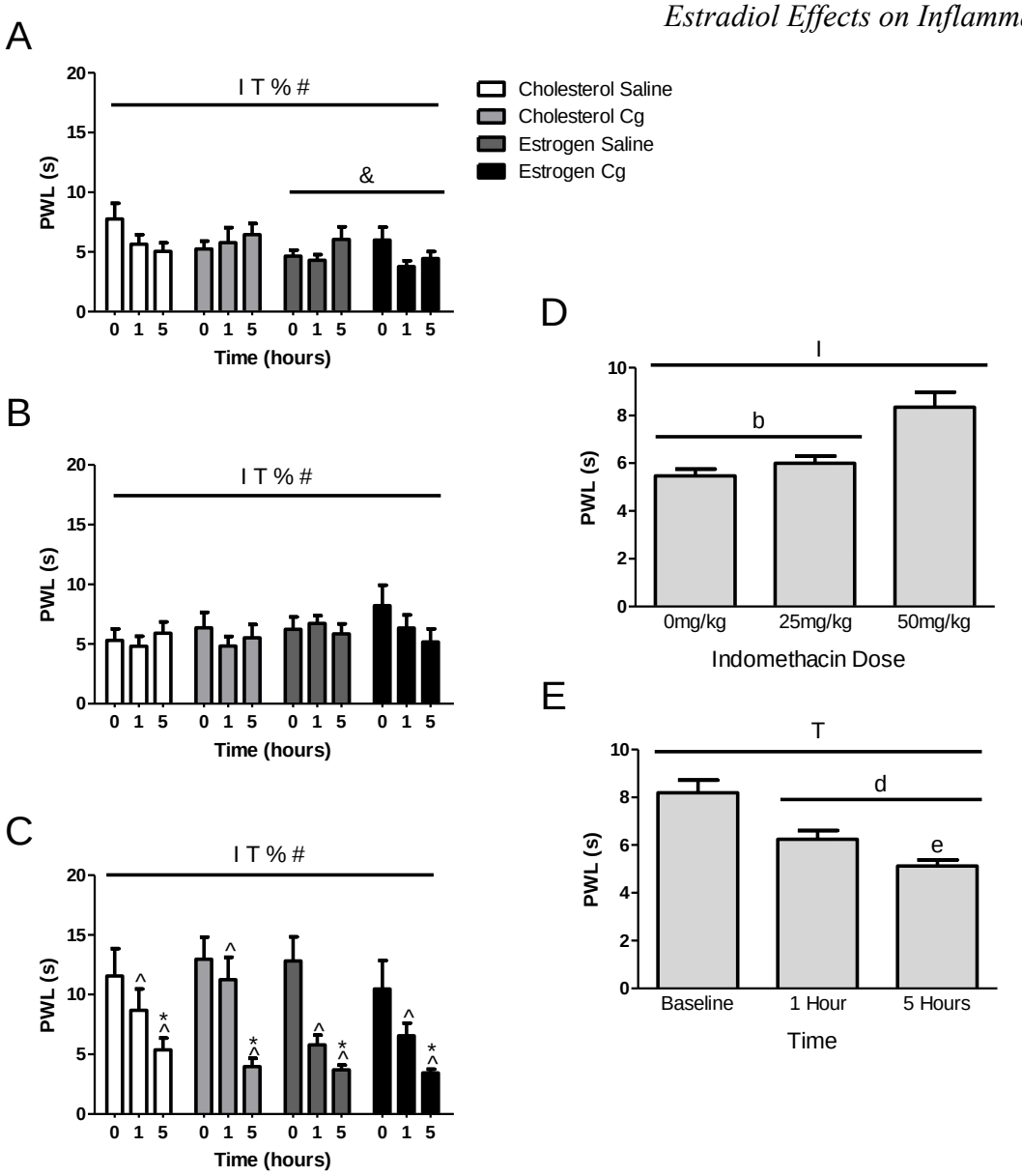

Figure 5.5. Effects of estradiol and indomethacin administration on contralateral PWL to low heat prior to, 1 hour, and 5 hours after Cg or saline administration. Data represent mean (±SEM) PWL in OVX C57BL/6N mice that received the control-dose (A), low-dose (B), and high dose (C) of indomethacin. Figures D-E represent significant main effects of drug treatment (D) and time (E). (I) represents a significant drug treatment main effect. (T) denotes a significant time main effect. (%) signifies a significant time by drug interaction. (#) represents a significant hormone by drug interaction. (b) indicates a significant difference to the high-drug-dose group. (d) signifies a significant difference to PWL at baseline. (e) denotes a significant difference to PWL 1 hour post-Cg or –saline administration. (&) represents a significant difference to the cholesterol group in the control-dose group. (^) signifies a significant difference to PWL prior to Cg- or saline treatment in the high-dose group. (*) represents a significant difference to PWL 1 hour post-treatment in the high-dose group. All p values < 0.05. *n*=7-8.

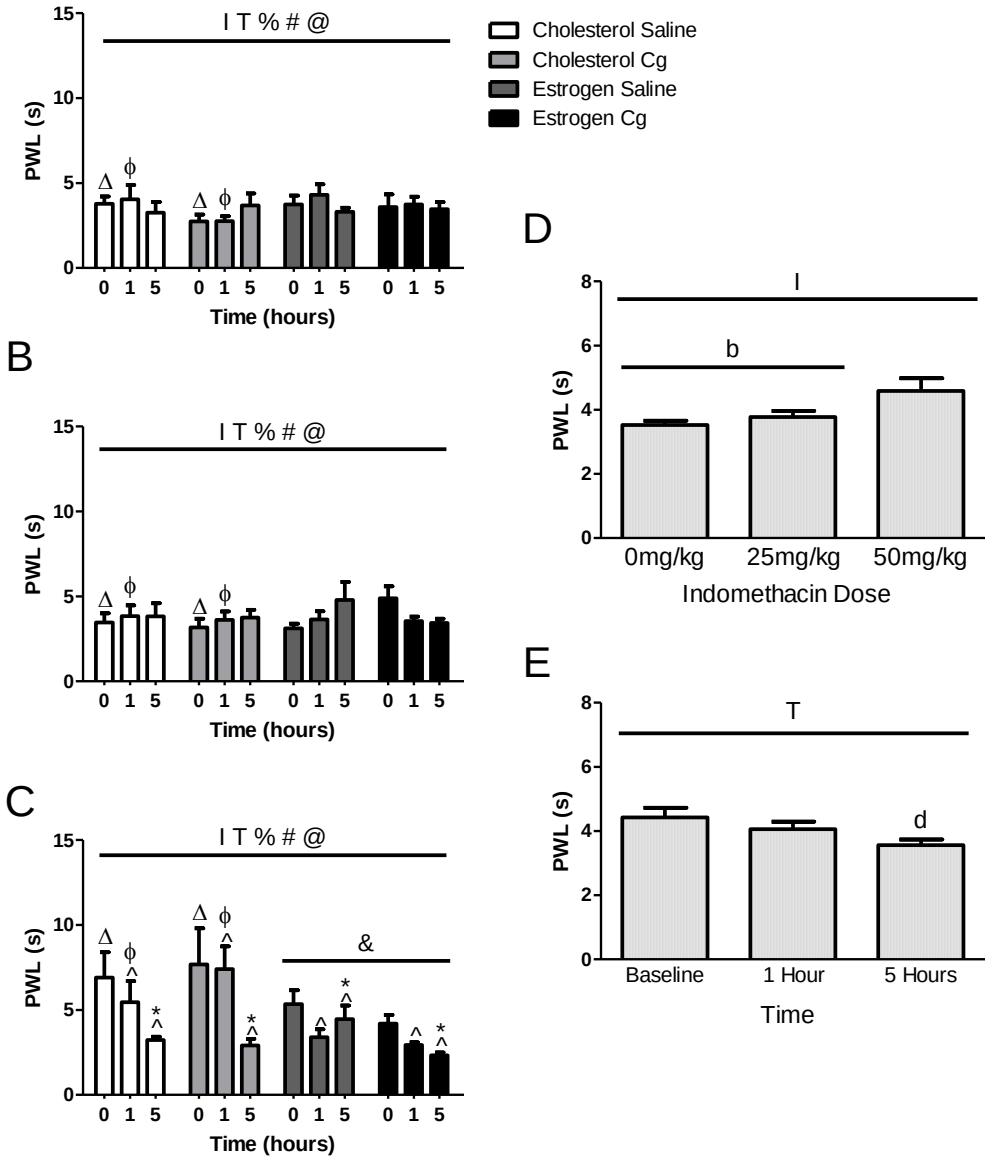

Figure 5.6. Effects of estradiol and indomethacin administration on contralateral PWL to medium heat prior to, 1 hour, and 5 hours after Cg or saline administration. Data represent mean (±SEM) PWL in OVX C57BL/6N mice that received the control-dose (A), low-dose (B), and high dose (C) of indomethacin. Figures D-E represent significant main effects of drug treatment (D) and time (E). (I) represents a significant drug treatment main effect. (T) denotes a significant time main effect. (%) signifies a significant time by drug interaction. (#) represents a significant hormone by drug interaction. (@) denotes a significant time by hormone by drug interaction. (b) indicates a significant difference to the high-drug-dose group. (d) signifies a significant difference to PWL at baseline. (&) represents a significant difference to the cholesterol group in the high-dose group. (^) signifies a significant difference to PWL prior to Cg- or saline treatment in the high-dose group. (*) represents a significant difference to PWL 1 hour post-treatment in the high-dose group. (Δ) signifies a significant difference to baseline PWL in the estradiol group. (Φ) denotes a significant difference to PWL 1 hour post-Cg or –saline treatment in the estradiol group. All p values < 0.05. *n*=7-8.

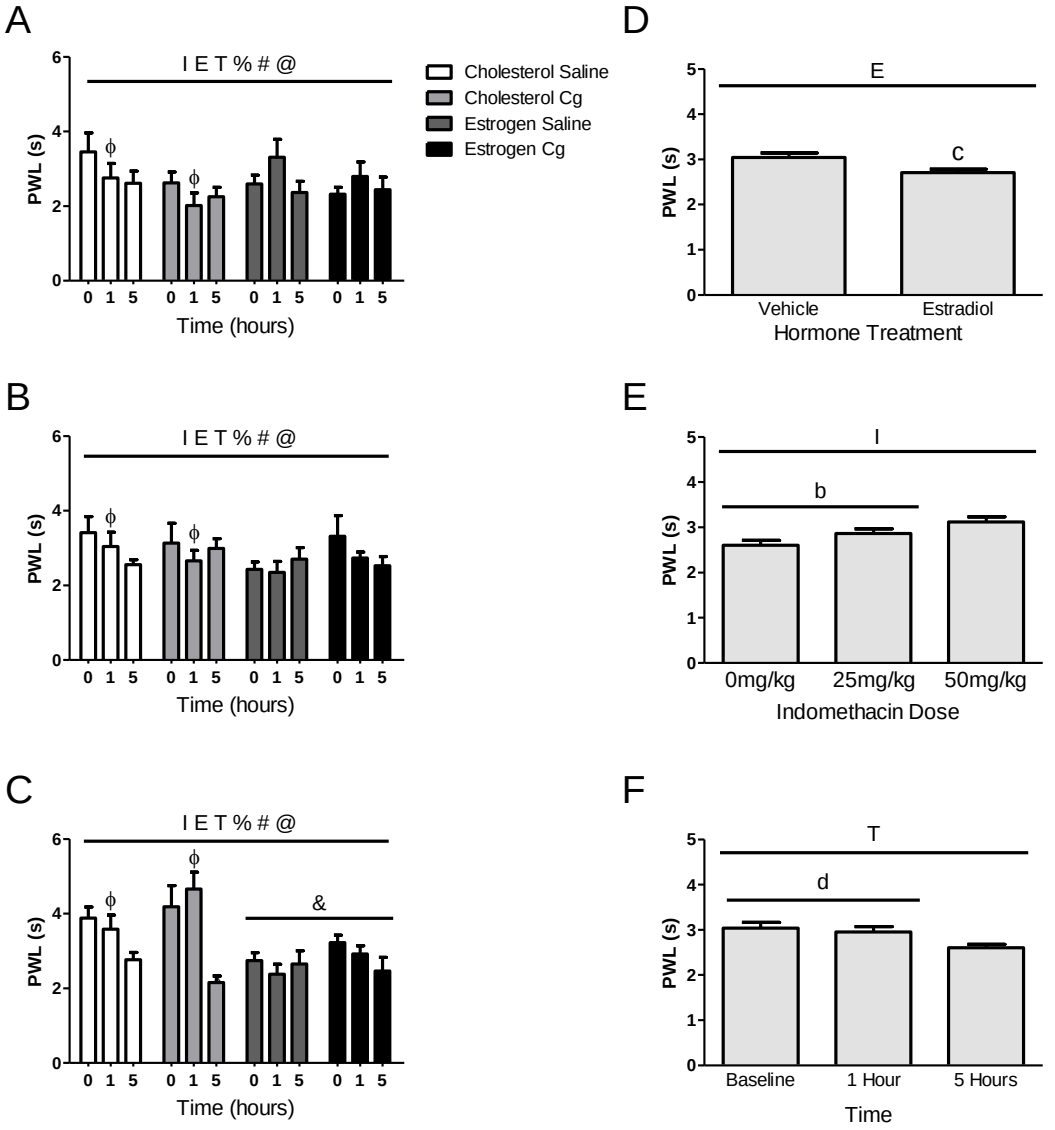

Figure 5.7. Effects of estradiol and indomethacin administration on contralateral PWL to high heat prior to, 1 hour, and 5 hours after Cg or saline administration. Data represent mean (±SEM) PWL in OVX C57BL/6N mice that received the control-dose (A), low-dose (B), and high dose (C) of indomethacin. Figures D-F represent significant main effects of hormone treatment (D), drug treatment (E) and time (F). (I) represents a significant drug treatment main effect. (E) signifies a significant main effect for hormone treatment. (T) denotes a significant time main effect. (%) signifies a significant time by drug interaction. (#) represents a significant hormone by drug interaction. (@) denotes a significant time by hormone by drug interaction. (b) indicates a significant difference to the high-drug-dose group. (c) denotes a significant difference to the vehicle group. (d) signifies a significant difference to PWL at baseline. (&) represents a significant difference to the cholesterol group in the high-dose group. (Φ) denotes a significant difference to PWL 1 hour post-Cg or –saline treatment in the estradiol group. All p values < 0.05. *n*=7-8.

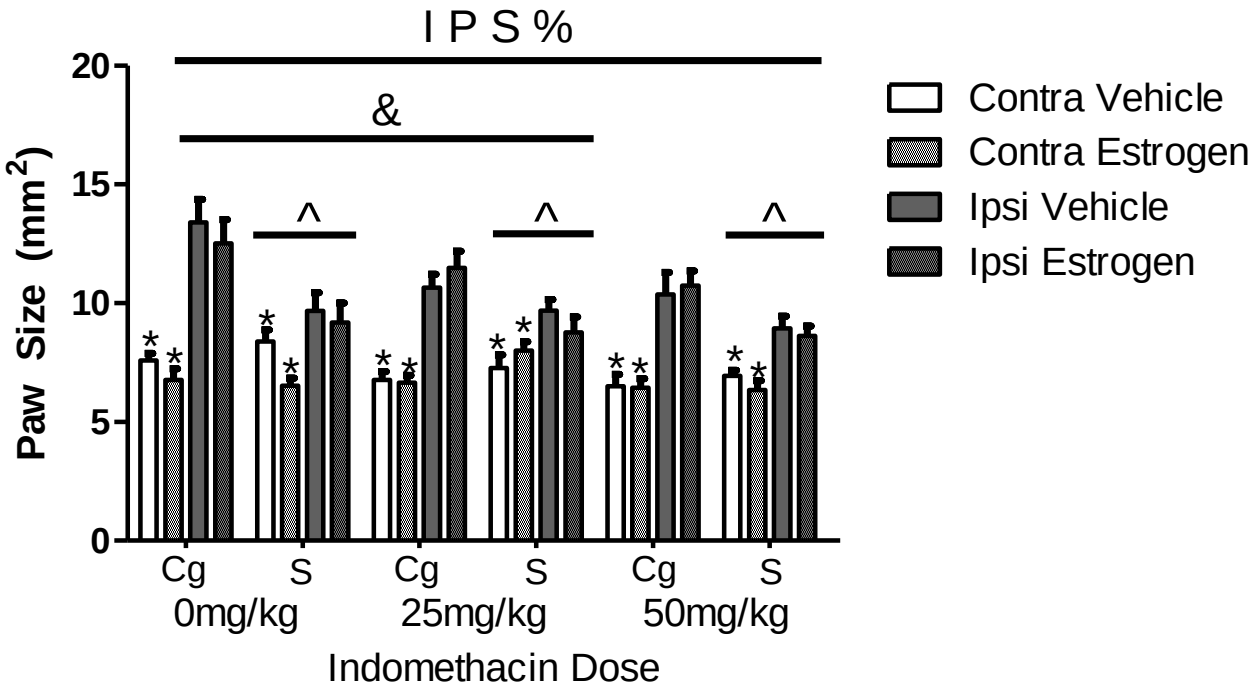

Figure 5.8. Effects of indomethacin and estradiol treatment on paw size after a 1% Cg or saline injection. Data represent mean paw size (±SEM) in all drug and estradiol-treated groups as well as their controls. (I) signifies a significant main effect for drug treatment. (P) represents a significant main effect for pain condition. (S) denotes a significant main effect for paw side (ipsilateral versus contralateral paw). (%) represents a significant paw side by pain treatment interaction. (&) signifies a significant difference to the high-drug-dose group. (^) represents a significant difference to the Cg-treated groups. (*) indicates a significant difference to contralateral paw size. All p values <0.01. *n*=7-8.

Chapter 6: Conclusions

The experiments presented in this thesis were conducted in order to elucidate the role 17β-estradiol plays on nociceptive behaviors and inflammatory mediators in a murine model. Previous experiments in this lab have shown that estradiol has anti-hyperalgesic effects on behavioral responses and cytokine production in rats. However, our results indicate that high doses of estradiol have hyperalgesic effects in mice. Contrary to what was predicted in our hypotheses, estradiol increased nociceptive behaviors in C57BL/6N mice. Furthermore, estradiol did not have a significant effect on cytokine levels at the site of injection or in the CNS. Our results also show that indomethacin blocked estradiol's effects to a low heat stimulus in the contralateral, but not ipsilateral paw, suggesting that estradiol may interact with prostanoid production. Together, our findings show that estradiol is effective at exacerbating inflammatory behaviors in mice, although this is not due to an increase in proinflammatory cytokines or a decrease in anti-inflammatory cytokines. Estradiol may be working by enhancing PG output, but this is not the sole mechanism at work.

The studies used to address Aim 1 provide evidence that, unlike in rats, estradiol causes an increase in pain behaviors in OVX mice and that this effect was strain dependent; estradiol showed an effect in C57BL/6N but not 129S6 mice. We found that high doses of estradiol exacerbate inflammatory behaviors to low, medium, and high heat stimuli up to 5 hours after insult with 1% Cg. Results also show that estradiol is effective at increasing inflammatory behaviors in the untreated paw, indicating a role of central sensitization in this model. These results contradict previous findings in our lab. Shivers (2009; personal communication) found that estradiol attenuates Cg-induced nociceptive behaviors in rats. Additionally, Kuba and colleagues (2006) found that estradiol also attenuates formalin-induced nociceptive behaviors in

rats. Our results, however, support research conducted by Kayser and colleagues (1996) that show elevated pain behaviors to mechanical stimulation in rats in the proestrus stage of the estrous cycle, a stage marked by high levels of estrogen. In addition, Calippe and colleagues (2008) found that estradiol causes increases in multiple inflammatory mediators, which could cause a subsequent increase in nociceptive behaviors. Additionally, and most importantly, the results obtained from this study support clinical evidence that shows exogenous estrogen causes an increase in pain states like migraine headaches and temporomandibular pain in human subjects (Martin & Behbehani. 2006; Warren & Fried, 2001). Studies should continue to be conducted in a murine model to investigate the mechanisms by which estrogen causes central sensitization and a subsequent increase in inflammation and pain behaviors due to the high correlation between effects seen in humans and those seen in mice.

Additionally, we found that, overall, estradiol did not exacerbate thermal hyperalgesia in 129S6 mice. This indicates that estradiol's effects are strain-dependent, and future studies can use the 129S6 strain as a contrast strain to compare physiological differences that may have lead to the different reaction to estradiol between strains. Another extension of our findings would be to utilize other strains to see if estradiol increases inflammatory behaviors in those strains as well. Furthermore, future studies could compare inflammatory behaviors in different stages of the estrous cycle of the mouse to see if endogenous hormone fluctuations have the same effect as exogenous hormone replacement. Additional studies could use estradiol replacement in gonadectomized male and female mice to see if the effect that estradiol has on pain behaviors in female mice are due to the activational or organizational effects of estrogen. This paradigm could also elucidate the effect that testosterone has on pain sensitivity. Studies by Aloisi and

colleagues (2003) show that behavioral responses to formalin significantly increase in male rats after gonadectomy, indicating a protective role for the endogenous male gonadal hormones.

The results of the experiment used to address Aim 2 suggest that estradiol is not increasing nociceptive behaviors in C57BL/6N mice solely through alterations of cytokine activity in the PNS or CNS. We found that 20% estradiol increased IL-1β, IL-5, GM-CSF, and TNF-α concentrations in the paw that was not treated with Cg. This indicates that estradiol works systemically to alter physiology. These results contradict previous literature that shows that estradiol alters both pro- and anti-inflammatory cytokine expression. Salem (2004) found that estradiol inhibits TNF-α and IFN-γ production while enhancing anti-inflammatory IL-10 and IL-4 production. Additionally, Ito and colleagues (2001) showed that estradiol decreases the number of TNF-α-producing cells in the CNS, causing a suppression of experimental autoimmune encephalomyelitis. Estradiol has also been shown to down-regulate IL1R, which decreases the nervous system's response to the inflammatory cytokine, IL-1β (Schaefer, et al., 2005). Although we found that 20% estradiol increased cytokine levels in the uninjected paw, a concomitant increase in nociceptive behaviors was not seen, so estradiol is likely working through an alternative mechanism to increase pain sensitivity in these mice. In addition, data show that the dose-response curve differed from the classic sigmoid curve and seemed to be an inverted-U shape. Inverted U dose-response characteristics are fairly common when investigating hormone effects on different behavioral tasks (Inagaki, Gautreaux, & Luine, 2010). This suggests that moderate doses estrogen produce optimal levels of receptor activation, while low and high doses are not effective.

In addition, we found that changes in PWL were accompanied by changes in cytokine levels in the PNS and CNS, which varied based on hormone treatment. At lower doses of

estradiol, nociceptive behavior correlated negatively with cytokine concentrations, supporting the theory that estradiol is working through cytokines to increase pain behaviors. When pretreated with higher doses of estradiol, however, cytokine concentrations were positively correlated with PWL, which may indicate that high doses of estradiol are effective at lowering cytokine levels in response to inflammation. This supports previous literature that has found that estradiol causes decreases in proinflammatory cytokine levels in response to inflammation (Schaefer, et al., 2005; Ito, et al., 2001). Taking into account that the higher doses of estradiol caused increases in overall pain behavior, this indicates that the sensitizing effects of high doses of estradiol are working through a pathway divergent from the cytokine pathways. It is possible that estradiol is affecting PG production directly, not via the cytokine system. One possibility is that estradiol increases COX production, stimulating more sensitizing PGs to form. Estradiol has been shown to influence the prostanoid cascade in the circulatory system. Li and Stallone (2005) and White and colleagues (2005) found that treatment with indomethacin, a COX blocker, prevented estradiol-induced contractions in the arterial system.

One way to determine estrogen's effect on PG synthesis is to measure serum or tissue PG levels directly. However, we could not test PG levels due to lack of enzyme-immunoassay reliability in our lab. Therefore, an alternative way to investigate estrogen's effects on prostanoid synthesis is to block the COX enzymes and abolish prostanoid synthesis altogether. Blocking spinal PG production is effective at reversing peripheral pain associated with tissue damage, and indomethacin has been shown to alleviate hyperalgesia (Moalem & Tracey, 2006). If estradiol is interacting with the prostanoid cascade, we would see no difference between estradiol- and vehicle-treated animals when treated with indomethacin.

The study used to address Aim 3 compared estradiol's effects on inflammatory behaviors in mice treated with high, low and control doses of indomethacin. The results indicate that indomethacin attenuates Cg-induced thermal hyperalgesia, which supports such research as conducted by Moalem and Tracey (2006) which show that indomethacin into an inflamed hindpaw alleviated hyperalgesia in nerve-injured rats. Additionally, our results indicate that estradiol may affect the prostanoid pathway, but this effect is not solely responsible for the hyperalgesic effects seen in the first 2 experiments. In response to a low-heat stimulus, indomethacin abolished the differences observed in PWL between estradiol- and cholesterol-treated mice in the untreated paw. This indicates that indomethacin's effects are working systemically, affecting the PNS on the side opposite to injury. Support of this notion comes from Bralley and colleagues (2008) and Gordon and colleagues (2007), who show that treatment with indomethacin works in both the treated and untreated side to reduce edema and neutrophil infiltration in a mouse model of ear inflammation. Additionally, we found that treatment with estradiol inhibited indomethacin effects on PWL to high heat stimuli in the contralateral paw. This result suggests that in response to a more severe injury, estradiol may be working through a prostanoid-independent system. This hypothesis is supported by research conducted by Hunter (2008; personal communication), who found that treatment with estradiol and COX inhibitors attenuated pain behavior more than estradiol treatment alone in response to formalin administration. An alternative explanation is that estradiol could be increasing NO levels. NO is released after noxious insult, and, like PGs, increases neuronal excitability, resulting in an increase in inflammatory mediator release from sensory neurons (Kidd & Urban, 2001). Recent literature supports that NO produced in the periphery also contributes to edema and hyperalgesia (Omote, et al., 2001). Specific studies have found that estradiol alters NO levels. For example,

Cuzzocrea and colleagues (2001) showed that 17β-estradiol inhibited the increase of inducible nitric oxide synthase (iNOS) activity subsequent to Cg-induced lung injury. Additionally, Dina and colleagues (2001) found that PG-induced inflammatory pain was NO-dependent, and they suggested that NO activity was estrogen-dependent. On the other hand, estradiol has been shown to cause an increase in NOS enzyme activity in the lung, showing that estradiol can cause increases in NOS activity (McCurnin, et al., 2009). Additionally, Karpuzoglu and colleagues (2006) show that estrogen treatment causes a large release of NO in mouse splenocyte cultures. This was also accompanied by up-regulated iNOS mRNA, iNOS protein, and COX-2 (Karpuzoglu, et al., 2006). Future studies should be done using this murine model of Cg-induced paw inflammation to elucidate the role NO plays in estradiol's hyperalgesic effects. NO and iNOS can be measured after estradiol treatment in inflamed tissue and in the spinal cord, and experimenters can also block NO synthesis pharmacologically. Another way in which the role of NO can be determined is to use the normal hormonal fluctuations in the mouse estrous cycle to determine if NO activity changes with different changes in hormone levels. Additional experiments should also be utilized to determine PGE_2 and PGI_2 levels in both serum and nervous system tissue.

Another alternative explanation to the results found in experiments addressing Aim 3 is that estradiol may be increasing bradykinin levels, resulting in an increase in sympathetic amine production. In rats, Cg-induced hyperalgesia causes an increase in bradykinin release and subsequent cytokine release and sensitization of the sensory neuron. In mice, however, bradykinin's sensitizing actions are not dependent on cytokine activation, and thus may be working to activate prostanoids and sympathetic amines (Cunha, et al., 2005). Although Cunha and colleagues (2005) found that indomethacin and IL-1ra attenuated Cg-induced

hypernociception, it did not completely abolish it, suggesting that Cg-induced inflammation is mediated by an alternative pathway, mainly the sympathetic component. In fact, researchers found that a sympathetic amine blocker also partially inhibited Cg-induced hyperalgesia (Cunha, et al., 2005).

Macrophage and glial activity is our body's immediate defense against infection and injury. These cells are major sources of many proinflammatory mediators, such as cytokines, bradykinin, and NO. Our studies suggest that estradiol's effects may, in part, be mediated by cytokines and changes in prostanoid synthesis. However, the main mechanism by which estrogen is increasing pain behaviors in mice is still under investigation. Further research into the role of bradykinin and NO in this model will offer a potential mechanism by which estradiol's effects occur.

As shown in the present study, estradiol is effective at increasing pain behaviors in female mice. Figure 6.1 shows the original proposed model hypothesized by the experimenters. Taking into account the results obtained from the present study, researchers propose a new model to account for the increase in thermal hyperalgesia caused by estrogen. Figure 6.2 shows the new proposed model by which estrogen may modulate the nervous system and result in an increase in pain. One mechanism by which estrogen may be working is via an up-regulation of NO in the CNS. NO increases neuronal excitability in the spinal cord, boosting glutamate release, and inflating the release of neuromodulators from the presynaptic terminals of the sensory neurons (McMahon, et al., 2005; Kidd & Urban, 2001). It is possible that estrogen is working via estrogen receptors on immune cells to cause an increase in NO release and subsequent glutamate release, exciting pain-relaying neurons in the spinal cord (McHugh & McHugh, 2000). Additionally, estrogen may activate immune cells in the periphery, causing an

increase in bradykinin release. Bradykinin has been shown to be a major inflammatory mediator (Milligan & Watkins), and estradiol may alter its release in such a way to create peripheral sensitization and subsequent central sensitization.

In summary, our research highlights the importance of studying inflammation and its mediators in female subjects. Pain perception is sexually dimorphic, and in order to alleviate pain syndromes in both males and females, it is imperative to understand the physiological differences in pain processing between these two populations. Studies such as the ones presented above will help improve pain treatment in women especially.

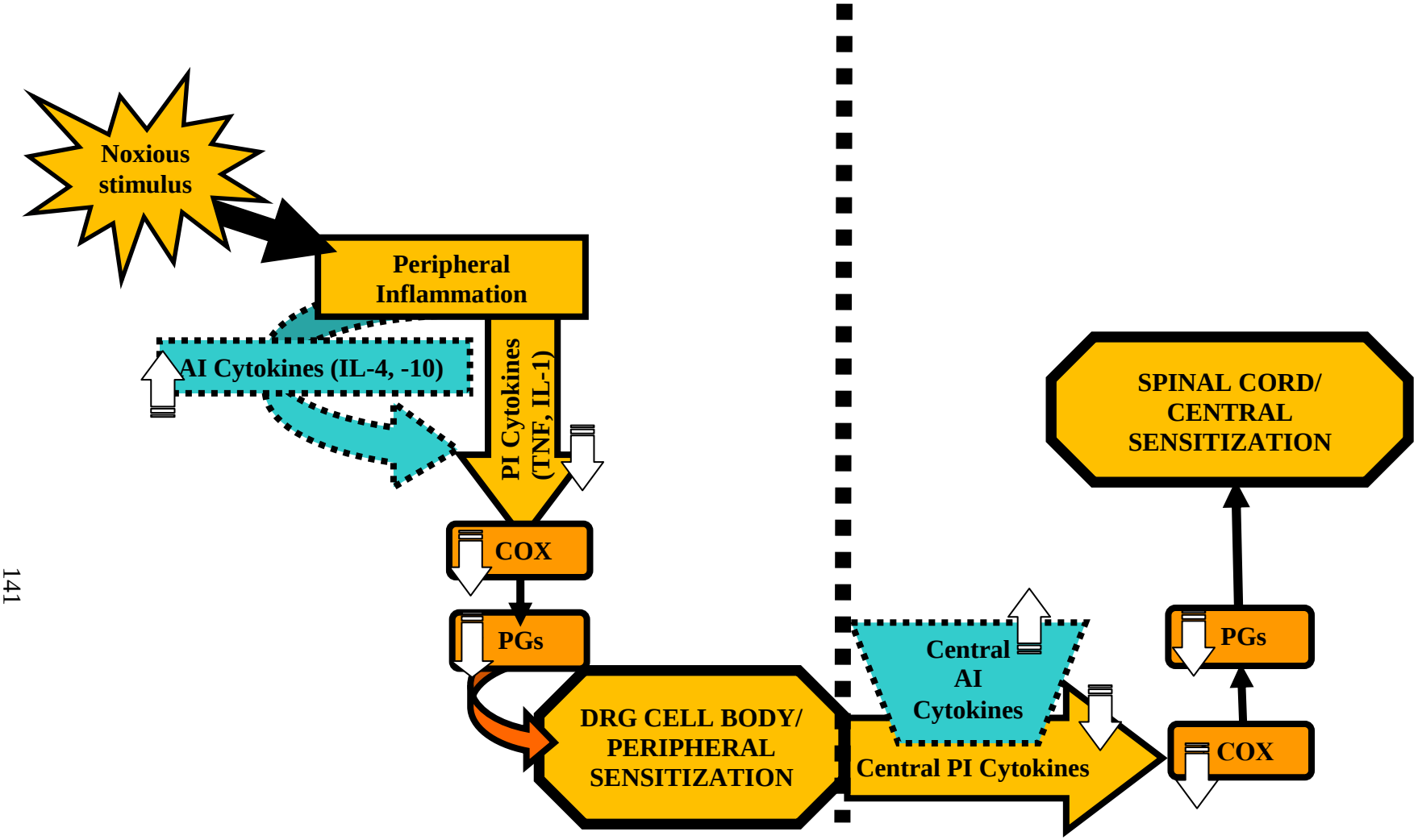

Figure 6.1. Oringinal proposed mechanisms for estrogen's attenuation of inflammation in the peripheral and central nervous systems. Estrogen may act to increase anti-inflammator y (AI) mediators such as AI cytokines or decrease proinflammatory (PI) mediators such as PI cytokines, COX, and PG. Each of these mechanisms would result in analgesia, and estrogen may be affecting both in the periphery and the central nervous system. White arrows indicate estrogen's proposed effects.

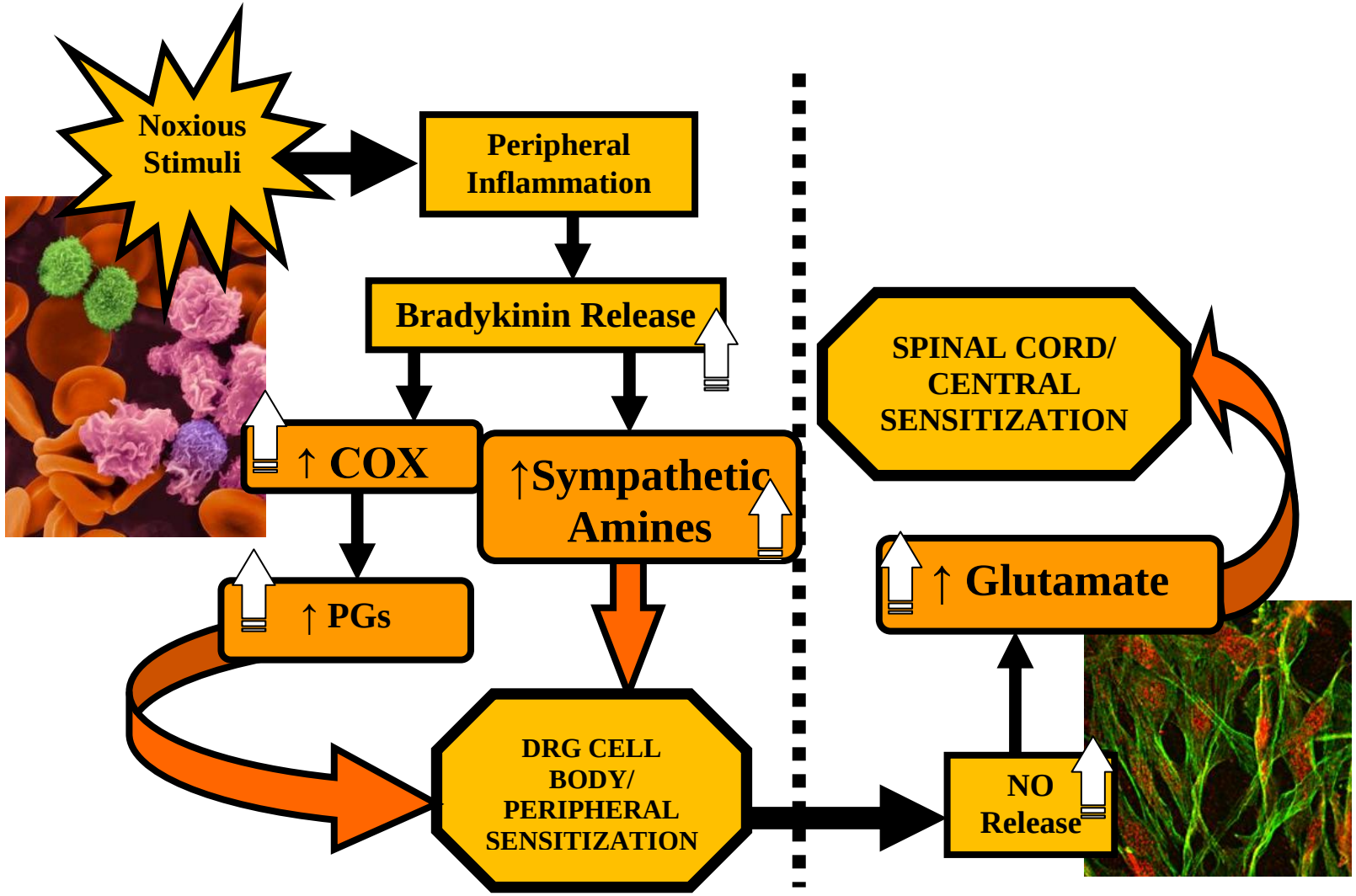

Figure 6.2. New Proposed mechanisms for estradiol's increase in inflammatory behaviors in C57BL/6N OVX mice. Taking into account the results found in our studies, estrogen is hypothesized to exacerbate hyperalgesia via up-regulation of inflammatory mediators in the peripheral and central nervous systems. Dark arrows represent inflammatory mediators activated by inflammation. White arrows represent estrogen's excitatory effects. Peripherally, estrogen increases NO and bradykinin release, causing an upregulation of prostanoid and sympathetic amine production, respectively. Centrally, estrogen is proposed to excite NO release from glial cells, leading to a subsequent increase in COX and PG production.

REFERENCES

Abrahamsen, B., Zhao, J., Asante, C.O., Cendan, C.M., Marsh, S., Martinez-Barbera, J.P., et al. (2008). The cell and molecular basis of mechanical, cold, and inflammatory pain. *Science, 321,* 702-705.

Aloisi, A. M., Ceccarelli, I., & Fiorenzani, P. (2003). Gonadectomy affects hormonal and behavioral responses to repetitive nociceptive stimulation in male rats. Annals of the New York Academy of Sciences*, 1007,* 232-237.

Angst, M.S., Clark, J.D., Carvalho, B., Tingle, M., Schmelz, M., & Yeomans, D.C. (2008). Cytokine profile in human skin in response to experimental inflammation, noxious stimulation, and administration of a COX-inhibitor: A microdialysis study. *Pain, 139(1),* 15-27.

Asquith, K.L., Ramshaw, H.S., Hansbro, P.M., Beagley, K.W., Lopez, A.F. & Foster, P.S. (2008). The IL-3/IL-5/GM-CSF common β receptor plays a pivotal role in the regulation of Th2 immunity and allergic airway inflammation. *The Journal of Immunology, 180(2),* 1199-1206.

Ba, Z.F., Lu, A., Shimizu, T., Szalay, L., Schwacha, M.G., Rue, III, L.R., et al. (2007). 17β-estradiol modulates vasoconstriction induced by endothelin-1 following trauma-hemmorage. *American Journal of Physiology—Heart and Circulation Physiology, 292,* 245-250.

Basbaum, A.I., & Jessell, T.M. (2000). The perception of pain. In E.R. Kandell, J.H. Schwartz, & T.M. Jessell, (Eds.), *Principles of Neural Science,* 4/e (pp.472-491). New York: McGraw-Hill.

Basbaum, A.I., & Woolf, C.J. (1999). Pain. *Current Biology, 9(12),* R429-R431.

Bennett, G.J. Animal models of pain. In: Kruger, L. (ed.) *Methods of Pain Research*, Boca Raton, FL: CRC Press; 2001: 67-92.

Berkley, K. J. (1997). Sex differences in pain. *Behavioral and Brain Sciences, 20,* 371-380.

Blaha, M.D., & Leon, L.R. (2008). Effects of indomethacin and buprenorphine analgesia on the postoperative recovery of mice. *Journal of the American Association for Laboratory Animal Science, 47(4),* 8-19.

Bradshaw, H.B., & Berkley, K.J. (2002). Estrogen replacement reverses ovariectomy-induced vaginal hyperalgesia in the rat. *Maturitas, 41,* 157-165.

Bralley, E.E., Greenspan, P., Hargrove, J.L., Wicker, L., & Hartle, D.K. (2008). Topical anti-inflammatory activity of *Polygonum cuspidatum* extract in the TPA model of mouse ear inflammation. *Journal of Inflammation, 5(1).*

Burgess, A.W., & Metcalf, D. (1980). The nature and action of granulocyte-macrophage colony stimulating factors. *Blood, 56,* 947-958.

Calippe, B., Douin-Echinard, V., Delpy, L., Laffargue, M., Lelu, K, Krust, A., et al. (2010). 17β-estradiol promotes TLR4-triggered proinflammatory mediator production through direct estrogen receptor-α signaling in macrophages in vivo. *Journal of Immunology, Epub ahead of print.*

Calippe, B., Douin-Echinard, V., Laffargue, M., Laurell, H, Rana-Poussine, V, Pipy, B., et al. (2008). Chronic estradiol administration in vivo promotes the proinflammatory response of macrophages to TLR4 activation: Involvement of the phosphatidylinositol 3-kinase pathway. *The Journal of Immunology, 180,* 7980-7988.

Campbell, I.K., Bendele, A., Smith, D.A., & Hamilton, J.A. (1997). Granulocyte-macrophage colony stimulating factor exacerbates collagen induced arthritis in mice. *Annals of the Rheumatic Diseases, 56,* 364-368.

Cata, J.P, Weg, H.-R., & Dougherty, P.M. (2008). Spinal injection of IL-2 or IL-15 alters mechanical and thermal withdrawal thresholds in rats. *Neuroscience Letters, 437,* 45-49.

Ceccarelli, I., Fiorenzani, P., Massafra, C., & Aloisi, A.M. (2003a). Long-term ovariectomy changes formalin-induced licking in female rats: the role of estrogens. *Reproductive Biology and Endocrinology, 1,* 24-33.

Ceccarelli, I., Scaramuzzino, A., Massafra, C., & Aloisi, A.M. (2003b). The behavioral and neuronal effects induced by repetitive nociceptive stimulation are affected by gonadal hormones in male rats. *Pain, 104,* 35-47.

Chen, S.X., Huang, F.Y., Tan, G.H., Wang, C.C., Huang, Y.H., Wang, H., et al. (2009). RNA interference against interleukin-5 attenuates airway inflammation and hyperresponsiveness in an asthma model. *Journal of Zhejiang University, Science B, 10(1),* 22-28.

Chichorro, J.G., Lorenzetti, B.B., & Zampronio, A.R. (2004). Involvement of bradykinin, cytokines, sympathetic amines and prostaglandins in formalin-induced orofacial nociception in rats. *British Journal of Pharmacology, 141,* 1175-1184.

Clemente, J.T., Parada, C.A., Arruda Veiga, M.C., Gear, R.W., & Tambeli, C.H. (2004). Sexual dimorphism in the antinociception mediated by kappa opioid receptors in the rat temporomandibular joint. *Neuroscience Letters, 372,* 250-255.

Costigan, M., & Woolf, C.J. (2000). Pain: Molecular mechanisms. *The Journal of Pain, 1(3),* 35-44.

Coutaux, A., Adam, F., Willer, J-C., & Le Bars, D. (2005). Hyperalgesia and allodynia: Peripheral mechanisms. *Joint Bone Spine, 72,* 359-371.

Cunha, F.Q., Poole, S,. Lorenzetti, B.B., Beiga, F.H., Ferreira, S.H. (1999). Cytokine-mediated inflammatory hyperalgesia limited by interleukin-4. *British Journal of Pharmacology, 126,* 45-50.

Cunha, T.M., Verri, Jr., W.A., Silva, J.S., Poole, S., Cunha, F.Q., & Ferreria, S.H. (2005). A cascade of cytokines mediates mechanical inflammatory hypernociception in mice. *Proceedings of the National Academy of Sciences, 102,* 1755-1760.

Cuzzocrea, S., Mazzon, E., Sautebin, L., Serraino, I., Dugo, L., Calabro, G., et al. (2001). The protective role of endogenous estrogens in carrageenan-induced lung injury in the rat. *Molecular Medicine, 7(7),* 478-487.

DeLeo, J.A., Colburn, R.W., & Rickman, A.J. (1997). Cytokine and growth factor immunohistochemical spinal profiles in two animal models of mononeuropathy. *Brain Research, 759,* 50-57.

Evans, R.W., & Becker, W.J. (2006). Migraine and oral contraceptives. *Headache, 46,* 328-331.

Frazen, R., Bouhy, D., & Schoenen, J. (2004). Nervous system injury: focus on the inflammatory cytokine 'granulocyte-macrophage colony stimulating factor'. *Neuroscience Letters, 361,* 76-78.

Gaumond, I., Arsenault, P., & Marchand, S. (2002). The role of sex hormones on formalin-induced nociceptive responses. *Brain Research, 958,* 139-145.

Gaumond, I., Arsenault, P., & Marchand, S. (2005). Specificity of female and male sex hormones on excitatory and inhibitory phases of formalin-induced nociceptive responses. *Brain Research, 1052,* 105-111.

Gold, M.S., Reichling, D.B., Shuster, M.J., & Levine, J.D. (1996). Hyperalgesic agents increase a tetrodotoxin-resistent Na^+ current in nociceptors. *Proceedings of the National Academy of Sciences., 93(3),* 1108-1112.

Gordon, J.S., Wolanin, P.M., Gonzalez, A.V., Fela, D.A., Sarngadharan, G., Rouzard, K., et al. (2008). Topical *N*-acetyl-*S*-cystein inhibits mouse skin inflammation, and unlike dexamethasone, its effects are restricted to the application site. *Journal of Investigative Dermatology,128,* 643-654.

Gupta, S., Mehrotra, S., Villalon, C.M., Perusquia, M., Saxena, P.R., VanDenBrink, A.M. (2007). Potential role of female sex hormones in the pathophysiology of migraine. *Pharmacology & Therapeutics, 113,* 321-340.

Hamilton, J.A., Whitty, G.A., Royston, A.K.M., Cebon, J., & Layton, J.E. (1992). Interleukin-4 suppresses granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor levels in stimulated human monocytes. *Immunology, 76,* 566-571.

Hanisch, U.-K. & Quirion, R. (1996). Interleukin-2 as a neuroregulatory cytokine. *Brain Research Reviews, 21,* 246-284.

Hao, S., Mata, M., Glorioso, J.C., & Fink, D.J. (2006). HSV-mediated expression of interleukin-4 in dorsal root ganglion neurons reduces neuropathic pain. *Molecular Pain, 2(6).*

Hart, P.E., Vitti, G.F., Burgess, D.R., Whitty, G.A., Piccoli, D.S., & Hamilton, J.A. (1989). Potential antiinflammatory effects of interleukin 4: Suppression of human monocyte tumor necrosis factor α, interleukin 1, and prostaglandin E_2. *Proc. Natl. Acad. Sci., 86,* 3803-3807.

Holt, S., Comelli, F., Costa, B., & Fowler, C.J. (2005). Inhibitors of fatty acid amide hydrolase reduce carrageenan-induced hind paw inflammation in pentobarbital-treated mice: comparison with indomethacin and possible involvement of cannabinoid receptors. *British Journal of Pharmacology, 146,* 467-476.

Hopkins, S.J. (2004). Central nervous system recognition of peripheral inflammation: a neural, hormonal collaboration. *Acta Biomedica, 2007,* 231-247.

Hull, E.M., & Dominguez, J.M. (2007). Sexual behavior in male rodents. *Hormones and Behavior, 52(1),* 45-55.

Hutchinson, M.R., Coats, B.D., Lewis, S.S., Zhang, Y., Sprunger, D.B., Rezvani, N., et al. (2008). Proinflammatory cytokines oppose opiod-induced acute and chronic analgesia. *Brain, Behavior, and Immunity, 22,* 1178-1189.

Inagaki, T., Gautreaux, C., & Luine, V. (2010). Acute estrogen treatment facilitates recognition memory consolidation and alters monoamine levels in memory-related brain areas. *Hormones and Behavior, 58(3),* 415-426.

Ito, A., Bebo, Jr., B.F., Matejuk, A., Zamora, A., Silverman, M., Fyfe-Johnson, A., et al. (2001). Estrogen treatment down-regulates TNF-α production and reduces the severity of experimental autoimmune encephalomyelitis in cytokine knockout mice. *The Journal of Immunology, 167(1),* 542-552.

Jonasson, Z. (2005). Meta-analysis of sex differences in rodent models of learning and memory: a review of behavioral and biological data. *Neuroscience and Biobehavioral Reviews, 28(8),* 811-825.

Kamachi, F., Ban, H.S., Hirasawa, N., & Ohuchi, K. (2007). Inhibition of lipopolysaccharide-induced prostaglandin E_2 productino and inflammation by the Na^+/H^+ exhchanger inhibitors. *The Journal of Pharmacology and Experimental Therapeutics, 321(1),* 345-352.

Karpuzoglu, E., Fenaux, J.B., Phillips, R.A., Lengi, A.J., Elvinger, G., & Ansar Ahmed, S. (2006). Estrogen up-regulates inducible nitric oxide synthase, nitric oxide, and cyclooxygenase-2 in splenocytes activated with T cell stimulants: role of interferon-gamma. *Endocrinology, 147(2),* 662-671.

Kayser, V., Berkley, K.J., Keita, H., Gautron, M., & Guilbaud, G. (1996). Estrous and sex variations in vocalization thresholds to hindpaw and tail pressure stimulation in the rat. *Brain Research, 742,* 352-354.

Kidd, B.L., & Urban, L.A. (2001). Mechanisms of inflammatory pain. *British Journal of Anaesthesia, 87(1),* 3-11.

Koch, A., Zacharowski, K., Boehm, O., Stevens, M., Lipfert, P., von Giesen, H.-J., et al. (2007). Nitric oxide and pro-inflammatory cytokines correlate with pain intensity in chronic pain patients. *Inflammation Research, 56,* 32-37.

Kouro, T. & Takatsu, K. (2009). IL-5- and eosinophil-mediated inflammation: from discovery to therapy. *International Immunology, 21(12),*1303-1309.

Kramer, S.D., & Testa, B. (2008). The biochemistry of drug metabolism-An introduction. *Chemistry & Biodiversity, 5(2),* 2465-2578.

Kuba, T., Kemen, L.M., & Quinones-Jenab, V. (2005). Estradiol administration mediates the inflammatory response to formalin in female rats. *Brain Research, 1047,* 119-122.

Kuba, T., & Quinones-Jenab, V. (2005). The role of female gonadal hormones in behavioral sex differences in persistent and chronic pain: Clinical versus preclinical studies. *Brain Research Bulletin, 66,* 179-188.

Kuba, T., Wu, H.-B. K., Nazarian, A., Festa, E.D., Barr, G.A., Jenab, S., et al. (2006). Estradiol and progesterone differentially regulate formalin-induced nociception in ovariectomized female rats. *Hormones and Behavior, 49,* 441-449.

LaCroix-Fralish, M.L., Tawfik, V.L., & DeLeo, J.A. (2005). The organizational and activational effects of sex hormones on tactile and thermal hypersensitivity following lumbar nerve root injury in male and female rats. *Pain, 114,* 71-80.

Lai, Y.G., Wang, Z.G., Feng, J.I., Wu, J.M., Chen, X., Zhen, L.I., et al. (2009). Animal study for airway inflammation triggered by gastroesophageal reflux. *Chinese Medical Journal, 122(22),* 2775-2778.

Lapchak, P.A., Araujo, D.M., Quirion, R., & Beaudet, A. (1991). Immunoautoradiographic localization of interleukin-2-like immunoreactivity and interleukin-2 receptors (Tac antigen-like immunoreactivity) in the rat brain. *Neuroscience, 44,* 173-184.

LaResche, L., Mancl, L., Sherman, J.L., Gandara, B., & Dworkin, S.F. (2003). Changes in temporomandibular pain and other symptoms across the menstrual cycle. *Pain, 106,* 253-261.

Lavich, T.R., Cordeiro, R.S.B., Silva, P.M.R., & Martins, M.A. (2005). A novel hot-plate test sensitive to hyperalgesic and non-opiod analgesics. *Brazilian Journal of Medical and Biological Research, 38,* 445-451.

Li, M., & Stallone, J.N. (2005). Estrogen potentiates vasopressin-induced contraction of female rat aorta by enhancing cyclooxygenase-2 and thromoxane function. *American Journal of Physiology. Heart and Circulatory Physiology*, *289*, 1542-1550.

Li, L., Fan, X., Warner, M., Xu, X.-J., Gustafsson, J.-A., & Wiesenfeld-Hallin, Z. (2009). Ablation of estrogen receptor α or β eliminates sex differences in mechanical pain threshold in normal and inflamed mice. *Pain, 143,* 37-40.

Loram, L.C., Fuller, A., Fick, L.G., Cartmell, T., Poole, S., & Mitchell, D. (2006). Cytokine profile during carrageenan-induced inflammatory hyperalgesia in rat muscle and hind paw. *The Journal of Pain, 8,* 127-136.

MacDonald, A.G., Murphy, E.A., Capell, H.A., Bankowska, U.Z., & Ralston, S.H. (1994). Effects of hormone replacement therapy in rheumatoid arthritis: a double blind placebo-controlled study. *Annals of the Rheumatic Diseases, 53,* 54-57.

Macfarlane, T.V., Blinkhorn, A., Worthington, H.V., Davies, R.M., & Macfarlane, G.J. (2002). Sex hormonal factors and chronic widespread pain: a population study among women. *Rheumatology, 41,* 454-457.

Mahmoud, H.H., Pui, C.H., Kennedy, W., Jaffe, H.S., Crist, W.M., & Murphy, S.B. (1992). Phase I study of recombinant human interferon gamma in children with relapsed acute leukemia. *Leukemia, 6,* 1181-1184.

Mannino, C.A., South, S.M., Inturrisi, C.E., & Quinones-Jenab, V. (2005). Pharmacokinetics and effects of 17β-estradiol and progesterone implants in ovariectomized rats. *The Journal of Pain, 6,* 809-816.

Mannino, C.A., South, S.M., Quinones-Jenab, V., & Inturrisi, C.E. (2006). Estradiol replacement in ovariectomized rats is antihyperalgesic in the formalin test. *The Journal of Pain,8,* 334-342.

Marshall, J.S., Leal-Berumen, I., Nielsen, L., Glibetic, M., & Jordana, M. (1996). Interleukin (IL)-10 inhibits long-term IL-6 production but not preformed mediator release from rat peritoneal mast cells. *The Journal of Clinical Investigation, 97(4),* 1122-1128.

Martin, V.T., & Behbehani, M. (2006). Ovarian hormones and migraine headache: Understanding mechanisms and pathogenesis—Part 2. *Headache, 46,* 365-386.

Martinez-Moczygemba, M. & Huston, D.P. (2003). Biology of the common β receptor-signaling cytokines: IL-3, IL-5, and GM-CSF. *Molecular Mechanisms in Allergy and Clinical Immunology, 112(4),* 653-665.

McHugh, J.M., & McHugh, W.B. (2000). Pain: Neuroanatomy, chemical mediators, and clinical implications. *American Association of Critical-Care Nurses Clinical Issues, 11(2),* 168-178.

McCurnin, D.C., Pierce, R.A., Willis, B.C., Chang, L.Y., Yoder, B.A., Yuhanna, I.S., et al. (2009). Postnatal estradiol up-regulates lung nitric oxide synthases and improves lung function in bronchopulmonary dysplasia. *American Journal of Respiratory and Critical Care Medicine, 179(6),* 492-500.

McMahon, S., Cafferty, W., & Marchand, F. (2005). Immune and glial cell factors as pain mediators and modulators. *Experimental Neurology, 192(2),* 444-462.

Mertz, P.M., DeWitt, D.L., Stetler-Stevenson, W.G., & Wahl, L.M. (1994). Interleukin 10 suppression of monocyte prostaglanding H synthase-2. *The Journal of Biological Chemistry, 269(33),* 21322-21329.

Meyer, R.A., Ringkamp, M., Campbell, J.N., & Raja, S.N. (2005). Peripheral mechanisms of cutaneous nociception. In S. McMahon & M. Koltzenburg (Eds.), *Wall and Melzack's Textbook of Pain, 5/e* (pp.3-27). New York: Elsevier Health Sciences.

Mijatovic, T., Kruys, V., Caput, D., Defrance, P., & Huez, G. (1997). Interleukin-4 and -13 inhibit tumor necrosis factor-α mRNA translational activiation in lipopolysaccharide-induced mouse macrophages. *The Journal of Biological Chemistry, 272(22),* 14394-14398.

Milligan, E.D., & Watkins, L.R. (2009). Pathological and protective roles of glia in chronic pain. *Nature Reviews, 10,* 23-36.

Moalem, G., & Tracey, D.J. (2006). Immune and inflammatory mechanisms in neuropathic pain. *Brain Research Reviews, 51,* 240-264.

Mogil, J.S., & Chandra, M.L. (2005). The case for the inclusion of female subjects in basic science studies of pain. *Pain, 117,* 1-5.

Ndengele, M.M., Cuzzocrea, S., Esposito, E., Mazzon, E., Di Paola, R., Matuschak, G.M., et al. (2008). Cyclooxygenases 1 and 2 contribute to peroxynitrate-mediated inflammatory pain hypersensitivity. *The FASEB Journal, Research Communication, 22,* 3154-3164.

Neumann, S., Doubell, T.P., Leslie, T., & Woolf, C.J. (1996). Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. *Nature, 384,* 360-364.

Omote, K., Hazama, K., Kawamata, T., Nakayaka, Y., Toriyabe, M., & Namiki, A. (2001). Peripheral nitric oxide in carrageenan-induced inflammation. *Brain Research, 912(2),* 171-175.

Paul, W.E. (1991). Interleukin-4: A prototypic immunoregulatory lymphokine. *Blood, 77(9),* 1859-1870.

Percegoni, N., Ferreira, A.C., Rodrigues, C.F., Rosenthal, D., Castelo Branco, M.T., Rumjanek, V.M., et al. (2009). Profile of serum IL-1beta and IL-10 shortly after ovariectomy and estradiol replacement in rats. *Hormone Metabolism Research, 41,* 50-54.

Poole, S., Cunha, F.Q., Selkirk, S., Lorenzetti, B.B., & Ferreira, S.H. (1995). Cytokine-mediated inflammatory hyperalgesia limited by interleukin-10. *British Journal of Pharmacology, 115,* 684-688.

Prusakiewicz, J.J., Duggan, K.C., Rouzer, C.A., & Marnett, L.J. (2009). Differential sensitivity and mechanism of inhibition of COX-2 oxygenation of arachidonic acid and 2-arachidonoylglycerol by ibuprofen and mefenamic acid. *Biochemistry, 48,* 7353-7355.

Ren, K., & Dubner, R. (1999). Inflammatory models of pain and hyperalgesia. *ILAR Journal Online: Animal Models of Pain, 40(3),* 1-7.

Riedel, W., & Neeck, G. (2001). Nociception, pain, and antinociception: Current concepts. *Zeitschrift für Rheumatologie, 60,* 404-415.

Riley III, J.L., Robinson, M.E., Wise, E.A., Myers, C.D., & Fillingim, R.B. (1998). Sex differences in the perception of noxious experimental stimuli: a meta-analysis. *Pain, 74,* 181-187.

Rittner, H.L., Machelska, H., & Stein, C. (2005). Leukocytes in the regulation of pain and analgesia. *Journal of Leukocyte Biology, 78,* 1215-1222.

Rittner, H.L., & Stein, C. (2005). Involvement of cytokines, chemokines, and adhesion molecules in opioid analgesia. *European Journal of Pain, 9,* 109-112.

Robertson, B., Xu, X.J., Hao, J.X., Wiesenfeld-Hallin, Z., Mhlanga, J., Grant, G., et al. (1997). Interferon-gamma receptors in the nociceptive pathways: role in neuropathic pain-related behaviors. *Neuroreport, 8,* 1311-1316.

Saab, C.Y., Waxman, S.G., & Hains, B.C. (2008). Alarm of curse? The pain of neuroinflammation. *Brain Research Reviews, 58,* 226-235.

Sabina, E.P., Chandel, S., & Rasool, M.K. (2008). Inhibition of monosodium urate crystal-induced inflammation by withaferin A. *Journal Journal of Pharmacy & Pharmaceutical Sciences: A Publication of the Canadian Society for Pharmaceutical Sciences, 11(4),* 46-55.

Salem, M.L. (2004). Estrogen, a double-edged sword: Modulation of TH1- and TH2-mediated inflammations by differential regulation of TH1/TH2 cytokine production. *Current Drug Targets-Inflammation and Allergy, 3,* 97-104.

Schaefer, T.M., Wright, J.A., Pioli, P.A., & Wira, C.R. (2005). IL-1β-mediated proinflammatory responses are inhibited by estradiol via down-regulation of IL-1 receptor type I in uterine epithelial cells. *The Journal of Immunology, 175(10),* 6509-6516.

Schafers, M., & Sorkin, L. (2008). Effect of cytokines on neuronal excitability. *Neuroscience Letters, 437,* 188-193.

Sommer, C., & Kress, M. (2004). Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia. *Neuroscience Letters, 361,* 184-187.

Song, P., Zhao, Z.Q., & Liu, X.Y. (2000). Expression of IL-2 receptor in dorsal root ganglion neurons and peripheral antinociception. *Neuroreport, 11(7),* 1433-1436.

Sternberg, E.M. (1997). Perspective series: Cytokines and the brain: Neural-immune interactions in health and disease. *The Journal of Clinical Investigation, 100,* 2641-2647.

Subramanian, S., Tovey, M., Afentoulis, M., Krogstad, A., Vandenbark, A.A., & Offner, H. (2005). Ethinyl estradiol treats collagen-induced arthritis in DBA/1LacJ mice by inhibiting the production of TNF-α and IL-1β. *Clinical Immunology, 115,* 162-172.

Süleyman, H., Demircan, B., Karagög, Y., Öztaşan, & Süleyman, B. (2004). Anti-inflammatory effects of selective COX-2 inhibitors. *Polish Journal of Pharmacology, 56,* 775-780.

Sweitzer, S.M., Arruda, J.L., & DeLeo, J.A. The cytokine challenge: Methods for the detection of central cytokines in rodent models of persistent pain. In: Kruger, L. (ed.) *Methods of Pain Research*, Boca Raton, FL: CRC Press; 2001: 109-132.

Tall, J.M., & Crisp, T. (2004). Effects of gender and gonadal hormones on nociceptive responses to intraplantar carrageenan in the rat. *Neuroscience Letters, 354,* 239-241.

Tall, J.M., Stuesse, S.L., Cruce, W.L.R., Crisp, T. (2001). Gender and the behavioral manifestations of neuropathic pain. *Pharmacology, Biochemistry, and Behavior, 68,* 99-104.

teVelde, A.A., Huijbens, R.J.F., Heije, K., de Vries, J.E., & Figdor, C.G. (1990). Interleukin-4 (IL-4) inhibits secretion of IL-1β, tumor necrosis factor α, and IL-6 in human monocytes. *Blood, 76,* 1392-1397.

Terner, J.M., Lomas, L.M., & Picker, M.J. (2005). Influence of estrous cycle and gonadal hormone depletion on nociception and opioid antinociception in female rats of four strains. *The Journal of Pain, 6,* 372-383.

Tober, K.L., Thomas-Ahner, J.M., Maruyama, T., & Oberyszyn, T.M. (2007). Possible cross-regulation of the E prostanoid receptors. *Molecular Carcinogenesis, 46,* 711-715.

Uceyler, N., & Sommer, C. (2008). Cytokine regulation in animal models of neuropathic pain and in human diseases. *Neuroscience Letters, 437,* 194-198.

Vale, M.L., Marques, J.B., Moreira, C.A., Rocha, F.A., Ferreira, S.H., Poole, S., et al. (2003). Antinociceptive effects of interleukin-4, -10, and -13 on the writhing response in mice and zymosan-induced knee joint incapacitation in rats. *The Journal of Pharmacology and Experimental Therapeutics, 304,* 102-108.

Valesecchi, A.E., Franchi, S., Panerai, A.E., Sacerdote, P., Trovato, A.E., & Colleoni, M. (2008). Genistein, a natural phytoestrogen from soy, relieves neuropathic pain following chronic constriction sciatic nerve injury in mice: anti-inflammatory and antioxidant activity. *Journal of Neurochemistry, 107(1),* 230-240.

Vannier, E., Miller, L.C., & Dinarello, C.A. (1992). Coordinated antiinflammatory effects of interleukin 4: Interleukin 4 suppresses interleukin 1 production but up-regulates gene expression and syntesis of interleukin 1 receptor antagonist. *Proc. Natl. Acad. Sci., 89,* 4076-4080.

Verri, Jr., W.A., Cunha, T.M., Parada, C.A., Poole, S., Cunha, F.Q., & Ferreira, S.H. (2006). Hypernociceptive role of cytokines and chemokines: Targets for analgesic drug development? *Pharmacology & Therapeutics, 112,* 116-138.

Vikman, K.S., Duggan, A.W., & Siddall, P.J. (2007). Interferon-γ induced disruption of GABAergic inhibition in the spinal dorsal horn *in vivo*. *Pain, 133,* 18-28.

Vikman, K.S., Hill, R.H., Backstrom, E., Robertson, B., & Kristensson, K. (2003). Interferon-gamma induces characteristics of central sensitization in spinal dorsal horn neurons in vitro. *Pain, 106,* 241-251.

Vikman, K.S., Siddall, P.J., & Duggan, A.W. (2005). Increased responsiveness of rat dorsal horn neurons in vivo following prolonged intrathecal exposure to interferon-gamma. *Neuroscience, 135,* 969-977.

Vincler, M., Maixner, W., Vierck, C.J., & Light, A.R. (2001). Estrous cycle modulation of nociceptive behaviors elicited by electrical stimulation and formalin. *Pharmacoloty, Biochemistry, and Behavior, 69,* 315-324.

Wagner, R., Janjigian, M., & Myers, R.R. (1998). Anti-inflammatory interleuki-10 therapy in CCI neuropathy decreases thermal hyperalgesia, macrophage recruitment, and endoneurial TNF-α expression. *Pain, 74*, 35-42.

Waldmann, T.A. (2006). The biology of interleukin-2 and interleukin-15: Implications for cancer therapy and vaccine design. *Nature Reviews. Immunology, 6(8),* 595-601.

Walker, J.S., & Carmody, J.J. (1998). Experimental pain in healthy human subjects: Gender differences in nociception and in response to ibuprofen. *Anesthesia and Analgesia, 86,* 1257-1262.

Warren, M.P., & Fried, J.L. (2001). Temporomandibular disorders and hormones in women. *Cells Tissues Organs, 169,* 187-192.

Watkins, L.R., & Maier, S.F. (1999). Implications of immune-to-brain communication for sickness and pain. *Proceedings from the National Academy of Sciences, 96,* 7710-7713.

Weiseler-Frank, J., Maier, S.F., & Watkins, L.R. (2004). Glial activation and pathological pain. *Neurochemistry International, 45,* 389-395.

Weiseler-Frank, J., Maier, S.F., & Watkins, L.R. (2005). Central proinflammatory cytokines and pain enhancement. *Neurosignals, 14,* 166-174.

White, R.E., Han, G., Dimitropoulou, C., Zhu, S., Miyake, K., Fulton, D., et al. (2005). Estrogen-induced contraction of coronary arteries is mediated by superoxide generated in vascular smooth muscle. *American Journal of Physiology: Heart and Circulation Physiology, 289(4)*, H1468-H1475.

Wiesenfeld-Hallin, Z. (2005). Sex differences in pain perception. *Gender Medicine, 2(3),* 137-145.

Woolf, C., & Costigan, M. (1999). Transcriptional and posttranslational plasticity and the generation of inflammatory pain. *Proceedings from the National Academy of Sciences, 96(14),* 7723-7730.

Xu, X.J., Hao, J.X., Olsson, T., Kristensson, K., van der Meide, P.H., & Wiesenfeld-Hallen, Z. (1994). Intrathecal interferon-gamma facilitates the spinal nociceptive flexor reflex in the rat. *Neuroscience Letters, 18,* 263-266.

Yao, M.Z., Gu, J.F., Wang, J.H., Sun, L.Y., Lang, M.F., Liu, Z.Q., et al. (2002). Interleukin-2 gene therapy of chronic neuropathic pain. *Neuroscience, 112(2),* 409-416.

Yao, M.Z., Gu, J.F., Wang, J.H., Sun, L.Y., Liu, H., & Liu, X.Y. (2003). Adenovirus-mediated interleukin-2 gene therapy of nociception. *Gene Therapy, 10,* 1392-1399.

Young, H.A., & Hardy, K.J. (1995). Role of interferon-gamma in immune cell regulation. *Journal of Leukocyte Biology, 58(4),* 373-381.

Zeilhofer, H.U., & Brune, K. (2006). Analgesic strategies beyond the inhibition of cyclooxygenases. *TRENDS in Pharmacological Science, 27(9),* 468-474.

Zhou, Y., Hancock, J.F., Lichtenberger, L.M. (2010). The nonsteroidal anti-inflammatory drug indomethacin induces heterogeneity in lipid membranes: Potential implication for its diverse biological action. *PLoS ONE, 5(1).*