

**The Effects of Estrogen on Cytokine Levels Before and
During Inflammatory Pain in Female Rats**

by

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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.

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ABSTRACT

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Epidemiological studies demonstrate that sex differences exist in pain perception and discrimination; females display greater sensitivity to noxious stimuli than males. Additionally, the female endocrinological profile is also shown to alter nociceptive responses to pain. For instance, 17β -estradiol reduces flinching responses after formalin administration. Furthermore, nociceptive responses to formalin are reduced during the proestrus stage of the estrous cycle. However, little is known about the specific biochemical mechanisms involved in estrogen's anti-hyperalgesic responses to pain. Research demonstrates that proinflammatory cytokines play a principal role in initiating pain responses. Cytokines activate cyclooxygenase (COX)-dependent prostanoid release. Additionally, glucocorticoids (i.e. corticosterone) are postulated to inhibit prostaglandin activation, and thus hamper inflammatory responses. The objective of this study is to determine if estrogen modulates inflammatory-induced pain by altering cytokine pathways and the hypothalamic-pituitary-adrenal (HPA) axis. Estrogen is postulated to hamper nociceptive responses by decreasing basal and inflammatory-induced cytokine levels at peripheral and central sites, and by increasing CORT activity. In intact female rats, hormone fluctuations across the estrous cycle led to changes in cytokine, but not

CORT, levels. In most cases, serum cytokine levels were altered in naïve rats, suggesting that in intact models the endocrinological profile influences pain responses by modulating cytokine levels before the introduction of painful stimuli. Following ovariectomy and/or adrenalectomy, 17β -estradiol altered peripheral and central cytokine levels both before and following formalin-induced pain. Estradiol's effects worked independent of CORT release, since estradiol's alteration of cytokine levels were also shown in adrenalectomized rats. Together, these results further suggest that estrogen's influence on cytokine levels contributes in part to anti-hyperalgesic effects within the formalin paradigm. Finally, 17β -estradiol lowered nociceptive responding in ovariectomized rats as measured by increased paw withdrawal latencies before and following carrageenan-induced pain. 17β -estradiol decreased paw inflammation and altered peripheral and central cytokine levels, which may be mechanisms through which estrogen lowers pain responses during the carrageenan paradigm. Although estradiol increased CORT levels in this model, heightened CORT activity did not always lead to a reduction in behavioral responses to pain. Taken together, our results suggest that estrogen's anti-hyperalgesic effects are mediated through down-regulation of the cytokine pathway in a mechanism independent of actions on the HPA axis.

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ABBREVIATIONS

ADX	Adrenalectomy
ACTH	Adrenocorticotrophic hormone
CCI	Chronic constriction injury
CFA	Complete Freund's adjuvant
Cg	Carrageenan
CIA	Collagen-induced arthritis
CNS	Central nervous system
CORT	Corticosterone
COX	Cyclooxygenase
CRH	Corticotropin-releasing hormone
DRG	Dorsal root ganglia
E	Estradiol
GC	Glucocorticoid
GDX	Gonadectomy
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp130	Glycoprotein 130
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
IFN	Interferon
IL	Interleukin
IL-1 ra	Interleukin-1 receptor antagonist
iNOS	Inducible nitric oxide synthase
Jak	Janus tyrosine kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
mPGES-1	Microsomal PGE synthase-1
NF- κ B	Nuclear transcription factor-kappaB
NGF	Nerve growth factor
NO	Nitric oxide
OVX	Ovariectomy
P	Progesterone
PAG	Periaqueductal gray
PG	Prostaglandin
PWL	Paw withdrawal latency
RA	Rheumatoid arthritis
STAT	Signal transducer and activator of transcription
SCI	Spinal cord injury
SNL	Spinal nerve ligation
TMD	Temporomandibular disorders
TMJ	Temporomandibular joint
TNF- α	Tumour necrosis factor-alpha
TNFR1	TNF receptor type 1
TNFR2	TNF receptor type 2
Trk	Tyrosine kinase
TRPV1	Transient receptor potential vanilloid-1
TTX-R	Tetrodotoxin-resistant
TTX-S	Tetrodotoxin-sensitive
WDR	Wide dynamic range

Chapter 1: Introduction

The sensation of pain is a useful response that aids in the survival of an organism. Pain serves as a way for the body to recognize impending damaging stimuli. The immediate activation of the pain pathway during acute pain is an adaptive response that has been evolved to enhance fitness and survival. However, organisms also experience long-lasting chronic pain that seems to have no immediate or evolutionary benefit; thus it is termed maladaptive. The maladaptive response alters the activation of the central nervous system (CNS), leading to the production of allodynia, the exaggerated response to non-noxious stimuli, and hyperalgesia, the increased sensitivity to pain.

Factors that contribute to pain have been studied extensively. From this research, apparent differences in pain discrimination and perception between sexes have been found. In order to understand the mechanisms underlying dimorphic responses to pain, current research is investigating the role of endogenous gonadal hormones. The regulatory actions of hormones may modulate biochemical mediators involved in inflammatory responses.

I. Background and Significance

Pain and Nociception

The term pain refers to an “unpleasant sensory and emotional experience associated with actual or potential tissue damage” (Merskey, 1991). Pain has been characterized into different categories, such as acute pain, in which actual or the potential threat of pain elicits protective functions and behaviors that remove the body from potentially damaging stimuli (Burian & Geisslinger, 2005; Millan, 1999; Riedel &

Neeck, 2001). Another type is inflammatory/subchronic pain, which arises from injury to inflammatory tissue and/or nerves (Millan, 1999). In contrast, chronic, or pathological pain, is prolonged in nature and serves no evolutionary benefit to the organism. Chronic pain usually involves damage or dysfunction of nerves (Millan, 1999). Examples of chronic pain are pains from terminal diseases (cancer pain), degenerative diseases (i.e. rheumatoid arthritis), and pain that persists after an injury heals (Millan, 1999).

Acute and inflammatory pain are physiological pain states that fall under the class called nociceptive pain or pain that arises from the stimulation of nerve fibers (Millan, 1999). Acute pain causes us to recognize and quickly remove an injurious stimulus, and to learn to avoid harm from the stimulus in the future (Basbaum & Woolf, 1999; Burian & Geisslinger, 2005; Wieseler-Frank, Maier, & Watkins, 2005). Inflammatory pain is more persistent in nature (Millan, 1999). During inflammatory pain, noxious (tissue-damaging) insult creates a region of hypersensitivity near the injured area (Basbaum & Woolf, 1999). Inflammatory pain associated with tissue injury often includes sensitization (Burian & Geisslinger, 2005). Sensitization is a course of action in nerve fibers that consists of 1) a decrease in threshold of activation, 2) an increase in response to specific stimuli, and 3) spontaneous activity that occurs in the absence of external stimulation (Burian & Geisslinger, 2005; Millan, 1999). Sensitization can occur at peripheral and central levels (Woolf, 1983).

The detection of the noxious stimulus and the neural response that incurs is termed nociception (from the Latin “nocere,” to hurt; Coutaux, Adam, Willer & LeBars, 2005; Woolf & Costigan, 1999). Nociception refers only to the specific activity of nerve pathways during pain, and does not include subjective pain sensations that contain

cognitive aspects along with the sensory experience (Kidd & Urban, 2001; Riedel & Neeck, 2001; Wieseler-Frank et al., 2005). Nociceptive signals are amplified by inflammation, the immune responses that occur following injury (Ferrero-Miliani et al., 2007; Wieseler-Frank et al., 2005). A cascade of soluble mediators become activated during inflammation, and they in turn cause redness via vasodilation, swelling via plasma extravasation, heat, pain, and loss of function in the affected area (Ferrero-Miliani et al., 2007). In response to inflammation, pain information travels from the periphery to the spinal cord via stimulation of nociceptors, afferent sensory inputs that become excited by noxious stimuli (Basbaum & Woolf, 1999).

Nociceptors are the endings of small-diameter nerve fibers (Coutaux et al., 2005). These peripheral sensory organs are typically classified according to their diameter, conduction velocity, and degree of myelination. Cutaneous C-fibers are unmyelinated, slowly conducting (0.4-2 m/s), and are about 0.4-1.2 μm in diameter, while minimally myelinated A δ -fibers are of intermediate velocity (4-30 m/s) and about 2-6 μm in diameter (Coutaux et al., 2005; Millan, 1999). Nociceptors form a functional “pain unit” with mast cells and capillary vessels in nearby tissue (McHugh & McHugh, 2000; Figure 1). Chemical mediators released during the inflammatory response activate the pain unit, and stimulate the nociceptors along with the involved localized tissue (McHugh & McHugh, 2000). Nociceptors can be activated by a variety of modalities, including mechanical, electrical, electromagnetic, thermal, and chemical stimuli, and are thus considered polymodal in nature (Coutaux et al., 2005). Most noxious stimuli (70%) are transmitted by C-fibers, while A δ -fibers are more responsive to high intensity stimuli (i.e. pinch; Basbaum & Woolf, 1999; McHugh & McHugh, 2000; Millan, 1999).

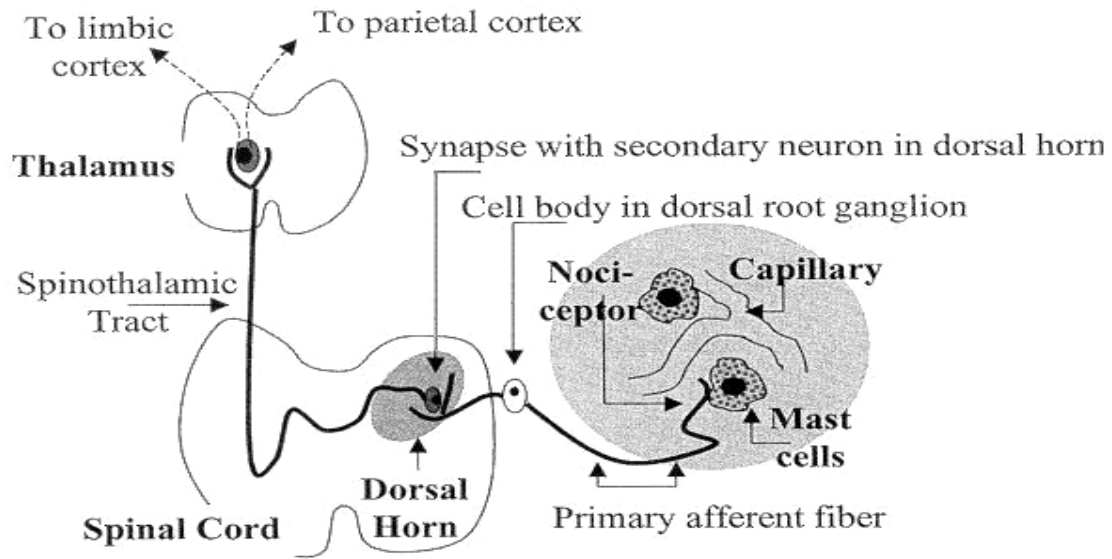


Figure 1. Organization of pain sensation. Nonspecific nociceptive fibers are found in tissues in proximity to capillaries and mast cells. All three of these elements respond to tissue injury in a coordinated fashion with the inflammatory process. Cell injury (leakage of proteases, decreases in interstitial pH), membrane disruption (consequent arachidonic acid metabolism), mast cell degranulation, plasma exudation (bradykinin formation) and capillary dilation all impose chemical and mechanical stimulation of the nociceptor. Impulses arrive at the cell body in the dorsal root ganglion and travel along projections to secondary afferent nerves in the dorsal horn. Impulses then cross to spinal thalamic tracts on the opposite side of the cord before ascending to the thalamus where pain begins to become a conscious event. Pain impulses are associated with meaning and responses as they are processed at higher cortical centers.
From: McHugh and McHugh: AACN CLIN ISSUES ADV PRACT ACUTE CRIT CARE, VOL 11(2) (2000) 168-178

The impulse activity of the nociceptor fibers converge on secondary neurons in superficial region of the dorsal horn, innervating cell bodies in lamina I and II of the spinal cord (Janig, 1987; McHugh & McHugh, 2000; Riedel & Neeck, 2001). The dorsal horn is the first region involved in the transfer of nociceptive stimuli to the central nervous system (CNS). Pain transmission crosses contralaterally and ascends via the spinothalamic tract to the nucleus ventralis posterolateralis (VPL) of the thalamus, the mesencephalon and the reticular formation of the brainstem, and finally to the limbic cortex and somatosensory cortex of the parietal lobe (Janig, 1987; McHugh & McHugh, 2000; Figure 1).

II. Mechanisms Regulating Pain: Anatomical Contributors

First-order nociceptive neurons and dorsal root ganglia

Environmental insult resulting in inflammation causes the release of chemicals from local macrophages, mast cells, immune cells, and injured cells, which then leads to changes in transduction mechanisms at nociceptor terminals (Costigan & Woolf, 2000). Normally, nociceptors have a high threshold for activation. However, after inflammation or the repeated presence of noxious stimuli, the peripheral terminal threshold drops, allowing lower intensity stimuli to activate the nociceptors in a phenomenon termed peripheral sensitization (Woolf & Costigan, 1999). Peripheral sensitization is an increased sensitivity of nociceptors at the site of injury that heightens pain perception and response (Millan, 1999; Riedel & Neeck, 2001). When the transduction sensitivity of the nociceptor terminals is reduced, the result is increased and more frequent action potentials in nociceptive neurons than that found during normal pain responses (Basbaum & Jessell, 2000; Millan, 1999; Woolf & Costigan, 1999).

Inflammation also leads to long lasting posttranslational and transcriptional changes in the dorsal root ganglia (DRG), the nodule on the afferent sensory fiber that contains the cell bodies of neurons (Gardner, Martin, & Jessell, 2000). Translational changes in the DRG result from retrograde transport of nerve growth factor (NGF) and other signal molecules (Ji et al., 2002; McMahon, Cafferty, & Marchand, 2005; Meyer, Ringkamp, Campbell, & Raja, 2006; Woolf & Costigan, 1999). Peripheral NGF contributes to changes in neuron sensitivity during inflammation by binding to TrkA receptors on afferent endings of nociceptors, where it is then transported retrogradely to the DRG (Kidd & Urban, 2001). Here, NGF activates p38 mitogen-activated protein

kinase (MAPK) enzymes, leading to an increase in transient receptor potential vanilloid-1 (TRPV1) expression. TRPV1 maintains inflammatory heat hypersensitivity once it is transported from the DRG to the nociceptive nerve endings (Ji et al. 2002).

Electrical activity also contributes to translational changes in the DRG. Sodium channels TTX-S (tetrodotoxin-sensitive) and TTX-R (tetrodotoxin-resistant) located in primary afferents play a key role in elevating action potentials that transfer nociceptive responses from the periphery to the spinal cord (Basbaum & Woolf, 1999; Woolf & Costigan, 1999). TTX current modulates peripheral terminals, and specifically TTX-R plays a particular role in contributing to nociception excitability within the DRG (Kidd & Urban, 2001; Woolf & Costigan, 1999).

Finally, inflammation hypersensitivity is also mediated by a “phenotypic switch” of low threshold, normally innocuous A β -sensory fibers (Neumann, Doubell, Leslie, & Woolf, 1996). During inflammation, A β -fibers begin to release C-fiber associated neuromodulators (i.e. substance P, brain-derived neurotrophic factor; Neumann et al., 1996). This transcriptional change leads to enhanced pain transmission to the spinal cord (Basbaum & Woolf, 1999; Neumann et al., 1996; Woolf & Costigan, 1999).

Second-order mechanisms contributing to nociception in the CNS

Primary afferents send their projections to the dorsal horn of the spinal cord, where the first relay of synaptic pain integration begins. Each laminae of the dorsal horn is topographically organized in the horizontal plane (Millan, 1999). A δ - and C- fibers mainly terminate in the superficial region of the spinal cord, innervating neurons in laminae I (the marginal zone) and II (substantia gelatinosa), while some fibers provide

lesser input to lamina V (Millan, 1999; Riedel & Neeck, 2001, Suzuki & Dickenson, 2005). Afferent fiber nociceptive input specifies the duration, intensity, and modality of painful stimuli to dorsal horn neurons (Costigan & Woolf, 2000).

Within the dorsal horn, three different types of neurons are predominantly involved in nociceptive input. Lamina I contains nociceptive-specific neurons that are activated primarily by high intensity noxious stimuli (Janig, 1987; Millan, 1999; Suzuki & Dickenson, 2005). These neurons are less capable of encoding stimulus intensity; however, they typically have long axons that project nociceptive information to several higher CNS regions, including the thalamus and periaqueductal gray (PAG; Millan, 1999; Todd, 2002). Lamina II consists mainly of small intrasegmental projection neurons that terminate locally and are involved in the integration and modulation of nociceptive inputs (Millan, 1999; Suzuki & Dickenson, 2005). Neurons in lamina V code innocuous to noxious information and are termed wide dynamic range (WDR; Janig, 1987; Suzuki & Dickenson, 2005). WDR neurons receive polymodal input from the skin, muscle, and viscera, and are responsible for coding stimulus intensity mediated by A δ -, C-, and A β -fibers (Millan, 1999; Janig, 1987).

Nociceptive activation of dorsal horn neurons leads to a progressive increase of action potentials generated by neurons after brief consecutive trains of C-fiber input (“windup;” Baranauskas & Nistri, 1998; Woolf & Costigan, 1999). Windup causes short lasting changes in neuronal excitability, allowing dorsal horn cells to respond to low intensity stimuli (Costigan & Woolf, 2000). NMDA-type glutamate receptors play a major role in this process. Removal of the Mg²⁺ block of the NMDA receptor increases the neuron’s response to successive trains of input (Woolf & Costigan, 1999).

Pain hypersensitivity from the periphery leads to substantial long-lasting increases in spinal excitability, known as central sensitization (Kidd & Urban, 2001). In central sensitization, glutamate and substance P are released from the central terminals of C-fibers during inflammation (Kidd & Urban, 2001). Neurotransmitter release leads to an increase in calcium ions and phosphorylation of the NMDA receptor, resulting in long lasting changes in dorsal horn excitation (Costigan & Woolf, 2000; Kidd & Urban, 2001). Since C-fiber input increases membrane excitability, posttranslational changes within the dorsal horn can last for several hours (Woolf & Costigan, 1999). As a result, dorsal horn neurons become more responsive to sub-threshold stimuli and produce exaggerated responses to innocuous stimuli (Kidd & Urban, 2001). In addition, the receptive field size for dorsal horn neurons increases and thereby exhibits a reduced threshold for activation by A β -fibers (Kidd & Urban, 2001; Millan, 1999). Allodynia (pain sensation in response to a previously non-noxious stimulus) and hyperalgesia (extreme sensitivity to pain) are features of central sensitization (Burian & Geisslinger, 2005; Woolf & Costigan, 1999).

III. Rodent models used to study pain

The formalin model of inflammatory/persistent pain

Rodent models are commonly used to study pain states analogous to persistent and chronic pain diseases observed in humans. The most common rodent models of persistent and chronic pain are thermal, chemical, electrical, or mechanical in nature (Bennett, 2001). These models are separated into acute, tonic/persistent inflammatory, and chronic, differentiated by their mechanism and duration of pain (Kuba & Quiñones-

Jenab, 2005). Acute models generally rely on brief, high-intensity stimuli (i.e. chemical, mechanical) to cause discomfort, while inflammatory pain models involve moderate, continuous pain usually elicited by an inflammatory agent (Bennett, 2001; Tjolsen et al., 1992). Chronic pain models are often used to mimic neuropathic pain states in humans, and rely on nerve injury to induce temporary or permanent allodynia or hyperalgesia around the site of injury (Kuba & Quiñones-Jenab, 2005).

The formalin test for nociception is a model of tonic/persistent pain. It is often used in pain studies because of its association with inflammation and tissue damage (Sternberg & Wachterman, 2000; Tjolsen et al., 1992). The formalin assay consists of an injection of diluted formaldehyde (1-10% in saline) into the hind paw that produces a biphasic behavioral and electrophysiological nociceptive response (Dickenson & Sullivan, 1987; Tjolsen et al., 1992). As shown in Figure 2, the formalin injection causes an initial five minutes of behavioral responses that include licking, flinching, or biting of the paw; these responses are termed Phase I (Damas & Liegeois, 1999; Dubuisson & Dennis, 1977). Following is a quiescent period, or interphase (10-15 minutes), where flinching responses greatly diminish. Thereafter, another phase of pain behavior, termed Phase II, lasts for about 30-40 minutes (Dickenson & Sullivan, 1987; Dubuisson & Dennis, 1977, Tjolsen et al., 1992). Dickenson and Sullivan (1987) used electrophysiology to reveal a relatively comparable biphasic pattern of activation in dorsal horn neurons following formalin administration, while Taylor, Peterson, and Basbaum (1995) demonstrated biphasic cardiovascular responses that correlate with nociceptive behavior.

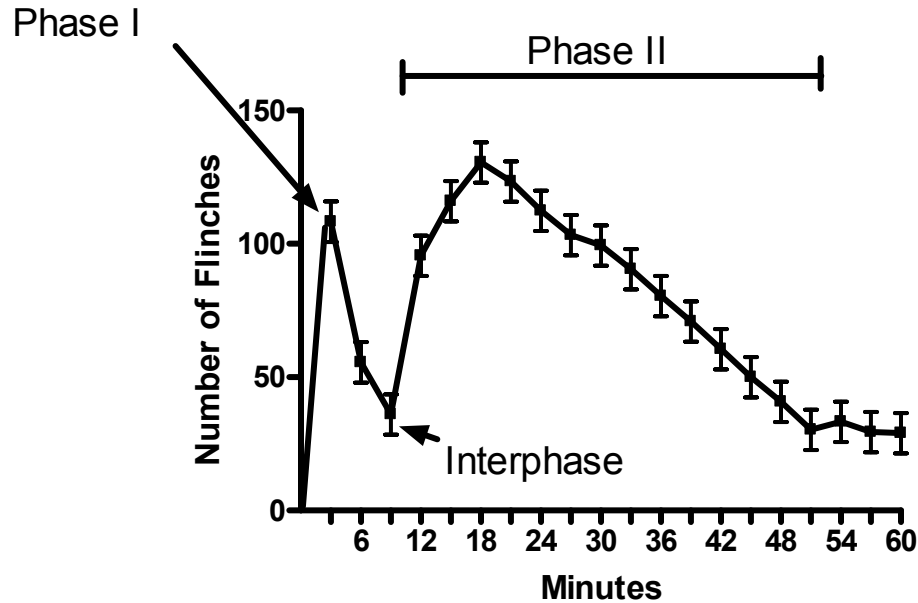


Figure 2. Nociceptive behavior resulting from diluted formalin injection into the hind paw. Across time, the biphasic flinching behavior is characterized as Phase I, for 5-10 minutes post formalin administration. Phase I results from the direct activation of peripheral nociceptors. Following Phase I, there is an interphase period, where relatively normal behavior occurs. Immediately following the interphase, Phase II begins and lasts for 20-50 minutes, where high frequency flinching is observed. Phase II results from continued afferent input from nociceptors as well as activation of NMDA and substance P receptors.

The biphasic nature of the formalin assay provides a method for evaluating acute (Phase I) and persistent (Phase II) pain in a single test that lasts about 60 minutes. The behaviors that ensue following formalin injection seem to depend on diverse mechanisms, since each phase can be inhibited by a different set of antagonists (Damas & Liegeois, 1999). The first phase of activity reflects direct activation of C-fiber sensory afferents by the noxious stimulant (Dubuisson & Dennis, 1977). The second phase is thought to depend on inflammatory responses to tissue injury initiated by C-fiber activity during Phase I, which then leads to NMDA- and substance P-mediated central

sensitization of dorsal horn neurons in the spinal cord (Hunskar & Hole, 1987; Taylor, Peterson, & Basbaum, 1995; Tjolsen et al., 1992). It has been shown that pretreatment of substance P and NMDA antagonists significantly reduces nociceptive responses during Phase II of the formalin test, further suggesting that NMDA and substance P are important mediators of behavioral responses to inflammation during this phase (Coderre & Melzack, 1992; Yamamoto & Yaksh, 1991).

In addition to the behavioral response, formalin also produces edema in the injected hind paw. Damas and Liegeois (1999) found that vascular changes induced by formalin are mediated by a release of neuropeptides, prostanoids, and mast cell amines. After a 5% dose of formalin was injected into the rat paw, edema appeared in two successive phases analogous to the behavioral reactions to formalin (Damas & Liegeois, 1999). A rapid increase in paw weight occurs during the first 20 minutes post formalin administration, followed by a 20 minute plateau, and then a second period of swelling (Damas & Liegeois, 1999). Edema was reduced by pretreatments of capsaicin (which depletes nerve terminals of substance P), the prostaglandin inhibitors indomethacin and NS-398, the 5-hydroxytryptamine (serotonin) inhibitor methysergide, and the antihistamine mepyramine (Damas & Liegeois, 1999). Mast cell amines are shown to play a greater role during the second phase of edema, since serotonin and histamine antagonists are more effective at reducing inflammation during the later time course than during the first (Damas & Liegeois, 1999).

The carrageenan model of inflammatory pain

Another common experimental method for inducing inflammation and hyperalgesia in rodents is through administration of carrageenan. Carrageenan is a glycoprotein derived from seaweed that is injected (in concentrations ranging from 1-3% in saline) in the rodent hind paw to produce a prolonged inflammatory response (Bennett, 2001). Carrageenan administration leads to biphasic events that result in the production of hyperalgesia, edema, erythema (redness), and localized hyperthermia in the affected area. The initial phase of inflammation (0-1 hrs post carrageenan injection) is due to activation of inflammatory agents such as histamine, bradykinin, nitric oxide, and 5-HT in the injected area (Crunkhorn & Meacock, 1971; Di Rosa et al., 1971; Garcia Leme et al., 1973; Hargreaves et al., 1988; Salvemini et al., 1996; Vinegar et al., 1987). The second sustained phase of swelling is correlated with the activation of neutrophils, cytokines, cyclooxygenase (COX)-2, and prostaglandins (Di Rosa et al., 1971; Loram et al., 2007; Seibert et al., 1994). This phase is usually termed the acute phase, and occurs 2-4 hours after injection (Hargreaves et al., 1988).

Figure 3 shows dose-dependent carrageenan-induced inflammation and hyperthermia in the injected paw over time. During carrageenan administration, hind paw swelling develops slowly, with maximum edema peaking after 2 ½ to 4 hours (Fig. 3A). Swelling is present until about 24 hours after carrageenan administration (Garcia Leme et al., 1973; Hargreaves et al., 1988). Paw hyperthermia follows a similar time course as edema formation, with the onset of local hyperthermia beginning at 1hr, and peaking 2-4 hrs after carrageenan injection (Hargreaves et al., 1988; Fig. 3B).

Carrageenan-induced hind paw pain can be reliably measured using the Hargreaves' test for thermal hyperalgesia (Bennett, 2001; Hargreaves et al., 1988). In the Hargreaves' test, rodents are placed in individual plexiglass cages set atop a clear glass platform. After an acclimation period, sensitivity to thermal stimuli is determined by directing radiant heat to the plantar surface of the hind paws and measuring (in sec) the time it takes for the rodent to withdraw its paw from the heat source. Thermal hyperalgesia (the increased sensitivity to a noxious thermal stimulus) is tested by comparing baseline paw withdrawal latency (PWL) to the readings taken after carrageenan administration. Changes in PWL to the heat source are then calculated. As shown in Figure 3C, Hargreaves et al. (1988) demonstrated that carrageenan elicits a decrease in PWL compared to saline-treated controls, and this effect is dose-dependant. Changes in PWL from carrageenan treatment are observed as early as 1 hour after injection, and a dose-related peak is present at about 2 ½ hours (Fig. 3C). Effects of carrageenan on PWL are greatly diminished 24 hours after administration (Hargreaves et al., 1988). However, using the von Frey model for mechanical hyperalgesia, it has been shown that carrageenan administration can lead to nociceptive responses that last up to 48 hours post injection (Loram et al., 2007). Taken together, the formalin and carrageenan assays are useful models for studying inflammatory pain states in rodents.

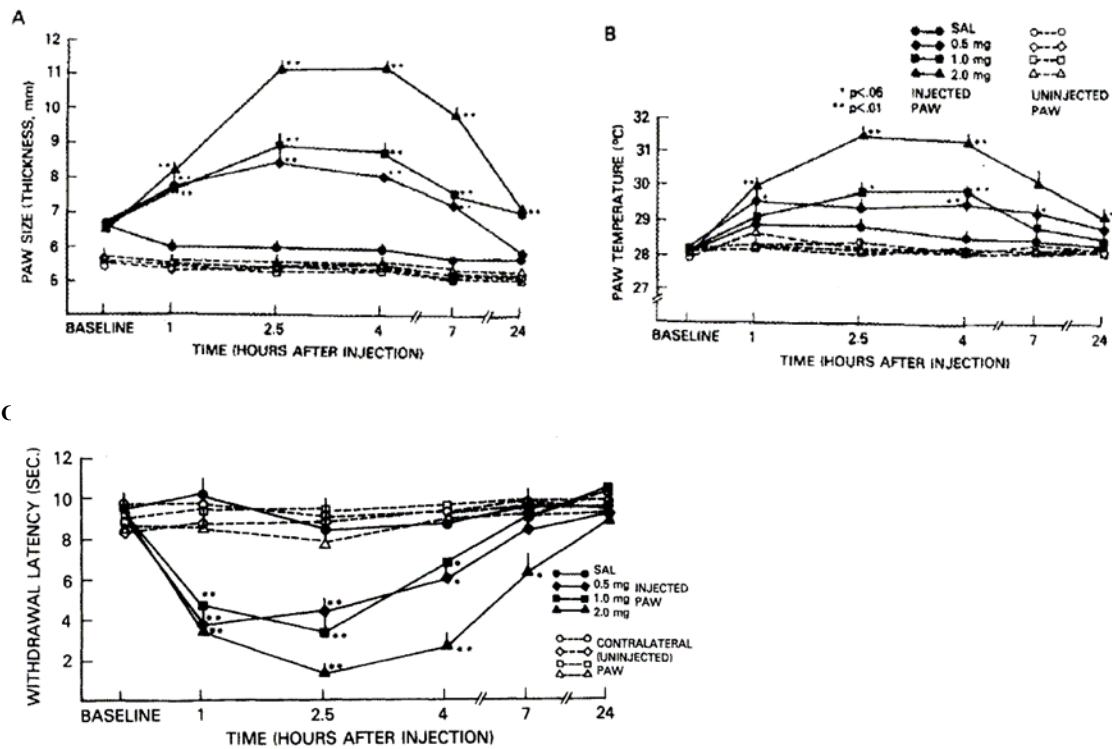


Figure 3. Dose-related effects of carrageenan. Rats (8/groups) were injected with either saline or 0.5, 1.0 or 2.0 mg of carrageenan into a hind paw (filled symbols), while the contralateral paw was untreated (corresponding open symbols). **A.** Effects of carrageenan on paw edema as measured by dorsal-plantar paw thickness using a vernier caliper. $**P < 0.01$. **B.** Effects of carrageenan on paw temperature measured with a contact thermocouple. $*P < 0.05$, $**P < 0.01$. **C.** Dose-related effects of carrageenan on paw withdrawal latency following exposure to radiant heat. $*P < 0.05$, $**P < 0.01$. Adapted from: Hargreaves et al.: PAIN, VOL 32 (1988) 77-88

IV. Modulators involved in nociception

Nociception results in the release of various neurochemicals that facilitate inflammatory processes (Basbaum & Woolf, 1999; Meyer et al., 2006). Inflammation associated with tissue damage or invasion by immune cells causes cell leakage into extracellular fluid, and leads to recruitment of inflammatory cells and neuroactive agents such as cytokines, chemokines, prostanoids, kinins (bradykinin), growth factors, and other inflammatory mediators at the site of injury (Ferrero-Miliani et al., 2007; Rittner & Stein, 2005; Woolf & Costigan, 1999). Most of these agents modulate nociceptors, cause posttranslational changes in the periphery and dorsal horn, and alter basal transduction levels that result in sensitization (Woolf & Costigan, 1999).

Cytokines

Cytokines are small proteins rapidly produced by resident cells in the periphery (mainly monocytes/macrophages) and by glial cells (astrocytes and microglia) in the CNS (Sommer & Kress, 2004; Wieseler-Frank et al., 2005). They play essential roles as mediators in pain, diseases, infections, and immune responses (Ferreira, 1993; Hopkins, 2007; Meyer et al., 2006; Moalem & Tracey, 2006; Rittner & Stein, 2005; Wieseler-Frank et al., 2005). Cytokines are produced on demand and act locally to induce or facilitate inflammatory and neuropathic pain and hyperalgesia (Sommer & Kress, 2004). Their activation or dysregulation has been involved in a variety of disease states such as multiple sclerosis, Chron's disease, and rheumatoid arthritis (Sommer & Kress, 2004). Peripheral cytokines have been implicated in playing a role in the initiation of inflammatory pain. After tissue injury, inflammatory responses are heightened when

leukocytes and regional cells produce cytokines and other proalgesic mediators (Rittner, Machelska, & Stein, 2005). Pain responses also lead to modulation of central cytokine production as well. Within the CNS, cytokines usually present at low levels during resting states are up-regulated by glial cells and infiltrating macrophages during injury and inflammation (Hopkins, 1997; Sternberg, 1997).

One of their characteristics is that unlike hormones, cytokines are pleiotropic and redundant in nature. Hence, a single cytokine can act on many different tissues and cells (pleiotropy), and several cytokines can have overlapping roles on a single target (redundancy; Kishimoto, Akira, Narazaki, & Taga, 1995). These characteristics may occur because of the molecular makeup of the cytokines' receptors (Kishimoto et al., 1995; Sommer & Kress, 2004). For instance, glycoprotein 130 (gp130), a transmembrane protein expressed in every tissue, has been shown to transduce signals for various cytokine receptor systems (Kishimoto et al., 1995). Therefore, cytokines can exhibit common characteristics in a variety of targets. However, these properties make it hard to classify a cytokine as either pro- or anti-inflammatory, since their functions may sometimes overlap.

The diverse molecular structure of cytokines has led them to be classified into four groups: growth factors, interleukins, interferons, and tumor necrosis factors (DeLeo, Colburn, & Rickman, 1997; Sommer & Kress, 2004). Several cytokines are considered proinflammatory, such as tumour necrosis factor-alpha (TNF- α), interleukin-1 beta (IL-1 β), and interleukin (IL)-6, while others are anti-inflammatory, such as interleukin (IL)-10 and interleukin (IL)-4. Cytokines also consist of chemokines, small chemoattractant cytokines that recruit leukocytes to the site of inflammation (Moalem & Tracey, 2006).

During inflammation, the production of cytokines occurs in a sequence, where TNF- α is produced first, then chemokines and other proinflammatory cytokines are produced to aid in the inflammatory response (Cunha, Poole, Lorenzetti, & Ferreira, 1992). This is followed by anti-inflammatory cytokine production that is enacted to inhibit further proinflammatory cytokine expression (Cunha, et al., 1992). Figure 4 gives an overview of cytokine activation during inflammation. As shown in Figure 4, cytokines trigger two basic hypernociceptive pathways: the prostanoid and the sympathetic (Ferreira, Lorenzetti, Cunha, & Poole, 1993; Rittner, et al., 2005). The biochemical mechanisms involved in hyperalgesia and analgesia following cytokine production will be discussed in the following sections.

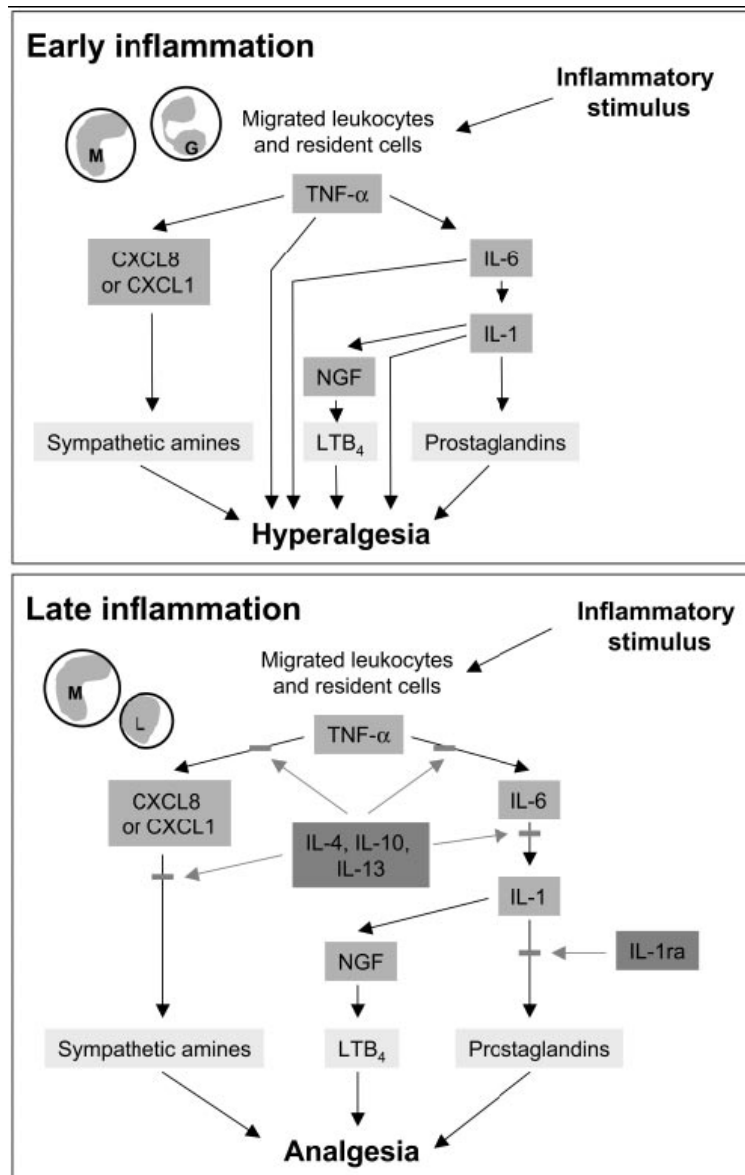


Figure 4. Pro- and analgesic mechanisms in inflammation. In early inflammation, an inflammatory agent stimulates the migration of leukocytes, e.g., granulocytes (G) and monocytes (M), into the inflamed tissue. Here, these leukocytes as well as resident cells initiate a cascade of cytokines including TNF- α and interleukins (ILs), chemokines [CXC chemokine ligand 8 (CXCL8), CXCL1], NGF, and secondary mediators, such as sympathetic amines, leukotriene B₄ (LTB₄), and prostaglandins, culminating in hyperalgesia. TNF- α , IL-6, IL-1, and bradykinin can also have direct hyperalgesic effects on nociceptors. During ongoing, late inflammation, lymphocytes (L) and monocytes/macrophages (M) start to produce anti-inflammatory cytokines, such as IL-4, IL-10, IL-13, and IL-1 receptor antagonist (IL-1ra). These cytokines inhibit the proinflammatory cytokines, such as TNF- α , IL-1, and IL-6, and block the cascade. *From: Rittner, Machelska, and Stein: JOURNAL OF LEUKOCYTE BIOLOGY, VOL 78(6) (2005) 1215-1222*

Proinflammatory cytokines

Proinflammatory cytokines (i.e. TNF- α , IL-6, IL-1) act synergistically to induce the expression of inflammatory mediators that are involved in nociceptive processes, such as prostaglandins and growth factors (DeLeo et al., 1997; Ferreira, Lorenzetti, Bristow, & Poole, 1988; Meyer et al., 2006; Moalem & Tracey, 2006). They begin early immune responses by recruiting and activating leukocytes and immune cells to the injury site (Moalem & Tracey, 2006; Wieseler-Frank et al., 2005). Proinflammatory cytokines also directly initiate the production of other cytokines, leading to the possibility of positive feedback if not regulated appropriately (DeLeo et al., 1997; Moalem & Tracey, 2006). Early research conducted by Cunha et al. (1992) and Ferreira et al. (1988) demonstrate that injection of the cytokines TNF- α and IL-1 β into the rat hind paw leads to increased nociceptive activity. Furthermore, after carrageenan administration, cytokine antagonists are able to reduce hyperalgesia in paw tissue, indicating that cytokine activation is important in the production of inflammatory pain (Cunha et al., 1992; Ferreira et al., 1988).

TNF- α

TNF- α plays a principal role in initiating the cascade of cytokines and other growth factors (Cunha et al., 1992; Sommer & Kress, 2004; Wieseler-Frank et al., 2005). It activates the release of interleukins and chemokines, resulting in the production of sympathetic amines, prostaglandins, and other mediators involved in inflammatory responses (Cunha et al., 1992; Inglis et al., 2005; Rittner et al., 2005; Figure 4). TNF- α can also act directly to trigger nociceptive responses (Cunha et al., 1992; Ferreira et al., 1988; Sorkin, Xiao, Wagner, & Myers, 1997; Zelenka, Schafers, & Sommer, 2005).

Electrophysiological research conducted by Sorkin et al. (1997) demonstrated that low dose topical application of TNF- α to the sciatic nerve induces high discharge rates in A δ - and C-fibers within one to three minutes of application. Intraneural and intraplantar TNF- α injections lead to mechanical and thermal hyperalgesia (Cunha et al., 1992; Zelenka et al., 2005). Clinically, it has been demonstrated that microglia release elevated levels of TNF- α during disorders of the nervous system, such as Alzheimer's and Parkinson's disease (Rosenberg, 2005; Sawada, Imamura, & Nagatsu, 2006).

The mechanisms in which TNF- α modulates nociception have been investigated. Schafers, Svensson, Sommer, and Sorkin (2003) determined that TNF- α -elicited hyperalgesia is mediated through p38 MAPK, an enzyme that plays a role in the maintenance of inflammation (Ji et al., 2002). Lumbar spinal cord and L5 and L6 DRG tissue were analyzed for p38 and TNF- α activation in rats that underwent spinal nerve ligation (SNL) alone or in combination with pretreatment of the TNF- α -antagonist etanercept. After SNL, p38 levels were elevated in spinal cord and DRG neurons, and phosphorylated p38 was found in TNF-positive DRG neurons. Etanercept pretreatment blocked p38 activation in DRG tissue. These results demonstrated that TNF- α and p38 activation play a significant role in the generation of mechanical allodynia after nerve injury (Schafers et al., 2003).

TNF- α exerts its biologic functions by binding to two receptor types, p55 TNF receptor type 1 (TNFR1) and the p75 TNF receptor type 2 (TNFR2). Inglis et al. (2005) found that these receptors are activated differently during chronic inflammation. Following administration of complete Freund's adjuvant (CFA), an inflammatory agent used in rodent disease models for arthritis, TNFR1 expression was increased in rat

ipsilateral and contralateral DRG neurons. TNFR2 expression increased as well, but only in non-neuronal macrophages found in the ipsilateral DRG. These results indicate that during hyperalgesia, TNF- α activates DRG neurons via the TNFR1 receptor, providing a direct role of TNF- α on inducing sensory neuron activity (Inglis et al., 2005). Further, macrophage TNFR2 expression demonstrates a second pathway by which TNF- α modulates neuronal functioning during chronic inflammation (Inglis et al., 2005).

IL-1

TNF- α release leads to the activation of interleukin (IL)-1. IL-1 is produced by many cell types, including monocytes, fibroblasts, and glial cells (Watkins & Maier, 1999). This cytokine exists in two forms, IL-1 α and IL-1 β . Early research conducted by Ferreira et al. (1988) demonstrated that during inflammation, IL-1 β , rather than IL-1 α , is highly involved in nociceptive responses. Since then, its role during inflammation has been investigated.

IL-1 β is a mediator of a variety of inflammatory processes (Ferrero-Miliani et al., 2007). It evokes hyperalgesia by initiating a signaling cascade that includes the stimulation of COX-2, and the subsequent release of prostaglandins (Zucali et al., 1986). Besides being synthesized in peripheral tissue, IL-1 β is produced by neurons and microglia in the brain. Rothwell (1991) reviewed that elevated IL-1 β levels produced in brain tissue and CSF during injury or inflammation, as well as in disease states such as Alzheimer's disease and meningitis, mediate chronic neuropathological changes in the CNS.

IL-1 β plays a direct role in inducing nociception as well. Fukuoka et al. (1994) showed that IL-1 β elicits nociceptive signaling in nerve fibers within 1 minute of

application. IL-1 β has been shown to elicit hyperalgesia when injected intraplantar in the rat hind paw and intraneural in the sciatic nerve (Ferreira et al., 1988; Zelenka et al., 2005). Inflammatory hypernociception elicited by carrageenan, bradykinin, IL-1 β , TNF- α , and lipopolysaccharide (LPS) are all inhibited by pretreatment with antibody against IL-1 β (Cunha, Cunha, Poole, & Ferreira, 2000). Application of antiserum against IL-1 β inhibits orofacial nociception in rats during Phase II of the formalin test (Chichorro, Lorenzetti, & Zampronio, 2004). IL-1 β induction also contributes to neuronal excitability within the CNS. Samad and colleagues (2001) showed that IL-1 β is the major cytokine involved in central COX-2 induction. Intrathecal IL-1 β administration led to increased spinal COX-2 mRNA and PGE₂ levels in dorsal horn neurons (Samad et al., 2001). Furthermore, COX-2 and PGE₂ levels were inhibited by intrathecal administration of recombinant IL-1 receptor antagonist (IL-1 ra). These results demonstrate that IL-1 β is involved in central prostanoid activation (Samad et al., 2001).

IL-6

The cytokine IL-6 is involved in many immunological processes, which include regulating B-cell differentiation and the acute phase response to injury (Kishimoto et al., 1995). It is produced by many cell types, such as mast cells, lymphocytes, neurons, and glial cells, and is up-regulated during Alzheimer's disease, Parkinson's disease, and HIV (Gadient & Otten, 1997; Moalem & Tracey, 2006). The actions of IL-6 depend on the presence of the membrane bound or soluble IL-6 receptor (IL-6R; Gadient & Otten, 1997). Obreja et al. (2002) demonstrated that the nociceptive actions of IL-6/IL-6R result from constitutive expression of gp130; intradermal administration of the IL-6/soluble IL-6R complex activates gp130. This in turn leads to the direct sensitization of

rat nociceptors to noxious heat, and to modulation of CGRP release (Obreja et al., 2002).

As shown in Figure 5, binding IL-6 to IL-6R triggers the homodimerization of gp130, resulting in two signal transduction cascades, one involving Janus tyrosine kinase (Jak), and the other involving Ras-dependent MAPK (De Jongh et al., 2003; Kishimoto et al., 1995). Both of these intracellular cascades are involved in nociception (De Jongh et al., 2003).

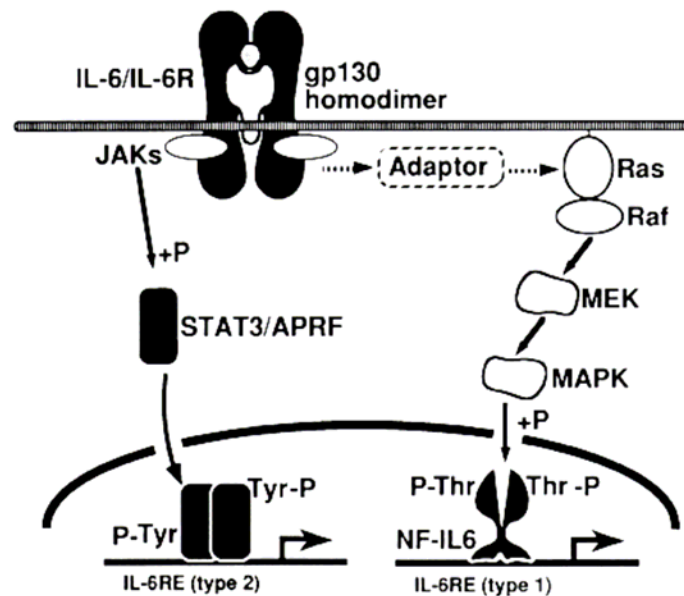


Figure 5. Two pathways from gp130 to acute phase protein gene expression. Homodimerization of gp130 activates the STAT3/APRF pathway as well as the MAPK cascade. An adaptor molecule not yet identified is postulated to link gp130 homodimer to Ras, leading to the critical threonine-phosphorylation of NF-IL6 through MAPK activation. The STAT3 dimer and the NF-IL6 dimer bind to type 1 and type 2 IL-6RE, respectively, of the acute-phase protein genes. *From: Kishimoto, Akira, Narazaki, and Taga: BLOOD, VOL 86(4) (1995) 1243-1254*

The role of IL-6 during inflammation is controversial. Clinically, IL-6 serum levels are elevated in patients suffering from neuropathies, autoimmune and chronic inflammatory conditions (Sommer & Kress, 2004). However, experimental studies

describe pro- and anti-inflammatory roles for this cytokine. For instance, formalin and carrageenan administration have been shown to elevate IL-6 serum levels in rats (Aloisi et al., 1995; Loram et al., 2007). Intraplantar, intracisternal, and intracerebroventricular injection of IL-6 leads to nociceptive behavioral responses in rats (Choi et al., 2003; Cunha et al., 1992; Oka, Oka, Hosoi, & Hori, 1995). IL-6 also modulates nociception by stimulating IL-1 β production in rats and prostaglandin production in mice (Cunha et al., 1992, 2005). In contrast, IL-6^{-/-} mice display higher withdrawal latencies after carrageenan administration than wild-types, and spinal IL-6 administration inhibits nociceptive signaling following SNL (Flatters, Fox, & Dickenson, 2003; Xu et al., 1997). These contradictory findings may be a result of methodology used between studies, or a pro- and anti-nociceptive role exhibited by IL-6 during various disease states. In this paper, IL-6 will be considered a proinflammatory cytokine.

GM-CSF

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a glycoprotein that is synthesized and secreted by activated T lymphocytes, macrophages, and cytokine-activated endothelial cells (Fibbe et al., 1989; Strieter et al., 1989). It is a major regulator that stimulates the proliferation and differentiation of granulocyte and macrophage colonies from precursor cells (Burgess & Metcalf, 1980). Production of GM-CSF occurs at basal levels in tissue, but studies have found that GM-CSF is up-regulated during acute phases of bacterial and viral infections, as well as after injection of lipopolysaccharide (LPS; Metcalf, 1973; Sheridan & Metcalf, 1972). This early research led to investigation of the role of GM-CSF in inflammation and autoimmunity.

Several studies have investigated inflammatory responses following GM-CSF administration. From this research, GM-CSF has been demonstrated to have proinflammatory actions in *in vivo* and *in vitro* models. For instance, in murine models, injection of GM-CSF *in vivo* leads to enhanced collagen-induced arthritis (CIA), while GM-CSF blockade by anti-GM-CSF monoclonal antibody ameliorated the development of CIA (Campbell et al, 1997; Cook et al., 2001). During LPS administration in mice, proinflammatory cytokine expression is augmented by the priming effect of GM-CSF on neutrophils, white blood cells involved in the acute phase of inflammation (Choi et al., 2008). Intraperitoneal injection of GM-CSF in mice leads to increased levels of neutrophils and peritoneal macrophages, demonstrating that GM-CSF regulates these cell types (Metcalf et al., 1987). Following peripheral nerve injury in mice, GM-CSF induced by TNF- α and IL-1 α leads to MAC-2 production, a macrophage activation marker involved in phagocytosis of myelin (Reichert, Saada, & Rotshenker, 1994; Saada, Reichert, & Rotshenker, 1996). *In vitro*, GM-CSF application induces proinflammatory cytokine production from human T lymphocytes and peripheral blood mononuclear cells (Eksioglu, Mahmood, Chang, & Reddy, 2007). The anti-inflammatory mediators IL-4 and dexamethasone (a synthetic glucocorticoid) inhibit the production of GM-CSF in LPS-treated human monocytes (Hamilton et al., 1992).

GM-CSF is involved in inflammatory pain states such as Multiple Sclerosis (Carrieri et al., 1998), rheumatoid arthritis, and Chron's disease [see Feldmann et al. (1996) and Panes et al. (2007) for review]. This cytokine may possibly enhance pain signaling from the periphery to the CNS, since it has the ability to cross the blood-brain and blood-spinal cord barriers (McLay, Kimura, Banks, & Kastin, 1997). More research

is warranted to investigate the potential role of down-regulating GM-CSF as a clinical treatment for inflammatory and autoimmune diseases.

IL-2

Interleukin (IL)-2, also known as T cell growth factor, is an immunoregulatory cytokine that stimulates the production of B, T, and Natural Killer cells (Hanisch & Quirion, 1996; Waldmann, 2006). IL-2 and its receptors are found in the dorsal root ganglia, as well as in many areas of the CNS, including the hippocampus, cerebellum, thalamus, and neostriatum (Lapchak et al, 1991; Song, Zhao, & Liu, 2000). During an immune response, antigens stimulate T_H1 cells to produce IL-2; in turn IL-2 induces the proliferation of T cells and the synthesis of cytokines such as TNF- α , IL-1, and IFN- γ (Hanisch & Quirion, 1996; Janssen et al., 1994). Although IL-2 stimulates proinflammatory cytokines, its role as an inflammatory mediator during pain processing remains controversial. For instance, IL-2 intraperitoneal and intrathecal injections lead to antinociception during the radiant heat test for thermal nociception (Song, Liu, & Zhao, 2002; Yao et al, 2003). However, IL-2 used clinically to treat metastatic renal cell carcinoma leads to significant toxicity in patients (Rosenberg et al., 1998). IL-2 plasma levels were correlated with elevated pain intensity in chronic pain patients (Koch et al., 2007). Further, in a murine model of herpes simplex virus type 1 infection of the corneas, systemic neutralization of IL-2 led to rapid regression of corneal inflammation (Tang, Chen, & Hendricks, 1997).

Cata and colleagues (2008) investigated the direct effects of IL-2 on pain processing. Rats were given an intrathecal injection of 500U (low dose) or 2000U (high dose) IL-2, and responses to mechanical and thermal nociceptive stimuli were analyzed.

Following 1 to 3 hours post injection, it was demonstrated that low dose IL-2 produced thermal antinociception, while the high dose produced thermal and mechanical hyperalgesia (Cata, Weng, & Dougherty, 2008). This research supports the notion that IL-2 plays a role in inflammation, and its effects are biphasic in nature. Further, these results may help explain why IL-2 is shown to have contradictory functions during inflammatory and neuropathic pain states. Due to the research previously mentioned, IL-2 will be referred to as a proinflammatory cytokine in this paper.

IFN- γ

The cytokine interferon-gamma (IFN- γ) is produced mainly by T cells, lymphocytes, and macrophages. Its production is stimulated by cytokines such as IL-2 and IL-12 (for review see Young and Hardy, 1995). IFN- γ has been shown to induce monocyte/macrophage differentiation and gene expression, and plays an important role in modulating immune and inflammatory responses (Farber, 1990; Perussia et al., 1983). The potential role of IFN- γ during pain processing comes from clinical and preclinical studies. Clinically, cancer patients who receive IFN- γ during therapy experience spontaneous pain after treatment (Mahmoud et al, 1992; Quesada et al, 1986). Experimentally, rats chronically treated with IFN- γ show reduced PWLs to mechanical hind paw stimulation (Vikman, Siddall, & Duggan, 2005). In rodents, intrathecal IFN- γ administration evokes pain related behaviors such as biting/ scratching and activation of the spinal nociceptive flexor reflex (Robertson et al., 1997; Xu et al., 1994). Knock-out mice with disruption to the IFN- γ receptor gene do not display nociceptive behaviors to intrathecal IFN- γ injections, supporting the involvement of this cytokine during chronic pain states (Robertson et al., 1997).

IFN- γ may modulate nociceptive transmission in the pain pathway, since its receptors are located in the superficial region of the dorsal horn, DRG, and lateral spinal nucleus (Robertson et al., 1997; Vikman et al., 1998). Chronic IFN- γ treatment leads to an increase in spontaneous synaptic activity in dorsal horn neurons, and enhanced responsiveness of DRG axons, *in vitro* (Vikman et al., 2003). *In vivo* studies suggest that IFN- γ contributes to central sensitization by reducing GABAergic inhibition in the dorsal horn during persistent pain (Vikman et al., 2007). Taken together, these findings demonstrate that IFN- γ contributes to pathological conditions within the nervous system.

Anti-inflammatory cytokines

The synthesis of proinflammatory cytokines is regulated by negative feedback systems, such as the production of anti-inflammatory cytokines (Wieseler-Frank et al., 2005). Anti-inflammatory cytokines inhibit proinflammatory cytokine function when their action is no longer needed (Wieseler-Frank et al., 2005). Specifically, anti-inflammatory cytokines counteract the effects of proinflammatory cytokines by suppressing genes that code for TNF- α , IL-1, and chemokines (Moalem & Tracey, 2006; Figure 4). Anti-inflammatory cytokines also display antinociceptive responses following tissue damage by opposing hyperalgesia at the site of injury (Millan, 1999).

IL-10

One notable anti-inflammatory cytokine is IL-10, a peptide produced by immune cells such as monocytes, mast cells, and T-lymphocytes (Wagner, Janjigian, & Myers, 1998). The IL-10 protein is a homodimer that exerts its effects by binding to the IL-10R1/IL-10R2 complex, and leads to the activation of the Jak/STAT (signal transducer

and activator of transcription) pathway (Commins, Steinke, & Borish, 2008). IL-10 lowers IL-1 β , IL-6, CXCL8 (IL-8), and TNF- α levels produced by activated macrophages, IFN- γ and IL-2 produced by T_H1 lymphocytes, and IFN- γ and TNF- α produced by Natural Killer cells (Commins et al., 2008; Fiorentino et al., 1991). Besides inhibiting cytokine production, IL-10 has also been shown to lower prostaglandin levels (Mertz et al., 1994). Poole et al. (1995) investigated the antinociceptive role of IL-10 in rats administered inflammatory agents. Rats pretreated with IL-10 were given intradermal injections of TNF- α , IL-1 β , or carrageenan. IL-10 administration significantly decreased cytokine- and carrageenan-mediated mechanical hyperalgesia (Poole et al., 1995).

Marshall et al. (1996) demonstrated that IL-10 modulates mast cell IL-6 production. In prostaglandin E₂-activated mast cells, the production of IL-6 was significantly inhibited by IL-10 (Marshall et al., 1996). In mast cells activated by anti-immunoglobulin (Ig) E (an antibody involved in histamine release), IL-10 significantly reduced IL-6 levels 18 hours, but not 10 minutes, following anti-IgE activation (Marshall et al., 1996). This study demonstrates that IL-10 inhibits mast cell-produced cytokines activated by inflammatory mediators such as prostaglandins and immunoglobins (Marshall et al., 1996). Furthermore, IL-10 does not immediately suppress proinflammatory cytokine activity, but rather selectively inhibits its long-term production (Marshall et al., 1996).

Research conducted by Wagner, Janjigian, and Myers (1998) showed that IL-10 administration decreases nociceptive responses and inflammatory agents produced during nerve injury. IL-10 was administered at the time of a chronic constriction injury (CCI)

lesion. CCI is a model of neuropathic pain that involves partial nerve injury, and results in nerve compression, thermal hyperalgesia, and inflammation (Seltzer, Dubner, & Shir, 1990; Wagner et al., 1998). Following CCI, thermal hyperalgesia was significantly decreased in rats pretreated with IL-10 (Wagner et al., 1998). Further, histological sections taken from the site of peripheral nerve injury showed that IL-10-treated rats had a lower recruitment of local macrophages as compared to controls (Wagner et al., 1998).

IL-4

IL-4 is an anti-inflammatory cytokine secreted by T-lymphocytes and mast cells that aids in regulating immune responses (Ben-Sasson et al., 1990; Paul, 1991; Verri et al., 2006). The analgesic role of IL-4 involves the inhibition of proinflammatory cytokines. For instance, IL-4 has been shown to reduce the production of TNF- α , IL-1 β , and IL-6 in activated human monocytes, suppress translational activation of TNF- α mRNA in murine macrophages, and suppress IL-1 β mRNA expression *in vitro* (Mijatovic et al., 1997; te Velde et al., 1990; Vannier, Miller, & Dinarello, 1992). Furthermore, IL-4 directly inhibits the induction of COX-2 and nitric oxide synthase, the enzymes responsible for PGE₂ and nitric oxide production, respectively (Fenton et al., 1992; Hart et al., 1989; Onoe et al., 1996). In inflammatory pain models, IL-4 has been demonstrated to inhibit hypernociception caused by intraplantar injections of carrageenan, bradykinin, or TNF- α in rats, and reduces the writhing response induced by intraperitoneal injection of acetic acid or zymosan in mice (Cunha et al., 1999; Vale et al., 2003).

Hao, Mata, Glorioso, and Fink (2006) demonstrated that IL-4 inhibits nociception during neuropathic pain as well; IL-4 inoculation reduced mechanical allodynia and

thermal hyperalgesia in rats following SNL; however, these antinociceptive responses dissipated over time. Also, IL-4 significantly reduced IL-1 β and PGE₂ expression (markers of neuropathic pain) in spinal cord tissue following SNL (Hao et al., 2006). Taken together, this study demonstrates that IL-4 reduces nociceptive pain responses, but does not prevent the ultimate development of neuropathic pain. Further, since a peripheral injection of IL-4 was able to block IL-1 β and PGE₂ spinal cord expression, axonal transport of IL-4 was shown to reduce spinal sensitization, a characteristic of chronic pain in the CNS (Hao et al., 2006).

Prostanoids and Cyclooxygenases

Prostanoid synthesis is an important mediator in pain production, not only in peripheral tissue but in the CNS as well (Burian & Geisslinger, 2005; Moalem & Tracey, 2006). Prostanoids lead to nociceptor activation and transmission in primary afferents by inducing second messenger pathways (cAMP and protein kinase A) that reduce nociceptor thresholds and increase membrane excitability (Ferreira & Nakamura, 1979; McCleskey & Gold, 1999). After injury, cellular phospholipids are converted into arachidonic acid by the action of the phospholipase A₂ (PLA₂) and PLC enzymes (Moalem & Tracey, 2006; Samad, Sapirstein, & Woolf, 2002; Vanegas & Schaible, 2001; Figure 6). Arachidonic acid is a fatty polyunsaturated acid that is converted to the prostanoids prostaglandin and thromboxane once it is released from the cell membrane (McHugh & McHugh, 2000; Moalem & Tracey, 2006; Samad et al., 2002).

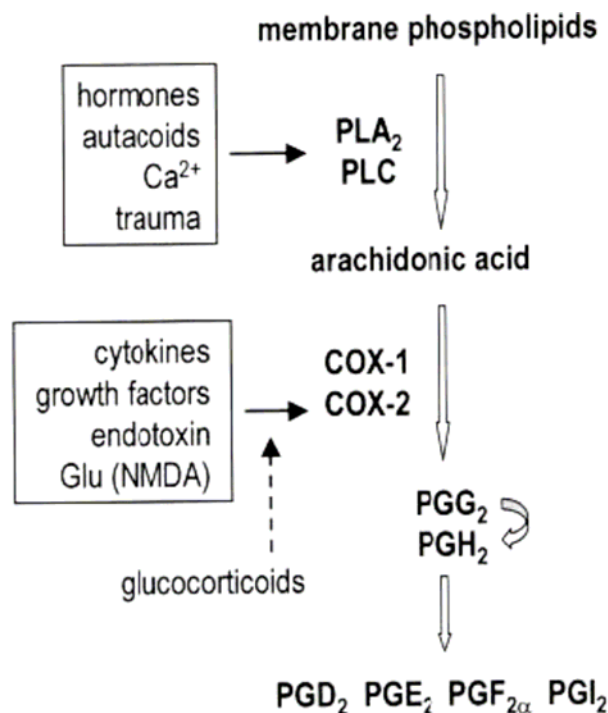


Figure 6. Steps in the synthesis of prostaglandins. Black full arrows indicate activation, and broken arrow indicates inhibition. *From: Vanegas and Schaible: PROG IN NEUROBIOL, VOL 64 (2001) 327-363*

Prostaglandins (PGs) are synthesized from arachidonic acid by cyclooxygenase (COX) enzymes (McHugh & McHugh, 2000; Vanegas & Schaible, 2001; Figure 6). The two COX isoforms, COX-1 and COX-2, are involved in different enzymatic functions. COX-1 is a constitutive enzyme that is mainly responsible for homeostatic prostaglandin production, whereas the COX-2 enzyme has a relatively low basal expression, and is up-regulated during inflammatory states by such factors as proinflammatory cytokines, growth factors, macrophages, and hormones (Moalem & Tracey, 2006; O'Banion, 1999; Vane, Bakhle, & Botting, 1998; Vanegas & Schaible, 2001; Figure 6).

COX-1 and COX-2 catalyze arachidonic acid in two different states: a cyclooxygenase reaction to form prostaglandin G synthase-2 (PGG₂) and a peroxidase

reaction to form prostaglandin H synthase-2 (PGH₂; Pace-Asciak & Smith, 1983; Samad et al., 2002). After PGH₂ is synthesized, it is further converted into the functional PG isoforms E₂, D₂, F_{2α}, and I₂ by tissue specific isomerases (PG synthases; Samad et al., 2002). microsomal PGE synthase-1 (mPGES-1), an inducible enzyme regulated by proinflammatory cytokines during inflammation, is responsible for converting PGH₂ into PGE₂ (Hopkins, 2007; Mosca et al., 2007; Murakami & Kudo, 2006).

PGE₂ mediates peripheral and central hypersensitivity during nociception and inflammation (Burian & Geisslinger, 2005; Moalem & Tracey, 2006; Zeilhofer & Brune, 2006). It lowers the threshold of activation of TRPV1 and TTX-R receptors after nociception. This in turn leads to sensitization and the allowance of pain causing agents, such as bradykinin, to reach threshold for initiating action potentials (Gold, Reichling, Shuster, & Levine, 1996; Tominaga et al., 1998). The hyperalgesic properties of PGE₂ have been demonstrated in experimental models. For instance, Malmberg & Yaksh (1995) found that formalin administration into the rat hind paw leads to elevated PGE₂ expression in the spinal cord. Intraplantar injection of PGE₂ causes nociceptive behavior (paw licking) and mechanical allodynia in mice (Kassuya, Ferreira, Claudino, & Calixto, 2007). Guay et al. (2004) found that PGE₂ levels increase in peripheral and CNS tissue after carrageenan-induced paw inflammation in the rat.

Estrogen has been shown to modulate prostaglandin levels and has thus been postulated to alter behavioral responses to pain via its regulation of the prostanoid pathway. Preliminary research in our lab shows that PGE₂ levels are altered across the estrous cycle, where rats in estrus (a stage with low levels of gonadal hormones) have lower PGE₂ levels than rats in metestrus and diestrus stages (Amador et al., personal

communication, 2009). Furthermore, Hunter et al. (in press) have shown that co-administration of 17 β -estradiol plus the COX inhibitor ibuprofen reduces flinching responses to formalin. Although PGE₂ levels were decreased due to estradiol administration, this difference failed to reach significance (Hunter et al., in press). Together, these studies indicate that a crosstalk may exist between ovarian hormone production and prostaglandin synthesis. It has been suggested that estradiol's anti-hyperalgesic effects may work in part by modulating COX-2-mediated mechanisms (Hunter et al., in press).

Glucocorticoids

The hypothalamic-pituitary-adrenal (HPA) axis provides negative feedback to immune challenges via the production of its end products, the glucocorticoids. Following a stress response, the HPA axis is activated, causing the release of corticotropin-releasing hormone (CRH) from the paraventricular hypothalamic nucleus (Sapolsky, 1992). CRH is a major secretagogue that regulates pituitary adrenocorticotrophic hormone (ACTH) release. ACTH results in the release of the adrenal steroid glucocorticoid (GC), a hormone often released in response to stress (Bethin, Vogt, & Muglia, 2000; Nelson, 2005). The two primary glucocorticoids are corticosterone (CORT), secreted largely in rats and mice, and cortisol, the primary GC secreted by humans (Nelson, 2005). GC production is up-regulated during nociception and inflammation; for instance, CORT is elevated in blood plasma following formalin administration (Taylor et al., 1998). One of the anti-inflammatory effects of GCs is its ability to attenuate mediators produced during the inflammatory response; in murine fibroblasts and human monocytes, GCs lower

inflammatory COX-2 production *in vitro* (O'Banion, Winn, & Young, 1992). GCs also inhibit the synthesis of prostaglandin synthase in human dermal fibroblasts stimulated with IL-1 (Raz, Wyche, & Needleman, 1989).

Besides inhibiting prostaglandin production, GCs regulate analgesia by modulating cytokine release (Sternberg, 1997). GCs activate the synthesis of the anti-inflammatory cytokines IL-4 and IL-10, as well as suppress proinflammatory cytokines IL-1, TNF- α , and IFN- γ (Webster, Tonelli, & Sternberg, 2002). Kapcala, Chautard, and Eskay (1995) showed that adrenalectomized (ADX) rats have increased mortality rates after IL-1 β and LPS administration; however CORT administration attenuates that lethality. Elevated serum CORT levels correlate with decreased TNF- α levels during LPS administration (Chensue et al., 1991). Conversely, cytokines modulate GC production as well. TNF- α and IL-1 β act as signaling molecules to activate the HPA axis during inflammation in an effort to restore homeostasis (for review see Pavlov et al., 2003). IL-6 has been shown to elevate ACTH levels in CRH knockout mice following acute restraint stress, and intravenous TNF- α administration leads to increased plasma ACTH levels, in part by triggering CRH release (Bethin et al., 2000; Watanobe & Takebe, 1992).

Furthermore, estrogen has been shown to modulate GC expression. In adrenalectomized-ovariectomized (ADX+OVX) rats, estradiol treatment leads to increased GC levels in brain and spinal cord tissue (Ferrini, Gonzalez, & De Nicola, 1993). Research conducted in our lab shows that estradiol treatment leads to decreased Phase II flinching responses after formalin administration in ADX and ADX+OVX female rats (Hunter et al., in press; Appendix A). Thus, the interplay between HPA

activation, hormones, and cytokine production may explain how immune and endocrine systems interact to restore homeostasis during inflammation (Sternberg, 1997).

V. Sex differences in pain

Epidemiological pain studies demonstrate that sex differences in pain perception exist; women are more likely to report pain as more persistent and of a longer duration than that of men (Fillingim & Maixner, 1995). For certain pain conditions, such as migraines, fibromyalgia, irritable bowel syndrome, and temporomandibular disorders (TMD), a higher prevalence has been reported in women; the frequency and severity of these pain syndromes accompany fluctuating hormonal levels at different stages in life (Gupta et al., 2007; LeResche et al, 1997; Nekora-Azak, 2004; Wiesenfeld-Hallin, 2005). Results from studies that experimentally induce pain demonstrate that women perceive noxious stimuli more intensely, and have a lower pain tolerance than men (Feine, Bushnell, Miron, & Duncan, 1991; Walker & Carmody, 1998; Wise et al., 2002).

Differences in pain perception may, on the other hand, involve sociocultural factors, such as expected gender-related responses to pain (Wiesenfeld-Hallin, 2005). However, animal studies demonstrate sex differences in pain behavioral responses (Table 1). Preclinical studies demonstrate that female rats subcutaneously injected with formalin in the hind paw exhibit greater or longer lasting behavioral nociceptive responses (i.e. flinching, licking) than male rats (Aloisi, Albonetti, & Carli, 1994; Gaumond, Arsenault, & Marchand, 2002). LaCroix-Fralish et al. (2005) examined tactile hypersensitivity in male and female rats in a rodent model of lower back pain. Following lumbar nerve root injury, female rats display increased hypersensitivity compared to males, demonstrating

that differences in mechanical allodynia are dependent on sex (LaCroix-Fralish et al., 2005). Cook and Nickerson (2005) found that female rats develop inflammation faster and exhibit greater hyperalgesia than males following CFA administration. A recent study by Li and colleagues (2009) demonstrates that following carrageenan-induced inflammation, wild-type female mice have lower PWL to mechanical stimulation than males; however no sex differences were observed in knock-out mice lacking estrogen receptors.

Further, GC production is expressed differently in men and women (Sapolsky, 1992). CORT is present at higher levels in female rats, and female mice generally release higher CORT levels than male mice in response to stress (Kitay, 1961; Wilder, 1995). Research conducted by Da Silva et al. (1993) demonstrated that gonadectomy leads to higher CORT levels in male mice than females following systemic inflammation. The opposite affect is seen in sham-operated mice, suggesting that the presence of female sex hormones regulates GC production during inflammation (Da Silva et al., 1993). Aloisi et al. (1996) demonstrated that after formalin administration, ACTH plasma levels were higher in female rats than in males; however there were no sex differences found in CORT production. The authors felt that CORT levels were equivalent in both sexes because the time of sacrifice preceded CORT release (Aloisi et al., 1996). Taken together, clinical and preclinical studies show that sex differences exist in pain discrimination and perception. The sexual dimorphism in pain perception may result from hormonal differences found between men and women, along with the interaction of sex steroids, inflammatory stimuli, and the HPA axis.

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Table 1
Sex differences in rodent models of inflammatory and chronic pain

Pain Assay	Male vs. Female Rodents	Doses	References
Formalin	Females display longer lasting licking and flexing behaviors	10%, 50μL	Aloisi et al., 1994
Formalin	Females have increased nociceptive behaviors	2%, 50μL	Gaumont et al., 2002
CFA	Females have increased hyperalgesia and faster development of inflammation	5mg/mL, 0.1mL	Cook and Nickerson, 2005
Lumber radiculopathy	Females display greater tactile hypersensitivity	N/A	LaCroix-Fralish et al., 2005
Carrageenan	Females have decreased PWL than males	2%, 20μL	Li et al., 2009
Formalin	GDX No differences in flinching behaviors between ♂ and ♀	2%, 50μL	Gaumont et al., 2002
CCI	No differences in hyperalgesic symptoms between ♂ and ♀	N/A	Tall et al., 2001

Legend. CCI, chronic constriction injury; CFA, complete Freund's adjuvant; GDX, gonadectomy; (♂) male; (♀) female.

Menstrual and Estrous Cycles

Ovarian hormone levels fluctuate during different phases of the menstrual cycle (Festa & Quiñones-Jenab, 2004; Kuba & Quiñones-Jenab, 2005; Martin & Behbehani, 2006; Figure 7A). In women, the menstrual cycle consists of the menses phase, followed by the follicular (a stage that occurs prior to ovulation) and luteal phases. Blood estrogen and progesterone levels are lowest during menses. Serum estradiol levels peak during the end of the follicular stage just prior to ovulation, whereas progesterone levels remain rather low (Martin & Behbehani, 2006; Figure 7A). Following ovulation, estrogen levels plateau during the mid-luteal phase and fall before menstruation. Progesterone levels peak during the middle of the luteal phase and are relatively high compared to estradiol levels. Progesterone levels then drop at the end of the luteal phase (Martin & Behbehani, 2006; Nelson, 2005; Figure 7A). The female menstrual cycle lasts for about 14 to 35 days (Brown, 1994).

Similarly, the rat estrous cycle consists of fluctuating ovarian hormone levels (Hurn and Macrae, 2000; Spornitz, Socin, & Dravid, 1999; Figure 7B). However, the estrous cycle lasts for only 4 to 5 days, and the pattern of the hormonal levels differ from that seen in women (Festa & Quiñones-Jenab, 2004; Spornitz et al. 1999). The estrous cycle is divided into four stages: proestrus, estrus, metestrus, and diestrus (Freeman, 1988). Estrogen levels begin to increase at the end of diestrus, peak and decline during proestrus, and remain low during estrus (Spornitz et al., 1999; Figure 7B). Progesterone levels peak during the end of metestrus and the early phase of diestrus (Figure 7B). Following is a second peak in progesterone levels during the end of proestrus, and a

decline during estrus (Marcondes, Bianchi, & Tanno, 2002; Nelson, 2005; Spornitz et al., 1999; Figure 7B).

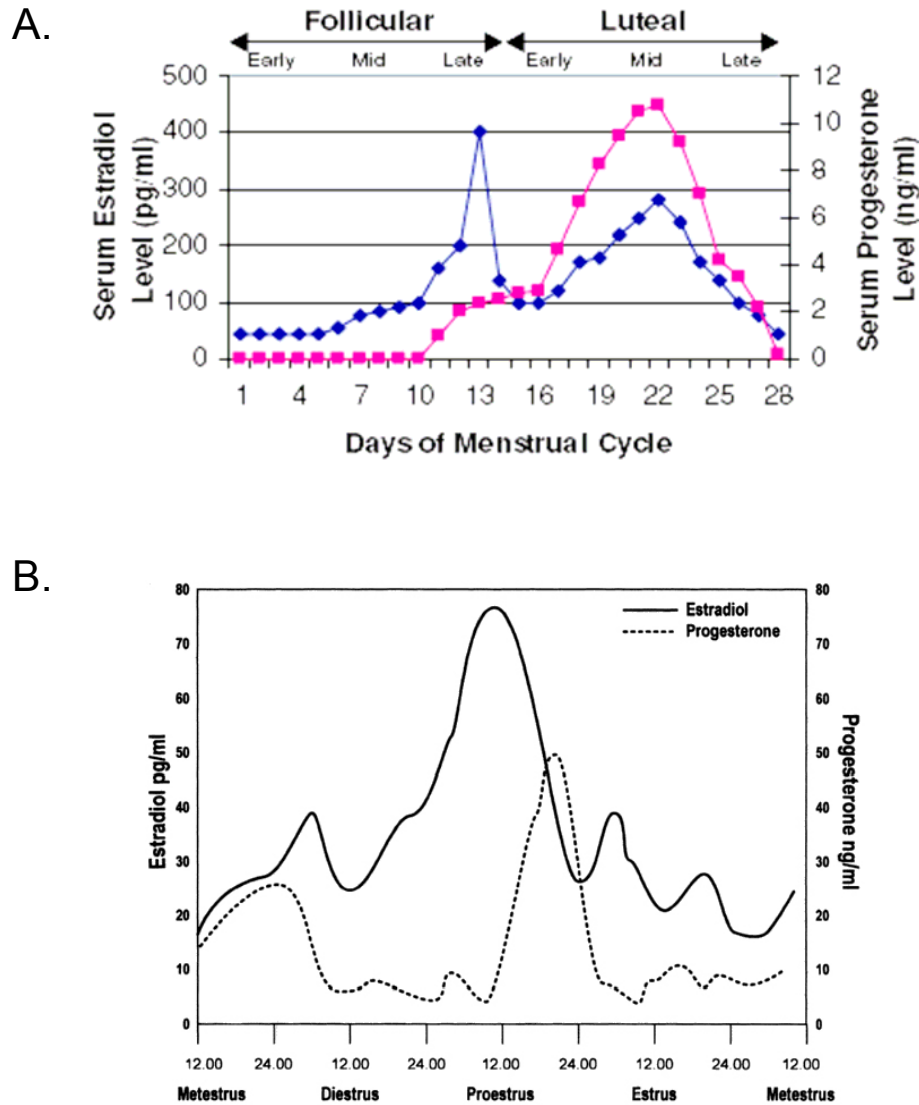


Figure 7. Stages of the human menstrual (A) and rat estrous (B) cycles. A. Changes in serum estrogen (◆) and progesterone (■) levels during a native menstrual cycle. Day 1 is the first day of menses and day 27 is the last day before the next menstrual period. The follicular phase includes all days prior to ovulation and the luteal phase includes all days after ovulation. The follicular and luteal phases can be divided into early, mid, and late time periods. *From:* Martin and Behbehani: HEADACHE, VOL 46 (2006), 365-386. B. Representative plasma levels of 17 β -estradiol (pg/mL) and progesterone (ng/mL) during the 4-day rat estrus cycle. *From:* Hurn and Macrae: JOURNAL OF CEREBRAL BLOOD FLOW & METABOLISM, VOL 20 (2000), 631-652.,

Clinical studies show that administration of estrogen and progesterone modulates pain perception, but these results are inconsistent. Early research conducted by Somerville (1972) demonstrated that estradiol administered shortly before menstruation delayed the onset of migraine headaches in women suffering from menstrual migraine (migraine headaches occurring around the onset of menstruation). However, in non-menstrual migraine sufferers, women taking oral contraceptives that contain estrogen reported that migraine symptoms are unchanged or worsened when using contraception (Cupini et al., 1995; Mueller, 2000; Ryan, 1978). Furthermore, in postmenopausal women, hormone replacement therapy improved migraine headaches in only 22% of cases (Hodson, Thompson, & al-Azzawi, 2000). Research conducted by MacDonald et al. (1994) showed that hormone replacement therapy increased well being and lumbar spine bone density in postmenopausal women suffering from rheumatoid arthritis. However, in a population study conducted by Macfarlane and colleagues (2002), no differences between hormonal factors and chronic pain were found amongst women. Taken together, clinical research contains many inconclusive results in reference to exogenous hormone administration and its effects on chronic pain.

Gonadectomy

Gonadectomized (GDX) rodents are used in preclinical pain studies to assess the role of endogenous hormones on nociception. For instance, the use of GDX rats in tonic and neuropathic pain paradigms show that intact females respond differently to pain administration compared to males; however, this difference is abolished after gonadectomy (Table 1; Gaumond et al., 2002; Tall et al., 2001). Similarly, gonadectomy

has been implemented to study the effects of exogenous estrogen and progesterone administration on pain responses (Table 2). In most cases, estradiol (E) replacement reduced ovariectomy-evoked vaginal hyperalgesia in rats (Bradshaw & Berkley, 2002). In a thermal acute pain paradigm, continuous administration of E, progesterone (P), or E+P administration did not alter tail-flick latencies in ovariectomized (OVX) rats as compared to OVX controls (Mannino, South, Inturrisi, & Quiñones-Jenab, 2005). During formalin administration, E replacement significantly reduced Phase II nociceptive flinching responses (Mannino, South, Quiñones-Jenab, & Inturrisi, 2007; Kuba, Kemen, & Quiñones-Jenab, 2005; Kuba et al., 2006). Furthermore, Kuba et al. (2006) demonstrated that following formalin administration, P reduced flinching during Phase I, and co-administration of E+P reversed the analgesic effects of estradiol during Phase II (Kuba et al., 2006). Although behavioral effects to pain may vary due to differences in the dose and duration of hormone replacement, as well as the pain assay employed, these results indicate that female gonadal hormones alter pain responses to nociceptive stimulation.

Estrous cycle effects on pain

Clinical research studies have found that hormonal fluctuations during a woman's menstrual cycle leads to variations in pain sensitivity. For instance, women report that the prevalence of migraines is significantly elevated during the onset of menstruation (Johannes et al., 1995; MacGregor, 2000). LeResche et al. (2003) examined changes in TMD in relation to the menstrual cycle. Participants reported that temporomandibular joint (TMJ) pain was elevated toward the end of the menstrual cycle and peaked during

menstruation (LeResche et al., 2003). Hellstrom and Anderberg (2003) demonstrated that women report chronic pain as being more severe during the menses and luteal phases of the menstrual cycle than during other phases. Also, Latman (1983) found that pain symptoms of rheumatoid arthritis were significantly decreased during the postovulatory phase of the menstrual cycle.

Rodent studies also demonstrate that female sex hormones modulate pain (Table 2). For example, Vincler, Maixner, Vierck, and Light (2001) used an electrical stimulation paradigm to test hind limb reflex responses in intact female rats. The reflex amplitude was greater when rats were in proestrus than in diestrus or estrus stages. This data indicates that rats were most responsive to stimuli when ovarian hormone levels were at their highest (i.e. during proestrus; Vincler et al., 2001).

Kayser et al. (1996) examined estrous variations in vocalization thresholds to hind paw and tail pressure stimulation in rats over a three-week period. During the somatic nociception experiment (hind paw and tail pressure stimulation), estrous cycle stage led to significant differences in paw and tail vocalization thresholds. Female rats in proestrus and estrus had lower thresholds than rats in diestrus, demonstrating that rats were more sensitive to pain while in these stages (Kayser et al., 1996; Table 2).

However, preliminary research in our lab shows contradictory behavioral responses to formalin administration during the estrous cycle stage as compared to the Vincler and Kayser studies (Amador et al., personal communication, 2009). Intact female rats were administered 5% formalin into their hind paw. No differences in flinching responses were observed during the 0-6 minutes after formalin injection (Phase I-acute pain; Appendix B). However, during Phase II (inflammatory pain), rats in the

proestrus stage exhibited less flinches than rats in metestrus (Table 2; Appendix B).

Furthermore, Fischer and colleagues (2008) demonstrated in a rodent model of formalin- and glutamate-induced TMJ pain that female rats experienced higher nociceptive responses (i.e. rubbing the orofacial region; head flinching) during diestrus than in the proestrus stage. These studies demonstrate that heightened levels of endogenous ovarian hormones during the proestrus stage lead to increased pain thresholds. The reasons for the discrepancies in these studies are unknown, but pain responses may vary from the time that estrous cycle stage was determined. Differences in pain behaviors may also result from the drastic shifts of ovarian hormones throughout the endocrinological profile. Taken together, research shows that hormonal fluctuations during the estrous cycle are involved in behavioral responses to pain.

Table 2
Hormonal effects on pain behaviors during nociception in female rodents

		Pain Assay	Behavior	References
OVX	E	Vaginal distention	↓ escape responses compared to OVX controls	Bradshaw and Berkley, 2002
		Thermal acute pain	no change in tail flick latencies compared to OVX controls	Mannino et al., 2005
		1% Formalin, 50μL	↓ Phase II flinching responses	Kuba et al., 2006
		5% Formalin, 50μL	↓ Phase II flinching responses	Kuba et al., 2005 Mannino et al., 2007
	P	Thermal acute pain	no change in tail flick latencies compared to OVX controls	Mannino et al., 2005
		1% Formalin, 50μL	↓ Phase I flinching responses	Kuba et al., 2006
	E+P	Thermal acute pain	no change in tail flick latencies compared to OVX controls	Mannino et al., 2005
		1% Formalin, 50μL	reversed E analgesic effects in Phase II (↑ flinching)	Kuba et al., 2006
	Estrous cycle	Electrical stimulation	↑ reflex amplitude during proestrus	Vincler et al., 2001
		Somatic nociception	lower pain thresholds in proestrus and estrus	Kayser et al., 1996
		TMJ nociception	↑ nociceptive pain responses during diestrus as compared to proestrus	Fischer et al., 2008
		5% Formalin, 50μL	↓ Phase II flinching during proestrus as compared to metestrus	Amador et al., personal communication, 2009

Legend. OVX, ovariectomy; E, estradiol; P, progesterone, CCI, chronic constriction injury; ↑, increase; ↓, decrease.

Estrogen effects on cytokine production: possible role for modulation in pain

Since estrogen has been shown to regulate pain responses, its analgesic effects may be a result of mediating cytokine production. Research indicates that estrogen modulates cytokine expression. Hu, Mitcho, and Rath (1988) demonstrated that peritoneal macrophages from OVX rats secrete lower amounts of IL-1 than macrophages taken from intact rats; further, estradiol administration led to higher IL-1 synthesis in OVX rats. However, Polan, Daniele, and Kuo (1988) found that high concentrations of estradiol and progesterone led to significant reductions in IL-1 activity in human peripheral monocytes. Schaefer et al. (2005) offer further support that estrogen decreases IL-1 production; estradiol inhibits IL-1 β mRNA expression, and lowers secretion of the chemokine CXCL8 in uterine epithelial cells. Estrogen has been shown to lower TNF- α , IL-6, and IL-1 β release in a dose dependent manner *in vitro* in peripheral blood samples obtained from postmenopausal women (Ralston, Russell, & Gowen, 1990; Rogers & Eastell, 2001). Estradiol replacement also lowers IL-6 production in OVX mice (Masiukiewicz, Mitnick, Grey, & Insogna, 2000), and activation of the estrogen receptor leads to IL-6 gene suppression (Liu, Liu, & Bodenner, 2005). Estrogen receptor agonists have been shown to attenuate TNF- α and IL-1 β in astrocytes following LPS treatment (Lewis et al., 2008). Salem (2004) reviewed that estrogen modulates T-cell mediated cytokine production; specifically estrogen has been shown to inhibit proinflammatory cytokines (i.e. TNF, IL-12, IFN- γ) secreted by T_H1 cells and stimulate the production of anti-inflammatory cytokines (i.e. IL-10 and IL-4) secreted by T_H2 cells (Salem, 2004). Thus, estrogen is shown to play a role in modulating cytokine levels.

Estrogen production alters cytokine expression during inflammatory responses as well. Estrogen prevents acute inflammation by inhibiting the production of the IL-1 β in a rat model of experimental colitis (Houdeau et al., 2007). Following collagen-induced arthritis in mice, administration of ethinyl estradiol lowered IL-6 and TNF- α secretion by T cells, and decreased mRNA expression for cytokines and chemokines in joint tissue (Subramanian et al., 2005). Also, E administered after trauma-hemorrhage injury lowers the release of IL-6 and TNF- α from Kupffer cells (liver macrophages) in male rats (Suzuki et al., 2006).

Santora et al. (2007) demonstrated that estrogen modulates cytokine production in a rodent model of rheumatoid arthritis (RA). The incidence of RA declines during pregnancy, when estrogen and relaxin hormones are elevated. OVX rats were treated with E and relaxin (RLX) after CFA-induced arthritis. The combination of E+RLX treatment decreased inflammation. TNF- α levels decreased more in E+RLX rats than treatment with E or RLX alone (Santora et al., 2007). Furthermore, IL-10 production increased in response to E and E+RLX treatment (Santora et al., 2007).

Johnson et al. (2006) found that estrogen lowers IL-1 β levels in the olfactory bulb of OVX adult (4 month old) rats after LPS administration. Furthermore, estrogen attenuates IL-1 β expression in the olfactory bulb and the pituitary gland in OVX senescent (9-11 month old) rats. This research suggests that estrogen differently modulates CNS tissue, and its effects on inflammatory cytokine expression are affected by age (Johnson et al., 2006).

Taken together, these results demonstrate that estrogen plays a role in altering cytokine levels, but the extent of this effect is unclear. Given that estrogen has been

shown to modulate cytokine expression *in vitro* and *in vivo*, there is apparently a strong correlation between hormonal release and cytokine expression. However, it is not yet understood if estrogen acts peripherally or centrally to modulate cytokine levels. It is also not clear if estrogen's effects on cytokines occur through direct regulation of the cytokine pathway or via indirect regulation through CORT release. Furthermore, hormonal levels present at different stages of the menstrual/estrous cycle may influence estrogen's actions on cytokine production. More research is warranted to understand this relationship.

VI. Proposed Model

We postulate that estrogen's anti-hyperalgesic effects at the peripheral and central levels on inflammatory pain occur through up-regulation of corticosterone release, which in turn down-regulates cytokine release, and thus, COX and prostaglandin activation, as shown in Figure 8. Specifically, estrogen may exert its effects at the peripheral level by reducing the production of proinflammatory cytokines, thus lowering the potentiation of DRG neurons. In turn, we postulate that estrogen increases anti-inflammatory cytokine and CORT levels, leading to further lowered pain responses. We further postulate that estrogen up-regulates CORT levels, and lowers proinflammatory cytokine levels in the central nervous system, leading to a decreased sensitization of dorsal horn neurons.

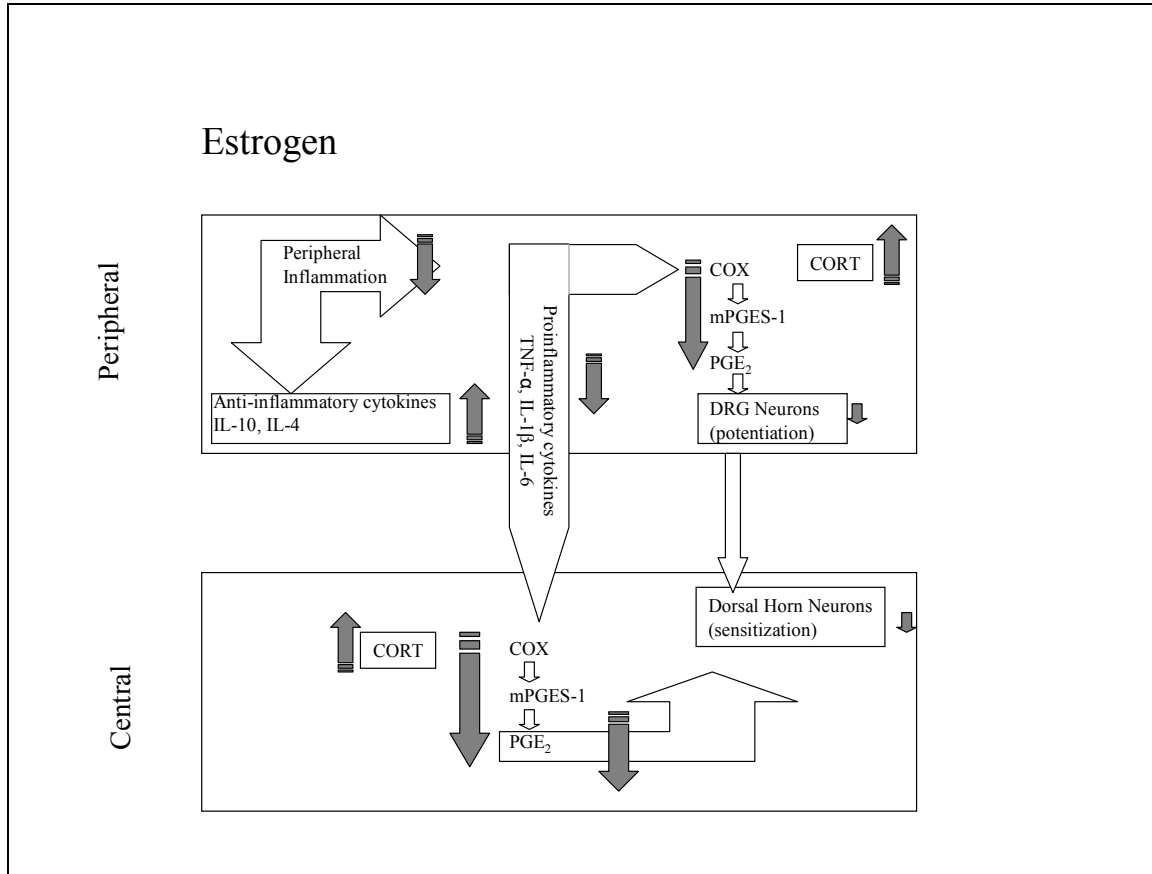


Figure 8. Proposed method by which estrogen modulates cytokine and CORT release. Estrogen may alter inflammation by down-regulating the production of proinflammatory cytokines, as well as COX and prostaglandin levels, in the peripheral and central nervous system. Estrogen may in turn increase anti-inflammatory cytokine and CORT activity, leading to anti-hyperalgesic responses in inflamed tissue. White arrows indicate cascades activated during inflammatory pain. Dark arrows indicate estrogen's activation and/or inhibition of pro- and analgesic mediators involved in inflammation.

Specific Aims

Clinical and preclinical studies have demonstrated that sex differences exist in pain discrimination and perception. Women display greater sensitivity to noxious stimuli, and report pain as more intense and of a longer duration than that of men. It has been suggested that gonadal hormones are the cause of dimorphic responses to pain. Certain pain conditions, such as migraines and temporomandibular disorders, have a higher prevalence in women, where the frequency or severity of these pain syndromes accompany fluctuating hormonal levels at different stages in life. Rodent studies have shown that female sex hormones modulate pain perception during formalin-induced nociception. For example, the estrous cycle has been shown to affect behavioral responses to inflammatory stimuli in female rats. Moreover, estradiol attenuates the tonic/persistent phase of pain after the administration of formalin. Thus, the fluctuations of estrogen and progesterone levels during the female reproductive cycle may be responsible for sex differences in behavioral responses to painful stimuli. However, more research is warranted to understand the biochemical role of female reproductive hormones on the control of inflammation.

Pain caused by inflammation is characterized by an increased sensitivity to mechanical and heat stimuli. Current research demonstrates that after tissue injury, proinflammatory cytokines, such as TNF- α and IL-6, play a principle role in initiating pain responses. Cytokine release activates cyclooxygenase (COX)-dependent prostanoid release. On the other hand, glucocorticoids, i.e. CORT, are postulated to inhibit prostaglandin (PG) activation, and thus hamper inflammatory responses. However, little

research investigates the interaction between estrogen and CORT on biochemical cytokine responses that lead to the production of pain.

To date, it is unclear if estrogen mediates its anti-hyperalgesic effects on inflammatory pain through direct or indirect regulation of the cytokine pathway, and if estrogen exerts its effects at PNS and/or CNS levels. Further, it is not known if estrogen mediates its anti-hyperalgesic effects in part through CORT activity. The objective of my dissertation is to test this postulate. We hypothesize that *estrogen's anti-hyperalgesic effects at the peripheral and central levels on inflammatory pain occur through up-regulation of corticosterone release, which in turn down-regulates proinflammatory cytokine release at CNS and PNS levels*. This hypothesis will be tested in two experimental models. First, we will determine if inflammatory responses change through the female's ovarian cycle. Second, we will directly test the effect of estrogen on ovariectomized (OVX; rats lacking endogenous hormones) and adrenalectomized (ADX; removal of adrenals) rats. To test these hypotheses, the following specific aims were proposed:

Aim 1: To determine if endogenous ovarian hormones cause changes in the production of cytokines at peripheral and central levels before and following inflammatory-induced pain (formalin). To this end, four experiments were designed to test if endogenous hormones affect cytokine production: **1A.** Measure cytokine levels in naïve intact female rats during different stages of the estrous cycle (proestrus, estrus, metestrus, and diestrus) to test if hormone fluctuations seen during the endocrinological profile alter cytokine levels before pain administration. **1B.** Measure cytokine levels in

formalin-treated intact female rats from each estrous cycle stage to determine if changes in the endocrinological profile will alter cytokine levels during inflammatory pain. **1C.** Measure paw size in both treatment groups to test if endogenous gonadal hormones reduce paw volume in peripheral tissue, which may be a mechanism through which female gonadal hormones decrease behavioral responses to pain. **1D.** Measure CORT levels in intact female rats to test if the presence of endogenous CORT leads to altered cytokine levels. We predict that elevated ovarian hormone levels during the proestrus stage will lower proinflammatory cytokine activity at both peripheral and central sites before and following inflammatory pain. We further predict that CORT will mediate these effects.

Aim 2: To determine if estrogen's effects on inflammatory pain (formalin) are in part mediated through interaction of corticosterone and cytokine activation.

To this end, four experiments were designed to determine the effect of gonadal and adrenal activity on cytokine levels before and during the formalin test: **2A.** Measure cytokine levels in naïve OVX Sham ADX, ADX Sham OVX, and ADX+OVX rats administered 20% 17 β -estradiol or vehicle (cholesterol) to determine if basal cytokine levels are altered by the presence or absence of ovarian and/or adrenal hormones. **2B.** Measure cytokine levels following formalin administration in the groups mentioned in 2A to determine if estradiol administration alters cytokine activity following inflammation-induced pain, and if these effects are mediated in part by CORT. **2C.** Measure paw size in all treatment groups to test if estradiol administration lowers peripheral inflammation at the site of injury. **2D.** Correlate cytokine and CORT levels to

further determine if the presence of endogenous CORT leads to altered cytokine levels before and following formalin administration. We predict that estrogen administration lowers basal and formalin-induced proinflammatory cytokine levels in both the peripheral and central nervous system. We further predict that estradiol administration decreases paw size before and following formalin-induced inflammation. We hypothesize that estrogen will up-regulate CORT release, which in turn down-regulates proinflammatory cytokine release, in the CNS and PNS of OVX rats. In ADX rats, we hypothesize that estradiol administration will lower cytokine production, but to a lesser extent than seen in rats whose adrenal glands are intact.

Aim 3: To determine if estradiol's anti-hyperalgesic effects on inflammatory pain occur in a more prolonged inflammatory-induced pain model (carrageenan), and if so, to test if estradiol administration lowers cytokine levels during this prolonged state. To this end, four experiments were designed to test the effects of estradiol on baseline cytokine levels and following 1, 5, and 24hrs post carrageenan or saline administration: **3A.** Utilize behavioral testing in carrageenan- and saline-treated OVX rats to determine if estradiol administration lowers sensitivity to pain following carrageenan-induced inflammation as compared to vehicle (cholesterol) controls. **3B.** Measure paw size in naïve, carrageenan-, and saline-treated OVX rats administered estradiol or vehicle to test if estradiol reduces paw volume in peripheral tissue, which may be a mechanism through which estrogen decreases behavioral responses to pain. **3C.** Measure cytokine levels in all treatment groups to test if estradiol administration alters cytokine levels before and after inflammatory pain. Further, cytokine levels were

tested 1, 5, and 24hrs post injection to test if estradiol's effects on cytokines can be extended for a prolonged period of time. **3D.** Measure CORT levels to test if endogenous CORT activity leads to altered cytokine levels. We hypothesize that estradiol will down-regulate cytokine levels in the CNS and PNS of OVX rats, and thus increase latency thresholds during behavioral testing. We further hypothesize that estradiol will reduce paw size following carrageenan-induced inflammation, and will up-regulate CORT levels which in turn will down-regulate proinflammatory cytokine release.

Chapter 2: Endogenous ovarian hormone influences on cytokine levels basally and in response to formalin administration.

1. Introduction

The female menstrual/estrous cycle contains fluctuations in ovarian hormones that correlate with specific stages of a cycle. In women, at the beginning of the menstrual cycle (the menses phase) estrogen and progesterone levels are at baseline. In the follicular phase, estrogen levels gradually rise, and then peak during ovulation (Martin & Behbehani, 2006). Progesterone levels rapidly increase and peak during the middle of the luteal phase, and at the end of this phase, both estrogen and progesterone levels decline (Martin & Behbehani, 2006). Similarly, rodents have an estrous cycle that last about 4 to 5 days in duration, and its stages are denoted as proestrus, estrus, metestrus, and diestrus (Freeman, 1988). Progesterone levels peak during the end of the metestrus stage (Hurn and Macrae, 2000). Furthermore, estrogen and progesterone levels peak during proestrus, and remain low during estrus (Spornitz et al., 1999).

Clinical research studies have found that hormonal fluctuations during a woman's menstrual cycle leads to variations in pain sensitivity. For instance, women report that the prevalence of migraines is significantly elevated during the onset of menstruation, and that temporomandibular joint (TMJ) pain peaks during menstruation (Johannes et al., 1995; LeResche et al., 2003; MacGregor, 2000). Women report chronic pain as being more severe during the luteal and menses phases of the menstrual cycle, when estrogen and progesterone levels are rather low (Hellstrom and Anderberg, 2003). Also, Latman (1983) found that pain symptoms of rheumatoid arthritis were significantly decreased during the postovulatory phase of the menstrual cycle.

Similarly, in animal models, female sex hormones modulate nociception; however, these results are often inconsistent. For example, Kayser et al. (1996) found that female rats in proestrus and estrus had lower vocalization thresholds to somatic nociception than rats in metestrus and diestrus. Vincler and colleagues (2001) found that rats in proestrus had higher reflex amplitude responses to electrical stimulation than rats in diestrus or estrus. These studies show that rats are most responsive to stimuli when ovarian hormone levels are at their highest (i.e. during proestrus). However, in a model of TMJ pain, diestrous female rats experienced higher TMJ nociception than rats in proestrus (Fischer et al., 2008). Further, preliminary research in our lab demonstrates that elevated levels of endogenous ovarian hormones lead to lower behavioral nociceptive responses (Amador et al., personal communication, 2009). For instance, during Phase II of the formalin test, rats in proestrus exhibit less flinching responses than rats in metestrus (Amador et al., personal communication, 2009; Appendix B). The reasons for the discrepancies in these studies are unknown, but pain responses may vary from the drastic shift of hormones seen during certain stages of the cycle.

Proinflammatory cytokines [i.e. tumour necrosis factor (TNF)- α , interleukin (IL)-6, interleukin (IL)-1] are pleiotrophic multifunctional proteins that are produced on demand and act locally to induce or facilitate inflammatory pain and hyperalgesia (Ferreira, 1993; Hopkins, 2007; Meyer et al., 2006; Moalem & Tracey, 2006; Rittner & Stein, 2005; Sommer & Kress, 2004; Wieseler-Frank et al., 2005). After tissue damage, leukocytes and regional cells produce cytokines and other proalgesic mediators near the site of injury, thus heightening inflammatory responses (Rittner, Machelska, & Stein, 2005). Cytokine activation leads to the up-regulation of inflammatory mediators that are

involved in nociceptive processes, such as prostaglandins and growth factors (DeLeo et al., 1997; Ferreira et al., 1988; Meyer et al., 2006; Moalem & Tracey, 2006). Pain responses also lead to modulation of central cytokine production as well. Within the CNS, activated glial cells and infiltrating macrophages up-regulate cytokine production during injury and inflammation (Hopkins, 1997; Sternberg, 1997). Further, peripheral cytokines activate central reactions to peripheral inflammation by stimulating the production of prostaglandin E₂, an inflammatory mediator whose properties allow for diffusion across the blood-brain barrier (Ek et al., 2001).

The synthesis of proinflammatory cytokines is regulated by negative feedback systems such as anti-inflammatory cytokines and glucocorticoids (GC, Sternberg, 1997; Wieseler-Frank et al., 2005). Anti-inflammatory cytokines (i.e. IL-10, IL-4) can lower inflammatory processes following tissue damage by suppressing the genes that code for proinflammatory cytokines, and by opposing hyperalgesia at the site of injury (Millan, 1999; Moalem & Tracey, 2006). GC also modulates cytokine production; elevated plasma GC levels result in IL-4 and IL-10 production, as well as suppression of IL-1 and TNF- α (Webster et al., 2002). Dexamethasone (a synthetic GC) decreases TNF- α production after LPS administration, while elevated endogenous corticosterone (CORT) levels correlate with decreases in TNF- α levels (Chensue et al., 1991).

Estrogen has been shown to play a role in the inhibition of proinflammatory cytokines as well. Estradiol benzoate suppresses early IL-1 β production in a rat model of experimental colitis (Houdeau et al., 2007). Estradiol replacement lowers IL-6 production in OVX mice, and activation of the estrogen receptor leads to IL-6 gene suppression (Liu, Liu, & Bodenner, 2005; Masiukiewicz et al., 2000). Taken together,

estrogen, GC, and anti-inflammatory cytokines have been shown to directly regulate proinflammatory cytokine production; however the interplay between these agents during inflammatory pain is poorly understood.

The formalin assay is a model of tonic/persistent pain that is often used in pain studies because of its association with inflammation and tissue damage (Sternberg & Wachterman, 2000; Tjolsen et al., 1992). Formalin injection into the rodent hind paw causes an initial five minutes of pain, termed Phase I, which leads to biting, flinching, and licking of the injected paw (Damas & Liegeois, 1999; Dubuisson & Dennis, 1977). Following is a quiescent period, and then a second phase of nociceptive behaviors, or Phase II, that lasts about 30-40 minutes (Dickenson & Sullivan, 1987; Dubuisson & Dennis, 1977, Tjolsen et al., 1992). Phase I is associated with acute pain, whereas Phase II is indicative of central sensitization (Dubuisson & Dennis, 1977; Hunskaar & Hole, 1987; Taylor, Peterson, & Basbaum, 1995; Tjolsen et al., 1992). Formalin challenge induces an array of inflammatory agents, namely proinflammatory cytokines, and was therefore used in this study to elicit cytokine production (Aloisi et al., 1995; Bianchi et al., 2004; Shi et al., 2006; Sweitzer et al., 1999; Zhou et al., 2008).

As mentioned previously, research conducted in our lab demonstrates that fluctuations of endogenous gonadal hormones produced during the estrous cycle alter behavioral nociceptive responses during Phase II of the formalin test (Amador et al., personal communication, 2009; Appendix B). Since exogenous estradiol replacement has been shown to lower cytokine levels, we investigated whether endogenous gonadal hormones alter inflammatory mediators before and after pain administration. Further, since CORT is elevated in blood plasma following formalin injection (Taylor et al.,

1998), we measured CORT serum levels to determine if the HPA axis may also be regulating cytokine production.

We hypothesize that fluctuations of endogenous ovarian hormones during the estrous cycle will regulate cytokine production in serum, DRG, paw, and spinal cord of naïve and formalin-treated rats. Furthermore, due to the distinct hormonal profile of each stage of the estrous cycle, a direct relationship of increases or decreases of serum ovarian hormones will correlate with changes in cytokine levels. Thus, during the proestrus stage, increases in gonadal hormones will decrease proinflammatory cytokine production at peripheral and central levels. On the other hand, during estrus, lowered ovarian hormone levels will lead to increased proinflammatory cytokine production. This study contains two aims, first to determine if basal cytokine levels are altered during the various stages of the estrous cycle. The second aim is to test if cytokine activation during formalin administration is in part mediated through changes in ovarian hormone production across the estrous cycle and/or via the endogenous release of CORT.

2. Materials and Methods

2.1 Animals

Eight-week old intact female Sprague-Dawley rats ($N = 40$) were purchased from Taconic (Germantown, NY). Rats were double-housed with a 12-hour light/dark photoperiod (lights on 8 A.M. EST), and food and water were available *ad libitum*. Animals were run in two separate cohorts. In order to determine basal cytokine and CORT levels in naïve rats, a separate cohort of animals were sacrificed without receiving formalin injections. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, Bethesda, MD) and was approved by the Institutional Animal Care and Use Committee at Hunter College of the City University of New York.

2.2. Smear cytology for determining estrous cycle stages

Estrous cycle stages were determined using vaginal smear cytology (Marcondes et al., 2002; Figure 9). Smears were obtained daily starting 10-12 days before testing, to assure that the rats were cycling normally. Every morning between 8:30 and 9:30 AM, vaginal secretions were collected with a cotton applicator soaked in distilled water by inserting the tip into the rat vagina, but not deeply. Vaginal fluid was then placed on a glass slide. Slides were dehydrated with ethyl alcohol, stained with cresyl violet acetate (Sigma-Aldrich, St. Louis, MO), and allowed to dry. Vaginal cells were observed under a light microscope, with a 20x objective lens. The determination of each stage was dependant on the proportion of three types of vaginal cells observed (Figure 9). Epithelial cells are round and nucleated, and demarcate proestrus; cornified cells are irregular without a

nucleus, and demarcate estrus; little round cells are leukocytes and are predominant during diestrus; while a mixed distribution of all cell types is classified as metestrus (Marcondes et al., 2002). Rats with an abnormal cycle (i.e. rats that remain in one cycle or who skipped stages) were not used in the study. On testing days, the estrous stage was determined about 2-3 hours before sacrificing.

2.3 Formalin administration

Using a 27 ½ -gauge needle, rats received a 50µL formalin injection (5% formaldehyde [Sigma-Aldrich, St. Louis, MO]; 95% sterile isotonic saline [0.9% Sodium Irrigation USP, Braun Medical, Irving, CA]) subcutaneously into the intraplantar region of their right hind paw while they were manually restrained. Formalin was administered between 9:00 AM and 3:00 PM.

2.4. Corticosterone Radioimmunoassay

Rats were sacrificed by rapid decapitation, following a brief (20 sec) exposure to CO₂. Naïve rats were sacrificed after removal from their home cage, while formalin-administered rats were sacrificed 60 minutes after injection to mimic the time that behavior during the formalin assay is collected. Trunk blood was collected in tubes containing K₂ EDTA (BD Vacutainer Systems, Franklin Lakes, NJ), and then centrifuged at 2,600 RPM for 30 minutes at 4° C. Serum was collected and stored at -80° C until assays were performed.

CORT was analyzed to assess the involvement of the HPA system during inflammatory pain. CORT serum levels were determined using Coat-A-Count

radioimmunoassay kits (Diagnostic Products Corporation, Los Angeles, CA). An automated gamma counter machine (WIZARD5DET1470015/4701845, PerkinElmer, Shelton, CT) was used to measure CORT levels. Intra-assay coefficients of variation averaged $10.0 \pm 1\%$, and values were expressed as ng/mL. To analyze if CORT production is influenced by endogenous fluctuations of hormones basally and following inflammatory pain, CORT serum levels were analyzed during each estrous cycle stage before and after formalin administration. Further, to determine if a relationship exists between CORT and cytokine production, CORT serum levels were correlated with cytokine serum levels in naïve and formalin-treated rats.

2.5 Tissue Collection and Homogenization

After rats were sacrificed, paw size in formalin-treated rats was determined using a dial caliper (General Tools, New York, NY) measuring the width and depth of paws in millimeters in order to measure hind paw swelling in the ipsilateral and contralateral paws following formalin administration. Similarly, to determine if hormonal fluctuations during the estrous cycle influence basal hind paw size, the right hind paw of naïve rats were analyzed. 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 then collected and immediately frozen. All tissue samples were stored at -80°C until use.

Tissue samples were then homogenized in lysing solution containing cell lysis buffer (from Cell Lysis Kit #171-304012, Bio-Rad, Hercules, CA), a protease inhibitor cocktail provided in the cell lysis kit, and 500mM phenylmethylsulfonyl fluoride (PMSF,

Sigma-Aldrich, St. Louis, MO) diluted in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO). Tissue samples were rapidly frozen at -80° C. Homogenates were thawed and then centrifuged at 5000 rpm for 15 minutes at 4° C. Supernatants were collected and stored at -80° C. Total protein concentration was determined using a Bradford kit from Bio-Rad Laboratories (Hercules, CA). All samples from the same area were homogenized at the same time.

2.6 Cytokine Multiplex Assay

Serum and tissue homogenates were analyzed for cytokine levels using the Bio-Plex Cytokine assay along with the Cytokine Reagent Kit (#171-K11070 and #171-304001, respectively, Bio-Rad, Hercules, CA). The cytokine assay consists of a multiplexed (IL-1 β , IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN- γ , and TNF- α) immunoassay containing premixed beads coated with target capture antibody. Simultaneous cytokine quantification was analyzed according to the manufacturer's protocol (for details see Hulse, Kunkler, Fedynyshyn, & Kraig, 2004). Afterwards, samples were processed using the Bio-Plex Suspension Array (Bio-Rad, Hercules, CA) system that allows for simultaneous quantification of a panel of cytokines. The array reader combines fluidics, two lasers, and real-time digital signaling to distinguish cytokines within each sample. Intra-assay coefficients of variance were below 20%. For tissue homogenates, cytokine concentrations obtained from the Bio-Plex software were divided by the protein concentrations measured for each sample. For serum, samples were diluted 1:4 with Rat Serum Diluent according to the manufacturer's protocol (Bio-Rad, Hercules, CA).

Cytokine values obtained from tissue samples are expressed as pg/ μ g of total tissue protein, while serum cytokine values are expressed as pg/mL.

2.7 Statistical Analysis

All data are expressed as mean $\pm$ SEM. One Way analysis of variance (ANOVA) was used to test for differences in cytokine levels and estrous cycle stage in serum, paw, spinal cord, and DRG tissue before and after formalin administration. To test for the spread of sensitization of the inflammatory stimulus, ipsilateral and contralateral DRG cytokine levels were compared using Repeated Measures ANOVA. Paw size represents the multiplication of the width and depth of each hind paw expressed in mm². Paw size measurements for naïve rats were analyzed using One Way ANOVA. Paw size comparing ipsilateral and contralateral paws following formalin administration were analyzed using Repeated Measures ANOVA. Factorial ANOVA was utilized to determine CORT serum levels in naïve and formalin treatment groups across the estrous cycle. For correlation analyses of CORT and cytokine serum levels during each estrous cycle stage, a linear regression analysis in GraphPad Prism 4.0 software was conducted. Fisher's Least Significant Difference (LSD) *post hoc* tests were performed when appropriate. For all comparisons, significance was at the $p < 0.05$ level.

3. Results

3.1 Gonadal hormones do not influence basal and formalin-treated paw size.

Figure 10A shows the right hind paw size (in mm²) of naïve rats in proestrus, estrus, metestrus, and diestrus stages. In naïve rats, the stage of the estrous cycle did not alter

paw size [$F(3, 18) = 1.31, p = 0.30$]. After formalin administration, as shown in Figure 10B, a main effect for formalin on paw size was observed [$F(1, 10) = 68.03, p < 0.01$]; ipsilateral paw size significantly increased after formalin injection compared to the contralateral paw in all groups ($p < 0.01$). However, hind paw inflammation in the ipsilateral paw was not altered by the estrous cycle stage that the rats were in [$F(3, 12) = 0.68, p = 0.58$].

3.2 The effect of endogenous gonadal hormones on cytokine levels

Proinflammatory cytokines

TNF- α

In naïve rats, as shown in Figure 11, TNF- α levels were not altered across the estrous cycle. Furthermore, in DRG tissue, TNF- α levels were below detection level by the cytokine assay. After formalin administration, (Fig.'s 12A-D) the estrous cycle stages did not alter TNF- α levels in any of the tissue samples tested. Since TNF- α levels were not up-regulated in the ipsilateral DRG, contralateral DRG levels were not analyzed (data not shown).

IL-1 β

As shown in Figure 13, a main effect for estrous cycle stage was observed in basal paw IL-1 β levels [$F(3, 11) = 4.97, p < 0.05$]; rats in estrus had significantly higher IL-1 β levels than rats in proestrus, metestrus, or diestrus ($p < 0.05$). In serum, a main effect for estrous cycle stage on IL-1 β levels was observed [$F(3, 13) = 5.17, p < 0.05$; Fig. 13B]; rats in diestrus had lower serum IL-1 β levels than rats in any other stage of the estrous

cycle ($p < 0.05$). Further, as shown in Figure 13C, in naïve DRG tissue a main effect for estrous cycle stage was observed [$F(3, 12) = 4.25, p < 0.05$]; rats in diestrus had higher IL-1 β levels than rats in proestrus, estrus, or metestrus ($p < 0.05$). However, in the spinal cord, no significant difference in basal IL-1 β levels across estrous cycle stages were observed (Fig. 13D).

In IL-1 β serum levels of formalin-treated rats, a main effect for estrous cycle stage was observed [$F(3, 14) = 4.25, p < 0.05$; Fig. 14B]; rats in metestrus had significantly lower IL-1 β serum levels than rats in proestrus or estrus ($p < 0.05$ for all comparisons). However, in the ipsilateral paw, DRG, or spinal cord, IL-1 β levels were not significantly altered when compared across different estrous cycle stages (Figures 14 A, C, and D, respectively). Further, in ipsilateral versus contralateral DRG, no significant difference in IL-1 β levels was observed (Fig. 14E).

IL-6

In naïve rats, basal IL-6 levels were not detected in paw tissue (Figure 15A). In serum, a main effect for estrous cycle stage on IL-6 levels was observed [$F(3, 14) = 5.85, p < 0.01$; Fig. 15B]; rats in diestrus had significantly lower IL-6 levels than rats in any other stage ($p < 0.05$). In DRG and spinal cord, basal IL-6 levels did not vary across the estrous cycle (Figures 15C and D, respectively).

Estrous cycle stage did not alter paw IL-6 levels in formalin-treated rats (Figure 16A). In serum, a main effect of estrous cycle stage on IL-6 levels was observed [$F(3, 11) = 4.90, p < 0.05$; Fig. 16B]; IL-6 levels were significantly lower when rats were in metestrus than in proestrus and estrus ($p < 0.05$ for all comparisons). In the ipsilateral

DRG, a main effect for estrous cycle stage on IL-6 levels was observed [$F(3, 10) = 6.93$, $p < 0.01$; Fig. 16C]; IL-6 DRG levels were highest in proestrus than in any other stage ($p < 0.05$). Spinal cord IL-6 levels were not altered by estrous cycle stage in formalin-treated rats (Figure 16D). As shown in Figure 16E, a main effect was observed between IL-6 levels in ipsilateral versus contralateral DRG tissue [$F(1, 7) = 5.31$, $p < 0.05$]. Ipsilateral DRG IL-6 levels were significantly higher than contralateral IL-6 levels ($p < 0.05$; Fig. 16E).

IL-2

In naïve rats, basal paw IL-2 levels were not altered across estrous cycle stage (Figure 17A). As shown in Figure 17B, in serum a significant main effect for estrous cycle stage on IL-2 levels was observed [$F(3, 12) = 7.54$, $p < 0.01$]; rats in metestrus had significantly lower serum IL-2 levels than rats in proestrus, while rats in diestrus had lower IL-2 levels than rats in other stages ($p < 0.05$ for both comparisons). Basal IL-2 levels were not altered across estrous cycle stage in the DRG or the spinal cord (Figures 17C and D, respectively). Following formalin administration, as shown in Figure 18, no significant differences in IL-2 levels in paw, serum, DRG, or spinal cord tissue were found across the estrous cycle. Further, IL-2 levels in contralateral DRG were not detected (data not shown).

GM-CSF

As shown in Figure 19A, in naïve rats paw GM-CSF levels were not altered across groups. In serum, a significant main effect for estrous cycle stage was observed [$F(3,$

14) = 9.83, $p < 0.01$; Fig. 19B]; rats in diestrus had significantly lower GM-CSF serum levels than rats in proestrus, estrus, or diestrus ($p < 0.05$). In DRG and spinal cord, no significant differences between the stages of the estrous cycle and GM-CSF levels were observed (Fig. 19C and D, respectively).

In formalin-administered rats, no differences in GM-CSF levels across the estrous cycle were observed in the ipsilateral paw and serum (Fig. 20A and B, respectively). GM-CSF levels were not detected in ipsilateral DRG or spinal cord (Fig. 20C and D, respectively). Further, GM-CSF levels in contralateral DRG were not detected (data not shown).

IFN- γ . As shown in Figures 21 and 22, IFN- γ levels were not altered in any samples tested in naïve or formalin-treated rats, respectively. Further, IFN- γ levels in paw tissue were not detected in either treatment group (Fig 21A and Fig 22A).

Anti-inflammatory cytokines

IL-10

In naïve rats, as shown in Figure 23, the estrous cycle did not alter basal IL-10 levels in any of the tested samples. After formalin administration, IL-10 levels were not significantly affected by estrous cycle stage in the ipsilateral paw, serum, ipsilateral DRG, or spinal cord (Fig.'s 24A-D). IL-10 levels in ipsilateral versus contralateral DRG were significantly altered [$F(1, 10) = 5.12$, $p < 0.05$];, *post hoc* analysis showed that during proestrus, IL-10 levels were higher in ipsilateral DRG as compared to contralateral DRG ($p < 0.05$; Fig. 24E).

IL-4

Paw IL-4 levels were not detected in naïve rats (Fig. 25A). As shown in Figure 25B, a main effect for estrous cycle stage on IL-4 serum levels was observed [$F(3, 10) = 24.02$, $p < 0.01$; Fig. 25B]; rats in diestrus had significantly lower IL-4 levels than rats in any other stage ($p < 0.01$). Further, rats in metestrus had lower IL-4 serum levels than rats in estrus ($p < 0.01$). Basal IL-4 DRG and spinal cord levels were not altered across the estrous cycle (Fig. 's C-D, respectively).

In formalin-treated rats, as shown in Figure 26, IL-4 levels were not detected in the ipsilateral paw (Fig. 26A). IL-4 serum levels were not altered across groups (Fig. 26B). Ipsilateral DRG IL-4 levels were not detected following formalin administration (Fig. 26C). Spinal cord IL-4 levels were not significantly different across the estrous cycle (Fig. 26 D). No IL-4 levels were detected in contralateral DRG tissue (data not shown).

3.3 The effect of estrous cycle stages on CORT levels in naïve and formalin groups

To test if female gonadal hormones alter glucocorticoids, CORT serum levels were analyzed in naïve and formalin-treated rats. As shown in Figure 27, a main effect for pain condition and estrous cycle stage on CORT levels was observed [Pain condition main effect: $F(1, 27) = 9.54$, $p < 0.01$; Estrous cycle stage main effect: $F(3, 27) = 2.98$, $p < 0.05$]; formalin-treated rats had significantly elevated CORT levels as compared to naïve rats ($p < 0.01$). Rats in diestrus had significantly higher CORT levels than rats in estrus ($p < 0.01$, Fig. 27). Post hoc analysis revealed that following formalin

administration, rats in diestrus had significantly elevated CORT levels as compared to rats in estrus ($p < 0.05$, Fig. 27).

3.4 Correlation analysis of corticosterone and cytokine serum levels

Correlation analyses of each cytokine to CORT during proestrus, estrus, metestrus, and diestrus are shown in Tables 3 and 4. In naïve rats during metestrus, a significant negative correlation between CORT and IL-4 levels was found [$r(5) = .92, p < 0.05$; Table 4]. Further, as shown in Table 3, correlation of the data revealed that CORT serum levels and TNF- α , IL-1 β , and IL-10 were significantly related during diestrus [TNF- α : $r(5) = .98, p < 0.01$ for a positive correlation; IL-1 β : $r(5) = .89, p < 0.05$ for a negative correlation; IL-10: $r(5) = .97, p < 0.01$ for a positive correlation, respectively].

Following formalin administration, as shown in Table 4, no significant correlation was found between CORT and cytokine serum levels during different stages of the estrous cycle.

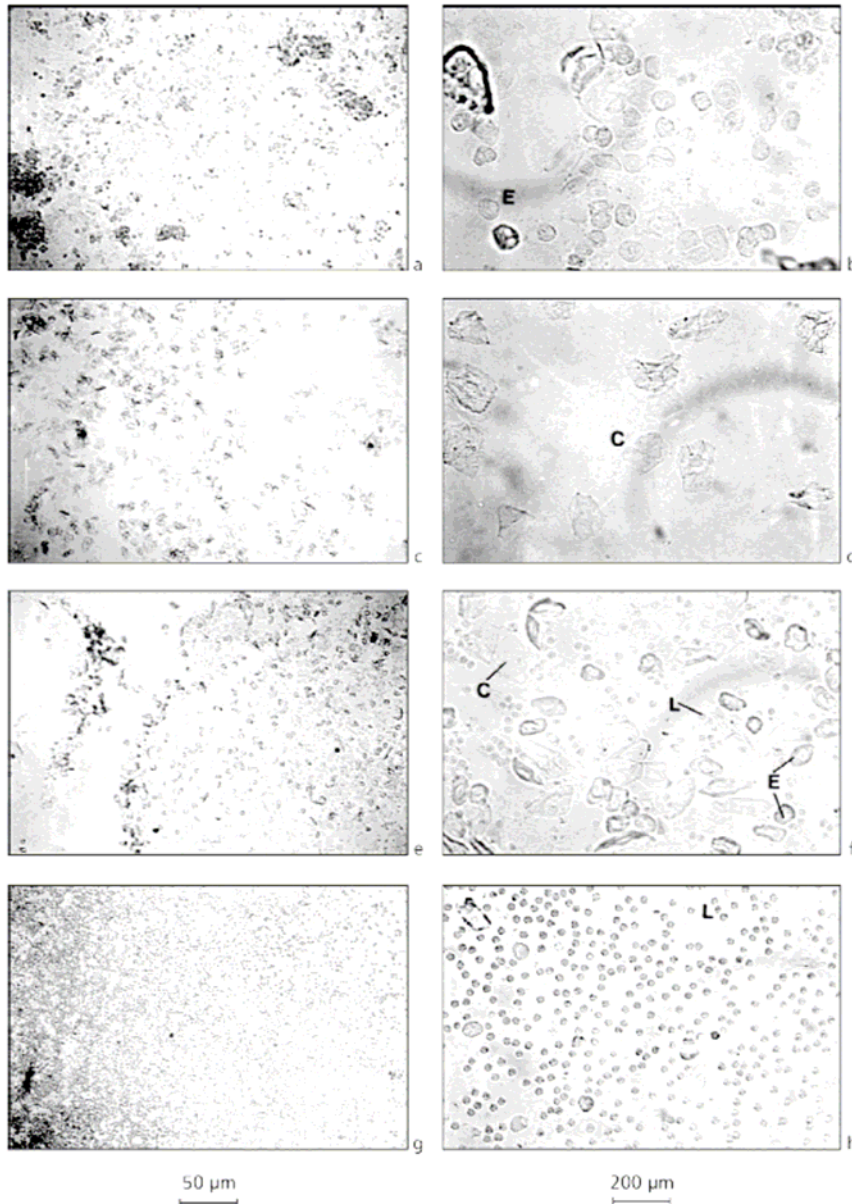


Figure 9. Photomicrographs of unstained vaginal smear from female rats at proestrus (a, b), estrus (c, d), metestrus (e, f), and diestrus (g, h). Leukocytes (L), epithelial (E) and cornified (C) cells are indicated. 50 x (left panel) and 200 x (right panel). Unstained native vaginal smear from female rats was observed under a light microscope, without the use of the condenser lens, with 10 and 40 x objective lenses. The proportion the three types of cells was used for the determination of the estrous cycle phases. Round and nucleated ones are epithelial cells (E); irregular ones without nucleus are the cornified cells (C); and the little round ones are the leukocytes (L). A proestrus smear consists of a predominance of nucleated epithelial cells (Figs. 9a, 9b); an estrous smear primarily consists of anucleated cornified cells (Figs. 9c, 9d); a metestrus smear consists of the same proportion among leukocytes, cornified, and nucleated epithelial cells (Figs. 9e, 9f); and a diestrus smear primarily consists of a predominance of leukocytes (Figs. 9g, 9h). From: Marcondes, Bianchi, and Tanner: BRAZ. J. BIOL., VOL 62 (4A) (2002) 609-614

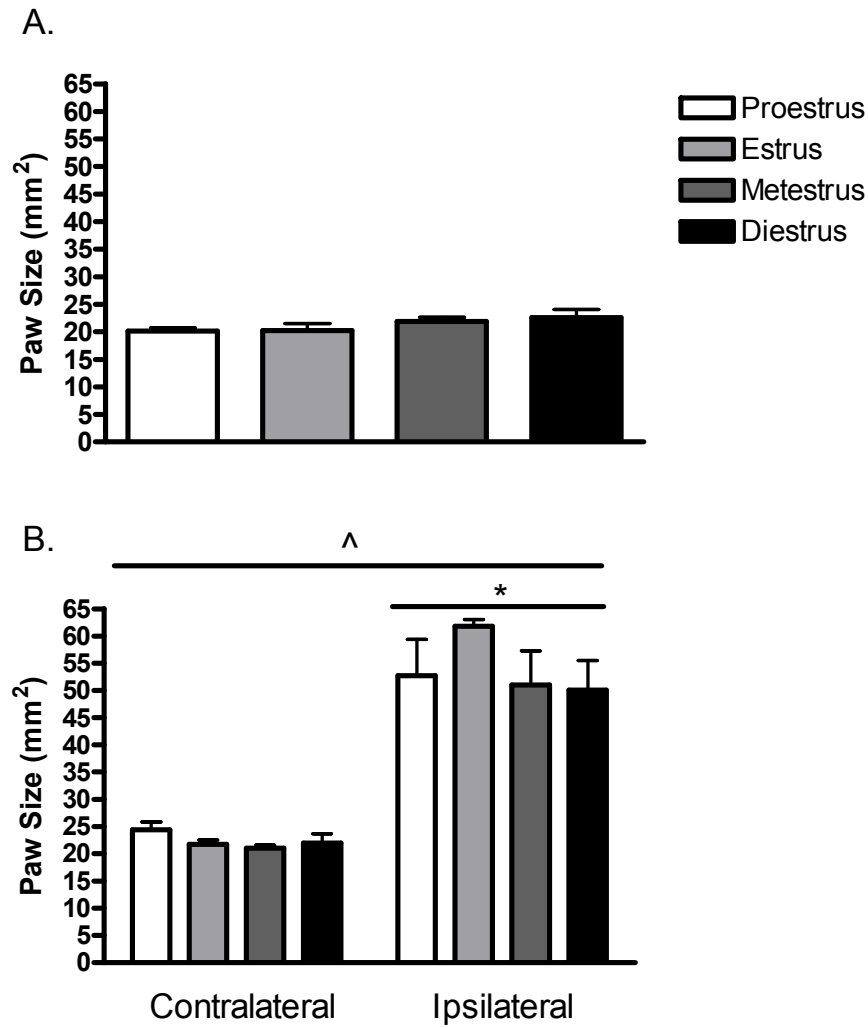


Figure 10. Paw size in naïve (A) and formalin-treated (B) intact female rats. Mean paw measurements ($\pm$ SEM) in mm² of female rats during the proestrus, estrus, metestrus, or diestrus stage. (^) Represents significant main effect of pain condition, $p < 0.01$. (*) Denotes significant increase in ipsilateral paw size as compared to contralateral paws following formalin administration ($p < 0.01$). $n = 4-7$ per group.

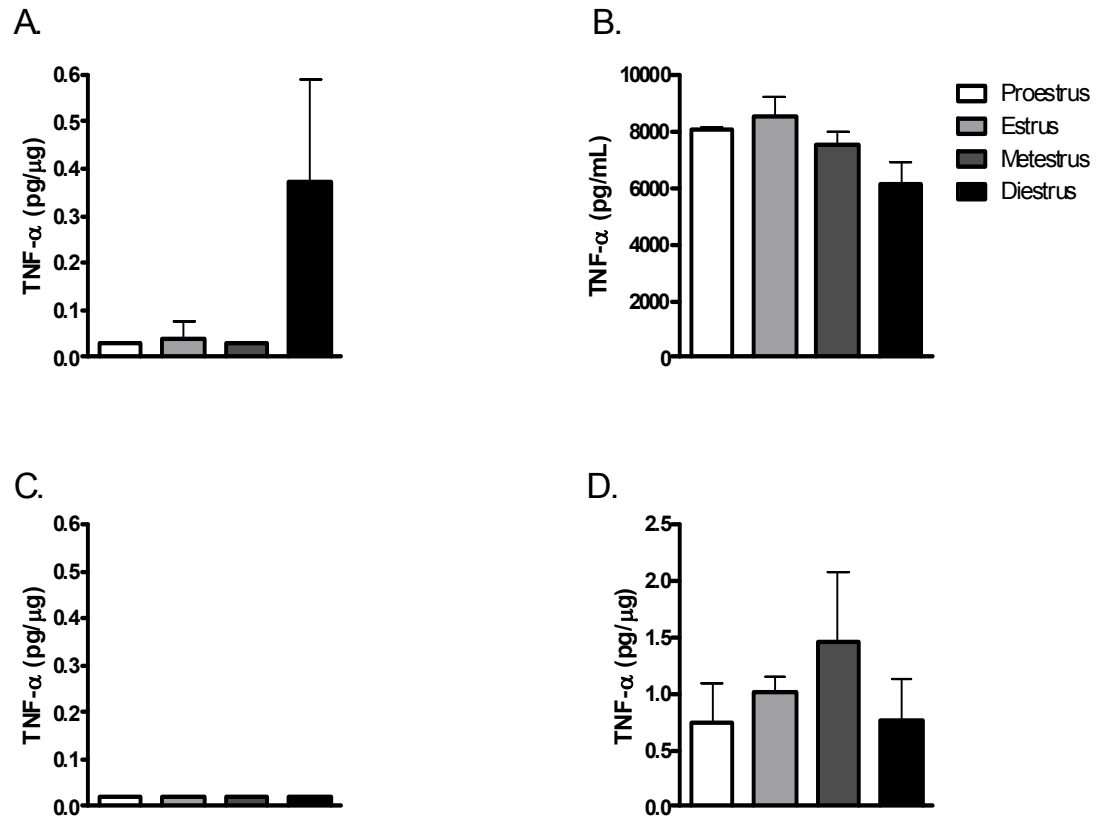


Figure 11. TNF- α levels in right paw (A), serum (B), DRG (C), and spinal cord (D) of intact female naïve rats. Mean TNF- α levels ($\pm$ SEM) in female rats during the proestrus, estrus, metestrus, or diestrus stage. No significant differences were observed. $n = 5$ per group.

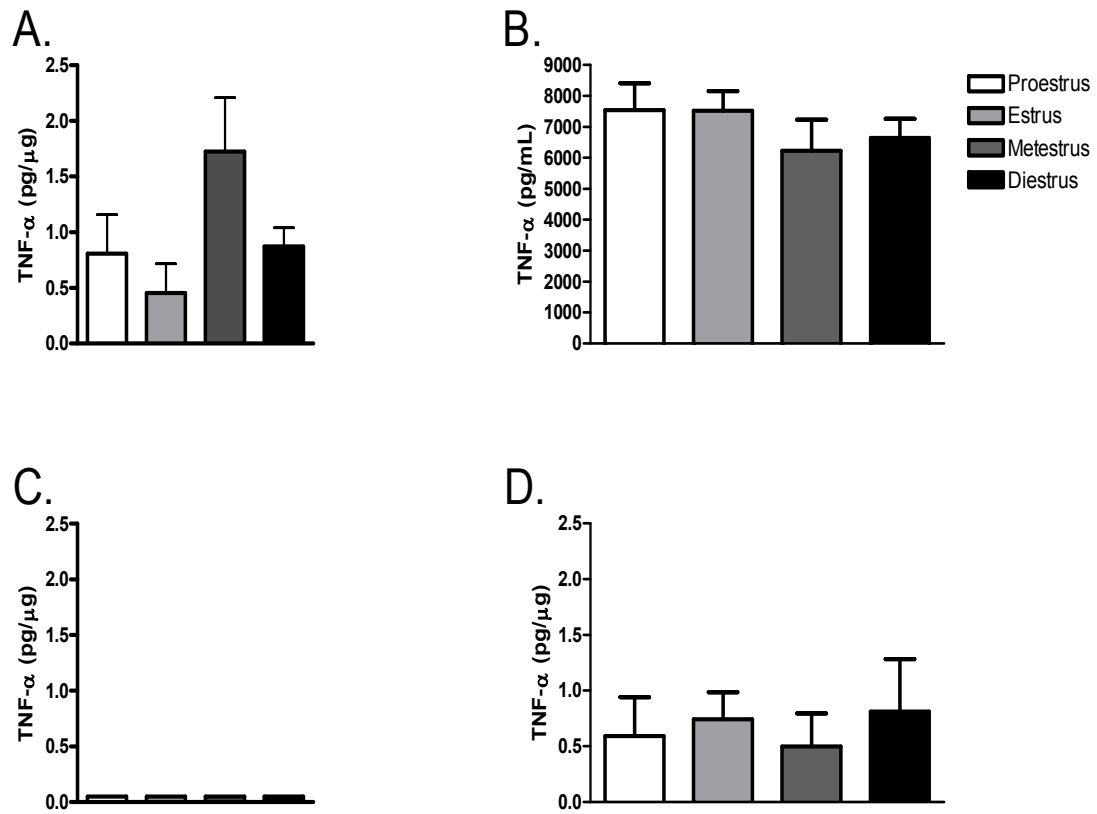


Figure 12. TNF- α levels in ipsilateral paw (A), serum (B), ipsilateral DRG (C), and spinal cord (D) of formalin-treated intact female rats. Mean TNF- α levels ($\pm$ SEM) in female rats during the proestrus, estrus, metestrus, or diestrus stage. No significant differences were observed. $n = 5$ per group.

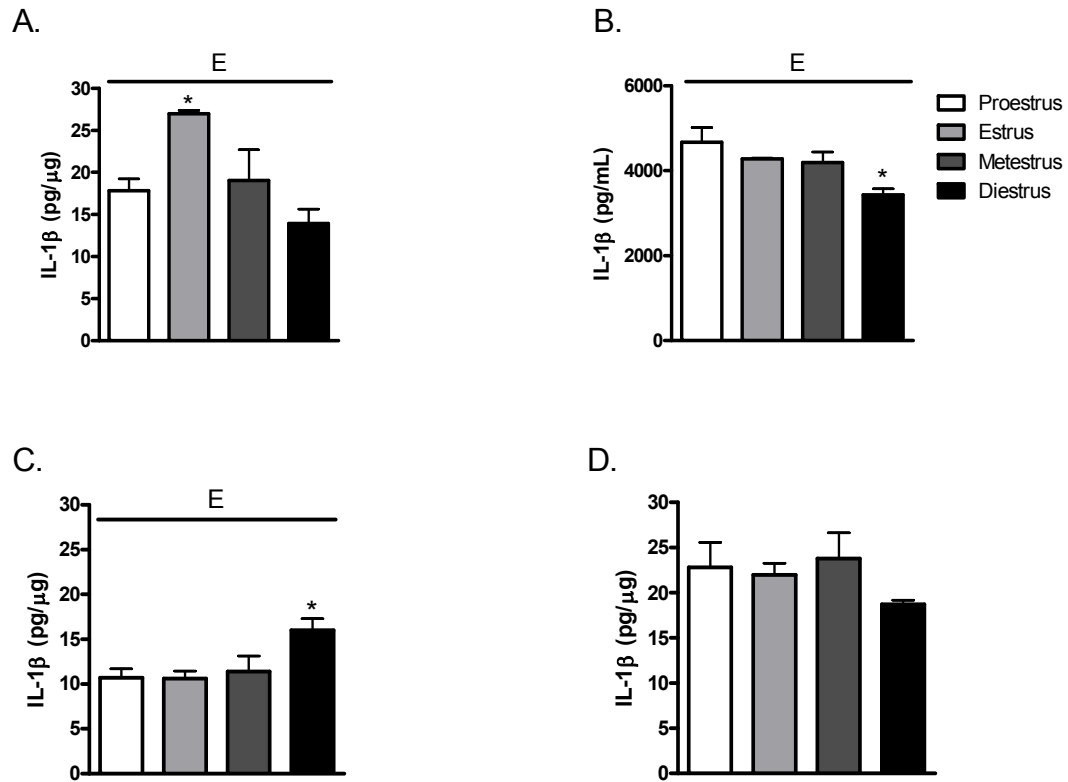


Figure 13. IL-1 β levels in right paw (A), serum (B), DRG (C), and spinal cord (D) of intact female naïve rats. Mean IL-1 β levels ($\pm$ SEM) in female rats during the proestrus, estrus, metestrus, or diestrus stage. (E) Represents significant main effect of estrous cycle stage ($p < 0.05$). (*) Denotes significant difference to all other stages ($p < 0.05$). $n = 5$ per group.

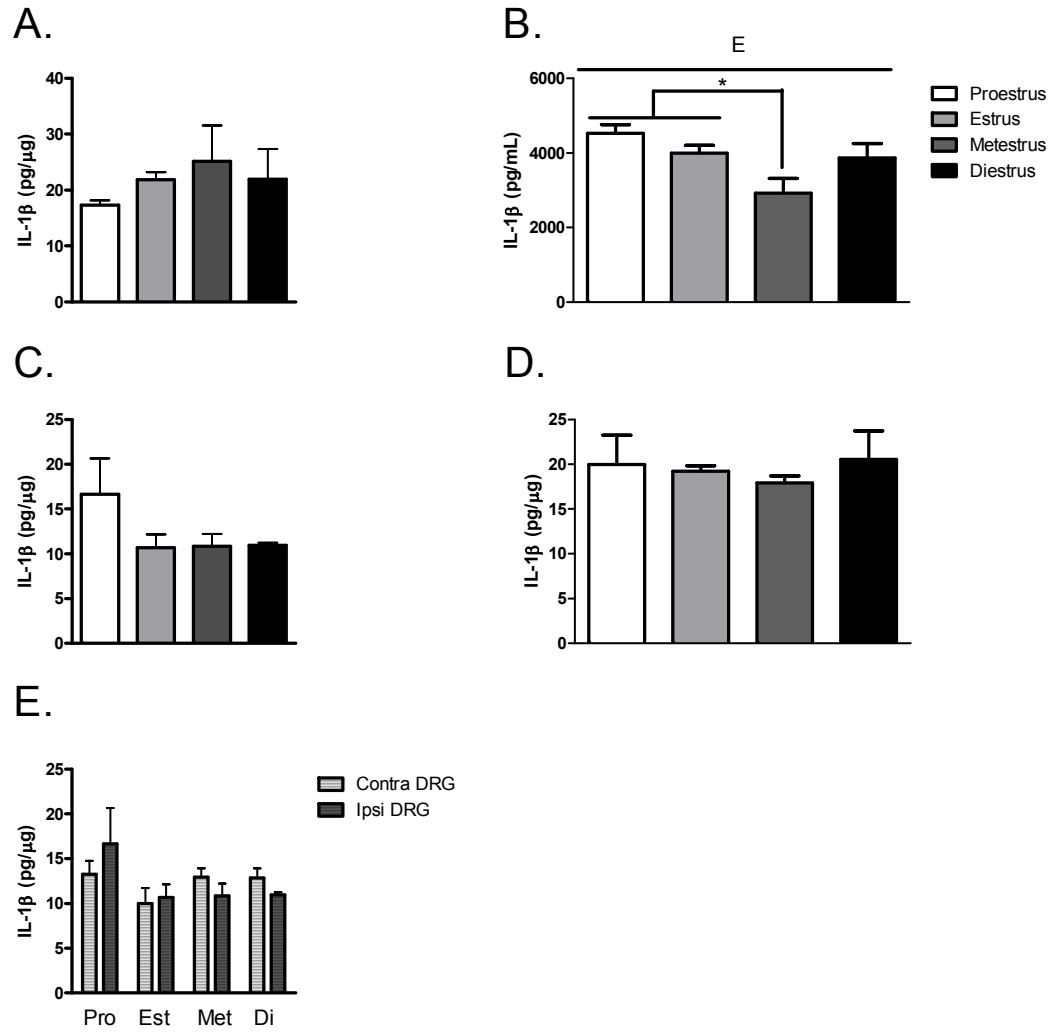


Figure 14. IL-1β levels in ipsilateral paw (A), serum (B), ipsilateral DRG (C), spinal cord (D), and ipsilateral versus contralateral DRG (E) of formalin-treated intact female rats. Mean IL-1β levels ($\pm$ SEM) in female rats during the proestrus, estrus, metestrus, or diestrus stage. (E) Represents significant main effect of estrous cycle stage ($p < 0.05$). (*) Denotes significant difference to proestrus and metestrus ($p < 0.05$). $n = 5$ per group.

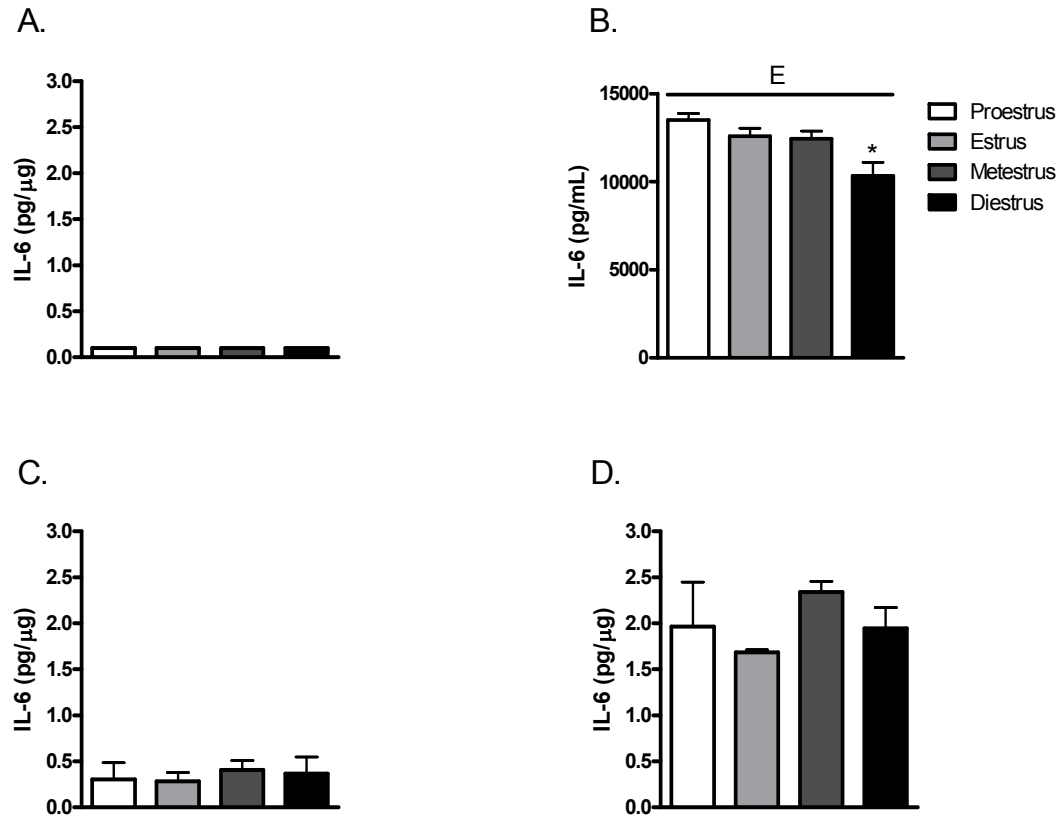


Figure 15. IL-6 levels in right paw (A), serum (B), DRG (C), and spinal cord (D) of intact female naïve rats. Mean IL-6 levels (± SEM) in female rats during the proestrus, estrus, metestrus, or diestrus stage. (E) Represents significant main effect of estrous cycle stage ($p < 0.01$). (*) Denotes significant difference to all other stages ($p < 0.05$). $n = 5$ per group.

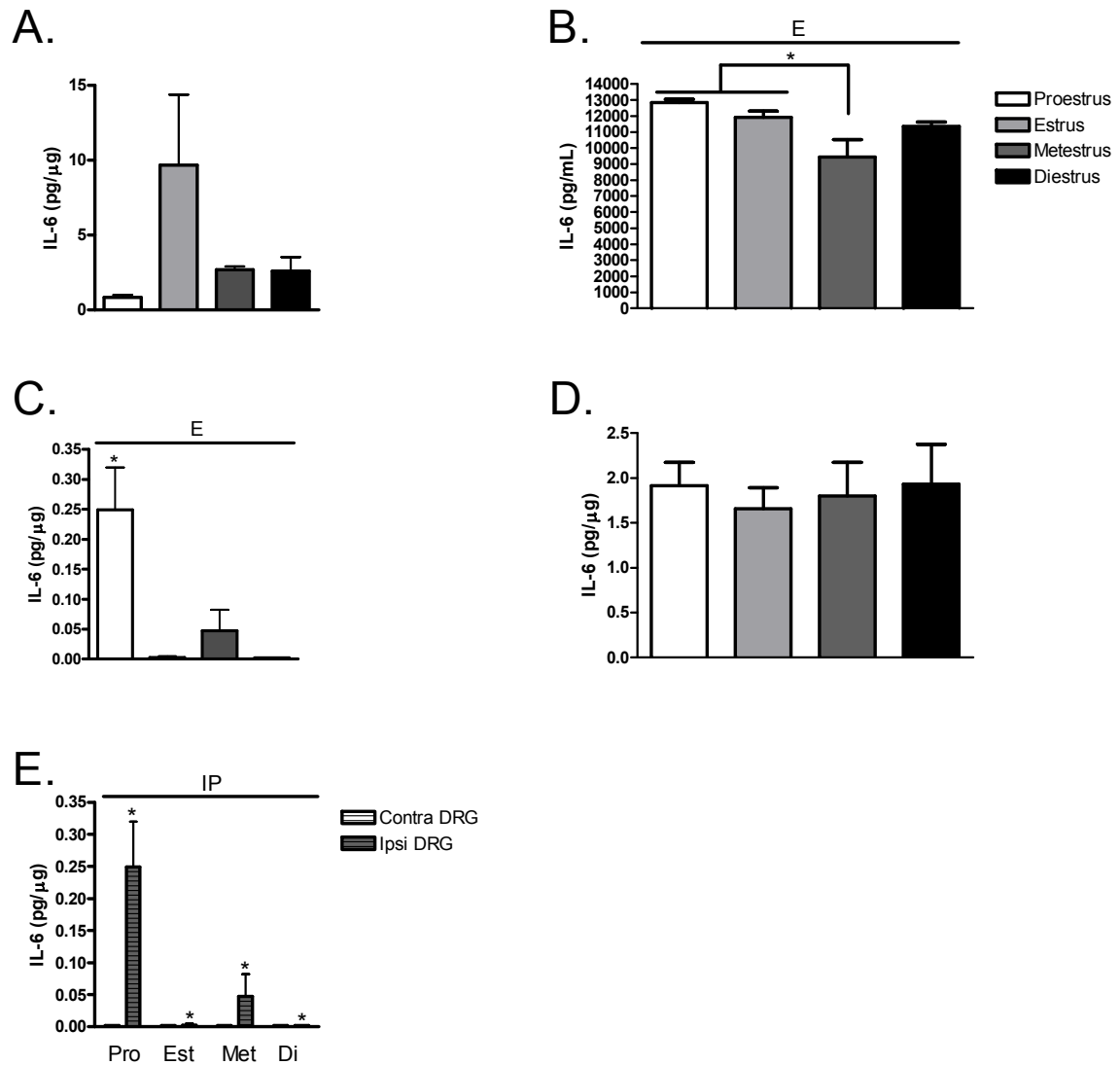


Figure 16. IL-6 levels in ipsilateral paw (A), serum (B), ipsilateral DRG (C), spinal cord (D), and ipsilateral versus contralateral DRG (E) of formalin-treated intact female rats. Mean IL-6 levels ($\pm$ SEM) in female rats during the proestrus, estrus, metestrus, or diestrus stage. In all figures, (E) Represents significant main effect of estrous cycle stage ($p < 0.05$). **Figure B:** (*) Denotes significant difference to proestrus and metestrus ($p < 0.05$). **Figure C:** (*) Denotes significant difference to all other stages ($p < 0.05$). **Figure E:** (IP) Denotes significant main effect between ipsilateral and contralateral DRG ($p < 0.05$). (*) Represents higher IL-6 levels in ipsilateral DRG as compared to contralateral DRG ($p < 0.05$). $n = 5$ per group.

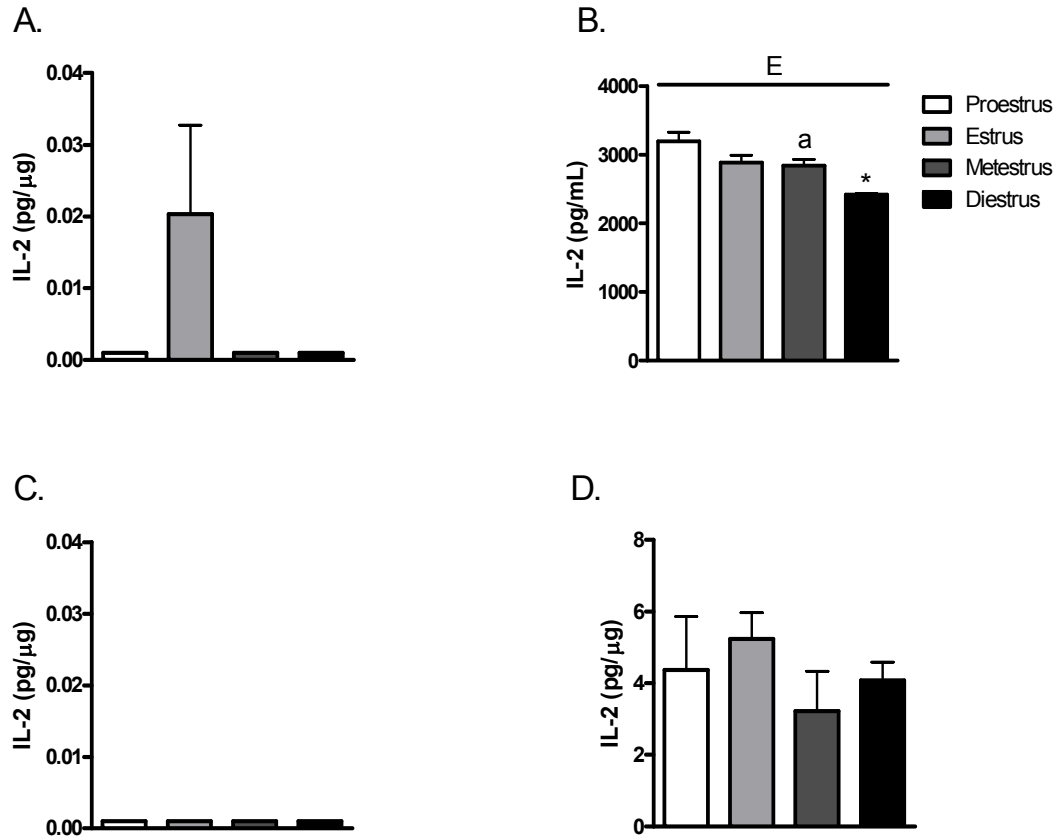


Figure 17. IL-2 levels in right paw (A), serum (B), DRG (C), and spinal cord (D) of intact female naïve rats. Mean IL-2 levels ($\pm$ SEM) in female rats during the proestrus, estrus, metestrus, or diestrus stage. (E) Represents significant main effect of estrous cycle stage ($p < 0.01$). (*) Denotes significant difference to all other stages ($p < 0.05$). (a) Denotes significant difference to proestrus ($p < 0.05$). $n = 5$ per group.

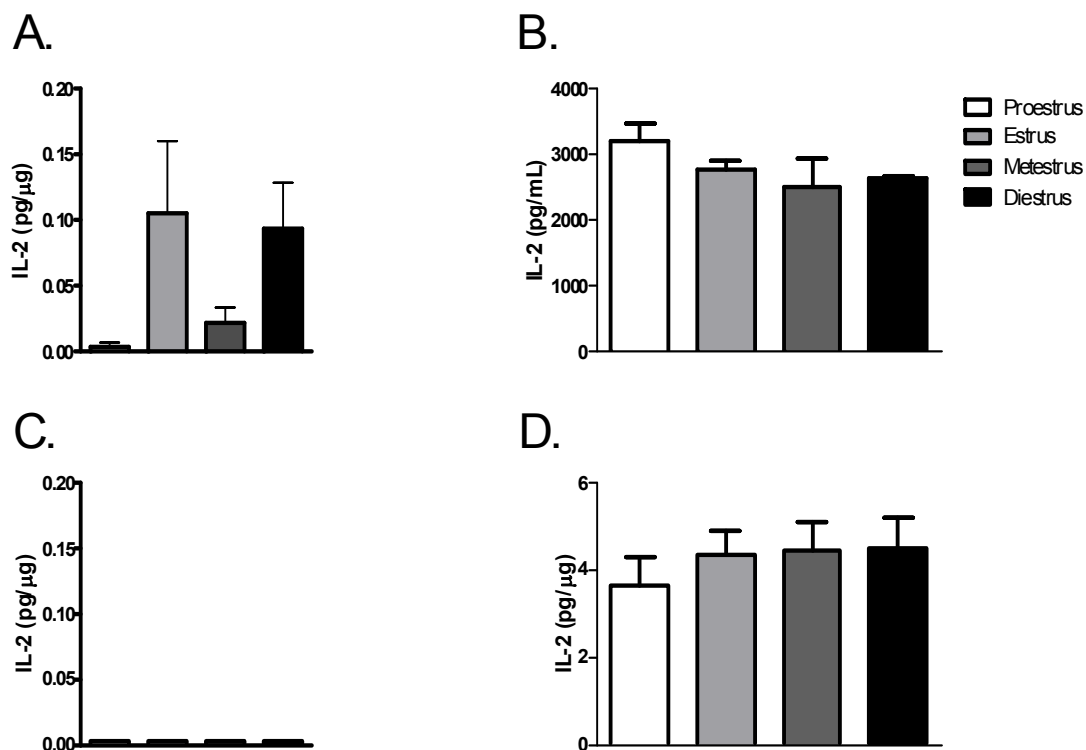


Figure 18. IL-2 levels in ipsilateral paw (A), serum (B), ipsilateral DRG (C), and spinal cord (D) of formalin-treated intact female rats. Mean IL-2 levels ($\pm$ SEM) in female rats during the proestrus, estrus, metestrus, or diestrus stage. No significant differences were observed. $n = 5$ per group.

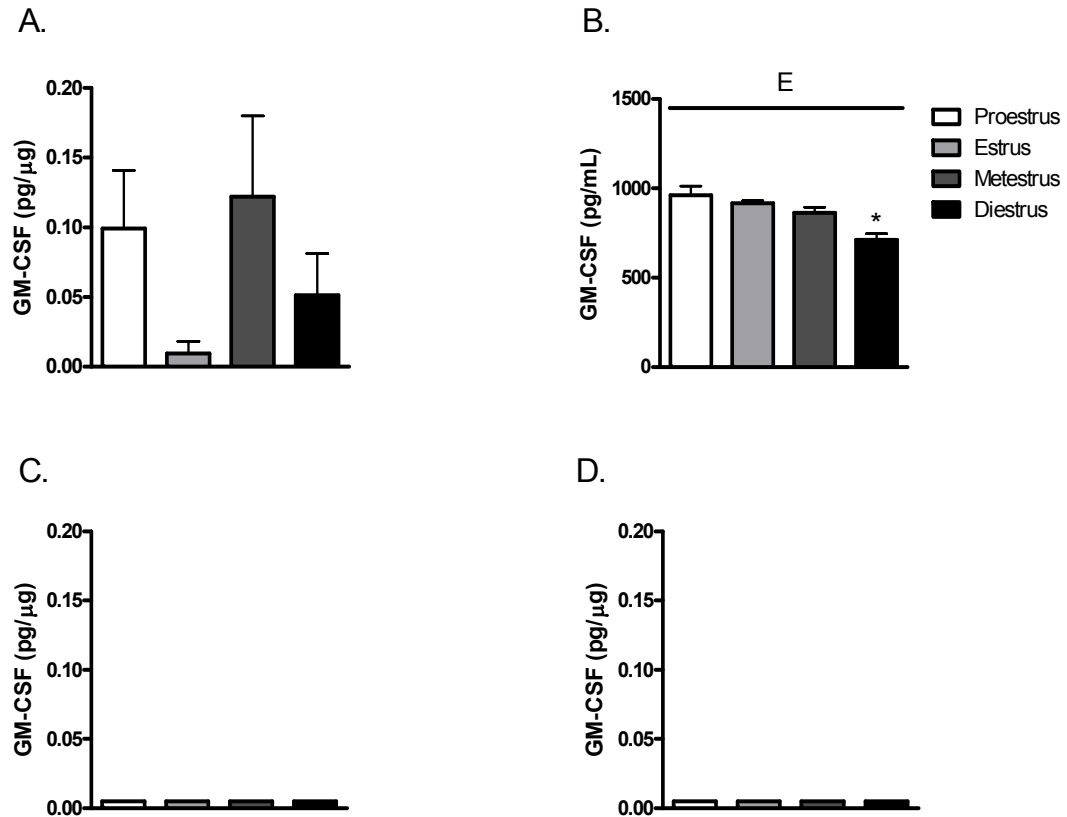


Figure 19. GM-CSF levels in right paw (A), serum (B), DRG (C), and spinal cord (D) of intact female naïve rats. Mean GM-CSF levels ($\pm$ SEM) in intact rats during the proestrus, estrus, metestrus, or diestrus stage. (E) Represents significant main effect of estrous cycle stage ($p < 0.01$). (*) Denotes significant difference to all other stages ($p < 0.05$). $n = 5$ per group.

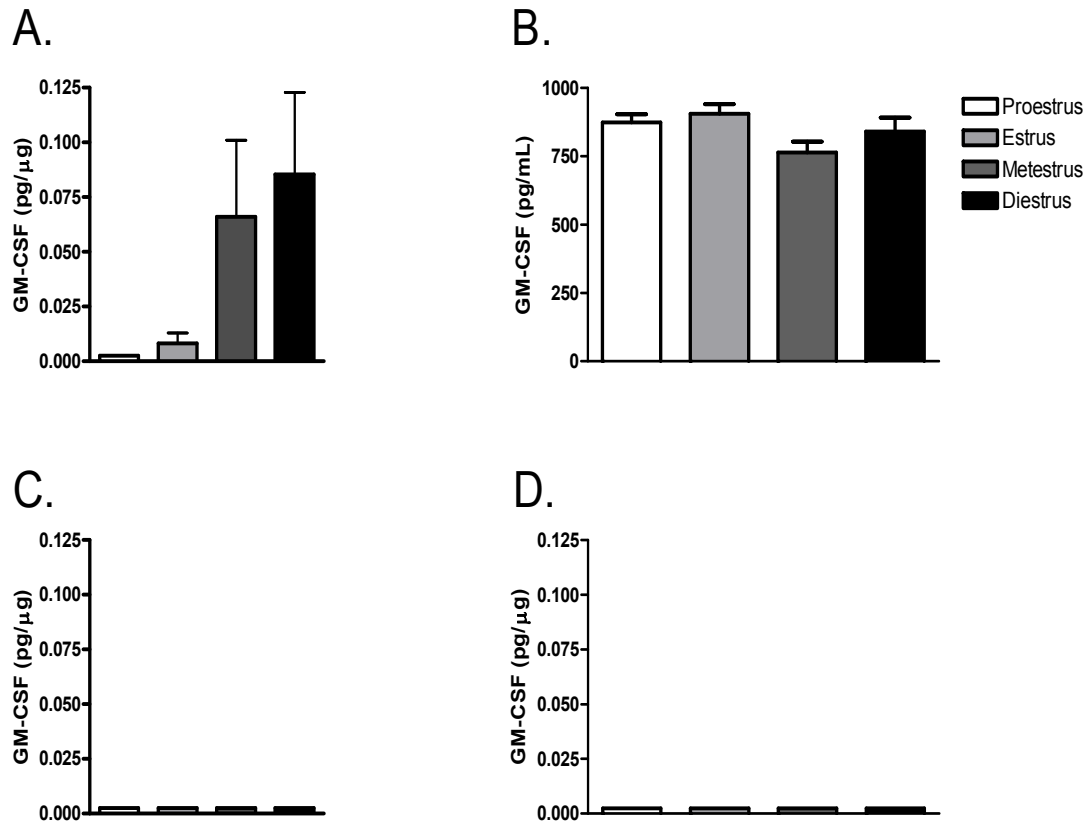


Figure 20. GM-CSF levels in ipsilateral paw (A), serum (B), ipsilateral DRG (C), and spinal cord (D) of formalin-treated intact female rats. Mean GM-CSF levels ($\pm$ SEM) in female rats during the proestrus, estrus, metestrus, or diestrus stage. No significant differences were observed. $n = 5$ per group.

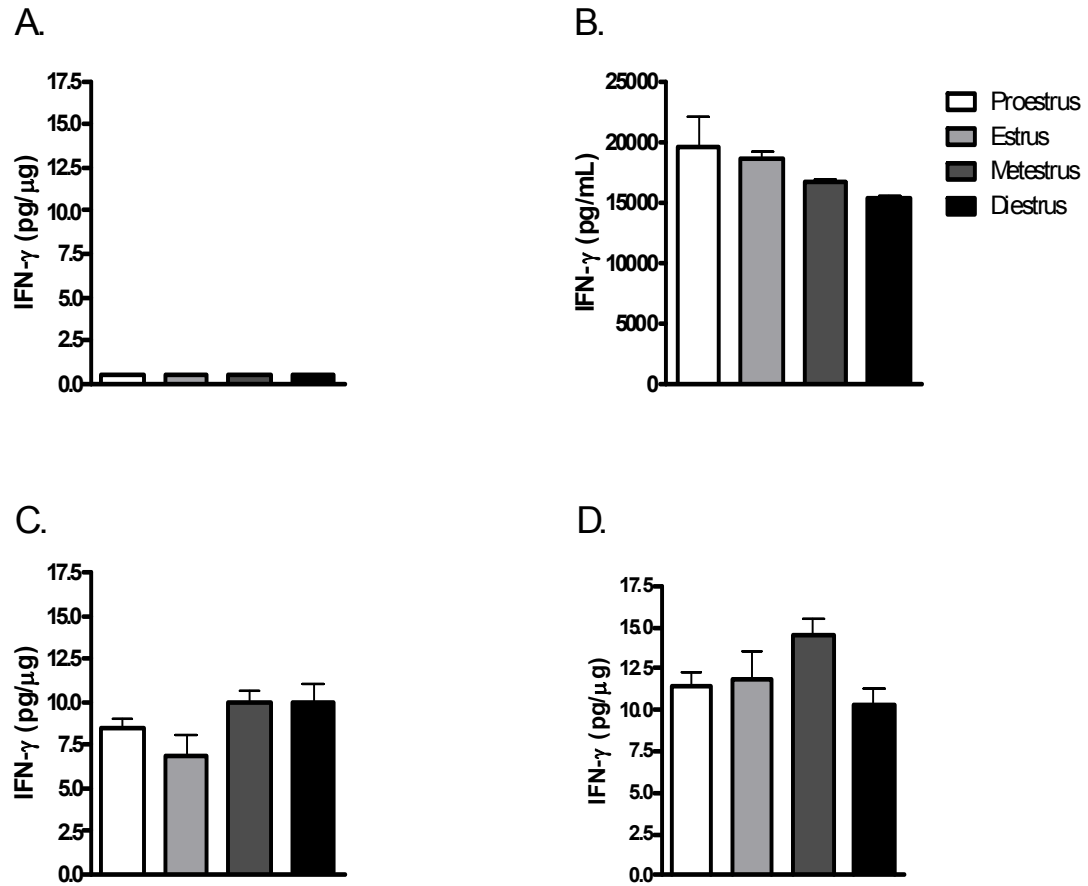


Figure 21. IFN- γ levels in right paw (A), serum (B), DRG (C), and spinal cord (D) of intact female naïve rats. Mean IFN- γ levels ($\pm$ SEM) in female rats during the proestrus, estrus, metestrus, or diestrus stage. No significant differences were observed. $n = 5$ per group.

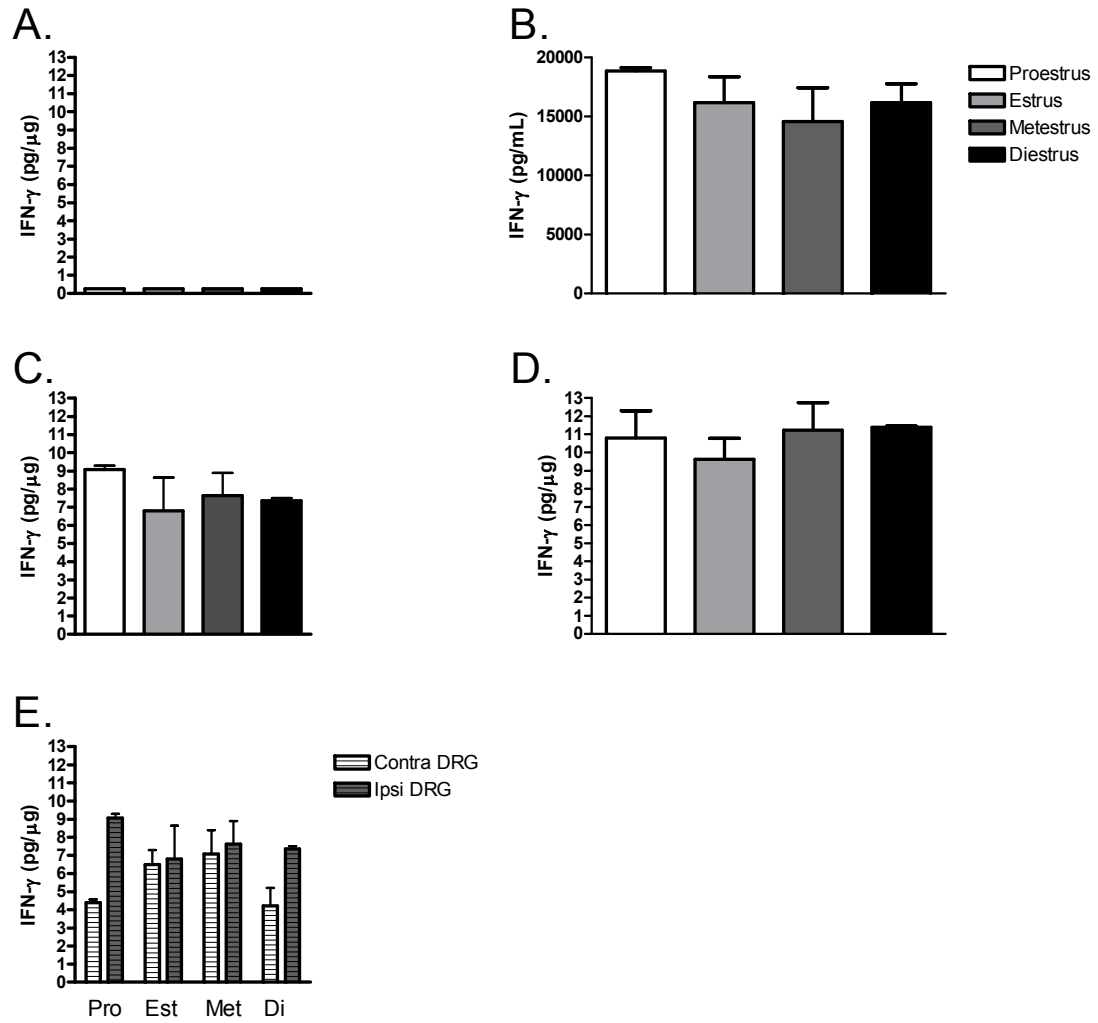


Figure 22. IFN- γ levels in ipsilateral paw (A), serum (B), ipsilateral DRG (C), spinal cord (D), and ipsilateral versus contralateral DRG (E) of formalin-treated intact female rats. Mean IFN- γ levels ($\pm$ SEM) in female rats during the proestrus, estrus, metestrus, or diestrus stage. No significant differences were observed. $n = 5$ per group.

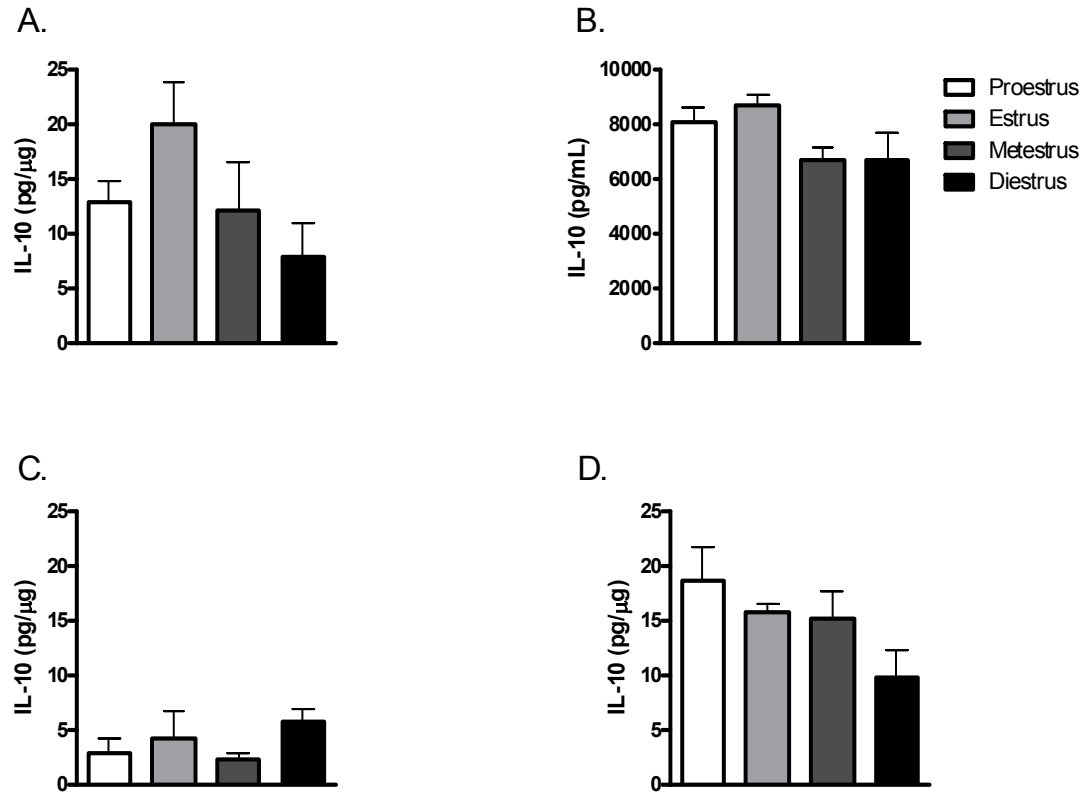


Figure 23. IL-10 levels in ipsilateral paw (A), serum (B), ipsilateral DRG (C), and spinal cord (D) of intact female naïve rats. Mean IL-10 levels ($\pm$ SEM) in female rats during the proestrus, estrus, metestrus, or diestrus stage. No significant differences were observed. $n = 5$ per group.

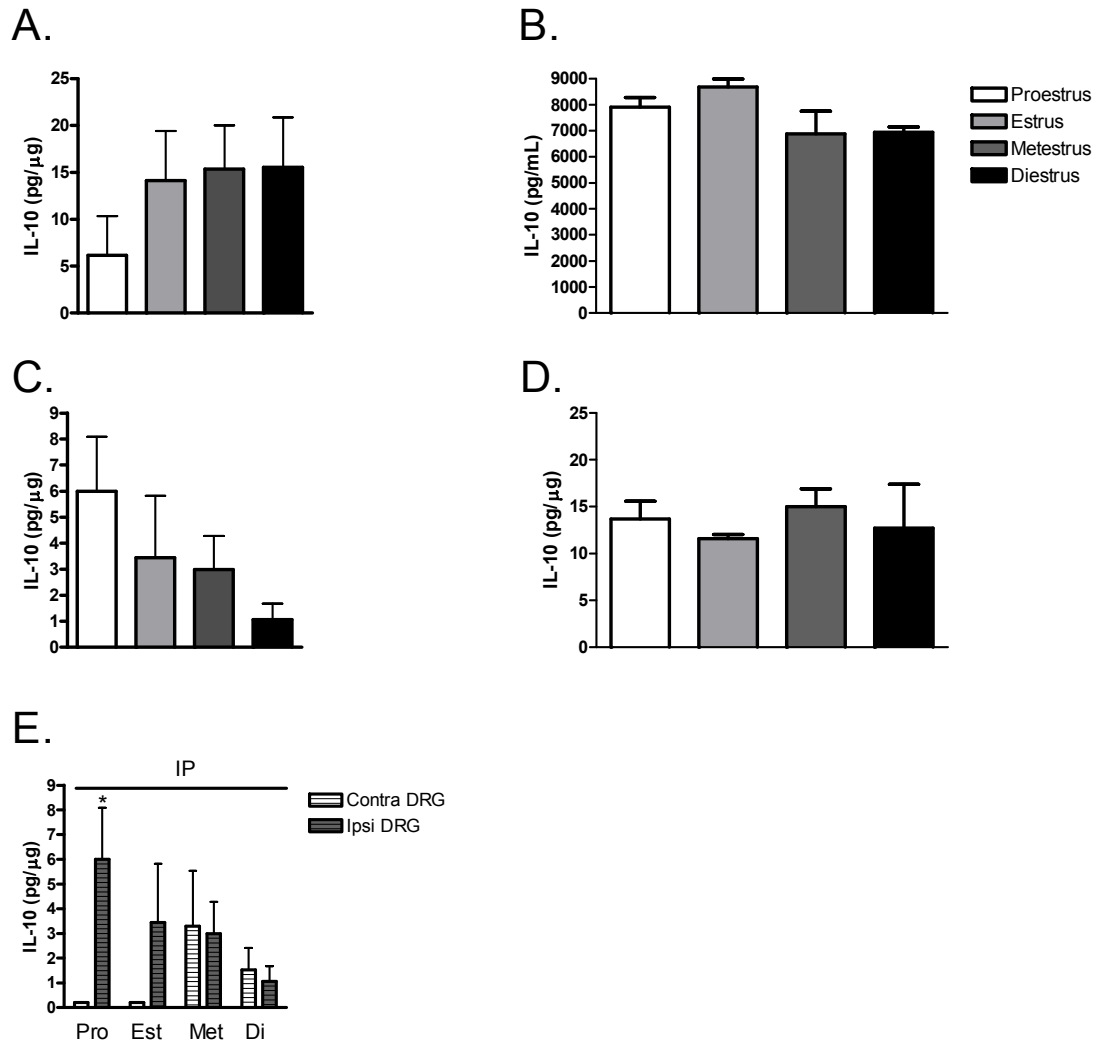


Figure 24. IL-10 levels in ipsilateral paw (A), serum (B), ipsilateral DRG (C), spinal cord (D), and ipsilateral versus contralateral DRG (E) of formalin-treated intact female rats. Mean IL-10 levels ($\pm$ SEM) in female rats during the proestrus, estrus, metestrus, or diestrus stage. (IP) Denotes significant main effect between ipsilateral and contralateral DRG ($p < 0.05$). (*) Represents significant difference to contralateral DRG, $p < 0.05$. $n = 5$ per group.

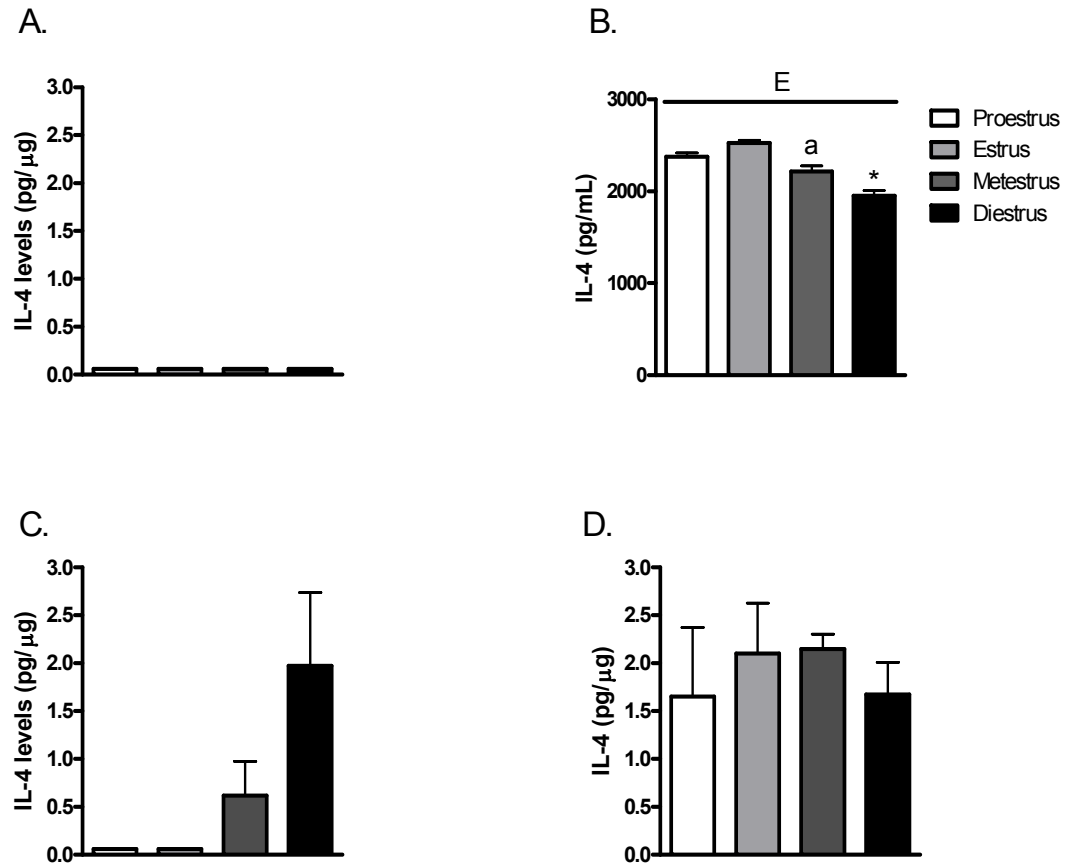


Figure 25. IL-4 levels in right paw (A), serum (B), DRG (C), and spinal cord (D) of intact female naïve rats. Mean IL-4 levels ($\pm$ SEM) in female rats during the proestrus, estrus, metestrus, or diestrus stage. (E) Represents significant main effect of estrous cycle stage ($p < 0.01$). (*) Denotes significant difference to all other stages ($p < 0.01$). (a) Denotes significant difference to estrus ($p < 0.01$). $n = 5$ per group.

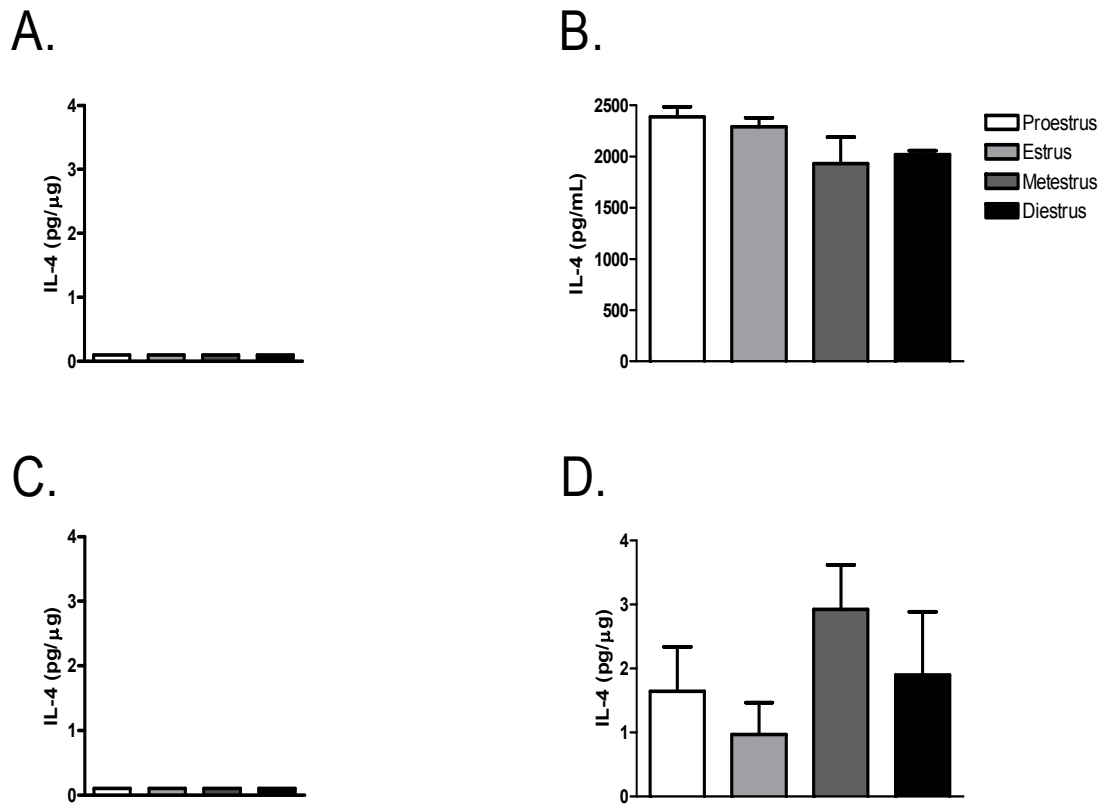


Figure 26. IL-4 levels in ipsilateral paw (A), serum (B), ipsilateral DRG (C), and spinal cord (D) of formalin-treated intact female rats. Mean IL-4 levels ($\pm$ SEM) in female rats during the proestrus, estrus, metestrus, or diestrus stage. No significant differences were observed. $n = 5$ per group.

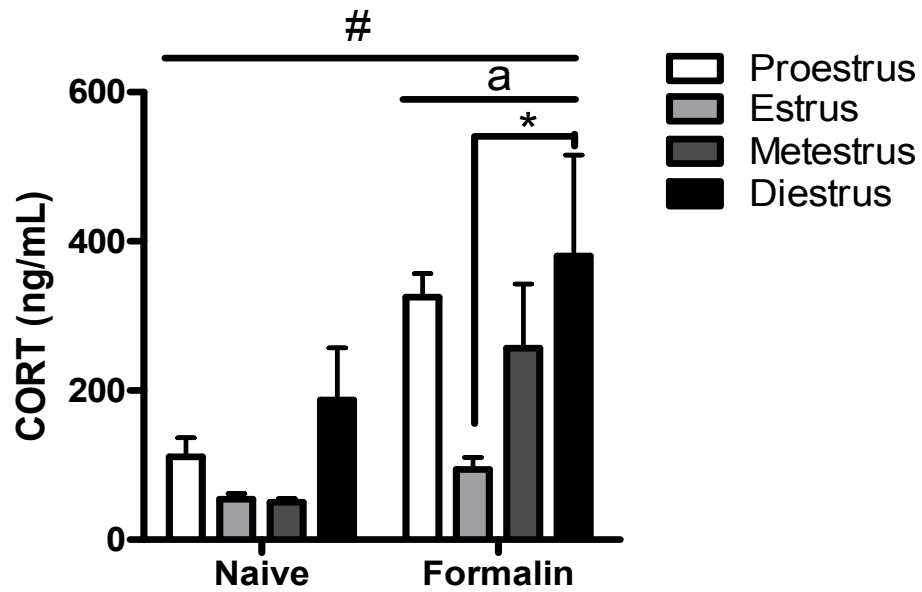


Figure 27. CORT levels in naïve and formalin-treated intact female rats. Mean CORT serum levels ($\pm$ SEM) in intact rats during proestrus, estrus, metestrus, or diestrus. (#) Represents significant main effect of pain condition, $p < 0.01$. (a) Represents statistically significant difference between naïve and formalin treatment groups, $p < 0.01$. (*) Represents significant difference between formalin-treated rats in diestrus as compared to rats in estrus, $p < 0.05$. $n = 6$ per group.

Table 3
Correlation analysis of CORT versus cytokine serum levels across the estrous cycle in naïve female rats

	Estrous cycle					Estrous cycle				
	stage	Correlation	<i>r</i>	<i>F</i>	<i>p</i>	stage	Correlation	<i>r</i>	<i>F</i>	<i>p</i>
06	Proestrus	CORT vs. TNF- α	0.64	0.71	0.56	Metestrus	CORT vs. TNF- α	0.71	3.11	0.18
		CORT vs. IL-1 β	0.76	4.13	0.14		CORT vs. IL-1 β	0.46	0.80	0.44
		CORT vs. IL-2	0.46	0.52	0.54		CORT vs. IL-2	0.54	1.22	0.35
		CORT vs. IL-6	0.80	3.54	0.20		CORT vs. IL-6	0.61	1.75	0.28
		CORT vs. GM-CSF	0.30	0.20	0.70		CORT vs. GM-CSF	0.69	2.63	0.20
		CORT vs. IFN- γ	0.17	0.06	0.83		CORT vs. IFN- γ	0.86	8.38	0.06
		CORT vs. IL-10	0.44	0.46	0.57		CORT vs. IL-10	0.67	2.45	0.22
		CORT vs. IL-4	0.98	23.55	0.13		CORT vs. IL-4	0.92*	16.24	0.03
	Estrus	CORT vs. TNF- α	0.04	0.004	0.96	Diestrus	CORT vs. TNF-α	0.98**	87.54	0.003
		CORT vs. IL-1 β	0.91	9.48	0.09		CORT vs. IL-1β	0.89*	11.33	0.04
		CORT vs. IL-2	0.92	11.64	0.08		CORT vs. IL-2	0.41	0.61	0.49
		CORT vs. IL-6	0.52	0.74	0.48		CORT vs. IL-6	0.22	0.15	0.72
		CORT vs. GM-CSF	0.06	0.007	0.94		CORT vs. GM-CSF	0.57	1.39	0.32
		CORT vs. IFN- γ	0.74	2.39	0.26		CORT vs. IFN- γ	0.50	1.02	0.39
		CORT vs. IL-10	0.40	0.38	0.60		CORT vs. IL-10	0.97**	54.68	0.005
		CORT vs. IL-4	0.80	3.53	0.20		CORT vs. IL-4	0.41	0.61	0.49

Legend. * $p < 0.05$. ** $p < 0.01$. $n = 5/\text{group}$.

Table 4
Correlation analysis of CORT versus cytokine serum levels following formalin administration across the estrous cycle

Estrous cycle stage	Correlation	<i>r</i>	<i>F</i>	<i>p</i>	Estrous cycle stage	Correlation	<i>r</i>	<i>F</i>	<i>p</i>
Proestrus	CORT vs. TNF- α	0.28	0.26	0.65	Metestrus	CORT vs. TNF- α	0.53	0.77	0.47
	CORT vs. IL-1 β	0.17	0.08	0.80		CORT vs. IL-1 β	0.88	6.59	0.12
	CORT vs. IL-2	0.10	0.04	0.86		CORT vs. IL-2	0.82	3.98	0.18
	CORT vs. IL-6	0.08	0.02	0.89		CORT vs. IL-6	0.87	6.18	0.13
	CORT vs. GM-CSF	0.57	1.46	0.31		CORT vs. GM-CSF	0.78	3.07	0.22
	CORT vs. IFN- γ	0.30	0.30	0.62		CORT vs. IFN- γ	0.85	5.18	0.15
	CORT vs. IL-10	0.20	0.11	0.76		CORT vs. IL-10	0.61	1.18	0.39
	CORT vs. IL-4	0.02	0.001	0.97		CORT vs. IL-4	0.82	4.07	0.18
Estrus	CORT vs. TNF- α	0.05	0.009	0.93	Diestrus	CORT vs. TNF- α	0.62	1.83	0.27
	CORT vs. IL-1 β	0.22	0.15	0.73		CORT vs. IL-1 β	0.55	1.31	0.34
	CORT vs. IL-2	0.20	0.12	0.75		CORT vs. IL-2	0.72	3.31	0.17
	CORT vs. IL-6	0.10	0.03	0.87		CORT vs. IL-6	0.74	3.60	0.15
	CORT vs. GM-CSF	0.10	0.04	0.85		CORT vs. GM-CSF	0.37	0.49	0.54
	CORT vs. IFN- γ	0.41	0.60	0.50		CORT vs. IFN- γ	0.65	2.15	0.24
	CORT vs. IL-10	0.20	0.13	0.74		CORT vs. IL-10	0.20	0.14	0.73
	CORT vs. IL-4	0.14	0.06	0.82		CORT vs. IL-4	0.33	0.38	0.58

Legend. *n* = 5/group.

4. Discussion

Previous research in this lab shows that during the formalin test for behavioral nociception, female rats flinch less during the proestrus stage as compared to metestrus (Amador et al., personal communication, 2009; Appendix B). In an effort to understand these behavioral responses, this study explored the role of endogenous female gonadal hormones on the regulation of cytokines. Estrous cycle effects on cytokine levels before and after formalin administration are summarized in Tables 5 and 6, respectively. It was demonstrated that changes in the female endocrinological profile during the estrous cycle altered cytokine levels. Specifically, in naïve rats, IL-1 β , IL-6, IL-2, GM-CSF, and IL-4 serum cytokine levels were lower during diestrus (Table 5). Basal GM-CSF and IL-4 serum levels were lower during metestrus as compared to proestrus and estrus stages, respectively (Table 5). In formalin-treated rats, IL-1 β and IL-6 serum levels were lower during metestrus as compared to proestrus and estrus stages (Table 6). Although cytokine levels were not lowered during proestrus, as was expected, alterations in cytokines throughout the endocrine profile indicate a mechanism through which female hormones may alter inflammatory mediators.

Endogenous ovarian hormone effects on paw size. Before injury, endogenous hormones did not change basal paw thickness; therefore gonadal hormones do not cause decreased vascular changes in the paw before the introduction of pain. As previously reported, formalin led to inflammation in the injected paw (Damas & Liegeois, 1999; Taylor et al., 1998). However, no link existed between hormone status during the estrous cycle and paw edema resulting from formalin administration. Therefore, this study

demonstrates that female gonadal hormones are not useful at lowering inflammatory effects at the site of injury.

Effects of endogenous ovarian hormones on basal cytokine levels. To this end, basal cytokine levels were assessed in serum as well as in peripheral and CNS tissue. As shown in Table 5, we demonstrate lower levels of IL-1 β , IL-6, IL-2, GM-CSF, and IL-4 basal serum levels in naïve rats during the diestrus stage. Furthermore, basal IL-2 and IL-4 serum levels were decreased during metestrus as compared to proestrus or estrus stages. Our study provides evidence that a relationship exists between hormonal levels found during the estrous cycle and cytokine serum production before the introduction of pain. Changes in basal cytokine levels may affect behavioral responses to pain by modulating cytokine levels during early stages of inflammation.

During diestrus, estrogen and progesterone serum levels start to rise in preparation of ovulation and behavioral estrus (Nelson, 2005). The fluctuation in estrogen and progesterone levels may in part be causing the decreased cytokine levels that were observed during this stage. In the paw and DRG, IL-1 β levels increased during estrus and diestrus, respectively (Table 5). This is a novel finding, since Houdeau and colleagues (2007) did not find changes in basal IL-1 β levels in peripheral tissue of naïve intact female rats. Further, most studies that investigate gonadal hormone effects on cytokines administer exogenous hormones and observe their actions after an inflammatory stimulus is introduced (Kuba et al., 2006; Lewis et al, 2008; Subramanian et al., 2005). Here we show that cytokine levels can be modulated by gonadal hormones before the introduction of inflammation or injury. The fact that serum cytokine levels decrease during diestrus suggests that under normal conditions ovarian hormones reduce

cytokine levels in an effort to lower production of inflammatory mediators before ovulation and estrus behavior occurs. Furthermore, since tissue IL-1 β levels were altered during estrous cycle stages that do not have similar gonadal hormone profiles suggests that sex steroids differentially regulate cytokine production at different peripheral sites.

Effects of endogenous hormones on cytokines during formalin-induced pain.

Preliminary research in our lab demonstrated that during the formalin test, rats in proestrus had decreased behavioral responses than rats in metestrus. In an effort to understand this finding, we examined cytokine levels following formalin administration to determine if endogenous hormones produced during proestrus were lowering cytokine activity, and thus behavioral responses, in this stage. Here we observed that serum levels of the proinflammatory cytokines IL-1 β and IL-6 were lower in metestrus than in proestrus and estrus stages (Table 6). This supports the notion that female gonadal hormones can influence cytokine production during inflammatory pain. However, the fact that cytokine levels were lower in metestrus instead of proestrus (when serum ovarian hormone levels are high) remains unclear.

Rodent models have shown that exogenous estradiol administration down-regulates the production of proinflammatory cytokines (Houdeau et al., 2007; Liu, Liu, & Bodenner, 2005; Polan et al., 1988; Ralston, Russel, & Gowen, 1990; Rogers & Eastell, 2001). However, the role of progesterone on cytokines is poorly understood since few research studies investigate its influence on inflammatory cytokine production. During metestrus, progesterone levels reach a second peak in which serum levels are elevated in comparison to estrogen (Kuba et al, 2005, Marcondes et al., 2002; Spornitz et al., 1999). It is suggested that this rise in progesterone is leading to the decreased proinflammatory

cytokine levels that were observed in this study. However, in a rat model of collagen-induced arthritis (CIA), progesterone administration did not lower proinflammatory cytokine levels (Ganesan et al., 2008). Houdeau and colleagues (2007) found that progesterone administration increased TNF- α tissue levels in an experimental model of colitis. Further, progesterone has been shown to increase nociceptive effects in rodent models of pain. For instance, in the formalin assay, progesterone administration lowers the analgesic effect of estrogen during Phase II (Kuba et al., 2006). Following CIA, estrogen treatment leads to reduced inflammation, however progesterone administered in combination with estrogen decreases these protective effects (Ganesan et al., 2008). Epidemiological pain studies demonstrate that sex differences in pain perception exist, where women are more likely to report pain as more persistent and of a longer duration than that of men (Fillingim & Maixner, 1995). It has been suggested that progesterone may counteract the protective role of estrogen during inflammatory pain, therefore explaining the sexual dimorphism in pain sensitivity (Ganesan et al., 2008). The link between progesterone and cytokine production needs to be further investigated to clarify the role of progesterone on nociceptive responses to pain.

In formalin treatment groups, IL-6 was up-regulated in the ipsilateral DRG across the estrous cycle, while during proestrus IL-10 levels were up-regulated in ipsilateral DRG as compared to contralateral DRG. However no other instances existed in which DRG cytokine levels were changed due to the estrous cycle. Surprisingly, cytokine levels were not modulated across the estrous cycle in paw and spinal cord tissue of formalin-treated rats, suggesting that ovarian hormones do not reduce inflammation by down-regulating cytokines at the site of injury or at CNS sites. Behaviorally, rats in

proestrus had less flinching responses during Phase II of the formalin test, a phase that is associated with central sensitization of dorsal horn neurons in the spinal cord (Taylor, Peterson, & Basbaum, 1995; Tjolsen et al., 1992). It was expected that spinal cord cytokine levels would be decreased during this stage, and would therefore offer a mechanism in which ovarian hormones reduce behavioral nociceptive responses. GCs produced during formalin administration may have masked fluctuations of cytokine levels throughout the estrous cycle in the tested tissue samples. For example, Nguyen and colleagues (1998) reported that in order to find an increase in brain IL-1 β levels following inescapable shock, rats in their study had to be adrenalectomized. Likewise, it may be necessary to adrenalectomize rats in this study to assess the role of female gonadal hormones on tissue cytokine levels without the inhibitory effects of CORT.

Estrous cycle effects on CORT levels. It was observed that estrous cycle stage does not influence CORT activity in naïve rats. Furthermore, we found that CORT concentrations reached its maximum levels during diestrus instead of proestrus in naïve and formalin treatment groups. These findings are in contrast to previous literature showing that rats reach maximum CORT levels during the proestrus stage (Atkinson & Waddell, 1997). This may be due to the time of sacrifice, since plasma concentrations of CORT reach peak heights during the evening of proestrus (Atkinson & Waddell, 1997).

Formalin administration led to heightened CORT serum levels as compared to naïve groups. We did observe that estrous cycle stage altered CORT levels following formalin-induced pain; rats in estrus had significantly lower CORT levels as compared to rats in diestrus. This suggests that endogenous gonadal hormones alter CORT levels following formalin administration.

CORT effects on cytokine activity. CORT release was demonstrated to correlate with cytokine levels in naïve, but not formalin-treated, rats. In naïve rats, correlation of the data revealed that during the diestrus stage elevated CORT levels correlated with decreased IL-1 β levels and elevated TNF- α and IL-10 levels (refer to Table 3). Furthermore, as discussed above, basal serum cytokine levels were significantly lower in diestrus as compared to proestrus, estrus, and metestrus stages (Table 5). It can be implied that in the diestrus stage the presence of basal CORT may have in part led to the inhibition of cytokines.

One way in which GCs exert their modulatory effects on cytokines is through inhibition of nuclear transcription factor-kappaB (NF- κ B), a transcription factor involved in regulating cytokine synthesis (Ghosh et al., 1998; McKay & Cidlowski, 1999). Furthermore, GCs have been shown to increase anti-inflammatory cytokine levels (Webster et al., 2002). This study suggests that CORT modulates basal cytokine levels in intact female rats. The fact that CORT does not, however, alter cytokine levels during formalin-induced pain demonstrates that in this model CORT is not mediating inflammatory effects through the cytokine pathway.

In conclusion, this study demonstrates that fluctuations in endogenous ovarian hormones seen during the female estrous cycle are associated with changes in cytokine levels. In most cases, gonadal hormones modulated basal cytokine production, suggesting that the estrous cycle may influence pain responses by modulating cytokine levels before the introduction of inflammation. In addition, the anti-inflammatory effects of the HPA axis may play a role in attenuating basal cytokine production. Future analysis of the direct effects of estrogen, progesterone, and the HPA axis should be assessed to

have a better understanding of the connection of these mediators on cytokine levels during formalin-induced pain.

Table 5
Summary of cytokine levels in paw, serum, DRG, and spinal cord of intact female naïve rats

Cytokine	Sample tested			
	Paw	Serum	DRG	Spinal cord
TNF- α	∅	∅	∅	∅
IL-1 β	↑ E	↓ D	↑ D	∅
IL-6	∅	↓ D	∅	∅
IL-2	∅	↓ D; ↓ M compared to P	∅	∅
GM-CSF	∅	↓ D	∅	∅
IFN- γ	∅	∅	∅	∅
IL-10	∅	∅	∅	∅
IL-4	∅	↓ D; ↓ M compared to E	∅	∅

Legend. P, E, M, and D denote proestrus, estrus, metestrus, and diestrus, respectively. (↑) denotes increase in cytokine levels during the stage labeled, $p < 0.05$. (↓) denotes decrease in cytokine levels during the stage labeled, $p < 0.05$. (∅) denotes no change in cytokine levels across the estrous cycle. $n = 5/\text{group}$.

Table 6
Summary of cytokine levels in paw, serum, DRG, and spinal cord of formalin-treated intact female rats

Cytokine	Sample tested				
	Paw	Serum	DRG	Spinal cord	Ipsi vs. Contra DRG
TNF- α	∅	∅	∅	∅	∅
IL-1 β	∅	↓ M compared to P & E	∅	∅	∅
IL-6	∅	↓ M compared to P & E	↑ P	∅	√
IL-2	∅	∅	∅	∅	∅
GM-CSF	∅	∅	∅	∅	∅
IFN- γ	∅	∅	∅	∅	∅
IL-10	∅	∅	∅	∅	√ in P
IL-4	∅	∅	∅	∅	∅

Legend. P, E, M, and D denote proestrus, estrus, metestrus, and diestrus, respectively. (↑) denotes increase in cytokine levels during the stage labeled, $p < 0.05$. (↓) denotes decrease in cytokine levels during the stage labeled, $p < 0.05$. (∅) denotes no change in cytokine levels across the estrous cycle. (√) denotes significant difference between ipsilateral and contralateral DRG, $p < 0.05$. $n = 5/\text{group}$.

Chapter 3: Effects of estrogen and endogenous corticosterone on basal and inflammatory responses.

1. Introduction

Inflammatory pain is a pain state often associated with physiological responses such as allodynia (increased responsiveness to non-noxious stimuli) and hyperalgesia (increased sensitivity to pain; Basbaum & Woolf, 1999; Burian & Geisslinger, 2005; Millan, 1999). Cytokines are small proteins that play a pivotal role in initiating signaling events involved in inflammatory pain (Sommer & Kress, 2004). They are synthesized from a variety of cell types that include mast cells and T cells in the periphery, and glial cells in the central nervous system (Moalem & Tracey, 2006; Wieseler-Frank et al., 2005). Proinflammatory cytokines such as TNF- α , IL-1 β , and IL-6 propagate pain signals by directly activating nerve cells following injury and insult (Cunha et al., 1992; Ferreira, Lorenzetti, Bristow, & Poole, 1988; Sorkin et al., 1997; Zelenka, Schafers, & Sommer, 2005). Cytokines further amplify inflammatory responses by stimulating and up-regulating the release of sympathetic amines and chemokines, as well as the prostanoid biosynthetic pathway, a system responsible for sensitizing peripheral and central nervous systems to impending noxious stimuli (Ferreira et al., 1988; Rittner, et al., 2005; Samad et al., 2002).

Cytokines are often regulated by negative feedback systems that hamper their production when its actions are no longer needed. Anti-inflammatory cytokines such as IL-10 and IL-4 limit the synthesis of proinflammatory cytokines (Cunha et al., 1999; Poole et al., 1995). Likewise, the female sex hormone estrogen has been shown to modulate cytokine activity. In peripheral blood samples obtained from postmenopausal

women, 17 β -estradiol lowers TNF- α , IL-6, and IL-1 β release in a dose-dependent manner *in vitro* (Ralston, Russell, & Gowen, 1990; Rogers & Eastell, 2001). Rodent models also support estrogen's role in regulating cytokine levels. Estradiol replacement lowers IL-6 production in OVX mice, while activation of the estrogen receptor leads to IL-6 gene suppression (Liu, Liu, & Bodenner, 2005; Masiukiewicz et al., 2000). Ethinyl estradiol lowers the secretion of IL-6 and TNF- α by T cells, and decreases mRNA for cytokines and chemokines in joint tissue, following collagen-induced arthritis in mice (Subramanian et al., 2005). In mouse splenic lymphocytes, estrogen treatment inhibits lipopolysaccharide (LPS)-induced IFN- γ production via selective regulation of microRNAs, small RNAs linked to homeostasis and immune responses (Dai et al., 2008). Following spinal cord injury in rats, pretreatment of 17 β -estradiol stimulates early cytokine release, which leads to improved locomotor activity and neuroprotection as compared to controls (Ritz and Hausmann, 2008). Further, in rat astrocyte cultures pretreated with 17 β -estradiol, LPS-induced protein expression of TNF- α and IL-1 β is attenuated by 44% and 27%, respectively (Lewis et al, 2008). Together, these studies demonstrate that estrogen plays an important role in cytokine regulation.

On the other hand, research shows that glucocorticoid (GC) hormones also contribute to the control of nociception and inflammation by reducing proinflammatory cytokine production (DeRijk et al., 1997; Sternberg, 1997). Elevated plasma GC levels suppress IL-1 and TNF- α , and increase anti-inflammatory cytokine IL-4 and IL-10 production (DeRijk et al., 1997; Sternberg, 1997). Chensue et al. (1991) found that during LPS administration, decreased TNF- α levels correlate with elevated corticosterone (CORT), the GC secreted in rodents. Kapcala et al. (1995) showed that adrenalectomized

(ADX) rats have increased mortality rates following IL-1 β administration, but CORT replacement attenuates that lethality. Injection of metyrapone plus aminoglutethimide (GC inhibitors) increases basal IL-1 β mRNA expression and alters IL-1 β levels following acute stress in the rat hypothalamus (Blandino Jr. et al., in press).

However, it is not clearly understood whether the HPA axis acts alone or in coordination with the HPG axis to suppress cytokine levels. Experimentally, estrogen has been shown to modulate GC expression; for instance, estradiol replacement increases CORT serum levels in rats (Niyomchai et al., 2005). In adrenalectomized-ovariectomized (ADX+OVX) rats, estradiol treatment leads to increased GC levels in brain and spinal cord tissue (Ferrini, González, & De Nicola, 1993). Da Silva et al. (1993) demonstrated that gonadectomy leads to elevated CORT levels in male mice as compared to females following systemic inflammation, whereas in sham-operated mice, females had higher CORT levels than males. This implied that the presence of gonadal hormones regulates GC production during inflammation (Da Silva et al., 1993).

Taken together, experimental observations suggest that pain perception may result from the interaction of sex steroids, inflammatory stimuli, and the HPA axis. However, there is little research investigating the specific interaction between estrogen and CORT on biochemical cytokine processes that lead to the inhibition of inflammatory pain.

The formalin test is a pain assay that leads to tissue damage, inflammation, and nociceptive behavioral responses (Sternberg & Wachterman, 2000; Tjolsen et al., 1992; Yashpal &Coderre, 1998). It consists of an injection of diluted formaldehyde into the rodent hind paw which produces biphasic behavioral responses that include licking, flinching, or biting of the paw (Tjolsen et al., 1992). The initial five minutes of

behavioral responses are termed Phase I and correspond with acute pain, while the next phase of pain behavior, termed Phase II, lasts for about 30-40 minutes and corresponds with tonic/persistent pain (Damas & Liegeois, 1999; Dickenson & Sullivan, 1987; Dubuisson & Dennis, 1977, Tjolsen et al., 1992). Paw oedema resulting from formalin administration is mediated by a release of neuropeptides, prostanoids, and mast cell amines (Damas & Liegeois, 1999). Yashpal andCoderre (1998) demonstrate that 5% formalin injection leads to a positive correlation between nociceptive behavioral responses and oedema in the rat hind paw. Thus, the formalin assay is a useful tool for modeling inflammatory pain responses in rats.

Research demonstrates that during formalin administration, estrogen replacement (via SILASTIC capsule) significantly reduces Phase II nociceptive flinching responses in ovariectomized (OVX) rats (Kuba et al., 2005, 2006; Mannino et al., 2007) Furthermore, Hunter et al (in press) have recently demonstrated that estradiol administration reduces flinching in ADX and ADX+OVX rats. However, it is not yet understood how estrogen is mediating its anti-hyperalgesic responses during these paradigms. Estrogen's anti-hyperalgesic effects during the formalin assay may be a result of directly inhibiting proinflammatory cytokine levels, since estrogen has been shown to regulate cytokine production. On the other hand, estrogen may work synergistically with the HPA axis to lower proinflammatory cytokine production, thus resulting in reduced nociceptive responses. We hypothesize that estrogen's anti-hyperalgesic effects on inflammation-induced pain occur through up-regulation of CORT release, which in turn down-regulates proinflammatory cytokine release, and thus, nociception. To examine this hypothesis, cytokine levels at peripheral and central sites were analyzed in naïve and formalin-treated

rats administered either estradiol or vehicle. To determine if activation of the HPA axis is necessary to mediate estrogen's anti-hyperalgesic responses, estradiol- and vehicle-treated OVX-SHAM ADX, ADX-SHAM OVX, and ADX+OVX rats were tested for CORT and cytokine levels before and after formalin administration.

2. Materials and Methods

2.1 Animals

Eight-week old OVX-SHAM ADX, ADX-SHAM OVX, and ADX+OVX female Sprague-Dawley rats ($n = 7-10$ per group) were purchased from Taconic (Germantown, NY). Rats were double-housed with a 12-hour light/dark photoperiod (lights on 8 A.M. EST), and food and water were available *ad libitum*. ADX animals were maintained on water supplemented with 0.9% sodium chloride. Rats weighed about 220 g at the time of sacrifice. Animal treatment was in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, Bethesda, MD) and was approved by the Institutional Animal Care and Use Committee at Hunter College of The City University of New York.

2.2 Estradiol replacement paradigm

Two weeks after ovariectomy and/or adrenalectomy, a SILASTIC capsule (1 cm, 0.058 in. ID x 0.077 in. OD, Dow Corning) was inserted into the nape of the rats' neck. The capsules contained either 100% cholesterol (5-Cholestin-3Beta-ol; Sigma-Aldrich, St. Louis, MO) for the vehicle group, or 20% 17-Beta-Estradiol (1, 3, 5 [10]-Estratriene-3, 17 Beta-diol; Sigma-Aldrich) in cholesterol for the experimental group. Rats were

randomly assigned to either the vehicle or estrogen experimental group; however, both rats in each cage received the same type of SILASTIC capsule. Adequate care was taken to minimize pain or discomfort. Rats were tested one week after hormone replacement to ensure that serum estradiol levels were maintained at a steady state, and that estradiol levels approximated peak estrogen levels seen during the proestrus stage of the rat estrous cycle (Mannino et al., 2005). Furthermore, previous literature has shown that 20% 17 β -estradiol administration reduces formalin-induced behavioral responses (Mannino et al., 2007). All animal procedures were approved by the Institutional Animal Care and Use Committee of Hunter College, CUNY.

2.3 Formalin administration

Using a 27 $\frac{1}{2}$ -gauge needle, rats received a 50 μ L formalin injection [5% formaldehyde (Sigma); 95% sterile isotonic saline (0.9% Sodium Irrigation USP, Braun Medical, Irving, CA)] subcutaneously into the intraplantar region of the right hind paw while they were manually restrained. Formalin was administered between 9:00 AM and 3:00 PM.

2.4 Tissue Collection and Homogenization

In order to mimic the time that behavior during the formalin assay is collected, rats were sacrificed by rapid decapitation one hour after formalin administration, following a brief (20 sec) exposure to CO₂. Naïve rats were immediately sacrificed after removal from their home cage. Trunk blood was collected in tubes containing K₂-EDTA (BD Vacutainer Systems, Franklin Lakes, NJ), and then centrifuged at 2,600 RPM for 30 minutes at 4° C. Serum was collected and stored at -80° C until assays were performed.

After rats were sacrificed, hind paw measurements were taken using a dial caliper (General Tools, New York, NY), measuring the width and depth in millimeters. Next, paws were removed post mortem, weighed, and rapidly frozen on dry ice. Spinal cord and dorsal root ganglia (DRG) tissue were collected from the lumbar region, and immediately frozen. All tissue samples were stored at -80° C until use.

For the tissue homogenization procedure, spinal cord, paw, and DRGs were then homogenized in lysing solution that contains cell lysis buffer (from Cell Lysis Kit #171-304012, Bio-Rad, Hercules, CA), a protease inhibitor cocktail provided in the cell lysis kit (Bio-Rad, Hercules, CA), and 500 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, St. Louis, MO) diluted in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO). Tissue samples were homogenized with a Polytron handheld homogenizer (Kinematica, Luzern, Switzerland) and were rapidly frozen at -80° C. Homogenates were thawed and then centrifuged at 5000 rpm for 15 minutes at 4° C. Supernatants were collected and stored at -80° C. Total protein concentration was determined using a Bradford kit from Bio-Rad Laboratories (Hercules, CA). In order to control for homogenate variability due to processing of lysing solution, each sample harvested from the same region were homogenized simultaneously.

2.5 Cytokine Multiplex Assay

The Bio-Plex Suspension Array (Bio-Rad, Hercules, CA) is a system that allows for simultaneous quantification of a panel of cytokines. Its array reader combines fluidics, two lasers, and real-time digital signaling to distinguish cytokines within each sample. Tissue homogenates and serum were analyzed for cytokine levels by using the Bio-Plex

Cytokine assay along with the Cytokine Reagent Kit (#171-K11070 and #171-304001, respectively, Bio-Rad, Hercules, CA). The cytokine assay consists of a multiplexed (IL-1 β , IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN- γ , and TNF- α) immunoassay containing premixed beads coated with target capture antibody. Simultaneous cytokine analysis was processed according to the manufacturer's protocol (for details see Hulse et al., 2004). Afterwards, samples were quantified using the Bio-Plex Suspension Array system and accompanying Bio-Plex Manager software (Bio-Rad, Hercules, CA). The limit of detection was <10 pg/mL, and intra-assay coefficients of variance averaged below 20% for each cytokine. Tissue cytokine concentrations were divided by the protein concentrations obtained from Bradford analysis. Because of variability between assays, tissue cytokine values were normalized to the percent change related to their respective hormone-vehicle treated groups. For serum, samples were diluted 1:4 with Rat Serum Diluent according to the manufacturer's protocol (Bio-Rad, Hercules, CA). Serum cytokine values are expressed as pg/mL. IL-4 levels were too low to detect in any samples tested, and were therefore not included in this study.

2.6 Corticosterone Radioimmunoassay

CORT serum levels were determined in estradiol- and vehicle-treated rats using Coat-A-Count radioimmunoassay kits (Diagnostic Products Corporation, Los Angeles, CA). An automated gamma counter machine (WIZARD5DET1470015/4701845, PerkinElmer, Shelton, CT) was used to quantify CORT levels. Intra-assay coefficients of variation averaged $10.0 \pm 1\%$, and values were expressed as ng/mL. CORT serum levels were analyzed before and after formalin treatment in estradiol- and vehicle-treated rats to

assess if CORT production is influenced by estrogen administration. Further, to determine if a relationship exists between CORT and cytokine production, CORT serum levels were correlated with cytokine serum levels in all treatment groups.

2.7 Statistical Analysis

Data are expressed as mean $\pm$ SEM. Cytokine values were analyzed using Factorial analysis of variance (ANOVA) to test for differences in cytokine levels, hormonal treatment, and surgery in serum, paw, spinal cord, and DRG tissue. To test for the spread of sensitization of the inflammatory stimulus, ipsilateral and contralateral DRG cytokine levels were compared using Repeated Measures ANOVA. Paw size is represented as the multiplication of the width and depth of each hind paw expressed in mm². For naïve rats, right hind paw size measurements were analyzed using One-Way ANOVA. In formalin-treated rats, paw size measurements were analyzed using Repeated Measures ANOVA to compare the ipsilateral to the contralateral hind paw in each treatment group. Factorial ANOVA was conducted to determine CORT serum levels in all treatment groups. CORT and cytokine serum levels were correlated using a regression analysis program in GraphPad Prism 5.00 Software. Fisher's Least Significant Difference (LSD) *post hoc* tests were performed when appropriate. For all comparisons, significance was at the $p < 0.05$ level.

3. Results

3.1 Estradiol administration does not influence paw size in ADX, OVX, and ADX+OVX treatment groups.

Figure 28 shows hind paw size (mm²) in naïve and formalin-administered rats across OVX, ADX, and ADX+ OVX treatment groups. In naïve rats, estradiol administration did not alter paw size when compared to vehicle controls in any of the groups tested [$F(1, 41) = 0.32, p = 0.57$; Figure 28A]. Further, surgical treatment (OVX, ADX, or ADX+OVX operation) did not alter paw size across groups [$F(2, 41) = 0.84, p = 0.44$; Figure 28A]. In formalin-treated rats, a main effect for pain condition on paw size was observed [$F(1, 43) = 255.93, p = 0.00$; Figure 28B]; formalin significantly increased ipsilateral paw size compared to the contralateral paw across all treatment groups ($p < 0.01$). However, no main effect for hormone or interaction between hormone and surgical condition on paw size was observed [Hormone: $F(1, 43) = 2.33, p = 0.13$; Hormone x surgery: $F(2, 43) = 0.53, p = 0.59$].

3.2 CORT serum levels in OVX, ADX, and ADX+OVX treatment groups.

To determine if estradiol administration affected CORT levels and to test for the efficacy of adrenalectomy, CORT levels were determined in naïve and formalin-treated OVX, ADX, and ADX+OVX rats following estradiol or vehicle treatment. In naïve and formalin-administered rats, as shown in Figure 29, a main effect of surgery was observed [$F(2, 59) = 40.57, p < 0.01$]. As expected, OVX rats had significantly higher CORT levels than ADX and ADX+OVX groups ($p < 0.01$; Figure 29). Post hoc analysis revealed that formalin-treated OVX rats had significantly elevated CORT levels as

compared to OVX naïve rats ($p < 0.01$, Fig. 29). Furthermore, in the formalin condition, rats administered estradiol had lower CORT levels than vehicle-treated rats ($p < 0.05$, Fig. 29).

3.3 The effect of estradiol administration on cytokine levels in OVX, ADX, and ADX+OVX treatment groups.

Proinflammatory cytokines

TNF- α

Figure 30 shows TNF- α levels in paw, serum, DRG, and spinal cord of naïve OVX, ADX, and ADX+OVX rats following hormone and surgical conditions. In naïve paw tissue, as shown in Figure 30A, no significant differences were observed. In basal serum, a main effect for surgical condition was observed [$F(2, 17) = 3.64, p = 0.05$]; TNF- α levels were higher in ADX+OVX groups as compared to OVX groups ($p < 0.05$; Figure 30B). An interaction between hormone x surgery was observed [$F(2, 17) = 7.18, p < 0.01$]; TNF- α serum levels were elevated in OVX rats administered estradiol, and lower in estradiol-treated ADX rats, as compared to vehicle controls ($p < 0.05$ for both analyses; Fig. 30B). Furthermore, post hoc analysis revealed that ADX+OVX rats had significantly higher TNF- α levels than vehicle-treated OVX rats ($p < 0.05$). In DRG tissue of naïve rats, as shown in Figure 30C, post hoc analysis revealed that ADX rats administered estradiol had higher TNF- α levels as compared to vehicle controls ($p < 0.05$). Furthermore, ADX estradiol-treated rats had higher DRG levels than any other treatment group ($p < 0.05$; Fig. 30C). As shown in Figure 30D, basal spinal cord TNF- α levels were not altered by surgical or hormone condition across groups.

The effect of estradiol and surgery on TNF- α levels following formalin administration are shown in Figure 31. In paw, a main effect for surgery and an interaction for hormone and surgery on TNF- α levels was observed [Surgery main effect: $F(2, 17) = 7.27, p < 0.01$; Hormone x surgery: $F(2, 17) = 7.27, p < 0.01$; Fig.31A]; ADX rats had elevated TNF- α levels than OVX and ADX+OVX groups ($p < 0.05$). ADX rats administered estradiol had higher paw TNF- α levels than vehicles, OVX, and ADX+OVX groups ($p < 0.01$ for all analyses, Fig. 31A). In serum of formalin-treated rats, as shown in Figure 31B, a main effect for surgery was observed [$F(2, 18) = 5.67, p < 0.05$]; ADX+OVX rats had higher serum TNF- α levels than OVX and ADX rats (OVX: $p < 0.01$; ADX: $p < 0.05$). Furthermore, post hoc analysis revealed that ADX+OVX rats administered estradiol had higher serum TNF- α levels than OVX vehicle-treated rats and ADX rats ($p < 0.05$; Fig. 31B). In ipsilateral DRG tissue of formalin-treated rats, as shown in Figure 31C, post hoc analysis revealed that ADX rats administered estradiol had lower DRG TNF- α levels than OVX rats administered estrogen ($p < 0.05$). In spinal cord, as shown in Figure 31D, TNF- α levels were lower in ADX rats administered estradiol than in vehicle controls ($p < 0.05$). In ipsilateral versus contralateral DRG tissue, as shown in Figure 31E, ADX rats administered estradiol had lower TNF- α levels in ipsilateral DRG as compared to OVX and ADX+OVX estradiol-treated rats ($p < 0.05$).

IL-1 β

Figure 32 shows IL-1 β levels in naïve estradiol- or vehicle-treated OVX, ADX, and ADX+OVX rats. In paw and DRG, as shown in Figures 32A-B, respectively, no

significant differences were observed. As shown in Figure 32C, ADX+OVX rats administered estradiol had higher spinal cord IL-1 β levels than OVX and ADX estradiol-treated rats ($p < 0.05$). Basal serum IL-1 β levels were not detected (data not shown).

Figure 33 shows IL-1 β levels in all treatment groups following formalin administration. In the paw, serum, and DRG, IL-1 β levels were not altered across any groups tested (Figures 33 A, B, and C, respectively). In the spinal cord, as shown in Figure 33D, a main effect of hormone was observed [Hormone main effect: $F(1, 17) = 4.99, p < 0.05$]; however post hoc analysis revealing lowered IL-1 β levels in estradiol treatment groups did not reach significance ($p = 0.054$). ADX rats administered estradiol had lower spinal cord IL-1 β levels than vehicles and estradiol-treated OVX rats ($p < 0.05$, Fig. 33D). No differences were observed between ipsilateral and contralateral DRG IL-1 β levels (Figure 33D).

IL-6

Basal IL-6 levels following hormone and surgical condition are shown in Figure 34. In the paw, a significant main effect of surgery and an interaction between hormone treatment x surgery on basal IL-6 levels was observed [Surgery: $F(2, 16) = 6.04, p < 0.05$; Hormone x surgery: $F(2, 16) = 6.04, p < 0.05$]. As shown in Figure 34A, ADX rats had higher paw IL-6 levels than OVX and ADX+OVX groups ($p < 0.05$). Estradiol-treated ADX rats had higher paw IL-6 levels than vehicle controls and OVX and ADX+OVX groups ($p < 0.01$; Fig. 34A). In serum, basal IL-6 levels were not altered across groups (Fig. 34B). In spinal cord, as shown in Figure 34D, a significant main effect of hormone, surgery, and an interaction between hormone x surgery on IL-6 levels

was observed [Hormone: $F(1, 18) = 4.42, p < 0.05$; Surgery: $F(2, 18) = 4.03, p < 0.05$; Hormone x surgery: $F(1, 18) = 4.03, p < 0.05$]; estradiol-treated rats had significantly higher IL-6 levels than vehicles ($p < 0.05$). OVX rats had higher spinal cord IL-6 levels than ADX and ADX+OVX rats ($p < 0.05$; Fig. 34). OVX rats administered estradiol had higher spinal cord IL-6 levels than vehicle, ADX, and ADX+OVX groups ($p < 0.01$). DRG IL-6 levels were not detected (data not shown).

Figure 35 shows IL-6 levels following formalin administration in all tested samples. As shown in Figure 35A, in the paw, no significant effect of hormone or surgical condition on IL-6 levels was observed. In serum, a main effect of surgery on IL-6 levels was observed [$F(2, 18) = 4.75, p < 0.05$]. ADX+OVX rats had significantly higher IL-6 serum levels than OVX and ADX rats ($p < 0.05$ for both comparisons, Fig. 35B). Post hoc analysis revealed that ADX+OVX rats administered estradiol had higher serum IL-6 levels than OVX and ADX vehicle treatment groups ($p < 0.05$, Fig. 35B). In DRG, as shown in Figure 35C, a main effect of hormone, surgery, and an interaction between hormone x surgery on DRG IL-6 levels was observed [Hormone: $F(1, 17) = 4.64, p < 0.05$; Surgery: $F(2, 17) = 4.06, p < 0.05$; Hormone x surgery: $F(2, 17) = 4.06, p < 0.05$]; however post hoc analysis revealing lowered IL-6 levels in estradiol-treated rats as compared to vehicles failed to reach significance ($p = 0.09$). OVX rats had lower DRG IL-6 levels than ADX and ADX+OVX groups ($p < 0.05$; Fig. 35C). Estradiol-treated OVX rats had lower DRG IL-6 levels than vehicle, ADX, and ADX+OVX groups ($p < 0.05$; Fig. 35C). In the spinal cord, post hoc analysis revealed that ADX rats administered estradiol had lower IL-6 levels than vehicle control and estradiol-treated

OVX rats ($p < 0.05$, Fig. 35D). Contralateral IL-6 levels were not detected, and were therefore not analyzed (data not shown).

IL-2

Basal IL-2 levels following hormone and surgical condition in all groups are shown in Figure 36. In the paw, post hoc analysis revealed that ADX rats administered estradiol had higher IL-6 levels than ADX+OVX estradiol-treated rats ($p < 0.05$; Fig. 36A). As shown in Figures 36B-D, no significant differences in IL-2 levels were observed in serum, DRG, or spinal cord, respectively.

Figure 37 shows IL-2 levels following formalin administration across treatment groups. In the paw, a main effect of surgery and an interaction between hormone x surgery was observed [Surgery main effect: $F(2, 18) = 13.15, p < 0.01$; Hormone x surgery main effect: $F(2, 18) = 13.15, p < 0.01$; Fig. 37A]; ADX rats had higher paw IL-2 levels than OVX and ADX+OVX groups ($p < 0.05$). Estradiol-treated ADX rats had higher paw IL-2 levels than vehicles, OVX, and ADX+OVX groups ($p < 0.01$; Fig. 37A). Furthermore, ADX+OVX rats administered estradiol had lower paw IL-2 levels than vehicle-treated rats ($p < 0.01$). As shown in Figure 37B, in serum a main effect of surgery was observed [$F(2, 18) = 3.99, p < 0.05$]; ADX+OVX rats had significantly higher IL-2 serum levels than OVX and ADX operated rats ($p < 0.05$). Furthermore, post hoc analysis revealed that vehicle-treated ADX+OVX rats had higher serum IL-2 levels than vehicle-treated OVX rats ($p < 0.05$; Fig. 37B).

In ipsilateral DRG, a main effect of hormone was observed [$F(1, 17) = 7.54, p < 0.05$; Fig. 33C]; estradiol administration lowered IL-2 levels in all groups as compared to

controls ($p < 0.05$). Post hoc analysis revealed that estradiol-treated ADX rats had lower DRG IL-2 levels than vehicle, OVX, and ADX+OVX groups ($p < 0.05$; Fig. 37C). In spinal cord, post hoc analysis revealed that ADX rats administered estradiol had lower IL-2 levels than vehicles and estradiol-treated OVX rats ($p < 0.05$; Fig 37D). As shown in Figure 37E, a significant difference between ipsilateral versus contralateral DRG IL-2 levels was observed [$F(1, 16) = 4.67, p < 0.05$]; however, post hoc analysis revealing lower IL-2 levels in ipsilateral DRG as compared to contralateral DRG did not reach significance ($p = 0.054$). An interaction between DRG IL-2 levels and hormone on IL-2 levels was observed [$F(1, 16) = 4.67, p < 0.05$]; in estradiol treatment groups, IL-2 levels were lower in ipsilateral DRG than in contralateral DRG ($p < 0.05$; Fig 37E). Post hoc analysis revealed that in the OVX group, contralateral DRG levels following estradiol administration were significantly higher than vehicle and ipsilateral DRG tissue ($p < 0.05$; Fig. 37E). Furthermore, estradiol-treated ADX rats had lower IL-2 levels in ipsilateral DRG as compared to contralateral DRG ($p < 0.05$; Fig. 37E).

GM-CSF

Serum GM-CSF levels in naïve and formalin treatment groups are shown in Figures 38 and 39, respectively. Serum GM-CSF levels were not altered by hormone or surgical condition in naïve or formalin-treated rats across any groups tested (Fig. 38 and 39). GM-CSF levels were not detected in paw, DRG, or spinal cord of any samples tested (data not shown).

IFN- γ

As shown in Figures 40A-B, basal IFN- γ levels were not altered across groups in paw or serum, respectively. In DRG, post hoc analysis revealed that estradiol-treated ADX+OVX rats have significantly lower IFN- γ levels than vehicles in all groups tested ($p < 0.05$). In spinal cord, IFN- γ levels were significantly lower in estradiol-treated ADX rats as compared to OVX rats administered estradiol ($p < 0.05$). Following formalin administration, as shown in Figure 41A, no significant differences in paw IFN- γ levels was observed. In serum, as shown in Figure 41B, a main effect for surgery on IFN- γ levels was observed [$F(2, 16) = 4.04, p < 0.05$]. ADX+OVX rats administered formalin had significantly higher IFN- γ serum levels than all treatment groups ($p < 0.05$; Fig. 41B). Post hoc analysis revealed that ADX+OVX rats administered estradiol had elevated serum IFN- γ levels as compared to OVX and ADX groups ($p < 0.05$; Fig. 41B). As shown in Figures 41C-E, no effects of hormonal treatment or surgery on IFN- γ levels were observed in the DRG and spinal cord.

Anti-inflammatory cytokine

IL-10

IL-10 was analyzed in order to observe the effects of estradiol treatment on anti-inflammatory cytokine levels before and after pain administration. Figure 42 shows basal IL-10 levels in all experimental groups. In the paw, as shown in Figure 42A, post hoc analysis revealed that ADX rats administered estradiol had higher paw IL-10 levels than OVX and ADX+OVX groups ($p < 0.05$). As shown in Figure 42B, serum IL-10 levels were not altered by hormone or surgical condition across groups. In DRG, post hoc

analysis revealed that OVX rats administered estradiol had lower IL-10 levels than ADX and ADX+OVX estradiol-treated rats ($p < 0.05$; Fig. 42C). In spinal cord, as shown in Figure 42D, post hoc analysis revealed that ADX rats administered estradiol had lower IL-10 levels than estradiol-treated OVX rats ($p < 0.05$).

Figure 43 shows IL-10 levels following formalin administration in all samples tested. In paw, a main effect of surgery and interaction between hormone x surgery on IL-10 levels was observed [Surgery: $F(2, 17) = 8.09, p < 0.01$; Hormone x surgery: $F(2, 17) = 8.09, p < 0.01$; Fig. 43A]; ADX rats had higher paw IL-10 levels than OVX and ADX+OVX rats ($p < 0.01$). ADX rats administered estradiol had significantly higher paw IL-10 levels as compared to vehicle and to OVX and ADX+OVX groups ($p < 0.01$). In serum of formalin-treated rats, a main effect of surgery on IL-10 levels was observed, as shown in Figure 43B [$F(2, 18) = 10.78, p < 0.01$]; ADX+OVX rats had higher IL-10 serum levels as compared to OVX and ADX groups ($p < 0.01$ for both comparisons). Post hoc analysis revealed that ADX+OVX rats administered estradiol had higher serum IL-10 levels than OVX and ADX groups ($p < 0.01$; Fig. 43B). Furthermore, vehicle-treated ADX+OVX rats had higher serum IL-10 levels than vehicle-treated OVX and ADX rats ($p < 0.05$; Fig. 43B). In ipsilateral DRG, no significant differences in IL-10 levels across groups were observed (Fig. 43C). As shown in Figure 43D, in spinal cord, a main effect of surgery and an interaction between hormone x surgery on IL-10 levels was observed [Surgery: $F(2, 16) = 8.85, p < 0.01$; Hormone x surgery: $F(2, 16) = 8.85, p < 0.01$]. IL-10 spinal cord levels were significantly higher in OVX rats as compared to ADX and ADX+OVX following formalin administration ($p < 0.01$; Fig. 43D). OVX rats administered estrogen had significantly higher spinal cord IL-10 levels as compared to

vehicle, ADX, and ADX+OVX groups ($p < 0.05$). Furthermore, ADX rats administered estradiol had significantly lower IL-10 levels than vehicle following formalin administration ($p < 0.05$; Fig. 43D). As shown in Figure 43E, in ADX+OVX rats administered estradiol, contralateral DRG IL-10 levels are lower than ipsilateral DRG levels ($p < 0.05$).

3.4 Correlation analysis of corticosterone and cytokine serum levels

Correlation analyses of OVX, ADX, and ADX+OVX cytokine and CORT serum levels were performed for all treatment groups tested. In naïve estradiol-treated ADX rats, a significant negative correlation between TNF- α serum levels and CORT was observed [$r(4) = 0.95, p < 0.05$]. However, in naïve OVX or ADX+OVX estradiol-treated rats, CORT levels did not correlate with any cytokine serum levels tested (data not shown). Further, in vehicle-treated naïve rats, no significant correlation between cytokine and CORT serum levels was observed (data not shown). Following formalin administration, no significant correlation was found between CORT and cytokine serum levels in any groups tested (data not shown).

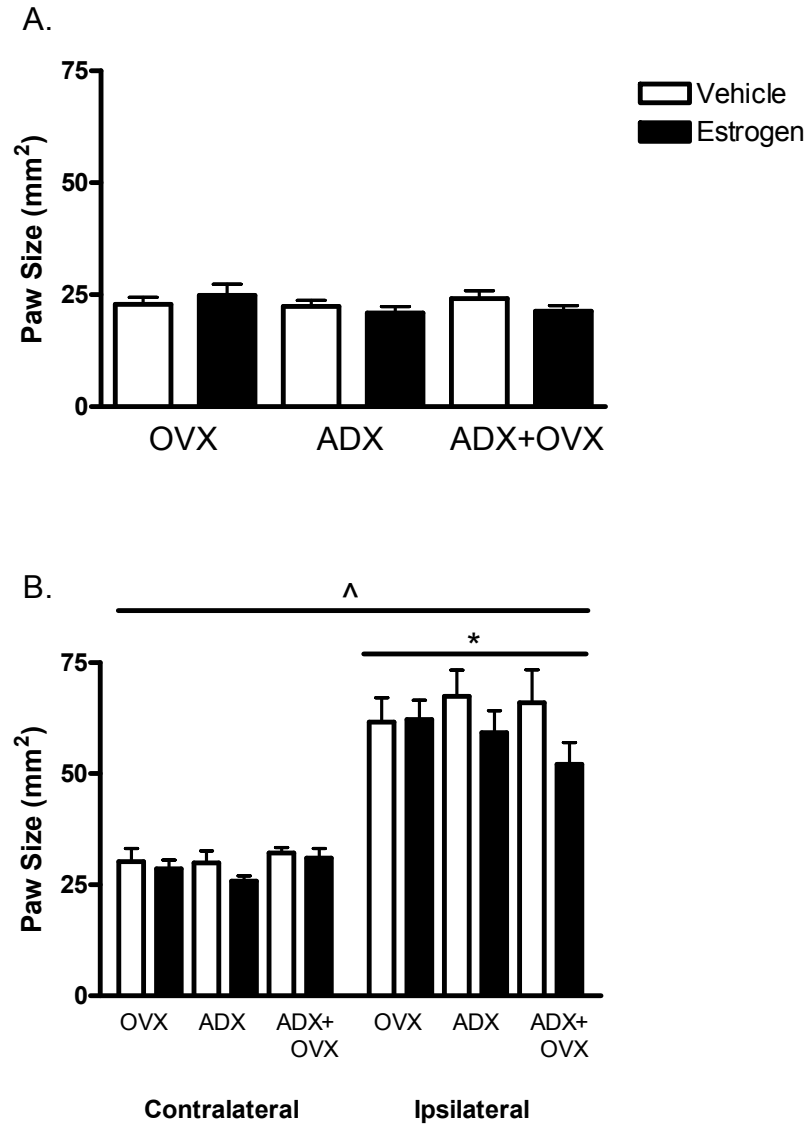


Figure 28. Paw size of naïve (A) and formalin-administered (B) OVX, ADX, and ADX+OVX operated rats in vehicle (white bars) and 17 β -estradiol (black bars) treatment groups. (^) Represents significant main effect of pain condition, $p < 0.01$. (*) Denotes statistically significant difference between ipsilateral and contralateral paw size following formalin administration, $p < 0.01$. Each treatment represents $n = 7-10$ per group.

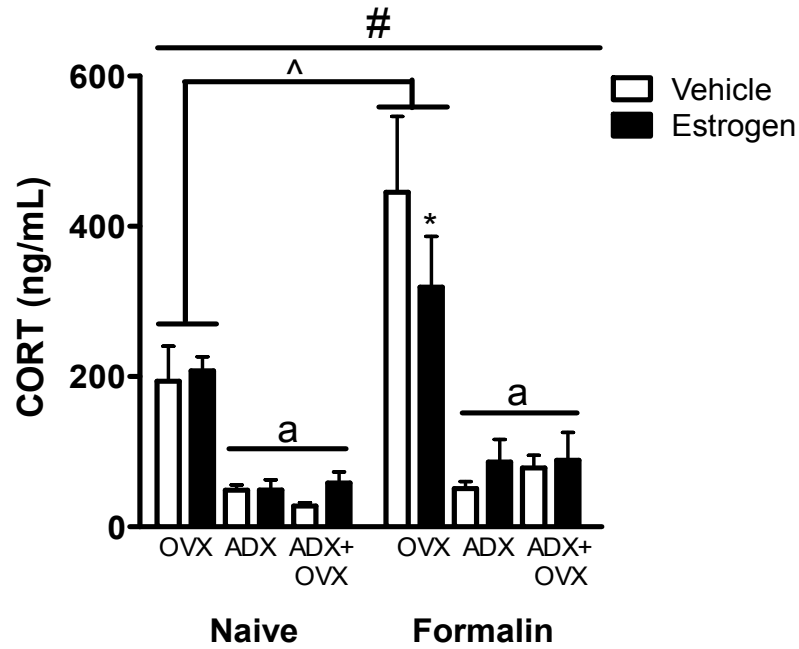


Figure 29. CORT serum levels in naïve and formalin-treated OVX, ADX, and ADX+OVX operated rats administered 17 β -estradiol or vehicle. CORT levels are expressed as mean $\pm$ SEM. White bars represent vehicle treated rats, while black bars represent rats treated with 17 β -estradiol. (#) Represents significant main effect of surgical condition, $p < 0.01$. (a) Denotes statistically significant difference to OVX groups, $p < 0.01$. (^) Represents significant difference between naïve and formalin-treated OVX groups, $p < 0.01$. (*) Represents significant difference to vehicle, $p < 0.05$. $n = 6$ per group.

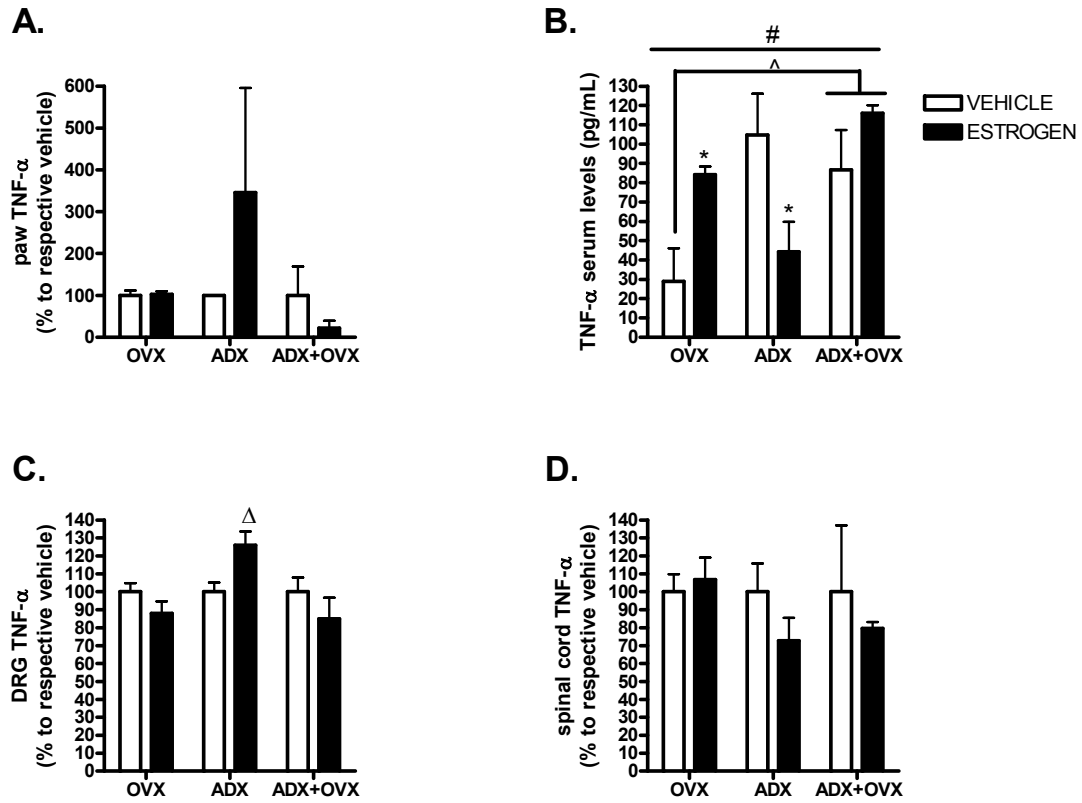


Figure 30. TNF- α levels in paw (A), serum (B), DRG (C), and spinal cord (D) of OVX, ADX, and ADX+OVX operated naïve rats administered 20% 17 β -estradiol (estrogen) or cholesterol (vehicle). For serum, data represents mean TNF- α levels ($\pm$ SEM). For tissue samples, data represents percent to respective vehicle. (#) Represents significant main effect of surgical condition. (*) Denotes significant difference to vehicle. (^) Denotes significant difference to indicated treatment group. (Δ) Denotes significant difference to all other treatment groups. For all analyses, $p < 0.05$. $n = 4$ per group.

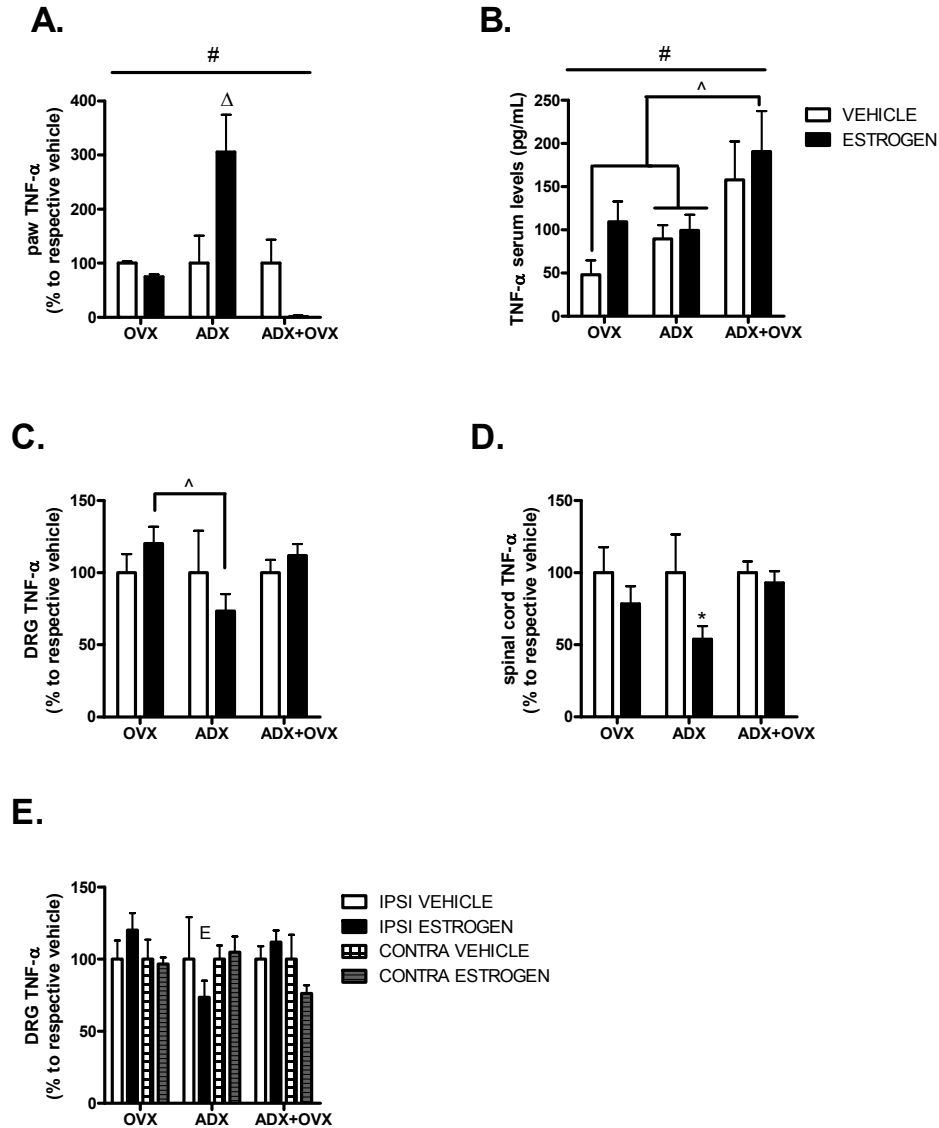


Figure 31. TNF- α levels in paw (A), serum (B), DRG (C), spinal cord (D), and ipsilateral and contralateral DRG (E) of estradiol- or vehicle-treated OVX, ADX, and ADX+OVX operated rats following 5% formalin administration. For serum, data represents mean TNF- α levels ($\pm$ SEM). For tissue samples, data represents percent to respective vehicle. (#) Represents significant main effect of surgical condition. (*) Denotes significant difference to vehicle. (^) Denotes significant difference to indicated treatment group. (Δ) Denotes significant difference to all other treatment groups. (E) Represents significant difference to ipsilateral OVX and ADX+OVX estrogen treatment groups. For all analyses, $p < 0.05$. $n = 4$ per group.

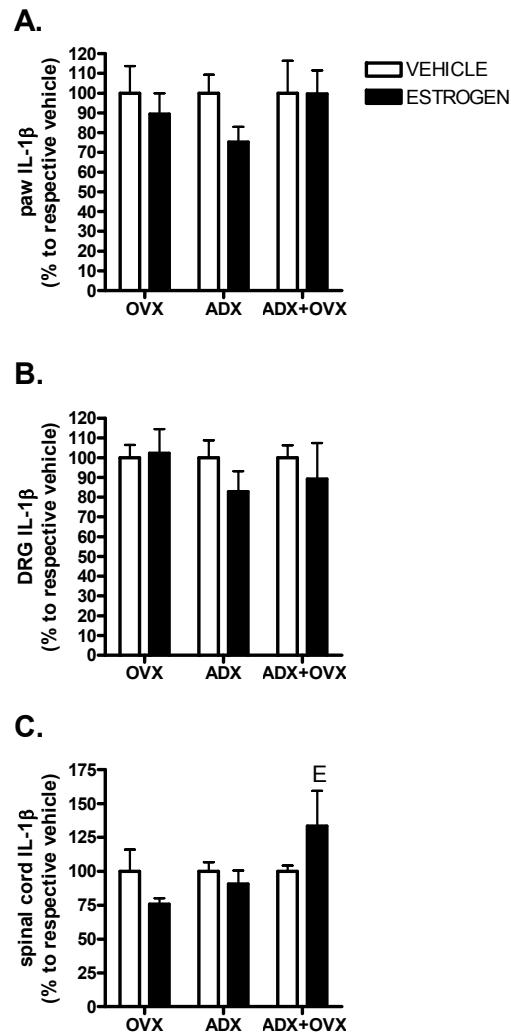


Figure 32. IL-1 β levels in paw (A), DRG (B), and spinal cord (C) of OVX, ADX, and ADX+OVX operated naïve rats administered 20% 17 β -estradiol (estrogen) or cholesterol (vehicle). For serum, data represents mean IL-1 β levels ($\pm$ SEM). For tissue samples, data represents percent to respective vehicle. (E) Represents significant difference to OVX and ADX estrogen treatment groups. Serum IL-1 β levels were not detected. For all analyses, $p < 0.05$. $n = 4$ per group.

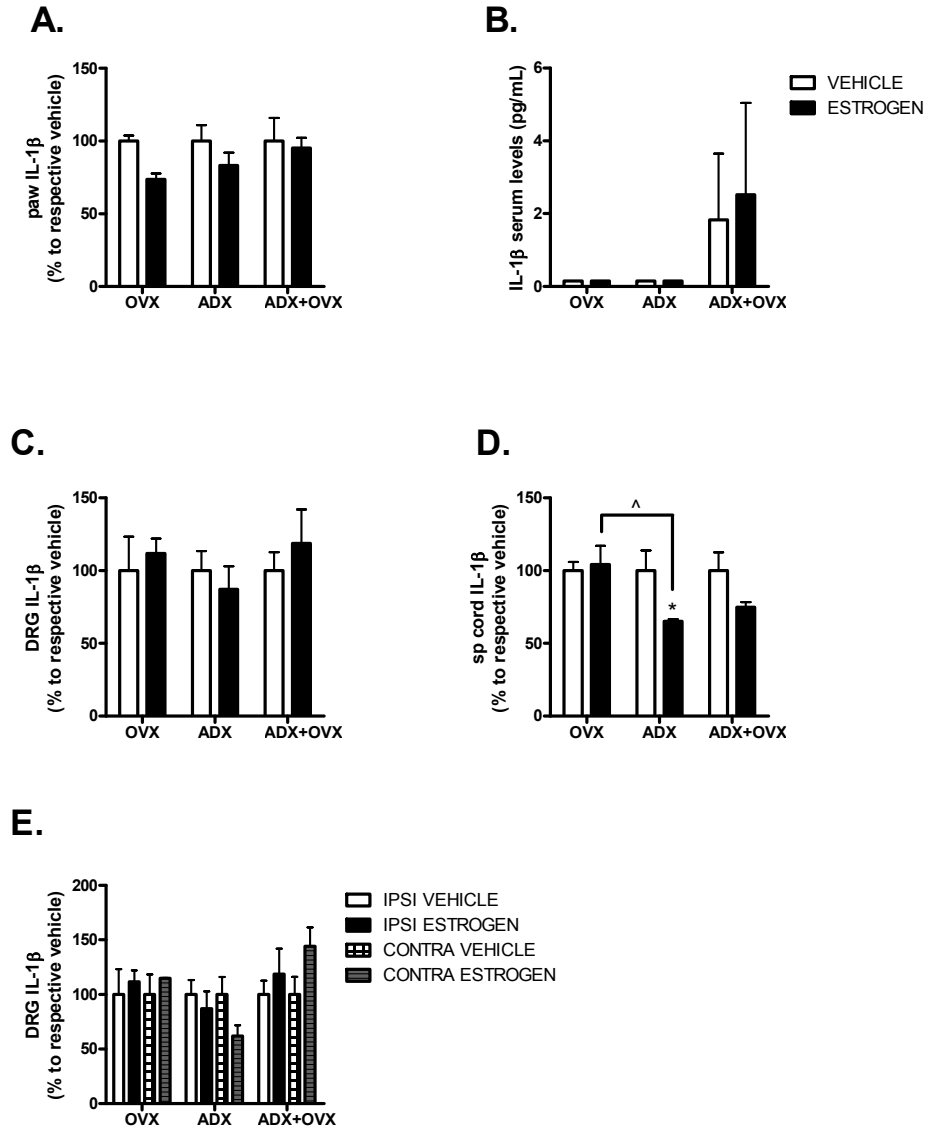


Figure 33. IL-1 β levels in paw (A), serum (B), ipsilateral DRG (C), spinal cord (D), and ipsilateral versus contralateral DRG (E) of estradiol- or vehicle-treated OVX, ADX, and ADX+OVX operated rats following 5% formalin administration. For serum, data represents mean IL-1 β levels ($\pm$ SEM). For tissue samples, data represents percent to respective vehicle. (*) Denotes significant difference to vehicle. (^) Denotes significant difference to indicated treatment group. For all analyses, $p < 0.05$. $n = 4$ per group.

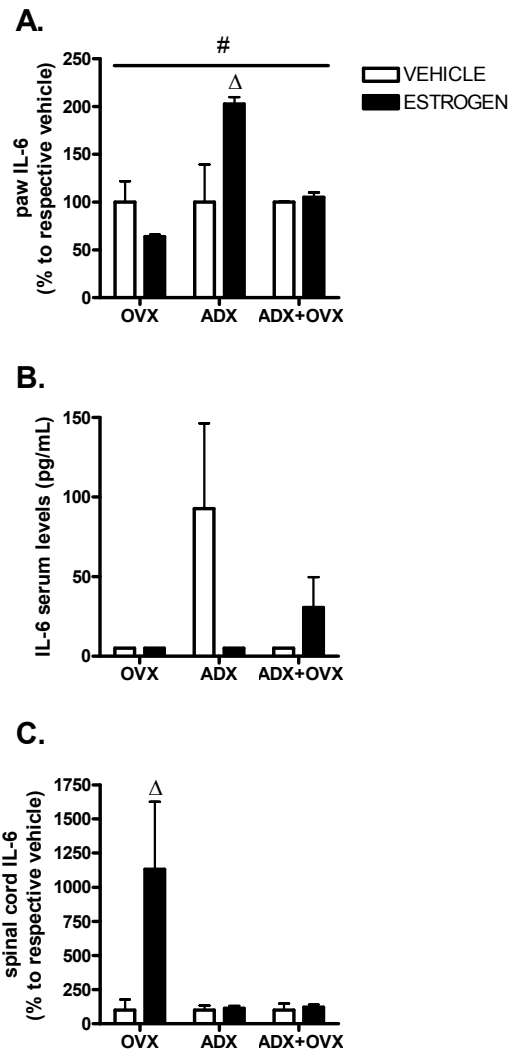


Figure 34. IL-6 levels in paw (A), serum (B), and spinal cord (C) of OVX, ADX, and ADX+OVX operated naïve rats administered 17 β -estradiol (estrogen) or cholesterol (vehicle). For serum, data represents mean IL-6 levels ($\pm$ SEM). For tissue samples, data represents percent to respective vehicle. (#) Represents significant main effect of surgical condition. (Δ) Denotes significant difference to all other treatment groups. DRG IL-6 levels were not detected. For all analyses, $p < 0.05$. $n = 4$ per group.

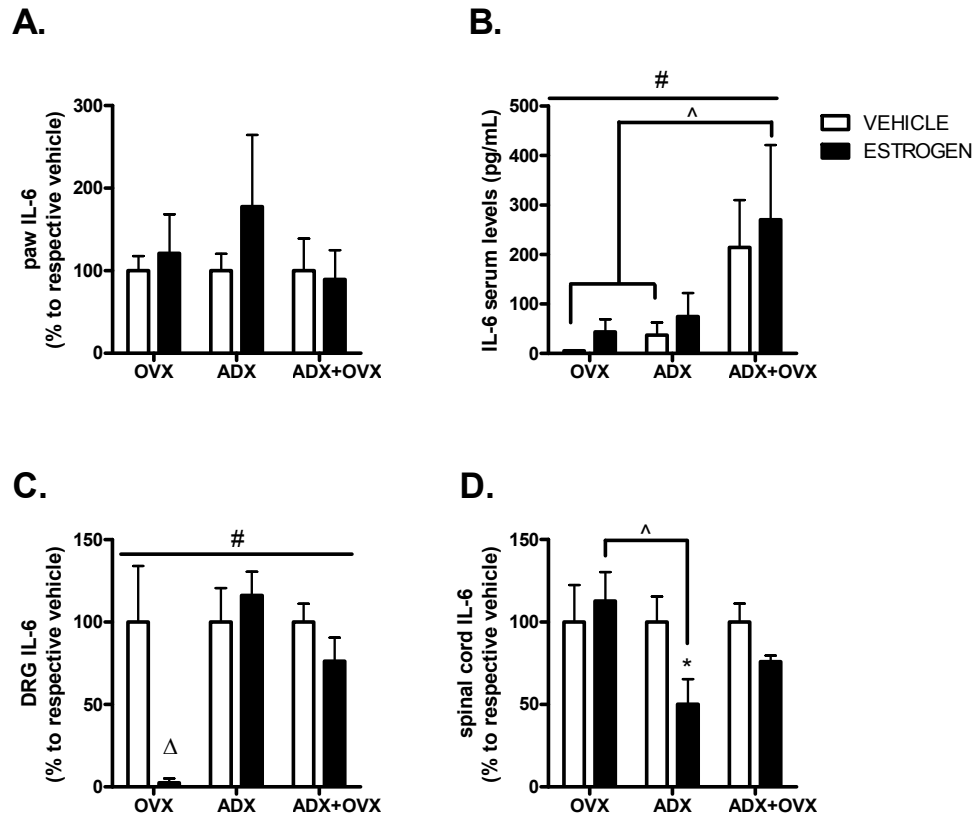


Figure 35. IL-6 levels in paw (A), serum (B), ipsilateral DRG (C), and spinal cord (D) of estradiol- or vehicle-treated OVX, ADX, and ADX+OVX operated rats following 5% formalin administration. For serum, data represents mean IL-6 levels ($\pm$ SEM). For tissue samples, data represents percent to respective vehicle. (#) Represents significant main effect of surgical condition. (*) Denotes significant difference to vehicle. (^) Denotes significant difference to indicated treatment group. (Δ) Denotes significant difference to all other treatment groups. For all analyses, $p < 0.05$. $n = 4$ per group.

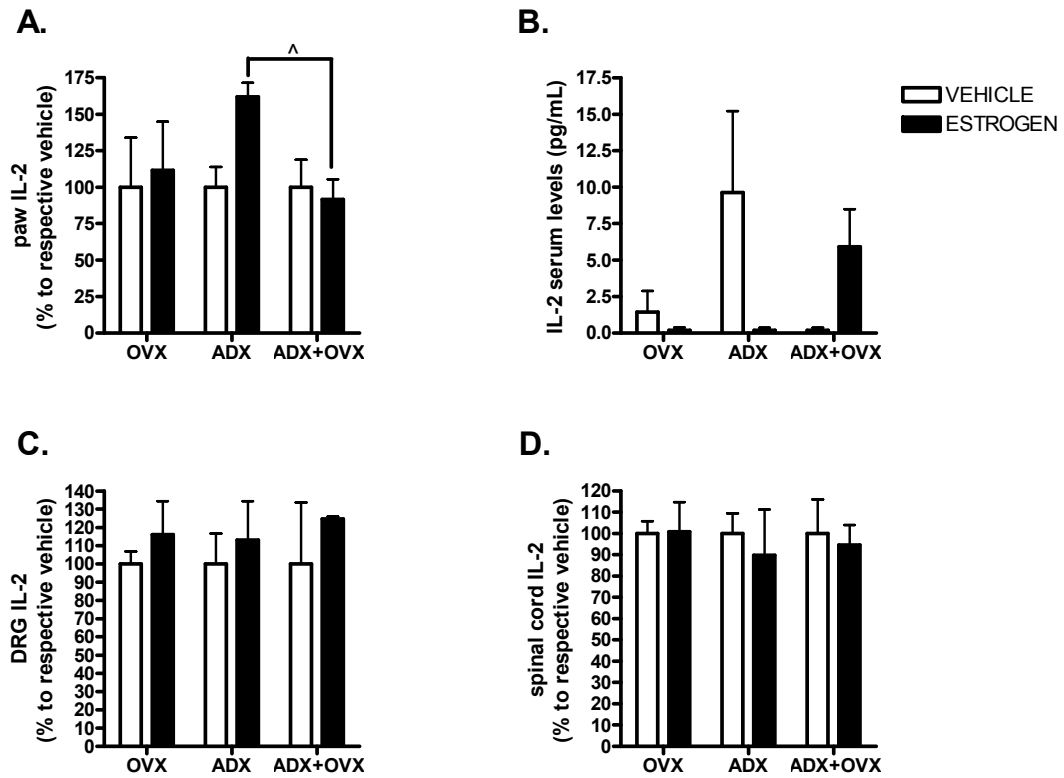


Figure 36. IL-2 levels in paw (A), serum (B), DRG (C), and spinal cord (D) of OVX, ADX, and ADX+OVX operated naïve rats administered 17 β -estradiol (estrogen) or cholesterol (vehicle). For serum, data represents mean IL-2 levels ($\pm$ SEM). For tissue samples, data represents percent to respective vehicle. (^) Denotes significant difference to indicated treatment group. For all analyses, $p < 0.05$. $n = 4$ per group.

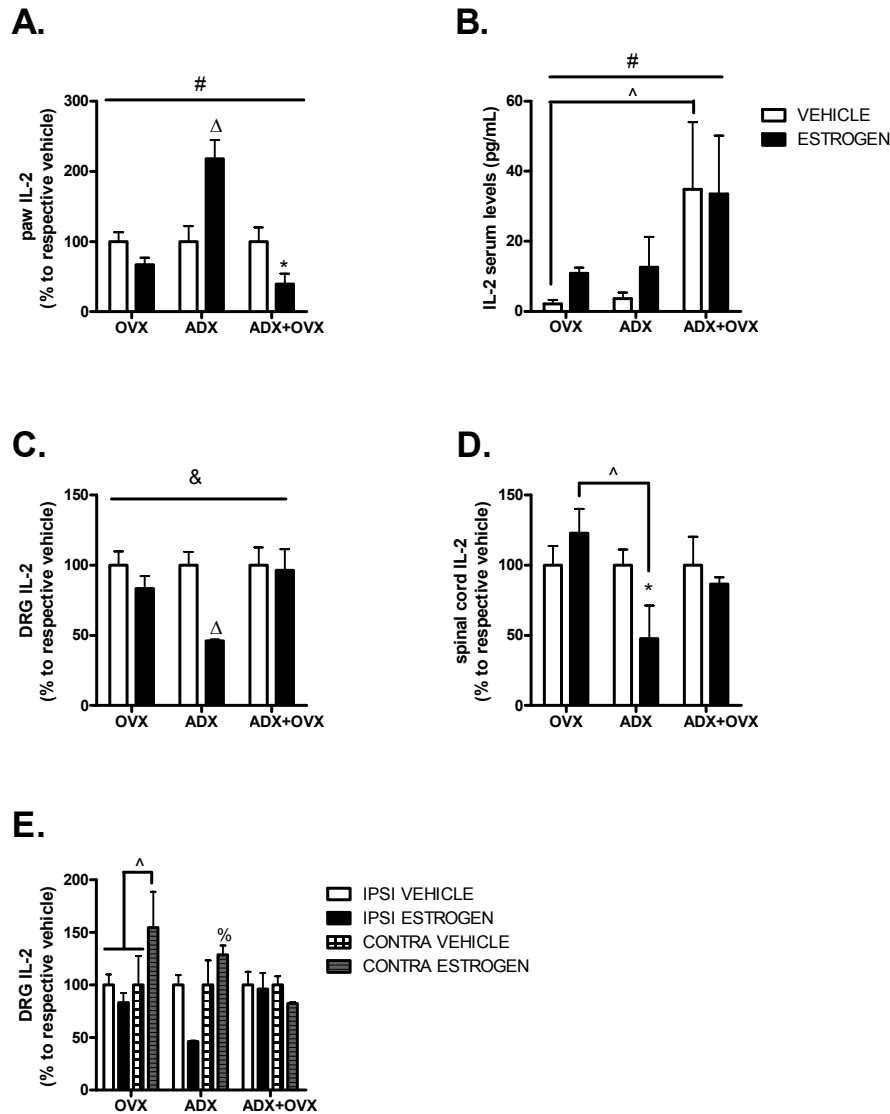


Figure 37. IL-2 levels in paw (A), serum (B), ipsilateral DRG (C), spinal cord (D), and ipsilateral versus contralateral DRG (E) of estradiol- or vehicle-treated OVX, ADX, and ADX+OVX operated rats following 5% formalin administration. For serum, data represents mean IL-2 levels ($\pm$ SEM). For tissue samples, data represents percent to respective vehicle. (#) Represents significant main effect of surgical condition. (&) Denotes significant main effect of hormone. (*) Denotes significant difference to vehicle. (^) Denotes significant difference to indicated treatment group. (Δ) Denotes significant difference to all other treatment groups. (%) Represents significant difference to ADX ipsilateral DRG following estradiol treatment. For all analyses, $p < 0.05$. $n = 4$ per group.

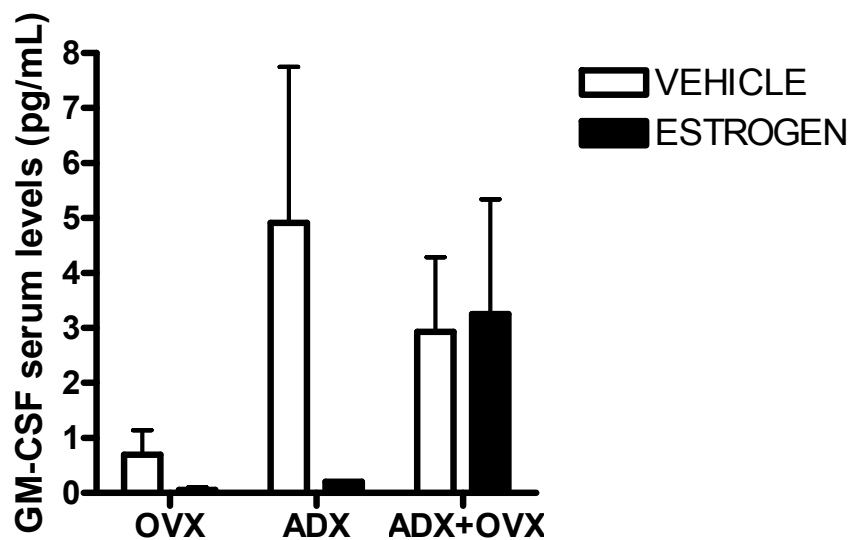


Figure 38. GM-CSF levels in serum of OVX, ADX, and ADX+OVX operated naïve rats administered 17 β -estradiol (estrogen) or cholesterol (vehicle). Data represents mean GM-CSF levels ($\pm$ SEM). No significant differences were observed. $n = 4$ per group.

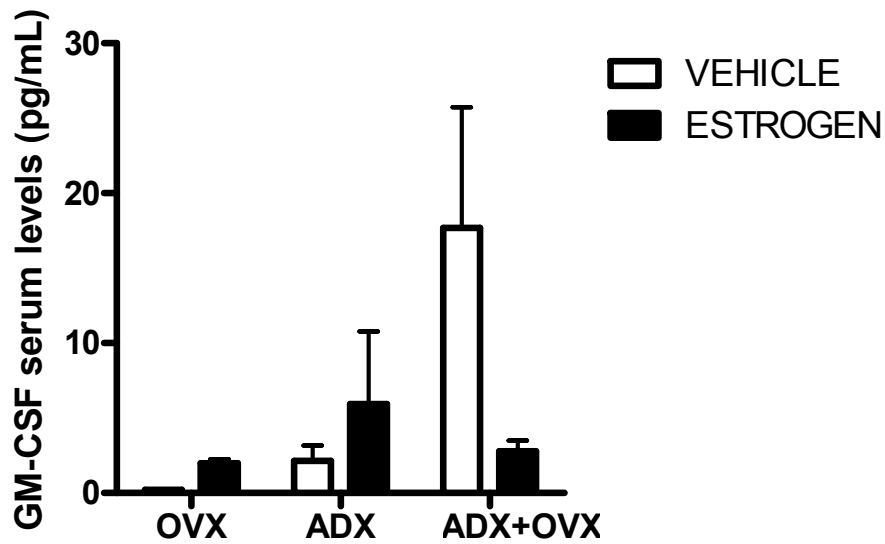


Figure 39. GM-CSF levels in serum of estradiol- or vehicle-treated OVX, ADX, and ADX+OVX operated rats following 5% formalin administration. Data represents mean GM-CSF levels ($\pm$ SEM). No significant differences were observed. $n = 4$ per group.

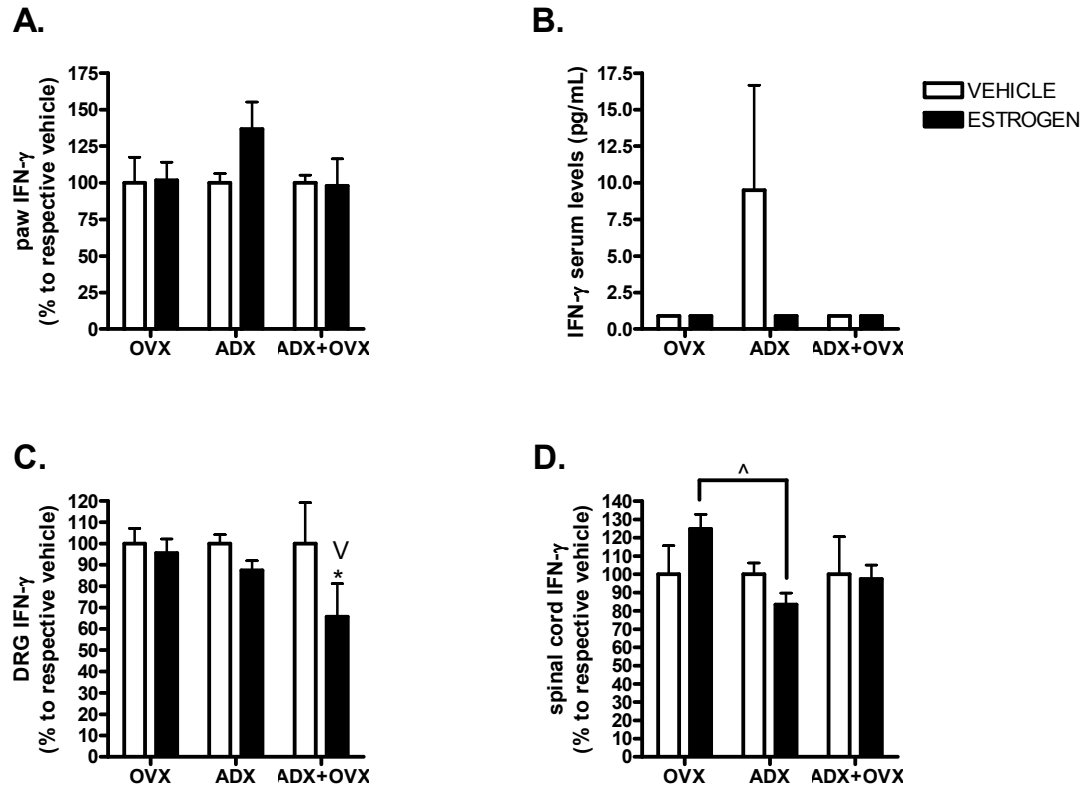


Figure 40. IFN- γ levels in paw (A), serum (B), DRG (C) and spinal cord (D) of OVX, ADX, and ADX+OVX operated naïve rats administered 17 β -estradiol (estrogen) or cholesterol (vehicle). For serum, data represents mean IFN- γ levels ($\pm$ SEM). For tissue samples, data represents percent to respective vehicle. (*) Denotes significant difference to vehicle. (^) Denotes significant difference to indicated treatment group. (V) Represents significant difference to vehicle OVX and ADX groups. For all analyses, $p < 0.05$. $n = 4$ per group.

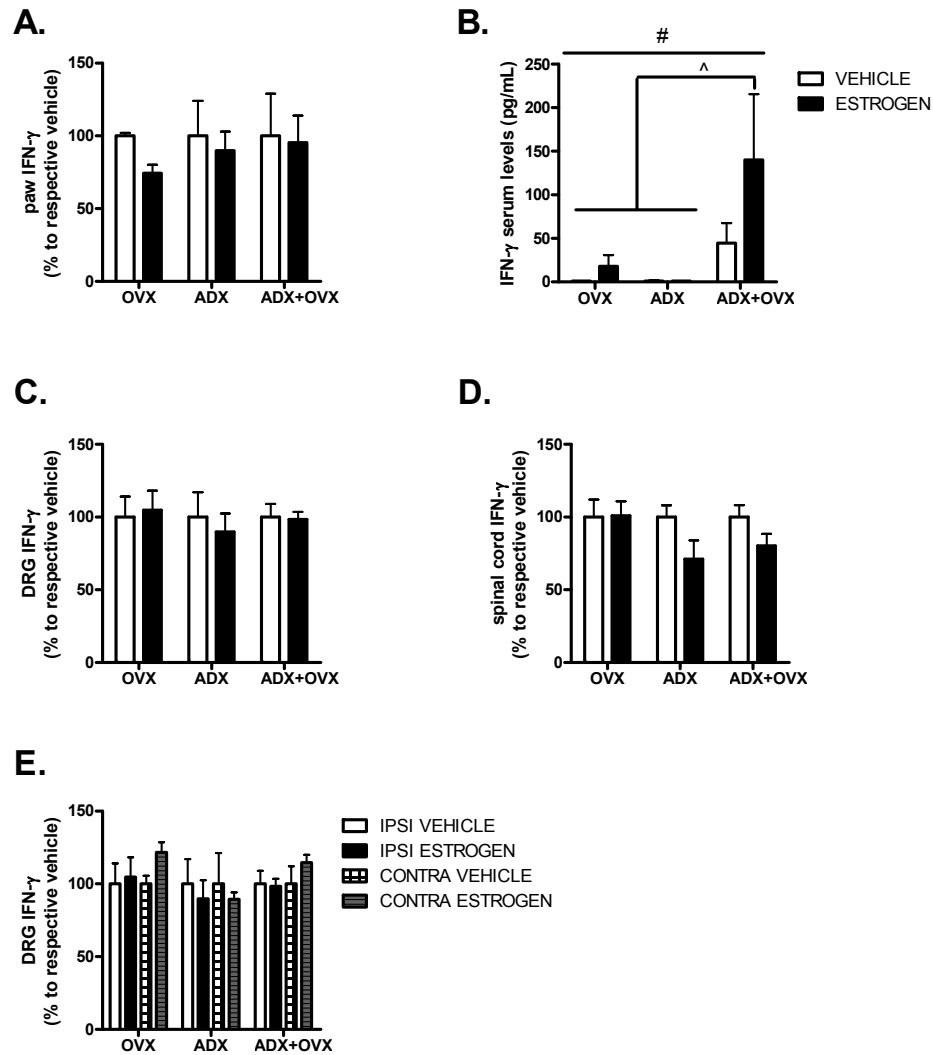


Figure 41. IFN- γ levels in paw (A), serum (B), ipsilateral DRG (C), spinal cord (D), and ipsilateral versus contralateral DRG (E) of estradiol- or vehicle-treated OVX, ADX, and ADX+OVX operated rats following 5% formalin administration. For serum, data represents mean IFN- γ levels ($\pm$ SEM). For tissue samples, data represents percent to respective vehicle. (#) Represents significant main effect of surgical condition. (^) Denotes significant difference to indicated treatment group. For all analyses, $p < 0.05$. $n = 4$ per group.

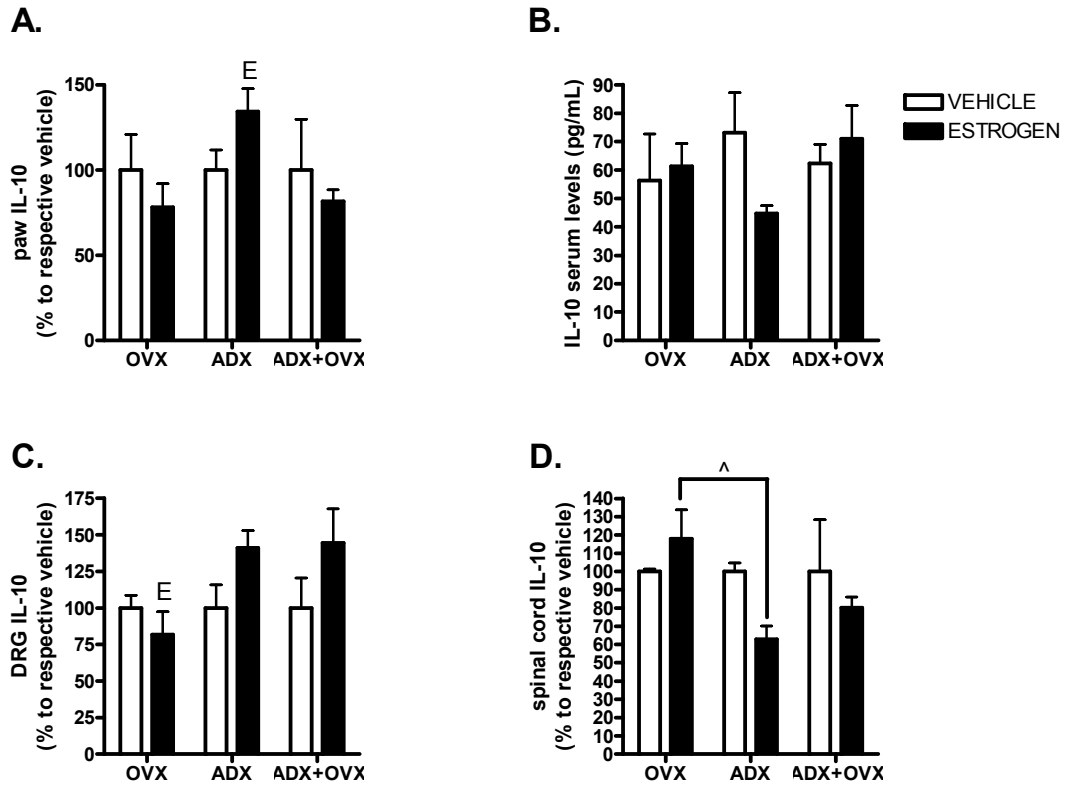


Figure 42. IL-10 levels in paw (A), serum (B), DRG (C), and spinal cord (D) of OVX, ADX, and ADX+OVX operated naïve rats administered 17 β -estradiol (estrogen) or cholesterol (vehicle). For serum, data represents mean IL-10 levels ($\pm$ SEM). For tissue samples, data represents percent to respective vehicle. (^) Denotes significant difference to indicated treatment group. In 42A, (E) represents significant difference to OVX and ADX+OVX estrogen treatment groups. In 42C, (E) represents significant difference to ADX and ADX+OVX estrogen treatment groups. For all analyses, $p < 0.05$. $n = 4$ per group.

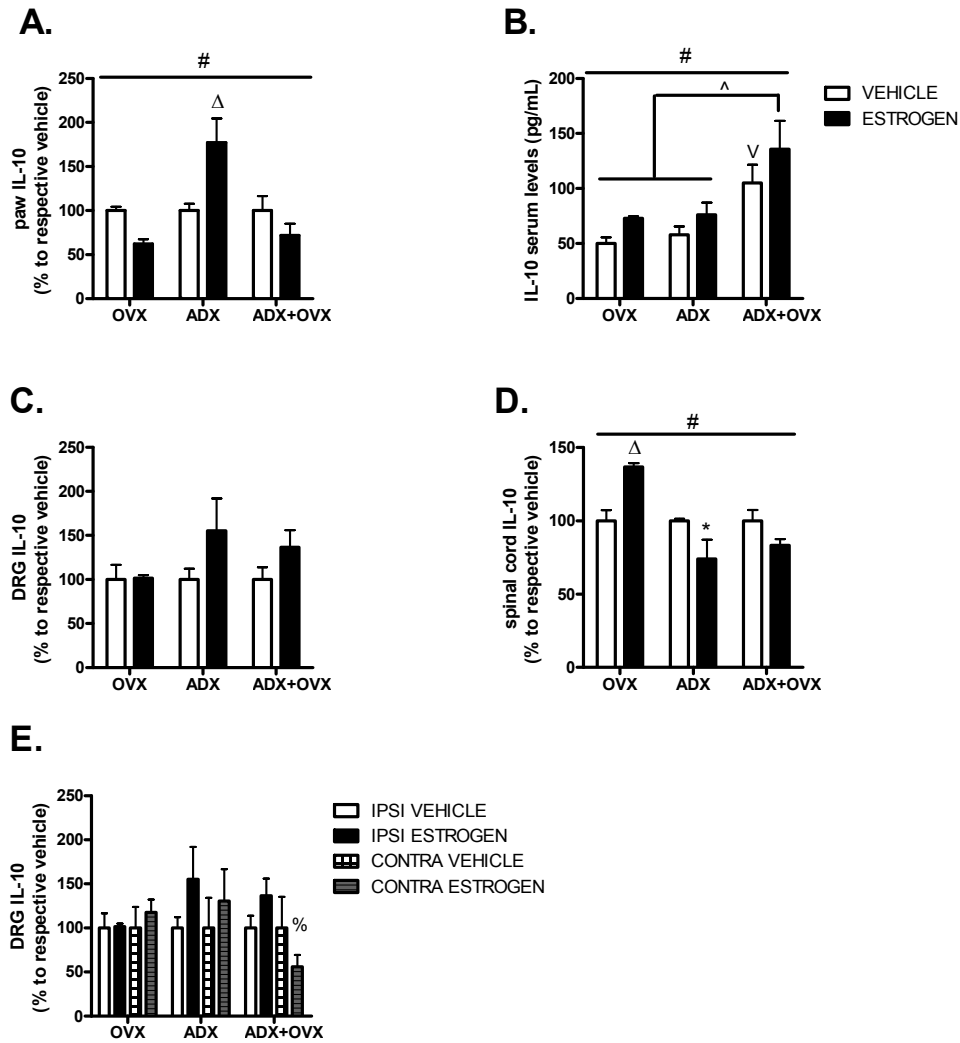


Figure 43. IL-10 levels in paw (A), serum (B), ipsilateral DRG (C), spinal cord (D), and ipsilateral versus contralateral DRG (E) of estradiol- or vehicle-treated OVX, ADX, and ADX+OVX operated rats following 5% formalin administration. For serum, data represents mean IL-10 levels ($\pm$ SEM). For tissue samples, data represents percent to respective vehicle. (#) Represents significant main effect of surgical condition. (*) Denotes significant difference to vehicle. (^) Denotes significant difference to indicated treatment group. (Δ) Denotes significant difference to all other treatment groups. (V) Represents significant difference to vehicle OVX and ADX+OVX groups. (%) Represents significant difference to ADX+OVX ipsilateral DRG following estradiol treatment. For all analyses, $p < 0.05$. $n = 4$ per group.

4. Discussion

Previous work in our lab shows that in OVX, ADX, and ADX+OVX groups, estradiol administration reduces formalin-induced behavioral responses as compared to vehicle-treated controls (Hunter et al., in press; Appendix A). To understand the influence of ovarian hormones on inflammatory pain mediators, the effects of 17 β -estradiol administration on paw size, cytokine levels, and corticosterone were observed. Estradiol was effective in altering TNF- α , IL-6, and IFN- γ levels in naïve rats, and TNF- α , IL-1 β , IL-6, IL-2, and IL-10 levels in formalin-treated rats as compared to controls. Estrogen administration did not alter paw size before or after formalin administration. Adrenalectomy was effective in lowering CORT serum levels as compared to ovariectomized rats. However, contrary to other reports (Ferrini et al., 1993; Niyomchai et al., 2005), exogenous estradiol administration did not increase serum CORT levels. . To this end, our study suggests that estradiol administration may reduce nociceptive behaviors in part by regulating cytokine production both basally and following inflammatory pain.

Estradiol effects on paw size and CORT levels. In naïve models, estradiol administration did not alter paw thickness, indicating that estradiol does not lead to vascular changes in paw size before inflammation. In formalin-treated rats, the fact that formalin administration increased paw size across groups demonstrated that it was effective at causing inflammation and edema in the injection paw as previously demonstrated (Damas & Liegeois, 1999; Taylor et al., 1998). However, estradiol did not lower mediators involved in plasma extravasation and vasodilation of paw tissue

following formalin injection. Therefore, estradiol does not reduce inflammation at the site of injury.

Adrenalectomy was effective at lowering CORT levels in both naïve and formalin-treated groups as compared to OVX rats. Furthermore, in OVX rats, formalin administration increased serum CORT levels as compared to naïve groups. However, estradiol administration did not increase serum CORT levels across groups. Ferrini et al. (1993) showed that estradiol treatment increases GC levels in CNS tissue of ADX+OVX rats. Previous work in our lab also demonstrated that estradiol administration increases CORT levels following ovariectomy and/or adrenalectomy; however elevated CORT was not shown to mediate estrogen's anti-hyperalgesic effects during the formalin test (Hunter et al., in press). In our study, estradiol decreased CORT levels in formalin-treated OVX rats, which is a finding contrary to what was expected. It may be necessary to increase our sample size in order to find increased CORT levels across groups. Here we also found that CORT and cytokine levels did not correlate in formalin treatment groups, suggesting that CORT activity does not in turn lead to decreased cytokine levels.

Effects of estradiol administration and surgical condition on basal cytokine levels. Summary tables of estradiol's effects on cytokine levels in both naïve and formalin-treated rats are shown in Tables 7 and 8, respectively. As shown in Table 7, estradiol treatment altered cytokine levels before pain administration. In serum, estradiol up-regulated TNF- α levels in OVX rats. These results support research demonstrating that estradiol replacement prevents the reduction of serum TNF- α levels normally seen following ovariectomy (Oztekin et al., 2006). Further, Percegoni and colleagues (2009) demonstrated that following estradiol administration, serum IL-1 β and IL-10 levels were

not significantly altered as compared to controls (Percegoni et al., 2009). In the spinal cord, estradiol administration up-regulated IL-6 levels as compared to vehicle controls. This indicates that estrogen increases basal cytokine activity in CNS tissue, which is in contrast to what has been observed in previous literature (Houdeau et al., 2007). Due to lack of research on cytokine activity and estradiol replacement in naïve models, it is difficult to make a direct comparison of prior research to this study.

In naïve ADX rats, basal TNF- α serum levels were down-regulated by estradiol administration. Furthermore, although ADX rats had reduced CORT levels, a negative correlation between TNF- α levels and CORT release was observed. Together this may indicate that reduced CORT activity leads to up-regulated cytokine release. However, estradiol was still effective at reducing pain responses to formalin, suggesting that the presence or absence of CORT is not necessary for estradiol's anti-inflammatory effects to occur.

Although estradiol altered basal TNF- α serum levels in ADX rats, no modulations of TNF- α serum levels was observed in ADX+OVX groups. Since ADX+OVX rats did not exhibit a modulation of TNF- α , it can be implied that changes in TNF- α levels observed in ADX rats are not entirely influenced by exogenous estradiol administration alone, and more of a result of interactions between estradiol and secretions of endogenous ovarian hormones, such as progesterone. In ADX rats, whose ovaries were left intact, the presence of progesterone may influence TNF- α production, since Oztekin and colleagues (2006) demonstrate that progesterone administration alters TNF- α serum levels. Furthermore, it has been shown that progesterone alters nociceptive responses during inflammatory pain (Fischer et al., 2008; Kuba et al., 2006), although most studies suggest

a hypernociceptive role of progesterone during pain administration (Ganesan et al., 2008; Kondo et al., 2006; Lacroix-Fralish et al., 2006). Indeed, progesterone, along with endogenous and exogenous estrogen, may work synergistically to modulate basal cytokine production. Unfortunately, little is known about the effects of progesterone on cytokine release and/or regulation. Thus, future studies are needed to determine if progesterone and/or progesterone plus estradiol regulate cytokine activity before or after pain administration.

Interestingly, basal cytokine activity did not follow the same pattern of release across groups. For instance, in the paw and DRG, estradiol administration led to heightened IL-6 and TNF- α levels in ADX rats, while in ADX+OVX models estradiol decreased DRG IFN- γ levels. Therefore, estrogen differentially regulates basal cytokine activity within these groups. Together, our research indicates that in naïve rats, estrogen alters cytokine levels before the introduction of pain, thus demonstrating that to a certain extent estradiol effects are independent from painful regulation.

Effects of estradiol treatment and surgical condition on formalin-induced cytokine activity. Cytokine levels were tested in NS tissue of estradiol-treated rats following formalin administration in an effort to understand how hormonal mechanisms are involved in regulating cytokines during pain responses. As summarized in Table 8, estradiol modulated TNF- α , IL-1 β , IL-6, IL-2, and IL-10 levels at peripheral and central sites. These results support literature demonstrating that estradiol treatment alters cytokine activity following pain administration (Dai et al., 2008; Houdeau et al., 2007; Johnson et al., 2006; Lewis et al., 2008; Subramanian et al., 2005). An interesting finding was the way that cytokine activity was altered. In OVX rats, estrogen increased the anti-

inflammatory cytokine IL-10 as compared to vehicle controls in spinal cord tissue.

Furthermore, estradiol administration did not alter proinflammatory cytokine levels as compared to vehicles in this treatment group. It can be postulated that in OVX models estrogen reduces behavioral responses to pain in part by increasing central anti-inflammatory cytokine activity. As reviewed by Salem (2004), estrogen had been shown to stimulate IL-10 release from T cells. Estradiol may be working in a similar fashion to up-regulate anti-inflammatory modulators during inflammation-induced pain.

Furthermore, estrogen effects on IL-10 levels are working independent of CORT release, since estradiol decreased serum CORT activity in OVX rats. No correlation between CORT and IL-10 activity was observed, which indicates that in OVX rats estrogen mediates its anti-hyperalgesic effects independent of CORT release.

Following adrenalectomy, estradiol increased TNF- α , IL-2, and IL-10 activity in paw tissue of ADX rats (Table 8). Similarly, Ritz and Hausmann (2008) demonstrated that estradiol administration increases cytokine levels following SCI, which in turn lead to increased behavioral effects. The authors postulated that estrogen aided in reducing pain responses by heightening cytokine activity, and thus speeding up recovery time, at the site of injury (Ritz and Hausmann; 2008). Interestingly, our study shows that increases in peripheral cytokine activity following estradiol administration were accompanied by decreases in central TNF- α , IL-1 β , IL-6, IL-2, and IL-10 cytokine levels in ADX rats (Table 8). It has previously been shown that during inflammatory pain, TNF- α release induces proinflammatory cytokine (i.e. IL-6, IL-1 β) activity (Cunha et al., 1992). In turn, IL-1 β has been shown to influence central sensitization by up-regulating prostaglandin levels in CNS tissue (Samad et al., 2001). It is postulated that in ADX rats,

estrogen lowers formalin-induced central sensitization by reducing TNF- α , and thus IL-1 β and IL-6 activity, at central sites. Together, these findings suggest that estrogen is working to up-regulate inflammatory effects at peripheral sites, which may possibly aid in reducing the spreading of inflammation, and thus the propagation of pain signals, to the CNS. In turn, this leads to reduced behavioral responses during the formalin test.

Surprisingly, in ADX+OVX rats, estradiol administration did not alter cytokine activity to a great extent. Estradiol was effective at lowering paw IL-2 levels as compared to vehicle controls (Table 8); however, no other cytokines were modulated. We expected that reduced flinching responses to formalin in the ADX+OVX groups were a result of estrogen's reduction of cytokine levels. Since estrogen did not influence cytokine activity in this treatment group, our findings suggest that another system may be working with estrogen to decrease behavioral responses to pain. For instance, adrenalectomy increases ACTH and β -endorphins, two hormones involved in reducing stress and inflammation (Nelson, 2005). Elevated release of these hormones may have worked synergistically with estrogen to decrease behavioral activity via a feedback loop used to control stress-mediated responses. Research on ACTH and β -endorphin hormone activity on inflammatory pain is currently under investigation.

In summary, our results suggest that estrogen's attenuation of behavioral responses to inflammation is in part mediated through a reduction of proinflammatory mediators that occur before and during nociceptive stimulation. Estrogen's effects are independent of CORT release, since estradiol's regulation of cytokine levels also occur in ADX rats. The influence of estrogen on cytokine levels is thought to contribute to anti-hyperalgesia within the formalin paradigm. Our results help to understand how estrogen

administration works to regulate inflammatory mechanisms involved in pain states,
which is a topic that is neglected in current literature.

Table 7
Summary of estradiol effects on cytokine levels in naïve OVX, ADX, and ADX+OVX rats

Cytokine	Surgery	Sample tested			
		Paw	Serum	DRG	Spinal Cord
TNF- α	OVX	∅	↑	∅	∅
	ADX	∅	↓	↑	∅
	ADX+OVX	∅	∅	∅	∅
IL-6	OVX	∅	∅	∅	↑
	ADX	↑	∅	∅	∅
	ADX+OVX	∅	∅	∅	∅
IFN- γ	OVX	∅	∅	∅	∅
	ADX	∅	∅	∅	∅
	ADX+OVX	∅	∅	↓	∅

Legend. (↓) denotes decrease in cytokine levels following estradiol treatment as compared to vehicle (cholesterol) controls. (↑) denotes increase in cytokine levels following estradiol treatment as compared to vehicle. (∅) denotes no change in cytokine levels as compared to vehicle. Significance is at $p < 0.05$ for all analyses. $n = 4/\text{group}$.

Table 8
Summary of estradiol effects on cytokine levels following formalin administration in OVX, ADX, and ADX+OVX rats

Cytokine	Surgery	Sample tested			
		Paw	Serum	Ipsilateral DRG	Spinal Cord
TNF- α	OVX	∅	∅	∅	∅
	ADX	↑	∅	∅	↓
	ADX+OVX	∅	∅	∅	∅
IL-1 β	OVX	∅	∅	∅	∅
	ADX	∅	∅	∅	↓
	ADX+OVX	∅	∅	∅	∅
IL-6	OVX	∅	∅	∅	∅
	ADX	∅	∅	↓	↓
	ADX+OVX	∅	∅	∅	∅
IL-2	OVX	∅	∅	∅	∅
	ADX	↑	∅	↓	↓
	ADX+OVX	↓	∅	∅	∅
IL-10	OVX	∅	∅	∅	↑
	ADX	↑	∅	∅	↓
	ADX+OVX	∅	∅	∅	∅

Legend. (↓) denotes decrease in cytokine levels following estradiol treatment as compared to vehicle (cholesterol) controls. (∅) denotes no change in cytokine levels as compared to vehicle. Significance is at $p < 0.05$ for all analyses. $n = 4/\text{group}$.

Chapter 4: Estradiol administration alters cytokine levels during carrageenan-induced inflammation.

1. Introduction

Environmental insult resulting in inflammation causes the release of chemicals from local macrophages, mast cells, immune cells, and injured cells, which then leads to changes in transduction mechanisms at nociceptor terminals (Costigan & Woolf, 2000). Noxious (tissue-damaging) insult creates a region of hypersensitivity near the injured area, known as inflammatory pain (Basbaum & Woolf, 1999). Inflammatory pain associated with tissue injury has a pathological character that consists of sensitization, a course of action in nerve fibers that involves a decrease in the threshold of activation, an increase in response to specific stimuli, and spontaneous activity (Burian & Geisslinger, 2005).

During inflammation, peripheral sensitization allows increased sensitivity of nociceptors at the site of injury that heightens pain perception and response (Millan, 1999; Riedel & Neeck, 2001). Pain hypersensitivity from the periphery leads to substantial long-lasting increases in spinal excitability, known as central sensitization (Kidd & Urban, 2001). During central sensitization, the receptive field size for dorsal horn neurons increases and exhibits a reduced threshold for activation by nociceptors (Kidd & Urban, 2001; Millan, 1999). Dorsal horn neurons become more responsive to sub-threshold stimuli and produce exaggerated responses to innocuous stimuli (Kidd & Urban, 2001).

Following tissue injury, proinflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, are involved in sensitizing nociceptive pathways (Cunha et al., 1992; Ferreira et al., 1988; DeLeo et al., 1997; Moalem & Tracey, 2006). They recruit and activate leukocytes

and immune cells at the site of injury (Moalem & Tracey, 2006; Wieseler-Frank et al., 2005). Further, cytokines act synergistically to induce the expression of inflammatory mediators that are involved in nociceptive processes, such as prostaglandins and growth factors (Ferreira, Lorenzetti, Bristow, & Poole, 1988; Meyer et al., 2006; Wieseler-Frank et al., 2005).

The synthesis of proinflammatory cytokines is regulated by various negative feedback systems, one of them being estrogen (Pavlov et al., 2003). Estradiol replacement has been shown to reduce proinflammatory cytokine production and increase anti-inflammatory cytokine levels (cytokines that inhibit inflammatory mediators) *in vivo* (Ganesan et al., 2008; Houdeau et al., 2007; Johnson et al., 2006; Santora et al., 2007; Suzuki et al., 2006). Corticosterone (CORT) has also been shown to reduce cytokine production during inflammation-induced pain (DeRijk et al., 1997; Sternberg, 1997). However, the interaction between estradiol and CORT effects on inflammatory responses is poorly understood.

Previous research conducted in our lab showed that estradiol replacement elicits anti-hyperalgesia during the formalin model of inflammatory pain (Kuba et al., 2005, 2006). To our knowledge, it is not published whether estrogen modulates nociception during a more prolonged inflammatory state, and if so does it work by altering cytokine production over time. We therefore used an estradiol-replacement paradigm to identify estrogen's role on cytokine production during carrageenan-induced inflammatory pain. The carrageenan test for cutaneous nociception is a common procedure used to evoke hind paw inflammation and hyperalgesia (Di Rosa et al., 1971; Garcia Leme et al., 1973; Hargreaves et al., 1988; Vinegar et al., 1987). It induces persistent pain that can last up

to 24 hours (Hargreaves et al., 1988). Carrageenan administration has been shown to elicit inflammatory mediators (i.e. cytokines and prostaglandins) that sensitize nociceptive pathways (Basbaum and Woolf, 1999; Cunha et al., 1992; Ferreira et al., 1988; Loram et al., 2007). Preliminary research in our lab found that estradiol replacement decreases nociceptive behaviors 1 and 5 hours post carrageenan administration in ovariectomized (OVX) rats (Hunter et al., personal communication, 2008). Therefore, we measured cytokine levels 1, 5, and 24 hours following carrageenan administration in estradiol-treated rats to determine if the anti-hyperalgesic effects of estrogen are a result of modulating cytokine levels over time. CORT levels were also measured to determine if estradiol's effects on pain are in part mediated through glucocorticoid production. We hypothesize that estradiol administration will lower proinflammatory cytokine levels in a time-dependent manner.

2. Materials and Methods

2.1. Animals

Eight-week old OVX female Sprague-Dawley rats ($N = 82$) were purchased from Taconic (Germantown, NY). Rats were double-housed with a 12-hour light/dark photoperiod (lights on 8 A.M. EST), and food and water was available *ad libitum*. Rats weighed 220-240 g at the time of sacrifice. 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. Estradiol replacement paradigm

Two weeks after ovariectomy, a SILASTIC capsule (1 cm, 0.058 in. ID x 0.077 in. OD, Dow Corning) was inserted into the nape of the rats' neck. The capsules contained either cholesterol (5-Cholestin-3Beta-ol; Sigma-Aldrich, St. Louis, MO) for the vehicle group, or 20% 17-Beta-Estradiol (1, 3, 5 [10]-Estratriene-3, 17 Beta-diol; Sigma-Aldrich) mixed with 80% cholesterol for the experimental group. Rats were randomly assigned to either the vehicle (100% cholesterol) or 17 β -estradiol (20% estradiol) experimental groups, however, both rats in each cage received the same type of SILASTIC capsule. Adequate care was taken to minimize pain or discomfort. All animal procedures were approved by the Institutional Animal Care and Use Committee of Hunter College, CUNY.

2.3. Experimental Procedures

Rats were sacrificed one week after SILASTIC surgery to ensure that serum estradiol levels were maintained at a steady state (Mannino et al., 2005). 100 μ L of carrageenan [1% lambda carrageenan, (Fluka BioChemika, Ronkonkoma, NY) suspended in 0.9% sterile isotonic saline (Sodium Irrigation USP; Braun Medical, Irving, CA)] or 100 μ L saline was injected subcutaneously into the intraplantar region of the right hind paw using a 27 $\frac{1}{2}$ gauge needle. For the behavioral test, each rat received only one injection, which was administered between 9AM to 3PM. Rats were assigned to six experimental groups ($n = 4-7$ per group): saline + 17 β -estradiol (SE), saline + vehicle (SV), carrageenan + 17 β -estradiol (CE), carrageenan + vehicle (CV), naïve + 17 β -estradiol (NE), or naïve + vehicle (NV), where naïve rats were not given an injection.

2.4. Nociceptive Behavioral Testing

The Paw Thermal Stimulator system (Department of Anesthesiology, University of California, San Diego, CA) is a device used to determine thermal nociceptive responses in the hind paw. Rats were weighed and then individually placed in plexiglass chambers (9 x 22 x 25 cm) arranged atop a clear glass surface. Glass surface temperature was maintained at $30^{\circ} \pm 0.1^{\circ}\text{C}$. The nociceptive heat source was emitted beneath the floor from a focused projection bulb mounted on ball bearing slides that allow for the stimulus to be delivered separately to the plantar surface of each hind paw. The stimuli used were 4.5 mV, 4.8 mV, and 5.3 mV in intensity, and are denoted as low, medium, and high, respectively. Paw withdrawal is detected by photodiode motion sensors mounted on the apparatus. In order to measure paw withdraw latencies (PWL) to heat, a timer automatically starts once the heat source is administered. The heat source and timer stop when the paw is withdrawn from the surface. The stimulus automatically shuts off after 20 seconds in order to avoid tissue damage. No tissue injury resulted during behavioral testing.

Rats were allowed to acclimate in the plexiglass chambers for 30 minutes in order to minimize novelty to the testing environment. Following acclimation, baseline readings to the three stimulus intensities were measured in the left and right hind paws. After baseline PWL was recorded, rats were injected in the right hind paw with either 100 μL 1% carrageenan or saline. Following injection, PWL to low, medium, and high heat intensities were measured in both the ipsilateral and contralateral hind paws. Separate groups were utilized to assess PWL at 1, 5, or 24 hours post carrageenan or saline

administration. For instance, for the 1 hour reading, rats were only tested at baseline and 1hr post injection before sacrifice.

2.5. Corticosterone Radioimmunoassay

Immediately after completion of testing, rats were sacrificed by rapid decapitation at 1, 5, or 24hrs post injection, following a brief (20 sec) exposure to CO₂. This procedure is in compliance with NIH guidelines. Naïve rats were sacrificed after removal from their home cage. Trunk blood was collected in tubes containing K₂ EDTA (BD Vacutainer Systems, Franklin Lakes, NJ), and then centrifuged at 2,600 RPM for 30 minutes at 4° C. Serum was collected and stored at -80° C until assays were performed.

CORT serum levels were measured in order to determine if estradiol's anti-hyperalgesic effects are in part mediated through up-regulation of glucocorticoids. CORT levels were analyzed using Coat-A-Count radioimmunoassay kits (Diagnostic Products Corporation, Los Angeles, CA) along with an automated gamma counter machine for quantification (WIZARD5DET1470015/4701845, PerkinElmer, Shelton, CT). Intra-assay coefficients of variation averaged $10.0 \pm 1\%$. CORT values are expressed as ng/mL.

2.6. Tissue Collection and Homogenization

After rats were sacrificed, paw size was determined using a dial caliper (General Tools, New York, NY) measuring the width and depth in millimeters in order to measure hind paw swelling in paws following carrageenan or saline administration. These measurements were later compared to the right hind paw sizes of naïve rats. Next, 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 then collected and immediately frozen. All tissue samples were stored at -80° C until use.

Spinal cord, DRG, and paw samples were homogenized in lysing solution containing cell lysis buffer (from Cell Lysis Kit #171-304012, Bio-Rad, Hercules, CA), a protease inhibitor cocktail provided in the cell lysis kit, and 500mM phenylmethylsulfonyl fluoride (PMSF, Sigma) diluted in dimethyl sulfoxide (DMSO, Sigma). Tissue samples were rapidly frozen at -80° C. Homogenates were thawed and then centrifuged at 5000rpm for 15 minutes at 4° C. Supernatants were collected and stored at -80° C. Total protein concentration was determined using a Bradford kit from Bio-Rad Laboratories (Hercules, CA). In order to reduce variation in homogenates, all samples from the same region were homogenized at the same time.

2.7. Cytokine Multiplex Assay

Tissue homogenates were analyzed for cytokine levels using the Bio-Plex Cytokine assay along with the Cytokine Reagent Kit (#171-K11070 and #171-304001, respectively, Bio-Rad, Hercules, CA). The cytokine assay is a multiplexed (IL-1 β , IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN- γ , and TNF- α) immunoassay consisting of premixed beads coated with target capture antibody. Simultaneous cytokine analysis was processed according to the manufacturer's protocol (for details see Hulse et al., 2004). Samples were quantified using the Bio-Plex Suspension Array system and accompanying Bio-Plex Manager software (Bio-Rad, Hercules, CA). The limit of detection was <10 pg/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/ μ g of total tissue protein.

2.8. Statistical Analyses

All data are expressed as mean $\pm$ SEM. Behavioral data: Repeated Measures ANOVA was used to analyze: 1.) baseline PWL following estradiol administration; 2.) effects of estradiol on PWL comparing baseline to PWL post injection; and 3.) effects of estradiol on PWL post injection over low, medium, and high heat intensities. Factorial ANOVA was used to analyze estradiol effects on PWL across time. Paw size data: For evaluation of paw size, the width and depth of each hind paw was multiplied and expressed in mm². One Way ANOVA was used to compare naïve paw size measurements to saline- and carrageenan-injected paws. Repeated Measures ANOVA was used to compare ipsilateral and contralateral paw size post carrageenan or saline administration. CORT data: One Way ANOVA was conducted to determine CORT serum levels in naïve rats. Factorial ANOVA was conducted to compare CORT levels between saline and carrageenan treatment groups at 1, 5, and 24hrs post injection. Cytokine data: One Way ANOVA was used to analyze cytokine values in naïve groups. Factorial ANOVA was conducted to test for differences in cytokine levels, hormonal treatment, and pain condition at all time points in paw, spinal cord, and DRG. Repeated Measures ANOVA was used to determine differences between cytokine levels in ipsilateral and contralateral DRG of carrageenan- injected rats to test for the spread of sensitization following pain

administration. Fisher's Least Significant Difference (LSD) *post hoc* tests were conducted when appropriate. For all comparisons, significance was at the $p < 0.05$ level.

3. Results

3.1 The effects of 17 β -estradiol on nociceptive behavioral responses

Effects of estradiol administration on baseline PWL in right and left paws. Figure 44 shows PWL for estradiol- or vehicle-treated OVX rats at low, medium, and high heat intensities, before saline or carrageenan administration. A significant main effect for hormone, heat intensity, and an interaction between hormone x heat intensity on PWL was observed [Hormone main effect: $F(1, 129) = 20.85, p < 0.01$; Heat Intensity main effect: $F(2, 258) = 262.80, p < 0.01$; Interaction: $F(2, 258) = 12.62, p < 0.01$]; rats administered estradiol had longer PWL than vehicle treated rats ($p < 0.01$). PWL to heat were different across all heat intensities ($p < 0.01$; Fig. 44). At low heat, estradiol-treated rats had longer PWL than vehicle-treated rats and rats tested at medium and high heat intensities ($p < 0.01$; Fig. 44).

Effects of estradiol administration on ipsilateral PWL at baseline and one, five, and 24 hours post saline or 1% carrageenan administration.

Low heat intensity. Figures 45 A-C show PWL to low heat in the ipsilateral paw at 1 (Fig. 45A), 5 (Fig. 45B), and 24 hours (Fig. 45C) post injection in estradiol- or vehicle-treated OVX rats. At 1hr, a main effect of hormone, time of testing post injection, and an interaction between hormone x time on PWL to low heat was observed [Hormone main effect: $F(1, 17) = 6.01, p < 0.05$; Time main effect: $F(1, 17) = 17.18, p$

< 0.01; Interaction: $F(1, 17) = 14.76, p < 0.01$; Fig. 45A]. Rats administered estradiol had longer PWL to low heat than controls ($p < 0.05$). Rats had shorter PWL to low heat 1 hour post injection than at baseline ($p < 0.01$). Rats administered estradiol had longer baseline PWL to low heat than vehicle-treated rats at baseline and rats tested 1hr post injection ($p < 0.01$). As shown in Figure 45B, at 5hrs a main effect for pain condition, time, and an interaction between hormone x time on PWL to low heat were observed [Pain condition main effect: $F(1, 18) = 5.59, p < 0.05$; Time main effect: $F(1, 18) = 12.24, p < 0.01$; Interaction: $F(1, 18) = 8.64, p < 0.01$]. Rats administered carrageenan had significantly shorter PWL than saline-treated rats ($p < 0.05$; Fig. 45B). PWL to low heat were shorter 5hrs post injection than at baseline ($p < 0.01$; Fig. 45B). Rats administered estradiol had longer baseline PWL to medium heat than vehicle-treated rats at baseline and rats tested 5hrs post injection ($p < 0.05$ for all comparisons; Fig. 45B). As shown in Figure 45C, at 24hrs, a main effect for hormone was observed [$F(1, 18) = 5.94, p < 0.05$]; rats administered estradiol had longer PWL to low heat than vehicle-treated rats ($p < 0.05$).

Medium heat intensity. Figures 45 D-F shows ipsilateral PWL to medium heat 1, 5, and 24hrs post injection, respectively. PWL to medium heat intensity was not altered across groups at baseline or 1hr post injection (Figure 45D). At five hours, a main effect for hormone and pain condition on PWL was observed [Hormone main effect: $F(1, 17) = 9.77, p < 0.01$; Pain condition main effect: $F(1, 17) = 4.61, p < 0.05$; Fig. 45E]. Rats administered estradiol had longer PWL to medium heat than vehicles ($p < 0.01$; Fig. 45E). Rats administered carrageenan had shorter PWL to medium heat than saline-

treated rats ($p < 0.05$; Fig. 45E). As shown in Figure 45F, no significant differences in PWL to medium heat were observed at baseline or 24hrs post injection.

High heat intensity. Figures 45 G-I show ipsilateral PWL to high heat 1, 5, and 24hrs post injection, respectively. As shown in Figure 45G, regardless of hormone and pain condition PWL to high heat did not differ at baseline or 1hr post injection. At 5hrs, as shown in Figure 45H, a main effect for time of testing post injection, and an interaction for time x pain condition was observed [Time main effect: $F(1, 20) = 11.52, p < 0.01$; Interaction: $F(1, 20) = 4.96, p < 0.05$]; PWL to high heat were shorter 5hrs post injection than at baseline ($p < 0.01$). At five hours post injection, carrageenan-treated rats had shorter PWL to high heat than saline controls ($p < 0.05$; Fig. 45H). As shown in Figure 45I, no significant difference between PWL at baseline and 24hrs to high heat was observed.

Effects of estradiol administration on contralateral PWL at baseline and one, five, and 24 hours post saline or 1% carrageenan administration.

Low heat intensity. Figure 46 A-C shows PWL to low heat in the contralateral paw at 1 (Fig. 46A), 5 (Fig. 46B), and 24hrs (Fig. 46C) post injection in estradiol- or vehicle-treated OVX rats. As shown in Figure 46A, at 1hr post injection an interaction for time x pain condition on PWL was observed [$F(1, 18) = 7.36, p < 0.05$]; rats administered carrageenan had significantly longer PWL to low heat 1hr after injection than at baseline ($p < 0.05$). At five hours, as shown in Figure 46B, a main effect for time on PWL was observed [$F(1, 20) = 4.99, p < 0.05$]; PWL to low heat were shorter 5hrs after injection than at baseline ($p < 0.05$). As shown in Figure 46C, at 24 hours a main

effect of time on PWL to low heat was observed [$F(1, 19) = 7.51, p < 0.05$]; rats had longer PWL 24hrs post carrageenan or saline administration than at baseline ($p < 0.01$).

Medium heat intensity. Figures 46 D-F show contralateral PWL to medium heat 1, 5, and 24hrs post injection, respectively. As shown in Figures 46 D-E, regardless of hormone or pain condition, no significant difference in PWL to medium heat was observed 1 or 5hrs post injection, respectively. At 24hrs, as shown in Figure 46F, a main effect for hormone on PWL was observed [$F(1, 19) = 5.66, p < 0.05$]. Rats administered estradiol had longer PWL to medium heat than vehicle-treated rats ($p < 0.05$, Fig. 46F).

High heat intensity. Figures 46 G-I show PWL to high heat in the contralateral paw at 1, 5, and 24 hours post injection, respectively. At high heat intensity, as shown in Figure 46G, a main effect for hormone on PWL was observed at baseline and 1 hr [$F(1, 17) = 5.49, p < 0.05$]; rats pretreated with estradiol had longer PWL to high heat than vehicle-treated rats ($p < 0.05$). As shown in Figure 46H, in the 5hr condition an interaction between hormone x pain condition on PWL was observed [$F(1, 18) = 4.72, p < 0.05$]. CE rats had longer PWL to high heat than CV rats ($p < 0.05$; Fig. 46H). 24 hours post injection, as shown in Figure 46I, no significant difference in PWL to high heat was observed, regardless of hormone treatment or pain condition.

Effects of 17 β -estradiol on PWL over heat intensities post saline or 1% carrageenan administration.

Ipsilateral paw. Figures 47 A-C show ipsilateral PWL to low, medium, and high heat intensities in estradiol- or vehicle-treated rats post injection. At 1hr, as shown in Figure 47A, a main effect for heat intensity on PWL was observed [$F(2, 36) = 30.73, p <$

0.01]. Ipsilateral PWL to low, medium, and high heat intensities were significantly different from each other across treatment groups ($p < 0.01$ for all comparisons, Fig. 47A). At 5hrs, as shown in Figure 47B, a main effect for pain condition and heat intensity on PWL was observed [Pain condition main effect: $F(1, 17) = 9.87, p < 0.01$; Heat intensity main effect: $F(2, 34) = 28.28, p < 0.01$]. Rats administered carrageenan had significantly shorter PWL than saline-treated rats ($p < 0.01$, Fig. 47B). PWL to low, medium, and high heat were significantly different across groups ($p < 0.05$ for all comparisons, Fig. 47B). At 24hrs, as shown in Figure 47C, a main effect for heat intensity was observed [$F(2, 36) = 35.97, p < 0.01$]; rats had significantly different PWL at each heat intensity ($p < 0.05$ for all comparisons). Although rats administered estradiol had longer PWL than vehicle-treated rats, this effect failed to reach significance ($p = 0.051$; Fig. 47C).

Contralateral paw. Figures 47 D-F show PWL in the contralateral paw to low, medium, and high heat intensities, respectively, in estradiol- or vehicle-treated rats post injection. As shown in Figure 47D, at 1hr a main effect for heat intensity on PWL was observed [$F(2, 34) = 41.46, p < 0.01$]; contralateral PWL to low, medium, and high heat intensities were significantly different from each other across treatment groups ($p < 0.01$ for all comparisons). At 5hrs, as shown in Figure 47E, a main effect for heat intensity on PWL was observed [$F(2, 38) = 31.43, p < 0.01$]; PWL to low, medium, and high heat were significantly different to each other across groups ($p < 0.01$). As shown in Figure 47F, a main effect for hormone and heat intensity on contralateral PWL was observed [Hormone main effect: $F(1, 17) = 5.31, p < 0.05$; Heat intensity main effect: $F(2, 34) = 32.43, p < 0.01$]; rats administered estradiol had longer PWL to heat than vehicle-treated

controls ($p < 0.05$). Rats had significantly different PWL to low, medium, and high heat intensities across treatment groups ($p < 0.01$ for all comparisons, Fig. 47F).

Effects of 17 β -estradiol on PWL across time following carrageenan or saline administration.

Low heat intensity. Figures 48A-B show the effects of estradiol administration at 1, 5, and 24hrs post injection in the ipsilateral (48A) and contralateral (48B) paws. In the ipsilateral paw, a main effect for pain condition and time of testing post injection on PWL was observed [Pain condition main effect: $F(1, 56) = 6.29, p < 0.05$; Time main effect: $F(2, 56) = 3.34, p < 0.05$; Fig. 48A]. Rats administered carrageenan had shorter PWL to low heat than saline-treated rats ($p < 0.01$; Fig. 48A). Rats had shorter PWL to low heat at 1hr than at 24hrs post injection ($p < 0.01$; Fig. 48A). An interaction between hormone x time was observed [$F(2, 56) = 3.26, p < 0.05$; Fig. 48A]; at 24hrs, estradiol-treated rats had longer PWL than rats treated with estradiol at 1 and 5hrs, and vehicle-treated rats at 1, 5, and 24hrs ($p < 0.05$). An interaction between pain condition x time was observed [$F(2, 56) = 4.88, p < 0.05$; Fig. 48A]; at 5 and 24hrs, carrageenan-administered rats had shorter PWL than saline controls ($p < 0.01$). In the contralateral paw, no significant difference in PWL to low heat following hormone or pain condition at 1, 5, or 24hrs was observed (Fig. 48B).

Medium heat intensity. PWL to medium heat in the ipsilateral and contralateral paws at 1, 5, and 24hrs post injection are shown in Figures 48C and D, respectively. As shown in Figure 48C, in the ipsilateral paw a main effect for hormone and pain condition on PWL was observed [Hormone main effect: $F(1, 56) = 8.01, p < 0.01$; Pain condition

main effect: $F(1, 56) = 6.36, p < 0.05$]; rats administered estradiol had longer PWL to medium heat than vehicle-treated rats ($p < 0.01$). Rats administered carrageenan had shorter PWL to medium heat than saline controls ($p < 0.01$; Fig. 48C). In the contralateral paw, as shown in Figure 48D, a main effect for hormone on PWL was observed [$F(1, 59) = 8.09, p < 0.01$]; rats administered estradiol had longer PWL to medium heat than vehicle controls ($p < 0.01$).

High heat intensity. Figures 48E and F show PWL to high heat at 1, 5, and 24hrs in the ipsilateral and contralateral paws, respectively. In the ipsilateral paw, as shown in Figure 48E, no significant difference on PWL following hormone or pain condition was observed. As shown in Figure 48F, in the contralateral paw a significant main effect of hormone was observed [$F(1, 54) = 6.53, p < 0.05$]; rats administered estradiol had longer PWL to high heat than vehicle-treated rats ($p < 0.05$).

3.2 Estradiol's effects in paw tissue during carrageenan-induced inflammation.

To test if estradiol treatment altered paw size following inflammation, right hind paw sizes (in mm²) of naïve rats were compared to paw sizes of rats injected with saline or 1% carrageenan measured at 1, 5, or 24 hours post injection (Fig 49A-C). As shown in Figures 49 A-C, a main effect for pain condition at 1, 5, and 24hrs was observed [Pain condition main effect at 1hr: $F(2, 29) = 68.20, p < 0.01$; Pain condition main effect at 5hrs: $F(2, 26) = 47.12, p < 0.01$; Pain condition main effect at 24hrs: $F(2, 26) = 11.18, p < 0.01$]; carrageenan treatment significantly increased ipsilateral paw size compared to naïve and saline groups ($p < 0.01$ for all analyses). For paw sizes measured 24 hours post injection, a main effect for hormone was observed; [$F(1, 26) = 5.21, p < 0.05$; Fig. 49C];

rats administered estradiol had significantly smaller paw sizes than vehicle-treated rats ($p < 0.01$).

To test if hormone and pain condition altered hind paw size in the contralateral paw, ipsilateral and contralateral paw sizes of carrageenan-administered estradiol- and vehicle-treated rats were compared to saline controls at one, five, and 24 hours post injection. As shown in Figure 49D, a main effect of hormone, pain condition, and time of testing post injection was observed [Hormone main effect: $F(1, 52) = 4.98, p < 0.05$; Pain condition main effect: $F(1, 52) = 117.92, p < 0.01$; Time main effect: $F(2, 52) = 6.72, p < 0.01$]; rats administered estradiol had smaller paw sizes than vehicle-treated rats ($p < 0.01$). Rats administered carrageenan had significantly larger paw sizes than saline controls ($p < 0.01$; Fig. 49D). Rats had significantly smaller paw sizes at 24hrs than at 1 or 5hrs post injection ($p < 0.01$; Fig. 49D). When comparing ipsilateral versus contralateral paw sizes, a main effect was observed [$F(1, 52) = 256.02, p < 0.01$]; ipsilateral paw size was larger than contralateral paw size across groups ($p < 0.01$; Fig. 49D).

An interaction between pain condition x time of testing post injection on paw size was observed [$F(2, 52) = 8.78, p < 0.01$; Fig. 49D]; at 1, 5, and 24hrs post injection, carrageenan administration led to significantly larger paw sizes than saline administration alone ($p < 0.01$). Further, an interaction between paw size x hormone x time of testing post injection was observed [$F(2, 52) = 3.34, p < 0.05$; Fig. 49D]; in the ipsilateral paw at 24hrs, rats administered estradiol had significantly smaller paw sizes than vehicle-treated rats ($p < 0.01$).

3.3 Estradiol effects on CORT serum levels before and after pain administration.

CORT levels were analyzed in serum of naïve, saline-, and carrageenan-treated rats administered 17 β -estradiol or vehicle. As shown in Figure 50A, in naïve OVX rats estradiol administration did not alter CORT serum levels as compared to NV rats [$F(1, 6) = 0.28, p = 0.62$]. Following saline or carrageenan administration, as shown in Figure 50B, a significant main effect for hormone and time of testing post injection was observed [Hormone main effect: $F(1, 34) = 9.87, p < 0.01$; Time main effect: $F(2, 34) = 20.67, p < 0.01$]; rats administered estradiol had higher CORT serum levels than vehicle-treated rats ($p < 0.01$). CORT serum levels were significantly different from each other at all time points tested post injection ($p < 0.01$ for all comparisons; Fig. 50B). An interaction between hormone x pain condition on CORT levels was observed [$F(1, 34) = 6.66; p = 0.01$]; saline-treated rats administered estradiol had higher CORT serum levels than SV, CV, and CE rats ($p < 0.05$ for all comparisons). Further, a significant interaction between hormone x pain condition x time of testing post injection was observed [$F(2, 34) = 5.56, p < 0.01$; Figure 50B]; at 1hr, SE rats had higher CORT serum levels than SV rats ($p < 0.05$); and CE rats had lower CORT serum levels than CV rats ($p < 0.01$). In carrageenan and saline treated rats 5hrs post injection, rats administered estradiol had higher CORT serum levels than vehicle treated rats ($p < 0.01$ for both comparisons; Figure 50B).

3.4 The effect of estradiol on cytokine levels in naïve, carrageenan-, and saline-treated OVX rats.

Proinflammatory Cytokines:

TNF- α

Estradiol's effects on TNF- α levels in both naïve and carrageenan-treated rats are shown in Figures 51 and 52, respectively. In naïve rats, as shown in Figure 51, estradiol treatment did not alter TNF- α levels in the paw, DRG, or spinal cord (Figures 51A, B, and C, respectively).

The effects of hormone and pain condition over time on TNF- α levels are shown in Figure 52. In the paw, as shown in Figure 52A, a main effect of pain condition and time of testing post injection was observed [Pain condition main effect: $F(1, 33) = 21.78$, $p < 0.01$; Time main effect: $F(2, 33) = 26.60$, $p < 0.01$]; rats administered carrageenan had higher TNF- α levels than saline-treated rats ($p < 0.01$). Rats tested 1hr post injection had higher TNF- α levels than at 5 and 24hrs post injection ($p < 0.01$; Fig. 52A). An interaction between pain condition x time was observed [$F(2, 33) = 13.00$, $p < 0.01$; Fig. 52A]; at 1hr, carrageenan-treated rats had higher paw TNF- α levels than saline-treated rats and TNF- α levels measured 5 and 24hrs post injection ($p < 0.01$ for all comparisons). An interaction between hormone treatment x pain condition was observed [$F(1, 33) = 5.84$, $p < 0.05$; Fig. 52A]; CE rats had higher paw TNF- α levels than SE, SV, and CV rats ($p < 0.05$ for all comparisons). An interaction between hormone x pain condition x time of testing post injection was observed [$F(2, 33) = 5.21$, $p < 0.05$; Fig. 52A]; at 1 hr estradiol-treated carrageenan rats had significantly higher paw TNF- α levels than CV rats, as well as all other treatment groups ($p < 0.01$ for all comparisons). Furthermore, at

1hr vehicle-treated carrageenan rats had significantly higher paw TNF- α levels than all groups except CE rats at 1hr ($p < 0.05$; Fig. 52A).

As shown in Figure 52B, in ipsilateral DRG a main effect for time of testing post injection and an interaction between pain condition x time was observed [Time main effect: $F(2, 32) = 6.14, p < 0.01$; Interaction: $F(2, 32) = 3.98, p < 0.05$]; rats had higher TNF- α levels in ipsilateral DRG at 5 hrs than at 1 and 24hrs post injection ($p < 0.05$). At 5hrs, carrageenan estradiol-treated rats had lower ipsilateral DRG TNF- α levels than saline controls ($p < 0.01$; Fig. 52B).

In the spinal cord, as shown in Figure 52C, an interaction between hormone x pain condition was observed [$F(1, 34) = 4.86, p < 0.05$]; vehicle-treated carrageenan rats had higher spinal cord TNF- α levels than SV, SE, and CE rats ($p < 0.05$). Post hoc analysis revealed that at 5hrs post injection, CV rats had higher TNF- α levels than saline controls ($p < 0.05$). Figure 52D shows ipsilateral and contralateral DRG TNF- α levels following carrageenan administration. As shown in Figure 52D, no significant differences between TNF- α levels in ipsilateral and contralateral DRG were observed.

IL-1 β

Figure 53 shows IL-1 β levels in paw (Fig. 53A), DRG (Fig. 53B) and spinal cord (Fig. 53C) of OVX estradiol- and vehicle-treated OVX naïve rats. As shown in Figure 53, no significant differences in basal IL-1 β levels following estradiol administration were observed in any tissue tested.

Figure 54 shows IL-1 β levels at 1, 5, and 24hrs post carrageenan or saline administration in estradiol- or vehicle-treated OVX rats. As shown in Figure 54A, in the

paw, a main effect of pain condition and time of testing post injection on IL-1 β levels was observed [Pain condition main effect: $F(1, 32) = 76.42, p < 0.01$; Time main effect: $F(2, 32) = 3.83, p < 0.05$]; rats administered carrageenan had higher paw IL-1 β levels than saline-treated rats ($p < 0.01$). At 24hrs post injection, rats had lower IL-1 β levels than rats tested 1hr post injection ($p < 0.05$; Fig. 54A).

In ipsilateral DRG, as shown in Figure 54B, a main effect for hormone, time of testing post injection, and an interaction for hormone x time was observed [Hormone main effect: $F(1, 35) = 12.24, p < 0.01$; Time main effect: $F(2, 35) = 4.83, p < 0.05$; Interaction: $F(2, 35) = 4.65, p < 0.05$]; rats treated with estradiol had lower ipsilateral DRG IL-1 β levels than vehicle-treated rats ($p < 0.01$). IL-1 β levels measured 24hrs post injection were significantly higher than at 1 and 5 hrs ($p < 0.05$; Fig. 54B). At 1 and 5hrs post injection, estradiol-treated rats had significantly lower ipsilateral DRG IL-1 β levels than vehicle-treated rats ($p < 0.01$; Fig. 54B). Further, an interaction between hormone x pain condition x time of testing post injection was observed [$F(2, 35) = 8.37, p < 0.01$; Fig. 54B]; at 1 and 5 hrs, CE rats had lower IL-1 β levels compared to CV rats ($p < 0.01$), whereas at 24hrs CE rats had higher IL-1 β levels than CV and SE rats ($p < 0.01$ for both comparisons).

In spinal cord, as shown in Figure 54C, no significant differences in IL-1 β levels across groups was observed. As shown in Figure 54D, a main effect was observed between ipsilateral versus contralateral DRG IL-1 β levels [$F(1, 18) = 37.68, p < 0.01$]; ipsilateral DRG IL-1 β levels were significantly higher than contralateral DRG ($p < 0.01$). Furthermore, in DRG, a main effect of time of testing was observed [$F(2, 18) = 6.00, p < 0.05$; Fig. 54D]; IL-1 β levels were higher at 24hrs than at 1 and 5hrs post injection ($p <$

0.01). Also shown in Figure 54D, an interaction between hormone x time of testing post injection on DRG IL-1 β levels was observed [$F(2, 18) = 8.595, p < 0.01$]; at 1 and 5hrs, estradiol treatment led to decreased IL-1 β levels as compared to vehicle treatment ($p < 0.05$), whereas at 24hrs estradiol treatment led to increased IL-1 β levels as compared to vehicles ($p < 0.05$). An interaction between DRG IL-1 β levels x hormone x time of testing was observed [$F(2, 18) = 4.01, p < 0.05$]; following estradiol-treatment, contralateral DRG IL-1 β levels were greater at 24hrs than at 1 and 5hrs post injection ($p < 0.05$).

IL-6

Basal IL-6 levels in OVX rats following estradiol or vehicle treatment are shown in Figure 55. In naïve rats, estradiol administration did not lead to altered IL-6 levels in paw, DRG, or spinal cord as compared to vehicle-treated rats (Fig. 's 55A, B, and C, respectively).

Figure 56 shows IL-6 levels 1, 5, and 24hrs post carrageenan or saline administration in estradiol- and vehicle-treated rats. As shown in Figure 56A, in paw a main effect for pain condition, time of testing, and an interaction between pain condition x time of testing post injection on IL-6 levels was observed [Pain condition main effect: $F(1, 33) = 42.03, p < 0.01$; Time main effect: $F(2, 33) = 7.61, p < 0.01$; Interaction: $F(2, 33) = 5.44, p < 0.01$]; paw IL-6 levels were significantly higher in carrageenan rats than saline controls ($p < 0.01$). IL-6 levels tested at 24hrs were significantly lower than at 1 and 5 hrs post injection ($p < 0.01$; Fig. 56A). At 1 and 5 hrs, carrageenan administration led to higher paw IL-6 levels than in saline controls ($p < 0.01$; Fig. 56A).

As shown in Figure 56B, in ipsilateral DRG no effects of pain condition, hormone, or time of testing post injection on IL-6 levels was observed. In spinal cord, as shown in Figure 56C, an interaction between hormone x pain condition was observed [$F(1, 35) = 4.80, p < 0.05$]; CV rats have higher IL-6 levels than SV rats ($p < 0.05$). As shown in Figure 56D, a main effect was observed between ipsilateral and contralateral DRG IL-6 levels [$F(1, 17) = 14.25, p < 0.01$]; IL-6 levels were higher in ipsilateral DRG than contralateral DRG ($p < 0.01$). Further, an interaction between hormone x time of testing post injection was observed [$F(2, 17) = 5.45, p < 0.05$; Fig. 56D], at 1hr, estradiol-treated rats had lower IL-6 levels than vehicle-treated rats ($p < 0.05$).

IL-2

Basal IL-2 levels following estradiol or vehicle treatment are shown in Figure 57. No significant differences in IL-2 levels in paw (Fig. 57A), DRG (Fig. 57B), or spinal cord (Fig. 57C) following hormone treatment was observed.

IL-2 levels at 1, 5, and 24hrs post carrageenan or saline injection are shown in Figure 58. In paw, a main effect for hormone, and an interaction between pain condition x time of testing post injection, was observed [Hormone main effect: $F(1, 34) = 5.86, p < 0.05$; Interaction: $F(2, 34) = 4.33, p < 0.05$; Fig. 58A]. Rats administered estradiol had lower IL-2 levels than vehicle-treated rats ($p < 0.01$; Fig. 58A). At 1hr, IL-2 levels were significantly lower in carrageenan rats than in saline controls ($p < 0.05$; Fig. 58A). Post hoc analysis revealed that at 1hr, SV rats had elevated paw IL-2 levels compared to all groups tested ($p < 0.05$).

In ipsilateral DRG, as shown in Figure 58B, no differences in IL-2 levels across groups were observed. In spinal cord, as shown in Figure 58C, a main effect for pain condition was observed [$F(1, 35) = 4.35, p < 0.05$]; however, post hoc analysis did not show significant differences between saline- and carrageenan-treated rats. An interaction between hormone x pain condition was observed [$F(1, 35) = 6.52, p < 0.05$]; CV rats had significantly higher spinal cord IL-2 levels than CE and SV rats ($p < 0.05$; Fig. 58C). An interaction between hormone treatment x pain condition x time of testing post injection was observed [$F(2, 35) = 3.41, p < 0.05$]; at 5hrs CV rats had higher IL-2 levels than any other treatment group ($p < 0.01$; Fig. 58C). As shown in Figure 58D, no differences in ipsilateral versus contralateral DRG IL-2 levels following hormone and carrageenan administration was observed.

GM-CSF

Figure 59 shows basal GM-CSF levels in naïve OVX rats following estradiol or vehicle treatment. No differences in basal GM-CSF levels were observed in the paw, DRG, or spinal cord (Figures 59 A, B, and C, respectively).

Figure 60 shows GM-CSF levels 1, 5, and 24hrs post injection following estradiol or vehicle treatment. As shown in Figure 60A, a main effect for time of testing post injection was observed [$F(2, 34) = 12.43, p < 0.01$]; OVX rats had significantly higher paw GM-CSF levels at 1hr than at 5 and 24hrs post injection ($p < 0.01$). In ipsilateral DRG, as shown in Figure 60B, a main effect for time of testing post injection was observed [$F(2, 35) = 6.62, p < 0.01$]; rats had significantly lower GM-CSF levels in ipsilateral DRG at 1hr than at 5 or 24hrs post injection ($p < 0.05$).

In spinal cord, as shown in Figure 60C, a main effect for pain condition was observed [$F(1, 34) = 5.88, p < 0.05$]; carrageenan-treated rats have higher spinal cord GM-CSF levels than saline controls ($p < 0.05$). An interaction between hormone x pain condition on spinal cord GM-CSF levels was observed [$F(1, 34) = 4.55, p < 0.05$; Fig. 60C]; CV rats have higher GM-CSF levels than SV, SE, and CE rats ($p < 0.05$). An interaction between pain condition x time of testing post injection was observed [$F(2, 34) = 5.02, p < 0.05$]; at 5hrs, carrageenan-treated rats have higher GM-CSF levels than saline controls and rats tested at 1 and 24hrs post injection ($p < 0.01$). An interaction between hormone x pain condition x time of testing on spinal cord GM-CSF levels was observed [$F(2, 34) = 4.94, p < 0.05$]; CV rats at 5hrs post injection had significantly higher spinal cord GM-CSF levels than all other treatment groups, with the exception of CV rats measured at 24hrs ($p < 0.05$). Further, at 24hrs CV rats had higher GM-CSF levels than SV rats ($p < 0.05$).

As shown in Figure 60D, a significant main effect between ipsilateral and contralateral DRG GM-CSF levels was observed [$F(1, 17) = 74.08, p < 0.01$]; GM-CSF levels were significantly higher in ipsilateral DRG than in contralateral DRG ($p < 0.01$). An interaction between GM-CSF DRG levels x time of testing post injection was observed [$F(2, 17) = 24.11, p < 0.01$; Fig. 60D]; at 5 and 24hrs post injection, GM-CSF levels were significantly higher in ipsilateral DRG than in contralateral DRG ($p < 0.05$).

IFN- γ

Basal IFN- γ levels in estradiol- and vehicle-treated OVX rats are shown in Figure 61. Hormone treatment did not lead to altered IFN- γ levels in paw, DRG, or spinal cord tissue (Figures 61A, B, and C, respectively).

IFN- γ levels following carrageenan or saline injection in estradiol- and vehicle-treated OVX rats are shown in Figure 62. In paw, as shown in Figure 62A, a main effect for time of testing post injection was observed [$F(2, 32) = 6.66, p < 0.01$]; rats have higher paw IFN- γ levels at 1 hr than at 5 and 24hrs post injection ($p < 0.05$). An interaction between pain condition x time on paw IFN- γ levels was observed [$F(2, 32) = 5.53, p < 0.01$; Fig. 62A]; at 1hr, saline controls had higher paw IFN- γ levels than at 5 and 24hrs post saline injection ($p < 0.01$). An interaction between hormone x pain condition x time was observed [$F(2, 32) = 3.57, p < 0.05$]; at 1hr CV rats had lower IFN- γ levels than SV rats ($p < 0.01$). Further, as shown in Figure 62A, at 24hrs CV rats had higher paw IFN- γ levels than SV rats ($p < 0.01$) and CE rats ($p < 0.05$).

Figures 62B and C show IFN- γ levels 1, 5, and 24hrs post injection in ipsilateral DRG and spinal cord, respectively. No significant differences in ipsilateral DRG or spinal cord IFN- γ levels following hormone or pain condition at any time tested were observed. Figure 62E shows IFN- γ levels in ipsilateral and contralateral DRG following carrageenan administration. A main effect of ipsilateral versus contralateral IFN- γ levels was observed [$F(1, 18) = 18.82, p < 0.01$]; IFN- γ levels were higher in ipsilateral DRG than in contralateral DRG ($p < 0.01$; Fig. 62D).

Anti-inflammatory cytokines:

IL-10

To examine the effects of estradiol administration on anti-inflammatory mediators, IL-10 levels were analyzed. Figure 63 shows IL-10 levels in naïve estradiol- and vehicle-treated OVX rats. No significant differences in paw or ipsilateral DRG IL-10 levels following estradiol administration were observed (Fig. 's 63A and B, respectively). However in spinal cord, as shown in Figure 63C, a main effect for hormone on basal IL-10 levels was observed [$F(1, 5) = 27.14, p < 0.01$]; estradiol replacement led to lower IL-10 levels as compared to vehicle treatment ($p < 0.01$).

Figure 64 shows IL-10 levels 1, 5, and 24hrs post carrageenan or saline injection in estradiol- and vehicle-treated OVX rats. In paw, as shown in Figure 64A, a main effect for pain condition and time of testing post injection was observed [Pain condition main effect: $F(1, 35) = 44.21, p < 0.01$; Time main effect: $F(2, 35) = 13.39, p < 0.01$]; rats administered carrageenan had higher paw IL-10 levels than saline controls ($p < 0.01$). Rats tested 1hr post injection had higher paw IL-10 levels than at 5 and 24hrs post injection ($p < 0.01$; Fig. 64A).

Figure 64B shows IL-10 levels in ipsilateral DRG post injection following estradiol or vehicle treatment. No significant differences in ipsilateral DRG IL-10 levels post hormone or pain condition over time was observed (Fig. 64B). In spinal cord, as shown in Figure 64C, an interaction between hormone x pain condition was observed [$F(1, 35) = 10.26, p < 0.01$]; CE rats had lower spinal cord IL-10 levels than CV rats ($p < 0.05$). However, SE rats had higher spinal cord IL-10 levels than SV rats ($p < 0.05$). Further, CV rats had higher spinal cord IL-10 levels than SV rats ($p < 0.01$). As shown in

Figure 54C, an interaction between hormone x time of testing post injection was observed [$F(2, 35) = 3.45, p < 0.05$]; rats administered estradiol had higher spinal cord IL-10 levels at 24hrs post injection than at 1hr post injection ($p < 0.05$). Post hoc analysis revealed that at 5hrs, CE rats had lower IL-10 levels than CV rats ($p < 0.05$).

Figure 64D shows IL-10 levels in ipsilateral and contralateral DRG post carrageenan administration in estradiol- and vehicle-treated rats. A main effect for ipsilateral versus contralateral IL-10 levels was observed [$F(1, 18) = 23.78, p < 0.01$]; IL-10 levels were higher in ipsilateral DRG than in contralateral DRG ($p < 0.01$).

IL-4

Basal IL-4 levels in estradiol- and vehicle-treated OVX rats are shown in Figure 65. No significant differences in IL-4 levels following estradiol administration were observed in paw, DRG, or spinal cord (Fig.'s 65A, B, and C, respectively).

Figure 66 shows IL-4 levels 1, 5, and 24hrs post carrageenan or saline administration in estradiol- and vehicle-treated OVX rats. As shown in Figures 66A and B, IL-4 levels were not altered following hormone or pain condition in paw or ipsilateral DRG, respectively. As shown in Figure 66C, in spinal cord a main effect for time of testing post injection on IL-4 levels was observed [$F(2, 33) = 3.76, p < 0.05$]; rats had higher spinal cord IL-4 levels 5hrs post injection than at 1 and 24hrs ($p < 0.05$). Further, an interaction between pain condition x time of testing post injection on spinal cord IL-4 levels was observed [$F(2, 33) = 9.01, p < 0.01$]; at 5hrs, carrageenan-treated rats had higher IL-4 levels than saline controls ($p < 0.01$). Carrageenan-treated rats also had higher spinal cord IL-4 levels than saline and carrageenan-treated rats at 1 and 24hrs post

injection ($p < 0.01$; Fig. 66C). As shown in Figure 66D, in ipsilateral and contralateral DRG IL-4 levels were not altered by hormone or pain condition at any time tested.

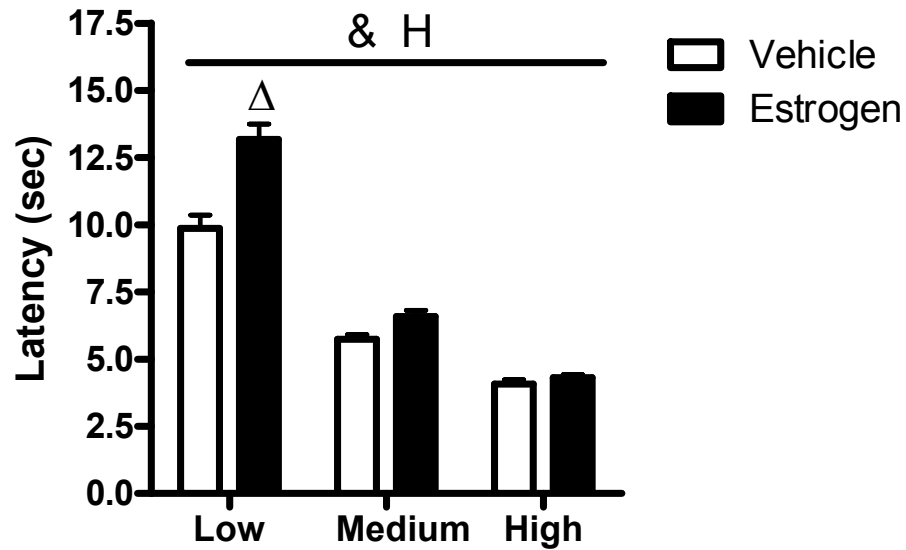


Figure 44. Effects of 17β -estradiol administration on baseline PWL. Data represents mean PWL ($\pm$ SEM) before carrageenan or saline administration at low (4.5mV), medium (4.9mV), and high (5.2mV) heat intensities. (&) Denotes significant main effect of hormone, $p < 0.01$. (H) Denotes significant main effect of heat intensity, $p < 0.01$. (Δ) Denotes significant difference to all other treatment groups, $p < 0.01$. $n = 34-38$ per group.

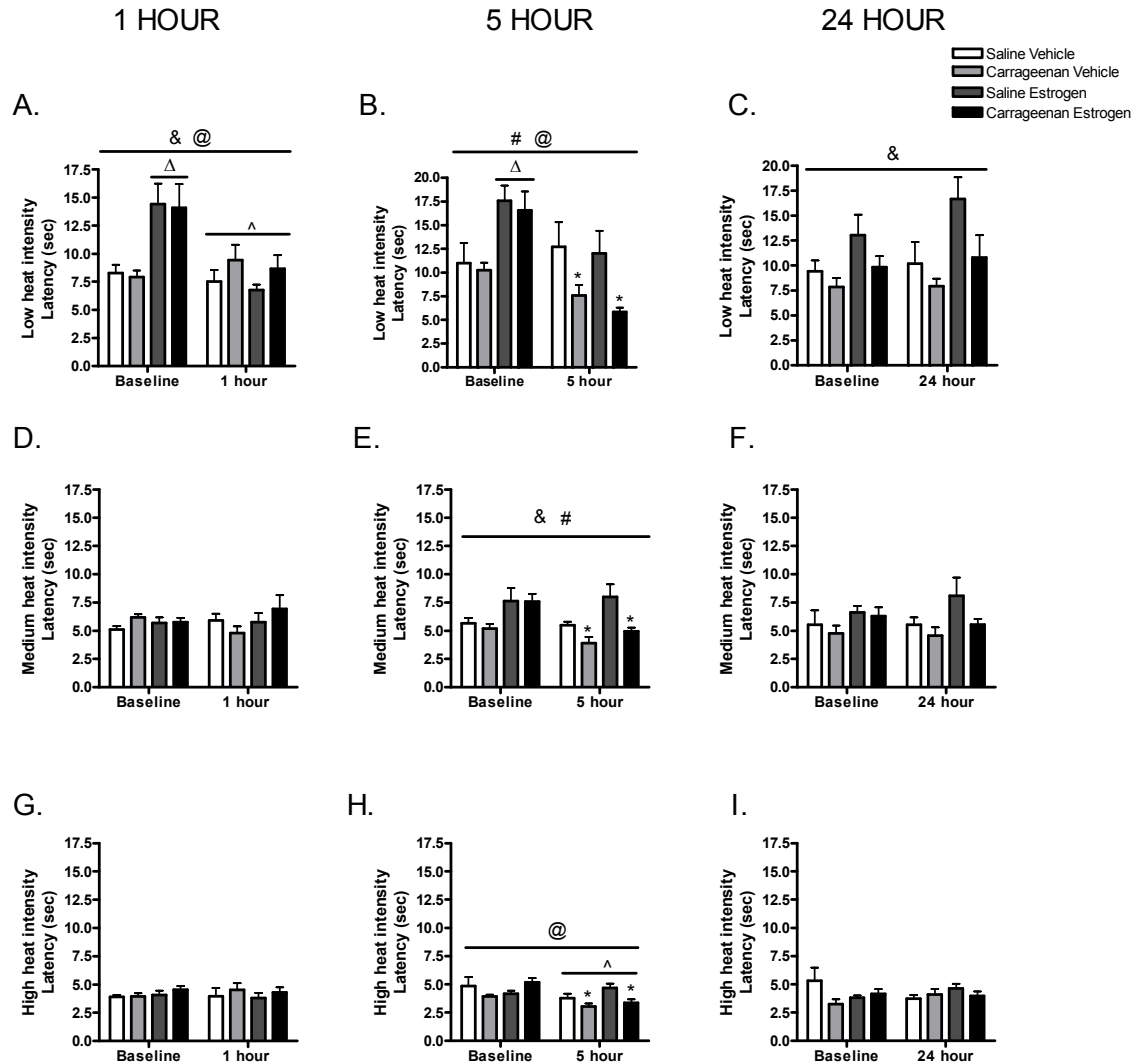


Figure 45. Effects of 17 β -estradiol on ipsilateral paw withdrawal latencies (PWL) at baseline and post 1% carrageenan or saline administration. Data represents mean PWL ($\pm$ SEM) for ipsilateral paw at 1, 5, and 24 hours compared to baseline at low (4.5 mV; **Fig.'s A-C**), medium (4.9 mV; **Fig.'s D-F**), and high (5.2 mV; **Fig.'s G-I**) heat intensities. (&) Denotes a significant hormone main effect, $p < 0.05$. (#) Denotes main effect of pain condition, $p < 0.05$. (@) Denotes time main effect, $p < 0.01$. (^) Denotes a significant difference in PWL compared to baseline, $p < 0.01$. (*) Represents significant difference to saline, $p < 0.05$. (Δ) Denotes significant difference to all other treatment groups, $p < 0.05$. $n = 4-7$ per group.

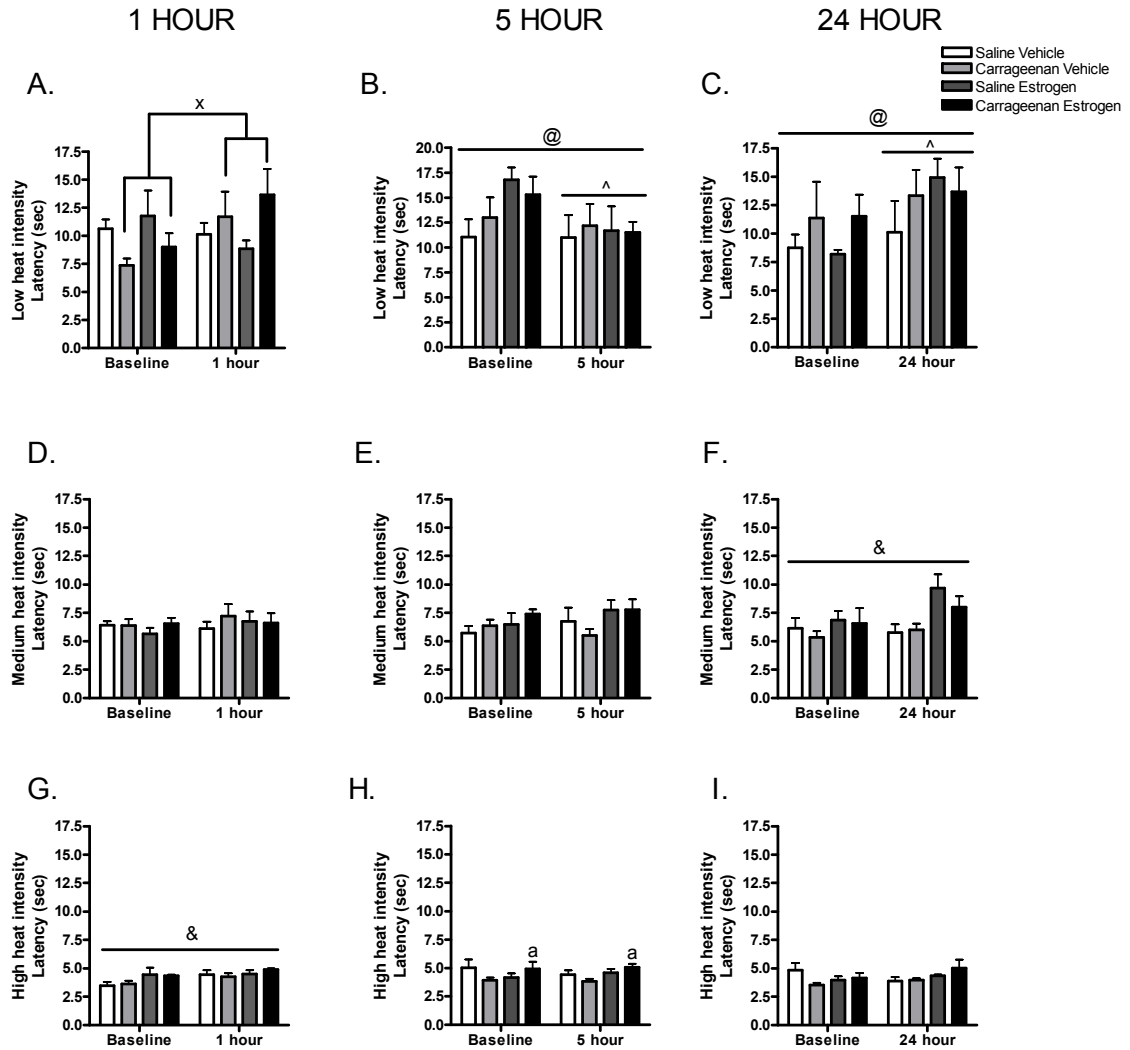


Figure 46. Effects of 17 β -estradiol on contralateral paw withdrawal latencies (PWL) at baseline and post 1% carrageenan or saline administration. Data represents mean PWL ($\pm$ SEM) for the contralateral paw at 1, 5, and 24 hours compared to baseline at low (4.5 mV; **Fig.'s A-C**), medium (4.9 mV; **Fig.'s D-F**), and high (5.2 mV; **Fig.'s G-I**) heat intensities. (&) Denotes a significant hormone effect, $p < 0.05$. (@) Denotes time main effect, $p < 0.05$. (^) Denotes a significant change in PWL compared to baseline, $p < 0.05$. (a) Represents a significant difference to carrageenan vehicle, $p < 0.05$. (x) Denotes significant difference in PWL between baseline and 1hr post carrageenan injection, $p < 0.01$. $n = 4-7$ per group.

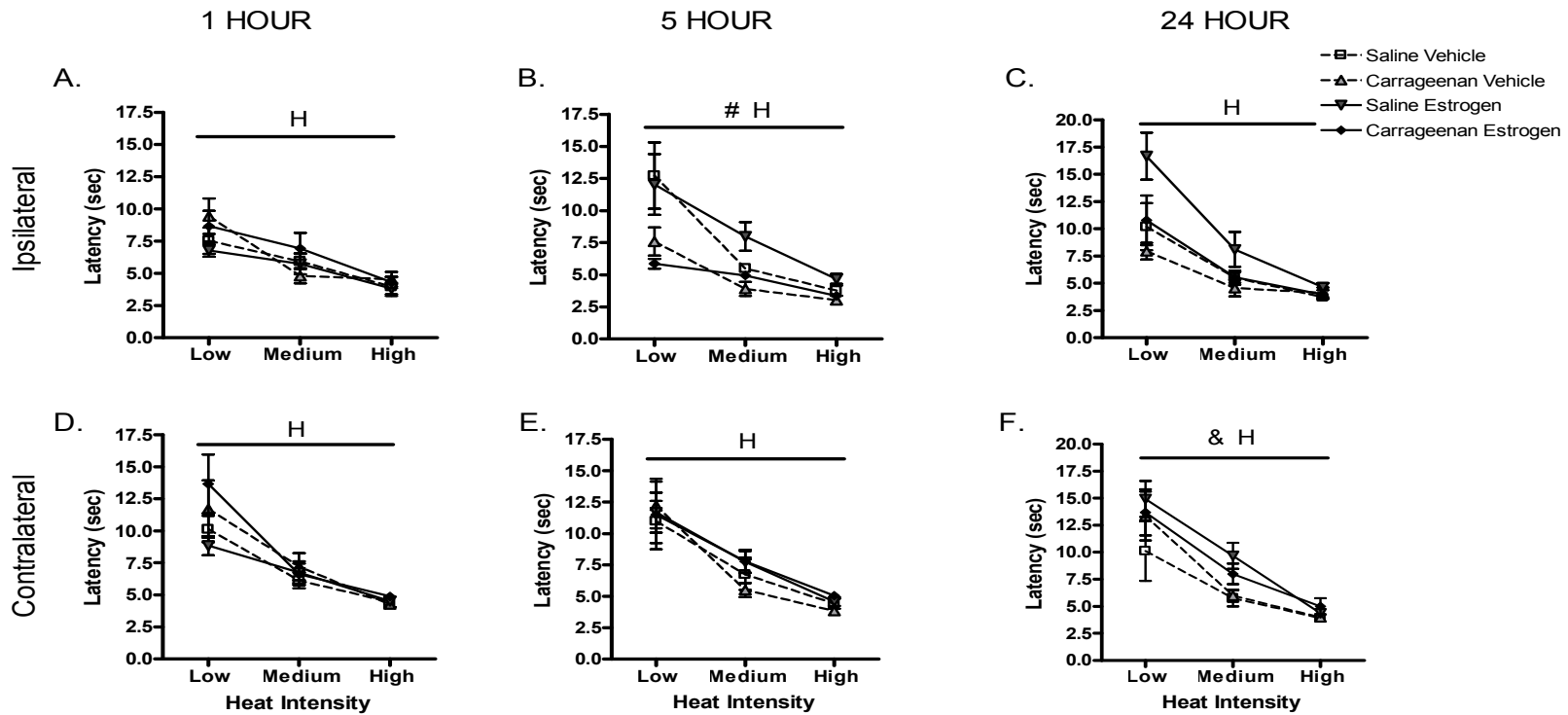


Figure 47. Effects of 17β -estradiol on paw withdrawal latencies (PWL) over heat intensity 1, 5, and 24 hours after 1% carrageenan or saline administration in the ipsilateral (A-C) and contralateral (D-F) paw. **Figures A-C.** Data represents mean ipsilateral PWL ($\pm$ SEM) 1, 5, and 24 hours post injection. **Figures D-F.** Data represents mean contralateral PWL ($\pm$ SEM) 1, 5, and 24 hours post injection. (H) Denotes a significant difference between low, medium, and high heat intensities, $p < 0.01$. (&) Represents a significant main effect of hormone, $p < 0.05$. (#) Denotes a significant main effect of pain condition, $p < 0.01$. $n = 4-7$ per group.

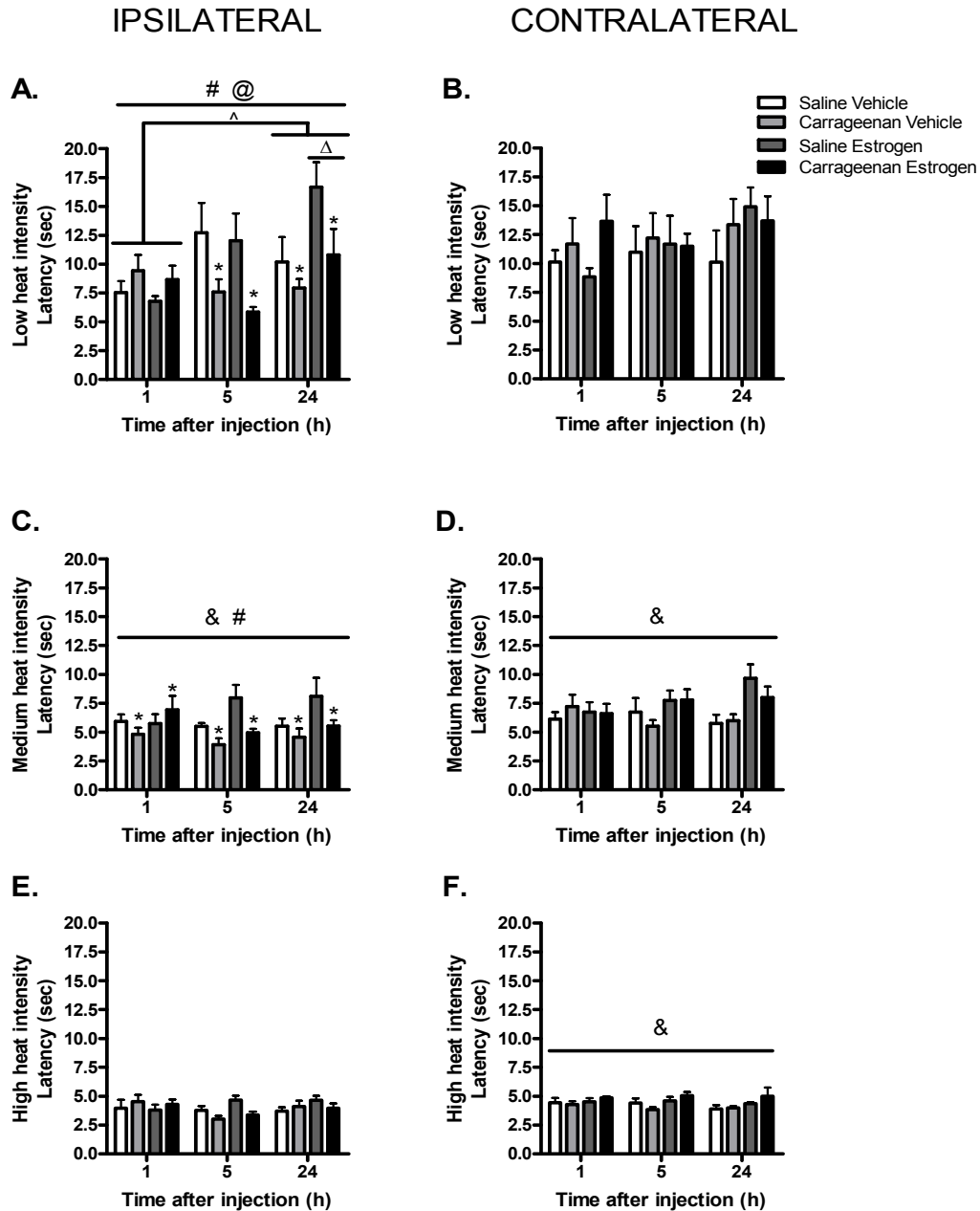


Figure 48. Effects of 17β -estradiol on PWL across time following carrageenan or saline administration. Data represents mean PWL ($\pm$ SEM) to low (Fig.'s A-B), medium (Fig.'s C-D) and high (Fig.'s E-F) heat intensities in the ipsilateral and contralateral paw. (#) Represents main effect of pain condition, $p < 0.05$. (&) Represents hormone main effect, $p < 0.05$. (@) Denotes time main effect, $p < 0.05$. (*) Represents significant difference to saline, $p < 0.01$. (Δ) Denotes significant difference to all other treatment groups, $p < 0.05$. (^) Denotes significant difference in PWL between 1 and 24hr groups, $p < 0.01$. $n = 4-7$ per group.

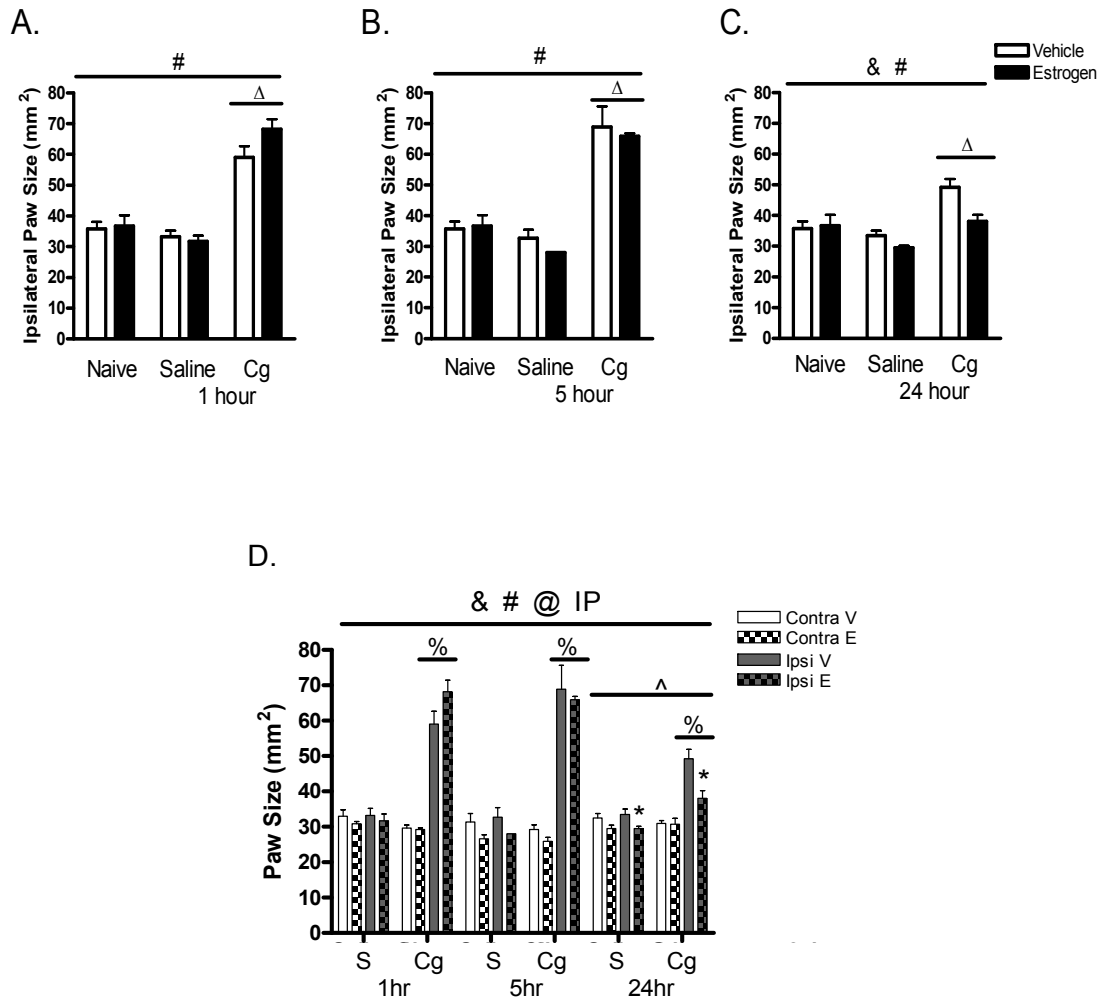


Figure 49. Changes in paw size following 17 β -estradiol treatment in naïve, saline-, and carrageenan-treated rats. In all figures, (&) denotes significant hormone main effect, $p < 0.05$; (#) denotes significant main effect of pain condition, $p < 0.01$. **Figures A-C.** Effects of carrageenan (cg) and estradiol on paw size at 1hr (**Fig. A.**), 5 hrs (**Fig. B.**), and 24 hrs (**Fig. C.**) post injection compared to naïve and saline treatment groups. (Δ) Denotes significant difference to naïve and saline-treated rats. **Figure D.** Effects of estradiol in the contralateral and ipsilateral paws following saline (s) or cg administration at 1, 5, and 24 hrs post injection. (@) Denotes time main effect, $p < 0.01$. (IP) Represents significant main effect between ipsilateral and contralateral paw size, $p < 0.01$. (^) Denotes significant difference of paw sizes at 24 hrs compared to 1 and 5 hrs post injection, $p < 0.01$. (%) Denotes significant difference to saline treatment and to the contralateral paw, $p < 0.01$. (*) Denotes significant difference to ipsilateral vehicle at 24hrs, $p < 0.01$. $n = 4-7$ per group.

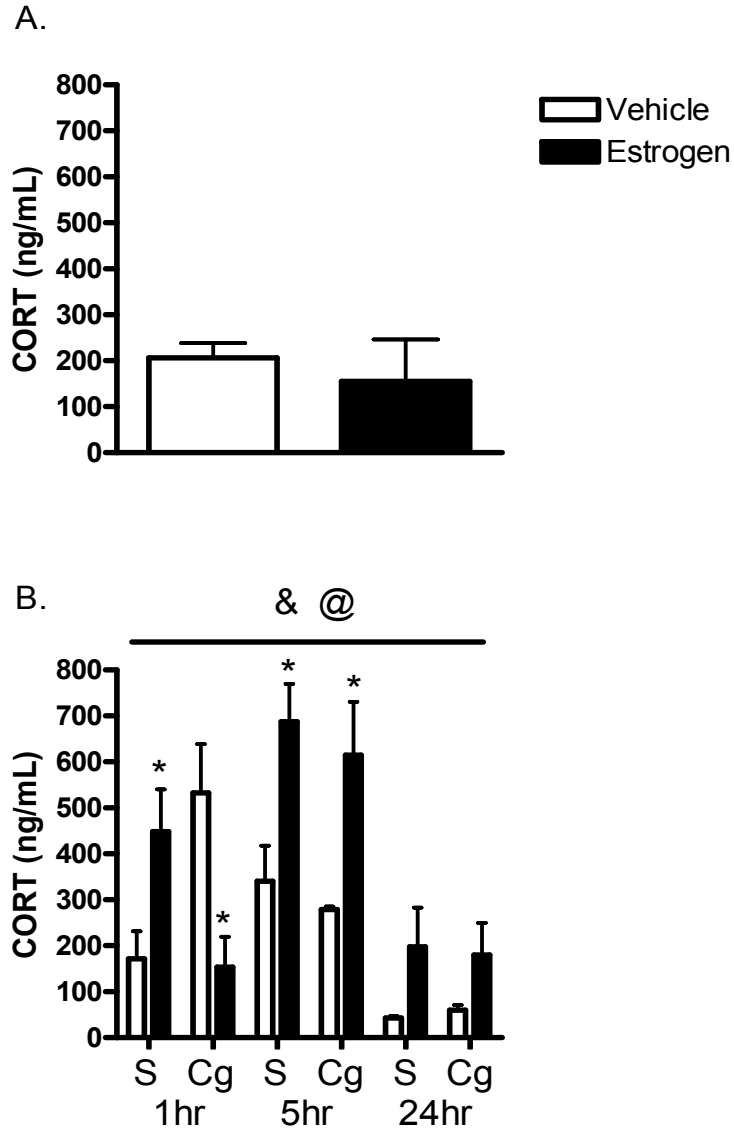


Figure 50. The effect of estradiol administration on corticosterone (CORT) levels. **Figure A.** Mean CORT levels ($\pm$ SEM) in OVX naïve rats following estradiol or vehicle administration. Here, estradiol does not alter CORT levels. **Figure B.** CORT levels in saline- and carrageenan-treated rats sacrificed at 1, 5, or 24 hours post injection. (&) Denotes significant hormone main effect, $p < 0.01$. (@) Denotes significant time effect, $p < 0.01$. (*) Represents significant difference to vehicle, $p < 0.05$. $n = 4$ per group.

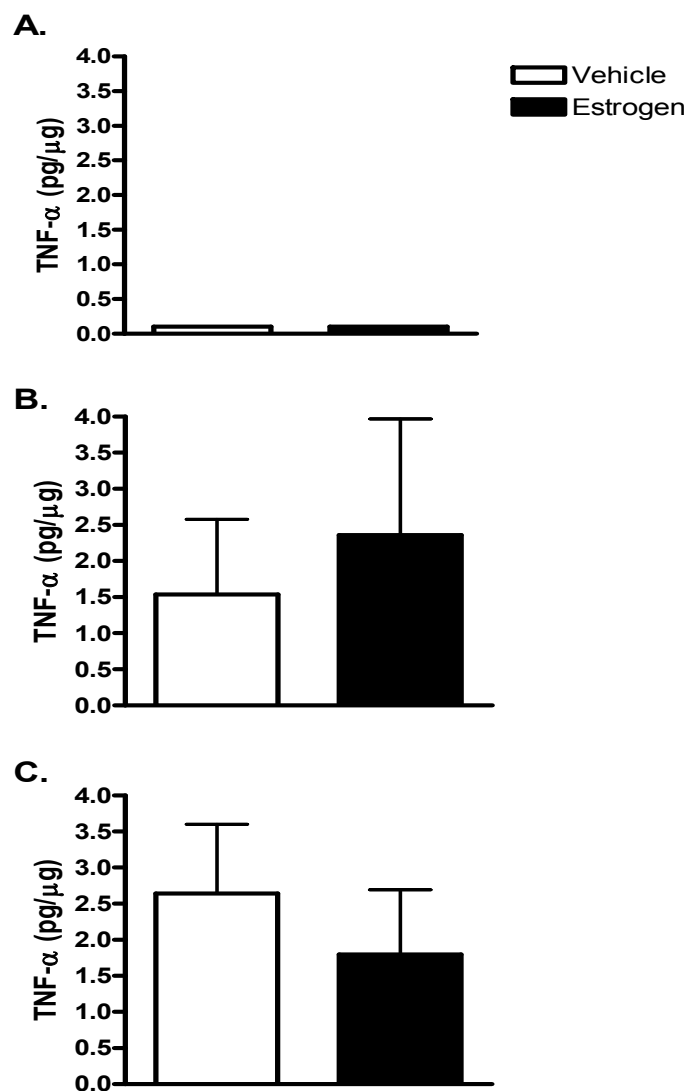


Figure 51. TNF- α levels in paw (A), DRG (B), and spinal cord (C) of OVX naïve rats administered 17 β -estradiol or vehicle (cholesterol). Data represents mean TNF- α levels ($\pm$ SEM). No significant difference in TNF- α levels following estradiol administration was observed. $n = 4$ per group.

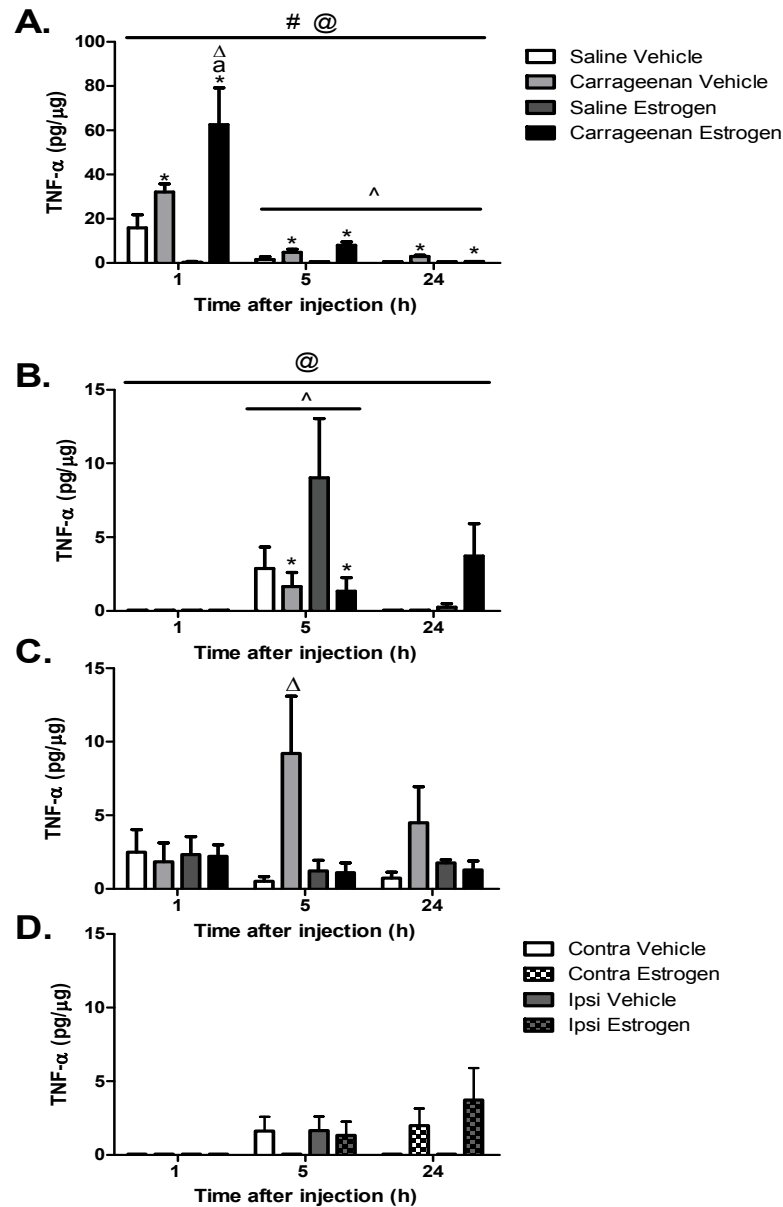


Figure 52. TNF- α levels measured 1, 5, and 24hrs post carrageenan or saline injection in estradiol- or vehicle-treated OVX rats. Data represents mean TNF- α levels ($\pm$ SEM). **Figures A-C.** TNF- α levels in paw (A), ipsilateral DRG (B), and spinal cord (C). (#) Represents main effect of pain condition, $p < 0.01$. (@) Represents time main effect, $p < 0.01$. (*) Represents significant difference to saline, $p < 0.05$. (^) Denotes significant difference in TNF- α levels at the specified time, $p < 0.05$. (a) Represents significant difference to carrageenan vehicle-treated rats, $p < 0.05$. (Δ) Denotes significant difference to all other treatment groups, $p < 0.01$. **Figure D.** TNF- α levels in ipsilateral and contralateral DRG following carrageenan administration in estradiol- and vehicle-treated rats. No significant differences were observed. $n = 4$ per group.

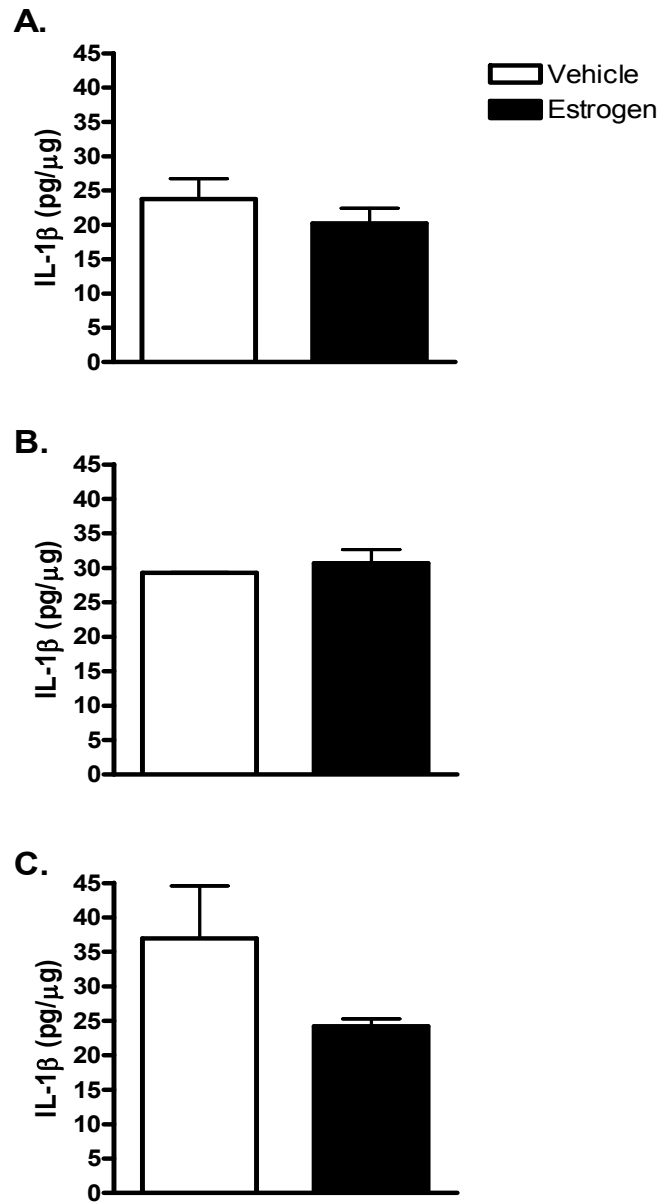


Figure 53. IL-1 β levels in paw (A), DRG (B), and spinal cord (C) of OVX naïve rats administered 17 β -estradiol or vehicle (cholesterol). Data represents mean IL-1 β levels ($\pm$ SEM). No significant difference in IL-1 β levels following estradiol administration was observed. $n = 4$ per group.

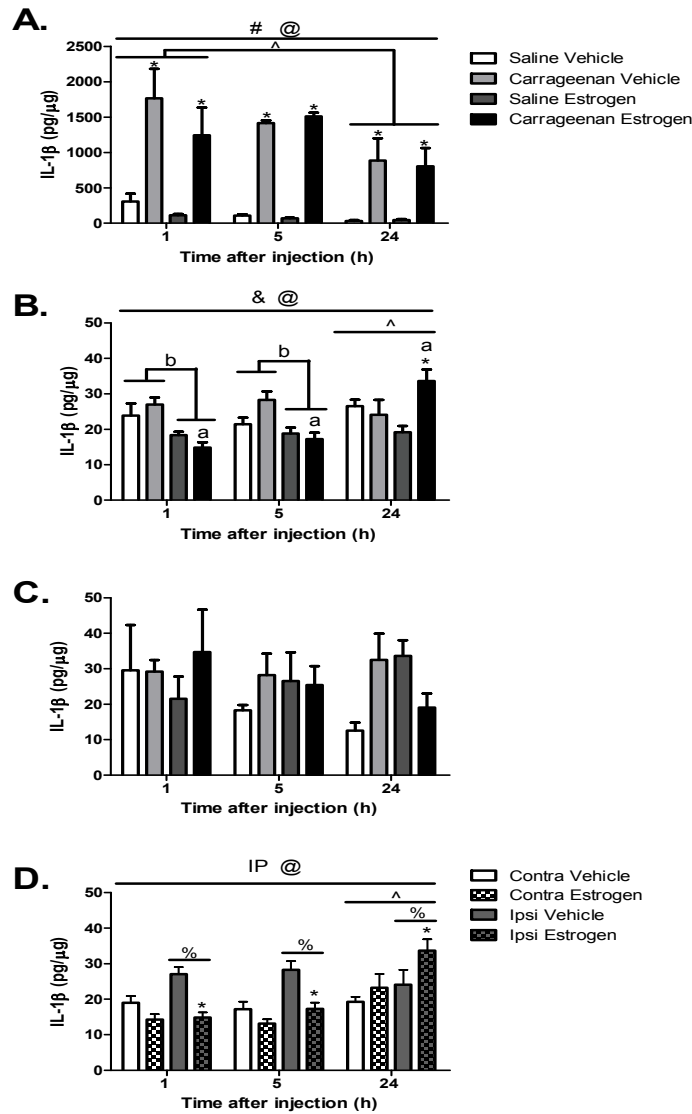


Figure 54. IL-1 β levels measured 1, 5, and 24hrs post carrageenan or saline injection in estradiol- or vehicle-treated OVX rats. Data represents mean IL-1 β levels ($\pm$ SEM). In all figures, (@) represents time main effect, $p < 0.05$. (^) Denotes significant difference in IL-1 β levels at the specified time, $p < 0.05$. **Figures A-C.** IL-1 β levels in paw (A), ipsilateral DRG (B), and spinal cord (C). (#) Represents main effect of pain condition, $p < 0.01$. (&) Represents hormone main effect, $p < 0.01$. (*) Represents significant difference to saline, $p < 0.05$. (a) Represents significant difference to carrageenan vehicle-treated rats, $p < 0.01$. (b) Denotes significant difference to vehicle treated rats at 1 and 5hrs, $p < 0.01$. **Figure D.** IL-1 β levels in ipsilateral and contralateral DRG following carrageenan administration in estradiol- and vehicle-treated rats. (IP) Denotes significant main effect between ipsilateral and contralateral DRG IL-1 β levels, $p < 0.01$. (%) Denotes higher IL-1 β levels in ipsi DRG as compared to contra DRG, $p < 0.01$. (*) Represents significant difference to vehicle, $p < 0.05$. $n = 4$ per group.

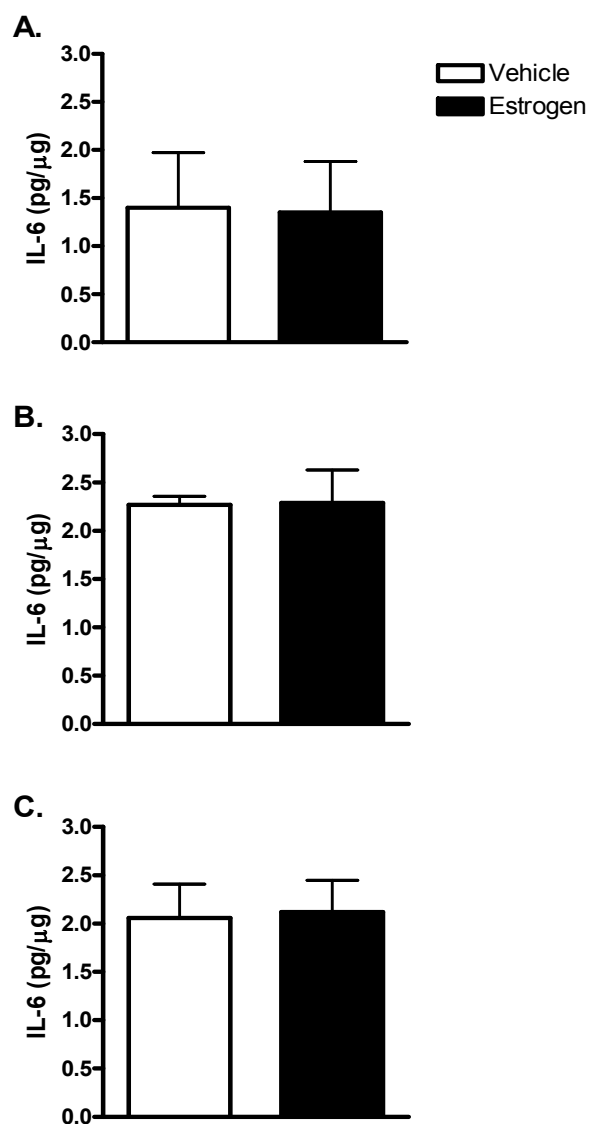


Figure 55. IL-6 levels in paw (A), DRG (B), and spinal cord (C) of OVX naïve rats administered 17 β -estradiol or vehicle (cholesterol). Data represents mean IL-6 levels ($\pm$ SEM). No significant difference in IL-6 levels following estradiol administration was observed. $n = 4$ per group.

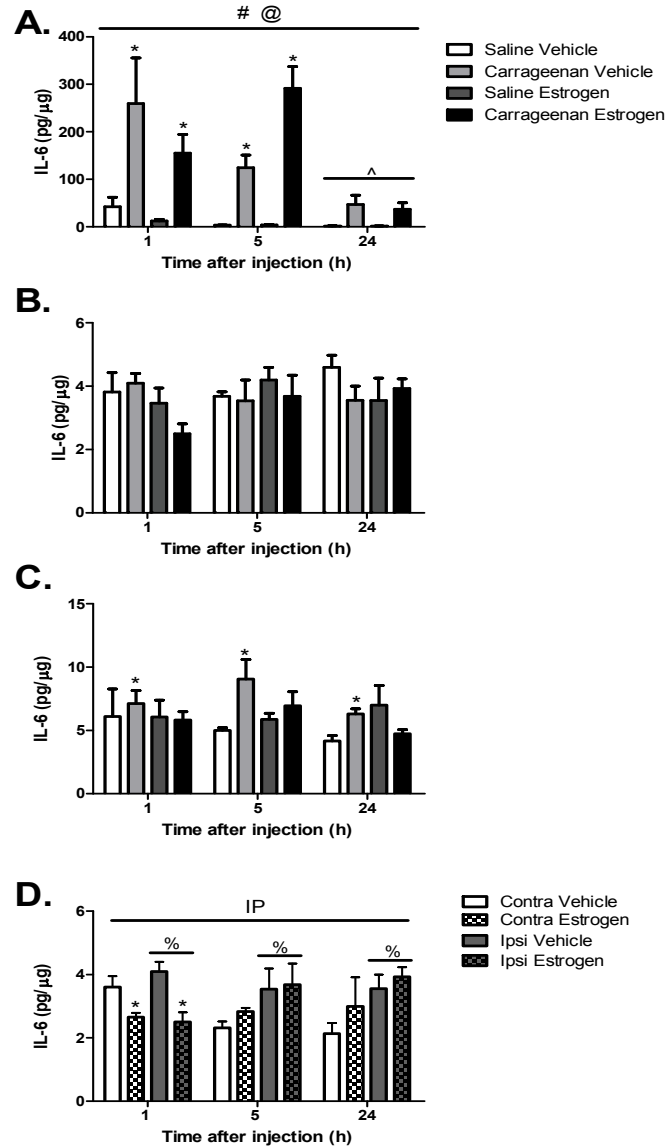


Figure 56. IL-6 levels measured 1, 5, and 24hrs post carrageenan or saline injection in estradiol- or vehicle-treated OVX rats. Data represents mean IL-6 levels ($\pm$ SEM). **Figures A-C.** IL-6 levels in paw (A), ipsilateral DRG (B), and spinal cord (C). (#) Represents main effect of pain condition, $p < 0.01$. (@) Represents time main effect, $p < 0.01$. (*) Represents significant difference to saline, $p < 0.05$. (^) Denotes significant difference in IL-6 levels at the specified time, $p < 0.01$. **Figure D.** IL-6 levels in ipsilateral and contralateral DRG following carrageenan administration in estradiol- and vehicle-treated rats. (IP) Denotes significant main effect between ipsilateral and contralateral DRG IL-6 levels, $p < 0.01$. (%) Denotes higher IL-6 levels in ipsi DRG as compared to contra DRG, $p < 0.01$. $n = 4$ per group.

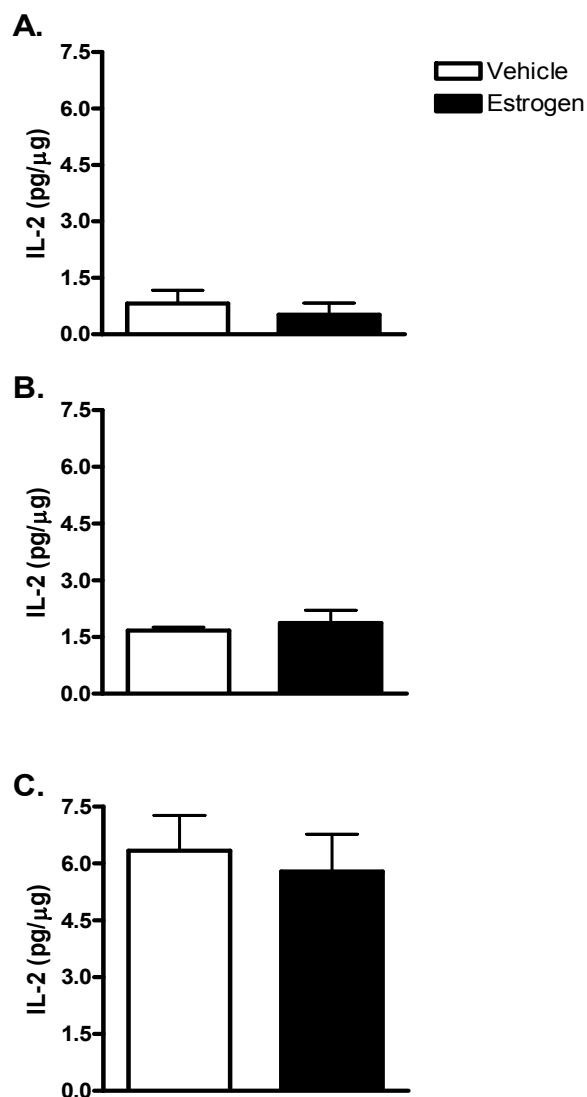


Figure 57. IL-2 levels in paw (A), DRG (B), and spinal cord (C) of OVX naïve rats administered 17 β -estradiol or vehicle (cholesterol). Data represents mean IL-2 levels ($\pm$ SEM). No significant difference in IL-2 levels following estradiol administration was observed. $n = 4$ per group.

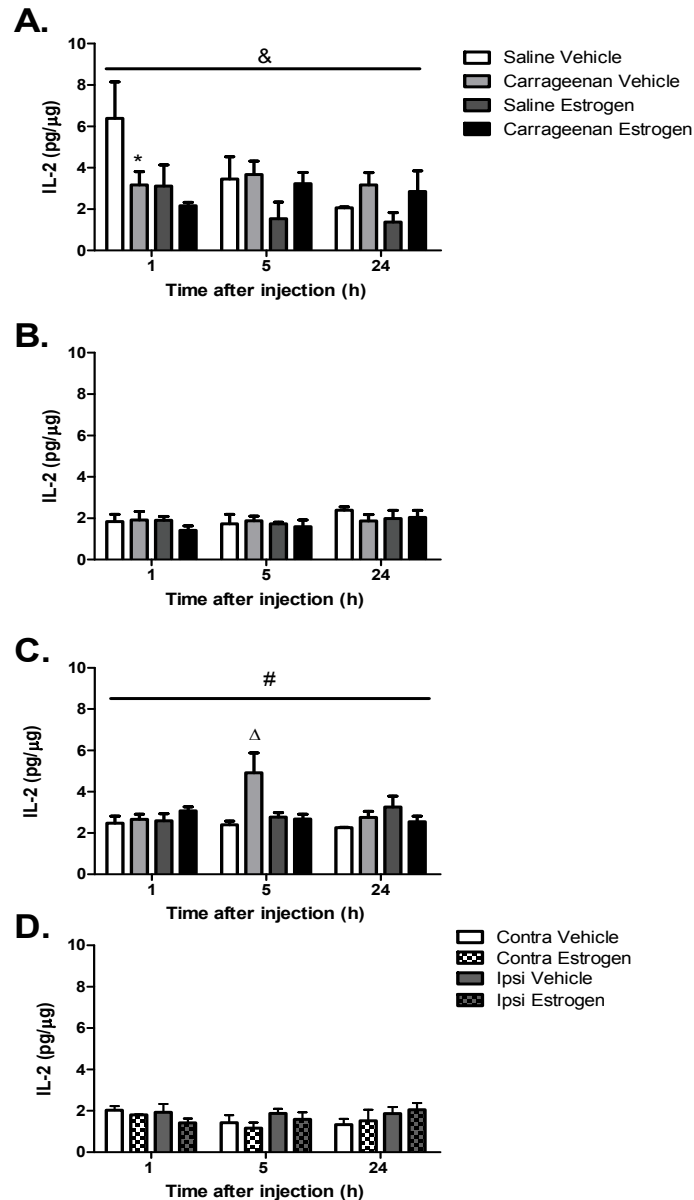


Figure 58. IL-2 levels measured 1, 5, and 24hrs post carrageenan or saline injection in estradiol- or vehicle-treated OVX rats. Data represents mean IL-2 levels ($\pm$ SEM). **Figures A-C.** IL-2 levels in paw (A), ipsilateral DRG (B), and spinal cord (C). (#) Represents main effect of pain condition, $p < 0.05$. (&) Represents hormone main effect, $p < 0.05$. (*) Represents significant difference to saline, $p < 0.05$. (^) Denotes significant difference in IL-2 levels at the specified time, $p < 0.01$. (Δ) Denotes significant difference to all other treatment groups, $p < 0.01$. **Figure D.** IL-2 levels in ipsilateral and contralateral DRG following carrageenan administration in estradiol- and vehicle-treated rats. No significant differences were observed. $n = 4$ per group.

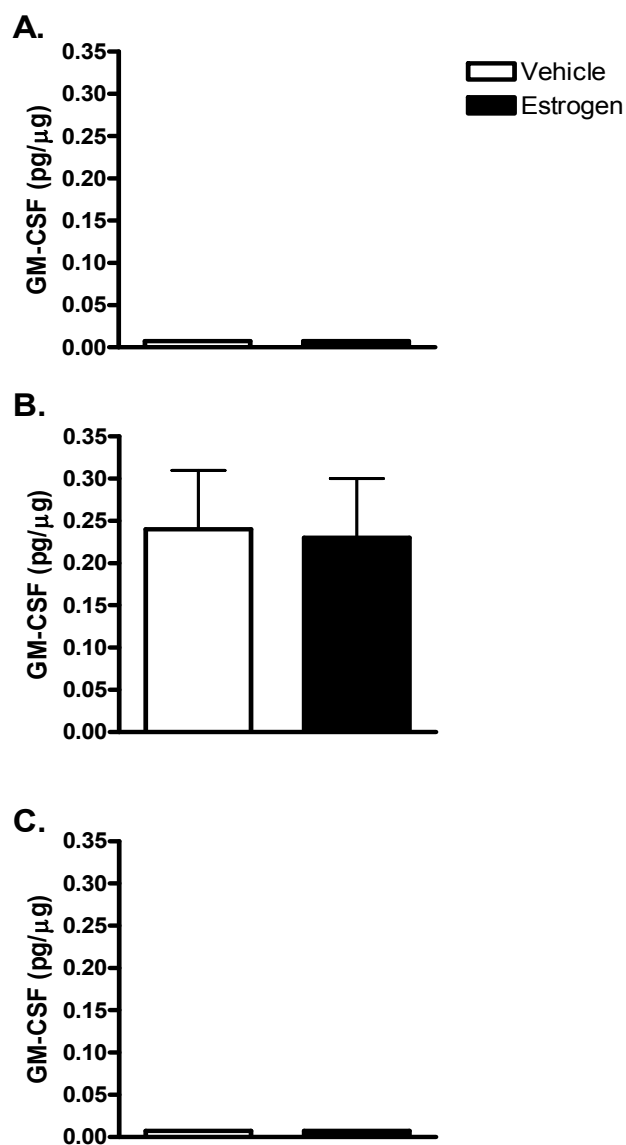


Figure 59. GM-CSF levels in paw (A), DRG (B), and spinal cord (C) of OVX naïve rats administered 17 β -estradiol or vehicle (cholesterol). Data represents mean GM-CSF levels ($\pm$ SEM). No significant difference in GM-CSF levels following estradiol administration was observed. $n = 4$ per group.

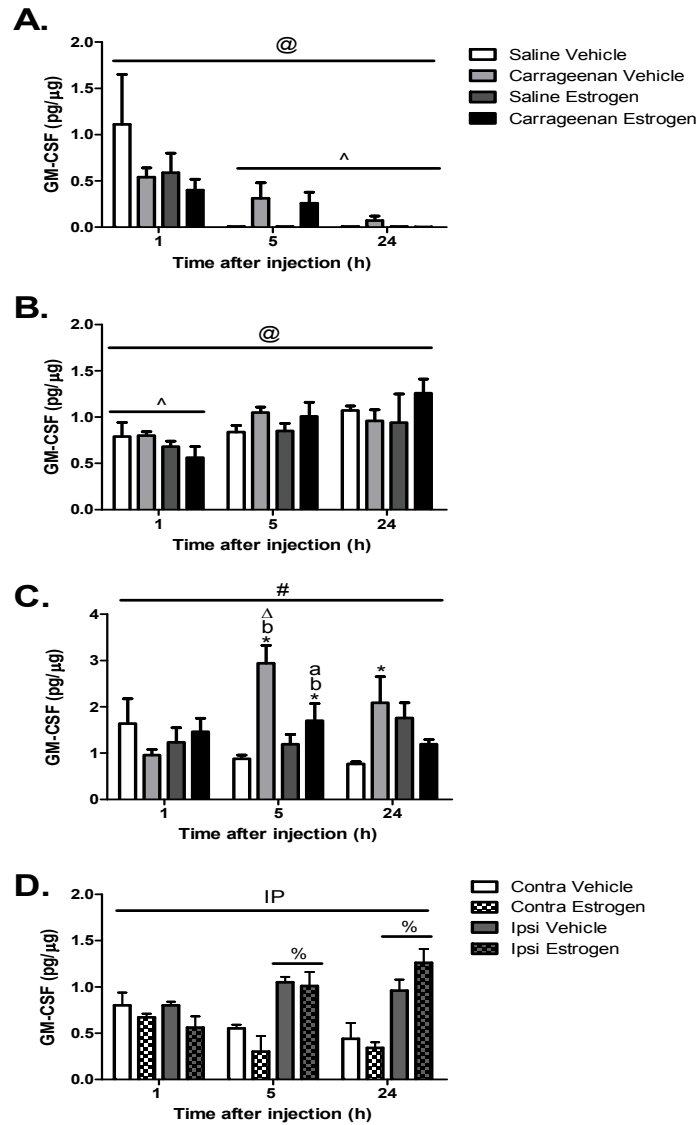


Figure 60. GM-CSF levels measured 1, 5, and 24hrs post carrageenan or saline injection in estradiol- or vehicle-treated OVX rats. Data represents mean GM-CSF levels ($\pm$ SEM). **Figures A-C.** GM-CSF levels in paw (A), ipsilateral DRG (B), and spinal cord (C). (#) Represents main effect of pain condition, $p < 0.05$. (@) Represents time main effect, $p < 0.01$. (^) Denotes significant difference in GM-CSF levels at the specified time, $p < 0.05$. (*) Represents significant difference to saline, $p < 0.05$. (a) Represents significant difference to carrageenan vehicle-treated rats, $p < 0.05$. (b) Denotes significant difference to saline or carrageenan estradiol-treated rats, $p < 0.05$. (Δ) Denotes significant difference to all other treatment groups (except carrageenan vehicle-treated rats at 24hrs), $p < 0.05$. **Figure D.** GM-CSF levels in ipsilateral and contralateral DRG following carrageenan administration in estradiol- and vehicle-treated rats. (IP) Denotes significant main effect between ipsilateral and contralateral DRG GM-CSF levels, $p < 0.01$. (%) Denotes higher GM-CSF levels in ipsi DRG as compared to contra DRG, $p < 0.01$. $n = 4$ per group.

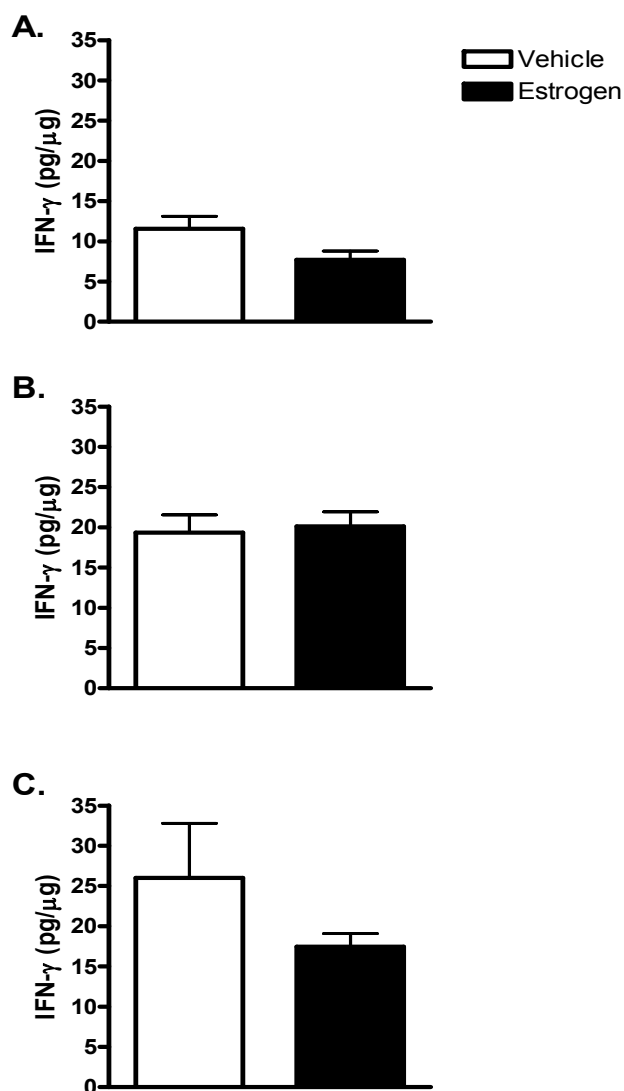


Figure 61. IFN- γ levels in paw (A), DRG (B), and spinal cord (C) of OVX naïve rats administered 17 β -estradiol or vehicle (cholesterol). Data represents mean IFN- γ levels ($\pm$ SEM). No significant difference in IFN- γ levels following estradiol administration was observed. $n = 4$ per group.

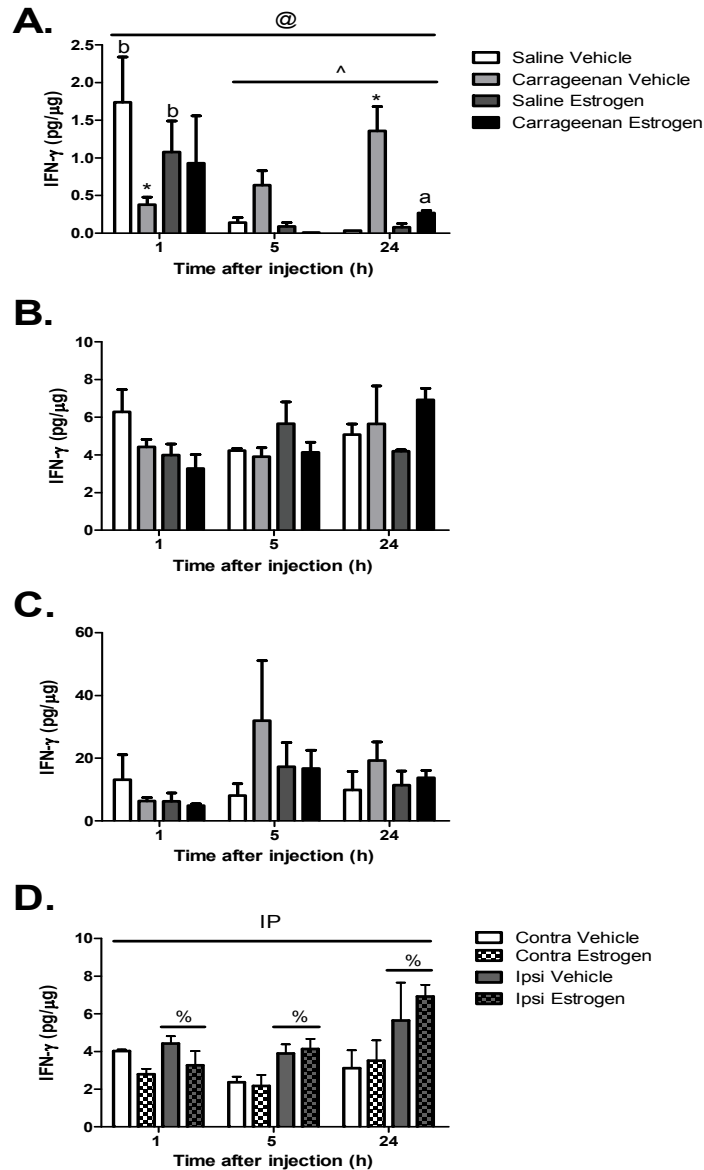


Figure 62. IFN- γ levels measured 1, 5, and 24hrs post carrageenan or saline injection in estradiol- or vehicle-treated OVX rats. Data represents mean IFN- γ levels ($\pm$ SEM). **Figures A-C.** IFN- γ levels in paw (A), ipsilateral DRG (B), and spinal cord (C). (@) Represents time main effect, $p < 0.01$. (^) Denotes significant difference in IFN- γ levels at the specified time, $p < 0.05$. (*) Represents significant difference to saline, $p < 0.01$. (a) Represents significant difference to carrageenan vehicle-treated rats, $p < 0.05$. (b) Denotes significant difference to saline treated rats at 5 and 24hrs, $p < 0.01$. **Figure D.** IFN- γ levels in ipsilateral and contralateral DRG following carrageenan administration in estradiol- and vehicle-treated rats. (IP) Denotes significant main effect between ipsilateral and contralateral DRG IFN- γ levels, $p < 0.01$. (%) Denotes higher IFN- γ levels in ipsi DRG as compared to contra DRG, $p < 0.01$. $n = 4$ per group.

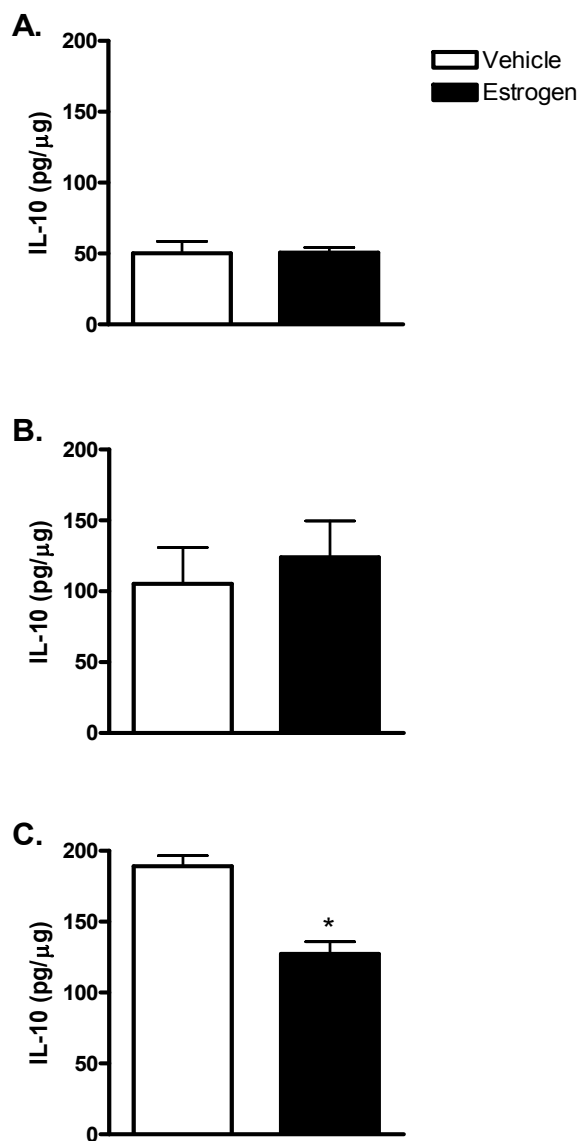


Figure 63. IL-10 levels in paw (A), DRG (B), and spinal cord (C) of OVX naïve rats administered 17 β -estradiol or vehicle (cholesterol). Data represents mean IL-10 levels ($\pm$ SEM). (*) Represents significant difference in IL-10 levels following estradiol administration, $p < 0.01$. $n = 4$ per group.

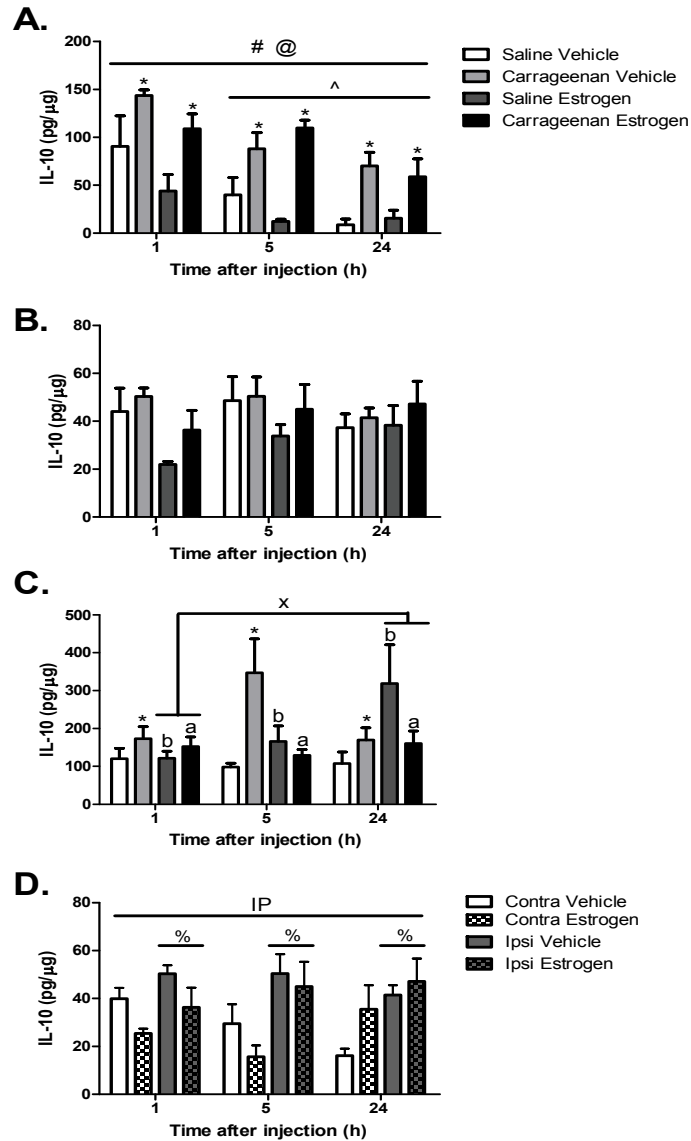


Figure 64. IL-10 levels measured 1, 5, and 24hrs post carrageenan or saline injection in estradiol- or vehicle-treated OVX rats. Data represents mean IL-10 levels ($\pm$ SEM). **Figures A-C.** IL-10 levels in paw (A), ipsilateral DRG (B), and spinal cord (C). (#) Represents main effect of pain condition, $p < 0.01$. (@) Represents time main effect, $p < 0.01$. (^) Denotes significant difference in IL-10 levels at the specified time, $p < 0.01$. (*) Represents significant difference to saline, $p < 0.01$. (a) Represents significant difference to carrageenan vehicle-treated rats, $p < 0.05$. (b) Denotes significant difference to saline vehicle-treated rats, $p < 0.01$. (x) Denotes significant difference between IL-10 levels in estradiol-treated rats at 1 and 24hrs, $p < 0.05$. **Figure D.** IL-10 levels in ipsilateral and contralateral DRG following carrageenan administration in estradiol- and vehicle-treated rats. (IP) Denotes significant main effect between ipsilateral and contralateral DRG IL-10 levels, $p < 0.01$. (%) Denotes higher IL-10 levels in ipsi DRG as compared to contra DRG, $p < 0.01$. $n = 4$ per group.

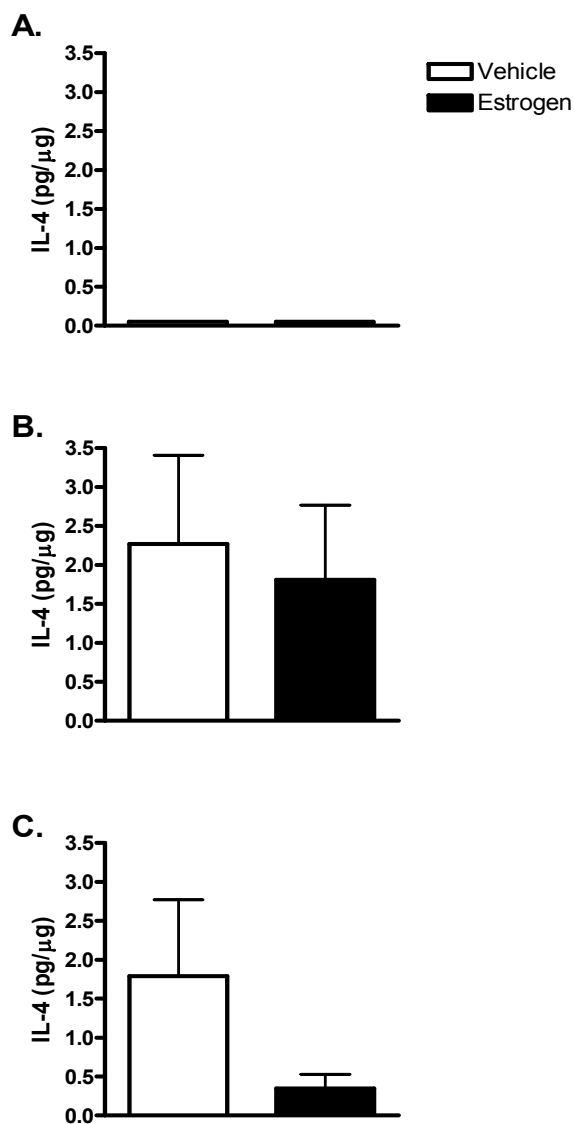


Figure 65. IL-4 levels in paw (A), DRG (B), and spinal cord (C) of OVX naïve rats administered 17 β -estradiol or vehicle (cholesterol). Data represents mean IL-4 levels ($\pm$ SEM). No significant difference in IL-4 levels following estradiol administration was observed. $n = 4$ per group.

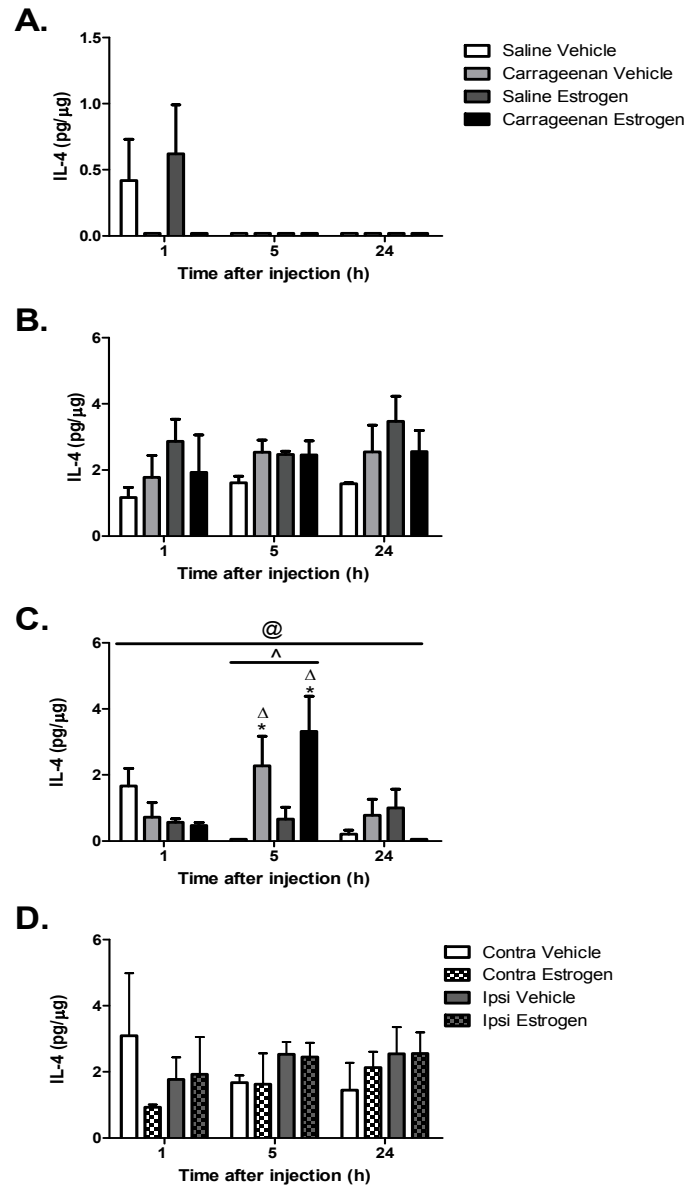


Figure 66. IL-4 levels measured 1, 5, and 24hrs post carrageenan or saline injection in estradiol- or vehicle-treated OVX rats. Data represents mean IL-4 levels ($\pm$ SEM). **Figures A-C.** GM-CSF levels in paw (A), ipsilateral DRG (B), and spinal cord (C). (@) Represents time main effect, $p < 0.05$. (^) Denotes significant difference in IL-4 levels at the specified time, $p < 0.05$. (*) Represents significant difference to saline, $p < 0.01$. (Δ) Denotes significant difference to saline- and carrageenan-treated rats at 1 and 24hrs, $p < 0.01$. **Figure D.** IL-4 levels in ipsilateral and contralateral DRG following carrageenan administration in estradiol- and vehicle-treated rats. No significant differences were observed. $n = 4$ per group.

4. Discussion

To understand the role of estrogen on cytokine levels during persistent inflammatory pain, we investigated estradiol effects on carrageenan-induced behavioral responses and inflammatory mediators over time. The results of this study extend preliminary findings indicating that estradiol administration alters nociceptive effects following carrageenan administration. First, estradiol administration increased PWL to heat before the administration of carrageenan, and lowered spinal cord IL-10 levels in naïve rats. Second, following carrageenan administration, pretreatment of estradiol increased PWL in ipsilateral and contralateral paws. Furthermore, estradiol administration lowered paw size, increased CORT, and altered cytokine levels following carrageenan or saline injection. Together, these results indicate that in this model exogenous estradiol administration is effective at hampering pain responses at the site of injury and via the cytokine pathway over a prolonged inflammatory state.

Estradiol effects on behavioral responses before and during carrageenan-induced pain. Previous work in our lab demonstrated that estradiol reduces nociceptive flinching responses during the formalin paradigm (Hunter et al., in press; Kuba et al., 2005, 2006). Further, preliminary findings using the carrageenan model showed that 20% estradiol administration increases PWL at baseline and 1 and 5hrs post carrageenan injection (Hunter et al., personal communication, 2008). Similarly, this study demonstrated that estradiol increased PWL both before and after pain administration. It was found that at baseline, estradiol-treated rats had increased sensitivity thresholds to heat. This demonstrates that even before the introduction of painful stimuli (i.e. carrageenan injection), estradiol administration decreases sensitivity to pain, suggesting

that previously reported estradiol anti-hyperalgesic effects are in part mediated before injury or nociception.

During carrageenan-induced inflammation, estradiol administration increased PWL to heat in both the ipsilateral and contralateral paws. Furthermore, estradiol administration increased PWL 24hrs post carrageenan injection, demonstrating that estradiol's anti-hyperalgesic responses can occur for an extended period of time. These finding supports research demonstrating that 17β -estradiol decreases carrageenan-induced inflammatory responses. For instance, estradiol administration lowers inflammation following pleurisy (lung injury; Cuzzocrea et al., 2000, 2001). Furthermore, Esposito and colleagues (2005) found that pretreatment with 17β -estradiol and raloxifene, a selective estrogen receptor agonist, attenuates carrageenan-induced inflammation as compared to controls. In contrast, Misiewicz et al. (1996) found that pretreatment with the estrogen antagonist tamoxifen decreased carrageenan-induced inflammatory responses. However, Misiewicz investigated inflammation in LEW/N female rats, which are relatively more susceptible to disease and thus may be more prone to the inflammatory effects of carrageenan than Sprague Dawley rats.

Estradiol effects on paw size. Estradiol administration did not alter paw size in naïve rats, suggesting that estradiol does not affect basal paw volume. In carrageenan-treated rats, it was shown that estradiol replacement decreases ipsilateral paw size 24hrs post carrageenan and saline injection. Furthermore, estradiol decreased contralateral paw size at all time points tested as compared to vehicles following carrageenan or saline administration. Similarly, Esposito et al. (2005) found that estradiol administration lowers paw edema resulting from carrageenan injection by decreasing

polymorphonuclear cell infiltration, COX-2 and iNOS (inducible nitric oxide synthase) activity in the inflamed tissue. Taken together, our results show that estrogen reduces inflammation in peripheral tissue, which may be a mechanism through which estrogen decreases behavioral responses to pain. For instance, PWL was higher in estradiol-treated rats 24hrs post injection than in vehicles in both the ipsilateral and contralateral paws. A reduction in paw edema following estradiol replacement explains this increased threshold to pain.

Estradiol effects on CORT release. Exogenous estradiol administration did not alter basal CORT levels as compared to vehicles. However, estradiol increased serum CORT levels in saline-treated rats 1hr post injection, and both saline and carrageenan-treated rats 5hrs post injection, supporting previous research that estradiol up-regulates GC release (Ferrini et al., 1993; Hunter et al., in press; Niyomchai et al., 2005). Surprisingly, CORT levels were lower in estradiol-treated rats than in vehicles 1hr after carrageenan administration. Reasons for this decrease in CORT are not clearly understood, since estradiol tends to up-regulate CORT levels. Behaviorally, at 1 hour post carrageenan injection, no differences in PWL were observed between rats pretreated with estradiol or vehicle, suggesting that increases or decreases in CORT did not feedback and regulate inflammatory responses to pain.

Although estradiol increased CORT levels, this did not always lead to a reduction in nociceptive behavioral responses. For instance, most effects of estradiol were seen 24hrs post injection. During this time point, CORT levels following estradiol administration were not significantly higher than CORT levels in vehicle-treated rats. Taylor et al. (1998) found that during the formalin paradigm, pretreatment with synthetic

CORT (i.e. dexamethasone) did not lower flinching responses as compared to controls. Furthermore, preliminary research in our lab demonstrates that removal of endogenous CORT through adrenalectomy does not inhibit estradiol's anti-hyperalgesic responses during the formalin test (Hunter et al., in press). Together, this suggests that estradiol effects on nociceptive responses are working independent of CORT release. Future research should investigate estradiol effects in ADX rats during carrageenan-induced pain to understand the relationship between gonadal and adrenal hormone activity in a persistent pain model.

The effects of estradiol on cytokine levels. Table 9 summarizes estradiol's effects on cytokine levels in naïve, carrageenan-, and saline-treated rats at peripheral and central sites. In naïve rats, estradiol down-regulated anti-inflammatory IL-10 spinal cord levels. However, in saline-treated rats, estradiol increased IL-10 spinal cord levels. The reason for this discrepancy is not clearly understood. Behaviorally, in the ipsilateral paw 24hrs post saline injection, saline-treated rats displayed higher PWL than all other groups (Fig. 48A). It may be suggested that estradiol's up-regulation of IL-10 increased latencies to heat in this group.

Following carrageenan administration, estradiol was effective at altering cytokine levels at peripheral and central sites. In the paw, estradiol down-regulated IFN- γ levels 24hrs post carrageenan administration (Table 9). In DRG, estradiol lowered IL-1 β levels 5 and 24hours post carrageenan administration. Surprisingly, estradiol up-regulated paw TNF- α levels at 1hr and IL-1 β DRG levels 24hrs following carrageenan injection. This may indicate that during carrageenan administration, estradiol is not effective at lowering inflammatory responses at peripheral sites. It should not be assumed, however, that

estradiol is propagating inflammatory signals. Ritz and Hausmann (2008) showed that estradiol increased proinflammatory cytokine levels following SCI, and this led to increased locomotor function as compared to controls. The authors suggested that estradiol mediated pain responses by preventing inflammatory stimuli from spreading to surrounding tissue and causing functional outcomes observed during SCI (Ritz & Hausmann, 2008). Likewise, it can be suggested here that at peripheral sites, estradiol is increasing paw and DRG cytokine levels in an effort to avoid the spreading of inflammatory responses into the CNS. Furthermore, within the spinal cord, estradiol decreased TNF- α , IL-2, IL-10, and GM-CSF levels 5hrs post carrageenan injection (Table 9). Decreases in spinal cord cytokine levels suggest that estradiol is down-regulating central inflammatory mediators, which may be a mechanism through which estradiol's attenuation of injury-induced inflammatory responses is occurring.

Taken together, the findings reported here extend preliminary research that estradiol evokes anti-hyperalgesic responses during the carrageenan paradigm. The effects of estradiol can be observed up to 24hrs following the initial application of inflammatory pain. Our results suggest that previously reported estradiol anti-hyperalgesic effects are in part mediated through down-regulation of proinflammatory cytokines. That is, in OVX female rats, estradiol decreases inflammatory mediators such that during injury responses in the inflammatory pathway are dampened and pain is diminished.

Table 9
Summary of estradiol effects on cytokine levels in naïve, carrageenan-, or saline-injected OVX rats in all samples tested

Cytokine	Group	Sample tested		
		Paw	Ipsilateral DRG	Spinal Cord
IL-10	Naïve	∅	∅	↓
TNF- α	Carrageenan	↑ (1)	∅	↓ (5)
	Saline	∅	↑ (5)	∅
IL-1 β	Carrageenan	∅	↓ (1, 5); ↑ (24)	∅
	Saline	∅	∅	∅
IL-6	Carrageenan	∅	∅	∅
	Saline	∅	∅	∅
IL-2	Carrageenan	∅	∅	↓ (5)
	Saline	↓ (1)	∅	∅
GM-CSF	Carrageenan	∅	∅	↓ (5)
	Saline	∅	∅	∅
IFN- γ	Carrageenan	↓ (24)	∅	∅
	Saline	∅	∅	∅
IL-10	Carrageenan	∅	∅	↓ (5)
	Saline	∅	∅	↑ (24)
IL-4	Carrageenan	∅	∅	↑ (5)
	Saline	∅	∅	∅

Legend. (↓) denotes decrease in cytokine levels following estradiol treatment as compared to vehicle (cholesterol) controls. (↑) denotes increase in cytokine levels following estradiol treatment as compared to vehicle. (∅) denotes no change in cytokine levels as compared to vehicle. (1) (5) (24) denotes cytokine levels were altered by estradiol treatment at 1hr, 5hrs, or 24hrs post injection, respectively. Significance is at $p < 0.05$ for all analyses. $n = 4/\text{group}$.

Chapter 5: Conclusion

The experiments presented in this thesis were conducted to understand the role of 17 β -estradiol on mediators involved in inflammatory pain. Previous experiments in this lab have shown that estradiol leads to anti-hyperalgesic behavioral effects to nociceptive stimuli. Our results indicate that the effects of estradiol on inflammatory pain responses are in part mediated via direct regulation of cytokines, which are involved in inflammatory responses. Contrary to what was predicted in our hypothesis, estrogen effects on cytokine levels can occur independent of CORT release. Estradiol decreases cytokine production both basally and following formalin- and carrageenan-induced inflammation. Together, our results indicate that altering cytokine signaling is critical to estradiol-mediated immunoprotective effects following inflammation and injury. Therefore, our research can be added to literature explaining one mechanism by which estrogen modulates inflammatory processes.

The first and second studies used to address Aim 1 provide evidence that circulating levels of endogenous hormones are effective at altering the cytokine pathway both before and following inflammatory pain. Changes in cytokine levels were seen at peripheral levels. We did not find changes in spinal cord cytokine concentrations either basally or following formalin injection in intact female rats, suggesting that ovarian hormone production does not influence central cytokine activity before or during pain. Perhaps influences of CORT activity inhibited cytokine production. As reviewed by Pavlov et al. (2003), peripheral cytokines send information to the CNS regarding inflammation and injury. In response to these inputs, the brain signals the HPA and HPG axes, as well as other immunomodulatory systems, to control inflammation via a negative

feedback loop. Estrogens and glucocorticoids exert anti-inflammatory effects by inhibiting the synthesis of proinflammatory cytokines (Pavlov et al., 2003). Therefore, in this study, some of the anti-inflammatory effects that could have been altered by estrogen and progesterone alone may not have been shown because of endogenous CORT release.

Contrary to what was expected, decreases in cytokine levels did not correlate with reduced nociceptive responses observed during the proestrus stage of the estrous cycle. For instance, in naïve groups, fluctuating levels of estrogen and progesterone led to lower serum cytokine levels in the diestrus stage. Furthermore, following formalin administration, serum cytokine levels were lower in metestrus as compared to proestrus or estrus stages. The fact that cytokine levels were down-regulated when ovarian hormone secretions were low indicates that during inflammatory-induced pain, gonadal hormones may be working through another system in order to alleviate pain. For instance, a number of studies have shown that nitric oxide (NO) and its isoforms are heavily involved in inflammatory hyperalgesia, and influence prostaglandin activity (Dina et al., 2001; Omote et al., 2001; Salvemini et al., 1996; Tao et al., 2004; Toriyabe et al., 2004). Estradiol and progesterone may modulate nociception during the formalin test by lowering NO activity. In support of this notion, Dina and colleagues (2001) found that inflammatory pain induced by prostaglandin production was dependent on NO release, which was suggested to be estrogen dependent. As discussed previously, Esposito et al. (2005) demonstrated that pretreatment with 17 β -estradiol decreased iNOS levels following carrageenan administration. Pretreatment with fulvestrant, a selective estrogen receptor- α antagonist, inhibits synthetic GC from reducing iNOS expression induced by carrageenan administration (Cuzzocrea et al., 2007). Furthermore, Amador

and colleagues found that neuronal NO synthase (nNOS) spinal cord levels were up-regulated during metestrus following formalin administration; however this failed to reach significance (Amador et al., personal communication, 2009). It was suggested that a larger sample size should be tested to investigate the effects of the endocrinological profile on NO production (Amador et al., personal communication, 2009). Taken together, gonadal hormone effects on NO activity offer another mechanism through which ovarian hormones can modulate inflammatory pain states. Alternatively, direct regulation of the prostaglandin system by endogenous gonadal hormone release may underlie some of the behavioral changes observed during the formalin assay.

Moreover, we did not test serum estrogen and progesterone levels in these models due to lack of reliability from radioimmunoassays in our lab. Therefore, another way to investigate estrogen and progesterone effects on cytokine activity is through a hormone replacement paradigm. Although replacement models should be used with caution when compared to intact models, they do offer a way to investigate specific levels of hormone activity in a more controlled model. For instance, in our lab Kuba and colleagues (2006) administered progesterone and estradiol in OVX rats and measured flinching activity during the formalin assay. As discussed previously, behavioral responses to formalin differed depending on hormone administration. Progesterone attenuated flinching during Phase I, while estradiol replacement reduced flinching during Phase II (Kuba et al., 2006). As a follow up to this study and to our work using intact rats, it would be interesting to measure cytokine levels both before and after formalin administration to test if exogenous progesterone activity alters nociception through modulation of the cytokine pathway.

The third and fourth studies used to address Aim 1 indicate that endogenous ovarian hormones do not influence paw size. Paw thickness was evaluated to determine if ovarian hormones alter inflammatory effects by reducing inflammation at the site of injury. Although estrogen and progesterone were effective at influencing serum cytokine production, demonstrating the effects of hormones on systemic cytokine release, they did not alter inflammatory effects (i.e. byproducts of inflammation) in paw tissue. CORT levels were heightened due to formalin administration as compared to naïve rats. Similarly, Hunter et al. (in press) showed that CORT levels were heightened by estradiol replacement during the formalin assay; however no correlation between CORT activity and behavioral responses to pain were observed. Taylor and colleagues (1998) stated that the formalin stimulus does increase both ACTH and CORT serum levels, but the release of CORT does not reduce nociceptive responses. In naïve models, we observed correlations between serum CORT and cytokine production, where in the diestrus stage CORT levels correlated with elevated TNF- α and IL-10 levels, as well as decreased IL-1 β levels. However, since we did not find a direct link between CORT activity and cytokine production during formalin administration, we cannot conclude that CORT activity is directly involved in cytokine release during inflammation in intact models.

The first study used to address Aim 2 showed that hormone administration altered basal cytokine production following ovariectomy and/or adrenalectomy. This offers an explanation as to why Hunter et al. (in press) found reduced nociceptive responding following formalin injection in these models (see Appendix A). In our study, altered serum TNF- α levels in naïve OVX and ADX rats suggests a mechanism through which estradiol modulates inflammatory effects before pain stimulation. Furthermore,

estradiol's effect on basal tissue cytokine levels suggests that estrogen is effective at modulating cytokine expression during resting states.

The second study used to address Aim 2 demonstrates that estradiol alters both peripheral and central cytokine activity following inflammatory pain. Our data shows that estrogen works differently at nervous system levels, and across surgical treatments, to modulate cytokine activity. For instance, increases in paw TNF- α , IL-2, and IL-10 levels following formalin administration were observed in ADX rats; however, cytokines were down-regulated at the spinal cord level. In OVX rats, estradiol elevated anti-inflammatory IL-10 spinal cord levels. Thus, regardless of surgical condition, estradiol's effect on cytokines at both peripheral and central sites in part leads to the antinociceptive behavioral responses observed within the formalin paradigm.

Furthermore in this study, estradiol-treated rats were analyzed as percentages to their respective vehicle due to sample variability. It may be necessary to increase the sample size in order to reduce variability and risks of Type I error. Once subjects are added to each study, a Bonferroni test can be utilized to correct for any false positives within the sample data.

The third study used to address Aim 2 does not reveal a relationship between estradiol administration and paw size. Paw thickness was not shown to differ from that of vehicle controls. The fourth study used to address Aim 2 revealed that CORT levels were heightened due to formalin treatment. Furthermore, in formalin-treated rats estradiol administration down-regulated CORT activity, a finding in contrast to previous research conducted in our lab (Hunter et al., in press). However, since Hunter et al. (in press) found that both acute (Phase I) and persistent (Phase II) nociceptive behaviors

were reduced from estradiol pretreatment regardless of the presence (or absence) of CORT, our model suggests that a decrease in these pain effects were due to estrogen's modulation of peripheral and central cytokines. CORT is not postulated to mediate these anti-hyperalgesic effects.

The first experiment used to address Aim 3 revealed that estradiol lowers behavioral responses to carrageenan-induced nociception. Behavioral data obtained measuring PWL indicates that estradiol increases latencies to heat before carrageenan administration. Furthermore, estradiol effects following carrageenan treatment were found in both the ipsilateral and contralateral paw at all heat intensities. Estradiol's ability to lower inflammatory responses is consistent with previous findings in our lab (Hunter et al., in press; Kuba et al., 2006). Our model was useful in demonstrating estrogen effects up to twenty-four hours following inflammatory pain. In turn, the second experiment addressing Aim 3 showed that pretreatment with estradiol reduced inflammation in the injected and contralateral paw. This result is consistent with previous research. For instance, estradiol decreases inflammation in the ipsilateral and contralateral hind paws following adjuvant-induced arthritis (Santora et al., 2007). Estradiol lowers paw and lung tissue damage and edema development following carrageenan administration (Esposito et al., 2005). Cuzzocrea and colleagues (2001) demonstrated that estrogen replacement decreases myeloperoxidase (MPO), ICAM-I, iNOS, and other indicators of lung inflammation in OVX rats following carrageenan-induced pleurisy. Furthermore, Ritz and Hausmann (2008) found that 17 β -estradiol decreased lesioning 1 week post spinal cord compression as compared to controls. Together, both studies tie in the fact that reduced inflammatory effects in the periphery

lead to reduced nociceptive responding. An extension of these findings would be to investigate estradiol regulation on MPO and other inflammatory mediators in paw tissue to observe regulation of this hormone on byproducts of inflammation in OVX rats.

The third and fourth experiments used to address Aim 3 revealed that estradiol is mediating carrageenan-induced nociception via modulation of the cytokine pathway, and this effect lasted up to 24hrs post carrageenan administration. Furthermore, estradiol increased CORT levels, although this did not necessarily lead to differences in nociceptive behaviors as compared to vehicles. Down-regulation of cytokine production was accompanied with lowered behavioral responses to nociceptive stimuli, thereby demonstrating a mechanism through which estradiol exerts its anti-hyperalgesic effects.

For future analysis, it may be useful to employ principal component analysis (PCA) to further investigate the effects of estrogen administration on grouped proinflammatory and anti-inflammatory cytokine levels. PCA has been used to extract the underlying variance of extensive data sets and thus summarizes the main aspects, or linear combinations, of the data. Although the sample size used here is too low for such an analysis, future research may be interested in utilizing PCA in order to reveal the underlying effects of estrogen on the combined proinflammatory and anti-inflammatory cytokines that were measured in this study.

Macrophage and glial cell activity are our body's early line of defense against disease and infection. These cells are a major source of proinflammatory mediators, namely cytokine production. Our study suggests that estradiol's effects during inflammatory pain are in part mediated by inhibiting macrophage and glial cytokine activity. However, the precise signaling mechanisms utilized by estrogen are still under

investigation. Proinflammatory cytokines activate signaling cascades such as p38 MAPK, NF- κ B, and Jak/STAT pathways, which all play important roles in immune cell function (Hsieh et al., 2007). During inflammation, increased activation of these intracellular pathways leads to the subsequent synthesis of inflammatory mediators through gene transcription and translation (Kaminska, 2005). Investigating estrogen effects on these pathways offers a potential mechanism through which inflammatory signals are being reduced in these models. In turn, further research on these downstream systems offers a potential target for therapeutic treatments to inflammatory disease.

Furthermore, estrogen receptors are found throughout the nervous system, and exist in regions involved in pain signaling, such as the DRG and the dorsal horn of the spinal cord (Bennett, Gustafsson, & Keast, 2003; Papka et al., 2001). The mechanisms through which estradiol modulates pain signaling at peripheral and central levels is still being researched. Our model found estrogen effects both peripherally and centrally. The fact that estradiol administration decreased paw, ipsilateral DRG, and spinal cord cytokine activity provides support that estradiol lowers the relay of pain signaling from the periphery to the CNS. It would be interesting to determine if estrogen activation at the receptor level leads to modulation of genomic pathways involved in inflammation. Follow up work should examine phosphorylated estrogen receptors in both the DRG and dorsal horn of the spinal cord, and examine its effects on downstream mediators during nociceptive signaling.

Another interesting study would be a side-by-side comparison of females versus males using both formalin and carrageenan models to analyze gonadal hormone influences on cytokine activity. Sex dimorphisms in behavioral responses to pain

continue to be an illusive area of research that needs further investigation. Basic research on cytokine effects using both sexes is limited. Aloisi, Muscettola, and Lupo (2001) investigated gonadal hormone production on formalin-induced IFN- γ release from splenocytes in male and female rats *in vitro*. It was demonstrated that splenocytes from females had higher IFN- γ production than males; however gonadectomy abolished this difference (Aloisi et al., 2001). Xu et al. (1997) examined carrageenan and neuropathic effects in male and female IL-6^{-/-} knockout mice. They found that inflammatory hyperalgesia did not differ due to sex; however during nerve injury females displayed more nociceptive behaviors than males (Xu et al., 1997).

In a recent clinical study, Cutolo and colleagues (2009) investigated the immunosuppressive drug Leflunomide (LEF-M), which is used for RA treatment. LEF-M effects on proinflammatory cytokine regulation in both male and female RA patients were examined. It was demonstrated that LEF-M lowered cytokine activity in males, whereas in females LEF-M increased proinflammatory cytokine activity. It was suggested that the presence of testosterone led to the stronger therapeutic efficacy of LEF in male patients (Cutolo et al., 2009). This study brings to light the importance of examining drug effects on inflammatory products in both sexes. It has often been suggested that testosterone offers protective effects to pain, thus explaining why males are at lower risk for developing certain pain conditions (i.e. RA, migraines) as compared to females. Furthermore, certain medications are postulated to work more effectively in male patients (for review see Cairns & Gazerani, in press). Taken together, continued research on the mechanisms underlying sex differences in pain needs further examination in order to help improve pain treatments in a clinical setting.

In summary, our research highlights the importance of investigating the development of pain in female subjects. Ovarian hormones are shown to influence pain behaviors. Therefore, in order to alleviate pain symptoms in a human population, it is essential to understand the interplay between pain production and hormone fluctuations throughout the reproductive cycle. This will permit for improved pain treatment and management in women.

In conclusion, as demonstrated in the present study, estradiol is effective at mediating cytokine activity at both peripheral and central sites. Figure 67 shows a proposed model by which estrogen may modulate its effects on inflammatory mediators. Since estradiol is decreasing cytokine levels, one mechanism through which estrogen may be working is by lowering intracellular systems activated by binding cytokines to their receptor sites. For instance, binding of TNF- α to its receptor induces p38 MAPK release (Schafers et al., 2003), a signaling pathway that leads to further cytokine production. IL-6 activates the Jak/STAT pathway, another intracellular system involved in inflammatory effects (De Jongh et al., 2003; Kishimoto et al., 1995). Estradiol treatment has been shown to down-regulate Kupffer cell p38 MAPK activation following trauma-hemorrhage (Hsieh et al., 2007). A similar mechanism on estradiol's effects during inflammatory pain is proposed. Specifically, estradiol attenuates cytokine release, which in turn lowers intracellular activation, and thus further cytokine production (Figure 67).

Furthermore, glial cells in the CNS release cytokines in response to peripheral and central inflammation and injury. As shown in Figure 67, estradiol is proposed to reduce central cytokine activity by lowering glial cytokine activity. Estrogen is also postulated

to lower inflammatory mediators, and thus prostaglandin synthesis, produced by cytokine release. An alternative model is that estrogen lowers NO production, which will in turn decrease inflammatory effects at both peripheral and central levels. The exact mechanism(s) by which estrogen alters immune/inflammatory functions remains to be further investigated.

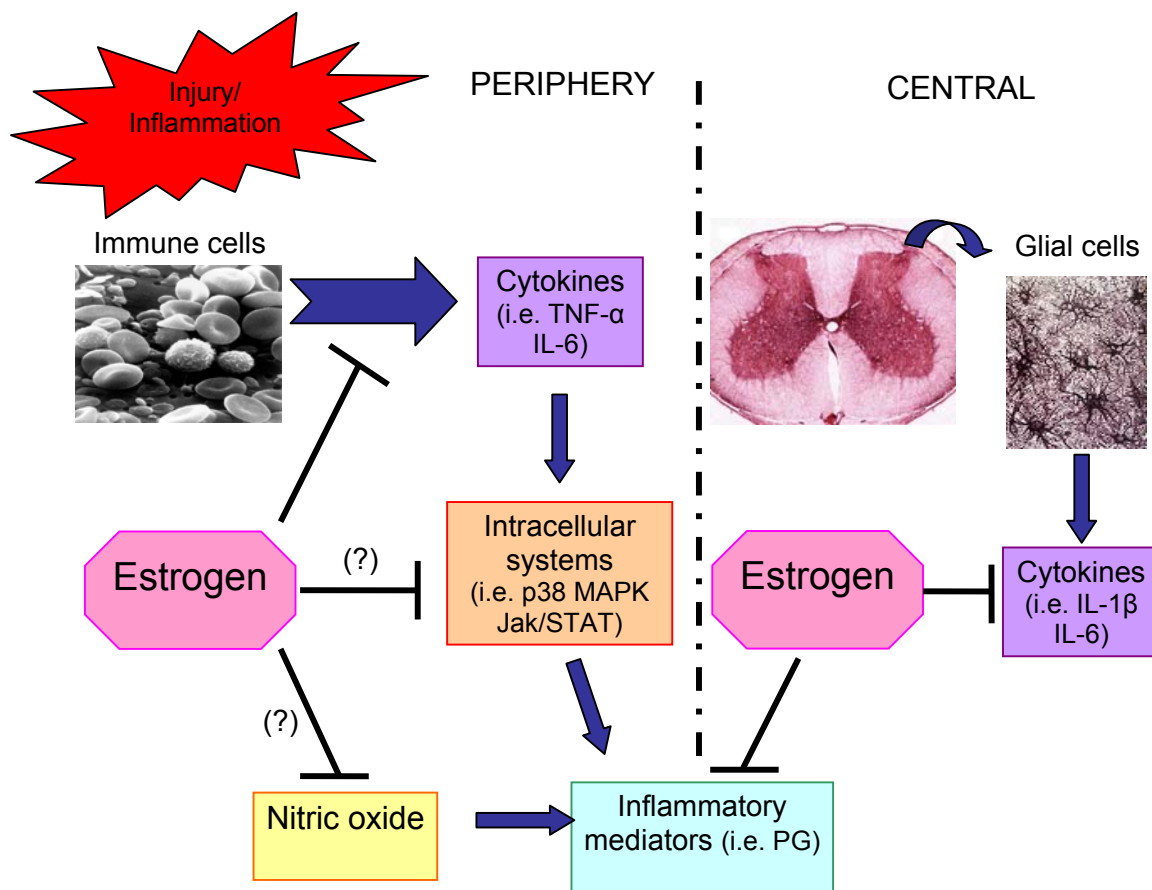
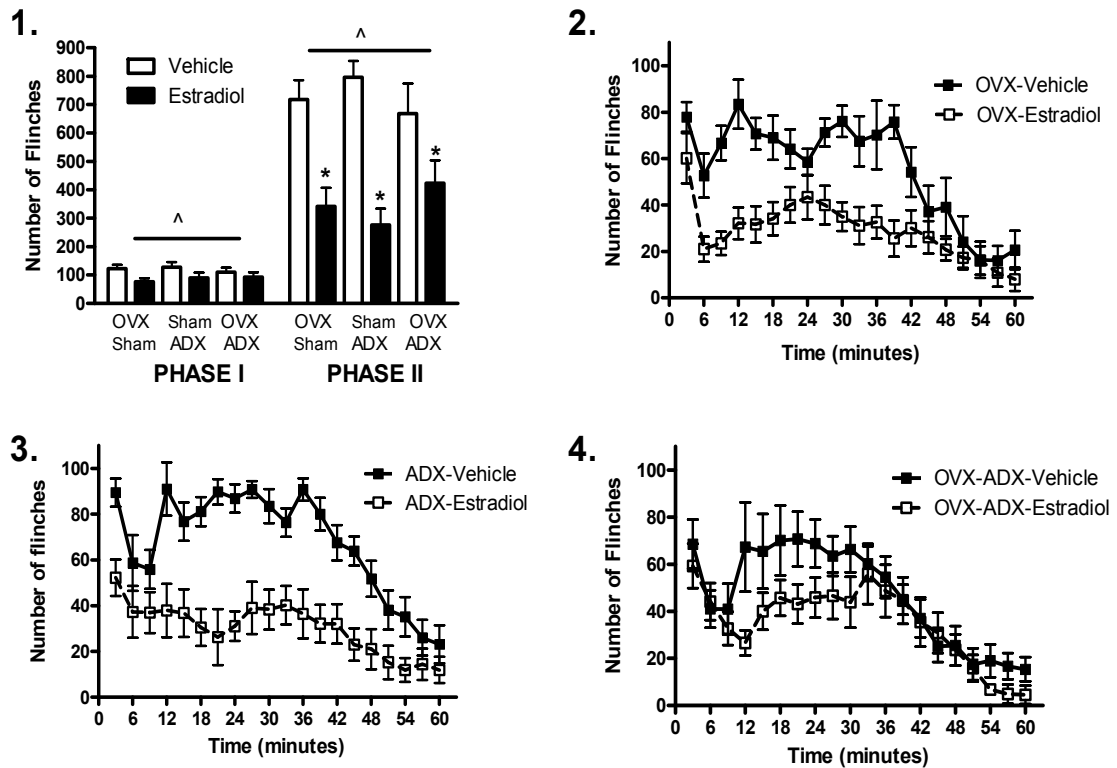


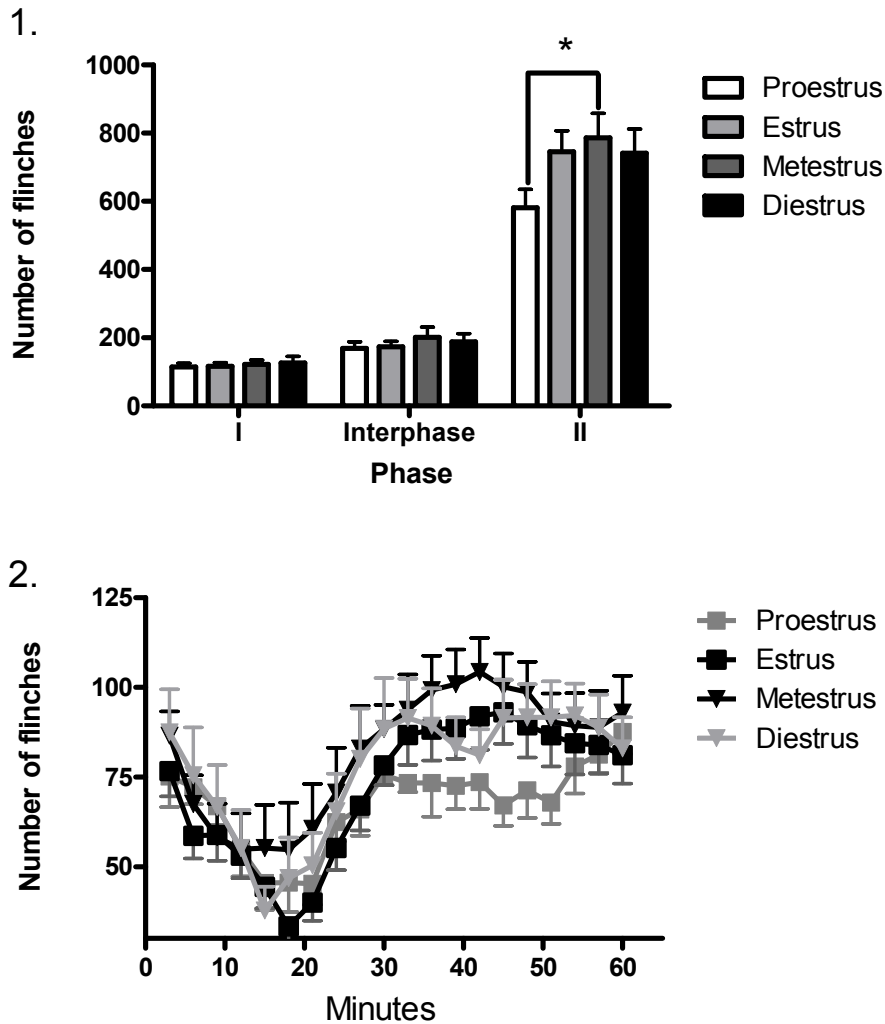
Figure 67. Proposed model by which estrogen reduces cytokines and other mediators involved in inflammatory processing. Estrogen is postulated to mediate inflammation via negative regulation of immune and glial cell cytokine signaling. Dark arrows represent inflammatory mediators activated by inflammation and/or injury, while (⊥) represents estrogen's inhibitory effects. In the periphery, estrogen lowers cytokine activity, and is further postulated to down-regulate cytokine intracellular systems (i.e. p38 MAPK pathways) and inflammatory mediators (i.e. prostaglandins) activated by the cytokine pathway. Centrally, estrogen is proposed to inhibit glial cell cytokine production as well as central inflammatory mediators activated by cytokines. Alternatively, estrogen is proposed to lower nitric oxide production at peripheral and central sites.

APPENDIX A



Appendix A. Effects of estradiol in OVX, ADX, and OVX+ADX rats on flinching behavioral responses after 5% formalin administration. 1. Data are presented as the mean of the sum of activity $\pm$ SEM in Phases I (0-9 min) and II (10- 60 min). Clear bars represent vehicle (sesame oil)-treated rats, and solid bars represent estradiol treatment. [^] Represents a significant hormone main effect. *Represents statistically significant differences between estradiol-treated groups and their respective vehicle-treated rats; $p < 0.05$ for all comparisons. **2. Time course of behavioral activation after hormone and vehicle treatment in OVX + sham-ADX rats. 3. Time course of behavioral activation after hormone and vehicle treatment in sham-OVX + ADX rats. 4. Time course of behavioral activation after hormone and vehicle treatment in OVX + ADX rats.** Data are represented as the mean $\pm$ SEM of flinching responses in 3-minute bins. Solid squares represent vehicle treatment and open squares represent estradiol administration. $n = 12$ to 15 per group. *Adapted from:* Hunter et al.: PAIN (2009) in press

APPENDIX B



Appendix B. Effects of estrous cycle stage on behavioral responses after (5%) formalin administration. 1. Data represents the cumulative mean of flinches (+/-SEM) in Phase I (0-5 min), Interphase (6-19 min) and Phase II (20-50 min) in animals during each stage of the estrous cycle. (*) Represents a significant group and phase effect. **2.** Time course of flinching responses in all estrous stages after (5%) formalin. Estrous cycle effects on nociceptive behavioral responses after 5% formalin. Time course of activation is represented as the mean of flinching responses in 3 minute bins across 60 minutes. $n = 10-12/\text{group}$. From: Amador et al. (2009) personal communication

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