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The role of COX-1 and COX-2 on estradiol's

anti-hyperalgesic effects on induced-inflammatory nociceptive responses

By

Deirtra Hunter

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THE CITY UNIVERSITY OF NEW YORK

Abstract

The role of COX-1 and/or COX-2 on estradiol's anti-hyperalgesic effects on induced-inflammatory nociceptive responses

by

Deirtra Hunter

Advisor: Professor Vanya Quinones-Jenab

Clinical and preclinical studies have demonstrated significant sex differences in the perception of inflammatory pain; females display higher nociceptive responses to inflammatory stimuli than male rats. Additionally, the complex endocrinological profile of females has been shown to impact their nociceptive responses. For example, estradiol reduces Phase II behavioral-nociceptive responses after formalin administration. However, little is known about the specific biological pathway(s) and/or mechanisms in which estradiol affects inflammatory pain responses. Current literature has established that cyclooxygenases and prostanoids are major pro-inflammatory mediators directly linked to inflammatory responses. Additionally, glucocorticoids, (i.e. corticosterone) negatively regulate inflammatory induced COX-2, resulting in attenuation of inflammatory responses. The objective of this study was to further understand how estradiol alters induced inflammatory responses by examining two physiological pathways (i.e. COX-1/COX-2 regulation of the prostanoid biosynthetic pathway and corticosterone regulation of the COX-1/COX-2 pathway) which may in part be responsible for these effects. Estradiol, IBU and NS398 but not SC560 were shown to significantly attenuate behavioral responses after formalin. Co-administration of estradiol and IBU or NS398 revealed an additive not potentiated increase in attenuating induced nociceptive behavioral responses suggesting that regulation of behavioral responses by estradiol may be occurring through a pathway independent

of the COX-prostanoid biosynthetic pathway. Furthermore, estradiol significantly reduced induced-thermal hyperalgesia as measured by decreased paw withdrawal latencies before and after carrageenan administration. Similar to observations observed using the formalin assay, estradiol's effects in the presence of either COX-inhibitor were additive after carrageenan administration providing further support for estradiol's regulation of induced-inflammatory nociceptive responses through an alternate pathway independent of the COX-prostanoid biosynthetic pathway. Surprisingly, significant attenuation of PWL before carrageenan suggests that estradiol is also effective in significantly attenuating induced acute nociceptive as well as inflammatory behavioral responses. Finally, estradiol-treated ADX animals showed significantly reduced flinching behaviors compared to untreated animals after formalin administration suggesting that corticosterone is not a mandatory mediator through which estradiol operates to attenuate induced nociceptive behavioral responses. In summary, our results suggest that estradiol's anti-hyperalgesic effects are not likely mediated in part through downregulation of COX activity and /or levels nor corticosterone downregulation of the COX-prostanoid biosynthetic pathway. Finally, estradiol's anti-hyperalgesic effects can be extended to the carrageenan induced thermal hyperalgesic pain model whereby estradiol was shown to significantly attenuate both induced acute and inflammatory nociceptive behavioral responses.

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I dedicate this dissertation project to my husband, Steve Romagnoli. His support, encouragement and love at the lowest points in my life made it possible for me to complete goals that I thought were out of reach.

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Table 1: Abbreviations

Adrenocorticotropic hormone	ACTH
Adrenalectomized	ADX
Arachidonic acid	AA
Aromatase-knockout	ArKO
Brain derived neurotrophic factor	BDNF
Central Nervous System	CNS
Collagen-induced arthritis	CIA
Complete Freund's adjuvant	CFA
Corticosterone	CORT
Cyclooxygenase-1	COX-1
Cyclooxygenase-2	COX-2
Cystolic PGES-1	cPGES-1
Delayed-type hypersensitivity	DTH
Descending facilitation	DF
Descending inhibition	DI
Dimethyl sulfoxide	DMSO
Dorsal root ganglia	DRG
E-prostanoid receptor	EP-receptor
Estradiol treatment	E2
Gamma-aminobutyric acid	GABA
Glucocorticoid	GC
Glucocorticoid receptor	GR
Gonadectomized	GDX
Guanine nucleotide binding protein	G protein
Hypothalamic-pituitary-axis	HPA
Ibuprofen	IBU
Estradiol receptor antagonist	ICI 182,780
Inducible nitric oxide synthase	iNOS
Inhibitory post synaptic current	IPSC
Interleukin-6	IL-6
Interleukin-18	IL-18
Intraperitoneal	IP
Intracisternal	IC
Lipocalin-type PGD synthase	L-PGDS
Lipopolysaccharide	LPS
Microsomal PGES-1	mPGES-1
Microsomal PGES-2	mPGES-2
Messenger ribonucleic acid	mRNA
N [2-(Cyclohexyloxy)- 4-nitrophenyl]methane sulfonamide (selective COX-2 inhibitor)	NS-398
N-methyl-D-aspartic acid	NMDA
Nerve growth factor	NGF
Neurotransmitter	NT

Nitric oxide	NO
Nitric oxide sythase	NOS
Non steroidal anti-inflammatory drugs	NSAID
Ovariectomized	OVX
Phosphoglucoisomerase	PGI
Pre-prodynorphin	PPD
Prostaglandin	PG
Prostaglandin D2	PGD_2
Prostaglandin E2	PGE_2
Prostaglandin E synthase	PGES
Prostaglandin $GF2_{\alpha}$	$PGF_{2\alpha,}$
Prostaglandin G2	PGG_2
Prostaglandin H2	PGH_2
Prostaglandin I2	PGI_2
Protein Kinase A	PKA
Protein Kinase C	PKC
Protein kinase C epsilon	PKCe
Paw withdrawal latencies	PWL
Phospholipase	PLA
Raloxifene	RAL
Real time polymeric chain reaction	RT-PCR
Rheumatoid arthritis	RA
5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl pyrazole (selective COX-1 inhibitor)	SC-560
Selective serotonin re-uptake inhibitor	SSRI
Tumor necrosis factor	TNF
Thromboxane A2	TXA_2

Introduction

The focus of this work was to identify specific biochemical pathways through which estradiol's antihyperalgesic effects on induced-inflammatory nociceptive behavioral responses are mediated. First, I will provide general background information on the psychological and physiological origin of pain. Secondly, I will introduce and summarize the roles of some major mediators involved in nociceptive inflammatory responses. Lastly, a review and summary of estradiol's effects on inflammatory nociceptive responses will be presented.

Pain sensation, although sometimes discomforting, often serves an adaptive function [1,2]. Stimulation of specialized nociceptors by a noxious stimuli serves to warn organisms of possible impending tissue damage [1,3]. The initial response to nociceptive stimuli is usually activated quickly, and can prevent or limit the extent of potential tissue damage. However, clinical pain that arises from either damage to the nervous system or chronic inflammation is pathologic and contributes no benefit to the individual [4].

Unfortunately, no simple equation(s) exist that can be used to determine the output of perceived pain based on the quality and magnitude of a stimulus input. Numerous variables contribute to nociceptive transmissions, responses and factors that modulate them. The extent that each individual variable influences nociception is thus far immeasurable. These variables have the ability to change an otherwise innocuous stimuli into a noxious one [5,6]; in addition, they can influence nociceptive responding such that when a noxious stimulus has been removed persistent signaling continues resulting in continued pain sensation [4]. This continued sensation is believed to stem from long term changes in the central nervous system contributing to hyperalgesia, a condition characterized by perception of abnormally high levels of pain in

response to normal noxious stimuli and/or allodynia whereby certain types of stimuli are perceived as painful which are not normally painful [2,7,8]. Different mechanisms that contribute to the final sensation ultimately determine whether stimuli will result in a pro- or anti-nociceptive transmission.

I. Background and Significance

What is pain?

Transmission of a nociceptive stimulus starts in the peripheral nervous system traveling through specialized afferent fibers [7]. As shown in Figure 1. A-delta and C fibers are the most common afferent types sensitive to noxious stimuli and are often classified as polymodal owing to the variety of stimuli that can activate them individually or simultaneously (i.e., thermal, mechanical or chemical) [9]. Nociceptors transduce noxious signals into depolarizing currents that are relayed to the dorsal horn in the spinal cord via A-delta and C fibers [1,3]. The dorsal horn in the spinal cord, is where most primary sensory nociceptive neurons terminate and is the first site of synaptic transfer in the nociceptive pathway. Most nociceptive neurons terminate in lamina II and V of dorsal horn [7]. It is here that nociceptive signals can be modified considerably by descending inhibition, descending facilitation [1] and local modulators [7]. Primary sensory neurons terminating at this site innervate both projection neurons and intrinsic dorsal horn interneurons. The modifiability of the signal in the dorsal horn makes this site and actions that take place here an extremely essential component of pain and pain hypersensitivity [9].

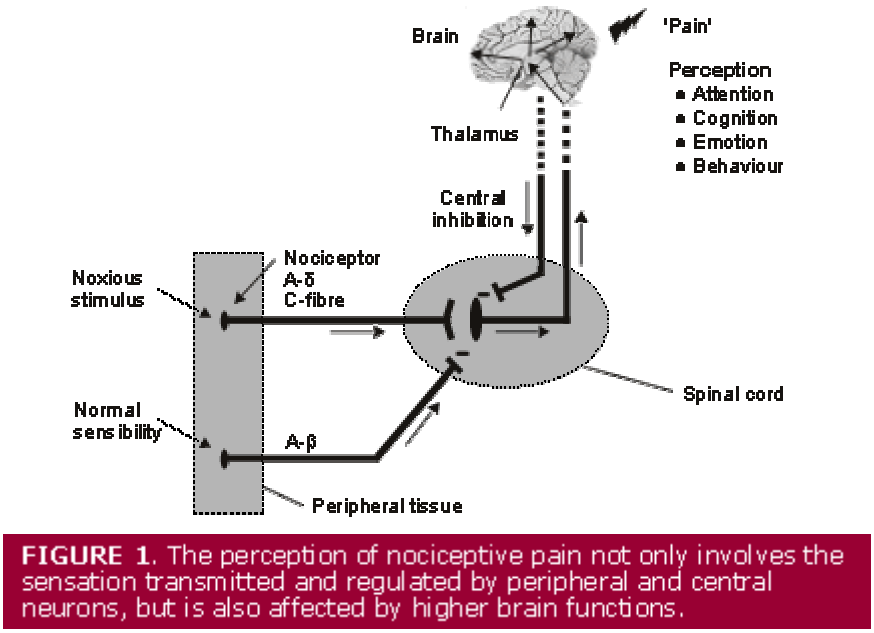

Figure 1. Shows the afferent pathway that the nociceptive signal travels. The first process involves Transduction, whereby nociceptors respond to noxious stimuli that initiate cellular changes resulting in stimuli being changed into electrical energy necessary to transmit pain. Transmission of pain begins when transduction is complete. During the first segment of transmission the impulse is carried along nociceptive fibers ascending to the dorsal horn of the spinal cord. This is followed by transmission from the spinal cord to the brain stem and thalamus. The thalamus acts as a relay station and sends the impulse to the cortex where it can be processed. Modulation of pain transmission can occur at various locations in the CNS. The pathways associated with modulation are referred to as descending pathways (central inhibition) since they originate in the brain stem and descend to the dorsal horn of the spinal cord. (Pasero et al., 1999)

Peripheral processing of physiologic pain

After an injury, tissue damage results in increased sensitivity of nociceptors at the site of injury through a process known as peripheral sensitization [3,7]. This, in turn, produces changes in the quantity, location and activity of ion channels in the injured tissue enhancing neural input

[2,3,10]; ultimately, this phenomena leads to lowering the threshold for depolarization of nociceptive neurons [3]. Two classes of receptors: ionotrophic, including N-methyl-D-asparate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA)/kainite (K) receptors, and metabotrophic, G-protein coupled receptors that are activated by glutamate, an excitatory amino acid are responsible for many of these changes [2,11]. Metabotrophic glutamate receptors are activated during increased spinal excitability associated with peripheral hyperalgesia [12]. Ionotrophic receptors are involved in spinal hyperexcitability occurring during central sensitization [13].

Inflammation, a physiological change that contributes to pain perception, is a response that occurs after tissue injury [7]. Inflammation is generated by local macrophages released at the site of damaged tissue [7] and characterized by the release of inflammatory response mediators such as Substance P, calcitonin gene related peptide, cytokines and other neuroactive agents from macrophages and non-inflammatory cells [3,14]. Amplification of inflammatory responses is also attributed to migrating blood cells. Together with bradykinin and prostaglandins, these chemical mediators act on the ion channels of nociceptors and specific receptors initiate a variety of signal transduction cascades resulting in immediate posttranslational modulation of effector molecules and changes in gene transcription [7]. Mediators and other chemicals act synergistically to maintain development of sensitization of nociceptive terminals and pain [7]. More specifically, two types of inflammatory responses exist. Acute inflammation is a short-term process characterized by classic signs of inflammation; swelling, redness, heat, pain and sometimes dysfunction of organs involved. Removal of the potentially harmful stimuli intiates down-regulation of this inflammatory response [15]. In contrast, chronic inflammation is not short-term but pathological. It is characterized by tissue

damage, local infiltration of mononuclear immune cells (monocytes, lymphocytes, macrophages and plasma cells), and attempts at healing (angiogenesis and fibrosis). In this case the stimulus is persistent, therefore down regulation of the inflammatory response does not occur [15].

Nerve signals that originate from the site of tissue or nerve injury can lead to long term changes in the Central nervous system (CNS) [16,17]. For example, behavioral models reveal that persistent afferent input generated from tissue injury contributes to abnormal pain states such as hyperalgesia and tactile allodynia. Hyperalgesia refers to a condition whereby a normally noxious stimulus is perceived as being abnormally more painful, allodynia refers to a condition whereby a normally innocuous stimulus is perceived as painful. Sensitization of the peripheral terminal is in part responsible for these abnormal pain states as well as to central sensitization in the spinal cord dorsal horn [18]. Sensitization is characterized by decreased thresholds which can result in augmented responses to suprathreshold stimuli and ongoing activity in pain pathways [19]. Although, it has been suggested that peripheral sensitization is responsible for hyperalgesia [20], Iskeda et al., (2006) recently revealed that if pain fibers are stimulated in a way that replicates activation by an inflammatory stimuli, amplification occurring in the spinal cord resembles long term potentiation. They also argued that long term potentiation was another way of producing hyperalgesia [21]. Peripheral sensitization is characterized by neighboring terminals of peripheral nociceptors at the site of injury and/or inflammation mass releasing prostanoids (PGs). These PGs act on their specific receptors resulting in the potentiation of the action of these primary sensory neurons. Peripherally, prostanoid signaling is mediated by second messengers (PKA, PKC) which regulate receptor and ion channel activity [22]. Understanding mechanisms associated with peripheral processing of physiological pain is

only one aspect of pain sensibility. A better understanding of this sensibility can be acquired by examining the cascade of events that follow peripheral processing of a noxious stimulus.

Central processing of physiologic pain

Central sensitization is the abnormal hyperexcitability of nociceptor neurons in the dorsal horn of the spinal cord [23]. Central sensitization occurs as a function of pharmacological and/or physiologic modulation associated with peripheral injury [23]. Release of neurotransmitter (NT) mediators contribute to changes in receptor sensitivity such as an increase in the number of nociceptive receptors responding to repeated activation. As a result, low intensity peripheral stimuli can activate afferent fibers that normally do not evoke pain under normal circumstances [14]. These NT's also act on ion channels and these joint actions activate intracellular signaling that induces cyclooxygenase gene expression and prostaglandin synthesis. Prostanoids act on both pre- and post-synaptic receptors modifying their functional properties and the excitability of dorsal horn neurons. These changes lead to central sensitization.

NMDA receptors are responsible for many changes associated with central sensitization [2,10,24]. Their contribution appears to enhance, extend the duration and modify the activity in the nociceptive circuitry in the spinal cord via respectively lowering of activation thresholds, increasing the response to a given stimulus and enlarging the size of the receptive field which serves to amplify existing stimulation [1,7]. After activation, a conformational change takes place that allows a greater influx of calcium and sodium into the cell. Extended depolarization by C-fiber stimulation leads to a phenomena referred to as "wind-up" characterized by a significant increase in the magnitude and duration of cell responses after NMDA activation to repetition of a given constant pain stimulus [6]. This in turn produces hypersensitivity of the

neurons to a pain stimulus, whereby spinal cord neurons possess increased excitability and sensitivity [6,7,25].

Acute and chronic pain are distinguished from each other by unique characteristics. Acute pain is intiated by tissue damage and is comprised of both phasic and tonic pain. It is characterized by quick responses, while chronic pain which can be conceptualized as the persistence of acute pain is characterized by a more pronounced and sustained modification in the activity of descending controls [26]. During acute pain, an adaptive reversible transient shift between the two modulating descending mechanisms involved in nociceptive transmission, i.e. descending inhibition (DI) which serves to attenuate sensitivity to a noxious stimuli and descending facilitation (DF), that increases sensitivity to a noxious stimuli [1,24]. In contrast, chronic pain is not transitory. Recent studies show that a reduction in descending tonic inhibitory controls (DI) as well activation of facilitatory systems (DF) contribute to various forms of chronic pain [27,28]. Chronic pain is associated with neuroplastic changes leading to longer lasting nociceptive responding to initial peripheral stimulation [14]. For the purposes of this project we will be examining acute and inflammatory pain which fall under the umbrella of nociceptive pain distinct from chronic pain. Nociception is a neurophysiological term that denotes activity in specific nerve pathways discussed earlier. These pathways transmit signals that function to protect the boy from potential tissue damage by warning or making aware the organism.

Formalin model for inflammatory/chronic pain

The most commonly used nociceptive assays in non-human animals are thermal, electrical, mechanical and chemical in nature [29]. Many traditional tests for pain rely on noxious stimuli that are brief in nature, escapable and result in a short lasting nociceptive

experience. Accordingly, they are referred to as phasic pain models [30]. Due to the short-lived experience it is often difficult to examine modulatory mechanisms that may be activated by the stimulus itself [31]. In contrast, models used to represent tonic pain usually result in a long lasting nociceptive experience characterized by continuous pain and often associated with injured tissue and/or inflammation [32]. This kind of model thereby induces a state that is more reflective of various types of clinical pain experienced by humans. The formalin assay is a behavioral model of persistent and inflammatory pain.

The formalin assay consists of an injection of diluted formaldehyde solution (usually 1-10%) into the dorsal surface of the hind paw of the rat resulting in licking, flexing and flinching behavior of that paw [32]. As shown in Figure 2, the time and frequency in which the animal performs these behaviors is scored as nociception. Seconds after the injection of formalin, an animal will flinch and lick its paw for approximately 5 minutes and then stop temporarily [29]. This period is referred to as Phase I and is thought to reflect acute nociceptive pain caused by formalin-evoked discharge in C-fiber nociceptors [17]. Phase I is also believed to be opioid mediated [29]. The period in which behavioral responding stops temporarily is referred to as the quiescent or interphase period believed to be mediated by descending inhibitory mechanisms [1]. Following interphase the rat will start flinching and licking the injected paw again at high frequencies for 15 minutes or more [33]; this phase is referred to as Phase II. Phase II is believed to be the result of mechanisms that resemble those responsible for central chronic and/or neuropathic pain. It is associated with responding from spinal cord nociceptive neurons that are activated by C-fiber discharge during the initial phase and is thought to be NMDA receptor-mediated [33-36]. For these reasons, the formalin assay is distinctive from other acute and

chronic nociceptive assays and provides a method of evaluating both acute and tonic pain in a single chemical test through its biphasic nature that lasts approximately 60 minutes.

Early studies support qualitative differences in mediation of the early and late phases of formalin responses. Specifically, the early phase of the formalin response is believed to result from the direct stimulation of nociceptors resulting in sensitization and firing of peripheral nociceptors, while late phase responses due to an ensuing inflammatory responses result from the sensitization of the dorsal horn neurons [31,37,38].

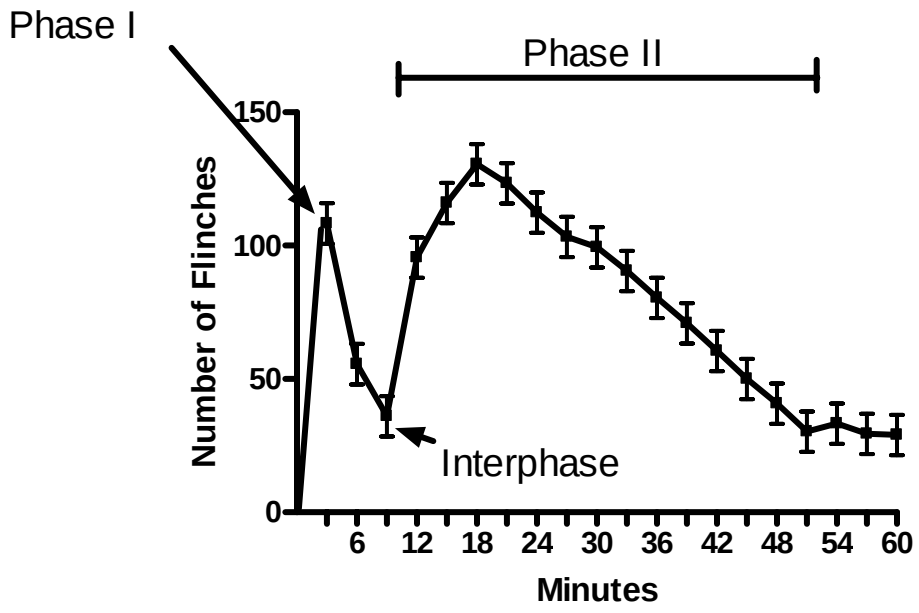

Figure 2. Nociceptive behavior resulting from diluted formalin injection to the right hind paw. Across time, the biphasic flinching behavior is characterized as Phase I, for 15 minutes or more. Phase I results from activation of peripheral nociceptors and direct monosynaptic activation of non-NMDA receptors by glutamate and NK1 receptors by substance P at the dorsal horn neuron. Following phase I, there is an interphase period, where no nociceptive activity is observed. Immediately following interphase, Phase II is characterized by continued afferent input from nociceptors as well as activation of NMDA receptors by continued glutamate release (This graph reflects data from control animals used in this study).

Based on early studies Yashpal et al., (1996) [36] suggested that persistent nociceptive responses due to formalin are a product of central sensitization via nociceptive inputs which impinge on the (CNS). Furthermore, although persistent nociceptive responses to formalin may be dependent on central sensitization, peripheral inflammation may also play a significant role in behavioral responses to higher concentrations of formalin. For example, Yashpal et al., (1996) noted that at higher concentrations of formalin, there was a greater dependence of nociceptive responses on peripheral inflammatory changes than changes associated with central sensitization [36]. Thus, at high concentrations of formalin, NSAIDs were more effective due to the large contribution of inflammatory mechanisms than lower concentrations of formalin which may rely more on central sensitization [39]. Yashpal et al. (1998) also examined differential effects of Phase I and Phase II with 1 and 5% formalin as well as comparing these two doses in the presence of dexamethasone and ibuprofen (anti-inflammatory drugs) [39]. Early phase nociceptive responses to either 1 or 5% formalin were not affected significantly by either drug [39]. In contrast, during Phase II, responses after 5% formalin administration were significantly reduced in a dose-dependent manner by both drugs, suggesting that nociceptive responses during Phase I are controlled by mechanisms different than those that contribute to Phase II. Additionally, a positive correlation between formalin effects, nociception and inflammatory responses at high concentrations of formalin (5%) were observed [39]. In summary, since NSAIDs (non steroidal anti-inflammatory drugs) do not affect the early phase but do affect the second phase, the role of peripheral inflammation has been suggested to be a major contributor to the late phase responses supporting the existence of different mechanisms that contribute to nociceptive responses in each phase following formalin administration.

Carrageenan model for persistent inflammatory pain

Another classic model to study inflammatory pain is referred to as the carrageenan model. It consists of an intraplantar injection of carrageenan in the hind paw [40-44]. This model has been used extensively to identify inflammatory mediators and screen a new family of anti-inflammatory drugs [40-42,44]. Carrageenan injection results in oedema (an increase in paw volume) and exacerbated sensitivity to mechanical and thermal stimuli referred to as hyperalgesia. Moreover, doses of carrageenan necessary to generate paw oedema and thermal hyperalgesia are similar [45,46] while mechanical hyperalgesia requires a much higher dose [42]. Carrageenan-induced inflammation is characterized by two phases, early (1-6 hrs) and late (12 and 24 hrs), respectively. The Hargreaves box, an automated detection testing paradigm can be used to ascertain nociceptive thresholds associated with paw withdrawal latencies after inflammatory induction. Using this method, Hargreaves et al., (1988) demonstrated that carrageenan-induced inflammation resulted in significantly shorter paw withdrawal latencies when compared saline treated paws. Dose-related hyperalgesia was detected as well its blockade with indomethacin or morphine [44]. Daher et al., presented evidence that indomethacin (non-steroidal anti inflammatory), in addition to its peripheral effect on oedema formation after carrageenan injection, also acts centrally to inhibit oedema [47]. These findings support a spinal mechanism for peripheral inflammation after carrageenan. Posadas et al., revealed that carrageenan-induced inflammatory responses are affected by age. Specifically, younger mice (3 to 4 weeks) displayed overall lower paw edema than older mice (5 to 8 weeks). Additionally, COX-1 levels were not modified after carrageenan but COX-2 levels were modified in the late phase [48].

Taken together, these studies show that both the formalin and carrageenan behavioral testing paradigms can be used to analyze different components of nociceptive processing and responding. Specifically, the two phases of the formalin model allow examination of variables that can effect or modify both induced acute and tonic pain respectively. While the early phase of the carrageenan model allows insight into induced acute, hyperalgesic and tactile allodynic responses all associated with tissue injury and/or inflammation.

These two testing models were utilized in the current study to address specific aims used to elucidate biochemical pathways that may in part contribute to estradiol's anti-hyperalgesic effects on induced-inflammatory nociceptive processing and responding.

II. Mediators of Inflammation

Prostaglandins & Cyclooxygenases

It has been widely established that prostaglandins (PG's) play a considerable role in the modulation of inflammatory responses associated with pain [1,14,49-54]**.** Prostaglandins (PG's) have been shown to have both analgesic and hyperalgesic properties [55] [43-45]. They enhance nociception produced by other chemical mediators [56-58]. As shown in Figure 3, the first step in the production of prostanoids is associated with the conversion of free arachidonic acid (AA) to PGG_2 and then to PGH_2, which is controlled by a pair of isoenzymes COX-1 and COX-2 jointly known as cyclooxygenase [59]. COX-1 differs from COX-2 in that COX-1 is constitutively expressed and COX-2 although constitutively expressed in brain cells [59] and the spinal cord [60,61], is also highly inducible in response to cytokines, growth factors or other inflammatory stimuli unlike COX-1 [56,62]. Moreover, the action of several tissue-specific synthases is responsible for the conversion of PGH_2 to various thromboxanes (TXA_2) and prostaglandins (PGD_2, $PGF_{2\alpha}$, PGE_2, PGI_2). Use of inhibitors for either of these PGH synthases

has been used to reveal their joint and individual roles in pain perception. Synthesis of prostanoids is controlled by humoral (originating from inflamed tissue) [52] and neural signals (activity in sensitized nerve fibers innervating the inflamed tissue) that input into the CNS [63].

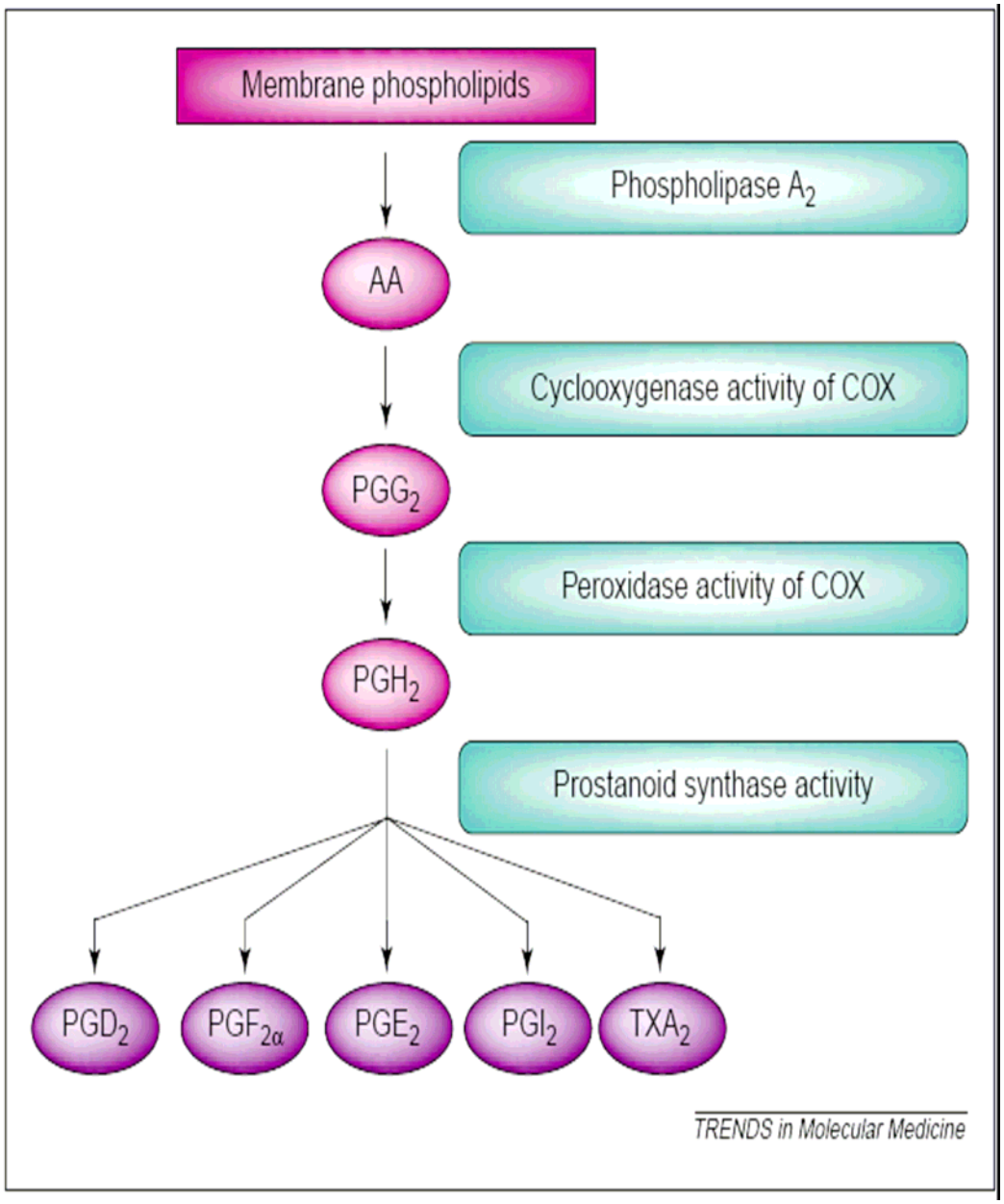

Figure 3. Prostanoid biosynthetic pathway. Following the release of arachidonic acid (AA) from cell membranes by phospholipase A2 (PLA), it is converted in a two step reaction, first to prostaglandin G_2 (PGG_2), then to PGH_2, by the action of cyclooxygenase (COX) enzyme. PGH_2 is then converted to various prostaglandins and the thromboxanes by the action of multiple tissue-specific synthases. Samad et al., 2002

Prostaglandins contribute to the development of pain both peripherally and centrally [15,52]. In the periphery, PG's generate peripheral sensitization by increasing the sensitivity of the peripheral terminals of nociceptors. This is achieved via a cascade of biochemical actions:

PKA mediated phosphorlylation of sodium channels and/or other receptors in terminal of nociceptors after activation by EP receptors (receptors specific for prostaglandin E_2), which increases the excitability of receptors resulting in a lowering of the pain thresholds [52]. These changes potentiate actions of chemical mediators such as heat, irritant molecules and bradykinin known for their pain producing stimulatory actions leading to peripheral sensitization [64,65].

The role of PG's in the inflammatory response has been revealed through the use of several animal behavioral models such as CFA (Complete Freund's adjuvant), carrageenan, zymosoan and formalin [40,66,67]. These models have contributed to the identification of specific mechanisms by which prostaglandins are synthesized and their underlying substrates. Inflammatory pain is caused by sensitization of peripheral and central nociceptive neurons and/or receptor activity which contributes differentially to inflammatory responses [15,35,40,67].

A number of studies have shown that PGE_2 is the dominant PG in spinal cord-mediated nociception. Specifically, PGE_2 is involved in spinal cord dorsal horn neuronal excitability and synaptic transmission [68-70]. PG's also increase neurotransmitter release from the central terminals of pain fibers in the spinal cord dorsal horn [52].

In the CNS, centrally administered PG's account for changes that produce significant alterations in nociceptive behavior including exaggerated responses to normally innocuous stimuli, i.e., allodynia, hyperalgesia [21,47,71]. Administration of PGE_2 antibodies [45] or deletion of PGE_2 receptor genes [72] inhibit hyperlagesic behavior after injury and provide support for a major role of PGE_2 mediation in hyperalgesia. PG's on a cellular level are able to increase transmission in the spinal cord in a variety of ways. For example, they can activate a non-selective cation channel resulting in direct depolarization of spinal cord neurons, and they can increase of neurotransmitters from central terminals of pain and reducing glycine levels

associated with endogenous analgesic effects in the descending modulatory pathway of pain control [73]. This markedly increases excitability of the spinal cord neurons [69].

PG's contribute to sensitization through activation of four receptors EP1-EP4 both peripherally and centrally [15]. Bar et al., (2004) examined the role of these individual receptors using comparisons of nociceptive input processing from a normal knee to an inflamed knee joint [74]. Recordings from nociceptive dorsal horn neurons established that under normal conditions activation of EP1, EP2 and EP4 receptors induce spinal hyperexcitability similar to PGE_2. Interestingly, after inflammation, responses by these receptors changed. EP1 was the only receptor that facilitated increased hyperexcitability whereas EP2 and EP4 had no additional effect. Surprisingly, an EP3α receptor agonist reduced responses to mechanical stimulation and reduced spinal hyperexcitability induced by PGE_2. Another study revealed that EP3 and IP receptor knockout mice displayed attenuated hyperalgesia after lipopplysaccharide administration [75]. Minami et al., (2001) found evidence that implicated EP1 and not EP3 receptors for their role in prostaglandin-induced spinal sensitization [76]. Together, these data present a new strategy for limiting pro-nociceptive effects of spinal PGE_2, possibly by activating an inhibitory EP receptor such as EP3.

Periaqueductal prostaglandin receptor stimulation has been associated with increases in formalin-induced nociceptive responses. Specifically, after formalin administration subsequent stimulation of these receptors increased glutamate and reduced GABA release [77]. Formalin administration produced similar biphasic fluctuations of both of NT's by increasing glutamate and reducing GABA [77]. When Misoprostol, a prostaglandin agonist and formalin were administered together there was a greater increase in glutamate followed by a larger decrease in the release of GABA [77]. Inhibitory glycine receptors in the dorsal horn have been identified as

target receptors for PGE_2 regulatory actions during peripheral inflammation as well. Blockade of these glycine receptors resulted in a significant reduction in glycine receptor mediated inhibitory post synaptic currents (IPSCs). Based on these findings, glycine activity has been implicated in providing a cellular and molecular basis for sensitization actions of PGE_2 in the dorsal horn [69].

Terminal prostanoid synthase activity is another site for analyzing the progression and modulation of inflammatory responses. Several proteins have been identified for their variable levels of PGE2 synthase activity, microsomal PGES-1 (mPGEs-1)[40], glutathione transferases [78], cytosolic PGES-1 (cPGES-1) [70] and mPGES-2 [79]. Among the PGES isoforms the physiological role of mPGES-2 remains unclear. However, it appears that cPGES-1 usually pairs with COX-1 and mPGES-1 with COX-2 [80,81]. mPGES-1 acts downstream of cyclooxygenase and specifically catalyzes the conversion of PGH2 to PGE2 under basal and inflammatory conditions[78]. mPGES-1 enzymes are induced by pro-inflammatory stimuli in both peripheral and central tissues [40,81,82]. Regulation of behavioral responses due to inflammation has been linked to activation of mPGES-1 [40,83]. Using knockout mice, Trebino et al., (2003) demonstrated that mice deficient in mPGES-1 have a milder reaction due to acid evoked responses and induction of arthrithis than do wild type mice. Additionally, they show significantly attenuated inflammatory responses [84]. Murakami et al., (2000) revealed that pro-inflammatory stimulation of various cultured cells leads to elevation of mPGES-1 directly associated with elevation of COX-2 expression and PGE_2 production [81]. Targeting this pro-inflammatory mediator as a means to identify new approaches to pain therapy can be successful.

Preferential coupling of mPGES-1 and COX-2 combined with growing concerns concurrent side effects associated with cyclooxygenases inhibition for pain therapy have contributed to research centered around the possibility of using mPGES-1 as a new target for

pain therapy [79]. Cheng et al., (2006) attempted to identify a mechanism that suppressed PGE_2 production without the adverse side effects that accompany COX inhibition mediated suppression of PGE_2 [83]. They revealed that knockout, mutation of PGH2 or deletion of the receptor for PGHS-2 accelerates thrombogenesis and elevate blood pressure. In contrast, they showed that deletion of mPGES-1 depressed PGE_2 expression and affected neither thromobogenesis nor blood pressure in mice [83]. Guay et al., (2004) examined changes in prostanoid tissue levels and in expression of terminal prostanoid synthases (mPGES-1 and mPGES-2) in both the CNS and inflamed peripheral tissue during carrageenan-induced paw inflammation in the rat [40]. They found that mPGES-1 expression was strongly upregulated in the brain and spinal cord during inflammation whereas no change was detected for the expression of cPGES, mPGES-2, and COX-1. They also showed that the carrageenan-induced edema in the paw elicits an early phase COX-2 induction that preceeded mPGES-1 and COX-2. Additionally, mPGES-1 remained elevated during the late phase with further increases in PGE_2 over the course of 24 hours. Taken together it was suggested that during peripheral inflammation, up-regulation of mPGES-1 contributes to COX-2 mediated PGE_2 production in the CNS [40]. Aside from PG's role as pro-inflammatory Cosme et al., (2000) demonstrated that PGE_2 mediation can also be anti-inflammatory. They found that PGE_2 inhibits local cytokine secretion by T-lymphocytes in inflamed colonic mucosal linings in human cells. They suggest that PGE_2 effects in this way are responsible for protecting mucosal integrity and preserving epithelial function [85].

Although PG's have been shown to promote both pro and anti-inflammatory actions, clearly PG's, their specific tissue synthases and enzymes responsible for their production have been established as playing a major role in nociceptive inflammatory processing and responding.

It is because of this role that PG's and biochemical components linked to their production were targeted as potential mediators in estradiol's effects on induced-inflammatory nociceptive responses in the present study.

Prostaglandins have also been recognized as significant mediators in the pathogenesis of rheumatoid arthritis (RA) [86]. Although the role of PGE_2 in RA has been established through widespread use of NSAIDs for treatment, the role of other PG's were relatively unknown. Honda et al., (2006) [86] examined the role of PHI_2 (prostacyclin) and PGE_2 in knock-out mice through collagen-induced arthritis (CIA). Mice deficient in the PGI (IP) receptor displayed significantly attenuated arthrithic scores compared to wild-type mice after CIA [86]. They also showed significant attenuations in pro-inflammatory cytokines. Moreover, inhibition or the loss of PGE_2 receptors subtypes EP2 or EP4 alone did not affect elicitation initiation of inflammation. However, inhibition of EP2 and EP4 simultaneously did result in partial suppression of CIA inflammatory symptoms. Taken together this data suggests a significant role for PGI_2 in RIA; thus inhibition of PGE_2 alone may not be enough to suppress RA symptoms [86].

PGD_2 another metabolite of PGH_2 is the most abundant prostanoid produced in the central nervous system [87,88] and has been shown to mediate neuroprotection similar to PGE_2, [89,90]. Lipocalin-type PGD synthase (L-PGDS) mediates the biosynthesis of PGD_2 in the central nervous system [91,92]. PGD_2 is able to exert its effect through two distinct G protein coupled receptors, DP and CRTH2 (DP2)[93,94] that have opposing effects on cyclic AMP production [90]. Currently, two types of PGD_2 synthases have been identified in the brain and mast cells [95].

Numerous studies have demonstrated PGD_2's role in modulating pain responses [93]. In contrast to PGE_2, PGD_2 shows little or no peripheral effect similar to PGE_2's ability to sensitize

afferent neurons to noxious stimuli [15]. Horiguchi et al., (1986) [67] found evidence to support separate mechanisms for nociceptive responses of PGD_2 and PGE_2 in mice. After intracisternal administration, both PGD2 and PGE_2 had biphasic effects on pain thresholds. However, after intracisternal injection of naloxone, hypoalgesia caused by higher doses of PGD_2 was blocked while hypoalgesia created by high doses of PGE_2 was not [67]. Ishizaka et al., (2001) showed an increase in L-PGDS levels after bacterial endotoxin lipopolysaccharide or pro-inflammatory cytokine enzyme administration in the cerebrospinal fluid [96]. Eguchi et al., (1991) used L-PGDS knockout mice to illustrate PGD_2's role in allodynia [97]. After intrathecal administration of PGE_2, allodynia failed to develop in the L-PGDS knockout mice in contrast to wild-type control mice. However, thermal hyperalgesia still developed in the knock-out mice [97].

Using western-blot and RT-PCR analysis after inflammation, Grill et al., (2006) demonstrated an enhanced expression of COX-2, mPGES-1 and L-PGDS mPNA and protein in the spinal cord [91]. They also observed that both PGE_2 and PGD_2 concentrations were significantly higher in mice that received endotoxin treatment (associated with spinal inflammatory responses) compared to the control group [91]. These findings suggest that PG biosynthesis is enhanced after endotoxin treatment. Additionally after endotoxin treatment they observed that a selective COX-2 inhibitor (lumiracoxib) significantly attenuated PGE_2 and PGD_2 release comparable to values seen in untreated mice [91]. Guay et al. (2004) recently observed an increase in PGD_2 levels in cerebrospinal fluid only during the early phase of inflammation (1-6 hours) after carrageenan administration [40]. Taken together these studies support a role for PGD_2 in inflammatory pain mechanisms. However, its involvement in hyperalgesia and other periphery inflammatory responses is not well documented. The relationship between prostanoids and cyclooxygenase contributes to their essential presence in establishing nociceptive

transmission/responses [56,62]. COX-1 and COX-2 are referred to as the rate limiting step in the process of prostanoid synthesis [52]. There is an abundance of literature that supports a major role for COX-2 in the mediation of inflammatory responses, but the role of COX-1 continues to be controversial.

Use of celecoxib, a selective COX-2 inhibitor [57] revealed that formalin-induced secondary hyperalgesia was prevented by a local pre-injection of celecoxib but not with a local post-injection. However, if celecoxib was administered spinally it inhibited formalin-induced secondary hyperalgesia with both pre- and post-injections [57]. It was suggested, that products of COX-2 are released immediately and followed by initial development of secondary hyperalgesia (pain sensitivity that occurs in surrounding undamaged tissue) since a local post injection does not inhibit development of secondary hyperalgesia [57].

Nogawa et al., (1997) [98] demonstrated that after induction of cerebral ischemia in rats, COX-2 mRNA was upregulated in the ipsilateral hemisphere but not in the contralateral hemisphere, twenty-four hrs after the ischemia procedure PGE_2 increased by 57% with a marked increase in the ischemic side [98]. Administration of a COX-2 inhibitor, NS-398 resulted in a reduction of PGE_2 as well as reduction in swelling associated with induced-cerebral ischemia. Upregulation of COX-2 corresponded to increased enzymatic activity reflected in an increase of PG levels [98].

Nantel et al., examined the regulation of COX-2 in paw oedema and mechanical hyperalgesia after carrageenan induction [42]. They found that carrageenan-induced mechanical hyperalgesia was associated with a greater increase in COX-2 and PGE_2 than paw oedema. Surprisingly, indomethacin administration blocked COX-2 induction in paw oedema but not in hyperalgesia. Authors suggested that a positive feedback–loop regulating COX-2 expression in

paw oedema might be responsible [42]. Also the more severe model of inflammation associated with hyperalgesia revealed a more widespread induction of COX-2 compared to oedema. (both oedema and hyperalgesic show COX-2 induction in the epidermis while hyperalgesia also showed induction in skeletal muscles and inflammatory cells). It was suggested that this wider distribution COX-2 induction may be responsible for production of a higher levels of inflammatory mediators that are high enough to drive the expression of COX-2 in the presence of indomethacin [42]. Another study showed pre-treatment of intrathecal lamotrigne (sodium blocker that suppresses glutamate) produced a time and dose dependent suppression of thermal and mechanical hyperalgesia while post treatment with lamotrigne only affected mechanical nociception [99]. This study implies that after the inflammatory response is initiated it may be harder to slow down the inflammatory processes with an inhibitory mediator.

Ichitani et al., (1997) [41] studied the localization of COX-1 and COX-2 in the spinal cord and dorsal root ganglia (DRG's) using in situ hybridization histochemistry after peripheral inflammation or axotomy in the rat. Although no COX-2 levels were detected under normal conditions in the spinal cord, carrageenan injection in the hind paw resulted in strong expression of COX-2 in non-neuronal cells in the grey and white matter along the leptomeninges and blood vessels [41]. These findings suggest COX-2 expression in non-neuronal cells contributes to PG production in and around the spinal cord associated with peripheral inflammation [41]. Furthermore, pretreatment with dexmethasone and indomethacin anti-inflammatory drugs reduced both phases of oedema in a dose dependent manner after subplantar carrageenan injection [43].

Yaksh et al., (2002) demonstrated that SC-560, a selective COX 1 inhibitor, had no effect on behavioral responses in the rat formalin test [100]. However, oral administration of celecoxib

(COX-2 inhibitor) and indomethacin (non-selective COX-1 & COX-2 inhibitor) depressed Phase II but not Phase I [100]. Furthermore, intrathecal administration of these two inhibitors respectively resulted in depression of nociceptive responses in both phases during the rat formalin test. This data suggest that PG's synthesized by COX 1 may not be involved in nociceptive transmission using the rat formalin assay but COX-2 may be a major mediator. In contrast, Tegeder et al., (2001) [101] suggested that formalin evoked rapid release of PGE_2 that may be primarily caused by COX-1 and not COX-2. Use of SC560, (a specific COX-1 inhibitor) was not only significantly effectively in reducing nociceptive behavior on the formalin assay but it completely abolished the formalin-evoked PGE_2 levels [49]. Surprisingly, celecoxib, a selective COX-2 inhibitor, was ineffective in significantly reducing nociceptive behavior and reducing preceding PGE_2 levels. Another study discovered that NS398, a selective COX-2 inhibitor only yielded antinociceptive activity at a dose of 27 mg/kg [102] which is probably no longer selective for COX-2 only using the formalin test. In contrast, diclofenac, a non-selective COX inhibitor [31] inhibited formalin-induced flinching behavior over the whole dose range tested, starting at a 1mg/kg dose in a dose dependent manner. Furthermore, a statistically significant reduction of flinching behavior only in Phase II was observed. These results suggest PG's mediating nociception in the formalin test of the rat are most likely produced by both COX-1 and COX-2 [31] and that COX-1 may play a larger role in nociceptive responding than previously thought.

Surprisingly, Yamamoto and Taguchi (1996) using the same model reported that intrathecally administered NS398 (a selective COX-2 inhibitor) inhibited formalin-induced flinching behavior in a dose dependent manner whereas I.P. injection of the same drug had no effect [103]. These data suggest COX-2 may play a pivotal role in formalin induced nociceptive

processing at the spinal cord level. Contrary to these findings, Dirig et al., (1993) suggested that PG's formed by COX-2 are of minor importance for the development of hyperalgesic responses in the formalin model since intrathecal administration of highly selective COX-2 inhibitors (SC 58125 and SC 58236) failed to inhibit flinching behavior [104].

Using the formalin and zymosan-evoked thermal hyperalgesia models for inflammatory pain, Hofacker et al., (2005) demonstrated that COX-1 and cystolic PGEs (cPGES) are constitutively expressed in the neuronal and non-neuronal cells of the dorsal and ventral horn of the spinal cords in adult rats [50]. A reduction of cPGES in the rat spinal cord reduced nociceptive behavior during both nociceptive models. These findings suggest a role for cPGES's in mediating early responses during spinal nociceptive processing [50]. Furthermore, Ballou et al., (2000) demonstrated that COX-1 was the primary COX isoform involved in spontaneous pain (hotplate assay) and slowly developing diffuse pain [105].

Nakano et al., (2007) examined the differential selectivity of COX-1 and -2 on the late phase of carrageenan-induced pleurisy (inflammation of the lining of the pleural cavity surrounding the lungs) via COX inhibitors [106]. They observed that aspirin, indomethacin and ketorolac (COX-1 inhibitor) significantly decreased the volume of pleural exudate but had no effect on COX-2 and mPGES-1 expression in the lymph nodes [106]. In contrast, selective COX-2 inhibitors; nimesulide and NS-398 not only enhanced COX-2 and mPGES-1 expressing cells but they also enhanced the extension of their dendritic processes and COX-2 levels ketorolac was able to antagonize these changes [106]. This data suggests that COX-1 and COX-2 may sometimes occupy antagonistic roles in the late phase of acute carrageenan-induced inflammation. In summary, mounting evidence suggest, a more significant role for COX-1's involvement in nociceptive and inflammatory responses than once believed.

The plethora of literature that demonstrates the pro-inflammatory role of COX as well as the relationship between cyclooxygenases (COX) and PG, whereby COX is the rate limiting enzyme responsible for PG production, implicates the COX-PG biosynthetic pathway as a potential target that may in part be mediated by estradiol. Ultimately, resulting in estradiol's attenuation of responding associated with inflammatory stimuli.

Corticosterone

Corticosterone, a steroid hormone secreted by the adrenal glands, under the control of adrenocorticotropic hormone (ACTH) is one of a group of glucorticoids known to inhibit inflammation [107]. This class of drugs is often used to treat immunoinflammatory diseases such as rheumatoid arthritis and tempromandibular arthritis in children [108-110]. Corticosterone release can be attributed to stress and/or nociceptive stimulation associated with the immune system, i.e. inflammatory responses [111,112]. Glucocorticoids negatively regulate inflammatory-induced COX-2 (reduction through gene expression) and therefore are known for their ability to attenuate inflammatory responses [52,63,113]. Hyperalgesic states have also been shown to be sensitive to glucocorticoids (GC) [114]. Zhang et al., (2004) demonstrated that both exogenous and endogenous glucocorticoids (GC's) affect spinal preprodynorphin (PPD) mRNA up-regulation, associated with dynorphin production, an opioid peptide thought to play an important role in the modulation of nociceptive neural networks at the level of the spinal cord. Using complete Freund's adjuvant (CFA, a behavioral pain assay that induces peripheral inflammation in the injected rat paw) revealed that adrenalectomized (ADX) rats showed a more intense hyperalgesia than control rats injected with CFA [114]. Implications of these findings is that endogenous GC's exert a powerful suppressive effect on CFA induced inflammatory hyperalgesia. In addition, pretreatment with dexamethasone (a synthesized GC) was able to

suppress the more intense hyperalgesia and upregulation of spinal PPD mRNA in ADX rats [114]. Wilson et al., (2000) used ADX male rats to successfully show that lack of adrenocorticoids influences the development of adjuvant arthritis and that glucocorticoids are extremely important in anti-arthrithic actions on pro-inflammatory mediators [115]. Specifically, ADX animals showed an increase in the frequency of paw oedema and hyperalgesia after adjuvant-induced arthritis was initiated compared to controls. Treatment with dexamethasone reversed the increase in hyperalgesia and oedema. Interestingly, celecoxib treatment (selective COX-2 inhibitor) was ineffective in inhibiting hyperalgesia and oedema [115].

Surprisingly, Vissers et al., (2004), demonstrated pain behavior decreased in ADX animals for both Phase I and II compared to sham ADX and non operated rats. Additionally, when naloxone (an opioid antagonist) was administered before the late phase no difference in pain behavior was observed in the sham ADX and non-operated animals but ADX animals pain reactivity, returned to levels comparable to the non-operated rats. It was suggested that the hypothalamo-pituitary adrenal (HPA) axis reduces pain using the formalin model via activation of endogenous opioid systems [116].

Xu et al., (2000) examined the frontal cortex of rats after complete Freud's adjuvant (CFA)–induced arthritic rats and found that compared to control rats, pain behavior scores increased significantly after CFA injection and these effects decreased markedly by injection of corticotrophin (corticotrophin hormone indirectly stimulates the release of corticosteroids) [117]. Moreover, after ADX surgery the decrease in pain behavior after CORT injection was partially prevented [117]. Recently, corticotrophin-releasing hormone has been shown to regulate IL-6 (a major pro-inflammatory cytokine) during inflammation. Jointly, these studies suggest that an

understanding of specific complex interactions between the HPA axis and immune system will contribute to a better understanding of mechanistic inflammatory processes and responses [118].

Interactions between cognitive factors such as emotional states and mood have been shown to influence nociceptive input such that the final transmission can be significantly affected via descending modulating pathways and/or HPA axis activation. One example is anxiety that accompanies the anticipation of a noxious stimulus, these phenomena can modify the intensity of the final nociceptive response dependent on a variety of coexisting factors but findings still remain inconsistent.

Fontella et al., (2005) reported the effects of chronic 17-β estradiol administration on OVX rats submitted to repeated restraint stress over forty days. Using the tail flick test, they noted that nociceptive threshold latencies were decreased in both groups (OVX, OVX + estradiol) [119]. More observations revealed that AMP hydrolysis in the spinal cord synaptosomes (ectonucleotidase activity believed to be associated with nociceptive processing) was decreased in OVX stressed rats compared to non-stressed rats [119]. Additionally, hormone replacement reversed these effects. These observations are evidence that repeated stress affects nociceptive sensitivity to noxious stimuli and can be modified by hormone replacement [119].

Another study used the formalin model and restraint to determine if inflammatory or stress induced increases in CORT levels reduced inflammation and pain related behaviors. To test this hypothesis they used adrenalectomized (ADX) animals to prevent activation of glucocorticoid and dexmethasone to saturate glucocorticoid receptors on nociceptive processing in the formalin test [112]. Although after formalin administration ACTH and CORT levels were significantly higher than restraint or saline injection, neither ADX nor dexmethasone changed behavioral or cardiovascular nociceptive responses. They concluded that formalin

administration is sufficient enough to activate corticosterone release via HPA axis but the release is not sufficient enough to feedback and reduce nociceptive processing [112]. Although formalin administration was associated with significant increases in ACTH and CORT compared to restraint or saline injections, it was suggested that these increases were associated with nociceptive input rather than stress. Aloisi et al., (1994) examined how analgesia induced by restraint stress would impact on subsequent pain induced hormonal responses in both male and female rats on the formalin test [120]. The data suggest that females exposed to stressful stimuli or subjected to stressful events show greater increases in plasma levels of ACTH and CORT than male rats. Furthermore, rats subjected to the formalin test after restraint procedures showed reductions in some behavioral responses, specifically licking and flexing that was limited to Phase II [120]. However, no differences were observed in Phase I. Only restraint procedures affected hormone levels not the combination of restraint and formalin testing. These results suggests that analgesia produced by restraint stress has little effect on nociceptive responding during formalin testing [120].

Evidence suggests that gender differences in pain evoked responses may be in part mediated by activation of the hypothalamic-pituitary adrenal (HPA) axis which is associated with ACTH and CORT release [75]. Sex hormones differentially affect hippocampal electrical activity which may ultimately contribute to the HPA axis's affect on final nociceptive responses [75]. For example, estradiol upregulates binding and expression of glucocorticoid receptors (GR) in the brain and GR binding in the dorsal horn [121]. There is also evidence that gonadal hormones can modulate adrenal corticosterone secretion and GR binding in neuroendocrine tissues. For example, females have higher secretory rates of adrenal steroids and higher ACTH output than males [122].

Da Silva et al., (1993) found that corticosterone levels were differentially affected by estradiol treatment [123]. Prior to any experimental manipulations, female and males did not differ significantly in their CORT levels. However, sham operated female rats had higher CORT levels than males and gonadectomy had opposing effects in the two genders. Specifically, CORT levels were reduced in females but significantly increased levels in males. In the chronic inflammatory model, OVX females with progesterone replacement comparable to normal physiological levels restores a response similar to that of intact females. However, oestradiol treatments did not affect CORT levels in OVX females. Release of interleukin-1 (a cytokine that is secreted by macrophages, monocytes and dendritic cells) is an important part of the inflammatory response of the body. Observation of interleukin-1 levels followed a similar pattern whereby females release more than males in both intact and gonadectomized mice with chronic inflammation [124]. This data suggest an inter-relationship between sex steroids, inflammatory stimuli and the HPA axis that contributes to females having a greater tendency than males to generate activating signals and a greater sensitivity to these factors [124]**.**

In contrast**,** Aloisi et al, (1996) discovered that corticosterone levels were not affected in either gender although ACTH levels increased as a result of both 1 and 10% formalin administration in females [125]. Authors suggested that the inconsistent increase in CORT levels compared to the increased ACTH may be due to sacrifice of animals too soon after formalin administration. Perhaps the biosynthetic pathway necessary to convert ACTH to CORT did not have time to culminate [125,126].

Finally, Alder et al., (1999) revealed that in response to hypoglycaemia (a pathological state produced by lower than normal levels of sugar (glucose) in the blood), patients with fibromyalgia had an impaired capacity to activate the HPA axis [127]. In summary, the HPA

axis (controls corticosterone release) and corticosterone are significant mediators in nociceptive processing and are affected differentially by sex hormones [127].

In summary, the HPA axis and corticosterone have been shown to modulate nociceptive responses. Additionally, a relationship between estradiol and corticosterone release and activity has been demonstrated. These findings make the HPA axis and corticosterone ideal targets to examine in the attempts of the current study to identify biochemical pathways that estradiol may be working through to attenuate induced-inflammatory nociceptive responding.

Other inflammatory mediators

Cytokines

Experimental studies in recent years have provided evidence that pro-inflammatory cytokines induce or increase inflammatory and neuropathic pain via their contribution to the generation of pain and hyperalgesia [128-130]. Cytokines positively regulate inflammatory-induced COX-2 [52,63]. Cytokines are small regulatory proteins that are produced by white blood cells and a variety of other cells including some in the nervous system [128]. If pro-inflammatory cytokines are blocked or anti-inflammatory drugs administered, in most cases neuropathic hyperalgesia is reduced in animal models [129]. Intraplantar injection of tumor necrosis factor-α-, a prototypic pro-inflammatory cytokine in rats, has been shown to induce mechanical allodynia and thermal hyperalgesia. In addition, correlations between tissue levels containing this cytokine with pain and hyperalgesia has been found in a number of painful diseases [130-132]. After administration of a cytokine antagonist, reduction of hyperalgesia using the carrageenan inflammation model was observed [133]. CytokinesIL-1β, and TNF also reduce nociceptive threshold through a prostaglandin dependent process.

Second messengers

Second messenger systems have also been shown to contribute to inflammatory pain responses [134]. Dina et al., (2001) found that inhibitors of PKCe, PKA and nitric oxide synthase (NOS) were able to antagonize epinephrine-induced hyperalgesia in males [134]. However, hyperalgesia induced by prostaglandin E_2 was dependent on PKA and NO in both sexes (meaning that blocking one or the other did not antagonize hyperalgesic effects) [134]. Gonadectomized (GDX) females displayed comparable second messenger contributions to that of males in epinephrine induced hyperalgesia. In addition, administration of oestradiol to GDX females succeeded in typical female second messenger participation [134]. Interestingly, a selective serotonin reuptake inhibitor (SSRI) fluoxetine, displayed marked anti-inflammatory action on carrageenan-induced paw inflammation. Fluoxetine was effective before, during and after carrageenan administration making it an ideal for clinical practice since pain therapy is usually implemented after the inflammatory process has developed [135]. There are also inherent differences associated with pain thresholds and ultimately the effectiveness of analgesics in scientific studies. Fecho at al, (2005) demonstrated that there were significant differences in basal and carrageenan-induced inflammatory pain sensitivity in Inbred Lewis, Fischer and Sprague Dawley rat strains [136].

III. Female behavioral responses to pain

Gonadal Hormones

It is widely accepted that pain affects men and women differentially; not only do females demonstrate significantly higher behavioral responses to chronic and inflammatory pain than males, but studies have also shown that women are more likely to report pain of a longer duration [137-141]. Furthermore, certain pain syndromes, such as joint pain and fibromyalgia

show prevalence rates in women increased with age implicating fluctuating hormonal levels that accompany women at different stages in life [142-146]. Bajaj et al., (2001) compared pain sensitivity in healthy women during different phases of their menstrual cycle to men. He found significant differences reflected in reduced pain pressure thresholds in the abdomen and lower back areas [147] These findings suggested that nociceptive differences may be attributed to enhanced nociception acting at both the peripheral and central level resulting in the hypersensitivity changes at the abdomen and lower back areas [147]. Recently, Aloisi et al., (2005) showed that patients suffering from chronic non-malignant pain had significantly lower estradiol levels and elevated cortisol levels compared to control. Interestingly, testosterone levels were comparable in both groups [146].

However, contradictory observations associated with sex differences and pain perception have been reported. For example, no sex differences were observed in pain perception associated with analgesic responses to ibuprofen [148]. Using post operative baseline pain after a molar extraction to examine this phenomenon, female's responses reflected significantly greater scores on perceived pain than males. Also, the pain intensity and pain relief scores overtime was not significantly different after any time point after drug administration (ibuprofen) and authors concluded there were no sex differences in analgesic responses using ibuprofen [148].

Rodent studies reveal similar sex differences in behavioral responses to persistent and chronic pain. Bradshaw et al., (2000) reported significant sex differences after induction of thermal hyperalgesia following administration of complete Freund's adjuvant (CFA) [39]. Ceccarelli et al., (2003) [149] showed that the licking response after formalin testing was higher in OVX than intact rats contrary to formalin induced flexing and flinching responses in Phase I

and II between these two groups. These findings support the hypothesis that neural circuits involved in the modulation of these behaviors are not only affected differently but that gonadal hormone depletion affects supraspinal circuits (circuits affecting licking response) more than spinal ones (circuits affecting flinching and flexing) [149].

Tall et al., (2004) revealed after intraplantar carrageenan nociceptive responses are significantly affected by sex and gonadal hormones in rats. Specifically, gonadally intact females displayed significant greater paw withdrawal latencies compared to males in both control and carrageenan treated groups, sex differences were abolished after gonadectomy surgery. Additionally, they observed that particular stages of the estrous cycle also influenced these thermal hyperalgesic responses. During proestrus, paw withdrawal latencies (PWL) were significantly higher than PWL in estrus or diestrus [150]. However, it has been suggested that failure to find sex differences, especially in males after gonadectomy may be due to differences in methodology. Specifically, it has been suggested that testing PWL prior to 4 hours post carrageenan [151] may contribute to missing significant chemical changes that alter PWL after carrageenan.

Using the formalin test numerous studies have demonstrated that female rats show more flinching responses than males and these differences have been attributed to hormonal effects [126,152-156]. Ovarian hormones have been implicated as being the basis for these differences between sexes and estradiol replacement has become another method of investigating the profound effects of this gonadal hormone on nociceptive processes.

Estradiol

Estradiol has been of particular interest based on the growing accumulation of studies supporting differential nociceptive responding in its presence. Mannino et al., (2006), revealed

that graded doses (5%-40%) of 17β-estradiol replacement in OVX rats reduced formalin-induced pain behaviors by 35% to 49% respectively [157]. Furthermore, 20% estradiol replacement achieved the maximum reduction in pain behaviors and the anti-hyperalgesic effect of 20% estradiol replacement was significant as early as 8 days after implantation and persist up to 21 days [157]. Other studies have shown estradiol replacement in rats reduced vaginal hyperalgesia in a menopause–associated dyspareunia model of pain and lowered autonomy scores after nerve injury [158,159]. Moreover, estradiol has been shown to decrease and increase latencies in the tail flick and threshold responses to hot plate assay [160,161] yet have no effect [162] in the electric foot shock assay.

Using Raloxifene (RAL) (a selective estradiol receptor modulator) or 17β-estradiol after carrageenan-induced acute inflammation Esposito et al., (2005) showed that RAL and 17β-estradiol decreased COX-2 and iNOS (inflammatory mediators) in inflamed areas and attenuated inflammation and tissue damage associated with oedema and pleurisy [163]. Furthermore, after induced inflammation RAL and 17β-estradiol not only restored normally inhibited peroxisome proliferators-activated receptor-γ expression (inflammatory marker) used by experimenters) they also increased cytoprotective heat shock proteins 72 which was suggested to be associated with an attenuated inflammatory response. Kuba et al., (2005) demonstrated that estradiol replacement in OVX rats attenuated Phase II behavioral responses, when high concentrations of formalin (5%) was used [164]. These findings suggest that estradiol effects are contingent on peripheral inflammation since effects of high concentrations of formalin are believed to rely on peripheral mechanisms [39]. In addition, use of α-estradiol (inactive isomer of estradiol) failed to produce the same effects as estradiol suggesting estradiol's actions in inflammatory responses most likely mediated through genomic estradiol receptor mediation [165].

While many studies support estradiol's analgesic effects on nocicepetive input and behavioral responses, some challenge it. One study revealed that tamoxifen (an estradiol antagonist which binds to the estradiol receptor but does not activate it but serves to block the natural effects of estradiol) decreased the duration of the estrous phase and it also inhibited the volume component of carrageenan-induced inflammatory responses by over 50% [166]. Based on these findings, Misiewicz et al., (1996) suggests that estradiol contributes to carrageenan-induced inflammation in female LEW/N rats [166].

In addition, a number of estrous cycle studies show that fluctuations in sex hormone levels have differential effects on nociceptive input and responses. For example, during proestrus, (stage when estradiol serum levels are at their highest) painful occurrences associated with a ureteral calculosis model decreased, where as after complete Freund's adjuvant or carrageenan, hyperalgesic responses increased [167,168]. Another study revealed that female rats in the proestrus stage exhibited increased hyperalgesia as compared to other stages of the cycle [139]. Moreover, female rats in proestrous exhibited increased hyperalgesia as compared with males [167]. Clemente et al., (2004) showed that females in diestrus exhibited significantly more pain responses than males [169]. Recently, Sunday et al., (2006) demonstrated that exogenous or endogenous ovarian hormones appear to alter cerebrovascular inflammation [170]. Using estradiol and progesterone replacement they examined their effects on inflammation in the cerebral vasculature , a major process in the development of ischemic brain injury[170]. After LPS-induced inflammation, estradiol treated OVX rats showed significantly attenuated levels of induced NOS (iNOS) and COX-2 compared to untreated OVX animals. In contrast, progesterone or medoxyprogesterone acetate (a synthetic) analog of progesterone intensified the inflammatory response to LPS. What is more, LPS induction of iNOS and COX-2 varied with

stages of the estrous cycle and levels were the highest during the estrus, when estradiol levels are their lowest and progesterone their highest [170].

Interestingly, Aloisi et al., (1994) demonstrated that the sex hormone effects were not sex specific; when gonadectomized (GDX) males were implanted with supplementary female sex hormones they were comparable to females in nociceptive responding [171]. In a 2004 study, Aloisi et al., (2004) examined the role of supraphysiological levels of testosterone on behavioral responses after formalin administration [153]. They found that although testosterone did not affect nociceptive input it did induce male-like responses in females. Gaumond et al., (2002) [156] compared intact males and females as well as (GDX) males and females. Results revealed significant differences between intact males vs. females. Overall, females show greater nociceptive responding than males and it was suggested that testosterone plays a protective role by reducing nociceptive responding in males as compared to females in both Phase I and Phase II of formalin testing [156]. No difference in behavioral responding between (GDX) females and males suggested that the differential effects observed in intact animals may be due to activational effects rather than organizational effects. They also found that intact compared to ovariectomized (OVX) females differed only during the interphase stage, and OVX, but not castration affected nociceptive responding during interphase [156]. Based on the theory that interphase responding is controlled by inhibitory systems not a cessation in nociceptive responses, authors suggested that increased chronic pain observed in females may be due to female sex hormones contributing to a deficiency in endogenous inhibitory pain mechanisms rather than increased nociceptive responding [155]. Endogenous inhibitory pain mechanisms may be a key player in mediating differential nociceptive responding between males and females and could be another potential target whereby estradiol's anti-hyperlagesic effects are mediated.

Estradiol has also been shown to exhibit close interactions with proteins involved in signal transduction mediated by neural growth factors or neurotransmitters. For instance, Bjorling and Wang, (2001) [172] found that estradiol has the ability to influence both the onset and course of neurogenic inflammation of the bladder in mice. Use of an estradiol receptor antagonist (ICI 182,780) resulted in a significant decrease in Neural Growth Factor (NGF) mRNA. NGF mRNA is associated with progressive modifications that contribute to neurogenic inflammation in bladders. Additionally, increased quantities of NGF mRNA [173] that directly stimulate peripheral afferent fibers have been linked to perceived pain [174]. Allen et al., (2005) examined the relationship between estradiol and brain derived neurotrophic factor (BDNF) in central systems since both have been associated with nociceptive processes during persistent and chronic pain [175]. Twenty-four hours after intraplantar formalin injection, BDNF gene expression was quantified in intact female rats during proestrus and diestrus and in estradiol treated or untreated OVX animals. They found that estradiol replacement increased BDNF mRNA in the spinal cord, cortex and hippocampus. Also, the up regulation of spinal BDNF gene expression was significantly increased by estradiol treatment [175].

In addition to published results from our lab, a growing amount of studies support a significant role of sex hormones nociceptive processing and responding. Specifically, estradiol has been noted for having both anti- and pro- inflammatory effects.

Specific effects of estradiol on inflammatory mediators

More recent studies have focused on identifying specific mechanisms by which sex hormones modulate nociceptive responses [155,156]. Multon et al., (2005) used aromatase-knockout (ArKO) mice lacking endogenous estradiol production to demonstrate that lack of estradiol increases pain in the trigeminal formalin model [176]. During interphase and the

subsequent Phase II of the test knockout mice that were treated with estradiol displayed significantly less grooming behavior than knockout mice not treated with estradiol. Surprisingly, immunhistochemical abnormalities associated with lack of endogenous estradiol were only partially reversed after estradiol administration [176]. These findings suggest that estradiol deprivation may contribute some permanent alterations in the Arko mice. Interestingly, Khaser et al., (2001) demonstrated that estradiol administration reversed suppressed nociceptive behavior in Phase II of the formalin test that usually accompanies gonadectomy plus vagotomy. Also, estradiol implants had no effect on nociception in gonad intact females [177].

Furthermore, estradiol has been shown to both increase and decrease COX and PGE_2 (pain mediators) levels. For example, estradiol alters PGE_2 synthesis in non central nervous system tissue via a decrease in PGE_2 synthesis in bovine endometrium [178-180]. Mitsutoshi et al., (2004) demonstrated that E_2 treatments resulted in significantly increased COX-2 mRNA levels in primary human uterine microvascular endothelial cells HUMEC [181]. Specifically, E_2 in various concentrations increased COX-2 mRNA by 2.3 fold to 2.4 fold. A time dependent increase of COX-2 mRNA levels was observed and administration of an estradiol antagonist ICI 182,780 fully reversed effects of E_2 on COX mRNA levels in 1hr and protein levels in 4 hrs [181]. Moreover, use of a COX-2 inhibitor NS-398 before treatment with estradiol completely abolished the induction of PGE_2 levels by E_2. These findings suggest that the presence of estradiol is positively correlated to increases in COX-2 mRNA levels [181]. Another study discovered that ovariectomy enhances renal cortical expression and function of cyclooxygenase-2, also supporting a relationship between estradiol release and cyclooxygenase [62].

Findings from our group have discovered a significant correlation between COX protein levels and PGE_2 synthesis in support of previous literature which postulates the presence of

PGE_2 is positively correlated with increased nociceptive responding and regulated by cyclooxygenases [52,182-184].

Ferrinin et al., (1995) [185] demonstrated that estradiol and corticoid treatment on OVX/ADX rats modulates binding to glucocorticoid receptors (cystolic) in the brain in opposite directions [185]. Estradiol treatment of rats that had been previously exposed to chronic CORT treatment showed a reversed effect in depressed glucocorticoid receptor (GR) levels associated with chronic corticosterone treatment. Estradiol treatment resulted in increased levels of GR [185]. Moreover, estradiol treatment increased mRNA for GR and binding to the GR receptor [185]. In conclusion, a clear association between estradiol and GR activity via increasing or decreasing GR levels was observed.

As mentioned earlier the presence of corticosterone has also been implicated as contributing to nociceptive responses. Using in vitro studies, Mohn et al., (2005) [186] showed that PGE_2 increases CORT release by adrenal gland. Others have shown that prostaglandins directly stimulate corticosteroidogenesis in adrenocortical tissues and cells [187-189]. Moreover, findings in our lab revealed that chronic estradiol significantly elevated CORT serum levels while COX-2 protein levels were 25% lower in estradiol-treated rats not receiving formalin compared to vehicle naïve rats. In chronic estradiol treated animals, a positive correlation between PGE_2 and CORT serum levels were found while a negative correlation between PGE_2 and COX-1 proteins levels was observed. Acute estradiol treated animals, displayed significantly elevated CORT but there were no changes in COX-1 or COX-2 levels. However, PGE_2 serum levels were higher in 5% formalin than naïve 1% treated rats. From these observations and previous studies it was suggested that corticosterone release may be regulated by inflammatory stimuli which potentiates prostaglandin release, further increasing

corticosterone. The administration of exogenous estradiol may mediate this system by regulating levels of PGE_2 and/or CORT release thereby mediating nociceptive responses to formalin. Kuba et al., (2005) suggested that there is a bi-directional relationship between CORT and PGE_2 such that an inflammatory stimulus regulates CORT release and subsequent increases in PGE_2 also causes CORT release.

Based on preliminary findings from our lab and a literary review of inflammatory mediators as well as estradiol's link to their nociceptive actions a model was proposed.

Proposed Model

We postulate that estradiols's hyperalgesic effects on inflammation-induced pain can in part be mediated through regulation of the COX-prostanoid biosynthetic pathway directly by estradiol or estradiol induction of corticosterone regulation of the COX-prostanoid biosynthetic pathway as shown in Figure 4. Specifically, estradiol's administration is believed to down-regulate COX-2 protein levels and enzyme activity either directly or through corticosterone regulation but not that of COX-1. It is also hypothesized that estradiol will increase corticosterone levels which have been shown to negatively regulate PG levels. This combined effect could account for the reduction in behavioral responses to formalin after the administration of estradiol. Furthermore, down regulation of COX-2 enzymes by estradiol will decrease prostaglandin serum levels in formalin-and carrageenan treated rats resulting in attenuation of behavioral responses to persistent pain that will closely parallel levels of prostaglandins. Findings from preliminary experiments show correlations suggesting that COX-2, prostaglandin and corticosterone may play a major role in estradiols analgesic effects.

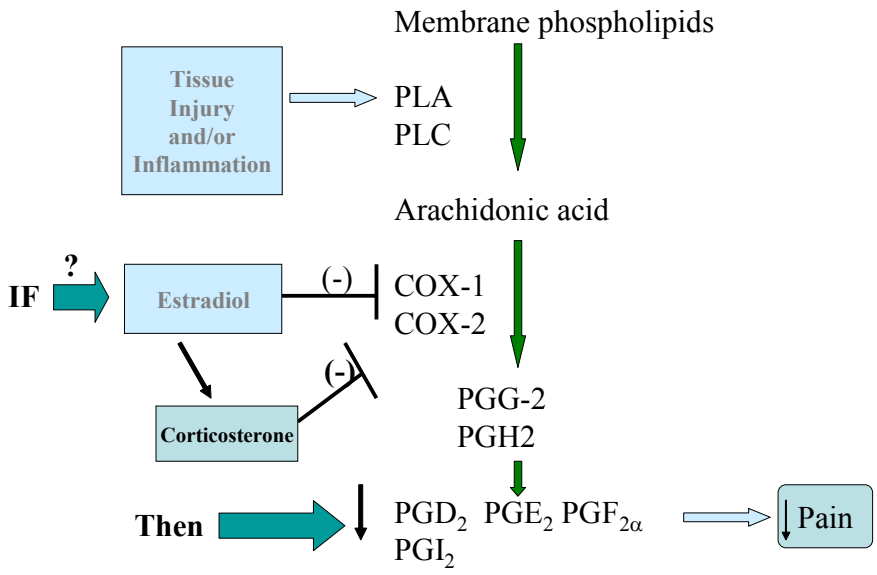

Figure 4. The proposed model suggests that both estradiol and corticosterone negatively regulate COX-2 and subsequent prostanoid production. Furthermore, estradiols anti-hyperalgesic effects on inflammatory pain are in part mediated through up regulation of corticosterone which negatively regulates prostaglandin synthesis via downregulation of COX-2 levels.

Specific AIMS:

Clinical and preclinical studies have demonstrated significant sex differences in the perception of chronic and inflammatory pain; females display higher nociceptive responses to inflammatory stimuli than male rats. Preliminary work in our lab has shown that hormone replacement of estradiol in female OVX rats attenuates nociceptive behavioral responding after formalin and carrageenan administration. Furthermore a positive correlation between corticosterone serum levels and prostaglandin release and PGE_2 and COX-2 protein levels were observed. These correlations suggested that COX-2, prostaglandin and corticosterone may play a major role in estradiol's analgesic effects to inflammatory-induced pain.

Elucidating the specific biological pathway in which estradiol imposes its affect on attenuating pain perception is particularly relevant. This work will serve to further the knowledge of dynamic hormonal interactions and their effect on persistent pain behaviors in the field of nociceptive (pain) processing; and it also has major clinical implications. For example, if hormonal changes which accompany the female reproductive cycle produce either pro-nociceptive or anti-nociceptive effects, differential pain treatment dependent on a patient's stage of the reproductive cycle may be needed to manage their pain. In addition, results from this work will help elucidate mechanism(s) involved in regulating pain responses, which in turn, will provide more rational and/or evidence for differential treatment between women and men.

` The specific aim of this project is to determine how estradiol reduces inflammatory pain responses. Specifically, through which biochemical pathway(s) are estradiol's effects mediated. Our hypothesis is that *estradiol's anti-hyperalgesic effects on inflammatory- induced pain are in part mediated through corticosterone and COX-2 regulation of the prostanoid biosynthetic pathway.* To test this hypothesis the following aims were proposed:

Specific Aim 1: To determine if estradiol's anti-hyperalgesic effects on behavioral responses to inflammatory stimuli (formalin) are in part mediated through deactivation/down regulation of COX-1 and COX-2. To test this hypothesis two experiments were designed: **1A.** Use pharmacological antagonists to block COX-2 and COX-1 activation in OVX rats that received either cholesterol (vehicle) or estradiol **1B.** Measure behavioral responses and prostaglandin levels after three different conditions: (1) Chronic estradiol (Silastic implant), (2) Administration of COX inhibitor (i.p injection) or (3) Co-administration of chronic estradiol and COX inhibitor. We predict that estradiol administration will down-regulate COX-2 protein levels and enzyme activity resulting in attenuated behavioral responding a reduced PGE_2 levels. Moreover, estradiol will interact with NS398 (selective COX-2 inhibitor) and/or ibuprofen (non-selective COX inhibitor) resulting in a potentiated attenuation of behavioral responses and PGE2 release than either estradiol or pharmacological agent alone would produce. We also predict that behavioral responses and PGE_2 but not PGD_2 serum levels will be reduced by estradiol.

Specific Aim 2: **To determine if estradiol's anti-hyperalgesic effects can be extended to different forms of inflammatory induced pain, (i.e. carrageenan-inflammatory model) and if so, are estradiol's anti-hyperalgesic effects on behavioral responses to this inflammatory stimuli in part mediated through deactivation/down regulation of COX-1 and COX-2.** To this end, three experiments were designed: 1A. Measure carrageenan-induced inflammatory behavioral responses in OVX rats that received either cholesterol (vehicle) or estradiol. 1B. Measure behavioral responses and prostaglandin levels after three different conditions: (1)

Chronic estradiol (Silastic implant), (2) Administration of COX inhibitor (i.p injection) or (3) Co-administration of chronic estradiol and COX inhibitor. We predict that estradiol administration will down-regulate COX-2 protein levels and enzyme activity resulting in attenuated behavioral responding and reduced PGE_2 levels. Moreover, estradiol will interact with NS398 (selective COX-2 inhibitor) and/or SC560 (a selective COX-1 inhibitor) resulting in a potentiated attenuation of behavioral responses and PGE2 release than either estradiol or pharmacological agent alone would produce. We also predict that behavioral responses and PGE_2 but not PGD_2 serum levels will be reduced by estradiol.

Specific Aim 3: To determine if estradiol's anti-hyperalgesic effects on behavioral responses to inflammatory stimuli are in part mediated through the induction of corticosterone release. To this end three experiments were designed: 3A. Levels of corticosterone will be measured after formalin and carrageenan administration. We predict that corticosterone will be dose-dependently increased only in estradiol-treated groups. 3B. The effect of estradiol on flinching responses after formalin administration will be determined in OVX, adrenalectomized (ADX), and OVX+ADX rats after estradiol or vehicle treatments. We predict that in ADX and OVX + ADX rats, estradiol will not produce anti-hyperalgesic effects after formalin administration. 3C. Measurement of PGE2 and PGD2 will be analyzed. We predict that PGE2 levels will be attenuated in OVX animals that recived chronic estradiol treatment but not ADX or ADX + OVX animals.

Methods

Animals

Eight-week-old ovariectomized (OVX) female Sprague-Dawley rats purchased from Taconic (Germantown, NY) were double-housed in 12-h light-dark cycle (lights on 8 AM) with food and water ad libithum. Animals were randomly assigned to experimental groups (n = 16 to 20 per group). At the time of nociceptive testing, rats weighed between 200 and 240g. Each study consisted of at least four different cohorts of rats. Animals were not reused for any study. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, Bethesda, MD and approved by the Institutional Animal Care and Use Committee at Hunter College of The City University of New York).

Reagents

17-β-estradiol 3-benzoate, cholesterol, sigma grade and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO). Ibuprofen, SC560, and NS398 were purchased from Cayman Chemical (Ann Arbor, MI). λ-Carrageenan was purchased from Fluka BioChemika (Ronkonkoma, NY). 0.09% Sodium Chloride solution was purchased from B. Braun Medical Inc. (Irvine CA). DMSO or 0.09% Sodium Chloride solution was used as a vehicle for all drugs. In Table: 2 we summarized the doses and manner of drug administration.

TABLE 2: Summary of doses and manner of administration for drugs used in this study.

Drug	Doses	Method of Adminstration
17-β-estradiol 3-benzoate	**10-30%**	**subcutaneous Silastic implant**
Cholesterol (vehicle for estradiol)	**100%**	**subcutaneous Silastic implant**
λ-Carrageenan	**1%**	**100µl intraplantar injection**
Formalin	**5%**	**50µl intraplantar injection**
Ibuprofen (non-selective COX inhibitor)	**40 or 100mg/kg**	**intraperitoneal injection**
SC560 (selective COX-1 inhibitor)	**20mg/kg**	**intraperitoneal injection**
NS398 (selective COX-2 inhibitor	**20 mg/kg**	**intraperitoneal injection**
DMSO (vehicle for antagonists)	**1mg/kg**	**intraperitoneal injection**

Estradiol replacement

Two weeks after ovariectomy, SILASTIC capsules (1 cm, 0.058 in. ID x 0.077 in. OD, Dow corning) were inserted into the nape of the animals neck. Capsules contained either vehicle (100% cholesterol) or estradiol (10-30% 17-β-estradiol: 80% cholesterol). This dose was chosen because it falls within the range of serum levels during the reproductive cycle and represents the maximally effective dose for attenuating formalin response without affecting basal activity [157,190,191,191].

Formalin apparatus

An automated flinch detecting system referred to as the "automated nociception analyzer" was used in the formalin nociceptive assay [192]. This instrument was purchased from the Department of Anesthesiology at the University of California, San Diego. All parameters of the program were set to default values [192]. Behavioral testing was conducted between 9:00 a.m. and 3:00 p.m.

Formalin assay

Formalin assay was carried out as previously described with minor modifications. Briefly, one week after hormone replacement, a soft metal band was placed on the right hind paw with the opening positioned at the plantar surface of the paw. To minimize the novelty of the testing environment and band, rats were placed inside the testing chamber for a total of 30 minutes prior to the formalin injection. Five-percent formalin, at a volume of 50µL, was injected intra-plantar on the banded right hind paw. Rats were then placed in the chamber and behavioral activity data collected at 1 min intervals for a total of 60 min after the formalin injection.

Carrageenan Apparatus

The Hargreaves' box (PAW thermal stimulator) was purchased from the Department of Anesthesiology at the University of California, San Diego. This apparatus consists of 6 plexiglass enclosures (21/8.5 cm). These enclosures are positioned on a heated glass surface that maintains a temperature of 30ºC ±1ºC. A mobile infrared heat lamp is able to focus different heat intensities ranging from 4.0-6.0mv. Three heat intensities were used respectively, 4.50 (low), 4.80 (medium) and 5.20mv (high). Here after these heat intensities will be referred to as low, medium and high (respectively). Behavioral testing was conducted between 9:00 a.m. and 3:00 p.m.

Carrageenan assay

Behavioral testing was done one week after hormone replacement animals. To minimize novelty of the new testing environment animals were placed into the testing chamber for a total of 30 minutes. After this acclimation period baseline latency to three heat intensities were determined (low, medium and high) determinations. Approximately 45 minutes after baseline assessments 100µl of 1% carrageenan was injected intra-plantar on the right hind paw. Rats were then placed back in the testing chamber. Underneath the targeted hind paw, a focused light source was then applied to the plantar surface of the injected or contralateral paw. The latency to withdrawal of the paw was automatically recorded [193]. Rats were free to remove their paw at the point of discomfort. Paw withdrawal latencies after carrageenan injection were measured for all three different heat intensities at one and five hours after carrageenan injection in the right and left paw.

Corticosterone and prostaglandin measurements

After behavioral testing rats were sacrificed by decapitation (following a brief exposure (20seconds to CO_2). Trunk blood was centrifuged at 3,000 RPM for 30 min at 4 ºC. Serum was collected and then stored at -80 ºC until used. Serum levels of corticosterone were detected using Coat-A-Count radioimmunoassay kits (Diagnostic Products Corporation, Los Angeles, CA). Intra-assay coefficients of variation averaged 10.0% ± 1.0%. PGE_2 and PGD_2 serum levels were detected by using enzyme immunoassay kits from Cayman Chemical (Ann Arbor, MI). Results for these assays were determined via a log-logit analysis within Graph Pad Prism

Software (San Diego, CA). Prostaglandin serum levels were expressed as pg/mL. Corticosterone serum levels were expressed as ng/mL.

Statistical data analysis

Two and three way ANOVAs were used to determine significant differences in behavioral and neurochemical measurements. Tukey's least significant difference post hoc testing was done when appropriate. For all analyses, significance was at the level of $p < 0.05$.

Chapter 2

Are estradiol's anti-hyperalgesic effects on behavioral responses to formalin in part mediated through deactivation/down regulation of COX-1 and/or COX-2?

Results

Effects of non-selective COX inhibition on estradiol- induced analgesia

As shown in Figure 5, a main effect of drug (ibuprofen) [F=2.398, p=0.050] and hormone [F=6.425, p=0.003] on formalin-induced behavioral responses was observed; ibuprofen (40 mg/kg) significantly decreased the number of flinching during Phase II (5B) after formalin administration when compared to the control group ($p<0.05$). Estradiol significantly decreased behavioral responses during both phases (5A, B) of formalin responses when compared to control groups (Phase I: $p<0.05$; Phase II: $p< 0.005$). As shown in Figure 6 and 7, both ibuprofen and estradiol reduced behavioral flinching across all treatment groups when compared to vehicle and control groups, respectively.

Effects of Ibuprofen on PGE_2 and PGD_2 serum levels

As shown in Figure 8A, a significant drug effect on PGE_2 serum levels was observed [F=8.661, p=0.001]; 100 mg/kg of ibuprofen significantly lowered PGE_2 serum levels when compared to control groups ($p<0.001$). Furthermore, a significant hormone effect was observed (t= 2.446, p=0.05); estradiol + 40 mg/kg Ibuprofen further decreased PGE2 levels when compared to vehicle-treated animals. As shown in Figure 8B, PGD2 serum levels were not altered by either estradiol or ibuprofen treatments.

Effects of ibuprofen on corticosterone serum levels

As shown in Figure 9, a significant drug effect was also observed on corticosterone serum levels [F=6.475, p=0.003]; 100 mg/kg of ibuprofen significantly increased corticosterone

serum levels when compared to control rats ($p<0.005$). Although estradiol + ibuprofen (100mg administration further increased corticosterone serum levels, it failed to reach statistical significance [$F=3.072$, $p=0.086$].

Effect of NS398 on estradiol- induced analgesia

As shown in Figure 10, a main effect of drug (NS398) [$F= 3.405$, $p=0.040$] and hormone [$F=11.368$, $p=0.000$] was observed; NS398 significantly decreased the number of flinches during Phase II (10B) but not Phase I (10A) when compared to vehicle treated rats ($p<0.05$). Estradiol significantly attenuated behavioral responses after formalin administration for both Phase I ($p<0.000$) and Phase II ($p<0.000$) when compared to control groups. However, no interaction between estradiol and NS398 was observed [$F=0.826$, $p=0.444$]. As shown in Figure 11, estradiol-treated animals showed overall reduced behavioral flinching throughout the time course compared to untreated animals.

NS398 effects on PGE_2 and PGD_2 serum levels

As shown in Figure 12A, a significant drug effect on PGE_2 serum levels was observed [$F=11.113$, $p=0.005$]. NS398 significantly lowered PGE_2 serum levels when compared to control or estradiol-treated groups ($p <0.01$). However, estradiol replacement did not produce a significant effect [$F=1.560$, $p=0.235$], nor estradiol plus NS398 administration [$F=1.284$, $p=0.279$]. As shown in Figure 12B, a main effect of NS398 on PGD2 serum levels was observed [$F=6.840$, $p=0.0225$]; PGD2 serum levels were significantly increased in NS398-treated animals when compared to vehicle-treated animals ($p<0.05$).

Effects of NS398 on corticosterone serum levels

As shown in Figure 13, a significant drug effect on corticosterone serum levels was observed ([F=0.030, p=0.038]; both NS398 and NS398 plus estradiol treatment significantly increased corticosterone serum levels when compared to control and estradiol-treated groups, ($p<0.05$ and $p<0.05$, respectively).

Effects of SC560 on estradiol- induced analgesia

As shown in Figure 14, a significant main effect of hormone was observed [F=15.692, p=0.000]. Formalin-induced flinching was significantly reduced by estradiol during both phases when compared to control groups (Phase I: $p<0.05$; Phase II: $p<0.000$). Although, estradiol + SC560-treated groups had significantly less number of flinches after formalin administration when compared to control groups this difference failed to reach significance. Furthermore, SC560 administration had no effect on either Phase I or Phase II. As shown in Figure 15, estradiol-treated animals showed reduced behavioral flinching compared to vehicle-treated animals.

Effects of SC560 on PGE_2 and PGD_2 serum levels

As shown in Figure 16A, neither SC560 nor estradiol treatments significantly altered PGE_2 serum levels [F=4.460, p=.054]. However, the estradiol treated SC560 group had lower PGe2 levels compared to the untreated group. As shown in Figure 16B, although estradiol-treated animals showed increased PGD2 serum levels, when compared to vehicle-treated control, they also failed to reach significance [F=3.49, p=0.081].

Effects of SC560 on corticosterone serum levels

As shown in Figure 17, there was a main effect for hormone on corticosterone serum levels [$F=6.834$, $p=0.018$]; estradiol and estradiol + SC560 increased corticosterone serum levels when compared to control or SC560 treatment ($p<0.05$ for all comparisons.)

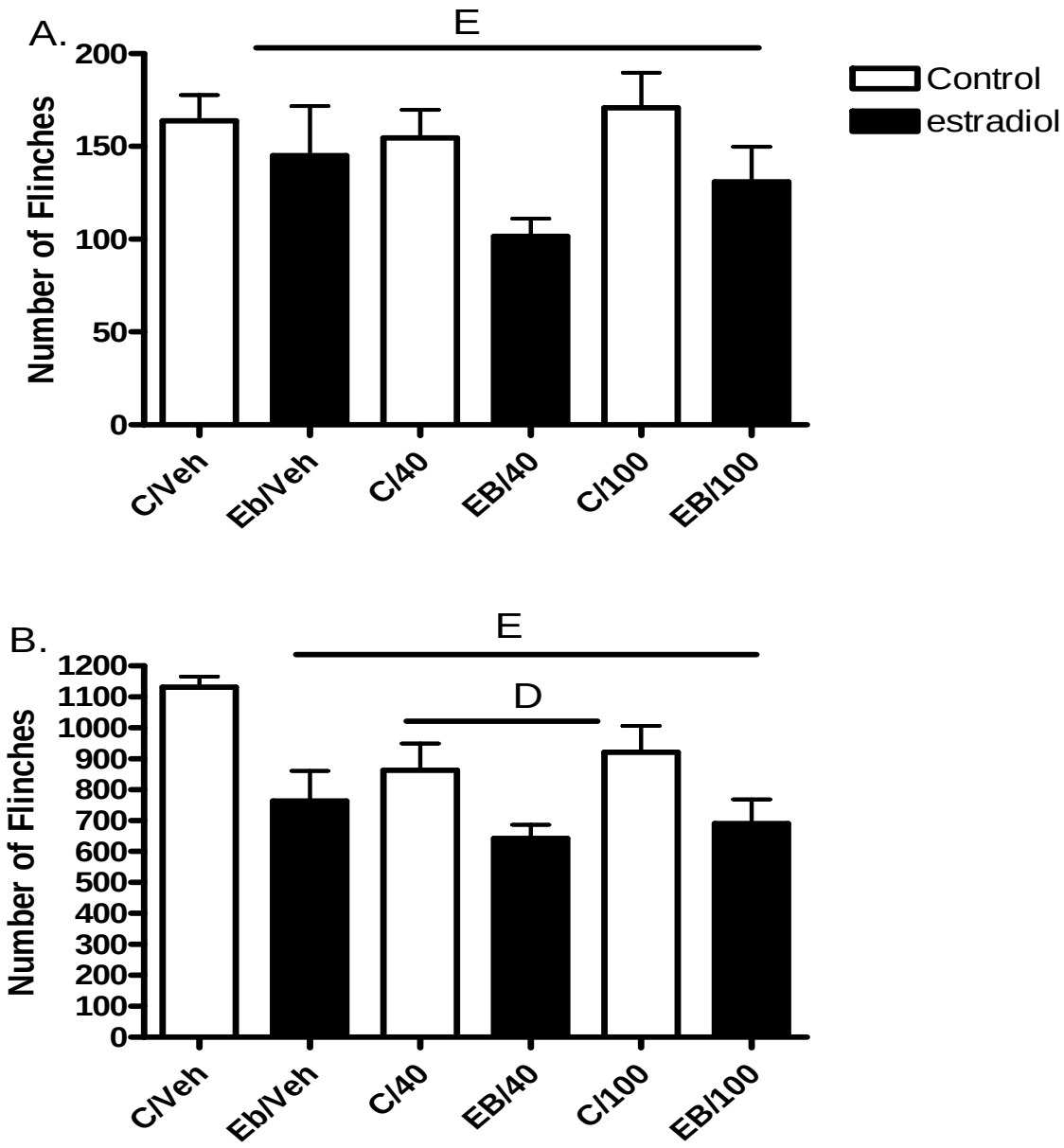

Figure 5: Effects of ibuprofen (0, 40 or 100mg/kg) and estradiol + Ibuprofen on behavioral flinching responses after (5%) formalin administration. A. Data represents the cumulative mean flinches (± SEM) during Phase I (0-6 min) and **B.** Phase II (9- 40 min) in estradiol or vehicle-treated animals after ibuprofen (0, 40, 100 mg/kg) or vehicle (DMSO) administration. Behavior was recorded for 60 minutes after formalin administration

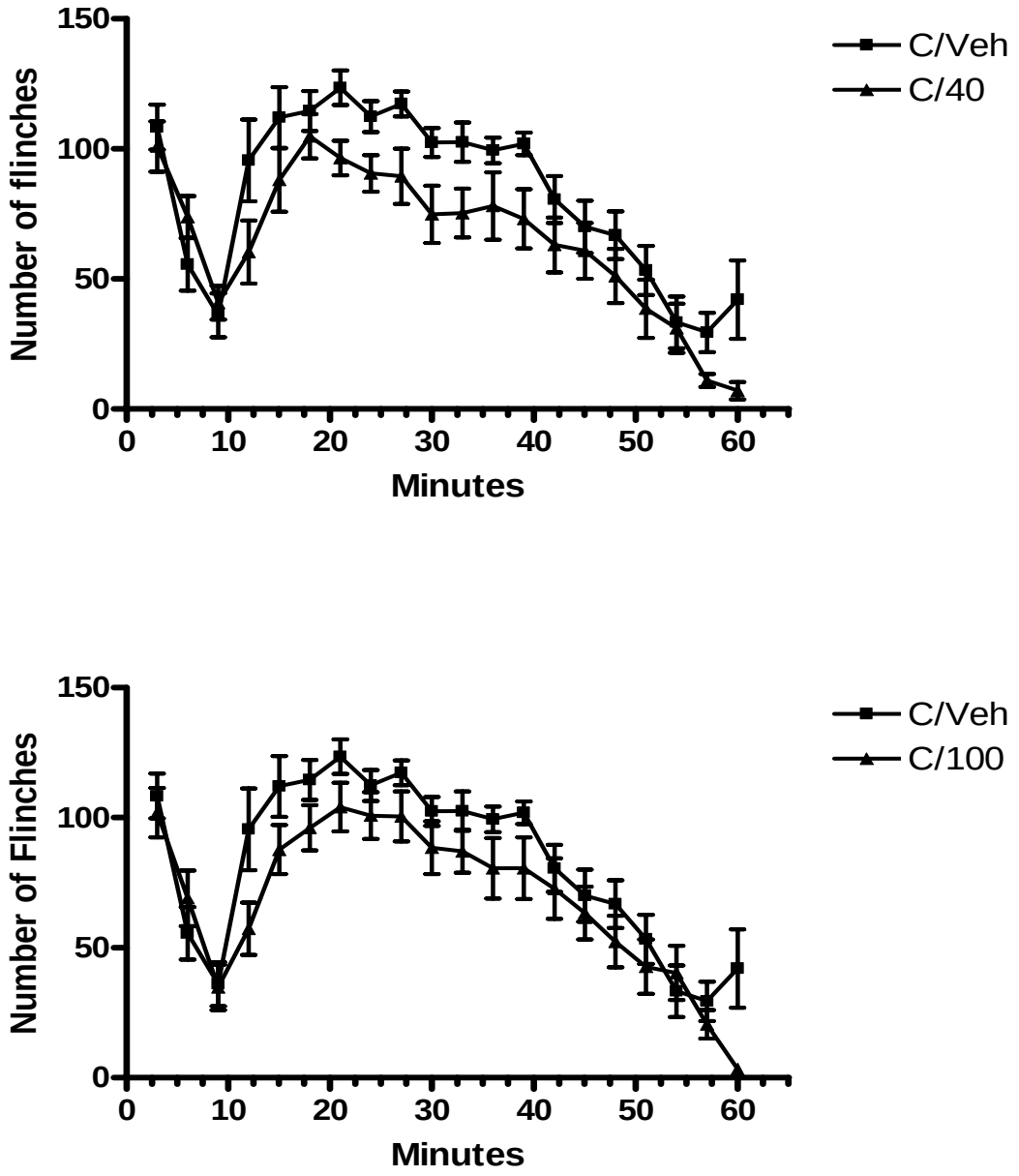

Figure 6: Time course of flinching responses in estrogen + ibuprofen treated rats after formalin administration (5%). Data represents the mean of flinching responses in 3 minute bins (n=9-11/dose).

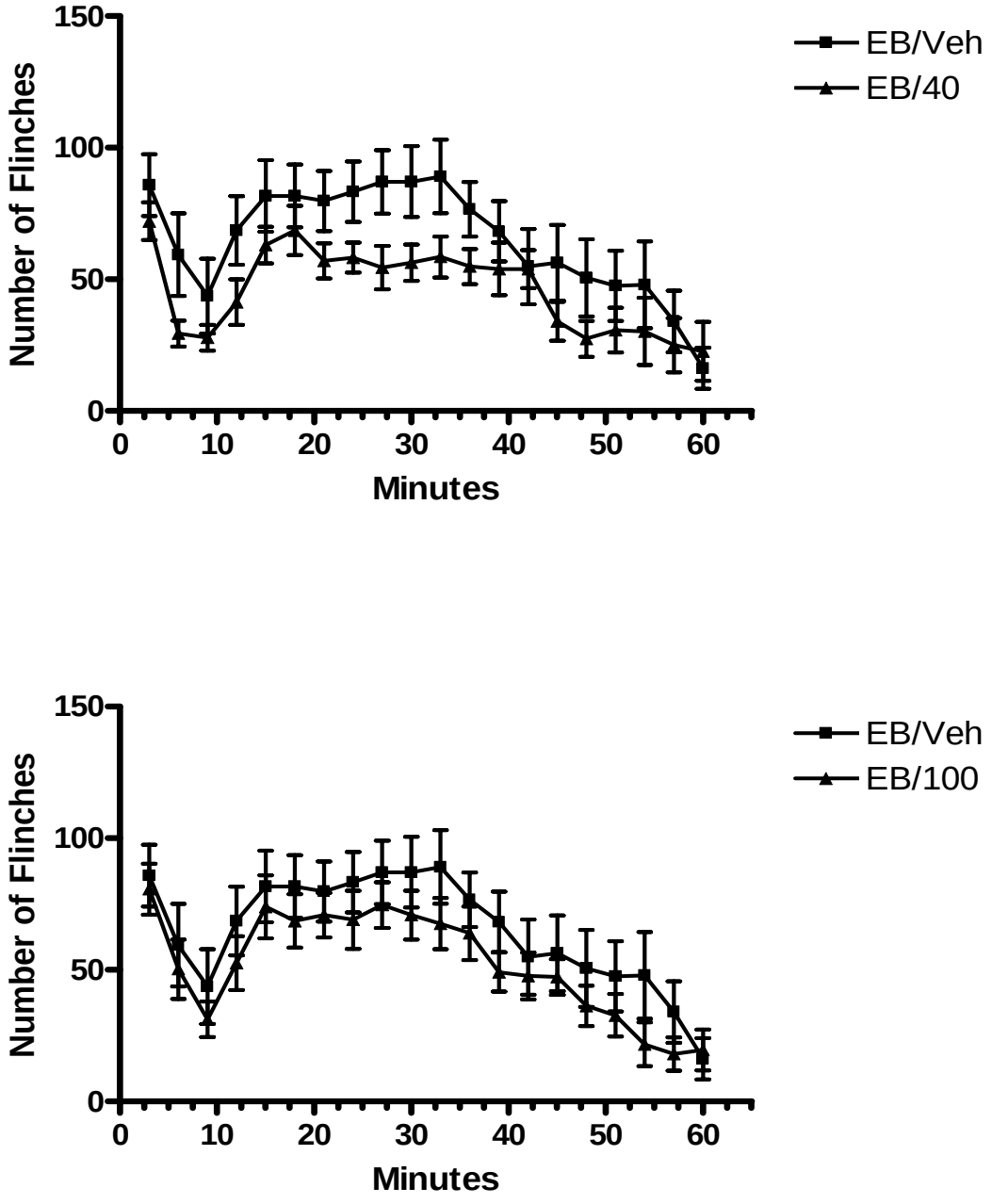

Figure 7: Time course of flinching responses in estradiol + ibuprofen treated rats after formalin administration (5%). Data represents the mean of flinching responses in 3 minute bins (9-11/dose).

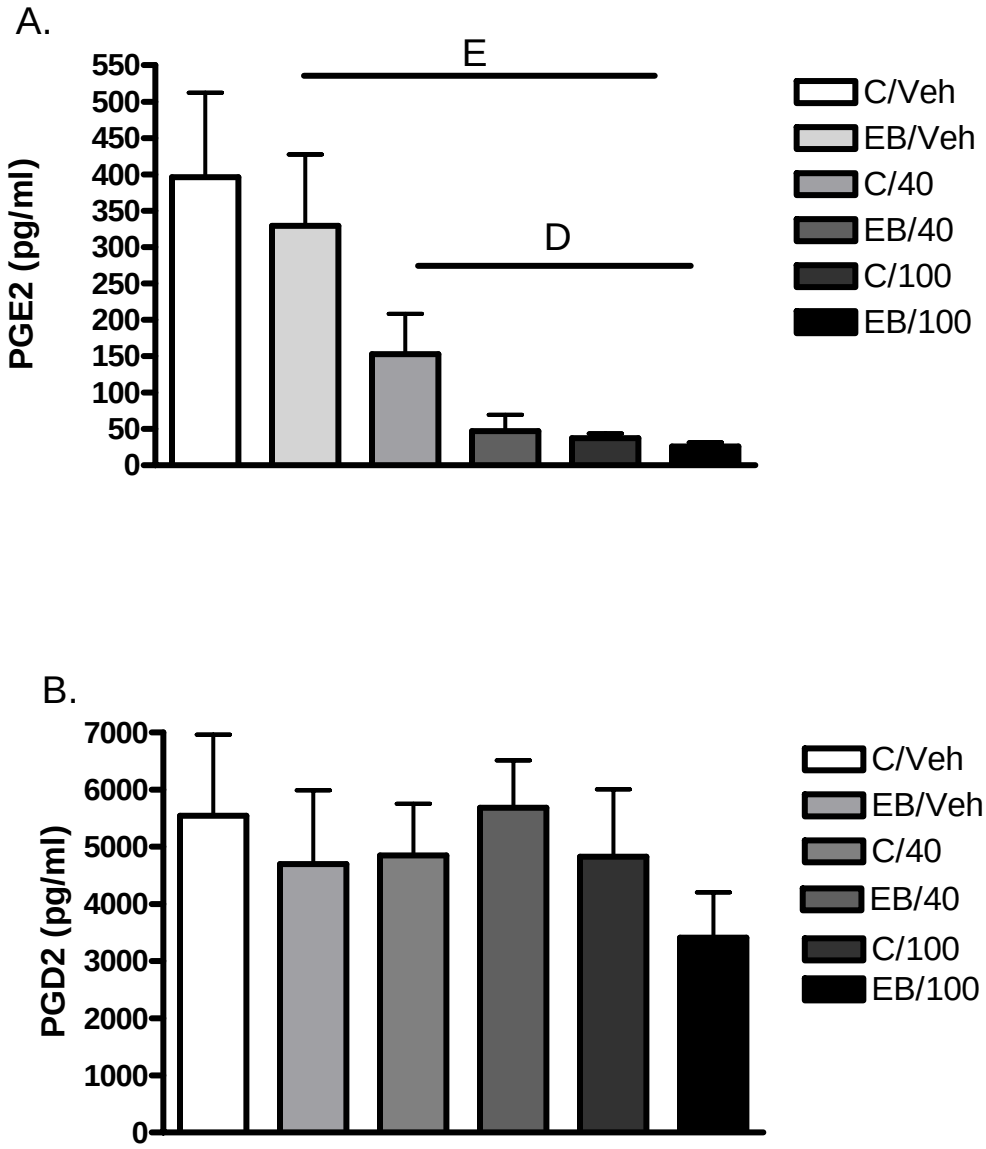

Figure 8. Effects of ibuprofen and estradiol on PGE_2 (A) and PGD_2 (B) serum levels. **A.** Data represents mean prostaglandin E2 serum levels (± SEM) at picograms per milliter. Each bar represents estradiol or vehicle-treated animals after ibuprofen (0, 40, 100 mg/kg) or vehicle (DMSO) administration (n = 4-5). **B.** Data represents mean prostaglandin D2 serum levels 60 minutes after formalin administration (n = 4-6). (E) Denotes a significant hormone effect ($p<0.05$) and (D) Denotes a significant drug effect ($p<0.05$).

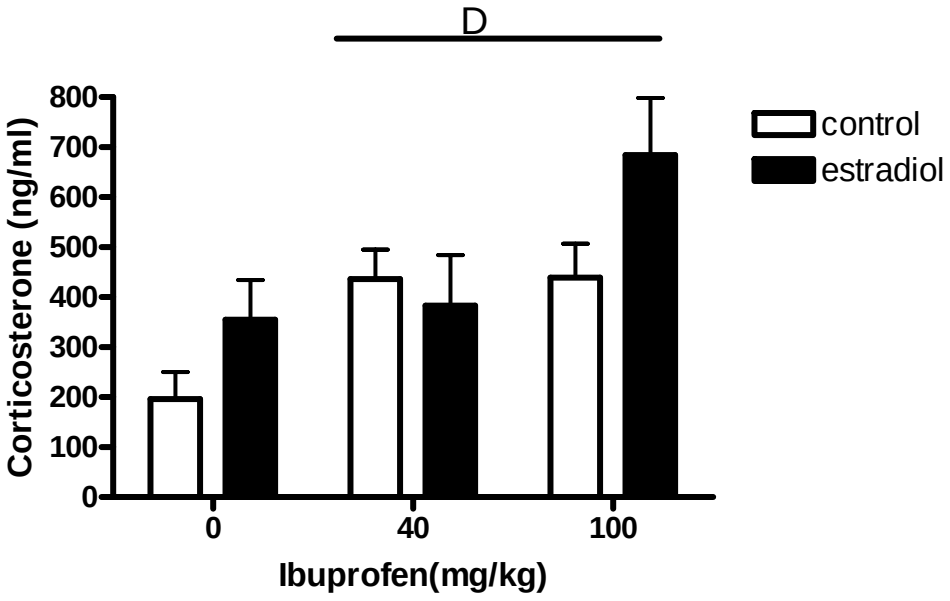

Figure 9. Effects of ibuprofen and estradiol on corticosterone serum levels. Data represents mean CORT serum levels (± SEM) measured in nanograms per milliters from trunk blood collected after formalin administration (n=8=11). (D) Denotes a significant main effect for drug treatment ($p<0.05$).

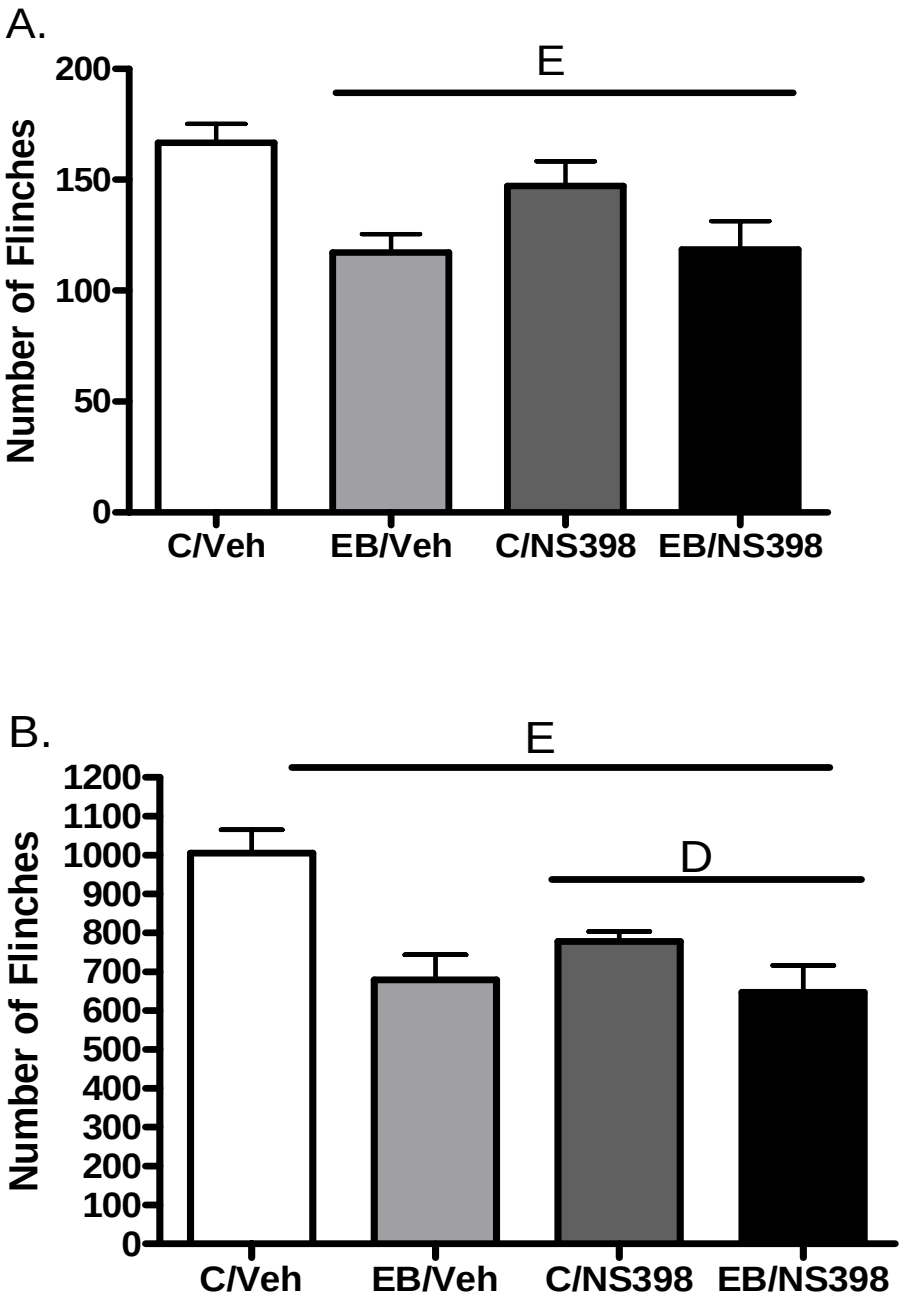

Figure 10: Effects of NS398 and estradiol on behavioral flinching responses after 5% formalin administration. (A.) Data represents the cumulative mean flinches (± SEM) during Phase I (0-6 min) and **(B.)** Phase II (9- 40 min) in estradiol or vehicle-treated animals after NS398 (20 mg/kg) or vehicle (DMS0) administration. Data is presented as the sum activity throughout 40 minutes of testing.

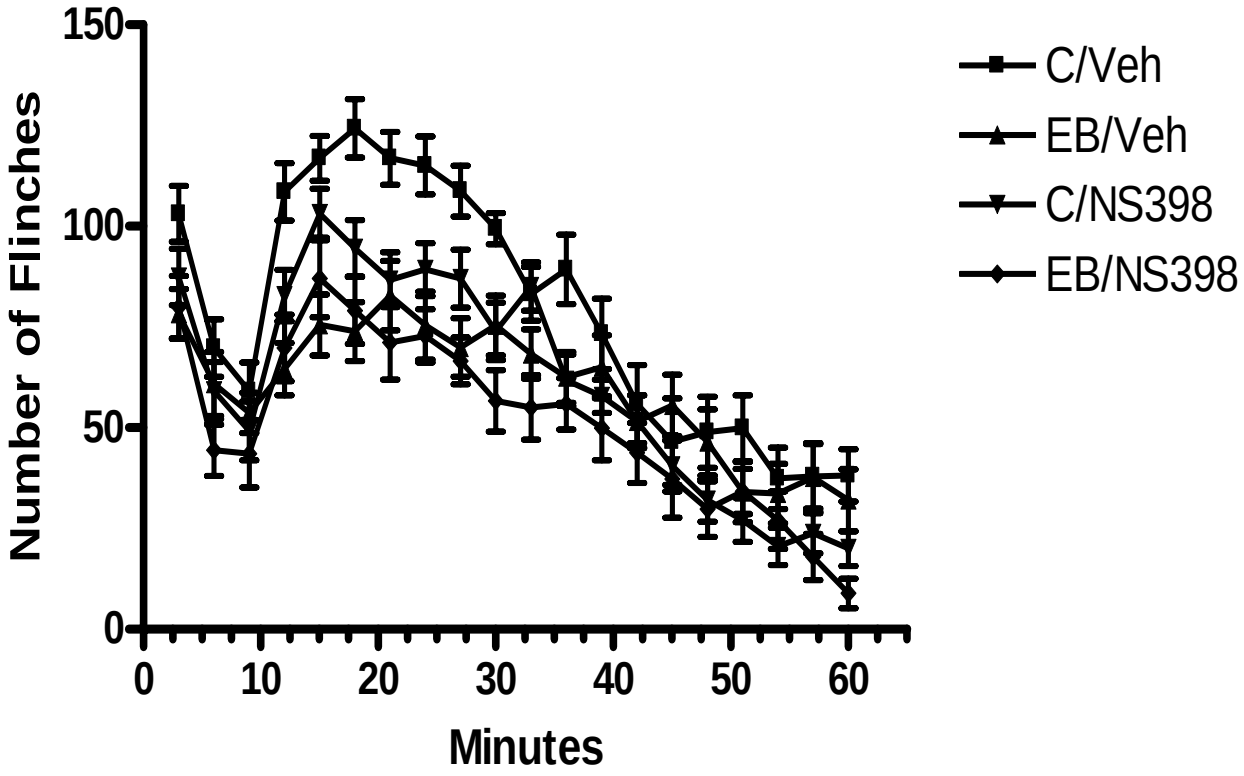

Figure 11: Time course of flinching responses in estradiol + NS398 treated rats after (5%) formalin administration B. Time course of activation is represented as the mean of flinching responses in 3 minute bins (n=12-15/dose).

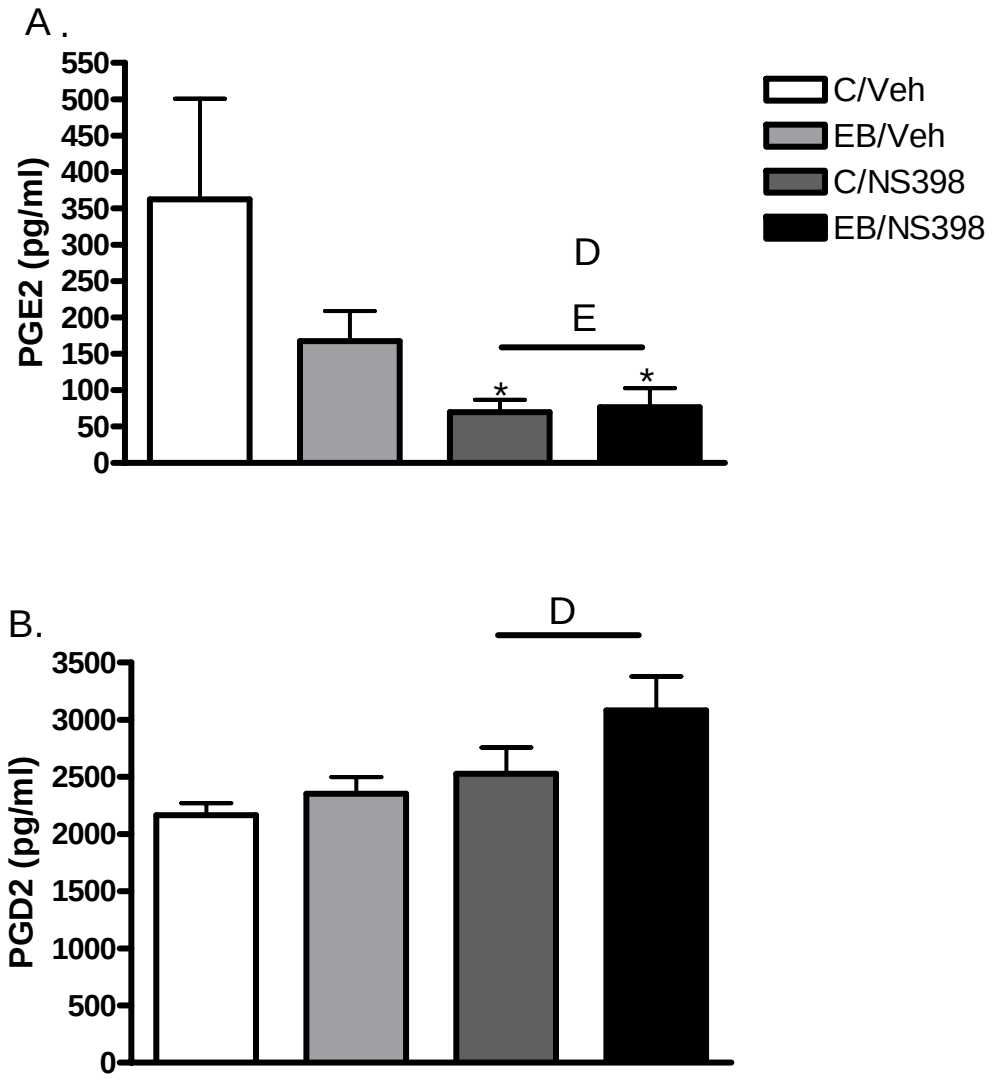

Figure 12. Effects of NS398 and estradiol on PGE_2 (A) and PGD_2 (B) serum levels. **A.** Data represents mean prostaglandin E2 serum levels at picograms per milliter after formalin administration. Each bar represents estradiol or vehicle-treated animals after NS398 (20 mg/kg) or vehicle (DMSO) administration (n = 4-5). **B.** Data represents mean prostaglandin D2 serum levels 60 minutes after formalin administration (n = 4). (#) Denotes a significant hormone effect. (D) Denotes a significant drug effect.

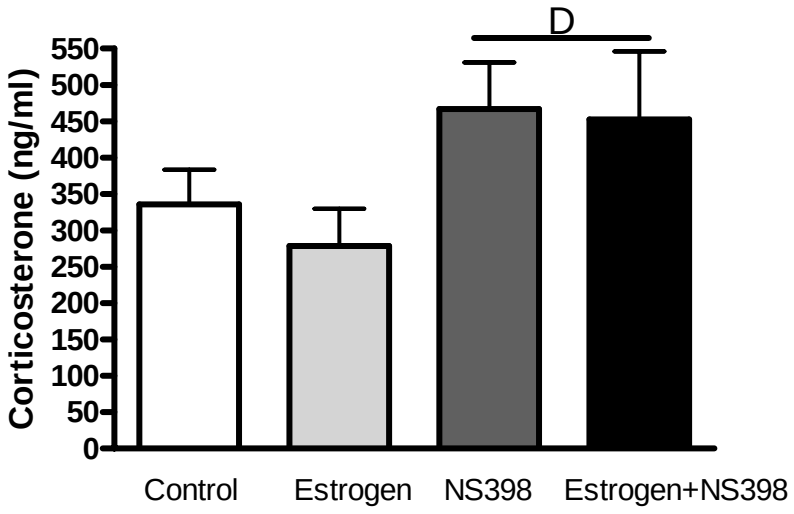

Figure 13. Effects of NS398 and estradiol on corticosterone serum levels. Data represents mean CORT serum levels (± SEM) measured in nanograms per milliters from trunk blood collected after formalin administration (n=8-9). (D) Denotes a significant main effect for drug ($p<0.05$).

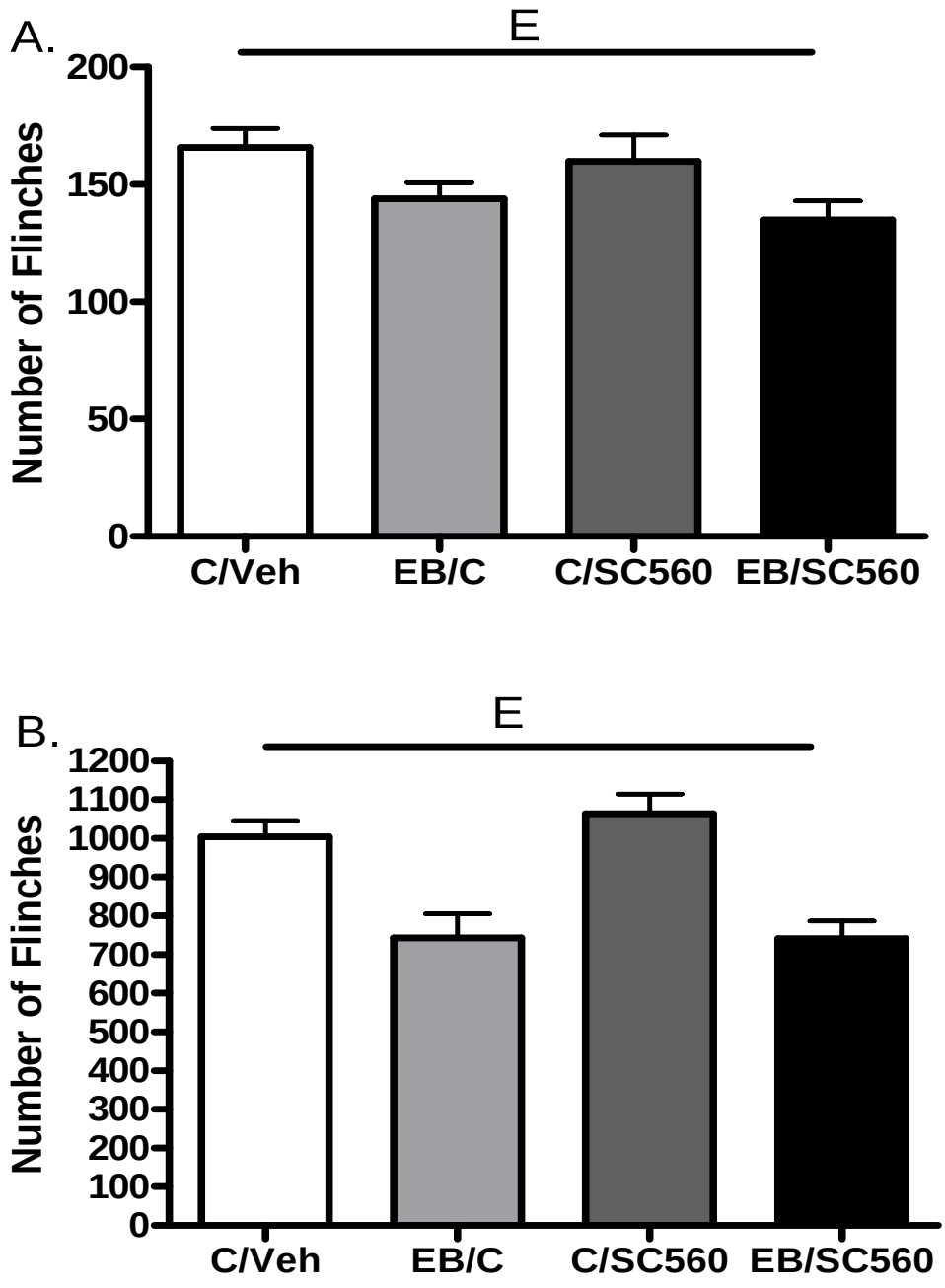

Figure 14. Effects of SC560 and estradiol on behavioral flinching responses after 5% formalin administration **A.** Data represents mean cumulative flinches (± SEM) in Phase I (0-6 min) and **B.** Phase II (9- 40 min) in estradiol or vehicle-treated animals after SC560 (20 mg/kg) or vehicle (DMSO) administration**.**

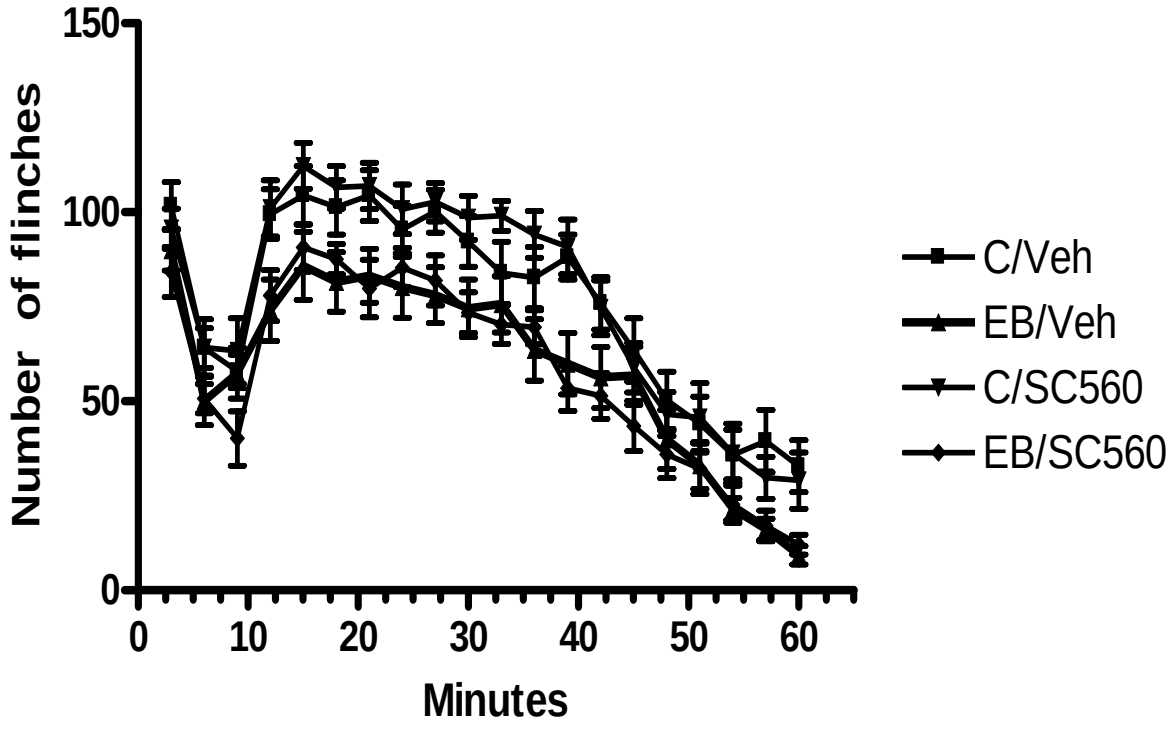

Figure 15. Time course of flinching responses in estradiol + SC560 treated rats after (5%) formalin administration. Time course of activation is represented as the mean of flinching responses in 3 minute bins (n=9-10/dose).

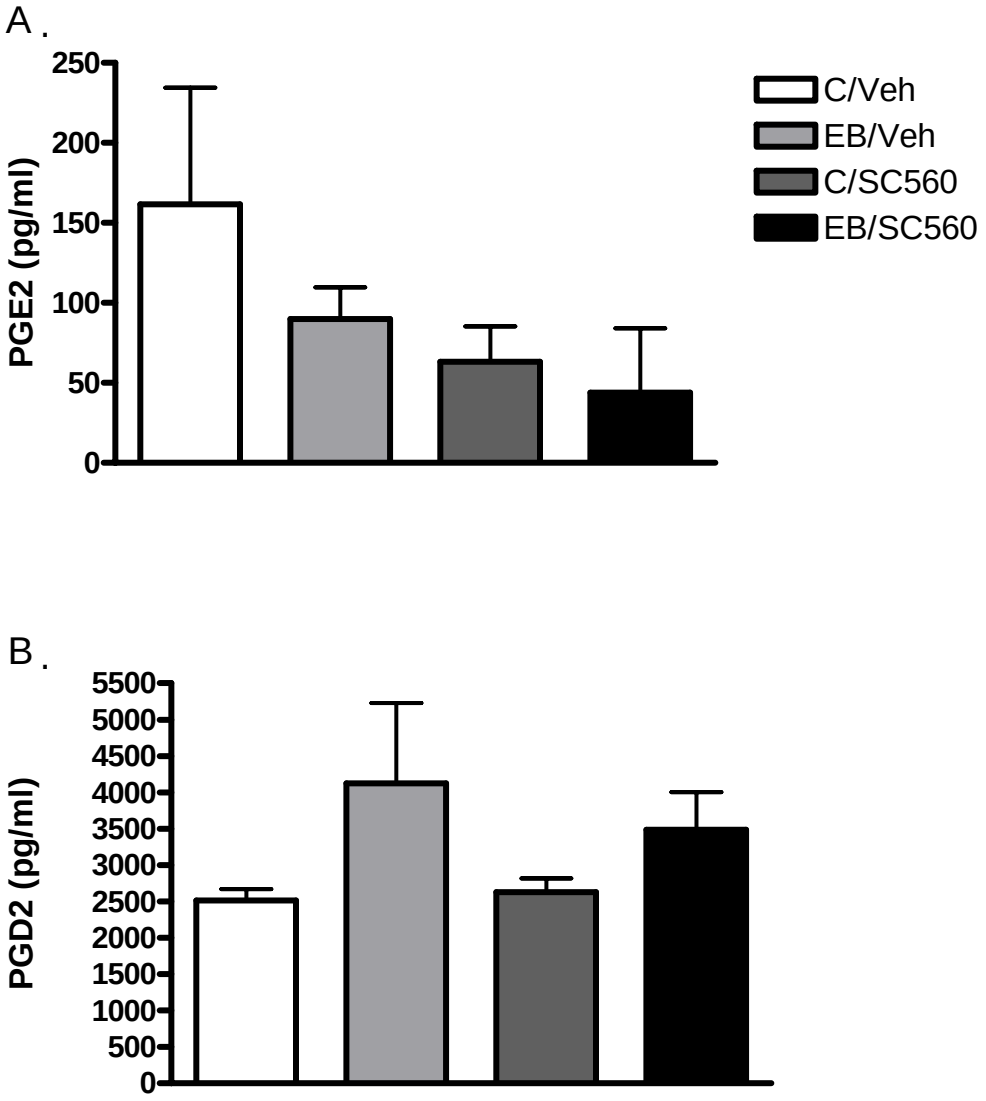

Figure 16. Effects of SC560 and estradiol on PGE2 (A) and PGD2 (B) serum levels **A.** Data represents mean prostaglandin E2 serum levels (± SEM) at picograms per milliter sixty minutes after formalin administration. Each bar represents estradiol or vehicle-treated animals after SC560 (20 mg/kg) or vehicle (DMSO) administration (n = 3-5). **B.** Data represents mean PGD_2 serum levels (± SEM) after formalin administration (n= 4-5).

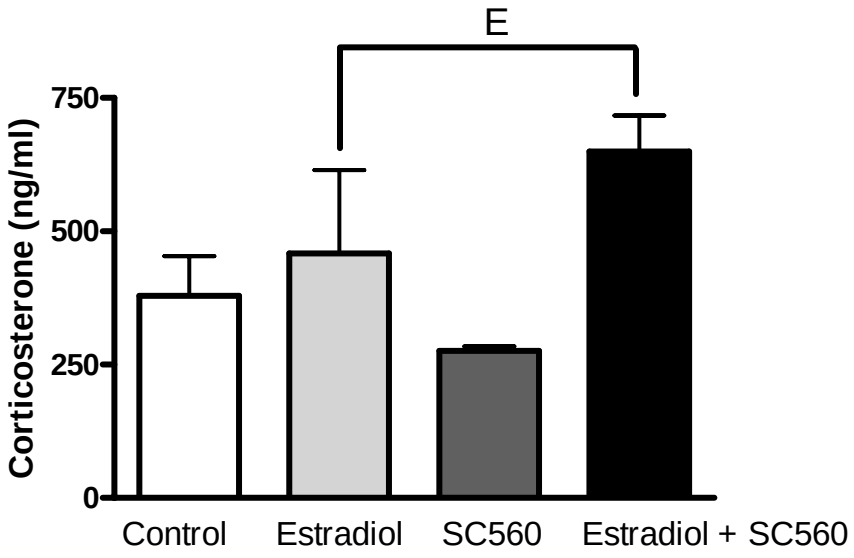

Figure 17. Effects of SC560 and estradiol on corticosterone serum levels Data represents mean CORT serum levels (± SEM) measured in nanograms per milliters from trunk blood collected sixty minutes after formalin administration (n=5-6). (E) Denotes a significant effect for hormone.

Discussion

The results of this study support and extend preliminary findings from previous studies in our lab [165,194,195]. First, estradiol replacement in female rats after OVX significantly reduces behavioral responses after formalin administration in not only Phase II but Phase I as well. Second, co-administration of a non-selective COX inhibitor, a selective COX-1 or COX-2 inhibitor + estradiol replacement resulted in a greater reduction of behavioral responses compared to either estradiol or COX inhibitor alone using the formalin pain model.

The present study demonstrated that estradiol replacement in OVX rats significantly reduces behavioral responses after formalin administration. Surprisingly, a significant attenuation was observed in both Phase I and Phase II. Both Mannino et al., (2006) and Kuba et al., (2005) observed this effect in Phase II only [157,196]. As discussed in the introduction, the formalin test produces bi-phasic flinching responses that are seen over 60 minutes after intraplantar administration of formalin. Phase I of the test represents behavioral responses corresponding to acute pain, whereas Phase II represents behavioral responses corresponding to injury-induced persistent pain which includes inflammatory pain. Although nociceptive behaviors seen in Phase II are due to central sensitization, peripheral inflammatory responses have been shown to contribute to this sensitization [197-200]. The significant effects of estradiol in attenuating behavioral responses during Phase I are not only novel but suggest that estradiol's effects can be extended to acute (nociceptive) as well as inflammatory pain responses.

COX inhibitors effects on inflammatory behavioral responses

Results showed a significant effect of Ibuprofen (a non-selective COX inhibitor) and NS398 (selective COX-2) but not SC560 (selective COX-1) on behavioral flinching in Phase II after

formalin administration. These findings are consistent with related literature whereby the role of COX-2 in inflammatory responses is highly supported and evidence suggesting a role for COX-1 in these reponses has been inconsistent. As mentioned earlier, cyclooxygenase (COX) is the rate limiting enzyme that catalyzes the conversion of arachdonic acid to prostaglandins (PG's) [22,62]. After injury, levels and activity of COX-2 proteins increases [61,201-203], suggesting a modulatory role of PG in spinal cord sensitization. Additionally, subcutaneous or IT administration of selective COX-2 inhibitors has been shown to suppress inflammatory-induced hyperalgesia [22,101,204]

COX inhibitors + estradiol effects on inflammatory behavioral responses

Overall, behavioral responses were attenuated to a greater degree when estradiol was co-administered with NS398 or Ibuprofen using the formalin assay. However, the increase in attenuation after co-administration of estradiol and COX inhibitors were either additive or sub additive suggesting independent pathways through which these individual mediators (estradiol or COX inhibitor) may be working through to attenuate behavioral responses after formalin administration. If these mediators were working in part through the same pathway, a more robust and/or synergized effect on behavioral responses should have been observed. This was not the case, thus these findings suggest that estradiol's analgesic effects after formalin administration are not likely mediated in part through down regulation of COX activity or levels.

Estradiol's effects after inflammatory stimulus on prostaglandin serum levels (PG)

After formalin administration, estradiol treated animals had lower PG serum levels compared to untreated animals but these differences failed to reach significance. As mentioned

earlier, the release of PG's have been linked to formalin-induced behavioral responses [101] as well as hyperalgesic states [73,205,206]. Significant attenuation of PG serum levels in the presence of estradiol would suggest at minimum, partial mediation in the PG bio-synthetic pathway but this was not the case here. Tenenbaum et al., (2007) demonstrated that estradiol significantly reduced LPS-induced increases of NO and TNF (pro-inflammatory mediators) but not PGE_2 in neuronal cultures [207]. These findings suggested that differences in PGE_2 serum levels may not always accompany estradiol's analgesic effects.

Estradiol + COX inhibitor effects after inflammatory stimulus on PGE_2 serum levels

Estradiol + 40mg/kg of IBU treated animals showed a significant reduction in PG serum levels compared to untreated 40mg/kg IBU after formalin. Additionally, untreated (no estradiol) animals that received NS398 or IBU showed a significant attenuation of PGE_2 serum levels compared to vehicle treated animals. Since SC560 did not reduce PGE_2 serum levels significantly after formalin, COX-2 but not COX-1 may contribute more to the development and or activity of pro-inflammatory mediators such as PG associated with these inflammatory responses. These findings are consistent with current literature.

Estradiol's effects after inflammatory stimulus on PGD_2 serum levels

PGD_2 serum levels were not significantly altered by estradiol but were increased compared to control groups. Overall PGD_2 serum levels increased in the presence of estradiol compared to attenuated levels of PGE_2 after estradiol. The current literature suggests two different mechanisms for nociceptive responses of PGD_2 and PGE_2 [52, 56]. Contrasting effects of estradiol on PGD_2 and PGE_2 in this study support two separate mechanisms.

Estradiol + COX- inhibitor effects after inflammatory stimulus on PGD_2 serum levels

Animals treated with NS398 showed significantly higher PGD_2 serum levels than either Ibuprofen or SC560 implicating a role for COX-2 in PGD_2 activity or synthesis. Interestingly, these findings are different from a recent study, where Grill et al., (2006) found after endotoxin treatment, lumiracoxib (a selective COX-2 inhibitor) significantly reduced PGD_2 serum levels. These differences could be a product of differential activation of inflammatory mediators by endotoxin. Although they observed an increase in PGD_2 levels after administration of endotoxin they did not see an increase in PG levels similar to the present study.

Effects of estradiol + COX inhibitor on corticosterone serum levels

Animals treated with estradiol + COX inhibitor (Ibuprofen, NS398 or SC560) after formalin were shown to have significantly higher corticosterone serum levels than untreated animals but these findings were not consistent.

Chapter 3

Can estradiol's analgesic/anti-hyperalgesic effects be extended to the carrageenan inflammatory pain model? Are estradiol's anti-hyperlagesic effects on behavioral responses to carrageenan in part mediated through deactivation/down regulation of COX-1 and COX-2?

Results

Effects of 0, 10, 20 and 30% estradiol on baseline levels of paw withdrawal latencies.

As shown in Figure 18, estradiol increased paw withdrawal latency (PWL) responses compared to vehicle at all doses. Overall, animals treated with 20% estradiol had higher withdrawal latencies. Additionally, 20% estradiol significantly increased PWL at the medium and high intensity ($p<0.05$). Although 20% estradiol increased PWL responses compared to vehicle at the lowest heat intensity, this difference failed to reach significance.

Effects of 0, 10, 20 and 30% estradiol on paw withdrawal latencies at one and five hours after 1% carrageenan administration at all heat intensities.

As shown in Figure 19A, one hour after carrageenan the injected paw shows a greater hyperalgesic effect than the uninjected paw. Overall, animals treated with etradiol had greater paw withdrawal latencies compared to untreated animals.

Effects of 0, 10, 20 and 30% estradiol on the injected paw withdrawal latencies (PWL) after 1% carrageenan administration at all heat intensities.

As shown in Figure 20A-C, a significant main effect for hormone [$F=7.119$, $p=0.01$] was observed; 20% estradiol was the most effective dose at across all times and all heat intensities. It was shown to significantly attenuate paw withdrawal latencies at both 1 and 5 hours ($p<0.05$) ($p<0.05$); respectively, and at all heat intensities ($p<0.05$).

Effects of 0, 10, 20, and 30% estradiol on contralateral PWL at after 1% carrageenan administration at all heat intensities.

In the left paw (Figure 21A-C), Overall, estradiol treated animals showed across all times and intensities. Similar to the injected paw the greatest increases were observed at the 20% estradiol dose.

Effects of 0, 10, 20, and 30% estradiol on PGE_2 and PGD_2 serum levels after carrageenan administration.

As shown in Figure 22A, although estradiol (10%) treatment reduced PGE2 serum levels, it failed to reach significance [F=1.50, p=0.253]. As shown in Figure 21B, there were no significant differences observed in PGD2 levels across the different doses.

Effects of 0, 10, 20, and 30% estradiol on corticosterone serum levels after carrageenan administration.

As shown in Figure 23, a significant effect for hormone in CORT serum levels was observed [F= 4.923, p=0.008]; Specifically, 30% estradiol significantly attenuated CORT serum levels when compared to 10% or 20% treatment ($p<0.05$ and $p<0.5$, respectively).

Effects of estradiol + vehicle (DMSO), SC560 (COX- 1 inhibitor), NS398 (COX-2 inhibitor) on baseline PWL.

Based on responses from the dose response curve (DRC), we used the 20% dose of estradiol replacement (the most effective dose) to determine if COX-2 activation will enhance, hinder or have no effect on estradiol's analgesic effects. Similar to results from the DRC,

estradiol (20%) altered baseline latencies at all three heat intensities (low: [F=3.789, p=0.05]; medium: [F=6.927, p=0.011]; high: [F=5.437, p=0.023]). Additionally, in the presence of estradiol, both SC560 and NS398 become effective in increasing withdrawal latencies at baseline.

Effects of estradiol + vehicle (DMSO), SC560 (COX 1 inhibitor), NS398 (COX-2 inhibitor) on PWL after carrageenan administration

As shown in Figure 25, at 5 hours the injected paw shows greater hyperalgesia than at 1 hour across all groups. Furthermore, animals treated with estradiol showed overall greater PWL responses compared to untreated animals.

Effects of estradiol + vehicle (DMSO), SC560 (COX- 1 inhibitor), NS398 (COX-2 inhibitor) on the injected PWL at each heat intensity.

As shown in Figure 26A-C, a significant effect for hormone [F=63.781, p=0.000]; drug [F=20.635, p=0.000] and an interaction between hormone and drug [F=3.560, p=0.036] was observed. Estradiol significantly increased PWL responses across all heat intensities ($p<0.05$). and all times ($p<0.05$). Both SC560 and NS398 significantly increased withdrawal latencies compared to control animals at one and five hours ($p<0.05$). Additionally, NS398 was more effective reflected in higher PWL responses and significantly different than SC560; ($p<0.05$). Furthermore, in the presence of estradiol; SC560 increased PWL responses significantly at baseline and one hour compared to PWL responses observed in the absence of estradiol ($p<0.05$). Similarly, NS398 effectiveness was enhanced in the presence of estradiol at both one and five hours ($p<0.05$).

Effects of estradiol + vehicle (DMSO), SC560 (COX 1 inhibitor), NS398 (COX-2 inhibitor) on contralateral PWL at each heat after carrageenan administration.

As shown in Figure 27A, a main effect for hormone [F=14.847, p=0.000] and drug [F=6.577, p=0.003] was observed. SC560 significantly increased PWL at baseline and one hour compared to vehicle treated groups ($p<0.05$). NS398 significantly increased PWL responses at one and five hours compared to vehicle treated groups ($p<0.05$). Estradiol was effective at all three channels ($p<0.05$).

Effects of estradiol + vehicle (DMSO), SC560 (COX 1 inhibitor), NS398 (COX-2 inhibitor) on PGE_2 and $PGD_{2\ serum}$ levels

As shown in Figure 28A. There was a main effect for drug [F=13.800, p=0.0000]; animals treated with SC560 and NS398 had significantly lower of prostaglandin E2 serum levels compared to vehicle treated animals ($p<0.0005$, $p<0.005$ respectively). As shown in Figure 27B, for PGD2 serum levels a main effect for hormone [F=15.128, p=0.0005] and drug [F=6.331, p=0.0005] was observed; estradiol treated animals had overall higher levels of PGD2 serum levels when compared untreated animals ($p<0.0005$). Moreover, SC560 treatment lowered levels of PGD2 serum levels compared to all other groups ($p<0.0002$).

Effects of estradiol + vehicle (DMSO), SC560 (COX-1 inhibitor), NS398 (COX-2 inhibitor) on corticosterone serum levels

As shown in Figure 29, no significant differences were observed between estradiol treated vs. untreated animals or differentially drug treated animals and their corticosterone serum level.

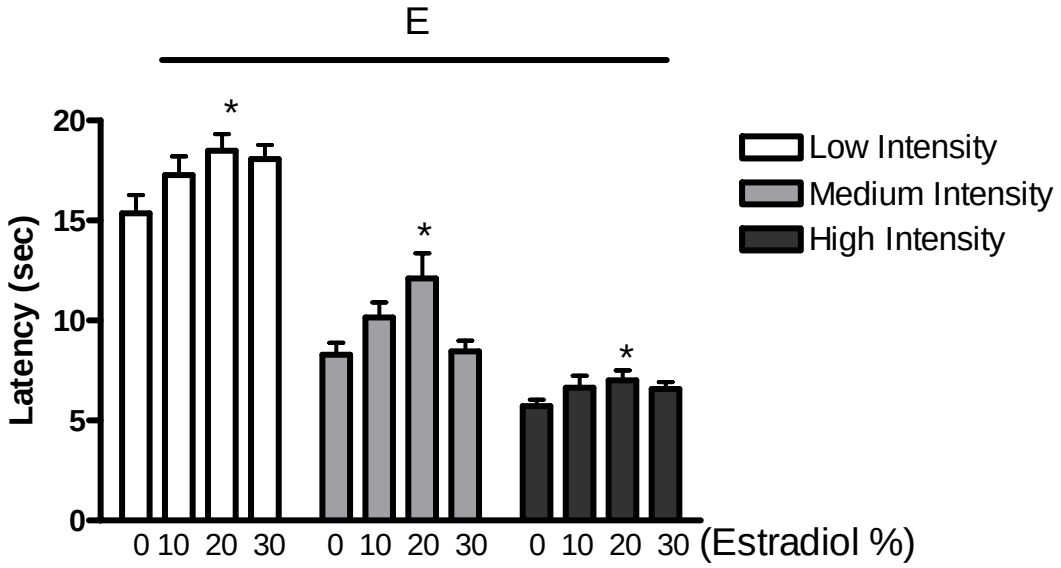

Figure 18. Effects of 0, 10, 20, and 30% estradiol on baseline levels of paw withdrawal latencies. Data represents mean paw withdrawal latencies (± SEM) thirty minutes before 1% carrageenan injection (n=9-10). (E) Denotes a significant hormone effect. (*) Denotes significantly different from control group.

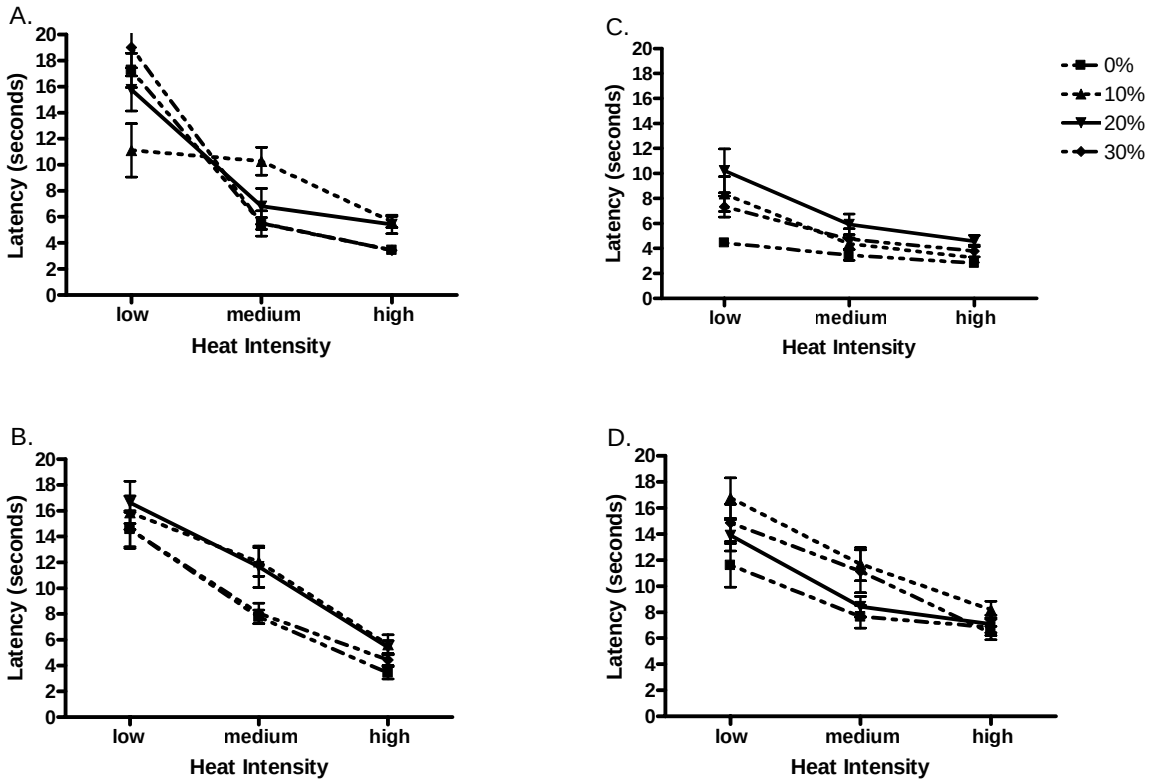

Figure 19. Effects of 0, 10, 20, and 30% estradiol on paw withdrawal latencies 1 and 5 hours after 1% carrageenan administration. A. Data represents cumulative mean withdrawal latencies (± SEM) 1 hour after 1% carrageenan injection at all heat intensities for the right paw. **B.** Represents mean withdrawal latencies (± SEM) 1 hour after 1% carrageenan injection at all heat intensities for the left paw. **C.** Represents mean withdrawal latencies (± SEM) 5 hours after 1% carrageenan injection at all heat intensities for the right paw. **D.** Represents mean withdrawal latencies (± SEM) 5 hour after 1% carrageenan injection at all heat intensities for the left paw (n=9-10).

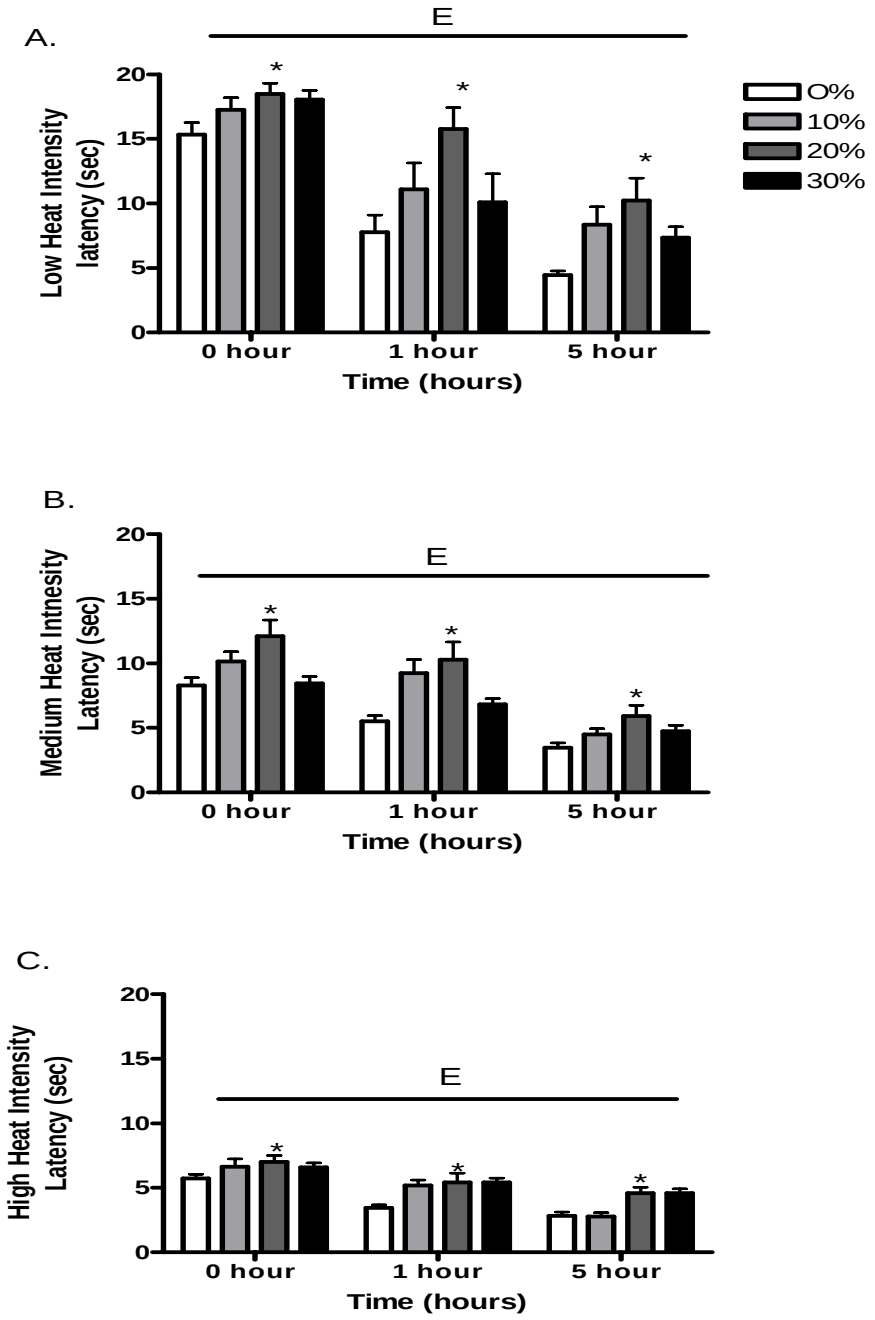

Figure 20. A-C Effects of 0, 10, 20, and 30% estradiol on the injected paw withdrawal latencies for each heat intensity after 1% carrageenan administration. A. Data represents cumulative mean withdrawal latencies (± SEM) for right paw at 1 and 5 hours after carrageenan at the low heat intensity (4.50mv). **B.** Represents cumulative mean withdrawal latencies at the medium heat intensity. (4.80mv). **C.** Represents cumulative mean withdrawal latencies (± SEM) at the high heat intensity (5.20mv). (E) Denotes a significant hormone effect ($p<0.05$).

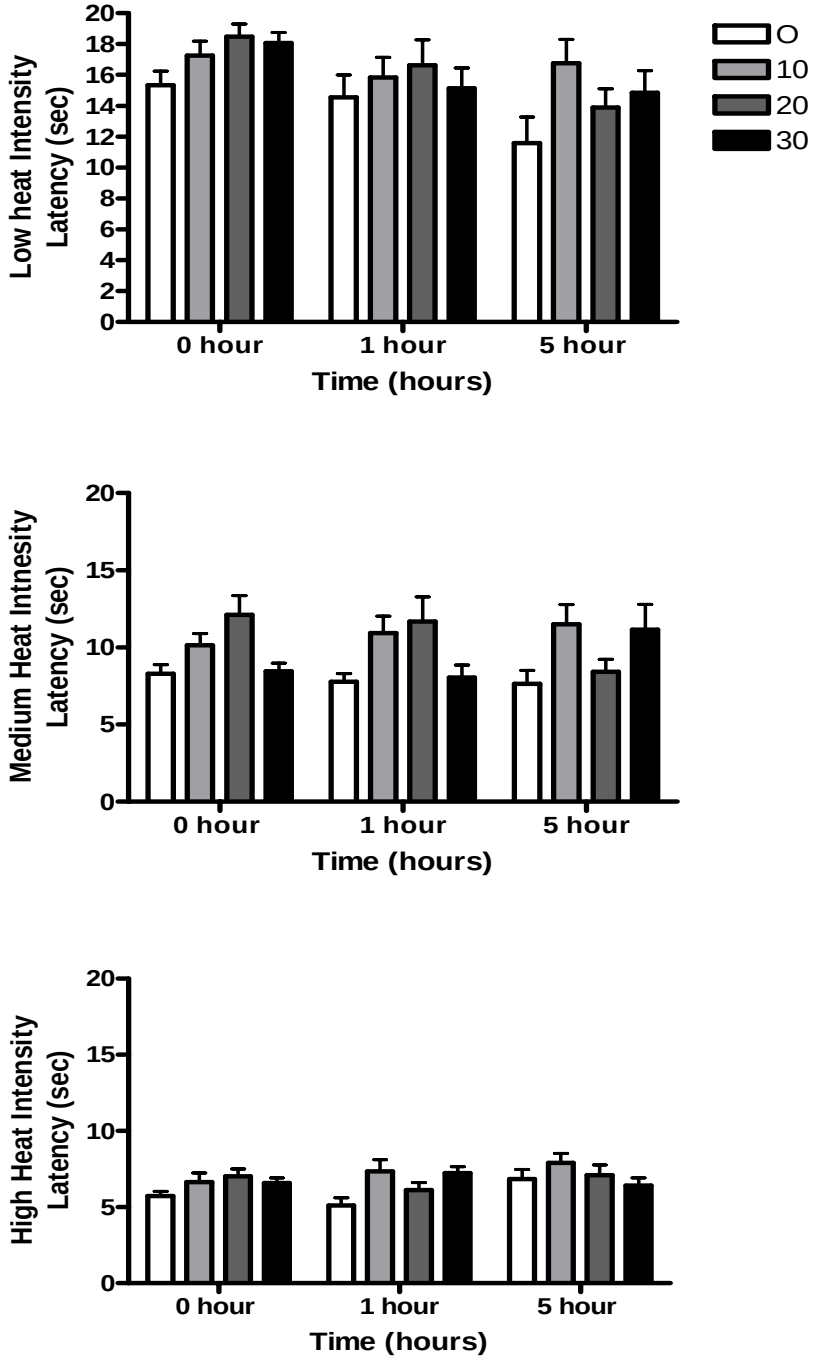

Figure 21. A-C Effects of 0, 10, 20, and 30% estradiol on the contralateral paw withdrawal latencies for each heat intensity 1 and 5 hours after 1% carrageenan administration. A. Data represents cumulative mean withdrawal latencies (± SEM) for left paw at 1 and 5 hours after carrageenan at the low heat intensity (4.50mv). **B.** Represents cumulative mean withdrawal latencies (± SEM) at the medium heat intensity (4.80mv) **C.** Represents cumulative mean withdrawal latencies (± SEM) at the high heat intensity (5.20mv).

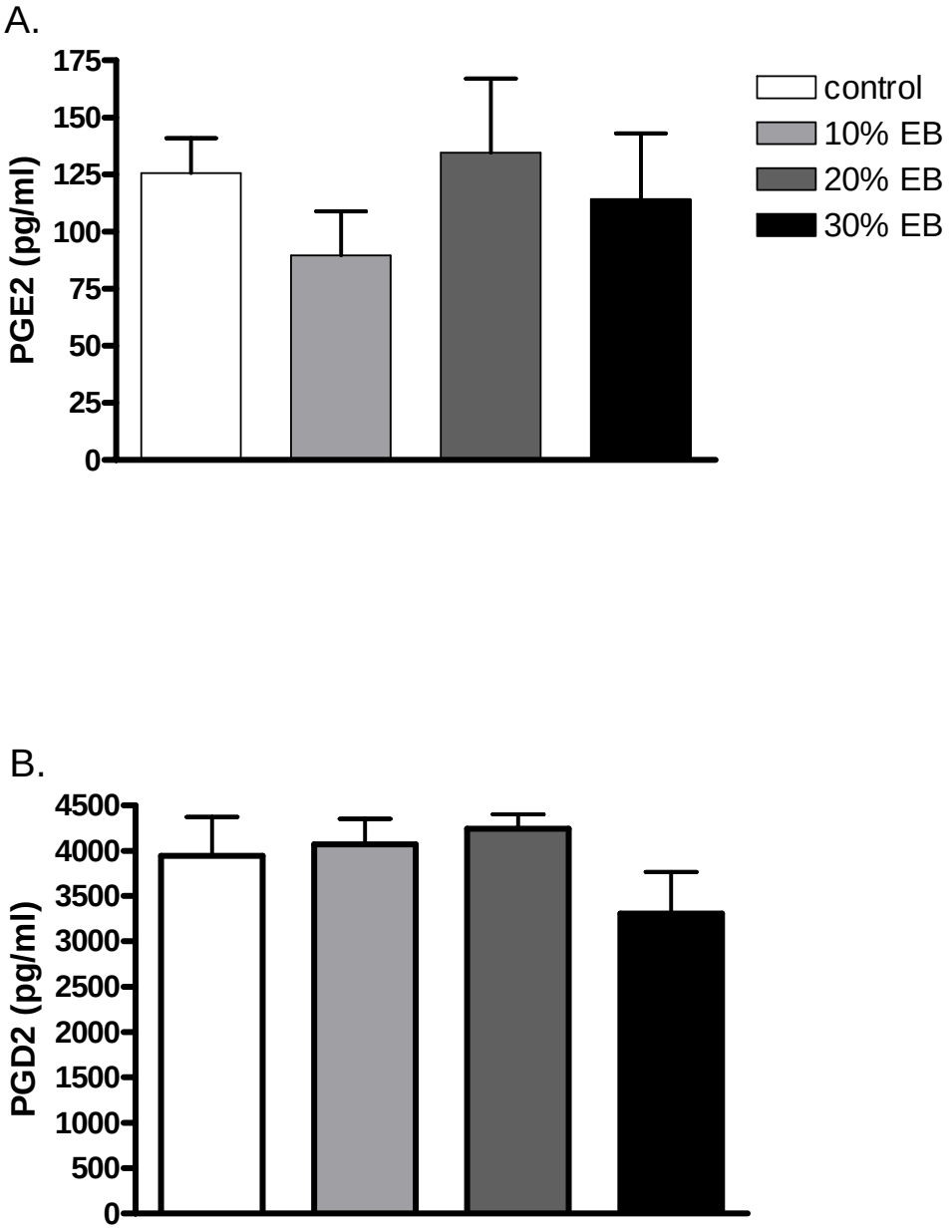

Figure 22. A. Effects of estradiol on PGE_2 (A) and PGD_2 (B) serum levels after carrageenan administration. **A.** Data represents cumulative mean prostaglandin E2 serum levels (± SEM) **B**. Data represents cumulative mean prostaglandin D2 serum levels (± SEM) at picograms per milliter 5 hours after carrageenan or vehicle (DMSO) administration (n=10).

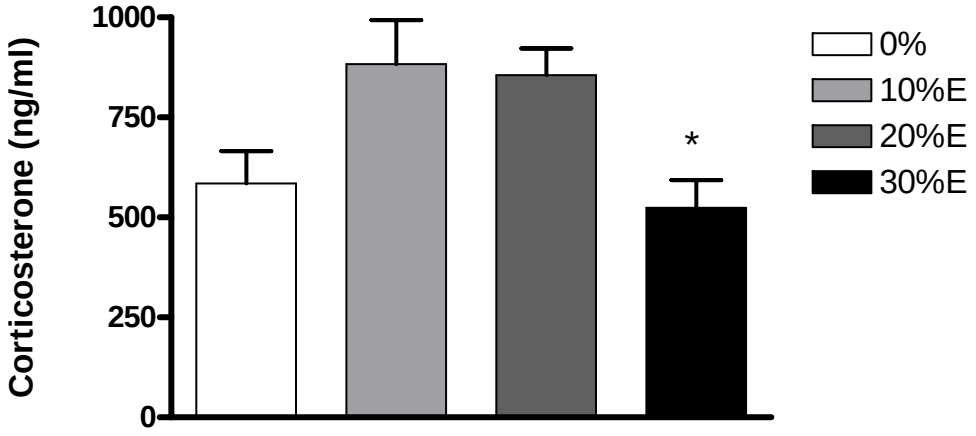

Figure 23. Effects of estradiol on corticosterone serum levels after carrageenan injection. Data represents cumulative mean CORT serum levels (± SEM) measured in nanograms per milliter from trunk blood collected 5 hours after carrageenan administration (n=7). (*****) Denotes significantly different from other estradiol treated groups.

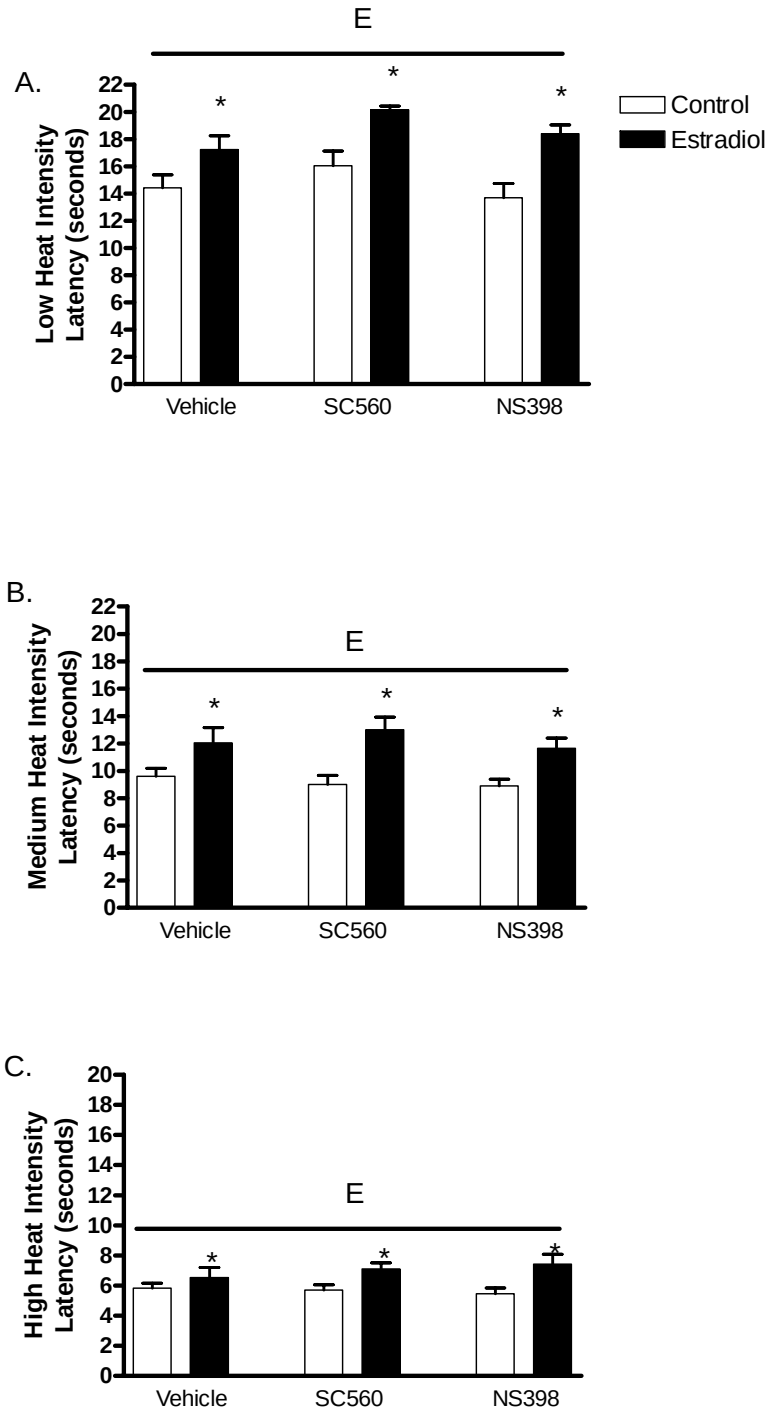

Figure 24. Effects of estradiol + Vehicle (DMSO), SC560 (COX 1 inhibitor), and NS398 (COX 2 inhibitor) on baseline paw withdrawal latencies. Data represents a cumulative mean sum of left and right paw withdrawal latencies 30 minutes before carrageenan injection at **A.** low heat intensity (4.50mv), **B.** medium heat intensity (4.88mv) and **C.** high heat intensity (5.20mv) (n=9-10). (E) Denotes a significant hormone effect ($p<0.05$).

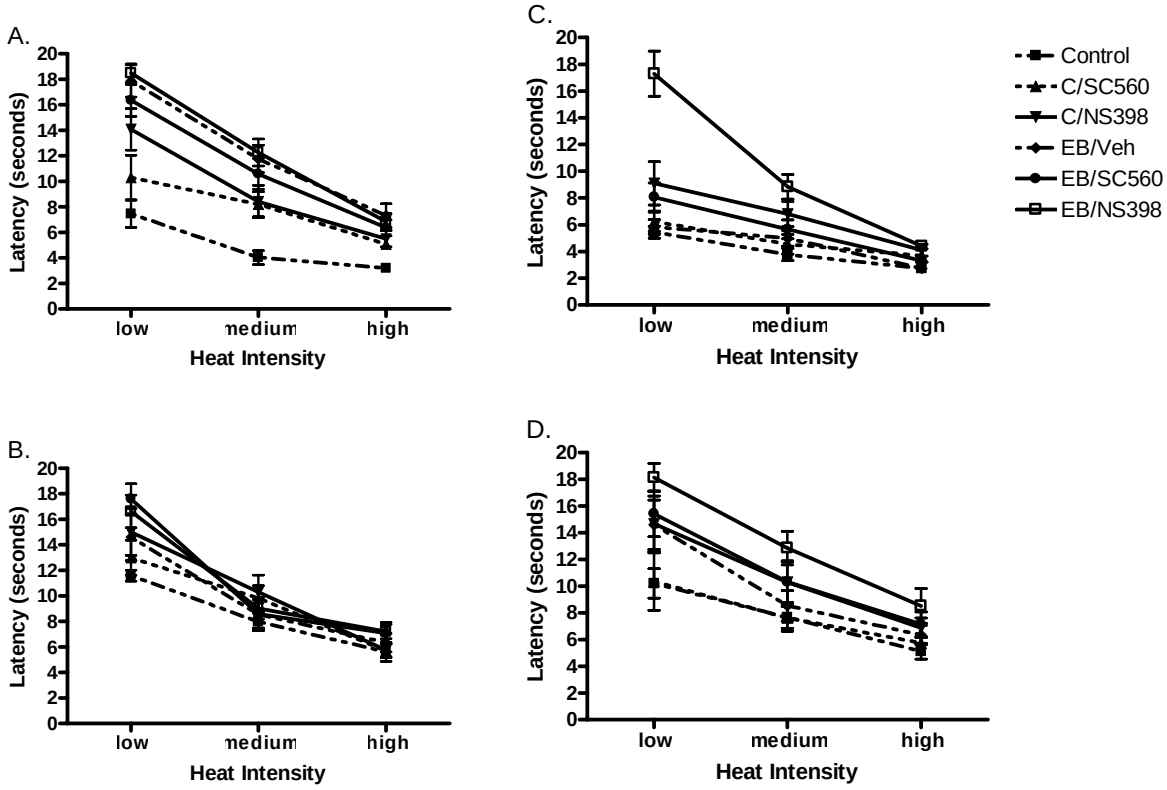

Figure 25. Effects of estradiol + Vehicle (DMSO), SC560 (COX 1 inhibitor), and NS398 (COX 2 inhibitor) on paw withdrawal latencies of 1 and 5 hours after 1% carrageenan administration. **A.** Data represents mean withdrawal latencies (± SEM) 1 hour after 1% carrageenan injection at all heat intensities for the injected paw. **B.** Data represents cumulative mean withdrawal latencies (± SEM) 1 hour after 1% carrageenan injection at all heat intensities for the contralateral paw. **C.** Data represents cumulative mean withdrawal latencies (± SEM) 5 hours after 1% carrageenan injection at all heat intensities for the injected paw. **D.** Data represents cumulative mean withdrawal latencies (± SEM) 5 hour after 1% carrageenan injection at all heat intensities for the contralateral paw (n=9-10).

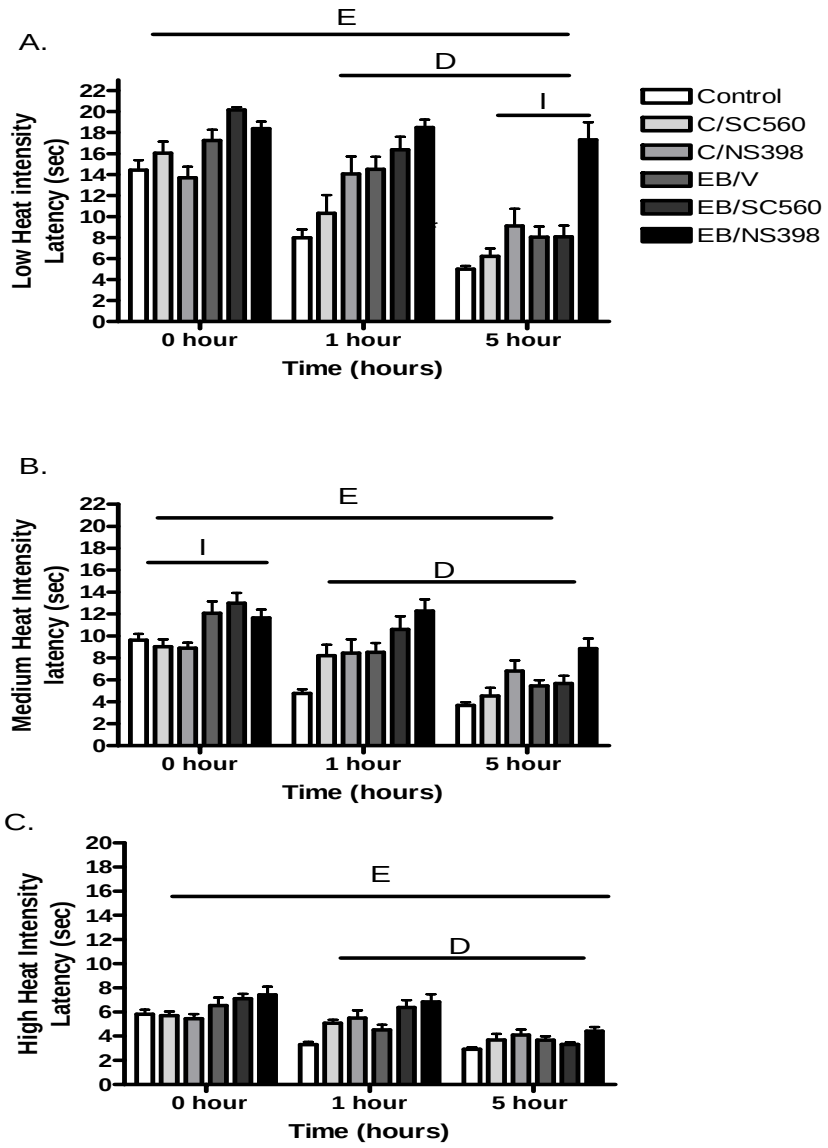

Figure 26. Effects of estradiol (20%) + Vehicle (DMSO), SC560 (COX 1 inhibitor), and NS398 (COX 2 inhibitor) on injected paw withdrawal latencies after 1% carrageenan administration. A. Data represents mean withdrawal latencies (± SEM) for injected paw at 1 and 5 hours after carrageenan at low intensity (4.50mv). **B.** Data represents cumulative mean withdrawal latencies (± SEM) at medium heat intensity (4.80mv) **C.** Data represents mean withdrawal latencies (± SEM) at high heat intensity (5.20mv). (E) Denotes a significant hormone effect. (D) Represents a significant drug effect. (I) denotes a significant interaction between drug and hormone.

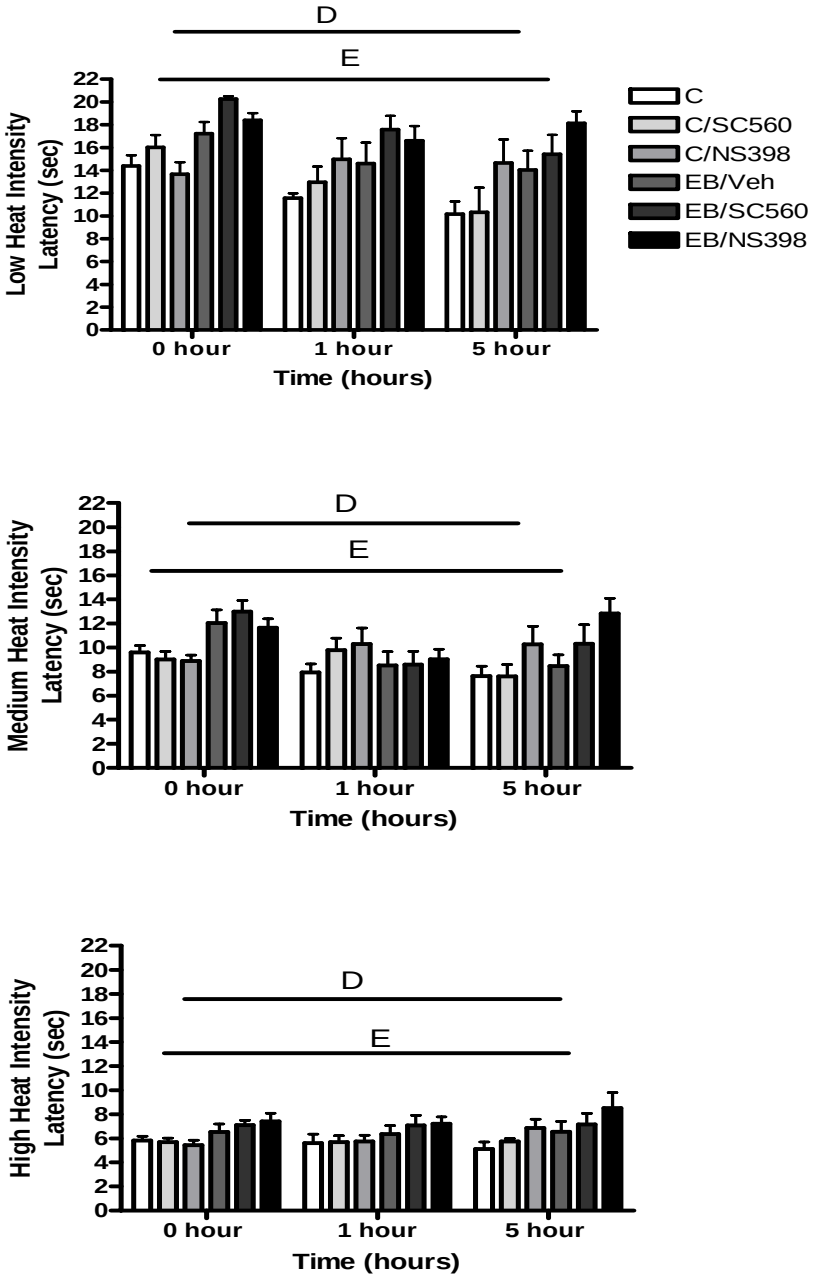

Figure 27. A-C Effects of estradiol + Vehicle (DMSO), SC560 (COX 1 inhibitor), and NS398 (COX 2 inhibitor) on the contralateral paw latencies after 1% carrageenan administration. A. Data represents mean withdrawal latencies for contralateral paw at 1 and 5 hours after carrageenan at low heat intensity (4.50mv). **B.** Data represents mean withdrawal latencies (± SEM) at medium heat intensity (4.80mv) **C.** Data represents mean withdrawal latencies at high heat intensity (5.20mv). (E) Represents a significant hormone effect. (D) Represents a significant drug effect; significance at ($p<0.05$).

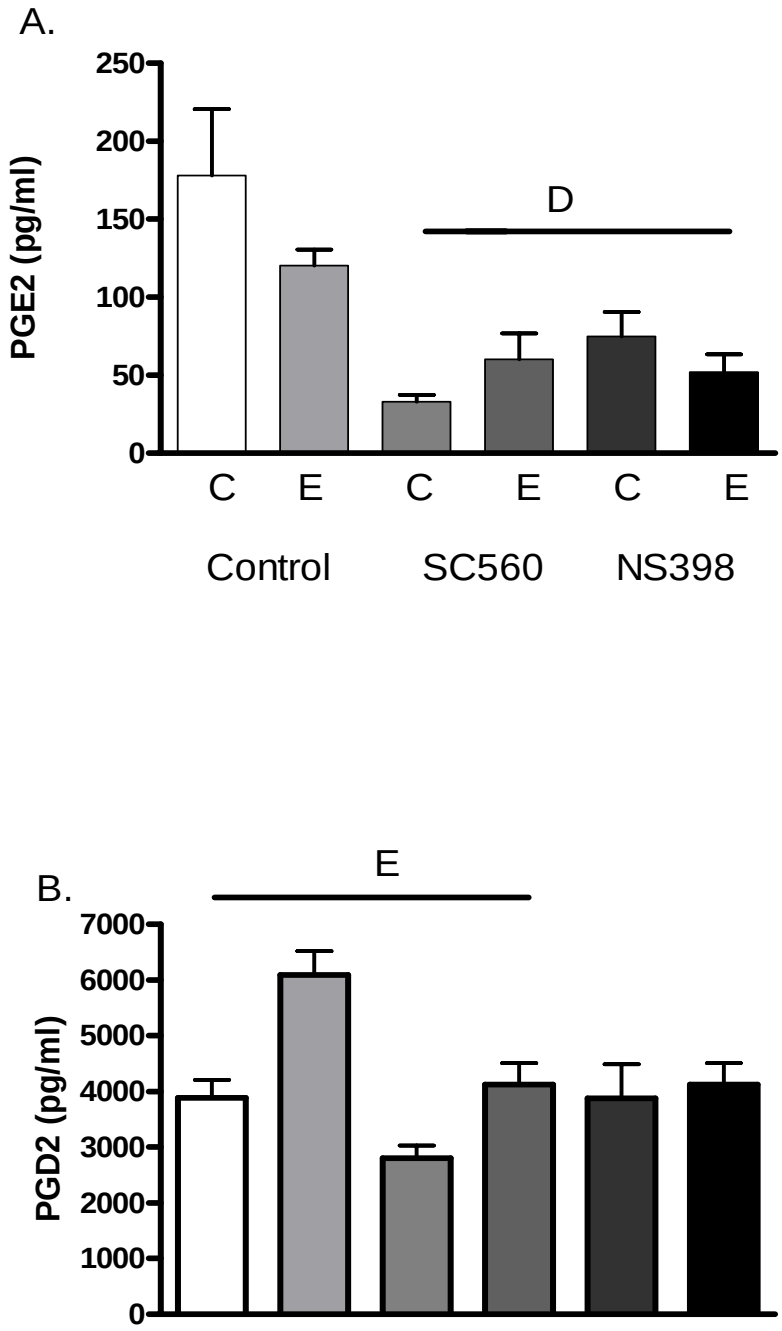

Figure 28. Effects of estradiol + Vehicle (DMSO), SC560 (COX 1 inhibitor), and NS398 (COX 2 inhibitor) on PGE_2 (A) and PGD_2 (B). **A.** Data represents cumulative mean prostaglandinE2 serum levels (± SEM) and **B.** Data represents cumulative mean prostaglandin D2 serum levels (± SEM) at picograms per milliter 5 hours after carrageenan or vehicle (cholesterol) administration (n = 4-5). (D) Denotes significance of $p<.0005$, (E) denotes a significant hormone effect ($p<0.05$).

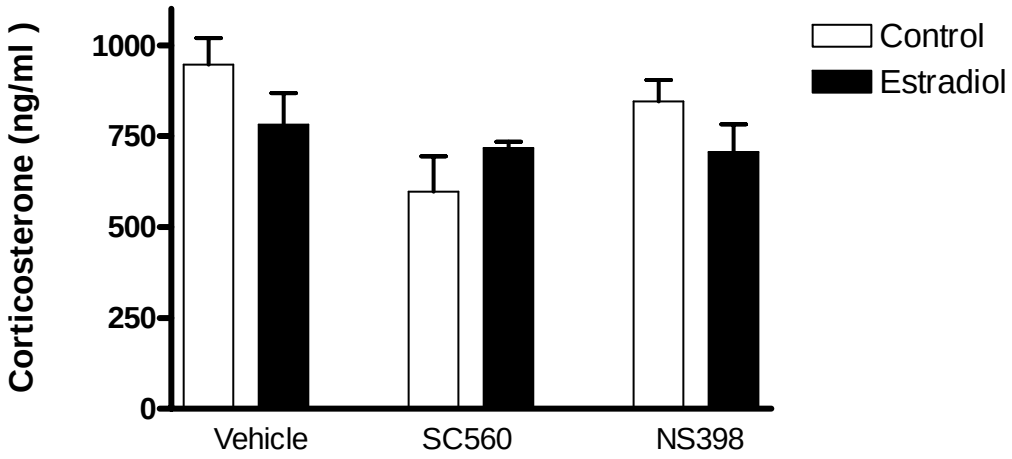

Figure 29. Effects of estradiol + Vehicle (DMSO), SC560 (COX 1 inhibitor) and NS398 (COX 2 inhibitor) on corticosterone serum levels. Data represents cumulative mean CORT serum levels (± SEM) measured in nanograms per milliter from trunk blood collected 5 hours after carrageenan administration. Each bar represents Vehicle (DMSO), SC560 or NS398 treated animals (n=7).

DISCUSSION

The results of this study extend preliminary findings from previous studies in our lab [165,194,195]. First, estradiol reduced induced-thermal hyperalgesia reflected in increased paw withdrawal latencies before and after carrageenan, a persistent inflammatory pain model. Second, estradiol replacement + COX-1 or COX-2 inhibitors resulted in an additive or sub additive but not potentiated reduction of behavioral responses compared to either estradiol or COX inhibitor alone. Thirdly, the contralateral paw (uninjected paw) also showed analgesic effects of estradiol. Additionally, animals treated with COX-inhibitors showed increased PWL responses at baseline and after carrageenan administration supporting their effectiveness on both nociceptive and inflammatory responses.

Estradiol replacement effects on inflammatory behavioral responses

After demonstrating analgesic and anti-hyperalgesic effects of estradiol on the formalin model these findings were extended to another model of persistent inflammatory pain, carrageenan. Similar to results found using the formalin test, estradiol attenuated carrageenan induced-thermal hyperalgesia reflected in increased PWL responses to heat stimulus after carrageenan administration. Moreover, estradiol's ability to attenuate inflammatory responses associated with carrageenan are consistent with the literature. Esposito et al., (2005) using the (carrageenan) model recently showed that use of a selective estradiol receptor modulator (Raloxifene) or 17β-estradiol reduced acute inflammation in both normal and OVX rats [163]. Compared to controls, estradiol treated rats had reduced inflamed tissue and/or damage in the injected paw [163]. Bradshaw et al., (2002) revealed that increased vaginal hyperalgesia induced by OVX surgery can be reversed with estradiol replacement [208] and high plasma levels of

estradiol have been associated with increased pain thresholds {1780, 1781, 8175, 1785}. Furthermore, Cuzzocrea et al., 2001 demonstrated that lack of endogenous estradiol enhances carrageenan-induced acute inflammation [209].

Unexpectedly, estradiol also increased heat thresholds before carrageenan administration. Animals treated with estradiol (20%) before carrageenan administration had overall higher PWL compared to untreated animals. This baseline effect of estradiol suggest that estradiol replacement increases sensitivity thresholds before an inflammatory stimulus is presented and is consistent with the Phase I effects observed using the formalin model. Interestingly, at the next highest dose (30% estradiol) PWL dramatically decreased for both low and medium heat intensities suggesting that at higher levels, estradiol's analgesic effect may cease and/or support a pro-nociceptive effect after carrageenan administration. These findings are in line with other studies that have shown that estradiol can also occupy a pro-nociceptive role [141,210-213]. Ryan SM et al., demonstrated that a specific amount of estradiol was able to restore opioid analgesia but additional estradiol suppressed this restoration [214].

The sensitivity of these responses to specific estradiol doses support receptor mediated effects. Moreover, Kuba et al., (2005) demonstrated that an inactive isomer of estradiol (17α-estradiol) failed to show the same attenuation in the chronic phase of the formalin response that 17β-estradiol significantly attenuated in OVX animals [165]. Furthermore, tamoxifen (a selective estradiol receptor mediator) was able to mimic estradiol's effects after formalin but co-administration with estradiol failed to reverse estradiol's antihyperalgesic effects after formalin suggesting activation by similar intracellular mechanisms, since co-adminstration of estradiol with progesterone (not a selective estradiol receptor) is able to reverse estradiol effects after formalin. Mannino et al., (2006) showed similar results [157,165]. Furthermore, estradiol's

analgesic effects were also observed in the left paw. Consistent with findings in the DRC experiment estradiol treated animals showed higher latency responses. In summary, estradiol-treated animals compared to controls were associated with the highest latencies before and after carrageenan administration. As would be expected, increasing heat intensity resulted in corresponding significantly lower PWL within all groups. However, out of the four doses used (0, 10 20, 30%) 20% estradiol remained the dose at which PWL's were the highest regardless of heat of intensity.

COX inhibitors effects on inflammatory behavioral responses

Similar to findings using the formalin pain model, carrageenan-evoked thermal hyperalgesia was significantly attenuated by NS398. Further suggesting that regardless of inflammatory stimulus COX-2 activity is involved in inflammatory induced behavioral responses. In contrast to the formalin assay, SC560 also significantly reduced induced-thermal hyperalgesia after carrageenan administration. This finding supports a major role for COX-1 in the mediation of persistent inflammatory responses associated with the carrageenan model and are consistent with other studies suggesting a larger role for COX-1 [50,100,101]. Toriyabe et al., (2004) recently examined the effects of COX-1 and COX-2 in peripheral PGE_2 and PGI_2 production after carrageenan-induced inflammation [215]. Consistent with findings in the present study they found that both isoforms contributed significantly to the resulting production of PG's at the site of inflammation. Inherent differences associated with these two pain models may account for contrasts observed in the effectiveness of either COX inhibitor to reduce nociceptive and/or hyperalgesic responses. Unlike formalin, carrageenan administration produces behavioral responses that are sustained for at least 48 hrs by the release of inflammatory mediators [42,216].

Because of this, carrageenan induced inflammation is extremely useful for accessing the contribution of mediators involved in the local vascular changes associated with inflammation [216]. Furthermore, responses in the uninjected paw can be accessed contributing to a better understanding of the analgesic effect of estradiol and COX inhibitors. Interestingly, both COX inhibitors were effective in increasing PWL responses in the absence of inflammation reflected in observations of the uninjected paw.

COX inhibitors + estradiol effects on inflammatory behavioral responses

Similar to results found using the formalin model, estradiol + either COX inhibitor resulted in a greater attenuation of carrageenan-induced thermal hyperalgesia reflected in higher PWL's compared to responses in the absence of estradiol. Again, these differences were additive. The greatest difference was observed after co-administration with NS398 (COX-2 inhibitor). The contralateral paw showed a similar trend to the injected paw such that estradiol + COX inhibitor resulted in significant increases in withdrawal latencies compared to either estradiol or COX-inhibitor alone. Moreover, both SC560 and NS398 were found to significantly increase PWL responses in the absence of inflammation. A "ceiling effect" of either estradiol and/or COX-inhibitor dose may be responsible for the differences in effectiveness of individual COX inhibitors used between the two models. Specifically, doses of COX inhibitors used may have reduced behavioral responses to a maximum level such that they could not be attenuated further by the addition of another anti-inflammatory mediator i.e. estradiol + COX inhibitor. Moreover, differential findings between the two models used may be associated with time course dependency of specific inflammatory mediators i.e. COX-2/COX-1. Toriyabe et al., (2004) established that in peripheral sites COX-1 upregulation peaked at two hours after carrageenan

administration whereas COX-2 upregulation was observed at three and six hours and upregulation of theses enzymes was not observed up to 6 hrs after carrageenan injection in the DRG [215]. Behavioral readings in the present study were taken once, one hour after formalin and twice, one and five hours after carrageenan. Specific time frames associated with up regulation of theses enzymes at different sites can be crucial in assessing/quantifying relevant data and should be considered in future studies using either the formalin or carrageenan model for inflammatory pain. In the present study, SC560 attenuation of PWL'S after carrageenan were present after one hour but not five hours reflecting time course effects of this drug. Furthermore, the intensity of the inflammatory stimulus carrageenan may also contribute to this difference.

Estradiol's effects after inflammatory stimulus on prostaglandin (PG) serum levels

After carrageenan administration, estradiol attenuated PG serum levels compared to control groups but again failed to reach significance. As mentioned earlier the release of PG's have been linked to formalin-induced behavioral responses [101] as well as hyperalgesic states [73,205,206]. Attenuation of these serum levels in the presence of estradiol suggests partial mediation in this pathway. However, estradiol effects may work through alternate pathways as well. Tenenbaum et al., (2007) demonstrated that estradiol significantly reduced LPS-induced increases of NO and TNF (pro-inflammatory mediators) but not PGE_2 in neuronal cultures [207]. This finding suggest that differences in PGE_2 levels may not always accompany estradiol's antihyperalgesic effects

Estradiol + COX inhibitor effects after inflammatory stimulus on PGE_2 serum levels

NS398 and SC560 significantly attenuated PGE_2 serum levels compared to the vehicle group suggesting a role for both COX-1 and COX-2 in PGE_2 production after carrageenan administration [215]. These findings are consistent with the behavioral data for carrageenan that support a role for both COX-1 and COX-2. Furthermore, their role in inflammatory pain appears to be partially mediated through PG modulations.

Estradiol's effects after inflammatory stimulus on PGD_2 serum levels

PGD_2 serum levels were not significantly altered by estradiol but were increased compared to controls. Overall PGD_2 serum levels increased in the presence of estradiol compared to attenuated levels of PGE_2 after estradiol. Evidence was found that suggest two different mechanisms for nociceptive responses of PGD_2 and PGE_2. Contrasting effects of estradiol on PGD_2 and PGE_2 in this study support two separate mechanisms.

Estradiol + COX- inhibitor effects after inflammatory stimulus on PGD_2 serum levels

Animals treated with NS398 showed significantly higher PGD_2 serum levels than either Ibuprofen or SC560 implicating a role for COX-2 in PGD_2 activity or synthesis. Interestingly, these findings are different from a recent study, where Grill et al., (2006) found after endotoxin treatment, lumiracoxib (a selective COX-2 inhibitor) significantly reduced PGD_2 serum levels. These differences could be a product of differential activation of inflammatory mediators by endotoxin. Although they observed an increase in PGD_2 levels after administration of endotoxin they did not see an increase in PG levels similar to the present study.

Effects of estradiol + COX inhibitor on corticosterone serum levels

After carrageenan, cortiscosterone serum levels were highest at the 10 and 20% dose compared to vehicle. At the 30% estradiol dose corticosterone serum levels were significantly decreased compared to other estradiol doses. This difference can be linked to the dose dependency of estradiol effects on inflammatory responses. Unlike formalin, no drug effects were observed on corticosterone serum levels after carrageenan. External and internal stress factors such as the dramatic change in environmental setting on testing day and types of inflammatory stimulus which were used have been shown to affect corticosterone serum levels to a varying degree. For example, DMSO alone has been shown to induce inflammatory responses and increase CORT serum levels significantly [217].

Chapter 4

Are estradiol's anti-hyperalgesic effects on behavioral responses to inflammatory stimuli (formalin) in part mediated through the induction of corticosterone release?

Results

Effects of ADX, OVX or OVX + ADX treatments and estradiol on behavioral flinching responses after 5% formalin administration

As shown in Figure 14A, a main effect of estradiol [F=22.5049, p=0.000] was observed. Estradiol significantly reduced behavioral flinching in both Phase 1 ($p<0.005$) and Phase 2 ($p<0.0001$). In summary, regardless of surgical treatment estradiol-treated animals showed significant reductions in behavioral flinching compared to vehicle-treated OVX, ADX and OVX + ADX animals.

Effects of ADX, OVX or OVX + ADX treatments and estradiol on PGE_2 and PGD_2 serum levels

As shown in Figure 15A, although a decrease in PGE2 levels was observed in estradiol-treated compared to untreated control animals this difference failed to reach statistical significance. However, a main effect for surgery on PGD2 serum levels (Figure 15B) was observed [F=4.476, p=0.020]; ADX animals showed a significant increase in PGD2 serum levels when compared to control animals ($p< 0.05$).

Effects of ADX, OVX or OVX + ADX treatments on corticosterone serum levels

As shown in Figure 16, a significant main effect for hormone [F=3.288, p= 0.07] and surgery [F= 15.416, p= 0.000] was observed; estradiol-treated animals had higher serum levels

than vehicle-treated animals ($p<0.05$). ADX ($p<0.0005$) and ADX +OVX ($p<0.0001$) animals had significantly lower corticosterone serum levels compared to the OVX treated animals.

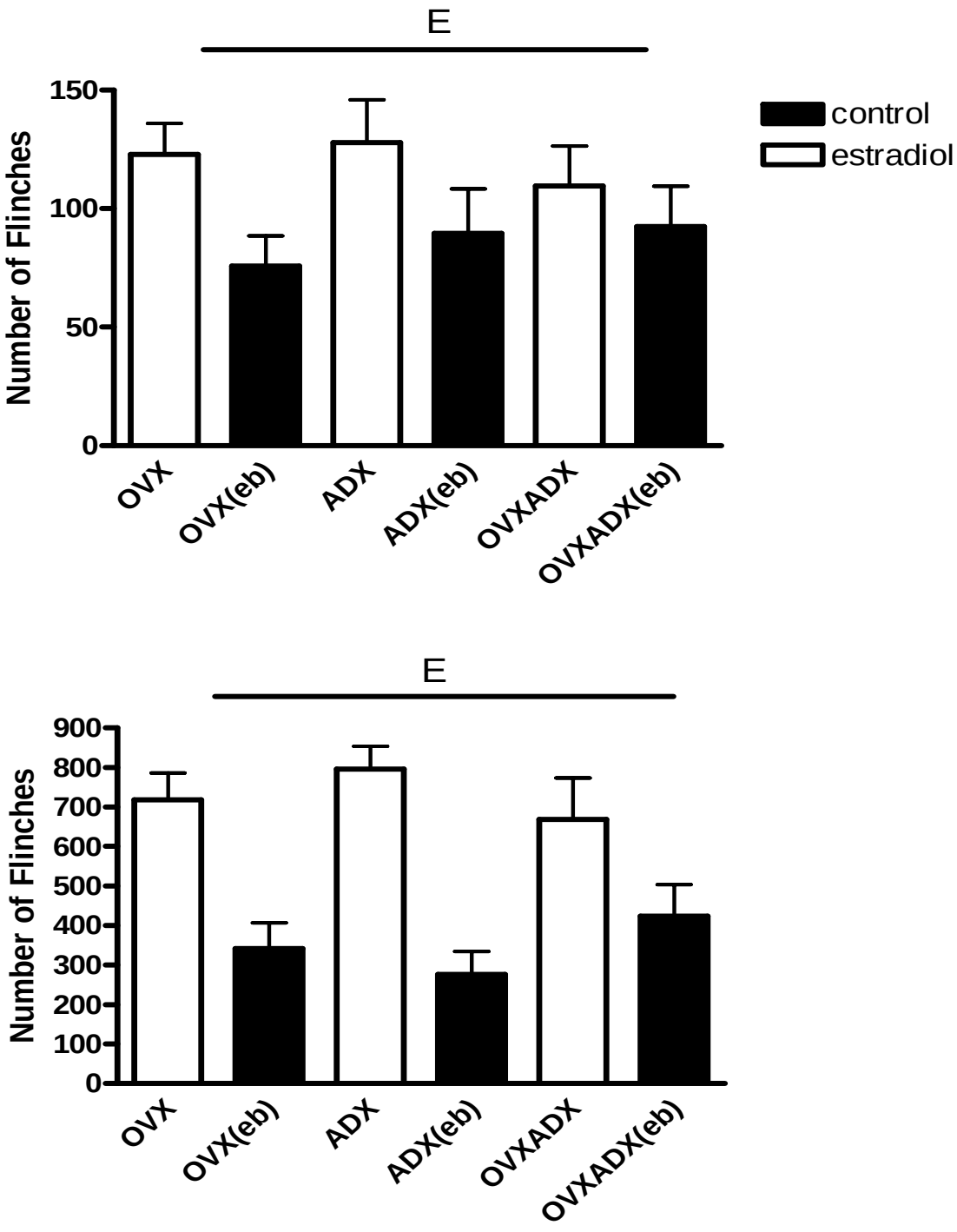

Figure 30. (A) Effects of different surgical procedures and estradiol on behavioral flinching responses after 5% formalin administration. Data represents mean cumulative flinches (± SEM) in Phase I (0-6 min) and II (9-40 min) in estradiol or vehicle-treated animals (n=9-10).

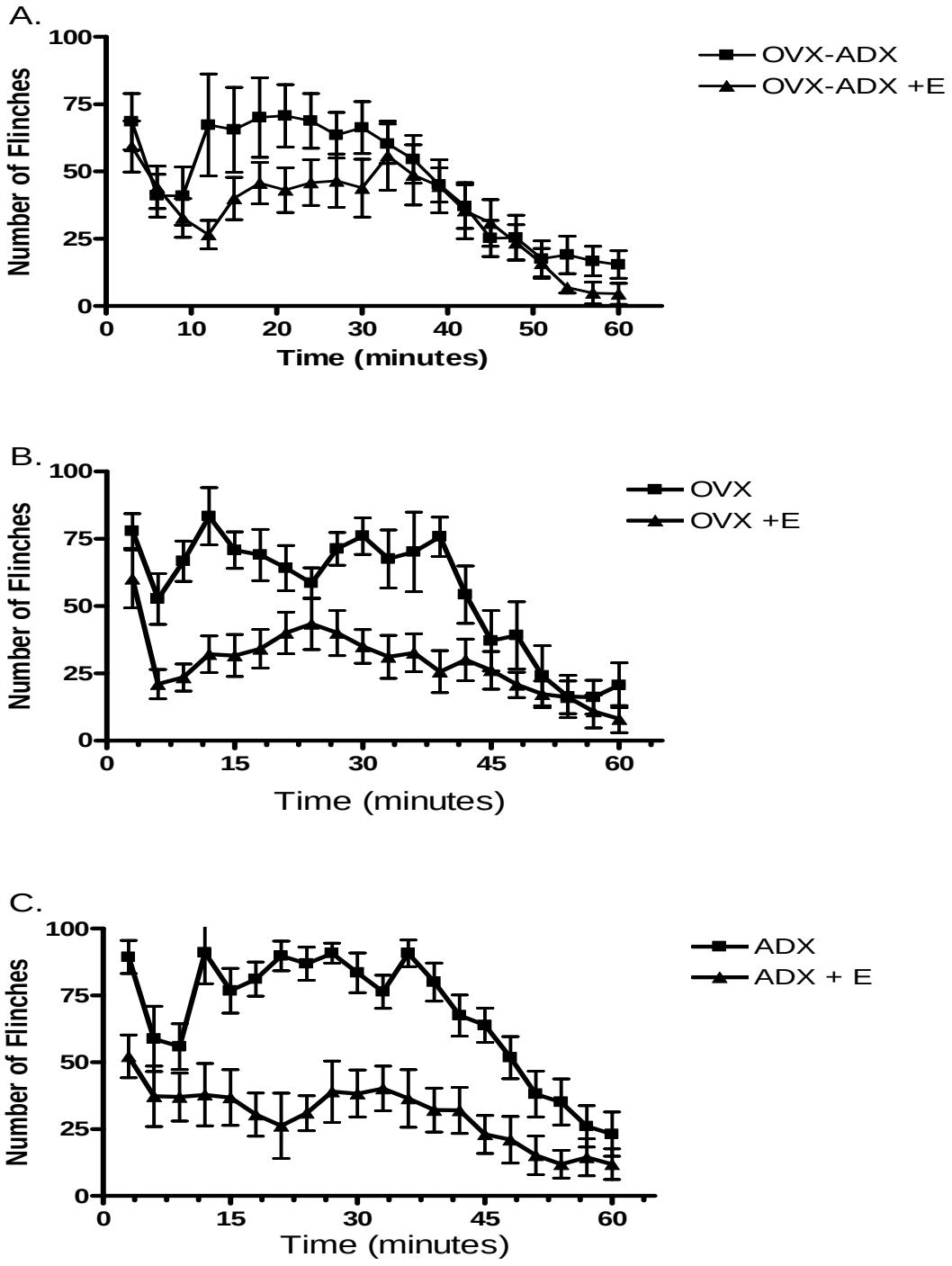

Figure 31: Time course of flinching responses in estradiol or vehicle treated rats after 5% formalin administration. A-C. Time course of activation is represented as the mean of flinching responses in 3 minute bins.

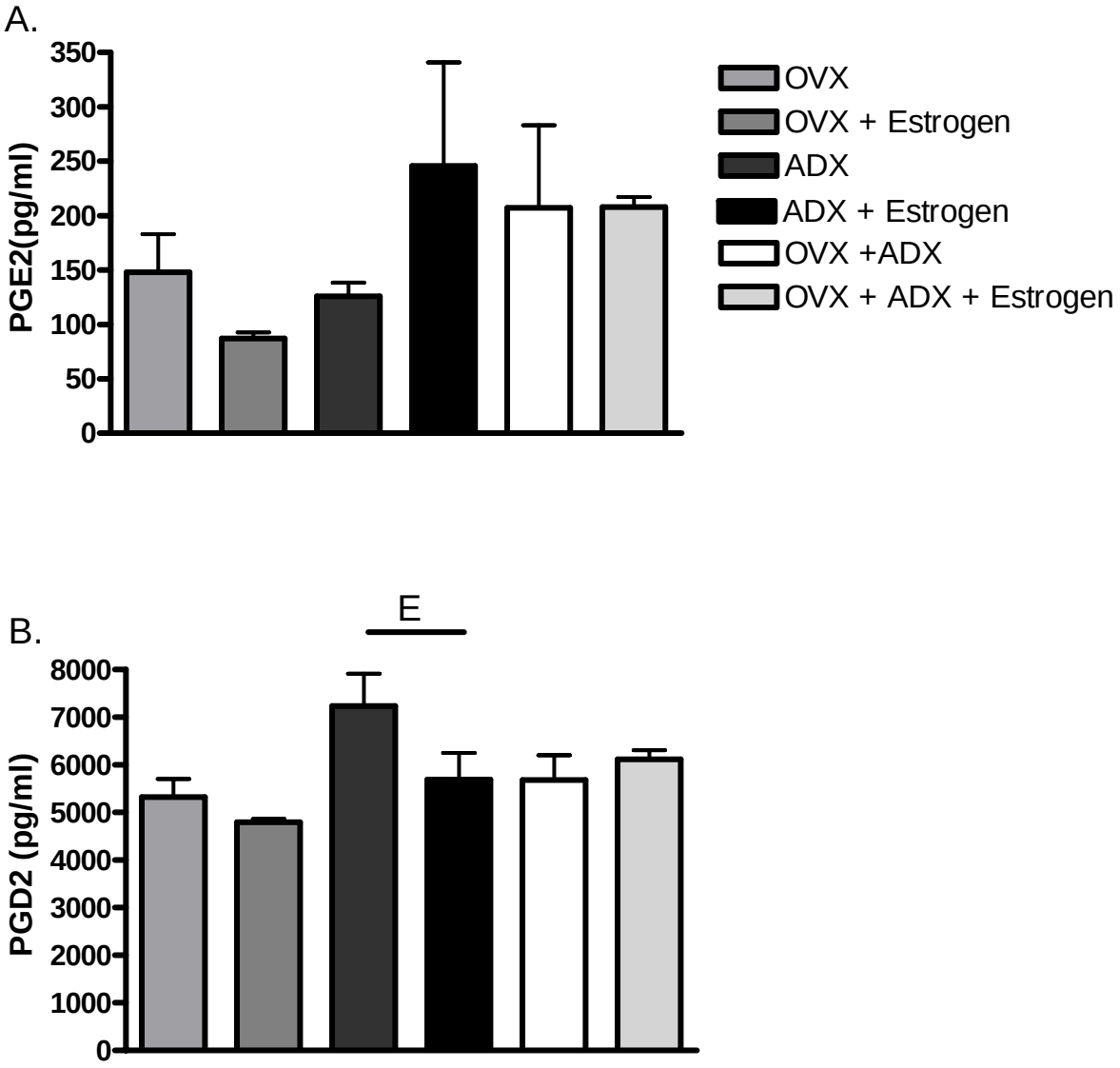

Figure 32. Effects of different surgical procedures and estradiol on prostaglandin E2 (A) and D2 (B) serum levels A. Data represents mean prostaglandin E2 serum levels (± SEM) at picograms per milliter after formalin administration. Each bar represents estradiol or vehicle-treated animals after (n = 4-5). **B.** Data represents mean prostaglandin D2 serum levels (± SEM) after formalin administration (n= 4-5).

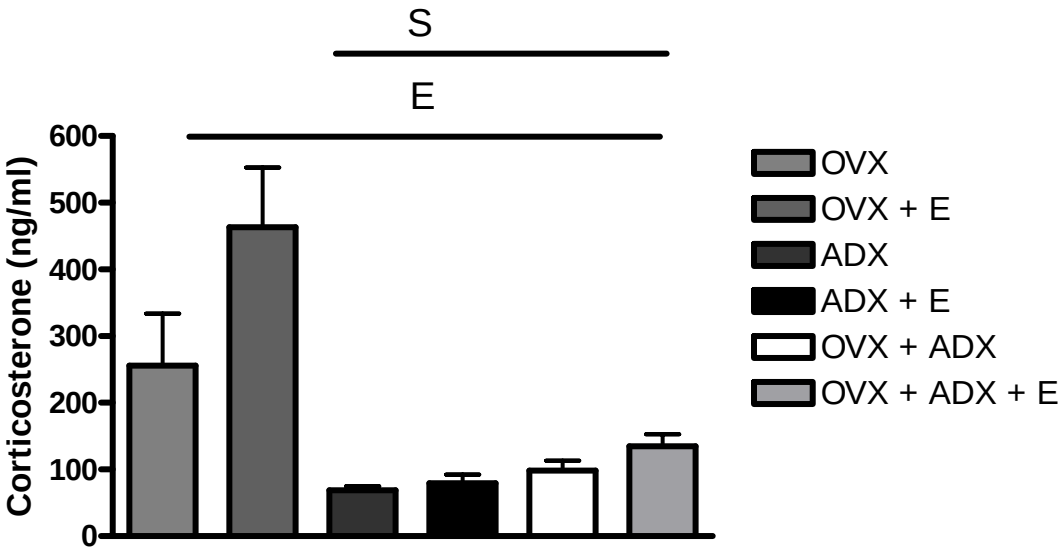

Figure 33. **Effects of different surgical procedures and estradiol on corticosterone serum levels.** Data represents mean CORT serum levels (± SEM) measured in nanograms per milliters from trunk blood collected after formalin administration (n = 7). (E) Denotes a significant hormone effect, (S) denotes a significant effect for surgical procedure.

DISCUSSION

Results from this study suggest that removal of the adrenal glands and subsequent lowered levels of circulating endogenous corticosterone levels did not inhibit estradiol's analgesic effects on behavioral responses after formalin. Therefore we conclude that estradiol's analgesic effects are not likely mediated through corticosterone regulation of PG synthesis.

Regardless of surgery (ADX or ADX + OVX), estradiol's analgesic and/or anti-hyperalgesic effects after formalin administration were still observed. This finding suggest that estradiol's analgesic effects are not dependent on adrenal functions or significantly lowered levels of endogenous circulating corticosterone and/or its release.

Since ADX animals also received a sham OVX operation their ovaries were still intact. Thus, all ADX animals maintained normal levels of endogenous estradiol in addition, estradiol treated animals had greater than normal levels of estradiol. Variables such as this in addition have been shown to be responsible for profound differences in nociceptive responses and processing such as the overall lowered responding of all animals in this experiment [139,149,212,218,219]. Visser et al., (2004) demonstrated that animals that who received ADX surgery showed overall lower behavioral responding during Phase I and Phase II compared to sham ADX and non-operated rats after formalin administration [116]. Dual surgery such as OVX + ADX surgery could magnify this effect contributing to a greater attenuation of behavioral responding. Both of these variables taken together can account for the overall lower responsiveness of animals in this group as a whole.

Estradiol's effects on ADX and ADX + OVX treated animals after inflammatory stimulus on PGE_2 serum levels

No significant difference in PGE_2 serum levels was observed between estradiol treated or untreated ADX or ADX + OVX animals. The loss of normal corticosterone level and activity does not appear to affect PGE_2 serum levels after estradiol treatment.

Estradiol's effects after inflammatory stimulus on PGD_2 serum levels

PGD_2 serum levels were not significantly altered by estradiol but were increased compared to controls. Furthermore, after ADX surgery estradiol treated animals showed a significant reduction in PGD_2 serum levels compared to the untreated group. Overall PGD_2 serum levels increased in the presence of estradiol compared to attenuated levels of PGE_2 after estradiol. Evidence was found that suggest two different mechanisms for nociceptive responses of PGD_2 and PGE_2. Contrasting effects of estradiol on PGD_2 and PGE_2 in this study support two separate mechanisms.

Effects of estradiol on Adrenalectomized (ADX) corticosterone serum levels

The effect of estradiol on corticosterone serum levels was not significantly different compared to untreated ADX and ADX +OVX animals. Although estradiol treated groups had greater corticosterone serum levels these differences failed to reach significance. Cuzzocrea et al., (2007) recently showed that an estradiol antagonist (ICI 182, 780) effectively inhibited anti-inflammatory actions of dexmethasone, a known anti-inflammatory glucocorticoid in carrageenan-induced pleurisy [216]. These findings suggest important cross talk between estradiol and glucorticoids for anti-inflammatory actions to proceed. In the present study the

increase of corticosterone serum levels observed in estradiol treated animals may be an example of this cross talk.

Table 3: Table of behavioral and inflammatory mediator results after inflammatory stimulus

	Drug	Hormone	Behavior	PGE_2	PGD_2	CORT
Formalin						
	BU	Vehicle Estradiol	, ,↓	, ,	—	`
	COX-2 NS398)	Vehicle Estradiol	, ,↓	, ,	`	`
	COX-1 SC560)	Vehicle Estradiol	 ,↓	, ,	—	 `
	ADX	Vehicle Estradiol	 ,↓	—	`	——
	ADX+OVX	Vehicle Estradiol	 ,↓	—	—	——
Carrageenan						
	COX-2 NS398)	Vehicle Estradiol	, ,↓	, ,	—	—
	COX-1 SC560)	Vehicle Estradiol	, @ 1 hr ,↓	, ,	`  `	—

Conclusion

In conclusion, the first study used to address Aim 1 suggests that estradiol is operating through a pathway independent of COX-2 regulation on prostanoid production. Behavioral data obtained using the formalin pain model revealed that the differences in significantly attenuating behavioral responses between estradiol treated vs. estradiol + COX inhibitor treated animals were additive. If estradiol were working through the COX-2/PG bio-synthetic pathway a more robust and/or synergized effect would be expected. Additionally, PG serum levels were not significantly reduced in the presence of estradiol and neither were corticosterone serum levels which can negatively regulate PG synthesis. Furthermore, the ineffectiveness of SC560 compared to the effectiveness of ibuprofen and NS398 suggest that behavioral responses after formalin are mediated by COX-2 but not COX-1. A novel finding from this study was the significant effect of estradiol on Phase I responses after formalin. These findings suggest that estradiol can attenuate both acute and inflammatory processes and responses after formalin.

The second study used to address Aim 2 revealed that estradiol's effects can be extended to another pain model, carrageenan. Estradiol (EB) was shown to dose dependently increase PWL responses with the 20% EB being the most effective dose. Similar to findings from the first study, behavioral responses observed after co-administration of EB + COX inhibitor(s) were additive not synergized compared to animals treated with only EB. These findings are consistent with findings in the first study and support the idea that estradiol's effects are in part mediated through a pathway independent of the COX-PG bio-synthetic pathway. Furthermore, hyperalgesic responses following carrageenan are in part mediated by both COX-1 and COX-2 reflected in the effectiveness of both of these inhibitors in significantly attenuating behavioral responding after carrageenan. Taken together these findings suggest differences in mechanisms

associated with these two pain models. In line with novel findings in the first study, estradiol was shown to significantly attenuate acute as well inflammatory responses reflected in the effectiveness of estradiol both before and after carrageenan administration. Further support of EB analgesic effect is reflected in responses observed in the left paw, in the absence of inflammation.

In contrast to findings using the formalin model, a significant interaction between COX inhibitor and estradiol was observed. This interaction appears to be demonstrated as an extension in the effectiveness duration of the two COX inhibitors investigated. Specifically, estradiol enhanced the effect of SC560 and NS398 such that a baseline effect was observed. This effect was not observed in animals that received COX-inhibitor alone. Additionally, the magnitude of the increase in PWL responses in animals that received EB + NS398 compared to animals that only received NS398 is greater than an additive effect. This interaction between EB + NS398 may work in a way that ultimately prolongs the anti-hyperalgesic effect. It is also consistent with earlier findings whereby only in the presence of EB do we see a baseline effect reflective of extending the effectiveness of the drug such that it works as an analgesic prior to the administration of an inflammatory stimulus i.e carrageenan.

The third study used to address Aim 3 revealed that corticosterone is not a necessary mediator that which estradiol uses to attenuate behavioral responses after formalin.

Based on the data collected in all three studies, it is unlikely that estradiol's effects are in part mediated through regulation of the PG biosynthetic pathway. There are a number of other inflammatory mediators that directly contribute to nociceptive and inflammatory responses after noxious stimuli such as: sympathetic amines, nerve growth factors, cytokines, chemokines, leukotriene B_4 and tumor necrosis factor. These bio-chemical mediators and pathways associated with their production and activation present alternative routes by which estradiol's effects may be

mediated. Further examination into these prospective alternatives can provide valuable insight into mechanisms associated with estradiol's effects on nociceptive and inflammatory processes.

Specifically, examination of estradiol's effects on inflammatory mediators that are upstream from cyclooxgenases and PG in the inflammatory processes may provide crucial insight into the origin of estradiol's effects. Numerous studies have examined the role that Nitric Oxide (NO) plays in nociceptive responding, especially inflammation. NO has been shown to have both peripheral and central effects on inflammatory responses [101,215,220]. After carrageenan-induced inflammation, spinal induced nitric oxide (iNOS) levels are increased and specific iNOS inhibitors reduce thermal hyperalgesia [220]. NO production is responsible for COX-1 activation and COX-2 upregulation after carrageenan. These changes result in PGE_2 and PGI_2 production at the inflammatory site [215]. Furthermore, recent studies have revealed an important relationship association between estradiol and NO. Esposito et al., (2005) demonstrated that a selective estradiol receptor modulator or 17β-estradiol decreased induced NO (iNOS) levels after carrageenan induced inflammation [163]. After LPS-induced inflammation, estradiol treated OVX rats showed significantly attenuated levels of iNOS and COX-2 compared to untreated rats. Moreover, NO is responsible for physiological changes associated with delaying the onset of migraines which are more prevalent in women. Interestingly, estradiol can counter these effects [221]. Moreover, NO has also been associated with corticosterone release. Recently, a mechanism of rapid release of corticosterone from the adrenal by NO has been uncovered [222]. This mechanism can deplete the corticosterone content in the adrenal by ~40% and is mediated by NO activated PGE_2 production. Focusing on NO levels may provide another medium in which to determine if corticosterone levels during inflammatory responses are changed in the presence of estradiol. In summary, examining estradiol replacement effects on either NO or NO synthase

actions may yield abundant information associated with the specific mechanism by which estradiol's anti-nociceptive actions modulate inflammatory responses.

As mentioned earlier, pro-inflammatory cytokines activated by peripheral tissue damage induce or increase inflammatory and neuropathic pain because they positively regulate inflammation-induced COX-2 [52,63]. In addition, correlations between tissue levels of cytokines with pain and hyperalgesia has been found in a number of painful diseases [130-132]. Estradiol has also been linked to attenuated levels and inhibition of pro-inflammatory cytokine levels or activity. Zhou et al., (2007) revealed that 17β-estradiol treatment inhibited the increased interleukin-18 (IL-18) expression associated with retinal ganglion cell apoptosis believed to be the leading cause of glaucoma [223]. Recently, Ma et al., (2007) examined local cytokine levels associated with delayed-type hypersensitivity (DTH) responses in intact females, males, OVX, and OVX + estradiol replacement. This study demonstrated that intact females had larger responses compared to males and OVX + estradiol replacement resulted in lower responses compared to intact females [224]. Overall, OVX females had the highest responses. Additionally, estradiol negatively regulates IL-6 which is associated with increases in DTH and positively regulates IL-4 associated with decreases in DTH [224]. These findings suggest positive direct and indirect regulation by estradiol on cytokine release and inflammatory responses. Cytokines activity and release make an ideal target for continued studies aimed at uncovering the mechanism by which estradiol is working through.

As demonstrated in the present study and current literature, estradiol is a very effective analgesic and anti-hyperalgesic. Its use as an alternative form of pain therapy promises to be bountiful. However, a lot more work will be required to ensure that its clinical use and benefits are maximized while unwanted side effects are minimized. A recent study showed how

differential effects of endogenous estradiol compared to estradiol replacement may also be responsible for unpredictable variations in nociception. Bradshaw et al., (2000, 2003) revealed that normal fluctuating hormonal levels such as those seen in rodents during estrous cycle compared to ovariectomized or ovariectomized with estradiol replacement were responsible for profound differential effects on neuronal activity as well as nociceptive responding [208,225]. This phenomenon makes it extremely complicated to make predictions based on anyone of these methods of examining estradiol effects. Specifically, it was noted that responses associated with different phases of the estrous cycle could not be directly correlated to responses achieved in the presence of estradiol replacement even if the estradiol dose was comparable to the percentage of endogenous hormone level associated with a specific stage. Based on these findings authors suggested that estradiol's unique effects are probably more closely related to changes in hormones levels that accompany normally cycling females rather than a specific dose or hormone percentage at a given time. More specifically they revealed that the difference between OVX and OVX + estradiol replacement was responsible for differences that ranged from increased inhibitory compared to excitatory responses to a reduction or increase in overall responses quantitative and qualitative. This is one of many aspects that will have to be addressed prior to use of estradiol replacement as a form of pain therapy.

Additionally, male subjects and immediate comparisons to male subjects continues to illuminate the origin of differential responding linked to sex hormones. By ensuring that both both sexes are used in studies targeted at illuminating the origins of estradiol's effects on inflammatory pain data collected can provide useful and indispensable insight to be applied to the population, both sexes. As has been the case in past and still today, studies examining of gonadal hormones effects on nociceptive processes show a lot of variability and lack of convergence.

Numerous factors continue to contribute to these inconsistencies often making it extremely difficult to set up experiments that actually examine and isolate the specific questions. For example, some studies show that testosterone yields a protective effects via anti-nociceptive actions while estradiol may be pro-nociceptive contributing to the difference in pain behavior in both human and animal studies [153,226]. Some studies suggest otherwise. For example, Caliborne et al., (2006) demonstrated that testosterone replacement in males restored spinal nociception, while estradiol in females attenuated this response [227]. Stoffel et al.,(2005) showed that both testosterone and estradiol do not consistently modulate analgesic effects in adult rats thus many measures have to be taken to be able to reliably predict the outcome of modifiers both exogenous and endogenous on analgesic effects [228].

Interestingly, Aloisi et al., (2004) established that supra-physiological levels of testosterone did not affect nociceptive input but did affect responding. Females treated with testosterone showed behavioral responses similar to those of males after formalin [229]. It was also suggested that differential behavioral responses observed between females and males during interphase (the stage of significantly reduced behavioral responding between phase I and Phase II believed to be controlled by inhibitory systems not a cessation in nociceptive responding) were controlled by descending inhibition, an endogenous inhibitor system that may be a key player mediating estradiol's analgesic effects [24]. Further investigation of the origin of differential responding between the sexes and descending inhibition can provide valuable insight and should be examined further.

In summary, the influence of sex hormones on the incidence and development of pain is omnipresent and much work remains to be done to elucidate the specific pathways responsible for these differences. By eliminating specific pathway(s) believed to be responsible for estradiol's

analgesic effects on pain behavior we move that much closer to identifying the mechanism(s) that are responsible for these effects. These findings can then be used to manage differential pain treatment dependent on a patient's sex and stage of the reproductive cycle.

Based on our findings, we propose the following model in which estradiol mediates behaviors following an inflammatory stimulus in the periphery by attenuating the release of IL -1β and TNF-α, pro-inflammatory mediators (Figure 29).

The introduction of an inflammatory stimulus activates inflammatory cells to release IL -1β and TNF-α which induce the release of other pro-inflammatory cytokines. These cytokines in turn induce synthesis and release of PG's via activation of the COX-2/mPGES-1 enzymatic pathways. Activation of PG receptors in the dorsal root ganglion alter neuronal activity at the dorsal horn that initiates changes in the spinal cord (central processing). These changes further enhance nociceptive impulse transmission by synthesizing and releasing pro-inflammatory cytokines and PG's. Reduced release of IL -1β and TNF-α by estradiol can result in attenuated inflammatory responses. An alternate model is estradiol reduces NO release after an inflammatory stimulus. NO directly down regulates COX-2 release following the same path as described above. Reduced COX-2 release will result in attenuated inflammatory responses.

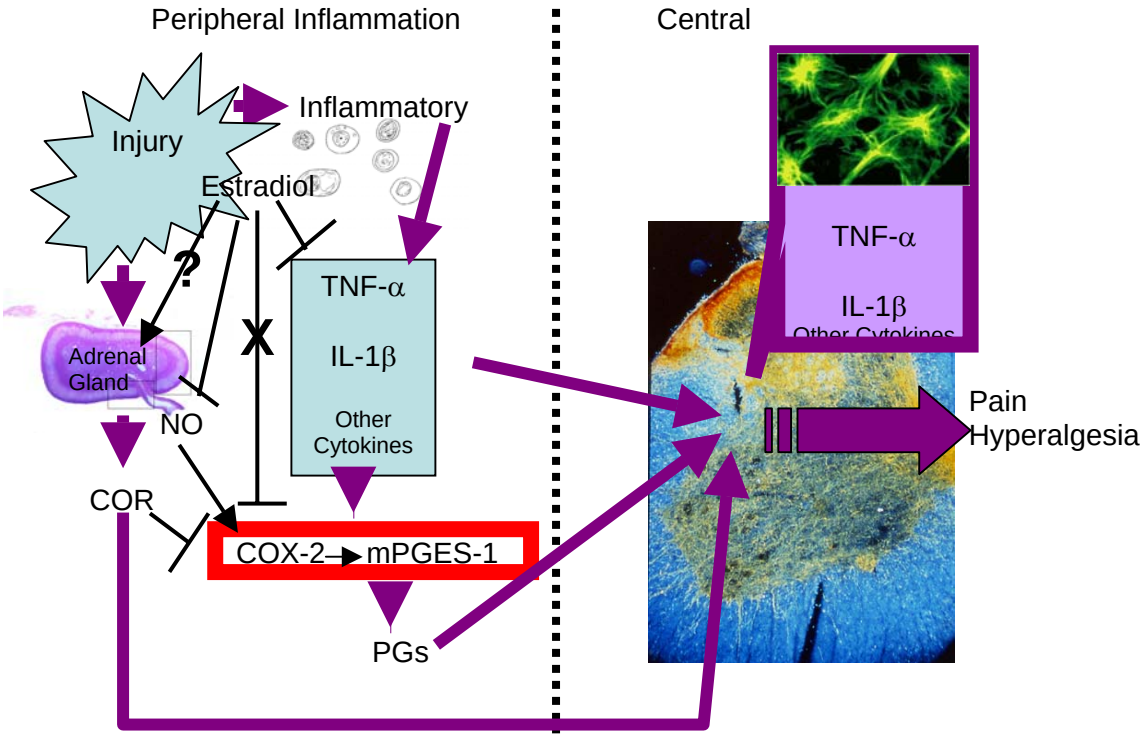

Figure 34.
Proposed model for possible mechanism (s) estradiol's anti-hyperalgesic operate through in mediating inflammatory behavioral responses.

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