The Role of Gonadal Hormones in Mediating Intracellular Cascades in Response to Persistent/Inflammatory Nociceptive Input

by

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Abstract

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Clinical and preclinical studies have found sex-specific differences in the discrimination and perception of nociceptive stimuli. The emerging picture from both clinical and preclinical studies suggests that the basis of these differences in nociceptive responses to such stimuli resides in the regulatory activity of gonadal hormones in the central nervous system. Published reports suggest that pain management targeted at female patients should consider hormonal factors during the female reproductive cycle.

We found that sex differences lie in the behavioral responses to persistent/inflammatory pain. Furthermore, gonadectomy of males and females alter corticosterone responses to formalin administration. However, although no differences were found in cyclooxygenase protein expression, we did find differences in prostaglandin serum levels between intact and gonadectomized animals. These findings indicate a possible mechanism in which corticosterone and/or endogenous gonadal hormones may modulate inflammatory pain responses through cyclooxygenase activation rather than transcriptional/translational effects.

We further demonstrated that estrogen and progesterone differentially mediate responses to formalin administration. While estrogen attenuates formalin-induced flinching during Phase II, progesterone attenuates Phase I activity. When co-administered, progesterone blocks estrogen-induced attenuation in Phase II. However, estrogen does not interfere with progesterone's activity in Phase I; suggesting that progesterone effects are independent from estrogen effects.

The administration of tamoxifen, a selective estrogen receptor modulator, resulted in the same Phase II attenuation as estradiol, while α-estradiol did not. These results indicate a genomic mechanism of action in which activation of intracellular estrogen receptors are required. Although both estrogen and progesterone receptors are present in the lumbo sacral region of the spinal cord, hormone replacement did not alter their levels. These indicate mechanisms further downstream than protein synthesis and or degradation mediating these effects. Furthermore, we demonstrate that with sustained release of hormone, via subcutaneous pellets rather than injection, estradiol exerts an anti-inflammatory action evident with 5% formalin and not with 1% formalin. Thus, suggesting that estrogen may be exerting its actions on inflammatory responses at the peripheral level of the nervous system. Since α-estradiol did not result in the same effect, we conclude that the anti-inflammatory activity is steroid receptor mediated. Because gonadal hormones differentially affect neural centers and are likely to play an important role in the expression and maintenance of pain states, females seeking pain management should consider hormonal factors during the female reproductive cycle.

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CHAPTER I

The sensation of pain serves as an alert to real or impending injury, which triggers the appropriate protective responses. This sort of protective pain is transient and acute in nature. However, when this pain outlives its usefulness as a warning system it may become chronic and debilitating. The switch to a chronically painful experience involves changes within the spinal cord and brain, ultimately altering nociceptive processing pathways within the central nervous system (CNS). These long-term changes within the CNS can contribute to an amplification and persistence of pain, a condition termed hyperalgesia.

Pain is a perception based on the activation of nociceptors, specialized sensory receptors that are activated by noxious insults to peripheral tissue. Nociception is the neural response to perceived or actual tissue damage. Harmful stimuli to the skin or subcutaneous tissue, such as joints or muscles, activate several classes of nociceptor terminals, including thermal, mechanical, and polymodal [201].

Nociceptors

Nociceptors predominantly have either thinly myelinated Aδ fibers or unmyelinated C-fibres, conducting at speeds of 5-30 m/s or less than 1 m/s, respectively [201]. These classes of nociceptors are distributed widely through the skin and deep tissues and will often work together in transmitting nociceptive information [201]. In general, exposing the skin to a noxious stimulus will result in myelinated Aδ fibres yielding the rapid, first phase of pain which is ‘sharp’ in nature. Unmyelinated C-fibres evoke the second wave of ‘dull’ pain that is felt after the noxious insult is removed [25].

Nociceptive afferents have been demonstrated to end predominantly in the dorsal horn of the spinal cord, receiving direct input from both Aδ and C-fibre mediated peripheral nociceptors [283], with a majority of these afferents terminating in the superficial region of the dorsal horn [201]. Activation of these fibres results in the release of the major excitatory neurotransmitter glutamate as well as substance P (SP), aspartate, calcitonin gene related peptide (CGRP) and nitric oxide (NO) at the dorsal horn [339].

Dorsal horn neuron responses to nociception

Under conditions of severe and persistent injury, pain hypersensitivity will result due to repetitive firing of C-fibres leading to a progressive increase in the response of dorsal horn neurons, a phenomenon called "wind-up". This phenomenon was first described by Mendell [198;199] as a frequency-dependent facilitation of spinal cord neuronal responses mediated by C-fibres. Stimuli that arrive to the spinal cord while there is ongoing activity from a previous stimuli, will sum to produce a more intense interneuron discharge in the dorsal horn [339].

In addition to wind-up, Woolf [331] described a distinct phenomenon he termed central sensitization, where the hyperexcitable responsiveness of spinal cord nociceptive neurons to later low and high threshold sensory input is modified [335]. As a neuroplasticity within the CNS, the threshold for activation of these neurons is decreased such that normally innocuous stimuli now cause pain, a condition termed allodynia. The repetitive C-fibre activation seen in wind-up leads to a stimulus dependent neuroplasticity that can result in central sensitization, which outlasts the initiating stimulus [167;332]. It is the high threshold and slowly conducting nature of C-fibre afferents, as compared to

Aδ fibres, which make it possible for wind-up to behave as a precursor for central sensitization [290]. [Fig. 1].

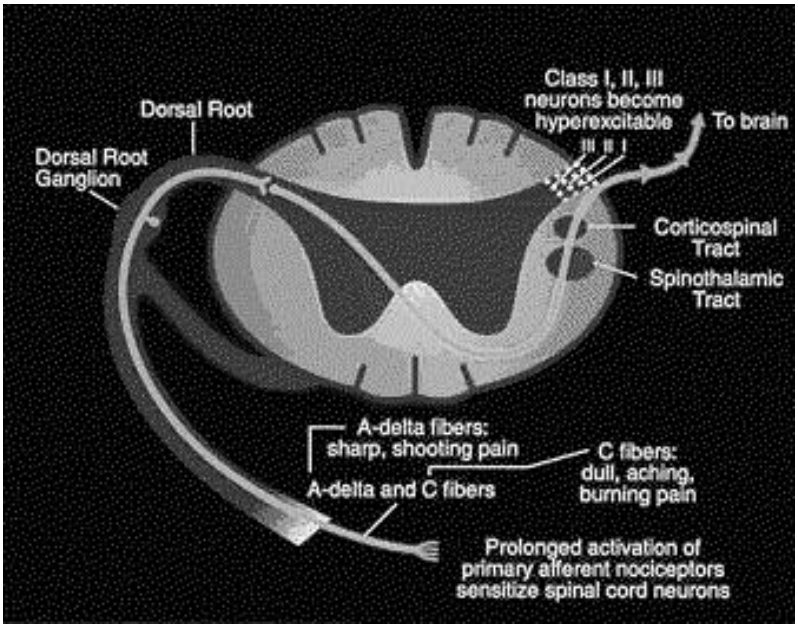


Figure 1
Nociceptive information is carried into the spinal cord via stimulation of Aδ and C-fibres. Stimulated Aδ and C fibres will ultimately synapse with neurons in the dorsal horn of the spinal cord. At the dorsal horn, nociceptive information is integrated, leading to a hyperexcitable state of the neurons. The behavioral manifestation of this excitable state is termed hyperalgesia [290].

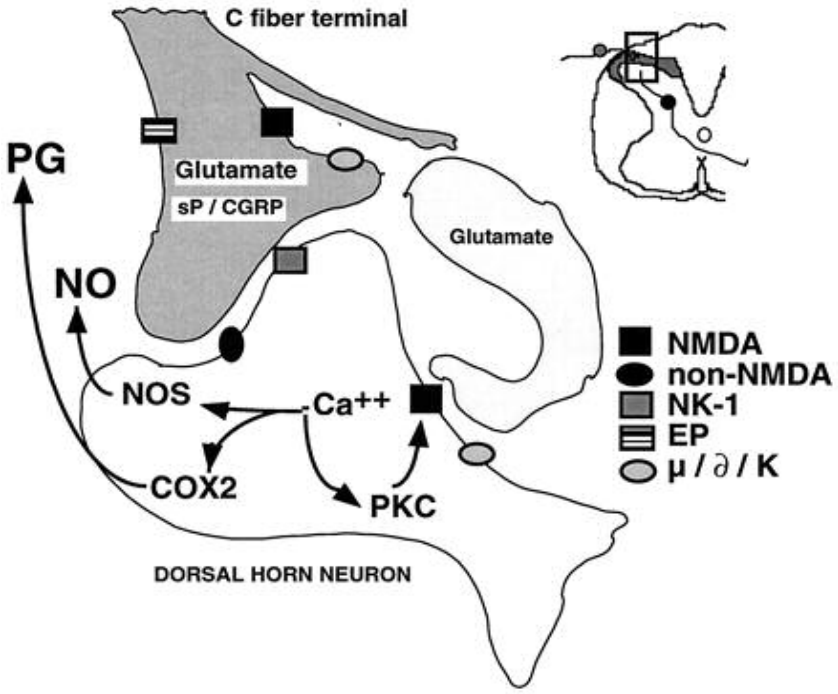
Mechanisms contributing to nociceptive responses at the spinal cord level

Glutamate, an excitatory amino acid, functions via the activation of two classes of receptors: ionotropic, including N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainite (K) receptors, the latter collectively referred to as non-NMDA receptors; and metabotropic, G-protein coupled, receptors. The NMDA receptor system is regarded as one of the most important structures in nociception with a major representation in the dorsal horn, as has been demonstrated by autoradiographic receptor binding techniques [139]. Metabotropic

glutamate receptors are engaged during increased spinal excitability that is associated with peripheral hyperalgesia [84]. The ionotropic class of receptors is involved in spinal hyperexcitability occurring during central sensitization [36]. Specifically, AMPA/K receptors mediate fast synaptic transmission showing extremely rapid activation and deactivation and showing desensitization with prolonged agonist exposure [295]. NMDA receptors, by comparison, have significantly slower activation and deactivation times, making it's involvement in certain types of synaptic plasticity like wind-up and central sensitization more feasible [191].

Repetitive C-fibre stimulation, like that seen after tissue injury, will release peptides, i.e. substance P (SP) and calcitonin gene related peptide (CGRP), as well as the excitatory amino acid glutamate, presynaptically, upon depolarization of the C-fibre terminal [82;134;166;339;343]. Evidence from electrophysiological studies demonstrates that sensory synaptic responses between primary afferent fibres and dorsal horn neurons are mediated by the excitatory transmitter glutamate via postsynaptic glutamate receptors [168;347]. Consistent with this, intrathecal (i.t.) administration of glutamate receptor agonists will evoke a potent spontaneous pain behavior and a subsequent thermal hyperalgesia and tactile allodynia [180]. Additionally, in electrophysiological studies of the flexor motoneuron, the output of the flexion reflex normally activated only by noxious stimuli is significantly attenuated by administration of glutamate receptor antagonists [173;334]. Taken together, the combined release of peptides and glutamate from active primary C-fibre afferents is responsible for inducing the hyperexcitable state of the neurons in the dorsal horn that characterize central sensitization [326]. [Fig. 2].

Interneurons excited by afferent barrage (repetitive C-fibre stimulation) also induce excitation in second order neurons via an NMDA receptor [339].



Adopted from Yaksh, 1999

Figure 2
Schematic representation of the dorsal horn system contributing to the processing of nociceptive information. Stimulated C fibres will result in the release of peptides (substance P/CGRP) as well as the excitatory amino acid, glutamate. Direct monosynaptic excitation of dorsal horn neurons occur via activation of non-NMDA receptors with glutamate, and the activation of NK1 receptors with substance P. Repetitive C fibre activation will result in excitation of interneurons and the further release of glutamate and excitation of NMDA receptors of the dorsal horn neuron. Depolarization of the dorsal horn neuron results in a Ca^{+2} influx that activates NOS and phosphorylating enzymes. Prostaglandins (PG) and nitric oxide (NO) are formed and released to act presynaptically, further increasing glutamate and peptide release. Phosphorylation of NMDA receptors leads to enhanced activation of the dorsal horn neuron.

The slow post-synaptic potential induced by neuropeptides, like SP, also contributing to the opening of voltage gated Ca^{+2} channels [72]. These slow potentials are additive and produce a cumulative depolarization which then activate the NMDA receptor resulting in an influx of calcium ions and an amplification of depolarization [72]. The net depolarization will then decay slowly resulting in a heightened response to

noxious stimuli, behaviorally manifested as hyperalgesia [72]. Blockade of the NK1 receptor by intrathecal antagonists or either down regulation of NK1 receptor expression by intrathecal treatment with NK1 receptor mRNA antisense has no effect on acute nociceptive thresholds, but reduces the second phase of the formalin response, which is thought to be mediated via NMDA receptor activation. Intrathecal injection of NK1 antagonist after phase 1 diminish their effect on the second phase [134;342]. This provides evidence that after an acute injury there is an initiating barrage of activity that leads to transmitter release leading to changes within the spinal cord that persist after the initial occupancy of the NK1 receptor by substance P. [Fig. 2].

The increase in intracellular Ca^{+2} leads to the activation of intracellular enzymes such as phospholipase A_2, NO synthase (NOS) and phosphorylating enzymes [339]. Cyclooxygenase (COX) products (prostaglandins, PG) and nitric oxide (NO) are then formed and released extracellularly, where they can facilitate further transmitter release from primary afferents, potentiating the injury response [128;181].

The intracellular increase in Ca^{+2}, as well as the activation of the NK1 receptor by SP, will also activate intracellular protein kinase C (PKC) which in turn phosphorolates NMDA receptors, further increasing intracellular Ca^{+2} [339]. The enhanced calcium influx increases the depolarization of the dorsal horn neuron [163]. The amplified activity in dorsal horn neurons after intradermal mustard oil or spinal NMDA is reduced by the local spinal delivery of PKC inhibitors [211;266]. [Fig. 2]

Endogenous opioid receptor system

Opiates are the most potent available analgesic compounds and are also strong addictive drugs. The opioid receptors were first discovered in 1973 [226;264;288]. Since this time, there has been the discovery of endogenous opioids, which fall into one of three categories, depending on their pharmacological properties: enkephalins, dynorphins and beta-endorphins, with enkephalins being the first endogenous opioid extracted [201].

Martin (1978) was the first to propose the existence of multiple opioid receptor types in an attempt to explain the complex agonistic/antagonistic properties of opioids. Three types of opioid receptors have since been identified and termed μ (mu), δ (delta), and κ (kappa), each binding differently with various opioids.

The μ-opioid receptor is the morphine receptor and has a high affinity for morphine and related opiate drugs [185;330]. The μ-opioid receptors are found in areas of pain processing, including the periaqueductal gray and the dorsal horn of the spinal cord [185;330]. The δ-opioid receptors are concentrated in the vas deferens as well as discrete areas of the CNS, in patterns similar to μ-opioid receptor distribution. The κ-opioid receptors have a distribution pattern distinct from μ and δ-opioid receptors and are sparsely found in periaqueductal gray, nucleus of the raphe, spinal trigeminal nucleus, and the dorsal horn of the spinal cord. κ-opioid receptors are thought to participate in spinal analgesia [185;330]. [Fig. 2].

Interactions of NMDA and opioid receptor system

There has been a close relationship found in the CNS between the NMDA receptor and the endogenous opioid system [58;265;306]. Several studies have shown that opioids directly or indirectly modulate NMDA receptor-mediated electrophysiological events, with μ and δ-opioid ligands either inhibiting or potentiating these events [58;265;306;349] and κ-opioids antagonizing NMDA receptor mediated currents [41;60]. Both cellular and intracellular mechanisms have been implicated in the role of opioids in regulating NMDA receptor activation [186;189;190]. For example, DAMGO and morphine, both μ-opioid receptor agonists, have been demonstrated to inhibit high voltage Ca^{+2} channel activity across cell membranes, however no direct association was made with Ca^{+2} channel activity and NMDA receptor activity [215;324]. In an NMDA antagonist sensitive model of pain, administration of the μ-opioid receptor agonist alfentanil reduced hyperalgesia [224].

Formalin assay as a model for tonic pain

As reviewed by Bennett (2001), the most commonly used nociceptive assays in nonhuman animals are thermal, electrical, mechanical and chemical. These assays are termed phasic/acute pain models, in which the noxious stimuli are brief in nature, escapable, high in intensity and usually employ a "cutoff" which represents a set time point or maximum stimulus intensity after which the noxious stimulus is removed from non-responding animals in order to avoid excessive tissue damage [272].

In contrast, models used to represent tonic pain are characteristically longer in duration, inescapable by the animal, moderate in intensity and often associated with

inflammation and/or tissue damage [272]. These reasons make tonic pain assays a more reasonable choice for modeling clinical pain conditions than do phasic models of pain [272]. The formalin assay is one such assay.

The formalin assay involves the injection of diluted formaldehyde solution (usually 1 – 10%) into the hindpaw of the rat resulting in a licking, flexing and flinching behavior of that paw [272]. The time and frequency with which the animal performs these behaviors is scored as nociception. Within seconds of the injection, the animal will flinch and lick its paw for approximately 5 minutes and will then stop temporarily. This is characterized as Phase I of the response. Phase I is thought to reflect acute nociceptive pain caused by a formalin-evoked discharge in C-fiber nociceptors and is thought to be an opioid mediated event [27]. The period following Phase I, where no nociceptive behavior is observed, is termed the quiescent (Q) phase, or interphase. Following, the rat will begin to flinch and lick again, at high frequencies, for 15 minutes or more [27]. This phase of nociceptive activity is termed Phase II. This Phase is thought to result from mechanisms resembling those of chronic and/or neuropathic pain, with particular respect to the responsiveness of spinal cord nociceptive neurons that are evoked by the C-fiber discharge during the initial phase [27]. Accordingly, this phase is thought to be NMDA receptor mediated [65;68;346].

The formalin assay, distinctive from other nociceptive assays, provides a method of evaluating both acute and tonic pain in a single chemical test due to its biphasic nature, lasting a total of approximately 60 minutes [Fig. 3]. An early component and a late component of the response are attributed to direct activation of nociceptors and central sensitization, respectively [64;299].

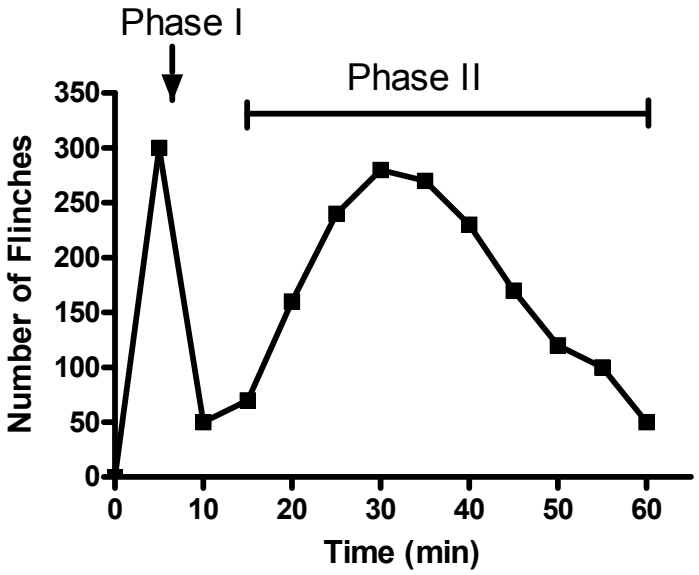


Figure 3
Nociceptive behavior resulting from diluted formalin injection to the right hind paw. Across time, the biphasic flinching behavior is characterized as Phase I, for 15 minutes or more. Phase I results from the activation of peripheral nociceptors and direct monosynaptic activation of non-NMDA 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 the interphase, Phase II begins and lasts for 20 – 50 minutes, where high frequency flinching is observed. Phase II results from continued afferent input from nociceptors as well as activation of NMDA receptors by continued glutamate release.

Formalin and NMDA Receptors

Haley et al. [117] found that administration of NMDA channel blockers, ketamine, MK-801 and AP5, during the second phase of neuron firing, dose dependently inhibited rate of firing. Administration of MK-801, 30 minutes prior to formalin injection, also caused a dose related inhibition of the second phase of neuron firing, but no effect on the first phase, an acute response thought to be mediated by the stimulation of peripheral nociceptors as opposed to changes in central processing of information at the spinal cord [217;291].

Central sensitization results in the expansion of the receptive field size of dorsal horn neurons as a result of C-primary afferent input [66;333]. Ren et al. [241] found that administration of the NMDA antagonist, MK-801, significantly reduced receptive field size of these neurons after administration of carrageenan, a solution that induces central sensitization via chemosensitive C-fibres.

Sex differences in pain

Females report a greater number of chronic pain conditions, as compared to males, including disorders such as fibromyalgia, temporomandibular disorder, chronic fatigue syndrome, headaches and irritable bowel syndrome [31;99;348]. Similarly, female rats demonstrate higher pain sensitivity compared to males in electric and chemical nociceptive assays [5;10;15;107;146]. Increased behavioral responses of females as compared to males in the formalin assay also suggest a role of hormonal environment [107]. In the phasic/acute pain models, however, male rats show greater pain responses involving reflexive withdrawal responses to noxious heat, with shorter latencies, compared to females [21;101;135;207;246]. Several factors may contribute to sex differences in the perception of pain, the most obvious being the role of sex steroid hormones, specifically estradiol and progesterone.

Estrous/Menstrual cycle effects on pain sensitivity

Clinical studies have shown that pain thresholds and ratings vary as a function of menstrual cycle [112;245], playing a role in the intensity of symptomatology in rheumatoid arthritis and fibromyalgia [76;219]. There are higher pain thresholds and

tolerance during the follicular phase and enhanced pain sensitivity during the luteal phase for most pain stimulation procedures with the exception of electrical pain, which tend to show the opposite [99;110;119].

Using acute pain assyas, pain thresholds of female rats vary as a function of estrous cycle with the strongest evidence suggesting that pain sensitivity peaks during late proestrus and early estrus when estradiol and progesterone are at their peak and then begin to reach low levels [115;188]. The role of estrous cycle is summarized in Table I. Briefly, Kayser et al. [144] demonstrated that vocalization thresholds to both tail and hindpaw stimulation were significantly lower during proestrus and estrous in female rats. Vincler et al. [313] reported greater sensitivity during proestrus with increased reflex amplitudes and decreased escape latencies to electrical stimulation. Data has also demonstrated the contrary, with pain sensitivity decreasing in these phases [101;239].

Gonadectomy effects in phasic/acute pain assays

Gonadectomy studies address the role of endogenous steroids in modulating pain responses (summarized in Table II). Gonadectomized (GDX) males have demonstrated increased nociceptive responses in the tail-flick assays [6;101;107]. However, GDX of male rats has no effect in the hotplate and electric foot shock assay [170;275]. The role of endogenous hormones in female rats is currently unclear. For example, ovariectomy decreases, increases or has no effect in pain threshold depending on the nociceptive assay [24;44;55;99;101;107;275]. Martinez-Gomez et al. [188] found that ovariectomy of female rats abolished fluctuations in tail flick latencies seen across the estrous cycle.

Hormone replacement effects in phasic/acute pain assays

Hormone replacement studies have examined the role that estradiol and progesterone play on pain thresholds. As summarized in Table III, the route and duration of administration, whether given alone or together, and the type of nociceptive assay employed, all greatly influence results.

Following estradiol administration alone in GDX males and females, there have been reports of increases, decreases, and no effects in nociceptive thresholds. For example, Stoffel et al. [275] report no effect in males and an increased threshold in females using the hotplate assay subsequent to estradiol administration. However, Gordon & Solimon [115] found a decreased threshold in females subsequent to estradiol administration in the hotplate assay. No effect was found in the electric foot shock assay when estradiol was administered in males and females [170]. In the tail-flick assay, contradicting results have also been found after estradiol administration, where estradiol either increased or decreased tail flick latencies [101;115].

As summarized in Table IV, studies examining the role of progesterone administration have also resulted in conflicting results. Overall, results on the effect of progesterone replacement vary with route of administration, dose and assay. Frye & Duncan [106] find a dose-dependent increase in tail-flick latency only when administered i.c.v., not when given s.c., i.v., or via implanted capsules. Gordon & Solimon [115] found that s.c. injections of progesterone (5 mg/kg/day for 7 days) increased latency in the tail-flick assay and decreased latency in the hotplate assay. Stoffel et al. [275] found an increase in threshold, when administered 500 μg progesterone every 4 days, in the hotplate assay.

As summarized in Table V, the effect of estradiol priming on progesterone's effects have also been studied in several nociceptive assays. Administration of both estradiol and progesterone have more consistently increased thresholds across several assays [115;275]. Dawson-Basoa & Gintzler [79] demonstrated that the administration of a pregnancy profile of both estradiol and progesterone in OVX females increased thresholds in the electric foot shock assay. These results were replicated in males as well [170].

Table I: List of estrous cycle effects on pain thresholds using various acute nociceptive assays

Estrous Phase	Estrus	Metestrus	Diestrus	Proestrus	Reference
Assay					
Hotplate	↓ threshold vs. diestrus		↑ threshold vs. estrus		[275]
Electric Foot shock		↓ threshold vs. proestrus		↑ threshold vs. metestrus	[162]
Tail-flick	↓ threshold vs. proestrus and diestrus	↓ threshold vs. proestrus and diestrus			[188]

Table II: Gonadectomy effects on pain thresholds using various acute nociceptive assays

	Pain Assay	Results	Reference
Male	Hotplate	No effect	[275]
	Electric foot shock	No effect	[170]
	Tail-flick	↑ sensitivity	[101]
Female	Hotplate	↑ sensitivity	[275]
	Electric Foot Shock	No effect	[24]
	Tail-flick	↑ sensitivity	[101]

Table III: List of estradiol replacement effects on pain thresholds using various phasic/acute nociceptive assays

	Pain Assay	Results	Estradiol Dose/Route	Reference
Male	Hotplate	No effect	10mm/100g b.wt. (s.c.)	[275]
	Electric foot shock	No effect	10mm/100g b.wt. (s.c.) Simulate pregnancy profile	[170]
Female	Hotplate	↑ threshold vs. GDX	One 1mm or 5mm capsule (s.c.)	[275]
		↓ threshold vs. GDX	50μg/kg/day for 7 days (s.c.)	[115]
	Electric foot shock	No effect	10mm/100g b.wt. (s.c.) Simulate pregnancy profile	[79]
	Tail-flick	↑ latency to withdrawal	50μg/kg/day for 7 days (s.c.)	[115]

Table IV: List of progesterone replacement effects on pain thresholds using various acute nociceptive assays

	Pain Assay	Results	Progesterone Dose/Route	Reference
Male	Electric foot shock	No effect	45mm implants Simulate pregnancy profile	[170]
Female	Hotplate	↑ threshold compared to GDX	500μg (s.c.) every 4 days	[275]
		↓ threshold compared to GDX	5mg/kg/day for 7 days (s.c.)	[115]
	Electric foot shock	No effect	45mm implants Simulate pregnancy profile	[79]

Table V: List of co-administration replacement effects on pain thresholds using various acute nociceptive assays

	Pain Assay	Results	Co-administration Dose/Route	Reference
Male	Electric foot shock	↑ jump threshold	E: 10mm/100g b.wt. (s.c.) P: 45mm implants	[170]
Female	Hotplate	↑ threshold compared to GDX	E: One 1mm or 5mm capsule (s.c.) P: 500μg (s.c.) every 4 days	[275]
		No effect	E: 50μg/kg/day for 7 days (s.c.) P: 5mg/kg/day for 7 days (s.c.)	[115]
	Electric foot shock	↑ jump threshold	E: 10mm/100 g b.wt. (s.c.) P: 45 mm implants	[79]
	Tail-flick	↑ latency to withdrawal	E: 50μg/kg/day for 7 days (s.c.) P: 5mg/kg/day for 7 days (s.c.)	[115]

Current state of the literature

As previously discussed, the literature is riddled with differences with respect to the effects of ovariectomy and hormone replacements. A majority of the results seem to indicate that ovarian hormones do indeed play a role in the modulation of nociception, as is indicative of ovariectomy studies that result in the abolition of nociceptive fluctuations with the estrous cycle. The inconsistencies are due to several variables, which include the following:

1. The regimen and manner of administration of hormone replacement vary between published studies. The duration of hormone replacement has been shown to be crucial in the lordosis literature, either inhibiting or synergizing behavior [103]. A systemic study using dose response curves and different assays are lacking.
2. The doses of administered hormone vary between studies.
3. The assays of nociception vary across studies.
4. The length after ovariectomy when the animals are tested vary across studies.

Additionally, few studies have examined the role of estradiol and progesterone in mediating chronic pain sensitivity in a female OVX rodent model.

Potential mechanisms underlying hormonal effects in a chronic pain assay

With evidence that estradiol and progesterone receptors exist throughout the CNS in regions involved in pain perception and inhibition, such as periaqueductal gray (PAG) and the spinal cord dorsal horn [11;142;222;301], endogenous steroids have been postulated to play a role in modulating pain pathways as well as contributing to both sex differences and hormonal modulation in pain perception. At the spinal cord level,

Williams et al. [325] found that cytosolic estradiol receptor levels progressively increased during the cycle from estrus and metestrus to diestrus and proestrus A.M., with a maximum at proestrus P.M. [325]. No differences in binding affinity, however, were seen at various stages of the cycle [325]. Similar trends were seen with serum estradiol levels across the estrus cycle [325].

Monks et al. [208] found that progesterone receptor concentrations fluctuated in concert with ovarian cyclicity during the estrus cycle in the rat lumbar spinal cord. This supports a role for estradiol-induced progesterone receptor in spinal cord neurons with respect to control for female sexual behavior [208]. Due to the scope of this dissertation work, we will only discuss mechanisms at the spinal cord level. Kastrup et al. (1999) found progesterone receptor expression in supraspinal locations involved in pain processing, however no progesterone receptor expression was found in the superficial dorsal horn. These findings suggest that the effect of progesterone on pain processing may be exerted through descending projections from supraspinal regions.

Precise mechanisms by which pain processing occurs in female rats are not firmly established. Endogenous gonadal hormones may influence CNS pathways involved in pain transmission, with the most obvious being at the primary afferent nerve fiber. Mechanisms of influence might include increased activity/sensitization of neurons, a diminished inhibitory control at the spinal level or at higher brain centers.

Dawson-Basoa & Gintzler [80;81] demonstrated that the analgesia found during pregnancy, and just prior to parturition, is due to the activation of both κ and δ-opioid receptors, in the spinal cord, by circulating levels of both estradiol and progesterone.

Blockade of either of these analgesic systems, but not the μ-opioid receptor system, will substantially attenuate the antinociception associated with either condition [81].

Amandusson et al. [11] found that there is a dense population of estradiol receptor immunoreactive neurons in the superficial laminae of the spinal and medullary dorsal horn, both target areas for primary afferent fibers responding specifically to noxious stimuli as well as the main locations of spinal opioid modulation of pain transmission. Furthermore, Amandusson et al. [12] have found that a majority of the enkephalinergic neurons in these laminae contain estradiol receptors and that estradiol administration has the ability to rapidly (4 hours later) induce an increase in the amount of enkephalin mRNA in the lumbar spinal cord of female rats. Studies have found that increasing levels of enkephalins are associated with less pain sensitivity [150;218;251]. Estradiol-induced increases in spinal enkephalin transcription in such a short period, may represent mechanisms by which pain sensitivity is influenced by estradiol during the menstrual/estrous cycle.

Dynorphin is an endogenous opioid peptide with a high affinity for the κ-opioid receptor. Up-regulation of preprodynorphin (PPD) mRNA in the spinal cord has been associated with an increase in neuronal activity and an increase in thermal hyperalgesia associated with inflammation [87]. An up-regulation of spinal cord dynorphin is correlated with increased pain thresholds in rats during pregnancy and partuition [113;196;197]. This effect is thought to be mediated through the interactions of elevated levels of both estradiol and progesterone and the κ- and δ-opioid systems [80;81]. Bradshaw et al. [43] found that subsequent to injection of complete Fruend's adjuvant

into the hindpaw, female rats in diestrus and proestrus showed significantly greater ipsilateral PPD mRNA induction compared with male rats.

Estradiol can also modulate the levels of neuropeptides that are involved in setting the level of pain transmission from the spinal cord to the brain, such as substance P and neurokinin A [89]. Duval et al. [89] show that plasma concentrations of both substance P and neurokinin A vary across estrous cycle. Plasma concentrations of substance P were positively correlated with 17 beta-estradiol levels and the absence of ovarian steroids lead to low levels of plasma Neurokinin A [88]. Furthermore, large fluctuations in hypothalamic and pituitary substance P and Neurokinin A contents were found in the female rat, across estrous, and strongly suggest the involvement of gonadal steroids in the regulation of these neuropeptides. Duval et al [88] also looked at neuropeptide levels in the cervical spinal cord of female rats, and found fluctuating levels of both neuropeptides across estrous cycle, with substance P and Neurokinin A contents falling with surges in 17 beta-estradiol, suggesting downregulation of the neuropeptides by 17 beta-estradiol. Furthermore, Villablanca & Hanley [312] found the administration of 17 beta-estradiol increased substance P receptor gene expression in the rat pancreatic acinar cell line, leading to increased specific binding of substance P. Substance P gene receptor expression was inhibited in the presence of a competitive estradiol receptor antagonist, tamoxifen, indicating an estradiol receptor-mediated genomic control pathway [312].

Several studies confirm that treatment with estradiol to ovariectomized rats increases hippocampal NMDA receptors, and that ovariectomy decreases NMDA receptor binding supraspinally [108;320;336]. Progesterone and co-administration of these steroids does not increase binding, suggesting that progesterone opposes the effect

of estradiol [74]. In intact rats, ovarian hormone withdrawal decreases hippocampal NMDA receptors and 17 beta-estradiol prevents this decrease [74;336]. With respect to the cortex and striatum, Cyr et al. [74] found that ovariectomy does not change NMDA receptor binding, however treatment with 17 beta-estradiol, progesterone and their combination decreases this binding.

Treatment of ovariectomized rats with tamoxifen or raloxifene resulted in increased hippocampal NMDA receptors, similar to that obtained by 17 beta-estradiol treatments [75]. Cortical and striatal NMDA receptor binding decreases with treatment of these estradiol receptor antagonists, resembling results subsequent to treatment with 17 beta-estradiol [75].

Serotonin receptors are present in both rat and human dorsal horn, particularly concentrated in superficial laminae I and II, areas involved in pain transmission [160]. During injury or inflammation, serotonin is released from platelets and mast cells and may activate G-protein coupled 5-HT1 and 5-HT2 receptors, inducing membrane depolarization and sensitizing nociceptors to heat and pressure stimuli and may also induce repetitive neuronal firing [85;166]. Gonadal hormones may also exert effects on the serotonergic system [32]. Marcus [187] found that peaks of estradiol are associated with decreased numbers of available serotonin receptors and a greater availability of serotonin at the spinal cord level. Following the estradiol peak, estradiol receptor affinity decreases and the number of available cortical serotonin receptors increases [34;298].

Genomic vs. non-genomic effects of sex steroid hormone

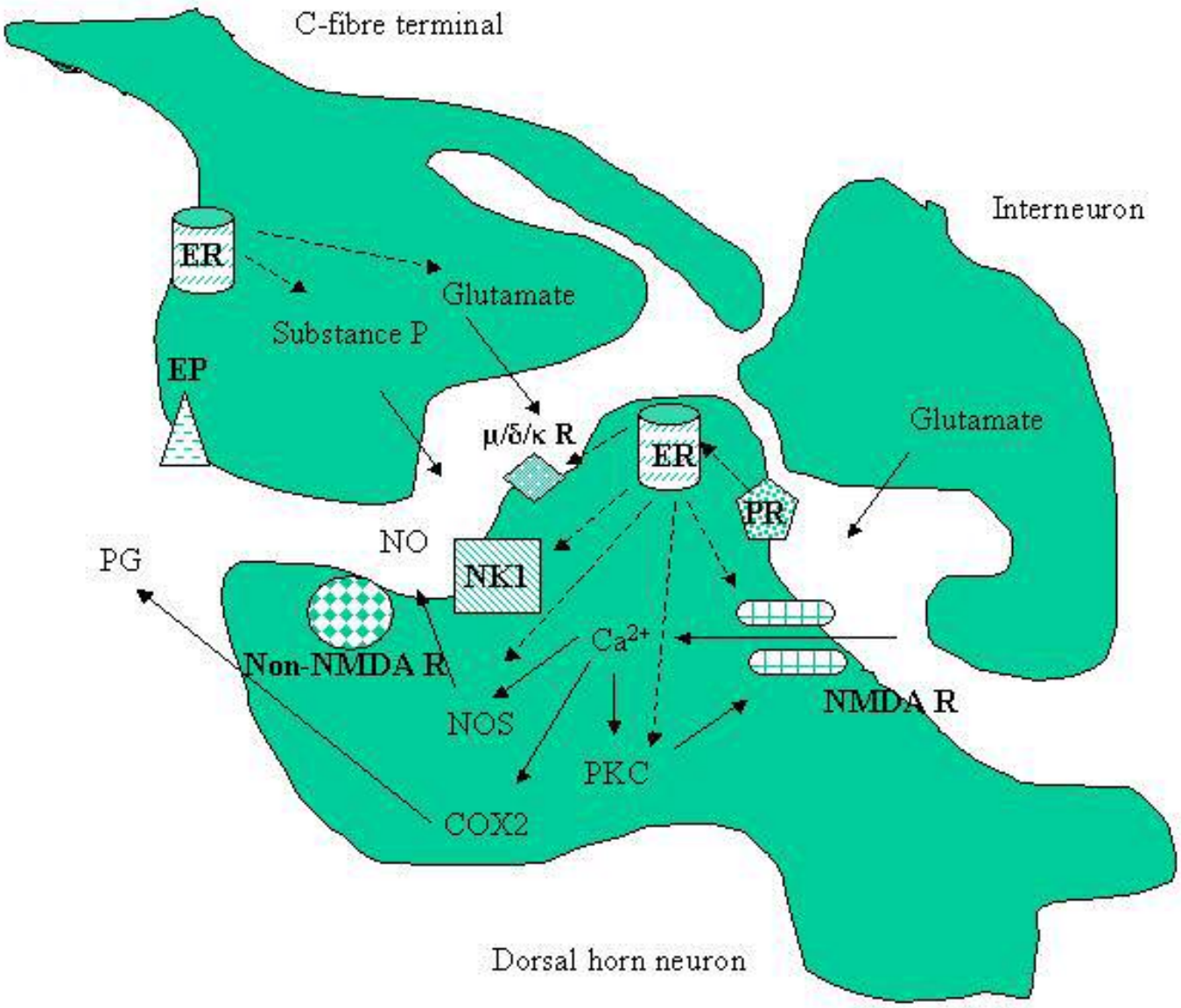
Customarily, those steroid actions that are delayed in onset and prolonged in duration are referred to as "genomic effects" whereas those actions that are rapid in onset and short in duration are referred to as "non-genomic effects" [194]. The non-genomic effects of sex steroid hormones include those occurring due to the recruitment of signaling pathways associated with ion channels and other cellular mechanisms occurring as a result of membrane receptor activation [319]. In the genomic model, intracellular steroid receptors are activated when steroid hormones diffuse into the target cell. The receptor and steroid complex now serves as a transcription factor regulating expression of a variety of functionally diverse genes, including those that encode surface receptors, like NMDA.

Progesterone has also been shown to produce rapid, non-genomic effects in reproductive tissues (within minutes) [18;37;53;171]. Progesterone has the ability to induce a very rapid increase of intracellular Ca^{+2} occurring within seconds of steroid addition to spermatozoa, and was found to be a dose-dependent reaction that is not blocked by RU-486, an antagonist of intracellular PR [17;38]. Similarly, estradiol has been shown to induce Ca^{+2} influx in cultured endometrial cells in less than 10 minutes [228].

Dorsal root ganglion neurons, located in the dorsal horn of the spinal cord, contain intracellular estradiol receptors, making genomic pathways leading to changes in gene transcription likely. Activation of these intracellular estradiol receptors may contribute to increased protein synthesis of NMDA and/or NK1 receptors at the cell membrane of the dorsal horn neuron.

Proposed Model

We postulate that the effects of estradiol and progesterone on the hyperalgesic response to formalin may occur via both genomic and non-genomic mechanisms. A rapid mechanism of modulating ion channel conductivity across the dorsal horn neuron membrane, specifically Ca^{2+} influx, triggers the second messenger cascade described earlier. Additionally, estradiol and progesterone may exert their effects via genomic mechanisms that involve a change in membrane protein levels (NMDA and/or NK1) and/or intracellular mechanisms necessary for the behavioral responses to injury (tonic pain) such as reduction of glutamate and substance P presynaptic release, PKC and/or NO syntheses activations. These changes are all proposed to occur at the dorsal horn of the spinal cord and may be the basis for the reported differences related to the estrous cycle that is demonstrated in behavioral and/or neurochemical responses to painful stimuli in rats [Fig. 4].



Modified from Yaksh (1999)

Figure 4
Potential mechanisms by which steroid hormones may modulate nociceptive responses in the spinal cord. Estradiol may exert its effects via the modulation of protein levels and/or activation of cellular mechanisms necessary for the behavioral responses to injury. These may include reduction of glutamate and substance P release presynaptically, reduction of PKC and/or NO mechanism activation intracellularly, and/or may affect NMDA/NK1 receptor activation or levels. Proposed mechanisms of action are diagrammed with a dotted line. (ER: estradiol receptor; PR: progesterone receptor; NOS: nitric oxide synthase; NO: nitric oxide; PG: prostaglandins; EP: prostaglandin receptor).

Specific AIMS:

Clinical data has shown a prevalence of pain disorders in the female population as compared to males. Research in this area will help elucidate the mechanisms by which pain responses are differentially mediated in females as compared to males. Understanding the mechanisms of these sex differences will allow for better treatment of females suffering from pain disorders in the clinical setting. The goal of this project is to determine the mechanisms by which both estradiol and progesterone regulate the nociceptive response in a persistent/inflammatory rodent model of pain. Furthermore, this project seeks to answer the role in which these endogenous hormones contribute to the prevalent sex differences seen in the response to pain in the clinical setting.

The scientific literature has reported discrepancies between the interactions of estradiol and progesterone and their effects on nociceptive behavior in females. The results of this study will provide relevant information on the analgesic potential of hormone replacement as a treatment method for clinical pain conditions in women prescribed orally administered contraceptives.

No studies have examined the role of estradiol and progesterone in modulating pain transmission via intracellular mechanisms including cyclooxygenase, and their products, in the spinal cord. Estradiol and progesterone mechanisms at the spinal level may regulate the behavioral response to chronic or inflammatory pain like that represented in the formalin assay. The specific aim of this project is to examine the role of estradiol and progesterone in mediating the behavioral response to formalin and the mechanisms via which they may be doing this.

We hypothesize that endogenous sex hormones play a role in decreasing nociceptive responses to persistent/inflammatory pain. Furthermore, that estradiol and progesterone exert their effects by acting via endogenous steroid receptor levels, activation of cyclooxgyenase or via mediation of prostaglandin levels. To test these postulates the following specific aims are proposed:

Specific Aim 1: We postulate that there will be a behavioral sex effect in response to formalin administration. Male and female rats, both intact and gonadectomized, will be tested after the administration of formalin.

Specific Aim 2: We postulate that estradiol and progesterone decrease inflammatory induced behavioral responses. First, we will perform a dose response curve of estradiol and progesterone, individually, in evaluating the formalin pain response. Finally, co-administration of estradiol and progesterone, at optimal doses, will determine their effects on the formalin pain responses.

Specific Aim 3: We postulate that estradiol and progesterone effects are membrane mediated. We will administer an estradiol receptor antagonist along with hormone replacement to determine if the pain response is mediated via the steroid receptor activation. Similarly, administration of 17α-estradiol, an isomer of estradiol that does not bind to the estradiol receptor, will be administered, and tested in the formalin assay.

Specific Aim 4: We postulate that estradiol and progesterone mediate these behavioral responses via spinal cyclooxygenase receptors. Western blot technique will be used to determine COX-1 and COX-2 receptor levels in the spinal cord. Following, we will use an enzyme activity assay to determine the level of prostaglandin available in the serum.

Chapter 2: The role of female gonadal hormones in behavioral sex differences in persistent and chronic pain: clinical vs. preclinical studies

Introduction

Clinical and preclinical studies have found sex-specific differences in the discrimination and perception of nociceptive stimuli. The emerging picture suggests that the biological basis of these differences in pain perception resides in the regulatory activity of estradiol and progesterone in the central nervous system. Since others have recently reviewed sex differences and the role of gonadal hormones in responses to acute pain [4;67;99], this review focuses on sex differences and the role of gonadal hormones in the behavioral response to persistent inflammatory and chronic pain stimuli.

Experimental animal models for persistent inflammatory and chronic pain

The measurement of "pain sensation" in animals is largely indirect, relying on aversive behavior in response to stimuli, as opposed to assessing the quality of the pain [27]. Pain models are traditionally divided into phasic/acute, tonic/persistent inflammatory, and chronic categories, distinguished by the duration and mechanism of the pain. Generally, phasic/acute tests require a high-intensity stimulus (such as thermal, mechanical, or chemical). Tonic/persistent inflammatory pain tests, those of long duration, use an irritant, a foreign chemical agent, as the nociceptive stimulus [27]. Whereas phasic/acute tests measure a threshold response, tonic tests quantitatively measure the resulting behavior after the stimulus (which varies in potency with time). Neuropathic pain models, on the other hand, use an injury to a peripheral nerve to

produce a temporary or permanent allodynia or hyperalgesia that develops over several days after the injury and can lead to chronic pain. Similar pain models include chronic inflammatory pain, which models human arthritis.

Table VI summarizes the most commonly used animal models of tonic/persistent inflammatory, chronic and neuropathic pain. These assays range from models of arthritis to more severe pain such as nerve compression and neuronal damage. Most of these models have been used to elucidate the contribution of gonadal hormones in mediating the prevalence of chronic pain conditions in the female population.

Table VI. Rodent models of persistent and chronic pain

Model of pain	*Method*	*Behavior*	*Disease model*	*References*
Persistent inflammatory and chronic pain				
Formalin	Injection of dilute formaldehyde (1%-10%) into hind paw or upper lip	Biphasic flinching response lasting a total of approximately 60 minutes; rubbing upper lip and head flinches for approximately 45 minutes	Acute pain (phase I); Inflammatory/chronic pain (phase II); Model of TMJ	[272] [62]
Capsaicin	Injection of pungent ingredient in hot chili peppers into hind paw or tail	Inflammation and hyperalgesia of paw or tail; lasts several hours	Mimics spinal mechanisms of chronic pain	[20]
Complete Freund's adjuvant (CFA)	Injection of CFA intravenously or into the hind paw	Inflammation of hind paw lasting 10 days; sensitive to heat & mechanical stimulation	Arthritis when injected i.v.; Edema in hind paw injection	[27]
Carrageenan	Injection of dilute carrageenan into the hind paw	Inflammation and hyperalgesia of paw; lasting several hours to a day	Inflammatory pain	[120]
Ureteral calculosis	Dental cement injected into ureter	Writhing behavior; episodes lasting from 45 minutes to over a course of up to 14 days; hyperalgesia	Ureteral calculosis	[111]
Neuropathic pain				
Chronic constriction model	Ligatures tied around sciatic nerve	Spontaneous pain via licking and shaking of affected hind paw; reduction in appetite and weight loss; hyperalgesia to heat, mechanical and chemical stimuli; pain present for 2-3 months	Nerve compression and mechanical neuronal damage	[28]
Partial nerve transection	Partial tight ligation of sciatic nerve	Spontaneous pain via licking and shaking of affected hind paw; reduction in appetite and weight loss; hyperalgesia to heat, mechanical and chemical stimuli; pain lasts 6+ months	Nerve compression and mechanical neuronal damage	[260]
Spinal nerve ligation	Ligation of L5 and L6 spinal nerves	Reduced innervation of hind paw; Hyperalgesia with heat and mechanical stimuli; mechano- and cold-allydonia; pain lasts 1-2 months	Nerve compression and mechanical neuronal damage	[147]

Sex differences in chronic pain

Clinical studies demonstrate sex differences in prevalence of persistent and chronic pain

As summarized in Table VII, epidemiological pain studies have found that women more frequently reported temporary and persistent pains than did men, and they were more likely to report pain of a longer duration [13;23;31;40;54;70;70;123;127;130;229;240;244;249;273;284;297;300;309]. For example, women had higher rates of chronic tension headache and migraine than men [233;235;236;238;321]. Reports also demonstrated that women experienced more nausea/vomiting and unilateral numbness/tingling with migraine [57;233;237;274;321]. Facial pain and tenderness in jaw muscles and the temporomandibular joint (TMJ) were reported more often in women than men [138;232;243;270;314;318]. Women also reported more musculoskeletal pain, with more intense and frequent pain, than did men [13;35;73;83;123;149;248;276;294;322]. Osteoarthritis, rheumatoid arthritis, and fibromyalgia were also more prevalent among women than men [257;261;308].

Table VII. Summary of some chronic pain disorders more prevalent in females than males

Pain disorder	*References*
Chronic tension headache	[235;236]
Migraine	[233;238;321]
Facial pain	[314]
Pain in the TMJ	[138;232;243;270;318]
Muskuloskelatal pain	[13;35;73;83;123;149;248;276;294;322]
Osteoarthritis	[257;261;308]
Rheumatoid arthritis	[257;261;308]
Fibromyalgia	[69;257;261;308]

TMJ, temporomandibular joint

Rodent studies demonstrate sex differences in behavioral responses to persistent and chronic pain

As summarized in Table VIII, after 10% or 2% formalin administration, female rats demonstrated increased licking and flexing behaviors during both behavioral phases as compared with males [5;107]. Female rats also demonstrated greater thermal hyperalgesia as a result of capsaicin injection [20]. Sex differences have been reported in thermal hyperalgesia after administration of complete Freund's adjuvant (CFA); female rats in proestrus displayed significantly increased hyperalgesia as compared with males [43]. Using the TMJ model of pain, Clemente et al (2004) [63] found that females in diestrus exhibited greater pain responses than males. In contrast, however, female paw withdrawal latencies were higher both before and 2 hours after carrageenan injection to the hind paw [279]. Additionally, 14 days after chronic constriction injury, female rats maintained higher latency values than males [280].

Taken together, both clinical and preclinical studies have demonstrated significant sex differences in perception of chronic and persistent inflammatory pain. Several explanations have been proposed to account for these differences [96;98]. A common hypothesis suggests that sex differences in the experience of pain reside in intrinsic hormonal differences [4;67;98;99].

Table VIII: Sex differences in inflammatory and chronic pain assays in rodents

Pain assay		*Female vs. male rats*	*Doses/volume*	*References*
Formalin	↑	Females display increased licking and flexing behavior during both phases	10%/50μl 2%/50μl	[5] [107]
Capsaicin	↑	Females display greater thermal hyperalgesia	0.1-3μg/100 μl	[20]
CFA	↑	Females (only in proestrus) display increased pain compared to males	100%/200 μl	[43]
Carrageenan	↓	Females display decreased pain before and 2 hours after injection compared to males	4%/150 μl	[279]
CCI	↓	Females display decreased pain responses to stimuli after nerve injury as compared with males	N/A	[280]

CFA, complete Freund's adjuvant; CCI, chronic constriction injury
↑ Increased nociceptive behavior
↓ Decreased nociceptive behavior

Menstrual/estrous cycle affects persistent inflammatory and chronic pain

Physiology of the menstrual/estrous cycle

During the menstrual/estrous cycle, serum levels of estradiol and progesterone fluctuate, as shown in Figure 1. In women at the beginning of the cycle, levels of estradiol and progesterone are both relatively low (Figure 1A). During the follicular phase, estradiol levels gradually increase, peaking prior to ovulation, and then moderately decrease during the luteal phase. Progesterone levels rapidly increase after ovulation, peaking during the middle of the luteal phase. Toward the end of the luteal phase, both estradiol and progesterone levels drastically decrease. This cycle ranges from 14 to 35 days in length [46]. Female rodents, by contrast, have an average estrous cycle of 4 days. Estradiol and progesterone levels peak during proestrus (Figure 1B) and then decline during early estrus. During diestrus, a second peak in progesterone levels occurs, but estradiol levels are moderately low in comparison with the proestrus peak [26;140].

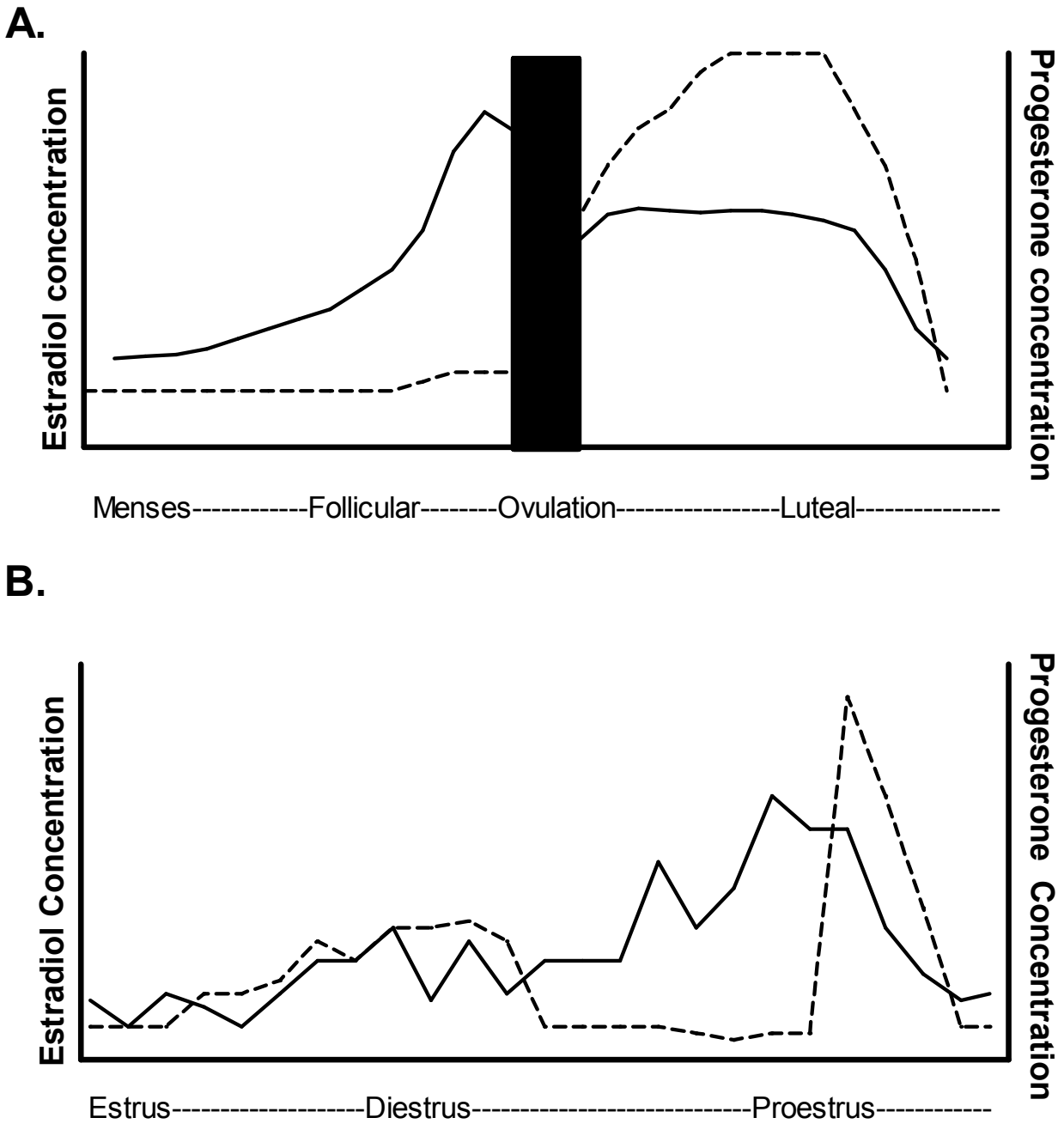


Figure 5
Schematic diagram of hormonal changes that occur during the human menstrual (A) and rat estrous (B) cycle. This figure has been adapted from data obtained by Carter (1993)[52]. Broken lines represent levels of progesterone while solid line represents levels of estradiol

Clinical studies demonstrate that the menstrual cycle affects the subjective experience of pain

As shown in Table IX, reports regarding pain sensitivity across the menstrual cycle have been conflicting, mainly as a result of variations in the different stimulus modalities employed [97;161]. At the end of the luteal phase, women reported higher rates of migraine headache, temporomandibular disorder (TMD), and lower back pain; they also reported a 16% to 42% increase over follicular phase pressure-pain thresholds [125;136;164;271]. Using a meta-analysis of experimentally induced pain, Riley et al (1999) [245] found similarly higher pain thresholds and tolerance in the follicular phase, the one exception being pain associated with electrical stimulation.

Table IX. Menstrual/estrous cycle-related effects on pain sensitivity

Type of study		*Reported effects*	*References*
Clinical			
Migraine headache	↑	Migraine increases at end of luteal phase compared with other phases	[271]
Musculoskeletal pain	↑	Intensity of pain associated with TMD increases at end of luteal	[164]
	↑	Pain during follicular and luteal phase	[136]
Chronic low back pain	↑	Pain increased in luteal and menstrual phase	[125]
Rodent			
1% Formalin	—	No effects across estrous cycle	[313]
CFA	—	No effects across estrous cycle	[242]
	↑	Hyperalgesia in proestrus compared with all other phases of cycle	[43]
Carrageenan	↓	Hyperalgesia in proestrus compared with estrus and diestrus	[279]
Ureteral calculosis	↓	Painful episodes decreased in proestrus/estrus as compared with metestrus/diestrus	[109]

TMD, temporomandibular disorder; CFA, complete Freund's adjuvant
↑ Increased painful incidence
↓ Decrease in painful incidence
— No effects seen across cycle

Rodent studies demonstrate that the estrous cycle can affect the response to persistent inflammatory and chronic pain stimuli

In rodents as in women, the estrous cycle variably affects behavioral responses to inflammatory stimuli (see Table IX). No estrous cycle effects were observed after 1% formalin administration or CFA [72;97] , but another study found higher hyperalgesic responses after CFA injection during proestrus as compared with other stages of the estrous cycle [43;242;313]. Carrageenan-injected hind paw latencies were significantly higher during proestrus than during estrus and diestrus [279]. Similarly, in a study using the artificial ureteral calculosis model, female rodents during proestrus experienced less pain than those in other stages of the estrous cycle [109]. A TMJ model of pain found that that females in proestrus exhibited fewer pain behaviors than those in diestrus [63].

Taken together, sex differences and menstrual/estrous cycle effects strongly suggest that the endocrinological profile of females affects their behavioral responses to pain. These postulates have been tested with two different approaches. First, studies have examined the role of endogenous hormones with side-by-side comparisons of intact versus gonadectomized (GDX) rats and cycling versus postmenopausal women. Second, individual contributions of estradiol and progesterone have been determined via hormone replacements.

Endogenous gonadal hormones affect pain

Clinical studies demonstrate that endogenous hormones affect persistent and chronic pain

Few studies have looked at how endogenous hormones affect pain perception and pain symptomatology in postmenopausal women. The 1994-1995 National Population Health Survey indicated that chronic pain was higher in women than males (20% versus 16%) and increased with age (aged 15 and over), with the most common types being back pain and arthritis/rheumatism [202]. For certain pain syndromes such as joint pain, chronic widespread pain, and fibromyalgia, prevalence rates were higher in cycling than postmenopausal women [164]. On the other hand, a variety of chronic pain conditions of the orofacial area—such as stomatodynia (burning mouth syndrome), atypical facial pain, and atypical odontalgia—preferentially affected perimenopausal and postmenopausal women as compared with premenopausal women [329]. Furthermore, these cranial and orofacial chronic pain conditions were more prevalent in ovariectomized (OVX) women than in a control population, and there was a correlation between stomatodynia or other orofacial pain and the presence of menopausal symptoms [92;116;174]. Symptoms of oral burning sensation in a proportion of these patients were alleviated after hormonal supplements [22;100;317].

Rodent studies demonstrate that endogenous hormones affect persistent and chronic pain

Few studies have aimed to determine the role of endogenous hormones in sex differences related to pain in rats (see Table X). After GDX, sex differences were abolished in paw withdrawal latencies and thermal hyperalgesia after carrageenan

treatment and chronic constriction injury, respectively (castrated males responded the same as OVX females) [279;280]. In female rats, ovariectomy has produced variable results: there have been reports of increases, decreases, or no change in responses to chronic and inflammatory pain after GDX. For example, while ovariectomy did not affect behavioral responses after 2% formalin administration, after 10% formalin administration OVX rats had significantly more licking of the injected paw than did intact females, with no significant change in the rate of flexing and flinching behaviors [55;107]. On the other hand, OVX rats had decreased thermal hyperalgesia after exposure to carrageenan [20].

In comparisons of OVX and intact female rats, there was no overall difference in paw withdrawal latencies after CFA, but hyperalgesia was lower in the OVX rats than in those in proestrus [43;242]. Because of the fluctuations in hormone levels across the estrous cycle, caution should be exercised in interpreting comparisons between OVX and intact female rats.

Table X. Effects of gonadectomy (GDX) on pain behaviors in persistent inflammatory and chronic pain assays

Pain assay		*Observation*	*Dose/volume*	*References*
Female vs. male				
Carrageenan	--	GDX abolished sex differences	4%/150 μl	[279]
CCI	--	GDX abolished sex differences in thermal hyperalgesia	N/A	[280]
CAST vs. intact				
Formalin	↑	Nociceptive responses in both phases in CAST	2%/50 μl	[107]
	↑	Nociceptive responses in both phases in CAST	1.5%/50 μl	[220]
Capsaicin	↑	Thermal hyperalgesia in CAST	0.1-3 μg/100 μl	[20]
OVX vs. intact				
Formalin	—	No effect in either phase	2%/50 μl	[107]
	↑	Increased licking response in both	10%/50 μl	[55]
Carrageenan	↓	Thermal hyperalgesia in OVX	0.1-3 μg/100 μl	[20]

CCI, chronic constriction injury; CAST, castration; OVX, ovariectomy
↑ Increased nociceptive behavior
↓ Decreased nociceptive behavior
— No difference in nociceptive behavior

Gonadal hormone replacement modulates persistent inflammatory and chronic pain behaviors

Clinical studies show that exogenous estradiol and progesterone administration affects persistent inflammatory and chronic pain responses

In postmenopausal women, exogenous estradiol or progesterone administration significantly increased reports of TMD [165]. Women receiving oral contraceptives had a 20% increase in the incidence of TMD and higher referral rates for treatment than did control groups [165]. Reports from open studies have shown an alleviation of symptoms of stomatodynia in a percentage of patients after hormonal supplements [22;100;317]. However, a recent study demonstrated no correlation between chronic widespread pain

and the use of oral contraceptives [175]. Thus, no consensus has been reached on the effects of oral contraceptives on chronic pain.

Rodent studies show that estradiol and progesterone replacement affects persistent and chronic pain responses

In rats, estradiol administration alleviated vaginal hyperalgesia in a model of menopause-associated dyspareunia, lowered autotomy scores after nerve injury and attenuated Phase II nociceptive behaviors in the formalin assay [44;153;296] (see Table XI). Progesterone administration also attenuated inflammatory responses after CFA and carrageenan injections [212;242].

Thus, in summary, results obtained after removal of endogenous gonadal hormones (either because of menopause in women or ovariectomy in female rats) and the administration of exogenous gonadal hormones, strongly support the hypothesis that gonadal hormones provide the biological basis for sex differences and estrous cycle effects in persistent inflammatory and chronic pain.

Table XI. Hormone replacement effects on pain behaviors in chronic pain assays

Pain assay		*Hormone replacement vs. GDX*	*Dose*	*References*
10% Formalin	↓	Testosterone to intact males decreases phase II response	5mg/kg	[8]
5% Formalin	↓	Estradiol to GDX females decreases phase II response	20% SILASTIC s.c. implants	[153]
CFA	↓	Inflammatory hyperalgesia in progesterone-treated groups	Six 3-cm SILASTIC s.c. implants	[242]
Carrageenan	↓	Inflammation decreased in progesterone-treated rats	1 mg/kg	[212]
Sciatic nerve injury	↓	Autotomy scores decreased in estradiol-treated rats	5 μg/day/rat	[296]
	↓	Testosterone replacement in GDX male rats decreases autotomy scores	500 μg/day/rat	[169]

GDX, gonadectomy; CFA, complete Freund's adjuvant; s.c., subcutaneous
↓ Hormone replacement decreased nociceptive responses

Gonadal hormone effects on acute vs. persistent inflammatory and chronic pain models

The studies reviewed in this paper show that gonadal hormones play a key role in modulating behavioral responses to pain. However, the current data suggest that the interactions between estradiol and progesterone are complex and do not affect the behavioral outcome to chronic and tonic pain in a straightforward manner. The reviews of acute pain responses also cite a plethora of results. For example, similar to the studies reviewed here, in acute pain studies, the estrous cycle has shown conflicting results with respect to influencing pain sensitivity [43;63;101;109;115;144;239;242;279;313]

Although chronic pain assays yield conflicting results after ovariectomy, acute pain assays show a general increase in nociception after ovariectomy [6;20;43;44;56;101;107;242;275]. Estradiol replacement yields variable results with regard to acute pain assays; however, progesterone replacement generally increases thresholds [44;79;115;275]. The few studies that have looked at the effects of estradiol and progesterone replacement and chronic pain have found a decrease in the pain response in female rats [44;212;242;296].

Many of the conflicting results may be due to the variability in assays employed, i.e., variability in temporal parameters of stimulation (brief vs. prolonged, acute vs. tonic), quality of the pain sensation (sharp vs. dull, pricking vs. aching, cutaneous vs. deep), and location of application (tail, hind paw, temporomandibular joint). Furthermore, the pain scores used vary as well (thresholds, numeric ratings, time spent in pain). All of these differences make comparisons across studies difficult.

Potential mechanisms underlying gonadal hormonal effects on persistent inflammatory and chronic pain

Corticosterone, a steroid hormone secreted by the adrenal gland, is an essential component of stress adaptation [77;90;210]. Basally and after different stressors (e.g., physical stress [204] and drug abuse [61;156]) female rats have higher corticosterone levels than male rats [3]. Furthermore, in male rats, formalin administration significantly increases serum levels of corticosterone [282].

Cyclooxygenase (COX) is the rate-limiting enzyme that catalyzes the conversion of arachidonic acid to prostaglandins [277]. Both isoforms of COX, COX-1 and COX-2,

are present in the peripheral and central nervous system [277]. Indomethacin (a non-selective COX-1 and COX-2 inhibitor), diclofenac and FR122047 (selective COX-1 inhibitors), and celecoxib (a selective COX-2 inhibitor) attenuate Phase II flinching responses after formalin administration [216;293;307;341]. This finding strongly suggests that both isoforms of COX mediate responses to inflammatory stimuli including formalin administration [91;129;195;303].

Dexamethasone, an anti-inflammatory glucocorticoid, attenuates complete Fruend's adjuvant-induced oedema formation [124;350], an attenuation correlated with the prevention of the induction of COX-2 mRNA by complete Fruend's adjuvant [124]. Thus, it has been postulated that glucocorticoids target COX-2 activation, which is necessary for the mediation of anti-inflammatory effects of glucocoritcoids [255].

Estradiol up-regulates COX-2 levels in endothelial cells of human umbilical vein, pig uterine stroma and epithelium, and rat endometrium and myometrium [2;42;59;262;337], whereas it down-regulates COX-2 levels in the bovine chondrocytes, epithelial cells, and renal cortical cells [209;277;338]. On the basis of these findings, we postulate that sex differences in inflammatory/chronic pain may in part be mediated through COX activation (via mRNA expression, protein level increase, and/or enzymatic activation) either by (1) endogenous sex differences in COX levels, (2) corticosterone regulation of COX levels, and/or (3) gonadal hormone regulation at either corticosterone or COX levels.

Clinical implications of the current literature

Although a clearer picture is emerging, one suggesting that dynamic endocrinological changes during the female cycle affect chronic pain responses, further research is required regarding how hormone concentrations affect these responses. Both estradiol and progesterone are simultaneously present during the reproductive cycle, but the extent to which the presence of both hormones modulates nociceptive behavioral responses in humans and rats remains to be further examined. Studies are therefore needed to delineate how these dynamic hormonal interactions affect chronic and/or persistent inflammatory pain behaviors. Furthermore, in the ovariecomty model, it is yet to be determined how hormonal administration paradigms and the length of time after ovariecomty affect chronic pain responses.

According to both the clinical and preclinical literature, pain management targeted at female patients should consider hormonal factors during the female reproductive cycle. For example, because hormonal changes that accompany the female menstrual cycle can be bi-directional (producing both pro-nociceptive and anti-nociceptive effects), consideration should be given to the patient's stage of the menstrual cycle and/or her age (pre-, peri-, or post-menopausal) when managing her pain. Studies of young adults showed that women who were using exogenous hormones in the form of oral contraception were less pain-sensitive than women not using hormones and generally did not differ from males in pain sensitivity [114;285;289]. Premenopausal women are under the influence of menstrual cyclicity, but oral contraception alters this cyclicity by preventing the mid-cycle luteinizing hormone (LH) surge, which has been found to lead to luteal phase pain sensitivity [29;30]. It is hard to ascribe these effects to one particular

hormone because oral contraception is usually a combination of estradiol and progesterone, the two of which can interact to modulate each other's effects [165].

Hormone replacement therapy (HRT), on the other hand, does not interfere with the menstrual cycle. In general, women receiving HRT have increased incidence of pain as compared with postmenopausal women not using HRT [47;165;328]. Fillingim and Ness (2000) propose that HRT alters nociceptive processing at multiple levels, including at peripheral, spinal, and more rostral central nervous system sites. These observations suggest the advisability of systematic collection of data regarding the individual hormonal changes and effects on chronic/inflammatory pain.

Another issue thus far receiving minimal attention is the possible therapeutic potential or effect of exogenously administered hormones in the treatment of pain. For example, the use of different combinations of estradiol and progesterone or hormone antagonists, such as tamoxifen, may potentiate or reduce the effectiveness of various analgesic treatments or the perception and reporting of chronic pain. Tamoxifen has been shown to be effective in the treatment of cyclical mastalgia in women [151;200]. With this evidence, it seems possible that these anti-estradiol compounds may be used as therapy in treating chronic pain conditions that are influenced by circulating hormones. These important clinical issues remain to be addressed.

Chapter 3: Gonadal hormone influences to intracellular mechanisms underlying sex differences in response to formalin injection

1. Introduction

Female rats display increased behavioral responses to persistent and inflammatory pain stimuli than male rats. For example, females show greater nociceptive responses following capcaisin, complete Freund's adjuvant and temporomandibular joint (TMJ) pain than male rats [3;8;20;30;43;47;79]. Similarly, after formalin administration, female rats also have increased nociceptive response than males [5;107]. Sex differences in inflammatory pain have been attributed to gonadal hormone effects; most evident during fluctuations in pain sensitivity across the female estrous cycle and after gonadectomy (reviewed in [4;67;98;99]. For example, hyperalgesia during proestrus is increased as compared with other phases of the cycle [43]. Conversely, carrageenan and ureteral calculosis models display a decrease in painful behavior during proestrus compared with other phases [109;279].

Although the role of endogenous hormones in acute pain responses has been extensively studied and reviewed in the literature, the few studies that have examined the role of endogenous hormones during inflammatory and chronic pain are not always consistent. For example, GDX abolished sex differences in paw withdrawal latencies and thermal hyperalgesia after carrageenan treatment and chronic constriction injury, respectively; castrated (CAST) males responded the same as ovariectomized (OVX) females [279;280]. However, the direction of gonadectomy effects is affected, in part, by the intensity of painful stimuli and/or pain model used; while OVX had no effect on

behavioral responses to 2% formalin, it significantly increased licking of the injected paw after 10% formalin [55;107]. On the other hand, gonadectomy decreased thermal hyperalgesia after exposure to carrageenan in female rats [20]. However, castration has produced consistent results; in both formalin and capsaicin models of inflammatory pain, castration increased nociceptive responses as compared to intact males [20;107;220].

Prostaglandins (PGs), especially prostaglandin E_2 (PGE2), are important mediators of inflammatory responses [302;305] where following a nociceptive stimuli, they are released at the site of injury [93;94;121;182;256;304;310]. This local release of PGE2 in the spinal cord has been implicated in the spinal sensitization process which occurs during Phase II of the formalin response [180;181]. Cyclooxygenase (COX) is the rate-limiting enzyme that catalyzes the conversion of arachidonic acid to prostaglandins during inflammation responses [277]. Both isoforms of COX (COX – 1 and COX – 2) mediate responses to inflammatory stimuli including formalin administration [91;129;195;303]]. For example, indomethacin (a non-selective COX-1 and COX-2 inhibitor), diclofenac and FR122047 (selective COX-1 inhibitors), or celecoxib (a selective COX-2 inhibitor) attenuate Phase II flinching responses after formalin administration [216;293;307;341]. Furthermore, anti-inflammatory glucocorticoids, attenuate complete fruend's adjuvant-induced oedema formation is in part mediated through COX-2 activation [124]. Thus, it has been postulated that anti-inflammatory responses after inflammatory stimuli involve the activation of COX enzymes by corticosterone, which in turn, release PGE2 [255].

This study has two aims, first to determine the role of sex and endogenous gonadal hormones on the nociceptive responses to 5% formalin administration. Secondly, to test

the hypothesis that sex and/or endogenous hormonal effects on inflammatory pain are in part mediated through endogenous differences in COX activation, COX protein levels, and/or release of prostaglandins.

2. Methods

2.1 Animals

8-week old intact and GDX male and female Sprague–Dawley rats were purchased from Taconic (Germantown, NY). Rats were double-housed with a 12-h light/12-h dark cycle (lights on 8 A.M. EST). Food and water were available *ad libitum*. Animals were randomly assigned to experimental groups (n=8-10/group) and run in two separate cohorts. In order to determine baseline corticosterone levels, separate cohorts of animals were sacrificed without receiving formalin injections. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, Bethesda, MD, USA) and approved by the Institutional Animal Care and Use Committee at Hunter College of The City University of New York.

2.2 Nociceptive testing

An automated nociceptive analyzer was used to determine overall behavioral activity after formalin administration [340]. All parameters of the computer program were set to default values (for details see [340]). Prior to formalin injection, a soft metal band was placed on the right hind-paw with the opening positioned at the plantar surface of the paw as previously described [153]. The formalin assay was always carried out between 9:00 am and 3:00 pm. Rats were placed inside the testing chamber for a total of

30 min prior to the formalin injection in order to minimize novelty of the testing environment and the band. Five percent formalin was injected intra-plantar on the banded right hind-paw in a volume of 50 µl. Rats were then replaced into the testing chamber and data collection commenced for a total of 60 minutes in one-minute intervals.

2.3 Radioimmunoassay and enzyme immunoassay

Sixty minutes after formalin injection, rats were sacrificed by decapitation, following a brief exposure to CO_2 (20 s), and trunk blood was collected in tubes containing K_2 EDTA. Blood was centrifuged at 3,000 RPM for 30 minutes at 4ºC. Serum was collected and stored at -80ºC until analyzed using Coat-A-Count radioimmunoassay kits for corticosterone (Diagnostic Products Corporation, Los Angeles, CA). Intra-assay coefficients of variation averaged 10.0 ± 1.0%. Serum was also analyzed for prostaglandin E_2 concentration using an enzyme immunoassay kit from Cayman Chemical (Ann Arbor, MI). Results for these assays were determined using a log-logit analysis within GraphPad Prism Software (CA.). Corticosterone serum levels were expressed as ng/mL and prostaglandin E_2 as pg/mL.

2.4 Western Blots

After decapitation, the lumbo-sacral region of the spinal cords were rapidly dissected and stored at -80ºC until use. The levels of COX 1and 2 were analyzed using Western blot techniques as previously described [153]. Briefly, 30 µg of protein samples were resolved in gradient SDS-PAGE gels (4-15%) and transferred to nitrocellulose membranes. Membranes were then blocked with 5% nonfat dry milk for 30 minutes and

incubated with COX-1 or COX-2 antibodies (1:1000; purchased from Cayman Chemical, Ann Arbor, MI) for 1 hour at room temperature or overnight at 4°C, respectively. After washing in TBST, membranes were incubated with appropriate secondary antibodies for 1 hour at room temperature. Band intensities were detected with an enhanced chemiluminescence kit from Amersham (Piscataway, NJ) and quantified with a Molecular Dynamic Computer Densitometer and Image Quant Program. α-tubulin antibody (1:1000) was used to normalize protein concentrations.

2.5 Data analysis

Behavioral data was analyzed as the mean number of flinches (± SEM) during Phase I (0-9 min) and Phase II (10-60 min) of the behavioral responses to formalin. Two-way ANOVAs were used to test for significant differences in the sum of the flinching response across SEX (male vs. female) and GROUP (intact vs. GDX) for Phase I and Phase II. Fisher's Least Significant Difference *post-hoc* testing was done when appropriate. To determine significant differences in serum levels of corticosterone or prostaglandin E2 for each treatment (formalin vs. no formalin), two-way ANOVAs were done [SEX (male vs. female) x GROUP (intact vs. GDX)]. To determine if significant differences were observed in COX-1 or COX-2 protein levels after each group, independent sample t-tests were used between groups. For all comparisons, significance was at the 0.05 level.

3. Results

Males display significantly increased nociceptive behavior in both Phase I and II of the behavioral responses as compared to females ([$F(1, 36) = 10.036$; $p < 0.01$] and [$F(1, 35) = 8.72$; $p < 0.01$], respectively; Figure 6). Furthermore, in both males and females, gonadectomy increased nociceptive behavior during Phase I as compared to respective intact groups ($F(1, 36) = 5.18$; $p = 0.02$; Figure 6).

In both naïve and formalin-treated groups, females had significantly higher serum levels of coricosterone than do males (Naive: $F(1, 31) = 13.40$; $p < 0.01$; Figure 7A; Formalin: $F(1, 35) = 42.66$; $p < 0.01$; Figure 7B). Furthermore, an interaction of gonadectomy and sex was also obtained in the formalin-treated group ($F(1, 35) = 15.47$; $p< 0.01$; Figure 7B); while OVX females have lower corticosterone levels than intact females ($p < 0.05$), CAST males have increased levels of corticosterone levels than do intact males ($p < 0.05$).

COX-1 and COX-2 protein levels in the spinal cord did not significantly differ either basally or after formalin administration in any of the experimental groups (Figure 8). Furthermore, in both naive and formalin-treated groups, gonadectomized male and female rats had significantly lower prostaglandin E2 levels when compared to their respective intact groups ($F(1, 22) = 8.23$; $p < 0.01$; Figure 9).

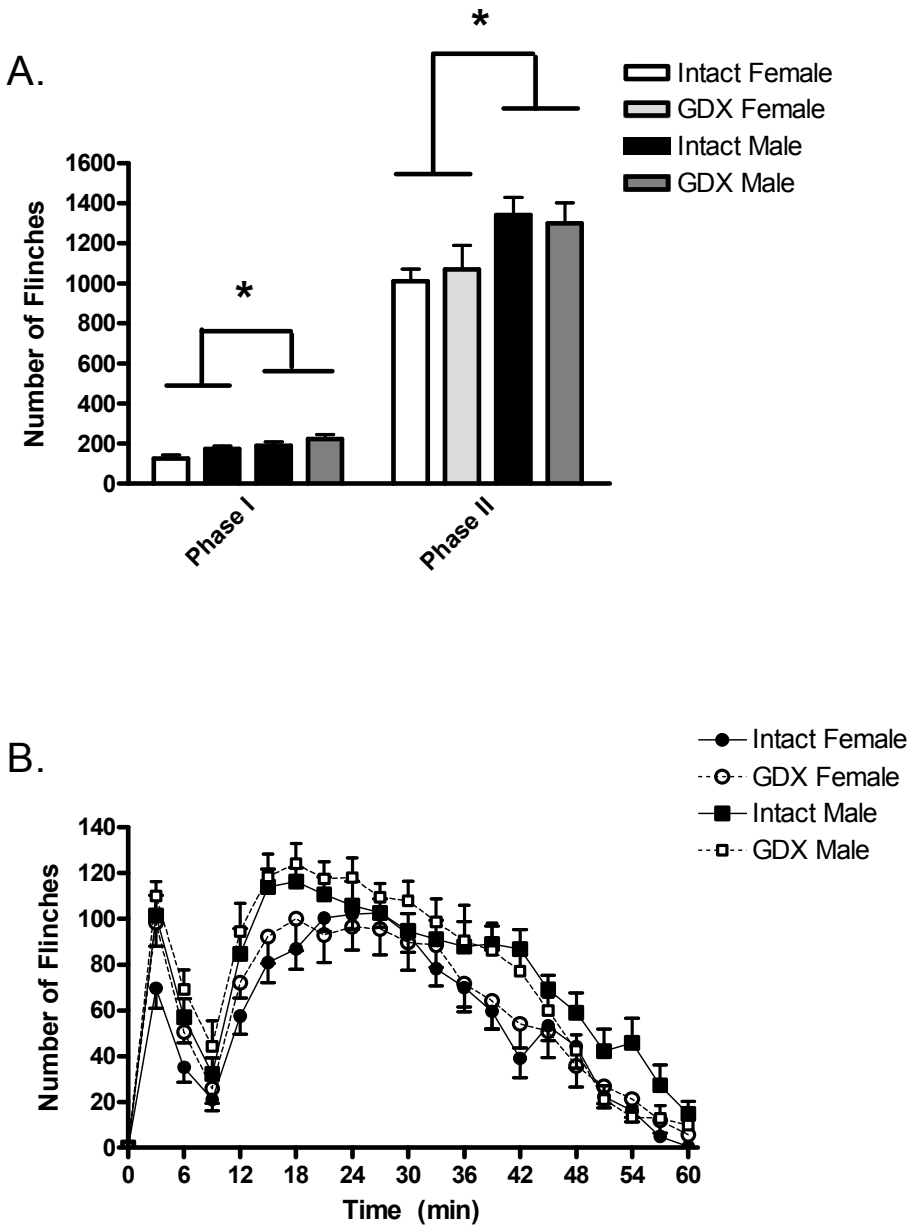


Figure 6
Sex and gonadectomy effects on the behavioral responses to formalin (5%) administration. (A) Mean flinches (± SEM) in Phase I (0-9 min) and II (10-60 min) in intact and gonadectomized (GDX) male and female rats (N=8/dose). (B) The time course of flinching responses after 5% formalin. Mean flinches (± SEM) are plotted in 3-min time bins. * Denotes a significant difference between males and females ($p<0.05$).

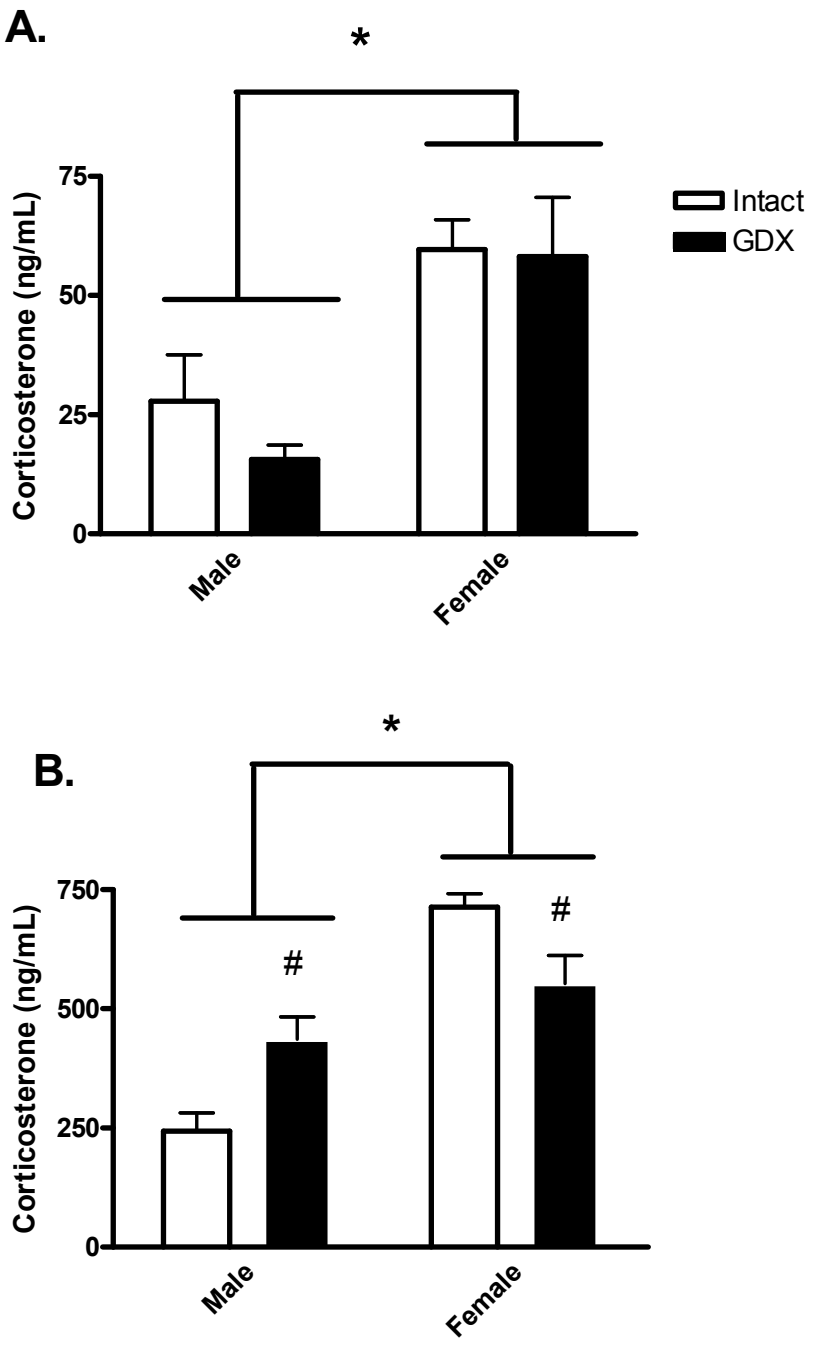


Figure 7
Sex and gonadectomy effects on serum corticosterone levels in (A) naïve and (B) 5% formalin treated rats. * Denotes a significant difference between males and females ($p<0.05$). # Denotes a significant differenct between intact and GDX animals ($p<0.05$).

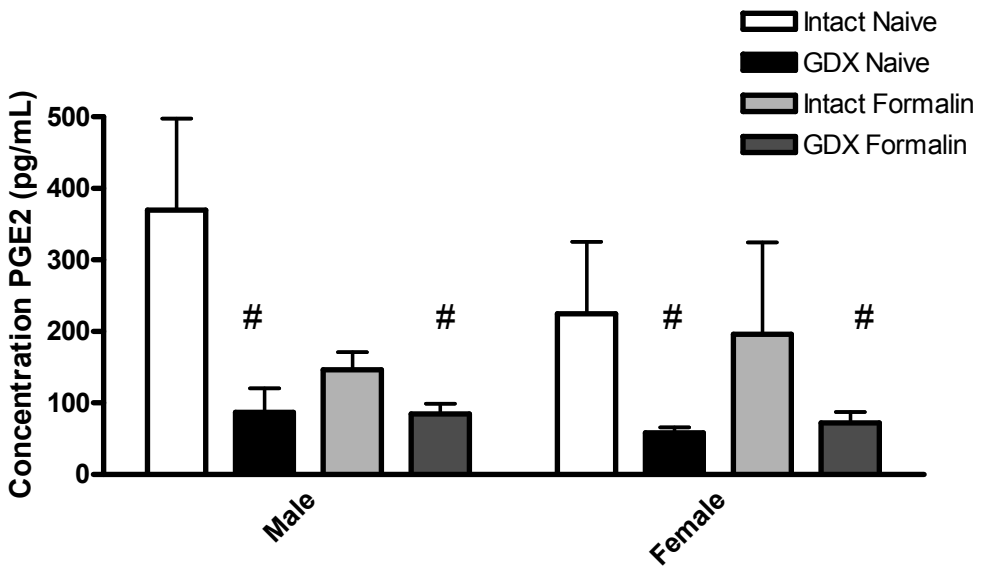


Figure 8
Sex and gonadectomy effects on prostaglandin E2 levels in naïve and 5% formalin treated rats.
# Denotes significant effect of GDX.

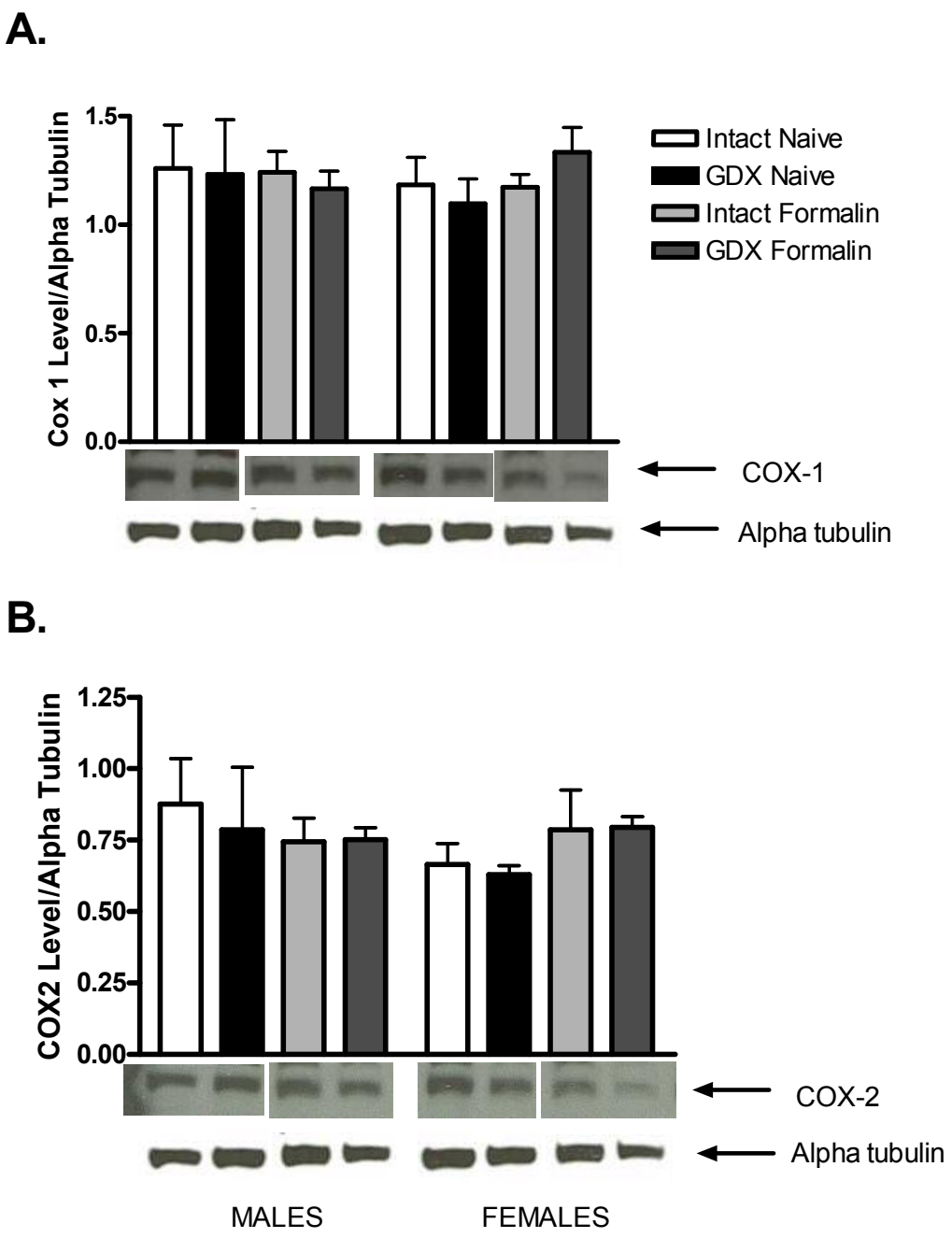


Figure 9
COX-1 (A) and -2 (B) expression in the spinal cord of naïve and formalin-treated male and female rats.

Discussion

Similar to the carrageenan and chronic constriction models, subsequent to 5% formalin administration, female rats display significantly lower nociceptive behavioral responses during Phase I and Phase II than males. However, Aloisi et al. (1994) [5] and Gaumond et al., (2002)[107] demonstrated that after 10% or 2% formalin, females displayed an increase in nociceptive responses when compared to males. These opposite results may in part be accounted for by several factors. First, scoring methods differed between experiments; both Aloisi et al. (1994) [5] and Gaumond et al. (2002) [107] scored each of the different components of the formalin nociceptive responses (i.e. licking, flexing and flinching) whereas we quantified the overall behavioral responses to formalin. Moreover, there are vendor and rat strain differences between all three studies, which have been shown to have an effect on nociceptive responses [51;126;205;206;340] . Aloisi et al. (1994) [5] used Wistar rats, while Gaumond et al. (2002) [107]used Charles River's Sprague-Dawley rats. Sprague-Dawleys from different vendors have different magnitude of blood pressure response after formalin administration [281;340]. Furthermore, rats of the same strain but from different vendors have different behavioral responses to stressful events [223;225].

Consistent with Cecarrelli et al (2003) [55], who demonstrated that after formalin administration an increased licking response in ovariectomized as compared to intact females, we showed that ovariectomy increased nociceptive response during Phase I of the response. However, ovariectomy decreased nociceptive responses after carrageenan injection [20]. Although it has been previously reported that castration increased nociceptive responses to capsaicin and formalin (1.5 or 2%) [20;107;220], herein,

castration had no effect on formalin-induced responses. Thus, suggesting that although endogenous hormones regulate the behavioral responses after inflammatory stimuli, the role of endogenous hormones on inflammatory pain responses may be confounded by the intensity, duration of the nociceptive stimulus, and/or sex.

Similar to previous reports by Taylor et al (1998) [282], formalin administration activates the release of corticosterone in male rats. Moreover, consistent with Aloisi et al. (1998) [9], females had higher corticosterone levels basally and subsequent to formalin administration than male rats. Furthermore, gonadectomy increased corticosterone levels in males, and decreased corticosterone levels in females; suggesting that estradiol, progesterone and testosterone differentially modulate corticosterone release in response to inflammatory nociceptive stimuli. In naive male and female rats, GDX altered corticosterone levels; with reports of GDX either increasing or decreasing corticosterone release [7;55;56;61;95;316]. Furthermore, while gonadectomy enhances stress-induced corticosterone secretion in male rats, it reduces such levels in female rats [258;317]. This has been attributed to a direct regulation of gonadal hormones on the HPA axis [49;118;172;234;311]. Exogenous administration of testosterone and estradiol male and female GDX rats, respectively, returned corticosterone levels to those of intact rats. Moreover, a hyperactive HPA activation has been reported in female rats when compared to males [49;118;172]. Thus, suggesting intrinsic differences in corticosterone release may contribute to the sexually dimorphic responses to inflammatory nociceptive stimuli.

Corticosterone has been shown to exert anti-inflammatory actions [19], and there is evidence that inflammation contributes to the nociceptive response in the formalin test

[291;344]. The inflammation associated with the injection of complete Freund's adjuvant was significantly attenuated with the administration of the glucocorticoid dexamethasone [124]. More recently, Zhang et al (2004) [350] demonstrated that endogenous glucocorticoids exerts a powerful suppressive effect on complete Freund's adjuvant induced inflammatory hyperalgesia. Indeed, we found that OVX females displayed a higher nociceptive response compared to intacts, during Phase I, and had significantly lower corticosterone levels than intacts. This data further suggest that corticosterone may play a significant role in mediating inflammatory nociceptive responses.

Although both isoforms of COX were present in the lumbo sacral region of the spinal cord, the pattern of expression was not sexually dimorphic. Furthermore, the observed sex differences and gonadectomy effects in formalin-induced behavior were not due to either basal or formalin-induced COX-1 or COX-2 protein levels. Indeed, in male rats, COX-2 protein synthesis remains unaltered up to 4 h after formalin administration [178;286;287]. Moreover, since no differences in COX-1 or COX-2 levels were observed after GDX, endogenous gonadal hormone regulation of COX protein levels may play a limited role.

Because we observed changes in prostaglandin E2 release after gonadectomy, we postulate that activation of COX-1 and COX-2 mediates gonadectomy effects on formalin induced behavioral responses. Indeed, activation of COX1/COX2 has been demonstrated to be an important step in the cascade of intracellular responses to formalin. For example, Tegeder et al. (2001) [287]demonstrated that after 5% formalin administration, PGE2 release coincided with nociceptive behavioral responses, returning to baseline levels 1-2 hours post formalin injection. Moreover, formalin-induced PGE2

increases and nociceptive behavioral responses were reduced after a COX 1 inhibitor administration, SC560, whereas the administration of a COX 2 inhibitor, celecoxib, had no effect on either PGE2 release or nociceptive behaviors [287]. Previous studies have shown, however, the effectiveness of COX-2 inhibitors in both formalin and other nociceptive models [216;267;287;293;307]. It is important to note that in this study, levels of PGE2 were analyzed 60 minutes after formalin administration rather than during the Phase I or Phase II responses, when behavioral sex differences were observed. Thus, a more detailed time course determination is needed to further elucidate if sex differences in PGE2 release occur at those time periods during the response.

Endogenous hormones have been shown to directly alter the synthesis of prostaglandins in non-central nervous system tissue; i.e., while progesterone increased, estradiol decreased PGF2 synthesis [122;148;183]. Taken together, we postulate that sex differences to inflammatory pain are in part mediated through gonadal hormone influences on COX activation, rather than fluctuations in protein expression levels. Moreover, we postulate that sex differences and endogenous hormone influence on corticosterone levels may further regulate COX activation.

Non steroidal anti-inflammatory drugs (NSAID's) are commonly used to treat pain by reducing prostaglandin synthesis via inhibition of cyclooxygenase [179]. Our study suggests that the efficacy of this treatment may be differentially affected by the reproductive stage of the female. Furthermore, because gonadal hormones influence corticosterone release after different stressors, NSAID efficacy may either be more or less effective to treat pain responses in women. This important clinical issue needs further study. A more systematic series of experiments are required in order to determine

the role that endogenous hormones play in regulating COX activity and the production of prostaglandins.

Chapter 4: Estradiol and progesterone differentially regulate formalin-induced nociception in ovariectomized female rats

Introduction

Numerous studies have reported that certain pain conditions such as migraine, temporomandibular disorders, neuropathic pain, some forms of arthritis, and fibromyalgia are significantly more prevalent in females than males [13;16;31;70;99;123;127;130;214;229;240;244;273;284;297]. Furthermore, studies have shown changes in sensitivity to pain throughout the different female reproductive stages, with a peak of prevalence in pain conditions occurring during the female's reproductive years [78]. Furthermore, across the menstrual cycle, sensitivity to pain is greater during the luteal than the follicular phase [48;99;110;119]. Similar to humans, rats demonstrate differences in nociception across the estrous cycle; nociceptive sensitivity peaks during late proestrus and is reduced during metestrus and diestrus [71;86;104;105;110;144;162;253].

Almost all of the animal studies have used acute nociception models, and only a few have addressed the role of gonadal hormones on behavioral responses to persistent and inflammatory pain. The formalin test, a test that measures nociceptive responses to a continuous noxious stimulus, produces a bi-phasic flinching response. Phase I represents behavioral responses corresponding to acute pain while Phase II represents behavioral activity corresponding to persistent and inflammatory pain. Using the formalin test, Gaumond et al. (2002) [107] and Aloisi et al. (1994) [5] demonstrated that female rats showed more flinching in both phases than did males. However, to date no studies have addressed the possible mechanisms by which estradiol and progesterone modulate the

acute and persistent phase of the formalin model. The goal of this study was to address the role of estradiol and progesterone in modulating the nociceptive response to formalin And further to determine if estradiol and progesterone receptors play important roles in modulating the behavioral response to formalin.

2. Materials and Methods

2.1 Animals

Eight-week-old ovariectomized (OVX) female Sprague-Dawley rats purchased from Taconic (Germantown, NY) were double-housed with a 12-hour light-dark cycle (lights on 8 AM). Food and water were available *ad libitum*. Animals were randomly assigned to experimental groups (n = 16 to 20 per group). Each study consisted of at least 4 different cohorts of rats. 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.

2.2 Reagents

β-estradiol 3-benzoate and progesterone were purchased from Sigma Aldrich (St. Louis, MO). Antibodies for estradiol receptor α (ERα), β (ERβ), and progesterone receptor (PR) were purchased from Santa Cruz Technologies (Santa Cruz, CA).

2.3 Estradiol and progesterone replacement paradigms

Two weeks after OVX, rats received subcutaneous injections of estradiol

10, 15, 20, 30 or 50 μg; 48 hours prior to testing) or progesterone (10, 50, 100 or 500 μg; 4 hours prior to testing). Two separate control groups (vehicle at 4hr or 48 hr prior to testing) were run to parallel the hormone administration paradigm. These doses fall within the range of serum levels that normally occur during the estrous cycle [102;132;227]. To determine if estradiol's effects were estradiol receptor (ER) mediated, OVX rats received estradiol (20 μg), tamoxifen (a selective estradiol receptor mediator, 15 mg/kg/mL), estradiol + tamoxifen, α-estradiol (an inactive isomer of estradiol, 20 μg), or vehicle (sesame oil) via subcutaneous injections 48 hours before testing. These doses of tamoxifen and α-estradiol have previously been shown to decrease lordosis behaviors in female rats by interfering with the binding of estradiol to its nuclear receptor [14].

2.4 Nociceptive testing

An automated nociceptive analyzer was used to determine overall behavioral activity after formalin administration [340]. All parameters of the computer program were set to default values (for details see [340]. Prior to formalin injection, a soft metal band was placed on the right hind-paw with the opening positioned at the plantar surface of the paw. The formalin assay was always carried out between 9:00 AM and 3:00 PM. Rats were placed inside the testing chamber for a total of 30 minutes prior to the formalin injection in order to minimize the novelty of the testing environment and the band. Formalin (5%, 2.5%, or 1%) was injected intra-plantar on the banded right hind-paw in a volume of 50 μL. Rats were then replaced into the testing chamber and data collection commenced for a total of 60 minutes at 1-minute intervals. To determine the effects of

hormonal replacement on baseline activity, flinching behavior was recorded for 1 hour in animals receiving either vehicle, estradiol (20 μg), or progesterone (50 μg and 500 μg).

2.5 Radioimmunoassay for plasma steroids

Rats were sacrificed by decapitation 60 minutes after formalin injection following abrief exposure to CO_2 (20 seconds). Trunk blood was collected and centrifuged at 3,000 rpm for 30 minutes at 4ºC. Serum was stored at -80ºC until analyzed using Coat-A-Count radioimmunoassay kits for estradiol or progesterone (Diagnostic Products Corporation, Los Angeles, CA). Intra-assay coefficients of variation averaged 10.0% ± 1.0%. Results for these assays were determined using a log-logit analysis within GraphPad Prism Software (San Diego, CA.). Estradiol and progesterone serum levels are expressed as pg/mL and ng/mL, respectively.

2.6 Western blots for steroid receptors

After decapitation, the lumbo-sacral region of the spinal cord was rapidly dissected and frozen in -80ºC until use. The levels of progesterone A and B (PR-A and PR-B) receptors and ER-α and ER-β receptors were analyzed using Western blot techniques as previously described [137]. Briefly, 20 μg of protein samples were boiled in Laemmli sample buffer containing 1% β-mercaptoethanol, resolved in a 4%-15% gradient SDS-PAGE, and transferred to nitrocellulose membrane. To determine the specificity of the bands, prefrontal cortex and nucleus accumbens of naïve female rats were run in parallel with the spinal cord extracts. Our lab has previously shown the presence of ER-α, ER-β, PR-A and PR-B in these areas of the brain (H.B.K. Wu et al.,

unpublished observations). Membranes were then blocked with 5% nonfat dry milk for 45 minutes followed by an incubation of ER-α or ER-β (1:500) or PR-A or PR-B (1:200) for 1 hour at room temperature. After washing in TBST, the membranes were incubated with the appropriate secondary antibodies for 1 hour at room temperature. Band intensities were detected with enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ) and quantified with a Molecular Dynamic Computer Densitometer and Image Quant Program. α-tubulin antibody (1:1000) was used to normalize protein concentrations.

2.7 Data analysis

Data were analyzed as the mean of flinches (± SEM) during Phase I (0-6 minutes) and Phase II (9 - 36 minutes) of the behavioral responses to formalin. Because after 36 minutes the rats' activity returned to baseline levels, Phase II is represented as 9 to 36 minutes. One-way ANOVAs were used to test for significant differences in the sum of the flinching responses across doses for Phase I and Phase II. Fisher's Least Significant Difference *post-hoc* testing was done when appropriate. To determine significant differences in serum levels of estradiol and progesterone across doses, one-way ANOVAs were done. To determine if significant differences were observed in receptor levels after each hormonal treatment, independent sample t-tests were used between control and each treatment group. Significance for all comparisons was at the 0.05 level.

3. Results

As summarized in Table XII, estradiol serum levels progressively increased with higher doses of estradiol administration [$F(4, 21) = 4.44$; $p < 0.05$]; rats treated with 20 and 30 μg of estradiol had significantly higher estradiol levels than vehicle-treated rats. Progesterone serum levels also increased dose dependently [$F(4, 37) = 14.87$; $p < 0.05$]; rats receiving 500 μg treatments had significantly higher levels of progesterone than those receiving vehicle.

As shown in Table XIII, neither 20 μg of estradiol nor 50 μg or 500 μg of progesterone had an effect on baseline flinching activity when compared to vehicle-treated rats. After 5% or 2.5% formalin administration [Figure 10A and 10B, respectively], none of the estradiol doses significantly affected total flinching activity. However, after 1% formalin administration [Figure 11], estradiol reduced flinching responses in Phase II in a dose-dependent manner [$F = (4, 82)$; $p < 0.05$]; 20 and 30 μg of estradiol significantly decreased the number of flinches ($p < 0.05$). Furthermore, both 20 μg and 30 μg of estradiol attenuated formalin-induced flinching responses across minutes 18 to 36 of Phase II. Only 500 μg of progesterone significantly attenuated formalin-induced (1%) flinching during Phase I [$F(4, 73) = 2.94$; $p < 0.05$; Figure 12]. Co-administration of 20 μg of estradiol and 50 μg of progesterone reversed estradiol-induced decreases in the number of flinching responses during Phase II ($p > 0.05$, Figure 13). However, this effect was not observed after co-administration of 20 μg of estradiol and 500 μg of progesterone [$F(5, 116) = 3.84$; $p < 0.05$; Figure 13]. Moreover, co-administration of 20 μg of estradiol and 500 μg of progesterone did not reverse

progesterone-mediated decreases in flinching responses during Phase I [$F(5, 123) = 5.55$; $p < 0.05$; Figure 13].

Estradiol alone, tamoxifen alone, and their co-administration significantly decreased flinching responses during Phase II [$F(3, 72) = 4.67$; $p < 0.05$; Figure 14]. On the other hand, α-estradiol administration did not significantly attenuate flinching responses during either Phase I or II ($p > 0.05$; Figure 14).

We did not detect changes in PR-B or ERα and β protein levels after any of the hormone replacement paradigms [Figure 15]. PR-A, although expressed in the prefrontal cortex and caudate putamen, was not detected in the spinal cord (data not shown).

Table XII: Estradiol and progesterone serum levels after hormone administration in OVX rats

Estradiol		**Progesterone**	
Dose (μg)	**Serum level (pg/mL)**	**Dose (μg)**	**Serum level (ng/mL)**
0	1.83 ± 0.25	0	8.54 ± 1.40
10	20.91 ± 1.83	10	10.34 ± 1.25
15	15.44 ± 3.22	50	9.99 ± 1.57
20	25.30 ± 8.39*	100	11.39 ± 0.81
30	35.04 ± 6.99*	500	19.48 ± 0.79*

* Indicates a significant difference as compared with controls ($p < 0.05$; n=10-16/group).

Table XIII: Baseline flinching activity of 8-week-old OVX Sprague-Dawley rats, by time and hormonal treatment

	Control	**Estradiol 20 μg**	**Progesterone 50 μg**	**Progesterone 500 μg**
Phase I$^{\Delta}$	64.58 ± 14.12	47.88 ± 8.71	79.83 ± 24.99	96.75 ± 57.45
Phase II$^{\Delta}$	231.17 ± 56.18	313.86 ± 39.34	310.83 ± 116.19	225.75 ± 38.06

$^{\Delta}$ Baseline activity was divided into Phase I (0-6 min) and Phase II (9-36 min) in rats that did not receive formalin injections. (N=8/group). Numbers represent the total number of flinches during that phase.

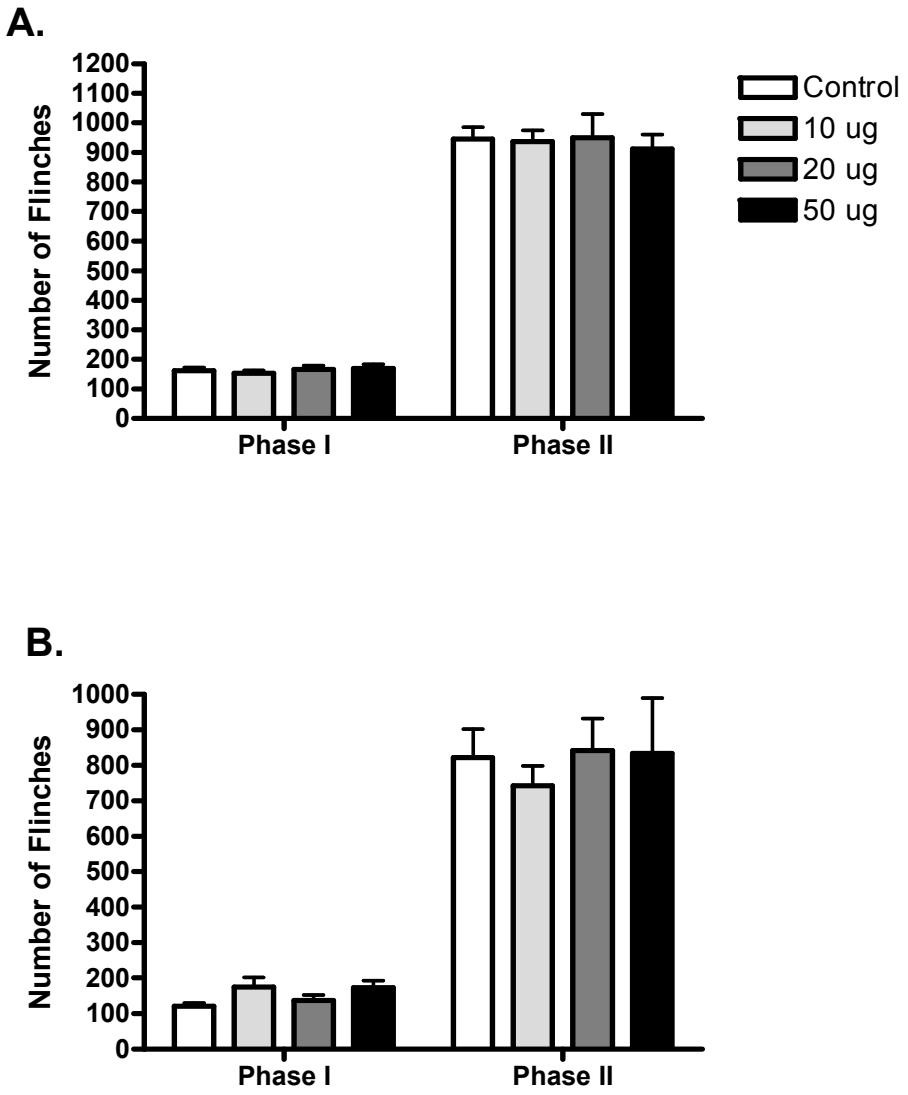


Figure 10
Lack of effect of estradiol on flinching responses after 5% formalin (A) and 2.5% formalin (B) administration. Mean flinches (± SEM) in Phase I (0-6 min) and II (9-36 min) in estradiol- or vehicle-treated animals (N=16-20/dose).

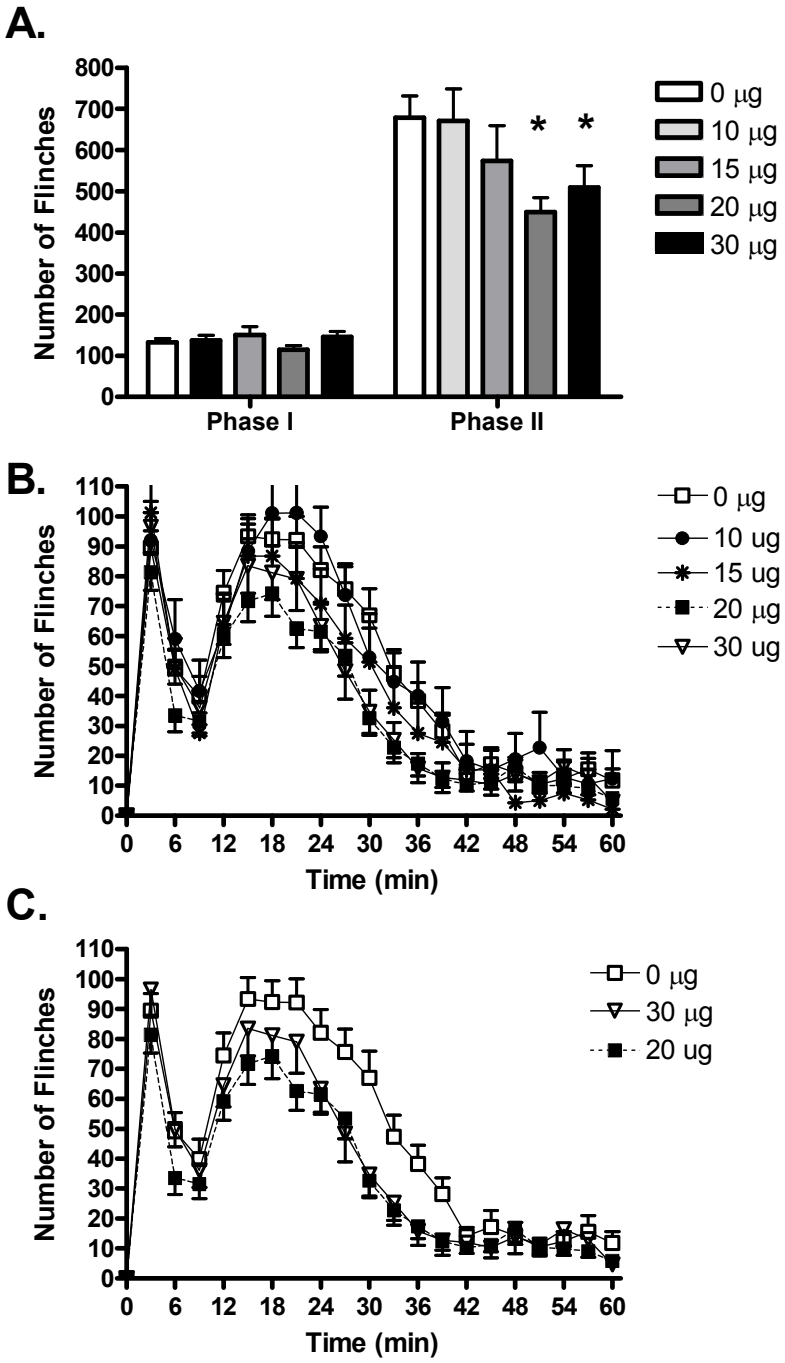


Figure 11
Effects of estradiol on flinching responses after 1% formalin administration. (A) Mean flinches (± SEM) in Phase I (0-6 min) and II (9-36 min) in estradiol- or vehicle-treated animals (N=15-20/dose). (B) Effects of estradiol replacement on the time course of flinching responses after 1% formalin. (C) Effects of estradiol (20 and 30 μg) on the time course of flinching responses after 1% formalin. Mean flinches (± SEM) are plotted in 3-min time bins.
* Denotes a significant difference of dose as compared with vehicle-treated animals ($p<0.05$).

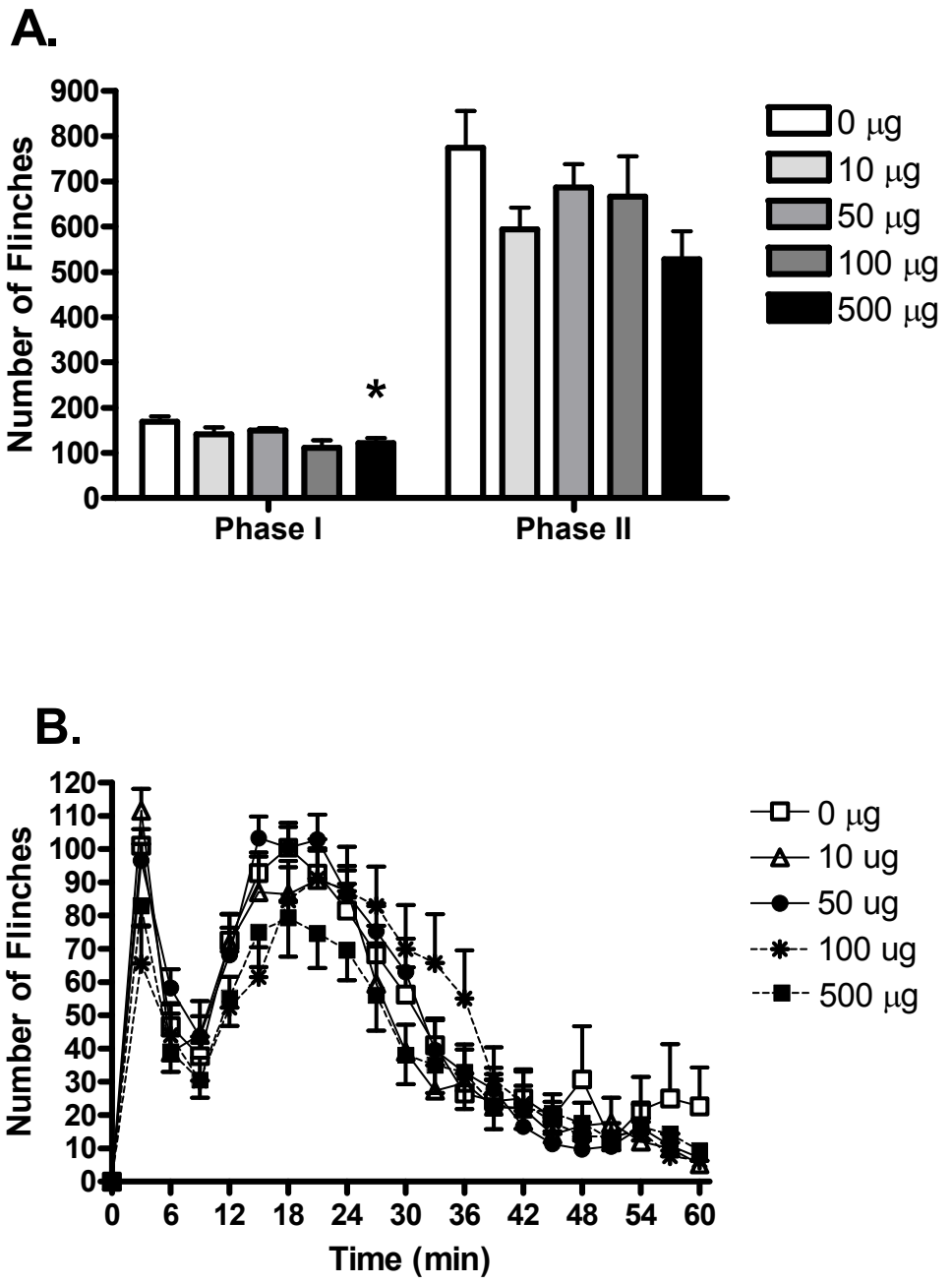


Figure 12
Effects of progesterone on flinching responses after 1% formalin administration. (A) Mean flinches (± SEM) in Phase I (0-6 min) and II (9-36 min) in progesterone- or vehicle-treated animals (N=15-20/dose). (B) Effects of progesterone replacement on the time course of flinching responses after 1% formalin. Mean flinches (± SEM) are plotted in 3-min time bins. * Denotes a significant difference of dose as compared with vehicle-treated animals ($p<0.05$).

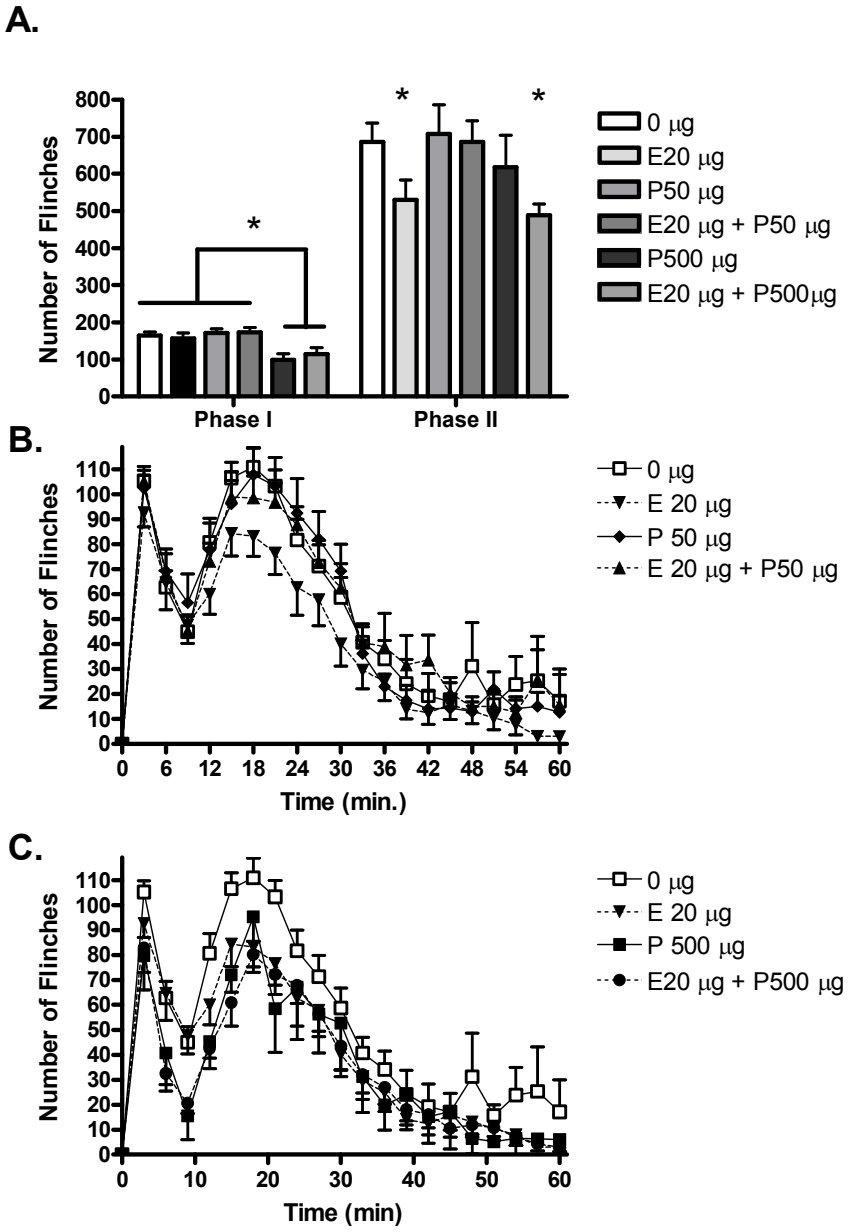


Figure 13
Effects of co-administration of estradiol and progesterone on flinching responses after 1% formalin administration. (A) Mean flinches (± SEM) in Phase I (0-6 min) and II (9-36 min) in estradiol- or vehicle-treated animals (N=15/dose). (B) Effects of estradiol (20 μg) + progesterone (50 μg) replacement on the time course of flinching responses after 1% formalin. (C) Effects of estradiol (20μg) and progesterone (500 μg) replacement on the time course of flinching responses after 1% formalin. Mean flinches (± SEM) are plotted in 3-min time bins. * Denotes a significant difference of dose as compared with all other dose-treated animals ($p<0.05$).

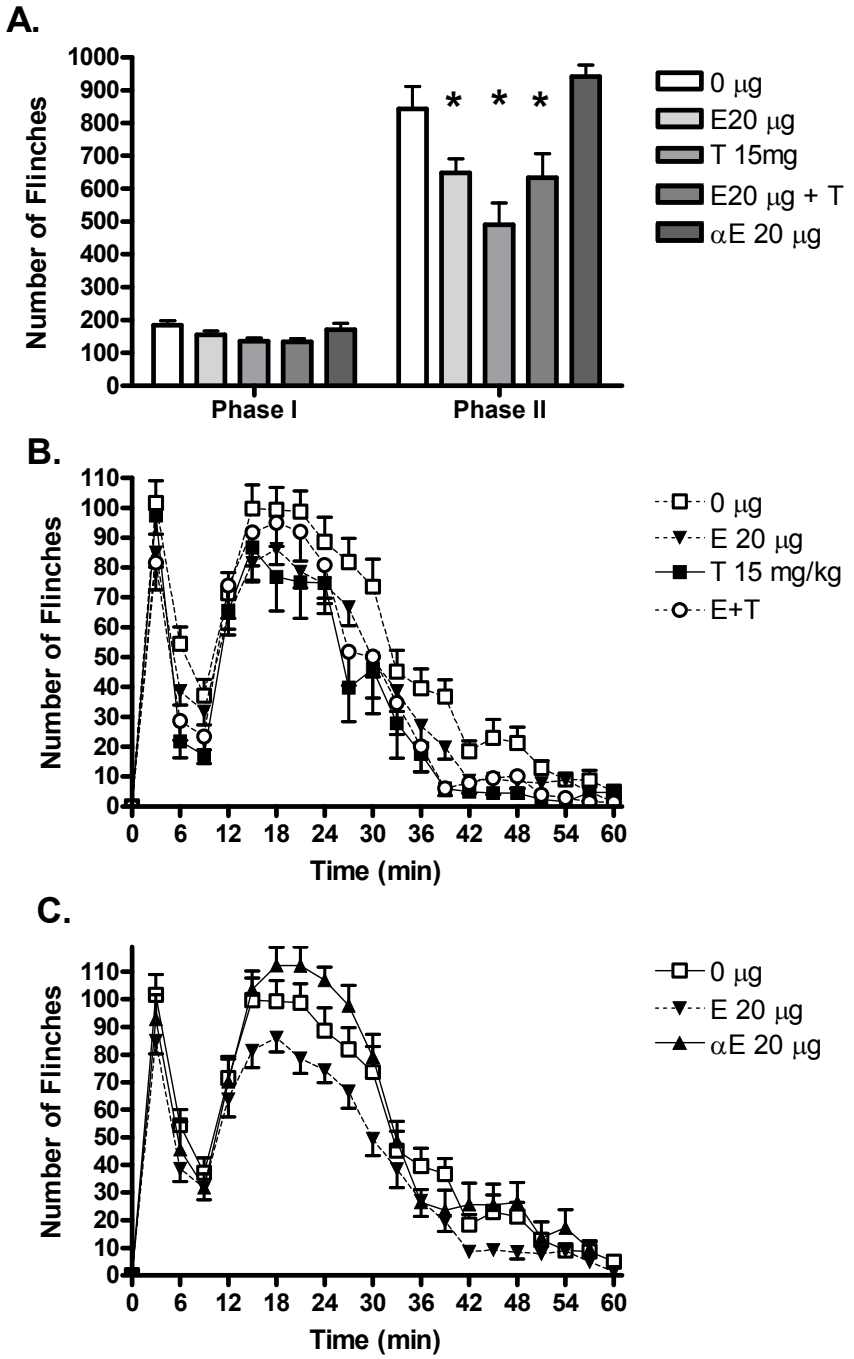


Figure 14
Effects of tamoxifen and α-estradiol on flinching responses after 1% formalin administration (A) Mean flinches (± SEM) in Phase I (0-6 min) and II (9-36 min) in OVX animals treated with β-estradiol (20 μg), tamoxifen (15 mg/kg), estradiol + tamoxifen, or αE (20 μg) (N=15/dose). (B) Effects of tamoxifen and estradiol + tamoxifen administration on the time course of flinching responses after 1% formalin. (C) Effects of αE administration on the time course of flinching responses after 1% formalin. * Denotes a significant difference of treatment as compared with vehicle-treated animals ($p<0.05$).

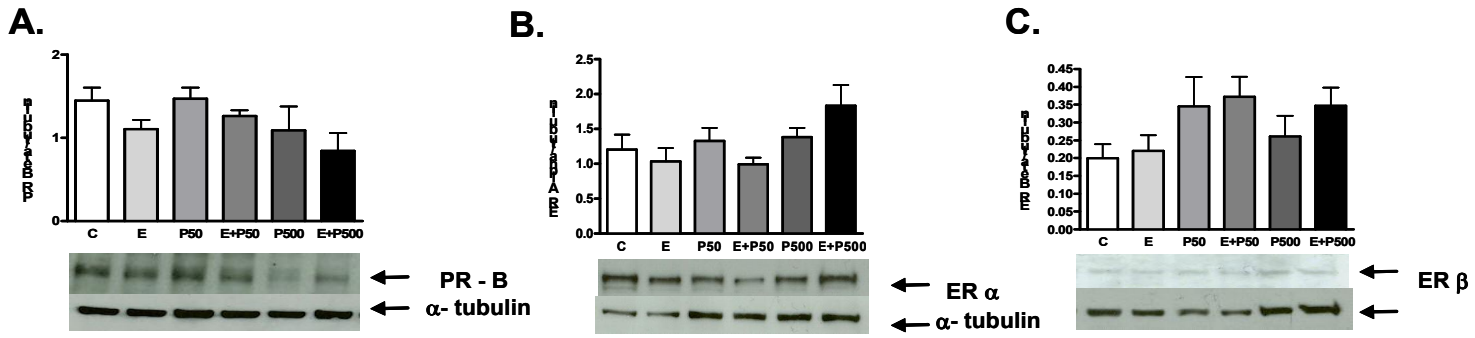


Figure 15
Detection of PR-B (A), ER-α (B) and ER-β (C) by Western blot analysis. Plotting ratio of receptor protein levels over α-tubulin levels across treatment groups. Receptor levels are shown as immunoblots and density of blots is represented in histogram form.

4. Discussion

To our knowledge, this is the first report of a systematic study of the effects of estradiol and progesterone in persistent/inflammatory pain in females. We found that estradiol dose-dependently was analgesic only during Phase II at the 1% formalin concentration. Analgesia produced by estradiol was not based on overall effects of estradiol on all behavior activity since it did not alter baseline activity. Moreover, recent studies from our group have demonstrated that at these doses, estradiol does not affect total locomotor, ambulatory, or rearing activities in OVX rats [213], a finding that suggests estradiol's effects are through nociceptive mechanisms rather than overall locomotor effects.

The role of estradiol in acute pain responses has been extensively studied. However, these studies have yielded either no change, an increase, or a decrease in nociception [101;115;170;275]. Our study, using a systematic approach, demonstrated that estradiol did not significantly affect behavioral responses during the Phase I response at 5%, 2.5%, or 1% of formalin). From this observation, we postulate that estradiol's effects are not ubiquitous to all pain behavioral responses and possibly involve specific mechanisms regulating persistent and inflammatory pain behaviors.

It has been postulated that nociceptive responses to either low or high concentrations of formalin activates different mechanisms of action [344]. While behavioral responses at low concentrations of formalin rely partially on central sensitization, but at higher concentrations of formalin, nociceptive responses rely on central sensitization and to a greater extent on peripheral inflammation [1;192;344;345]. Moreover, 1% formalin produces less late-phase C-fiber activation than does 5%

formalin administration [231;281]. As a consequence, we hypothesize that transient or pulsatory increases in estradiol serum levels during the female reproductive cycle may affect pathways related to central sensitization after a painful stimulus and to a lesser extent, late-phase C fiber activation.

Estradiol has also been shown to be analgesic in other models of persistent and inflammatory nociception. For example, estradiol decreased the number of autotomy scores in a model of neuropathic pain after sciatic nerve injury [296]. Similarly, during proestrus, when estradiol serum levels are at their highest, nociceptive responses decreased in both a ureteral calculosis model and a carrageenan hyperalgesic model [109;279]. In our study, the effective analgesic doses of estradiol (20 and 30 μg) fell within estradiol serum levels observed during late proestrus, whereas the non-effective doses (10 and 15 μg) fell within those observed during diestrus. From these observations, we postulate that fluctuation of estradiol serum levels during the estrous cycle modulates nociceptive responses to persistent/inflammatory pain and central sensitization.

α-estradiol, an inactive isomer of estradiol, did not significantly affect flinching responses during either Phase I or II after formalin administration, suggesting that estradiol's effects are mediated through the activation of specific estradiol receptor-mediated mechanisms. Similar to estradiol, tamoxifen also decreased the flinching response only during Phase II of the formalin model. Tamoxifen has been shown to reduce carrageenan-induced hyperalgesic responses in intact female rats [203]. Despite its known antagonistic effect in mammary tissue and its inhibition of lordosis activity, tamoxifen also mimics the effect of estradiol at certain doses in the brain and other

tissues [14]. Thus, these observations lead us to postulate that tamoxifen acts as an estradiol agonist in modulating formalin-induced nociceptive responses. However, the fact that tamoxifen did not synergize with estradiol suggests that both tamoxifen and estradiol may activate similar spinal cord cellular mechanisms to affect formalin-induced behavioral responses. Furthermore, since tamoxifen's and estradiol's agonistic effects are not additive, it is feasible that the response to either one alone is maximal. Because of recent concerns about the long-term risks of estradiol replacement therapy in postmenopausal women, there has been an increase in the clinical use of SERM (selective estradiol receptor modulator) [14;122] Tamoxifen has also been extensively used for treatment and prevention of breast cancer [14]. The results presented herein strongly suggest that the activation of estradiol receptor-mediated mechanisms is involved in the regulation of estradiol's effects.

Through immunocytochemical analysis, both isoforms of the estradiol receptors (ER-α and ER-β) have been shown to be expressed in different cell types in the lumbar spinal cord, including interneurons of the dorsal horns [263], astrocytes [131], and ependymal cells [50]. We extended these observations by demonstrating that in the spinal cord, ERα and ERβ have similar molecular weights as in the brain. However, although ERα and β are constitutively expressed in the spinal cord, hormone replacement did not affect their protein levels. The effects of tamoxifen suggest that, although receptor activation is required, they are not transcriptional in nature.

Most reports have suggested that analgesia occurs in female rats when circulating levels of progesterone are high [86;104;105;113;188;193;239;250;268]. Similar to these reports, we postulate that progesterone administration decreased the cumulative number

of flinches only during Phase I with the highest dose tested. However, although progesterone attenuated the peak responses during Phase II, it failed to reach significance when the cumulative number of flinches was analyzed. Progesterone has been shown to attenuate inflammatory responses in studies using other tonic pain models, i.e., CFA and carrageenan [212;242]. Discrepancy between these studies and ours may reside in the manner of administration or in the progesterone doses used. For example, Ren et al. (2000) [242] administered progesterone via Silastic capsules and Nakagawa et al. (1979) [212] administered progesterone via subcutaneous injections using higher doses (1 mg vs. 500 μg). Moreover, the degree of the inflammatory stimuli differs in all 3 studies. Thus, it is feasible that the higher intensity of the inflammatory stimuli may also affect the role that progesterone plays in analgesic responses of the female rat.

In this study, we demonstrated the presence of a constitutive PR-B isomer in the spinal cord. Most CNS areas express estradiol-inducible PR [33;39;143;177]. However, the evidence for estradiol inducibility of PR in the spinal cord of OVX rats is limited and controversial. Using immunocytochemical analysis, Monks et al. (2001) [208] demonstrated that exogenous administration of estradiol induced progesterone receptor expression in the lumbar region of the spinal cord. On the other hand, Labombarda et al. (2000) [159] demonstrated that spinal cord PRs do not exhibit a strict estradiol dependency. Moreover, spinal PR mRNA levels were not up-regulated by estradiol, in contrast to what is observed in many brain areas and in the uterus [158]. Similar to Labombarda et al., we demonstrated that PR was not strictly estradiol-dependent. Our observations and those of others [159;208] suggest that the presence of constitutive PRs

in the pain processing areas of the spinal cord may be a functional mechanism of action mediating some of the observed effects of progesterone in pain modulation.

Females have a complex endocrinological profile wherein estradiol and progesterone, under the regulation of hypothalamic and pituitary hormones, fluctuate throughout the estrous cycle/menstrual cycle. In both rodents and humans, the estrous cycle/menstrual cycle affects behavioral responses to inflammatory stimuli (reviewed in [154]). During proestrus, when both estradiol and progesterone are at the highest levels of the cycle, higher hyperalgesic responses are observed after CFA injection [43]; however, carrageenan injected hind paw latencies decrease [279], and fewer painful episodes occur during proestrus than during other stages of the cycle using the artificial ureteral calculosis model [109]. Ren at al. (2000) [242] found that during lactation, when progesterone serum levels are high and estradiol levels are low, endogenous progesterone attenuates persistent inflammatory hyperalgesia in female rats. In our study, when progesterone was co-administered with estradiol, it dose-dependently attenuated the analgesic activity of estradiol during Phase II responses. The reported differences in inflammatory/persistent pain responses during the estrous cycle thus may be based on progesterone's null effect (at the high dose) or inhibitory effects (at the middle dose) on estradiol's activity related to inflammation-induced behaviors. Animals receiving both estradiol and progesterone, however, continued to demonstrate an analgesic response during Phase I. Thus, progesterone's regulation of acute pain responses may be estradiol-independent.

Taken together, our results suggest that the biological basis for sex-specific differences in pain responses resides in the interactions between estradiol and

progesterone and the activation of pain-related pathways in the CNS. Although these effects could be supra spinal, because of the presence of estradiol and progesterone receptors it is feasible that some gonadal hormone effects may occur at the spinal cord level. We further postulate that the reported differences in pain reports in women using different steroid treatments, as well as at different stages of their menstrual cycles, reside in interactions between estradiol and progesterone. These ovarian hormone interactions may lead to under- or over-medication to relieve pain. This important clinical issue in females is currently under investigation.

Chapter 5: Estradiol administration mediates the inflammatory response to formalin in female rats

Introduction

Epidemiological pain studies have found that women are more likely than men to report a variety of temporary and persistent pains with more frequency and longer duration [13;16;23;40;54;70;123;127;130;229;240;273;284;300;309]. Similarly, in rats, females display higher pain scores in chronic and inflammatory pain models than do males [5;20;43;107;279;280]. Ovarian hormones have been postulated to be the basis for this health disparity between the sexes. For example, during proestrus (when estradiol serum levels are at their highest) painful episodes in an artificial ureteral calculosis model decreased, whereas hyperalgesic responses increased after either complete Freund's adjuvant or carrageenan [43;109;279]. Estradiol replacement reduced vaginal hyperalgesia in a menopause-associated dyspareunia model of pain and lowered autotomy scores after nerve injury [44;296]. These findings suggest that circulating estradiol may, in part, mediate some inflammatory responses in rats.

Formalin administration in the hind paw of rats is a commonly used model to study inflammatory and persistent pain responses [230]. Although persistent nociceptive responses to formalin may partly be due to central sensitization, responses to high concentrations of formalin may rely to a greater extent on peripheral inflammation [327;344;345]. In view of these different mechanisms regulating formalin responses, this

study aimed to determine the role of estradiol in peripheral versus central inflammatory responses in ovariectomized rats.

Eight-week-old ovariectomized Sprague-Dawley rats (Taconic, Germantown, NY) were double-housed under a 12-h light/12-h dark cycle (lights on at 8:00 a.m.) with food and water available *ad libitum*. Two weeks after ovariectomy, SILASTIC capsules (1 cm, 0.058 in. ID X 0.077in. OD, Dow Corning) were inserted into the nape of the animal's neck and contained either vehicle (100% cholesterol), estradiol (20% β-estradiol 3-benzoate: 80% cholesterol), or α-estradiol (an inactive estradiol isomer; 20% α-estradiol: 80% cholesterol). These doses have been shown to fall within the range of serum levels during the reproductive cycle [227]. Observed levels of serum estradiol fall within those observed in approximate days 15 to 22 during pregnancy [45]. Mannino et al. (2003) [184]have shown that 20% estradiol implants are maximally effective in attenuating the formalin response during Phase II. Furthermore, that group showed the time course of this effect remained stable from day 7 through day 21 after the implant [31].

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. One- or five-percent formalin, at a volume of 50 μL, was injected intra-plantar on the banded right hind paw. Rats were then placed in the testing chamber and behavioral activity data were collected at one-

minute intervals for a total of 60 minutes after the formalin injection. An automated flinch detecting system was used in the formalin nociceptive assay [340]. All parameters of the program were set to default values [340]. Behavioral testing was conducted between 9:00 a.m. and 3:00 p.m.

Sixty minutes after formalin injection, rats were sacrificed by decapitation, following a brief exposure to CO_2 (20 s), and trunk blood was collected. Blood was then centrifuged at 3,000 RPM for 30 minutes at 4º C. Serum was collected and stored at -80º C until analyzed with Coat-A-Count radioimmunoassay kits for estradiol (Diagnostic Products Corporation, Los Angeles, CA). Intra-assay coefficients of variation averaged 10.0% ± 1.0%. Results for these assays were determined via a log-logit analysis within GraphPad Prism Software (San Diego, CA). Estradiol serum levels were expressed as pg/mL.

One-way ANOVAs were used to test for significant differences in the sum of the flinching response across treatments for Phase I (0-9 min) and Phase II (10-60 min) after formalin administration. Statistical significance in estradiol serum levels across treatment groups was also analyzed with use of one-way ANOVAs. Fisher's least significant difference *post hoc* testing was done when appropriate. For all analyses, significance was at the level of $p < 0.05$.

The serum estradiol levels of rats receiving the same hormone replacement paradigm did not significantly differ from one another across formalin concentrations (Vehicle, $t(21) = 1.99$, $p > 0.05$; Estradiol, $t(21) = -0.17$, $p > 0.05$). Therefore, for

purposes of analysis, these values were combined. Serum levels of estradiol significantly increased after estradiol replacement; animals receiving 20 % estradiol had significantly higher levels of estradiol than vehicle-treated groups [$F(3, 52) = 18.545$; $p < 0.00$); Table XIV]. No significant differences between vehicle- and α-estradiol-treated groups were observed [$F(2, 52) = 18.545$; $p = 0.80$)]. After injection of 5% formalin, estradiol-treated animals displayed a significant attenuation in the flinching response during Phase II as compared with vehicle-treated animals [$F (2, 35) = 3.252$; $p = 0.01$; Figure 16]. However, animals receiving α-estradiol had flinching responses similar to those in vehicle-treated animals [$F (2, 35) = 3.252$; $p > 0.05$; Figure 16; Table XV]. After injection of 1% formalin, there were no significant differences between vehicle- and estradiol-treated animals in the number of flinches during Phase I and II [$F (2, 35) = 2.52$; $p > 0.05$); Table XV]. In estradiol-treated animals, no significant effect was observed during Phase I after either 1% or 5% formalin administration. (Table XV).

Table XIV. Estradiol serum concentration in female rats receiving estradiol or α-estradiol.

Treatment	Estradiol serum levels
Vehicle	22.82 ± 10.35
Estradiol	216.65 ± 36.83*
α-Estradiol	11.73 ± 5.01

Levels represented as mean ± SEM pg/mL (N=8/group). * Denotes significant difference between vehicle- and estradiol-treated groups

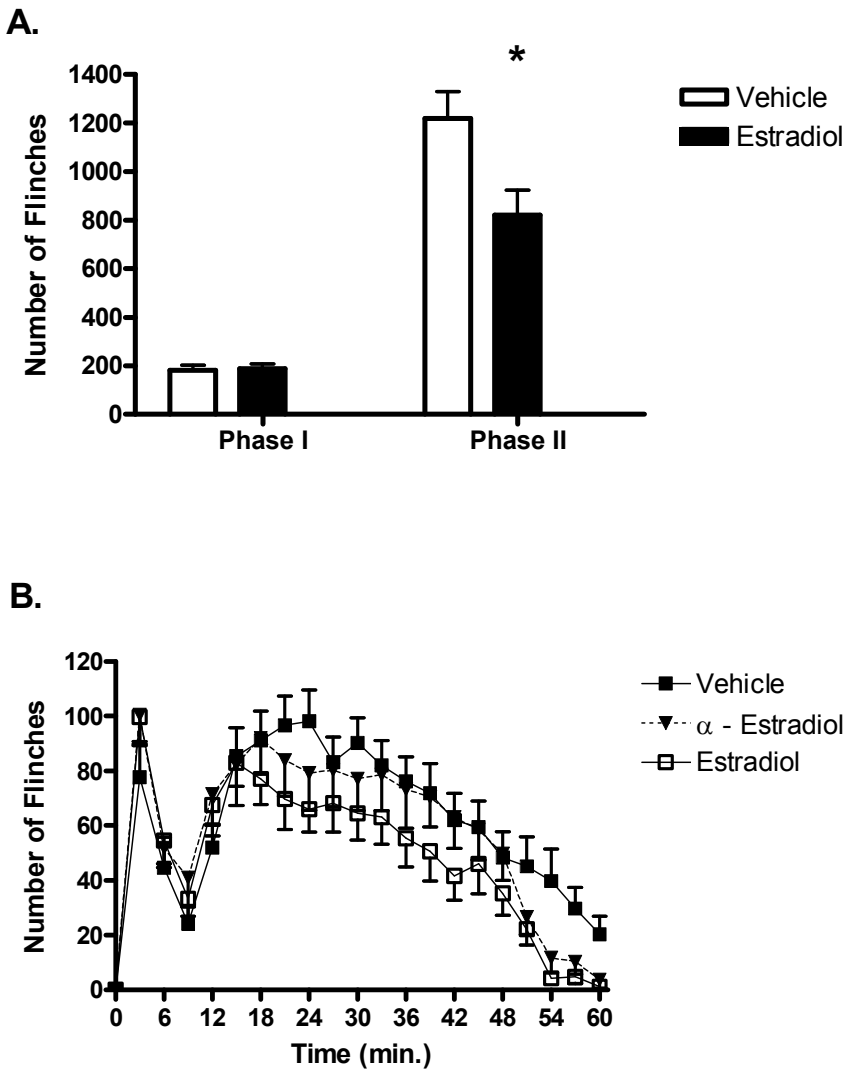


Figure 16.
Effects of estradiol implant on flinching behavior after 5% formalin administration (A) Total sum of flinches (± SEM) across phase I (0-9 min) and phase II (10-60 min) in vehicle- and estradiol-treated animals after 5% formalin administration. (B) α – estradiol and estradiol replacement effects across time (N=8/group). *Denotes significant difference ($p < 0.05$).

Table XV. Flinching activity after 1% and 5% formalin administration.

Treatment	Phase I	Phase II
1% Formalin Vehicle	152.58 ± 12.98	673.00 ± 64.09
Estradiol	204.81 ± 20.52	583.60 ± 41.51
5% Formalin Vehicle	181.72 ± 20.83	1219.00 ± 110.63
α-estradiol	196.54 ± 25.72	1096.90 ± 142.55

Levels represented as mean flinches ± SEM (N=8/group).

Most of the current pain literature has addressed the role of sex steroids in behavioral responses to acute nociceptive stimuli, but the results have been contradictory. Specifically, estradiol has been shown both to increase and to decrease threshold responses to hot plate and latencies in the tail flick assay [115;275]. On the other hand, estradiol administration had no effect in the electric foot shock assay in females [79]. Consistent with Dawson-Basoa and Gintzler (1993), we found that estradiol had no effect on the behavioral responses during the acute phase of the test, Phase I. Concentration and manner of administration of estradiol and pain assays employed differ between these studies. For example, whereas Stoffel et al. (2003) used implants (1-mm or 5-mm capsule), Gordon and Soliman (1996) administered subcutaneous injections for 7 days (50µg/kg/day). Dawson-Basoa and Gintzler (1993) administered subcutaneous silastics of estradiol to mimic pregnancy levels. Thus, a more systematic study is needed to address whether the manner of administration and/or concentration of estradiol has an effect on acute pain responses.

Estradiol reduces responses during Phase II only at a high concentration of

formalin administration. To the best of our knowledge, this report is unique in demonstrating the effects of estradiol on formalin responses in female rats. Estradiol's analgesic effect on formalin responses is consistent with results of previous studies using other inflammatory models; in both a vaginal hyperalgesia and autotomy model, estradiol decreased pain responses [44;296]. Since 5%, but not 1%, formalin activates peripheral inflammatory mechanisms [344], it is feasible that estradiol may be exerting its actions on inflammatory responses at the peripheral level of the nervous system. In fact, estradiol receptors are present on small-diameter dorsal root ganglion (DRG) neurons in the peripheral [145;221;269;278].

It has been shown that α-estradiol, an inactive isomer of estradiol, failed to stimulate sexual behavior and uterine growth in female rats [141;176;323]. This failure, in part, has been attributed to the inability of α-estradiol to activate nuclear/genomic estradiol receptor mechanisms in female rats (α-estradiol is a short acting estradiol that has been previously shown to activate only membrane receptors) [292;315]. Furthermore, estradiol receptors have a much higher affinity for estradiol than for α-estradiol (Kuiper et al., 1997) [157]. Thus, the inability of α-estradiol to alter formalin-induced behavioral responses suggests that estradiol's anti-inflammatory effects are through the activation of classical nuclear estradiol receptor mechanisms. This possibility needs further testing.

Consistent with other findings is the hypothesis that estradiol fluctuations in the female menstrual and reproductive cycle have an impact on inflammatory pain responses in the clinical setting [97;125;136;161;164;271]. A study by Vincler et al [313] found no estrous cycle effects on the behavioral response to 1% formalin. However, to date, no studies have demonstrated estrous cycle effects on the response to 5% formalin. It is at this higher concentration of formalin, when peripheral inflammatory mechanisms are activated, that variations in responses may be seen across the estrous cycle. Furthermore, estradiol replacement during menopause or oral contraceptive treatment or both have been postulated to affect behavioral pain responses in females [22;100;165;317]. Our observations suggest that consideration of estradiol levels in female patients needs to be addressed for treatment of inflammatory pain.

Chapter 6: Conclusion

The experiments presented in this thesis demonstrate sex differences in the behavioral response to persistent pain, and possible mechanisms contributing to these disparities. Males display significantly higher nociceptive responses in both Phase I and Phase II of the formalin model as compared to females. Furthermore, gonadectomy increased nociceptive responses in Phase I as compared to intact groups, and had no observed effects during Phase II of the response. Our results have been added to the findings in the literature, as shown in Table XVI.

Table XVI. Sex differences in inflammatory and chronic pain assays in rodents

Pain assay		*Female vs. male rats*	*Doses/volume*	*References*
Formalin	↑	Females display increased licking and flexing behavior during both phases	10%/50µl 2%/50µl	[5] [107]
	↓	Females display decreased flinching behaviors in both phases	5%/50 µl	[152]
Capsaicin	↑	Females display greater thermal hyperalgesia	0.1-3µg/100 µl	[20]
CFA	↑	Females (only in proestrus) display increased pain compared to males	100%/200 µl	[43]
Carrageenan	↓	Females display decreased pain before and 2 hours after injection compared to males	4%/150 µl	[279]
CCI	↓	Females display decreased pain responses to stimuli after nerve injury as compared with males	N/A	[280]

Based on these findings, circulating levels of endogenous hormones appear to contribute to the observed differences. Specifically, in females, there is a role estradiol and progesterone in mediating inflammatory pain responses. Estradiol and progesterone both altered the number of flinches after formalin administration. Estradiol significantly reduced the overall number of flinches during Phase II of the formalin response, while

progesterone attenuated Phase I of the response. After co-administration, progesterone reverses estradiol's analgesic effect in Phase II, however estradiol did not reverse progesterone's analgesic activity in Phase I. These effects have been added to Table XVII. Furthermore, estradiol administration resulted in a threshold effect, rather than a strictly dose-dependent effect on the formalin-induced flinching response. Both 20 and 30 μg of estradiol administration, via subcutaneous injection, resulted in a similar attenuation in the flinching response, not significantly different from one another.

Table XVII. Hormone replacement effects on pain behaviors in chronic pain assays

Pain assay		*Hormone replacement vs. GDX*	*Dose*	*References*
10% Formalin	↓	Testosterone to intact males decreases phase II response	5mg/kg	[8]
5% Formalin	↓	Estradiol to GDX females decreases phase II response	20% SILASTIC s.c. implants	[153]
1% Formalin	↓	Estradiol to GDX females decreases phase II responses	20 μg s.c. injection 48 hrs. prior to formalin	[155]
	↓	Progesterone to GDX females decreases phase I responses	500 μg s.c. injection 4 hrs. prior to formalin	[155]
CFA	↓	Inflammatory hyperalgesia in progesterone-treated groups	Six 3-cm SILASTIC s.c. implants	[242]
Carrageenan	↓	Inflammation decreased in progesterone-treated rats	1 mg/kg	[212]
Sciatic nerve injury	↓	Autotomy scores decreased in estradiol-treated rats	5 μg/day/rat	[296]
	↓	Testosterone replacement in GDX male rats decreases autotomy scores	500 μg/day/rat	[169]

Similar to estradiol, tamoxifen decreases the number of formalin-induced flinches during Phase II while α-estradiol did not affect formalin-induced responses. When tamoxifen was co-administered with estradiol, tamoxifen failed to reverse estradiol's effect, suggesting that both tamoxifen and estradiol activate similar intracellular mechanisms.

Finally, those nociceptive processes activated after formalin administration differ between low and high concentrations; NSAID's attenuate flinching responses at high concentrations and fail to do the same at lower concentrations, indicating the involvement of peripheral inflammation at high concentrations [344]. Estradiol's effects are more evident at higher concentrations of formalin than at lower, thought to be exerting its effects on inflammatory rather than central sensitization responses. We show that the administration of estradiol via a subcutaneous implant attenuated the persistent phase of the formalin response only at a high concentration of formalin (5%). An inactive isomer of estradiol, α-estradiol, failed to result in the same attenuation.

Although our studies demonstrated that females displayed fewer nociceptive responses than males, contradictory to epidemiological studies, several explanations can account for this. First, correlational analysis has determined that females are more likely than males to report persistent pains [247]. Furthermore, in a recent book by Fillingim (2000) [96], reasons for gender differences in reported pain are discussed. Of these, women and men differ in perception and evaluation of symptoms as well as action taken. It is also suggested that women report symptoms more willingly, and that they recall health problems to a greater extent than mend do [35]. Finally, it is suggested that

women exert more active care, that is, women get an earlier diagnosis due to more frequent medical care contacts [35].

Our study demonstrates that at higher formalin concentrations, 5%, only SILASTIC estradiol replacement attenuates the persistent phase of the response. On the contrary, pulsatile administration, like that seen with a subcutaneous injection, attenuates the persistent phase of low formalin concentration responses, those seen after 1%. SILASTIC estradiol replacement results in a 100 fold higher serum estradiol level than levels found subsequent to subcutaneous injection. A high serum concentration in SILASTIC replacement, that remains constant, may result in genomic alterations that do not occur with a pulsatile estradiol replacement, where the hormone surges and then quickly metabolizes in the serum. Yashpal and Coderre (1998) demonstrate that higher concentrations of formalin are NSAID sensitive while lower concentrations are NSAID insensitive, postulating the involvement of peripheral inflammatory mechanisms in the response to higher concentrations. This mechanistic difference may account for the varying effects of constant estradiol release versus a short-term effect, where constant high concentrations of estradiol may allow for protein synthesis in the periphery, alterations in receptor activity and/or the alteration of prostaglandin levels and/or activity. These possibilities are further discussed below.

Corticosterone, a steroid hormone secreted by the adrenal gland, is an essential component of stress adaptation and is released in response to formalin administration. Both naïve and formalin treated female rats displayed significantly higher levels of corticosterone than male rats. There is evidence that corticosterone levels vary across the estrous cycle and after gonadectomy [7;55;56;61;95;316] where GDX of male rats

increases corticosterone levels compared to intacts, and GDX of females does the opposite [258;259]. We found that subsequent to formalin administration, gonadectomized males had significantly higher levels than intact males, and gonadectomized females had significantly lower levels than intact females. These effects suggest that endogenous hormones may modulate the corticosterone response to pain.

Ovariecomtized females receiving subcutaneous injections of estradiol did not display significant differences in corticosterone levels across doses subsequent to formalin administration (data not shown). However, when animals were implanted with estradiol pellets, corticosterone levels were significantly elevated in estradiol treated animals as compared to controls subsequent to 1% formalin administration. This effect was not seen subsequent to 5% formalin administration.

With evidence that estradiol and progesterone receptors exist throughout the CNS in regions involved in pain perception and inhibition, such as PAG and the spinal cord dorsal horn, endogenous steroids have been postulated to play a role in modulating pain pathways as well as contributing to both sex differences and hormonal modulation in pain perception. Precise mechanisms by which pain processing occurs in female rats are not firmly established. Endogenous gonadal hormones may influence CNS pathways involved in pain transmission at several different levels; the scope of this dissertation was to determine intracellular mechanisms of the lumbo sacral region of the spinal cord whereby estradiol and progesterone may control inflammatory pain responses. Because we demonstrate estradiol and progesterone receptors are present in the lumbo sacral region of the spinal cord, genomic pathways leading to changes in gene transcription are likely. The activation of these receptors may contribute to protein synthesis, thereby

affecting pain transmission. However, we demonstrate that estradiol and progesterone effects are not through up-regulation of their levels, rather possibly through differences in their activity levels. Furthermore, this thesis did not measure phosphorylated steroid receptors in the spinal cord. Both estrogen and progesterone receptors may have been phosphorylated after activation, making the detection of differences in their levels difficult. As a follow up to this work, it would be interesting to measure phosphorylated levels of both estrogen and progesterone receptor in the lumbo-sacral region of the spinal cord.

Cyclooxygenase (COX) is the rate-limiting enzyme that catalyzes the conversion of arachidonic acid to prostaglandins [277]. Both isoforms of COX, COX-1 and COX-2, are present in the peripheral and central nervous system [277]. The levels of COX-1 and COX-2 proteins were unaltered after formalin, indicating that observed behavioral differences were not related to protein synthesis. Although COX-2 protein is induced by inflammation, protein levels have been shown to be unaltered after formalin for up to 4 hours. In addition, these findings are consistent with reports [133;343] that found no significant increases in COX2 protein levels following injections of inflammatory stimuli as compared to either baseline or sham animals. Additionally, no differences in protein levels were seen after GDX of either male or female rats, suggesting that endogenous gonadal hormones may play a limited role in regulating COX protein levels. As a follow-up to this research, it is suggested to measure cylooxygenase protein levels 6 or more hours following the formalin injection.

Prostaglandins, especially PGE2, are important mediators of inflammation, where following nociceptive stimuli, they are released at the site of injury. Cyclooxygenase

(COX) protein is responsible for the catalytic conversion of arachidonic acid into prostaglandins (PG). After a formalin injection, levels of PGE2 are raised and coincide with formalin induced flinching behavior. Levels of PGE2 release return to baseline when the behavioral response is over, 1-2 hours post formalin injection, whereas saline-treated animals demonstrate baseline activity for 6 hours. The local release of PGE2 in the spinal cord has been implicated in the spinal sensitization process occuring during Phase II of the formalin response and is associated with the induction of hyperalgesia and allodynia [252;302;305].

Corticosterone has been shown to exert anti-inflammatory actions by targeting cyclooxygenase. In cultured cells, glucocorticoids down-regulated COX-2 expression; similarly, COX-2 expression substantially increases in ADX rats [255;350]. Although we did not see differences in protein levels of COX, differences may lie in activity of COX-1 and/or COX-2. The activity of the protein is measured by the levels of prostaglandins released [133]. Our measurement of PGE2 came only at the end of the formalin assay, 1 hour after injection. Based on results from Tegeder et al [287], PGE2 activity coincides with formalin-induced behavior and returns to baseline. Therefore, behavioral differences may be explained in COX activity, specifically PGE2 release, during the time course of the formalin response. The underlying mechanism for our behavioral effects may lie in differences seen across the 60-minute response. As a follow-up to this work, it would be interesting to sacrifice animals at 5-10 minute time points following the formalin injection and measure PGE2 levels. Sex differences in behavioral responses to formalin may lie in sex differences in PGE2 release during the formalin response.

It has been demonstrated that endogenous hormones affect levels of PGE2. For example, progesterone stimulates PGE2 synthesis and the removal of progesterone cessates its secretion [148;254]. Estradiol, on the other hand, has been shown to inhibit the secretion of prostaglandin [183]. Based on these observations, our animals that received subcutaneous estradiol displayed an analgesic effect, which was reversed with progesterone administration in phase II, appearing to occur through the regulation of prostaglandin levels.

The repetitive C-fibre activation occuring subsequent to formalin injection excites NMDA receptors on the dorsal horn neuron. The resulting influx of CA+2 ions activates cyclooxygenase, which in turn produces prostaglandins, including PGE2, released extracellularly, facilitating further transmitter release from primary afferents, potentiating the injury response.

Alternatively, at the NMDA receptor, circulating female hormones, estradiol and progesterone, may be having an effect. Several studies confirm that treatment with estradiol to OVX rats increases hippocampal NMDA receptors and that OVX decreases NMDA receptor binding suraspinally [74;75]. Progesterone and co-administration of these steroids does not increase binding, suggesting that progesterone opposes the effects of estradiol. Similarly, treatment with tamoxifen or raloxifene, SERMS, resulted in increased hippocampal NMDA receptors. These data suggest that endogenous estradiol and progesterone may also regulate the NMDA receptor protein levels at the dorsal horn of the spinal cord. An increase in NMDA receptors may result in an increase CA+2 influx subsequent to nociceptive input.

Non-genomic activities of estradiol and progesterone are likely as well. These non-genomic effects, rapid in onset and short lasting, include the hormonal regulation of intracellular Ca+2 levels. Both estradiol and progesterone have the ability to induce a rapid increase of intracellular Ca+2 levels, found in spermatozoa and endometrial cells. These same effects may be occurring in the dorsal horn neuron, contributing to changes in the downstream cascades in nociceptive processing.

In addition to pain processing within the spinal cord, supraspinal areas may be responsible for the observed effects with estradiol and progesterone administration, as descrirbe earlier. The periaqueductal grey matter descends directly to the dorsal horn and may be influenced by estradiol and progesterone. There are studies that confirm the existence of estrogen and progesterone receptors in this area [11;142;221;299], making it feasible that these circulating hormones may be influencing supraspinal mechanisms which then descend into the dorsal horn of the spinal cord.

According to both the clinical and preclinical literature, pain management targeted at female patients should consider hormonal factors during the female reproductive cycle. For example, because hormonal changes that accompany the female menstrual cycle can be bi-directional (producing both pro-nociceptive and anti-nociceptive effects), consideration should be given to the patient's stage of the menstrual cycle and/or her age (pre-, peri-, or post-menopausal) when managing her pain. It is hard to ascribe effects to one particular hormone because both are present and can interact to modulate each other's effects [165]. Furthermore, oral contraceptives and hormone replacement therapy (HRT) should be considered. Observations from clinical studies

suggest the need for a systematic collection of data regarding the individual hormonal environment and its effects on chronic/inflammatory pain.

Based on our findings, we propose the following model in which estradiol and progesterone mediate behaviors following an inflammatory stimulus (Figure 17). The introduction of an inflammatory stimulus activates the release of corticosterone levels. Serum corticosterone levels are modulated by endogenous hormones, where estradiol and corticosterone levels are positively correlated. Corticosterone, which targets COX-1/COX-2, decreases the enzyme's activity, thereby decreasing production of prostaglandin, specifically PGE2. Therefore, there is a negative correlation between estradiol and COX-1/COX-2 activation. Progesterone blocks the analgesic activity of estradiol, through either level of corticosterone release and/or COX-1/COX-2 activation.

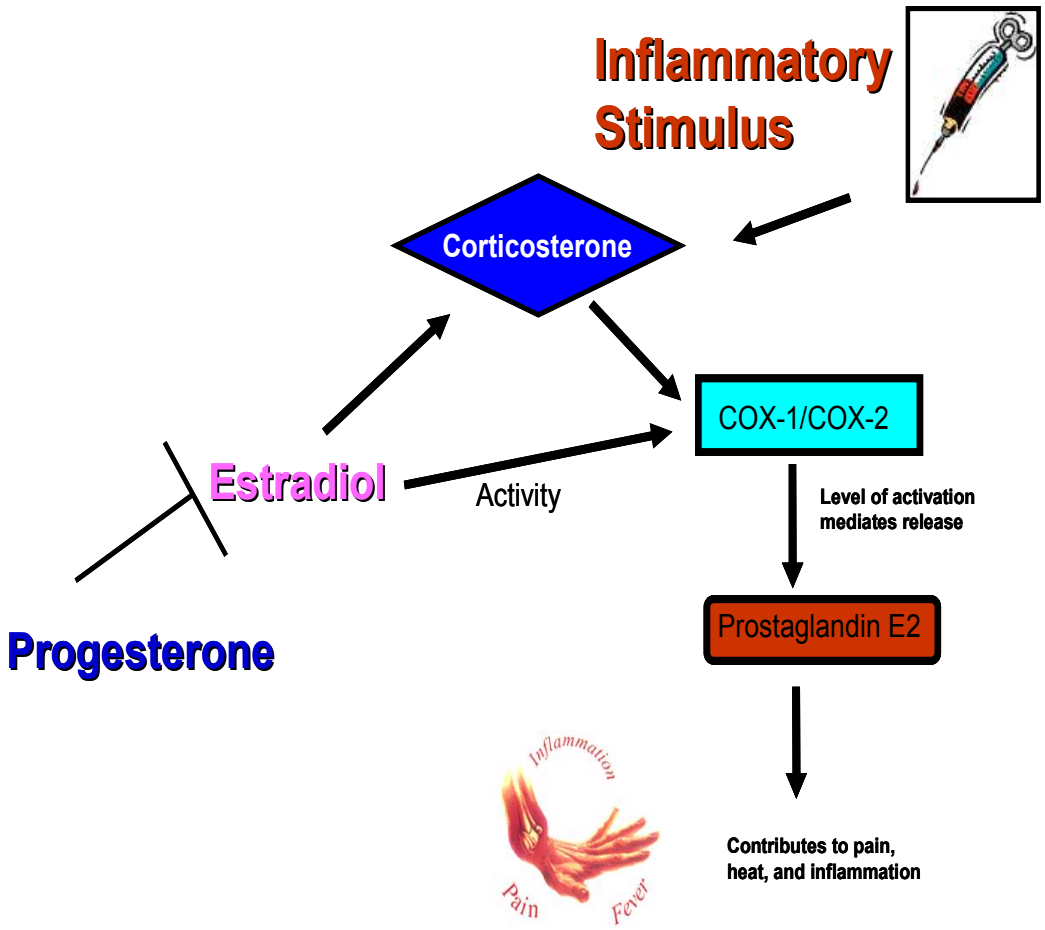


Figure 17.
Proposed model of estradiol and progesterone's role in mediating inflammatory behavioral responses.

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