SEX STEROIDS MODULATE NOCICEPTION, AND ESTROGEN MODIFIES SUPRASPINAL NOCICEPTIVE ACTIVATION

BY


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## *ABSTRACT*

Chronic pain leads neurological disorders in the US both in terms of numbers of cases and economic cost. The understanding of sex differences in the sensory experience of pain is emerging. For example, the incidence of many, but not all, chronic pain disorders is higher in women than men. There is considerable evidence that sex steroids can modulate pain processing and may contribute to sex differences in chronic pain. However, there are no reports of direct, systematic investigation into the anatomical sites of action in the nervous system of the enhancement of pain by estrogen. Thus, this research program aimed to determine the potential contribution of activational effects of sex hormones to pain sensation and was designed to test the overall hypothesis that sex differences in pain sensation are due, at least in part, to activational effects of sex hormones on peripheral, spinal and/or supraspinal sites involved in the transmission and perception of pain. To test this hypothesis, established rodent models of pain were employed, and biochemical and behavioral end points were quantified.

Initial experiments used gonadectomy and testosterone supplementation to manipulate androgen status in adult, male rats, testing the impact of androgen manipulation on behavioral responses in several pain models. Results revealed activational effects on pain of phasic thermal and persistent neuropathic origin, but perhaps not persistent inflammatory origin, providing evidence that androgens can contribute to sex differences in pain sensation.

Further experiments used ovariectomy and estradiol supplementation to manipulate estrogen status in adult, female rats, testing its impact on nociception-related

behavior and neuronal activation in response to formalin-evoked inflammatory nociception. Results of behavioral analyses showed that estradiol increases persistent, inflammatory nociception in an activational manner. Results of quantification of nociception-evoked neuronal activation (measurement of numbers of neurons expressing the protein Fos) showed that estrogen enhances inflammatory nociception primarily at higher brain centers, specifically the nucleus accumbens and the ventral hippocampal CA1 region.

These activational effects of estrogen may contribute to sex differences in pain sensation and/or the disproportionate severity or incidence of some pain syndromes in women.

To my loving parents, Gail and Leland Ralya.

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## *LIST OF ABBREVIATIONS*

| | |
|---|---|
| 5HT | 5-hydroxytryptamine |
| AF-1 | Activation function-1 |
| AF-2 | Activation function-2 |
| AMP | Adenosine monophosphate |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| ANOVA | Analysis of variance |
| AP | Action potential |
| AP-1 | Activator protein-1 |
| APtN | Anterior pretectal nucleus |
| AR | Androgen receptor |
| ASIC | Acid sensing ion channel |
| ATP | Adenosine triphosphate |
| BDNF | Brain-derived neurotrophic factor |
| cAMP | Cyclic adenosine monophosphate |
| CFA | Complete Freund's adjuvant |
| CGRP | Calcitonon gene-related peptide |

| | |
|---|---|
| CNS | Central nervous system |
| COX | Cyclooxygenase |
| CREB | Cyclic AMP response element binding protein |
| DAB | Diaminobenzidine |
| DAPI | 4’-6-diamidino-2-phenylindole |
| DEG/ENaC | Degenerin/epithelial sodium channel |
| DG | Dentate gyrus |
| DHT | 5α-dihydrotestosterone |
| DLF | Dorsolateral funiculus |
| DNA | Deoxyribonucleic acid |
| DRG | Dorsal root ganglia |
| E2 | 17β-estradiol |
| eNOS | Endothelial nitric oxide synthase |
| ERE | Estrogen response element |
| ERK | Extracellular signal-regulated kinase |
| ER | Estrogen receptor |
| FITC | Fluorescein isothiocyanate |

| | |
|---|---|
| FPA | Force Plate Actimeter |
| FSH | Follicle-stimulating hormone |
| GABA | Gamma-amino butyric acid |
| GDX | Gonadectomy or gonadectomized |
| GnRH | Gonadotropin releasing hormone |
| GPCR | G-protein coupled receptor |
| $H^+$ | Proton |
| HRP | Horseradish peroxidase |
| HVA | High-voltage-activated |
| IHC | Immunohistochemistry |
| LH | Luteinizing hormone |
| LRN | Lateral reticular nucleus |
| MAPK | Mitogen-acitvated protein kinase |
| MEK | Mitogen-activated ERK kinase |
| mRNA | Messenger ribonucleic acid |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NE | Norepinephrine |

| | |
|---|---|
| NGC | Nucleus reticularis gigantocellularis |
| NGCα | Nucleus reticularis gigantocellularis pars alpha |
| NGF | Nerve growth factor |
| NIH | National Institutes of Health |
| NK-1 | Neurokinin-1 |
| NMDA | N-methyl-D-aspartate |
| NO | Nitric oxide |
| NPG | Nucleus paragigantocellularis |
| NRM | Nucleus raphe magnus |
| NSAID | Non-steroidal anti-inflammatory drug |
| OR | Opioid receptor |
| OVX | Ovariectomy or ovariectomized |
| PAG | Periaqueductal grey |
| PBS | Phosphate-buffered saline |
| PBST | Triton X-100 in phosphate-buffered saline |
| pERK | Phospho-extracellular signal-regulated kinase |
| PFA | Paraformaldehyde |

| | |
|---|---|
| PFC | Prefrontal cortex |
| PKA | cAMP-dependent protein kinase |
| PKC | Protein kinase C |
| PLSD | Protected least significant difference |
| RVM | Rostral ventromedial medulla |
| SEM | Standard error of the mean |
| SHBG | Sex hormone-binding globulin |
| SNARE | N-ethylmaleimide-sensitive factor attachment protein receptors |
| SNI | Spared nerve injury |
| SNS | Sensory neuron-specific sodium channel |
| SP | Substance P |
| SSRI | Selective serotonin reuptake inhibitor |
| TBST | Tween-20 in Tris-buffered saline |
| TCA | Tricyclic antidepressant |
| TP | Testosterone propionate |
| TRP | Transient receptor pontential |
| TRPV1 | Transient receptor pontential vanilloid 1 |

| | |
|---|---|
| TRPV2 | Transient receptor pontential vanilloid 2 |
| TRPV4 | Transient receptor pontential vanilloid 4 |

# *CHAPTER ONE*

# *BACKGROUND AND SIGNIFICANCE*

### *1.1 Introduction*

Chronic pain leads neurological disorders in the US in terms of the total number of cases and their annual economic cost (Carey, 2005; Loeser, 2001; Stucky et al., 2001). Interestingly, recently emerging studies have begun to characterize sex differences in the sensory experience of pain. For example, the incidence of many chronic pain disorders, but not others, is higher in women than men, and for experimentally delivered stimuli, females have lower thresholds, higher pain ratings, and less tolerance of noxious stimuli than males (Berkley, 1997). Many painful disorders show prevalence in women, and examples include migraine headache with aura, temporomandibular joint disorder, fibromyalgia, and pain of psychological origin (e.g. depression) (Berkley, 1997). Women more often report multiple or recurrent pains than men, especially in certain body locations and at certain ages (James et al., 1991). Many painful disorders vary in their incidence, disappearance, and prevalence as a function of menstrual stage, puberty, pregnancy, menopause, and age (Berkley, 1997). For example, the incidence of migraine headaches and temporomandibular disorders varies with menstrual stage (Meisler, 1999). This is indirect clinical evidence that ovarian hormones such as estrogen may alter pain sensitivity. There is also experimental evidence for this as well. Female rats have heightened sensitivity to acute mechanical nociceptive stimuli during proestrus (Kayser et al., 1996), and the density of primary afferent innervation of the genitalia of female rats is greatest during proestrus (Robbins et al., 1992). The formalin test is an established model of persistent inflammatory pain (Porro & Cavazzuti, 1993; Tjolsen et al., 1992). The pain response (nociceptive behaviors) to a formalin stimulus has previously been shown to be greater in female rats than males (Aloisi et al., 1994). In addition, female

rats show less morphine-induced analgesia than males (Bodnar et al., 1988), and opioid receptor-mediated anti-nociception differs between men and women (Zubieta et al., 2002), indicating the opioidergic system may play an important role in sex differences in pain. Sex differences in analgesia are modulated by estrogen & other steroids (Berkley, 1997). Cicero et al. (1996) showed that male rats were more sensitive than females to morphine-induced anti-nociception, and that this difference was mediated by both spinal and supraspinal mechanisms. They also concluded that this difference was due to organizational effects rather than acute, activational effects of gonadal steroids (see p. 28-9). However, other reports have concluded that gonadal steroids can affect morphine-induced anti-nociception in an activational (acute) manner. Highly relevant to this research proposal, responses to inflammatory pain, both clinically and experimentally, vary with the estrous cycle of the female (Bradshaw et al., 2000; Fillingim et al., 1997; Hellstrom & Anderberg, 2003; Isselee et al., 2002; Lautenbacher & Rollman, 1993; LeResche et al., 2003; Somerville, 1972; Tall & Crisp, 2004).

Depression is about twice as prevalent in women than men (Bromberger, 2004). This gender difference emerges at the onset of puberty (menarche), and indicates a possible role for gonadal steroids such as estrogen (Bromberger, 2004; Steiner et al., 2003). Women are at heightened risk of depression and other mental disorders during postpartum time periods (Steiner et al., 2003), and the apparent higher vulnerability of women to depression could, at least ostensibly, be explained by large or rapid changes in ovarian hormone levels that occur during puberty, postpartum, perimenopause, or premenstrually (Bromberger, 2004). Since these large hormonal changes coincide with changes in mood, a causal link can logically be, and has been, suggested (Steiner et al.,

2003). However, there is little evidence directly linking these hormone levels and depressed mood (Bromberger, 2004). There is an association of depression with pain, as discussed below. Thus, estrogen could contribute to the incidence of depression by modulation of pain or through other mechanisms related to limbic system function (Allen & McCarson, 2005).

Pain is defined by the International Association for the Study of Pain as an unpleasant sensory and emotional experience associated with tissue damage or described in terms of such damage. Thus, pain is a complex conscious experience that involves not only the transduction of noxious environmental stimuli, but also emotional and cognitive processing by the brain (Julius & Basbaum, 2001). Pain normally serves as a warning mechanism to living beings of potential or real tissue damage. The nervous system involved in the processing of nociceptive stimuli mediates protective mechanisms such as reflex withdrawal from the harmful stimuli. This natural defense system is fast-acting and can be adaptive. Sometimes the pain outlasts the injury, outliving its usefulness as a warning/protective system (Julius & Basbaum, 2001). The pain can then become chronic and potentially debilitating, a maladaptive process involving changes in the brain, spinal cord, and primary sensory neuron (nociceptor) (Julius & Basbaum, 2001). Chronic pain can result in anxiety, depression, and reduced quality of life (Hunt & Mantyh, 2001). Chronic pain often is accompanied by depression, and vice versa, indicating an association between the two disorders (Blackburn-Munro & Blackburn-Munro, 2001; Fishbain et al., 1997; King, 1991), and leading to speculation that chronic pain could be a variant of depression (Blumer & Heilbronn, 1982). Fishbain et al. (1997) concluded that depression was more likely a consequence of chronic pain than vice versa, supporting the

consequence or scar (predisposition to depression) hypotheses of chronic pain-associated depression. Recent studies have characterized hippocampal responses to pain that mimic those induced by stress and depression (Duric & McCarson, 2005, 2006a, 2006b).

Numerous reports have addressed the modulation by estrogen of biochemicals involved in nociceptive neuronal signaling or behavioral responses. However, there are no reports of direct, systematic investigation into identification of the anatomical sites of action in the nervous system of the enhancement of nociception and pain by estrogen.

## *1.2 Neuronal pain signaling*

### *1.2.1 Pathways - Overview*

A complex system of many neurons is involved in the sensing and processing of noxious stimuli (See **Figure 1** for a diagrammatic overview). Noxious stimuli are detected by primary afferent neurons also known as nociceptors. These neurons arise from the trigeminal or dorsal root ganglia (DRG) where their cell bodies reside. The nociceptors then form synapses with secondary afferent neurons in the spinal cord. These secondary spinal neurons make up the spinothalamic tract, or anterolateral system, which extend up the spinal cord and terminate in the thalamus and other midbrain centers. The thalamus then acts as an integrator and relay for sensory information and sends third order neurons projecting to the cortex. The spinothalamic tract also sends collateral branches to the lateral parabrachial nucleus in the pons, which in turn sends projections to the amygdala, one part of the limbic system.

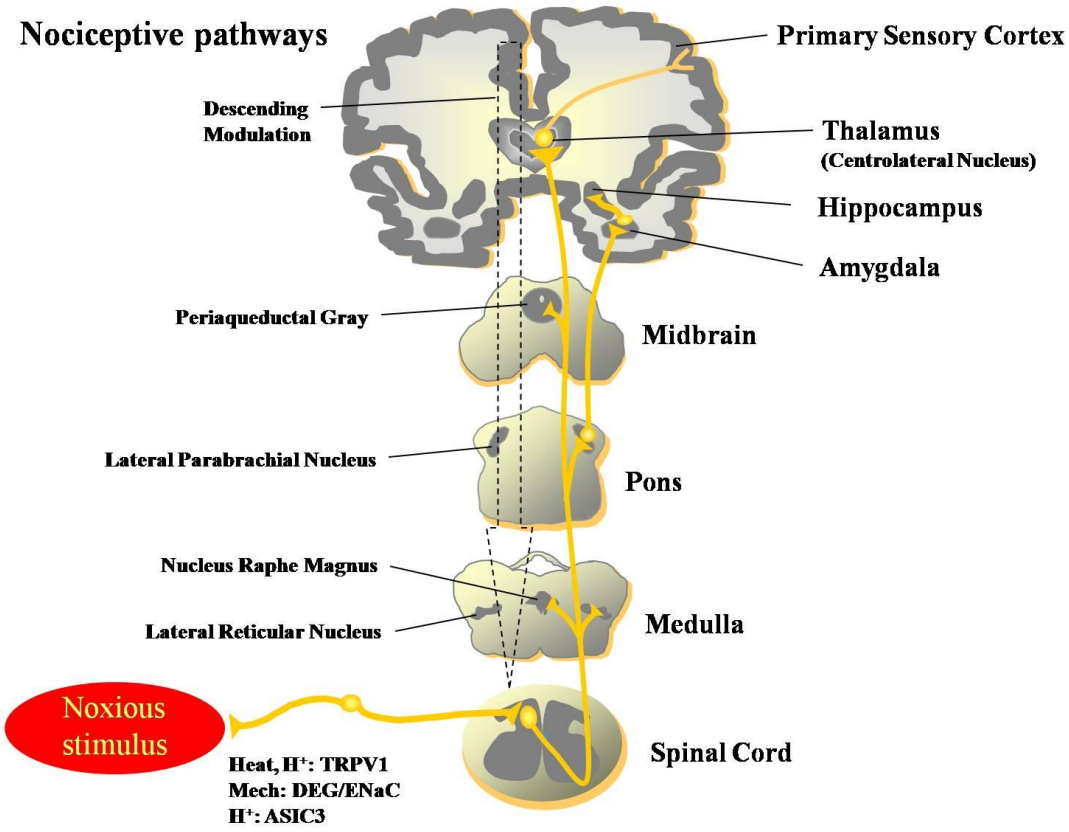


**Figure 1.** Neuronal pathways of nociception/pain.

### *1.2.2 Primary afferent nociceptors*

Primary afferent neurons that innervate the head and body have their cell bodies in the trigeminal or DRG, respectively, and are the first nerves to be activated by noxious stimuli. Primary afferent nociceptors are primarily made up of two types of nerve fibers: Aδ and C fibers. They are described as being thinly or unmyelinated, respectively, and mediating fast and slow pain, respectively, a phenomenon related to their conduction velocities. Fast (or first) pain is described as sharp or cutting, and slow (or second) pain described as more diffuse and dull. Nociceptors are further characterized by the type of noxious stimulus they are most sensitive to (adequate stimulus). Both Aδ and C fibers can be activated by mechanical stimuli. Aδ fibers are further divided based on their differential abilities to also be stimulated by thermal stimuli. Most C fibers are polymodal, responding to both thermal and mechanical stimuli. Some are only activated by mechanical stimuli. In addition, most C fibers are also activated by noxious chemicals such as acid or capsaicin. There are also "silent" nociceptors that are recruited during sensitization due to tissue damage. It is possible that this is attributable to recruitment of Aβ sensory fibers (Millan, 1999).

### *1.2.3 Spinal cord pain processing*

Processing of nociception is critical at the synapse between the primary afferent neuron and the secondary neuron residing in the dorsal horn of the spinal cord. Somatosensory and nociceptive neurons from the hind limbs (legs) terminate in the lumbar segments of the spinal cord. Aδ fibers terminate primarily in laminae I and V of

the spinal cord dorsal horn on secondary fibers that project to the thalamus and lateral parabrachial nucleus. C fibers are divided into two classes based on their content of neuropeptides, substance P (SP) and calcitonin gene-related peptide (CGRP). C fibers that contain these peptide neurotransmitters synapse in the most superficial layers of the dorsal horn, lamina I and outer lamina II, largely on second order neurons that project to higher pain processing centers in the brain, including the amygdala via a synapse in the lateral parabrachial nucleus. The other, nonpeptidergic C fibers synapse in the inner lamina II, primarily on interneurons, which have short, intraspinal projections. Wide dynamic range neurons, with their cell bodies in lamina V, receive input from Aδ and C nociceptors, as well as Aα/β somatosensory fibers. These wide dynamic range neurons then project up to the thalamus.

### *1.2.4 Spinal nociceptive reflexes*

The spinal cord serves a protective role by mediating the flexor (and crossed-extension) reflex. This spinal nociceptive reflex is mediated by polysynaptic pathways involving interneurons. In this phenomenon, cutaneous Aδ nociceptors are activated by a noxious stimulus, and synapse on interneurons in the dorsal horn. Several interneurons are activated sequentially, which leads to an excitatory synapse onto flexor motor neurons, which causes withdrawal of the body part from the noxious stimulus. There is also reciprocal innervation of the extensor muscle via inhibitory interneurons to facilitate the movement. Sometimes there is innervation to a contralateral limb to mediate what is referred to as the crossed-extension reflex. Spinal animals have been used in the

laboratory to demonstrate these simple reflexes so they are not obscured by modulation from higher CNS levels.

### *1.2.5 Thalamic nociceptive relays*

Spinothalamic neurons are heavily myelinated and project up the spinal cord to the intralaminar nuclei of the thalamus via the paleospinothalamic tract. This tract also sends collateral branches to the amygdala via the parabrachial nucleus of the pons and to the hypothalamus. There are nociceptive projections from the spinal cord to the nucleus reticularis gigantocellularis (NGC) in the medulla, which relays projections to the thalamus (Peschanski & Besson, 1984). The thalamus acts as an integrator and relay for sensory information and sends ("third order") neurons projecting to the primary sensory cortex and cingulate gyrus, as well as diffuse areas of the cortex associated with a poorly localized sensation of pain. In addition, the thalamus has reciprocal communication with the hypothalamus.

### *1.2.6 Limbic system contributions to the negative affective component of pain*

The limbic system is the key center for processing the affective, or emotional, component of pain. It is integrally involved in the regulation of many emotions, such as love, fear, and depression, and their autonomic responses. This system has many components, including cortical areas, the hippocampal formation, septal complex nuclei, the amygdala, nucleus accumbens, and hypothalamus. The hypothalamus coordinates

and mediates mental and physiological, and especially autonomic, responses associated with emotions. The amygdala is needed for expression of emotions, and evokes both emotional feeling and autonomic responses. Projections from the amygdala have a prominent effect on hypothalamic function. The hippocampal formation plays a predominant role in stress responses. There are extensive connections feeding back from the cortex to limbic structures, especially direct projections from the prefrontal areas to the amygdala and hypothalamus. Much of the behavioral information known about emotions pertains to the amygdala and prefrontal cortex. There is also extensive communication of the hippocampus with cortical areas and hypothalamus.

### *1.2.7 Descending modulation of pain*

There is much documented knowledge of descending inhibition of spinal nociceptive transmission from supraspinal centers. Major centers in the descending inhibitory pathways include the periaqueductal grey (PAG), nucleus raphe magnus (NRM), nuclei reticularis gigantocellularis (NGC), gigantocellularis pars alpha (NGCα), paragigantocellularis (NPG), and anterior pretectal nucleus (APtN). Of these, the PAG and NRM are the most thoroughly described. The PAG receives input from the frontal and insular cortex, amygdala, and hypothalamus (Beitz, 1982b; Mantyh, 1983). Major brainstem inputs that activate PAG neurons come from the nucleus cuneiformis and pontine reticular formation (Basbaum & Fields, 1984). The PAG sends serotonin (5HT) and neurotensin projections to the rostral medulla (Beitz, 1982a, 1982c). The rostral ventromedial medulla (RVM) in particular is a major site for projecting neurons to the

dorsal horn, mainly via the dorsolateral funiculus (DLF), making it an important relay for the descending inhibition. The NRM is one important part of the RVM, and sends 5HT-neurons, which may also contain substance P (Hokfelt et al., 1978), to the spinal cord (Basbaum & Fields, 1984). The NGC, NGCα, and NPG also project to the spinal cord, possibly releasing 5HT or norepinephrine (NE), although the neuronal sources for NE from the brainstem to the spinal cord are not well known (Basbaum & Fields, 1984). The PAG also has direct connections with the spinal cord (Mantyh & Peschanski, 1982), but the medullary relays are necessary for descending inhibition. Descending neurons impinge upon inhibitory interneurons in the spinal dorsal horn that release opioids, especially enkephalins but also endorphins (Basbaum & Fields, 1984). These opioids have both presynaptic and postsynaptic inhibitory effects on primary afferent and secondary spinothalamic projection neurons, respectively.

There is also supraspinal descending *facilitation* of nociceptive neurotransmission in the spinal cord that has been reported, especially for rodent hind paw responses. Zhuo and Gebhart (1992) showed that electrical or glutamate stimulation of the NGC and NGCα can produce facilitation or inhibition of spinal transmission in response to noxious heating of the hind paw, depending on intensity of stimulation of the nuclei. Mustard oil-induced mechanical allodynia in rat hind paws was blocked by spinalization or lidocaine injection into the nucleus raphe magnus (NRM) or lateral reticular nucleus (LRN) of the medulla (Mansikka & Pertovaara, 1997). Spinalization also raised mechanical thresholds for hind paw withdrawal reflexes in inflamed and non-inflamed paws (Mansikka & Pertovaara, 1997). It is also evident that the hind limb and tail reflexes are differentially modulated by supraspinal influences, a phenomenon which may be further dependent on

the type of noxious stimulus (Aimone & Gebhart, 1986; Bian et al., 1998; Mansikka & Pertovaara, 1997). This information robustly implicates supraspinal sites (including the NRM, LRN, NGC, and NGCα) in descending facilitation of nociceptive signaling in the spinal cord (particularly the lumbar region).

### *1.3 Molecules & mechanisms*

#### *1.3.1 Peripheral nociceptor activation*

Primary afferent nociceptors detect noxious thermal, mechanical, and chemical stimuli. They do this via receptors on their distal terminal. The main receptor responsible for detection of noxious heat is transient receptor potential vanilloid 1 (TRPV1), a member of the transient receptor potential (TRP) family. The TRPV1 channel is a non-selective cation channel that spans the cell membrane, and is activated by capsaicin as well. It is expressed on C and type II Aδ fibers, which respond to heat with a threshold of ~45 °C as well as to capsaicin (Julius & Basbaum, 2001). Type I Aδ fibers have a higher thermal threshold, ~52 °C, and do not respond to capsaicin (Julius & Basbaum, 2001). It is not clear what receptor(s) is responsible for high-threshold thermal detection in these fibers; it is apparently not TRPV1, but could be a similar vanilloid receptor-like channel, TRPV2 (a.k.a. VRL-1) (Caterina et al., 1999). There are also noxious cold-activated fibers, but their mechanism of activation is unknown and different than that of heat detection. Alterations in sodium, potassium, or calcium currents have been speculated as potential mechanisms for this activation (Askwith et al., 2001; Reid & Flonta, 2001; Suto & Gotoh, 1999).

Receptors for mechanical sensation have been extensively studied in *Drosophila* and *C. elegans* (Syntichaki & Tavernarakis, 2004). These studies have provided promising candidates for receptors of noxious mechanical stimulation in mammals, most likely in the degenerin/epithelial sodium channel (DEG/ENaC) family. Also, TRPV4 is a mechanosensitive channel in mammals that has been implicated in hypotonic nociceptive detection (Alessandri-Haber et al., 2003).

Other receptors on nociceptors can be activated by chemicals such as protons ($H^+$). For example, TRPV1 and members of the acid sensing ion channel (ASIC) family are important $H^+$ receptors. Protons also potentiate responses to capsaicin and heat, and, thus, take part in the phenomenon of convergence at the molecular level by modulating TRPV1. This is an important part of the inflammatory sensitization that follows tissue damage, as tissue acidosis ensues. Receptors for many other chemicals exist on the nerve terminal that excite or sensitize (decrease threshold of) the neuron. Such chemicals that are important for mediating (peripheral) inflammatory sensitization make up the so-called "inflammatory soup," and include bradykinin, prostaglandins, 5HT, ATP, and nerve growth factor (NGF). Part of this sensitization is neurogenic inflammation, which is an efferent function of the nociceptors. This involves the release of SP and CGRP, which causes vasodilation and extravasation. Substance P also activates mast cells or neutrophils to release inflammatory mediators such as histamine. Activation of cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) pathways also contributes to peripheral sensitization by enhancement of TRPV1 function (Varga et al., 2006). Following inflammation, TRPV1 and sensory neuron-specific sodium channel (SNS) expression is up-regulated in the primary afferent (Michael & Priestley, 1999; Tate et al.,

1998). Extracellular signal-regulated kinase (ERK) may also be involved in peripheral sensitization of primary afferent nociceptors (Dai et al., 2002).

*1.3.2 Neurotransmission and intracellular signaling*

Glutamate and its receptors, as well as the tachykinin substance P and its neurokinin-1 (NK-1) receptor, are perhaps the most important signaling molecules for synaptic transmission of nociception. Glutamate is the major excitatory neurotransmitter of the peripheral and central nervous system, and has many receptors that are metabotropic or ionotropic. Glutamate and SP are released by the primary afferent nociceptors at their synapses in the spinal cord. NK-1 is expressed mainly in the superficial laminae (I and II) of the spinal cord (Mantyh et al., 1989; Moussaoui et al., 1992), and is a G-protein coupled receptor that signals through $G_{\alpha q/11}$. This activates phospholipase C (Hanley et al., 1980), which in turn mobilizes internal $Ca^{2+}$ stores (Streb et al., 1983) and activates protein kinase C (Kishimoto et al., 1980). The $Ca^{2+}$, via interaction with Ca-calmodulin kinases, may also activate phosphorylation of other proteins, such as cyclic AMP response element binding protein (CREB), and, thus, stimulate the immediate early gene *c-fos* (Miyamoto, 2006). Gamma-amino butyric acid (GABA) and type A & B GABA receptors are important for pre- and postsynaptic inhibition of signal transmission via interneurons, and opioid receptors (OR) are important for pre- and postsynaptic descending inhibition of nociceptive signaling.

Cell-surface receptors that mediate neuronal transduction can be either ionotropic or metabotropic [the G-protein coupled receptors (GPCR)]. Ionotropic receptors are ion

channels that directly regulate ion flux across the cell membrane. Metabotropic receptors have indirect effects on ion flux via second messengers that modulate ion channels. Once excitatory neuronal cell-surface receptors are activated, they depolarize the cell membrane potential, or electrochemical gradient. This is done by altering the permeability/flux of ions across the membrane through ion channels. If the stimulus is great enough, the depolarization is sufficient to raise the membrane potential to the threshold, the point at which voltage-gated ion channels open. Especially important for this are voltage-gated sodium ($Na^+$) channels, which further depolarize the neuron and propagate an action potential (AP) by allowing $Na^+$ inflow. Voltage-gated potassium ($K^+$) channels then open, allowing $K^+$ to flow out, deactivating $Na^+$ channels, and repolarizing the cell. When the AP reaches the synaptic terminal, the depolarization opens voltage-gated $Ca^{2+}$ channels, followed by $Ca^{2+}$ activation of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) such as synaptobrevin. Synaptobrevin works with synapsin to mediate exocytosis of vesicles containing neurotransmitters. The neurotransmitter then binds to post-synaptic receptors on the secondary neurons and produces either an excitatory or inhibitory post-synaptic potential. Neurotransmitters can also bind to pre-synaptic "autoreceptors" that regulate neurotransmitter release.

The immediate early gene *c-fos* is rapidly and transiently expressed in neurons following stimulation. The protein product of *c-fos*, Fos, forms dimers with Jun proteins to make activator protein-1 (AP-1) complexes, which act as transcription factors regulating expression of downstream genes. Fos has been used extensively as a neuronal marker of nociceptive activation in brain and spinal cord pathways (Bon et al., 2002;

Ceccarelli et al., 1999; Doyle & Hunt, 1999; Harris, 1998; Herrera & Robertson, 1996; Kaneko et al., 2000; Khanna et al., 2004; Kovacs, 1998; Menetrey et al., 1989; Presley et al., 1990; Sandner et al., 1993) Fos expression in neurons is most effectively manifested by robust, prolonged and/or repeated activation. The expression of Fos in the spinal dorsal horn following noxious simulation is mainly in lamina I, IIo, V, and VI, corresponding to the distribution of nociceptor terminals, and is different than that following non-noxious stimulation (Harris, 1998). In addition, the number of Fos-positive neurons in the spinal cord follows a dose-response relationship with formalin injection into the hind paw of rats (Kaneko et al., 2000).

ERK is a mitogen-acitvated protein kinase (MAPK), with phospho-ERK (pERK) being an indicator of cellular activation, and can be activated by, among other things, inflammatory cytokines (Alberts et al., 2002). ERK1 and ERK2 are activated by the upstream mitogen-activated ERK kinase (MEK) (Cano & Mahadevan, 1995; Seger & Krebs, 1995). They mediate short-term effects by phosphorylating kinases, receptors, and ion channels, and long-term effects by activating transcription factors such as CREB (Impey et al., 1999). The Src family of cytoplasmic tyrosine kinases includes Src itself and, via phosphorylation, activates kinase pathways, including ERK (Alberts et al., 2002). An overview of intracellular signaling including ERK and Fos is shown in **Figure 2**.

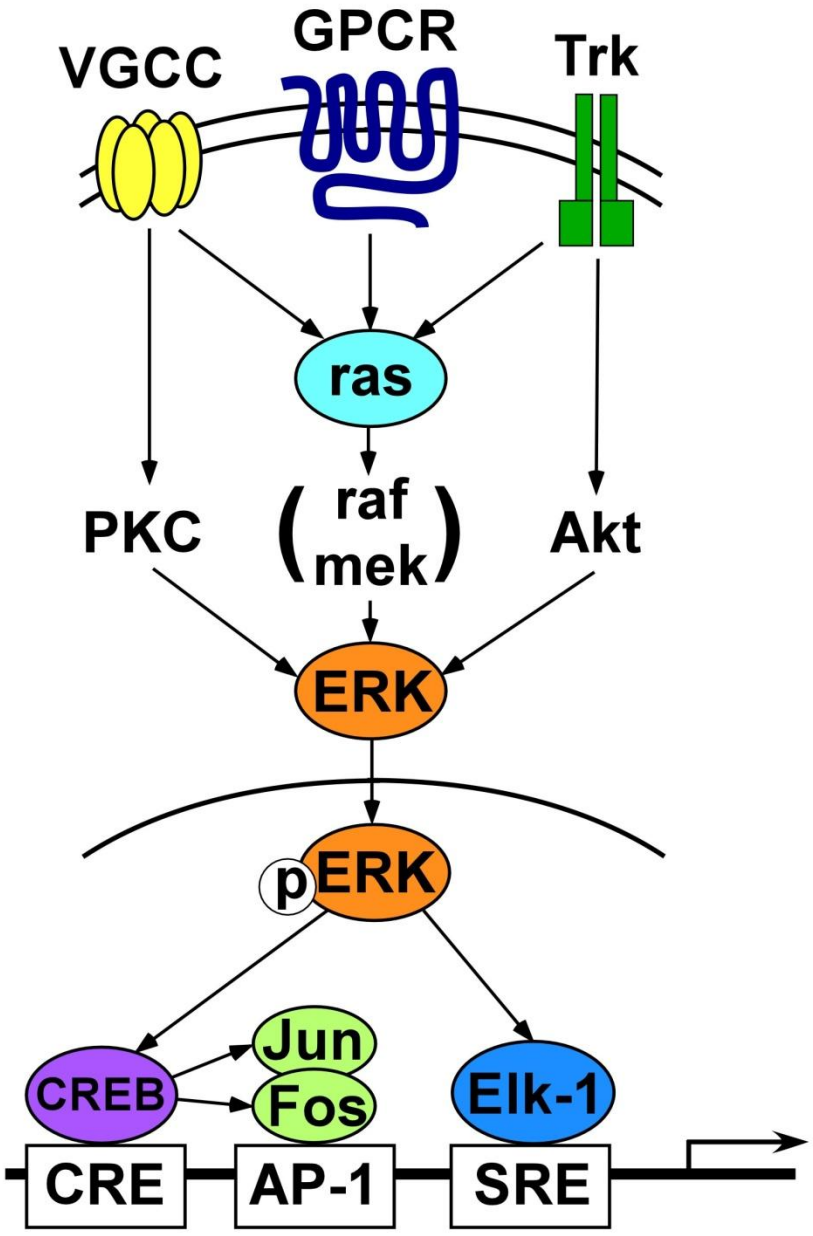


**Figure 2.** Fos & ERK signaling cascade.

*1.3.3 Central sensitization*

The phenomenon of central sensitization occurs during persistent inflammatory or neuropathic pain, and consists of facilitation of excitatory signaling and decreased inhibition (Woolf & Salter, 2000). This produces pain responses to normally innocuous stimuli (allodynia) and hyperalgesia in areas outside the tissue injury. A major component of this central sensitization is up-regulation of the N-methyl-D-aspartate (NMDA) receptor for glutamate. The enhancement of NMDA activity in the spinal cord could be due to the tyrosine kinase Src (Yu et al., 1997) or PKC, for example. Two mechanisms contribute to NMDA-mediated sensitization: 1. Reduced $Mg^{2+}$ blockade of NMDA channels, which is done by PKC, and 2. Enhanced NMDA function by converging signaling cascades from GPCRs, such as NK-1 or mGlu receptors, and receptor tyrosine kinases, for example the trkB receptor for brain-derived neurotrophic factor (BDNF) (Woolf & Salter, 2000). A point of convergence for these may be PKC, which indirectly potentiates NMDA currents in hippocampal neurons by activating Src (Lu et al., 1999). The MAPK pathway is also implicated in NMDA-mediated central sensitization (Woolf & Salter, 2000). In addition, PKC regulates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (glutamate receptor) channel membrane insertion in the spinal cord (Li et al., 1999). Another component of central sensitization is decreased spinal inhibition, which may be largely mediated by GABAergic interneurons (Woolf & Salter, 2000). In addition to modulation of central neurons, following inflammation, A-fibers begin expressing substance P and BDNF, which may increase the ability of stimulation of peripheral neurons to contribute to central sensitization (Woolf & Salter, 2000).

### *1.4 Experimental models of persistent pain*

Subcutaneous injection of formalin is a commonly used model of persistent inflammatory pain (Tjolsen et al., 1992). It is usually used in mice and rats, and may provide a better approximation of clinical chronic pain than traditional phasic nociceptive tests, such as acute radiant thermal, hot-plate, or mechanical paw withdrawal tests. Formalin is a dilute solution of formaldehyde, and, therefore, is a cross-linking agent at amino groups, disrupting proteins (Harvey, 1975; Metz et al., 2004). This induces tissue changes that are typical of inflammation such as mast cell degranulation, edema, and invasion of granulocytes. Formalin induces a biphasic pain-related behavioral response, separated by a quiescent interphase. The first (early) phase is due to C-fiber activation, while the second (late) phase is due to peripheral inflammation and central sensitization. This central sensitization is instigated by C-fiber activity, and is due to functional changes in the spinal cord (Coderre et al., 1990; Hunskaar & Hole, 1987). The behavioral responses that can be evaluated include lifting, licking, biting, and flinching of the injected paw. The flinching response seems to be a consistent component of the response, and is easy to observe and quantify. The other behavioral responses have been more problematic, and it has been suggested that flinching is less influenced by conditions that affect non-nociceptive behavior, and, thus, is a better end-point (Tjolsen et al., 1992).

### *1.5 Therapeutic approaches to the control of pain*

Current therapies for pain by and large still consist of either opioids (narcotics) or non-steroidal anti-inflammatory drugs (NSAIDs). Opioids target the opioid receptors, and their primary sites of analgesia are thought to be in the PAG and NRM. NSAIDs inhibit the cyclooxygenase (COX) enzymes, and newer NSAIDs are selective for certain subtypes of COX enzymes. Despite their wide use as analgesics, opioids and NSAIDs have quite a few deficiencies and problematic adverse effects (Schmidt, 2003). Opioids carry with them adverse effects such as drowsiness, dizziness, and vomiting, as well as significant toxicity and propensity for tolerance and addiction (Schmidt, 2003). NSAIDs can cause gastrointestinal ulcers and bleeding, and long-term use can produce liver and kidney toxicity. In addition, NSAIDs and opioids are sometimes ineffective at providing complete analgesia, especially for moderate-severe pain, and opioids can be cross-tolerant to morphine for chronic pain (Schmidt, 2003). Thus, there are significant needs for development of analgesics that have reduced adverse effects and provide more personalized treatment of specific pain states. Clinical treatment with "antidepressants" such as selective serotonin reuptake inhibitors (SSRIs) or tricyclic antidepressants (TCAs) has provided analgesia for some chronic pain conditions, regardless of presence of depression (Guay, 2001). Many analgesics, anti-inflammatory drugs, and anti-migraine drugs are currently under development, some of which have novel mechanisms of action. Some of these emerging therapeutic strategies may be more appropriate for treating women with pain or depression.

### *1.6 Estrogen and its mechanistic contribution to pain sensation*

#### *1.6.1 Biology of estrogen*

Estrogens are endogenous steroid hormones that have various physiological functions. In females, these functions include developmental effects such as sexual differentiation, control of ovulation, and actions on processes that effect body composition, for example mineral, carbohydrate, protein, and lipid metabolism (Loose-Mitchell & Stancel, 2001). The most abundant and potent estrogen occurring in humans is 17β-estradiol (estradiol, E2), followed by estrone and estriol. The most common pharmacological uses of estrogens are oral contraception and hormone replacement therapy in postmenopausal women (Loose-Mitchell & Stancel, 2001).

#### *1.6.2 Estrogen metabolism*

Steroid hormones are made from cholesterol (See **Figure 3**), and synthesis of steroids (steroidogenesis) takes place in the adrenal gland, ovaries, testes and placenta. Cholesterol can come from the diet or can be synthesized from acetate. Androstenedione is a common precursor for the sex hormones (androgens and estrogens), and can be converted into testosterone, an androgen, or estrone and then estradiol. Testosterone then can be converted into 5α-dihydrotestosterone (DHT) or E2. The conversion of adrostenedione to estrone and of testosterone to E2 is catalyzed by aromatase. Aromatase is a cytochrome P450 monooxygenase enzyme complex (CYP19A1), which uses NADPH and molecular oxygen as cofactors (Simpson et al., 1994). Aromatase is located in the endoplasmic reticulum of ovarian granulosa cells, testicular sertoli and leydig cells,

stromal cells of adipose tissue, placental syncytiotrophoblasts, bone, skin, hair, and most areas of the brain (Simpson et al., 1999). Aromatase is expressed in neurons but not glial cells (Lephart, 1996). Since aromatase is found in the nervous system, circulating androgens can be converted to estrogens and, thus, exert estrogenic effects on the local nervous tissue.

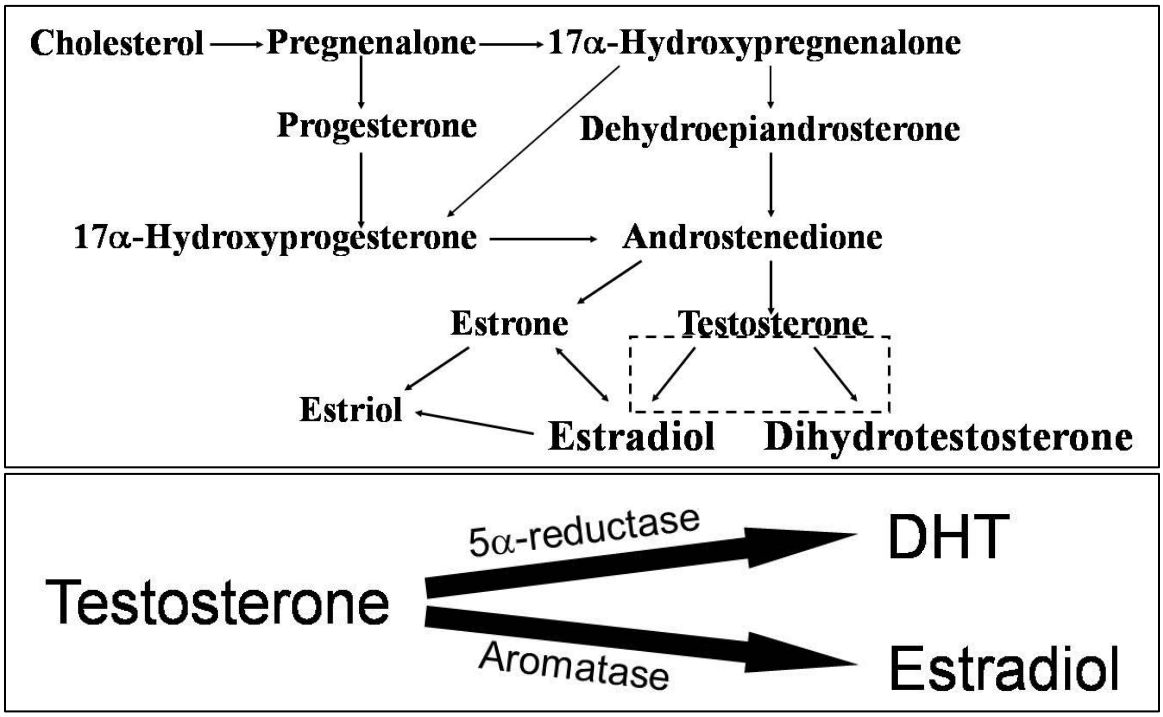


**Figure 3.** Estrogen and androgen steroid synthesis.

Ovaries are the primary source of estrogen in premenopausal women. In the ovaries, theca cells secrete androgens, which are then converted to estrogens, especially E2, and secreted by the granulosa cells. Gonadal hormone synthesis and aromatase activity is stimulated by gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary that bind to GPCRs that stimulate cAMP production. Secretion of LH and FSH is stimulated by release of gonadotropin releasing hormone (GnRH) from the hypothalamus, which is regulated by feedback of estrogen. In men and postmenopausal women, the primary source of estrogen is the adipose tissue stroma, which produces estrone (Loose-Mitchell & Stancel, 2001).

Most circulating estrogens are bound to serum albumin with low affinity, or sex hormone-binding globulin (SHBG) with high affinity. Rats do not express a protein equivalent to SHBG. Estrogens are metabolized in the liver by oxidation or conversion to glucuronides or sulfates, and excreted in the urine. In adult female rats, the half-life of serum estradiol is about 4 minutes (Noe et al., 1992). This short half-life is consistent with the observation by Lesclous et al. (2001) that endogenous serum estradiol levels drop markedly within 4 days of ovariectomy (OVX) in adult Sprague-Dawley rats.

### *1.6.3 Mechanisms of estrogen signaling*

Two main mechanisms of estrogen action are "genomic" and "nongenomic." Genomic actions are more delayed and prolonged, whereas nongenomic actions are rapid and more transient. Classical estrogen actions are characterized as genomic and are carried out by the nuclear estrogen receptors (ERs), members of the nuclear

steroid/thyroid receptor superfamily. Nongenomic actions may be carried out by ERs or a putative membrane-bound G-protein coupled estrogen receptor, mER (Qiu et al., 2003). Estrogen actions via second messengers may have either genomic or nongenomic mechanisms, each of which may be from classical ERs or the putative mER. An overview of estrogen signaling mechanisms is shown in **Figure 4**.

Two subtypes of nuclear ER have been identified: ERα and ERβ. These ERs are transcription factors that, upon ligand binding, form homo- or hetero-dimers, translocate to the nucleus, and regulate transcription of target genes by binding to specific DNA elements. These DNA elements are called estrogen response elements (ERE), and are *cis*-acting enhancers located in promoter regions of target genes. The DNA-bound ER dimer interacts with the transcription apparatus either directly or indirectly via cofactors (Hall et al., 2001). The major functional domains of the ERs, from amino- to carboxyl-terminus, are a ligand-independent transcription activation function-1 (AF-1) domain, a DNA binding domain with two zinc fingers essential for binding, a hinge, and a ligand-dependent transcription activation function-2 (AF-2) (Nadal et al., 2001). The ERs can also regulate transcription of genes at sites other than EREs. This is done by interaction of the activated ER with other transcription factors, such as the activator protein-1 (AP-1) complex of Fos/Jun, thereby up-regulating transcription (Hall et al., 2001). However, the human ERβ cannot activate transcription at AP-1 sites, due to its lack of the AF-2 domain (Hall et al., 2001). In addition, ERs can be activated by phosphorylation due to extracellular growth factor activation of intracellular kinases, and this ligand-independent activation modulates binding to EREs and transcription (Hall et al., 2001). The distribution of ERα and ERβ in the body differ greatly, with moderate to high expression

of ERα in pituitary, kidney, epididymis, and adrenal, moderate to high expression of ERβ in prostate, lung, and bladder, and overlapping high expression in brain, ovary, testis, and uterus (Kuiper et al., 1997; Kuiper et al., 1998; Shughrue et al., 1996).

There are limited and controversial findings on estrogen action at the cell membrane. Some reports suggest the presence of a novel membrane-bound estrogen receptor, mER, while others demonstrate membrane locality and function of classical ERs. Early studies showed antibody labeling of ERα at or near the cell surface, and in membranes of Chinese hamster ovarian cells transfected with ERα and ERβ, estradiol activated $G_{\alpha q}$ and $G_{\alpha s}$, which rapidly stimulated inositol phosphate production and adenylyl cyclase activity (McEwen & Alves, 1999). Also, ERα and ERβ have nongenomic activity in caveolae, stimulating eNOS activity (Chambliss et al., 2002). In contrast, there are reports of a novel mER that is coupled to $G_{\alpha q}$ and stimulates phospholipase C, leading to stimulation of PKCδ and PKA (Qiu et al., 2003; Qiu et al., 2006). This activity was coupled to rapid desensitization of $GABA_B$ and μ-opioid receptors in hypothalamic neurons (Qiu et al., 2003; Qiu et al., 2006). Furthermore, a non-steroidal compound, STX, has been synthesized (Tobias et al., 2006), which selectively stimulates this mER and has no binding affinity for nuclear ERα or ERβ. STX was more potent than E2 at mediating this desensitization, and both E2 and STX were fully efficacious in ERα,β knock-out mice (Qiu et al., 2006).

In addition to these findings, there are several second messenger systems that could mediate estrogen effects. One of these is increased cAMP. Estrogen increased cAMP levels in MCF-7 or uterine cells in culture and, in some brain regions, increased phosphorylation of CREB via a cAMP-dependent mechanism (Aronica et al., 1994; Gu et

al., 1996; Zhou et al., 1996). This represents a pathway for gene regulation by estrogen through phosphorylation of DNA-binding proteins such as CREB. Mitogen activated protein kinase (MAPK) activity is another possible second messenger mechanism of estradiol action. Several reports have shown that ERs (and androgen receptors) directly interact with and stimulate Src, and stimulate ERK phosphorylation (Harrington et al., 2006; Kousteni et al., 2001; Migliaccio et al., 2000). There is also evidence that estrogen-dependent ERK-1 and ERK-2 phosphorylation may not involve a classical ER (McEwen & Alves, 1999). Yet another second messenger pathway for estrogen action is via calcium homeostasis. In one example, estradiol rapidly inhibits L- and N-type high-voltage-activated (HVA) $Ca^{2+}$ channel currents (Lee et al., 2002; Mermelstein et al., 1996). This effect was nongenomic and seemed to be mediated by a unique ER on the cell surface (McEwen & Alves, 1999). There are also reported estrogen effects on calcium currents that appear to be due to intracellular, genomic action, requiring protein synthesis (McEwen & Alves, 1999).

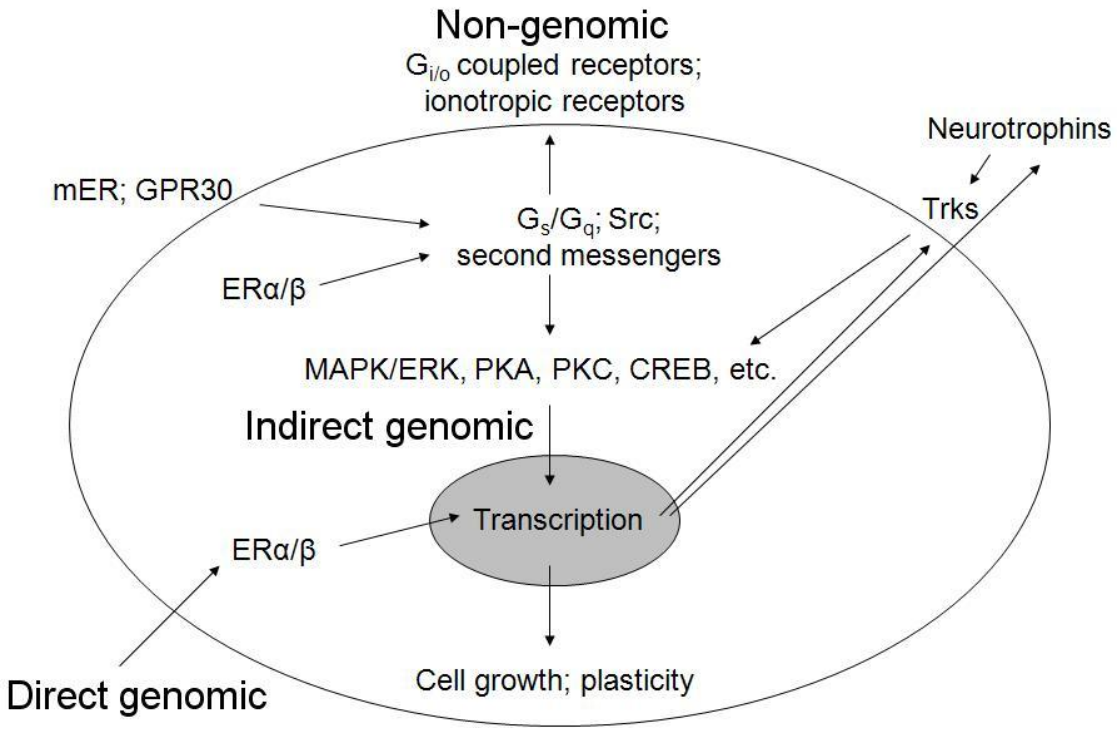


**Figure 4.** Estrogen signaling pathways/mechanisms.

*1.6.4 Estrogens and the nervous system*

There are estrogen receptors (ER) (Kato et al., 1994) and androgen receptors (AR) distributed throughout the (rat) central and peripheral nervous system, largely in neurons (Melcangi et al., 2005; Patrone et al., 1999; Simerly et al., 1990). The distribution of ERα and ERβ within the central nervous system is differential, and initial reports indicated that ERβ was more widely distributed, with overlapping areas (Shughrue et al., 1997). There is much higher expression of ERα mRNA in the amygdala and ventromedial hypothalamic nucleus, whereas ERβ mRNA has higher expression in cerebellum, hypothalamus, cerebral cortex, and hippocampus. In the hippocampus, ERα is expressed, but at a much lower level, and both ERs are distributed evenly in the frontal

cortex (Shughrue et al., 1997). The bed nucleus of the stria terminalis, preoptic area, and medial amygdala co-express ERα protein and ERβ mRNA, while in other regions such as the arcuate, ventromedial nucleus and cortical amygdala, co-expression of ERα and ERβ is very rare (Woolley, 1999). A moderate amount of cells expressing ERβ were found in the spinal cord lamina II, and a scattered expression of ERα was found in laminae I, II, VI, VII, and X (Shughrue et al., 1997). Sohrabji et al. (1994) showed that ERα is expressed in small DRG neurons, while ERβ is expressed in large, medium, and small DRG neurons (Taleghany et al., 1999).

Estrogens have numerous and varied effects on nervous system functions. For instance, estrogens affect verbal and spatial memory, motor skills, and appear to have neuroprotective effects in culture and in conditions such as Alzheimer's disease and Parkinson's disease, among others (Gillies et al., 2004; McEwen & Alves, 1999). Sex differences in and estrogen effects on the serotonergic, cholinergic, dopaminergic, and noradrenergic systems may all contribute to affective state, movement disorders, and cognitive function (McEwen & Alves, 1999).

There is permanent developmental sexual differentiation of the nervous system, influencing both neuronal wiring and responses to hormonal regulation of gene expression (McEwen & Alves, 1999; Morris et al., 2004). Many of the effects of estrogens on the nervous system differ qualitatively or quantitatively between the sexes, implying that they may be due to sexual differentiation during pre- or post-natal development and/or by different levels of sex hormones (McEwen & Alves, 1999). In fact, estrogen itself imparts many developmental (organizational) effects and transient (activational) effects during adulthood on the nervous system (Becker et al., 2005; Morris

et al., 2004). In many regions of the developing and adult brain, estrogen has actions on structural development, synaptogenesis, and morphological plasticity (Beyer, 1999). Estrogen promotes neurite outgrowth, dendritic spine formation, and synapse formation (Murphy et al., 1998; Toran-Allerand, 1991; Woolley & McEwen, 1993). Estrogen regulates neurogenesis in the dentate gyrus of the hippocampus, which produces new neurons in adulthood, and this effect fluctuates with the estrous cycle (Tanapat et al., 1999; Woolley & McEwen, 1992). A possible consequence of post-menopausal loss of estrogen in women is loss of synaptic connections in the hippocampus (Woolley & McEwen, 1992). In the anteroventral periventricular nucleus, males express more preproenkephalin mRNA than females, and the opposite is true for prodynorphin; females that are androgen sterilized at birth have male patterns of neuropeptide gene expression, indicating an important organizational role of estrogen (McEwen & Alves, 1999). On the other hand, expression of ERα in the hippocampus and dorsal raphe nucleus appears to be similarly regulated by estradiol in both sexes (Alves et al., 1998; Weiland et al., 1997).

There are numerous reports on the effects of gonadal hormones on sensory and nociceptive sensitivity, and the nervous system components that have these functions. Robinson and Short reported that the skin of the nipples, areolae, and breast had no sex differences in sensitivity prepubertally, while postpubertally sensitivity increased markedly in women but not men, indicating a role of increasing gonadal hormones (Robinson & Short, 1977). In rodents, estrogen injection increased the receptive field size of trigeminal mechanoreceptors (Bereiter et al., 1980). Estrogen and inflammation increases the excitability of rat temporomandibular joint afferent neurons in an additive manner (Flake et al., 2005). Dina et al. (2001) showed gender differences in PKC, PKA,

and NO signaling in epinephrine-induced hyperalgesia that were estrogen dependent. Estrogen has a vasodilatory action in vascular endothelium, regulating synthesis of NO, and thus may have a vascular influence in syndromes such as migraine headache. In the DRG, estrogen up-regulates expression of the trkA (tyrosine kinase) receptor for nerve growth factor (NGF), a neurotrophin, suggesting that estrogen may contribute to normal or pathological neuronal function (Sohrabji et al., 1994). Lee et al. (2002) showed that estradiol rapidly inhibited L- and N-type high-voltage-activated (HVA) $Ca^{2+}$ channel currents in DRG neurons, an effect that was greater in cells from female rats than males.

In the spinal cord, effects of gonadal hormones on pain have been mostly studied with relevance to pregnancy. Increased pain thresholds during pregnancy in rats and humans appear to involve an opioid-mediated mechanism, activated by sex hormones (Dawson-Basoa & Gintzler, 1993). Estradiol and progesterone activate a spinal dynorphin/κ-opioid analgesic system both pre-synaptically and post-synaptically (Aloisi, 2000; Dawson-Basoa & Gintzler, 1993). In female rats, ovariectomy and estradiol and/or progesterone treatment had variable effects on opioid anti-nociception, but E2 tended to decrease μ-opioid anti-nociception (Stoffel et al., 2005). In intact male rats, intracerebroventricular estrogen decreased the formalin-induced paw jerk and increased the licking response; both effects were blocked by opioid receptor antagonists (Aloisi & Ceccarelli, 2000; Ceccarelli et al., 2004). Amandusson et al. (1999) demonstrated that in female rats ERs are expressed in the same area of the spinal cord as enkephalin-expressing neurons, and that estrogen increases enkephalin mRNA levels in the spinal cord of OVX rats. Allen and McCarson (2005) demonstrated that estradiol potentiated increased BDNF gene expression in the spinal cord by formalin in OVX rats.

Gonadal hormones appear to have a significant effect on thalamic transmission (Aloisi, 2000). In certain areas of the brain, females exhibit greater activation from the same painful stimuli than males, suggesting differential supraspinal processing (Aloisi, 2000). Some effects of estrogen on pain processing may be due to actions at supraspinal sites, as discussed above. Estrogen has also been reported to have rapid effects on neuronal activity in the hippocampus, hypothalamus, cerebellum, cortex, and amygdala (McEwen & Alves, 1999).

### *1.7 Conclusion*

Multiple sites may exist for the enhancement of pain by estrogen. However, there is evidence to suggest that sex differences in, or estrogen modulation of, pain may be primarily due to supraspinal processes. Limbic system sensitization plays an important role in conditions such as multiple chemical sensitivity and depression, which are more prevalent in women, as well as post-traumatic stress disorder. The limbic system may similarly have a pivotal role in chronic pain, especially if levels of estrogen are associated with painful or stressful conditions. The observation that pain of psychological origin is more prevalent in women may support the importance of estrogen in modulating the limbic processing of pain. Importantly, the modulation of pain by estrogen appears to depend on the route of administration, dosing regimen, and pain model being used.

The major focus of this dissertation project was to determine the possible sites of action for estrogen contribution to sex differences in pain. To anatomically distinguish sites of estrogen enhancement of nociception, estrogen replacement was used in OVX

female rats. The formalin test was used as a model of persistent inflammatory pain, and biochemical and behavioral end-points were quantified. The results of this study implicate possible locations for estrogen enhancement of inflammatory pain. This information ultimately will shape the anatomical locations and biomolecular mechanisms that could be targeted by novel pain therapies for women.

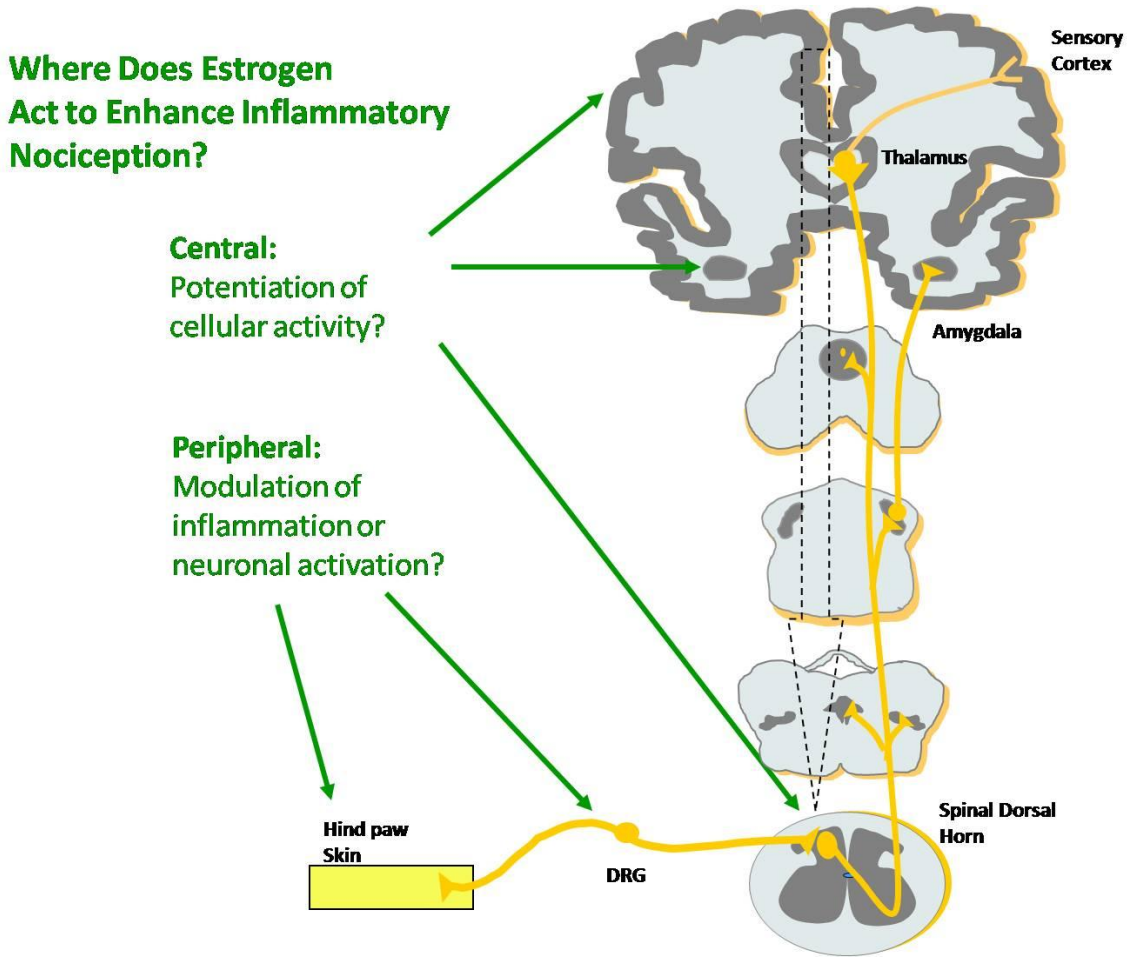


**Figure 5.** Schematic of anatomical sites where estrogen may alter inflammatory nociception.

# *CHAPTER TWO*

# *STATEMENT OF PURPOSE*

**This dissertation project had the purpose of testing the overall hypothesis that sex hormones have activational effects on pain that contribute to sex differences in the experience of chronic pain.**

Chronic pain leads neurological disorders in the US in terms of the total number of cases and their annual economic cost (Carey, 2005; Loeser, 2001; Stucky et al., 2001). Sometimes pain outlasts its usefulness as a warning/protective system (Julius & Basbaum, 2001), and becomes chronic and potentially debilitating through maladaptive processes. Chronic pain can result in anxiety, depression, and reduced quality of life (Hunt & Mantyh, 2001).

The incidence or severity of many chronic pain disorders, but not others, is higher in women than men, and for experimentally delivered stimuli, females have lower thresholds, higher pain ratings, and less tolerance of noxious stimuli than males (Berkley, 1997). In addition, chronic pain often is accompanied by depression, and vice versa, indicating an association between the two disorders (Blackburn-Munro & Blackburn-Munro, 2001; Fishbain et al., 1997; King, 1991), and depression is about twice as prevalent in women than men (Bromberger, 2004).

Many painful disorders vary in their incidence, disappearance, and prevalence as a function of menstrual stage, puberty, pregnancy, menopause, and age (Berkley, 1997). This is indirect clinical evidence that ovarian hormones such as estrogen may alter pain sensitivity. There is also experimental evidence for this as well. Highly relevant to this research proposal, responses to inflammatory pain, both clinically and experimentally, vary with the estrous cycle of the female (Bradshaw et al., 2000; Fillingim et al., 1997;

Hellstrom & Anderberg, 2003; Isselee et al., 2002; Lautenbacher & Rollman, 1993; LeResche et al., 2003; Somerville, 1972; Tall & Crisp, 2004).

Numerous reports have addressed the modulation by estrogen of biochemicals involved in nociceptive neuronal signaling or behavioral responses. However, there are no reports of direct, systematic investigation into identification of the anatomical sites of action in the nervous system of the enhancement of nociception and pain by estrogen. Thus, this research program had the purpose of determining the potential contribution of activational effects of sex hormones to sex differences in pain conditions, and was specifically designed to test the overall hypothesis that ***sex differences in pain sensation are due, at least in part, to activational effects of sex hormones on peripheral, spinal and/or supraspinal sites involved in the transmission and perception of nociceptive stimuli.*** To test this hypothesis, established models of pain were employed, and biochemical and behavioral end points were quantified. The resulting data provide novel and significant information pertaining to sex hormone modulation of nociceptive processing, and establish specific CNS sites where estrogen may enhance pain sensation in females. Ultimately, these findings may have impact human health by helping identify novel strategies for the management of pain syndromes that are more prevalent or intense in women.

***2.1 Specific Aim #1:*** *Determine whether short-term exposure to androgens during adulthood alters pain-related behavior.*

Several emerging lines of evidence support the existence of sex differences in the sensory experience of pain. For example, the incidence or severity of many chronic pain

disorders is higher in women than men, but men experience gender-selective pain syndromes as well. Additionally, sex differences in nociception and analgesia have been demonstrated, as has their modulation by estrogens, androgens and other steroids. Gonadal steroids such as estrogens and androgens may produce these differences through effects on sexual development or through activational effects in the adult animal. However, little is known about the activational effects of male gonadal hormones on nociception. Previous reports have suggested that males are less sensitive to stimulus-evoked nociception. These observations suggest that male gonadal hormones have anti-nociceptive, activational effects in males.

To test the activational effects of androgen on nociception, experiments in this aim investigated the activational effects of male gonadal hormones on nociception-related responses to phasic or persistent noxious stimuli in male rats. We hypothesized that *male gonadal hormones produce anti-nociceptive effects*. To this end, studies in this aim used adult male rats and evaluation of pain-related behavioral responses in several well-characterized rodent models of phasic or persistent pain. Concurrently, male gonadal hormones were manipulated by gonadectomy and/or testosterone replacement. The goal of these experiments was to reveal what activational effects androgens have on pain of phasic or persistent origin. Thus, these studies were carried out to gain insight into the specific contribution of male gonadal hormones to sex differences in pain sensation.

***2.2 Specific Aim #2:*** *Determine whether short-term exposure to estrogen during adulthood increases nociception.*

Estrogens have been demonstrated to have activational, often pro-nociceptive (pain-potentiating), effects on sensory function. Previous results from our laboratory suggested that sustained, long-term estrogen replacement in ovariectomized females enhances formalin-induced pain-related behavior. We hypothesized that *short-term estrogen replacement in ovariectomized females would similarly enhance formalin-evoked persistent inflammatory nociception.* Ultimately, this aim set out to provide supporting evidence of the overall hypothesis that sex differences in pain sensation are due, at least in part, to activational effects of estradiol.

To determine whether activation in response to inflammatory pain was increased by acute, activational effects of estrogen, experiments in this aim examined the intensity of nociceptive behaviors evoked by formalin (a model of persistent inflammatory pain). These nociception-related behaviors were measured as a biological proxy for the degree of the overall nociceptive state. The formalin test is a well-established, widely used model of persistent inflammatory pain (Porro & Cavazzuti, 1993; Tjolsen et al., 1992). The noxious formalin stimulus results in peripheral inflammation and central sensitization that more closely approximates the persistent pain experienced with chronic inflammatory conditions than traditional phasic nociceptive tests. These experiments quantified nociception-related behaviors, especially flinching of the inflamed hind paw, and other behaviors monitored by actometry, following formalin injection, and investigated whether short-term estrogen exposure increased these behavioral responses in adult, female rats.

Activational effects of estrogen on this persistent, inflammatory nociception were investigated using ovariectomy and a single injection of estradiol in adult female rats. A

preliminary experiment was designed to establish the efficacy of a single injection of estradiol (by measuring uterus weight), which would be the estrogen manipulation model for experiments in this aim and future experiments. Estrogen manipulation was designed to be relatively short-term with respect to timing prior to application of the formalin pain model. These estrogen manipulations were designed with the purpose of emulating the short-term fluctuations in symptoms of chronic pain that occur in synchrony with the menstrual cycle.

The goal of experiments in this aim was to establish evidence for short-term, activational estradiol-mediated enhancement in the inflammatory pain model. With this established, experiments could then move forward to investigate where in the nervous system this modulation takes place.

***2.3 Specific Aim #3:*** *Determine whether short-term exposure to estrogen during adulthood increases nociception in peripheral, spinal and/or supraspinal sites of the nervous system.*

Previous reports and results from Specific Aim #2 suggest that elevated serum estrogen levels enhance persistent inflammatory nociception. However, there were no reports of any direct, systematic investigation into identification of the anatomical sites of estrogens' action in the nervous system to enhance nociception and pain. We hypothesized that *sex differences in pain sensation are due, at least in part, to activational effects of estradiol on peripheral, spinal and/or supraspinal sites involved in the transmission and perception of nociceptive stimuli*. Emphasis was placed on determining which specific sites largely contribute to estrogen-induced enhancement of

nociception. Thus, studies in this aim had the purpose of determining whether estrogen increased neuronal activation in specific, nervous system sites driven by inflammatory nociception.

These experiments measured the levels of biomarkers of neuronal activation evoked by intraplantar formalin, a stimulus that models the persistent inflammatory pain experienced with chronic pain conditions. Two widely-used biological proxies for the degree of nociceptive activation or throughput (Fos or phospho-ERK) were measured in the spinal cord, amygdala, hippocampus and nucleus accumbens as initial sites of investigation. Initially, the spinal cord was chosen as a site of investigation because it is the site of the "first synapse" where signals from the primary afferent neurons are processed. Subsequently, supraspinal sites were investigated based on their being limbic structures associated with the regulation of affect or nociception.

Activational effects of estrogen on this neuronal activation were investigated using ovariectomy and a single injection of estradiol in adult, female rats. Estrogen manipulation was designed to be relatively short-term with respect to timing prior to application of the formalin pain model. As in the previous aim, these estrogen manipulations were designed to emulate short-term cyclic fluctuations in chronic pain symptoms across the menstrual cycle.

# *CHAPTER THREE*

## *GONADECTOMY INCREASES MECHANICAL NOCICEPTION IN A NEUROPATHIC PAIN MODEL, AND TESTOSTERONE DECREASES THERMAL NOCICEPTION: STUDIES IN MALE RATS*

### *3.1 Abstract*

Experiments in this chapter investigated the effect of male gonadal hormones on phasic or persistent noxious stimuli in male rats.

Little is known about the activational effects of male gonadal hormones on nociception. Previous reports have suggested that males tend to be less sensitive to acute nociceptive stimuli. We hypothesized that *endogenous testosterone and/or its metabolites, dihydrotestosterone (DHT) or estradiol (E2), have anti-nociceptive, activational effects in males*. To test this hypothesis, persistent pain models used included spared nerve injury (SNI) and formalin injection; phasic stimuli were thermal (analgesiometer) and, following SNI, mechanical (von Frey). To test the effect of androgen on phasic thermal sensitivity, adult male rats were placed into three groups: 1) gonadectomized (GDX); 2) GDX receiving 20 mg testosterone propionate (TP) pellets implanted subcutaneously (GDX + 20 mg TP); and 3) gonadally intact naïve controls. One week after GDX, behavioral evaluation of acute nociceptive thermal hind paw withdrawal thresholds was performed using a thermal analgesiometer. Serum levels of DHT and E2 were subsequently determined. In other experiments, the effect of gonadectomy was investigated in the formalin model and the spared nerve injury (SNI) model; male rats were either GDX or gonadally intact. Dilute formalin was injected into the plantar hind paw, and paw flinches were quantified. For SNI, mechanical thresholds were determined over several days following surgery. Results of the testosterone replacement experiment, as well as a separate, one-week time-course experiment, showed no difference in thermal withdrawal latency between GDX and naïve rats. However, GDX + 20 mg TP rats had longer withdrawal latencies than naïve or GDX rats. Serum

DHT levels in naïve and GDX + 20 mg TP rats were not different, but were higher than GDX rats. Importantly, serum E2 levels were not changed by any manipulation. These results suggest that testosterone or DHT has an activational, hypoalgesic effect on acute thermal nociception in males that is not likely due to the effects of E2 produced by metabolism of testosterone. No effect of androgen was observed on formalin-induced nociceptive behavior. Also, gonadectomy in the context of the SNI model increased mechanical nociceptive sensitivity contralateral to the SNI, indicating an anti-nociceptive effect of male gonadal hormone(s) possibly through modulation of descending control. These observations taken together demonstrated androgen had a hypoalgesic effect on phasic thermal stimulus and an anti-nociceptive effect on phasic mechanical nociception contralateral to a persistent neuropathic pain model. Thus, it is evident that androgen has activational effects on pain of phasic thermal and persistent neuropathic origin, but perhaps not persistent inflammatory origin. These results provide evidence that androgens, as well as estrogens, may contribute to sex differences in pain sensation.

### *3.2 Introduction*

Chronic pain leads neurological disorders in the United States in terms of incidence and annual economic impact (Carey, 2005; Loeser, 2001; Stucky et al., 2001). Several emerging lines of evidence support the existence of sex differences in the sensory experience of pain. For example, the incidence or severity of many chronic pain disorders is higher in women than men, and for experimentally delivered stimuli, females have lower thresholds, higher pain ratings, and develop less tolerance toward noxious stimuli than males (Berkley, 1997). Many painful disorders show prevalence in women (*e.g.* migraine headache, fibromyalgia, irritable bowel syndrome, temporomandibular

joint disorder), but men experience gender-selective pain syndromes as well (*e.g.* cluster headache, Raeder's paratrigeminal syndrome, post-herpetic neuralgia, ankylosing spondylitis) (Berkley, 1997). Gonadal steroids such as estrogens and androgens may produce these differences through effects on sexual development or through activational effects in the adult animal.

While sex differences in the prevalence and intensity of pain syndromes have gained increased attention, very little is currently understood about the specific biological mechanisms underlying the modulatory roles of gonadal hormones such as androgens. Studies of gonadal steroid modulation of pain sensation to date have largely focused on the activational effects of estrogen in adult females; little is known about the activational effects of male gonadal hormones on nociception. Sex differences in nociception and analgesia have been demonstrated, as has their modulation by estrogens, androgens and other steroids. Previous reports suggest that males are less sensitive to stimulus-evoked nociception. For example, Lautenbacher and Rollman (1993) reported women having lower pain thresholds and higher pain ratings than men for electrocutaneous stimuli, and Barrett et al. (2002) demonstrated that male rats had higher mechanical nociceptive thresholds than females. Studies indicate that male rats develop more robust morphine-induced anti-nociception than females (Cicero et al., 1996; Wang et al., 2006). Numerous other reports conclude that gonadal steroids can affect nociception or opioid-induced anti-nociception in an activational manner. Specifically, androgen manipulation activationally increases nociceptive withdrawal latencies of rats or birds receiving transient thermal stimuli (Edinger & Frye, 2005; Forman et al., 1989; Frye & Seliga,

2001; Hau et al., 2004), and testosterone administration alters morphine-evoked thermal anti-nociception in a sexually dimorphic manner in rats (Stoffel et al., 2003).

Androstenedione is a common precursor for the sex hormones (androgens and estrogens), and can be converted into testosterone (an androgen) via 17β-hydroxysteroid dehydrogenase or into estrone via aromatase and then estradiol (E2) via 17β-hydroxysteroid dehydrogenase. Testosterone then can be converted into either 5α-dihydrotestosterone (DHT), the most potent androgen, via 5α-reductase or E2 via aromatase. All of these steroids are found in circulating blood, and thus are available to affect all tissues, including all locations of the nervous system. This arrangement of testosterone metabolism means that when testing the effects of testosterone, it is important to consider the possible effects of its major metabolites such as DHT and E2.

The above (and other) observations suggest that testosterone and/or its metabolites, DHT or E2, have anti-nociceptive, activational effects in males. Accordingly, the current study used adult male Sprague-Dawley rats and behavioral evaluation of acute nociceptive thermal hind paw withdrawal thresholds following gonadectomy and testosterone replacement to characterize the specific contribution of male gonadal hormones to sex differences in pain sensation. The effect of gonadectomy on formalin-induced persistent nociception was investigated. In addition, the effects of gonadectomy on thermal and mechanical sensitivity following the spared nerve injury (SNI) model were investigated.

The SNI model is a model of persistent neuropathic pain. It was developed by Decosterd & Woolf (2000) as a modification of other partial denervation models. The

model involves unilateral surgical transection of the common peroneal and tibial branches of the sciatic nerve, leaving the sural branch intact. The SNI model produces immediate (next day) and long-lasting mechanical hyperalgesia on the affected hind paw (Decosterd & Woolf, 2000). One study investigated sex differences in the SNI model, but reported no differences (Bourquin et al., 2006).

The formalin test is an established, widely used model of persistent inflammatory pain (Porro & Cavazzuti, 1993; Tjolsen et al., 1992). It is usually used in mice and rats, and may provide a better approximation of clinical chronic pain than traditional phasic nociceptive tests, such as acute radiant thermal, hot-plate, or mechanical paw withdrawal tests. Formalin induces a biphasic pain-related behavioral response, separated by a quiescent interphase. The first (early) phase is due to C-fiber activation, while the second (late) phase is due to peripheral inflammation and central sensitization. The behavioral responses that can be evaluated include lifting (flexing), licking, biting, and flinching of the injected paw.

Some previous studies have investigated sex differences or sex hormone effects on nociceptive behavior with the formalin model (Aloisi et al., 1994; Aloisi & Ceccarelli, 2000; Aloisi et al., 2004; Aloisi et al., 2000; Aloisi et al., 1995; Ceccarelli et al., 2004; Nayebi & Ahmadiani, 1999), with mixed results; some reported no effect, whereas some reported effects that were contradictory to each other.

### *3.3 Materials & methods*

This study was conducted using male Sprague-Dawley rats (Harlan, Indianapolis, IN). Animal facilities were temperature- and humidity-controlled with a 12-h dark–light cycle and food and water ad libitum. All surgical procedures and animal handling were performed in accordance with National Institutes of Health laboratory care standards and were approved by the University of Kansas Medical Center Animal Care and Use Committee. All testing, surgical procedures and handling of rats were performed during the light cycle.

In one experiment, male Sprague-Dawley rats (~10-13 weeks old, 290-360 g, Harlan, Indianapolis, IN) were randomly assigned to three groups: 1) gonadectomized (GDX; n = 4); 2) GDX receiving 20 mg testosterone propionate (TP) pellets implanted subcutaneously at the nape of the neck (GDX + 20 mg TP; n = 3); and 3) gonadally intact naïve controls (n = 5). One week after gonadectomy and hormone manipulation, behavioral evaluation of acute thermal nociceptive withdrawal threshold was performed using a thermal paw analgesiometer. Rats were housed one per cage beginning at least three days before nociceptive testing. Immediately following behavioral evaluation, rats were decapitated and trunk blood collected for serum steroid (DHT and E2) analysis. Results from rats in this experiment are included in the report of DHT levels in naïve, GDX and GDX + TP rats in the text and **Figure 7**.

A separate pilot experiment was conducted to determine the testosterone dose that yielded serum DHT concentrations similar to normal endogenous levels. Sixteen male Sprague-Dawley rats (~10-13 weeks old, 290-360 g, Harlan, Indianapolis, IN) were left

gonadally intact (naïve; n = 4) or were gonadectomized and received either no further hormonal manipulation (n = 3) or subcutaneous doses of 1, 5 or 10 mg TP (GDX + TP; n = 3 per group) in pelleted form. One week after gonadectomy and hormone manipulation, rats were decapitated and trunk blood collected for serum DHT analysis. Results from rats in this experiment are included in the report of DHT levels in naïve, GDX and GDX + TP rats in the text and **Figure 7**.

In a separate experiment, male Sprague-Dawley rats (~7-9 weeks old, 190-230 g, Harlan, Indianapolis, IN) were divided into two groups: 1) gonadectomized (GDX; n = 6); and 2) gonadally intact naïve controls (n = 5). A time-course of acute nociceptive thermal withdrawal threshold was conducted for one week following GDX using a thermal paw analgesiometer, as described below. Naïve control rats were housed two per cage, and GDX rats were housed one per cage for days 1-4 post-surgery and two per cage otherwise. Another set of rats was used for a similar time-course experiment, except the control group in this experiment received sham GDX surgery instead of being naïve. This set of rats was also used later for the spared nerve injury (SNI) experiment.

*3.3.1 Spared nerve injury (SNI)*

Left-side spared nerve injury (SNI) surgery was performed on rats according to methods described by Decosterd & Woolf (2000). Rats were anesthetized with a mixture of ketamine (64 mg/kg, i.p.; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (5.3 mg/kg; i.p.), and an incision was made in the biceps femoris muscle exposing the sciatic nerve. The common peroneal and tibial nerves were tight-ligated with 4.0 silk and cut

distal to the ligation, and 2-4 mm were removed from the remaining distal portion. The sural nerve was left intact. Muscle and skin were sutured closed separately.

Thermal withdrawal latencies for all four paws and tail were determined ~12 days after SNI using a thermal analgesiometer as described below. Rats were gonadectomized or left intact 8-9 days before SNI.

A time course of mechanical paw withdrawal threshold was performed in nerve-injured rats using Von Frey testing (described below). One week prior to baseline measurements, rats were gonadectomized or left intact. Baseline readings were taken 1 or 2 days prior to SNI surgery, and days represented are relative to SNI surgery. Mechanical withdrawal thresholds for hind paws were determined daily (except Day 2) up to 8 days, beginning one day after SNI. Mechanical withdrawal thresholds for all four paws were also determined ~12 days after SNI. Hind paws were tested on the lateral aspect and front paws were tested on their central portion.

*3.3.2 Hormone manipulation*

For gonadectomy (GDX), rats were anesthetized with a mixture of ketamine (64 mg/kg, i.p.; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (5.3 mg/kg; i.p.). Under aseptic conditions, a ventral midline incision (~1.5 cm long) was made into the lower abdominal cavity. Both testes were externalized, vas deferens and blood vessels ligated with suture just proximal to the testes, and testes removed with scissors. Adipose tissue, vas deferens, and blood vessels were reinserted into the abdominal cavity. Muscle and skin were closed with suture. For rats receiving testosterone replacement, an incision (~0.5-1 cm) was made in the skin at the nape of the neck during the anesthesia procedure

for gonadectomy. In these rats, pellets containing 1, 5, 10 or 20 mg testosterone propionate (TP; 21-day sustained release; Hormone Pellet Press, Leawood, KS) were inserted s.c., and skin closed with suture [method modified from Allen and McCarson (2005)]. The vehicle for 1, 5 or 10 mg TP pellets was cholesterol; the 20 mg TP pellet did not contain any vehicle. Gonadectomized or gonadally intact naïve controls did not receive any sham surgeries or steroid/vehicle. The day of GDX surgery was defined as day zero for all experiments, with the exception of the spared nerve injury (SNI) experiment.

### *3.3.3 Determination of serum dihydrotestosterone and estradiol content*

Serum levels of dihydrotestosterone (DHT) and estradiol (E2) were determined using radioimmunoassay kits according to the manufacturers' instructions (DSL-9600, Diagnostic Systems Laboratories, Webster, TX; and KE2D1, Diagnostic Products Corporation, Los Angeles, CA, respectively). Briefly, DHT was extracted from serum with 98% n-hexane: 2% ethanol, dried under nitrogen gas, and reconstituted in sample diluent. For E2, serum was added directly to assay tubes. All steroid samples and standards were assayed in duplicate. Both kits used $^{125}$I-labeled steroid for competitive binding to an antibody.

### *3.3.4 Nociceptive behavioral testing*

#### *3.3.4.1 Thermal withdrawal threshold*

Rats were placed in a clear plastic chamber on a thermal analgesiometer (University of California San Diego) with the glass surface thermostatically controlled at 30 °C and allowed to acclimate to the environment for approximately 10 minutes

[method adapted from Hargreaves et al. (1988)]. The radiant heat source of the analgesiometer was calibrated to elicit a latency time of approximately eight seconds for baseline or naïve rats, with a 20-second cut-off time to prevent tissue injury. Rats from each group were randomized, and multiple rats were tested at the same time, all within an hour. Three readings were taken on each body part plantar surface, allowing at least two minutes between testing of the same part. Except where individual paws are specified, the left and right hind paws were tested, and the resulting six readings were then averaged into one latency value per rat. For the testosterone replacement experiment, rats were tested on the seventh day after hormone manipulation. For the time-course experiments, baseline readings were taken 1-3 days before GDX surgery. Testing on the tail for the SNI experiment was done on approximately the middle 1/3 of the length of the tail.

*3.3.4.2 Formalin testing*

Male rats (the same set used in the thermal threshold time-course experiment) were gonadectomized (GDX) or left intact. Fifteen to 17 days after GDX, rats were injected with 100 µL of 5% formalin into the plantar side of the right hind paw. Flinches of the injected paw were counted 0-7 ("early" phase) and 30-41 (peak of the "late" phase) minutes post-injection. Statistical testing for differences of GDX vs. Naïve was done for each minute or for the sums over each time period using Student's t-test. n = 5 Naïve, 6 GDX.

*3.3.4.3 Mechanical withdrawal threshold (Von Frey)*

Rats were placed individually in plastic cages that allowed animals to move freely and that sat upon a wire mesh platform. Semmes-Weinstein monofilaments were applied

perpendicularly to the plantar surface with sufficient force to cause a slight bending of the monofilament in increasing, then decreasing order of intensity, according to an up-down paradigm [adapted from Brennan et al., (1996)] Monofilaments were applied 1-2 times and held for approximately five seconds. A withdrawal of the paw indicated a positive response. When a positive response was elicited 100% of the time from a monofilament, monofilaments were then presented in sequentially decreasing intensity. The monofilament with an intensity that elicited a response ~50% of the time was considered the threshold.

*3.3.5 Data analyses*

All data are shown as mean ± SEM. For the time courses of thermal withdrawal latencies, data were analyzed using a two-way, repeated-measures ANOVA with a post hoc (Holm-Sidak) multiple comparisons test. For DHT and E2 concentration data, and for data on the effects of testosterone treatment on thermal withdrawal latencies, data were analyzed using a one-way ANOVA with a post hoc (Tukey-Kramer) multiple comparisons test. For data on the effect of GDX on the formalin test, and with SNI, single-day thermal and mechanical thresholds, data were analyzed by Student's t-test. Data for the mechanical threshold time course with SNI were analyzed by two-way ANOVA with a post hoc (Holm-Sidak) multiple comparisons test. For all analyses, the significance level was set to $p \leq 0.05$.

### *3.4 Results*

Thermal nociceptive testing was performed daily (except for day five) for one week following GDX. Rats receiving sham GDX surgery (n = 6) did not show any change in thermal paw withdrawal latency at one day after surgery compared to the baseline latency of the same rats one day before surgery. Two-way repeated measures ANOVA revealed a significant main effect of time ($F[6, 70] = 12.89$, $p < 0.001$), but not GDX ($F[1, 75] = 0.47$, $p = 0.510$) on paw withdrawal latency. Results revealed a subtle but significant drift to longer thermal paw withdrawal latencies over the week-long time course for both naïve (n = 5) and GDX (n = 6) rats compared to their respective baseline latencies (**Figure 6**). This drift in responsiveness could be due to habituation/adaptation to testing over the week, or physical growth of the rats or changes in hind paw skin/footpad thickness. There was no difference in withdrawal latency between naïve and GDX rats on each day tested during the week-long time course. This finding implies the presence or absence of gonads as a factor does not change the acute thermal nociceptive paw withdrawal threshold during this first post-surgery week.

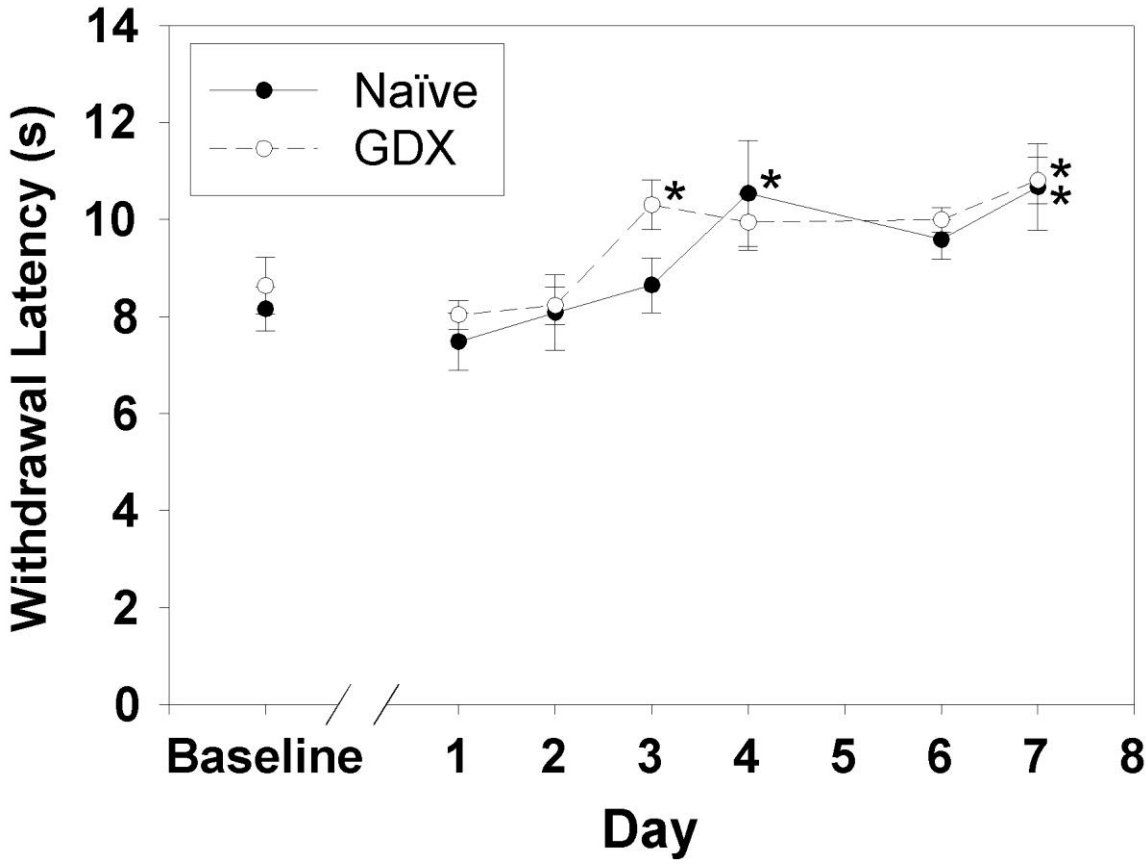


**Figure 6.** Time course of thermal paw withdrawal latency in gonadectomized (GDX) male rats. Rats were gonadectomized or left intact (Naïve, left panel; Sham GDX, right panel). Thermal withdrawal latencies were determined daily (except day 5) and averaged for left and right hind paws using a thermal analgesiometer. Results revealed increasing thermal paw withdrawal latencies in both naïve (n = 5) and GDX (n = 6) rats compared to their respective baseline latencies. Note that there was no difference in withdrawal latency between naïve and GDX rats on each day tested. These data indicate GDX did not affect withdrawal latencies. (*$p \leq 0.05$ vs. respective baseline, two-way repeated-measures ANOVA, Holm-Sidak post-hoc test). Data represent the mean ± SEM.

After one week of various hormonal manipulations, serum DHT and E2 concentrations of the rats were determined (**Figures 7 and 8**, respectively). The mean serum DHT concentration of naïve rats was 108.1 ± 27.2 pg/mL, which is in accordance with previously published levels for intact adult male rats (Overpeck et al., 1978). One-way ANOVA indicated a significant effect of treatment on serum DHT concentration ($F[5, 22] = 4.40$, $p = 0.006$). Tukey-Kramer post hoc analysis revealed that the serum

DHT levels of GDX rats receiving 20 mg TP pellets (GDX + 20 mg TP; n = 3) were not different from the endogenous levels of naïve controls (n = 9), yet the serum DHT levels in GDX rats (n = 7) were lower than those of either naïve or GDX + 20 mg TP rats (**Figure 7**).

| Treatment: | Serum DHT (pg/mL) |
|---|---|
| Naïve | 108.1 ± 27.2$^{\dagger}$ |
| GDX | 25.6 ± 2.1* |
| GDX + 1 mg TP | 23.5 ± 2.5 |
| GDX + 5 mg TP | 38.4 ± 8.2 |
| GDX + 10 mg TP | 59.1 ± 15.3 |
| GDX + 20 mg TP | 153.1 ± 18.9$^{\dagger}$ |

**Figure 7.** Table of serum dihydrotestosterone (DHT) concentrations in gonadectomized (GDX) male rats following androgen manipulation. After one week, results demonstrated that GDX reduced DHT levels compared to naïve. Note that the DHT level in GDX rats receiving 20 mg testosterone propionate (TP) was not different from the endogenous level of naïve controls, but was higher than that of GDX rats. These data indicate hormone manipulation significantly altered serum DHT levels. (*$p \leq 0.05$ vs. naïve. $^{\dagger}p \leq 0.05$ as compared vs. GDX, one-way ANOVA, Tukey-Kramer post hoc test; n = 9 naïve, n = 7 GDX, n = 3 each for all other groups). Data represent the mean ± SEM.

Analysis of serum estradiol levels revealed a mean serum E2 concentration in naïve rats of 3.96 ± 0.45 pg/mL, which is in accordance with previously published levels for intact adult male rats (Overpeck et al., 1978). One-way ANOVA indicated no significant effect of treatment on serum E2 concentration ($F[2, 9] = 2.93$, $p = 0.104$) of naïve (n = 5), GDX (n = 4), or GDX + 20 mg TP (n = 3) rats (**Figure 8**).

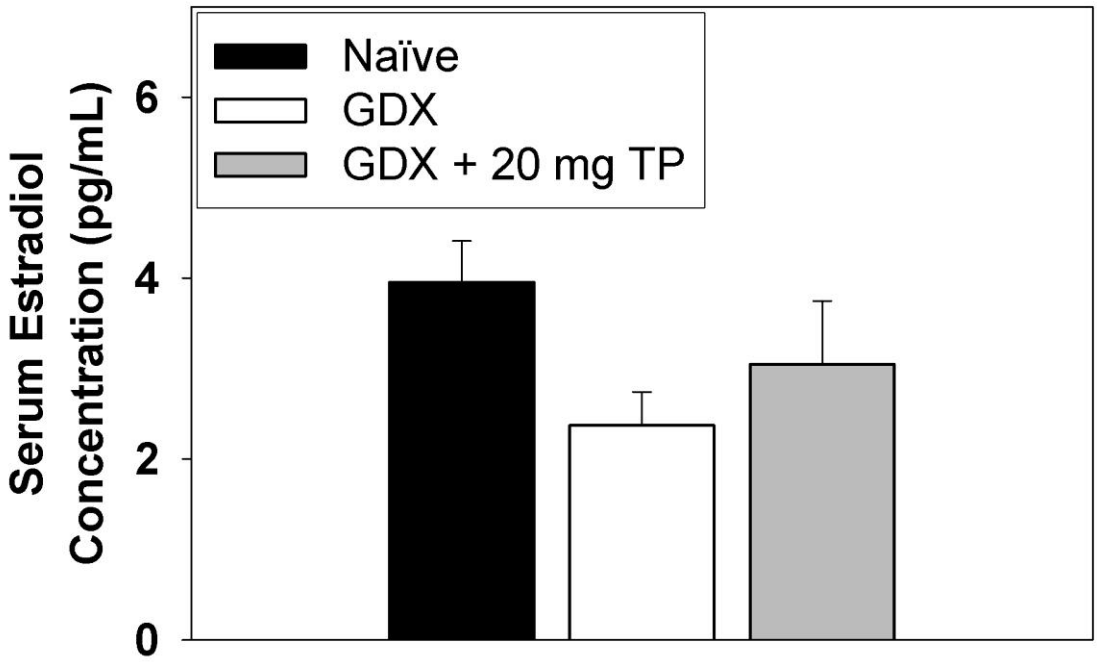


**Figure 8.** Serum estradiol (E2) concentrations in gonadectomized (GDX) male rats following androgen manipulation. Note that, after one week, serum E2 concentrations were not different for naïve (n = 5), GDX (n = 4), or GDX + 20 mg TP (n = 3) rats. These results confirm hormone manipulation did not alter serum E2 levels. ($p > 0.05$, one-way ANOVA). Data represent the mean ± SEM.

After one week of hormone manipulation, there was no significant difference in hind paw thermal withdrawal latency between GDX (n = 4) and naïve (n = 5) rats (**Figure 9**). One-way ANOVA indicated a significant effect of treatment on hind paw withdrawal latency ($F[2, 9] = 11.09$, $p = 0.004$), and Tukey-Kramer post hoc analysis revealed that GDX + 20 mg TP (n = 3) rats had a significantly longer thermal withdrawal latency (higher threshold) than naïve or GDX rats.

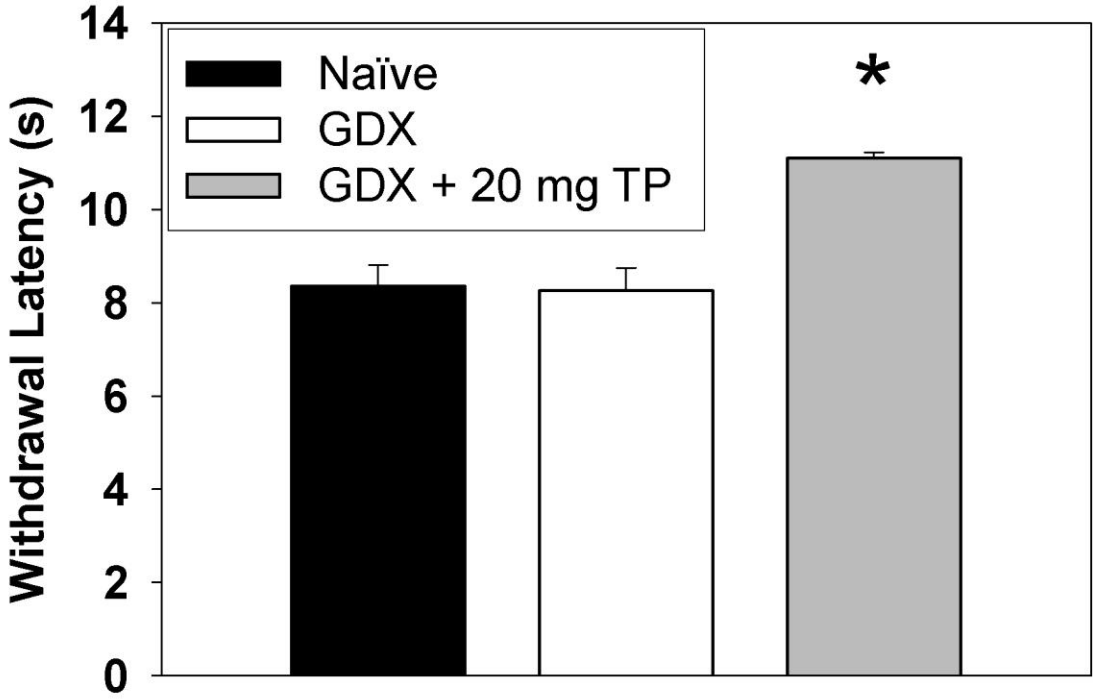


**Figure 9.** Thermal paw withdrawal latency in male rats of varying androgen status. Results showed that, after one week, whereas there was no difference in latency between gonadectomized (GDX; n = 4) and naïve rats (n = 5), administration of testosterone propionate (TP) pellets to GDX rats (n = 3) significantly increased thermal withdrawal latency. (*$p \leq 0.05$ vs. naïve or GDX, one-way ANOVA, Tukey-Kramer post hoc test). Data represent the mean ± SEM.

Hind paw flinches in rats injected with formalin were quantified in the early phase and peak of the late phase (**Figure 10**). Note there were no group differences between GDX (n = 6) and Naïve (n = 5) for any minute or summed over each time period (Student's t-test). These results indicate GDX did not affect formalin-induced flinching behavior.

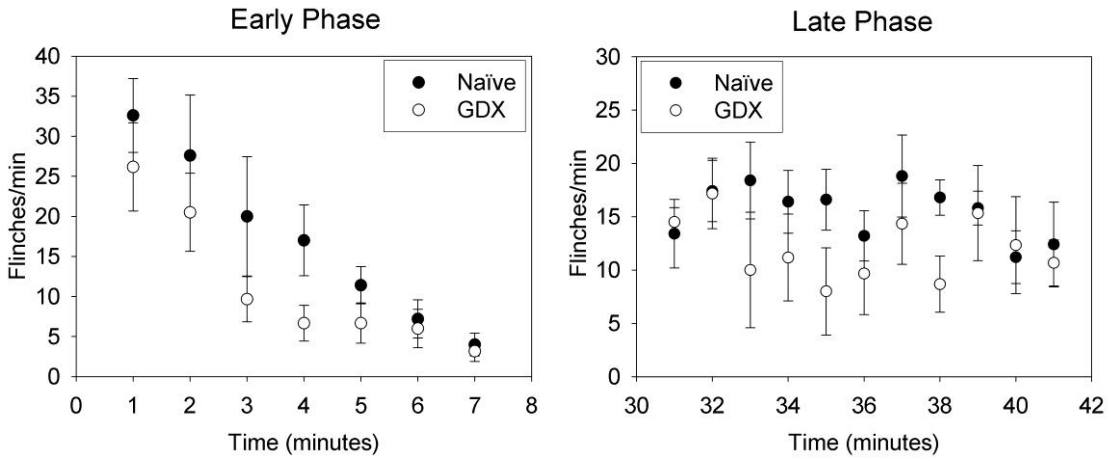


**Figure 10.** Effect of gonadectomy on flinching behavioral response in male rats during early (left) and late (right) phases following hind paw injection of 5% formalin. Flinches of the injected hind paw were counted 0-7 and 30-41 minutes post-injection of 100 µL 5% formalin into the plantar right hind paw. Note there were no group differences GDX vs. Naïve for any minute or summed over each time period (Student's t-test). Data represent the mean ± SEM; n = 5 Naïve, 6 GDX.

Thermal withdrawal latencies for all four paws and tail were determined ~12 days after SNI using a thermal analgesiometer (**Figure 11**). Results revealed that there was no difference in withdrawal latency between Sham GDX (n = 6) and GDX (n = 6) rats. These data indicate GDX did not affect thermal withdrawal latencies (thresholds) in the SNI model (Student's t-test).

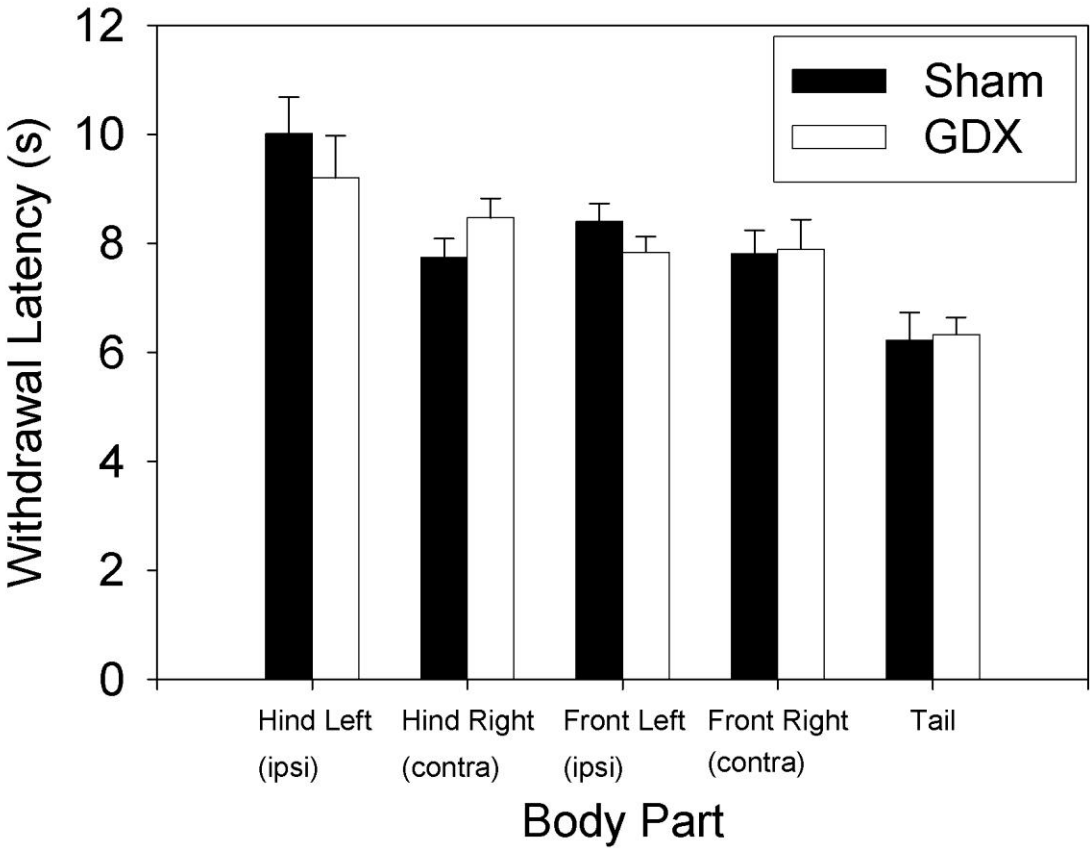


**Figure 11.** Thermal withdrawal latency in gonadectomized (GDX) male rats with left-side spared nerve injury (SNI). Rats were gonadectomized or left intact. Thermal withdrawal latencies for all four paws and tail were determined ~12 days after SNI using a thermal analgesiometer. Results revealed that there was no difference in withdrawal latency between Sham GDX (n = 6) and GDX (n = 6) rats. These data indicate GDX did not affect withdrawal latencies. ($p > 0.05$, Student's t-test). Data represent the mean ± SEM.

Results of Von Frey testing on hind paws (**Figure 12**) revealed that, ipsilateral to SNI, there was no difference in mechanical threshold between Sham GDX and GDX rats on each day tested or overall (two-way ANOVA with Holm-Sidak post-hoc test; n per group by day: Baseline = 6, Day 1 = 5, Day 3 = 5, Days 4-8 = 6). However, contralateral to the SNI, rats that received GDX overall had lower thresholds than Sham GDX. Also, ipsilateral to the SNI, thresholds were lower overall vs. Baseline, while there was no difference on the contralateral side, indicating efficacy of the SNI model. These data indicate GDX decreased withdrawal thresholds contralateral but not ipsilateral to SNI. Results of testing on day ~12 post-SNI revealed that there was no difference in withdrawal threshold between Sham GDX (n = 6) and GDX (n = 6) rats (Student's t-test) for all four paws (**Figure 13**). These data indicate no effect of GDX on mechanical withdrawal threshold was present at this time point.

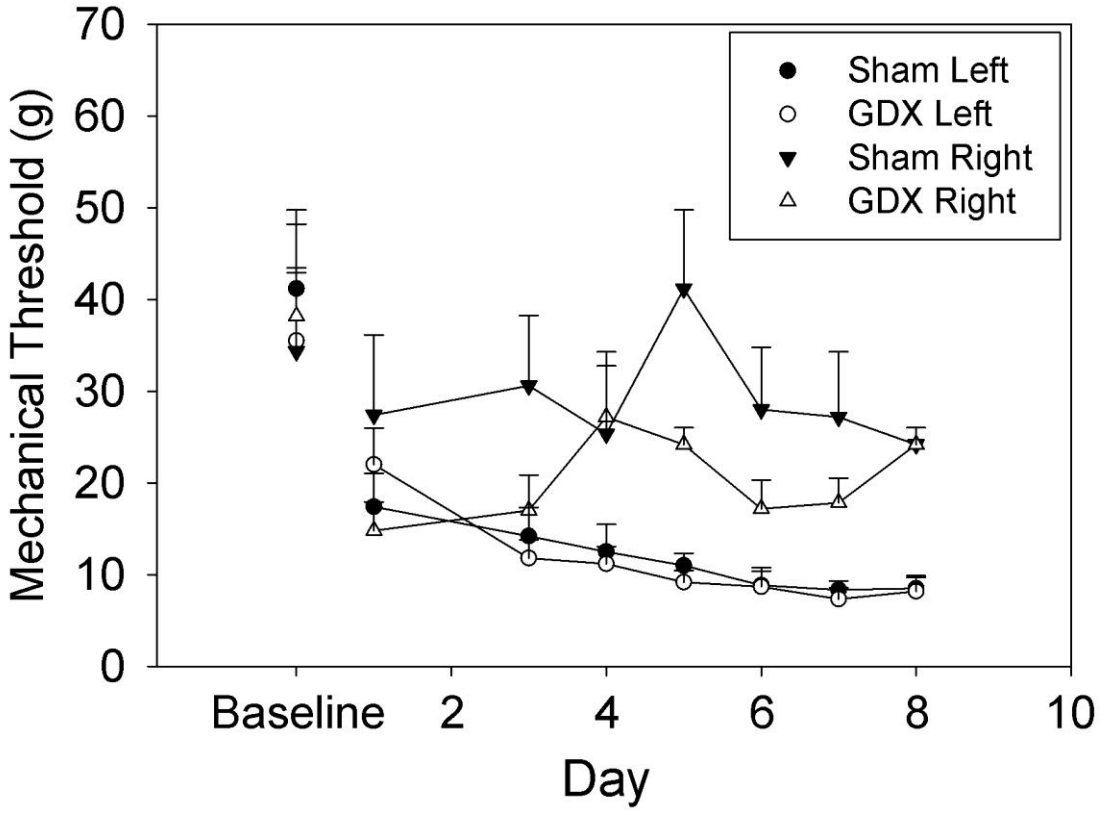


**Figure 12.** Time course of mechanical paw withdrawal threshold in gonadectomized (GDX) male rats with left-side spared nerve injury (SNI). One week prior to Baseline readings, rats were gonadectomized or left intact. Mechanical withdrawal thresholds for hind paws were determined daily (except Day 2), beginning one day after SNI, using Von Frey monofilaments. Baseline readings were taken 1 or 2 days prior to SNI surgery, and days represented are relative to SNI surgery. Results revealed that, ipsilateral to SNI, there was no difference in mechanical threshold between Sham GDX and GDX rats on each day tested or overall. However, contralateral to the SNI, rats that received GDX overall had lower thresholds than Sham GDX. Also, ipsilateral to the SNI, thresholds were lower overall vs. Baseline, while there was no difference on the contralateral side, indicating efficacy of the SNI model. These data indicate GDX decreased withdrawal thresholds contralateral but not ipsilateral to SNI. These data may indicate male gonadal hormones either inhibit descending facilitation or promote descending inhibition contralateral to SNI. (Significance level set to $p \leq 0.05$, two-way ANOVA with Holm-Sidak post-hoc test; n per group by day: Baseline = 6, Day 1 = 5, Day 3 = 5, Days 4-8 = 6). Data represent the mean ± SEM.

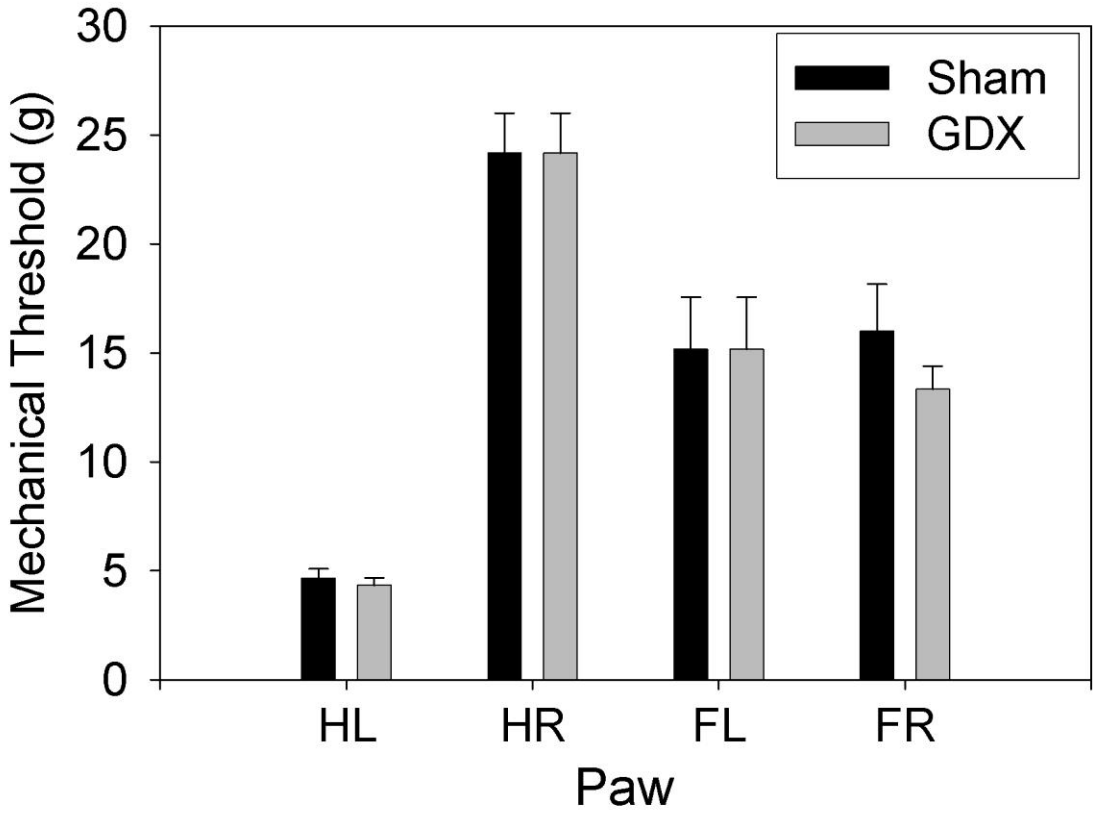


**Figure 13.** Mechanical withdrawal threshold in gonadectomized (GDX) male rats with left-side spared nerve injury (SNI). Rats were gonadectomized or left intact. Mechanical withdrawal thresholds for all four paws were determined ~12 days after SNI using Von Frey monofilaments. Results revealed that there was no difference in withdrawal threshold between Sham GDX (n = 6) and GDX (n = 6) rats. These data indicate GDX did not affect withdrawal threshold. ($p > 0.05$, Student's t-test). HL = hind left; HR = hind right; FL = front left; FR = front right. Data represent the mean ± SEM.

### *3.5 Discussion*

Numerous previous studies have reported sex differences in nociception, with many reporting females as having lower thresholds or higher intensity of nociception [see reviews (Berkley, 1997; Bodnar et al., 1988)]. Women had lower thresholds and higher pain ratings compared with men for electrocutaneous stimulation (Lautenbacher & Rollman, 1993). Female rats had lower mechanical nociceptive thresholds than males (Barrett et al., 2002). On the contrary, there are reports of females having lower sensitivity to experimentally delivered stimuli. For example, Tall et al. (2001) reported female rats as having longer paw withdrawal latency to radiant thermal stimulation than males, and Wang et al. (2006) reported female rats had less thermal hyperalgesia (longer paw withdrawal latency) in the complete Freund's adjuvant (CFA) model than males.

There are reports of sex differences in opioid- and non-opioid-induced analgesia, the majority reporting opioid-induced analgesia as being greater in male than female rodents [see reviews (Craft, 2003a, 2003b)]. Morphine's anti-nociceptive effect was greater in male than female rats on hot plate, tail flick, and abdominal constriction tests (Cicero et al., 1996), and morphine's anti-hyperalgesic effect in the CFA model and anti-nociceptive effect were greater in male than female rats with radiant thermal stimulus to the paw (Wang et al., 2006). In mice as well, morphine anti-nociception on tail flick latencies was greater in males than females (Candido et al., 1992).

The differences in pain sensation and analgesia between the sexes may be due to a plethora of causal factors, but fewer research reports to date have focused on the effects of circulating androgens than estrogens in altering pain sensation. This study was

designed to characterize the effects of exogenous testosterone replacement in gonadectomized adult male rats on acute nociceptive thermal hind paw withdrawal thresholds in order to explore specific components of sex differences in pain sensation contributed by male gonadal hormones. This report provides the novel information of paw withdrawal latencies in male rats followed over a time course of one week after gonadectomy, and of the effect of testosterone replacement on paw withdrawal latencies using a radiant thermal analgesiometer.

Several studies addressing thermal nociception in males corroborate the hypothesis that testosterone can have hypoalgesic effects. For example, GDX of adult male rats did not change thermal paw withdrawal latency, but neonatal GDX of male or testosterone androgenization of female rats indicated an organizational hypoalgesic effect of testosterone (LaCroix-Fralish et al., 2005). However, that study did not directly investigate the activational effects of testosterone on thermal paw withdrawal latencies of adult rats, as did the current study. Testosterone, DHT or 3α-androstanediol-3α-diol increased tail flick and hot plate latencies in GDX male rats (Edinger & Frye, 2005). In male rats, testosterone fully reversed GDX-induced hyperalgesia on a tail flick test and partially reversed that of a hot plate test (Forman et al., 1989). Similar to the findings of the current study, Frye and Seliga (2001) reported GDX itself did not affect nociceptive threshold, but testosterone replacement in GDX rats increased the tail flick threshold.

Activational effects of testosterone on anti-nociception have been observed as well. Morphine anti-nociception was more potent on a hot plate test in gonadally intact male rats and testosterone-treated GDX than GDX rats (Stoffel et al., 2003), and morphine was more potent in intact than GDX male mice (Candido et al., 1992), although

inhibition of the 5α-reductase enzyme was found to potentiate morphine anti-nociception in the tail flick test (Verdi & Ahmadiani, 2007). Also, Thompson et al. (2008) reported that testosterone was required for spinal $\alpha_2$-adrenoreceptor-mediated anti-nociception on a tail flick test in male rats. Another report demonstrated GDX of male rats increased nociceptive behaviors in the formalin model of persistent inflammatory pain, indicating an anti-nociceptive effect of androgen (Aloisi & Ceccarelli, 2000). However, in another study using the formalin model, testosterone had a pro-nociceptive effect in male rats (Nayebi & Ahmadiani, 1999). Moreover, the formalin model – a persistent pain model – may employ additional or alternative mechanisms of nociceptive signaling than those of acute (phasic) thermal stimulation. None of these studies on testosterone's effect on nociception were done with radiant thermal stimulation of the paw, as was the current report. The paw withdrawal test is a refinement of the tail flick test which possesses several distinctions from the tail flick test, including the following: stimulation is on glabrous skin, the animal is not restrained, higher precision of stimulus location, and possible differences in the degree or nature of supraspinal modulation (Bennett, 2001).

Sex-related differences in acute nociceptive threshold have largely been attributed to mechanisms regulated by circulating estrogens, and many studies on the somatosensory effects of estrogens have reported pro-nociceptive effects. Despite this prevalent interpretation, E2 reportedly can have anti-nociceptive effects as well. For example, Forman et al. (1989) reported ovariectomized (OVX) rats receiving estradiol had longer tail flick latencies than OVX controls, and E2-treated OVX rats had lower thermal nociceptive sensitivity than OVX rats on a hot plate test (Stoffel et al., 2003), although this is somewhat contrary to results of a hot plate test reported by Forman et al.

(1989). Nonetheless, the hypoalgesic effect on thermal nociception currently observed with testosterone replacement is opposite in direction to what is typically observed following E2 administration. Serum E2 levels were measured in this study to exclude the possibility that the testosterone-evoked hypoalgesia was mediated via altered circulating estradiol levels (since TP can be metabolized into E2). The results of this study showed that serum E2 concentrations were not significantly altered following hormone manipulation (**Figure 8**) , and remained at levels consistent with previous reports for adult male rats (Overpeck et al., 1978). This observation, concurrent with the observed change in nociceptive withdrawal behavior (**Figure 9**), suggests that the hypoalgesic effect was not mediated by alteration of serum E2.

The hypoalgesic effect of testosterone observed in the current study could be due to direct (there are androgen receptors throughout the nervous system (Melcangi et al., 2005; Simerly et al., 1990) or indirect (possibly via the hypothalamic-pituitary-gonadal and/or -adrenal axes (Viau, 2002) effects on the nervous system responsible for nociception. Since gonadectomy itself did not alter withdrawal latencies, but testosterone replacement did, one possible interpretation is that androgen-induced hypoalgesic effects may be counterbalanced or masked by other endogenous gonadal factors. For example, inhibin, activin and follistatin are known hormones produced in the testes that regulate endocrine function in the brain, such as levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH). It is possible that these or other (perhaps unknown) gonadal factors directly or indirectly affect nociception. In fact, receptors for LH and activin are found in the brain, including the limbic system [reviewed by Vadakkadath Meethal and Atwood (2005)].

Serum DHT concentrations in the 20 mg TP-supplemented rats were consistent with those previously reported for intact adult male rats (Overpeck et al., 1978). Thus, the dose of TP supplementation results in serum DHT levels that mimic normal endogenous serum concentrations. However, episodic, endogenous testosterone concentrations, as opposed to constant, exogenous release may make a difference in the net effect of androgens on nociceptive sensation. Endogenous testosterone release in rats is pulsatile in manner, and serum levels are episodic, with a daily periodicity (Mock et al., 1978; Sodersten et al., 1980; Sodersten et al., 1983). The testosterone pellets used in this study may produce androgen levels with less temporal variability than endogenous levels (*i.e.* that do not pulsate or fluctuate episodically over a day) and thus may produce different effects than the endogenous, episodic androgen levels (Hutchison & Goldman, 1975; Moger, 1976).

As mentioned before, a previous report demonstrated a pro-nociceptive effect of testosterone in the formalin model of persistent inflammatory pain in male rats (Nayebi & Ahmadiani, 1999), and another study showed an anti-nociceptive effect of androgen (Aloisi & Ceccarelli, 2000). However, the findings of the current study indicated no effect of male gonadal hormones on formalin-induced flinching behavior. Compared to the current study, the previous reports used different formalin stimuli, longer periods during which rats were GDX before testing, and different pain scoring procedures than the current study, all of which may contribute to the contrast of the results of these studies. In a gonadectomy study performed on male and female rats, Aloisi et al. (2000) reported that gonadal hormones in both sexes inhibited paw flexion duration following formalin, but did not affect the paw-jerk response. They concluded that the effects of the

gonadal hormones on flexion posturing were independent of the [degree of] nociceptive [peripheral/spinal] input [to supraspinal processing and behavior], since flexion and licking are regarded as being more susceptible to supraspinal modulation than is flinching. This indicated the hormones acted supraspinally. The question – perhaps a rhetorical question – is which behavior(s) best represent the overall nociceptive experience. Indeed, flinching is viewed as less influenced by conditions that affect non-nociceptive behavior, and, thus, a more meaningful end point (Tjolsen et al., 1992). Another report found that GDX in males increased licking, flexion duration, and flinching in the formalin first phase and increased flexion duration in the second phase (Aloisi & Ceccarelli, 2000). In yet another study (Aloisi et al., 2004), females had higher licking duration than males following formalin. Licking duration was reduced by testosterone in females to a "male-like" level; the authors speculated that this was [indirect] evidence that testosterone limits the licking response in males. Supraphysiological testosterone added to intact males did not alter licking, but this negative result could be due to a ceiling effect. Ultimately, the results of the current study do not support androgen activational effects as a contributing factor to sex differences in inflammatory nociception.

Since the introduction of the SNI model, there have been a very limited number of studies into sex differences or sex steroid effects on the mechanical hyperalgesia induced by SNI. Bourquin et al. (2006) reported no sex difference in mechanical threshold following SNI. They did find a slight sex difference in ipsilateral paw withdrawal frequency to the same stimulus over a range of intensities, with males being more sensitive. However, this slight sex difference was present at baseline before SNI, and the

authors stated that the effect of SNI was equal for both genders. They reported no sex difference for the contralateral paw. The findings of the current study are different than the previous study in that they show an effect of gonadectomy. Taken together, these studies imply that gonadectomy has effects on nociception that may not contribute to sex differences.

The current data indicate GDX decreased withdrawal thresholds contralateral but not ipsilateral to SNI. These data may indicate male gonadal hormones either inhibit descending facilitation or promote descending inhibition contralateral to SNI. However, the implications of this apparent androgen effect are limited by the lack of sex difference observed in the SNI model. This lack of sex-difference context for the SNI model seems to extend to clinical observations, which have largely failed to demonstrate a sex difference in incidence of neuropathic pain following injury, [for example, see (Ciaramitaro et al., 2010)]. These observations suggest that pain of neuropathic origin is not typically altered by gender or sex hormones, despite the large body of evidence for women (and girls) having, in general, higher incidence, severity, and frequency of persistent body pains (Berkley, 1997; Unruh, 1996). Gialloreti et al. (2010) reported the incidence of post-herpetic neuralgia in patients with herpes zoster was higher in females than males. However, this neuropathic pain originates with viral infection and may sensitize the nervous system through different mechanisms than other injuries, such as the SNI model. Thus, the current study adds novel evidence that androgen may have an anti-nociceptive effect in neuropathic pain states, at least in the SNI model.

The findings of the current study indicate GDX did not affect thermal withdrawal latencies (thresholds) in the SNI model. This was no surprise because GDX itself did not

alter thermal thresholds in the absence of the SNI model (**Figure 9**), and the SNI model does not alter thermal thresholds (Decosterd & Woolf, 2000).

In conclusion, the results of this study demonstrate that exogenous replacement of testosterone in gonadectomized male rats increased thermal nociceptive withdrawal latencies over those observed in naïve or surgical control rats. This finding suggests that testosterone or DHT has an activational, hypoalgesic effect on acute thermal nociception in males that is not likely mediated by metabolism of testosterone to estrogen.

The observations in this chapter, taken together, demonstrated androgen had a hypoalgesic effect on phasic thermal stimulus and an anti-nociceptive effect on phasic mechanical nociception contralateral to a persistent neuropathic pain model. Thus, it is evident that androgen has activational effects on pain of phasic thermal and persistent neuropathic origin, but perhaps not persistent inflammatory origin. These results provide evidence that androgens may contribute to sex differences in pain sensation.

# *CHAPTER FOUR*

## *ESTRADIOL INCREASES PERSISTENT NOCICEPTION-RELATED BEHAVIOR: STUDIES CHARACTERIZING COMPLEX BEHAVIORAL RESPONSES TO FORMALIN INJECTION IN FEMALE RATS*

### *4.1 Abstract*

These experiments characterized nociception-related behaviors, especially paw flinching, and actometry end points following formalin injection, and investigated the activational effect of estradiol upon them. Many studies have demonstrated activational effects of estrogen, including modulation of pain sensation in females. Previous results from our laboratory suggest that sustained, long-term estrogen replacement in ovariectomized females enhances formalin-induced pain-related behaviors. We hypothesized that *a single injection of estradiol, mimicking a proestrus surge, would similarly enhance formalin-evoked persistent inflammatory nociception*. Thus, adult female rats were ovariectomized and, six days later, injected subcutaneously with 10 μg/kg estradiol benzoate or vehicle. Twenty-four hours later, 100 μL of 5% or 50 μL of 1.25% formalin was injected into the right hind paw. Spontaneous hind paw flinches were then quantified, or behaviors were monitored by actometry. A preliminary experiment verified that a single injection of estradiol, which would be the estrogen manipulation model for this and future experiments, had an uterotrophic effect. Results revealed no difference due to estradiol in quantity of paw flinching at 1.25% formalin, but an estradiol-induced increase in flinching at 5% formalin. Actometry revealed a nociception-related increase in behaviors in a 4.4–5.4 Hz frequency band (potentially reflecting increased licking, whisking, or respiration), and increased total distance traveled between formalin-injected and control groups at 5% but not 1.25% formalin. Estradiol injection reduced behaviors in a 0.3-1.5 Hz frequency band in rats receiving 1.25% formalin; this may reflect alteration of locomotion during nociception. Behaviors represented by these frequency and distance measures, or their detection, may be stimulus

intensity-dependent. Taken together, these data indicate that a proestrus-like surge in serum estrogen can modify inflammatory nociception-related behaviors and, thus, presumably nociception in an activational manner.

### *4.2 Introduction*

Estrogens appear to have activational, often pro-nociceptive (pain-potentiating), effects on innervation, synapse formation, and sensory function. However, there are no reports of direct, systematic investigation into identification of the anatomical sites of action in the nervous system of the enhancement of nociception and pain by estrogens. Thus, we hypothesized that sex differences in pain sensation are due, at least in part, to activational effects of estradiol on peripheral, spinal and/or supraspinal sites involved in the transmission and perception of nociceptive stimuli. To determine whether activation in response to inflammatory pain was increased by acute, activational effects of estrogen, the current study addressed the intensity of nociceptive behaviors evoked by formalin (a model of persistent inflammatory pain).

The formalin test is an established, widely used model of persistent inflammatory pain (Porro & Cavazzuti, 1993; Tjolsen et al., 1992). It is usually used in mice and rats, and may provide a better approximation of clinical chronic pain than traditional phasic nociceptive tests, such as acute radiant thermal, hot-plate, or mechanical paw withdrawal tests. Formalin induces a biphasic pain-related behavioral response, separated by a quiescent interphase. The first (early) phase is due to C-fiber activation, while the second (late) phase is due to peripheral inflammation and central sensitization. The behavioral responses that can be evaluated include lifting (flexing), licking, biting, and flinching of the injected paw. The flinching response seems to be a consistent component of the response, and is easy to observe and quantify. The other behavioral responses have been more problematic, and it has been suggested that flinching is less influenced by

conditions that affect non-nociceptive behavior, and, thus, is a better end-point (Tjolsen et al., 1992).

### *4.3 Materials & methods*

Adult female Sprague-Dawley rats (~11 weeks old, 200-230 g, Harlan, Indianapolis, IN) were used in these experiments. A set of formalin-only experiments was conducted in gonadally intact females and characterized the dose-response relationship and time course of the flinching response to formalin. In addition, formalin-only actometry experiments used rats that were OVX for a period of one week before nociceptive behavioral testing, but received no further manipulation.

For studies investigating effects of estrogen, rats were divided into two groups: 1) ovariectomized (OVX) receiving estradiol, and 2) OVX receiving an equivalent volume of vehicle. Estradiol (E2) supplementation was followed 24 hours later by nociceptive behavioral evaluation. Flinching behavior was quantified in rats that received 1.25% or 5% formalin injection. Another actometry experiment studied the effect of E2 on 1.25% formalin-induced behavior.

In a separate experiment, twenty-four hours after E2 injection (seven days after OVX), rats were weighed then decapitated, adipose tissue was removed from uteri, and uteri were excised at the base and weighed wet.

#### *4.3.1 Hormone manipulation*

For ovariectomy (OVX), rats were anesthetized with a mixture of ketamine (64 mg/kg, i.p.; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (5.3 mg/kg; i.p.). Under aseptic conditions, a ventral midline incision (~1.5 cm) was made into the lower abdominal cavity. Both ovaries were externalized, fallopian tubes and blood vessels ligated with suture just proximal to the ovaries, and the ovaries excised. Adipose tissue,

fallopian tubes, and blood vessels were reinserted into the abdominal cavity. Muscle and skin layers were individually closed with suture. Six days later, rats (except those in formalin-only actometry experiments) received a single subcutaneous injection into the left flank of 10 μg/kg estradiol benzoate (Sigma, E-9000) at 10 μg/mL in vehicle (10% ethanol/90% corn oil), or an equivalent volume of vehicle. This dose of E2 was chosen to mimic the surge in E2 observed during proestrus in rats [see (Butcher et al., 1974; Medlock et al., 1991; Nag & Mokha, 2006; Viau & Meaney, 1991; Zoubina et al., 2001)]. Rats in the formalin-only actometry experiments did not receive any injection of estradiol or vehicle.

### *4.3.2 Nociceptive behavioral analysis*

#### *4.3.2.1 The Formalin Test*

Approximately 24 hours after estradiol or vehicle injection (or seven days after OVX), rats received a unilateral injection of 100 μL of 5% or 50 μL of 1.25% formalin into the plantar hind paw as a model of persistent inflammatory pain. Spontaneous hind paw flinches were quantified post-injection.

#### *4.3.2.2 Actometry*

For force plate actometry experiments, behavioral responses were recorded for one hour before, and for one hour immediately after formalin injection. Detailed recordings of the rats' movements were collected during these periods using a force-plate actometer (Bioanalytical Systems, Inc.). Rats in the 1.25% formalin experiments were acclimated to the actometer environment over a 5 day period prior to the experiment. Rats in the 5% formalin experiments were not acclimated. Rats in "Sham" groups were

manually restrained and hind paw handled as if injecting, but received no needle prick or injection.

*4.3.3 Data analyses*

Force-plate actometry data were collected, and distance metrics and frequency spectra power analyses performed, using FPA analysis software provided by the manufacturer (Bioanalytical Systems, Inc.). Frequency/power data were exported to Microsoft Excel to compile group values, for analysis of power spectra within selected frequency bands, and for general statistical analyses and plotting. All data represent the mean ± SEM. Data were analyzed by Student's t-test or ANOVA statistical analyses with the significance level set to $p \leq 0.05$.

### *4.4 Results*

Results of the uterus measurement (**Figure 14**) revealed rats receiving estradiol (OVX + E2) had significantly higher uterus weight than controls receiving vehicle (OVX) (Student's t-test, Welch-corrected; n: Vehicle = 17, E2 = 18).

Data in **Figure 15** show the dose-response relationship of hind paw flinching behavioral response to formalin injection in gonadally intact female rats. The number of flinches quantified during the 30-40 minute post-injection period were 37.6 ± 10.1, 104.3 ± 16.7 and 132.3 ± 15.9 for 1.25, 2.5 and 5%, respectively (n = 4 each). These data demonstrate the degree of flinching response to different levels of formalin stimulus.

In the time-course experiment (**Figure 16**), gonadally intact rats (n = 8) were injected with 50 µL of 1.25% formalin into the right plantar hind paw, and paw flinches were quantified 0-60 minutes post-injection. These data show the dynamic pattern of flinching response quantity over time. These data are in accordance with previously published time courses of flinching behavior following formalin injection (Tjolsen et al., 1992) and demonstrate the typical biphasic response.

Results of E2 manipulation revealed that, for 1.25% formalin (**Figure 18**), total flinches (in the 30-40 minute time frame) for rats receiving estradiol (OVX + E2) were not different than controls receiving vehicle (OVX) (Student's t-test, n = 10 per group). In contrast, for 5% formalin (**Figure 19**), rats receiving estradiol (OVX + E2) had significantly more total flinches than controls receiving vehicle (OVX) (Student's t-test, n = 12 per group).

Further analysis was performed to examine the effect of E2 quantified hind paw flinches 0-60 minutes post-injection for 1.25% formalin. Total flinches over the entire 60 minutes were: Vehicle 286.4 ± 15.1; E2 289.6 ± 16.1. When flinching behavior was broken down into Early (0-5 min), Inter- (5-15 min) and Late (15-60 min) phases, total flinches for rats receiving estradiol (OVX + E2) were not different than controls receiving vehicle (OVX) for any time period or for the entire 60 minutes (Data not shown; Student's t-test, n = 10 per group).

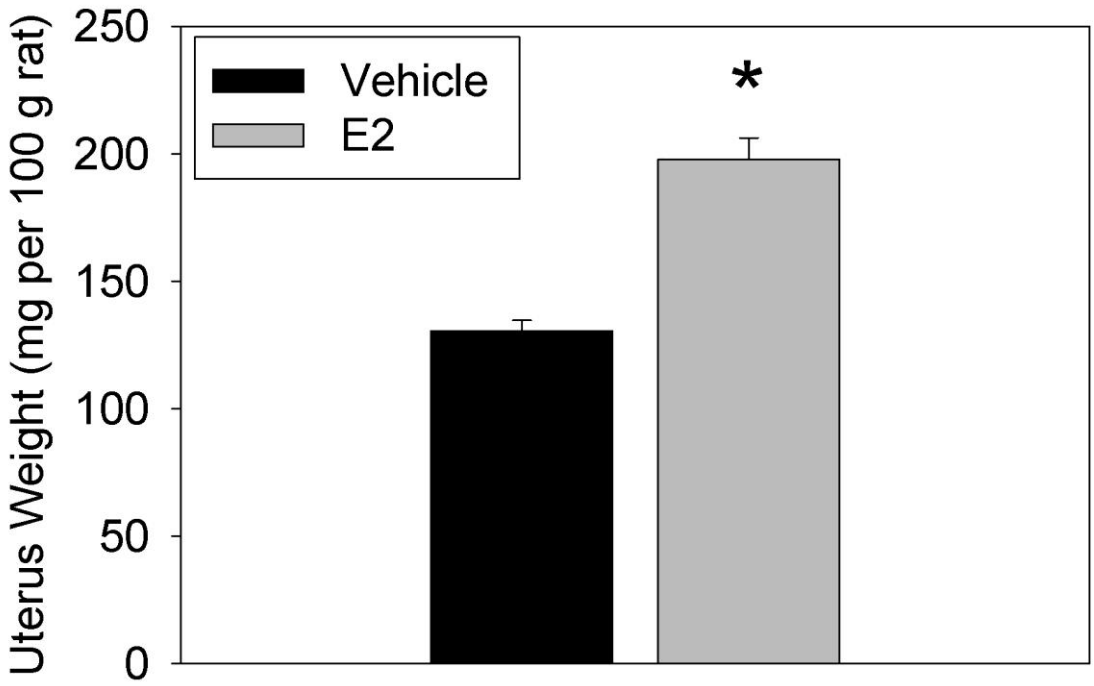


**Figure 14.** Effect of estradiol on uterine weight in ovariectomized (OVX) female rats. Rats were ovariectomized and, six days later, received a single s.c. estradiol (10 µg/kg) or vehicle injection. Twenty-four hours after estradiol (E2) injection (seven days after OVX), rats were weighed then decapitated, adipose tissue was removed from uteri, and uteri were excised at the base and weighed wet. Note that rats receiving estradiol (OVX + E2) had significantly higher uterus weight than controls receiving vehicle (OVX). These data demonstrate that the single dose of E2 produced an estrogenic effect. (*$p < 0.0001$, unpaired Student's t-test, Welch-corrected; n: Vehicle = 17, E2 = 18). Data represent the mean ± SEM.

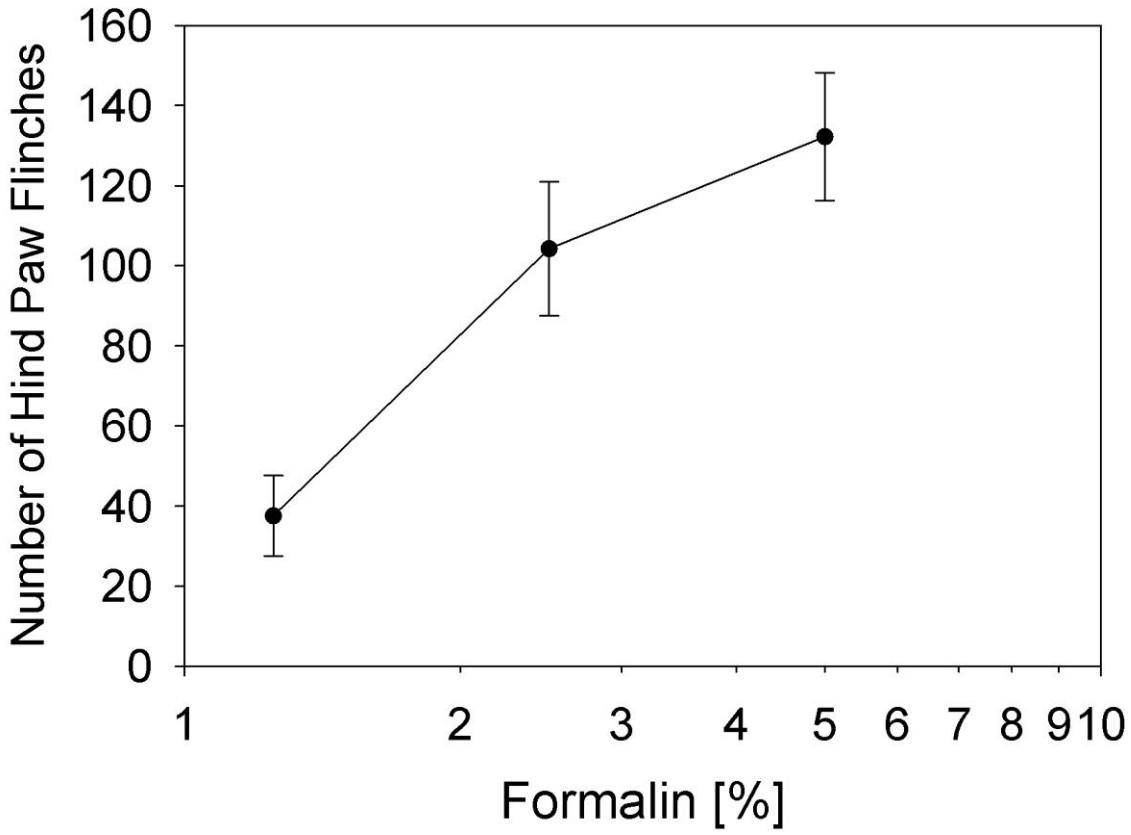


**Figure 15.** Dose-response relationship of hind paw flinching behavioral response to formalin injection in female rats. Rats were injected with 50 μL of 1.25, 2.5 or 5% formalin into the right plantar hind paw. Spontaneous hind paw flinches were quantified 30-40 minutes post-injection (during the peak of the late-phase behaviors). These data characterize the degree of flinching response to different levels of formalin stimulus. Data represent the mean ± SEM (n = 4 per group).

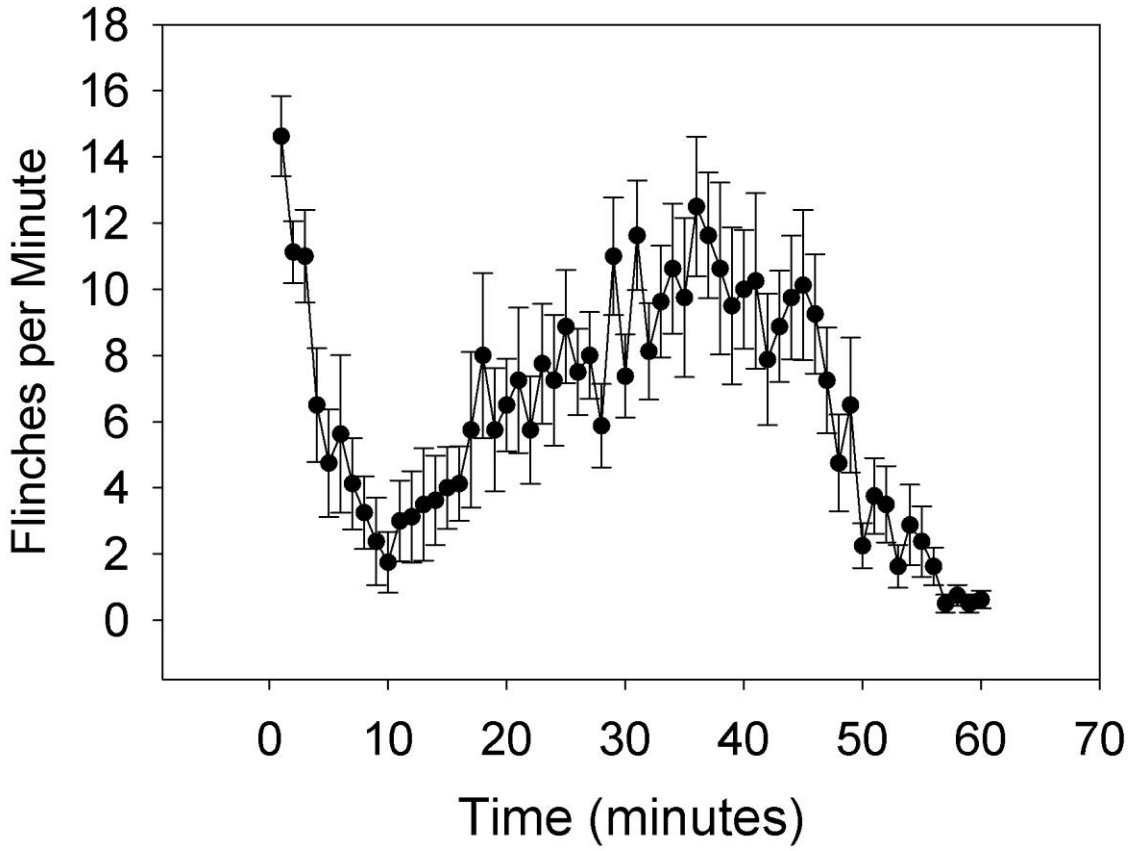


**Figure 16.** Time course of hind paw flinching behavioral response to formalin injection in female rats. Rats were injected with 50 µL of 1.25% formalin into the right plantar hind paw. Spontaneous hind paw flinches were quantified 0-60 minutes post-injection. These data characterize the flinching response over time. Data represent the mean ± SEM (n = 8).

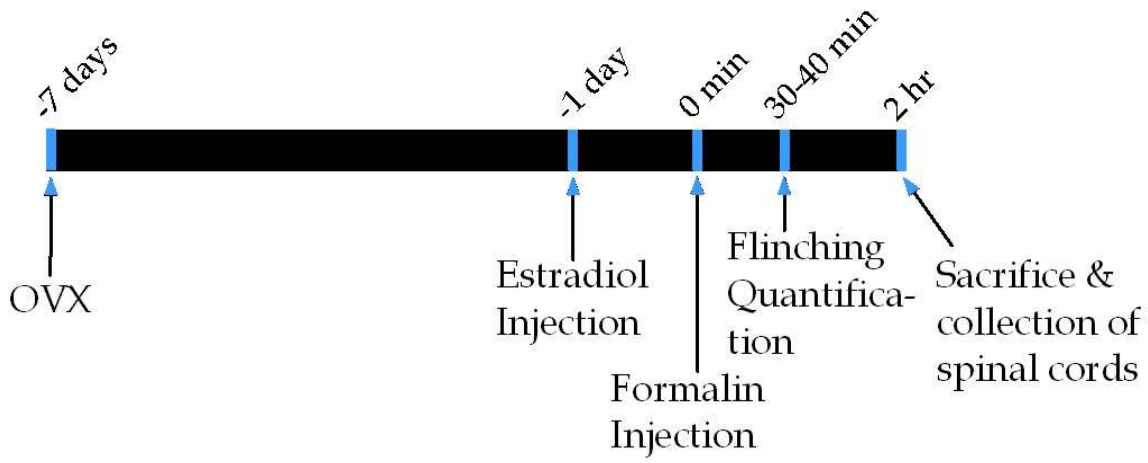


**Figure 17.** Experimental timeline for studying the effect of estradiol on flinching behavior following formalin injection. Rats were ovariectomized and, six days later, received a single s.c. estradiol (10 µg/kg) or vehicle injection. Twenty-four hours after estradiol (E2) injection (seven days after OVX), rats were injected with formalin into the right plantar hind paw. Spontaneous hind paw flinches were quantified 30-40 minutes post-injection. Two hours after formalin injection, rats were anesthetized, perfused, and spinal cords removed by laminectomy for Fos immunohistochemistry.

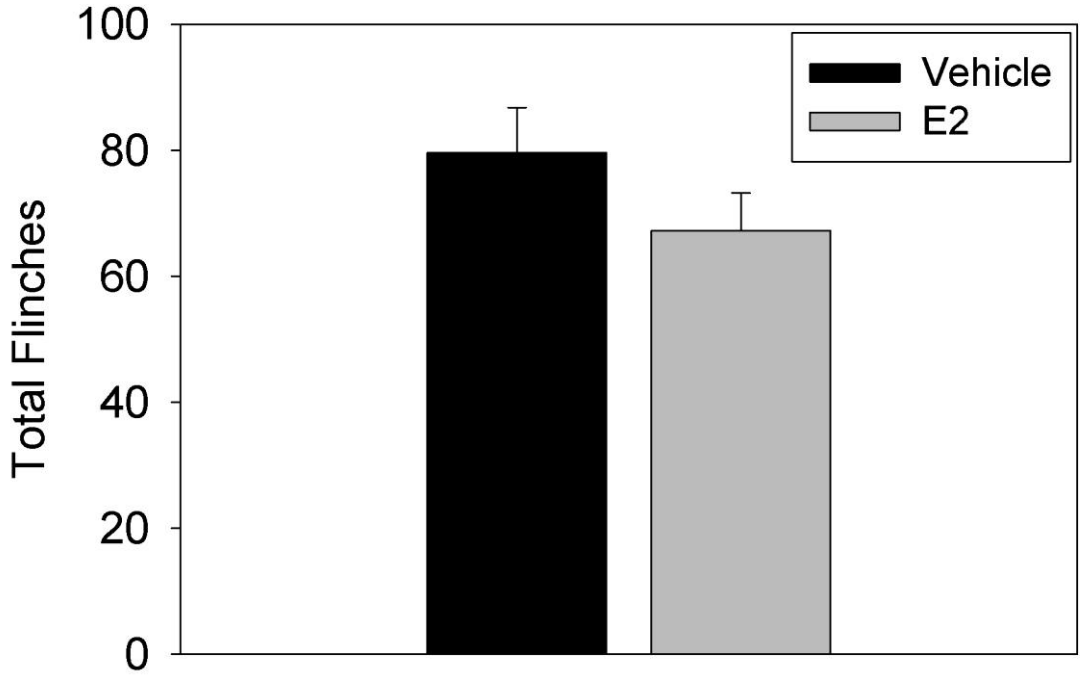


**Figure 18.** Effect of estradiol on 1.25% formalin-evoked spontaneous flinching behavior 30-40 minutes post-formalin. Rats were ovariectomized and, six days later, received a single s.c. estradiol (10 μg/kg) or vehicle injection. Twenty-four hours after estradiol (E2) injection (seven days after OVX), rats were injected with 50 μL of 1.25% formalin into the right plantar hind paw. Spontaneous hind paw flinches were quantified 30-40 minutes post-injection (during the peak of the late-phase behaviors). Note that total flinches for rats receiving estradiol (OVX + E2) were not different than controls receiving vehicle (OVX). Data represent the mean ± SEM (Significance level set to $p \leq 0.05$, unpaired Student's t-test, n = 10 per group).

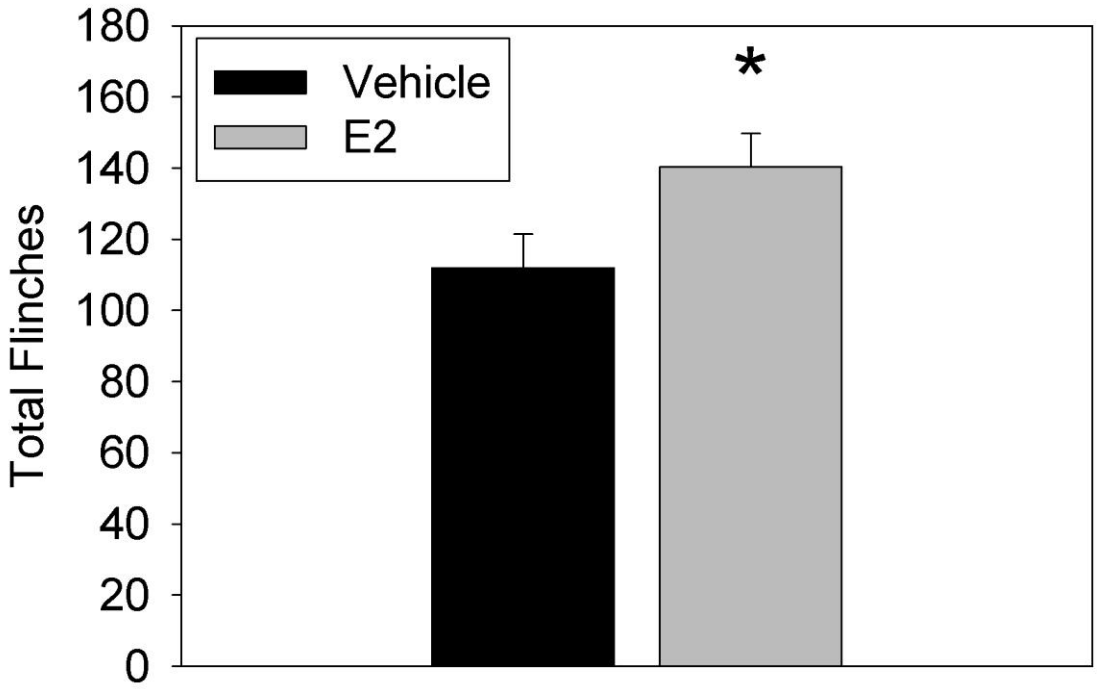


**Figure 19.** Effect of estradiol on 5% formalin-evoked flinching behavior. Rats were ovariectomized and, six days later, received a single s.c. estradiol (10 μg/kg) or vehicle injection. Twenty-four hours after estradiol (E2) injection (seven days after OVX), rats were injected with 100 μL of 5% formalin into the right plantar hind paw. Spontaneous hind paw flinches were quantified 30-40 minutes post-injection (during the peak of the late-phase behaviors). Note that rats receiving estradiol (OVX + E2) had significantly more total flinches than controls receiving vehicle (OVX). Data represent the mean ± SEM (*$p \leq 0.05$, unpaired Student's t-test, n = 12 per group).

**Figure 20** shows representative force/time recordings from rats that were either injected with 50 μL 1.25% formalin into the left hind paw (right) or received sham restraint (left), then were placed in the actometer for one hour. These force/time traces demonstrate activity of the rats and that the force on the plate changed over time as the subject manifested either restricted body movements (such as grooming) or moved about the behavioral arena (such as locomotion). These changes in force over time are related to the specific patterns of movement that comprise behavior, and are the basis for Fourier-transformation of the data into frequency/power spectra. .

**Figure 21** shows Fourier-transformed power spectra of actometer force/time recordings of female rats that were either injected with 50 μL of 1.25% formalin into the right hind paw or received sham restraint, then were placed in the actometer for one hour. Rats had been acclimated to the actometer environment prior to the experiment. Fourier-transform power spectral analysis was performed on the force/time traces of actometry over the entire 60-minute period following formalin injection. Results revealed similar power spectra for Sham (n = 20) and 1.25% formalin-treated (n = 20) rats (**Figure 21**). Data represent mean power spectra. Rats in the Sham and Formalin groups were the same rats which were monitored in the actometer first without formalin (Sham) then after formalin injection (Formalin); rats in each treatment group were comprised of rats that had been OVX 7 days prior to the experiment, which received injection of either E2 (n = 10) or vehicle (n = 10) 24 hours before the experiment.

**Figure 22** shows Fourier-transformed power spectra of actometer force/time recordings of female rats that were either naïve or injected with 50 μL of 5% formalin into the left hind paw, then were placed in the actometer for one hour. Results revealed

potentially qualitatively different power spectra for Naïve (n = 6) and 5% formalin-treated (n = 6) rats. Data represent mean power spectra. Rats in the Naïve and Formalin groups were the same rats which were monitored in the actometer first without formalin (Naïve) then after formalin injection (Formalin). Rats had not been acclimated to the actometer environment.

The mean power spectrum for Sham (n = 20) rats was subtracted from that of 1.25% formalin-treated (n = 20) rats. Results revealed a difference power spectrum with relatively small magnitudes (**Figure 23**). The mean power spectrum for Naïve (n = 6) rats was subtracted from that of 5% formalin-treated (n = 6) rats. Results revealed a difference power spectrum with relative larger magnitudes at some frequencies than those at 1.25% formalin (**Figure 24**).

Three categories of frequency band (range) were empirically designated for the power spectra, based on the difference power spectrum of 5% formalin. The frequency band designations were: Low: 0.3-1.5 Hz; Peak: 4.4-5.4 Hz; High: 2.4-9.3 Hz; they are represented by horizontal lines and shown with difference power spectra for 1.25% and 5% formalin in **Figures 25 and 26**, respectively.

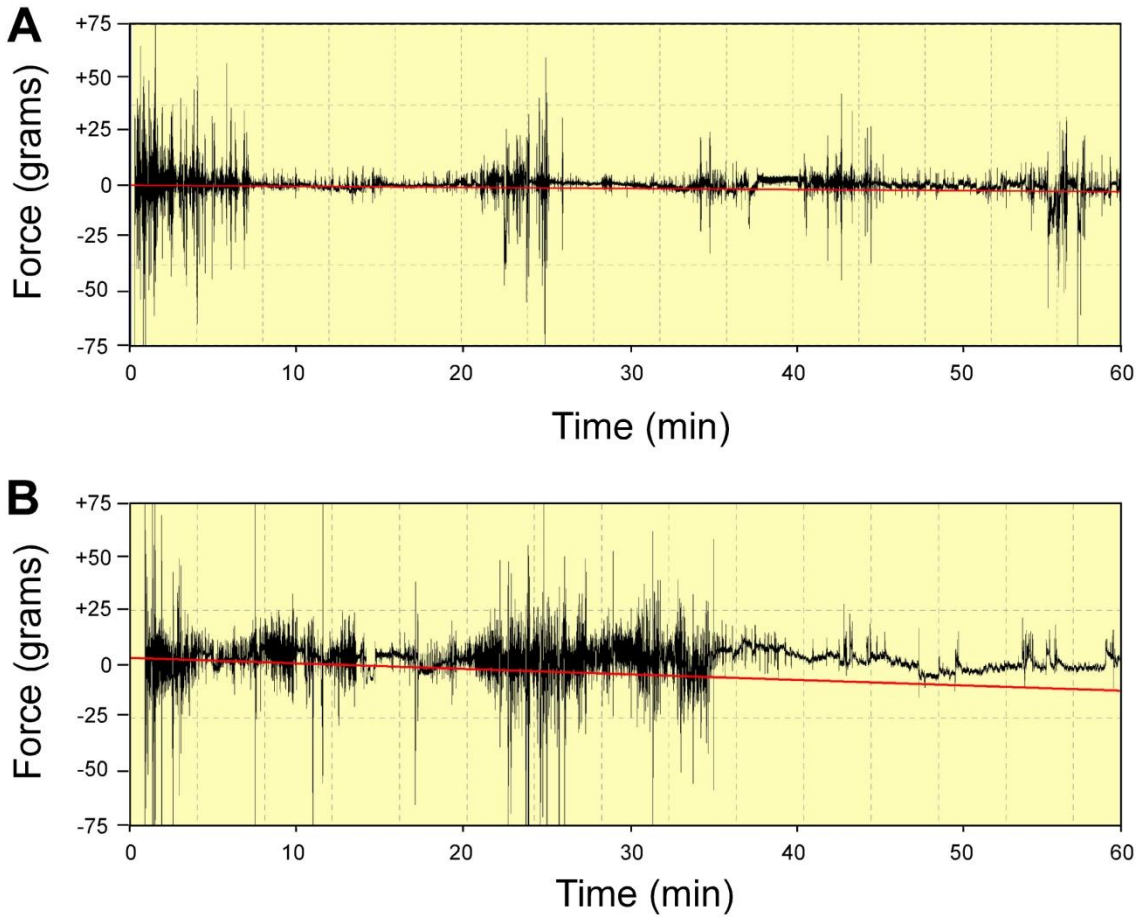


**Figure 20.** Representative actometer force/time recordings for rats without or with formalin injection. These are representative force/time recordings from rats that received either **A)** sham restraint or **B)** injection of 50 µL of 1.25% formalin into the left hind paw and then placed in the actometer for one hour.

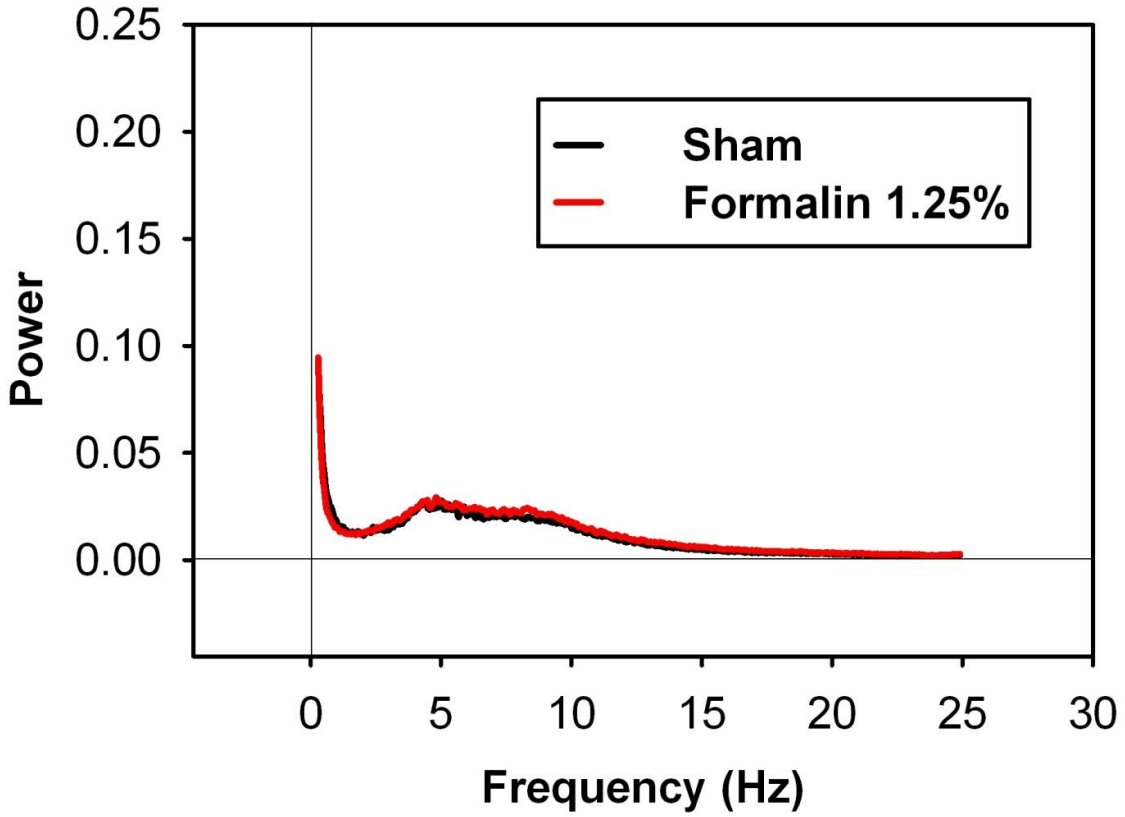


**Figure 21.** Fourier-transformed power spectra of actometer force/time recordings of female rats following hind paw injection of 1.25% formalin. Rats were either injected with 50 µL 1.25% formalin into the right hind paw or received sham restraint, then were placed in the actometer for one hour. Rats had been acclimated to the actometer environment over a 5 day period prior to the experiment. Results revealed similar power spectra for Sham (n = 20) and formalin-treated (n = 20) rats. Data represent mean power spectra. [Rats in the Sham and Formalin groups were the same rats which were monitored in the actometer first without formalin (Sham) then after formalin injection (Formalin); rats in each treatment group were comprised of rats that had been OVX 7 days prior to the experiment, which received injection of either E2 (n = 10) or vehicle (n = 10) 24 hours before the experiment.]

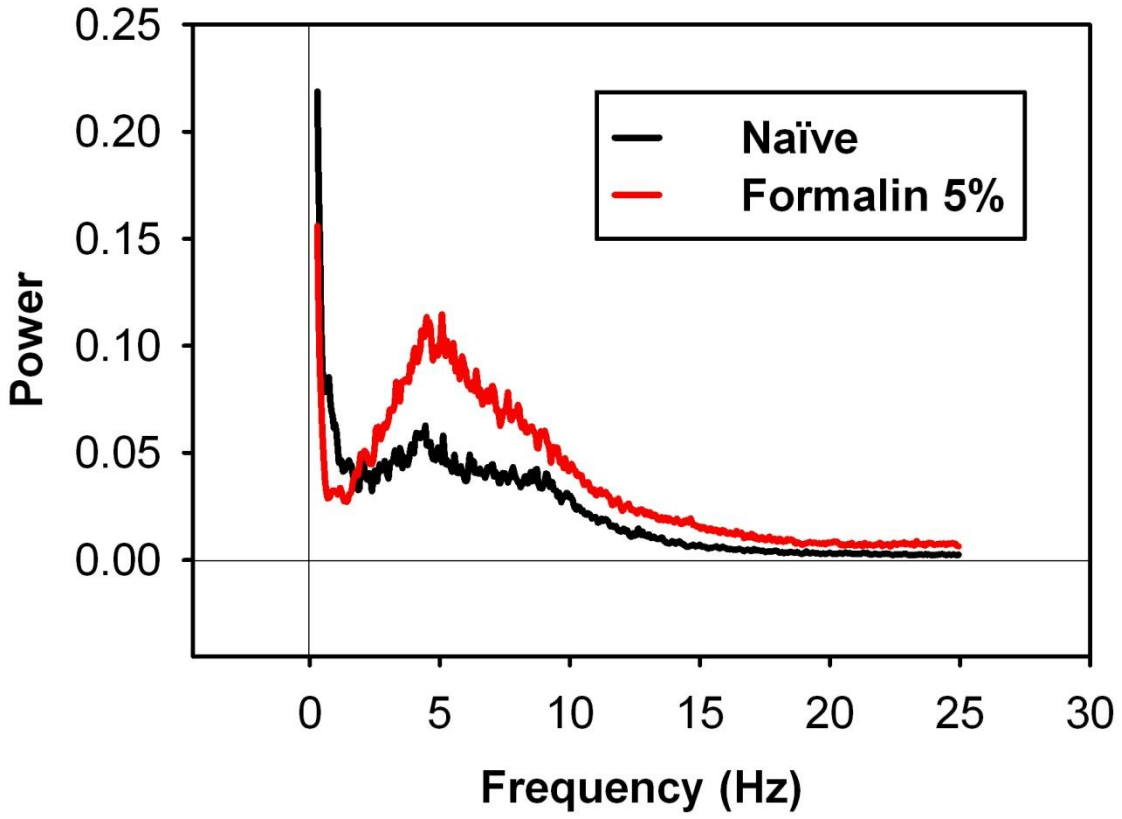


**Figure 22.** Fourier-transformed power spectra of actometer force/time recordings of female rats following hind paw injection of 5% formalin. Rats were either naïve or injected with 50 µL 5% formalin into the left hind paw, then were placed in the actometer for one hour. Results revealed potentially qualitatively different power spectra for Naïve (n = 6) and formalin-treated (n = 6) rats. Data represent mean power spectra. [Rats in the Naïve and Formalin groups were the same rats which were monitored in the actometer first without formalin (Naïve) then after formalin injection (Formalin). Rats had not been acclimated to the actometer environment.]

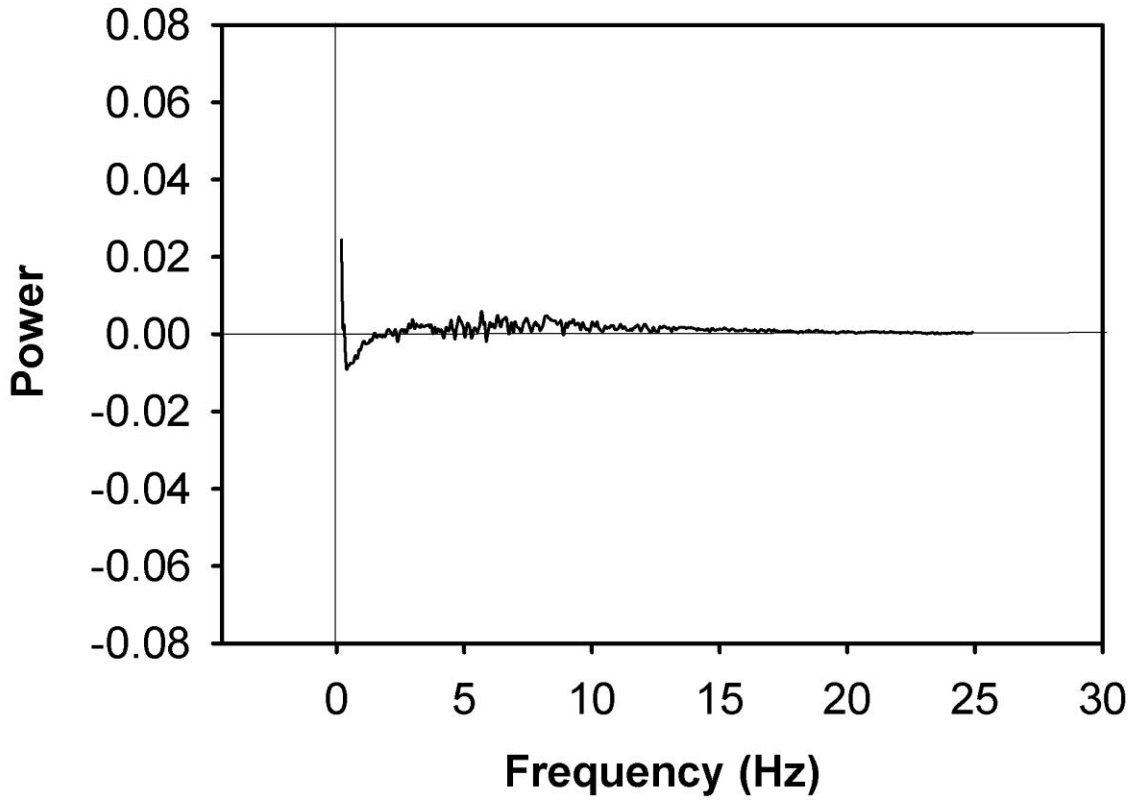


**Figure 23.** Fourier-transformed difference power spectrum of actometer force/time recordings of female rats following hind paw injection of 1.25% formalin. Rats were either injected with 50 µL 1.25% formalin into the right hind paw or received sham restraint, then were placed in the actometer for one hour. Rats had been acclimated to the actometer environment over a 5 day period prior to the experiment. The mean power spectrum for Sham (n = 20) rats was subtracted from that of formalin-treated (n = 20) rats. Results revealed a difference power spectrum with relatively small magnitudes. Data represent the mean power spectrum. [Rats in the Sham and Formalin groups were the same rats which were monitored in the actometer first without formalin (Sham) then after formalin injection (Formalin); rats in each treatment group were comprised of rats that had been OVX 7 days prior to the experiment, which received injection of either E2 (n = 10) or vehicle (n = 10) 24 hours before the experiment.]

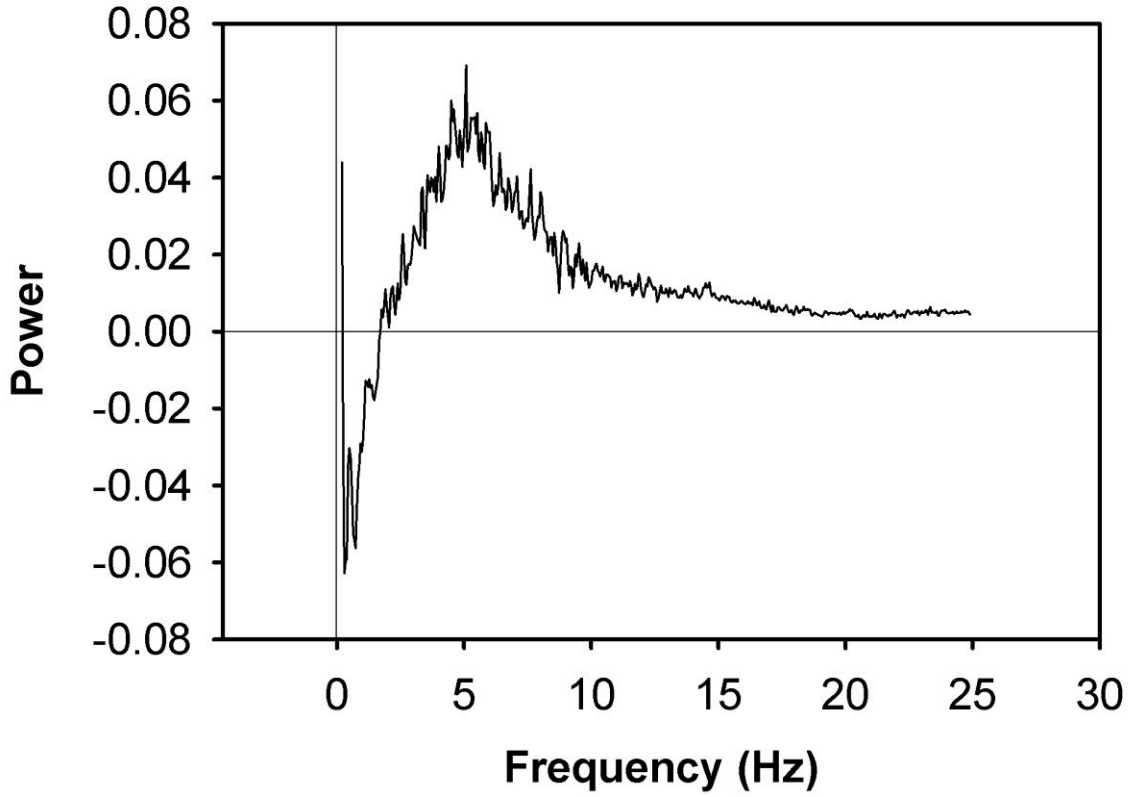


**Figure 24.** Fourier-transformed difference power spectrum of actometer force/time recordings of female rats following hind paw injection of 5% formalin. Rats were either naïve or injected with 50 µL 5% formalin into the left hind paw, then were placed in the actometer for one hour. The mean power spectrum for Naïve (n = 6) rats was subtracted from that of formalin-treated (n = 6) rats. Results revealed a difference power spectrum with potentially significant magnitudes. Data represent the mean power spectrum. [Rats in the Naïve and Formalin groups were the same rats which were monitored in the actometer first without formalin (Naïve) then after formalin injection (Formalin). Rats had not been acclimated to the actometer environment.]

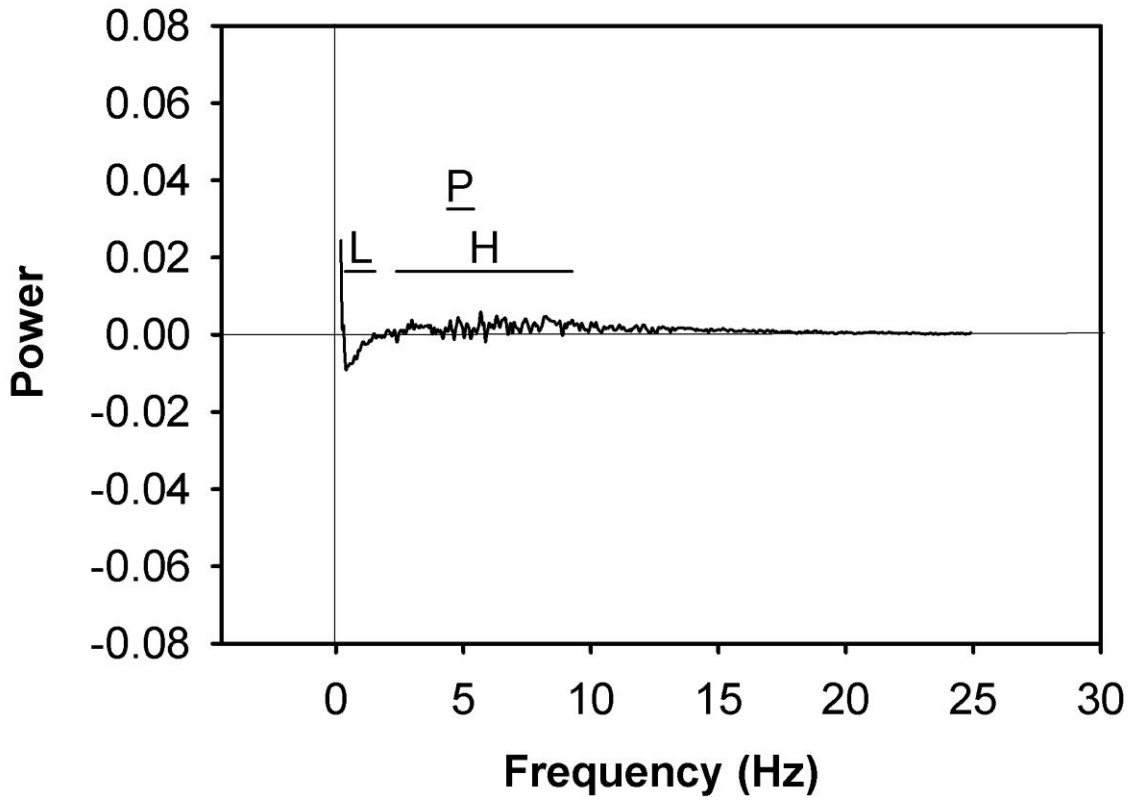


**Figure 25.** Designations of frequency bands of actometry power spectra for quantification of behaviors, superimposed on the difference power spectrum of 1.25% formalin. Horizontal lines represent frequency band (range) designations of the power spectra, shown here with the Fourier-transformed difference power spectrum of actometer force/time recordings of female rats following hind paw injection of 1.25% formalin. Bands designations were largely based on the difference power spectrum of 5% formalin. The frequency band designations are: Low (L): 0.3-1.5 Hz; Peak (P): 4.4-5.4 Hz; High (H): 2.4-9.3 Hz.

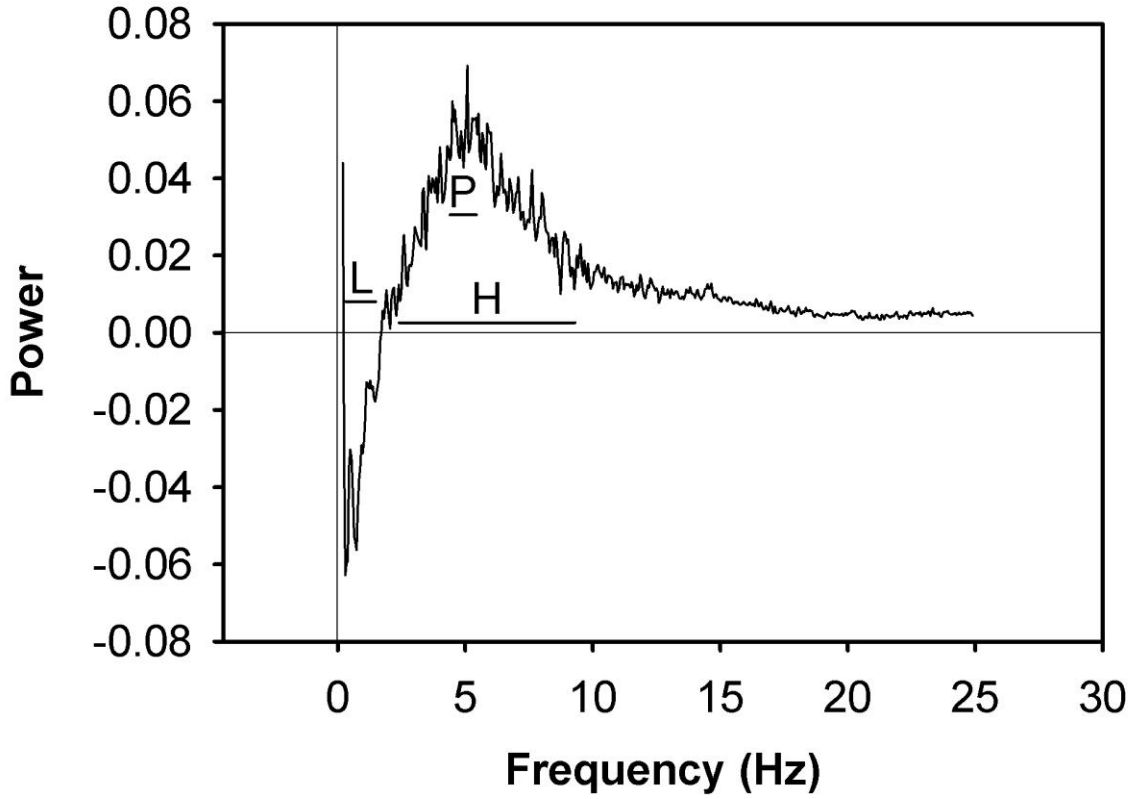


**Figure 26.** Designations of frequency bands of actometry power spectra for quantification of behaviors, superimposed on the difference power spectrum of 5% formalin. Horizontal lines represent frequency band (range) designations of the power spectra, shown here with the Fourier-transformed difference power spectrum of actometer force/time recordings of female rats following hind paw injection of 5% formalin. Band designations were largely based on the difference power spectrum of 5% formalin. The frequency band designations are: Low (L): 0.3-1.5 Hz; Peak (P): 4.4-5.4 Hz; High (H): 2.4-9.3 Hz.

Power spectra were summed over the designated frequency bands. Results (**Figure 27**) revealed no differences in power for Sham (n = 20) vs. 1.25% formalin-treated (n = 20) rats for Low, Peak or High frequency ranges (Student's t-test). In contrast, results for 5% formalin revealed a difference in power for Sham (n = 6) vs. formalin-treated (n = 6) rats at the Peak frequency band (**Figure 28**), but not at the Low or High frequency band (Student's t-test). These data demonstrate that 5% formalin induced an increase in a behavior(s) that is represented by power in the 4.4-5.4 Hz frequency band.

**Figure 29** shows Fourier-transformed power spectra of actometer force/time recordings of female rats following hind paw injection of 1.25% formalin. Rats had been acclimated to the actometer environment prior to the experiment. Power spectra were summed over selected frequency bands, which were: Low: 0.3-1.5 Hz; Peak: 4.4-5.4; Hz High: 2.4-9.3 Hz. Results revealed lower power for E2-treated (n = 10) vs. vehicle-treated (n = 10) rats in the Low frequency band, but no difference in the Peak or High frequency bands (Student's t-test). These data demonstrate estradiol decreased a behavior(s) that is represented by power in the Low frequency band.

**Figure 30** shows representative spatial plots from actometer force/time recordings of female rats over one hour. These data demonstrate movement of the rats across the force plate arena and that movement changed over time. Movement across the (X,Y) coordinates of the force plate grid provide the basis for the analysis of total distance traveled.

Analysis of total distance traveled from actometer recordings was performed, revealing no difference in total distance traveled for Sham (n = 20) vs. 1.25% formalin-treated (n = 20) rats (**Figure 31, panel A**). However, results revealed that 5% formalin-treated (n = 6) rats had higher total distance traveled over Naïve (n = 6) rats (**Figure 31, panel B**) (paired Student's t-test).

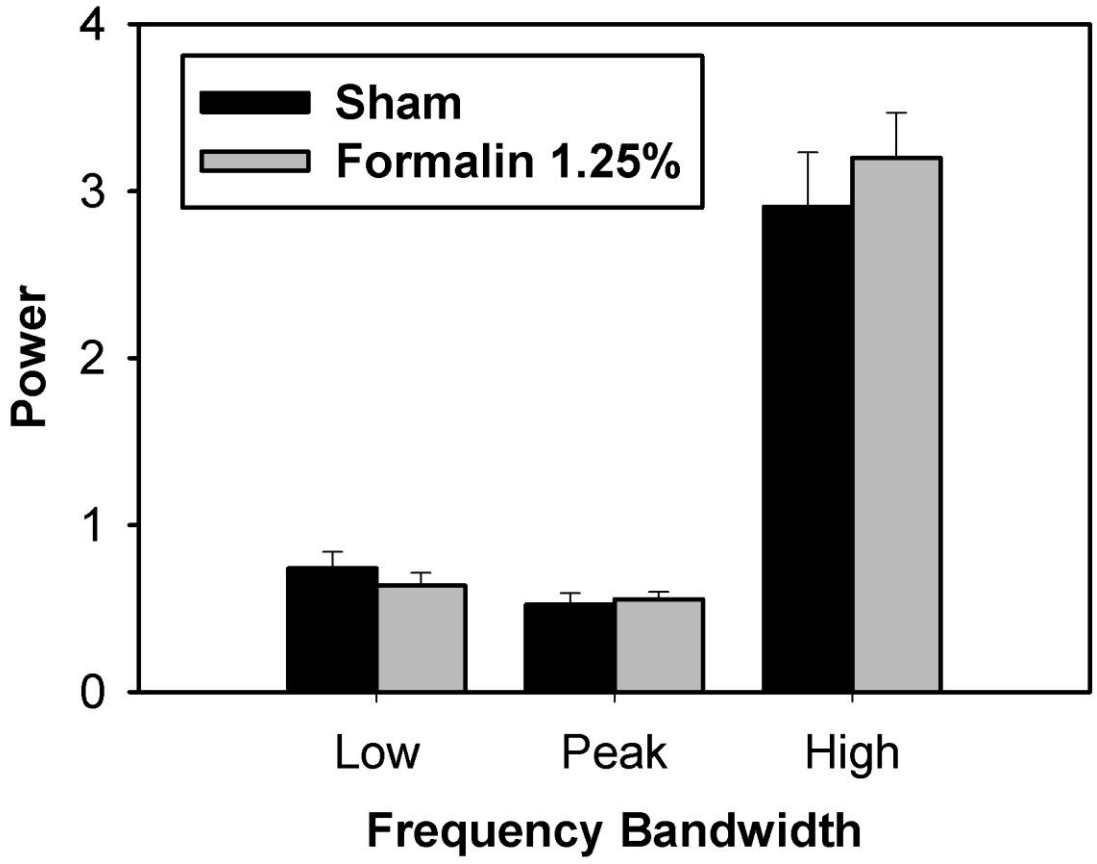


**Figure 27.** Analysis of power over frequency bands in rats following 1.25% formalin. Rats were either injected with 50 μL 1.25% formalin into the right hind paw or received sham restraint, then were placed in the actometer for one hour. Rats had been acclimated to the actometer environment over a 5 day period prior to the experiment. Power spectra were summed over the designated frequency bands. The frequency band designations are: Low: 0.3-1.5 Hz; Peak: 4.4-5.4; Hz High: 2.4-9.3 Hz. Results revealed no differences in power for Sham (n = 20) vs. formalin-treated (n = 20) rats. Data represent the mean ± SEM (Significance level set to $p \leq 0.05$, unpaired Student's t-test). [Rats in the Sham and Formalin groups were the same rats which were monitored in the actometer first without formalin (Sham) then after formalin injection (Formalin); rats in each treatment group were comprised of rats that had been OVX 7 days prior to the experiment, which received injection of either E2 (n = 10) or vehicle (n = 10) 24 hours before the experiment.]

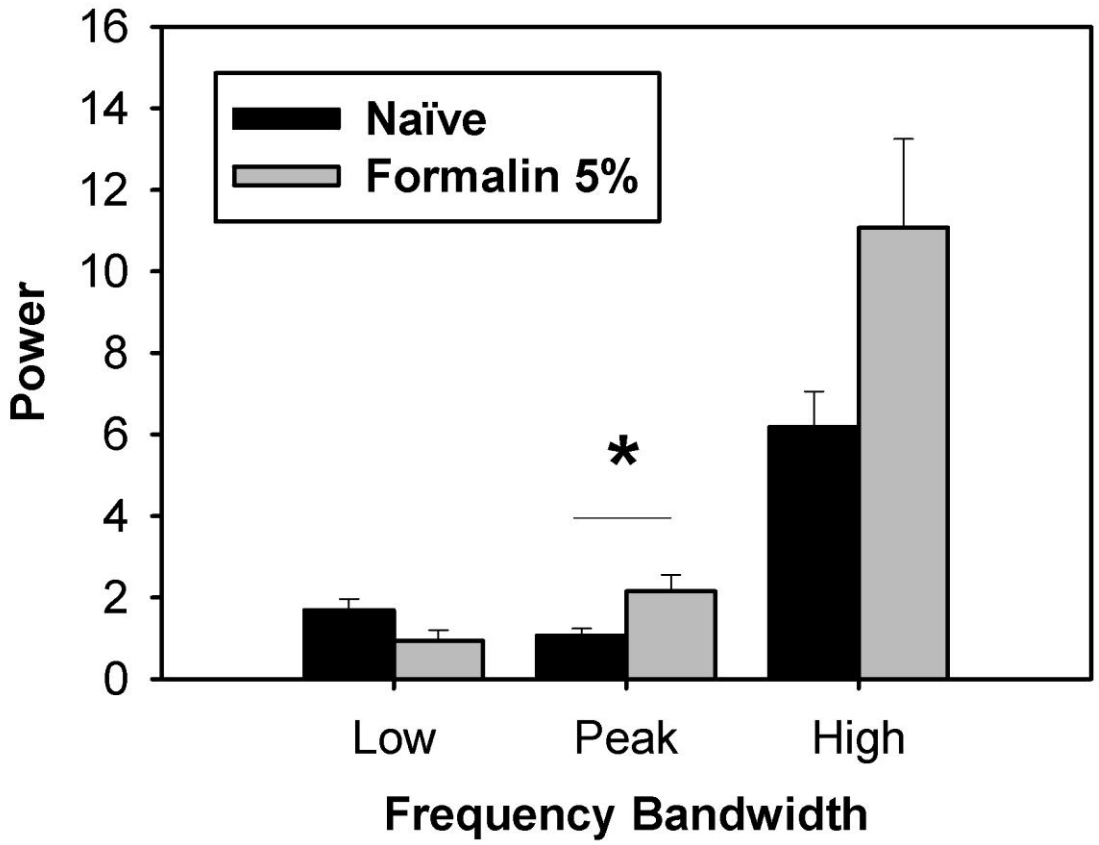


**Figure 28.** Analysis of power over frequency bands in rats following 5% formalin. Rats were either injected with 50 µL 5% formalin into the left hind paw or were naïve, then were placed in the actometer for one hour. Power spectra were summed over the designated frequency bands. The frequency band designations are: Low: 0.3-1.5 Hz; Peak: 4.4-5.4; Hz High: 2.4-9.3 Hz. Results revealed a difference in power for Sham (n = 6) vs. formalin-treated (n = 6) rats at the Peak frequency band, but not at the Low or High frequency band. These data suggest 5% formalin induced an increase in a behavior(s) that is represented by power in the Peak frequency band. Data represent the mean ± SEM (*$p \leq 0.05$, unpaired Student's t-test). [Rats in the Naïve and Formalin groups were the same rats which were monitored in the actometer first without formalin (Naïve) then after formalin injection (Formalin). Rats had not been acclimated to the actometer environment.]

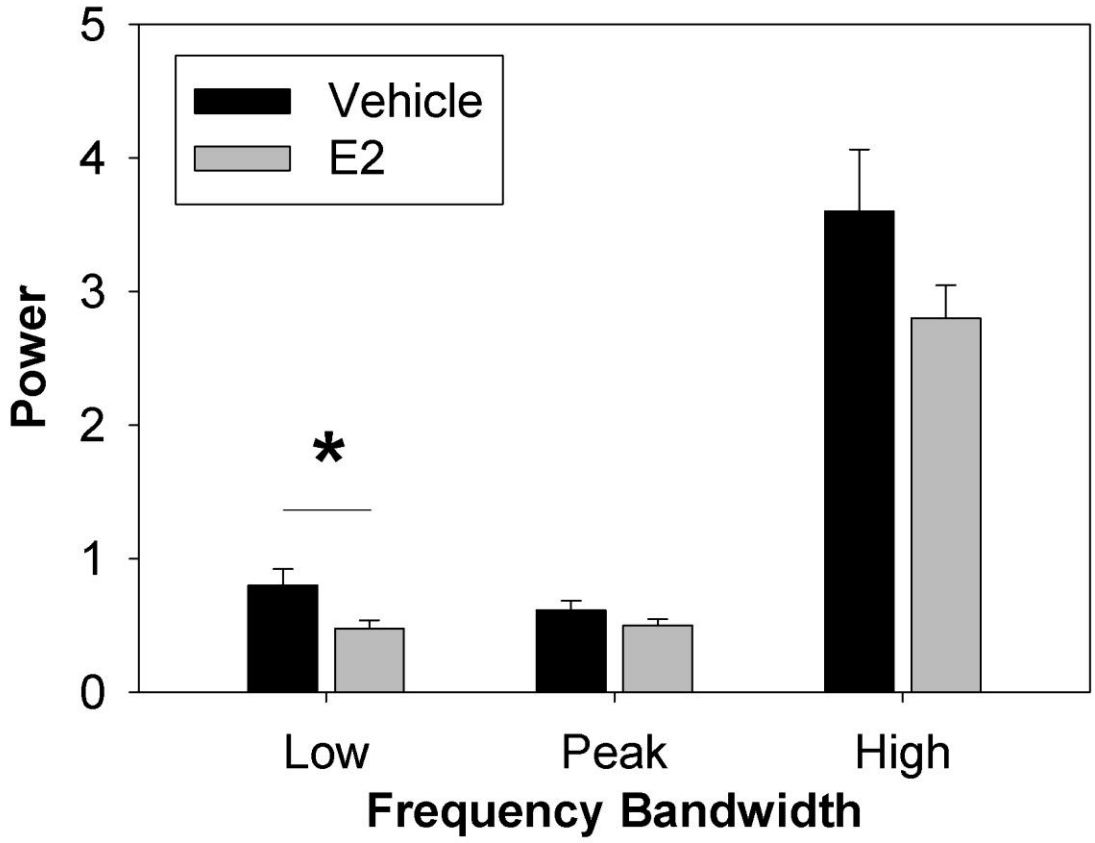


**Figure 29.** Effect of estradiol on Fourier-transformed power spectra of actometer force/time recordings of female rats following hind paw injection of 1.25% formalin. Rats were ovariectomized (OVX) and, six days later, received a single s.c. injection of 10 μg/kg estradiol (E2) or vehicle. Twenty-four hours after estradiol (E2) injection (seven days after OVX), rats were injected with 50 μL 1.25% formalin into the right hind paw, then were placed in the actometer for one hour. Rats had been acclimated to the actometer environment over a 5 day period prior to the experiment. Power spectra were summed over the designated frequency bands. The frequency band designations are: Low: 0.3-1.5 Hz; Peak: 4.4-5.4; Hz High: 2.4-9.3 Hz. Results revealed a difference in power for vehicle (n = 10) vs. E2-treated (n = 10) rats in the Low frequency band, but not Peak or High frequency bands. These data suggest estradiol decreased a behavior(s) that is represented by power in the Low frequency band. Data represent the mean ± SEM (*$p \leq 0.05$, unpaired Student's t-test).

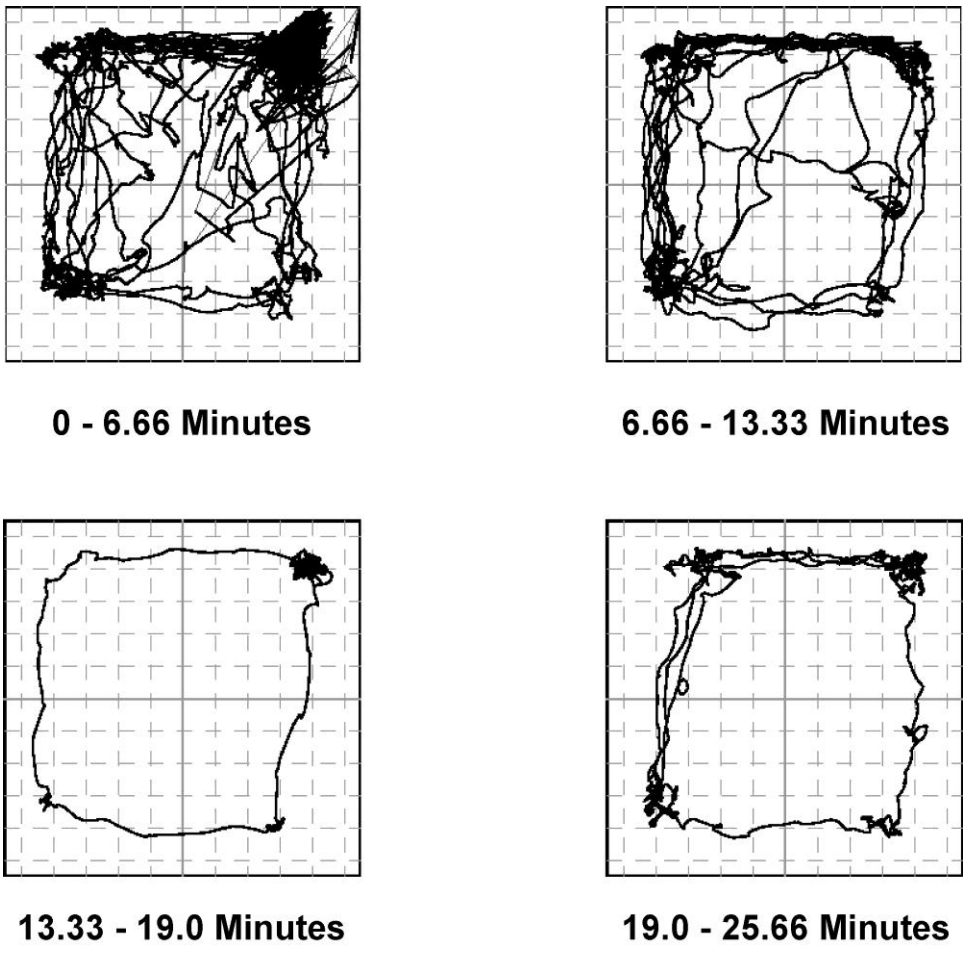


**Figure 30.** Representative examples of recordings of position/time data of female rats for one hour. Data were collected using a BASi force-plate actometer. Analyses of total distance traveled was calculated based on the change in position of the center of force recordings over time. Note that the position traces reflect the initial exploratory behavior in the force-plate arena, which diminishes over time.

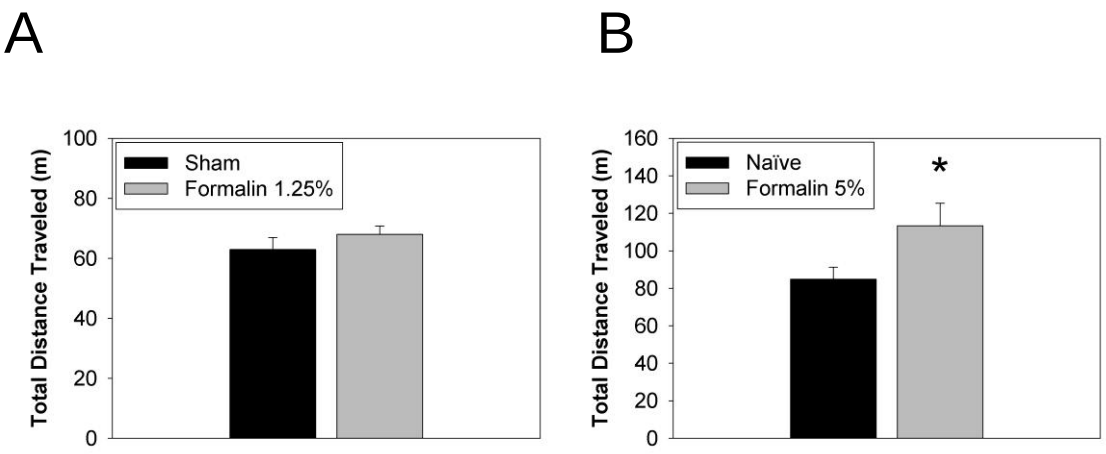


**Figure 31.** Analysis of total distance traveled from actometer force/time recordings of female rats following hind paw injection of formalin. A) 1.25% formalin: Rats were either injected with 50 µL 1.25% formalin into the right hind paw or received sham restraint, then were placed in the actometer for one hour. Results revealed no difference in total distance traveled for Sham (n = 20) vs. formalin-treated (n = 20) rats. [Rats in the Sham and Formalin groups were the same rats which were monitored in the actometer first without formalin (Sham) then after formalin injection (Formalin); rats in each treatment group were comprised of rats that had been OVX 7 days prior to the experiment, which received injection of either E2 (n = 10) or vehicle (n = 10) 24 hours before the experiment. Rats had been acclimated to the actometer environment over a 5 day period prior to the experiment.] B) 5% formalin: Rats were either injected with 50 µL 5% formalin into the right hind paw or were naïve, then were placed in the actometer for one hour. Results revealed 5% formalin-treated (n = 6) rats had higher total distance traveled over Naïve (n = 6) rats. Data represent the mean ± SEM (*$p \leq 0.05$, paired Student's t-test). [Rats in the Naïve and Formalin groups were the same rats which were monitored in the actometer first without formalin (Naïve) then after formalin injection (Formalin). Rats had not been acclimated to the actometer environment.]

### *4.5 Discussion*

These experiments characterized nociception-related behaviors following formalin injection, and investigated the activational effect of estradiol upon these behaviors. Behaviors examined were flinching of the inflamed hind paw and two force plate actometry end points: total distance traveled and Fourier-transformed power spectra. To test acute effects of estrogen, behavioral analyses with the formalin model were performed 24 hours after a single injection of estradiol.

Results of the uterus measurement (**Figure 14**) revealed that ovariectomized, adult rats receiving administration of a single dose of estradiol, which mimics the proestrus surge of estrogen in intact female rats [see (Butcher et al., 1974; Medlock et al., 1991; Nag & Mokha, 2006; Viau & Meaney, 1991; Zoubina et al., 2001)], had significantly higher uterus weight than controls receiving vehicle. These data demonstrated that the single dose of E2 produced an estrogenic (uterotrophic) effect, which was evident 24 hours after E2 injection (Student's t-test, Welch-corrected; n: Vehicle = 17, E2 = 18). The uterotrophic effect of this E2 dose in OVX rats has been reported previously (Zoubina et al., 2001). These results confirmed the efficacy of using this estrogen dose and timing regimen for the experimental design.

Data in **Figure 15** show the dose-response relationship of hind paw flinching behavioral response to formalin injection in gonadally intact female rats. The number of flinches quantified during the 30-40 minute post-injection period were 37.6 ±10.1, 104.3 ±16.7 and 132.3 ±15.9 for 1.25, 2.5 and 5%, respectively (n = 4 each). These data demonstrate the degree of flinching response to different levels of formalin stimulus.

In the time-course experiment (**Figure 16**), gonadally intact rats (n = 8) were injected with 50 μL of 1.25% formalin into the right plantar hind paw, and paw flinches were quantified 0-60 minutes post-injection. These data show the dynamic pattern of flinching response quantity over time. These data are in accordance with previously published time courses of flinching behavior following formalin injection (Tjolsen et al., 1992) and demonstrate the typical biphasic response.

Results of estradiol manipulation confirmed the hypothesis that a single injection of estradiol significantly enhances late-phase formalin-induced hind paw flinching at 5% formalin (**Figure 19**); in contrast, no difference was observed at 1.25% (**Figure 18**), even upon further investigation of an entire hour post-injection (data not shown). These results showed that a proestrus-like surge in serum estradiol levels can increase pain-related behavior evoked by persistent inflammatory nociception. This observation is consistent with several previous reports suggesting that elevated serum estrogen levels can enhance persistent inflammatory nociception (Bradshaw et al., 2000; Fillingim et al., 1997; Hellstrom & Anderberg, 2003; Isselee et al., 2002; Lautenbacher & Rollman, 1993; LeResche et al., 2003; Meisler, 1999; Somerville, 1972; Tall & Crisp, 2004).

Previous reports have investigated the effect of gonadal hormones on persistent inflammatory nociception, but few have directly manipulated estrogen. In a gonadectomy study performed on male and female rats, Aloisi et al. (2000) reported that gonadal hormones in both sexes inhibited paw flexion duration following formalin, but did not affect the paw-jerk response. They concluded that the effects of the gonadal hormones on flexion posturing were independent of the [degree of] nociceptive [peripheral/spinal] input [to supraspinal processing and behavior], since flexion and

licking are regarded as being more susceptible to supraspinal modulation than is flinching. This indicated the hormones acted supraspinally. Another report found that GDX in males increased licking, flexion duration, and flinching in the formalin first phase and increased flexion duration in the second phase (Aloisi & Ceccarelli, 2000). In other studies (Aloisi et al., 1994; Aloisi et al., 2004), females had higher flexion and licking duration than males following formalin. Licking duration was reduced by testosterone in females to a "male-like" level (Aloisi et al., 2004); the authors speculated that this was [indirect] evidence that testosterone limits the licking response in males. Supraphysiological testosterone added to intact males did not alter licking, but this negative result could be due to a ceiling effect. Another study showed gonadectomy for 3 months (i.e. longer than the current study) in female rats increased rubbing of the formalin-injected lip, but no change in flinches of an injected paw (Pajot et al., 2003). A different study demonstrated that E2 increases formalin-induced paw licking behavior in male rats (Ceccarelli et al., 2004) – possibly corroborating the current findings – but was in males, not females. A few studies showed an anti-nociceptive effect of estradiol administration on formalin-related behaviors, but, in contrast to the current study, these were done with longer, continuous supplementation of estradiol in younger rats (Kuba et al., 2005; Kuba et al., 2006; Mannino et al., 2007). The current study demonstrated that E2 supplementation increased formalin-induced flinching behavior in females – a finding that is difficult to directly compare or contrast with any of these previous studies because they differ in the hormone manipulation, formalin dose, age and sex of rats. In addition, Aloisi et al. (1995) reported that sex differences found in formalin-induced behaviors were dependent on the stimulus level (formalin dose). Other reports have demonstrated

stimulus-intensity dependence for sex differences in behavioral responses to other noxious stimuli [for review, see (Aloisi, 1997)]. This stimulus-intensity dependence is a characteristic that likely extends to sex-hormone modulation of nociception – an idea to which the findings of the current study point, with the positive result at 5% but negative result at 1.25% formalin. Another caveat to consider when performing and analyzing studies of sex hormone modulation in pain-related behavior is the sex differences themselves, which may or may not be due to differences in response to the pain, but to the other experimental parameters (Aloisi, 1997). Thus, the current results add to the existing body of data pertaining to sex hormone modulation of persistent inflammatory nociception by demonstrating an effect of acute, direct manipulation of estradiol in female rats on behavior evoked by 5% formalin.

The force/time traces demonstrate activity of the rats and that the force on the plate changed over time. These changes in force over time are related to behavior and are the basis for Fourier-transformation of the data.

Fourier-transformation-based power spectral analysis was performed on the force/time traces of actometry over the entire 60-minute period following formalin injection. Based on the difference in power spectra between control rats and those injected with formalin, three specific frequency bands (ranges) were designated for further comparison of behavioral power. The frequency band designations were: Low: 0.3-1.5 Hz; Peak: 4.4-5.4 Hz; and High: 2.4-9.3 Hz (**Figures 25 and 26**). The power differences were more pronounced in the 5% formalin-injected rats, which formed the primary basis for the selection of these specific power bands.

Results for 5% formalin revealed an increase in power for formalin-treated rats in the Peak frequency band (**Figure 28**), but not at the Low or High frequency band. These data demonstrate that 5% formalin induced an increase in a behavior(s) that is represented by power in the 4.4-5.4 Hz frequency band. This increase was accompanied by an apparent loss of power in the low-frequency band.

Specific behaviors are associated with power in restricted frequency bands. The few well-defined examples include licking (4-7 Hz), respiration (1-2 Hz), heartbeat (6-8 Hz), and whisking (5-7 Hz). Locomotion is typically large amplitude and very low frequency (< 2 Hz). Accordingly, the increased power in the 4.4-5.4 Hz frequency band may represent increased licking and whisking behaviors, and perhaps an increased respiratory rate in 5% formalin-injected rats. The concomitant loss of power in the low frequency band may reflect a shift in frequency in locomotive movements, which may be altered by flinching behaviors being superimposed on normal locomotion.

In the E2-supplementation experiment with actometry, estradiol injection lowered power in the Low (0.3-1.5 Hz) frequency band with 1.25% formalin, indicating estrogen decreased a behavior(s) represented in the Low frequency band (**Figure 29**). However, 1.25% formalin itself did not change this value (**Figure 27**). This frequency band corresponds to locomotive behaviors in the rat; a loss of power here may indicate that estrogen modifies locomotive behaviors during formalin-evoked nociception. Whereas estrogen did not enhance the numbers of 1.25% formalin-evoked flinches, it may nonetheless modify the locomotive behaviors that accompany inflammatory nociception. For example, freezing behavior may be enhanced, or the frequency (Hz) of locomotion between flinches increased (out of the low frequency band) in these subjects.

Spatial plots from actometer force/time recordings of female rats over one hour demonstrate movement of the rats across the force plate area and that movement changed over time (**Figure 30**). Movement across different portions of the force plate grid provides the basis for the analysis of total distance traveled and demonstrate the utility of the actometer.

Analysis of total distance traveled from actometer recordings revealed that 5% formalin-treated rats had higher total distance traveled over Naïve rats (**Figure 31, panel B**), indicating this formalin stimulus level evoked more locomotion than rats that were not enduring a noxious stimulus. The increased locomotion observed with formalin may be an escape-related behavior whereby the rats to try to avoid the noxious stimulus.

Actometry also revealed behavioral differences in total distance traveled between formalin-injected and control groups at 5%, but not 1.25% formalin. Total distance traveled may provide an adequate measure of pain-related behavior at 5% formalin, but any interpretation of total distance measures with regard to nociception-related behavior must consider the importance of stimulus-intensity effects. These results suggest a stimulus-intensity threshold associated with formalin-evoked measures of total distance traveled. Alternatively, the actometry methods used may not be sensitive enough to detect any putative, subtle changes in these behaviors elicited by lower formalin doses.

Not many studies have investigated locomotor activity or exploration after formalin. A very relevant study characterized many behaviors over a time course and several concentrations of formalin in adult rats (Abbott et al., 1999); the report demonstrated a formalin concentration-dependent decrease in “active behaviors.”

However, when comparing the highest concentration vs. the next lower concentration used, "active behaviors" in the first part of the second phase increased – opposite the trend. In addition, the highest concentration used in that study was 2%, which is lower than the concentration in the current study (5%) where a difference in distance traveled was observed, making it difficult to extrapolate the results for comparison. Also, locomotor activity (walking) was summed with other "active behaviors" for analysis. One study in P25 rat pups showed increased locomotion with increasing formalin (Teng & Abbott, 1998). Another report showed a decrease in "exploratory behavior" due to formalin in males (Abbott et al., 1995), but it is unclear whether "exploratory behavior" is equivalent to locomotion. All of these previous reports observed the time spent in (or frequency of occurrence of) the behavioral category, which may be a different measure of ambulation than distance traveled (as in the current study) since there may be differences or variations in the rate (speed) of locomotion, between subjects or over time. One study investigated distance traveled as well as locomotion following formalin and demonstrated an increase in these end points compared to baseline (Mena et al., 1996), which supports the findings of the current study that showed increased distance traveled following 5% formalin. On another point of interest, it is evident from these studies that pharmacological manipulations within the formalin model can bring about modulation of these end points that does not correlate with pain scores. For example, in one study analgesics decreased the pain score but increased locomotor activity (Abbott et al., 1995); in a different study, a low or high dose of naloxone both decreased locomotor activity even though the low dose induced analgesia and the high dose caused hyperalgesia (Mena et al., 1996). Differences in experimental parameters used can, therefore,

potentially complicate or limit interpretation of results for locomotor-type end points with regard to any differences, or the power to detect such differences, in intensity of the nociceptive experience. In addition, there are sex differences in the behavioral responses to noxious stimuli (Aloisi, 1997), and the current study used only female rats. For these reasons, caution must be used when using these actometry end points as specific measures of pain-related behaviors.

All these data taken together indicate that estrogen can increase nociception-related behaviors and, thus, presumably nociception in an activational manner; however effects of estradiol on nociception (and/or related behaviors) are dependent on the intensity of noxious stimulus. Thus, it is evident that estrogen, by acting in an acute, activational manner, may contribute to the higher burden of chronic pain states in women.

# *CHAPTER FIVE*

## *ESTRADIOL DOES NOT ALTER A MARKER OF NOCICEPTION IN SPINAL CORD FOLLOWING FORMALIN INJECTION IN FEMALE RATS: STUDIES OF FOS EXPRESSION*

### *5.1 Abstract*

Many studies have demonstrated activational effects of estrogen, including modulation of pain sensation in females. Previous results from our laboratory demonstrated that a single injection of estradiol in ovariectomized female rats, mimicking a proestrus surge, enhances formalin-evoked pain-related behaviors. We hypothesized that *estradiol would increase a formalin-evoked marker of nociception*, Fos (the protein product of the immediate-early gene *c-fos*), *in the spinal cord*. This study was designed to explore whether the appearance of estrogen's modulation of Fos was dependent on the intensity of the inflammatory formalin stimulus. Fos expression in spinal cord neurons was characterized following formalin injection using (fluorescent) immunohistochemistry in female rats, and the effect of estradiol on this Fos expression was also investigated. Thus, adult female rats were ovariectomized and, six days later, injected subcutaneously with 10 µg/kg estradiol benzoate or vehicle. Twenty-four hours later, solutions containing formalin (1.25% or 5.0%) were injected into the right hind paw. Following formalin injection, rats were anesthetized, and lumbar spinal cords were removed by laminectomy. The number of neurons in the lumbar spinal cord dorsal horn expressing Fos was quantified by immunohistochemistry. Results revealed that formalin evoked concentration-related numbers of Fos-positive neurons in the ipsilateral lumbar spinal dorsal horn. A time-course experiment showed overall Fos expression in laminae I-VI at two hours to be approximately as high if not higher than earlier time points. No differences due to estradiol were observed in the number of Fos-positive neurons in laminae I-VI for 1.25% or 5% formalin. This implied that estradiol was not modulating

nociception at the level of the peripheral nervous system or spinal cord, indicating other anatomical sites may be more likely targets of estrogen modulation.

### *5.2 Introduction*

Estrogens appear to have activational, often pro-nociceptive (pain-potentiating) effects on innervation, synapse formation, and sensory function. Several previous reports suggest that elevated serum estrogen levels enhance persistent inflammatory nociception. However, there are no reports of direct, systematic investigation into identification of the anatomical sites of estrogens' action in the nervous system to enhance nociception and pain. We hypothesized that sex differences in pain sensation are due, at least in part, to activational effects of estradiol on peripheral, spinal and/or supraspinal sites involved in the transmission and perception of nociceptive stimuli. To determine whether spinal activation– as an initial site of investigation – in response to inflammatory pain was increased by estrogen, the current study addressed the levels of Fos (a biomarker of neuronal activation) in the spinal cord dorsal horn evoked by intraplantar formalin (a model of persistent inflammatory pain).

The rostro-caudal distribution of Fos-positive neurons across spinal segments following plantar hind paw formalin injection is highest at the L3-L5 levels (Presley et al., 1990). The current study was designed to focus investigation of hind paw inflammation-driven changes in neuronal activation in as restricted and relevant an area of the spinal cord as possible. Accordingly, Fos expression was measured primarily at the L4-L5 spinal segments.

The distribution of Fos-positive neurons across spinal dorsal horn laminae following noxious stimulation has been previously described (Harris, 1998; Hunt et al., 1987; Kaneko et al., 2000; Khanna et al., 2004; Presley et al., 1990) and is suggested to

correspond to different functions of the laminae. A genetic study showed that variations in the degree of nociception-related licking behavior across mouse strains correlated with the number Fos-positive neurons in spinal laminae V-VI (Bon et al., 2002). These previous descriptions of the laminar organization and Fos expression in the spinal cord provided rationale for the method of quantification of Fos within divided groups of the dorsal laminae used in the current study.

A previous time course study showed that maximum Fos-positive neuron counts in the dorsal spinal cord following formalin injection occurred 2 hours after initiation of the inflammatory stimulus (Presley et al., 1990). The current study set out to verify that this 2 hour time point would be optimal for Fos quantification.

Previous studies have demonstrated stimulus-intensity dependence of the level of Fos immunoreactivity in the spinal cord following formalin injection (Fukuda et al., 2001; Kaneko et al., 2000; Khanna et al., 2004).

### *5.3 Materials & methods*

Adult female Sprague-Dawley rats (~75 days old, 200-230 g, Harlan, Indianapolis, IN) were divided into two groups: 1) ovariectomized (OVX) receiving estradiol, and 2) OVX receiving an equivalent volume of vehicle. Estradiol supplementation was followed 24 hours later by an injection of either 100 μL of 5% formalin or 50 μL of 1.25% formalin into the right plantar hind paw as a model of persistent inflammatory pain.

#### *5.3.1 Hormone manipulation*

For ovariectomy (OVX), rats were anesthetized with a mixture of ketamine (64 mg/kg, i.p.; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (5.3 mg/kg; i.p.). Under aseptic conditions, a ventral midline incision (~1.5 cm) was made into the lower abdominal cavity. Both ovaries were externalized, fallopian tubes and blood vessels ligated with silk suture just proximal to the ovaries, and the ovaries excised. Adipose tissue, fallopian tubes, and blood vessels were reinserted into the abdominal cavity. Muscle and skin layers were individually closed with suture. Six days later, rats received a single subcutaneous injection into the left flank of 10 μg/kg estradiol benzoate (Sigma, E-9000) at 10 μg/mL in vehicle (10% ethanol/90% corn oil), or an equivalent volume of vehicle. This dose of estradiol was chosen to mimic the surge in estradiol observed during proestrus in rats [see (Butcher et al., 1974; Medlock et al., 1991; Nag & Mokha, 2006; Viau & Meaney, 1991; Zoubina et al., 2001)].

*5.3.2 Spinal cord collection*

Two hours after formalin injection (or 3 minutes, 1 or 2 hours for the time course), rats were anesthetized with ketamine/xylazine, and perfused transcardially with ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS, pH 7.4. Lumbar spinal cords were subsequently removed by laminectomy. Spinal cords were post-fixed in PFA at 4 °C, then in 30% sucrose at 4 °C before freezing.

*5.3.3 Immunohistochemistry for Fos*

For fluorescent immunohistochemistry, frozen lumbar spinal cords were cut into 20 μm-thick sections and placed on charged glass microscope slides. Slides were washed in 0.4% Triton X-100 in PBS (PBST), pH 7.4, blocked with 5% normal donkey serum plus 1% BSA in PBST, incubated with primary rabbit anti-Fos polyclonal IgG antibody (1:1000, Calbiochem, Ab-5, Cat. No. PC38) overnight at 4 °C. They were then incubated with secondary fluorescein (FITC)-conjugated donkey anti-rabbit IgG antibody (1:200, Jackson ImmunoResearch, Code No. 711-095-152) for 1 hour at room temperature, then mounted with Vectashield Hard Set mounting medium with DAPI (Vector Laboratories, Cat. No. H-1500). Sections were fluorescently co-labeled for NeuN to verify neuronal identification (not shown). Slides were then viewed on a Nikon 80i fluorescent microscope system, and neurons with Fos-positive immunofluorescence counted in ipsilateral laminae I-VI of sections at least 60 μm apart in spinal segments L4-L5, with at least five sections counted and averaged for each rat. Laminar divisions and spinal segments were based on gross landmarks of the lumbar enlargement according to Paxinos

& Watson (2005). Comparison of Fos immunoreactivity with DAPI staining during counting was used to confirm that only neurons displaying intact nuclei were quantified.

*5.3.4 Data Analyses*

All data represent the mean ± SEM. Data were statistically analyzed using Student's t-tests with the significance level set to $p \leq 0.05$.

### *5.4 Results*

Formalin-evoked inflammatory nociception of the hind paw elicited robust Fos expression in neurons throughout the ipsilateral, lumbar spinal cord dorsal horn two hours after injection into the hind paw (**Figures 32, 35 and 36**). **Figure 32** shows representative photomicrographs of fluorescent immunohistochemical staining for Fos in lumbar spinal cord dorsal horns following injection of 100 μL of 5% formalin into the right hind paw. Formalin-evoked Fos expression was visible throughout the upper laminae of the ipsilateral dorsal horn (for laminar divisions, see **Figure 33**); Fos staining in the contralateral dorsal horn was sparse. Also, there was a prevalence of Fos-positive neurons in the medial part of the dorsal horn and less so laterally (see **Figure 32**). Additionally, results revealed that the magnitude of the number of Fos-positive neurons in the ipsilateral spinal cord dorsal horn evoked by plantar formalin was proportional to the stimulus intensity (**Figures 35 and 36**).

A time course of 1.25% formalin-evoked Fos immunohistochemistry in the spinal dorsal horn rats is shown in **Figure 34**. Results demonstrated the changing pattern of Fos expression over time at 3 minutes, 1 and 2 hours. Ipsilateral to the inflamed hind paw, there was a generally increasing number of Fos-positive neurons over the observed time, for estradiol- or vehicle-treated rats. Overall, Fos expression in laminae I-VI at two hours was higher than earlier time points. Relatively lower numbers of Fos-positive neurons were found on the contralateral side, accompanied by a smaller range of expression over time. Results suggest a peak in contralateral Fos expression at 1 hour.

Comparing rats receiving estradiol (OVX + E2; n = 6) with those receiving vehicle (OVX + Veh; n = 5), data showed that estradiol administration did not alter the number of Fos-positive neurons in spinal cord laminae I-II, III-IV, V-VI, or total across laminae I-VI for 5% formalin (**Figure 35**). The hypothetical possibility of a ceiling effect on the number of Fos-positive neurons at 5% formalin prompted further investigation of the same comparison at a lower, 1.25%, formalin dose. This experiment revealed that 1.25% formalin also elicited robust Fos expression in the ipsilateral dorsal horn, with a pattern similar to that observed with 5% formalin, except relatively lower in magnitude. At this 1.25% formalin stimulus intensity, results again revealed no difference due to estradiol for any of the laminar regions examined (**Figure 36**; n = 8 per group).

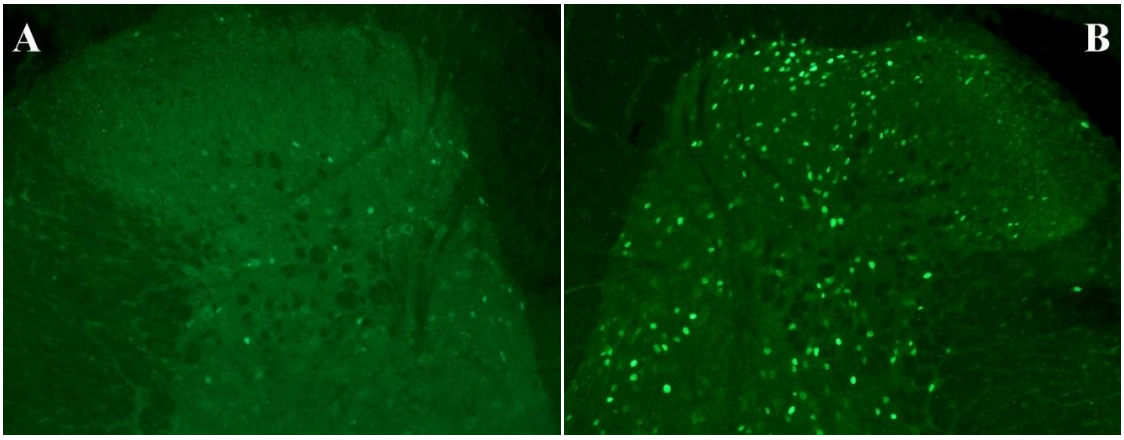


**Figure 32.** Representative photomicrographs of fluorescent immunohistochemical staining for Fos in lumbar spinal cord dorsal horns following formalin. Spinal cords were collected two hours after injection of 100 µL of 5% formalin into the right hind paw. Lumbar spinal cords were cut into 20 µm-thick sections, probed with primary rabbit anti-Fos antibody (1:1000, Calbiochem), and labeled with FITC-conjugated donkey anti-rabbit antibody (1:200, Jackson). Note that formalin-evoked Fos expression is visible throughout the upper laminae of the ipsilateral (right) dorsal horn (panel B); Fos staining in the contralateral dorsal horn (panel A) is sparse. Digital photomicrographs taken at 10X magnification.

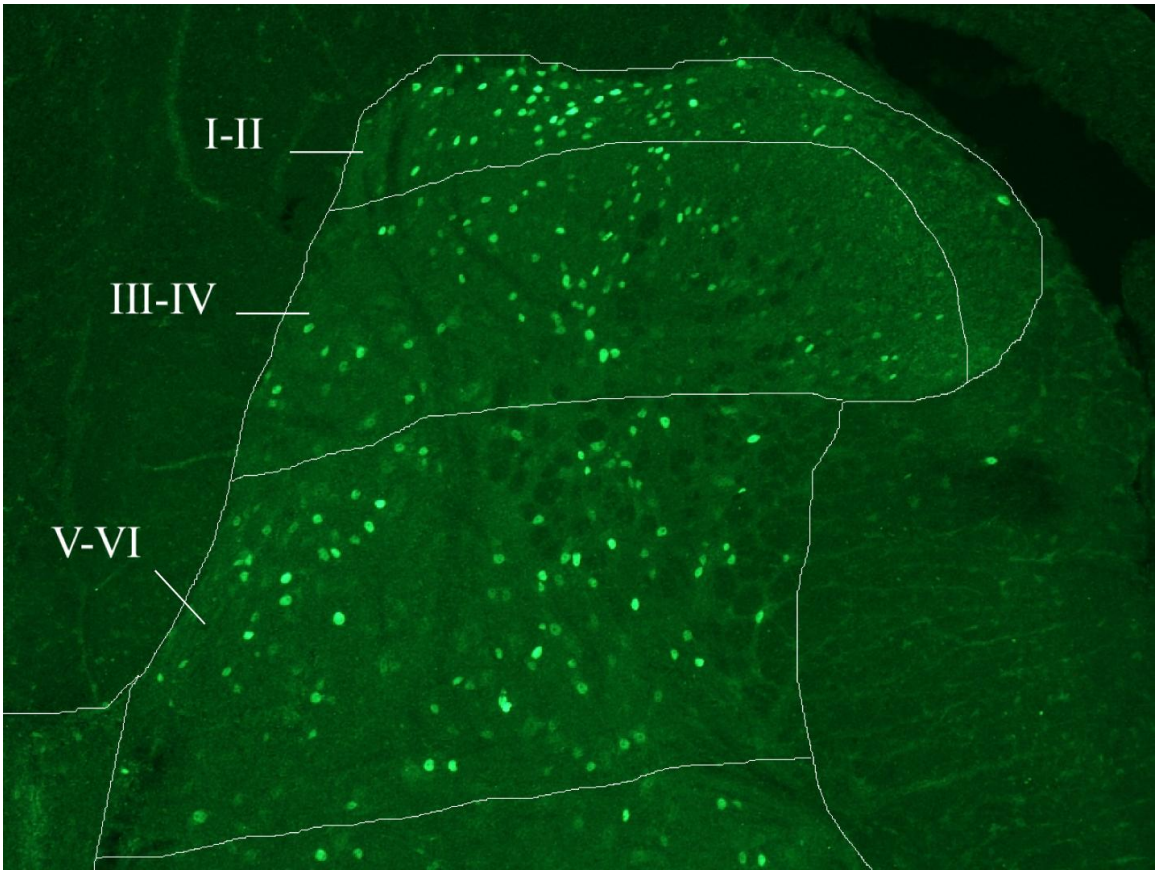


**Figure 33.** Representative example of spinal cord dorsal horn immunohistochemistry for Fos, showing laminar divisions. Digital photomicrograph taken at 10X magnification.

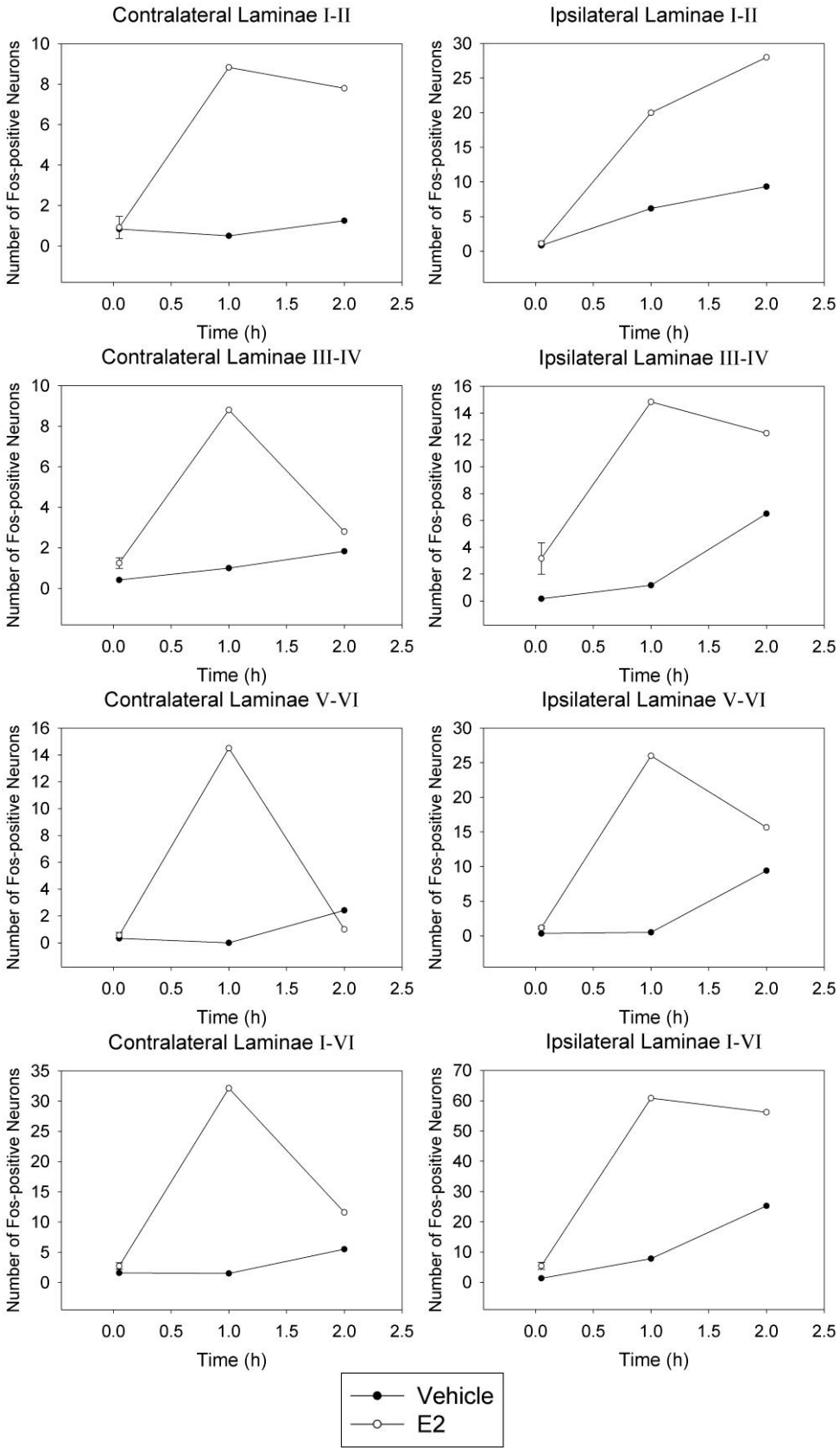


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**Figure 34.** Time course of 1.25% formalin-evoked Fos immunohistochemistry in the spinal dorsal horn. Female rats were ovariectomized (OVX) and, six days later, received a single s.c. injection of 10 µg/kg estradiol (E2) or vehicle. Twenty-four hours after estradiol (E2) injection (seven days after OVX), rats were injected with 50 µL of 1.25% formalin into the right hind paw, and spinal cords were collected 0.05, 1 and 2 hours later. Following immunohistochemical staining for Fos, sections in spinal segments L4-L5 were viewed on a fluorescent microscope, and neurons in laminae I-VI of the spinal cord dorsal horn exhibiting NeuN- and Fos-positive immunoreactivity and DAPI staining were counted. Data represent the mean (± SEM for $n \geq 3$; n: Vehicle: 0.05 h = 2, 1 h = 1, 2 h = 2; E2: 0.05 h = 3, 1 h = 1, 2 h = 1).

| | Number of Fos-positive Neurons | |
|---|---|---|
| Laminae | Vehicle | E2 |
| I-II | 26.4 ± 7.5 | 33.4 ± 5.4 |
| III-IV | 21.9 ± 3.9 | 25.1 ± 4.7 |
| V-VI | 40.3 ± 4.2 | 36.3 ± 4.1 |
| I-VI Total | 88.6 ± 14.1 | 94.7 ± 13.4 |

**Figure 35.** Effect of estradiol on 5% formalin-evoked Fos immunohistochemistry in the ipsilateral spinal dorsal horn. Rats were ovariectomized (OVX) and, six days later, received a single s.c. injection of 10 µg/kg estradiol (E2) or vehicle. Twenty-four hours after estradiol (E2) injection (seven days after OVX), rats were injected with 100 µL of 5% formalin into the right hind paw, and spinal cords were collected two hours later. Following immunohistochemical staining for Fos, sections in spinal segments L4-L5 were viewed on a fluorescent microscope, and neurons in laminae I-VI of the ipsilateral spinal cord dorsal horn exhibiting NeuN- and Fos-positive immunoreactivity and DAPI staining were counted. Note that estradiol administration did not alter Fos expression in any of the laminar regions examined. Data represent the mean ± SEM (Significance level set to $p \leq 0.05$, unpaired Student's t-test; n: Veh = 5; E2 = 6).

| | Number of Fos-positive Neurons | |
|---|---|---|
| Laminae | Vehicle | E2 |
| I-II | 17.5 ± 2.9 | 16.5 ± 0.9 |
| III-IV | 15.1 ± 2.6 | 12.4 ± 2.2 |
| V-VI | 25.6 ± 2.6 | 21.0 ± 3.3 |
| I-VI Total | 59.6 ± 6.8 | 49.9 ± 5.6 |

**Figure 36.** Effect of estradiol on 1.25% formalin-evoked Fos immunohistochemistry in the ipsilateral spinal dorsal horn. Rats were ovariectomized (OVX) and, six days later, received a single s.c. injection of 10 µg/kg estradiol (E2) or vehicle. Twenty-four hours after estradiol (E2) injection (seven days after OVX), rats were injected with 50 µL of 1.25% formalin into the right hind paw, and spinal cords were collected two hours later. Following immunohistochemical staining for Fos, sections in spinal segments L4-L5 were viewed on a fluorescent microscope, and neurons in laminae I-VI of the ipsilateral spinal cord dorsal horn exhibiting NeuN- and Fos-positive immunoreactivity and DAPI staining were counted. Note that estradiol administration did not alter Fos expression in any of the laminar regions examined. Data represent the mean ± SEM (Significance level set to $p \leq 0.05$, unpaired Student's t-test; n = 8 per group).

### *5.5 Discussion*

Formalin-evoked inflammatory nociception of the hind paw elicited robust Fos expression in neurons throughout the ipsilateral, lumbar spinal cord dorsal horn two hours after injection (**Figures 32, 35 and 36**). The magnitude of the number of Fos-positive neurons in the ipsilateral spinal cord dorsal horn evoked by plantar formalin was directly related to the stimulus intensity. This result is consistent with many previous reports that have characterized the activation of neuronal Fos expression as a biomarker of nociceptive activation. Several previous studies have demonstrated the level of Fos immunoreactivity in the (dorsal) spinal cord to be stimulus-intensity dependent for formalin (Fukuda et al., 2001; Kaneko et al., 2000; Khanna et al., 2004) and other noxious stimuli (Abbadie et al., 1994; Bester et al., 1997; Bullitt et al., 1992; Buritova et al., 1998; Hunt et al., 1987; Willcockson et al., 1995).

The time-course experiment showed overall Fos expression in laminae I-VI at two hours to be approximately as high, if not higher, than earlier time points (**Figure 34**), an observation consistent with previous reports in the literature (Presley et al., 1990). This result verified 2 hours post-injection as an optimal time point for further experiments involving Fos quantification for investigating potential estradiol effects on spinal nociceptive neuronal activation.

The overall medial-lateral distribution pattern of Fos expression in the dorsal spinal cord was more medial than lateral (unpublished observations; for example, see **Figure 32**), which is similar to the pattern of spinal Fos expression after formalin observed by Presley et al. (1990). This pattern of Fos expression in spinal neurons relates

to the known somatotopic input to the spinal cord from hind paw peripheral afferents (Bullitt, 1991; Presley et al., 1990).

Elevated Fos expression levels were associated with formalin-evoked nociception overall. Contrary to the working hypothesis, the administration of estradiol did not significantly alter numbers of nociception-evoked Fos-positive neurons in the ipsilateral lumbar spinal cord dorsal horn at either formalin concentration (**Figures 35 and 36**). Our previous results have demonstrated that estrogen supplementation enhances formalin-evoked flinching behavior (see Chapter 4, **Figure 19**). Together, these observations support the conclusion that, whereas estrogen enhances pain-related behaviors evoked by persistent inflammatory nociception, estrogen may not significantly modulate the intensity of nociceptive neuronal activation in the peripheral nervous system (primary sensory neurons) or spinal cord. Conversely, this finding suggests that estrogen may be enhancing inflammatory nociception primarily via actions at other anatomical sites, such as supraspinal sites, where pain sensation is modified subsequent to spinal transmission. The convergence of the neuronal activity that provides the substrate for interpretation of pain intensity and subsequent motor responses may occur at sites farther along nociceptive neuronal pathways than the first synaptic connection in the spinal cord dorsal horn. Alternatively, peripheral or spinal enhancement of nociception within the context of the formalin model may not be manifested as a change in spinal Fos. One potential explanation for this is the manifestation of a "ceiling effect" whereby the dynamic range of Fos expression in the "positive" spinal nociceptive neurons is limited, and is maximized following moderate nociceptive activation, without additional cells available for "recruitment" under the effects of estrogen. However, this interpretation is less likely

because, based on the established data demonstrating the relationship of Fos expression in spinal neurons with noxious peripheral stimulation, and the fact that peripheral nociceptors must signal through the spinal cord, if there was peripheral modulation of nociception, it would likely be seen as a change in the degree of spinal Fos-positive neurons.

In conclusion, these data demonstrate the response of spinal neurons to noxious, persistent, inflammatory stimulation as manifested by the expression of Fos, a biomarker of neuronal activation. Acute estradiol treatment did not alter the number of Fos-positive neurons in the spinal cord within two stimulus intensities. Thus, it appears that the peripheral nervous system and spinal cord may not be primary targets for estrogen acting to increase inflammatory nociception.

# *CHAPTER SIX*

## *FORMALIN HAS MINIMAL DETECTABLE EFFECT ON AND ESTROGEN DOES NOT MODIFY SPINAL ERK ACTIVATION IN FEMALE RATS*

### *6.1 Abstract*

These experiments were undertaken to characterize the activation of ERK (as phospho-ERK, pERK) as an alternative to Fos as a marker for neuronal activation in the spinal cord following formalin injection. We hypothesized that *estradiol would increase pERK in the spinal cord*. Accordingly, adult female rats were ovariectomized and, six days later, injected subcutaneously with 10 µg/kg estradiol benzoate or vehicle. Twenty-four hours later, solutions containing 1.25% or 5.0% formalin were injected into the right hind paw. Following formalin injection, spinal cords were collected. The number of neurons in the lumbar spinal cord dorsal horn expressing pERK was quantified by immunohistochemistry. Alternatively, fresh-frozen lumbar enlargements of spinal cords were dissected into dorsal, ipsilateral quarters and homogenized for pERK quantification by western blotting. Results of immunohistochemistry revealed that minimal numbers of pERK-positive neuronal cell bodies were visible in all laminar regions examined, they had minimal change in number over the time course, and no difference due to estradiol was observed. Western blot analysis showed no difference in pERK activation between no-formalin control and any time point following formalin, and no change between time points. These observations led to the conclusion that quantification of ERK activation by either of these methods was not adequately sensitive to use as a marker for neuronal activation in the spinal cord under these conditions.

### *6.2 Introduction*

Estrogens appear to have activational, often pro-nociceptive (pain-potentiating) effects on innervation, synapse formation, and sensory function. Several previous reports suggest that elevated serum estrogen levels enhance persistent inflammatory nociception. However, there are no reports of direct, systematic investigation into identification of the anatomical sites of estrogens' action in the nervous system to enhance nociception and pain. We hypothesized that sex differences in pain sensation are due, at least in part, to activational effects of estradiol on peripheral, spinal and/or supraspinal sites involved in the transmission and perception of nociceptive stimuli. To determine whether spinal activation– as an initial site of investigation – in response to inflammatory pain was increased by estrogen, the current study addressed the levels of pERK (a biomarker of neuronal activation) in the spinal cord dorsal horn evoked by intraplantar formalin (a model of persistent inflammatory pain). Previous studies have observed activation of ERK (as pERK) in the spinal cord following noxious stimulation, including formalin, although with mixed results (Fukuda et al., 2009; Ji et al., 1999; Li et al., 2010; Svensson et al., 2006).

### *6.3 Materials & methods*

Adult female Sprague-Dawley rats (~ 11-12 weeks old, 200-230 g, Harlan, Indianapolis, IN) were divided into two groups: 1) ovariectomized (OVX) receiving estradiol, and 2) OVX receiving an equivalent volume of vehicle. Estradiol supplementation was followed 24 hours later by an injection of either 100 µL of 5% or 50 µL of 1.25% formalin into the right plantar hind paw as a model of persistent inflammatory pain. Separate sets of animals were used for these experiments; the rats treated with 100 µL of 5% formalin were prepared for western blot analysis following investigation of Fos immunohistochemistry in similarly-treated animals (see Chapter 5). Subsequently, a lower concentration of formalin was selected to address perceived limitations of the 5% stimulus (see Chapter 5 discussion, page 127). Accordingly, immunohistochemical analysis of spinal pERK in rats treated with 50 µL of 1.25% formalin was conducted in alternate sections from the same rats used for analysis of Fos immunostaining (see Chapter 5).

#### *6.3.1 Hormone manipulation*

For ovariectomy (OVX), rats were anesthetized with a mixture of ketamine (64 mg/kg, i.p.; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (5.3 mg/kg; i.p.). Under aseptic conditions, a ventral midline incision (~1.5 cm) was made into the lower abdominal cavity. Both ovaries were externalized, fallopian tubes and blood vessels ligated with suture just proximal to the ovaries, and the ovaries excised. Adipose tissue, fallopian tubes, and blood vessels were reinserted into the abdominal cavity. Muscle and skin layers were individually closed with suture. Six days later, rats received a single

subcutaneous injection into the left flank of 10 μg/kg estradiol benzoate (Sigma, E-9000) at 10 μg/mL in vehicle (10% ethanol/90% corn oil), or an equivalent volume of vehicle. This dose of estradiol was chosen to mimic the surge in estradiol observed during proestrus in rats [see (Butcher et al., 1974; Medlock et al., 1991; Nag & Mokha, 2006; Viau & Meaney, 1991; Zoubina et al., 2001)].

### *6.3.2 Spinal cord collection*

#### *6.3.2.1 For immunohistochemistry*

Three minutes (or 0.05, 1 or 2 hours for the time course) after injection of 50 μL of 1.25% formalin, rats were anesthetized with ketamine/xylazine, and perfused transcardially with ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS, pH 7.4. Lumbar spinal cords were subsequently removed by laminectomy. Spinal cords were post-fixed in PFA at 4 °C, then in 30% sucrose at 4 °C before freezing.

#### *6.3.2.2 For western blotting*

Either without formalin injection or 5 or 40 minutes after injection of 100 μL of 5% formalin into the right hind paw, rats were decapitated. Spinal cords were then removed by hydraulic extrusion with 0.9% saline, and immediately frozen.

### *6.3.3 Immunohistochemistry for pERK*

Frozen, fixed lumbar spinal cords were cut into 20 μm-thick sections and placed on charged glass microscope slides. For fluorescent immunohistochemistry, slides were washed in 0.1% Tween-20 in Tris-buffered saline, pH 7.6 (TBST), blocked with 5%

normal donkey serum in TBST, and incubated with primary rabbit anti-pERK monoclonal IgG (1:100, Cell Signaling Technology, #4370) overnight at 4 °C. Then they were incubated with secondary fluorescein (FITC)-conjugated donkey anti-rabbit IgG antibody (1:200, Jackson ImmunoResearch, Code No. 711-095-152) for 1 hour at room temperature and then mounted with Vectashield Hard Set mounting medium with DAPI (Vector Laboratories, Cat. No. H-1500). Sections were fluorescently co-labeled for NeuN to verify neuronal identification (not shown). Slides were then viewed on a Nikon 80i fluorescent microscope system, and neurons with Fos- or pERK-positive immunofluorescence counted in ipsilateral laminae I-VI of sections at least 60 μm apart in spinal segments L4-L5, with at least five sections counted and averaged for each rat. Comparison of pERK immunoreactivity with DAPI staining during counting was used to confirm that only neurons displaying intact nuclei were quantified.

*6.3.4 Western blotting for pERK*

Lumbar enlargements of fresh-frozen spinal cords were dissected into ipsilateral, dorsal quarters, homogenized and lysed. Homogenates were blotted by electrophoresis, then probed with primary rabbit anti-pERK antibody (1:200, #4376, Cell Signaling Technology), and labeled with HRP-conjugated bovine anti-rabbit secondary antibody (1:2000, #sc2374, Santa Cruz Biotechnology) and chemiluminescent substrate. Then blots were stripped and re-probed for total ERK with rabbit anti-ERK primary antibody (1:1000, #9102, Cell Signaling Technology) and labeled with HRP-conjugated bovine anti-rabbit secondary antibody (1:10000, #sc2374, Santa Cruz Biotechnology) and chemiluminescent substrate. Blots were exposed to photographic film, and the film

developed and digitally photographed. Ovariectomized rats that received estradiol or vehicle were included together within all of the treatment groups/time points.

*6.3.5 Data analyses*

All data represent the mean ± SEM. Data were statistically analyzed by Student's t-test or one-way ANOVA with the significance level set to $p \leq 0.05$. Photomicrographs of western blots were analyzed using quantitative densitometry to measure the area under the density curves for each relevant band in the western blots using ImageJ software (NIH).

### *6.4 Results*

Immunohistochemistry following formalin showed there were a few pERK-expressing neurons visible, mostly in the upper laminae, of the ipsilateral (right) dorsal horn (**Figure 37**), although positive pERK staining was sparse in general across laminae I-VI.

**Figure 38** shows the results of a pilot study investigating the time course of pERK immunohistochemistry in the spinal dorsal horn evoked 3 minutes, 1 and 2 hours after 1.25% formalin. Minimal numbers of pERK-positive neuronal cell bodies were found in laminae I-II, III-IV, V-VI or total across I-VI, and they had minimal change in number over the time course (n: Vehicle: 0.05 h = 3, 1 h = 1, 2 h = 2; E2: 0.05 h = 3, 1 h = 1, 2 h = 1). At the three-minute time point after 1.25% formalin, estradiol did not alter the number of pERK-positive neurons in laminae I-II, III-IV, V-VI or total across I-VI (**Figure 39**;Student's t-test; n = 3 per group).

**Figure 40** shows photomicrographs of representative western blots for ERK in spinal cord homogenates following 5% formalin. They demonstrate that pERK1, pERK2, total ERK1 and total ERK2 were all present and detectable by the western blotting method. Results of western blotting revealed no effect of formalin on pERK levels in the dorsal spinal cord at five or forty minutes (**Figure 41**;one-way ANOVA; n = 8 per group).

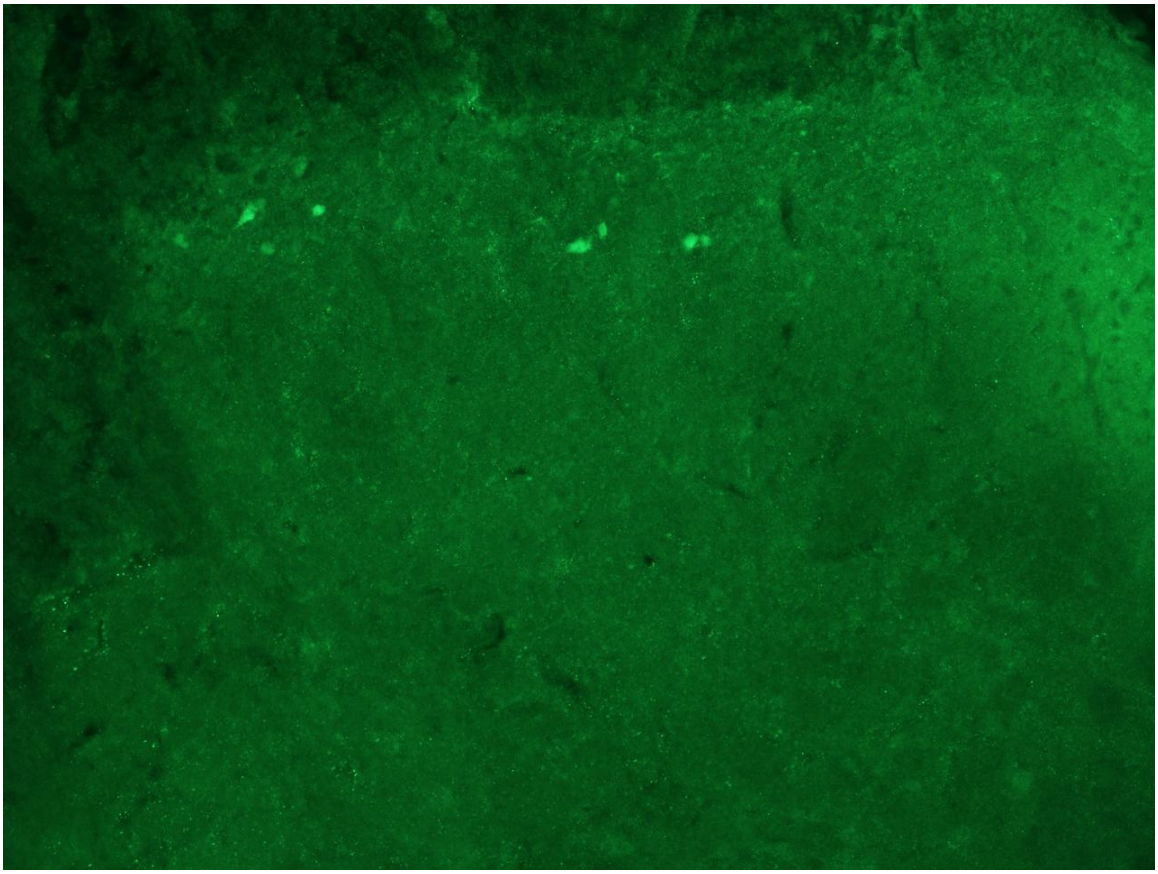

**Figure 37.** Representative photomicrograph of fluorescent immunohistochemical staining for pERK in ipsilateral lumbar spinal cord dorsal horn following formalin. Spinal cords were collected three minutes after injection of 100 µL of 5% formalin into the right hind paw. Subsequently, 20 µm-thick sections were cut from the lumbar enlargement, probed with primary rabbit anti-pERK antibody (1:100, #4370, Cell Signaling Technology), and labeled with FITC-conjugated donkey anti-rabbit antibody (1:200, Jackson). Note there were few pERK-expressing neurons visible in the upper laminae of the ipsilateral (right) dorsal horn, although positive pERK staining was sparse throughout the upper dorsal horn. Digital photomicrograph taken at 10X magnification.

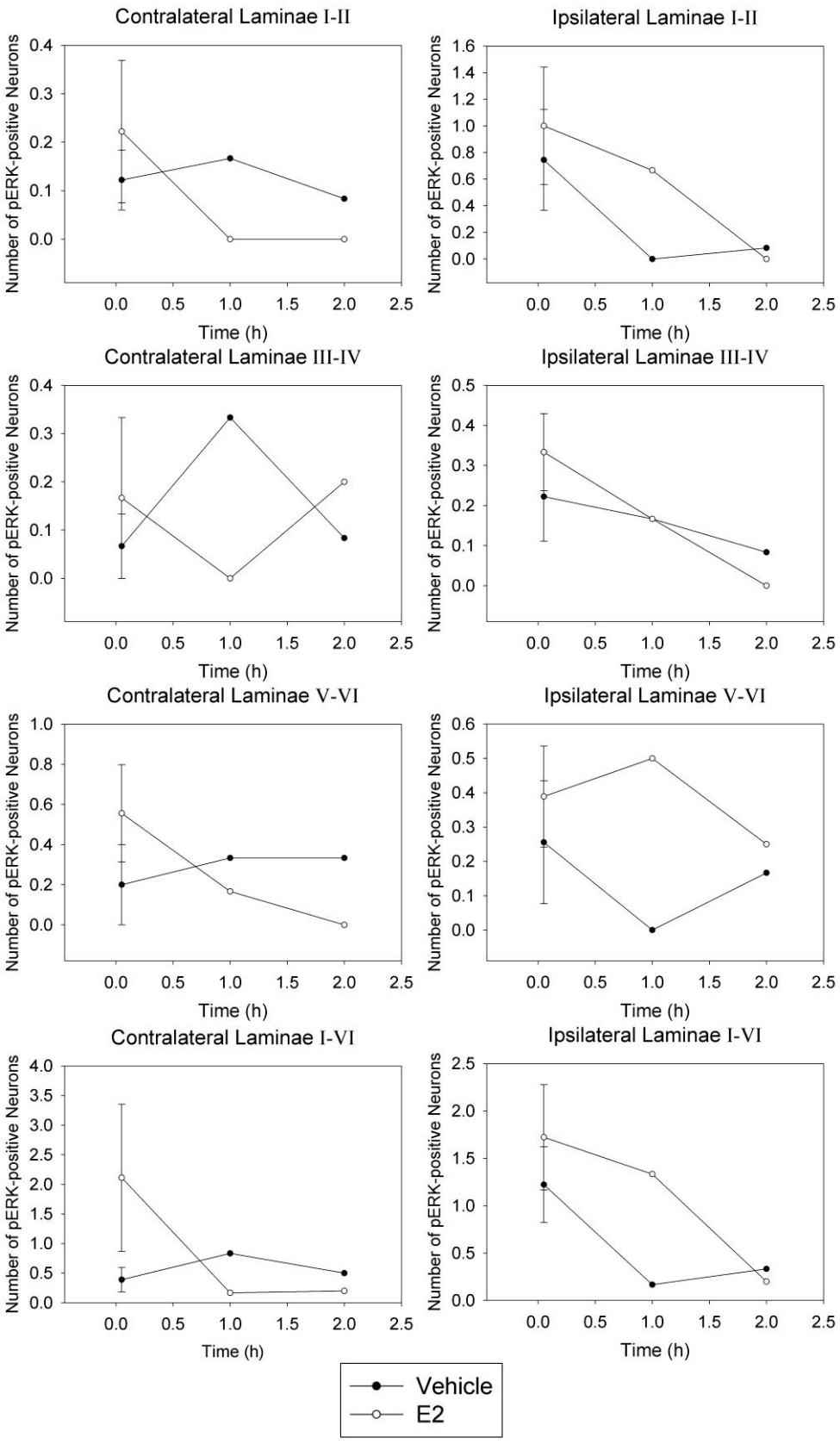


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**Figure 38.** Time course of 1.25% formalin-evoked pERK immunohistochemistical staining in the spinal dorsal horn. Female rats were ovariectomized (OVX) and, six days later, received a single s.c. injection of 10 µg/kg estradiol (E2) or vehicle. Twenty-four hours after estradiol (E2) injection (seven days after OVX), rats were injected with 50 µL of 1.25% formalin into the right hind paw, and spinal cords were collected 0.05, 1 and 2 hours later. Following immunohistochemical staining for pERK in spinal segments L4-L5, sections were viewed on a fluorescent microscope, and neurons in laminae I-VI of the spinal cord dorsal horn exhibiting NeuN- and pERK-positive immunoreactivity and DAPI staining were counted. Data represent the mean (± SEM for $n \geq 3$; n: Vehicle: 0.05 h = 3, 1 h = 1, 2 h = 2; E2: 0.05 h = 3, 1 h = 1, 2 h = 1).

| | Number of pERK-positive Neurons | |
|---|---|---|
| Laminae | Vehicle | E2 |
| I-II | 0.7 ± 0.4 | 1 ± 0.4 |
| III-IV | 0.2 ± 0.1 | 0.3 ± 0.1 |
| V-VI | 0.3 ± 0.2 | 0.4 ± 0.1 |
| I-VI Total | 1 ± 0.4 | 2 ± 0.6 |

**Figure 39.** Effect of estradiol on 1.25% formalin-evoked pERK immunohistochemistry in the ipsilateral spinal dorsal horn. Female rats were ovariectomized (OVX) and, six days later, received a single s.c. injection of 10 µg/kg estradiol (E2) or vehicle. Twenty-four hours after estradiol (E2) injection (seven days after OVX), rats were injected with 50 µL of 1.25% formalin into the right hind paw, and spinal cords were collected 3 minutes later. Following immunohistochemical staining for pERK in spinal segments L4-L5, sections were viewed on a fluorescent microscope, and neurons in laminae I-VI of the ipsilateral spinal cord dorsal horn exhibiting NeuN- and pERK-positive immunoreactivity and DAPI staining were counted. Minimal numbers of pERK-positive neurons were observed in all laminae, and estradiol did not alter the number of pERK-positive neurons in any of the laminar regions examined. Data represent the mean ± SEM (Significance level set to $p \leq 0.05$, unpaired Student's t-test; n = 3 per group).

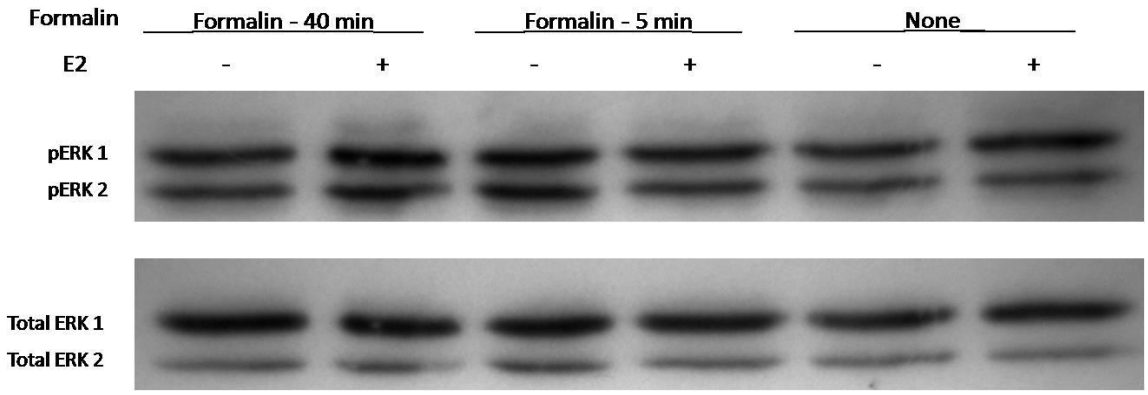


**Figure 40.** Photographs of representative western blots for ERK in ipsilateral dorsal spinal cord following 5% formalin. Female rats were ovariectomized (OVX) and, six days later, received a single s.c. injection of 10 µg/kg estradiol (E2) or vehicle. Twenty-four hours after estradiol (E2) injection (seven days after OVX), spinal cords were collected either without formalin injection or 5 or 40 minutes after injection of 100 µL of 5% formalin into the right hind paw. Ipsilateral dorsal quarters of the lumbar enlargements of spinal cords were homogenized and lysed. Homogenates were blotted by electrophoresis, then probed with primary rabbit anti-pERK antibody (1:200, #4376, Cell Signaling Technology), and labeled with HRP-conjugated bovine anti-rabbit secondary antibody (1:2000, #sc2374, Santa Cruz Biotechnology) and chemiluminescent substrate. Blots were then stripped and re-probed for total ERK with rabbit anti-ERK primary antibody (1:1000, #9102, Cell Signaling Technology) and labeled with HRP-conjugated bovine anti-rabbit secondary antibody (1:10000, #sc2374, Santa Cruz Biotechnology) and chemiluminescent substrate. Blots were exposed to photographic film, and the film developed and digitally photographed and analyzed.

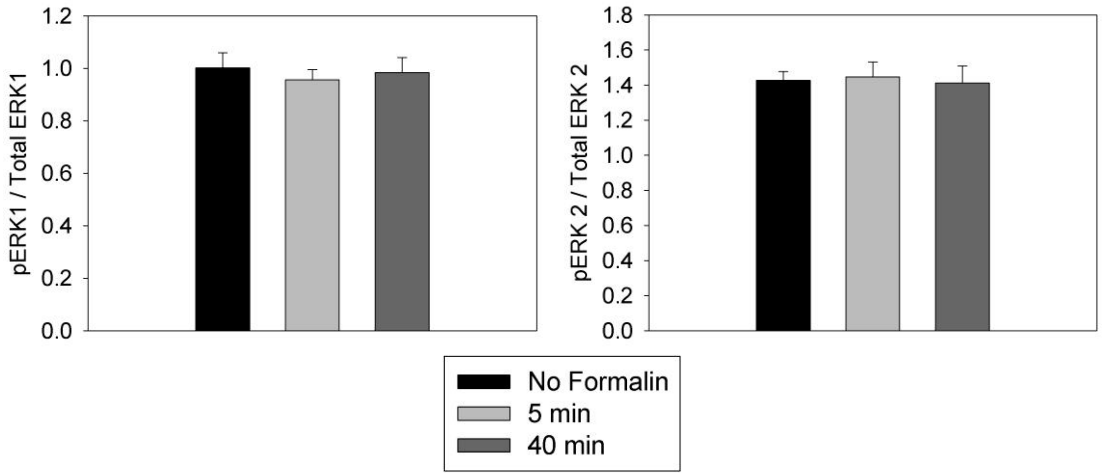


**Figure 41.** Time course of ERK activation in ipsilateral dorsal spinal cord following 5% formalin. Female rats were ovariectomized (OVX) and, six days later, received a single s.c. injection of 10 µg/kg estradiol (E2) or vehicle. Twenty-four hours after estradiol (E2) injection (seven days after OVX), spinal cords were collected either without formalin injection or 5 or 40 minutes after injection of 100 µL of 5% formalin into the right hind paw. Ipsialteral dorsal quarters of the lumbar enlargements were homogenized and lysed. Homogenates were blotted by electrophoresis, then probed with primary rabbit anti-pERK antibody (1:200, #4376, Cell Signaling Technology), and labeled with HRP-conjugated bovine anti-rabbit secondary antibody (1:2000, #sc2374, Santa Cruz Biotechnology) and chemiluminescent substrate. Blots were then stripped and re-probed for total ERK with rabbit anti-ERK primary antibody (1:1000, #9102, Cell Signaling Technology) and labeled with HRP-conjugated bovine anti-rabbit secondary antibody (1:10000, #sc2374, Santa Cruz Biotechnology) and chemiluminescent substrate. Blots were exposed to photographic film, and the film developed and digitally photographed. The digital photographs were analyzed by densitometry using ImageJ software. Results revealed no effect of formalin on pERK levels at five or forty minutes. Data represent the mean ± SEM (Significance level set to $p \leq 0.05$, one-way ANOVA; n = 8 per group).

### *6.5 Discussion*

Following formalin, minimal numbers of pERK-positive neurons were found in laminae I-VI, and they did not change in number over the time course examined (**Figures 37, 38 and 39**). In addition, results of western blotting revealed no effect of formalin on pERK levels in the dorsal spinal cord (**Figure 41**). These observations led to the conclusion that quantification of ERK activation by either of these methods, IHC or western blotting, was not adequately sensitive to observe nociception-evoked changes in neuronal activation in the spinal cord under these conditions.

These results are difficult to compare to one previous study of ERK activation in the superficial spinal cord following noxious stimulation with formalin (Ji et al., 1999). An immunohistochemical time course in that study showed activation of pERK above baseline that peaked at three minutes post-formalin and then fell to lower levels for up to an hour later. They showed a similar time course of spinal pERK after capsaicin injection, another inflammatory, noxious chemical. The results of that study suggest that pERK could be a useful and sensitive marker for nociceptive activation in the spinal cord following this kind of stimulation. However, their method of pERK quantification may have (it is unclear) summed the total number of positive neurons over eight sections per rat, as opposed to taking the average number per section, as did the current study. In addition, that study only had 2-3 rats per group, and used a larger section thickness (30 µm) than the current study (20 µm). These factors make it difficult to compare these studies and complicate the interpretation of whether either method of pERK quantification is an adequate proxy for the degree of spinal activation.

Another, later study more clearly demonstrated activation of pERK in the spinal cord after formalin injection compared with baseline control using western blotting (Svensson 2006). Despite this, similar to the current study, a different immunohistochemical study failed to demonstrate a difference in spinal pERK following formalin compared to control, although it measured pERK at a later time point (Fukuda et al., 2009).

Given the rapid onset of ERK activation, pERK levels may be more representative of the noxious stimulation that occurs in that early time period, such as the mechanical nociception due to the needle prick or the initial, direct chemical activation of nociceptors. Furthermore, pERK is likely less representative of the degree of activation that occurs at later time points during persistent, inflammatory nociception, when sensitization of the spinal cord is being manifested. Although activation of ERK may have a role in the early response to noxious stimulation that leads to the later sensitization (Alter et al., 2010; Choi et al., 2006; Ji et al., 1999; Karim et al., 2006; Karim et al., 2001; Polgar et al., 2007; Svensson et al., 2006), this does not necessarily mean that it is an adequate marker for the degree of activation that occurs during the later period, after the nervous system has been sensitized. For example, spinal pERK was not elevated three days after formalin injection, when formalin-induced secondary mechanical hyperalgesia was present (Li et al., 2010). Thus, pERK may not be the best marker for the degree of nociceptive activation that occurs with persistent, noxious, inflammatory stimuli.

In conclusion, approaches used in this study to address pERK, including both immunohistochemical and western-blot techniques, revealed that estradiol did not modify nociception-evoked levels of ERK activation in the ipsilateral lumbar spinal cord dorsal

horn. This result indicates that, at this formalin stimulus condition, estrogen modulation of spinal pERK expression was not apparent. Our previous results have demonstrated that estrogen supplementation enhances formalin-evoked flinching behavior (see Chapter 4, **Figure 19**). Taken together, these observations support the conclusion that while estrogen enhances pain-related behaviors evoked by persistent inflammatory nociception, estrogen may not significantly alter the intensity of neuronal activation of primary sensory or spinal neurons. Interpretation of this negative result is hindered by the limitation that formalin elicited minimal pERK-positive neurons in the spinal cord. Beacuse of this, peripheral or spinal enhancement of nociception within the context of the formalin model may not be manifested as a measurable change in spinal pERK, possibly because the range of pERK expression elicited by formalin is too small to observe any modulation by estrogen.

Finally, the findings of this study and the previous study addressing Fos activation suggested that estrogen may primarily be enhancing inflammatory nociception via actions at supraspinal sites where pain sensation is modified subsequent to spinal transmission. Based on this possibility, the subsequent study was designed to address the activation of several candidate higher brain centers by formalin and the modification of its effects by estrogen.

# *CHAPTER SEVEN*

## *ESTROGEN MODIFIES PERSISTENT INFLAMMATORY NOCICEPTION IN THE BRAIN: STUDIES OF FOS EXPRESSION IN SPECIFC BRAIN REGIONS FOLLOWING FORMALIN INJECTION IN FEMALE RATS*

### *7.1 Abstract*

This study explored whether nociceptive activation of higher brain centers is increased by estrogen supplementation. Previous results from our laboratory and other studies have indicated estrogen can enhance formalin-evoked pain-related behaviors. We hypothesized that *an estradiol surge would modify formalin-evoked levels of a marker of nociception, Fos, in supraspinal brain regions*. Using immunohistochemistry for Fos, brain structures investigated were some known to be important for processing the affective component of pain: hippocampus, amygdala and nucleus accumbens. To test this hypothesis, adult female rats were ovariectomized and, six days later, injected subcutaneously with 10µg/kg estradiol benzoate or vehicle. Twenty-four hours later, 50 µL of 1.25% or 100 µL of 5% formalin was injected into the right hind paw. Two hours following formalin injection, brains were removed and frozen. Brains were subsequently cut into coronal sections, and the number of Fos-expressing neurons in the nucleus accumbens, amygdala and hippocampus was quantified by immunohistochemistry. Pilot studies hand-quantified fluorescent immunohistochemistry of Fos at control and different doses of formalin, and revealed no differences due to estrogen. An improved method of immunohistochemistry (colorimetric with image analysis) was then employed which had the purpose of establishing a relationship of Fos expression to formalin dose and control conditions. Results revealed that formalin (1.25%) alone increased Fos in the right nucleus accumbens; no changes due to formalin alone were observed in hippocampal dentate gyrus or ventral CA1, or central amygdala. Formalin (1.25%) increased Fos in left nucleus accumbens in the presence of estradiol, but did not change Fos in absence of E2. Estradiol decreased Fos in right ventral CA1 in the presence of 5% formalin; but

estradiol made no difference in the absence of formalin. Also, estradiol did not alter Fos expression in the dentate gyrus or central amygdala and, thus, may not modify nociception in those regions. Overall, these findings support the hypothesis that estrogen enhances inflammatory nociception at supraspinal sites.

### *7.2 Introduction*

Previous investigations in our laboratory and several previous reports have demonstrated that elevated serum estrogen levels enhance persistent inflammatory nociception [see Chapter 4, (Bradshaw et al., 2000; Fillingim et al., 1997; Hellstrom & Anderberg, 2003; Isselee et al., 2002; Lautenbacher & Rollman, 1993; LeResche et al., 2003; Meisler, 1999; Somerville, 1972; Tall & Crisp, 2004)]. However, there are no reports of direct, systematic investigation into identification of the anatomical sites of estrogens' action in the nervous system to enhance nociception and pain. Previous findings indicated no difference due to estradiol in number of Fos-expressing neurons in the dorsal spinal cord following intraplantar formalin, suggesting supraspinal sites of action (see Chapter 5). We hypothesized that sex differences in pain sensation are due, at least in part, to activational effects of estradiol on supraspinal sites involved in the transmission and perception of nociceptive stimuli. The relationship of Fos expression to intraplantar formalin stimulus has been described for some supraspinal sites, including the hippocampal dentate gyrus (inverse) and ventral CA1 (inverse) (Khanna et al., 2004). To determine if supraspinal activation in response to inflammatory pain is increased by estrogen, the current study quantified the number of Fos-expressing neurons in the amygdala, hippocampus and nucleus accumbens following intraplantar formalin (a model of persistent inflammatory pain) as initial sites of investigation.

These sites were selected for investigation based on the predominance of inflammatory nociception-evoked changes in neurokinin-1 receptor (NK-1) and brain-derived neurotrophic factor (BDNF) gene expression previously determined in the McCarson laboratory. While investigating the effects of formalin-evoked nociception on

the plasticity of gene expression in the rat brain, Amy Allen, Ph.D., discovered that chemogenic inflammatory stimuli alter BDNF and NK-1 receptor gene expression in limbic brain structures associated with the regulation of affect (data from Dr. Allen's results are represented in **Figure 42**). The direction of these changes (increase versus decrease) were not consistent across all brain regions studied; NK-1 and BDNF gene expression was robustly down-regulated in the hippocampus and amygdala following formalin-evoked nociception, but were markedly increased in the nucleus accumbens following the same treatment. These changes suggest communication between signaling pathways that regulate BDNF and NK-1 receptor gene expression in limbic brain structures during inflammatory nociception. The regional differences in BDNF and NK-1 receptor gene expression may underlie long-term changes in sensory processing and mood associated with chronic pain.

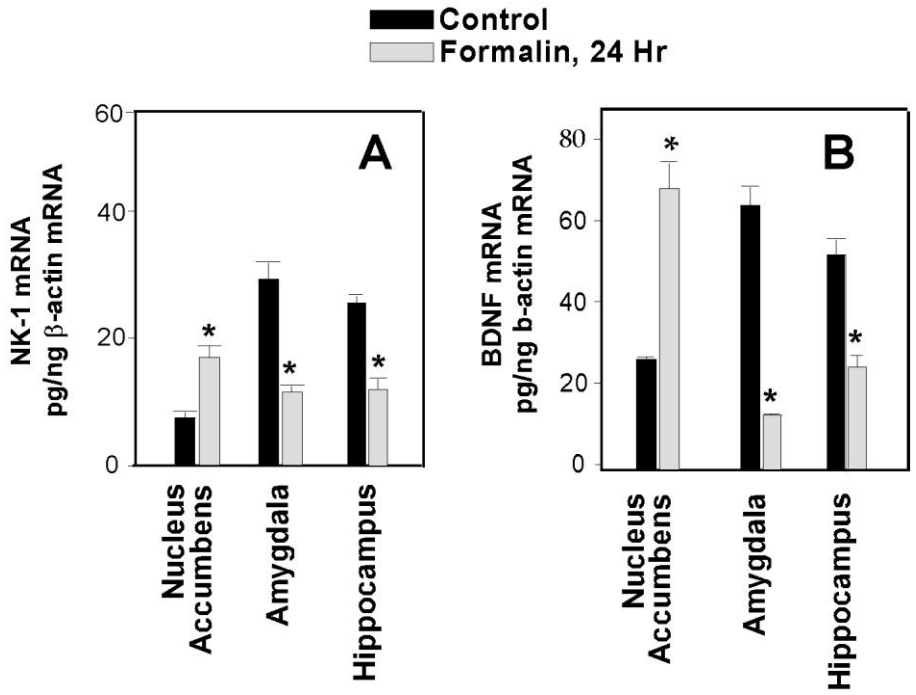


**Figure 42.** Histogram showing BDNF and NK-1 receptor mRNA levels in the nucleus accumbens, amygdala, and hippocampus of male rats after an inflammatory stimulus. A) BDNF gene expression levels B) NK-1 receptor gene expression levels. Note that formalin treatment for 24 hours resulted in a significant increase in BDNF and NK-1 gene expression in the nucleus accumbens and significant decreases in the amygdala and hippocampus. All values are shown as pg specific mRNA/ng β-actin mRNA (Mean ± S.E.M.; n = 6) *$p < 0.05$ vs. control rats (ANOVA and Fisher's PLSD test).

Based on these observations, the amygdala, hippocampus and nucleus accumbens were selected for analysis of Fos expression following formalin-evoked inflammatory nociception. In addition, these structures have functional roles in processing negative affect or nociception, and undergo structural and functional changes in affective disorders (Ferre et al., 2007; Khanna & Sinclair, 1992; Knyihar & Csillik, 2006; Koyanagi et al., 2008; Magnusson & Martin, 2002; Mayberg, 1997; Millan, 1999; Neugebauer, 2007; Neugebauer et al., 2004; Phelps & LeDoux, 2005; Ploghaus et al., 2001; Porro, 2003; Price, 2000, 2002; Price et al., 2006; Sheline et al., 1996; Zubieta et al., 2003; Zubieta et al., 2001). Also, there is considerable plasticity in these structures both with regard to

nociceptive/affective function as well as interaction with estrogen (Cooke & Woolley, 2005; Desmond & Levy, 1997; Maren, 2005; McEwen, 2001; McEwen & Magarinos, 2001; Neugebauer, 2007; Neugebauer et al., 2004; Price, 2000, 2002; Price et al., 2006; Woolley, 1998, 2000; Woolley & McEwen, 1992), making these structures logical choices for investigating potential estrogen modulation of nociception.

### *7.3 Materials & methods*

Adult female Sprague-Dawley rats (~11 weeks old, 200-250 g, Harlan, Indianapolis, IN) were used in these experiments. Two types of experiments were performed: formalin-only or estradiol-supplementation experiments. Rats in the formalin-only experiments were ovariectomized but received no further hormone manipulation; for the colorimetric immunohistochemistry experiments, data from rats that received vehicle in the estradiol-supplementation experiments were included in the formalin-only experimental results.

Rats in the estradiol-supplementation experiments were divided into four groups: 1) ovariectomized (OVX) receiving estradiol (E2) and no formalin (restraint-only); 2) OVX receiving an equivalent volume of vehicle and no formalin (restraint-only); 3) OVX receiving E2 and formalin injection; and 4) OVX receiving vehicle and formalin injection. Some preliminary experiments did not include the two restraint-only treatment groups (groups 1 and 2 above).

#### *7.3.1 Hormone manipulation*

For ovariectomy (OVX), rats were anesthetized with a mixture of ketamine (64 mg/kg, i.p.; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (5.3 mg/kg; i.p.). Under aseptic conditions, both ovaries were externalized and excised. Muscle and skin layers were individually closed. Six days later, rats in the estradiol-supplementation experiments received a single subcutaneous injection into the left flank of 10 μg/kg estradiol benzoate (Sigma, E-9000) at 10 μg/mL in vehicle (10% ethanol/90% corn oil), or an equivalent volume of vehicle.

*7.3.2 Pain model (Formalin injection)*

Approximately 24 hours after estradiol or vehicle injection (or seven days after OVX), rats received an injection of 50 μL of 1.25% formalin or 100 μL of 5% formalin into the right plantar hind paw as a model of persistent inflammatory pain. Restraint-only control rats were manually restrained and hind paw handled as if injecting, but received no needle prick or injection.

*7.3.3 Tissue collection*

*7.3.3.1 Brains*

Two hours after formalin injection, rats were anesthetized with ketamine/xylazine, and perfused transcardially with ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS, pH 7.4. Brains were subsequently removed and post-fixed overnight in PFA at 4 °C, then in 30% sucrose at 4 °C for a few days before freezing.

*7.3.3.2 Uteri*

Approximately 24 hours after estradiol or vehicle injection (seven days after OVX), a separate group of rats were weighed then decapitated, adipose tissue was removed from uteri, and uteri were excised at the base and weighed wet.

*7.3.4 Immunohistochemistry for Fos*

Frozen brain coronal sections at approximate Bregma +2.3 through +0.8 mm, −1.6 through −3.1 mm, and −4.8 through −6.3 mm [based on "The Rat Brain in

Stereotaxic Coordinates" by Paxinos & Watson (2005)] were prepared at 20 μm using a cryostat and mounted on microscope slides.

*7.3.4.1 Fluorescent immunohistochemistry*

Coronal sections were collected at Bregma −1.6 through −3.1 mm (amygdala and rostral hippocampus for dentate gyrus and CA1/2/3), and −4.8 through −6.3 mm (caudal hippocampus for ventral dentate gyrus and ventral CA3) [based on "The Rat Brain in Stereotaxic Coordinates" by Paxinos & Watson (2005)]. Sections were probed with primary rabbit anti-Fos antibody (1:1000, Calbiochem), and labeled with FITC-conjugated donkey anti-rabbit antibody (1:200, Jackson), as described for spinal cord sections (see Chapter 5). Sections were then viewed on a fluorescent microscope, and neurons in CA1, CA2, CA3 and dentate gyrus (DG) of the hippocampus and basolateral and basomedial (combined), central and lateral nuclei of amygdala exhibiting NeuN- and Fos-positive immunoreactivity and DAPI staining were counted for the entirety of each region, with 2-8 sections averaged per rat. Anatomical regions were defined based on Paxinos & Watson (2005).

*7.3.4.2 Colorimetric immunohistochemistry and automated image analysis*

Coronal sections were collected at Bregma +2.3 through +0.8 mm (nucleus accumbens), −1.6 through −3.1 mm (hippocampal dentate gyrus and central amygdala), and Bregma −4.8 through −6.3 mm (hippocampal ventral CA1). Sections were incubated one hour at room temperature with a rabbit polyclonal anti-Fos primary antibody (1:1000, Calbiochem, La Jolla, CA). Sections were then incubated with secondary antibody (1:200, goat anti-rabbit, Vector Laboratories, Burlingame, CA)for 30

minutes at room temperature, coupled with avidin DH: biotinylated horseradish peroxidase H complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA), and visualized with diaminobenzidine (ImmPACT DAB Peroxidase Substrate, Vector Laboratories, Burlingame, CA), according to our optimized protocol based on the manufacturer's instructions. Anatomical regions were defined based on Paxinos & Watson (2005). Digital photomicrographs of each region were taken at 40X magnification using a Nikon 80i light microscope. One image sampling area was chosen for each region of interest as follows: A) nucleus accumbens: dorsomedial to the anterior commissure so that the anterior commissure is just out of frame; B) central amygdala: to include the lateral-most portion of the central nucleus; C) dentate gyrus (anterodorsal hippocampus): to include both aspects of the granular layer at the ventromedial "corner" of the dentate gyrus near the 3$^{rd}$ ventricle; and D) ventral CA1 (posterior hippocampus): within one to two frames dorsal of the "turn" of the granular layer where its orientation changes from more medio-lateral to dorso-ventral (or vice versa). Images were always oriented with the shorter (top-bottom) dimension approximately parallel with the dorso-ventral axis. An automated image analysis macro with the software ImageJ was used, which included, for all images, thresholding of intensity – based on the modal pixel intensity value of the entire image – and positive area size limits (to exclude areas too small or large to be neuronal nuclei) before automated counting. The threshold for positivity was determined empirically and set to pixel intensity ≤ (darker than) 81% of the modal pixel intensity value of the (each) entire image. The positive area size limits were set to include pixel area sizes that were calculated to be equivalent to the area occupied by circles with diameters of 5-15 μm.

Limitations were also made on the relative circularity of shape of these areas. Immuno-positive cells were counted and averaged per image for sections (at least 60 μm between sections) within the rostral-caudal extent for each supraspinal site (one image per region of interest, per section) from each rat, with 3-18 sections averaged per rat. The average number of immuno-positive cells in sections without primary antibody was subtracted from other average values for each rat. After group averaging, data were normalized to the control group value for the left or right side independently of one another. Data are expressed as the mean ± SEM for each group.

*7.3.5 Statistical analyses*

All data represent the mean ± SEM. Data were statistically analyzed by Student's t-test or one-way ANOVA with a post hoc (Holm-Sidak) multiple comparisons test, with the significance level set to $p \leq 0.05$.

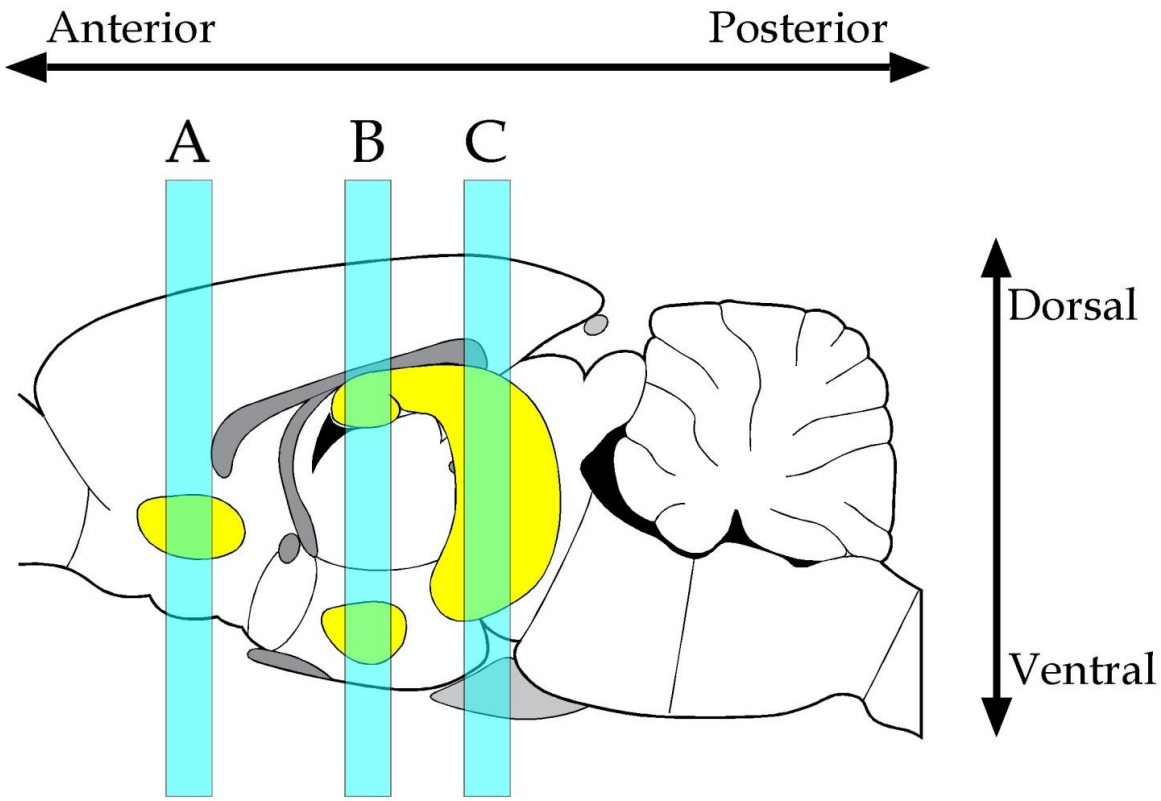


**Figure 43.** Illustration of sampling locations and anatomy for automated image analysis of Fos immunohistochemistry. Sagittal view of rat brain showing where coronal sections were sampled through three main areas: A) Bregma +2.3 through +0.8 mm (Nucleus Accumbens), B) Bregma −1.6 through −3.1 mm (hippocampal dentate gyrus and central amygdala), and C) Bregma −4.8 through −6.3 mm (hippocampal ventral CA1). Coordinates based on "The Rat Brain in Stereotaxic Coordinates" by Paxinos & Watson (2005).

## *7.4 Results*

### *7.4.1 Fluorescent immunohistochemistry for Fos*

The current study used fluorescent immunohistochemistry to investigate the regulation of Fos expression in brain regions evoked by two intensities of a nociceptive formalin stimulus compared with a restraint-only control. This project was also designed to investigate the impact of acute E2 supplementation on Fos expression in the same brain structures. Brain structures investigated were CA1, CA2, CA3 and dentate gyrus of the hippocampus, as well as basolateral and basomedial (combined), central and lateral nuclei of the amygdala. The results of this study demonstrated the presence of Fos-positive neurons in all of the regions investigated (**Figure 44**). Analysis of the numbers of Fos-positive cells revealed that formalin did not change Fos expression in the amygdala or hippocampus (**Figures 45 and 46**). In addition, the results of this experiment did not reveal any estradiol-evoked modification of Fos expression in the amygdala or hippocampus following 1.25% formalin injection in the hind paw (**Figures 47 and 48**). As is typical for sections stained by fluorescent IHC, the fluorescent labeling of the Fos targets remained visible for only a short period of time (days) before their integrity was lost). For this reason, the resulting sample numbers in this experiment were small, and may have precluded the observation of significant formalin- or estradiol-evoked effects. Accordingly, fluorescent IHC was not pursued further in favor of colorimetric IHC, which would provide relatively longer-lasting stained sections – a quality desired for a more comprehensive and time-consuming investigation of Fos staining throughout these and additional structures.

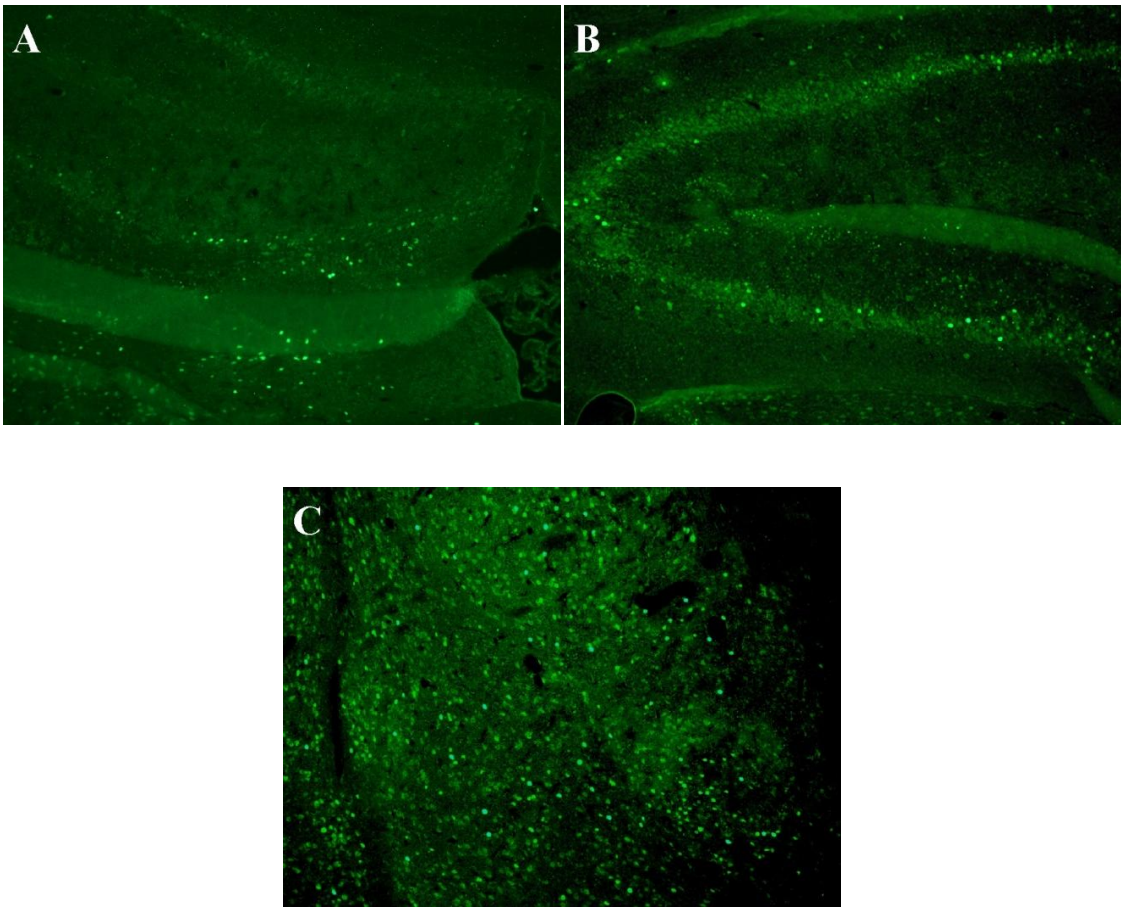


**Figure 44.** Representative photomicrographs of fluorescent immunohistochemical staining for Fos in rat brain following formalin-evoked inflammatory nociception. Brains were collected two hours after injection of 50 µL of 1.25% formalin into the right hind paw. Brains were cut into 20 µm-thick sections, probed with primary rabbit anti-Fos antibody (1:1000, Calbiochem), and labeled with FITC-conjugated donkey anti-rabbit antibody (1:200, Jackson). Note that Fos expression was visible in the granular layer of the hippocampal dentate gyrus (A) and CA1/2/3 regions (B) as well as the lateral and central amygdala (C). Digital photomicrographs taken at 10X magnification.

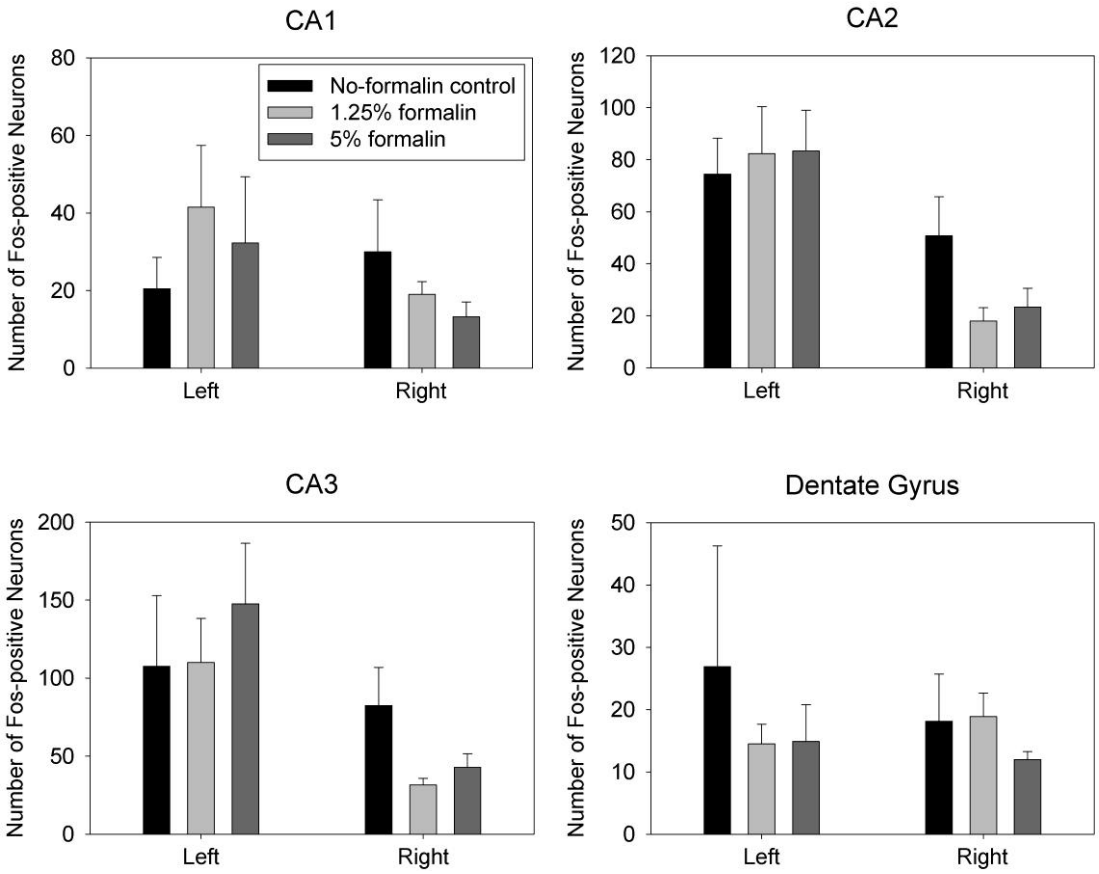


**Figure 45.** Fos immunohistochemistry in the hippocampus following 1.25% or 5% formalin-evoked inflammatory nociception. Data collected using manual counting of fluorescence-positive cell bodies. Female rats were ovariectomized (OVX) and, seven days later, rats were either injected into the right hind paw with 50 µL of 1.25% formalin, 100 µL of 5% formalin or received sham restraint only (no formalin), and brains were collected 2 hours later. Following immunohistochemical staining for Fos, 20-µm sections were viewed on a fluorescent microscope, and neurons in CA1, CA2, CA3 and dentate gyrus (DG) of the hippocampus exhibiting NeuN- and Fos-positive immunoreactivity and DAPI staining were counted. Note that Fos-positive neurons were present in each of the regions examined. Data represent the mean ± SEM (n: CA1 & DG: 4 per group; CA2 & CA3: Left = 3, Right = 4).

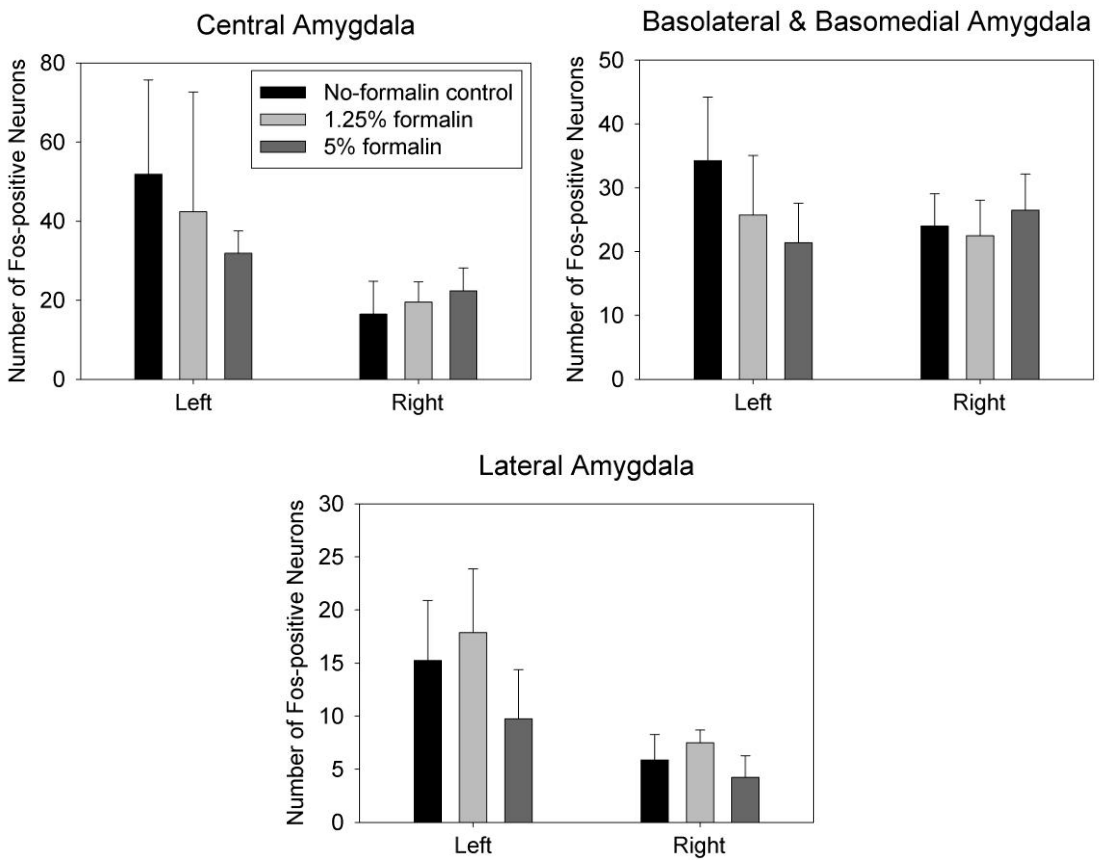


**Figure 46.** Fos immunohistochemistry in the amygdala following 1.25% or 5% formalin-evoked inflammatory nociception. Data collected using manual counting of fluorescence-positive cell bodies. Female rats were ovariectomized (OVX) and, seven days later, rats were either injected into the right hind paw with 50 µL of 1.25% formalin, 100 µL of 5% formalin or received sham restraint only (no formalin), and brains were collected 2 hours later. Following immunohistochemical staining for Fos, 20-µm sections were viewed on a fluorescent microscope, and neurons in basolateral & basomedial (combined), central and lateral amygdala exhibiting NeuN- and Fos-positive immunoreactivity and DAPI staining were counted. Note that Fos-positive neurons were present in each of the regions examined. Data represent the mean ± SEM (n = 4 per group).

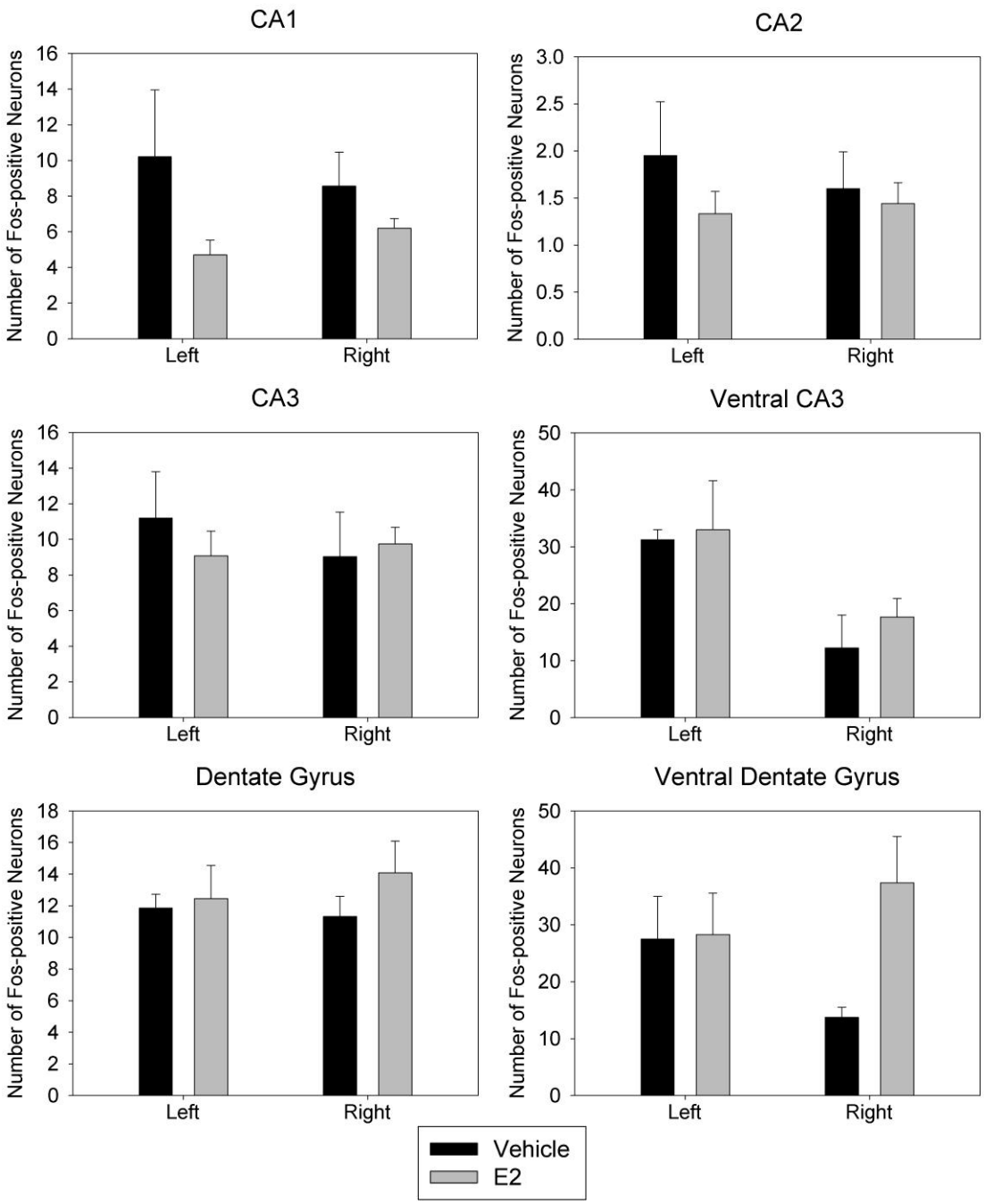


**Figure 47.** Effect of estradiol on Fos expression in hippocampus following 1.25% formalin-evoked inflammatory nociception. Data collected using manual counting of fluorescence-positive cell bodies. Female rats were ovariectomized (OVX) and, six days later, received a single s.c. injection of 10 µg/kg estradiol (E2) or vehicle. Twenty-four hours after estradiol (E2) injection (seven days after OVX), rats were injected with 50 µL of 1.25% formalin into the right hind paw, and brains were collected 2 hours later. Following fluorescent immunohistochemical staining for Fos, 20-µm sections were viewed on a fluorescent microscope, and neurons in CA1, CA2, dorsal CA3, ventral CA3, dorsal dentate gyrus (DG) and ventral dentate gyrus of the hippocampus exhibiting NeuN- and Fos-positive immunoreactivity and DAPI staining were counted. Data represent the mean ± SEM (Significance level set to $p \leq 0.05$, unpaired Student's t-test; n: CA1, CA2, dorsal CA3, dorsal DG: 6 per group; ventral CA3: Left – Vehicle = 2, E2 = 6, Right – Vehicle = 2, E2 = 5; ventral DG: Vehicle = 2, E2 = 5).

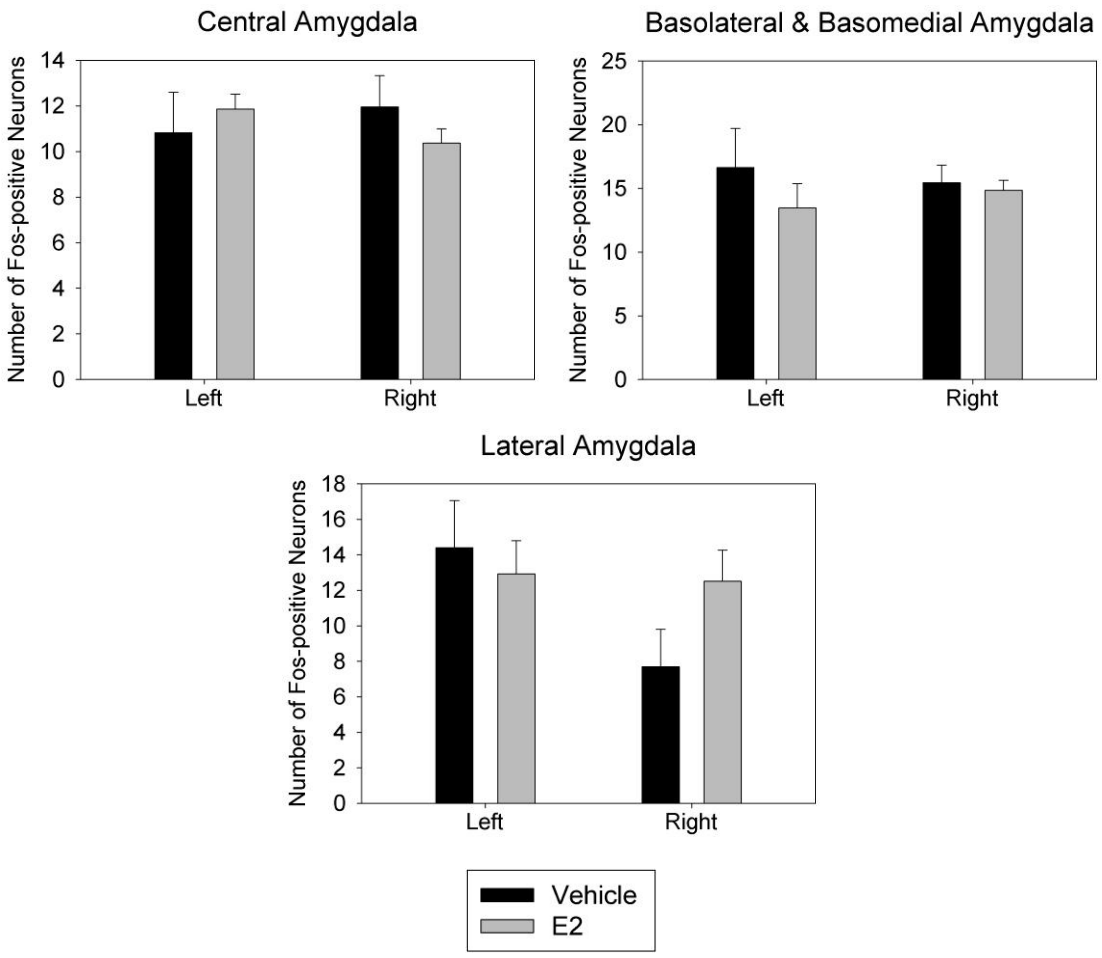


**Figure 48.** Effect of estradiol on Fos expression in amygdala following 1.25% formalin-evoked inflammatory nociception. Data collected using manual counting of fluorescence-positive cell bodies. Female rats were ovariectomized (OVX) and, six days later, received a single s.c. injection of 10 µg/kg estradiol (E2) or vehicle. Twenty-four hours after estradiol (E2) injection (seven days after OVX), rats were injected with 50 µL of 1.25% formalin into the right hind paw, and brains were collected 2 hours later. Following immunohistochemical staining for Fos, 20-µm sections were viewed on a fluorescent microscope, and neurons in basolateral & basomedial (combined), central and lateral amygdala exhibiting NeuN- and Fos-positive immunoreactivity and DAPI staining were counted. Data represent the mean ± SEM (Significance level set to $p \leq 0.05$, unpaired Student's t-test; n = 6 per group).

*7.4.2 Colorimetric immunohistochemistry for Fos*

The continuation of the study used colorimetric IHC to investigate the relationship between Fos expression in brain regions and the inflammatory nociception evoked by two levels of formalin stimulus versus restraint-only controls. Brain regions investigated were nucleus accumbens, central amygdala, dentate gyrus and ventral CA1 of hippocampus. Results of colorimetric IHC showed that 1.25% formalin increased Fos-positive neurons in the right nucleus accumbens. There was no change in the number of Fos-positive neurons for 1.25% or 5% formalin in the dentate gyrus, ventral CA1, central amygdala, or left nucleus accumbens (**Figure 52**).

This project also investigated the impact of acute E2 supplementation on Fos expression in the (same four) aforementioned brain structures. The results of this analysis revealed that in the left nucleus accumbens, 1.25% formalin increased the number of Fos-positive neurons in E2-treated, but not vehicle-treated, rats. This group with 1.25% formalin and E2 also had higher numbers of Fos-positive neurons than that of the control group receiving sham restraint and no E2 (vehicle). No other differences were found between any treatment groups for the dentate gyrus, ventral CA1, central amygdala, or right nucleus accumbens (**Figure 53**).

Furthermore, the results of this study also revealed that, in the right ventral CA1, E2 treatment decreased the number of Fos-positive neurons in 5% formalin-treated rats. Also, the group receiving 5% formalin and no E2 (vehicle) treatment had more Fos-positive neurons than the group that received sham restraint and E2, but this comparison is not relevant to the hypothesis. No other differences were found between any treatment

groups for the dentate gyrus, central amygdala, nucleus accumbens, or left ventral CA1 (**Figure 54**).

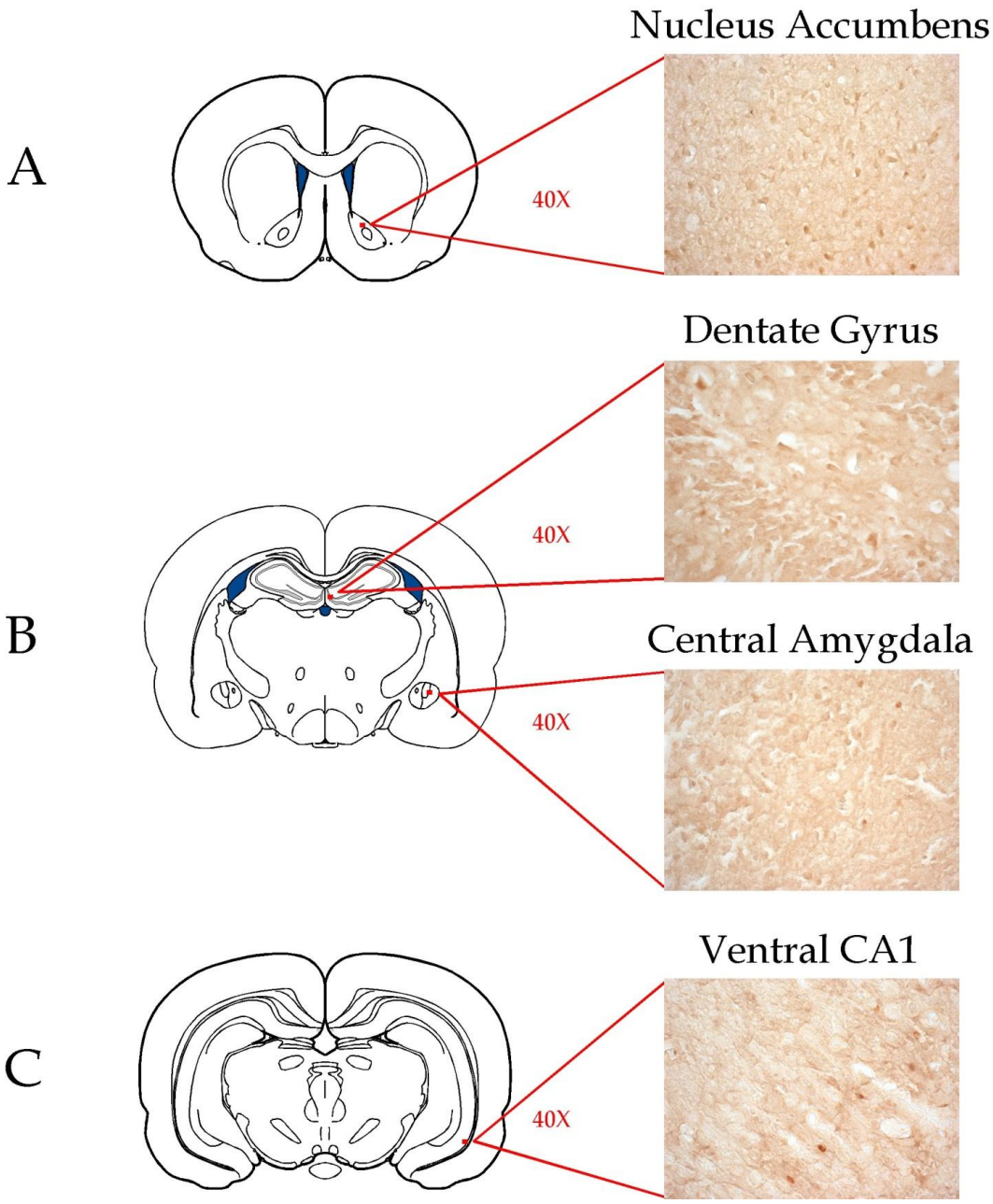


**Figure 49.** Illustration of sampling regions and anatomy for automated image analysis of Fos immunohistochemistry & representative photomicrographs of immunohistochemical staining for Fos. Illustrations (left) of coronal sections through sampling locations [modified from "The Rat Brain in Stereotaxic Coordinates" by Paxinos & Watson (2005)]. The box indicates the approximate image sampling area for each structure. Coronal sections at A) Bregma +2.3 through +0.8 mm (Nucleus Accumbens), B) Bregma −1.6 through −3.1 mm (hippocampal dentate gyrus and central amygdala), and C) Bregma −4.8 through −6.3 mm (hippocampal ventral CA1) were prepared at 20 μm using a cryostat and visualized with diaminobenzidine. Digital photomicrographs (right) of each region were captured at 40X magnification using a Nikon 80i light microscope.

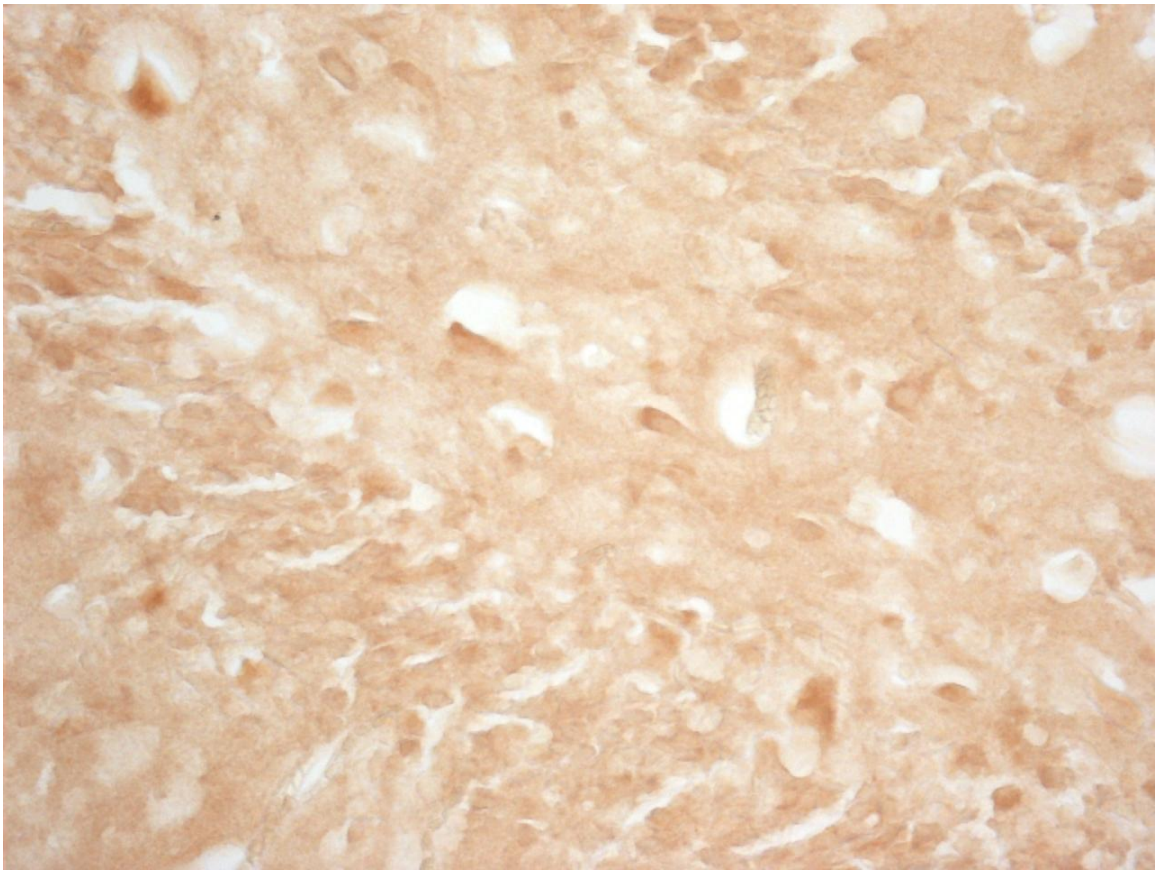

**Figure 50.** Representative photomicrograph of DAB immunohistochemical staining for Fos in brain (right dentate gyrus) following formalin. Sections were prepared at 20 μm and probed with a rabbit anti-Fos primary antibody (1:1000, Calbiochem). Sections were then labeled with secondary antibody (1:200, goat anti-rabbit, Vector Laboratories), and visualized with avidin DH: biotinylated horseradish peroxidase H complex (Vectastain Elite ABC Kit, Vector Laboratories) and diaminobenzidine. Digital photomicrograph taken at 40X magnification.

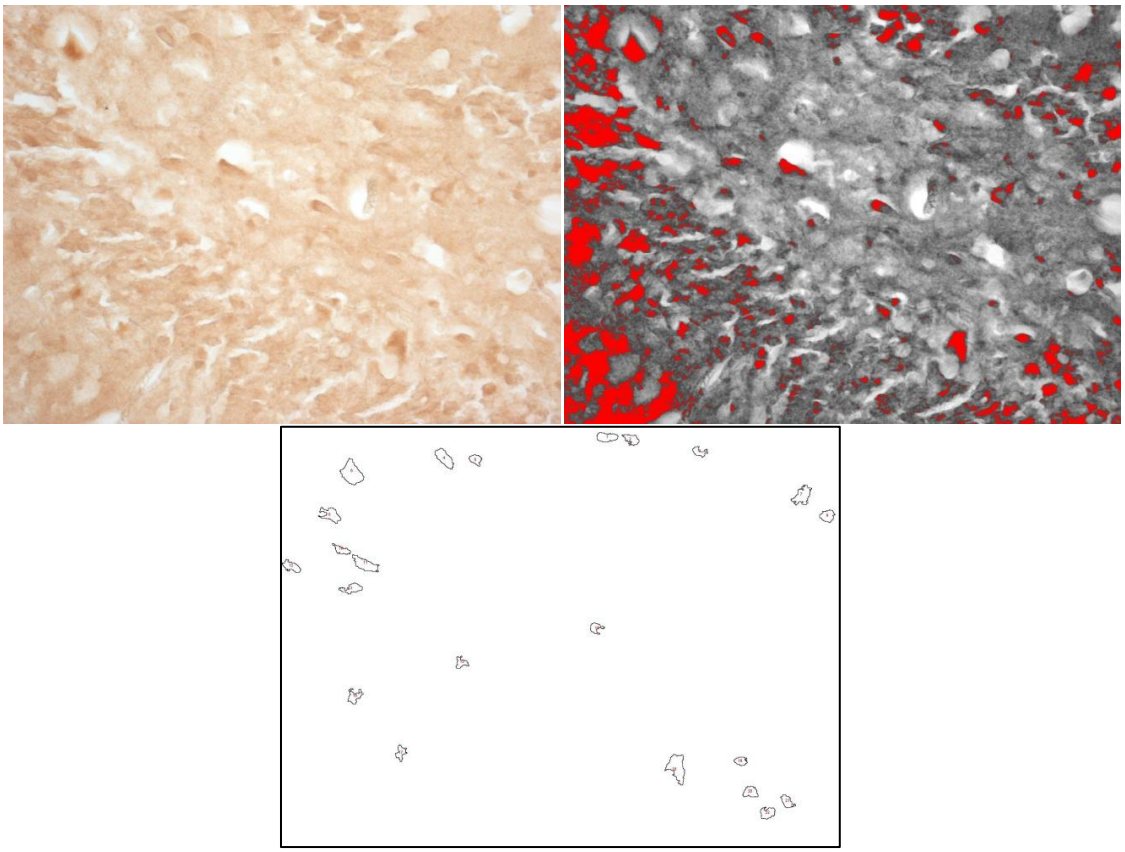

**Figure 51.** Example of automated image analysis. Panels (A) through (C) show the same representative photomicrograph at various steps through the automated image analysis method. Following immunohistochemical staining for Fos with DAB, digital photomicrographs of each region were taken at 40X magnification (A). An automated image analysis macro with the software ImageJ was used, which included, for all images, thresholding of intensity (B) and positive area size limits (to exclude areas too small or large to be nuclei) before automated counting (C). A) An original example photomicrograph. B) After thresholding. Red areas are "positive." C) Image of outlines of the final, positive areas resulting from automated counting.

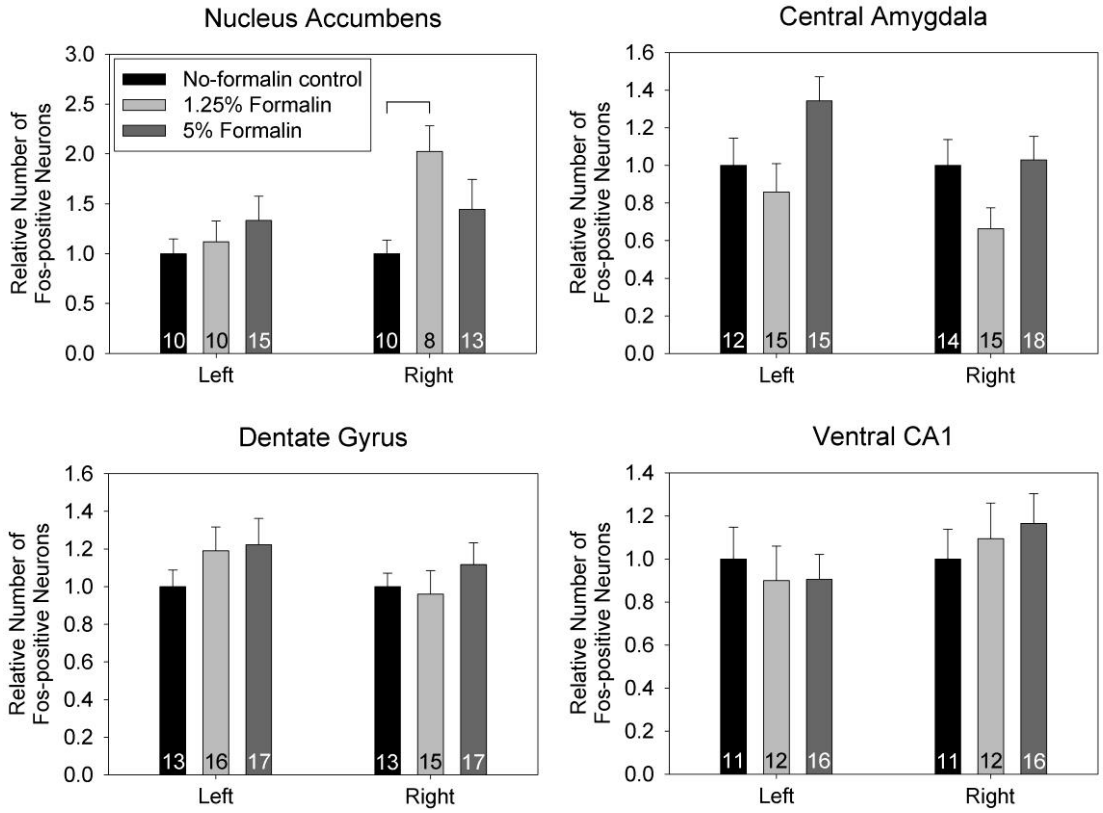


**Figure 52.** Fos immunohistochemistry in brain regions following no injection, 1.25% or 5% formalin. Data collected using automated image analysis of DAB staining. Female rats were ovariectomized (OVX) and, seven days later, rats were either injected into the right hind paw with 50 µL of 1.25% formalin, 100 µL 5% formalin or received sham restraint only (no formalin), and brains were collected 2 hours later. Sections were prepared at 20 µm, and, following immunohistochemical staining for Fos with DAB, digital photomicrographs of the left and right dentate gyrus were taken at 40X magnification. An automated image analysis macro with the software ImageJ was used for automated counting of Fos-positive cells. Note that 1.25% formalin increased Fos-positive neurons in the right nucleus accumbens. There was no change in Fos-positive neuron counts for any other stimulus in any other region examined. Data represent the mean ± SEM (Statistically significant comparisons are marked with brackets; $p \leq 0.05$, one-way ANOVA with Holm-Sidak post-hoc test. The number of rats in each group is labeled in each histogram bar.).

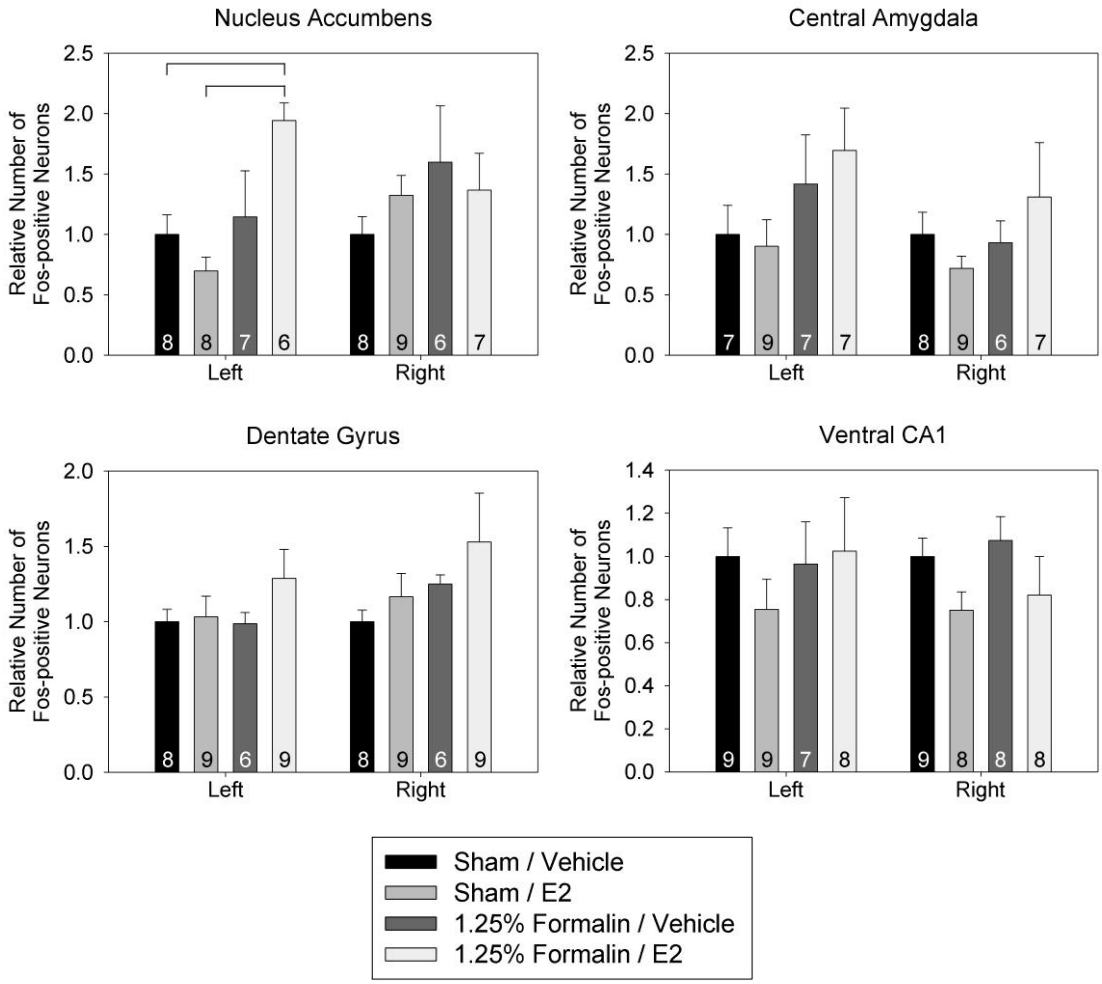


**Figure 53.** Effect of estradiol on Fos expression in brain regions following 1.25% formalin. Data collected using automated image analysis of DAB staining. Female rats were ovariectomized (OVX) and, six days later, received a single s.c. injection of 10 µg/kg estradiol (E2) or vehicle. Twenty-four hours after estradiol (E2) injection (seven days after OVX), rats were injected with 50 µL of 1.25% formalin into the right hind paw, and brains were collected 2 hours later. Sections were prepared at 20 µm, and, following immunohistochemical staining for Fos with DAB, digital photomicrographs of the left and right dentate gyrus were taken at 40X magnification. An automated image analysis macro with the software ImageJ was used for automated counting of Fos-positive cells. Note that, in the left nucleus accumbens, 1.25% formalin increased Fos-positive neuron counts in E2-treated rats. This 1.25%/E2 group also had higher counts than that of the control group receiving sham restraint and no E2 (vehicle). No other differences were found between any treatment groups for any of the regions examined. Data represent the mean ± SEM (Statistically significant comparisons are marked with brackets; $p \leq 0.05$, one-way ANOVA with Holm-Sidak post-hoc test. The number of rats in each group is labeled in each histogram bar.).

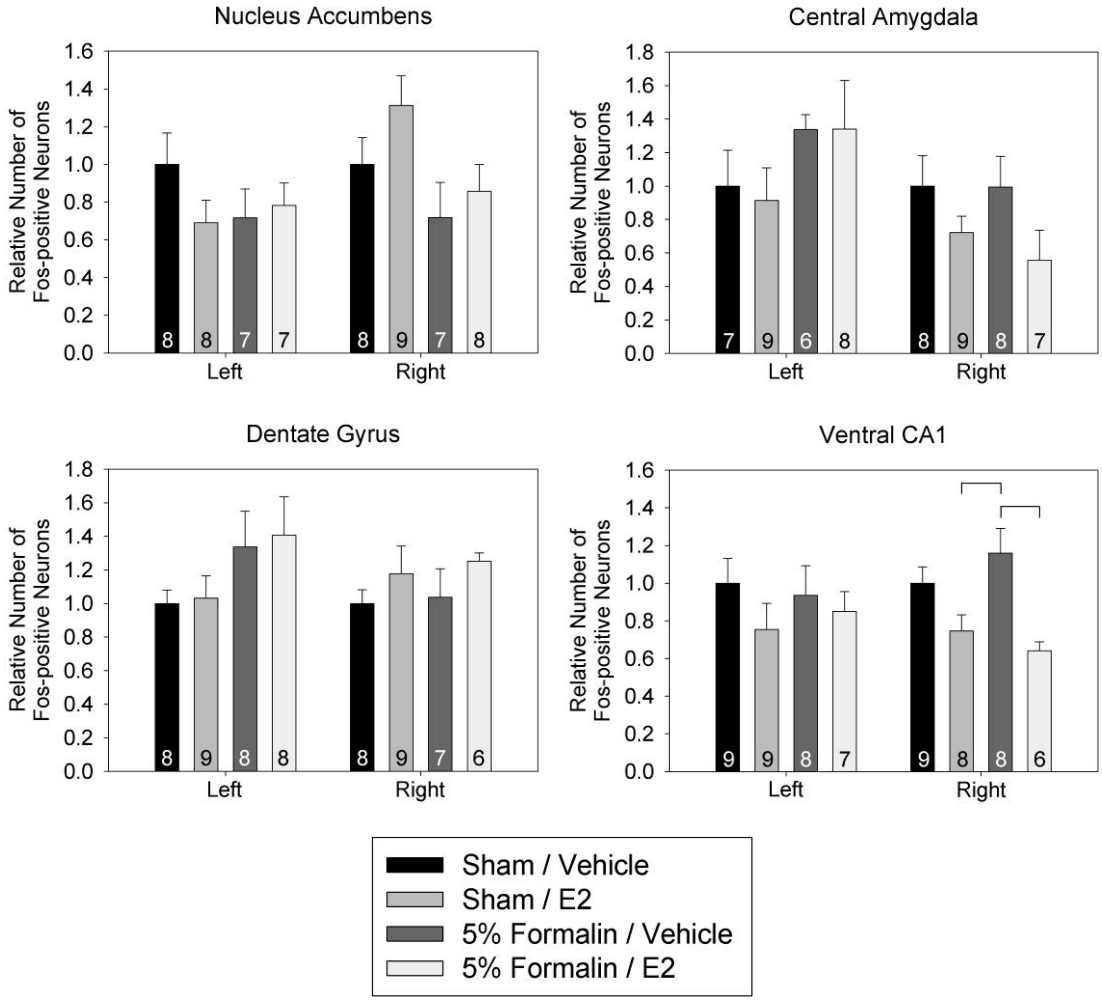


**Figure 54.** Effect of estradiol on Fos expression in brain regions following 5% formalin. Data collected using automated image analysis of DAB staining. Female rats were ovariectomized (OVX) and, six days later, received a single s.c. injection of 10 µg/kg estradiol (E2) or vehicle. Twenty-four hours after estradiol (E2) injection (seven days after OVX), rats were injected with 100 µL of 5% formalin into the right hind paw, and brains were collected 2 hours later. Sections were prepared at 20 µm, and, following immunohistochemical staining for Fos with DAB, digital photomicrographs of the left and right dentate gyrus were taken at 40X magnification. An automated image analysis macro with the software ImageJ was used for automated counting of Fos-positive cells. Results revealed that, in the right ventral CA1, E2 treatment decreased Fos-positive neuron counts in 5% formalin-treated rats. Also, the group receiving 5%/vehicle treatment had higher counts than the sham/E2 group. No other differences were found between any treatment groups for any of the regions examined. Data represent the mean ± SEM (Statistically significant comparisons are marked with brackets; $p \leq 0.05$, one-way ANOVA with Holm-Sidak post-hoc test. The number of rats in each group is labeled in each histogram bar.).

## *7.5 Discussion*

The current data add significantly to our understanding of the alteration of central nervous system activation by persistent inflammatory nociception. Potential central sites of nociception-evoked activation have been suggested by previous Fos-labeling (Bon et al., 2002; Ceccarelli et al., 1999; Doyle & Hunt, 1999; Harris, 1998; Herrera & Robertson, 1996; Kaneko et al., 2000; Khanna et al., 2004; Kovacs, 1998; Menetrey et al., 1989; Presley et al., 1990; Sandner et al., 1993) and gene expression studies (Allen & McCarson, 2005; Duric & McCarson, 2005, 2006b, 2007; McCarson & Krause, 1994, 1995; Neugebauer et al., 2004). The current study investigated formalin-evoked Fos expression in supraspinal regions that had not been previously investigated in peer-reviewed publications, such as the nucleus accumbens, as well as some that had, such as the hippocampal dentate gyrus and ventral CA1 and central amygdala (Aloisi et al., 2000; Aloisi et al., 1997; Ceccarelli et al., 1999; Khanna et al., 2004; Neugebauer et al., 2004).

### *7.5.1 Fluorescent immunohistochemistry for Fos*

The current study used fluorescent immunohistochemistry to investigate the regulation of Fos expression in brain regions evoked by two intensities of a nociceptive formalin stimulus compared with a restraint-only control. This project was also designed to investigate the impact of acute E2 supplementation on Fos expression in the same brain structures. Brain structures investigated were CA1, CA2, CA3 and dentate gyrus of the hippocampus, as well as the basolateral and basomedial (combined), central and lateral nuclei of the amygdala. The results of this study demonstrated the presence of Fos-positive neurons in all of the regions investigated. These results also revealed that

formalin did notchange Fos expression in the amygdala orhippocampus and that estradiol did not modify the Fos expression in the amygdala or hippocampus evoked by 1.25% formalin injection in the hind paw. Due to limitations, fluorescent IHC was not pursued further in favor of colorimetric IHC.

*7.5.2 Colorimetric immunohistochemistry for Fos:*

The use of automated, quantitative image analysis for colorimetric immunohistochemistry provides a more unbiased, and likely more precise, quantification method than manual counting. Computer image analysis can arguably more accurately and reproducibly (precisely) make a determination of intensity relative to the threshold of intensity for positivity, for each instance and from sample to sample. It also allows for high-throughput analysis, as multiple images can be analyzed in a relatively short time period compared to manual counting.

This study was the first of its kind to report Fos expression in the nucleus accumbens following intraplantar formalin-evoked nociception. Current results demonstrated that inflammatory nociception evoked by intraplantar injection of 1.25% formalin increased Fos-positive neurons in the right nucleus accumbens (**Figure 52**). This may indicate that an increase in Fos in the nucleus accumbens reflects increased activation (nociception). The increase in Fos in the accumbens is consistent with previous gene expression studies. In a related study, the number of Fos-positive neurons in the core of nucleus accumbens was reported as higher than control following injection of formalin into low back skin or muscle (Ohtori et al., 2000). However, this study was performed in anesthetized rats, complicating comparison to the current study.

Nonetheless, it supports the findings of the current study that formalin-evoked nociception increases Fos expression in the nucleus accumbens. Moreover, Dr. Allen's dissertation showed that NK-1 and BDNF gene expression were markedly increased in the nucleus accumbens 24 hours following intraplantar formalin (**Figure 42**), supporting a role of this structure in pain processing that involves increased expression of gene products that typically mediate neuronal excitation. The change in expression of these genes in the nucleus accumbens may also partially represent plasticity-mediated sensitization of the central nervous system that occurs with persistent nociception, and similar changes may occur in chronic pain conditions. The results of the current study support the addition of the nucleus accumbens to the list of brain structures that manifest altered gene (Fos) expression from nociceptive activation.

The current study did not demonstrate a formalin-induced change in Fos expression in the dentate gyrus or ventral CA1; these results were not consistent with previous reports, which demonstrated formalin-induced decreased Fos expression in these regions (Ceccarelli et al., 1999; Khanna et al., 2004). One report mentioned Fos expression "tended" to decrease in the dorsal dentate gyrus and ventral CA1 (in OVX females) following formalin, but statistical significance of these comparisons were not reported (Aloisi et al., 2000); thus, those data may support the current negative findings. Allen & McCarson (2005) found that formalin injection decreased BDNF expression in whole hippocampus, but only in rats with E2 supplementation, not ovariectomized ones. Dr. Allen showed in her dissertation that hippocampal NK-1 receptor expression was also decreased by formalin (**Figure 42**).

The current study did not find a change in Fos expression in the central amygdala due to formalin. This is consistent with one previous publication which found no change in *c-fos* mRNA in the central amygdala following intraplantar formalin, whereas changes were demonstrated in the basolateral and lateral amygdala (Nakagawa et al., 2003). In contrast, the number of Fos-positive neurons in the central amygdala was reported as higher than control following injection of formalin into low back skin or muscle (Ohtori et al., 2000). However, this study was performed in anesthetized rats, and control conditions were different, complicating comparison to the current study. Additionally, Traub et al. (1996) showed that noxious colorectal distention increased Fos expression in the central amygdala. However, that pain model may activate different neural substrates or pain-processing mechanisms than the formalin model, as the processing in the amygdala differs with the type of painful stimulus (Neugebauer, 2007). In a model of arthritic persistent pain, expression of metabotropic glutamate receptors were up-regulated in the central amygdala, demonstrating plasticity induced by the model consistent with pharmacological observations of enhanced sensitivity (Neugebauer et al., 2004). NK-1 and BDNF gene expression were robustly down-regulated in the amygdala following formalin-evoked nociception (**Figure 42**). Therefore, pain of different origins is capable of modulating gene expression in the central amygdala, but the current study corroborates the previous report in that Fos expression in the central amygdala was not altered by the formalin model.

It was observed in the left nucleus accumbens that inflammatory nociception evoked by intraplantar injection of 1.25% formalin increased Fos-positive neuron counts in E2-treated, but not vehicle-treated, rats (**Figure 53**). This observation indicated a

"permissive" effect of E2 on nociceptive activation of these neurons, supporting an E2-facilitated activation of the left nucleus accumbens by formalin. This result supports the hypothesis suggesting the existence of supraspinal sites of action, namely the nucleus accumbens, through which E2 potentiates inflammatory nociception. However, caution should be used when interpreting the direction of change (increase or decrease) on nociception in the left accumbens, because, even though the current results indicated an increase in Fos in the right accumbens correlated with nociception (**Figure 52**), the relationship of Fos expression to nociceptive neuronal function may not necessarily be the same for left and right accumbens. The current study implicates the nucleus accumbens as a supraspinal site of action of E2-mediated increased persistent inflammatory nociception.

Notably, the results of this study revealed that, in the right ventral CA1, E2 treatment decreased the number of Fos-positive neurons in 5% formalin-treated rats (**Figure 54**). This indicates that E2 modified nociception-related neuronal activity in the ventral CA1; thus the ventral CA1 was a probable supraspinal site of action where E2 increases nociception. Therefore, the current results further support the hypothesis that E2 increases inflammatory nociception by acting at supraspinal sites. Allen & McCarson (2005) found that formalin injection decreased BDNF expression in whole hippocampus, but only in rats with E2 supplementation, not ovariectomized ones. This indicated an interaction of E2-mediated signaling with formalin-evoked nociceptive processing that suppressed the level of BDNF in the hippocampus. Because E2 changed BDNF and Fos expression in the same direction (decrease), their expression levels may have similar functional implications for the hippocampus, or neurons within the hippocampus.

However, the function of these BDNF- or Fos-positive neurons must be delineated before solid conclusions can be made in terms of contribution to overall nociception. Dr. Allen showed in her dissertation that hippocampal NK-1 receptor expression was also decreased by formalin (**Figure 42**). Previous reports have demonstrated formalin-induced decreased Fos expression in the ventral CA1 (Ceccarelli et al., 1999; Khanna et al., 2004). Thus, it appears that a decrease in Fos in the ventral CA1 may indicate the occurrence of pro-nociceptive processing in the ventral CA1. These arguments support the conclusion that, since E2 decreased formalin-evoked Fos expression in the ventral CA1, estradiol increased pro-nociceptive processing in the ventral CA1.

These findings support the hypothesis that activational effects of estrogen may contribute to sex differences in chronic pain syndromes by acting at supraspinal sites in the CNS, such as the nucleus accumbens and hippocampal ventral CA1.

# *CHAPTER EIGHT*

# *CONCLUSIONS*

### *8.1 Effects of androgens on pain-related behaviors*

These experiments used gonadectomy alone or with testosterone supplementation to manipulate androgen status in an acute manner in adult, male rats, and subsequently tested the impact of this androgen manipulation on behavioral responses in several pain models. Results of androgen manipulation revealed that androgen has activational effects on pain of phasic thermal and persistent neuropathic origin, but perhaps not persistent inflammatory origin. These results provide evidence that androgens, as well as estrogens, may contribute to sex differences in pain sensation. The lack of evidence for androgen-mediated effects on nociceptive behavior in the formalin model prompted subsequent focus on estrogen manipulation in females.

### *8.2 ERK as a marker of nociceptive activation*

Levels of ERK in the spinal cord were quantified at several time points following injection of formalin into the rat hind paw, using immunohistochemistry for pERK-positive neurons or western blotting for pERK. Observations of levels of ERK led to the conclusion that quantification of ERK activation by either of these methods was not adequately sensitive to use as a marker for neuronal activation, or estrogen modulation thereof, in the spinal cord under the conditions used.

### *8.3 Estrogen modifies formalin-evoked flinching but not spinal Fos expression*

These experiments used ovariectomy with estradiol supplementation to manipulate estrogen status in an acute manner in adult, female rats, and subsequently tested the impact of this estrogen manipulation on behavioral responses (paw flinching and actometry end points) to formalin injection into the hind paw – a model of persistent, inflammatory pain. Results of the behavioral analyses indicate that a proestrus-like surge in serum estrogen can modify (increase) inflammatory nociception-related behaviors and, thus, presumably nociception in an activational manner. However effects of estradiol on nociception (and/or related behaviors) may be dependent on the intensity of noxious stimulus. Results of estradiol manipulation confirmed the hypothesis that a single injection of estradiol significantly enhances late-phase formalin-induced hind paw flinching at 5% formalin (**Figure 19**). By demonstrating an effect of acute, direct manipulation of estradiol in female rats on behavior evoked by 5% formalin, these results support the conclusion that estradiol activationally increases persistent, inflammatory nociception.

However, the administration of estradiol did not significantly alter numbers of nociception-evoked Fos-positive neurons in the ipsilateral lumbar spinal cord dorsal horn at either formalin concentration (**Figures 35 and 36**). Together, these observations support the conclusion that, whereas estrogen enhances pain-related behaviors evoked by persistent inflammatory nociception, estrogen may not significantly modulate the intensity of nociceptive neuronal activation in the peripheral nervous system (primary sensory neurons) or spinal cord. Thus, it appears that the peripheral nervous system and

spinal cord may not be primary targets for estrogen acting to increase inflammatory nociception.

The current findings also suggest that estrogen may be enhancing inflammatory nociception primarily via actions at other anatomical sites, such as supraspinal sites, where pain sensation is modified subsequent to spinal transmission. Accordingly, subsequent investigation was aimed at determining modulatory effects of estradiol at supraspinal sites.

### *8.4 Selection of supraspinal sites for investigation*

Hypothetically, limbic and cortical areas involved in affective processing may be important for sex differences or sex hormone modulation of pain. This notion, in part, guided the selection of supraspinal sites for investigation in this project. The incidence of affective/emotional disorders in women and the cyclic nature of their onset, severity and duration suggest that hormonal modulation in affective centers could be a mechanism that enhances pain sensation. Sensitization of the limbic system by estrogen and a heightened impact on the affective component of pain may underlie the disproportionate burden of mood disorders and certain chronic pain syndromes (e.g., depression and fibromyalgia) in women.

Synaptic plasticity associated with long-term potentiation occurs in limbic centers such as the amygdala (Maren, 2005) and hippocampus. There is extensive literature on plasticity and long-term potentiation in the hippocampus. The amygdala is deeply involved in emotional processing (Phelps & LeDoux, 2005). Limbic structures have

functional roles in processing negative affect or nociception and undergo structural and functional changes in affective disorders (Ferre et al., 2007; Khanna & Sinclair, 1992; Knyihar & Csillik, 2006; Koyanagi et al., 2008; Magnusson & Martin, 2002; Mayberg, 1997; Millan, 1999; Neugebauer, 2007; Neugebauer et al., 2004; Ploghaus et al., 2001; Porro, 2003; Price, 2000, 2002; Price et al., 2006; Sheline et al., 1996; Zubieta et al., 2003; Zubieta et al., 2001). Also, there is considerable plasticity in these structures both with regard to nociceptive/affective function as well as interaction with estrogen (Cooke & Woolley, 2005; Desmond & Levy, 1997; McEwen, 2001; McEwen & Magarinos, 2001; Neugebauer, 2007; Neugebauer et al., 2004; Price, 2000, 2002; Price et al., 2006; Woolley, 1998, 2000; Woolley & McEwen, 1992), making these structures logical choices for investigating potential estrogen modulation of nociception.

### *8.5 Estrogen and nociception in the hippocampal ventral CA1*

Results showed that estradiol decreased Fos in right ventral CA1 in the presence of 5% formalin (**Figure 54**); no difference in Fos due to estradiol in the absence of formalin was observed. These results, combined with the observations of E2-mediated increased flinching behavior and lack of E2 effect on spinal Fos expression (as discussed above), support the hypothesis that E2 increases persistent, inflammatory nociception primarily at supraspinal sites of action. Furthermore, the ventral CA1 is a probable target for this pro-nociceptive effect of E2.

The finding of an E2-induced difference in Fos in the ventral CA1 elicited by 5% formalin, but not 1.25% formalin, is consistent with the observation of an E2-induced

increase in flinching at 5% formalin but not 1.25% formalin. These results indicate E2 increases inflammatory nociception in a stimulus-intensity dependent manner. This could mean there is a threshold effect for E2 to influence nociception, especially for any particular structure. E2 may affect neurons, signaling mechanisms, pathways, or synapses that are involved in processing of higher, but not lower, intensity stimulation.

Estradiol, in the presence of 5% formalin, changed Fos expression in the same direction (decreased) as formalin was previously reported to have in the ventral CA1 (Khanna et al., 2004), thus appearing to have a pro-nociceptive effect there. Also, reduced activity (reduced Fos immunoreactivity) in the ventral CA1 is consistent with mechanisms of depression; persistent inflammatory nociception or immobilization stress will drive down NK-1 and BDNF gene expression throughout the hippocampus (Duric & McCarson, 2005).

The lack of a formalin-only-induced change in Fos in ventral CA1 in the current data limit interpretation of the estradiol-induced change in Fos. Differences in control group conditions and findings of sex differences in previous reports [see (Aloisi et al., 2000; Aloisi et al., 1997; Ceccarelli et al., 1999; Khanna et al., 2004)] make it difficult to provide context for interpretation of the current Fos expression data in terms of influence (interaction) of E2 or gonadal hormones on (with) formalin-/noxious stimulus-induced nociception or related neuronal activity.

Finally, from these data we can at least conclude that the ventral CA1 has modified neuronal activity due to E2. Whether this activity is pro- or anti-nociceptive is unclear. In all, these data support the existence of supraspinal site(s) of E2-mediated

modulation of/increases in nociception. The ventral CA1 is implicated as such a site of E2-mediated modulation; or E2-mediated modulation could be due to action at another (supraspinal) site that modifies activity in the ventral CA1.

The CA1 subdivision is the main output of the hippocampus (Witter, 2006). Major output targets of the CA1 include the cingulate cortex, anterior thalamus, prefrontal cortex, mammillary bodies, subicular complex, nucleus accumbens, and entorhinal cortex. In addition, the anterior thalamus in turn projects to cingulate cortex.

The cingulate cortex is a very important pain and affective processing center (Price et al., 2006; Rainville, 2002; Zubieta et al., 2003; Zubieta et al., 2001). It receives spinothalamocortical nociceptive input. The experience of pain involves interaction of processing at many nervous system sites, including the cingulate cortex. There is correlation between the activity in the cingulate and ratings of pain intensity as well as ratings of pain unpleasantness (affect) (Rainville, 2002). Also, specific manipulation of pain affect produced specific modulation of activity in the cingulate and pain unpleasantness (Rainville, 2002). Several areas within the cingulate cortex undergo pain-related activation. Part of the cingulate may also contribute to motor control during pain. The cingulate appears to be a center for processing motor, behavioral, cognitive, and affective responses to pain, as well as modulation of pain. Thus, the cingulate cortex is a key area for processing nociception and behavior in animals and pain affect in humans. Therefore, modulation of output to the cingulate, from places such as the CA1, likely has an impact on the (affective) processing of pain/nociception.

The CA1 region of the hippocampus has direct & indirect (through the subicular complex, entorhinal cortex, or cingulate cortex) output to the prefrontal cortex (PFC) (Conde et al., 1995; Laroche et al., 1990; Tierney et al., 2004). The direct pathway is excitatory and can undergo long-term potentiation (Laroche et al., 1990). The prefrontal cortex has functions in the affective and cognitive aspects of pain (Price et al., 2006; Price & Drevets, 2010), and afferent pathways to the PFC from limbic structures such as the CA1 may regulate sensorimotor gating (Miller et al., 2010). Thus, modulation of CA1 activity, as shown by an estradiol-mediated effect in the current study, possibly modulates signaling in the PFC and, through this, impacts nociception and potentially pain sensation. Modulation of the PFC is another possible avenue through which estradiol could mediate increases in nociception.

The CA1 output to nucleus accumbens also has high significance in terms of potential impact on pain processing. The accumbens is another structure that has important function in the processing of pain and affect (see p. 188). Thus, modulation of output from the CA1 to the accumbens is another possible way estrogen could increase nociception.

In addition, hippocampal output to the forebrain and hypothalamus may influence descending modulation of nociception. These targets of hippocampal output have descending projections to the periaqueductal gray that could serve to integrate and relay descending modulation of nociception (Marchand & Hagino, 1983).

Electrophysiological and pharmacological data reveal decreased activity in (some) CA1 neurons in response to noxious stimulation (Khanna & Sinclair, 1992).

Pharmacological blockade in the hippocampus that is relevant to this phenomenon decreased nociception-related behavior (Rodgers & Brown, 1976). Other evidence implies that CA1 neurons may also have an opioid-mediated inhibitory role in pain processing (Roumy & Zajac, 1998). These observations are consistent with a decrease in Fos expression in the CA1 being correlated with increased nociception, as in the current study with E2 and in the report by Khanna et al. (2004).

### *8.6 Estrogen and nociception in the nucleus accumbens*

It was observed in the left nucleus accumbens that inflammatory nociception evoked by intraplantar injection of 1.25% formalin increased Fos-positive neuron counts in E2-treated, but not vehicle-treated, rats (**Figure 53**). This observation indicated a "permissive" effect of E2 on nociceptive activation of these neurons, supporting an E2-facilitated activation of the left nucleus accumbens by formalin. Formalin (1.25%) alone increased Fos expression in the right nucleus accumbens in the formalin-only study, indicating the involvement of the nucleus accumbens (core) in nociceptive function (at least in formalin-induced nociceptive processing). However, caution should be used when interpreting the direction of change (increase or decrease) pertaining to nociception in the left accumbens, because the relationship of Fos expression to nociceptive neuronal function may not necessarily be the same for left and right accumbens. Nonetheless, this result supports the hypothesis suggesting the existence of supraspinal sites of action, namely the nucleus accumbens, through which E2 potentiates inflammatory nociception.

Thus, the current study implicates the nucleus accumbens as a supraspinal site of action of E2-mediated increased persistent inflammatory nociception.

The nucleus accumbens is part of a neural network loop with the pallidum, thalamus, and prefrontal cortex that overlaps and is interconnected with a loop involving the amygdala, striatum, pallidum, and thalamus (Price & Drevets, 2010). The accumbens (ventral striatum) receives direct input from the spinal cord (Newman et al., 1996) and amygdala (Price & Drevets, 2010). The amygdala also receives direct projections from the spinal cord (Newman et al., 1996). These observations support the assertion that the limbic system (including the accumbens) plays an important role in mediating pain.

Recent studies have established a function of the nucleus accumbens in pain modulation. Through indirect connection with the PFC, which participates in descending modulation of pain, the accumbens could participate in the descending modulation of nociception.

The nucleus accumbens clearly regulates outflow of pain-modulatory information from the limbic system. This influence can be inhibitory or facilitatory, and both excitatory (e.g. NMDA) and inhibitory (e.g. dopamine receptor $D_2$, cholecystokinin, endogenous opioid) mechanisms exist within the nucleus accumbens. The nucleus accumbens is an important pain & affect processing center (Knyihar & Csillik, 2006; Koyanagi et al., 2008; Magnusson & Martin, 2002; Millan, 1999; Ohtori et al., 2000; Zubieta et al., 2001, 2002). Pharmacological studies support the existence of adenosine and opioid signaling in the accumbens that contribute to nociceptive sensory and affective processing (Ferre et al., 2007; Zubieta et al., 2001). The nucleus accumbens

(core) appears to have an anti-nociceptive function in processing nociception elicited by formalin injection (Altier & Stewart, 1997; Lapeyre et al., 2001; Magnusson & Martin, 2002; Taylor et al., 2003). Magnusson & Martin (2002) demonstrated that blockade of activity by microinjection of local anesthetic into the accumbens core, but not shell, enhanced formalin-induced nociception.

Fos expression could reflect activity in cells of either excitatory or inhibitory function, or both. Thus, if estradiol increases nociception, estradiol-mediated increased activity (Fos) in the nucleus accumbens could represent increased activity of inhibitory inter-neurons within the accumbens, resulting in decreased output of neurons with anti-nociceptive function and, therefore, a pro-nociceptive effect. Alternatively, estradiol may be increasing nociception by acting on a different supraspinal structure, and the increased activity (Fos) in the accumbens may represent increased compensatory, anti-nociceptive processing.

The E2-facilitated increase in Fos expression in the accumbens evoked by 1.25% formalin, with no estradiol-mediated change in the degree of flinching behavior, may mean E2 increases nociception, but that this effect is masked by nociceptive or behavioral output processing at other centers. Also, the lack of an E2-mediated change in Fos expression elicited by 5% formalin, along with an E2-mediated increase in flinching, may mean E2 does not modulate nociception in the accumbens at higher concentrations (stimulus levels), or that E2 does modulate nociception in the accumbens, but this effect is not manifested as a change in Fos expression. This dichotomy may speak to a limitation of the use of Fos immunohistochemistry as a proxy for the degree of activity, at least in this structure.

### *8.7 Fos as a marker of nociceptive activation*

There are limitations for using Fos expression or behavior as proxies of the nociceptive experience. The response level or range of Fos expression in a given site in the nervous system may limit the ability to observe modulation of neuronal activity. There are thresholds or ceilings to the expression of Fos elicited by a stimulus. There may be differences in these thresholds or ceilings on Fos expression in each respective structure versus other structures or versus behavioral output. The level of Fos expression represents activation of a given structure, whereas behavior represents the net output of all processing in all relevant nervous system centers. Also, it is possible that modulation of behavior could occur without modulation of overall nociception, or vice versa.

The specific contributions of each of the brain regions where Fos expression is altered by formalin-evoked inflammation to the overall perception of pain have not been fully elucidated. Accordingly, the functional implications of Fos expression in these brain areas remain somewhat undefined. Fos-expressing neurons in each location could have differential effects (e.g. excitation/inhibition, nociception/anti-nociception), and it remains unclear whether Fos expression only reflects afferent input, or represents altered sensitivity to the input and/or the degree of activity (output) of the labeled neurons. In addition, estrogen may be acting directly in these structures, or indirectly by acting on other centers that modulate neuronal activity in these structures.

Potential limitations of the brain Fos study include the following: 1) "signal" might be diluted with sections over the rostral-caudal extent of a structure, if Fos-expressing neurons (or neurons that respond to stimulus/modulated by E2) are not

homogeneously spread throughout the structure (i.e., located within a smaller rostral-caudal extent than that examined). 2) Also, one sampling region is only part of the structure and may not represent neuronal/Fos activation for the whole structure (as opposed to counting Fos-positive neurons across the entire structure within each section). These two aspects represent differences between the current study and other previous studies.

Why does E2 modulation of gene expression occur in sites where no modification of nociception-evoked Fos was observed? Nociception-evoked gene expression in the spinal cord dorsal horn has been shown to be modulated by estrogen status. For example, basal and nociception-evoked expression of the brain-derived neurotrophic factor (BDNF) gene in the spinal cord is modified by estrogen (Allen & McCarson, 2005).

First, the observations of BDNF (and NK-1) were made with a different (longer OVX, longer & constant E2) estrogen treatment than current experiments where Fos was quantified. These differences in experimental parameters could bring about different modulation of the nervous system, and, thus, the observed dichotomy in these end points.

In addition, observations of BDNF (and NK-1) were made at 24 hours following formalin; observation of Fos was made at 2 hours. Used at these different time points, they (or any measures) may be markers of different phenomena. For example, different sensitizing changes to the nervous system may be present at 24 hours; these changes might be more representative of sensitivity to *subsequent* stimulation. The expression of Fos at 2 hours is likely more representative of sensitization that takes place during the *initial* inflammatory insult. Both aspects are important in terms of estrogen sensitizing

the nervous system, but modulation that impacts the initial insult (which is probably in place beforehand) is perhaps more powerful or important regarding sensitization than modulation that only occurs upon subsequent stimulation.

Perhaps its most distinguishing property, the quantification of Fos is a marker of function; i.e., it represents the degree of neuronal activity or metabolism. BDNF and NK-1 are biomolecules that have function in neuronal signaling, but, standing alone, are not functional markers. Compensatory changes could take place in other facets of signaling or neuronal function, a possibility which blurs the interpretation of such end points.

Obviously, there are inherent obstacles to the use of biomolecular end points (such as these) as proxies of the degree on neuronal activity. It is important to recognize that any potential relationship between immediate-early gene expression and other, subsequent events like signaling cascade modification or regulation of evoked gene expression remains largely undefined. Ultimately, perhaps the most interpretable measures of neuronal activity are electrophysiological recording or functional imaging.

### *8.8 Comparison with previous reports*

Differences in control group conditions and findings of sex differences in previous reports make it difficult to give context to interpretation of the current Fos expression data in terms of (regarding) influence (interaction) of E2 or gonadal hormones on (with) formalin-/noxious stimulus-induced nociception or related neuronal activity. There are differences in the manipulation of control groups between the current study and

other relevant studies, and among other studies; for example, some control groups received essentially no manipulation (Ceccarelli et al., 1999), paw manipulation but no needle prick/injection (current study), needle prick into the paw but no injection (Aloisi et al., 2000), or injection of saline (Khanna et al., 2004). Another difference between this study and others is the sex of the subjects; the current study was performed with females, but the study by Khanna et al. (2004) was with males. Relevant to this, sex differences have been found in the end points used (e.g. hippocampal Fos expression) following stimulation with formalin (Aloisi et al., 1997; Ceccarelli et al., 1999).

Another aspect that sets the current study apart from others is the timing of the estradiol manipulation. Previous studies typically used longer durations of ovariectomy and longer treatment with estradiol before the nociceptive stimulus was applied. For example, work in Dr. Amy Allen's dissertation used an OVX duration of two weeks and then sustained estradiol treatment for two weeks before application of formalin. The current study used an OVX period of six days and then a single injection of estradiol 24 hours before formalin, in order to model more acute effects of estradiol. Different dose and timing of estradiol manipulation regimens may employ different estrogen signaling mechanisms (e.g. genomic vs. rapid, membrane signaling) and potentially different effects on neuronal activity.

Other bodies of work have focused on organizational differences, which take place during development and result in permanent sex differences. For example, studies have shown that aspects of opioid anti-nociception were modified by organizational effects of sex hormones (Cataldo et al., 2010; Craft & Ulibarri, 2009). Investigations

such as these into this organizational type of sex steroid effect are in contrast with the plasticity-driven, activational effects that are the focus of this dissertation project.

Some other studies have used chronic inflammation models of chronic pain, such as subcutaneous injection of complete Freund's adjuvant (CFA), to study nociceptive activation and plasticity in specific sites of the nervous system. The CFA model does not evoke stereotyped behaviors like the formalin model does, but manifests hyperalgesia to subsequent phasic stimulation. This is in contrast to the use of formalin in this project; formalin produces persistent activation, but is used as an acute stimulus.

Similar to this dissertation project, Dr. Amy Allen's dissertation work investigated estradiol modulation of gene expression in response to noxious stimulation with formalin in specific nervous system sites. Dr. Allen demonstrated some estradiol-mediated changes in gene expression in relevant brain regions (Allen & McCarson, 2005).

### *8.9 Future studies*

As with most scientific inquiries, results of this project open the door for more inquiry. Future or further studies could microinject E2 into the structures investigated herein to determine how E2 modulation in these structures affects nociception and related behavior. Recapitulation of the behavioral enhancement or changes in Fos expression by local E2 effects would confirm these structures as sites of action for E2-mediated enhancement of nociception.

To better define the contribution of each structure to formalin-induced nociception and behavior, as well as give more context to Fos expression in these areas, experiments could modify activity in specific sites using microinjection of local anesthetic or agonists or antagonists of specific neuronal signaling molecules.

More studies are needed to determine the function of Fos-positive neurons in these structures. For example, pertinent information would be the mode of output of these neurons (excitatory or inhibitory), their connectivity, and the function of their projection targets.

The current project focused on investigating sites involved in emotional/affective processing. Alternatively, future investigation of potential sites of action of estradiol could be sensory/discriminative sites, such as the ventral posterolateral thalamus, primary or secondary sensory cortex, etc. In addition, other sites involved in affective processing of pain as well as higher level integration of sensory, cognitive and affective components of pain (e.g. cingulate cortex, prefrontal cortex) could be investigated.

Investigations could be undertaken into the signaling mechanisms of estrogen-mediated modulation of nociception in specific nervous system sites that have been identified as participating in such modulation.

Other investigations, similar to this project, could be made into identifying nervous system sites where androgens may impart modulation on nociception.

### *8.10 Final conclusions*

The results of this research project demonstrate that nociception-evoked levels of Fos expression in higher brain centers, unlike those in the spinal cord, are modified by estrogen status in female rats. Together with observations that estrogen enhances pain-related behaviors, these results suggest that estrogen enhances inflammatory nociception via actions at higher brain centers.

The results of these studies reveal the nucleus accumbens and the ventral CA1 region of the hippocampus as supraspinal structures where estrogen modifies nociception-evoked neuronal activity. Both centers are involved in the regulation of affect, and thereby may contribute to the emotional component of pain sensation. Modulation of pain processing in these structures may also contribute to sex differences in the sensory and affective experiences of pain.

The results of these studies also demonstrate that activational, rather than organizational, effects of estrogen at supraspinal sites of action modify nociception. These activational effects may contribute to sex differences in pain sensation and/or the disproportionate severity or incidence of some pain syndromes in women.

Experiments in this dissertation represent only a small glimpse of what impact sex steroids are capable of having on pain processing. They demonstrate what impact sex steroids *can* have on processing pain of specific types. This knowledge still needs to be reconciled with the impact that sex steroids *do* have on pain processing. There are limitations which prevent the making of generalizations based on these, or any, data pertaining to sex steroid modulation of pain. Many factors that contribute to these

limitations include, but are not limited to, the timing with respect to the noxious stimulation, duration and level of steroid presence, the type (modality) of pain, location on the body, etc., as well as more generic factors, such as sex, age, genetics, and lifestyle.

Certainly, the knowledge gained from this research contributes to a more complete understanding of modulation of pain by sex hormones. Knowledge of the specific roles of sex hormones in pain perception will eventually lead to more efficacious and gender-oriented therapies for chronic pain syndromes in men and women.

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