# Influences of the Female Reproductive Cycle on

# Inflammatory Induced Pain Responses

By

Nicole J. Amador

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of
the requirements for the degree of Doctor of Philosophy,
The City University of New York
2009

©2009

Nicole J. Amador

All rights Reserved

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

Vanya Quinones-Jenab, Ph.D

________________ __________________________

Date Chair of Examining Committee

Maureen O'Connor

________________ __________________________

Date Executive Officer

Vanya Quinones-Jenab, Ph.D.
Shirzad Jenab, Ph.D.
Jim Gordon, Ph.D.
Victoria Luine,Ph.D.
Ann Ho, Ph.D.
Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

Abstract

Influences of the Female Reproductive Cycle on Inflammatory Induced Pain Responses

by

Nicole J. Amador

Advisor: Professor Vanya Quiñones-Jenab

Clinical and preclinical studies have demonstrated significant sex differences in the perception of inflammatory pain; females display higher nociceptive responses to inflammatory stimuli than male rats. Additionally, the complex endocrinological profile of females has been shown to impact their nociceptive responses. For example, estradiol reduces Phase II behavioral-nociceptive responses after formalin administration. However, little is known about the specific biological pathway(s) and/or mechanisms in which cycling endogenous female sex hormones affect inflammatory pain responses. Current literature has established that cyclooxygenases and prostanoids are major pro-inflammatory mediators directly linked to inflammatory responses. Additionally, glucocorticoids, (i.e. corticosterone) negatively regulate inflammatory induced COX-2, resulting in attenuation of inflammatory responses. The objective of this study was to further understand how fluctuations of endogenous female sex hormones alter inflammatory-induced responses by examining two physiological pathways (i.e. NO/COX-2 regulation of the prostanoid biosynthetic pathway and corticosterone regulation of the NO/COX pathway) which may in part be responsible for these effects. Endogenous peaks of estrogen and progesterone during proestrus, were shown to significantly attenuate behavioral responses after formalin administration. This attenuation of behavioral responding was accompanied by a significant increase in $PGD_2$ serum levels. Cortiscosterone serum levels were unaffected after formalin administration suggesting that regulation of behavioral responses by

endogenous hormones may be occurring through a pathway independent of the corticosterone biosynthetic pathway. COX-2 and nNOS levels in the spinal cord were not significantly affected by the estrous cycle, suggesting that regulation of behavioral responses by endogenous hormonal fluctuations may be occurring through a pathway independent of the NO/COX biosynthetic pathway.

Furthermore, although no estrous cycle effects were seen in paw withdrawal latency after carrageenan administration, we observed estrous cycle effects in the contralateral paw at baseline and one hour post-injection. Rats in proestrus showed a significant reduction in thermal-induced hyperalgesia as measured by increased paw withdrawal latency. Although no significant differences were seen in $PGD_2$ serum levels, rats in estrus had significantly higher $PGE_2$ serum levels after carrageenan administration. A significant decrease in PWL was observed in rats during estrus, a time of the lowest levels of fluctuating hormones. These results suggest that hormonal troughs during the cycle may affect inflammation through the PG biosynthetic pathway. Finally, during ages when animals are considered “middle aged” attenuation in inflammatory induced behavior was observed. This finding was accompanied by significant decreases in $PGE_2$ and $PGD_2$ levels and a significant increase in corticosterone serum levels. Taken together these results suggest a relationship between endogenous hormonal fluctuations, corticosterone release and PG activity. In summary, our results suggest that endogenous hormonal peaks and troughs effects on inflammation may be mediated through the regulation of the NO/COX-2/prostanoid biosynthetic pathway.

**ACKNOWLEDGEMENTS**

Getting to this point in my career took much help, encouragement, and support from many around me. The last five years have been made possible though the invaluable efforts of my mentor, Dr. Vanya Quiñones-Jenab. She has not only been a mentor but a friend who constantly provides the support that is needed to get through this program. I thank Dr. Shirzad Jenab for putting up with all of my "western" related questions and concerns. Without him my project would have never come to completion. The two of them bring a smile to my face every day with their wit and sarcasm, in addition to giving me solutions to each and every dilemma that I encounter. Both have mentored me throughout my time in their lab, teaching me the ropes of science while reminding me to smile at the same time. I am forever grateful for both the career and personal lessons that they have taught me, I will always carry those with me. I would also like to thank Dr.Victoria Luine for her input and advice during my experiment and writing this thesis. Dr. Jim Gordon, our neighbor, has made it very easy for me to drop into his lab and ask him questions at any time of the day or night, and for that I am grateful. Dr. Ann Ho, I thank you for your invaluable input into the completion of my thesis and its defense.

I owe a huge thank you to everyone who has been or still is a member of the Quiñones-Jenab lab. They were always there to listen to me, whether it was to complain, to ask questions, to get advice, or just to chat about non-science related things! The support of my colleagues, who have become my friends, have made this trip quite memorable and most of all bearable at rough times. Each member has helped me in some aspect of my life and experiments. The bonds that I have made with each of them are like nothing else I have ever experienced, and I am forever grateful for that.

I want to thank all of my friends that have stood by my side through this experience of graduate school. Through the ups and the downs they have always shown me their love and support, which made this whole process much easier. I can not imagine doing this without them. Most of the time they have no idea what I am doing in the lab, but that does not deter them from being a base of my support system. I can not thank each and everyone one of them for inspiring me to become who I am, I love you all with all my heart.

Lastly, I must thank my family for their constant love and motivation. My brother and my sister, for dealing with my cranky moods and still loving me through it all. For my sister, for understanding that I must defend my dissertation two days before her wedding, and loosening up on the maid of honor duties during this difficult time! I am so thankful for the both of you, and love you more than you know.

I dedicate this thesis to my parents, Diane and Aristides. Their support and guidance in my life, starting from day one, has allowed me to accomplish a goal that I once thought was unreachable. Their constant encouragement, support, and most of all, unconditional love, have been a crucial factor in my life. Without them I would be lost. I look up to the both of you, and hope I have made you proud. I am forever grateful for all that you have done for me, and for paving the road that has lead me to the completion of my dissertation.

***TABLE OF CONTENTS***

Abstract…………………………………………………………………………………...iv

Acknowledgements………………………………………………………………………vi

Table of Contents………………………………………………………………………..viii

List of Figures…………………………………………………………………………….x

List of Tables……………………………………………………………………………..xii

Abbreviations……………………………………………………………………………xiii

Chapter 1: Introduction……………………………………………………………………1

I. Background and Significance…………………………………………………………..2

A. Peripheral processing of physiological pain …………………………………...……2
B. Inflammation……………………………………………………………………….......4
C. Central processing of physiological pain………………………….....................5
D. Hyperalgesia and Sensitization…………………..…………………………...…7
E. Formalin model for inflammatory/chronic pain………………………………..…9
F. Carrageenan model for persistent inflammatory pain……………………….…...12

II. Modulators of Inflammatory Pain……………………………………………………..14

A. Prostaglandins ……………………..……………………………………………15
B. Cyclooxygenase……………………………………..…………………...23
C. Corticosterone……………………………………………………………………26
D. Nitric Oxide………………………………………………………………………...31

III. Sex differences in pain…………………..……………………………….……………...33

A. Menstrual/estrous cycle effects persistent inflammatory pain...…………………....34
B. Hormonal fluctuations across the female endocrinological profile.......................39
C. Age studies with rodents…………………………………………………………41
D. Affects of estrogen and progesterone on inflammatory mediators………………43

IV. Proposed model……………………………………………………………………….….47

A. Specific Aims………………………………………………………….....…......48

B. Methods………………………………………………………………….……...52

Chapter 2: Are endogenous gonadal hormonal fluctuations occurring during the female estrous cycle altering formalin induced inflammatory responses……………………………............57

V. Results………………………………………………………………..…….……57

A. Discussion…………………………………………………...…………….…64

Chapter 3: Can endogenous gonadal hormone fluctuations analgesic/anti-hyperalgesic effects be extended to the carrageenan inflammatory pain model? Are estrogen and progesterone's effect on behavioral responses to carrageenan in part mediated through deactivation/down-regulation of COX-2?.........................................................................................................70

VI. Results…………………………………………….…………….............70

A. Discussion…………………………………….…………………………79

Chapter 4: Are endogenous gonadal hormonal fluctuations throughout life altering formalin induced inflammatory responses to pain?...........................................................................83

VII. Results ………………………………………………………………..……...….83

A. Discussion……………………………………………………………………...87

Chapter 5: Are endogenous estrogen and progesterone's effects on behavioral responses to inflammatory stimuli in part mediated through the induction of corticosterone release?............91

VIII. Results…………………………………………………………………………91

A. Discussion……………………………………………………………………….95

IX. Conclusion…………………………………………………………………......99

X. New Proposed Model……………………………...…………………………...113

XI. References……………………………………………...………………………114

# LIST OF FIGURES

**Figure 1**: Afferent pathway of a nociceptive signal…………………………………………...3

**Figure 2**: Nociceptive behavior after formalin injection……………………………………11

**Figure 3**: Prostanoid biosynthetic pathway…………………………………………………..17

**Figure 4**: Schematic representation of the peripheral and central action of prostanoids………19

**Figure 5**: The endocrine and neural systems stimulated by stress…...…………………………27

**Figure 6**: The different neurotransmitter systems during each phase of menstrual cycle……...35

**Figure 7:** Depiction of the changes in ovarian hormones during different stages of the rat estrous cycle……………………………………………………………………………...…36

**Figure 8**: Cells present in vaginal smears in the four different phases of the estrous cycle ……………………………………………………………………...……...37

**Figure 9**: The proposed model ………………………………………………….…………...47

**Figure 10**: Time course of flinching responses in all estrous stages after (5%) formalin administration …………...…………………………………………………..........................59

**Figure 11:** Effects of estrous cycle stage on behavioral flinching responses after 5% formalin administration……………………………………………………………………………....59

**Figure 12:** Effects of estrous cycle stage on $PGE_2$ (A) and $PGD_2$ (B) serum levels after formalin administration …………………..…………………………………..……………………..60

**Figure 13**: Effects estrous cycle stage on nNOS levels in the spinal cord after formalin administration…..........................................................................................……………………61

**Figure 14**: Effects estrous cycle stage on COX-2 protien levels in the spinal cord after formalin administration ……………………………………………………………...…………....62

**Figure 15:** Effects of estrous cycle stage on baseline levels of right paw withdrawal latencies………………………………………………………………………………………..72

**Figure 16**: Effects of estrous cycle stage on baseline levels of conrtalateral paw withdrawal latencies. ……………………………………………………………………………………...72

**Figure 17 A-D**: Effects of estrous cycle stage on paw withdrawal latencies 1 and 5 hours after 1% carrageenan administration ……………………………………………………………..…….73

**Figure 18 A-C**: Effects of estrous cycle stage on injected paw withdrawal latencies for each heat intensity after 1% carrageenan administration…………………………………………………..74

**Figure 19 A-C**: Effects of estrous cycle stage on contralateral paw withdrawal latencies for each heat intensity after 1% carrageenan administration …..…………………………………………75

**Figure 20 A-B**: Effects of estrous cycle stage on PGE2 (A) and PGD2 (B) serum levels after carrageenan administration ……………………………………………...……………...76

**Figure 21:** Effects estrous cycle stage on nNOS levels in the spinal cord after carrageenan administration………………………………………………………………………………77

**Figure 22**: Time course of flinching responses in all age groups after (5%) formalin administration..…………………………………………………………………...………84

**Figure 23**: Effects of age on behavioral flinching responses after 5% formalin administration………………………………………………………………………………84

**Figure 24 A-B**: Effects of age on PGE2 (A) and PGD2 (B) serum levels after formalin administration………………………………………………………………………………85

**Figure 25**: Effect of estrous cycle stage on corticosterone serum levels after formalin administration ……………………………………………………………………...…………92

**Figure 26**: Effects of estrous cycle stage on corticosterone serum levels after carrageenan administration ……………...……………………………………………………………93

**Figure 27**: Effects of age on corticosterone serum levels after formalin administration………94

**Figure 28**: Proposed model……………………………………………………………………113

## LIST OF TABLES

**Table 1:** Abbreviations……………………………………………………………………xiii

**Table 2:** Correlations of estrous cycle rats after formalin administration…………. …………..63

**Table 3:** Correlations of estrous cycle rats after carrageenan administration…………………..78

**Table 4:** Correlations of aged rats after formalin administration………………………………86

**Table 1: Abbreviations**

| | |
|---|---|
| Adrenocorticotropic hormone | ACTH |
| Adrenalectomized | ADX |
| Arachidonic acid | AA |
| Aromatase-knockout | ArKO |
| Brain derived neurotrophic factor | BDNF |
| Central Nervous System | CNS |
| Complete Freund's adjuvant | CFA |
| Corticosterone | CORT |
| Cyclooxygenase-1 | COX-1 |
| Cyclooxygenase-2 | COX-2 |
| Cystolic PGES-1 | cPGES-1 |
| Descending facilitation | DF |
| Descending inhibition | DI |
| Dimethyl sulfoxide | DMSO |
| Dorsal root ganglia | DRG |
| E-prostanoid receptor | EP-receptor |
| Estradiol treatment | E2 |
| Gamma-aminobutyric acid | GABA |
| Glucocorticoid | GC |
| Glucocorticoid receptor | GR |
| Gonadectomized | GDX |
| Guanine nucleotide binding protein | G protein |
| Hypothalamic-pituitary-axis | HPA |
| Estradiol receptor antagonist | ICI 182,780 |
| Inducible nitric oxide synthase | iNOS |
| Inhibitory post synaptic current | IPSC |
| Interleukin-6 | IL-6 |
| Intraperitoneal | IP |
| Lipocalin-type PGD synthase | L-PGDS |
| Lipopolysaccharide | LPS |
| Microsomal PGES-1 | mPGES-1 |
| Microsomal PGES-2 | mPGES-2 |
| Messenger ribonucleic acid | mRNA |
| N [2-(Cyclohexyloxy)- 4-nitrophenyl]methane sulfonamide (selective COX-2 inhibitor) | NS-398 |
| N-methyl-D-aspartic acid | NMDA |
| Neurotransmitter | NT |
| Nitric oxide | NO |
| Nitric oxide synthase | NOS |

| | |
|---|---|
| Non steroidal anti-inflammatory drugs | NSAID |
| Ovariectomized | OVX |
| Phosphoglucoisomerase | PGI |
| Pre-prodynorphin | PPD |
| Prostaglandin | PG |
| Prostaglandin D2 | $PGD_2$ |
| Prostaglandin E2 | $PGE_2$ |
| Prostaglandin E synthase | PGES |
| Prostaglandin $GF2_\alpha$ | $PGF_{2\alpha,}$ |
| Prostaglandin G2 | $PGG_2$ |
| Prostaglandin H2 | $PGH_2$ |
| Prostaglandin I2 | $PGI_2$ |
| Protein Kinase A | PKA |
| Protein Kinase C | PKC |
| Paw withdrawal latencies | PWL |
| Phospholipase | PLA |
| Real time polymerase chain reaction | RT-PCR |
| Rheumatoid arthritis | RA |
| 5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl pyrazole (selective COX-1 inhibitor) | SC-560 |
| Tumor necrosis factor | TNF |
| Thromboxane A2 | $TXA_2$ |

**Introduction**

The sensation of pain, although sometimes discomforting, often serves as an adaptive function, signaling or warning an organism of actual or potential tissue damage caused by noxious stimuli (Millan, 1999; Ren and Dubner, 1999a). Signals sent by stimulated nociceptors evoke the appropriate behavioral and automatic responses used to provide protection to the organism. These initial responses to pain are protective in that they are activated quickly in an attempt to impede or limit the extent of potential tissue damage. However, when these responses extend beyond that of a warning system, they become maladaptive and systematically debilitating, resulting in chronic, persistent and long-term pain syndromes that have no biological function (Zimmermann, 2001).

Unfortunately, no simple equation(s) exist that can be used to determine the output of perceived pain based on the quality and magnitude of a stimulus input. Numerous variables contribute to nociceptive responses, transmissions, and factors that modulate them. The extent that each individual variable influences nociception is thus far immeasurable. These variables have the ability to change an otherwise innocuous stimuli into a noxious one (Dickenson, 1997; Dickenson and Sullivan, 1987b).

Chronic pain causes alterations in neuronal structure and connections resulting in increased excitatory activity and decreased inhibitory mechanisms in the molecular pain pathway, termed neuronal plasticity (Stucky et al., 2001; Woolf C.J. and Salter M.W., 2000). In turn, this plasticity exerts effects on the nociceptive processing pathways of the central nervous system (CNS), therefore inducing states of allodynia and hyperalgesia (Millan, 1999).

The differences between acute and chronic pain lie in their characteristics. Acute pain is initiated by tissue damage and is comprised of both phasic and tonic pain. It is characterized by

quick responses, while chronic pain which can be conceptualized as the persistence of acute pain is characterized by a more pronounced and sustained modification in the activity of descending controls (Fishman and Teichera, 2003). Quick responses are seen during acute pain, while a more distinct and enduring behavioral modification is seen during chronic pain (Fishman and Teichera, 2003; Millan, 1999; Velle, 1987). Two reversible transient descending mechanisms modulate acute pain; descending inhibition (DI) which decreases sensitivity to noxious stimuli and descending facilitation (DF) which increases sensitivity to these stimuli (Millan, 1999). By contrast, chronic pain is not short lasting. A reduction in descending tonic inhibitory controls (DI) as well as activation of facilitatory systems (DF) contribute to various forms of chronic pain (Colvin and Power, 2005; Pan, 2004). The prolonged responses to nociceptive stimuli seen in chronic pain are a result of neuroplastic change in the nervous system (Ghafoor, 2003). For the purposes of this project, we will be examining acute and inflammatory pain which fall under the umbrella of nociceptive pain distinct from chronic pain. Nociception is a neurophysiological term that denotes activity in specific nerve pathways discussed earlier. These pathways transmit signals that function to protect the body from potential tissue damage by warning the organism of danger.

## I. Background and Significance

### Peripheral Processing of Physiological Pain

Pain sensation begins with the activation of a primary sensory neuron (nociceptor) by noxious stimuli (Sherrington, C. S., 2006). Nociceptors have a particular characteristics that separates them from other sensory nerve fibers; they are polymodal, transducing noxious mechanical, chemical, or thermal information into electrical signals (Costigan and Woolf, 2000;

Julus and Basbaum, 2001). Two primary afferents have been characterized; rapidly conducting, thinly myelinated Aδ fibers, and slowly conducting, unmyelinated C-fibers, eliciting the first phase of 'sharp' pain and then a second wave of 'dull' pain respectively (Julus and Basbaum, 2001; Millan, 1999).

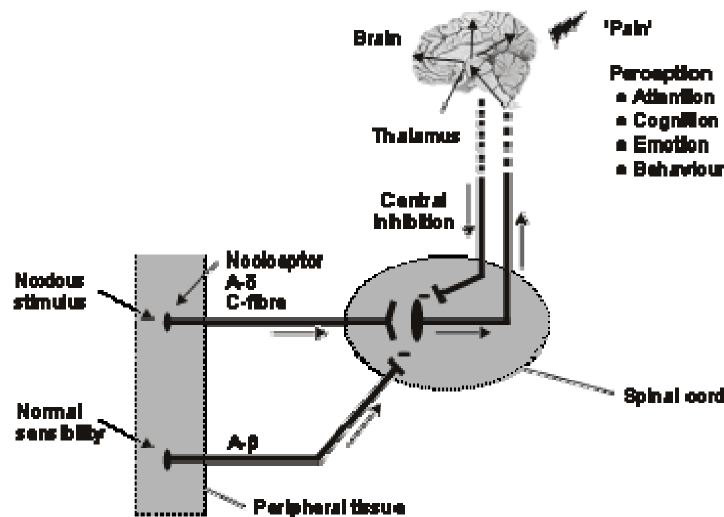



**Figure 1. Shows the afferent pathway that the nociceptive signal travels. The first process involves Transduction, whereby nociceptors respond to noxious stimuli that initiate cellular changes resulting in stimuli being changed into electrical energy necessary to transmit pain. Transmission of pain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

As shown in Figure 1, Aδ- and C-fiber-mediated peripheral nociceptors terminate in the dorsal horn of the spinal cord where their sensory inflow is transferred to second-order neurons in the spinal cord in a highly organized and topographic manner (Costigan and Woolf, 2000; Markenson, 1996; Meyer, R. A., Ringkamp, M., Campbell, J. N., and Raja, S. N., 2006; Millan, 1999). The receiving neurons include projection cells with axons that travel into the white matter and project information to various parts of the brain, and interneurons whose axons

remain in the spinal cord and contribute to local neuronal circuits (Riedel and Neeck, 2001; Todd, A. J. and Koerber, H. R., 2006). This is the site in which the transfer of information about the onset, duration, location and quality of the peripheral noxious stimuli occurs (Woolf C.J. and Salter M.W., 2006).

**Inflammation**

Inflammation is a physiological response to tissue damage in the peripheral nervous system and is a main contributor in pain perception (Costigan and Woolf, 2000; Pasero, 2004). Inflammation is generated by local macrophages released at the site of damaged tissue (Costigan and Woolf, 2000). At the site of tissue damage there is a release of a mixture of cytokines and other neuroactive agents (i.e. bradykinin, leukotrienes, serotonin, histamine, substance P (SP), thrombozanes, adenosine, ATP, prostaglandin (PG), and free radicals) from damaged cells and inflammatory cells (Costigan and Woolf, 2000; Millan, 1999). Mediators act on various receptors and ion channels of the nociceptors, causing various signal transduction cascades that result in modulation of effector molecules and changes in gene transcription (Costigan and Woolf, 2000). The actions of the mediators sensitize nociceptors to further neural input. More specifically, two types of inflammatory responses exist. Acute inflammation is a short-term process characterized by classic signs of inflammation; swelling, redness, heat, pain and sometimes dysfunction of organs involved (Costigan and Woolf, 2000; Millan, 1999). Removal of the potentially harmful stimuli intiates down-regulation of this inflammatory response (Wall, P. D. and Melzack, R., 1994). In contrast, chronic inflammation is not short-term but pathological. It is characterized by tissue damage, local infiltration of mononuclear immune cells (monocytes, lymphocytes, macrophages and plasma cells), and attempts at healing

(angiogenesis and fibrosis). In this case the stimulus is persistent; therefore down regulation of the inflammatory response does not occur (Wall and Melzack, 1994).

## Central Processing of Pain

### Spinal Cord Level

The spinal cord receives a specific pattern of inputs from the primary afferent nociceptors which respond to tissue-damaging stimuli (Todd and Koerber, 2006; Woolf C.J. and Salter M.W., 2006). The dorsal horn contains the first synapse in the ascending pathways that conveys peripheral sensory information to the third-order nociceptive neurons in the brain which project to the cortex where the perception of pain occurs (Riedel and Neeck, 2001; Todd and Koerber, 2006). At the dorsal horn, nociceptive fiber activation results in the release of the major excitatory neurotransmitter glutamate, (SP), calcitonin gene related peptide (CGRP), aspartate, and nitric oxide (NO), important mediators of spinal pain transmission (Todd and Koerber, 2006; Urban and Gebhart, 1999; Yaksh, 1999). The receiving interneurons in the dorsal horn divide into two classes: inhibitory which use γ-aminobutyric acid (GABA) or glycine, and excitatory which are glutamatergic (Todd and Koerber, 2006). Glutamate is an excitatory amino acid that activates two classes of receptors which become engaged during increased spinal excitability: metabatrophic G-protein coupled receptors alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainite (K) and ionotrophic receptors, N-methyl-D-aspartate (NMDA) (Morgan et al., 1999; Ren and Dubner, 1999a; Ren and Dubner, 1999b; Todd and Koerber, 2006). While the AMPA/K receptors desensitize with prolonged agonistic exposure, mediating fast synaptic transmissions by activating and deactivating rapidly, the NMDA receptors have a significantly slower activation and deactivation time (McBain and Mayer, 1994;

Trussel et al., 1994). The NMDA receptor system is a critical part of the nociceptive pathway as it is activated during peripheral hyperalgesia and accountable for the changes seen during central sensitization (Costigan and Woolf, 2000; Morgan et al., 1999; Ren and Dubner, 1999a; Ren and Dubner, 1999b).

**Brain Level**

Once nociceptive signals are processed in the dorsal horn, projection neurons carry the information to higher brain areas (Almeida and Lima, 1997; Berkley and Hubscher, 1995; Bester et al., 1995; Burstein et al., 1990; Calvino, 2006; Millan, 1999). Evidence from electrophysiological studies shows that various neuropepetides and glutamate actions are responsible for the transfer of information to the brain (Li et al., 1996; Millan, 1989; Ossipov et al., 1997). Specifically, glutamate facilitates the transfer from the spinothalamic tract to the thalamus and from the spinomesencephalic tract to the periaqueductal grey (PAG) while other neuropeptides (i.e. SP) play a role in relaying information to the brain (Azkue et al., 1997; Jensen and Yakish, 1992). Two different pathways project nociceptive information: the monosynaptic, going directly to higher cerebral structures and the other projecting the medial, pontomedullary reticular formation and to thalamocortical circuits (Bushnell, M. C. and Apkarian, A. V., 2006; Millan, 1999). The spinothalamic tract is the pathway critical for the experience of pain. It first innervates the thalamus and consequently the postcentral gyrus of the cortex (Apkarian et al., 1992; Berkley and Hubscher, 1995; Casey et al., 1996; Millan, 1999). The thalamus is also a major relay station in the emotional and cognitive processing of pain that is targeted by other nociceptive tracts (Millan, 1999). The thalamus relays this information to various cortical regions via a complex pattern of connections (Millan, 1999)

**Hyperalgesia and Sensitization**

A leftward shift of the stimulus-response function relating pain to stimulus intensity is evident during states of hyperalgesia, a hallmark feature of tissue injury and inflammation (Meyer et al., 2006). Hyperalgesia causes a decrease in threshold for pain and an increase in responses to suprathreshold stimuli at both the site of injury (primary) and surrounding areas (secondary). Evidence suggests that primary hyperalgesia to heat and mechanical injury is mediated by sensitization of peripheral nociceptors (LaMotte et al., 1982; Meyer and Campbell, 1981). Whereas hyperalgesia involves the subjective response, sensitization involves the neuronal responses. The release of cellular mediators during inflammation sensitizes nociceptors to further neural input (DeLeo, 2006). Sensitization of primary afferent nociceptors results in a decreased threshold for responses, increased response to suprathreshold stimuli, and spontaneous neuronal activity (Meyer et al., 2006; Pasero, 2004; Ren and Dubner, 1999a; Ren and Dubner, 1999b).

Secondary hyperalgesia is a consistent feature of chronic pain characterized by enhanced pain to only mechanical stimuli (Ali et al., 1996). It is a centrally controlled phenomenon, which includes an enhanced responsiveness of the CNS neurons to mechanical stimuli after cutaneous injury (LaMotte et al., 1991). When the CNS does not receive input from nociceptors at the time of acute injury, hyperalgesia does not occur (del Olmo et al., 2006). The peripheral signal for pain involves nociceptors, and under certain conditions, other receptor types that are not normally associated with pain but have developed the capacity to evoke pain such as a condition termed central sensitization, arising because of the changes in central neuronal responding to low-threshold mechanoreceptors. Central sensitization is the abnormal hyperexcitability of

nociceptor neurons in the dorsal horn of the spinal cord (Beydoun and Backonja, 2003). Central sensitization occurs as a function of pharmacological and/or physiologic modulation associated with peripheral injury (Beydoun and Backonja, 2003). The release of neurotransmitter (NT) mediators contribute to changes in receptor sensitivity such as an increase in the number of nociceptive receptors responding to repeated activation. As a result, low intensity peripheral stimuli can activate afferent fibers that do not evoke pain under normal circumstances (Ghafoor, 2003). These NTs also act on ion channels, and these joint actions activate intracellular signaling that induces cyclooxygenase (COX) gene expression and PG synthesis. Prostanoids act on both pre- and post-synaptic receptors modifying their functional properties and the excitability of dorsal horn neurons. These changes lead to central sensitization (del Olmo et al., 2006).

Alterations occurring at the slower acting NMDA receptors account for the central sensitization whereby there is a decrease in the thresholds and enlargement of receptive fields causing an increase in behavioral responding to a stimulus (Millan, 1999; Ren and Dubner, 1999a; Ren and Dubner, 1999b). This sustained NMDA output caused by extended depolarization from C-fiber stimulation results in an additional release of neuromodulators (glutamate, SP, CGRP) leading to a significant increase in the cellular responses of neurons in the dorsal horn, a phenomenon termed “wind-up” (Costigan and Woolf, 2000; Morgan et al., 1999; Woolf C.J. and Salter M.W., 2006). The neuromodulators induce slow post-synaptic potentials which contribute to the opening of $Ca^{+2}$ voltage gated channels at the NMDA receptor resulting in an influx of calcium ions and the amplification of the depolarization (McBain and Mayer, 1994). Ongoing activities in the spinal cord from a previous stimuli in conjunction with new stimuli that arrive are summed together resulting in a more intense interneuron dorsal horn

discharge (Yaksh, 1999). Taken together, these long term changes within the CNS contribute to increased responsiveness to innocuous stimuli or continued responding once the noxious stimulus is removed (Dickenson and Sullivan, 1987a; Zimmermann, 2001).

## Models Used to Study Inflammatory Pain

### Formalin model for inflammatory/chronic pain

The most common nociceptive assays used in non-human animal models are thermal electrical, mechanical and chemical (Bennett, G. J., 2001). Most traditional nociceptive tests (i.e. the tail flick and hot plate tests) rely on noxious stimuli that are brief in nature, escapable, and based on a physical stimulus of high intensity. They usually employ a "cutoff" which represents a set time point or maximum stimulus intensity after which the noxious stimulus is removed from non-responding animals in order to avoid excessive tissue damage (Bennett, 2001). In contrast, models used to represent tonic pain are longer in duration characterized by continuous pain often associated with inflammation and/or tissue damage (Sternberg, W. F. and Wachterman, M. W., 2000). The tonic pain models are therefore a more practical model for clinical pain conditions than the phasic assays. The formalin model is a tonic pain model of persistent inflammatory pain.

The formalin assay involves the administration of diluted formaldehyde solution (usually 1-10%) into the dorsal surface of the hind paw of the rat which results in behaviors such as licking, flexing and flinching of the paw (Sternberg and Wachterman, 2000). The nociceptive score is a result of the time and frequency with which the animal performs these behaviors. Within seconds of the formalin injection, the animal begins to flinch and lick its paw for approximately 9 minutes and then temporarily stop the behavior (Bennett, 2001). This behavior

is characterized as Phase I of the nociceptive response, reflecting opioid mediated acute nociceptive pain caused by a formalin-evoked discharge in C-fiber nociceptors (Yaksh, 1997). The period directly following Phase I is termed the quiescent or interphase in which no behaviors are observed, which is mediated by descending inhibitory mechanisms (Millan, 1999) The rat will then begin to flinch and lick the injected paw once again at higher frequencies for 15+ minutes. This phase of activity is termed, Phase II (Bennett, 2001). Phase II activity results from mechanisms resembling those of chronic and/or neuropathic pain associated with responding from spinal cord nociceptive neurons evoked by C-fiber discharge during the initial phase and is thought to be NMDA receptor-mediated (Bennett, 2001). Phase II is believed to be the result of mechanisms that resemble those responsible for central chronic and/or neuropathic pain. (Ceccarelli et al., 2003; Coderre and Yashpal, 1994; Yashpal et al., 1995).

The formalin assay is distinct from other nociceptive assays because of its triphasic nature lasting a total of ~60 minutes (Figure 2). It allows for a method of evaluating both acute and tonic pain in a single chemical test. The early and late components of the response are attributed to direct activation of nociceptors and central sensitization respectively (Coderre et al., 1990; Vaccarino and Chorney, 1994). The early phase (0-5 min) appears to be caused by C-fibre activation as a result of the peripheral stimulus. The interphase (6-20 min) is the period following the early phase and characterized by a quiescent period of relatively low responding after the formalin injection (Hacimuftuoglu et al., 2006). The late phase (~20-50 min) appears to be dependent on both the peripheral tissue inflammation reaction and the functional changes that occur in the dorsal horn of the spinal cord (Tjolsen et al., 1993).

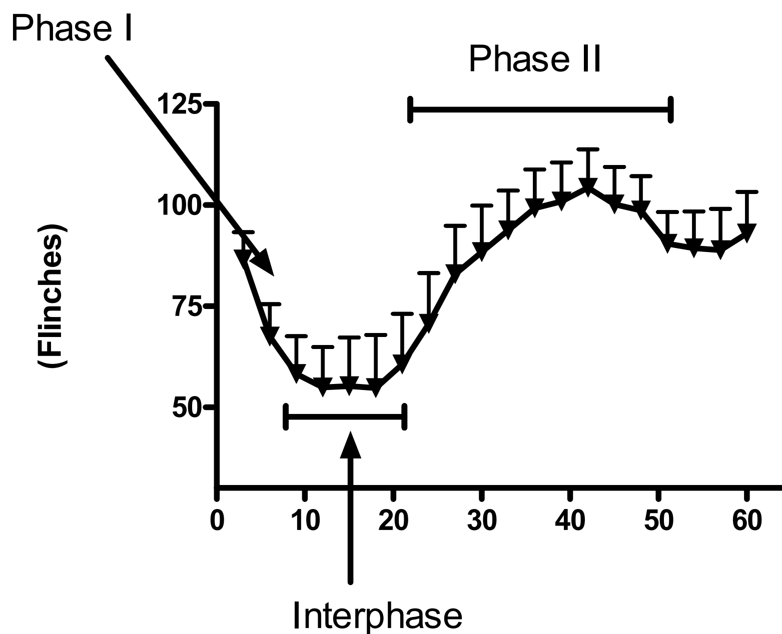



**Figure 2. Nociceptive behavior resulting from diluted formalin injection to the right hind paw. Across time, the biphasic flinching behavior is characterized as Phase I, (0-5min). Phase I results from activation of peripheral nociceptors and direct monosynaptic activation of non-NMDA receptors by glutamate and NK1 receptors by substance P at the dorsal horn neuron. Following phase I, there is an interphase period, where no nociceptive activity is observed. Immediately following interphase, Phase II is characterized by continued afferent input from nociceptors and activation of NMDA receptors by continued glutamate release.**

Several studies support the qualitative differences in mediation of the early and late phases of the formalin responses. Administration of non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to reduce nociceptive behavior during Phase II, while the Phase I was unaffected (Hunskaar S and Hole K, 1987). Yahspal et al. (1998) examined the effects of Phase I and Phase II responses after both 1% and 5% formalin administration in addition to comparing these responses after the administration of two anti-inflammatory drugs (dexmethasone and ibuprofen) (Yashpal and Coderre, 1998). Early phase nociceptive responses were not significantly altered by either drug after 1% or 5% formalin (Yashpal and Coderre, 1998). However, during Phase II, responses after 5% formalin administration were significantly affected in a dose-dependent manner by both drugs, suggesting that nociceptive responding in

each phase is controlled by different mechanisms. Additionally, a positive correlation between formalin effects, nociception, and inflammatory responses at high concentrations of formalin (5%) were observed (Yashpal and Coderre, 1998). In summary, since NSAIDs do not affect the early phase but do affect the late phase, the role of peripheral inflammation has been suggested to be a major contributor to the late phase responses, supporting the existence of different mechanisms that contribute to nociceptive responses in each phase following formalin administration.

**Carrageenan model for persistent inflammatory pain**

Another classic model used to study inflammatory pain is referred to as the carrageenan model (Guay et al., 2004; Hargreaves et al., 1988; Henriques et al., 1987). This model has been used to identify inflammatory mediators and test various anti-inflammatory drugs. Carrageenan is a glycoprotein derived from seaweed that is injected (in concentrations ranging from 1-3% in saline) in the dorsal surface of the rodent hind paw to produce a prolonged inflammatory response (Bennett, 2001). Carrageenan administration leads to biphasic events that result in the production of hyperalgesia, edema, erythema (redness), and localized hyperthermia in the affected area in a dose-dependent manner. The carrageenan assay involves the administration of carrageenan into the dorsal surface of the hind paw of the rat resulting a behaviors such as licking, flexing and flinching of the paw (Guay et al., 2004; Hargreaves et al., 1988; Nantel et al., 1999). The injected paw becomes oedemic (increased in paw volume) and an increase in sensitivity to mechanical and thermal stimuli is present (hyperalgesia). The doses necessary for generating paw oedema and thermal hyperalgesia are similar (Portanova et al., 1996; Zhang et al., 1997), while mechanical hyperalgesia requires a much higher dose (Nantel et al., 1999).

There are two phases involved in carrageenan induced inflammation, early (1-6 hours) and late (12 and 24 hours). The initial phase of inflammation (0-1 hrs post carrageenan injection) is due to activation of inflammatory agents such as histamine, bradykinin, nitric oxide, and 5-HT in the injected area (Crunkhorn & Meacock, 1971; DiRosa et al., 1971; Garcia Leme et al., 1973; Hargreaves et al., 1988; Salvemini et al., 1996; Vinegar et al., 1987). The second sustained phase of swelling is correlated with the activation of neutrophils, cytokines, prostaglandins, and cyclooxygenase-2 (COX-2; Di Rosa et al., 1971; Loram et al., 2007; Seibert et al., 1994; Vinegar, 1971). This phase is usually termed the acute phase, and occurs 2-4 hours after injection (Hargreaves et al., 1988).

The automated detection testing paradigm used is the Hargreaves box, which assesses nociceptive thresholds associated with paw withdrawal latencies after induction of inflammation. In the Hargreaves' test, rodents are placed in individual plexiglass cages set atop a clear glass platform. After an acclimation period, sensitivity to thermal stimuli is determined by directing radiant heat to the plantar surface of the hind paws and measuring (in sec) the time it takes for the rodent to withdraw its paw from the heat source. Thermal hyperalgesia, the increased sensitivity to a noxious thermal stimulus, is tested by comparing baseline paw withdrawal latency (PWL) to the readings taken after carrageenan administration. Changes in PWL to the heat source are then calculated. Hargreaves et al., (1988) demonstrated a significant reduction in paw withdrawal latencies after carrageenan induced inflammation when compared to saline treated paws (Hargreaves et al., 1988). Dose-related hyperalgesia was detected and blocked with the NSAID, indomethacin or morphine (Hargreaves et al., 1988). Indomethacin acts peripherally and centrally by inhibiting oedema, supporting a spinal mechanism for peripheral inflammation following carrageenan administration (Daher and Tonussi, 2003). Carrageenan-induced

inflammatory responses are also affected by age (Posadas et al., 2004). Younger mice (3-4 weeks) displayed an overall lower paw edema than older mice (5-8 weeks). It was also shown that COX-1 levels were not altered after carrageenan but COX-2 levels were modified after injection (Posadas et al., 2004).

Taken together, these studies demonstrate that both the formalin and carrageenan behavioral testing paradigms can be used to analyze different components of nociceptive processing and responding. Specifically, the two phases of the formalin model allow examination of variables that can effect or modify both induced acute and tonic pain respectively. The early phase of the carrageenan model allows insight into induced acute, hyperalgesic and tactile allodynic responses all associated with tissue injury and/or inflammation. These two testing models were utilized in the current study to address specific aims and evaluate the effects of endogenous hormone fluctuation on inflammatory-induced nociceptive processing and responding

**II. Modulators of Inflammatory Pain**

The increase in intracellular $Ca^{2+}$ that occurs at the NMDA receptor site during inflammation results in the activation of intracellular enzymes, phospholipase $A_2$ ($PLA_2$) and nitric oxide synthase (NOS) (Yaksh, 1999). The actions of $PLA_2$ enzymes leads to the release of arachidonic acid (AA) phospholipids (McMahon S.B., David, L. I., Bevan, S., and Bevan, S., 2006; Meyer et al., 2006). AA is then metabolized by COX enzymes, resulting in an expression of its metabolites which have diverse physiological roles in mediating pain and inflammation (McMahon S.B. et al., 2006). Two forms of COX enzymes exist, COX-1 and COX-2, which are responsible for catalyzing the synthesis of a specific AA metabolite, PGs, and are referred to as

the rate liming step in prostanoid synthesis. (McMahon S.B. et al., 2006; Meyer et al., 2006; Millan, 1999; Yaksh, 1999). COX-1 and COX-2 differ from each other in that COX-1 is a constitutive isoform expressed in almost all tissues mediating many physiological responses by producing PGs required for cellular functioning (Millan, 1999). In contrast, COX-2 is primarily found in cells involved in inflammation and pain in the brain and spinal cord (Beiche et al., 1996; Breder et al., 1995) receiving nociceptive input (Millan, 1999) and thereby can be regulated in response to inflammatory conditions (Beiche et al., 1996; McMahon S.B. et al., 2006). COX-2 is the isoform that is primarily responsible for the synthesis of PGs involved in both acute and chronic inflammatory pain states, producing much larger potentially pathological amounts of PG as compared to COX-1 (McMahon S.B. et al., 2006; Meyer et al., 2006; Millan, 1999).

**Prostaglandins**

It has been established that PGs play a significant role in modulation of inflammatory responses associated with pain (Bos et al., 2004; Meyer et al., 2006; Millan, 1999; Samad et al., 2002; Vengas and Schaible, 2001; Willingale et al., 1997). PGs are a metabolite of AA and have their hyperalgesic effect not by activating nociceptors directly, but by sensitizing the nociceptors to noxious stimuli via the activation of chemical mediators (Meyer et al., 2006; Patrignani et al., 2005; Veiga et al., 2004; Yamamoto and Nozaki-Taguchi, 2002). The initial step in the formation of PGs is the conversion of free AA to $PGG_2$, which then is reduced to $PGH_2$ by COX-1 and COX-2 enzymes (Figure 3). Tissue specific synthases then convert $PGH_2$ into various isoforms of PGs and thromoboxanes (i.e. $PGD_2$ $PGF_{2\alpha}$, $PGE_2$, $PGI_2$, and $TXA_2$) (Millan, 1999; Samad et al., 2002; Vengas and Schaible, 2001) These are all produced during

inflammation and exert their biological actions through seven-transmembrane domain, G-protein coupled receptors: DP, FP EP (EP1, EP2, EP3, EP4), IP, and TP receptors. (McMahon S.B. et al., 2006; Samad et al., 2002). The specific PGs likely to have a role in inflammation, sensitization and hyperalgesia are $PGD_2$ $PGE_2$, and $PGI_2$. $PGE_2$ is of particular interest; $PGH_2$ is converted to $PGE_2$ by the membrane bound prostaglandin E synthase-q (mPGES)-1 (Grill et al., 2006) and exerts its effects via its actions on the four EP receptors both peripherally and centrally (Engblom et al., 2002; Millan, 1999; Samad et al., 2002). In situ hybridization studies show the expression of EP1, EP3, and EP4 mRNAs in primary sensory neurons, specifically in the dorsal root suggesting involvement of $PGE_2$ mediated peripheral sensitization (McMahon S.B. et al., 2006; Samad et al., 2002). Receptor activation leads sensitization in the terminals caused by an increase in concentration of cyclic AMP (cAMP) by adenylate cyclase activation leading to greater sodium currents.

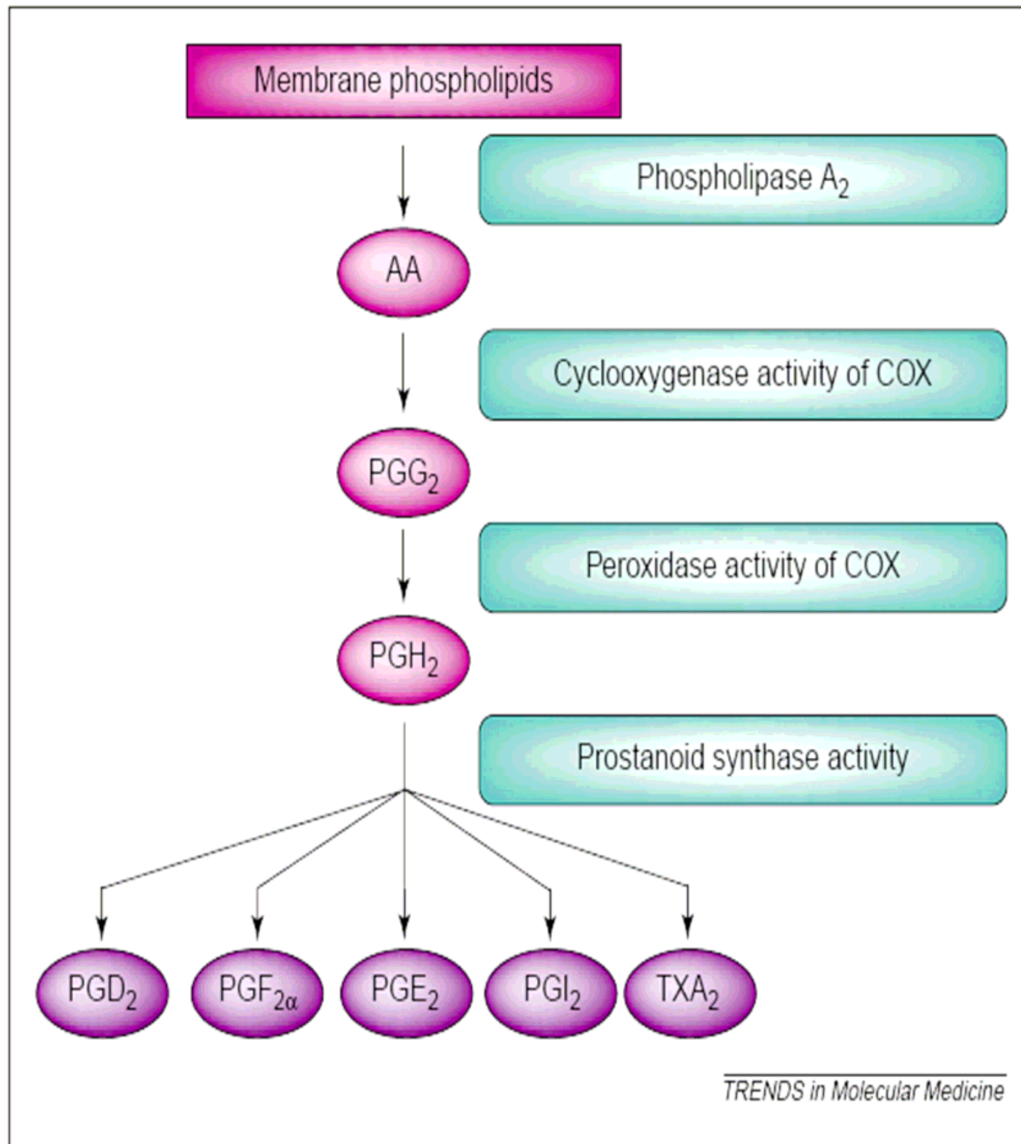



**Figure 3. Prostanoid biosynthetic pathway. Following the release of arachidonic acid (AA) from cell membranes by phospholipase A2 (PLA), it is converted in a two step reaction, first to prostaglandin $G_2$ ($PGG_2$), then to $PGH_2$, by the action of cyclooxygenase (COX) enzyme. $PGH_2$ is then converted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

The role of $PGE_2$ in the inflammatory response has been revealed through the use of several animal behavioral models such as those involving an administration of Complete Freund's Adjuvant (CFA), carrageenan, zymosan, and formalin. The use of these models has contributed to the identification of the specific mechanisms by which PGs exert their effects.

Inflammatory pain is caused by sensitization of peripheral and central nociceptive neurons and/or receptor activity which contributes differentially to inflammatory responses (Guay et al., 2004; Horiguchi et al., 1986b; Wall and Melzack, 94; Yashpal et al., 1995).

PGs exert their actions and contribute to the development of pain via their actions both peripherally and centrally (Figure 4) (Samad et al., 2002). In the periphery, PGs reduce pain threshold and potentiate the actions of noxious stimuli. PGs are released in great amounts during inflammation acting on their specific binding site on the nociceptor, producing a protein kinase A (PKA)-mediated phosphorylation of sodium channels and other receptors in the terminals of nociceptors after activation by EP receptors, thereby increasing the sensitivity of the peripheral terminals of the nociceptors (Engblom et al., 2002; Millan, 1999; Samad et al., 2002). These changes potentiate actions of chemical mediators such as heat, irritant molecules, and bradykinin known for their pain-producing stimulatory actions leading to peripheral sensitization (Gold, 1999; Khaser, 1998). Reductions in pain thresholds occur and are potentaited the actions of various pain producing stimuli.

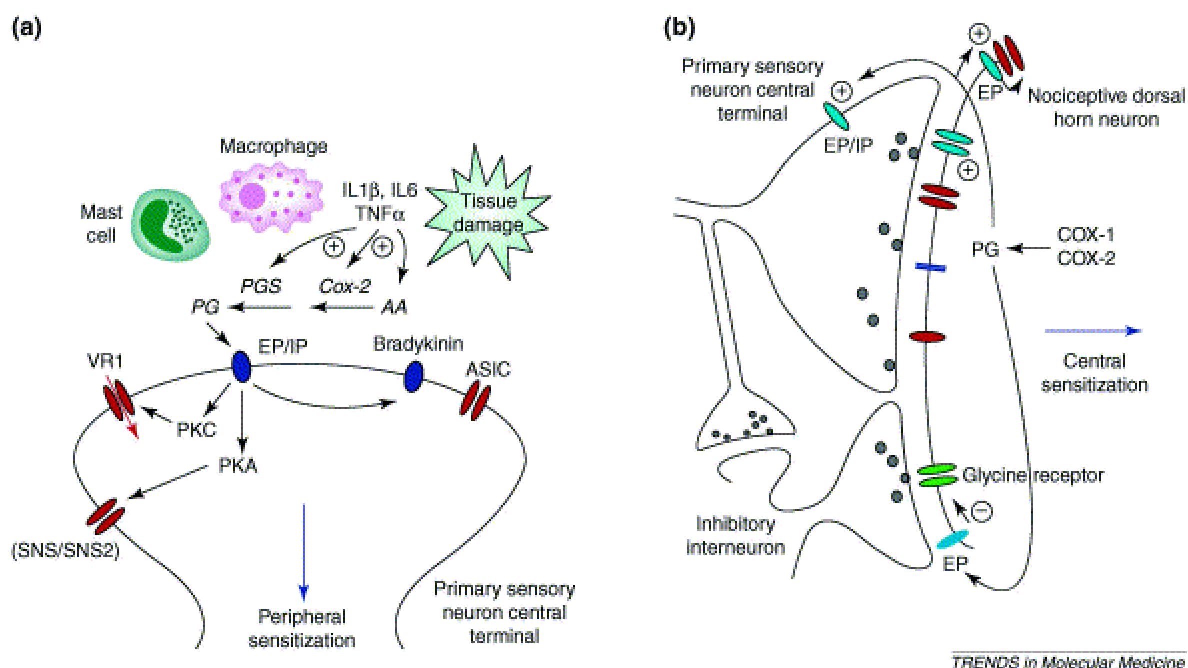



**Figure 4. Schematic representation of the peripheral and central action of prostanoids. (a) The peripheral terminal of a nociceptor neighboring the site of injury/inflammation where prostanoids are massively produced and released to act on their specific receptors. Prostanoid signaling is mediated by second messengers such as protein kinase A (PKA) and PKC, which regulate the activity of many receptors and ion channels and contribute to the potentiation of primary sensory neurons leading to peripheral sensitization. (b) The central terminal of a nociceptor where neurotransmitters/neuromodulators are released and act on receptors and ion channels in the dorsal horn neurons to activate intracellular signaling and induce cyclooxygenasse 2 (COX-2) gene expression and prostaglandin synthesis. Prostaglandins in turn, act on pre- and post-synaptic receptors. This signaling mechanism in which prostanoids constitute a key mediator contributes to the regulation of the functional properties and membrane excitability of dorsal horn neurons leading to central sensitization. Abbreviations: AA, arachidonic acid; IL, interleukin; TNFα, tumor necrosis factor α. Samad et al., 2002**

Centrally, the spinal cord dorsal horn is the second site where PG exerts its pain sensitizing effects (Figure 4). PGs act on the primary sensory neuron central terminals where neuromodulators and neurotransmitters are released and act on receptors in the dorsal horn neurons resulting in induction/modification of COX-2 gene expression and PG synthesis (McMahon S.B. et al., 2006; Samad et al., 2002). Their actions contribute to the functioning and thresholds of these neuronal membranes in the dorsal horn, ultimately leading to central sensitization (Engblom et al., 2002; Minami, 1994; Samad et al., 2002). $PGE_2$ increases transmitter release at these terminals, (Malmberg et al., 1995) work on a non-selective cation

channel to depolarize dorsal horn neurons, (Baba et al., 2001; Ren and Dubner, 1999a) and reduce glycine-mediated inhibition in the spinal cord (Ahamadi et al., 2002). Centrally administered PGs account for changes that produce significant alterations in nociceptive behavior including exaggerated responses to normally innocuous stimuli, i.e., allodynia and hyperalgesia (Daher et al., 2003; Ikeda et al., 2006; Minami, 1994). Administration of $PGE_2$ antibodies (Portanova et al., 1996) or deletion of $PGE_2$ receptor genes (Camacho-Arroyo et al., 2003) inhibit hyperlagesic behavior after injury and provide support for a major role of $PGE_2$ mediation in hyperalgesia.

Glycine is one of the major inhibitory transmitters and by reducing its actions, excitability increases at the site (Samad et al., 2002). Recently, Reinold et al. (2005) discovered that the inhibitory actions of $PGE_2$ are mediated through a single receptor (EP2 receptor) and are restricted to specific glycine receptors with the α3 subunit. EP2 receptor deficient mice or α3 subunit receptor deficient mice lack both PG-mediated inhibition of glycine actions in the dorsal horn and the pronociceptive effects of spinal $PGE_2$ (Reinold et al., 2005). Periaqueductal prostaglandin receptor stimulation is associated with increases in formalin-induced nociceptive responses. Specifically, after formalin administration, subsequent stimulation of these receptors increased glutamate and reduced GABA release (Oliva, Patrizia05). Formalin administration produced similar biphasic fluctuations of both of NTs by increasing glutamate release and reducing GABA release (Oliva, Patrizia05). When Misoprostol, a PG agonist, and formalin were administered together there was a greater increase in glutamate followed by a larger decrease in the release of GABA (Oliva, Patrizia05). Inhibitory glycine receptors in the dorsal horn have been identified as target receptors for $PGE_2$ regulatory actions during peripheral inflammation as well. Blockade of these glycine receptors resulted in a significant reduction in glycine receptor

mediated inhibitory post synaptic currents (IPSCs). Based on these findings, glycine activity has been implicated in providing a cellular and molecular basis for the sensitizating action of $PGE_2$ in the dorsal horn (Ahamadi et al., 2002).

The actions of all PGs remains to be clarified, but $PGE_2$ is of particular interest. It appears to be the most predominant form of pronociceptive PG contributing to hyperalgesia via its modification of COX-2 activity through activation of EP receptors (EP1, EP2, EP3, and EP4) (Millan, 1999). Recordings from nociceptive dorsal horn neurons established that under normal conditions activation of EP1, EP2, and EP4 receptors induce spinal hyperexcitability similar to $PGE_2$, but after inflammation, the responses changed (Ren and Dubner, 1999a). EP1 was the only receptor that was associated with an increase in excitability, while EP2 and EP4 had no additional effects. Surprisingly, Reinold et al. (2005), showed that mice deficient in the EP2 receptor (EP2-/- mice) completely lacked spinal $PGE_2$ – evoked hyperalgesia (Reinold et al., 2005). After treatment with a noxious stimulus, deficient mice exhibited short-lasting peripheral hyperalgesia but lacked a second phase of pain, which is the phase of spinal origin (Reinold et al., 2005). Whole-cell patch-clamp and intracellular recording techniques have been used to show that bath-applied $PGE_2$ induced membrane depolarization in the majority of deep dorsal horn neurons. Baba et al. (2001) demonstrated that both and EP1 and an EP3 agonist had little effect on membrane current, but that the agonist for EP2 mimicked the effect of $PGE_2$ suggesting that $PGE_2$ acts via an EP2- like receptor directly depolarizing spinal neurons.

$PGE_2$ regulates the sensitivity of “silent” nociceptors that under normal conditions are difficult to activate and mechanically insensitive to even very strong stimuli (Koltzenburg M. et al., 1992). After exposure to $PGE_{2,}$ the thresholds are greatly decreased resulting in sensitization to noxious stimuli. $PGE_2$ may contribute to excitatory mechanisms through suppression of $K^+$

conductance resulting in increasing $Na^+$ and $Ca^+$ conductance (Millan, 1999) effecting activation of capsaicin receptors TRPV1 (Caterina et al., 1997) and tetrodotoxin-resistant $Na^+$ channels. An application of $PGE_2$ to dorsal root ganglion cells results in an increase current response rate to noxious stimuli (Lopshire and Nicol, 2007). These tetrodotoxin-resistant $Na^+$ channel location in the DRGs are pivotal in giving rise to nociceptive unmylenated C and Aδ nerve fibers. The $PGE_2$ modulation of the channels involves the activation of adenylyl cyclase and increases in cAMP, leading to a PKA phosphorylation of the channels (McMahon S.B. et al., 2006). Centrally, $PGE_2$ appears to work at the spinal cord dorsal horn producing hyperalgesia via its reduction of glycine in the superficial layers of the dorsal horn and direct depolarization of deep dorsal horn neurons (McMahon S.B. et al., 2006; Mouihate et al., 2004; Samad et al., 2002).

An alternative pathway of PG metabolism is the conversion of $PGD_2$ by PGD synthases. In the CNS, the biosynthesis of $PGD_2$ is mediated by the lipocalin-type PGD sythase (L-PGDS) (Grill et al., 2006). The bioactivity of this PG is mediated by two G protein coupled receptors, DP and CRTH2 (Gervais et al., 2001) that have opposing effects on cyclic AMP production (Liang et al., 2005). $PGD_2$ may be involved in hyperalgesia, although currently little is known about the regulation of $PGD_2$ biosynthesis in the spinal cord during inflammation (Hata and Breyer, 2004). $PGD_2$ shows little or no peripheral effect similar to $PGE_2$'s ability to sensitize afferent neurons to noxious stimuli (Wall and Melzack, 94). Horiguchi et al., (1986) found evidence to support separate mechanisms for nociceptive responses of $PGD_2$ and $PGE_2$ in mice (Horiguchi et al., 1986b). After intracisternal administration, both $PGD_2$ and $PGE_2$ had biphasic effects on pain thresholds. However, after intracisternal injection of naloxone, hypoalgesia caused by higher doses of $PGD_2$ was blocked while that effect what not seen after high doses of $PGE_2$ (Horiguchi et al., 1986b). Ishizaka et al. (2001) demonstrated an increase in

proinflammatory cytokine enzyme levels of L-PGDS in the cerebrospinal fluid after administration of a bacterial endotoxin lipopolysaccharide (LPS), (Ishizaka et al., 2001). Guay et al. (2004) showed an increase in $PGD_2$ levels in the cerebrospinal fluid only during the early phase of inflammation (Guay et al., 2004). Real time polymeric chain reaction (RT-PCR) and western-blot analysis reveal an enhanced expression of COX-2, mPGES-1, L-PGDS mPNA and protein in the spinal cord after inflammation (Grill et al., 2006). $PGE_2$ and $PGD_2$ concentrations in the mouse spinal cord were significantly greater in endotoxin treatment groups than in the control group, suggesting enhanced PG biosythesis in the spinal cord after treatment (Grill et al., 2006). An addition of the selective COX-2 inhibitor (lumiracoxib) significantly attenuated $PGE_2$ and $PGD_2$ release in endotoxin treatment groups to values similar to those in the control group (Grill et al., 2006). Taken together these studies support a role for $PGD_2$ in inflammatory pain mechanisms. However, its involvement in hyperalgesia and other periphery inflammatory responses is not well documented.

**Cyclooxygenase**

The relationship between PGs and COX is essential in the establishment of nociceptive transmissions and responses. Many studies in the literature support a major role for COX-2 in mediating inflammatory responses, but the role of COX-1 remains debatable. Yamamoto et al. (2002) investigated the role of PGs synthesized by COX-1 versus those synthesized by COX-2 using a behavioral nociceptive assay, the formalin test (Yamamoto and Nozaki-Taguchi, 2002). Behaviors associated with phase II of inflammatory pain were decreased only in rats treated with an oral administration of the selective COX-2 inhibitor, celecoxib and indomethacin (non-selective COX-1 and COX2 inhibitor). Those who were treated with these inhibitors

intrathecally, showed depression of nociceptive responses in both phases during the rat formalin test. Animals treated with the selective COX-1 inhibitor, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole (SC-560), responded similarly to untreated animals (Yamamoto and Nozaki-Taguchi, 2002). These data strongly suggest that PG's synthesized by COX-1 are not involved in nociceptive transmission during the rat formalin test, while the inhibition of COX-2 actions seems to be the main mechanism by which NSAIDs produce analgesic effects during the formalin test. Veiga et al. (2004) demonstrated that celecoxib (COX-2 inhibitor), given intrathecally, produced an analgesic response in the periphery only when administered before introduction of the noxious stimulus (formalin) (Veiga et al., 2004). Once the inflammatory process begins, peripheral inhibition of COX-2 does not inhibit the development of secondary hyperalgesia, but spinal blockade is still effective (Veiga et al., 2004). This provides evidence that COX-2 products may be released at the site of inflammation immediately after the formalin injection. Central sensitization seems to be independent of continuity of peripheral inputs.

After the induction of cerebral ischemia in rats, COX-2 mRNA up-regulation is seen in the ischemic but not the contralateral hemisphere, with $PGE_2$ being elevated 57% in the same hemisphere 24 hours later (Nogawa et al., 1997). $PGE_2$ levels were reduced in animals pre-treated with NS-398, a COX-2 inhibitor (Nogawa et al., 1997). These data suggest that that COX-2 message and protein up-regulation is associated with increased tissue concentration of $PGE_{2,}$ indicating that COX-2 mRNA is translated into a functional enzyme. Thus, cerebral ischemia leads to up-regulation of COX-2 mRNA, protein, and reaction products.

COX-1 may play a larger role in mediating some of the above mentioned nociceptive and inflammatory responses mentioned above. Several studies have questioned the role of COX-2 in

this pain pathway. Dou et al. (2004) reported that COX-1 was the only COX isoform expressed in dorsal root ganglion (DRG) neurons. Yamamoto and Nozaki-Taguchi (1996) reported that the intrathecally-administered NS-398 inhibited formalin-induced flinching behavior in a dose-dependant manner whereas the intraperitoneal injection of this selective inhibitor had no such effect (Yamamoto and Nozaki-Taguchi, 1996). In line with these findings, Euchenhofer et al. (1998) showed that NS-398 only yielded its antinociceptive activity at a dose so high (27 mg/kg) that it most likely lost its selectivity. The non-selective COX inhibitor diclofenac, reduced formalin-induced flinching behavior in a dose dependant manner starting with 1mg/kg (Euchenhofer et al., 1998).

Using a pain model to mimic both spontaneous and slowly developing diffuse pain (the stretching test), Ballou et al. (2000) showed that COX-1 was the primary COX isoform that is involved in this test (Ballou et al., 2000). Studies using in situ-hybridization, an immunohistological analysis, demonstrate that COX-1 and cystolic PGEs (cPGES) are coupled and expressed in the spinal cord of adult rats (Hofacker et al., 2005). A reduction of cPGES in the rat spinal cord resulted in a significant reduction in nociceptive behaviors (Hofacker et al., 2005). This data suggests that cPGES also play an important role in mediating early responses during nociceptive responses. Furthermore, Tonioka et al. (2000) showed that after injection of formalin, rapid $PGE_2$ release is in part mediated by COX-1 (Tanioka et al., 2000).

The administration of selective COX-1 inhibitor (SC-560) reduced nociceptive behavior during the formalin assay in addition to completely abolishing formalin-evoked $PGE_2$ levels (Tegeder et al., 2001). More recently, Zhang et al. (2007) reported an increase in COX-1 expression by microglia in the spinal cord beginning one day after formalin injection. (Zhang et

al., 2007). Collectively, these results suggest that COX-1 may play a larger role in nociceptive responding that previously thought.

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

**Corticosterone**

Corticosterone (CORT), the steroid hormone released by the cortex of the adrenal glands, is released in response to, and under the control of, adrenocorticotropic hormone (ACTH). CORT is included in the class of corticosteroids the glucocorticoids (GCs), which are released during the stress-response and known to inhibit inflammation. The release of CORT begins when a stressor is perceived in the brain. The corticotrophin-releasing hormone (CRH) is released from the hypothalamus and stimulates the pituitary to release ACTH which then stimulates the adrenals to release CORT (Figure 5).

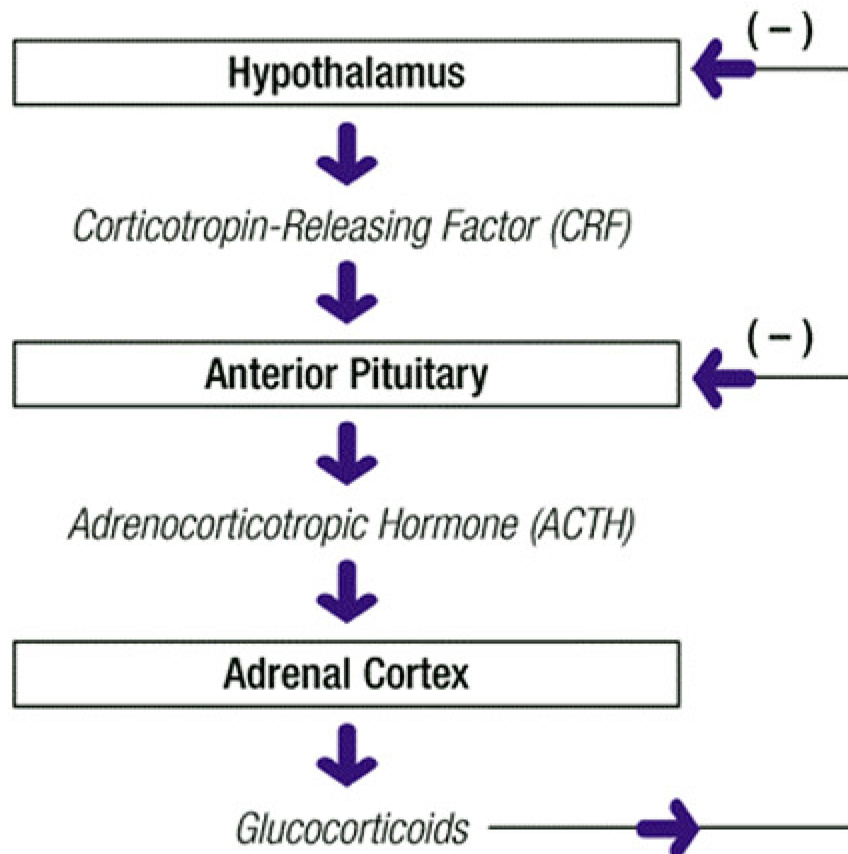



**Figure 5. The endocrine and neural systems stimulated by stress. Inflammation induces the release of CRF from the hypothalamus, which enter the hypothalamic-pituitary portal circulation. It triggers the anterior pituitary to release ACTH, which then stimulates glucocorticoid release from the adrenal cortex. Arabashi et al. 2005.**

Glucocorticoids (GC) are among the most important drugs used to treat rheumatic diseases such as arthritis and temporomandibular arthritis due to their potent immunosuppressive and anti-inflammatory effects (Arabshashi et al., 2005; Theile et al., 2005). Although corticosteroids are used for treating these inflammatory diseases, the mechanisms and underlying actions are still in question. It has been posited that they are released during stress and/or nociceptive stimulation associated with the immune system (Taylor et al., 1998) promoting mechanisms important for normal resolution of inflammation via macrophage phagocytosis of leukocytes undergoing apoptosis (Harbuz, 2002; Harbuz et al., 1999). GCs negatively regulate the inflammatory-induced COX-2 cascade via reduction through gene expression which results

in an attenuation of the inflammatory response (Engblom et al., 2002; O'Banion et al., 1992; Samad et al., 2002). With the use of adrenalectomized (ADX) male rats, Wilson et al., (2000) successfully showed that GCs are extremely important in anti-arthrithic actions on pro-inflammatory mediators (Yokoro et al., 2003). ADX animals demonstrated an increase in the frequency of paw oedema and hyperalgesia after adjuvant-induced arthritis was initiated compared to controls. Treatment with dexamethasone reversed this increase in hyperalgesia and oedema. Interestingly, celecoxib treatment (selective COX-2 inhibitor) was ineffective in inhibiting hyperalgesia and oedema (Yokoro et al., 2003).

Panayi (1992) demonstrated that treating rheumatoid arthritic (RA) patients with drugs blocking the synthesis of endogenous GCs in the adrenal cortex results in flares in disease activity (Panayi, 1992). Takasu et al. (1990) reported that patients who had undergone adrenalectomies developed RA (Takasu et al., 1990). Rodent studies show that adrenalectomy results in an earlier onset of inflammatory disease (Harbuz et al., 1999). This data demonstrate the fundamental importance of the hypothalamic-pituitary-adrenal axis (HPA axis) and the actions of corticosterone in regulating inflammation.

Hyperalgesic states are sensitive to GCs (O'Banion et al., 1992; Zhang et al., 2004). Zhang et al., (2004) investigated the effects of GCs on spinal preprodynorphin (PPD) mRNA up-regulation that occurs during peripheral inflammation induced by injecting CFA, (a behavioral pain assay that induces peripheral inflammation in the injected rat paw) into the hind paw of ADX rats (Zhang et al., 2004). PPD mRNA regulation is associated with dynorphin production; an opioid peptide that is thought to play an important role in the modulation of nociceptive neural networks at the level of the spinal cord. The ADX group displayed a more intense hyperalgesia with a reduction in the up-regulation of PPD mRNA than did control rats injected

with CFA and this intensified reaction could be significantly suppressed by a subcutaneous treatment with a synthesized GC, dexamethasone (Zhang et al., 2004). The implication of these findings is that endogenous GCs exert a powerful suppressive effect on CFA induced inflammatory hyperalgesia.

Surprisingly, Taylor et al (1998) reported that ADX (preventing activation of glucocorticoid receptors) and or high-doses of dexamethasone (saturating glucocorticoid receptors) had no effect on behavioral responses to formalin-induced inflammation (Taylor et al., 1998). This study suggests that although the pituitary-adrenocortical system was activated in the rat following administration of formalin, the resulting release of CORT does not feed back to attenuate the responses associated with inflammation and hyperalgesia. Another study using ADX animals' demonstrated a decrease in pain behavior for both Phase I and II compared to sham ADX and naive rats. Moreover, when naloxone (an opioid antagonist) was administered, no difference in pain behavior was observed in the sham ADX and naive animals, while ADX animals pain reactivity returned to levels comparable to the naive rats. These results suggest that HPA axis reduces pain using the formalin model via activation of the endogenous opioid systems (Vissers et al., 2004).

Evidence suggests that gender differences in pain evoked responses may be in part mediated by activation of the HPA axis which is associated with ACTH and CORT release (Teyler et al., 1980). Sex hormones differentially affect hippocampal electrical activity which may ultimately contribute to the HPA axis effect on final nociceptive responses (Teyler et al., 1980). For example, estradiol upregulates binding and expression of glucocorticoid receptors (GR) in the brain and GR binding in the dorsal horn (Ferrini et al., 1993a). There is also evidence that gonadal hormones can modulate adrenal CORT secretion and GR binding in

neuroendocrine tissues. For example, females have higher secretory rates of adrenal steroids and higher ACTH output than males (Ferrini et al., 1993b).

Da Silva et al., (1993) found that CORT levels were differentially affected by estradiol treatment (Da Silva and Hall, 1992). Prior to any experimental manipulations, female and males did not differ significantly in their CORT levels. However, sham operated female rats had higher CORT levels than males and GDX had opposing effects in the two genders. Specifically, CORT levels were reduced in females but significantly increased in males. In the chronic inflammatory model, OVX females with progesterone replacement comparable to normal physiological levels restores a response similar to that of intact females. However, estradiol treatments did not affect CORT levels in OVX females. Release of interleukin-1 (IL-1) (a cytokine that is secreted by macrophages, monocytes and dendritic cells) is an important part of the inflammatory response of the body. Observation of IL-1 levels followed a similar pattern whereby females release more than males in both intact and GDX mice with chronic inflammation (Kaga and Berkun, 1954). This data suggest an inter-relationship between sex steroids, inflammatory stimuli and the HPA axis that contributes to females having a greater tendency than males to generate activating signals and a greater sensitivity to these factors (Da Silva et al., 1993).

In contrast, Aloisi et al, (1996) discovered that CORT levels were not affected in either gender, although ACTH levels increased as a result of both 1 and 10% formalin administration in females (Aloisi et al., 1996). Authors suggested that the inconsistent increase in CORT levels compared to the increased ACTH may be due to sacrifice of animals too soon after formalin administration. Perhaps the biosynthetic pathway necessary to convert ACTH to CORT did not have time to culminate (Aloisi, 1997; Aloisi et al., 1996).

Finally, Alder et al., (1999) revealed that in response to hypoglycaemia (a pathological state produced by lower than normal levels of sugar (glucose) in the blood), patients with fibromyalgia had an impaired capacity to activate the HPA axis (Shiraishi et al., 2005). The HPA axis and CORT are significant mediators in nociceptive processing and are affected differentially by sex hormones (Shiraishi et al., 2005).

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

**Nitric Oxide**

Nitric oxide (NO) is an important intracellular mediator that is produced in a wide variety of cell types both peripherally and centrally. NO is generated from L-arginine after the activation of the enzyme nitric oxide synthase (NOS) by calcium. Three isoforms of NOS exist; neuronal (nNOS ), inducible NOS (iNOS), and endothelial NOS (eNOS). nNOS and eNOS are present in the spinal cord and brain and are both $Ca^{+2}$/calmodulin dependant while iNOS is functionally $Ca^{+2}$-independent and normally expressed in macrophages and inflammatory cells (Meyer et als, 06; Millan, 1999). Once formed, NO exerts its actions intracellularly at its site of origin and act as a vehicle of intracellular communication. After it diffuses to its site of action, it stimulates guanylate cyclase to produce cyclic GMP, which then causes alterations of various intracellular processes (i.e. activation of protein kinases, ion channels, phosphorilation, SP,

CGRP) and acts by activating COX enzymes and this affects PG synthesis (McMahon S.B. et als, 06).

It has been suggested that the $Ca^{+2}$/calmodulin-dependant spinal nNOS plays an important role in inflammatory hyperalgesia (Tao et al., 2004). Tao et al. (2004) demonstrated that spinal cord nNOS levels are up-regulated after peripheral inflammation (Tao et al., 2004). Mice lacking nNOS displayed normal hyperalgesia only in early phases of pain after carrageenan administration (Tao et al., 2004). Garthwaite el al. (1998) demonstrated that in the hippocampus, NO synthesis appears to be initiated by NMDA receptor-mediated increases in $Ca^{+2}$. Spinal delivery of NO synthesis inhibitors blocked hyperalgesia induced by activation of spinal NMDA receptors or by tissue injury (Malmberg and Yaksh, 1993), in turn, suggesting that NO production activates NMDA receptors in the spinal cord which stimulate $Ca^{+2}$-dependant increase in cGMP.

Intravenous administration of non-selective cNOS and iNOS (constitutive and inducible NOS) inhibitors given one hour before or after carrageenan administration, inhibits paw oedema (Salvemini et al., 1996). A selective iNOS inhibitor, N-iminoethyl-l-lysine (L-NIL), inhibited paw oedema only at 5-10 hours after carrageenan administration (Salvemini et al., 1996). This study suggests that NO produce by cNOS is involved in the development of early inflammation while, NO produced by iNOS is involved in the maintenance of inflammatory pain.. Using microdialysis, Omote et al. (2001) demonstrated that the early phase of carrageenan-induced release of NO is regulated by cNOS while nNOS and iNOS are responsible for NO release in the later phase.

Toriyabe et al. (2004) investigated the effects of NO release on central and peripheral COX expression/activation and production of PGs after carrageenan-induced inflammation

(Toriyabe et al., 2004). The carrageenan-induced inflammation increased peripheral concentrations of NO, $PGE_2$, and $PGI_2$, which all were completely inhibited by the NOS inhibitor, L-NMMA (Toriyabe et al., 2004). Administration of L-NMMA inhibited the up-regulation of COX-2 thereby affecting PG synthesis (Toriyabe et al., 2004). Administration of a COX-1 inhibitor (SC-560) and COX-2 inhibitor (NS-398) resulted in a reduction of PG concentrations, but had no effect on NO concentrations.

Surprisingly, Clancy et al. (2000) reported that exposure of resting macrophages to NO-enhanced PGE2 release was inhibited by indomethacin (non-selective COX inhibitor) while selective COX-2 inhibitor (NS-398) had no such affect (Clancy et al., 2000). This group has also reported that NO activated COX-1, but inhibited COX-2 derived PG production when using COX-deficient cell lines.

Taken together, these findings support the hypothesis that PG production is in part mediated by NO (Toriyabe et al., 2004). Although the significance of the spinal pools of NO and their mechanisms of action remain to be elucidated, it is likely that NO has an important role in spinal mediated processes of sensitization that underlie painful states.

**Sex differences in pain**

Clinical and preclinical studies demonstrate significant sex differences in the perception of chronic and persistent inflammatory pain (Fillingim, 2000; Fillingim, R. B. and Maixner, W., 2000; Maixner, W., Sigurdsson, A., Fillingim, R. B., Lundeen, T., and Booker, D., 1995). Females report lower pain thresholds, a greater ability to discriminate painful sensations, higher pain ratings, and an overall lower tolerance for pain than males. Furthermore, epidemiological pain studies have also shown that females report a greater number of chronic pain conditions,

such as headaches, chronic fatigue, fibromyagia, and irritable bowel syndrome than males (Bingefors and Isacson, 2004; Kohlmann, 2003; Schuna, 2002; Shaver, 2004). Similarly, female rats show a higher pain sensitivity in studies testing nociception with chemical, heat, and electrical nociceptive assays (Aloisi et al., 1994; Arjune and Bodnar, 1989; Gaumond et al., 2002; Kepler et al., 1991). For example, after either 2% or 10% administration of formalin, female rats demonstrated increased licking and flexing behaviors during both behavioral phases as compared with males (Aloisi et al., 1994; Gaumond et al., 2002). The complexity of the female endocrinological profile is likely to have an influence on their nociceptive response. A common hypothesis is that sex differences in the experience of pain reside in intrinsic biological differences (i.e., changes in pain responses associated with hormonal fluctuations), yet little is known of the role of endogenous ovarian hormone fluctuations and their effect on the control of inflammatory pain (Kuba and Quinones-Jenab V., 2005).

**Menstrual/estrous cycle effects on persistent inflammatory and chronic pain**

Plasma concentrations of several hormones such as estradiol and progesterone, systematically fluctuate during the menstrual/estrous cycle. The nature and timing of such variations for the menstrual cycle are shown in Figure 6. The menstrual cycle is divided into two phases. The follicular phase, including all days from the first day of menstrual bleeding to the day before ovulation, and the luteal phase, including all days from the first day of ovulation to the last day before the next menstrual period. In women, the start of the cycle begins with low levels of both progesterone and estradiol. Estradiol gradually increases during the follicular phase, peaking prior to ovulation and then slowly decreasing during the luteal phase. Progesterone levels increase post ovulation with its peak during the middle of the luteal phase.

Both of these hormones drastically decrease toward the end of the luteal phase. On average this cycle occurs every 28 to 29 days although there are several inter several inter- and intra-individual variations in the length of this cycle.

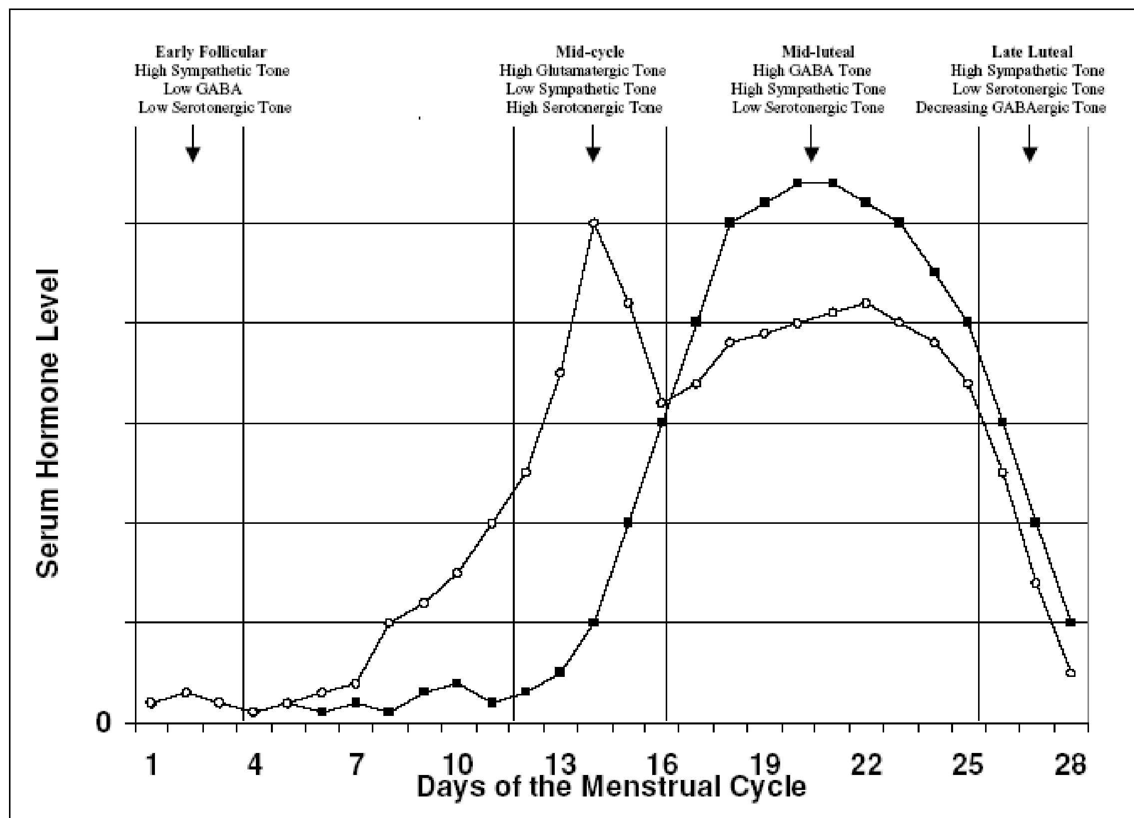



**Figure 6. Activity of different neurotransmitter systems during each phase of menstrual cycle. Increased activity is relative to the activity experienced during other phases of the menstrual cycle. Serum levels of estrogen (-o-) and progesterone (-■-) are displayed in the graph (Martin and Behbehani, 2006).**

Contrastingly, female rodents have an estrous cycle of 4 days. As shown in Figure 7, estradiol and progesterone levels are at their peak during the proestrus phase then decline during early estrus. Estradiol levels tend to stay low in comparison to the second peak of progesterone that occurs during late metestrus into early diestrus.

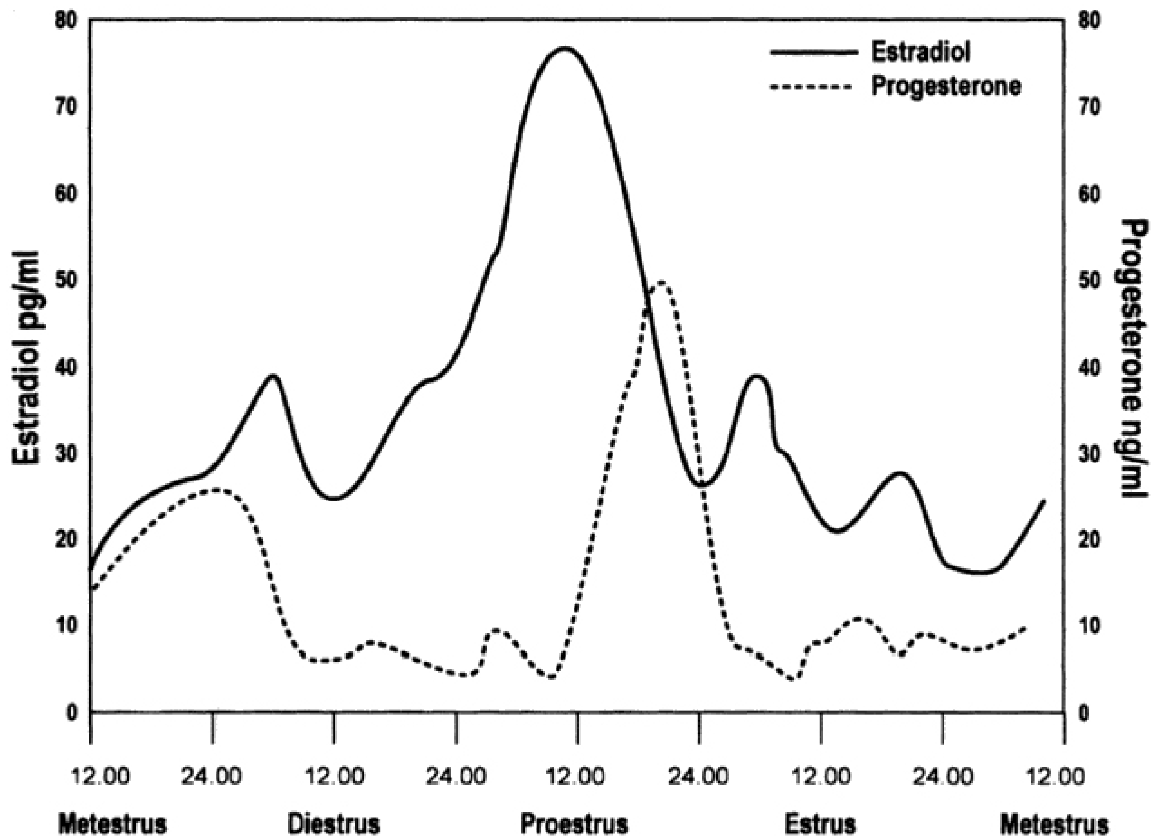



**Figure 7. Depiction of the changes in ovarian hormones during different stages of the rat estrous cycle. Note that serum levels of estradiol (solid line) peaking during the mid- to late morning of the proestrus stage and fall during the afternoon and evening. There are moderate elevations in serum levels of estradiol and progesterone (dashed line) during diestrus stages. 0 hours represents 12:00 AM (Martin and Behbehani, 2006).**

We have used the estrous cycle as a measure of endogenous hormonal fluctuations and their effects on inflammation, which may be a reason why our results are not always consistent will previous studies using OVX and hormone treatments. In rats, during the 4 day estrous cycle, the secretion rate of estrogen into ovarian venous plasma, is lowest on estrus, begins to rise significantly by late metestrus through the morning of diestrus, and reaches its peak concentrations by the afternoon of proestrus (Knobil, E and Neill, J. D., 1988). At its highest peak in proestrus, concentrations of estrogen in peripheral plasma reach 42 pg/ml (Knobil and Neill, 1988). At its lowest concentrations in estrus, they are about at 5pg/ml (Knobil and Neill, 1988). There are two peaks of progesterone secretion into ovarian veinis blood during the cycle.

The first peak occurs during the afternoon of metestrus and the second peak occurs during the late afternoon of proestrus (Knobil and Neill, 1988). At its highest peak in proestrus, concentrations of progesterone in peripheral plasma reach 58pg/ml. (Knobil and Neill, 1988). At its lowest concentrations in estrus, they are about at 2pg/ml (Minami, 2001).

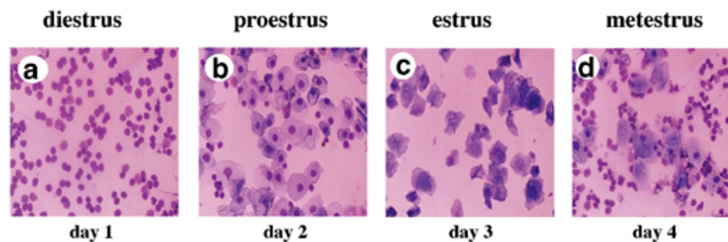



**Figure 8. Cells present in vaginal smears in the four different phases of the estrous cycle; diestrus (a) proestrus (b) estrus (c) and metestrus (d) are shown. The approximate days required to progress through each phase is indicated at the bottom of each panel. (Richard V Mettus and Sushil G Rane, 2003).**

The phases of estrus in rats can be determined by examination of cells obtained from vaginal smears by light microscopy after staining. As shown in Figure 8., during diestrus, leukocytes are the predominant cell population observed while during proestrus, both leukocytes and well-formed nucleated epithelial cells appear in approximately equal numbers. The period of estrus is indicated by the predominance of large, cornified (without nuclei) squamous-type epithelial cells, whereas metestrus is represented by approximately equal numbers of large cornified epithelial cells and leukocytes.

Clinical studies demonstrate the fluctuations of both physiological and emotional symptoms associated with pain throughout the menstrual cycle. The Riley et al. (1999) meta-analytic review of pain found similarly higher pain thresholds and tolerance during the follicular phase and enhanced pain sensitivity during the luteal phase after experimentally induced pain with one exception, pain associated with electrical stimulation tending to show the opposite (Riley et al., 1999). LeResche et al. (2003) investigated the effects of hormonal fluctuations

during the menstrual cycle on temporomandibular (TMD) pain and found that TMD pain was highest at times of low estrogen with a secondary peak around the time of ovulation (LeResche et al., 2003). Women with interstitial cystitis show increased sensitivity to bladder pain sensation during the perimenstrual period of the cycle when there is a rapid change in hormone levels (Powell-Boone et al., 2005). Hellstrom and Anderberg (2003) showed increased pain perception in women during the menstrual and luteal phases in women with chronic low pack pain (Hellstrom and Anderberg, 2003). In contrast, muscosckeletal pain has been shown to increase in the follicular and luteal phases (Isselee et al., 2002). These results are contrasting but it has been argued these discrepancies are a result of the different modalities in use for each experiment (Fillingim et al., 1997).

Rodent studies have also demonstrated that the estrous cycle can affect an animal's response to pain. The same inconsistencies occur in the way that the estrous cycle affects behavioral responses to inflammatory stimuli. No estrous cycle effects were observed after unilateral hindpaw injections of CFA (Ren et al., 2000). However, another study found higher hyperalgesic responses during proestrus when compared to all other phases (Bradshaw et al., 2000). In a study using the artificial ureteral calculosis model, female rodents in the proestrus and estrus cycle day had a decrease in painful episodes when compared to females in metestrus and diestrus (Giamberardino et al., 1997). Similarly, after a hindpaw injection of carrageenan, a decrease in hyperalgesia was observed in the proestrus group when compared to the estrus and diestrus groups (Tall and Crisp, 2004b). A temporomandibular joint (TMJ) model of pain, where formalin is administered by a TMJ injection, found that females in proestrus show a decrease in pain behavior when compared to females in diestrus. Contrary to these finding, no estrous cycle

effects were observed after either 1% or 5% administration of formalin into the hind paw (Kim et al., 1999; Vincler et al., 2001).

More recently Vierck et al. (2008) investigated the influence of sex and ovarian hormones on formalin-induced TMJ nociception in rats (Vierck et al., 2008). They found that the nociceptive behavior in females during proestrus was similar to that of the male rats. Furthermore, females in the diestrus phase showed behavior similar to OVX rats, both significantly greater that the females in the proestrus phase (Vierck et al., 2008). Taken together, sex differences and menstrual/estrous cycle effects strongly suggest a significant role for the female endocrinological profile in a female's behavioral response to pain.

**Hormonal fluctuations across the female endocrinological profile**

Inflammatory pain disorders increase with aging and coincide with a marked increase in both hyperalgesia and allodynia (Gagliese and Melzack, 1997; Helme et al., 1989). The physiological mechanisms underlying persistent nociceptive disorders during reproductive life events (e.g., menarche, menstrual cycles, pregnancy, perimenopause, and menopause) are poorly understood. Menarche refers to the onset of menstruation during puberty, when the cycles are often anovulatory. Serum estradiol levels range from 10 to 156pg/mL, and do not reach levels similar to the adult menstrual cycle for several years (Martin and Behbehani, 2006b). During the adult menstrual cycle serum estradiol levels range from 25 to 400 pg/mL peaking just prior to ovulation and fall just prior to menstruation (Martin and Behbehani, 2006b). Progesterone levels range from <2 to 10 pg/mL peaking during the mid luteal phase. (Martin and Behbehani, 2006b). Perimenopause occurs 2-8 years prior to menopause. Santoro et al. found higher levels of urinary estrogen metabolites and lower levels of progesterone metabolites in perimenopausal

women when compared to younger women (Santoro et al., 1996). Menopause is defined as the "permanent cessation of menstruation determined after 12 consecutive months of amenorrhea without pathological or physiological cause" by the World Health Organization. Serum levels of estradiol drop to a range of 10-20 pg/mL (Martin and Behbehani, 2006b). The varying levels of hormones throughout these reproductive life events affect the perception of pain.

These hormonal fluctuations may be responsible for some of the variations and changes in responses to pain throughout life. For example, the incidence of chronic inflammatory conditions such as polymyalgia rheumatica (PMR) and temporal arthritis (TA) is five times more likely in people ages 70-79 than people ages 50-59 with women being affected 1.5 times more frequently than men (Imrich et al., 2006). Natural aging has been accompanied by reductions in various hormonal levels associated with the increase of pro-inflammatory cytokines, such as TNF and IL-6 (Straub et al., 1998). The onset of these diseases occur at ages >50 suggesting that processes that occur as a result of aging may share in the pathogenesis of these diseases. Studies reveal that women are affected by migrane three times more than men, and they appear to be modulated by changes in ovarian hormones throughout the female life span (Martin and Behbehani, 2006a). The onset of migraines often occurs during menarche, improving during pregnancy which is marked by high levels of estrogen and progesterone, and tend to remit after menopause (Lipton et al., 2001; Sances et al., 2003). Decreasing serum estradiol levels during the perimenstrual time trigger migraine headaches, while higher levels during pregnancy and hormone replacement therapy are preventative (Martin and Behbehani, 2006b). Preliminary evidence suggesst that the increase in progesterone levels during the mid luteal phase, can also be preventative for migraines when compared to other times of the cycle (Martin and Behbehani, 2006b).

**Age studies with rodents**

Most of the studies on age differences in antinociceptive responsivity and pain inhibition have focused on the opioid system and the response to opioid agonists and antagonists. Studies have shown that there is a general decline in this system with age, because of the decrease in the concentration of opioid receptors in several areas of the brain (Gagliese and Melzack, 1997) although tests of age differences in antinocicption produced by opioids agonists have inconsistent results (Gagliese and Melzack, 1997). These results suggest that increases in age may be associated with a decrease in sensitivity to opioid agonists.

The data that is available for rodents are mostly based on tests using phasic or brief, transient pain and to date, widely accepted criteria for age classification are not available (Gagliese and Melzack, 2000). The most commonly used animal model is the tail-flick test that involves exposure of the tail to a heat source and measures simple reflexive behavior, or nociceptive sensitivity (Dubner, 1994). Results found using this particular test have been inconsistent, several studies found no age differences with the tail flick test (Girardot and Halloway, 1985; Hamm and Knisely, 1986; Hamm, Knisely and Watson, 1986; Crispt et al., 1994; Ghirardi, 1994; Goicoechea et al, 1997). Goettl et al. (2000) and others found age differences in the tail flick test but only at high intensity with aged animals showing a longer tail-flick latency, while other have found decreases in latency with age (Goettl et al. 2000, Islam et al, 1993, Crisp et al., 1994). The exact reasons for these inconsistencies remain unclear, although it has been suggested that the methodological differences may account for the differences.

Other pain assays have been used to test organized unlearned behavior such as the hot-plate test which measures vocalization and the jump-flinch test. Typically what is observed in

these tests is an increase in the intensity or duration of the stimulation required to elicit a response increases with age (Hess and Roth, 1981; Nicak, 1971; Lippa et al, 1980; Gordon et al., 1978; Giuliani et al 1994). A variable that must be looked at in these tests is the ability to perform behaviors, and in these studies the performance of the older rats may be impaired by a decrease in muscle strength and coordination and an increase in weight in relation to the younger rats (Cambell, et al. 1980; Atlun et al 2007). More recently, Finkel et al. (2006) used mice to investigate age differences in current vocalization in a novel nociceptive assay. They found that current vocalization to stimuli changes over the lifespan in a curvliniear fashion, changing in a U-shaped pattern over the lifespan (Finkel et al, 2006).

There are only a few studies to date that have looked at age effects of behavioral models of inflammatory induced pain. Gagliese and Melzack (1999) investigated the relationship between age and pain behaviors after injection of formalin. Their model of tonic tissue injury and inflammation shows that behavioral responses to formalin increase though early adulthood (>3 months), peaking at mid life (18 months), and then decrease thereafter (24 months) (Gagliese and Melzack, 1999). The scores of the young (3 months) and old (24 months) groups do not differ from one another, suggesting that there may be an age-associated change in the sensitivity to tonic pain, with a peak sensitivity in mid life, supporting the idea of the U-shaped pattern mentioned above.

There have been no studies done to date investigating the relationship between age, hormonal levels, and responses to inflammatory induced pain. In the current study, we begin to explore the possibility of hormone interactions in several inflammatory pain pathways.

**Effects of estrogen and progesterone on inflammatory mediators**

Little research has looked at the relationship between endogenous hormonal fluctuations and their effects on inflammatory mediators such as COX-2, NO, PGE2, PGD2, and CORT. This thesis focuses on these central processes and how they are affected by normally fluctuating hormones throughout the female endocrinological profile. Multon et al., (2005) used aromatase-knockout (ArKO) mice lacking endogenous estradiol production to demonstrate that lack of estradiol increases pain in the trigeminal formalin model (Multon et al., 2005). The estradiol-treated knockout mice displayed significantly less grooming behavior than the saline group during both the interphase and Phase II. Surprisingly, there was only a partial reversal of the immunhistochemical abnormalities associated with lack of endogenous estradiol with exogenous estradiol administration (Multon et al., 2005). These findings suggest that estradiol deprivation may contribute to some permanent alterations in the ArKo mice. Khaser et al., (2001) demonstrated that estradiol administration reversed suppressed nociceptive behavior in Phase II of the formalin test that usually accompanies GSX plus vagotomy. Interestingly, estradiol implants had no effect on nociception in gonadally intact females (Khaser et al., 2001). More recently, Li et al., (2009) used estrogen receptor knock out mice to study the responses to carrageenan-induced inflammation. They found that in normal wild-type mice, females had significantly lower paw withdrawal thresholds than males (Li et al., 2009). However, significantly elevated response threshold was observed in knock out female mice, which eliminated sex differences in nociception. These results suggest that sex differences in pain threshold may be eliminated in mice lacking either the estrogen alpha or beta receptors.

Various studies have shown that exogenous estrogen increases COX and PGE2 levels. For example, estradiol alters $PGE_2$ synthesis in non-CNS tissue via a decrease in $PGE_2$ synthesis

in bovine endometrium (Mann, 2001; Scaramuzzi et al., 1977). Mitsutoshi et al. (2004) demonstrated that estradiol (E2) treatments resulted in a significant increase in COX-2 mRNA levels in primary human uterine microvascular endothelial cells HUMEC (Tamura et al., 2004). A time-dependent increase of COX-2 mRNA levels was observed and administration of estrogen antagonist ICI 182,780 fully reversed the effects of E2 on COX mRNA levels in 1hr and protein levels in 4 hrs (Tamura et al., 2004). Moreover, the use of a COX-2 inhibitor (NS-398) before treatment with estrogen completely abolished the induction of PGE2 levels by E2. More recently, Cuzzocrea et al., (2007) found that the administration of an estrogen receptor antagonist (ICI 182,780) reverses the anti-inflammatory effect of dexamethasone, a synthetic GC in OVX rats after carrageenan-induced lung inflammation (Cuaaocrea 2007). They also showed that administration of ICI 182, 780 significantly inhibited the ability of dexamethasone to reduce iNOS and COX espression. These findings suggest that the presence of estrogen is positively correlated to increases in COX-2 mRNA levels.

Evidence suggests that the gender differences in pain responses may be in part mediated by the activation of the HPA axis during inflammation. It has been shown that CORT release varies depending on gender, suggesting gender differences in pain may be a result of differences in this pathway (Teyler et al., 1980). Sex hormones differentially affect the hippocampal electrical activity which may ultimately contribute to the HPA axis effect on final nociceptive responses. Ferrini et al., (1993) reported that estrogen upregulates the binding and the expression of GC receptors in the brain and in the dorsal horn (Ferrini et al., 1993a). Evidence suggests that gonadal hormones modulate CORT secretion and receptor binding with females having greater rates of adrenal steroid secretion in addition to higher ACTH output than that seen in males (Ferrini et al., 1993b). Da Silva et al. (1993) found that CORT levels were

differentially affected by sex (Da Silva et al., 1993). Prior to any experimental manipulations, the female and male mice did not differ significantly in their CORT levels. However, CORT levels were higher in the sham operated females than in the males, while GDX had opposite results in the two genders. CORT levels were reduced in females and significantly increased in the males. More recently, Mannino et al. (2006) demonstrated that formalin-induced increases in serum CORT are attenuated in OVX control rats when compared with OVX rats treated with 20% 17β-estradiol (Mannino et al., 2006).

On the contrary, Aloisi et al, (1996) revealed that ACTH levels increases after formalin administration in females, while CORT levels were not affected in either gender (Aloisi et al., 1996) It was suggested that the inconsistent findings may be in part due to the timing of sacrifice being to soon after formalin administration. The conversion of ACTH to CORT along this biosynthetic pathway may take longer than this expreriment allowed (Aloisi et al., 1996; Hope BT et al., 2007).

Using in vitro studies, Mohn et al., (2005) showed that $PGE_2$ increases CORT release by adrenal gland. Others have shown that PGs directly stimulate corticosteroidogenesis in adrenocortical tissues and cells (Kudo et al., 1991; Nishi et al., 1999). Moreover, findings in our lab revealed that chronic estradiol significantly elevated CORT serum levels while COX-2 protein levels were 25% lower in estradiol-treated rats not receiving formalin compared to vehicle naïve rats (Kuba et al., 2005). In chronic estradiol-treated animals, a positive correlation between $PGE_2$ and CORT was found while a negative correlation between $PGE_2$ and COX-1 proteins levels was observed. Acute estradiol-treated animals displayed significantly elevated CORT but there were no changes in COX-1 or COX-2 levels. However, $PGE_2$ serum levels were higher in 5% formalin than naïve 1% treated rats. From these observations and previous

studies, it was suggested that CORT release may be influenced by inflammatory stimuli which potentiates PG release, further increasing corticosterone levels. The administration of exogenous estradiol may alter this system by changing levels of $PGE_2$ and/or CORT release thereby mediating nociceptive responses to formalin. Kuba et al., (2005) suggested that there is a bi-directional relationship between CORT and $PGE_2$ such that an inflammatory stimulus that regulates CORT release and subsequent increases in $PGE_2$ also causes increases CORT release. Taken together these results suggest a relationship between estrogen and corticosterone release and activity. These varying hormonal levels appear to affect HPA axis functioning, which make the HPA axis and CORT ideal targets to examine in the current study to identify biochemical pathways in which fluctuating hormones may be working to attenuate induced-inflammatory nociceptive responding.

**Proposed Model**

We postulate that peaks of endogenous estrogen peaks will mediate inflammation induced pain through down regulation of COX-2 protein levels and enzyme activity either directly or through NO, which in turn will negatively regulate PG levels resulting in a decreased behavioral response to pain. It is also hypothesized that estrogen will increase corticosterone levels which have been shown to negatively regulate PG levels. This potentially combined effect could account for the reduction in the behavioral responses during times when estrogen levels are at their highest. Furthermore, down regulation of COX-2 enzyme by estrogen will decrease PG serum levels in rats treated with an inflammatory stimulus. The treatment will result in an attenuation of behavioral responses to persistent pain that will closely parallel levels of prostaglandin. We postulate that during times when progesterone levels are peaking, this whole

estrogen cascade will be inhibited by the presence of progesterone. Findings from preliminary experiments show correlations suggesting that COX-2, PGs and CORT may play a major role in the analgesic effect of circulating gonadal hormones.

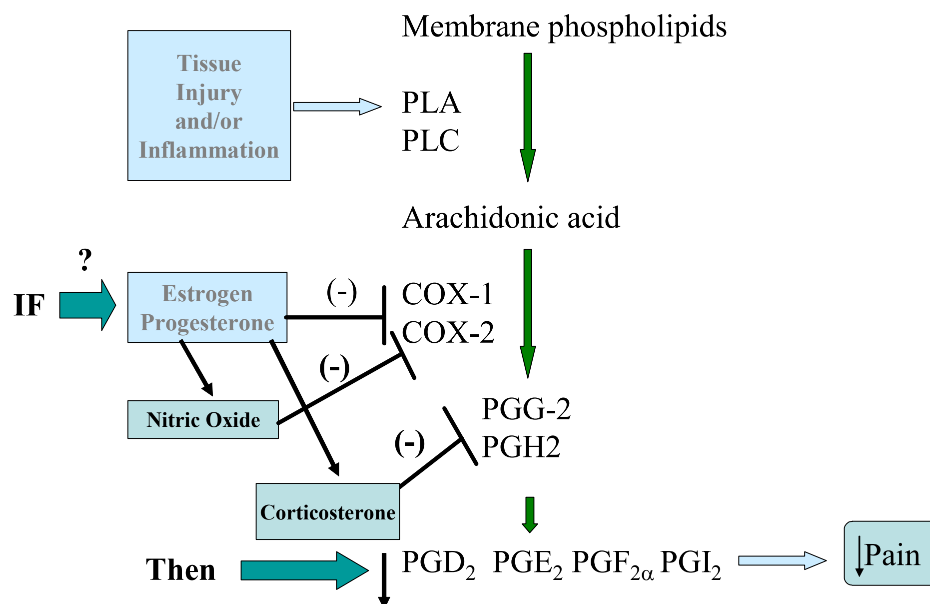



**Figure 9. The proposed model suggests that peaks in endogenous estrogen negatively regulate COX-2 and subsequent prostanoid production. Furthermore, estrogen will mediate inflammation induced pain through down regulation of COX-2 protein levels and enzyme activity either directly or through Nitric Oxide, which in turn will negatively regulate PG levels resulting in a decreased behavioral response to pain. It is also hypothesized that estrogen will increase corticosterone levels which have been shown to negatively regulate PG levels.**

**Specific Aims:**

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

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

The specific aim of this project is to determine how endogenous gonadal hormonal fluctuations alter inflammatory pain responses. We hypothesize that fluctuations of estrogen and progesterone in the female reproductive cycle will affect inflammatory responses. Specifically,

in stages where estrogen and progesterone levels peak, we predict to see an attenuation of inflammatory-induced behavioral pain responses. In stages where estrogen and progesterone levels drop, we predict that behavioral responses to inflammatory pain will be increased. Secondly, we hypothesize that intracellular steps associated with inflammation (CORT, $PGE_2$, $PGD_2$, NO, and COX pathways) will also be regulated by estrogen and progesterone fluctuations. Similar to inflammatory-induced behavioral pain responses, when estrogen and progesterone levels peak, physiological responses to inflammation will be reduced, but when estrogen and progesterone levels drop physiological responses to inflammation will increase. Specifically, through which biochemical pathway(s) estrogen and progesterone operate? Our hypothesis is that *estrogen and progesterone's analgesic effects on inflammation-induced pain are in part mediated through corticosterone and NO/ COX-2 regulation of the prostanoid biosynthetic pathway.*

**Specific Aim 1: To determine if endogenous gonadal hormone fluctuations occurring during the female estrous cycle alter formalin-induced inflammatory responses to pain.** To test this hypothesis, two experiments will determine the effect of circulating gonadal hormones on behavioral and physiological responses to pain. **1A.** To test the behavioral effects of estrous cycle stage on formalin-induced inflammation, one experiment was designed using intact adult rats. After determination of cycle stage (proestrus, estrus, metestrus, diestrus), behavioral responses to formalin-induced inflammation will be assessed. **1B.** Measure prostaglandin serum levels, nNOS and COX-2 levels in the four estrous cycle stages after formalin administration. We predict that during times when endogenous estrogen and progesterone peaks (proestrus stage), we will see an increase in nNOS levels which in turn may down-regulate COX-

2 protein levels and enzyme activity resulting in attenuated behavioral responding and reduced $PGE_2$ levels. Moreover, we predict that high levels of estrogen and progesterone will reduce behavioral responses and $PGE_2$ serum levels, but not $PGD_2$, serum levels.

**Specific Aim 2**: **To determine if the endogenous gonadal hormone fluctuations effects can be extended to different forms of inflammatory induced pain, (i.e. carrageenan-inflammatory model) and if so, are estrogen and progesterone's anti-hyperalgesic effects on behavioral responses to this inflammatory stimuli in part mediated through deactivation/down regulation of COX-2.** To this end, two experiments were designed: **1A.** Measure carrageenan-induced inflammatory behavioral responses in intact female rats after determination of cycle stage (proestrus, estrus, metestrus, diestrus). **1B.** Measure prostaglandin and nNOS levels in the four estrous cycle stages after formalin administration. We predict that during times when endogenous estrogen and progesterone peaks (proestrus stage), we will see an increase in nNOS levels which in turn may down-regulate COX-2 protein levels and enzyme activity resulting in attenuated behavioral responding and reduced $PGE_2$ levels. Moreover, we predict that high levels of estrogen and progesterone will reduce behavioral responses and $PGE_2$ serum levels, but not $PGD_2$, serum levels.

**Specific Aim 3: To determine if endogenous gonadal hormone fluctuations throughout life alter formalin-induced inflammatory responses to pain. 3A.** To test the behavioral effects of endogenous hormonal fluctuations throughout life on formalin-induced inflammation one experiment was designed using intact female rats. To this end, responses to formalin-induced inflammation in rats of varying ages (peri-adolescent, adolescent, adult, and aged) will be

assessed. **3B.** Measure prostaglandin serum levels in the four age groups after formalin administration. We predict during ages when endogenous estrogen and progesterone peak, COX-2 protein levels and enzyme activity will be down regulated, resulting in attenuated behavioral responding a reduced $PGE_2$ levels. Moreover, we predict that at rats at ages having higher levels of estrogen and progesterone will reduce behavioral responses and $PGE_2$ serum levels, but not $PGD_2$, serum levels.

**Specific Aim 4: To determine if endogenous estrogen and progesterone's effects on behavioral responses to inflammatory stimuli are in part mediated through the induction of corticosterone release.** To this end three experiments were designed: **4A**. Following formalin administration, serum levels of corticosterone will be measured in intact female rats after determination of cycle stage (proestrus, estrus, metestrus, diestrus). 4**B.** Following carrageenan administration, serum levels of corticosterone will be measured in intact female rats after determination of cycle stage (proestrus, estrus, metestrus, diestrus). **4C.** Following formalin administration, serum levels of corticosterone will be measured in intact female rats of varying ages (peri-adolescent, adolescent, adult, and aged). We predict that corticosterone serum levels will fluctuate according to the levels of the endogenous gonadal hormones, thereby having an affect on various pain mediators. To this end, we predict that during times of high estrogen and progesterone, corticosterone serum levels will be at their highest. producing anti-hyperalgesic effects after formalin and carrageenan administration

**Methods:**

***Animals***

Three weeks to eleven month old intact female Sprague-Dawley rats purchased from Taconic (Germantown, NY) were double-housed in a Plexiglas chamber (20 x 20 x 41) layered with beta chips in a 12-h light-dark cycle (lights on 8 AM) with food and water adlibithum. Animals were randomly assigned to experimental groups (*n* = 16 to 20 per group). Each study consisted of at least three different cohorts of rats. Animals were not reused for any study. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, Bethesda, MD and experiments were approved by the Institutional Animal Care and Use Committee at Hunter College of The City University of New York). For the age studies, ages of the rats were divided into peri-adolescent (3 weeks), adolescent (6 weeks), adult, (8 weeks) and aged (10-11 months). At the time of nociceptive testing, rats weighed between 100g and 400g.

***Estrous Cycle Stage Determination***

After a two-day acclimation period, three cohorts of 8-week old female rats were weighed, handled, and lavaged daily for two weeks prior to experimentation. At the time of nociceptive testing, rats weighed between 200g and 240g. Female rats (n=8-14/group) were assigned to the one of the four phases of the estrous cycle based on the vaginal smears taken on the day of testing. Estrous cycle stage was monitored by analysis of cell types in vaginal lavages. Vaginal lavages were collected 30 minutes after the lights went on and were allowed to dry on microscope slides. Slides were then fixed with ethanol and stained with cresyl violet acetate (Sigma, St. Louis, MO). Cells were classified into four indentifiable stages: (proestrus

(predominance of mature epithelial cells), estrus (fully cornified epithelial cells), metestrus (cornified cells with few leukocytes), and diestrus (predominance of leukocytes with a small number of epithelial cells) [Freeman 94]. Animals that could not be readily staged, or that did not produce two consecutive cycles, were not included in any statistical analysis.

For the age studies, ages of the rats were divided into peri-adolescent (3 weeks), adolescent (6 weeks), adult, (8 weeks) and aged (10-11 months). At the time of nociceptive testing, rats weighed between 100g and 400g.

***Formalin apparatus***

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

***Formalin assay***

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

***Carrageenan Apparatus***

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

***Carrageenan assay***

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

***Corticosterone and prostaglandin measurements***

After behavioral testing, rats were sacrificed by decapitation following a brief exposure (20seconds) to $CO_2$. Trunk blood was collected in tubes containing $K_2EDTA$. Blood was

centrifuged at 3,000 RPM for 30 min at 4 ºC. Serum was collected and then stored at -80 ºC until used. Serum levels of corticosterone were detected using Coat-A-Count radioimmunoassay kits (Diagnostic Products Corporation, Los Angelos, CA). Intra-assay coefficients of variation averaged 10.0% ± 1.0%. $PGE_2$ and $PGD_2$ serum levels were detected by using enzyme immunoassay kits from Cayman Chemical (Ann Arbor, MI). Results for these assays were determined via a log-logit analysis within Graph Pad Prism Software (San Diego, CA). Prostaglandin serum levels were expressed as pg/mL. Corticosterone serum levels were expressed as ng/mL.

***Western Blots***

After decapitation, the lumbo-sacral region of the spinal cords were rapidly dissected and stored at -80°C until use. The tissue was then homogenized using a Polytron homogenizer (Kinematica, Luzern, Switzerland) in lysis buffer (50 mM Tris-HCL (pH 7.5), 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1% ipegal, 1% sodium deoxycholate) containing a mixture of protease inhibitors (pepstatin, leupeptin, 1M DTT, apoprotinin, 100 mM PMSF, 50 mM NaF and 1 mM $Na_3VO_4$). Protein concentration was then determined using a Bradford kit (Bio-Rad Laboratories, Hercules, CA). For Western Blot analysis, equal amounts of protein (20-50 μg) were boiled in Laemmli buffer (Bio-Rad Laboratories, Hercules, CA) containing 5 % β-mercaptoethanol for 5 min, then loaded onto SDS-PAGE and electrophoresed. Protein in the gels were then transferred onto nitrocellulose membranes overnight at 4° C. Membranes were then rinsed with trizma-buffered saline with 10% tween (TBS-T) and blocked with 5 % non-fat dry milk in TBS-T at room temperature for 60 min. Membranes were then washed three times at 5 min/wash and once at 10 min and probed with specific primary antibodies. Membranes were incubated with either COX-2 or nNOS antibodies (1:500; purchased from Cell Signaling

Technology, Danvers, MA) overnight at 4°C. After washing in TBST, membranes were incubated with appropriate secondary antibodies for 1 hour at room temperature. Membranes were washed as previously described and incubated with their appropriate secondary antibody for 60 min, and washed once more as previously described. Membranes were washed a final time in TBS-T for 10 min to reduce the possibility of background, then treated with an enhanced chemiluminescence kit (ECL; Amersham Pharmacia, Piscataway, NJ) to detect antibody binding. Membranes were then exposed to film (Kodak) and the resulting films were scanned and quantified with a computer-densitometer and Image Quant Program (Molecular Dynamics). All membranes were re-probed with α-tubulin (1:1000) for 60 min to assess normalized protein levels. The ratio of phosphorylated or total proteins over α-tubulin levels was used to calculate the protein levels.

**Statistical analysis**

Behavioral data was analyzed as the mean number of flinches (+/- SEM) during Phase I (0-5 minute), the Interphase (6-19 minutes), and Phase II (20-40 minutes). Repeated measures, one or two way, and factorial analysis of variance were used to determine significant differences in behavioral and neurochemical measurements. To determine the significant differences in serum levels of corticosterone or prostaglandin E2 and D2, one way analysis of variance were used. Western blot data were presented as a ratio of specific protein levels to α-tubulin or IgG levels as arbitrary densitometric units. Unpaired-samples t-test or one way analysis of variance was used. For post hoc analysis, Fischer LSD tests were conducted when applicable. For all analyses, significance was at the level of $p < 0.05$. Fishers's least significant difference post hoc testing was done when appropriate.

# CHAPTER 2

Are endogenous gonadal hormonal fluctuations occurring during the female estrous cycle altering formalin induced inflammatory responses to pain?

## Results

### *Behavioral effect of cycle stage on formalin induced inflammation*

As shown in Figure 10, the effects of estrous cycle on flinching responses are shown in three minute bouts across time. The mean for proestrus rats during bouts 11-16 was 75.05 when compared to those in metestrus with a mean of 99.51. The increasing differences in these means are apparent across time.

Although the estrous cycle did not alter behavioral responses during Phase I or the Interphase, a significant interaction between the stage of the estrous cycle and formalin responses was seen in Phase II [$F\ (2,86)=363.46$; $p=.0001$]. As shown in Figure 11, a significant phase effect was observed ($p<0.01$). A significant phase x group interaction was observed in Phase II [$F\ (2,86)=2.741$; $p<0.05$]. Animals in proestrus have significantly lower number of flinching responses when compared to the metestrus phase ($p<0.01$).

### *Effects estrous cycle stage on $PGE_2$ and $PGD_2$ serum levels after formalin administration.*

Shown in Figure 12A, a significant main effect of estrous stage on $PGE_2$ levels was observed [$F\ (3,27)=3.163$; $p=0.041$]; rats in estrus had significantly lower $PGE_2$ serum levels when compared to rats in metestrus or diestrus ($p<0.05$) for all comparisons. As shown in Figure 12B, a significant effect of estrous stage in $PGD_2$ serum levels was also observed [F (3, 14)

=5.58; p=.009]; rats in proestrus had significantly higher $PGD_2$ serum levels when compared to all other groups ($p<0.01$ for all comparisons).

***Effects estrous cycle stage on nNOS levels in the spinal cord after formalin administration.***

Although estrous cycle stage did not have an significant effect on nNOS levels after formalin, we observed the greatest means of nNOS levels in the spinal cord during metestrus. Animals in metestrus had 6.70 as mean level of nNOS when compared to proestrus (0.942) and estrus (0.689). As shown in Figure 13, we did not detect significant changes in nNos in the spinal cord after formalin administration [$F(7, 24)=9.65$; $p=0.005$]. No significant effects of estrous cycle were seen both the formalin treated and the naïve rats.

***Effects estrous cycle stage on COX-2 levels in the spinal cord after formalin administration.***

As shown in Figure 14, COX-2 levels in the spinal cord did not change as a result of estrous cycle after formalin administration [$F(3, 12)=36.70$; $p=0.000$]. Although, animals in proestrus had higher mean level of COX-2 protein (0.363) than estrus (0.212), metestrus (0.214) and diestrus (0.226), it failed to reach significance.

***Correlations of estrous cycle rats after formalin administration; Phase III behavior, CORT, $PGE_2$, $PGD_2$, COX-2, and nNOS.***

All correlations for formalin treated rats in each stage of the cycle are shown in Table 2.

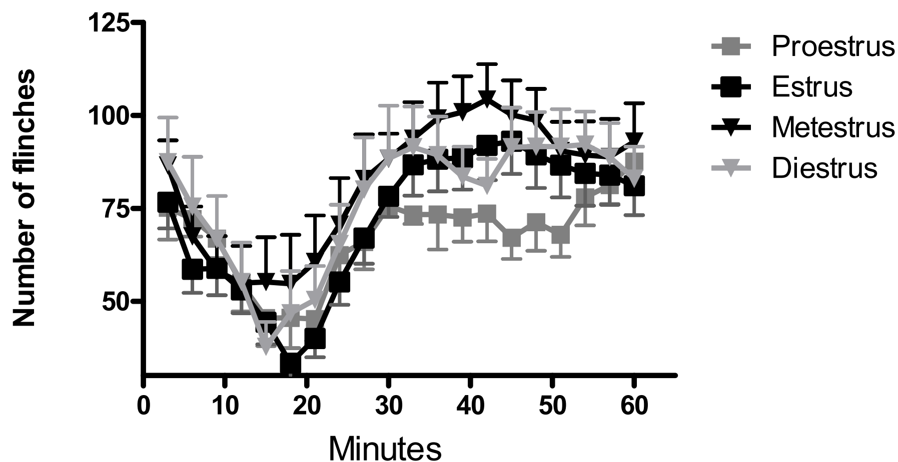



**Figure 10. Time course of flinching responses in all estrous stages after (5%) formalin administration.** Time course of activation is represented as the mean of flinching responses in 3 minute bins (N=10-12/group).

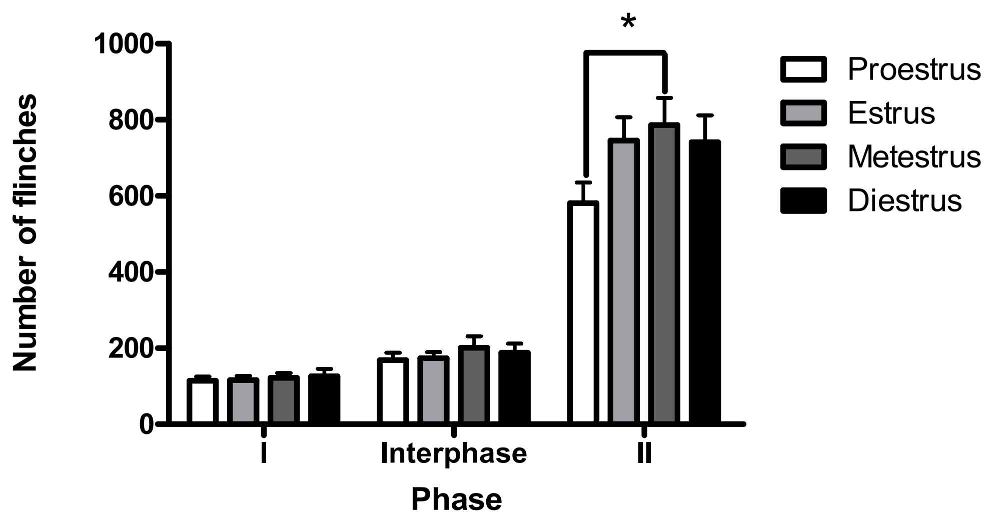
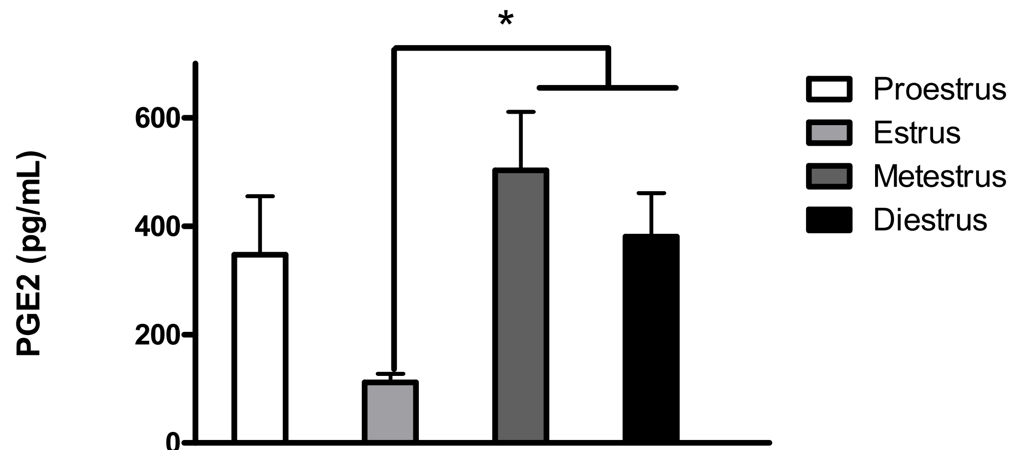



**Figure 11. Effects of estrous cycle stage on behavioral flinching responses after 5% formalin administration.** Data represents the cumulative mean of flinches (+/-SEM) in Phase I (0-5 min), Interphase (6-19 min) and Phase II (10-50 min) in animals during each stage of the estrous cycle. (*) Represents a significant group and phase effect. (N=10-12/group).

A.




B.

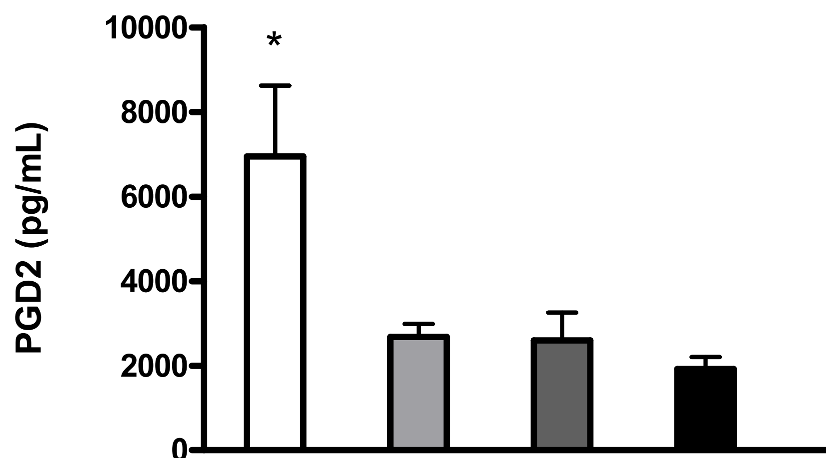



**Figure 12 A-B. Effects of estrous cycle stage on $PGE_2$ (A) and $PGD_2$ (B) serum levels after formalin administration** **A.** Data represents mean prostaglandin $E_2$ serum levels (± SEM) at picograms per milliter sixty minutes after formalin administration (N = 4-6/group). (*) denotes significantly lower $PGE_2$ serum levels in the estrous group when compared to the metestrus and diestrus groups **B.** Data represents mean prostaglandin $D_2$ serum levels (± SEM) after formalin administration (N= 4-6/group). (*) denotes significantly higher $PGD_2$ serum levels in the proestrus group when compared to all other groups.

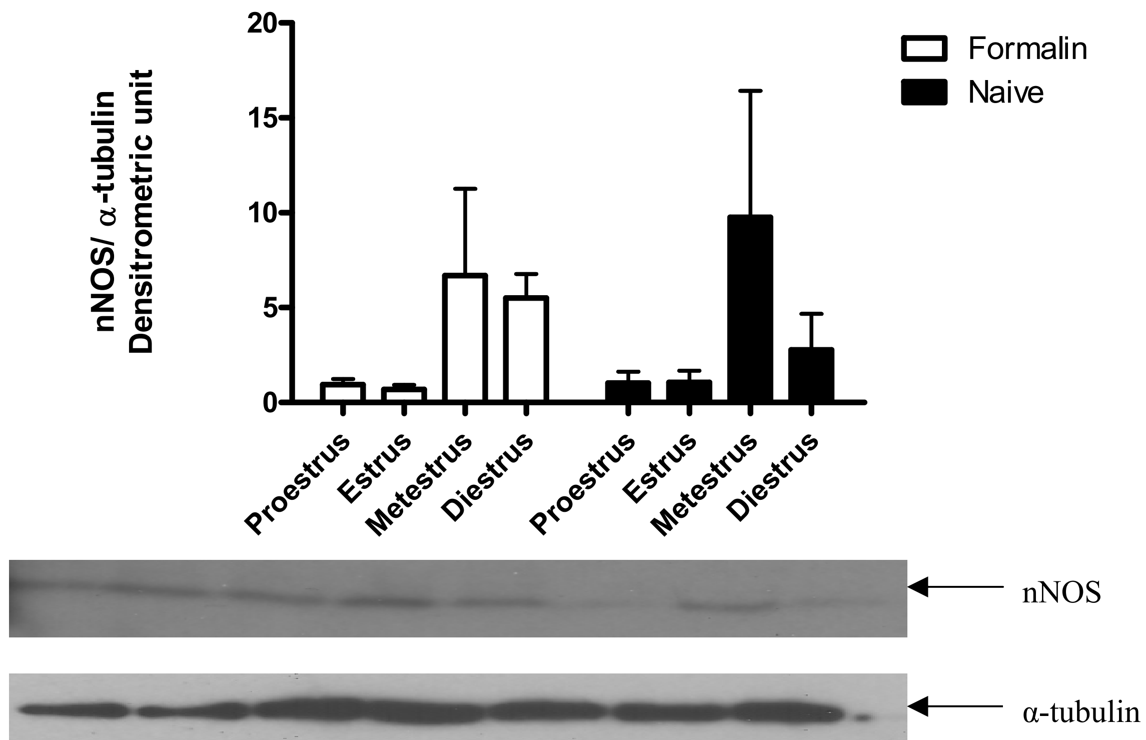
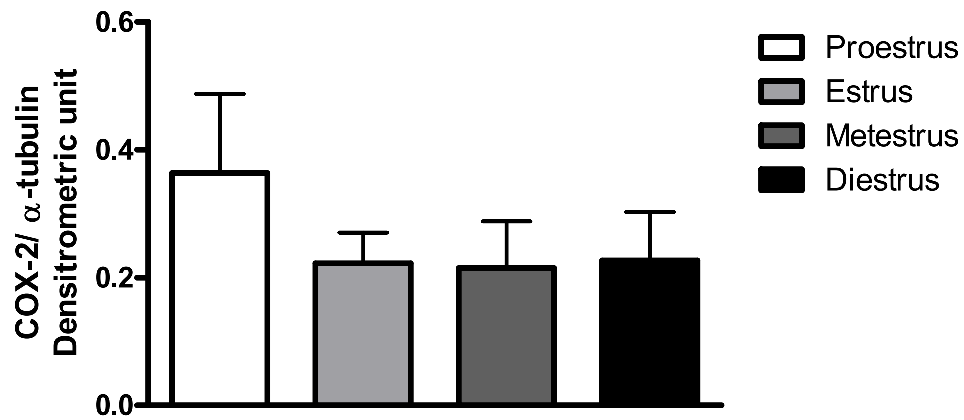



**Figure 13. Effects estrous cycle stage on nNOS levels in the spinal cord after formalin administration.** Data represents nNOS levels over α-tubulin (N=4/group).



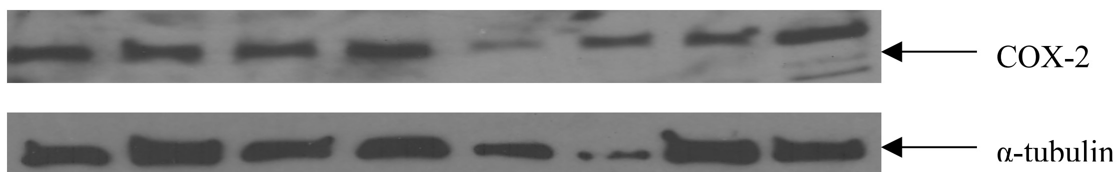




**Figure 14. Effects estrous cycle stage on COX-2 protien levels in the spinal cord after formalin administration.** Data represents COX-2 protein levels over α-tubulin (N=4/group).

| | **Phase II** | **CORT** | **PGE2** | **PGD2** | **COX-2** | **nNOS** |
|---|---|---|---|---|---|---|
| **Phase II** | | r=0.071 P=0.790 | r=0.344 P=0.210 | **r=0.559 P=0.031** | r=0.000 P=0.967 | r=0.534 P=0.196 |
| **CORT** | r=0.000 P=0.790 | | r=0.329 P=0.231 | r=0.055 P=0.854 | r=0.348 P=0.204 | r=0.045 P=0.885 |
| **PGE2** | r=0.329 P=0.210 | r=0.000 P=0.231 | | r=0.199 P=0.499 | r=0.104 P=0.716 | r=0.326 P=0.236 |
| **PGD2** | **r=0.055 P=0.031** | r=0.199 P=0.854 | r=0.000 P=0.499 | | r=0.028 P=0.549 | r=0.002 P=0.861 |
| **COX-2** | r=0.348 P=0.967 | r=0.105 P=0.204 | r=0.167 P=0.716 | r=0.028 P=0.549 | | |
| **nNOS** | r=0.045 P=0.196 | r=0.326 P=0.885 | r=0.045 P=0.236 | r=0.002 P=0.861 | | |

**Table 2.** Correlations of estrous cycle rats after formalin administration, Phase II behavior, CORT, $PGE_{2,}$ and $PGD_{2.}$

**Discussion**

The results of this study support and extend preliminary finding from previous studies in our lab (Kuba et al., 2005a; Kuba and Quinones-Jenab V., 2005; McClung CA et al., 2005). The present study demonstrated that although no significant effects were seen in Phase I or the Interphase after formalin administration, females in proestrus had significantly reduced behavioral responding compared to females in metestrus after Phase II. As discussed in the introduction, the formalin test produces tri-phasic flinching responses that are seen over 60 minutes after intraplantar administration of formalin. Phase I of the test represents behavioral responses corresponding to acute pain, whereas Phase II represents behavioral responses corresponding to injury-induced persistent pain which includes inflammatory pain. Although nociceptive behaviors seen in Phase II are due to central sensitization, peripheral inflammatory responses have been shown to contribute to this sensitization (Dale et al., 2005; Groth and Aanonsen, 2002; Li et al., 2006; Oka et al., 2007).

**Behavioral effect of cycle stage on formalin induced inflammation**

Results showed a significant decrease on behavioral flinching during proestrus in Phase II after formalin administration. Our results are inconsistent with other studies using the intraplantar formalin and CFA tests, observing no estrous cycle effects in any of the phases (Kim et al., 1999; Vincler et al., 2001). Our findings are consistent with a study finding that females in proestrus showed a decrease in pain behavior after a TMJ formalin administration (Tall and Crisp, 2004a).

Previous studies in our lab have demonstrated similar results. Kuba et al. (2006) reported a decrease in behavioral responding in the last phase of the formalin assay (Kuba et al., 2005a). OVX females treated with 20 and 30μg dose of estradiol showed an attenuation of flinching after

formalin administration. They also found that co-administration of estradiol and progesterone, results in a reversal of estradiol-induced decreases in flinching. Rats in proestrus have been compared to OVX animals treated with 20% 17 ß-estradiol. (Mannino et al., 2006). Mannino et al., (2006) demonstrated that OVX rats treated with 20% 17 ß-estradiol had similar behavior as female rats in proestrus after an inflammatory stimulus. Although, they reported no change in flinching behavior after OVX animals were treated with progesterone (Kuba et al., 2005a; Mannino et al., 2006).

The interaction of these two endogenous cycling hormones may have an effect on the behavioral responses to inflammation. As discussed in the introduction, the highest peaks of endogenous estrogen and progesterone occur during proestrus (Knobil and Neill, 88). There is also a second peak of progesterone during the middle of metestrus. We are seeing a significant peak in pain behavior during metestrus and a significant attenuation of the behavior in proestrus. These results support the idea that estrogen attenuates pain while progesterone negates estrogen's anti-inflammatory properties as reported by Kuba et al. (2005). The significant effect of endogenous estrogen peaks during the estrous cycle attenuating while peaks in progesterone increasing behavioral responses after formalin administration into the hindpaw is a novel finding. Our findings suggest that peaks in endogenous estrogen and progesterone may affect inflammatory pain pathways.

**Effects estrous cycle stage on $PGE_2$ and $PGD_2$ serum levels after formalin administration.**

After formalin administration, rats in estrous had significantly lower $PGE_2$ levels than rats in metestrus and diestrus. As mentioned earlier the release of PG's have been linked to formalin-induced behavioral responses (Tegeder et al., 2001) as well as hyperalgesic states (Camacho-Arroyo et al., 2003; Rackman and Ford-Hutchinson, 1983; Scheuren et al., 1997).

Attenuation of these serum levels in the stage where estrogen and progesterone levels are falling from their peaks, suggests a partial regulation of estrogen and progesterone in inflammatory mediated responses. However these endogenous hormones may work through alternate pathways as well. Tenenbaum et al., (2007) demonstrated that the administration of an exogenous hormone, estradiol, significantly reduced LPS-induced increases of NO and TNF (pro-inflammatory mediators) but not $PGE_2$ in neuronal cultures (Tenenbaum et al., 2007). We found the highest $PGE_2$ levels during metestrus, the stage where progesterone peaks, leading us to postulate that progesterone may have the opposite effects of estrogen on inflammatory pain. This finding suggests that differences in $PGE_2$ levels may not always accompany estradiol's antihyperalgesic effects but may accompany progesterone's effect of inflammation.

In the presents study, rats in proestrus had significantly higher $PGD_2$ levels than all other groups after formalin administration. The role of $PGD_2$ and its involvement in hyperalgesia and other periphery inflammatory responses is not well documented. It has been postulated that at the spinal cord level, $PGD_2$ blocks the $PGE_2$-evoked pain responses-implying that endogenous $PGD_2$ may play an inhibitory role in the appearance of spinal cord nocicpetion under physiological conditions (Minami et al., 1999). Our results support these findings; rats in proestrus have significantly higher $PGD_2$ levels and a significant decrease in inflammatory-induced behavior. Evidence from this study suggests that two different mechanisms may be responsible for nociceptive responses of $PGE_2$ and $PGD_2$. These findings support the notion that expression of $PGD_2$ may play a role in the resolution of inflammation (Scher JU, 2009) Ohkubo et al. (1983), discovered that peripheral $PDG_2$ expression elicited hyperalgesia while central PGD2 showed an analgesic effect (Ohkubo T, 1983). Interestingly, these findings are different from a recent study, where Grill et al., (2006) found after endotoxin treatment, lumiracoxib (a

selective COX-2 inhibitor) significantly reduced $PGD_2$ serum levels (Grill et al., 2006). These differences could be a product of differential activation of inflammatory mediators by endotoxin. Although they observed an increase in $PGD_2$ levels after administration of endotoxin they did not see an increase in PG levels similar to the present study. Together, these and our findings may support a role for $PGD_2$ involvement in estrogen and progesterone's effects on inflammatory pain.

**Effects estrous cycle stage on nNOS levels in the spinal cord after formalin administration.**

We did not detect changes in nNos in the spinal cord after formalin administration. Although significance of the spinal pools of NO and their mechanisms of action remain to be elucidated, it is likely that NO has an important role in spinal mediated processes of sensitization that underlie painful states. As mentioned in the introduction, it has been suggested that the $Ca^{+2}$/calmodulin-dependant spinal nNOS plays an important role in inflammatory hyperalgesia (Tao, 2004). Tao et al. (2004) demonstrated that spinal cord nNOS levels are up-regulated after peripheral inflammation (Tao F, 2004).

In the present study it appears that levels of nNOS are greatest in metestrus in both formalin treated and naïve animals. Cuzzocrea et al., (2001) showed that estradiol treatment inhibits the increase of inducible nitric oxide synthestase (iNOS) activity after inflammation, suggesting that in the cycling rat, this hormone, plays a key role in the increased sensitivity to the inflammatory injury seen in the OVX rat (Cuzzocrea et al., 2001). The high levels of nNOS that we observed in metestrus accompanied the significant increase in both $PGE_2$ and flinching behavior when compared to animals in proestrus. This supports the findings of Dina et al., (2001), who reported that hyperalgesia induced by PGE2 was dependent on NO although they

suggested that this may be estrogen dependent (Dina et al., 2001). Our findings suggest that progesterone may be altering this inflammatory pathway. Taken together these findings support the idea that cycle stage may have an effect on inflammatory mediators.

**Effects of estrous cycle stage on COX-2 levels in the spinal cord after formalin administration.**

COX-2 levels in the spinal cord did not change as a result of estrous cycle after formalin administration. Although animals in proestrus had higher levels of COX-2 protein levels, it failed to reach significance. Various studies have shown that exogenous estrogen increases COX levels. To our knowledge, there is no literature on endogenous hormonal fluctuations effect on spinal COX after an inflammatory stimulus. Our study does support the findings from various studies looking at estradiol's effects on COX expression after inflammation. Mitsutoshi et al., (2004) demonstrated that E2 treatments resulted in a significant increase in COX-2 mRNA levels in primary human uterine microvascular endothelial cells HUMEC (Tamura et al., 2004). A time dependent increase of COX-2 mRNA levels was observed and administration of estrogen antagonist ICI 182,780 fully reversed the effects of E2 on COX mRNA levels in 1hr and protein levels in 4 hrs. More recently, Cuzzocrea et al., (2007) found that the administration of an estrogen receptor antagonist significantly inhibited the ability of dexamethasone to reduce iNOS and COX expression (Cuzzocrea et al., 2007). These findings suggest that the presence of estrogen is positively correlated to increases in COX-2 mRNA levels.

Taken together, most of the literature investigates exogenous estradiol and progesterone's effects on the inflammatory pain pathway. We have used the estrous cycle as a measure of endogenous hormonal fluctuations and their effects on inflammation, which may be a reason why our results are not always consistent will previous studies using OVX and hormone

treatments. In rats, during the 4 day estrous cycle, the secretion rate of estrogen into ovarian venous plasma, is lowest on estrus, begins to rise significantly by late metestrus through the morning of diestrus, and reaches its peak concentrations by the afternoon of proestrus (Knobil and Neill, 88). At its highest peak in proestrus, concentrations of estrogen in peripheral plasma reach 42 pg/ml. (Knobil and Neill, 1988). At its lowest concentrations in estrus, they are about at 5pg/ml (Knobil and Neill, 1988). There are two peaks of progesterone secretion into ovarian vein blood during the cycle. The first peak occurs during the afternoon of metestrus and the second peak occurs during the late afternoon of proestrus (Knobil and Neill, 88). At its highest peak in proestrus, concentrations of progesterone in peripheral plasma reach 58pg/ml (Knobil and Neill, 1988). At its lowest concentrations in estrus, they are about at 2pg/ml (Knobil and Neill, 1988).

## Chapter 3

Can analgesic/anti-hyperalgesic properties of endogenous gonadal hormones be extended to the carrageenan inflammatory pain model? Are estrogen and progesterone's effects on behavioral responses to carrageenan mediated through deactivation/down regulation of COX-2?

### Results

***Effects of cycle stage on baseline levels of paw withdrawal latencies.***

There was a significant main effect for heat intensity on paw baseline withdrawal latency in both the right (Figure 15) and left (Figure 16) paws [$F (3, 33) = 32.516$; $p<.000$] and [$F (3, 34) = 28.098$; $p<.000$] respectively. PWL was decrease as heat intensity increased in all groups.

***Effects of estrous cycle stage on paw withdrawal latencies 1 and 5 hours after 1% carrageenan administration.***

As shown in Figure 17A-D, one and five hours after carrageenan administration, the injected paw shows a greater hyperalgesic effect than the uninjected paw.

***Effects of estrous cycle stage on the injected paw withdrawal latencies (PWL) after 1% carrageenan administration at all heat intensities.***

As show in Figures 18 ABC, carrageenan administration reduced latencies to withdrawal in the injected paw at all three heat intensities; low heat stimuli [$F (3, 33) = 471.127$; $p=0.000$] medium heat stimuli [$F (3,33) = 38.2035$; $p<0.000$] and high heat stimuli [$F (3,32) = 31.253$; $p=0.000$]. Furthermore, post hoc analysis showed a time effect in which at 5 hours post injection, all groups had lower paw withdrawal latencys than at 1 hour ($p< 0.05$).

***Effects of estrous cycle phase on contralateral PWL at all heat intensities.***

As shown in Figure 19 ABC, after carrageenan administration an increase in paw withdrawal latencies occur in the left (uninjected) paw at the low heat stimuli [F (3,34)

=3.143.709; p=0.049]. However, no significant differences were seen in the uninjected paw after medium heat stimuli. At the highest heat intensity the paw withdrawal latency was affected by the stage of the cycle. Specifically, differences in paw withdrawal latency at baseline and after 1 hour post carrageenan injection [F (6,68) =3.009; p=0.011]. Animals in estrus had significantly higher paw withdrawl latencies when compared the animals in the metestrus group (p<0.05), while at 1hour animals in estrus had significantly shorter latencies than animals in metestrus or diestrus (p<0.05 for all comparisons).

***Effects estrous cycle stage on $PGE_2$ and $PGD_2$ serum levels after carrageenan administration.***

As shown in Figure 20A, a significant effect for estrous stage on $PGE_2$ levels after carrageenan administration was observed [F (3, 12) =3.54; p=0.048]. Animals estrus had significantly higher $PGE_2$ levels when compared to those in metestrus or diestrus (p<0.05 for all comparisons). As shown in Figure 20B, estrous cycle stage had no effect on $PGD_2$ levels after carrageenan administration.

***Effects estrous cycle stage on nNOS levels in the spinal cord after carrageenan administration.***

As shown in Figure 21, we observed a higher mean for nNOS levels in the spinal cord during metestrus (0.07) when compared to proestrus (0.04), estrus (0.02), diestrus (0.04). Although we observed the differences in the means, we did not detect significant changes in nNos levels in the spinal cord after carrageenan administration [F (7, 24) =9.65; p=0.005]. No significant effects of estrous cycle were seen in the carrageenan treated and the naïve rats. Correlations of estrous cycle rats after carrageenan administration; PWL at 5 hours post injection, CORT, $PGE_{2,}$ $PGD_{2,}$ and nNOS. All correlations for carrageenan treated rats in each stage of the cycle are shown in Table 3.

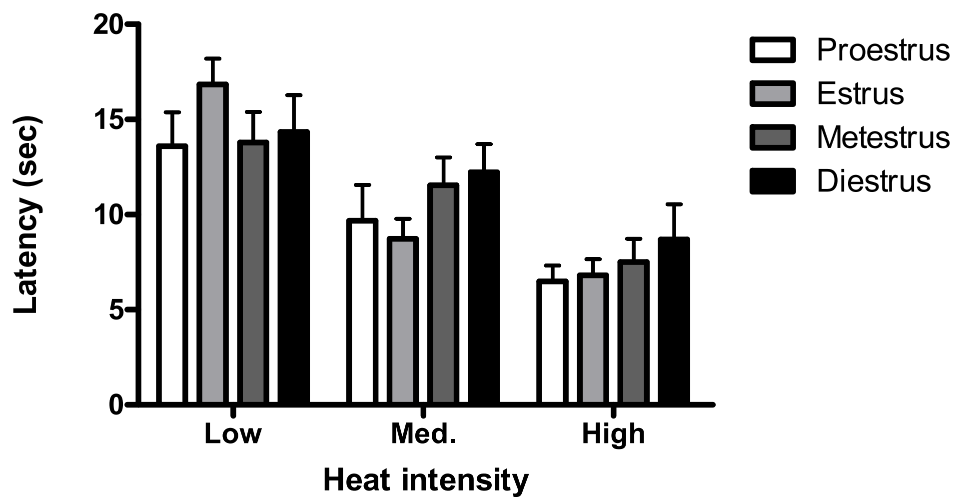



**Figure 15. Effects of estrous cycle stage on baseline levels of right paw withdrawal latencies.** Data represents mean paw withdrawal latencies (± SEM) thirty minutes before 1% carrageenan injection (N=10-12/group).

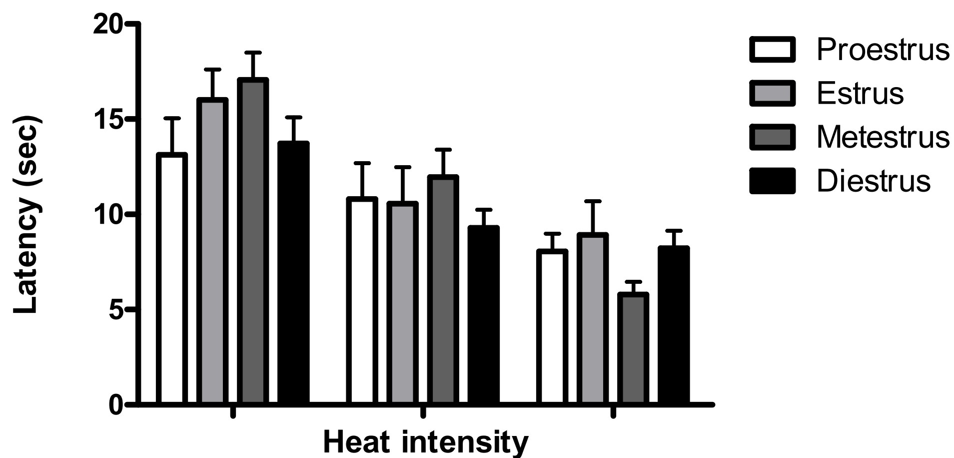



**Figure 16. Effects of estrous cycle stage on baseline levels of contralateral paw withdrawal latencies.** Data represents mean paw withdrawal latencies (± SEM) at the low heat intensity (4.50mv),medium heat intensity (4.80mv) and at the high heat intensity (5.20mv) (N=10-12/group).

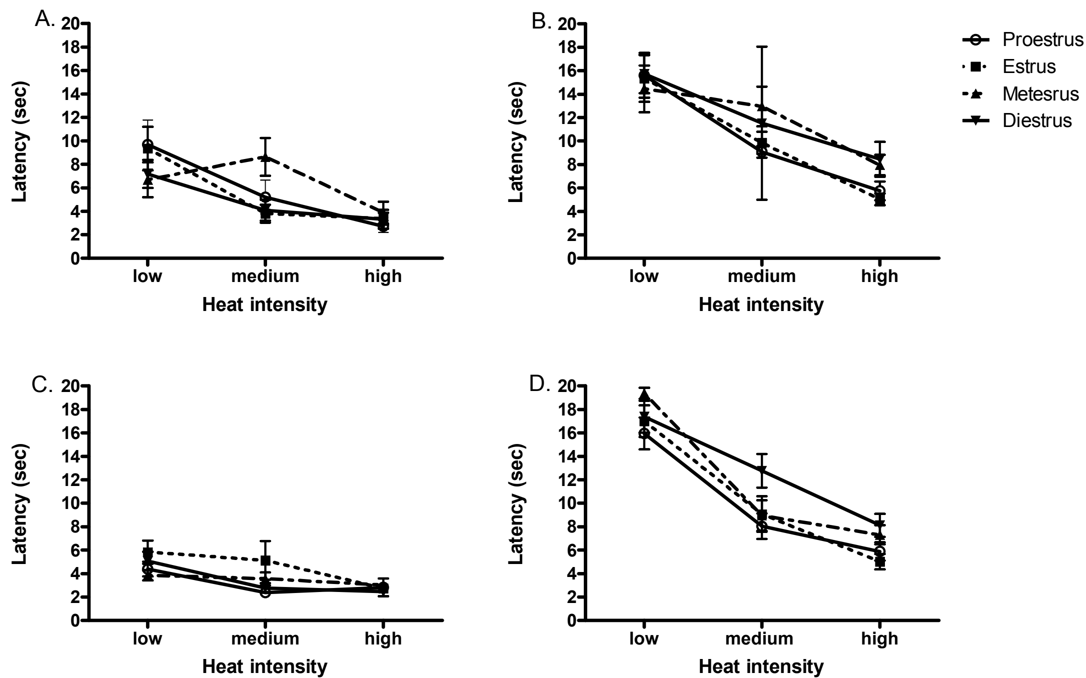



**Figure 17 A-D. Effects of estrous cycle stage on paw withdrawal latencies 1 and 5 hours after 1% carrageenan administration. A.** Data represents cumulative mean withdrawal latencies (± SEM) 1 hour after 1% carrageenan injection at all heat intensities for the right paw. **B.** Represents mean withdrawal latencies (± SEM) 1 hour after 1% carrageenan injection at all heat intensities for the left paw. **C.** Represents mean withdrawal latencies (± SEM) 5 hours after 1% carrageenan injection at all heat intensities for the right paw. **D.** Represents mean withdrawal latencies (± SEM) 5 hour after 1% carrageenan injection at all heat intensities for the left paw (N=10-12/group).

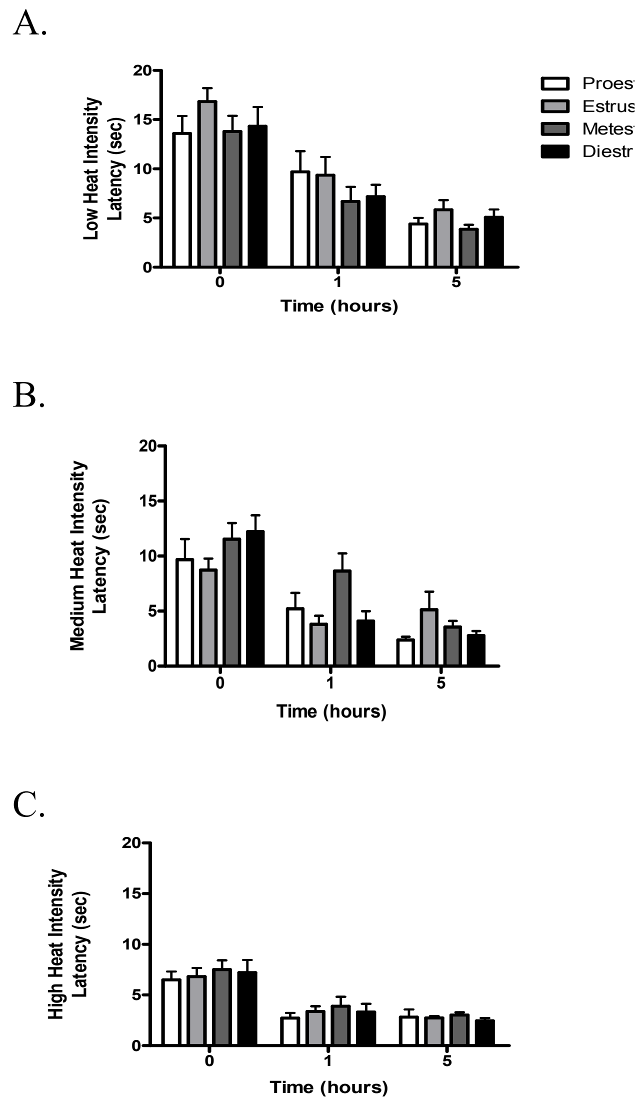
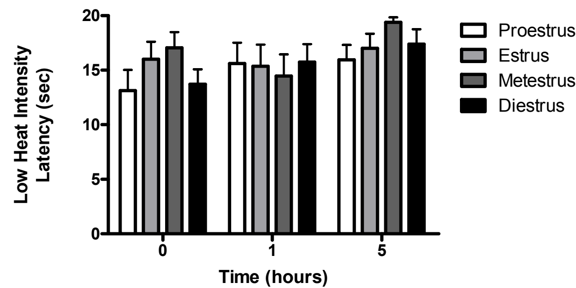
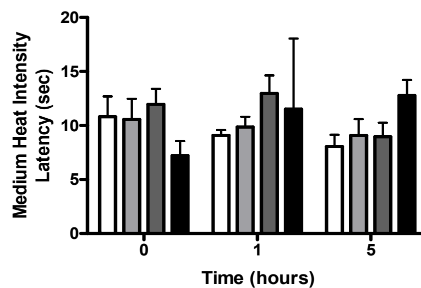



**Figure 18. A-C Effects of estrous cycle stage on injected paw withdrawal latencies for each heat intensity after 1% carrageenan administration. A.** Data represents cumulative mean withdrawal latencies (± SEM) for right paw at 0, 1, and 5 hours after carrageenan at the low heat intensity (4.50mv). **B.** Represents cumulative mean withdrawal latencies (± SEM) at the medium heat intensity (4.80mv) **C.** Represents cumulative mean withdrawal latencies (± SEM) at the high heat intensity (5.20mv). (N=10-12/group).

A.




B.




C.

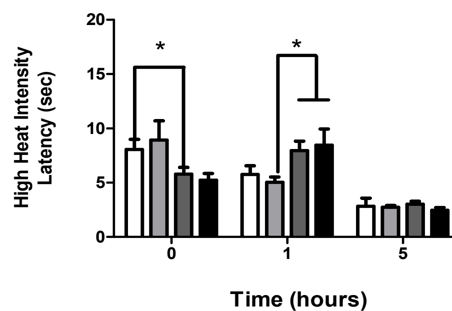



**Figure 19. A-C Effects of estrous cycle stage on contralateral paw withdrawal latencies for each heat intensity after 1% carrageenan administration. A.** Data represents cumulative mean withdrawal latencies (± SEM) for the left paw at 0, 1, and 5 hours after carrageenan at the low heat intensity (4.50mv). **B.** Represents cumulative mean withdrawal latencies (± SEM) at the medium heat intensity (4.80mv) **C.** Represents cumulative mean withdrawal latencies (± SEM) at the high heat intensity (5.20mv). (*) Represents a significant stage and time interaction effect (N=10-12/group).

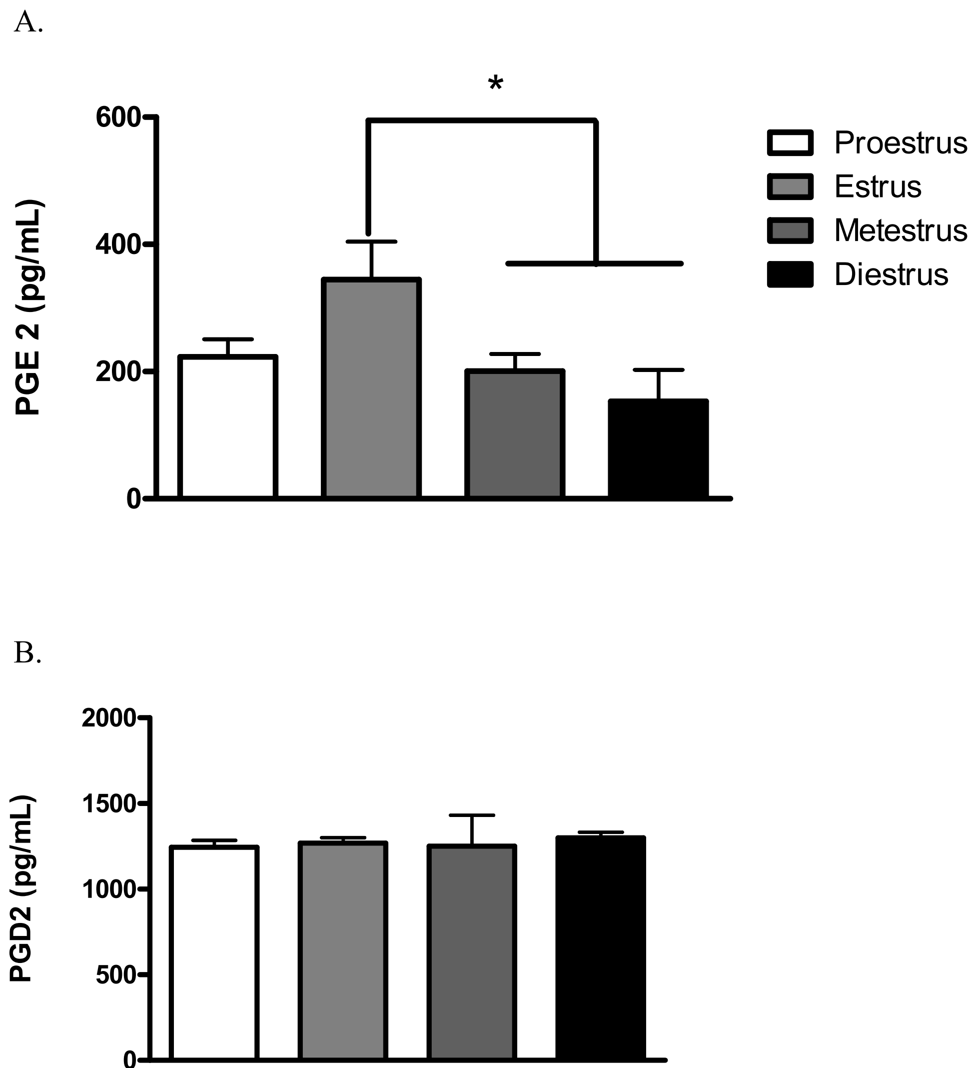



**Figure 20 A-B. Effects of estrous cycle stage on PGE2 (A) and PGD2 (B) serum levels after carrageenan administration A.** Data represents mean prostaglandin E2 serum levels (± SEM) at picograms per mililiter sixty minutes after carrageenan administration (N = 4-6/group). (*) Denotes significantly higher PGE2 serum levels in the estrous group when compared to the metestrus and diestrus groups **B.** Data represents mean prostaglandin $D_2$ serum levels (± SEM) after formalin administration (N= 4-6/group).

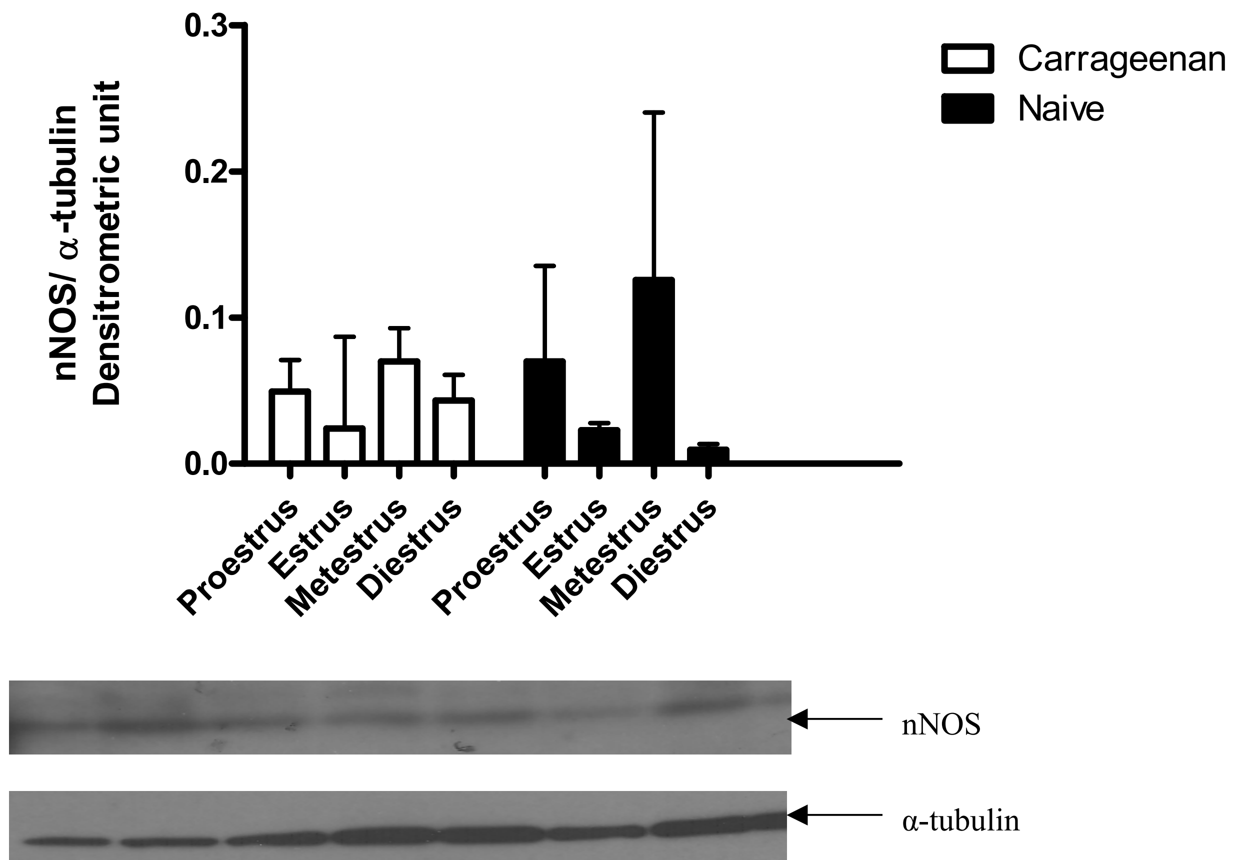



**Figure 21. Effects estrous cycle stage on nNOS levels in the spinal cord after carrageenan administration.** Data represents nNOS levels over α-tubulin (N=4/group).

| | PWL 5 hours | CORT | PGE2 | PGD2 | nNOS |
|---|---|---|---|---|---|
| **PWL 5 hrs.** | | r=0.285 P=0.225 | r=0.130 P=0.648 | r=0.329 P=0.233 | r=0.179 P=0.510 |
| **CORT** | r=0.275 P=0.225 | | r=0.418 P=0.107 | r=0.155 P=0.567 | r=0.365 P=0.165 |
| **PGE2** | r=0.130 P=0.648 | r=0.418 P=0.107 | | r=0.114 P=0.673 | r=0.031 P=0.898 |
| **PGD2** | r=0.329 P=0.233 | r=0.155 P=0.567 | r=0.114 P=0.673 | | r=0.179 P=0.510 |
| **nNOS** | r=0.179 P=0.510 | r=0.365 P=0.165 | r=0.032 P=0.898 | r=0.179 P=0.510 | |

**Table 3.** Correlations of estrous cycle rats after carrageenan administration; PWL at 5 hours post injection, CORT, $PGE_2$, $PGD_2$, and nNOS.

## Discussion

### Effects of cycle stage on baseline levels of paw withdrawal latencies.

After demonstrating the effects of cycle stage on the formalin model, these findings were extended to another model of persistent inflammatory pain, carrageenan. Overall baseline levels of PWL before carrageenan administration in both the right and left paw showed no group differences in behavioral responding. Unexpectedly, no significant differences in PWL between groups were seen after carrageenan administration. At the medium heat 5 hour test, females in proestrus appeared to have a lower PWL that all other groups, although his failed to reach significance. These findings extend work previously done in our lab, demonstrating that estradiol administration dose dependently produces antihyperalgesic effects during inflammatory pain. Tall and Crisp (2004) found that females in proestrus had significantly higher PWL when compared to females in estrus and diestrus two hours after carrageenan administration (Tall and Crisp, 2004a). Our findings support theirs but in contrast to Tall and Crisp's findings, we demonstrated an estrous cycle effect on contralateral PWL; at baseline, animals in estrus had significantly higher paw withdrawl latencies when compared the animals metestrus, while at 1 hour animals in estrus had significantly shorter latencies than animals in metestrus or diestrus. This finding could be the result of the short estrous cycle in the female rat; post-mortem lavage samples were taken and several of the rats had moved on to the next stage or were in-between stages of the cycle after the 5 hour test. The difference could also be seen because they only did one test at one heat intensity at 2 hours post 4 % injection. It is feasible as shown by our results that the intensity of the thermal inflammatory stimulus affects behavioral responses. Tall and

Crisp (2004) also grouped animal diestrus and metestrus together, this is important to examine. We are finding effects in metestrus, where a second progesterone peak occurs during the cycle. If diestrus and metestrus are gouped together the effects of the progesterone peak may be negated by the low levels of hormones seen during diestrus. We felt that it was important to separate the two stages, to observe the effects of the hormonal fluctuations that occur during each stage of the estrous cycle. During the beginning of the estrus stage of the cycle we see low levels of estrogen and progesterone continually decreasing after they have peaked in proestrus (Knobil and Neill, 1988). It is possible that at baseline, endogenous hormones were just beginning to peak, which would explain why the PWL was higher in the estrous group. While at one hour the estrogen and progesterone levels might have gone down even further explaining why during the 1 hour testing animals in estrus had significantly lower PWL when compared to animals in metestrus and diestrus.

Studies in our lab have also found baseline effects of estradiol administration before carrageenan, showing an overall higher PWL compared to untreated animals. Furrthermore, Cuzzocrea et al., 2001 demonstrated that the lack of endogenous estrogen enhances carrageenan-induced acute inflammation in the rat lung (Cuzzocrea et al., 2001). Although, they only used the estrus phase of the cycle, comparing it to OVX and OVX + estradiol treatement groups when coming to this conclusion. Our findings support the idea that endogenous and exogenous sex hormones affect pain differently after carrageenan administration due to the constant variation in endogenous hormone levels in the estrous cycle.

**Effects estrous cycle stage on $PGE_2$ and $PGD_2$ serum levels after carrageenan administration.**

After carrageenan administration, rats in estrous had significantly higher $PGE_2$ levels than rats in metestrus and diestrus. As mentioned earlier the release of PG's has been linked to formalin-induced behavioral responses (Tegeder et al., 2001) as well as hyperalgesic states (Camacho-Arroyo et al., 2003; Rackman and Ford-Hutchinson, 1983; Scheuren et al., 1997). An increase of these serum levels in the stage where estrogen and progesterone are coming down from their peaks, suggests a partial mediation in this pathway. However these endogenous hormones may work through alternate pathways as well. Tenenbaum et al., (2007) demonstrated that the administration of an exogenous hormone, estradiol, significantly reduced LPS-induced increases of NO and TNF (pro-inflammatory mediators) but not $PGE_2$ in neuronal cultures (Tenenbaum et al., 2007). In the present study, $PGE_2$ levels were not affected by peaks in endogenous estrogen during proestrus although we observed a significant increase in $PGE_2$ when estrogen and progesterone levels are dropping, in estrus. Although the presence of estrogen during proestrus had no effect on PG levels, the drop in endogenous hormones during the estrous cycle appears to alter estrogen's anti-hyperalgesic effects.

No differences were seen in plasma levels of $PGD_2$ after carrageenan administration. The role of $PGD_2$ and its involvement in hyperalgesia and other periphery inflammatory responses is not well documented. Although, Horiguchi et al., (1986) found evidence to support separate mechanisms for nociceptive responses of $PGE_2$ and $PGD_2$ in mice (Horiguchi et al., 1986b). This finding suggests that differences in $PGD_2$ levels may not always accompany estradiol's antihyperalgesic effects. Evidence of this study suggests that two different mechanisms may be responsible for nociceptive responses of $PGE_2$ and $PGD_2$. Interestingly, these findings are different from a recent study, where Grill et al., (2006) found after endotoxin treatment, lumiracoxib (a selective COX-2 inhibitor) significantly reduced $PGD_2$ serum levels (Grill et al.,

2006). These differences could be a product of differential activation of inflammatory mediators by endotoxin. The present study observed significant effects of estrous cycle on PGD2 levels after formalin administration but not after carrageenan administration. This could be in part due to the time of sacrifice, where PG's may be active soon after the inflammatory insult, explaining why we observed effects in $PGD_2$ after1 hour and not after 5 hours. Thus, it is feasible that the magnitude or intensity of the inflammatory stimulus-carrageenan produces more severe and longer lasting inflammatory responses-which in turn, alters estrogen and progesterone's effects of mechanisms controlling inflammatory responses.

**Effects estrous cycle stage on nNOS levels in the spinal cord after carrageenan administration.**

We did not detect changes in nNos in the spinal cord after carrageenan administration. No significant effects of estrous cycle were seen in the carrageenan treated and the naïve rats. Although it appears that nNOS levels are once again highest in rats during metestrus. Using microdialysis, Omote et al. (2001) demonstrated that the early phase of carrageenan-induced release of NO is regulated by nNOS while nNOS and iNOS are responsible for NO release in the later phase (Omote et al., 2001). We observed significantly lower PWL and significantly higher PGE2 plasma levels of rats in metestrus when compared to rats in estrus. The increased nNOS levels observed during metestrus can be the result of the peak in progesterone during this stage of the estrous cycle. In the previous study, using formalin, we observed similar effects on behavior, PG and nNOS. Thus, we postulate that the NOS-COX-PG pathway is mediated by endogenous hormonal fluctuations during the female estrous cycle.

# CHAPTER 4

Are endogenous gonadal hormonal fluctuations throughout life altering formalin induced inflammatory responses to pain?

***Results***

***Behavioral effect of age on formalin induced inflammation***

Figure 22, shows the analysis of 3 minute bouts over the 60 minute time post injection for each group. We observed a decrease in the mean number of flinches for the adult group (19.26) when compared to all others; peri-adolescent (37.37), adolescent (30.18), and aged (43.10) during the time of the interphase.

As shown in Figure 23, there are no significant differences between age groups in Phase I, the Interphase and Phase II after formalin administration.

***Effects of age on PGE2 and PGD2 serum levels after formalin administration.***

As seen in Figure 24A, after formalin administration a significant age effect on $PGE_2$ serum levels was seen [F (3,19) =4.23172; p=0.0189]. The peri-adolescent and adolescent rats had significantly higher $PGE_2$ serum levels than aged rates ($p<0.01$) for all comparisons. As shown in Figure 24B, a significant age effect in $PGD_2$ serum levels was observed after formalin administration [F (3, 19) =3.4461; p=0.037413]; the peri-adolescent rats had higher $PGD_2$ serum levels that than adult or aged rats ($p< 0.02$ for all comparisons).

***Correlations for all age groups after formalin administration; Phase III behavior, CORT, $PGE_2$, and $PGD_2$.***

All correlations for formalin treated rats in each age group are shown in Table 4.

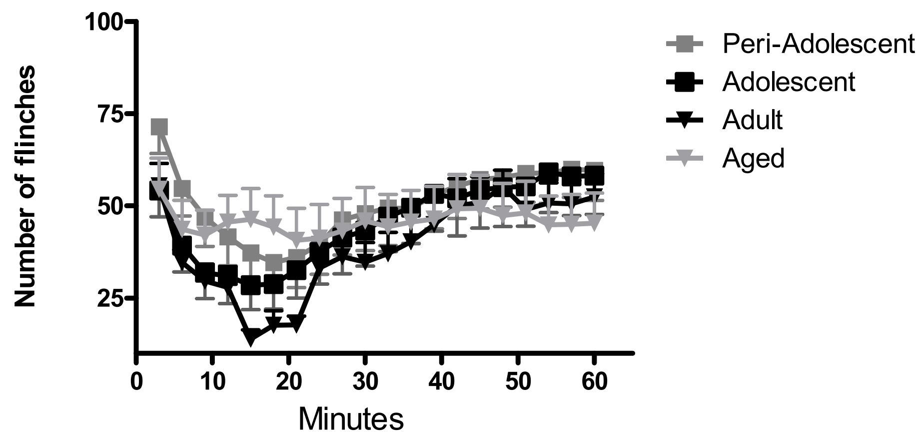



**Figure 22. Time course of flinching responses in all age groups after (5%) formalin administration.** Time course of activation is represented as the mean of flinching responses in 3 minute bins (N=10-12/group).

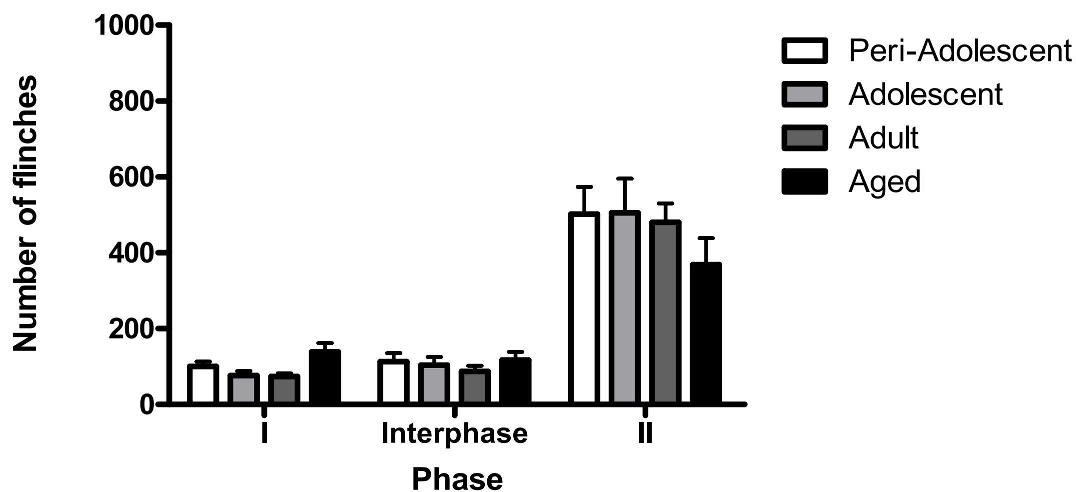
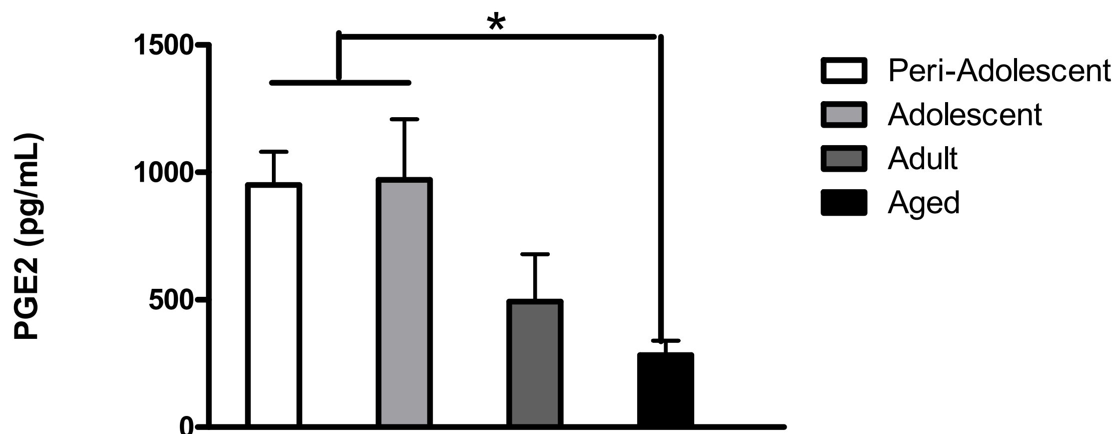



**Figure 23. Effects of age on behavioral flinching responses after 5% formalin administration.** Data represents the cumulative mean of flinches (+/-SEM) in Phase I (0-5 min), Phase II (6-19 min) and Phase 3 (10-50 min) in animals at four different ages (N=10-12/group).

A.




B.

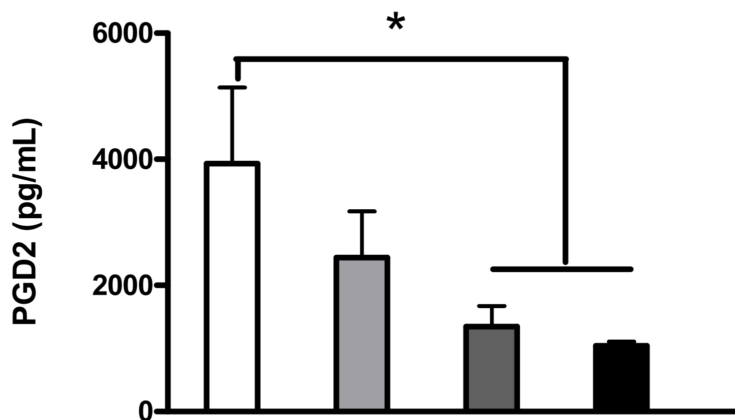



**Figure 24A-B. Effects of age on PGE2 (A) and PGD2 (B) serum levels after formalin administration**
**A.** Data represents mean prostaglandin E2 serum levels (± SEM) at picograms per milliliter sixty minutes after formalin administration (N= 4-6/group). (*) Denotes significantly lower $PGE_2$ serum levels in the aged group when compared to the peri-adolescent and adolescent groups. **B.** Data represents mean prostaglandin $D_2$ serum levels (± SEM) after formalin administration (N= 4-6/group). (*) Denotes significantly higher $PGD_2$ serum levels in the proestrus group when compared to the adult and aged groups.

| | **Phase II** | **CORT** | **PGE2** | **PGD2** |
| --- | --- | --- | --- | --- |
| **Phase II** | | r=0.105<br>P=0.645 | r=0.045<br>P=0.837 | r=0.257<br>P=0.2615 |
| **CORT** | r=0.105<br>P=0.645 | | r=0.110<br>P=0.640 | r=0.279<br>P=0.223 |
| **PGE2** | r=0.045<br>P=0.837 | r=0.110<br>P=0.640 | | r=0.416<br>P=0.061 |
| **PGD2** | r=0.257<br>P=0.2615 | | r=0.416<br>P=0.061 | |

**Table 4.** Correlations of aged rats after formalin administration; Phase II behavior, CORT, $PGE_{2,}$ and $PGD_{2.}$

## Discussion

### Behavioral effect of age on formalin induced inflammation

There have been no studies done to date, investigating the relationship between age, hormonal levels, and responses to inflammatory induced pain. In the current study, we begin to explore the possibility of hormone interactions several inflammatory pain pathways. The results of this study extend preliminary finding from previous studies (Gagliese and Melzack, 1999). The present study demonstrated that no significant effect of age on inflammatory induced behavior. Aged females had a decrease in responding in Phase II, although this did not reach significance. As discussed in the introduction, the formalin test produces tri-phasic flinching responses that are seen over 60 minutes after intraplantar administration of formalin. Phase I of the test represents behavioral responses corresponding to acute pain, whereas Phase II represents behavioral responses corresponding to injury-induced persistent pain which includes inflammatory pain. Although nociceptive behaviors seen in Phase II are due to central sensitization, peripheral inflammatory responses have been shown to contribute to this sensitization (Dale et al., 2005; Groth and Aanonsen, 2002; Li et al., 2006; Oka et al., 2007). Investigating the effects of endogenous hormonal fluctuations throughout life altering behavioral responses after formalin administration is a novel way to approach hormonal effects on pain.

### Behavioral effect of age on formalin induced inflammation

Results showed no significant changes in behavioral flinching in any of the Phases after formalin administration. Our results differ from those seen in Galgleise and Melzack (1999), where adult female rats had significantly higher behavioral responses to formalin, than the young and aged rats (Gagliese and Melzack, 1999). As discussion in the introduction, the data that is

available for rodents are mostly based on tests using phasic or brief, transient pain and to date, a widely accepted criteria for age classification are not available (Gagliese and Melzack, 2000). Results found using this particular test have been inconsistent, several studies found no age differences with the tail flick test (Crisp T, 1994; Ghirardi O, 1994; Girardot MN and Holloway FA, 1985; Hamm and Knisely, 1986). Goettl et al. (2000) found age differences in the tail flick test, but only at high intensity with aged animals showing a longer tail-flick latency while other have found decreases in latency with age (Goettl VM, 2000; Islam et al., 1993).

Rodents are typically considered “aged” when they are approximately 2 years old, “middle aged” are 16-18 months and “young” are 3-4 months when used for studies of memory (Frick KM., 2009). For our studies, ages of the rats were divided into peri-adolescent (3 weeks), adolescent (6 weeks), adult (8 weeks) and aged (10-11 months). This could be responsible for the surprising results that we observed. In rats, reproductive decline begins at 9-12 months of age (Finch C.E. et al., 1984); by 12 months approximately 70% of female rats cycle irregularly or are acyclic and nearly 75% of females become acyclic by 24 months (Markowska A.L. and Breckler, 1999). Our lab was not under a grant that allowed for the purchace of “aged” animals, therefore we used retired breeders. Although the definition of “aged” remains to be elucidated, our “aged” animals appear to be closer in age to “middle aged” animals, explaining why their inflammatory induced behavior was reduced. If they were to still be considered “middle age” estrogen and progesterone would still be present at its normal fluctuations, perhaps effecting responses to inflammatory pain. It is also a possibility that the behavioral responses of our “aged” group was a consequence of impaired motor abilities or increased weight compared to the younger rats. This hypothesis supports the findings of Campbell et al., (1980) who found no age

differences of measures of reflexive behavior but reported an age-related decline in the ability to perform the actual behaviors (Wallace, 1980).

The apparent inconsistency between the present results and those reported previously may also be due to the test of nociception employed, as most test do not involve persistant pain comparable to the formalin test. This could be a critical variable because age differences seen in humans, may also depend on the type of pain measured (Gagliese, 2009). Data from humans suggest age-related increase in pain threshold, decrease in pain tolerance, but no change in the intesnsity of post operative and chronic pain (Gagliese, 2009). It is not suprising then that the results for age studies and pain are inconsitent across various models of pain and nociception in the rat.

**Effects of age on PGE2 and PGD2 serum levels after formalin administration.**

Our results show that after formalin administration, $PGE_2$ serum levels are significantly higher during peri-adolescence and adolescence when compared to aged rats. As mentioned earlier the release of PG's have been linked to formalin-induced behavioral responses (Tegeder et al., 2001) as well as hyperalgesic states (Camacho-Arroyo et al., 2003; Rackman and Ford-Hutchinson, 1983; Scheuren et al., 1997). An increase in these serum levels at ages where estrogen and progesterone are lower, suggests a partial mediation in this pathway. However these endogenous hormones may work through alternate pathways as well.

In the present study, peri-adolescent rats had significantly higher $PGD_2$ levels than adult and aged rats. This supports the findings of Horiguchi et al., (1986), reporting evidence that suggested separate mechanisms for $PGE_2$ and $PGD_2$ (Horiguchi et al., 1986a). The role of $PGD_2$ and its involvement in hyperalgesia and other periphery inflammatory responses is not well documented. As previously discussed, it has been postulated that at the spinal cord level, $PGD_2$

blocks the $PGE_2$-evoked pain responses-implying that endogenous $PGD_2$ may play an inhibitory role in the appearance of spinal cord nocicpetion under physiological conditions (Minami et al., 1996). Evidence of this study suggests that two different mechanisms may be responsible for nociceptive responses of $PGE_2$ and $PGD_2$. Interestingly, the peri-adolescent group had an increase in both $PGE_2$ and $PGD_2$ when compared to the aged group. The increase in both PG's in the peri-adolescent group may be a result of low levels of endogenous estrogen and progesterone, causing the negation of $PGE_2$ pro-inflammatory effects by $PGD_2$. These findings support the notion that expression of $PGD_2$ may play a role in the resolution of inflammation (Scher and Pillinger, 2009). Ohkubo et al. (1983), discovered that peripheral PDG2 expression elicited hyperalgesia while central $PGD_2$ showed an analgesic effect (Ohkubo T, 1983). Although, the present study shows that an increase in these PG's did not accompany any differences in behavioral responding to formalin-induced inflammation. Our findings may support a role for $PGE_2$ and $PGD_2$'s involvement in estrogen and progesterone's effects on pain.

## Chapter 5

Are endogenous estrogen and progesterone's effects on behavioral responses to inflammatory stimuli in part mediated through the induction of corticosterone release?

### *Results*

#### *Effects of estrous cycle stage on CORT serum levels after formalin administration.*

As shown in Figure 25, no significant differences in corticosterone serum levels during each phase of the estrous cycle after formalin administration was seen. Although the differences failed to reach significance, animals in the estrus stage had lower mean CORT serum levels (171.10) after formalin administration when compared to proestrus (362.39), metestrus (385.64), and diestrus (482.67) [$F(3, 20) = 1.723$; $p = 0.194$].

#### *Effects of estrous cycle stage on CORT serum levels after carrageenan administration.*

As shown in Figure 26, a significant effect of estrous cycle on corticosterone serum levels after carrageenan administration was observed [$F(3,17) = 4.3402$; $p = 0.019156$]. Rats in proestrus had a significantly lower corticosterone serum level than all other groups ($p < 0.05$ for all comparisons).

#### *Effects of age on CORT serum levels after formalin administration.*

As shown in Figure 27, a significant effect for age in CORT serum levels was observed [$F = (3,26)\ 4.14$; $p = 0.051$]; aged rats showed an increase in CORT serum levels when compared to all other groups ($p < 0.01$)

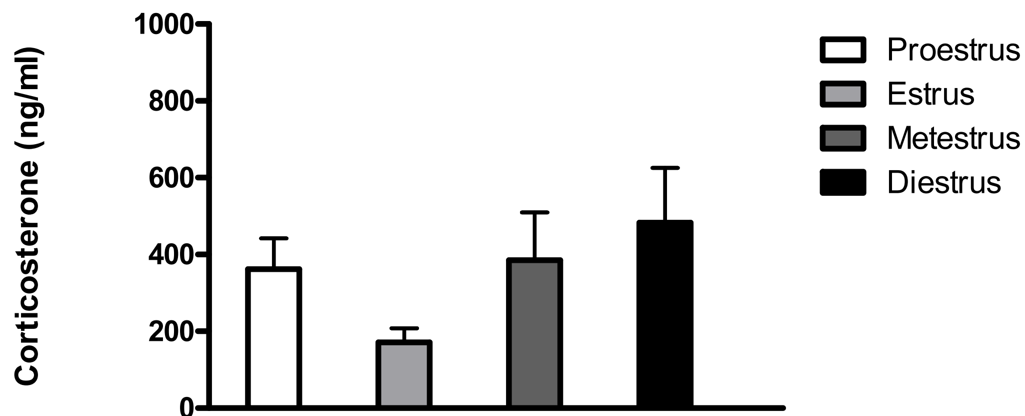



**Figure 25**. **Effects of estrous cycle stage on corticosterone serum levels after formalin administration.** Data represents mean CORT serum levels (± SEM) measured in nanograms per milliters from trunk blood collected after formalin administration (N= 7).

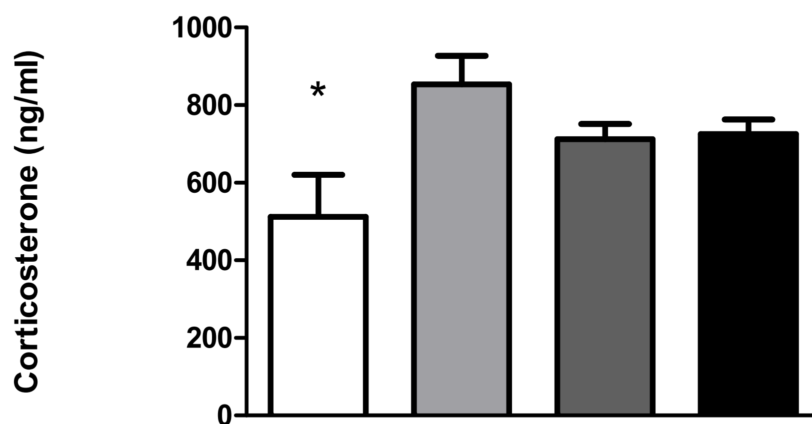



**Figure 26**. **Effects of estrous cycle stage on corticosterone serum levels after carrageenan administration.** Data represents mean CORT serum levels (± SEM) measured in nanograms per milliters from trunk blood collected after carrageenan administration (N= 7/group). (*) Denotes a significantly lower corticosterone serum level in the proestrus group when compared to all other groups.

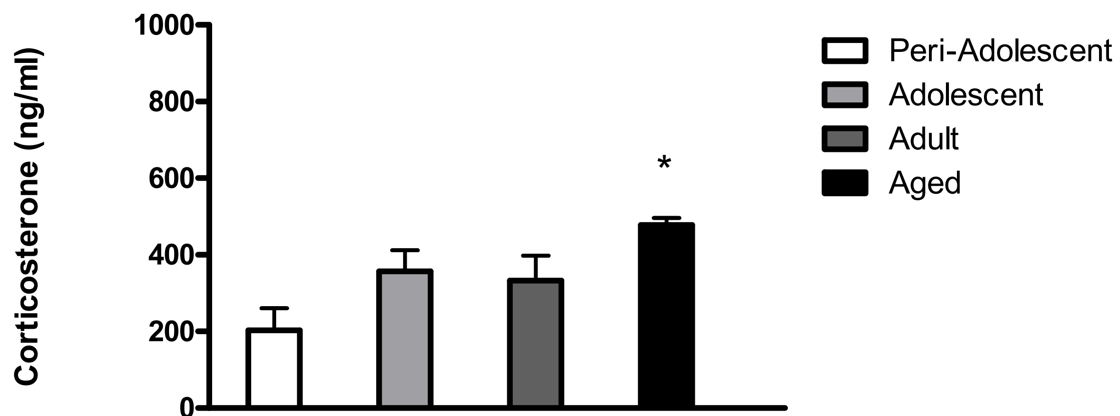



**Figure 27**. **Effects of age on corticosterone serum levels after formalin administration.** Data represents mean CORT serum levels (± SEM) measured in nanograms per milliters from trunk blood collected after formalin administration (N= 7/group). (*) denotes a significantly higher corticosterone serum level in the aged group when compared to all other groups.

**Discussion**

Corticosterone has been shown to exert anti-inflammatory actions, and evidence shows that inflammation contributes to the nociceptive response in the formalin test (Engblom et al., 2002; O'Banion et al., 1992; Samad et al., 2002). Glucocorticoids negatively regulate the inflammatory-induced COX-2 cascade (reduction through gene expression) which results in an attenuation of the inflammatory response (O'Banion et al., 1992). In summary, the HPA axis and corticosterone have been shown to modulate nociceptive responses. Additionally, a relationship between estradiol and corticosterone release and activity has been demonstrated. These findings make the HPA axis and corticosterone ideal targets to examine in the attempts of the current study to identify biochemical pathways that estradiol may be working through to attenuate induced-inflammatory nociceptive responding.

**Effects of estrous cycle stage on CORT serum levels after formalin administration.**

Results show no significant differences in corticosterone serum levels during each phase of the estrous cycle after formalin administration. Although it failed to reach significance, animals in the estrus stage had lower corticosterone serum levels after formalin administration when compared to all other groups. Previous findings have shown a crosstalk between estradiol and the hypothalamic-pituitary-adrenal (HPA) axis (Weiser, 2008), although we failed to see any effect of estrous cycle on corticosterone activity. Perhaps, levels of estrogen used in studies that have gotten these effects, are higher than levels seen in the intact rat. However, more recently we showed that estradiol’s antihyperalgesic effects on formalin-induced flinching are independent of HPA axis activation; namely, in both ADX and OVX+ADX rats, estradiol administration reduced flinching responses to the same level observed in OVX rats. On the contrary, Aloisi et al, (1996) revealed that ACTH levels increasesafter formalin administration in

females, while CORT levels were not affected in either gender (Aloisi et al., 1996). It was suggested that the inconsistent findings may be in part due to the timing of sacrifice being to soon after formalin administration. The conversion on ACTH to CORT along this biosynthetic pathway may take longer than this expreriment allowed (Aloisi et al., 2003; Aloisi et al., 1996). Our experiment may have not allowed enough time for the conversion, but estrogen and progesterone may have effected ACTH levels, which may partly explain the reduction in inflammatory-induced behavior seen during proestrus.

**Effects of estrous cycle stage on CORT serum levels after carrageenan administration.**

We observed a significant effect of estrous cycle on corticosterone serum levels after carrageenan administration. Rats in proestrus had a significantly lower corticosterone serum level than all other groups. This finding is surprising and is not consistent with previous results in our lab. Moreover, findings in our lab revealed that chronic estradiol significantly elevated CORT serum levels while COX-2 protein levels were 25% lower in estradiol-treated rats not receiving formalin compared to vehicle naïve rats (Kuba et al., 2005b). We are seeing the opposite effects in both the formalin and carrageenan studies.

The disparities that are being reported may be in part due to differences in exogenous estradiol and endogenous hormonal fluctuations. However, even though corticosterone is known to exert anti-inflammatory actions (Barnes et al., 1993), our results suggest that expression of corticosterone is not nessesary for mediating inflammatory-induced flinching responses in the intact female rat (Barnes and Adcock, 1995). Perhaps the biosynthetic pathway necessary to stimulate ACTH to produce CORT did not have time to culminate in the formalin study, where animals were sacrificed 1 hour post injection compared to the 5 hour post injection done with carrageenan. Yet to be elucidated is the extent to which estrogen and progesterone regulation of

cytokines and/or HPA axis coupled with activation of the COX-2-PG pathway mediates inflammatory responses.

**Effects of age on CORT serum levels after formalin administration.**

Results demonstrated that aged rats showed an increase in corticosterone serum levels when compared to all other groups after formalin administration. The behavioral responses of the aged group after formalin administration were lower than all the other groups. This supports the idea that an increase in corticosterone serum levels attenuate pain. Ferrini et al., (1993) reported that estrogen upregulates the binding and the expression of glucocorticoid receptors in the brain and in the dorsal horn (Ferrini et al., 1993a). Evidence suggests that gonadal hormones modulate corticosterone secretion and receptor binding with females having greater rates of adrenal steroid secretion in addition to higher ACTH output that seen in males (Ferrini et al., 1993b). If in fact our “aged” rats fall into the “middle age” range, this result would be consistent with previous findings. If the “aged” females had regularly cycling estrogen and progesterone, it may have resulting in an increase in corticosterone levels which then in turn attenuated behavioral responses to inflammatory stimuli.

Kuba et al., (2005) suggested that there is a bi-directional relationship between CORT and $PGE_{2}$, such that an inflammatory stimulus that regulates CORT release and subsequent increases in $PGE_2$ also causes CORT release (Kuba et al., 2005b). Our findings support this study, in which significant increases in corticosterone levels in the aged group were accompanied by significant decreases in $PGE_2$ levels in the same group. We also observed a decrease in inflammatory induced behavior in the aged group, although it failed to reach significance. Taken together these results suggest a relationship between endogenous hormonal fluctuations, corticosterone release and PG activity. These results support previous studies; Aloisi et al,

(1996) discovered that corticosterone levels were not affected in either gender although ACTH levels increased as a result of both 1% and 10% formalin administration in females (Aloisi et al., 1996; Barnes and Adcock, 1995). Several groups have suggested that the inconsistent increase in CORT levels compared to the increased ACTH may be due to sacrifice of animals too soon after formalin administration (Aloisi et al., 1996; Forabosco et al., 1992). Perhaps the biosynthetic pathway necessary to convert ACTH to CORT did not have time to culminate in the formalin study, where animals were sacrificed 1 hour post injection compared to the 5 hour post injection done with carrageenan.

**Conclusion**

In conclusion, the first study used to address Aim 1 suggests that endogenous hormonal fluctuations during the phases of the estrous cycle are working through a pathway of COX-2 regulation on prostanoid production. The use of the formalin model has never before shown an effect of cycle stage on inflammatory pain behavior. Data obtained using the formalin pain model revealed that during proestrus, the stage of the estrous cycle with the highest peaks of estrogen and progesterone, behavioral responses to inflammatory stimuli are attenuated. An additional novel finding from this study was the significant effect of progesterone's second peak in metestrus, causing an increase in behavioral responding after formalin administration. These findings suggest the combination of endogenous estrogen and progesterone during certain stages can attenuate behavioral responses after formalin, while the presence of endogenous progesterone alone, may have the opposite effects. Perhaps, during proestrus, endogenous estrogen attenuates the inflammatory response, but findings using the intact female rat may not be as robust as those seen studies using OVX+estradiol because of the presence of progesterone during this peak of estrogen. If in fact, the peak of progesterone during metestrus was responsible for the increase in the behavioral responding, it appears that progesterone may weaken or counteract estrogens anti-hyperalgesic effects during proestrus.

In the second study used to address Aim 1, we observed a significant increase in $PGD_2$ serum levels in the group that had a significant decrease in behavior, during proestrus. Interestingly, we observed a significant increase in $PGE_2$ serum levels during metestrus, the stage of the cycle with the highest behavioral responses after formalin. It has been postulated that at the spinal cord level, $PGD_2$ blocks the $PGE_2$-evoked pain responses. Our findings provide

evidence of the anti-hyperalgesic effects that are present during proestrus, when estrogen and progesterone are both at their highest levels along with $PGD_2$ being at it highest level, but surprisingly having the opposite effect when only progesterone is peaking and $PGE_2$ is at its highest levels. The levels of $PGE_2$ and $PGD_2$ after inflammation may explain how estrous cycle stage is mediating this process. With estrogen and progesterone peaking during proestrus we saw a significant decrease in behavior and an increase in $PGD_2$. In contrast, during the peak of progesterone we observed a significant increase in behavior and an increase in $PGE_2$ serum levels. It appears that the two hormones may be affecting PGs along two different pathways.

If the combination of peaks in estrogen and progesterone during proestrus are working through the NO/COX-2/PG bio-synthestic pathway, we might expect to see these effects along with an alteration in COX-2 levels. Our study did show an increase in COX-2 levels in the proestrus group, but this finding failed to reach significance. Perhaps if more animals were added to each stage, the result would become more robust. Several studies support this idea; Grill et al., (2006) found after endotoxin treatment, lumiracoxib (a selective COX-2 inhibitor) significantly reduced $PGD_2$ serum levels (Grill et al., 2006). More recently, Cuzzocrea et al., (2007) found that the administration of an estrogen receptor antagonist significantly inhibited the ability of dexamethasone to reduce iNOS and COX expression (Cuzzocrea et al., 2007). To our knowledge, there is no literature on endogenous hormonal fluctuations effects on spinal COX-2 levels after administration of an inflammatory stimulus. Our findings support studies that used exogenous hormones, and also provided novel evidence for progesterone's pro-inflammatory actions. Our results may not be as robust as those using exogenous estrogen replacement, due to the peak of estrogen and progesterone at the same time in the intact cycling animal. Estrogen's anti-inflammatory effects may be partially reversed by progesterone in the intact rat.

In the present study it appears that levels of nNOS are greatest in metestrus in both formalin treated and naïve animals, although it failed to reach significance. The high levels of nNOS that we observed in metestrus accompanied the significant increase in both $PGE_2$ and flinching behavior when compared to animals in proestrus. Cuzzocrea et al., (2001) showed that estradiol treatment inhibits the increase of inducible nitric oxide synthestase (iNOS) activity after inflammation while others have reported that hyperalgesia induced by $PGE_2$ was dependent on NO although they suggested that this may be estrogen dependent (Dina et al., 2001). Our findings suggest that in the cycling rat, progesterone may play a key role in the increased sensitivity to the inflammatory injury via its actions on the $PGE_2$ pathway, perhaps via NOS. Once again, if each group had a greater number of animals with less variability, our results might have reached significance. Taken together these results support the hypothesis that fluctuations in estrogen and progesterone have an effect on inflammatory mediators. We provided evidence for the separate actions of endogenous estrogen and progesterone, and their effects on inflammation.

The first study used to address Aim 2 showed that our findings from the first Aim may not extend to other inflammatory stimuli. We did not observe any significant differences in PWL between groups after carrageenan administration. At the medium heat 5 hour test, females in proestrus appeared to have a lower PWL that all other groups, although this failed to reach significance. Previous findings in our lab have shown that estradiol (EB) dose dependently increases PWL responses with the 20% EB being the most effective dose. We may have not gotten the effect in the intact rat, with the peaks and troughs of the endogenous hormones that differ from a dose of estrogen in an OVX+estradiol rat.

Surprsingly, we demonstrated an estrous cycle effect on contralateral PWL. At baseline, animals in estrus had significantly higher paw withdrawl latencies when compared the animals metestrus, while at 1 hour animals in estrus had significantly shorter latencies than animals in metestrus or diestrus. Animals in estrus having the shorter PWL at 1 hour, is consistent with our hypothesis, demonstrating that in the absence of estrogen, the intact female shows hyperalgesic behavior. This finding could be the result of the 4 day estrous cycle in the female rat; post-mortem lavage samples were taken and several of the rats had moved on to the next stage or were in-between stages of the cycle after the 5 hour test. In addition, the hyperalgesic responses seen in the contralateral paw during estrus may be in part mediated by absence of estrogen and progesterone. Taken together these findings suggest differences in mechanisms associated with these two pain models.

In the second study used to address Aim 2, we found that rats in estrus had significantly higher $PGE_2$ levels than rats in metestus and diestrus. An increase of these serum levels in the stage where estrogen and progesterone are coming down from their peaks, suggests a partial mediation in this pathway. The estrous group also showed a decrease in PWL at 1 hour which may be a result of the increases in $PGE_2$. The drop in endogenous hormones during the estrous cycle appears to alter estrogen's anti-hyperalgesic effects. It appears, that in the absence of the endogenous estrogen peak, there are greater levels of $PGE_2$ accompanied by an increase in behavioral responding to this inflammatory stimulus. An alternate explanation for these results is that rats may have moved from estrus on to metestrus, when the second progesterone peak occurs. If the rat had entered metestrus during the study, our results would be consistent with our findings using formalin.

Although we observed significant effects of estrous cycle on $PGD_2$ levels after formalin administration, we saw no effects after carrageenan administration. This could be in part due to the time of sacrifice, where PG's may be active soon after the inflammatory insult, explaining why we observed effects in $PGD_2$ after formalin (1 hour) and not 5 hours after the carrageenan insult. Thus, it is feasible that the magnitude or intensity of the inflammatory stimulus, produces more severe and longer lasting inflammatory responses which in turn, alters the estrous cycle's effects of mechanisms controlling inflammation.

In the third study used to address Aim three, we did not observe any chances in nNOS levels in the spinal cord after carrageenan administration. Although, it appears that nNOS levels are once again highest in rats during metestrus. The results appear to be similar in both the formalin and carrageenan studies, providing a reason to add animals to these groups which may result in more robust effects and less variability. Taken together, we observed that at times with the lowest estrogen and progesterone levels, there is an increase in hyperalgisic responses which may mediated through the NO/COX/PG bio-synthetic pathway.

The results from Aim 4 demonstrated no significant effect of age on inflammatory induced behavior. Aged females had a decrease in behavioral responding during Phase II, although this failed to reach significance. As discussed in the introduction, the data that is available for rodents are mostly based on tests using phasic or brief, transient pain and to date, a widely accepted criteria for age classification are not available (Gagliese and Melzack, 2000). Recently Frick et al., (2009) reviewed age studies in rodents and concluded that rodents are typically considered "aged" when they are approximately 2 years old, "middle aged" are 16-18 months and "young" are 3-4 months when used for studies of memory (Frick KM., 2009). For our studies, ages of the rats were divided into peri-adolescent (3 weeks), adolescent (6 weeks),

adult (8 weeks) and aged (10-11 months). The way in which we grouped the animals may be responsible for the surprising results that we observed. Our “aged” group was closer in age to what is now classified as “middle aged” which would have a great effect on our study.

We found a significant increase in $PGE_2$ serum levels in peri-adolescent and adolescent animals, while $PGD_2$ was significantly higher only in the peri-adolescent group. During this time in life, younger rats have lower estrogen levels than those seen in adult animals, which may explain why the $PGE_2$ levels were higher in these groups. This provides evidence suggesting separate mechanisms for $PGE_2$ and $PGD_2$, given that no behavioral effects were seen in the group with the highest $PGE_2$ and $PGD_2$ levels. Interestingly, the peri-adolescent group had an increase in both $PGE_2$ and $PGD_2$ when compared to the aged group. The increase in PG’s in the peri-adolescent group may be a result of low levels of endogenous estrogen and progesterone, causing the negation of $PGE_2$ pro-inflammatory effects by $PGD_2$. These findings support the notion that expression of $PGD_2$ may play a role in the resolution of inflammation (Scher and Pillinger, 2009).

The first study used to address Aim 5 revealed that corticosterone may not be a mediator which the estrous cycle uses to alter responses to inflammation. Results show no significant differences in corticosterone serum levels during each phase of the estrous cycle after formalin administration. Although it failed to reach significance, animals in the estrus stage had lower corticosterone serum levels after formalin administration when compared to all other groups. This observation is in line with the hypothesis that the absence of endogenous estrogen results in an increase in pro-inflammatory responses to pain.

We observed a significant effect of estrous cycle on corticosterone serum levels after carrageenan administration. Surprisingly, rats in proestrus had a significantly lower

corticosterone serum level than all other groups. This decrease in corticosterone during proestrus, suggests that expression of corticosterone may not nessessary for mediating inflammatory-induced flinching responses in the intact female rat. Many studies have reported an increase in corticosterone levels after administration of estradiol, although in the present study we have found that during proestrus, when estrogen and progesterone are present in the intact female, no such effect is observed. As previously reported, we did not observe an attenuation in behavioral responding for the proestrus group after carrageenan. Due to the time of sacrifice, 5 hours post-injection, this group could have entered estrus, which would be a time of the lowest estrogen and progesterone levels. This may also explain why the proestrus group had the lowest levels of corticosterone.

Results demonstrated that aged rats showed an increase in corticosterone serum levels when compared to all other groups after formalin administration. The behavioral responses of the aged group after formalin administration were lower than all the other groups. If in fact these animals are in "perimenopause" higher levels of urinary estrogen and lower levels of progesterone would be seen when compared to younger animals. If estrogen is higher in this group, and estrogen alone is mediating its anti-hyperalgesic effects through corticosterone, our findings support the idea that an increases in endogenous estrogen accompanies increases corticosterone serum levels which in turn, attenuate pain.

Based on the data collected in all five studies, it is likely that endogenous hormonal effects are in part mediated through regulation of the PG biosynthetic pathway. There are a number of other inflammatory mediators that directly contribute to nociceptive and inflammatory responses after noxious stimuli such as: mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/ERK), PKA, PKC, cAMP response element binding (CREB), and cyclic

adenosine monophosphate (cAMP) (Bester et al., 1995). These bio-chemical mediators and pathways associated with PG production and activation present alternative and perhaps extended routes by which endogenous hormonal effects may be mediated. Further examination into these prospective alternatives can provide valuable insight into mechanisms associated with estrogen and progesterone's effects on nociceptive and inflammatory processes.

Specifically, examination of endogenous estrogen and progesterone's effects on inflammatory mediators which are downstream from the NO/COX and PG pathway in the inflammatory processes may provide crucial insight into the extent of the hormonal effects. Chemical mediators released during inflammation not only activate nociceptors, but may lead to changes in the sensory neurons rather than directly activating them. Prostanoid signaling is mediated by second messengers such as protein kinase A (PKA) and PKC, which regulate the activity of many receptors and ion channels and contribute to the potentiation of primary sensory neurons leading to peripheral sensitization (Samad et al., 2002). Inflammatory mediators such as $PGE_2$ have been shown to activate PKA and PKC (Bester et al., 1995). Specifically, the $PGE_2$ modulation of the channels involves the activation of adenylyl cyclase and increases in cAMP, leading to a PKA phosphorylation of the channels (Bester et al., 1995). PKA phosphorilation then sensitizes nociceptors to heat by modulating activity of tetrodotoxin-resistant sodium currents, resulting in an increase in the pain response.

Estrogen has been shown to mediate genomic mechanisms by its binding to membrane-bound receptors that are coupled to various intracellular second messenger systems: MAPK/ERK, PKA, PKC, CREB) (Martin and Behbehani, 2006a). The molecular products of the secondary messenger systems activated by endogenous hormones may then modulate transcription of specific genes involved in the PG pathway. Singer et al. (1999) demonstrated that the inhibition

of MAPK signaling with a MAPK inhibitor or the anti-estrogen (ICI 182, 780), blocked estrogen's neuroprotective affects in cortical neurons (Singer et al., 1999). More recently, Wang et al. (2006) demonstrated that E2 supplementation in the OVX rat decreased the activation of p38 MAPK, which is activated after injury (Wang et al., 2006). It appears that estrogen is working through excitation and/or inhibition of the MAPK/ERK signaling pathway. It would be interesting to probe for MAPK/ERK, PKA, PKC, and CREB to observe the changes in this system in the presence or absence of endogenous hormones after an inflammatory insult and how these potential changes impact the expression of PGs.

In addition to evaluating these second messengers, it would be interesting to investigate if the activation and/or inhibition of MAPK/ERK leads to a change in expression of genes associated with PG conversion. If endogenous estrogen is exerting its anti-hyperalgesic effects via PG's, it is possible that the activation of second messengers by estrogen is expressing proteins that may alter PG gene transcription. Several studies have found that after treatment with a MAPK/ ERK inhibitor, cells failed to release several pro-inflammatory mediatiors including $PGE_2$. Moon and Befus (2008) demonstrated that NO increases COX-2-dependent $PGD_2$ through the p38 MAPK pathway (Moon and Befus, 2008). LPS treatment induces MAPK-activated PGDS expression in macrophages, which further supports the idea that this may be a pathway in which estrogen may exert its antihyperalgesic effects (Joo et al., 2009). Taken together, these findings demonstrate the MAPK/ERK pathway's involvement in inflammatory mediation via PGs. If estrogen and/or progesterone are exerting their effects via PGs, it would be note worthy to look at endogenous hormonal fluctuations and their effects on the MAPK/ERK pathway.

Perhaps, endogenous hormones activate a second messenger cascade that effects the production of PG synthases, via transcription of a gene that translates to a protein for a PG enzime. PG synthases, PTGES, PTGES2, and PTGES3 are responsible for converting $PGH_2$ into $PGE_2$, while PGDS is responsible for the conversion of $PGH_2$ to $PGD_2$ (Bester et al., 1995). Kamei et al. (2004) showed a reduction in pain hypersensitivity and inflammation in mice lacing PGE synthase-1. Furthermore, other studies have reported similar responses to inflammation in animals with a disruption in this particular synthase (Kamei et al., 2004; Langenbach et al., 1995). Rajakariar et al. (2007) reported the effects of the PGD synthase activation on the resolution of acute inflammation, providing further evidence for this pathway being involved in both pro- and anti-inflammatory processes (Rakajumar et al., 1987). After analyzing the results of the present study, edogenous hormones appear to exert their effect via the alteration of prostaglandin biosynthesis pathways. It would be promising to investigate the expression of the PGE and PGD synthases in the presence of endogenous hormonal peaks and troughs during the estrous cycle, and how exactly these hormones alter PG expression and activation.

As demonstrated in the current literature, estradiol is a very effective analgesic and anti-hyperalgesic. Its use as an alternative form of pain therapy promises to be bountiful. However, more work will be required to ensure that its clinical use and benefits are maximized while unwanted side effects are minimized. Our results begin to uncover and advance the understanding of the mechanisms in which endogenous hormones exert their effects on inflammation. Bradshaw et al., (2000, 2003) revealed that normal fluctuating hormonal levels such as those seen in rodents during estrous cycle compared to ovariectomized or ovariectomized with estradiol replacement were responsible for

profound differential effects on neuronal activity as well as nociceptive responding (Bradshaw and Berkley, 2003; Bradshaw and Berkley, 2000). This phenomenon makes it extremely complicated to make predictions based on anyone of these methods of examining hormonal effects. Specifically, it was noted that responses associated with different phases of the estrous cycle could not be directly correlated to responses achieved in the presence of estradiol replacement even if the estradiol dose was comparable to the percentage of endogenous hormone level associated with a specific stage.

It would be beneficial to measure port-mortem hormone serum levels in addition to the vaginal smears taken on the day of testing. This would provide critical information when using a test that spans hours, because the rat is likely to have alterations in hormonal levels during the experiment. Using analysis of cell types in vaginal lavages before testing and hormonal serum level analysis after testing, would aid in clarification of hormonal levels throughout the assay.

Based on these findings it is suggested that endogenous hormonal effects are probably more closely related to changes in hormones levels that accompany normally cycling females rather than a specific dose or hormone percentage at a given time. This is one of the many aspects that will have to be addressed when discussing findings with OVX+estrogen treated rats and results seen in the intact female rat.

Additionally, male subjects and immediate comparisons to male subjects continues to illuminate the origin of differential responding linked to sex hormones. By ensuring that both sexes are used in studies targeted at examining the origins of estrogen and progesterone's effects on inflammatory pain data collected can provide useful and indispensable insight to be applied to the population, both sexes. As has been the case in

past and still today, studies examining of gonadal hormones effects on nociceptive processes show a lot of variability and lack of convergence. Numerous factors continue to contribute to these inconsistencies often making it extremely difficult to set up experiments that actually examine and isolate the specific questions. For example, some studies show that testosterone yields a protective effects via anti-nociceptive actions while estradiol may be pro-nociceptive contributing to the difference in pain behavior in both human and animal studies (Aloisi et al., 2004; Yeung and Rudy, 1980). Some studies suggest otherwise. For example, Caliborne et al., (2006) demonstrated that testosterone replacement in males restored spinal nociception, while estradiol in females attenuated this response (Claiborne et al., 2006). Stoffel et al.,(2005) showed that both testosterone and estradiol do not consistently modulate analgesic effects in adult rats thus many measures have to be taken to be able to reliably predict the outcome of modifiers both exogenous and endogenous on analgesic effects (Stoffel et al., 2003).

In summary, the influence of sex hormones on the incidence and development of pain is ubiquitous and much work remains to be done to elucidate the specific pathways responsible for these differences. By further investigating the specific pathway(s) believed to be responsible for the effects of estrogen and progesterone on inflammation we move that much closer to identifying the mechanism(s) that are responsible for these effects. These findings can then be used to manage differential pain treatment dependent on a patient's sex and stage of the reproductive cycle.

According to both clinical and preclinical literature, pain management targeted at female patient should consider hormonal factors during the female reproductive cycle. For example, because hormonal changes that accompany the female menstrual cycle can

be bi-directional (producing both pro-nociceptive and anti-nociceptive effects), consideration should be given to the patient's stage of the menstrual cycle and/or her age when managing her pain. It is difficult to ascribe effects to one particular hormone because both are present and appear to interact to modulate eachother's effects. Futhermore, oral contraceptives and hormone replacement therapy (HRT) should be considered. Observations from clinical studies suggest a need for a systematic collection of data reguarding the individual hormonal environment and its effects on inflammation.

Based on our findings, we propose the following model in which estrogen and progesterone mediate behaviors following an inflammatory stimulus via activation of second messengers which bind to DNA regulatory regions such as CREB, PKA, PKC, and MAPK/ERK, affecting the synthesis and/or the actions of PGs.

The introduction of an inflammatory stimulus activates inflammatory cells to increase in intracellular $Ca^{2+}$ that occurs at the NMDA receptor site during inflammation results in the activation of intracellular enzymes, phospholipase $A_2$ ($PLA_2$) and nitric oxide synthase (NOS). The actions of $PLA_2$ enzymes leads to the release of arachidonic acid (AA) phospholipids. AA is then metabolized by COX enzymes, resulting in an expression of its metabolites. The initial step in the formation of PGs is the conversion of free AA to $PGG_2$, which then is reduced to $PGH_2$ by COX-1 and COX-2 enzymes. Endogenous hormones may activate a second messenger cascade that effects the production of PG synthases, via transcription of a gene that translates to a protein for a PG enzime. Tissue specific PG synthases (PTGES, PTGES2, PTGES3 and PGDS) then convert $PGH_2$ into various isoforms of PGs and thromoboxanes (i.e. $PGD_2$ $PGF_{2\alpha}$, $PGE_2$, $PGI_2$, and $TXA_2$). These are all produced during inflammation and exert their biological actions through

seven-transmembrane domain, G-protein coupled receptors: DP, FP EP (EP1, EP2, EP3, EP4), IP, and TP receptors. Reduction in the activation or expression of pro-inflammatory PGs in conjunction with activation of anti-inflammatory PGs will result in attenuated inflammatory responses.

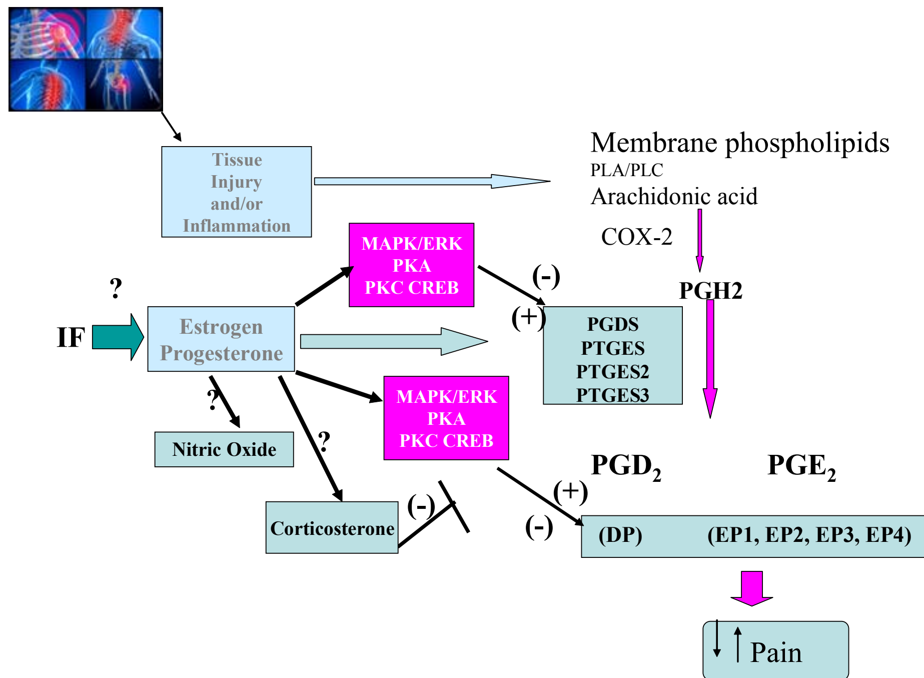



**Figure 28.** Proposed model of endogenous estrogen and progesterone's role in mediating inflammatory behavioral responses.

# Reference List

Ahamadi, S., Lippross, S., Nehuber, W., Zeilhofer, H., 2002. PGE2 selectively blocks inhibitor glycinergic neurotransmission onto the superficial dorsal horn neurons, Nature Neuroscience 5.

Ali, Z., Meyer, R.A., Campbell, J.N., 1996. Secondary hyperalgesia to mechanical but not heat stimuli following a capsaicin injection in hairy skin., Pain 68: 401-411.

Almeida, A., Lima, D., 1997. Activation by vutaneous or visceral noxious stimulation of spinal neurons projecting to the medullary dorsal reticular nuceus in the rat: c-fos study., Eur J Neurosci 9: 686-695.

Aloisi, A.M., 1997. Sex differences in pain-induced effects on the septo-hippocampal system, Brain Res.Rev. 25: 397-406.

Aloisi, A.M., Albonetti, M.E., Carli, G., 1994. Sex differences in the behavioural response to persistent pain in rats, Neurosci Lett 179: 79-82.

Aloisi, A.M., Ceccarelli, I., Fiorenzani, P., 2003. Gonadectomy affects hormonal and behavioral responses to repetitive nociceptive stimulation in male rats, Ann N Y Acad Sci. 1007: 232-237.

Aloisi, A.M., Ceccarelli, I., Fiorenzani, P., De Padova, A.M., Massafra, C., 2004. Testosterone affects formalin-induced responses differently in male and female rats, Neurosci Lett 361: 262-264.

Aloisi, A., Albonetti, M., Carli, G., 1996. Formalin-induced changes in adrenocorticotropic hormone and corticosterone plasma levels and hippocampal choline acetyltransferase activity in male and female rats., Neuroscience 74: 1019-1024.

Apkarian, A.V., Stea, R.A., Manglos, S.H., Szevernyi, N.M., King, R.B., Thomas, F.D., 1992. Persistant pain inhibits contralateral somatosensory cortical activity in humans., Neurosci.Lett. 140: 141-147.

Arabshashi, B., Dewitt, E., Cahill, A., Kaye, R., Baskin, K., Towbin, R., Cron, R., 2005. Utility of corticosteroid injection for temporomandibular arthritis in children with juvenile idiopathic arthrit, Arthritis Rheum. 52: 3563-3569.

Arjune, D., Bodnar, R.J., 1989. Post-natal morphine differentially affects opiate and stress analgesia in adult rats, Psychopharmacology (Berl). 98: 512-517.

Azkue, J.J., Knopfel, T., Kuhn, R., Mateos, J.M., Grandes, P., 1997. Distribution of the metabatropoc glutamate receptor subtype mGluR5 in the rat midbrain periaqueductal grey and relationship with ascending spinofugal afferents., Neurosci.Lett. 228: 1-4.

Baba, H., Kohno, T., Moore, K.A., Woolf C.J., 2001. Direct Activation of Rat Spinal Dorsal Horn Neurons by Prostaglandin E2, Journal of Neuroscience 21: 1750-1756.

Ballou, L., Botting, R., Goorha, S., Zhang, J., Vane, J., 2000. Nociception in cyclooxygenase isozyme-deficient mice, Proc Natl Acad Sci U.S.A 97: 10272-10276.

Barnes, P.J., Adcock, I., 1995. Anti-inflammatory actions of steroids: molecular mechanism, Trends Pharmacol Sci 14: 436-441.

Beiche, F., Scheuerer, S., Brune, K., Gesslinger, G., Gopelt-Struebe, M., 1996. Upregulation of cyclooxygenase-2 mRNA in the rat spinal chord following peripheral inflammation, FEBS Letters 8390: 165-169.

Bennett, G. J. Animal Models of Pain. In: Kruger, L., ed. Methods of Pain Research. Boca Ratan, FLA: CRC Press; 2001: 67-92.

Bennett, M., 2001. Pain assessment and management in pediatric intensive care, Pediatric nursing 13: 26-29.

Berkley, K.J., Hubscher, C.H., 1995. Are there separate central nervous system pathways for touch and pain?, Nature Medicine 1: 766-773.

Bester, H., Menendez, L., Besson, J.M., Bernard, J.F., 1995. Spino (trigemino) parabrachiohypothalamic pathway: electrophysiological evidence for and involvement in pain processes., Journal of Neurophysiology 73: 568-585.

Beydoun, A., Backonja, M.M., 2003. Mechanistic Stratification of Antineuralgesic agents, Pain Symtom Management 25: S18-30.

Bingefors, K., Isacson, D., 2004. Epidemiology, co-morbidity, and impact on health-related quality of life of self-reported headache and musculoskeletal pain - a gender perspective, Eur J Pain 8: 435-450.

Bos, C., Richel, D.J., Ritsema, T., Peppelenbosch, M., Versteeg, H.H., 2004. Prostanoids and prostanoid receptors in signal transduction., Int J Biochem and Cell Bio 36: 1187-1205.

Bradshaw, H., Miller, J., Ling, Q., Malsnee, K., Ruda, M.A., 2000. Sex differences and phases of the estrous cycle alter the response of spinal cord dynorphin neurons to peripheral inflammation and hyperalgesia, Pain 85: 93-99.

Bradshaw, H., Berkley, K., 2003. The influence of ovariectomy with or without estrogen replacement on responses of rat gracile nucleus neurons to stimulation of hindquarter skin and pelvic viscera, Brain Res. 986: 82-90.

Bradshaw, H., Berkley, K., 2000. Estrous changes in response properties of rat gracile nucleus neurons to stimulation of the skin and pelvic viscera, J Neurosci. 20: 7722-7727.

Breder, C.D., deWitt, D., Kraig, R.P., 1995. Characterization of inducible cyclooxygenase in rat brain, Journal Comp Neurol 355: 296-315.

Burstein, R., Dado, R.J., Giesler, G.J., 1990. The Cells of origin of the spinothalamic tract of the rat: a quantitative reexamination, Brain Res 511: 329-337.

Bushnell, M. C.; Apkarian, A. V. Representation of Pain in the Brain. In: McMahon S.B.; Koltzenburg M., eds. Textbook of Pain. London: Churchill Livingston; 2006: 107-124.

Calvino, B., 2006. Neural basis of pain., Psychol Neuropsychiatr Vieil 4: 7-20.

Camacho-Arroyo, I., Gonzalez-Arenas, A., Gonzalez-Aguero, G., Guerra-araiza, C., Gonzalez-Moran, G., 2003. Changes in the content of progesterone receptor isoforms and estrogen receptor alpha in the chick brain during embryonic development, Comparative Biochemistry and Physiology 136: 447-452.

Casey, K.L., Minoshima, S., Morrow, T.J., Koeppe, R.A., 1996. Comparison of human cerebral activation pattern during cutaneous warmth, heat pain, and deep cold pain., Journal of Neurophysiology 76: 571-581.

Caterina, M.J., Schumacher M., Tominaga, M., Rosen, T.A., Levine, J.D., Julus, D., 1997. The capsaicin receptor: a heat activated ion channel in the pain pathway., Nature 389: 816-824.

Ceccarelli, I., Fiorenani, P., Massafra, C., Aloisi, A.M., 2003. Long-term ovariectomy changes formalin-induced licking in female rats; the role of estrogens, Reprod Biol Endocrinol 1: 1-24.

Claiborne, J., Nag, S., Mokha, S.S., 2006. Activation of opioid receptor like-1 receptor in the spinal cord produces sex-specific antinociception in the rat: estrogen attenuates antinociception in the female, whereas testosterone is required for the expression of antinociception in the male., J Neurosci 26: 13048-13053.

Clancy, R., Varenika, B., Huang, W., Ballou, L.R., Attur, M., Amin A.R., Abramson, S.B., 2000. Nitric Oxide Synthase/COX crosstalk: Nitric Oxide Activates COX-1 But Inhibits COX-2-Derived Prostaglandin Production., Journal of Immunology 165: 1582-1587.

Coderre, T.J., Vaccarino, A.L., Melzack, R., 1990. Central nervous system plasticity in the tonic pain response to subcutaneous formalin injection., Brain Res 535: 155-158.

Coderre, T.J., Yashpal, K., 1994. Intracellular messengers contributing to persistent nociception and hyperalgesia induced by L-glutamate and substance P in the rat formalin pain model, Eur J Neurosci 6: 1328-1334.

Colvin, L., Power, I., 2005. Neurobiology of chronic pain states, Anaesthesia and intensive care medicine 6: 10-13.

Costigan, M., Woolf, C., 2000. Pain: Molecular Mechanisms, The Journal of Pain 1: 35-44.

Crisp T, S.J.H.D.P.V.U.M.G.TL., 1994. Age-related changes in the spinal antinociceptive effects of DAGO, DPDPE and beta-endorphin in the rat., Brain Res 18: 282-286.

Cuzzocrea S, Mazzon E, Sautebin L, Serraino I, Dugo L, Calabro G, Caputi AP, Maggi A, 2001. The protective role of endogenous estrogens in carrageenan-induced lung injury in the rat., Mol Med. 478.

Cuzzocrea, S., Bruscoli, S., Crisafulli, C., Mazzon, E., Agostini, M., Muia, C., Esposito, E., Di Virgilio, R., Meli, R., Vegeto, E., Maggi, A., Riccardi, C., 2007. Estrogen receptor antagonist fulvestrant (ICI 182,780) inhibitis the anti-inflammatory effect of glucocorticoids, Mol Pharmacol. 71: 132-144.

Da Silva, J.A., Hall, J.A., 1992. The effects of gender and sex hormones on outcome in rheumatoid arthritis, Baillieres Clin Rheumatol 6: 196-219.

Da Silva, J., Peers, S., Perretti, M., Willoughby, D., 1993. Sex steroids affect glucocorticoid response to chronic inflammation and to interleukin-1., J Endocrinol 136: 389-397.

Daher, J B and Tonussi, C R, 2003. A spinal mechanism for the peripheral anti-inflammatory actiom of indomethacin, <[11] Journal Name> 962: 207-212.

Dale, C., Pagano, R.L., Rioli, V., Hyslop, S., Giorgi, R., Ferro, E., 2005. Antinociceptive action of hemopressin in experimental hyperalgesia, Peptides 26: 431-436.

del Olmo, N., Miguens, M., Higuera-Matas, A., Torres, I., Garcia-Lecumberri, C., Solis, J.M., Ambrosio, E., 2006. Enhancement of hippocampal long-term potentiation induced by cocaine self-adminsitration is maintained during teh extinction of this behavior, Brain Reseach 1116: 120-126.

DeLeo, J.A., 2006. Basic Science of Pain, Journal of Bone and Joint Surgery 88A : 58-62.

Dickenson, A.H., 1997. NMDA receptor antagonists: interactions with opioids, Acta Anaesthesiologica Scandinavica 41: 112-115.

Dickenson, A.H., Sullivan, A.F., 1987a. Peripheral origins and central modulation of subcutaneous formalin-induced activity of rat dorsal horn neurons., Neurosci.Lett. 16: 207-211.

Dickenson, A.H., Sullivan, A.F., 1987b. Subcutaneous formalin-induced activity of dorsal horn neurons in the rat: Differential response to an intrathecal opiate administered pre or post formalin., Pain 30: 349-360.

Dina, O.A., Aley, K.O., Isenberg, W., Messing, R.O., Levine, J.D., 2001. Sex hormones regulate the contribution of PKCepsilon and PKA signalling in inflammatory pain in the rat., Eur J Neurosci 13: 2227-33.

Engblom, D., Ek, M., Saha, S.E.-D.A., Jakobsson.PJ, Bomqvist, A., 2002. Prostaglandins as inflammatory messengers across the blood brain barrier, J Mol Med 80: 5-15.

Euchenhofer, C., Maihofner, C., Brune, K., Tegeder, I., Geisslinger, G., 1998. Differential effect of selective cyclooxygenase-2 (COX-2) inhibitor NS 398 and diclofenac on formalin-induced nocicption in the rat., Neurosci.Lett. 248: 25-28.

Ferrini, M., & I.Elman, A., +1, 1993a. Estradiol increases glucocorticoid binding and glucocorticoid induction of ornithine decarboxylase in the rat spinal cord., Life Sci 52: 677-685.

Ferrini, M., Gonzalez, S., Antakly, T., DeNicola, A., 1993b. Immunocytochemical localization of glucocorticoid receptors in the spinal cord: effects of adrenalectomy, glucocorticoid treatment, and spinal cord transection., Cell Mol Neurobiol 13: 387-397.

Fillingim, R.B., 2000. Sex, gender and pain: women and men really are different, Curr.Rev.Pain 4: 24-30.

Fillingim, R.B., Maixner W., Girdler, S.S., Light, K.C., Harris, M.B., Sheps, D.S., Mason, G.A., 1997. Ischemic but not thermal pain sensitivity varies across the menstrual cycle, Psychosom Med 59: 512-520.

Fillingim, R. B.; Maixner, W. Sex-Related Factors in Temporomandibular Disorders. In: Fillingim, R. B., ed. Sex, Gender and Pain. Seattle: IASP Press; 2000: 309-326.

Finch C.E., Felicio, L.S., Mobbs, C.V., Nelson, J.F., 1984. Ovarian and steroidal influences on neuroendocrine again processes in female rodents, Endocr.Rev. 5: 467-497.

Fishman, S., Teichera, D., 2003. Challenges and choices in drug therapy for chronic pain, Cleve Clinical Journal of Medicine 70: 119-121.

Forabosco, A., Criscuolo, M., Coukos, G., Uccelli, E., Weinstein, R., Spinato, S., Botticelli, S., Volpe, A., 1992. Efficacy of hormone replacement therapy in postmenopausal women with oral discomfort, Oral Surg Oral Med Oral Pathol Oral Radiol Endod 73: 570-574.

Frick KM., 2009. **Estrogens and age-related memory decline in rodents: what have we learned and where do we go from here?**, Horm Behav. 55: 2-23.

Gagliese, L., 2009. Pain and Aging: The emergence of a New Subfield of Pain Research, The Journal of Pain 10: 343-353.

Gagliese, L., Melzack, R., 1997. Chronic Pain in Elderly People, Pain 70: 3-14.

Gagliese, L., Melzack, R., 1999. Age differences in the response to the formalin test in rats., Neurobiol.Aging 20: 699-707.

Gagliese, L., Melzack, R., 2000. Age differences in nocicpetion and pain behaviors in the rat, Neurisci Behav Rev. 24: 843-854.

Gaumond, I., Arsenault, P., Marchand, S., 2002. The role of sex hormones on formalin-induced nociceptive responses, Brain Res 958: 139-145.

Gervais, F.G., Cruz, R.P., Chateauneuf, A., Gale, S., Sawyer, N., Nantel, F., Metters, K.M., O'neill, G.P., 2001. Selective modulation of chemokinesis, degranulation, and apoptosis in eosinophils through the PGD2 receptors CRTH2 and DP., Journal of Allergy Clinial Immunology 108: 982-988.

Ghafoor, V.L., 2003. Treatment of Chronic Pain, Journal of Pharamacy Prcatice 16: 249-260.

Ghirardi O, C.A.R.M.A.L., 1994. Effect of long-term acetyl-L-carnitine on stress-induced analgesia in the aging rat., Exp Gerontol. 29: 569-574.

Giamberardino, M.A., Affaitati, G., Valente, R., Iezzi, S., Vecchiet, L., 1997. Changes in visceral pain reactivity as a function of estrous cycle in female rats with artificial ureteral calculosis, Brain Res 744: 234-238.

Girardot MN, Holloway FA, 1985. Effect of age and long-term stress experience on adaptation to stress analgesia in mature rats: role of opioids., Behavioral Neuroscience 99: 411-422.

Goettl VM, L.A.N.N.H.M., 2000. GM1 ganglioside restores abnormal responses to acute thermal and mechanical stimuli in aged rats., Brain Res. 858: 380-385.

Gold, M., 1999. Tetrodoxin-resistant sodium currents and inflammatory hyperalgesia, Proc Natl Acad Sci U.S.A 42: 111-112.

Grill, M., Peskar, B.A., Schuligoi, R., Amann, R.P., 2006. Systematic inflammation induces COX-2 mediated pristaglandin D2 biosynthesis in mice spinal cord., Neuropharmacology 50: 165-173.

Groth, R., Aanonsen, L., 2002. Spinal brain-derived neurotrophic factor (BDNF) produces hyperalgesia in normal mice while antisense directed against either BDNF or trkB, prevent inflammation-induced hyperalgesia, Pain 100: 171-181.

Guay, J., Bateman, K., Gordon, R., Macini, J., Riendeau, D., 2004. Carrageenan-induced Paw Edema in Rat Elicits a Predominant Prostaglandin E2 Response in the Central Nervous System Associated with the Induction of Microsomal PGE2 Synthase-1., Journal of Biological Chemistry 279: 24866-24872.

Hamm, Knisely, 1986. The analgesia produced by food deprivation in 4-month old, 14-month old, and 24-month old rats., Life Sci 39: 1509-1515.

Harbuz, M.S., 2002. Neuroendocrine function and chronic inflammatory stress., Exp Physiology 87: 519-525.

Harbuz, M.S., Chover-Gonzalez, A.J., Gilbert-Rahola, J., Jessop, D.S., 1999. Is there a deficit in cortisol production in rheumatoid arthritis?, Rheumatology 38: 298-302.

Hargreaves, K, Dubner, R, Brown, C, Joris, F, and Joris, J, 1988. A new sensitive method for measuring thermal nocicpetion in cutaneous hyperalgesia, <[11] Journal Name> 32: 77-88.

Hata, A. N and Breyer, R. M, 2004. Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation, <[11] Journal Name> 103: 147-166.

Hellstrom, B., Anderberg, U.M., 2003. Pain perception across the menstrual cycle phases in women with chronic pain, Percept Mot Skills 96: 201-211.

Helme, R., Katz, B., Gibson, S., Corran, T., 1989. Can psychometric tools be used to analyze pain in a geriatric population?, Clin.Exe.Neurol. 26: 113-117.

Henriques, MG, Silva, PM, Martins, MA, Flores, CA, Cunha, FK, Assreuy-Filho, J, and Cordeiro, RS, 1987. Mouse paw edema. A new model for inflammation, <[11] Journal Name> 20: 243-249.

Hofacker, A., Coste, O., Ngyuen, H.-V., Marian, C., Klaus, S., Gesslinger, G., 2005. Down regulation of cytolic prostaglandin E2 synthase results in decreased nociceptive behavior in rats, journal neuroscience 25: 9005-9009.

Hope BT, Nagarkar D, Leonard S, Wise RA, 2007. Long-term upregulation of protein kinase A and adenylate cyclase levels in human smokers, J Neurosci 27: 1964-1972.

Horiguchi, S., Ueno, R., Hyodo, M., Hayaishi, O., 1986a. Alterations in nociception after intracisternal administration of prostaglandin D2, E2 or F2 alpha to conscious mice., Eur J Pharmacol 122: 173-179.

Horiguchi, S, Ueno, R, Masayoshi, H, and Hayaishi, O, 1986b. Alterations in nociception after intracisternal administration of prostaglandin D2, E2 or F2 to conscious mice, <[11] Journal Name> 122: 173-179.

Hunskaar S, Hole K, 1987. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain., Pain 30: 103-114.

Ikeda, H., Stark, J., Fischer, H., Wagner, M., Drdla, R., Jager, T., Sandkuhler, J., 2006. Synaptic amplifier of inflammation in the spinal dorsal horn, Science 312: 1662.

Imrich, R., Bozak, V., Rovensky, J., 2006. Polymyalgia Rheumatica and the Temporal Arteritis: The Endocrine Relations and the Pathogeneisis. Review., Endocrine Regulations 40: 83-89.

Ishizaka, M., Ohe, Y., Senbongi, T., Wakabayashi, K., Ishikawa, K., 2001. Inflammatory stimuli increase prostaglandin D synthase levels in cerbrospinal fluid of rats., Neuroreport 12: 1161-1165.

Islam, A.K., Cooper, M.L., Bodnar, R.J., 1993. Interactions among aging, gender and gonadectomy effects upon morphine antinociception in rats, Physiol.Behav. 54: 45-53.

Isselee, H., De Laat, A., De Mot, B., Lysens, R., 2002. Pressure-pain threshold variation in temporomandibular disorder myalgia over the course of the menstrual cycle, J Orofac Pain 16: 105-117.

Jensen, T.S., Yakish, T.L., 1192. The antinociceptive activity of excitatory amino acids in the rat brainstem: an anatomical and pharmacological analysis., Brain Res 569: 255-267.

Joo, M., Kwon, M., Cho, Y.J., Hu, N., Pedchenko, T.V., Sadikot, R.T., Blackwell, T.S., Christman, J.W., 2009. LPS-dependent interaction between PU.1 and cJun determines production of lipocalin-type prostaglandin D synthase and prostaglandin D2 in macrophages, Am J Physiol Ling Cell Mol Physiol 296: 771-779.

Julus, D., Basbaum, A.I., 2001. Molecular Mechanisms of Nociception, Nature 413: 203-210.

Kaga, J., Berkun, M., 1954. The reward value of running activity, J.Comp.Physiol.Psychol. 47: 108.

Kamei, D., Kiyofumi, Y., Takegoshi, Y., Mikami-Nakanishi, M., Nakatani, Y., Sachiko, O., Hidekazu, Y., 2004. Reduced pain hypersensitivity and inflammation in mice lacking microsomal prostaglandin E synthase-1., J Biol Chem .

Kepler, K.L., Standifer, K.M., Paul, D., Kest, B., Pasternak, G.W., Bodnar, R.J., 1991. Gender effects and central opioid analgesia, Pain 45: 87-94.

Khaser, S.G., 1998. A tetrodoxin-resistant sodium currunt mediates inflammatory pain in the rat, Neurosci.Lett. 256: 17-20.

Khaser, S., Isenber, W., Miao, F., Gear, R., Green, P., Levine, J., 2001.
Gender and Gonadal hormone effects on vagal modulation of tonic nociception, Journal of Pain 2: 91-100.

Kim, S.J., Calejesan, A.A., Li, P., Wei, F., Zhou, M., 1999. Sex differences in late behavioral response to subcutaneous formalin injection in mice., Brain Res 829: 158-189.

Knobil, E.; Neill, J. D. The Physiology of Reproduction. 1988.

Kohlmann, T., 2003. Musculoskeletal pain in the population, Schmerz 17: 405-411.

Koltzenburg M., Kress, M., Reeh, P., 1992. The nociceptor sensitization by bradykinin does not depend on sympathetic neurons., Neuroscience 46: 465-473.

Kuba, T., Jenab, S., Quinones-Jenab, V., 2005a. Endogenous gonadal hormones mediate sex differences in formalin-induced behavioral responses through prostaglandin E2 release, Journal of Pain submitted.

Kuba, T., Kemen, L.M., Quinones-Jenab V., 2005b. Estradiol administration mediates the inflammatory response to formalin in female rats, Brain Res. 1047: 119-122.

Kuba, T., Quinones-Jenab V., 2005. The role of female gonadal hormones in behavioral sex differences in persistent and chronic pain: clinical vs. preclinical studies, Brain Res Bull 66: 179-188.

Kudo, M., Kudo, T., Matsuki, A., 1991. Role of prostaglandin E1 in steroidogenesis by isolated rat adrenal cells, Masui 40: 1819-1824.

LaMotte, R.H., Shain, C.N., Simone, D.A., Tsai, E.F., 1991. Neurogenic hyperalgesia: psychophysical studies of underlying mechanisms., Journal of Neurophysiology 66: 190-211.

LaMotte, R.H., Thalhammer, J.G., Torebjork, H.E., Robinson, C.J., 1982. Peripheral neural mechanisms of cutaneous hyperalgesia following mild injury by heat., Journal of Neuroscience 2: 765-781.

Langenbach, R., Morham, S.G., Tiano, C.D., Ghanayerm, B.I., Chulada, P., Mahler, J., Lee, C., Goulding, E., Kluckman, K., Kim, H., Smithies, O., 1995. Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration., Cell 83: 483-492.

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

Li, C., Zhang, X., Matthews, E., Li, K., Kurwa, A., Boroujerdi, A., Gross, J., Gold, M., Dickenson, A., Feng, G., Luo, Z., 2006. Calcium channel alpha2delta1 subunit mediates spinal hyperexcitability in pain modulation, Pain 125: 20-34.

Li, J.L., Ding, Y.Q., Shingemoto, R., Mizuno, N., 1996. Distribution of trigeminothalamic and spinothalamic tract neurons showing substance P receptor-like immunoreactivity in the rat., Brain Res 719: 207-212.

Liang, X, WU, L, Hand, T, and Andreasson, K, 2005. Prostaglandin D2 mediates neuronal protection via the DP1 receptor, <[11] Journal Name> 92: 477-486.

Lipton, R., Stewart, W.F., Diamond, S., Diamond, M., Reed, M., 2001. Prevalence and burden of migraine in the United States; data from the American Migraine Study II., Headache 41: 646-657.

Lopshire, J.C., Nicol, G., 2007. Activation and recovery of the PGE2-mediated sensitization of the capsaicin response in the rat sensory neuron., Medical Neurobiology Program .

Maixner, W.; Sigurdsson, A.; Fillingim, R. B.; Lundeen, T.; Booker, D. Regulation of acute and chronic orofacial pain. In: Friction, J. R.; Dubner, R. B., eds. Orofacial pain and temporomandibular disorders. New York: Raven Press; 1995: 85-102.

Malmberg, A.B., Yaksh, T.L., 1993. Spinal nitric oxide synthesis inhibition blocks NMDA-induced thermal hyperalgesia and produces antinociception in the formalin test in rats, Pain 54: 291-300.

Malmberg, A., Hamberger, A., Hedner, T., 1995. Effects of prostaglandin E2 and capsaicin on behavior and cerebrospinal fluid amino acid concentrations of unanesthetized rats: a microdialysis study., Journal Neurochem 65: 2185-2193.

Mann, G.E., 2001. Hormone control of prostaglandin F(2 alpha) production and oxytocin receptor concnetrations in bovine endometrium in explant culture, Domest Anim Endocrinol 20: 217-226.

Mannino, C.A., South, S.M., Quinones-Jenab V, Inturrisi, C., 2006. Estradiol Replacement in Ovariectomized Rats is Antihyperalgesic in the Formalin Test., Pain In Press.

Markenson, J.A., 1996. Mechanisms of chronic pain., American Journal of Medicine 101: 6S-18S.

Markowska A.L., Breckler, S.J., 1999. Behavioral biomakers of aging: illistration of a multivariate approach for detecting age-related behavioral changes., J Gerontol A Bio Scie Med Sci 54: 549-566.

Martin, V.T., Behbehani, M., 2006a. Ovarian Hormones and Migraine Headache: Understanding Mechanisms and Pathogenesis--Part 1, Headache 46: 3-23.

Martin, V.T., Behbehani, M., 2006b. Ovarian Hormones and Migrane Headache: Understanding Mechanisms and Oathogenesis- Part 2, Headache 46: 365-386.

McBain, C.J., Mayer, M.L., 1994. N-methyl-D-aspartic acid receptor structure and function, Physiological Review 74: 723-760.

McClung CA, Sidiropoulou K, Vitaterna M, Takahashi JS, White FJ, Cooper DC, Nestler EJ, 2005. Regulation of dopaminergic transmission and cocaine reward by the Clock gene, Proc Natl Acad Sci 102: 9377-9381.

McMahon S.B.; David, L. I.; Bevan, S.; Bevan, S. Inflammatory mediators and modulators of pain. In: McMahon S.B.; Koltzenburg M., eds. Textbook of Pain. London: Churchill Livingstone; 2006: 49-72.

Meyer, R.A., Campbell, J.N., 1981. Myelinated nociceptive afferents account for the hyperalgesia that follows a burn to the hand, Science 213: 1527-1529.

Meyer, R. A.; Ringkamp, M.; Campbell, J. N.; Raja, S. N. Peripheral mechanisms of cutaneous nociception. In: McMahon S.B.; Koltzenburg M., eds. Textbook of Pain. London: Elsevier Churchill Livingstone; 2006: 3-34.

Millan, M.J., 1989. Kappa-opioid receptor-mediated antinociception in the rat. l. Comparative actions of mu- and kappa-opioids against noxious thermal, pressure and electrical stimuli, Pharmacol.Exp.Ther. 251: 334-341.

Millan, M.J., 1999. The induction of pain: an integrative review, Prog.Neurobiol. 57: 1-164.

Minami, I., Okuda-Ashitaka, E., Hori, Y., Sakuma, K., Sugimoto, I., Sukimura, K., Mishina, M., Ito, S., 1999. Involvement of primary afferent C-fibers in touch evoked pain (alloydynia) induced by prostaglandin E2, European Journal neuroscience 11: 1849-1856.

Minami, T., 1994. Characterization of EP-receptor subtypes involved in allodynia and hyperalgesia induced by intrathecal administration of prostaglnadin E2 mice, Br.J.Pharmacology 112: 735-740.

Minami, T., 2001. Characterization of EP receptor subtypes responsible for prostaglandin E2-induced pain responses by use of EP1 and EP3 receptor knockout mice , Br J Pharmacol 133: 438-444.

Minami, T., Okuda-Ashitaka, E., Mori, H., Ito, S., Hayaishi, O., 1996. Prostaglandin D2 inhibitsy prostaglandin E2-induced allodynia in concious mice, Pharmacology 278: 1146-1152.

Mohn, C.E., Fernandez-Solari, J., De Laurentiis, A., Prestifilippo, J.P., de la Cal, C., Funk, R., Bornstein, S.R., McCann, S.M., Rettori, V., 2005. The rapid release of corticosterone from the adrenal induced by ACTH is mediated by nitric oxide acting by prostaglandin E2, Proc Natl Acad Sci 102: 6213-6218.

Moon, T.C., Befus, A.D., 2008. Exogenous nitric oxide regulates cyclooxygenase-2 expression and prostaglandin D(2) generation through p38 MAPK in mouse bone marrow-derived mouse cells., Free Radic Biol Med 45: 780-788.

Morgan, D., Cook, C.D., Picker, M.J., 1999. Sensitivity to the discriminative stimulus and antinociceptive effects of m opioids: role of strain of rat, stimulus intensity and intrisic efficacy at the m opioid receptor, J Pharmacol Exp Ther 289: 965-975.

Mouihate, A., Boisse, L., Pittman, Q.J., 2004. A novel antipyretic action of 15-deoxy-D 12, 14,-Prostaglandin J2 in the rat brain., Journal of Neuroscience 24: 1312-1318.

Multon S, Pardutz A, Mosen J, Hau MT, Defays, C., HOnda S, Harada, N., Bohotin, C., Franzen, R., Schoenen J, 2005. Lack of estrogen increases pain in the trigeminal formalin model: a behavioural and immunocytochemical study of transgenic ArKO mice. . 2005 Mar;114(1-2):257-65., Pain 114: 257-265.

Nantel, F., Denis, D., Gordon, R., Northey, A., Cirino, M., Metters, KM., and Chan, CC., 1999. Distribution and regulation of cyclooxygenase-2 in carrageenan-induced inflammation, <[11] Journal Name> 128: 853-859.

Nishi, A., Snyder, G.L., Higashi, H., Nairn, A.C., Greengard, P., 1999. Requirement for DARPP-32 in mediating the effect of dopamine D2 activation, Eur.J.Neurosci. 11: 2589-2592.

Nogawa, S., Zhang, F., Ross, E., Iadecola, C., 1997. Cyclo-Oxygenase-2 Gene Expression in Neurons Contributes to Ischemic Brain Damage, The journal of neuroscience 17: 2746-2755.

O'Banion, M., Winn, V., Young, D., 1992. cDNA cloning and functional activity of glucorcorticoids regulated inflammatory cyclooxygenase., Proc Acad Sci USA 89: 4892.

Ohkubo T, S.M.T.H.I.R., 1983. Effect of prostaglandin D2 on pain and inflammation., Jpn J Pharmacol 33: 264-266.

Oka, Y., Ibuki, T., Matsumura, K., Namba, M., Yamakazi, Y., Poole, S., Tanaka, T., Kobayashi, S., 2007. Interleukin-6 is a candidate molecule that transmits inflammatory information to the CNS, Neuroscience 145: 530-538.

Oliva, Patrizia. Role of periaquedductal grey prostaglandin receptors in formalin-induced hyperalgesia. European Journal of Pharmacology . 2005.
Ref Type: In Press

Omote, K., Hazama, K., Kawamata, M., Kawamata, T., Nakayaka, Y., Toriyabe, M., Namiki, A., 2001. Peripheral Nitric Oxide in Carrageenan-Induced Inflammation., Brain Res 912: 171-175.

Ossipov, M.H., Lopez, Y., Bian, D., Nichols, M.L., Porreca, F., 1997. Synergistic antinociceptive interactions of morphine and clonidine in rats with nerve ligation injury., Anesthesiology 86: 196-204.

Pan, Z., 2004. An intensified descending pain-facilitating pathway, Pain and anesthesia 1: 121-125.

Panayi, G.S., 1992. Neuroendocrine modulation of disease expression in rheumatoid arthritis., EULAR Congress Reports 2: 2-12.

Pasero, C., 2004. Pathophysiology of Neuropathic Pain, Pain Management Nursing 5: 3-8.

Patrignani, P., Tacconelli, S., Sculli, M., Capone, M., 2005. New Insights into COX-2 biology and inhibition, Brain Research Reviews 48: 352-359.

Portanova, JP, Zhang, Y, Anderson GD, Hauser SD, Masferrer, JL, Seibert, K, Gregory SA, and Isakson, PC, 1996. Selective neutralization of prostaglandin E2 blocks inflammation, hyperalgesia and interleukin 6 production in vivo, <[11] Journal Name> 184: 883-891.

Posadas, I, Bucci, M, Roviezzo, F, Rossi, A, Parente, L, Sautebin, L, and Cirino, G, 2004. Carrageenan-induced mouse paw oedema is bi phasic, age-weight dependent and displays differential nitric oxide cyclooxygenase-2 expression, <[11] Journal Name> 142: 331-338.

Powell-Boone, T., Ness, T.J., Cannon, R., Lloyd, L.K., Weigent, D.A., Fillingim, R.B., 2005. Menstrual cycle affects bladder pain sensation in subjects with interstitial cystitis., Jounral of Urology 174: 1832-1836.

Rackman, A., Ford-Hutchinson, A., 1983. Inflammation and pain sensitivity: effects of leukotrienes D4, B4 and prostaglandin E1 in the rat paw, Prostaglandins 25: 193-203.

Rakajumar, G., Chiu, P., Johnson, B., Mishra, R.K., 1987. 17B estradiol induced increase in brain dopamine D2 receptor: antagonism by MIF-1, Peptides 8: 997-1002.

Reinold, H., Ahmadi, S., Depner, U.B., Layh, B., Heindi, C., Hamza, H., Pahl, A., Brune, K., Narumiya, S., Muller, U., Zeilhofer, H.U., 2005. Spinal inflammatory hyperalgesia is mediated by prostaglandin E receptors of the EP2 subtype., Journal of Clinical Investigation 115: 673-679.

Ren, K., Dubner, R., 1999a. Central Nervous System Plasticity and Persistent Pain, Journal Of Orofacial Pain 13: 155-163.

Ren, K., Dubner, R., 1999b. Inflammatory models of pain and hyperalgesia, ILAR Journal 40.

Ren, K., Wei, F., Dubner, R., Murphy, A., Hoffman, G.E., 2000. Progesterone attenuates persistent inflammatory hyperalgesia in female rats: involvement of spinal NMDA receptor mechanisms, Brain Res 865: 272-277.

Riedel, W., Neeck, G., 2001. Nociception, pain and antinociception: Current concepts, Z Rheumatology 60: 405-415.

Riley III, J.L., Robinson, M.E., Wise, E.A., Price, D.D., 1999. A meta-analytic review of pain perception across the menstrual cycle, Pain 81: 225-235.

Salvemini, D., Wang, Z.Q., Wyatt, P.S., Bourdon, D.M., Marino, M.H., Manning, P.T., Currie, M.G., 1996. Nitric ocide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation., Br J Pharmacol 118: 829-838.

Samad, T., Sapirstein, A., Woolf, C., 2002. Prostanoids and pain: unraveling mechanisms and revealing therapeutic targets, Trends Molecular Medicine 8: 390-396.

Sances, G., Granella, F., Nappi, R.E., 2003. Course of migraine during pregnancy and post pardum;  a prospective study., Cephalalgia 23: 197-205.

Santoro, N., Brown, J., Adel, T., Skurnick, J.H., 1996. Characterization of reproductive hormonal dynamics in the perimenopause, Journal of Clinical Endocrinology and Metabolism 81: 1495-1501.

Scaramuzzi, R.J., Baird, D.T., Boyle, H.P., Land, R.B., Wheeler, A.G., 1977. The secretion of prostaglandin F from the autotransplanted uterus of the ewe, J Reprod Fertil 49: 157-160.

Scher JU, P.M., 2009. The Anti-Inflammatory Effects of Prostaglandins, Journal of Inversigative Medicine .

Scher, J.U., Pillinger, M.H., 2009. The anti-inflammatory Effects of Prostaglandins, J Investig Med .

Scheuren, N., Neupert, W., Ionac, M., Neuhuber, W., Brune, K., Geisslinger, G., 1997. Peripheral noxious stimulation releases spinal PGE2 during the first phase in the formalin assay of the rat, Life Sci 60: PL295-300.

Schuna, A.A., 2002. Autoimmune rheumatic diseases in women, J Am Pharm Assoc (Wash) 42: 612-623.

Shaver, J., 2004. What about an "E" campaign?, Nurs Outlook 52: 275-276.

Sherrington, C. S. The Integrative Action of the Nervous System. New York: Scribner; 1906.

Shiraishi, Y., Asano, K., Nakajima, T., Oguma, T., Suzuki, Y., Shiomi, T., Sayama, K., Niimi, K., Wakaki, M., Kagyo, J., Ikeda, E., Hirai, H.Y.K., Ishizaka, A., 2005. Prostaglandin D2-induced Eosinophilic Airway Inflammation Is Mediated by CRTH2 Receptor., Journal of Pharmacology and Experimental Theraputics 312: 954-960.

Singer, C.A., Figueroa-Masot, X.A., Batchelor, R.H., Dorsa, D.M., 1999. The mitogen-activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons., J Neurosci 19: 2463.

Sternberg, W. F.; Wachterman, M. W. Experimental studies of sex-related factors influencing nociceptive responses: nonhuman animal research. In: Fillingim, R. B., ed. Sex, Gender, And Pain. Seattle: IASP Press; 2000: 71-88.

Stoffel, E.C., Ulibarri, C., Craft, R.M., 2003. Gonadal steroid hormone modulation of nociception, morphine antinociception and reproductive indices in male and female rats, Pain 103: 285-302.

Straub, R., Konecna, L., Hrach, S., 1998. Serum dehydroepiandrosterone (DHEA) and DHEA sulfate are negatively correlated with serum interleukin-6 (IL-6), and DHEA inhibits IL-6 secretion from mononuclear cells in man in vitro: Possible link between endoctinosenescence and immunosenescence., J Clin Endocrinol Metab 83: 2012-2017.

Stucky, C.L., Gold, M., Zhang, X., 2001. Mechanisms of pain, PNAS 98: 11845-11846.

Takasu, N., Komiya, I., Nagasawa, Y., Asawa, T., Yamada, T., 1990. Exacerbation of autoimmune thyroid dysfuntion after unilateral adrenalectomy in patients with Cushing's syndrome due to adreocortical adenoma., New England Journal of Medicine 322: 1702-1712.

Tall, J.M., Crisp, T., 2004a. Effects of gender and gonadal hormones on nociceptive responses to intraplantar carrageenan in the rat, Neurosci Lett 354: 239-241.

Tall, JM and Crisp, T, 2004b. Effects of gender and gonadal hormones on nocicpetive responses to intraplantar carrageenan in the rat, <[11] Journal Name> 354: 239-241.

Tamura, M., Deb, S., Sebastian, S., Okamura, K., Bulun, S.E., 2004. Estrogen up-regulates cyclooxygenase-2 via estrogen receptor in human uterine microvascular endothelial cells., Fertility and Sterility 81: 1351-1356.

Tanioka, T., Nakatani, Y., Semmyo, N., Murakami, M., Kudo, I., 2000. Molecular identification of cytosolic prostaglandin E2 synthase that is functionally coupled with cyclooxygenase-1 in immediate prostaglandin E2 biosynthesis, J Bio Chem 275: 32775-32782.

Tao F, T.Y.Z.C.D.S.L.W.R.S.J.RA., 2004. Differential roles of neuronal and endothelial nitric oxide synthases during carrageenan-induced inflammatory hyperalgesia., Neuroscience 128: 421-430.

Tao, F., Tao, Y.-X., Zhao, C., Dore, S., Liaw, W.J., Raja, S.N., Johns, R.A., 2004. Differential foles of neuronal and endothelial nitric oxide synthases during carrageenan-induced inflammatory hyperalgesia., Neuroscience 128: 430.

Taylor, B.K., Akana, S.F., Peterson, M.A., Dallman, M.F., Basbaum, A.I., 1998. Pituitary-Adrenocortical Responses to Persistent Noxious Stimuli in the Awake Rats: Endogenous Corticosterone Does Not reduce Nociception in the Formalin Test, Endocrinology 139: 2407-2413.

Tegeder, I., Niederberger, E., Vetter, G., Brautigam, L., Geisslinger, G., 2001. Effects of selective COX-1 and -2 inhibition on formalin-evoked nociceptive behaviour and prostaglandin E2 release in the spinal cord, J Neurochem 79: 777-786.

Tenenbaum, M., Azab, A., Kaplanski, J., 2007. Effects of estrogen against LPS-induced inflammation and toxicity in primary glial and neuronal cultures, J Endotoxin R. 13: 158-166.

Teyler, T., Vardis, R., Lewis, D., Rawitch, A., 1980. Gonadal steroids: effects on excitability of hippocampal pyramidal cells., Science 209: 1017-1018.

Theile, K., Buttgereit, F., Huscher, D., Zink, A., 2005. Current use of glucocorticoids in patients with rheumatoid arthritis in Germany, Arthritis Rh.eum 53: 740-747.

Tjolsen, A., Berge, O.G., Hunskaar, S., Rosland, J.H., Hole, K., 1993. The formalin test: an evaluation of the method, Pain 51: 5-17.

Todd, A. J.; Koerber, H. R. Neuroanatomical substrates of spinal nociception. In: McMahon S.B.; Koltzenburg M., eds. Textbook of pain. London: Elsevier Churchill Livingstone; 2006: 73-90.

Toriyabe, M., Omote, K., Kawamata, T., Namiki, A., 2004. Contribution of Interaction between Nitric Oxide and Cyclooxygenases to the Production of Prostaglandins in Carrageenan-induced Inflammation., Anesthesiology 101: 983-990.

Trussel, L.O., Raman, I.M., Zhang, Y.J., 1994. AMPA receptors and rapid synaptic transmission, Seminar in Neuroscience 6: 71-79.

Urban, M.O., Gebhart, G.F., 1999. Central mechanisms in pain., Med Clin North Am. 83: 585-596.

Vaccarino, A.L., Chorney, D.A., 1994. Descending modulation of central neural plasticity in the formalin pain test, Brain Res 666: 104-108.

Veiga, A.P.C., Duarte, I.D.G., Avila, M.N., da Motta, P.G., Tatsuo, M.A.K.F., Francischi, J.N., 2004. Prevention by celecoxib of secondary hyperalgesia induced by formalin in rats, Life Sci 75: 2807-2817.

Velle, W., 1987. Sex differences in sensory functions, Perspectives in Biology and Medicine 30: 491-522.

Vengas, H., Schaible, H.-G., 2001. Prostaglandins and cycloxygenases in the spinal cord, Progress in neurobiology 64: 327-363.

Vincler, M., Maixner, W., Vierck, C.J., Light, A.R., 2001. Estrous cycle modulation of nociceptive behaviors elicited by electrical stimulation and formalin, Pharmacol Biochem Behav 69: 315-324.

Vissers, K.C., De Jongh, R.F., Crul, B.J.P., Vinken, P., Meert, T.F., 2004. Adrenolectomy affects pain behavior of rats after formalin injection., Life Sci. 74: 1243-1251.

Wall, P. D.; Melzack, R. Textbook of Pain. New York: Churchill Livingstone; 1994.

Wallace JE, K.E.C.B., 1980. Animal models of declining memory in the aged: short-term and spatial memory in the aged rat., J Gerontol. 35: 355-363.

Wang, M., Tsai, B.M., Reiger, K.M., Brown, J.W., Meidrum, D.R., 2006. 17-beta-estradiol decreases p38 MAPK-myocardial inflammation adn dysfunction folllowing acute ischemia, J Mol Cell Cardiol 40: 205-212.

Weiser, M.J.W.C.D.F.R.J.H., 2008. Estrogen receptor beta in the brain: From form to function, Brain Res.Rev. 57: 308-319.

Willingale, H.L., Gardiner, N.J., McLymont, N., Grubb, G., Grubb, B.D., 1997. Prostanoids synthesized by cyclo-oxygenase isoforms in rat spinal cord and their contribution to the development of neuronal hyperexcitability., British Journal of Pharmacology 122: 1593-1604.

Woolf C.J., Salter M.W., 2000. Neuronal Plasticity: Increasing the Gain in Pain, Science 288: 1765-1768.

Woolf C.J.; Salter M.W. Plasticity and pain: role of the dorsal horn. In: McMahon S.B.; Koltzenburg M., eds. Textbook of Pain. London: Elsevier Churchill Livingstone; 2006: 91-105.

Yaksh, T.L., 1997. Pharmacology and mechanisms of opioid analgesic activity, Acta Anaesthesiologica Scandinavica 41: 94-111.

Yaksh, T.L., 1999. Spinal systems and pain processing: development of novel analgesic drugs with mechanistically defined models, Trends in Phamacological Sciences 20: 329-337.

Yamamoto, T., Nozaki-Taguchi, N., 1996. Analysis of the effects of cyclooxygenase (COX) - 1 and COX-2 in spinal nociceptive transmission using indomethacin, a non-selective COX inhibitor, and NS-398, a COX-2 selective inhibitor., Brain Res 739: 104-110.

Yamamoto, T., Nozaki-Taguchi, N., 2002. The Role of Cyclooxygenase-1 and -2 in the rat formalin test, Anesthesiology Analog 94: 962-967.

Yashpal, K., Coderre, T.J., 1998. Influence of formalin concentration on the antinociceptive effects of anti-inflammatory drugs in the formalin test in rats: separate mechanisms underlying the nociceptive effects of low- and high-concentration formalin, Euro J Pain 2: 63-68.

Yashpal, K., Pitcher, G.M., Parent, A., Quirion, R., Coderre, T.J., 1995. Noxious thermal and chemical stimulation induce increases in 3G-phorbol 12,13-dibutyrate binding in spinal cord dorsal horn as well as persistent pain and hyperalgesia which is reduced by inhibition of protein kinase C, Clin J Neuroscience 15: 3263-3272.

Yeung, J.C., Rudy, T.A., 1980. Sites of antinociceptive action of systemically injected morphine:involvement of supraspinal loci as revealed by intracerebroventricular injection of naloxone., J Pharmacol Exp Ther. 215: 626-632.

Yokoro, CM, Tatsuo, MAKF, Pereira, LSM, Alves, DLF, and Francishi, JN, 2003. Role of endogenous glucocorticoids in hyperalgesia and edema in old arthritic rats, <[11] Journal Name> 36: 77-83.

Zhang, F., Wan, Y., Zhan, Z.K., Light, A.R., Fu, K.Y., 2007. Peripheral Formalin Injection Iduced Long Lasting Increases in Cyclooxygenase 1 Expression by Microglia in the Spinal Cord., Pain 8: 110-117.

Zhang, R.X., Lao, L., Qiao, J.T., Malsnee, K., Ruda, M.A., 2004. Endogenous and exogenous glucocorticoid suppresses up-regulation of preprodynorphin mRNA and hyperalgesia in rats with peripheral inflammation, Neurosci Lett 359: 85-88.

Zhang, Y, Shaffer, A, Portanova, J, Seibert, K, and Isakson, PC, 1997. Inhibition of cyclooxygenase-2 rapidly reverses inflammatory hyperalgesia and peostaglndin E2 production, <[11] Journal Name> 283: 1069-1075.

Zimmermann, M., 2001. Pathobiology of neuropathic pain, European Journal of Pharamcology 429: 23-37.