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The Role of Gonadal Hormones on Opioid Receptor Protein Density in Arthritic Rats

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List of Abbreviations

CAS	castrated
CFA	Complete Freund's Adjuvant
DOR	delta opioid receptor
g	gram
GDP	guanosine diphosphate
GDX	gonadectomized
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
h	hour
IP	immunoprecipitation
KOR	kappa opioid receptor
MB	midbrain
mg	milligram
mL	milliliter
mm	millimeter
MOR	mu opioid receptor
ORL1	orphan receptor-like 1
OVX	ovariectomized

PDYN	prodynorphin
PENK	proenkephalin
POMC	proopiomelanocortin
S.E.M.	standard error of the mean
μL	microliter
VEH	vehicle

Abstract

THE ROLE OF GONADAL HORMONES ON OPIOID RECEPTOR PROTEIN DENSITY IN ARTHRITIC RATS

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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The majority of research on the endogenous opioid system has focused on various pain assays and the efficacy of different opioid agonists. However, minimal attention has been focused on the effects of gonadal hormones and their impact on the opioid peptide system. The present study was designed to determine the effects of modulation of gonadal hormones on the opioid receptor protein levels in Complete Freund's Adjuvant (CFA)-treated arthritic and non-arthritic male and female Lewis rats. Midbrain and spinal cord tissues were collected for comparison of the μ , δ , and κ receptor protein levels in arthritic and non-arthritic animals. Male gonadectomy did not dramatically impact opioid receptor protein levels, whereas female gonadectomy resulted in significant increases in opioid receptor protein levels. Furthermore, it was determined

that MOR protein levels were greatest in male rats, while KOR protein levels were greatest in female rats regardless of arthritis condition or gonadal hormones.

Introduction

History of Opioids

Opium, a substance derived from the poppy plant, *Papaver somniferum*, has been used throughout history and in almost every culture to alleviate pain and other ailments. Although much of the early history of opium is unclear, it appears that cultures that used the juices from the unripe seed of the poppy plant understood its power. Historical references indicate that the Sumerians were the first civilization to use opium, possibly first used by priests as a euphoriant in religious ceremonies (Aragon-Poce et al., 2002). Opium's euphoria and analgesic properties undoubtedly led to its popularity over many of the herbal drugs used prior to opium. By the thirteen century A.D. opium use had spread from the Middle East, to Greece, Rome, Asia, and throughout Europe.

After opium had been used for centuries to treat ailments such as headaches, surgical pain, and to produce constipation, its active ingredient was isolated in 1806. German scientist, Friedrich Serturner, discovered the specific alkaloid in opium responsible for its profound effects and named it morphine, after the Greek god of dreams, Morpheus (Brownstein, 1993). Scientists continued to explore the alkaloids found in the juices of *Papaver somniferum* and discovered substances like codeine, thebaine, and papaverine; however the greatest advances in understanding the effects of

opiates occurred after scientists began to understand and modify the chemical structure of these alkaloids.

Unfortunately, the analgesic benefits of treatment with opium, morphine, and codeine were plagued by their ability to produce tolerance and addiction. Heroin was the first attempt at producing a drug that was able to alleviate pain like morphine, but without causing addiction. However, after Bayer released heroin in 1898, its claims of providing non-addictive pain relief were proven false (Corbett et al., 2006). This search to synthetically produce substances with similar or greater analgesic effects not only led to the discovery of numerous other opioid drugs, like methadone, oxycodone, and fentanyl, but also provided a view into the mechanisms that produced the desired analgesia. The synthetic opioid nalorphine provided the initial insight into the mechanism of opioid analgesia. Scientists discovered that nalorphine was able to produce analgesia, while antagonizing the analgesic effects of morphine. The mixed effect of nalorphine was the action of a mixed agonist-antagonist, which indicated the presence of multiple receptors within the opioid system (Brownstein, 1993).

Opioid Receptors

As more opioid agonists, antagonists, and mixed agonist-antagonists were developed and studied the theory of multiple opioid receptors was reaffirmed. The initial evidence for multiple receptors came from Gilbert and Martin (1976) where they tested morphine- and nalorphine-like drugs on chronic spinal dogs. Ultimately, it was determined that there were three opioid receptors working at the same time and named

them according to their agonists, μ for morphine, κ for ketazocine, and σ for N-allylnormetazocine (SKF 10047) (Martin et al., 1976). This discovery was followed by the subsequent proposal of the δ receptor in order to explain the activity of enkephalin, a peptide known at the time to interact with opioid receptors (Lord, 1977).

The overall understanding of the opioid receptors was greatly advanced when the receptor were successfully cloned in the 1990's. In 1992, the δ receptor (DOR) was first to be cloned. (Evans et al., 1992; Kieffer et al., 1992). Then, in 1993, the μ receptor (MOR) (Chen et al., 1993a,b; Fukuda et al., 1993; Thompson et al., 1993) and κ receptor (KOR) (Li et al., 1993; Meng et al., 1993; Minami et al., 1993; Nishi et al., 1993). However, the σ receptor, originally discovered by Martin et al. is no longer considered an opioid receptor since it lacks the stereoselectivity and antagonism by opioid antagonists that is characteristic of opioid receptors (Corbett et al., 2006). Then an orphan opioid receptor, with almost 70% sequence homology to the other opioid receptors, was identified in 1997 (Henderson and McKnight, 1997). After the discovery of the 'orphan' receptor, called opioid receptor-like receptor (ORL1), the list of accepted opioid receptors remains: μ , δ , κ , and ORL1.

Endogenous Opioid Peptides

Discovering the presence of multiple opioid receptors was pivotal in the advancement of understanding of the opioid receptor system. Unfortunately, this did not explain why the juices of the poppy plant had such a profound interaction with the opioid

receptors and were able to produce its popular analgesic effect. These interactions were explained by the discovery of the endogenous opioid peptides, including the dynorphins, enkephalins, and endorphins. In 1975, Hughes et al. observed that a substance from guinea pig brain acted as an agonist at opioid receptors, but its effect was antagonized by naloxone. This substance was later determined to contain a mixture of the first two endogenous opioid peptides, methionine-enkephalin and leucine-enkephalin (shortened to [Met⁵]- and [Leu⁵]-enkephalins) (Hughes et al., 1975). Following the discovery of the enkephalins, opiate-like activity was observed in peptides found in pituitary glands, which led to another class of endogenous opioid peptides, the dynorphins (Goldstein et al., 1975). The last class of endogenous opioid peptides, the endorphins, was discovered with β -endorphin isolated from human and camel pituitary glands (Li and Chung, 1976).

The endogenous opioid peptides are derived from larger peptides found within the body. Proenkephalin (PENK), Prodynorphin (PDYN), and Pro-opiomelanocortin (POMC) function as precursor peptides for enkephalins, dynorphins, and B-endorphin, respectively (Przewlocki R. and Przewlocki B., 2001). Proenkephalin is the source of [Met⁵]- and [Leu⁵]-enkephalins and several longer peptides, including Peptide E, BAM 22P, and Metorphamide (Corbett et al., 2006). PENK-containing neurons are expressed in neuronal and nonneuronal sites throughout the central and peripheral nervous system (Przewlocki R. and Przewlocki B., 2001). [Met⁵]- and [Leu⁵]-enkephalins derived from PENK have high affinities for DOR. Prodynorphin (PDYN) functions as a precursor peptide for the dynorphins, alpha and beta neoendorphin, and as the primary source of [Leu⁵]-enkephalin. PDYN-containing neurons are localized in areas of the brain and

peripheral nervous system associated with nociception (Watson et al., 1981). Opioid fragments of PDYN have the greatest affinity for KOR, but also display significant affinity at MOR and DOR (Corbett et al., 1993).

The peptide pro-opiomelanocortin (POMC) is the source of β -endorphins (1-27) and several non-opioid peptides. POMC is also the precursor to adrenocorticotropin hormone (ACTH), melanocyte-stimulating hormone (MSH), and β -lipotropin. β -endorphin peptides bind with similar affinity to MOR and DOR and with a lower affinity to KOR. β -endorphin peptides are present in the nucleus arcuatus of the mediobasal hypothalamus and function in pain response (Przewlocki R. and Przewlocki B., 2001).

G Protein Superfamily

MOR, DOR, KOR, and ORL-1 opioid receptors are members of the G protein coupled receptor superfamily. G proteins are heterotrimers composed of α , β , and γ subunits bound to either a guanosine diphosphate (GDP) or guanosine triphosphate binding protein (GTP) (Shaqura et al., 2004). Opioid receptors are coupled to G protein heterotrimers, which become activated through the binding of a opioid ligand to the receptor (Forse, 2000). Once activated the G protein changes conformation, resulting in a decreased affinity for the GDP binding protein (Neer, 1995). After releasing GDP, the α subunit is activated by binding a GTP, causing the α subunit to dissociate from the $\beta\gamma$ subunit. Eventually, the GTP is hydrolyzed from the α subunit by the enzyme GTPase,

resulting in a single GDP. The α and $\beta\gamma$ subunits are then able to reassociate, become inactive, and return to the receptor (Neer, 1995).

During the G protein cycle once the α and $\beta\gamma$ subunits have dissociated, each subunit is able produce its effect. The effect of the G proteins can be quite broad; influencing adenylate cyclase, kinases, phospholipases, and ion-channel exchanges (Forse, 2000). It is the effect of the GPCR that can result in the diminished pain sensation caused by the opioid system (Shaqura et al., 2004).

Pain

Advances in the study of the opioid system have resulted in dramatic improvements of our understanding of pain mechanisms and methods of pain treatment. Pain can be classified into two groups, either acute pain or chronic pain. Acute pain is associated with tissue damage or injury that results from trauma or surgery. This form of pain is essential as part of a survival instinct to protect from further damage and to allow for recovery. However, chronic pain occurs as the result of disease, such as autoimmune mediated diseases. Some of the most common examples of diseases that can cause chronic pain are rheumatoid arthritis, systemic lupus erythematosus (SLE), and Graves' disease (Druckman, 2001).

Treatment of chronic pain diseases differs from that of acute pain in that many of the mechanisms behind the disease and its continuation are unknown. Furthermore, many of the mechanisms and processes that occur as a result of acute pain have been established, such as the increased activity of particular endogenous opioid peptides

(Millan et al., 1986). Due to the uncertainty surrounding chronic pain conditions, researchers are attempting to elucidate a greater understanding of the pain associated with these diseases. The use of inflammatory agents, including Complete Freund's Adjuvant, carrageenan, and formalin, are employed to simulate the chronic pain state in laboratory animals.

Complete Freund's Adjuvant (CFA) is commonly used as the model for simulating chronic pain in male and female rats. Rats treated with CFA experience symptoms including inflammation, joint deterioration, and hyperalgesia, creating a diseased state comparable to Rheumatoid arthritis in humans (Colpaert, 1987). The inflammation and hyperalgesia produced by CFA results from alterations in several neuropeptide systems (Cox, 2004). Opioid systems are among those affected by administration of CFA, causing changes in the receptors, peptides, and mRNAs involved in producing opioid nociception (Millan, 1986).

The polyarthritic state produced in the weeks following CFA administration results in significant effects on functions of the opioid systems. Increased levels of prodynorphin and proenkephalin are observed in the spinal and supraspinal neurons, which correspond to increased levels of the mRNAs encoding prodynorphin and proenkephalin (Holtt et al., 1987; Iadarola et al., 1988;). Chronic inflammatory conditions have also resulted in reductions in [Met⁵]-enkephalin-like peptides and levels of proenkephalin A mRNA in the dorsal root ganglia of arthritic rats (Pohl et al., 1997).

Gonadal Hormones

Whether the pain results from natural or experimental methods of pain, there are several factors that influence every aspect from sensation to analgesia. The presence of sex differences between male and female subjects is among these factors. Gonadal hormones in male and female animals are responsible for both organizational and activational effects. Organizational effects of steroid hormones occur in the late prenatal and early postnatal development of rats (Cicero et al., 2002). These effects include producing sexually appropriate physiology and behavior and making the brain sensitive to the activational effects of sex hormones during puberty. The activational effects do not appear until adulthood, when they are responsible for maintaining typical male and female sexual and other behaviors (Cicero et al., 2002).

Many investigations attempting to determine the influence of the gonadal hormones on the body have been conducted with varying results. Gonadectomy and administration of estrogens or androgens are commonly used to study sex differences in laboratory animals. Krzanowska et al. (2002) and Cicero et al. (2002) successfully eliminated sex differences in morphine antinociception through the neonatal castration of male rats and by the administration of androgens to neonatal female rats. However, adult gonadectomy in both male and female rats did affect morphine analgesia, indicating that the organizational effects of sex hormones may be responsible for sex differences. Researchers examining the activational effects of gonadal hormones have discovered less consistent results in both males and females, showing increases, decreases, and no change in opioid analgesia after adult gonadectomy (Turner et al., 2002).

Androgens, the primary gonadal hormones present in males, are produced primarily in the testes and to a lesser degree in the adrenal cortex (Aloisi, 2003). The primary androgens are testosterone and dihydrotestosterone. Although, generally considered male sex hormones, androgens are present in much lower levels in females. Female androgens are secreted from the ovaries and adrenal cortex in small amounts. This is also true for presence of the female gonadal hormones found in low concentrations in males (Craft et al., 2004). Aside from the role of testosterone in the production of male characteristics, it appears that testosterone provides some anti-inflammatory function. Lower levels of gonadal and adrenal androgens have been observed in male and female patients with rheumatoid arthritis when compared to control subjects (Navarro et al., 1998; Cutolo et al., 2000). This anti-inflammatory effect of androgens has also been noted as a common factor in other chronic inflammatory diseases, like, fibromyalgia, systemic lupus erythematosus (SLE), and Graves' disease (Aloisi, 2003).

There are two groups of hormones that comprise the female gonadal hormones, estrogens and progestins. The estrogens (estradiol, estriol, and estrone) and progestins (progesterone) are both produced primarily by the ovaries (Simpson, 2003). Unlike the androgens, estrogens are often linked to increased pain sensitivity in females. Korzun et al. (2000) observed increased pain, perceived stress, and depression associated with fibromyalgia during the luteal phase of the menstrual cycle of patients corresponding to high levels of estrogen and progesterone. Estrogens are also responsible for various non-

reproductive effects, including bone formation, cardiovascular function, metabolic rate, water and salt balance, and hemostasis (Amandusson et al., 1999).

Female hormone levels fluctuate at a much greater degree in females than males, due to processes such as the menstrual cycle, pregnancy, and menopause. Compared to males, the female reproductive system undergoes a much greater dynamic of change as the female progresses through life. Rat ovarian cycles consist of an estrus, or ovulation phase, lasting about 12 hours; then metestrus, or luteal phase, lasting for one day; then diestrus, or follicular phase, lasting 1-2 days; followed by proestrus, lasting about 12 hours (Mahesh, 1985). Compared to males, cycling females demonstrate the greatest sex differences when in the estrus phase, indicated by low estradiol and progesterone levels (Craft et al., 2004).

Pregnancy and menopause represent the extremes of the female hormonal spectrum. Pregnant females experience increased levels of progesterone and estrogen. Conversely, during menopause, females experience a dramatic decrease in the ovarian production of estrogens (Aloisi, 2003), which makes the aromatization of testosterone to estradiol the primary source of estrogens (Craft et al., 2004). After menopause, females rely on the extragonadal aromatization of androgens, like testosterone, androstenedione, dehydroepiandrosterone (DHEA), and DHEA sulphate (DHEAS) for the production of estrogens (Zhao et al., 2005).

Correlations between gonadal hormone levels and pain suggest that both androgens and estrogens have important influences in pain modulation. In humans, this is seen in the significantly higher prevalence of autoimmune disease in human females

than males (Druckmann, 2001). Also, injection of estradiol into male rats resulted in higher levels of formalin-induced licking than in control rats (Aloisi and Ceccarelli, 2000), suggesting a pronociceptive effect of estrogen. Given the dramatic sex differences seen in prevalence of chronic pain diseases it seems likely that the ability of the gonadal hormones results from their impact on the opioid system.

Rationale for Study and Hypotheses

The majority of research on the endogenous opioid system has examined various pain assays and the efficacy of different opioid agonists. However, minimal attention has been focused at the effects of the gonadal hormones and their impact on the opioid peptide system. Sex differences in response to pain have been observed in both human and animal models (Przewlocki R. and Przewlocki B., 2001). Most studies have concluded that opioid analgesia is greater in males than females and also that females exhibit a greater nociceptive and hyperalgesic response to inflammatory agents and nociceptive stimuli (Wiesenfeld-Hallin, 2005). Many studies have found that these sex differences have been eliminated through the manipulation of gonadal steroids through castration in males and testosterone administration in females (Cicero et al., 2002; Krzanowska et al., 2002).

The present study was designed to determine any effects of the gonadal hormones on the opioid receptor protein levels of CFA on treated male and female Lewis rats. Following a paw pressure pain assay, the midbrain and spinal cord tissues were collected for comparison of MOR, DOR, and KOR protein levels. Receptor protein levels should

provide an estimate of the receptor density, which should be decreased in animals experiencing pain. It was hypothesized that male rats would feel less pain than female rats regardless of the presence of gonadal hormones and gonadectomy, which would result in a decreased pain threshold in male arthritic and nonarthritic rats.

Materials and Methods

Subjects

Hormonally intact male and female Lewis rats approximately 60 days of age were obtained from Charles Rivers Laboratories (Raleigh, NC). Castrated (CAS) and ovariectomized (OVX) rats were also obtained from Charles Rivers Laboratories (Raleigh, NC), with the surgeries being performed when the rats were approximately 50 days of age. All rats were allowed to acclimate to the vivarium until approximately 70 days of age, at which time the experiment commenced. Rats were individually housed, had free access to food and water, and were maintained on 12/12h light/dark cycle.

CFA Administration

On Day 1 animals were injected intradermally in the base of the tail with 0.1 ml of 5.0 mg/ml Complete Freund's Adjuvant (CFA) (heat-killed *Mycobacterium*; Difco Laboratories, Detroit, MI) or 0.1 ml of vehicle (VEH) (mineral oil).

Paw Thickness and Nociceptive Sensitivity Testing.

Paw thickness (mm) of the left and right hindpaws was determined with a digital caliper. Nociceptive sensitivity was assessed on Day 19 by determining thresholds in response to mechanical pressure applied to the hindpaws of the rats. Rats were lightly restrained in a

towel and a mechanical stimulus was applied with an analgesy meter (Ugo Basile, Varese, Italy), a device with a dome-shaped plastic tip (diameter = 1 mm) that applies a linearly increasing pressure (g) to the dorsal surface of the hindpaw, with the tip applied to the region of the paw just proximal to the third digit. On three separate occasions prior to Day 12 each rat was habituated to the paw pressure procedure. Habituation involved wrapping the rat lightly in a towel and placing one hindpaw on the plinth under the dome-shaped plastic tip. Increasing pressure was then applied until a withdrawal response was initiated. This habituation procedure was repeated for the other hindpaw. On Day 19, paw pressure thresholds were determined for both the left and right hindpaws with the order of testing counterbalanced across animals.

Estrous Cycle Phase Determination

Estrous cycle phase determination was determined in female rats by vaginal lavage on Day 19. The stages were identified based upon the type of cells detected. Proestrus phase was signified by a majority of round, nucleated cells, and an absence of leukocytes. Estrous phase was signified by a majority of cornified cells, and an absence of leukocytes. Metestrus phase was signified by the presence of round nucleated cells, cornified cells, and leukocytes. Diestrus phase was signified by the presence of all three cell types seen in metestrus, however, the number of cells in this phase was greatly reduced.

Tissue Collection

Following nociceptive testing, the rats were killed by rapid decapitation 19 days after complete Freund's adjuvant inoculation. Midbrain and spinal cord tissues were removed and immediately frozen at -80°C.

Tissue Preparation

For each assay, spinal cord and midbrain tissue were thawed and homogenized in 10 ml cold IP buffer with protease inhibitors, followed by centrifugation at 3,500 x g for 12 minutes at 4°C. The supernatant was transferred into clean 27 x 115 mm plastic centrifuge tubes and centrifuged at 20,000rpm for 33 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in IP/detergent buffer, using 350 µl for spinal cord tissue and 425 µL for midbrain tissue. To solubilize the pellets, they were left on ice and vortexed vigorously every 5 minutes for 1 hour. Tissues were then centrifuged at 12,000 rpm for 15minutes at 4°C. The supernatant was pipetted into 17 x 100 mm plastic culture tubes. Protein assays were conducted with the Bio-Rad Detergent Compatible assay kit. Protein samples were aliquotted and stored at -80°C.

Western Immunoassays

Electrophoresis was performed with a standard Laemmli method. Samples were diluted 1:1 with 2X sample buffer and loaded into a 1.5 mm 8% SDS-PAGE gel. Protein (8 µg) was loaded into each gel for each of the receptors, MOR, DOR, and KOR. Following

protein separation, transfer onto Immobilon-P PVD membrane (Millipore, Bedford, MA) was performed by the tank method. Blots were reversibly stained with Ponceau solution (Sigma Chemical Co.) blocked at least one hour in casein blocker in TBS (Pierce, Rockford, IL). The blots were incubated in the appropriate opioid receptor antibody concentrations overnight at room temperature (KOR, 1:2000 in casein, DOR, 1:10,000 in casein, MOR, 1:125 in casein). After washing three times in Tris-buffered saline plus 0.5% Tween 20 (TBST) for 5 minutes, the blots were incubated in horseradish peroxidase-conjugated goat anti-rabbit IgG antiserum (Sigma Chemical Co.) at 1:50,000 dilution in casein for one hour at room temperature. Blots were washed again three times in TBST and then incubated for 5 minutes in SuperSignal CL-HRP chemiluminescence (Pierce, Rockford, IL). Blots were then exposed on XAR-2 film (Eastman Kodak, Rochester, NY). Bands were quantified with scanning densitometry, and comparisons of the optical density values were performed by unpaired t-tests.

Antibodies

Anti-MOR-antibody (anti-acetyl-EAETAPLP-amide, C-terminal) was obtained from Research and Diagnostic Antibodies (Berkley, CA). Anti-DOR (anti-acetyl-CGRQEPGSLRRPRQA-amide, C-terminal, anti-KOR (anti-acetyl-SREKDRNLRRITKL-amide, C-terminal) antibodies were obtained from Biosource International (Camarillo, CA).

Data Analysis

Following quantification of bands with scanning densitometry, the optical density ratios were calculated by dividing the respective GAPDH optical densities by the optical density of MOR, DOR, and KOR. The optical densities were reported as mean \pm S.E.M. and tested for significance ($p < 0.05$) with the independent sample t-test in SPSS, Version 12.0 for Windows.

Results

3.1. Paw pressure testing prior to tissue collection

Male and female Lewis rats were tested to determine the effect of gonadectomy in vehicle and CFA treated rats using the paw pressure test. CFA-treated (arthritic – “Arth”) animals, denoted by black bars, had significantly lower pain thresholds in both males and females regardless of the presence of gonadal hormones (Figure 1). A significant increase in the pain threshold of arthritic female rats was observed after ovariectomy. No significant differences were observed between intact and castrated non-arthritic or arthritic male rats. No significant difference was observed between non-arthritic intact and ovariectomized females.

3.2. Effect of arthritis on female rats.

Figure 2, Panels A, B, and C shows the optical density ratios of MOR, DOR, and KOR standardized to the GAPDH loading control. Non-arthritic rats are shown in white bars and arthritic rats in black bars. In Fig. 2.A no significant differences were observed in the midbrain (“Brain”) of female Lewis rats when tested for MOR protein. Receptor protein levels in the spinal cord (“Cord”) of non-arthritic gonadectomized (“GDX”) female rats were significantly lower ($p < 0.05$) than in the arthritic gonadectomized females. No significant differences were observed in DOR protein levels in the midbrain

or spinal cord tissues of non-arthritic and arthritic animals regardless of gonadectomy (Panel B).

In Panel C, no significant differences were observed in the KOR protein of the midbrain tissue in intact and gonadectomized females. However, there were significant differences between the nonarthritic and arthritic spinal cord tissue of both intact and gonadectomized female rats. In the case of the intact rats, the development of arthritis resulted in a significant decrease in KOR protein in the cord. Gonadectomized rats conversely had lower levels of KOR protein in the non-arthritic rats compared to the intact non-arthritic rats. The induction of arthritis in the gonadectomized animals significantly increased KOR protein level in the spinal cord.

3.3 Effect of arthritis on male rats.

Figure 3, panels A, B, and C show the optical density ratios of MOR, DOR, and KOR proteins, respectively, standardized to the GAPDH loading control in male Lewis rats. In Panel A, the MOR protein level of the intact arthritic males is significantly higher than the intact non-arthritic midbrain tissue, but no difference is observed in the midbrain tissue of the castrated (“GDX”) male rats. In the male spinal cord tissue, the MOR protein levels showed no significance differences between intact or castrated rats.

There were no significant differences observed in the male DOR protein levels in the midbrain and spinal cord tissue, as seen in Panel B. (in arthritic or non-arthritic rats). The KOR protein level (Panel C) was not significantly different in the intact male midbrain tissue, although there is a trend for arthritis to decrease KOR protein levels in

the midbrain. Gonadectomized arthritic males displayed higher KOR protein levels than the gonadectomized non-arthritic rats. Significantly higher levels of KOR protein were found in the spinal cord tissue of the intact non-arthritic male rats than the intact arthritic rats. Among the castrated arthritic and non-arthritic male spinal cord tissue no significant differences were observed.

Sex Differences in MOR, DOR, and KOR

Male and female data were directly compared in Fig 4, Panels A, B, and C to determine variations in MOR, DOR, and KOR protein levels. These figures display the male and female data comparing the midbrain (“MB”) and cord tissues of arthritic and non-arthritic animals and of intact and gonadectomized animals. Fig 4.A shows the comparison of the MOR protein, in males and females, which shows significantly higher ($p < 0.05$) protein levels in the midbrain tissue of intact non-arthritic and arthritic male rats and also in nonarthritic gonadectomized male rats versus female rats. The midbrain (MB) data also indicated a near significant difference between the MOR protein levels of male and female arthritic gonadectomized rats ($p = 0.05$). Male MOR protein levels were significantly higher in all spinal cord tissue versus that of female rats.

No significant differences were observed between male and female midbrain DOR protein levels, as seen in Fig B. In the spinal cord, intact female rats had significantly higher DOR protein levels in both nonarthritic and arthritic rats compared to male rats. However, no significant differences were observed in the spinal cord DOR protein levels of arthritic and nonarthritic animals after gonadectomy, although the trend

remains that the female DOR proteins are higher than male in cord. According to Fig 4., although intact male and female rats had similar KOR protein in MB when no arthritis was present, gonadectomy and arthritis significantly reduced KOR proteins in males, but had no effect on female MB KOR protein. Female KOR protein levels were significantly higher in all spinal cord tissue versus that of male rats.

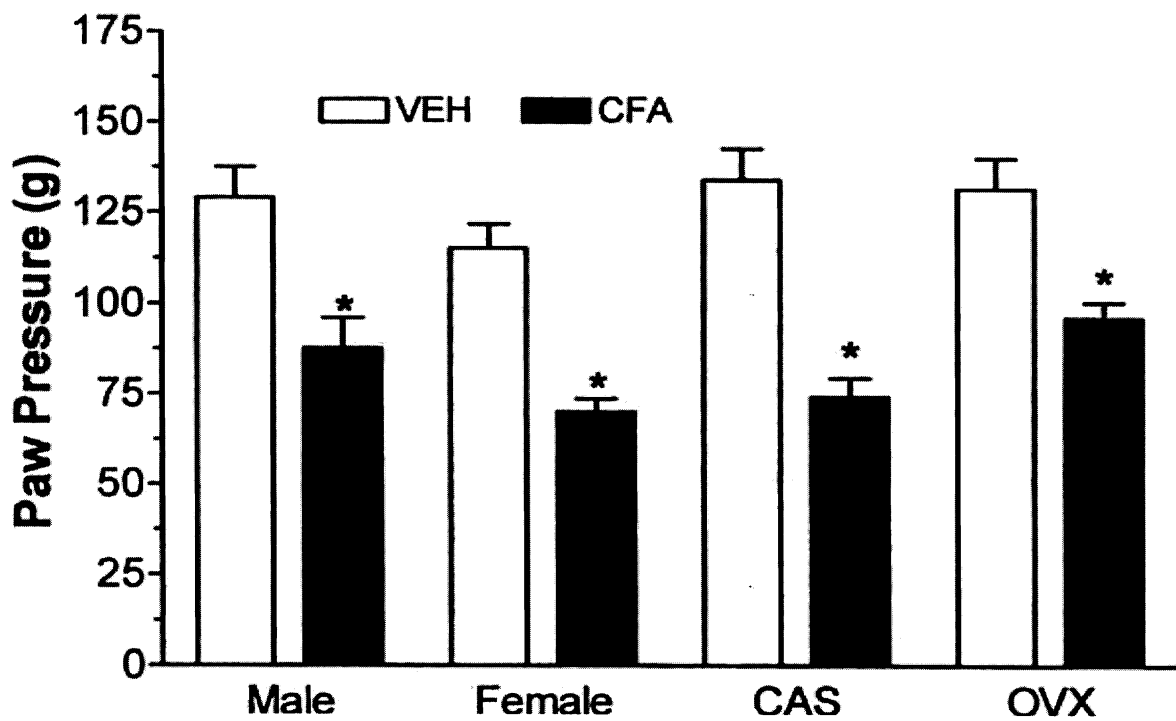
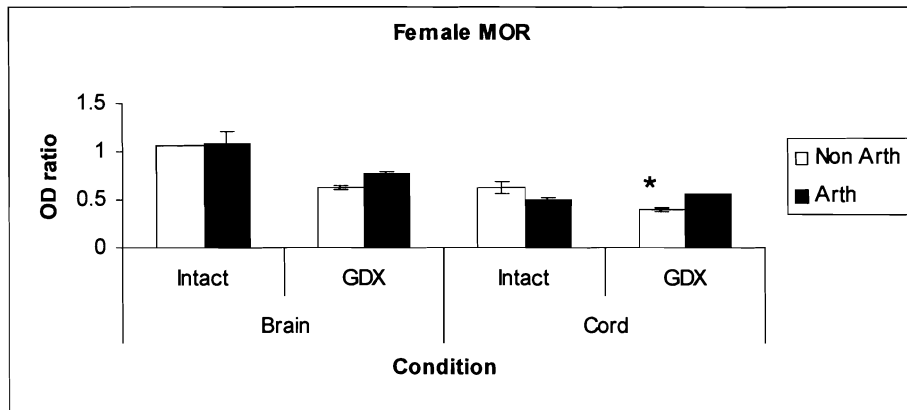
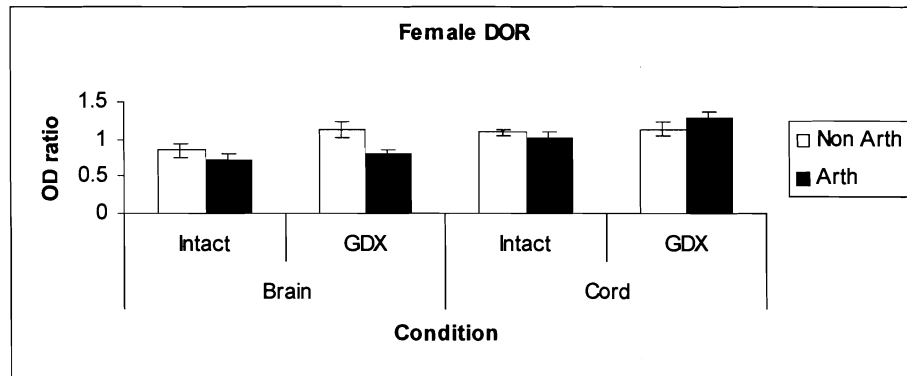


Figure 1. The effect of gonadectomy and CFA treatment in male and female rats on paw pressure thresholds on day 19. Brackets indicate S.E.M. Asterisk (*) indicates a significant difference (Bonferroni's post hoc test) from same day VEH treatment group.

Panel A



Panel B



Panel C

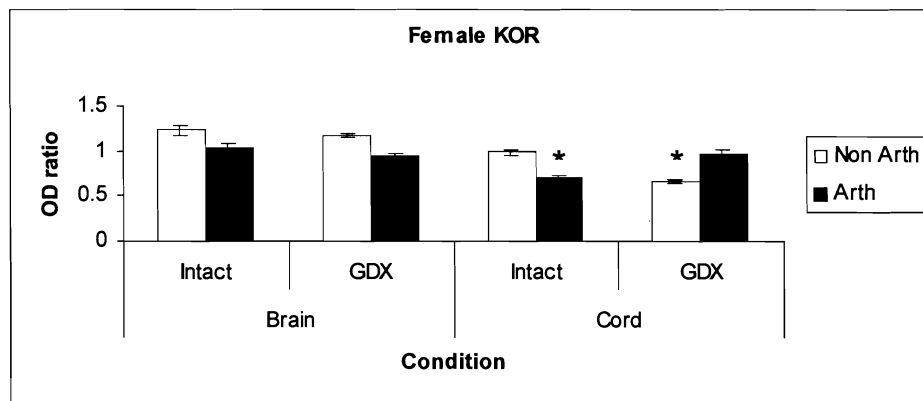
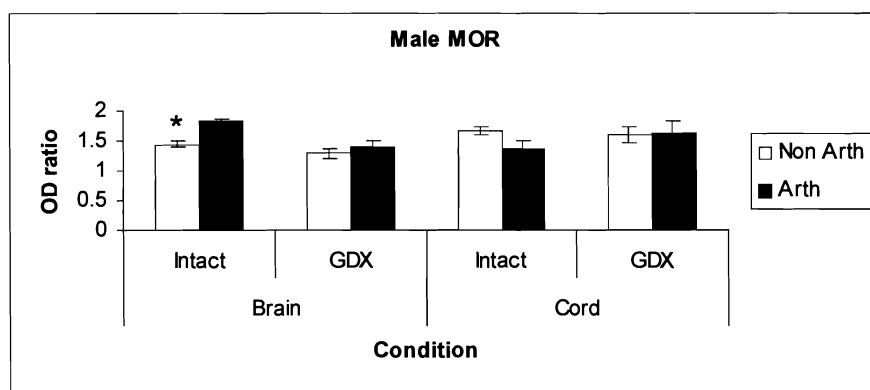
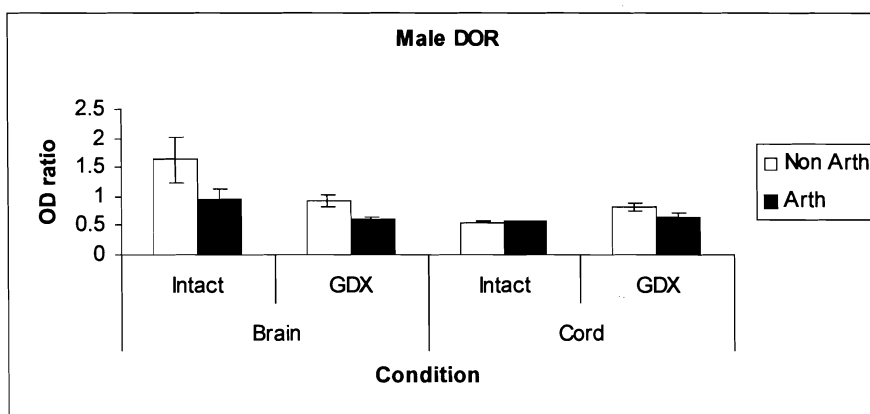


Figure 2. Effect of arthritis on MOR, DOR, and KOR protein levels in female rats are shown in Panels A, B, and C, respectively. The arthritic state was induced by an intradermal injection of Freund's adjuvant. The midbrain and spinal cord tissue were removed immediately after decapitation and frozen at -80°C . Bars represent the mean \pm S.E.M. of 3 female rats. * Indicates significant difference between non-arthritic and arthritic animals: $P < 0.05$ by an independent samples t-test.

Panel A



Panel B



Panel C

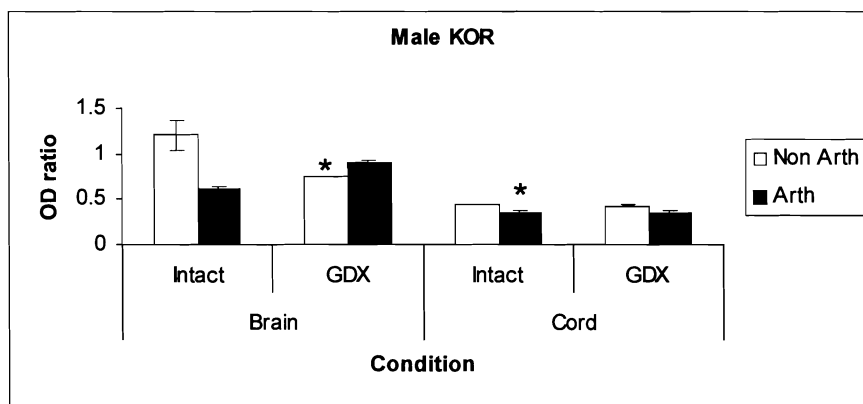
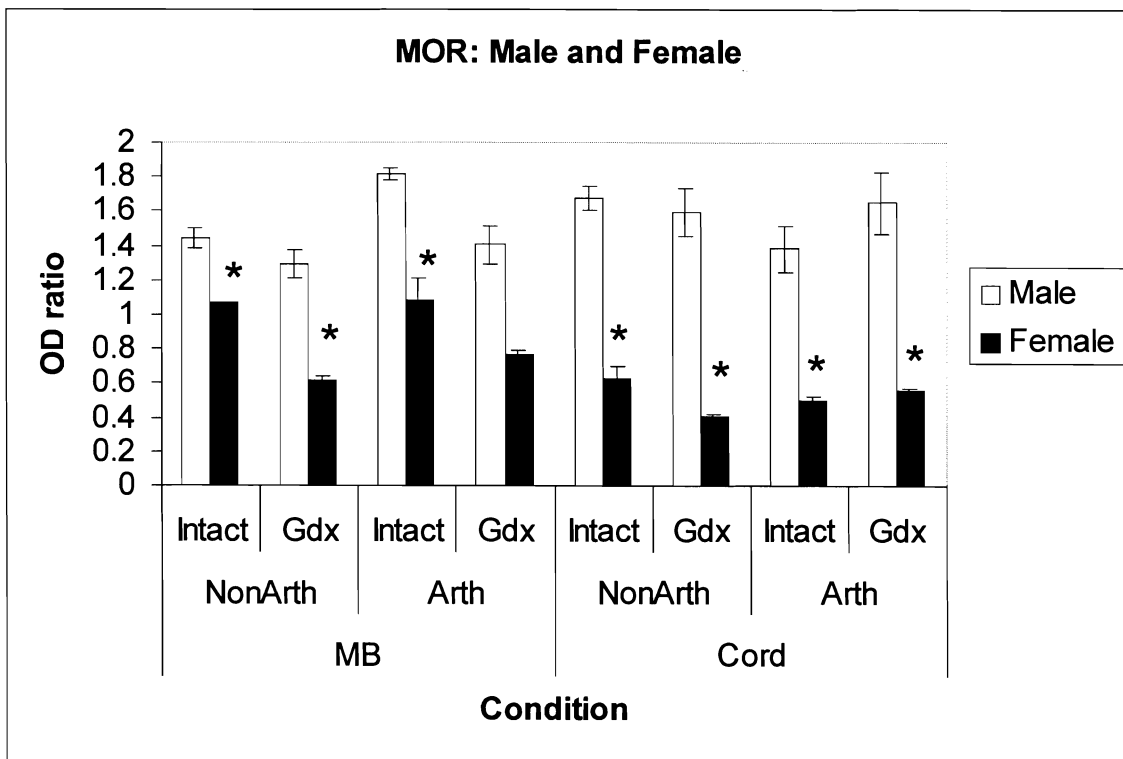
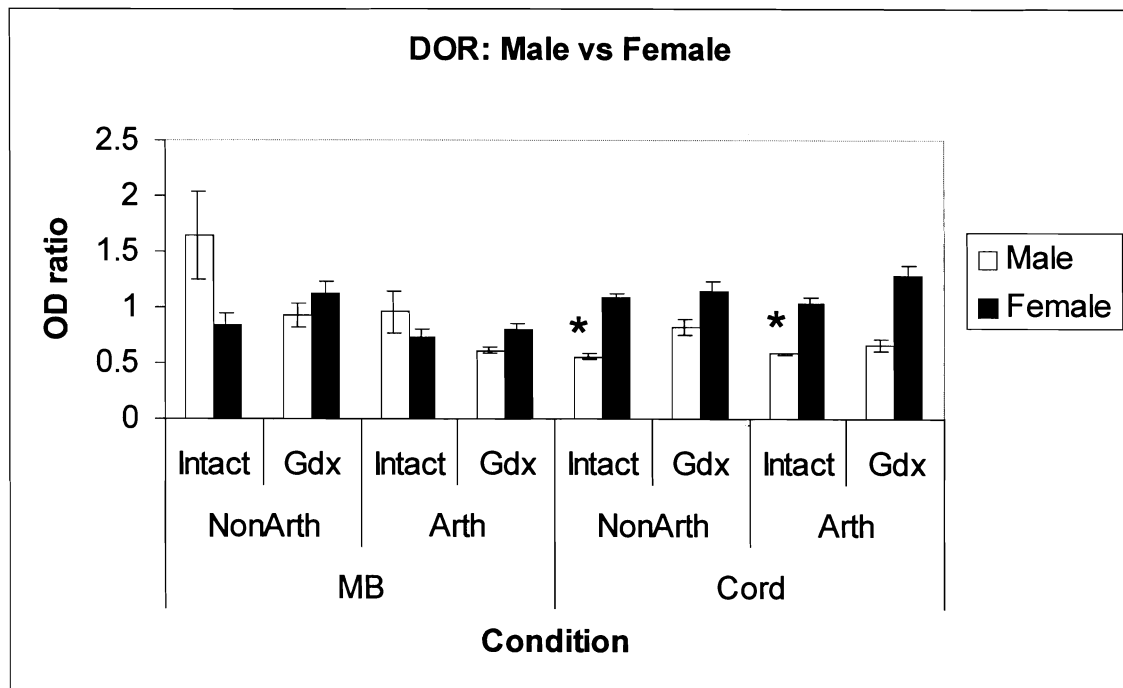


Figure 3. Effect of arthritis on MOR, DOR, and KOR protein levels in male rats are shown in Panels A, B, and C, respectively. The arthritic state was induced by an intradermal injection of Freund's adjuvant. The midbrain and spinal cord tissue were removed immediately and stored at -80°C . Bars represent the mean \pm S.E.M. of 3 male rats. * Indicates significant differences between non-arthritic and arthritic animals: $P < 0.05$ by an independent samples t-test.

Panel A



Panel B



Panel C

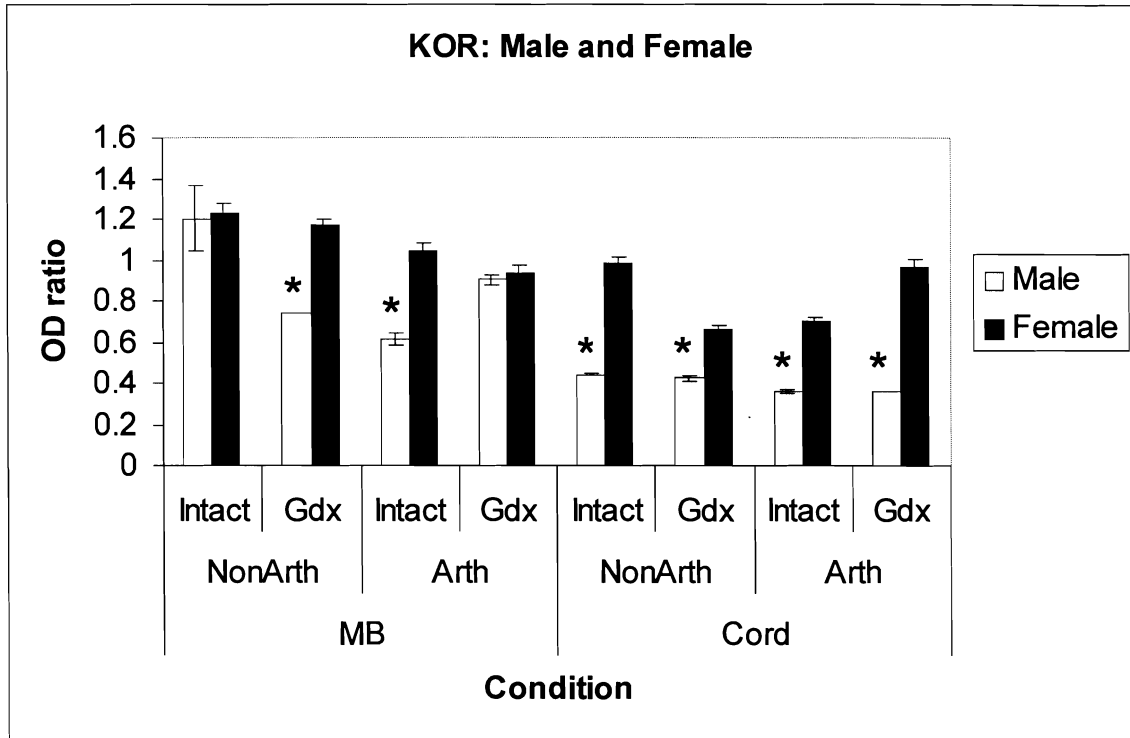


Figure 4. Sex Differences in MOR, DOR, and KOR. Effect of arthritis and gonadectomy on MOR, DOR, and KOR protein levels in male and female rats are shown in Panels A, B, and C, respectively. The arthritic state was induced by an intradermal injection of Freund's adjuvant. The midbrain and spinal cord tissue were removed immediately after decapitation and frozen at -80°C . Bars represent the mean \pm S.E.M. of 3 male or female rats. * Indicates significant differences between non-arthritic and arthritic animals: $P < 0.05$ by an independent samples t-test.

Table 1. Optical Densities on MOR, DOR, and KOR. The data from scans of optical densities for the opioid receptor proteins standardized as a ratio to GAPDH are shown in Table 1. The data were the source for Figures 2-4, previously presented. Values in bold have significantly lower means than the opposite sex (independent samples t-test).

^A Significant difference vs. Intact Non-Arthritic of the same sex and location

^B Significant difference vs. Intact Arthritic of the same sex location

^C Significant difference vs Gonadectomized Arthritic of the same sex and location

Mu	Brain		Cord	
	Male	Female	Male	Female
NonArth				
Intact	1.442 ± 0.11 ^B	1.070 ± 0.00	1.670 ± 0.14	0.630 ± 0.12
GDX	1.290 ± 0.15	0.616 ± 0.10 ^A	1.593 ± 0.28	0.401 ± 0.04 ^C
Arth				
Intact	1.818 ± 0.07	1.086 ± 0.24	1.382 ± 0.27	0.498 ± 0.05
GDX	1.403 ± 0.22	0.769 ± 0.05	1.648 ± 0.37	0.553 ± 0.02
Delta	Brain		Cord	
	Male	Female	Male	Female
NonArth				
Intact	1.638 ± 0.78	0.844 ± 0.20	0.559 ± 0.05	1.095 ± 0.08
GDX	0.921 ± 0.21	1.127 ± 0.22	0.819 ± 0.14	1.135 ± 0.19
Arth				
Intact	0.960 ± 0.37	0.725 ± 0.15	0.581 ± 0.03	1.207 ± 0.13
GDX	0.611 ± 0.05	0.810 ± 0.09	0.659 ± 0.11	1.280 ± 0.20
Kappa	Brain		Cord	
	Male	Female	Male	Female
NonArth				
Intact	1.203 ± 0.33	1.228 ± 0.11	0.443 ± 0.01	0.986 ± 0.07
GDX	0.743 ± 0.00	1.170 ± 0.05	0.425 ± 0.03 ^A	0.667 ± 0.04 ^C
Arth				
Intact	0.618 ± 0.06	1.045 ± 0.07	0.358 ± 0.02 ^A ^C	0.702 ± 0.04 ^A
GDX	0.904 ± 0.05 ^B	0.940 ± 0.07	0.361 ± 0.01	0.963 ± 0.08

Discussion

Historically, most studies investigating sex differences on chronic pain have focused primarily on the use of opioid agonists and their relative efficacies based on the presence or absence of pain or gonadal hormones. The present study examined the role of gonadal hormones on opioid receptor protein levels in intact and gonadectomized, non-arthritic and CFA-induced arthritic Lewis rats. The presence of the gonadal hormones was demonstrated to have an important effect on the pain sensation in both male and female vehicle and CFA-treated rats. Within a single sex, the effect of gonadectomy also showed to be an important variable in the modulation of pain for males and females. Significant sex differences were observed in MOR and KOR protein levels, but not DOR protein.

The effects of CFA-induced hyperalgesia resulted in significantly decreased pain thresholds in males and females, compared to vehicle treated animals in the paw pressure test (Fig 1). The spinal cord tissue of female Lewis rats may have a greater role in modulating pain sensations than midbrain tissues. CFA-treated female rats exhibited an increase in pain threshold after ovariectomy (Fig. 1), which directly corresponds to a significant increase in KOR protein levels in the spinal cord of arthritic ovariectomized females (Fig 2, Panel C; Fig 4, Panel C).

High densities of KOR have been found in the lumbosacral region of the spinal cord of both rats and humans (Gouarderes et al., 1985). KOR protein levels of female intact non-arthritic cord tissue are significantly greater than that of the intact arthritic female; however, this difference is reversed by ovariectomy (Fig 2, Panel C). This trend toward increased pain threshold was also demonstrated in MOR protein (Fig 2, Panel A), indicating a significant effect of the gonadal hormones in female pain modulation. The present findings also correlate with previous studies that observed an upregulation of dynorphin in the spinal cord of rats during pregnancy and parturition (Gintzler and Bohan, 1990; Medina et al., 1993).

Androgens often function as natural anti-inflammatory hormones by suppressing both humoral and cellular immune responses (Stoffel et al., 2005). The role of the androgens in pain modulation has primarily been investigated by testing opioid analgesia in sham and gonadectomized animals. These studies have demonstrated mixed results after male gonadectomy, including increases, decreases, and no change. In the present study gonadectomy of males produced a stabilizing effect on opioid receptor protein in the midbrain and spinal cord of nonarthritic and arthritic rats. That is, MOR and KOR protein levels in the spinal cord tissue of nonarthritic male rats were significantly higher than in arthritic rats, however this difference was eliminated by the removal of the gonadal hormones (Fig 3, Panels A and C). Gonadectomy produced similar results in the μ receptor protein level in the male midbrain tissue as well (Figure 3, Panel A). However, the effects of gonadectomy in males was much less profound than in females. The data on the MOR protein in the midbrain along with MOR and KOR spinal cord

receptor protein levels demonstrate the potential impact of the male gonadal hormones on opioid receptor proteins and suggests that gonadectomy may increase nociception.

Similarly, Stoffel et al. (2005) reported decreased antinociception after gonadectomy in male rats treated with μ and κ opioids, but not δ opioids. The small magnitude of change observed in opioid receptor proteins in males following gonadectomy may have little impact on pain perception.

The presence of sex differences in opioid antinociception has been observed in both rodent (Kepler et al., 1989; Cicero et al., 1996, 1997; Kest et al., 1999) and non-human primate models (Negus and Mello, 1999). In the present study, sex differences were observed in both the μ and κ opioid receptor protein levels. The spinal cord tissue of male rats, regardless of the presence of gonads or arthritis displayed significantly greater levels of MOR protein levels than females (Fig. 4, Panel A). Male MOR protein levels were significantly greater in all tested midbrain tissue, except for the near significant difference ($p = 0.05$) seen in arthritic gonadectomized male (Fig. 4, Panel A). These results are consistent with previous studies that suggest greater opioid antinociception from μ agonists in male rats.

Despite these findings, KOR protein levels appear to have a significantly greater influence on pain sensation in female rats. Female KOR protein levels are significantly greater than males in all spinal cord tissue. KOR protein levels in female midbrain tissue were also significantly higher in the non-arthritic ovariectomized and arthritic intact female rats (Fig. 4, Panel C). According to previous studies, gonadectomized females displayed decreased opioid antinociception compared to intact females (Cicero et al.,

1996; Craft et al., 1996; Kest et al., 1999), which may have resulted from using μ -selective agonists, such as morphine, fentanyl, and hydromorphone. The present study indicates the possibility of greater opioid analgesia in females with κ -selective agonists.

It is also important to mention that rodent strain may also function in modulating sensitivity to opioid-induced antinociception and determining the magnitude of sex differences. In previous studies, F344 and Lewis strains demonstrated greater sex differences than the Sprague-Dawley strain in potency and effectiveness of μ -opioid agonists (Bartok and Craft, 1997; Barrett et al., 2001; Cook et al., 2000). The significantly greater KOR protein levels seen in the Lewis rats used in the present study, may correlate to the slightly more sensitive effects of U50,488, a kappa agonist, on female Lewis rats compared to male Lewis rats observed in a previous study (Barrett et al., 2001).

The overall findings of the present study provide strong evidence for the importance of the gonadal hormones in the modulation of pain sensation in arthritic and nonarthritic Lewis rats, especially in the female rat. The removal of gonadal hormones appears to have opposite effects in males and females; causing decreased pain in females and increased pain in males. Under these conditions, pain sensation in males or females does not appear to be modulated by delta opioid receptor proteins. The presence of significantly greater MOR protein levels in male rats is in agreement with previous studies. However, a major implication of the present study is the greater levels of KOR protein in female rats, possibly indicating that usage of more κ -selective opioid agonists,

like U50,488, butorphanol and pentazocine, may demonstrate greater efficacy in providing pain relief to females. The administration of κ -selective opioid agonists may also eliminate many of the negative effects of μ -opioid agonists that women experience, including nausea, emesis, and constipation.

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Vita

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