



Sex differences and phases of the estrous cycle alter the response of spinal cord dynorphin neurons to peripheral inflammation and hyperalgesia

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Abstract

The neuromodulatory interactions of sex steroids with the opioid system may result in sex differences in pain and analgesia. Dynorphin is an endogenous kappa-opioid peptide that is upregulated in an animal model of peripheral inflammation and hyperalgesia and is possibly regulated by circulating levels of sex steroids. The present study compared behavioral responses of male, cycling female, and gonadectomized Sprague–Dawley rats in a model of persistent pain. Cycling female rats were behaviorally tested over a 14-day period, and their estrous cycles were monitored by daily vaginal smears. Thermal hyperalgesia was measured by paw withdrawal latencies taken prior to and 24–72 h after rats received a unilateral hindpaw injection of complete Freund's adjuvant (CFA). Prior to CFA administration, there was no significant difference in paw withdrawal latencies between male rats, cycling female rats, and ovariectomized female rats. Following CFA administration, female rats in proestrus exhibited significantly increased hyperalgesia compared with male rats, ovariectomized female rats, and female rats in other estrous stages ($P \leq 0.05$). Levels of spinal preprodynorphin (PPD) mRNA induction in the L4–L5 segments were assessed by Northern blot analysis. PPD mRNA expression ipsilateral to the injected paw was significantly higher in female rats in diestrus ($P \leq 0.05$) and proestrus ($P \leq 0.01$) compared with rats in estrus and intact male rats. Ovariectomized rats had significantly higher levels of PPD mRNA expression compared with intact male rats ($P \leq 0.05$). However, castrated male rats had significantly lower levels of PPD mRNA expression than intact male rats ($P \leq 0.05$). PPD mRNA expression was not altered on the contralateral side of the spinal cord in any group. These results suggest a hormonal regulatory influence on the response of spinal cord dynorphin neurons to chronic inflammation and furthermore, that the association of the endocrine and opioid systems have the ability to influence an animal's sensitivity to pain. Published for the International Association for the Study of Pain by Elsevier Science B.V.

Keywords: Preprodynorphin; Hormones; Opioid; Pain response

1. Introduction

Sex differences, developmental in origin, and enhanced by the changing hormonal milieu, shape the circuitry of the nervous system. Many neural pathways are differentially influenced when exposed to steroid hormones (for review see: Breedlove, 1992; Toran-Allerand, 1995). Also, the response of an organism to environmental stressors can be sex-dependent (Sternberg and Liebeskind, 1995; Sternberg et al., 1995; Mogil and Belknap, 1997). Sex differences in the perception and modulation of pain in both animal and human studies have been previously reviewed (Berkley, 1997). These sex differences may be related to the effect of steroid hormones on the developing and adult nervous

systems' response to pain. Therefore, an understanding of possible neuromodulatory roles of sex steroid hormones on the opioid system is important in understanding sex differences in pain modulation.

Dynorphin is an endogenous opioid peptide with a high affinity for the kappa-opioid receptor. Up-regulation of preprodynorphin (PPD) mRNA in the spinal cord has been associated with an increase in neuronal activity and an increase in thermal hyperalgesia associated with inflammation (Dubner and Ruda, 1992). However, there is also evidence to suggest that dynorphin may play a role in antinociception. An up-regulation of dynorphin in the spinal cord coincides with an elevation in pain thresholds in rats during pregnancy and parturition (Gintzler and Bohan, 1990; Medina et al., 1993a,b). This effect is thought to be mediated through the interactions of elevated levels of estrogen and progesterone with the kappa and delta opioid systems (Dawson-Basoa and Gintzler, 1996, 1998). Clinical

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evidence has shown that sensitivity thresholds in women in response to a variety of painful stimuli is dependent upon menstrual cycle phase, tissue type, and plasma sex steroid levels (Giamberardino et al., 1997a,b; Fillingim et al., 1997, 1998). Furthermore, clinical studies suggest that women obtain greater pain relief from kappa-opioid analgesics than men (Gear et al., 1996a,b).

Investigating possible sex and estrous cycle differences in the mechanisms associated with the up-regulation of spinal PPD mRNA expression may provide insight into the neuro-modulatory role of sex steroids on pain and analgesia. This study examined the hypothesis that sex differences exist in the neuronal response to peripheral inflammation and hyperalgesia and that these differences are reflected in spinal PPD mRNA expression and inflammation-induced thermal hyperalgesia. We would predict that these sex differences may be dependent upon natural hormonal variations associated with the estrous cycle and that gonadectomy would alter the natural hormonal effect on the processing of noxious stimuli and the perception of pain.

2. Methods

2.1. General experimental design

Behavioral responses to thermal stimuli delivered to the hindpaw were assessed in rats before and after unilateral injection of complete Freund's adjuvant (CFA) into the hindpaw. Rats were sacrificed and the lumbar region of the spinal cord was isolated, RNA was extracted, and processed for Northern blot analysis to examine the expression of PPD mRNA. In these experiments, 70 cycling and 18 ovariectomized female rats as well as 32 intact and 6 castrated male rats were originally studied. The final experimental groups are listed below.

2.2. Subjects

All experiments were performed using adult male and female Sprague–Dawley rats (males: 225–375 g; females: 175–200 g). Experimental groups included in our analysis consisted of, cycling female rats ($n = 18$), ovariectomized female rats ($n = 12$), intact male rats ($n = 18$), and castrated male rats ($n = 6$). Gonadectomized rats (castrated rats: 300–325 g; ovariectomized rats: 275–325 g) were received from the Harlan Sprague–Dawley Company. The animals were kept on a 12/12 h light–dark cycle (07:00 h to 19:00 h) and provided with food and water ad libitum. The animal protocol was reviewed and approved by the NIDCR Animal Care and Use Committee. The procedures in this study followed the NIH Guidelines for the Care and Use of Laboratory Animals. Treatment of animals in this study conforms to the International Association for the Study of Pain Ethical Guidelines (Zimmerman, 1983).

2.3. Estrous cycle determination

Estrous phase was determined by daily vaginal lavage within 3 h of lights-on using the methods of Long and Evans (1922). Approximately 0.10 ml of distilled water was forced into the vaginal canal using a small plastic pipette and instantly retrieved and transferred to a clean slide. Each sample was viewed and cytology was categorized using the system outlined by Freeman (1994). Briefly, the 2 days of diestrus are characterized by a predominance of leukocytes, proestrus is characterized by a predominance of round nucleated epithelial cells and an absence of leukocytes, and estrus is characterized by large numbers of cornified squamous epithelial cells. Female rats that displayed a 4-day estrous cycle were used in this study.

2.4. Behavioral assessments

Rats were tested for paw withdrawal latency to a noxious radiant heat stimulus (Hargreaves et al., 1988) prior to and 24 h after receiving a 0.2 ml unilateral subcutaneous injection of the inflammatory agent complete Freund's adjuvant into the plantar surface of the left hindpaw (1:1, CFA/saline). All comparison groups were tested at the same time of day, between 3 and 5 h after lights-on.

Paw withdrawal latency was calculated by combining and averaging the mean latencies of three stimulus presentations to each hindpaw prior to CFA treatment. After CFA treatment, relative thermal hyperalgesia was determined by difference scores of the mean latencies of three stimulus presentations to each hindpaw (contralateral minus ipsilateral hindpaw).

2.5. Tissue collection

Rats were sacrificed 24–72 h after CFA injection. Cycling female rats were sacrificed between 7 and 9 h after lights-on. At the time of sacrifice, rats were overdosed with sodium pentobarbital injected intraperitoneally (50 mg/kg, Abbott Laboratories, Chicago, IL). The L4 and L5 dorsal root ganglion (DRG) were exposed and their roots were traced up to the entry point in the spinal cord. An 8 mm piece of the lumbar spinal cord containing the L4–L5 segments was removed, and tissue was cut along the midline into ipsilateral and contralateral sides. Identical landmarks were used for tissue removal in each experimental group. Tissue dissections for each group of experiments were performed by the same individual. Tissue was frozen at -70°C until RNA isolation.

2.6. Northern blot procedures

RNA was isolated using the TRI Reagent method (Molecular Research Center Inc., Cincinnati, OH). RNA absorbance at 260 nm was measured on a spectrophotometer to obtain a yield in $\mu\text{g}/\mu\text{l}$. Equal amounts (10 μg) of denatured total RNA were separated on 1% agarose-formaldehyde gels

and transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH) by capillary transfer overnight at room temperature with $20 \times$ SSPE. RNA was cross-linked to the membrane in a UV Stratalinker (Stratagene, La Jolla, CA). Blots were baked for 1–2 h at 60°C in a vacuum oven. Blots were hybridized to a 1.7 kb *EcoRI/PstI* rat preprodynorphin genomic clone fragment. The probe was labeled with [α - ^{32}P]dCTP (New England Nuclear, Boston, MA) by nick translation (Amersham Life Sciences, Piscataway, NJ) and 2×10^6 cpm/ml of labeled probe was added to 10 ml of prehybridization buffer containing 1 ml of 50% dextran sodium sulfate (Pharmacia Biotech, Uppsala, Sweden). Blots were hybridized overnight at 42°C and washed three times at 42°C with $2 \times$ SSPE/0.1% SDS; the final wash was done at 60°C for 30 min.

RNA loading of the blots was normalized by reprobing with a glyceraldehyde phosphate dehydrogenase oligonucleotide (GAPDH) (Oncogene Research Products, Cambridge, MA). GAPDH was used as a normalization control because its expression is not regulated by the methods used in this study. The GAPDH oligo was labeled with [α - ^{32}P]dATP using 3' end labeling (Boehringer Mannheim, Indianapolis, IN).

Autoradiographs were produced by exposing the labeled membranes to Biomax film (Eastman Kodak Co., Rochester, NY) with intensifying screens at -70°C . Blots were then exposed to phosphor screens and quantified according to a phosphorimager system analysis protocol (Molecular Dynamics, Sunnyvale, CA).

For the estrous stage blots, spinal cords from three male rats were pooled together to provide enough sample so that the same control mRNA could be loaded on all the estrous stage blots. For the experimental female rats in the estrous stage blots, a single animal was represented in each lane. For the gonadectomy blots, spinal cords from three to four rats per lane were pooled together. Different groups of control male rats were used in the ovariectomy and castration blots. Levels of PPD mRNA expression in all experimental groups were standardized to the intact male's contralateral levels, which were normalized as 100%.

2.7. Statistical analysis

To establish a standardized way of comparing results across experimental groups, levels of PPD mRNA expres-

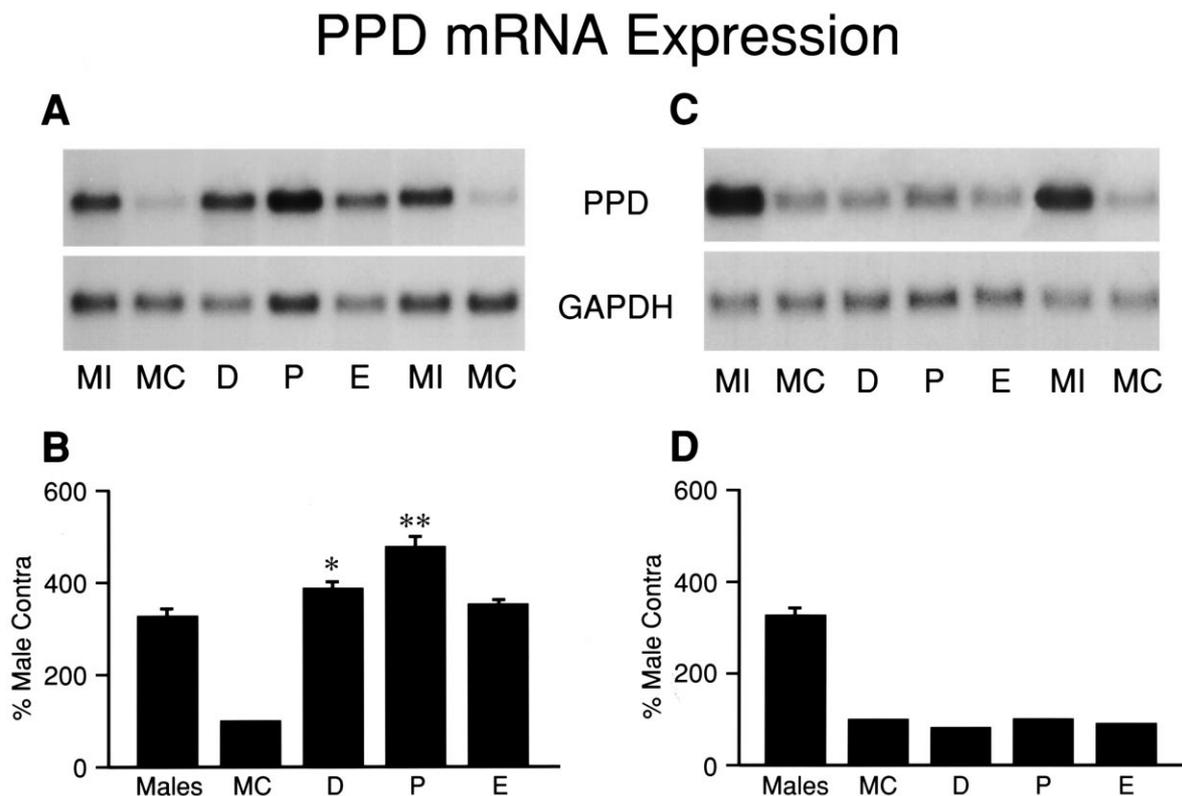


Fig. 1. Northern blot analysis of preprodynorphin (PPD) mRNA expression in the ipsilateral (A) and contralateral (C) lumbar spinal cord 24 h after CFA-induced hindpaw inflammation. (B) Increases in PPD mRNA induction resulting from inflammation and hyperalgesia were significantly greater in female rats in diestrus (D) and proestrus (P) ($*P \leq 0.05$, $**P \leq 0.01$) compared with that observed on the ipsilateral side in male rats (MI). Female rats in estrus (E) and male rats had equivalent values. In (B), each bar represents the mean value of four animals, normalized to GAPDH, and expressed as a percentage of the contralateral side of the male rats (MC). There was no difference in contralateral PPD mRNA expression among male rats and cycling female rats, as shown in (D); for the female rats, each bar represents a single animal.

PPD mRNA Expression

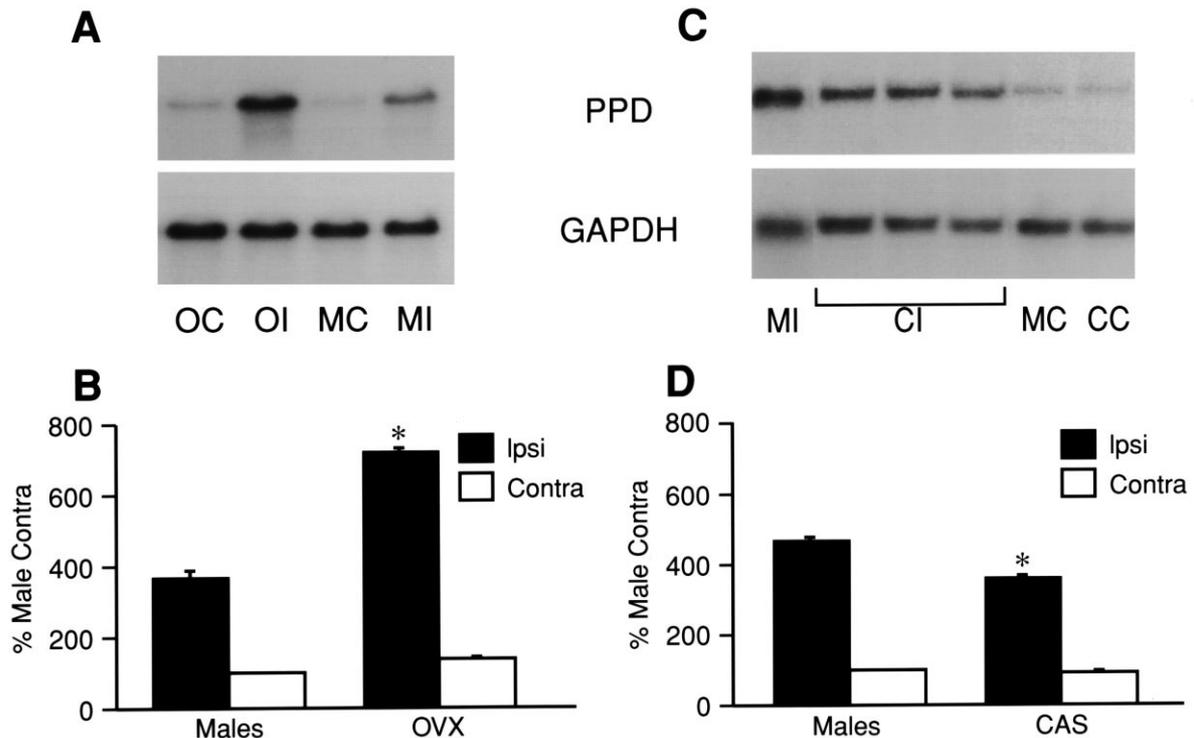


Fig. 2. Northern blot analysis of PPD mRNA expression in the spinal cord of intact male rats versus ovariectomized (OVX) female rats (A) and castrated (CAS) male rats (C). The induction of ipsilateral PPD mRNA following inflammation was significantly greater in the OVX female rats compared with intact male rats ($n = 6$, $*P \leq 0.05$) (B). (D) Ipsilateral PPD mRNA induction was significantly lower in the CAS male rats ($n = 6$) compared with intact male rats, 24 h after CFA injection ($*P \leq 0.05$). MI, male ipsilateral; MC, male contralateral; OI, ovariectomized ipsilateral; OC, ovariectomized contralateral; CI, castrated ipsilateral; CC, castrated contralateral.

sion in each group was standardized to the intact male's contralateral levels, which were normalized as 100% expression. This approach avoided potential estrous cycle differences in mRNA expression. PPD mRNA intensity percentage scores from four animals in each estrous group were averaged from individual blots and compared with blots from intact males. Percentage scores of PPD mRNA for ovariectomized and castrated rats ($n = 6$ per group) were also determined relative to intact males.

Baseline paw-withdrawal latencies for cycling female rats, ($n = 18$; diestrus: $n = 6$, proestrus: $n = 6$, and estrus: $n = 6$) ovariectomized females, ($n = 6$) and intact male rats ($n = 6$) were determined prior to CFA administration. Post-CFA paw-withdrawal latencies were determined using five rats in each of these experimental groups. From the post-CFA groups behaviorally tested, four rats per group were used to determine PPD mRNA intensity percentage scores. Separate groups of ovariectomized females were used to test behavioral responses and PPD mRNA.

Behavioral and PPD mRNA expression data were analyzed using repeated measures ANOVA with post-hoc Fisher's least significant difference analysis and *t*-tests. Graphical representations are shown as means \pm SEM.

3. Results

3.1. Preprodynorphin (PPD) mRNA after unilateral hindpaw CFA injection

A representative Northern blot of PPD mRNA expression from the ipsilateral spinal cord of intact male rats and cycling female rats is shown in Fig. 1A. The phase of the estrous cycle did not influence the constitutive expression of dynorphin mRNA on the contralateral side of the spinal cord after hindpaw CFA injection (Fig. 1C,D). The levels of dynorphin mRNA were similar for male rats and female rats on the contralateral side. However, following CFA treatment differences in dynorphin mRNA expression were noted between male and female rats as well as across the phases of the estrous cycle. Female rats in diestrus and proestrus showed significantly greater ipsilateral PPD mRNA induction compared with male rats. Female rats in proestrus showed the greatest induction in PPD mRNA levels at 478% ($**P \leq 0.01$, Fig. 1B) compared with PPD mRNA expression levels for male rats at 327%. Female rats in estrus and intact male rats had equivalent levels of PPD mRNA induction.

The effect of gonadectomy on dynorphin mRNA induction following hindpaw peripheral inflammation and hyperalgesia was also examined. Ovariectomized female rats at 722% expression had significantly higher levels of PPD mRNA in the spinal cord ipsilateral to the inflamed hindpaw compared with intact male rats, at 369% ($*P \leq 0.05$, Fig. 2A,B). Castrated male rats showed lower levels of PPD mRNA expression in the ipsilateral spinal cord compared with intact males (362% compared with 469% $*P \leq 0.05$, Fig. 2C,D). There were no differences in contralateral PPD mRNA expression among the gonadectomized groups (Fig. 2A–D).

3.2. Hindpaw withdrawal latencies before and after CFA in intact and gonadectomized rats

Prior to hindpaw CFA injection, there were no sex or estrous cycle differences in paw withdrawal latencies to a noxious radiant heat stimulus (Fig. 3A). However, in female rats in proestrus, compared with intact male rats, thermal hyperalgesia was significantly greater 24 h after unilateral hindpaw CFA injection ($*P \leq 0.05$, Fig. 3B). Female rats in either diestrus or estrus did not differ from those observed in the male rats. Thermal hyperalgesia in ovariectomized female rats did not differ from the intact male rats (Fig. 3B).

4. Discussion

In the present study, sex differences in levels of PPD mRNA induction and behavioral sensitivity thresholds following peripheral inflammation and hyperalgesia were related to the hormonal milieu. The greatest changes in PPD mRNA induction levels in the intact animal appeared

to follow the natural progression of the rise and fall of sex steroids in the female rat. During diestrus when sex steroids (estrogen more than progesterone) are beginning to rise, PPD mRNA induction levels were significantly higher than those observed in the male rats. PPD mRNA induction levels were highest during the afternoon of proestrus when circulating estrogen is highest, yet before progesterone has peaked. During the afternoon of estrus, when circulating sex steroids are at their lowest levels (Freeman, 1994), PPD mRNA levels were equivalent to those of the males. These data suggest a relationship between sex steroids and PPD mRNA induction in response to peripheral inflammation and hyperalgesia.

The mechanisms by which post-inflammatory changes in spinal PPD mRNA expression occur over the estrous cycle are unclear. An example of how the hormonal and opioid systems exert regulatory control over each other can be found in the hypothalamus. Under normal conditions, the opioid system exerts a tonic inhibition on luteinizing hormone secretion in female animals which depends upon the hormonal feedback loop between the ovaries and the hypothalamus (Kordon, 1994). The mechanism for this control is not well understood, although neurons in the anteroventral periventricular nucleus of the hypothalamus have been shown to co-express opioid and steroid receptors (Simerly et al., 1996). It is possible such co-expression of receptors is also present in the spinal cord, however, much less is known about sex steroid modulation of the opioid system in the spinal cord. Estrogen receptors have been identified in the rat spinal cord (Amandusson et al., 1995) and those in the lumbar spinal cord (in laminae I–V) have been shown to vary in density across the estrous cycle, becoming most dense during proestrus (Williams et al.,

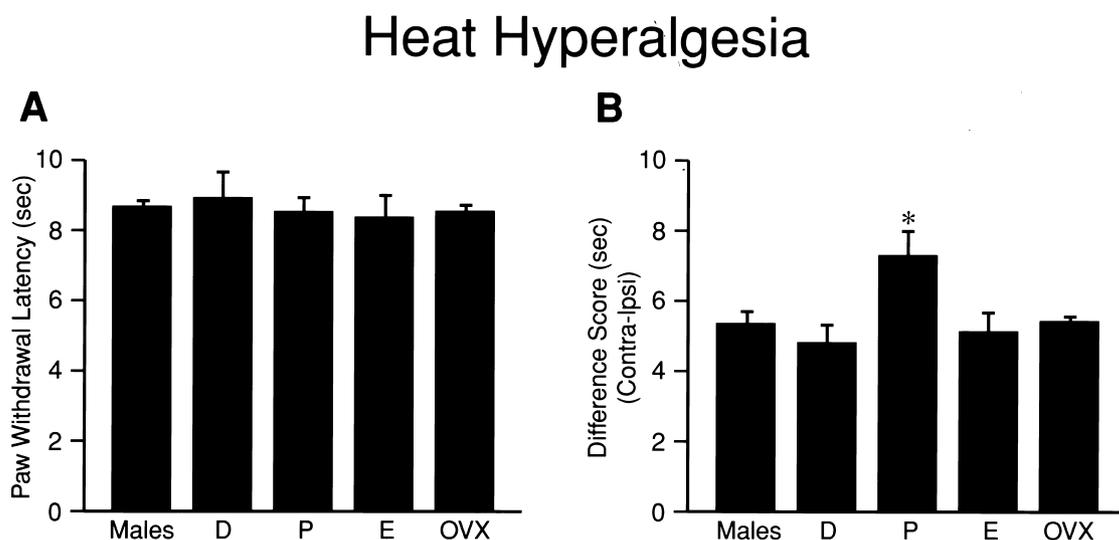


Fig. 3. Paw withdrawal latency to a noxious radiant heat stimulus before (A) and after (B) unilateral hindpaw CFA injection. (A) Normal hindpaws exhibited no difference in paw withdrawal latencies between intact male rats (Males), diestrus (D), proestrus (P), estrus (E), or ovariectomized (OVX) female rats ($n = 6$ per group). (B) Following CFA injection, hindpaw hyperalgesia was significantly greater in female rats in proestrus compared with male rats ($*P \leq 0.05$). Paw withdrawal latencies in female rats in diestrus and estrus as well as ovariectomized females did not differ from those of intact male rats ($n = 5$ per group).

1997). This is evidence that there are cellular and molecular changes in the female lumbar spinal cord which coincide with the estrous cycle. Glutamate, acting through NMDA receptors, has been shown to play a pivotal role in the up-regulation of dynorphin in the CFA-induced inflammatory pain model used in this study (Dubner and Ruda, 1992). Estrogen has been shown to potentiate glutamate actions in multiple sites in the nervous system through, as yet unknown mechanisms (for review see: Smith, 1991; Wooley, 1998). Therefore, if the changes in PPD mRNA expression observed after inflammation across the estrous cycle are related to hormonal fluctuations, it is possible that they are mediated by changes in sex steroid interactions with the glutamate and opioid systems.

One way to investigate the effect of sex steroids on a system is by challenging and observing the system before and after removal of the ovaries and testes. In male animals, PPD mRNA levels after inflammation were significantly lower in castrated rats compared with intact males. By contrast, in the female animals, PPD mRNA levels after inflammation were significantly higher after ovariectomy compared with the intact male rats suggesting that the observed increases in PPD mRNA were not solely related to elevated estrogen levels that occur during proestrus. In gonadectomized animals, the loss of the ovaries and testes and the resultant changes which occur in the natural hormonal feedback loops appear to create unique and complex conditions which may affect the response to persistent pain differently in males and females. To further address these phenomena, future studies should include the addition of dose–response sex steroid replacement regimens to investigate how individual hormones affect this system.

Previous research has shown that ovariectomy significantly increases PPD mRNA levels in the anterior pituitary in female animals, with these levels being further enhanced by tamoxifen administration, an antagonist that binds to estrogen receptors (Spampinato et al., 1995), suggesting a regulatory control of estrogen on PPD mRNA expression. Holtzman and colleagues (1997) observed that estrogen treatment significantly increased preproenkephalin (PPE) mRNA expression in the ventromedial nucleus of the hypothalamus in ovariectomized animals. However, they found that spinal cord PPE mRNA was only increased by estrogen manipulation in the ovariectomized animals after formalin-induced inflammation compared with the control rats, with the highest PPE mRNA content per cell in formalin-treated rats given steroid replacement. These data, taken together with data from the present study, suggest that endogenous opioid peptide modifications in the spinal cord by steroid fluctuations and manipulations in female animals may be most prominent following a noxious stimulus.

Dynorphin expression in the lumbar spinal cord increases during parturition and coincides with an increase in the pain threshold. It has been hypothesized that this effect may be due to the interactions of elevated levels of sex steroids with the kappa and delta opioid systems (Dawson-Basoa and

Gintzler, 1993, 1996, 1998). Our behavioral findings differ in part with this idea since the increase in thermal hyperalgesia observed during proestrus occurred during the time in the estrous cycle when estrogen levels were at their peak. There are two primary factors that likely explain these differences. Firstly, the behavioral modalities used to test hindpaw sensitivity are different (foot shock versus radiant heat) and the processing of these two types of stimuli are likely different. Secondly, the hormonal status during parturition and proestrus are distinct and perhaps cannot be compared since noxious stimulation inputs during natural reproductive states may not be comparable to that observed during hindpaw inflammation. Furthermore, the synergistic involvement of the delta opioid system may be the determining factor in the increase in pain threshold observed during parturition (Dawson-Basoa and Gintzler, 1998) and sex differences in opioid antinociception may be assay, dose and/or time dependent (Bartok and Craft, 1997). Multiple observation intervals may be required to detect any functional changes that may coincide with the levels of spinal cord PPD mRNA seen in this study. It is noted that assessments of thermal hyperalgesia may lack the sensitivity required to determine sex and estrous cycle differences in the response to peripheral inflammation and hyperalgesia.

The present findings suggest that the cyclic fluctuations of ovarian hormones in the female and the loss of sex steroids in both males and females plays a neuromodulatory role on spinal cord PPD mRNA expression after a persistent inflammatory stimulus. These data support the hypothesis that the ovarian cycle and the manipulation of ovarian and testicular hormones influence and are influenced by the opioid system. Furthermore, this evidence suggests a complex regulatory system involving the interactions of sex, level of circulating hormones, and class of sensory input on pain and analgesia.

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