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THE PERIAQUEDUCTAL GRAY: A SITE ELICITING GENDER DIFFERENTIATED RESPONSES IN OPIOID ANALGESIA

by

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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

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Abstract

THE PERIAQUEDUCTAL GRAY: A SITE ELICITING GENDER DIFFERENTIATED RESPONSES IN OPIOID ANALGESIA

by Eliza Krzanowska

Advisor: Professor Richard J. Bodnar, Ph.D.

Sex differences in antinociception are commonly observed following systemic and intraventricular administration of opioids with male rats displaying more marked antinociceptive responses than female rats. The purpose of this dissertation research was to identify a potential brain site and mechanisms which elicit these sex differences. We hypothesized that the ventrolateral periaqueductal gray (vlPAG) is subject to sex differences in opioid antinociception due to its common critical roles in both antinociceptive and reproductive behaviors. We also examined whether these sex differences are mediated by either activational or organizational effects of gonadal hormones which are respectively altered by adult neonatal or neonatal gonadectomy. Thus, the first experiment evaluated sex differences and adult gonadectomy effects in morphine antinociception elicited from the vlPAG. To assess generalizability of these effects, the second experiment evaluated sex and adult gonadectomy differences upon antinociception elicited by the opioid agonists, beta-endorphin and D-Proendomorphin-2 in the vlPAG. Sex differences in motor activity elicited by these two agonists were also assessed. The third and final experiment evaluated sex and neonatal gonadectomy differences in vlPAG morphine antinociception.

Male rats displayed significantly greater magnitudes of antinociception elicited by morphine and beta-endomorphin in the vlPAG than female rats. In contrast, D-Pro2-endomorphin-2 antinociception in the vlPAG on the tail-flick test was significantly greater in female rats than in males, but these effects appeared secondary to sex-specific alterations in motor activation elicited by D-Pro2-endomorphin-2 in the vlPAG.

Adult gonadectomy failed to alter antinociception elicited by morphine in the vlPAG in males, but slightly enhanced morphine antinociception in female rats. In marked contrast, neonatal gonadectomy in males produced a pattern of vlPAG morphine antinociception that was indistinguishable from that of sham-treated females. Moreover, neonatal androgenization of females increased the magnitude of vlPAG morphine antinociception to a level similar to that of sham-operated males. Collectively, these results strongly implicate vlPAG and organizational effects of gonadal hormones in mediating sex differences in opioid antinociception in rats.

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Glossary of Abbreviated Terms

EAA – excitatory aminoacid
Ac-RYYRWK-NH2 – acetyl-Arg-Tyr-Arg-Trp-Lys-NH2
ACTH - adrenocorticotropin
BFNA – beta-funaltrexamine
CCWS – continuous cold-water swims
CLIP – corticotropin-loke intermediate lobe peptide
DADL – D-Ala2, D-Leu5-enkephalin
DALCE – D-Ala2, Leu5, Cys6-enkephalin
DAMGO – D-Ala2, Met-Phe4, Gly-ol5-enkephalin
DHT – dihydrotestosterone
DLF – dorso-lateral funiculus
DPDP – D-Pen2, D-Pen5-enkephalin
DSLET – D-Ser2, Leu5-enkephalin
DRN – dorsal raphe nucleus
HPA – hypothalamic-pituitary-adrenal axis
icv – intracerebroventricular
ICWS – intermittent cold-water swims
LC – locus coeruleus
MIH – Mullerian inhibitory hormone
M6G – morphine-6beta-glucuronide
mPOA – medial pre-optic area
MSH – melanocyte-stimulating hormone
NalBzOH – naloxone benzoylhydrazone
NMDA – N-methyl-D-aspartate
NOR-BNI – nor-binaltorphamine
NRM – nucleus raphe magnus
NRGC – nucleus reticularis gigantocellularis
NSAID – non-steroid anti-inflammatory drug
NTI – naltrindole
NTII – naltrindole isothiocyanate
NTS – nucleus tractus solitarius
ORL1 – orphanin-opioid receptor
PAG – periaqueductal gray
PIA – pregnancy-induced antinociception
POMC – proopiomelanocortin
PPE – preproenkephalin
RVM – rostral ventromedial medulla
s.c. – subcutaneous
SPA – stimulation-produced analgesia
TDF – testis determining factor
TP – testosterone prioprionate
U50,488H – kappa agonist
U69,593 – kappa agonist
VCS – vaginocervical stimulation

vlPAG – ventro-lateral periaqueductal gray
VMH – ventromedial nucleus of the hypothalamus

CHAPTER 1

Introduction

Over the past three decades an endogenous opioid system mediating pain-inhibition has been identified (Basbaum and Fields, 1984), and its neuroanatomical, neurophysiological, and neurochemical substrates have been described, based primarily on research with young male rodents. Recently however, it has become increasingly clear that there are considerable individual differences in sensitivity to painful stimuli and in antinociceptive processing in both humans and other animals. The experience of pain and antinociceptive responsiveness can be shaped and modulated by a variety of organismic variables, such as sex, age and genetic make-up of the individual, by environmental factors, such as diet and diurnal stage, and by psychological influences, such as anxiety and stress (see reviews: Bodnar, Romero, and Kramer, 1988; Fillingim and Maixner, 1995; Kanarek and Homolesky, 2000; Kavaliers and Innes, 1987; Mogil, Chesler, Wilson, Juraska and Sternberg, 2000).

Among those organismic variables that influence pain inhibition, sex and sex-related hormonal factors have recently received considerable attention. Converging evidence from human and animal studies has convincingly demonstrated that there are significant sex-related differences in prevalence of endogenous pain states, in nociceptive sensitivity, in antinociceptive responsivity to naturally-occurring environmental stressors, and in pharmacological responses to opioid and nonopioid antinociceptive drugs. Specifically, findings from human studies have shown that women experience a greater number of endogenous pain states than men and are more likely to suffer from painful medical conditions (Unruh, 1996). Women also appear more sensitive to experimentally-induced

noxious stimuli (Riley, Robinson, Wise, Myers and Fillingim, 1998). Sex differences in human responses to antinociceptive medications have also been observed, with women generally displaying more robust antinociception to morphine and other opioid and nonopioid antinociceptive drugs (e.g., DeKock and Scholtes, 1991; Gear, Miaskowski and Levine, 1996).

Evidence from rodent studies demonstrates that female animals typically display lower basal nociceptive thresholds than male animals (see review: Mogil et al., 2000). Female animals also show significantly lower magnitudes of antinociception following acute exposure to several forms of opioid- and non-opioid mediated environmental stressors (Bodnar et al., 1988; Kavaliers, 1988). In contrast with findings from most human studies, female rats and mice also display significantly less robust antinociceptive responses to morphine and several other opioid agonists (Bartok and Craft, 1997; Cicero, Nock and Meyer 1996; Kepler, Kest, Kiefel, Cooper and Bodnar, 1989; Kepler, Standifer, Paul, Kest, Pasternak and Bodnar, 1991). Some, but not all, of the above antinociceptive responses are sensitive to adult gonadectomy, steroid replacement therapy, and changes in the female estrous cycle.

Since sex differences in opioid-mediated antinociception have important clinical and therapeutic relevance, the present research focused upon identification of potential sites and mechanisms which are subject to sex-related effects in opioid antinociception. The sites of action at which morphine-induced antinociception is sensitive to sex differences are presumed to be central, given the effectiveness of the ventricular route of administration (Kepler et al., 1989). The ventro-lateral periaqueductal gray (vlPAG) has emerged as one of the sites that may elicit sex effects in opioid antinociception because it

plays critical roles in both antinociception and reproductive behavior. The vlPAG is a major part of the endogenous pain inhibitory system, with multiple and reciprocal connections with the limbic system, spinal cord, and frontal lobe (Basbaum and Fields, 1984). Stimulation of the vlPAG elicits potent supraspinal opioid antinociception, primarily through mu opioid receptor subtypes (Bodnar,Williams, Lee and Pasternak, 1988; Fang, Fields and Lee, 1986; Rossi, Pasternak and Bodnar, 1994). The vlPAG also plays a crucial role in female rat reproductive behaviors, particularly through its interaction with estradiol-containing hypothalamic loci. Further, the vlPAG mediates interactions between sex hormones and opioid peptides by regulating the transcription of the preproenkephalin gene by estradiol (Lauber, Romano, Mobbs, Howells and Pfaff, 1990; Pfaff, 1999; Romano, Harlan, Shivers, Howells and Pfaff, 1988).

The mechanism by which sex differences in opioid antinociception are mediated is currently not well understood. However, the interaction between the central opioid receptors mediating antinociception and gonadal hormones provides one plausible explanation as to why such effects occur. There are two ways in which gonadal hormones could exert their modulatory effects upon sex differences in opioid antinociception: acute activational effects which would be affected by adult gonadectomy (Phoenix, Goy, Gerall and Young, 1959), and long-term organizational effects which would be affected by neonatal gonadectomy (Phoenix, Goy, Gerall and Young, 1959).

Given these considerations, the primary hypothesis of this research was that opioid antinociception elicited from the vlPAG is subject to sex differences. Therefore, the first specific aim was to evaluate whether sex differences in antinociceptive responses occur following microinjections of morphine into the vlPAG in intact and gonadectomized

male and female rats, on the tail-flick test and the jump test. To explore the generalizability of sex differences to other opioid agonists, the second specific aim was to employ an analogous protocol to evaluate sex effects in antinociception in response to vlPAG microinjections of either beta-endorphin or D-Pro2-Endomorphin 2, and to examine sex differences in motor responses to these two opioid agonists. Finally, to elucidate a potential organizational mechanism mediating sex differences in the vlPAG, the third specific aim was to evaluate antinociceptive responses to vlPAG morphine in intact male rats, intact female rats, neonatally castrated male rats, and neonatally androgenized female rats.

The following background review is meant to provide an underlying conceptual basis for the proposed study by covering the following research areas: 1) characterization of the endogenous pain inhibitory system with special emphasis on the role of the vlPAG, opioid peptides, and opioid receptors in mediating supraspinal antinociception; 2) characterization of the role of the vlPAG and opioid peptides and agonists upon female rat reproductive behavior; 3) sex differences in pain experience, pain sensitivity and antinociception in humans; 4) characterization of sex-related aspects of pain processing and pain inhibition in rodents, including: a) sex differences in nociceptive sensitivity, b) sex differences in stress-induced antinociception, c) female –specific environmental forms of antinociception, including vaginocervical stimulation-induced antinociception and pregnancy-induced antinociception and d) sex-differences in opiod antinociception, including sex differences in morphine antinociception and sex differences in response to opioid drugs other than morphine.

Background

Pain Inhibitory Opioid Systems and the PAG

The Periaqueductal Gray

The midbrain periaqueductal gray (PAG), so named because it consists mostly of cell bodies surrounding the cerebral aqueduct, has a well documented role in many life functions important for survival, including autonomic regulation, reproduction, antinociception and defense. Recent studies with excitatory aminoacids (EAA) have revealed that these functions are organized along overlapping longitudinal columns of neurons that coordinate different behavioral and physiological strategies for coping with different life-threatening, stressful or painful situations (Bandler and Shipley, 1994). Thus, injections of EAA into the areas of the PAG ventrolateral and lateral to the cerebral aqueduct have been shown to induce diametrically different patterns of behavioral and autonomic adjustments. Excitation of the posterior ventrolateral column produces quiescence, hyporeactivity, hypotension, bradycardia and opioid analgesia, a pattern of reactions that is consistent with a passive coping strategy that follows deep injury or a defeat in a social encounter. In contrast, excitation of the lateral column of the PAG elicits a defensive type of behavior characterized by hypertension, tachycardia and non-opioid analgesia, a pattern of responses that is very similar to that of a threatened, attacked or superficially injured animal. Further, the type of defensive strategy produced by the stimulation of the lateral column depends on the region of the PAG excited. Stimulation of the anterior and intermediate lateral PAG produces a confrontational defensive reaction which is characterized by facing the threatening stimulus, boxing and biting, and is associated with decreased blood flow to limbs and viscera and increased

blood flow to the face. Conversely, stimulation of the posterior lateral PAG elicits a flight reaction, which is associated with decreased blood flow to the face and viscera and increased blood flow to limbs.

Recent neuroanatomical tracing studies provide neuroanatomical evidence for the columnar organization within the PAG. These studies show that the pattern of afferent and efferent connections of the PAG can be clearly dissociated along the lateral, ventrolateral, and also dorsomedial and dorsolateral columns (Bandler and Shipley, 1994). For example, whereas the lateral PAG column receives somatotopically organized spinal and spinal trigeminal inputs; the ventrolateral PAG column receives a convergent somatic and visceral input which does not show topographic organization. Similarly, although both the lateral and ventrolateral columns project to ventromedial and ventrolateral medulla, only the ventrolateral column projects to the periambigual lateral medulla that sends outputs to the heart. Further, extensive cortical inputs to the PAG exist that are also organized along mostly complementary, anterior-posterior columns that extend along the longitudinal axis of the PAG. Moreover, the PAG inputs from the subcortical medial preoptic area (mPOA) and central nucleus of the amygdala show remarkable degrees of topographical complementary organization, with the mPOA sending its inputs primarily to the dorsomedial and lateral columns, and the amygdala to the lateral and ventrolateral columns (Bandler and Shipley, 1994).

Pain Inhibition and the PAG

One of the most important advances in pain research has been the discovery and characterization of the endogenous opioid-mediated pain-inhibitory system (Basbaum and Fields, 1984). This system originates in the neurons of the vl PAG in the midbrain

which project to nuclei in the rostral ventral medulla (RVM), including the nucleus raphe magnus (NRM) and nucleus reticularis gigantocellularis (NRGC). The medullary neurons, in turn, project to the dorsal horn of the spinal cord, via a pathway in the dorso-lateral funiculus (DLF), where they specifically inhibit incoming nociceptive input. Additional neural substrates supporting this pain-inhibitory system have been identified, including the pontine locus coeruleus (LC), the pontine parabrachial area, and several medullary nuclei lateral to the NRM (i.e., nuclei reticularis magnocellularis, raphe pallidus, and paragigantocellularis lateralis and ventralis)(Beitz, Mullet and Weiner, 1983), which all receive projections from the PAG and project to to the spinal cord. Further, reciprocal connections between the LC and the RVM exist, suggesting that noradrenergic projections are also important in pain inhibition (Clark and Proudfit, 1991; Ennis and Aston-Jones, 1987).

Pain and some environmental stressors normally activate this system and produce antinociception through the release of endogenous opioid peptides, biogenic amines and other neurotransmitters (Basbaum and Fields, 1984). However, the system can also be activated by electrical stimulation of any of its component nuclei or by microinjections of minute amounts of morphine or other opiates (Hertz, Albus, Metys, Schubert and Teschemacher, 1970; Mayer and Liebeskind, 1974; Reynolds, 1969; Tsou and Jang, 1964). Both of these manipulations appear to exert their effects by stimulation of the neural circuits of the pain-inhibitory system originating in the vlPAG (Basbaum and Fields, 1984).

The vlPAG is the site which is most sensitive to stimulation-produced antinociception (SPA) and to the antinociceptive effects of opiates (Lewis and Gebhart,

1977; Yaksh, Yeung and Rudy, 1976). The vlPAG has a large number of high-affinity opioid receptors as well as substantial levels of opioid peptides, particularly met-enkephalin and leu-enkephalin (Atweh and Kuhar, 1977; Hokfelt, Ljungdahl, Terenius, Elde and Nilsson, 1977; Moss, Glazer and Basbaum, 1983).

Electrical stimulation of the vlPAG in awake rats produces antinociception so profound that abdominal surgery can be performed without pharmacological anesthesia (Reynolds, 1969). This antinociception can be blocked by the general opioid antagonist, naloxone (Cannon, Prieto, Lee and Liebeskind, 1982). Electrical stimulation of the vlPAG in humans produces naloxone-reversible antinociception that can alleviate intractable pain (Hosobuchi, Adams and Linchitz, 1977; Kumar, Wyant and Nath, 1990). Although electrical stimulation of the RVM and other sites rostral to the PAG is also known to produce antinociception in humans, this effect appears to be mediated via the vlPAG (Rhodes, 1979).

The vlPAG also appears to be the region of maximum sensitivity to the effects of opiates. Microinjection of small amounts of morphine into the vlPAG produces potent, dose-dependent antinociception that can be reversed by the opiate receptor antagonist naloxone (Yakhs, Yeung and Rudy, 1976; Yeung and Rudy, 1980). Evidence from neurophysiological studies suggests that stimulation-produced and morphine-induced antinociception from the vlPAG operate through a common neural mechanism since both are found to exert their effect by inhibiting the firing of dorsal horn neurons, and both are abolished byselective transection of the DLF (Basbaum, Morley, O'Keefe and Klanton, 1977; Liebeskind, Guilbaud, Besson and Oliveras, 1973). Effective sites for stimulation-produced and opiate-induced antinociception from the PAG overlap, with the maximum

sensitivity observed in the ventrolateral region (vlPAG) (Lewis and Gebhart, 1977). Further, cross-tolerance between stimulation-produced antinociception and morphine develops, such as that rats made tolerant to morphine display reduced stimulation antinociceptive responses and vice versa (Mayer and Hayes, 1975).

As mentioned earlier, significant evidence supports the involvement of both serotonergic and noradrenergic systems in the mediation of supraspinal opioid antinociception. Microinjection of serotonin into the RVM produces significant antinociception (Llewlyn et al., 1983, 1984) while the general setotonergic antagonist, methysergide administered into the RVM blocks antinociception elicited by electrical stimulation of the vlPAG (Aimone and Gebhart, 1986). Moreover, morphine antinociception elicited from the vlPAG is significantly attenuated by pretreatment with methysergide or with the selective 5HT2A (ritanserin) or 5HT3 (ICS205930) serotonergic antagonists in the RVM (Kiefel, Cooper and Bodnar, 1992). Further, intrathecal administration of either methysergide or the noradrenergic antagonist, phentolamine, significantly reduces the antinociception elicited by stimulation of the PAG (Hammond and Yaksh, 1984) or intracerebral (either PAG or RVM) morphine administration (Jensen and Yaksh, 1986).

Pain Inhibition and Endogenous Opioid Peptides

The mechanism by which nociceptive stimuli and electrical stimulation activate pain inhibitory systems and produce antinociception is not completely understood, but is known to involve the release of endogenous opioid peptides and biogenic amines which, acting through inhibitory interneurons, activate pain control circuits originating in the vlPAG. Morphine and other exogenous opioids are believed to exert their

antinociceptive effects by mimicking the actions normally performed by endogenous opioids (Basbaum and Fields, 1984).

Three major families of opioid peptides have been known for some years: endorphins, enkephalins, and dynorphins. Each family is derived from one of three distinct precursor molecules and all share a common opiate-characteristic pentapeptide core (Tyr-Gly-Gly-Phe-Met/Leu) (Akil et al., 1984, Snyder and Childers, 1979). These precursor molecules are proopiomelanocortin (POMC), which produces the opioid peptide, beta-endorphin, proenkephalin which yields met-enkephalin and leu-enkephalin, and prodynorphin, which yields alpha- and beta-neoendorphin, dynorphin A and dynorphin B (Akil et al., 1984; Mc Dowell and Kitchen, 1987; Sherman, Akil and Watson, 1989; Snyder and Childers, 1979). Each of these threee families of opioid peptides has a characteristic anatomical distribution and differential affinity for mu, delta, and kappa opioid receptor binding sites (Akil et al., 1984; Hollt, 1986; Snyder and Childers, 1979).

Recently, a novel opioid peptide nociceptin/orphanin FQ, its receptor, and its precursor, named pro-nociceptin/orphanin FQ have been identified, giving rise to a fourth distinct opioid peptide class (Meunier, 1997; Meunier et al., 1995; Reinscheid, 1995). Two other novel opioid peptides, Endomorphin-1 and Endomorphin-2 have also been isolated. Both of these peptides have very high affinity for mu receptors, and are believed to be a product of a common, although as yet unidentified, precursor (Martin-Schild, Zadina, Gerall, Vigh and Kastin, 1997; Zadina, Hackler, Ge and Kastin, 1997;). The following sections describe the anatomical and biochemical specificity of each opioid peptide family.

POMC. POMC has been identified as the precursor of the opioid peptide, beta-endorphin, and of the two non-opioid hormones, adrenocorticotropin (ACTH) and melanocyte-stimulating hormone (MSH) (Roberts and Herbert, 1977). POMC also gives rise to the no-nopioid, corticotropin-like intermediate lobe peptide (CLIP). Beta-endorphin is a 31-amino-acid C-terminal peptide of beta-lipotropin with very potent opioid properties which has high affinity for mu and delta receptors and much lower affinity for kappa receptors (Akil et al., 1984; Simon and Hiller, 1994). The beta-endorphin cell bodies have a limited brain distribution and are concentrated primarily in the arcuate nucleus of the medial basal hypothalamus, from which they send long projections to the preoptic area, the locus coeruleus and the vlPAG. A somewhat smaller concentration of beta-endorphin cells is found in the nucleus tractus solitarius (NTS), which sends its projections to the medullary NRM, reticularis gigantocellularis, paragigantocellularis, and reticularis lateralis (Bloom et al., 1978; Simon and Hiller, 1994; Snyder and Childers, 1979).

Beta-endorphin produces potent antinociception following intrathecal, ventricular and intracerebral administration (Loh, Tseng and Li, 1976; Tseng, Cheng and Fujimoto, 1983; Tseng, Wei, Loh and Li, 1980). Early studies seemed to indicate that the antinociceptive effects of morphine and beta-endorphin are mediated by the same mechanisms, more recent research suggests that the neuroanatomical and pharmacological actions of morphine and beta-endorphin can be dissociated. It has been proposed that beta-endorphin antinociception may be mediated through a distinct opioid binding site, the epsilon receptor (Houghten, Johnson and Pasternak, 1984; Schultz, Wuster and Hertz, 1981; Suh and Tseng, 1988). Although the existence of the epsilon

receptor has not yet been verified, evidence suggests that the mechanism of beta-endorphin antinociception is distinct from that of morphine. Specifically, it has been determined that, whereas ventricular beta-endorphin antinociception can be blocked with spinally administered naloxone, morphine antinociception is not affected by similar manipulations (Tseng and Fujimoto, 1985). Subsequent studies showed that ventricular morphine antinociception is mediated by spinal serotonergic and noradrenergic pathways, whereas ventricular beta-endorphin antinociception is mediated by spinal met-enkephalin release (Suh, Fujimoto and Tseng, 1989; Suh, Tseng and Li, 1988; Tseng and Tang, 1990). Met-enkephalin antibody blocks antinociception elicited by ventricular beta-endorphin but not ventricular morphine (Tseng and Suh, 1989). Ventricular morphine and beta-endorphin do not develop antinociceptive cross-tolerance (Suh and Tseng, 1990), and are differentially affected by pentobarbital anesthesia (Smith, Robertson, Monroe, Leedham and Cabral, 1992). Morphine and beta-endorphin also appear to engage different mechanisms of action when injected directly into the vlPAG. Although antinociception induced by either morphine or beta-endorphin in the vlPAG can be blocked by either general or mu-selective antagonists, the dose-inhibition curves are not parallel, suggesting involvement of separate populations of receptors (Monroe, Hawranko, Smith and Smith, 1996; Smith et al., 1992). Further, vlPAG morphine and beta-endorphin antinociceptive responses are differentially affected by barbiturate anesthesia in that barbiturate anesthesia increases morphine antinociception but decreases beta-endorphin antinociception (Smith et al., 1992).

Proenkephalin. The proenkephalin precursor gives rise to multiple copies of met-enkephalin and leu-enkephalin, as well as to several other enkephalins, including peptide

E, F and B (Mc Dowell and Kitchen, 1987). Met-enkephalin has very high affinity for delta receptors, ten-fold lower affinity for mu receptors and very small affinity for kappa receptors (Akil et al., 1984). Enkephalin neurons are very widely distributed throughout the entire neuraxis, from the telencephalon to the spinal cord, in which they most typically form local circuits or function as interneurons. The areas with the highest concentrations of enkephalins include the reticular formation, hypothalamus, hippocampus, substantia nigra, amygdala, striatum, frontal, pyriform and entorhinal cortices, anterior olfactory nucleus, lateral septum, posterior pituitary gland, and the vlPAG (Hokfelt, Elde, Johansson, Telenius and Stein, 1977; Khachaturian, Lewis, Schafer and Watson, 1985).

Prodynorphin. The prodynorphin precursor is cleaved to produce four major peptides, that are named dynorphin A, dynorphin B, alpha-neodynorphin and beta-neodynorphin, as well as leu-enkephalin (Akil et al., 1984; Simon and Hiller, 1994). All dynorphin peptides have high affinity for kappa receptors but they also bind to mu and delta receptors with lower affinity (Akil et al., 1984). Dynorphin neurons have an almost identical anatomical distribution to that of enkephalin neurons. The areas with the highest concentration of dynorphins are the posterior pituitary, hypothalamus (particularly the paraventricular nucleus), amygdala, septum, spinal cord, midbrain, and striatum (Khachaturian et al., 1982; Simon and Hiller, 1994; Snyder and Childers, 1979).

Pronociceptin/orphanin FQ. The only known product of the pronociceptin/orphanin precursor is nociceptin/orphanin FQ which, unlike other opioid peptides, has a C-terminal phenylalanine rather than the opioid-like pentapeptide sequence. However, the overall amino acid sequence of nociceptin/ orphanin FQ displays

significant homology with classical opioid peptides, suggesting a common ancestral link (Meunier et al., 1995; Reinscheid, 1995). Nociceptin/orphanin FQ binds to its own receptor, ORL1, but has negligible affinity for mu, delta and kappa receptors. Similarly, beta-endorphin, enkephalins and dynorphins have very low affinity for the ORL1 receptor. The anatomical distribution of nociceptin/orphanin FQ is also distinct from that of other opioid peptides. Northern blot analyses showed the nociceptin/ orphanin FQ gene to be primarily expressed in the amygdala and subthalamic nuclei with lower expression levels also observed in hypothalamus, substantia nigra and thalamus (Meunier, 1997; Nothacker et al., 1997; Pan, Xu and Pasternak, 1996). In situ hybridization revealed highest hybridization signals in the PAG, central tegmental field, nucleus of the lateral lemniscus, superior olive and spinal trigeminal nucleus of the brainstem (Houtani, Nishi, Takeshima, Nukada and Sugimoto, 1996; Meunier, 1997).

Endomorphin-1 and Endomorphin-2. These two recently identified opioid tetrapeptides are structurally distinct from the other opioid peptapeptides and appear to be the products of an unknown common precursor (Martin-Schild et al., 1997; Zadina et al., 1997). Both endomorphin-1 and endomorphin-2 display very high selectivity for the mu receptor and have been proposed to be natural ligands at the mu opioid binding site (Zadina et al., 1997). Endomorphin-1 and Endomorphin-2 significantly increase nociceptive thresholds following both spinal and supraspinal administration which are differentially blocked by mu and mu1 opioid antagonists (Goldberg et al., 1998; Przewlocka, Mika, Labuz, Toth and Przewlocki, 1999; Sakurada, Zadina and Kastin, 1999; Stone, Fairbanks and Laughlin, 1997; Tseng et al., 2000). CXBK mice, deficient in mu opioid receptors, fail to display endomorphin-1 or endomorphin-2 antinociception

(Goldberg et al., 1998). Whereas endomorphin-1-induced antinociception is selectively blocked by mu, but not kappa or delta opioid antagonists, endomorphin-2-induced antinociception is blocked by both mu and kappa opioid antagonists as well as antisera directed against dynorphin A (1-17)(Tseng et al., 2000). Electrical stimulation of primary spinal afferent neurons releases endomorphin-2 (Williams, Wu, Dun, Kwok and Dun, 1999) while endomorphin ligands inhibit activity of medullary neurons (Chu, Xu, Li and Wang, 1999). Endomorphin-2-induced antinociception is modulated by the proteolytic enzyme, dipeptyl peptidase IV. A dipeptidyl peptidase IV inhibitor enhances endomorphin-2 antinociception and also by itself produces an opioid-sensitive antinociception (Shane, Wilk and Bodnar, 1999). An enzyme-resistant analog of endomorphin-2, D-Pro2-Endomorphin-2, produces more potent and longer-lasting opioid-sensitive antinociception following ventricular administration (Shane et al., 1999). The immunocytochemical distributions of endomorphin-1 and endomorphin-2 fibers are highest in the areas involved in pain inhibition, including the vlPAG, RVM, locus coeruleus, and the spinal cord (Martin-Schild et al., 1997; Martin-Schild, Gerall, Kastin and Zadina, 1998; Martin-Schild, Gerall, Kastin and Zadina, 1999).

Pain Inhibition and Opioid Receptor Subtypes

The opioid pain-inhibitory system mediates antinociception through a number of pharmacologically-distinct opioid receptor types acting at both spinal and supraspinal levels (Yaksh, 1984 a, 1984 b). Three major types of opioid receptors: mu, delta, and kappa have been identified and characterized biochemically, pharmacologically, anatomically and molecularly (Chen, Mesick, Hurley and Yu, 1993; Evans, Keith, Morrison, Magendoza and Edwards, 1992; Kieffer, Befort, Gaveriaux-Ruff and Hirth,

1992; Mansour, Khachaturian, Lewis, Akil, and Watson, 1988; Martin, Eades, Thompson, Huppler and Gilbert, 1976; Minami et al., 1993; Pasternak,1993; Pasternak and Wood, 1986; Yasuda et al., 1993). The use of selective opioid agonists and antagonists has facilitated the study of the roles of each opioid receptor type in mediating opioid antinociception. The uses of antisense probes and CXBK and knock-out mice deficient in the specific opioid receptor binding sites have further elucidated these roles.

Recently, a fourth distinct opioid receptor type has been isolated and cloned (Bunzow et al., 1994; Chen et al., 1994; Fucuda et al., 1994; Mollereau et al., 1994; Wang et al., 1994). This receptor displays a remarkable structural similarity to other opioid receptors and hence has been named ORL1 (Opioid Receptor-Like). However, the pharmacological effects of the ORL1 binding appear to be quite distinct from those of other opioid receptors in that it has both antiopioid and anti-antinociceptive properties (King, Rossi, Chang, Williams and Pasternak, 1997; Meunier et al., 1995; Reinscheid et al., 1995; Rossi, Leventhal and Pasternak, 1996;). The following section provides a brief review of the opioid receptor types and their roles in mediating supraspinal and spinal antinociception.

Mu Receptors. Mu opioid receptors are primarily distributed in the forebrain, midbrain, and hindbrain, but can also be found in the spinal cord (Mansour et al., 1988). D-ala2, Me-Phe4,Gly(ol)5 (DAMGO) is a selective agonist at the mu site while beta-funaltrexamine (B-FNA) is a selective antagonist. The mu receptor has been subdivided into mu 1 and mu 2 subtypes (Pasternak and Wood, 1986). The mu 1 subtype has a very high affinity for both opiates and enkephalins, binding morphine, enkephalin peptides, and beta-endorphin with equal affinity. Naloxonazine is a selective antagonist at this site.

The mu 2 subtype preferentially binds morphine but it also binds enkephalins with low affinity (Pasternak and Wood, 1986; Wolozin and Pasternak, 1981).

Mu 1 receptors are primarily implicated in mediating supraspinal antinociception (Bodnar, Williams,. Lee and Pasternak, 1988; Fang, Fields and Lee, 1986; Ling and Pasternak, 1983; Rossi, Pasternak and Bodnar, 1993). Microinjections of morphine, DAMGO, beta-endorphin, or the endogenous mu opioid peptide endomorphin-1 into the PAG or RVM produces potent antinociception which can be blocked by either B-FNA or naloxonazine (Bodnar et al., 1988; Rossi, Pasternak and Bodnar, 1994; Smith, Robertson, Monroe, Leedham and Cabral, 1992; Zadina, Hacler, Ge and Kastin, 1997). CXBK mice, which display a selective mu1 receptor deficit, display poor antinociceptive sensitivity to intraventricularly-administered DAMGO and morphine (Moskowitz and Goodman, 1988; Vaught, Mathiasen and Raffa, 1988). Similarly, intraventricular morphine fails to produce antinociception in knock-out mice that lack the mu opioid receptor (MOR-1) (Matthes et al., 1996). Furthermore, treatment with antisense oligodeoxynucleotide directed against the MOR-1 gene blocks morphine antinociception elicited from the vlPAG in rats (Rossi, Pan, Cheng and Pasternak, 1994).

Mu 2 receptors appear to be responsible for mu- related spinal antinociception. Antinociception produced by intrathecal morphine administration can be blocked with B-FNA but not with naloxonazine (Paul, Bodnar, Gistrak and Pasternak, 1989; Pick, Roques, Gacel and Pasternak, 1992). Further, CXBK and mu-deficient mice display normal antinociceptive responses to intrathecally administered morphine (Vaught et al., 1988).

Recently, the existence of a novel splice variant of the mu opioid receptor has been proposed based on the observations of dissociations of pharmacological actions of morphine and its potent metabolite, morphine-6beta-glucoronide (M6G) (Inturrisi et al., 1983; Rossi, Brown, Leventhal, Yang and Pasternak, 1996). M6G and morphine antinociception have also been dissociated using mu-receptor deficient approaches (Rossi, Standifer and Pasternak, 1995; Standifer, Rossi and Pasternak, 1996;). The actions of M6G are believed to be mediated by a separate splice variant of the MOR-1 clone which also mediates antinociceptive actions of heroin, fentanyl, 6-acetylmorphine and etonitazine, but not the actions of morphine (Rossi et al., 1996). M6G appears to be active in mediating both supraspinal and spinal antinociception since potent antinociception can be elicited following administration of M6G into the ventricles, into the vlPAG, or intrathecally (Pasternak, Bodnar, Clark and Inturrisi, 1987; Paul, Standifer, Inturrisi and Pasternak, 1989).

Delta Receptors. Delta opioid receptors are primarily found in the forebrain with a secondary, smaller distribution in the diencephalon, brainstem and spinal cord (Mansour et al., 1988). The delta receptor has been subcharacterized in two distinct subtypes, delta 1 and delta 2 (Negri, Potenza, Corsi and Melchiorri, 1991). Selective agonists and antagonists have been developed for delta, delta 1 and delta 2 receptors. D-Ser2, Leu5-enkephalin-Thr6 (DSLET) and D-Ala2, D-Leu5-enkephalin (DADL) are general agonists at the delta opioid site while ICI 174864 is a general antagonist. The delta 1 receptor subtype is characterized by the agonist actions of (D-Pen 2- D-Pen5)-enkephalin (DPDPE), and the antagonist actions of D-Ala2, Leu5, Cys6-enkephalin (DALCE). The delta 2 receptor is characterized by the agonist actions of D-Ala2 deltorphin II

(Deltorphin) and the antagonist action of naltriben or naltrindole 5'- isothiocyanate (5'-NTII) (Jiang, Takemori, Sultana, Portoghese, Bowen, Mosbergand Porreca,1991; Mattia, Vanderah, Mosberg and Porreca, 1991).

Both DSLET and DADL produce antinociception following intracerebral administration (Bodnar et al., 1988; Heyman Mulvaney, Mosbert and Porreca, 1987), but these effects cannot be conclusively attributed to the activity at either delta 1 or delta 2 receptor since DSLET and DADL also bind with relatively high affinity to mu1 sites (Itzah and Pasternak, 1987; Pasternak and Wood, 1986). Although DPDPE and deltorphin II both produce antinociception following ventricular administration (Mattia et al., 1991; Porreca et al., 1987) only deltorphin II, but not DPDPE, is effective in eliciting antinociception directly from the PAG (Rossi, Pasternak and Bodnar, 1994). This antinociception can be blocked with 5'NTII but not with DALCE (Jiang et al., 1991). In addition, CXBK mice which may also have a supraspinal delta 2 receptor deficit fail to display antinociception following intraventricular administration of deltorphin but not DPDPE (Raffa, Martinez and Porreca, 1992). These data suggest that only delta 2 receptors are involved in mediating supraspinal antinociception from the vlPAG while both delta 1 and delta 2 receptors are involved in producing spinally-mediated antinociception.

Kappa Receptors. Kappa receptors are most densely distributed in the telencephalon, diencephalon, neural lobe of the pituitary gland and the nucleus tractus solitarius, with moderate binding in the brainstem and the dorsal horn of the spinal cord (Mansour et al., 1988). The general kappa agonists U50, 488H and ethylketocyclazocine, and the general kappa antagonist nor-binaltorphamine (NOR-BNI) have been developed

(Portoghese, Lipkowski and Takemori, 1987; VanVoigtlander, Lahti and Ludens, 1983). The kappa receptor has been subdivided into kappa 1, kappa 2, and kappa 3 subtypes (Zukin, Eghbali, Olive, Unterwald and Tempel, 1988), with U69,593 as the selective agonist and NOR-BNI as the selective antagonist at the kappa 1 site. The kappa 3 site has been identified using naloxone benzolhydrazone (NalBzoH) (Clark, Liu, Price, Hersh, Edelson and Pasternak,1989; Paul, Levison, Howard, Pick, Hahn and Pasternak, 1990).

Kappa opioid agonists are involved in mediating spinal antinociception but they do not appear to mediate supraspinally-mediated antinociception. Although potent antinociception can be produced following ventricular administration of the general kappa agonists ethylketocyclazocine and U50,488H, neither of them is effective when microinjected directly into the vlPAG (Bodnar, Paul and Pasternak, 1991; VanVogtlander et al., 1983).

The Orphan Receptor. The recently isolated orphan receptor has been localized in many discrete areas of the central nervous system with the most dense concentrations found in the cortical and cortico-limbic areas, hypothalamus, brain stem and the dorsal and ventral horns of the spinal cord (Bunzow et al., 1994; Fukuda et al., 1994; Mollereau et al., 1994). The mRNA of the ORL-1 receptor has also been visualized in many areas outside of the central nervous system, including the intestine, skeletal muscle, vas deferens and spleen (Halford, Gebhardt and Carr, 1995). The only known agonist of the ORL-1 receptor is its natural ligand nociceptin/orphanin FQ. The peptide acetyl-Arg-Tyr-Arg-Trp-Lys-NH2 (Ac-RYYRWK-NH2) has recently been reported to be a putative antagonist at the ORL-1 site (Berger et al., 1999). Nociceptin/Orphanin FQ was initially

reported to possess pronociceptive properties when administered intraventricularly (Meunier et al., 1995; Reinscheid et al., 1995; Rossi et al., 1996), but subsequent investigations determined that its supraspinal actions are better characterized as anti-opioid, that is inhibiting opioid antinociception (Mogil et al., 1996). In contrast, this peptide produces antinociception following intrathecal administration, suggesting that it has opioid-like antinociceptive properties at the spinal site (King et al., 1997; Rossi et al., 1996).

Female Reproductive Behavior, Opioid Systems and the PAG

In addition to its role in pain inhibition, the PAG plays an important role in mediating sexual behaviors in the female rat (Pfaff, 1999). The PAG is a crucial component of the neural circuit that controls the expression of lordosis. Lordosis is a hormone-sensitive stereotyped postural behavior exhibited by the sexually-receptive female rat in response to cutaneous stimulation applied by the male rat during mating. The basic neural circuit of the lordosis reflex consists of ascending pathways from the spinal cord which transmit somatosensory information about cutaneous stimulation, from descending motor pathways from the PAG which control the execution of the lordosis response, and from the descending neuroendocrine input from the hypothalamus that provides the necessary hormonal facilitation for this response to occur (Pfaff, 1999; Pfaff, Schwartz-Giblin, McCarthy, Kow, 1994).

The PAG is an important relay station that receives and integrates all of these inputs. The sensory information relevant for induction of lordosis reaches the PAG via the anterolateral branch of the spinothalamic pathway where it becomes integrated with the information about the hormonal status of the female. This information is provided by the

ventromedial hypothalamus (VMH) and medial preoptic area (mPOA) which express estrogen receptors. Acting through estrogen receptors, estrogen induces expression of the gene for the progesterone receptor, which binds circulating progesterone. Genomic effects of the combined estrogen and progesterone binding provide a strong facilitating effect upon the PAG which then activates the medullary reticular neurons that control the motor output of the lordosis reflex (Pfaff, 1999; Pfaff, Schwartz-Giblin, McCarthy, Kow, 1994). Acting in response to the somatosensory and hormonal information, the PAG also activates descending pain inhibitory systems which induces stimulus-dependent antinociception and neutralizes potentially painful and aversive copulatory stimuli (Komisaruk and Whipple, 1995; also see review: Komisaruk and Whipple, 2000).

The pivotal role of the PAG in mediation of the lordosis reflex is well documented. Electrical stimulation of the PAG facilitates lordosis, whereas lesions placed in the PAG significantly suppress this response (Sakuma and Pfaff, 1979a; Sakuma and Pfaff, 1979b). Bilateral lesions placed in the caudal PAG and its lateral surround virtually eliminate the lordosis response, presumably by eliminating its connections with the VMH (Hennesey, Camak, Gordon and Edwards, 1990). In contrast, lesions placed in the VMH do not immediately abolish lordosis but result in gradual diminution and eventual loss of this behavior (Pfaff and Sakuma, 1979). This evidence suggests that, whereas the PAG is necessary for execution of lordosis, the VMH provides tonic hormone-related facilitation of this response.

There is considerable evidence that opioids influence expression of lordosis behavior and that the PAG may be one of the sites at which these actions are mediated. Endogenous opioid peptides and their receptors are localized in the VMH and mPOA

regions of the rat hypothalamus and their expression is regulated by estrogen. One of these peptides, met-enkephalin, is induced in the VMH as part of genomic effects of estrogen and progesterone binding, and appears to facilitate the lordosis reflex (Romano, Harlan, Shivers, Howells and Pfaff, 1988; Romano, Mobbs, Lauber, Howells and Pfaff, 1989). In intact female rats, the synthesis of proenkephalin varies as a function of the normal estrous cycle, and is highest during the proestrus phase (Funabashi, Brooks, Kleopoulus et al., 1995). Ovariectomized females treated with estrogen show dose-dependent increases in the synthesis of proenkephalin in the VMH, and a corresponding increase in the lordosis behavior (Lauber, Romano, Mobbs, Howells and Pfaff, 1990). It appears that met-enkephalin actions upon the target neurons in the PAG is primarily inhibitory and estrogen-dependent. Met-enkephalin was found to inhibit PAG neurons in electrophysiological studies using a tissue slice preparation. Further, met-enkephalin action upon PAG neurons was increased in a dose-dependent manner in estrogen-primed ovariectomized females, but not in ovariectomized females which were not primed with estrogen (Ogawa et al., 1994).

Expression of beta endorphin and mu receptors in the mPOA of rats is also regulated by estrogen, and seems to be associated with inhibition of lordosis behavior (Sirinathsinghji, 1986). In intact female rats, content of mPOA beta-endorphin fluctuates across the estrous cycle with highest levels observed during the diestrus and lowest during the proestrus phases (Knuth, Sikand, Casaneuva, Havlicek and Friesen, 1983). These changes in beta-endorphin levels in the mPOA are closely correlated with changes in mu receptor density in this region as the highest concentration of mu receptors are found during the metestrus and diestrus phases and lowest during the proestrus phase

(Hammer, 1990). Ovariectomized females treated with estrogen alone show a decrease in the synthesis of proopiomelanocortin as well as reductions in beta-endorphin content (Wardlaw, Thoron and Frantz,1982; Wilcox and Roberts, 1985). However, upregulation of mu receptors and the concurrent increase in the synthesis of proopiomelanocortin have been observed in ovariectomized females treated with both estrogen and progesterone (Hammer, Zhou and Cheung, 1994).

Evidence from pharmacological studies supports the role of opioids in mediating female reproductive behaviors. Central administration of selective opioid agonists can either facilitate or inhibit lordosis behavior, depending on the receptor type and hormonal status of the female. Administration of the selective delta receptor agonist DPDPE or the selective kappa receptor agonist U50-488H into the lateral ventricles facilitates lordosis in ovariectomized female rats primed with estradiol and progesterone or with estradiol alone (Pfaus and Pfaff, 1992). Facilitation of the lordosis response has also been observed following the general delta opioid agonist DSLET and the selective kappa agonist leumorphin (Pfaus and Gorzalka, 1987; Suda et al., 1986). In contrast, intraventricular administration of the selective mu agonist DAMGO inhibits the lordosis response in ovariectomized females primed with estradiol and progesterone, but not in ovariectomized females primed with estradiol alone (Pfaus and Pfaff, 1992). Morphine injected into the VMH or the PAG, or beta-endorphin injected into the medial preoptic area, VMH, lateral ventricles, PAG or mesencephalic reticular formation also inhibit lordosis (Vathy, van der Plas, Vincent and Etgen , 1991; Wiesner and Moss, 1989). The inhibitory effect of beta-endorphin can be blocked by naloxazone, suggesting that mu 1 receptors are involved in mediating this effect (Vathy et al., 1991). Taken together, these

results suggest that activation of delta or kappa receptors exerts a facilitatory effect on lordosis, whereas activation of mu receptors has an inhibitory effect. Further, whereas both estradiol and progesterone are required for the inhibitory action of mu receptor stimulation upon lordosis, the presence of estradiol alone is sufficient for the facilitatory effect of delta or kappa receptor stimulation.

The brain site (s) mediating the modulatory effects of opioids upon lordosis behavior has not been identified. Potential sites of action for the inhibitory effect of mu opioid agonists on lordosis include the medial preoptic area, bed nucleus of stria terminalis and the PAG, all of which contain mu opioid binding sites (Mansour et al, 1987; Pfaus and Pfaff, 1992) Potential sites of action for the facilitatory action on lordosis by delta or kappa opioids include the cortex, dorsal and ventral striatum, MPOA, VMH, suprachiasmatic nucleus and the PAG, all of which contain both delta and kappa opioid binding sites as well as the bed nucleus and stria terminalis, diagonal band and medial amygdala which contain delta opioid binding sites alone (Mansour et al, 1987; Pfaus and Pfaff, 1992) . Thus, the PAG has been implicated as one of the potential sites that may mediate both facilitatory and inhibitory actions of opioid agonists on lordosis. Administration of naloxone into the lateral ventricles or the PAG inhibits lordosis in females primed with estradiol and progesterone, while infusion of naloxone into the PAG facilitates lordosis in females primed with estrogen alone (Lindblom, Forsberg and Sodersten, 1986; Sirinathsinghji, 1984).

Sex Differences in Pain Experience, Pain Sensitivity and Antinociception in Humans

Evidence from a number of recent studies suggests that sensitivity to pain and responsiveness to various pharmacological agents may differ between women and men. Women report more pain, in more body parts, and of greater severity and longer duration (Unruh, 1996). Women are more likely to suffer from painful medical conditions, including chronic tension-type headache, migraine without aura, fibromyalgia, causalgia, post-herpetic neuralgia, rheumatic arthritis, lupus erythmatosus, temporomandibular disorders, and irritable bowel syndrome (Berkley, 1997; Le Resche, 1999; Unruh, 1996). Women are frequently found to have lower pain thresholds and tolerance to experimentally-induced noxious stimuli, particularly to mechanical pressure and electrical stimulation (Fillingim and Maixner, 1995; Lautenbacher and Rollman, 1993; Riley et al., 1998). While sex differences in nociceptive sensitivity to thermal stimuli are less consistently observed, women tend to manifest greater temporal summation of thermal pain (Riley et al., 1998). Preexisting painful medical conditions, such as tuberoinfidibular disorders, fibromyalgia and headache tend to decrease thermal pain thresholds in both sexes, but more so in women than in men (Fillingim, Edwards and Powell, 1999). Likewise, psychological factors, such as stress and anxiety, may differentially affect women and further potentiate sex differences in endogenous pain complaints and in sensitivity to exogenous nociceptive stimuli (Fillingim, Keefe, Light, Booker and Maixner, 1996; Rollman, 1995). For example, as compared to men, women tend to show greater stress responses on the sympatho-adrenal and hypothalamic-pituitary-adrenal (HPA) axes, which in turn increase inflammatory responses and

exacerbate symptoms of inflammatory diseases such as rheumatic arthritis and lupus erythmatosus (Da Silva, 1995; Ng, Callister, Johnson and Seals, 1993; Peskind et al.,1995).

Menstrual cycle effects may modulate both endogenous pain complaints and experimentally-induced pain sensitivity in women. Healthy women report significantly more idiopathic pain complaints during the late luteal (premenstrual) phase of the cycle (Boyle, Berkowitz and Kelsey, 1987; Huerta-Franco and Malacara, 1993), while women with preexisting painful medical conditions, such as headache, rheumatic arthritis, fibromyalgia or irritable bowel syndrome, tend to experience symptom exacerbation during that phase (Heitkemper, Jarrett, Cain, Shaver, Walker and Lewis, 1995;Keenan and Lindamer, 1992; Ostensen, Rugelsjoen and Wigers, 1997; Valentino, Savastano and Tommaselli, 1993). The luteal phase of the menstrual cycle is also associated with increased pain sensitivity to most types of experimentally- induced pain (Fillingim, Girdler, Booker, Light, Harris and Maixner, 1995; Riley et al., 1999).

Exogenous hormones have been reported to reduce sex differences and menstrual effects in the perception of pain. For example, several studies reported that oral contraceptives eliminate both sex differences and menstrual cycle differences in experimentally-induced pain sensitivity to electrical and thermal stimulation (Goolkasian, 1980; Tedford, Warren and Flynn, 1977). Oral contraceptive use may also be associated with decreased sensitivity to endogenous pain states, such as muscle pain following exercise (Hapidou and Rollman, 1998; Thompson, Hyatt, DeSouza and Clarkson, 1997).

Data from a limited number of clinical studies using a postoperative model of pain indicate sex differences in antinociceptive effects of opioid drugs with most studies

showing more robust antinociceptive responses in women than in men (Miaskowski and Levine, 1996). For example, women have been found to report less pain and require less morphine following abdominal surgery (DeKock and Scholtes, 1991). Women also manifest more potent antinociception in response to the kappa receptor agonists pentazocine, butorphanol, and nalbuphine when these agents are administered to treat postoperative dental pain (Gear, Gordon, Heller, Paul, Miaskowski and Levine, 1996; Gear, Miaskowski, Gordon, Paul, Heller and Levine, 1996). In contrast, men undergoing colonoscopy have been found to display greater antinociception than women to morphine-like synthetic opioid meperidine, to the benzodiazapine midazolam, or to the combination of both these agents (Froechlich et al., 1997).

Sarton and coworkers (Sarton et al., 2000) have recently compared the antinociceptive effects of morphine in healthy male and female volunteers using a model of experimentally-induced acute pain. Women displayed greater antinociception than men despite the fact that comparable levels of plasma concentration of morphine and its metabolites were observed in both sexes. However, men showed faster speed of onset and offset of antinociception than women. It should be noted that the reports of greater sensitivity to opioids in women as compared to men conflict with the results of extensive animal studies, which consistently show the opposite effect. The reason for these discrepancies is not well understood but may be at least partially related to methodological differences which, for obvious ethical considerations, cannot easily be avoided. The following sections provide a review of rodent research on sex-related differences in pain processing and antinociception.

Sex Differences in Pain Sensitivity and Antinociception in Rodents

Sex Differences in Pain Sensitivity

Consistent with the results of human studies, sex-related effects have been observed in nociceptive sensitivity in rodents, which are the primary species studied for these behaviors. Female rats typically display significantly lower thresholds in response to noxious electric shock than male rats (see review: Mogil et al., 2000). Estrous cycle effects may modulate these responses since some studies report significant fluctuations in shock sensitivity accros the estrous cycle, with females tested in estrus generally displaying decreased pain thresholds relative to females tested in other phases of the estrous cycle (Drury and Gold, 1978; Kepler et al., 1991)

Sex differences in shock sensitivity can be abolished by neonatal castration of male rats or neonatal androgenization of female rats (Beatty and Fessler, 1976). Adult gonadectomy of either male or female rats is typically ineffective in altering pain thresholds to electric shock (see review: Mogil, 2000).

Female rats are also found to be more sensitive to chemical and inflammatory pain, such as produced by acetic acid injection (abdominal constriction test), kidney stones (cystitis) and formalin injection (Aloisi, Albonetti and Carli, 1994; Aloisi, Albonetti and Carli, 1996; Baamonde, Hidalgo and Andres-Trelles, 1989; Bon, Lanteri-Minet, Mentrey and Berkley, 1997; also see review: Mogil, 2000).

In contrast, sex differences in basal nociceptive sensitivity to thermal stimulation, as measured by the tail-flick or the hot-plate tests, are less consistently observed. As reviewed (Mogil et al., 2000), only six out of 23 studies that examined male and female threshold responses on the hot-plate test reported significant sex effects and all six

suggested greater sensitivity in females. On the tail-flick test, sex differences were found in only 10 out of 23 studies, with two reporting greater sensitivity in females and eight in males. Adult gonadectomy effects were examined in several of these studies but failed to produce any significant alterations in male or female sensitivity to nociceptive heat stimulation on either the hot-plate or tail-flick test. Estrous cycle effects in thermal sensitivity of females have also been reported. Two studies reported greater thermal sensitivity of females in proestrous compared to those in other phases of the cycle (Frye, Cuevas and Kanarek, 1993; Kepler et al., 1989), whereas females tested during estrous or metestrous were most sensitive to noxious heat in one study, which specifically investigated the effect of estrous cycle on thermal sensitivity in female rats (Martinez-Gomez, Cruz, Salas, Hudson and Pacheco, 1994).

Sex Differences in Stress-Induced Antinociception

Sex differences have also been examined using opioid and nonopioid forms of stress-induced antinociception. Opioid and nonopioid mediation of antinociceptive responses by various stressors can be dissociated by manipulating the parameters of the stressors. For example, whereas an intermittent cold water swim (ICWS) elicits an antinociceptive response which is cross-tolerant with morphine antinociception and blocked by naloxone, continuous cold water swim (CCWS) produces antinociception which is neither cross-tolerant with morphine antinociception nor reversed by treatment with naloxone (Bodnar, Kelly, Spiaggia, Ehrenberg and Glusman, 1978; Bodnar, Kelly, Steiner and Glusman, 1978; Girardot and Holloway, 1984; Romero, Kepler and Bodnar, 1987). Similarly, a forced 3-minute swim in 32 degrees centigrade water results in opioid antinociception which is reversed by naloxone, whereas a forced 3-minute swim in 15 degrees centigrade

water produces nonopioid antinociception which is insensitive to naloxone, but blocked with the competitive NMDA receptor antagonist MK-801. Further, a forced 3-minute swim in 20 degree centigrade water induces a mixed opioid/nonopid antinociception which can be blocked with a combination of naloxone and MK-801 (Marek, Mogil, Sternberg, Panocka and Liebeskind, 1992). Pharmacologically-distinct, opioid and nonopioid forms of stress-induced antinociception can also be produced in rodents by varying the parameters of certain ethologically relevant aversive environmental stimuli such as novelty, immobilization (restraint), exposure to a predator, exposure to biting flies, or exposure to a parasite-infected mating partner (Kavaliers and Colwell, 1991; Kavaliers, Colwell and Choleris, 1998; Kavaliers and Innes, 1987; Kavaliers and Innes, 1988).

Both forms of stress-induced antinociception are sensitive to sex differences. For example, female rats display significantly less opioid mediated ICWS antinociception and nonopioid mediated CCWS antinociception than male rats (Romero and Bodnar, 1986). Analogous sex differences have been observed in stress antinociception in response to various ethologically- relevant environmental stressors (Kavaliers and Innes, 1987; Kavaliers and Innes, 1988; Kavaliers and Colwell, 1991).

Estrous cycle effects were examined but were not evident in either ICWS or CCWS swim-induced antinociception (Romero and Bodnar, 1986), or in antinociception induced by immobilization stress (Kavaliers and Innes, 1987), although such effects were observed in stress-induced antinociceptive responses elicited by a tail-shock (Ryan and Maier, 1988). Adult castrations and ovariectomies significantly reduced the magnitudes of both ICWS and CCWS antinociception relative to intact males and females, with a

rank order potency of intact males>> intact females = castrated males> ovariectomized females (Romero, Kepler, Cooper, Komisaruk and Bodnar, 1987). The effectiveness of adult gonadectomy in altering the pattern of sex differences in CCWS and ICWS antinociception suggests an activational role of gonadal hormones in mediating opioid and nonopioid forms of stress-induced antinociception. Consistent with this hypothesis, steroid replacement therapy with testosterone propionate or estradiol benzoate reversed the effects of gonadectomy in castrated males and ovariectomized females on both CCWS and ICWS antinociception. In contrast, analogous steroid treatment was ineffective in altering the antinociceptive responses of intact male and female rats (Romero, Cooper, Kepler, Komisaruk and Bodnar, 1988). These data suggest that activational effects of gonadal hormones mediate the observed sex differences in swim antinociception, and are necessary for the full expression of antinociceptive responses in both male and female rats.

Sex differences in stress-induced antinociception are not limited to the magnitude of the effects but extend to the underlying neurochemical mechanisms mediating these differences. Mogil (Mogil, Sternberg, Kest, Marek and Liebeskind, 1993) proposed that nonopioid stress-induced antinociception in female mice is mediated by a distinct, estrogen-dependent pain inhibitory system which engages an unknown neurochemical pathway that does not involve either opioid receptors or NMDA receptors. Although a forced 3-minute swim in 15 degrees centigrade water produced comparable levels of non-opioid, naloxone-insensitive antinociception in intact and gonadectomized male and female mice, MK-801 was effective in blocking this antinociceptive effect in intact and castrated male mice but not in intact female mice. Interestingly, adult-ovariectomized

female mice displayed the male pattern of NMDA-mediated antinociception, but estrogen replacement therapy restored the pattern of insensitivity to NMDA antagonism in these females. Further, neonatal exposure of female mice to testosterone produced the male pattern of NMDA-mediated swim-stress antinociception in adulthood, which was maintained even in the presence of estrogen (Sternberg, Mogil, Kest, Page, Leong, Yam and Liebeskind, 1995). These data indicate that an additional pain-inhibitory mechanism may be present in females that is formed in the period of early ontogeny in the absence of organizational effects of male steroid hormones, and is independent of both opioid and NMDA mechanisms.

Female-Specific Environmental Forms of Antinociception

Vaginocervical Stimulation-Induced Antinociception. It has been demonstrated in female rats and women, that vaginocervical stimulation (VCS) produces significant antinociception across a variety of nociceptive measures (Komisaruk and Whipple, 1986; Whipple and Komisaruk, 1985), without producing suppression of motor responses or anesthesia (Komisaruk and Wallman, 1977). In women, VCS-induced antinociception is associated with pleasurable experience, sometimes leading to orgasm (Whipple and Komisaruk, 2000).

VCS-induced antinociception is spinally mediated and depends upon intact afferent input via both the pelvic and hypogastric nerves, which enervate the cervix and the vaginal canal, and the uterus and cervix, respectively. Simultaneous transection of the hypogastric and pelvic nerves blocks VCS-induced antinociception (Cunningham et al., 1991). The magnitude of VCS-induced antinociception is proportional to the amount of pressure applied to the vagino-cervical area, with increasing amounts of pressure

producing progressively greater suppression of nociceptive responses (Crowley, Rodriguez-Sierra and Komisaruk, 1977; Rothfeld, Gross and Watkins, 1985).

The underlying neurochemical mechanisms mediating VCS-induced antinociception are test- specific and can be either opioid or non-opioid. Pretreatment with naloxone reduces VCS-induced antinociception on the tail-flick but not on the tail-shock test, suggesting that the involvement of the opioid system in the mediation of the VCS-induced antinociception may depend on the nature of the nociceptive stimulus employed (Hill and Aylliffe, 1981; Levine et al., 1984). Much evidence also supports the participation of both serotonergic and noradrenergic systems in the mediation of vaginocervical antinociception. Norepinephrine and serotonin are released into the spinal cord during vaginocervical stimulation (Steinman, Komisaruk, Yaksh and Tyce, 1983). Further, VCS-induced antinociception is significantly attenuated by pretreatment with pharmacological antagonists of either norepinephrine or serotonin (Steinman et al., 1983). These findings indicate that VCS-induced antinociception, at least partially, utilizes the neural pathways of the endogenous opioid pain inhibitory system originating in the vlPAG (Komisaruk and Whipple, 1995).

The question of whether VCS-induced antinociception is a form of stress-induced antinociception has received some attention in light of the fact that, in addition to antinociception, VCS typically produces an increase in heart rate, blood pressure and pupil diameter, which are consistent with strees-induced activation of the sympathetic nervous system (Szechtman, Adler and Komisaruk, 1985; Whipple, Ogden and Komisaruk, 1992). However, it has been shown that these physiological changes are mediated by separate mechanisms and they are neither necessary nor sufficient for the

VCS-induced analgesia to occur (Catelli, Sved and Komisaruk, 1987; Komisaruk and Whipple, 2000). While emphasizing these differences, Komisaruk and Whipple (2000) nonetheless suggest that VCS-induced antinociception is a form of stress-induced antinociception provided that it is acknowledged "..... that pleasure can be a stressor, stress can be pleasurable, and vaginal stimulation can produce a pleasurable stress-induced analgesia".

Pregnancy-Induced Antinociception. Another example of analgesia unique to females is pregnancy-induced antinociception (PIA) (Gintzler, 1980), which has been documented in females of many mammalian species, including humans. This antinociception becomes activated during the latter stages of pregnancy in response to the rising levels of circulating estrogen and progesterone, and is associated with the abrupt increase of pain thresholds in preparation for labor and delivery (Cogan and Spinnato, 1986; Gintzler, 1980). An additional increase in pain thresholds occurs during parturition and appears to be due to vaginocervical mechanostimulation resulting from the cervical contractions and distention of the reproductive canal during birth (Whipple, Josimovich and Komisaruk, 1990). PIA is attenuated by transection of the hypogastric nerve but is unaffected by adrenalectomy or hypophysectomy (Gintzler, Peters and Komisaruk, 1983; Baron and Gintzler, 1984 and 1998). Opioid antagonists block PIA, indicating the opioid mediation of this antinociceptive response (Gintzler, 1980). Spinal delta and kappa, but not mu opioid receptors have been implicated in the mediation of PIA (Dawson-Basoa and Gintzler, 1997). Further, PIA is reduced by intrathecal alpha 2 noradronergic antagonists, suggesting that the noradrenergic systems also participate in the mediation of this antinociceptive response (Liu and Gintzler, 1999).

Simulation of the hormonal profile of pregnancy with 17-beta estradiol and progesterone has been previously shown to be effective in inducing pregnancy-like antinociception in pseudopregnant rats and in nonpregnant ovariectomized females (Dawson-Basoa and Gintzler, 1997;Gintzler and Bohan, 1990; Medina, Dawson-Basoa and Gintzler, 1993). Liu and Gintzler (Liu and Gintzler, 2000) recently reported that the analogous hormonal regimen of 17-beta estradiol and progesterone was also effective in eliciting robust antinociception in castrated male rats. However, although the magnitude of this pregnancy-simulated antinociception in castrated males was almost identical to this observed in females, the underlying neurochemical mechanisms were sex-specific and could be dissociated. In ovariectomized females, this hormonally-induced antinociception depended upon the synergistic action of spinal kappa and delta opioid receptors, but not mu receptors. In contrast, pregnancy-simulated antinociception in castrated males involved additive actions of spinal kappa and mu opioid receptors, but not delta receptors. Further, expression of these hormonally-induced antinociceptive responses was dependent upon synergy of spinal opioid receptors and alpha 2 noradrenergic receptors in ovariectomized females but not males. These data suggest that pregnancy-induced antinociception engages distinct pain inhibitory pathways in female rats that are sexually- dymorphic and not present in male rats.

Sex Differences in Opioid Antinociception

Sex Differences in Morphine Antinociception. Considerable evidence from animal studies indicates significant sex differences in antinociceptive sensitivity to morphine with male rodents typically displaying significantly greater magnitudes of systemic morphine-induced antinociception than female rodents (Baamonde, Hidalgo

and Andres-Trelles, 1989; Candido et al., 1992; Cicero et al, 1996; Kavaliers and Innes, 1987). Moreover, a similar pattern of sex differences is observed in the reinforcing properties of morphine and in the development of morphine tolerance and dependence with males showing greater sensitivity than females (Cicero, Ennis, Ogden and Meyer, 2000; Craft, Stratman, Bartok, Walpole and King, 1999). It appears that the observed sex differences in morphine antinociception are centrally-mediated since they are also observed following microinjections into the lateral ventricles or RVM (Boyer, Morgan and Craft, 1998; Kepler et al., 1989).

The sex differences in antinociception are not test-specific since they have been consistently documented across many somatic and visceral measures of nociception (Baamonde, Hidalgo and Andres-Trelles, 1989; Cicero et al., 1996; Kepler et al., 1989). Further, they cannot be explained by differences in the pharmokinetics of morphine because comparable levels of serum concentrations of morphine and its half-life elimination from blood and brain have been observed in male and female rats (Cicero, Nock and Meyer, 1997, but see Candido et al., 1992). Instead, these sex differences appear to be due to an intrinsic, enhanced central nervous system sensitivity to morphine in males than in females (Cicero et al., 1997). Sex differences may exist in the underlying neurochemical mechanism mediating morphine antinociception. For example, it has been demonstrated that morphine antinociception in male mice involves activation of NMDA receptors while morphine antinociception in females is not as strongly dependent on the NMDA system for its expression (Lipa and Kavaliers, 1990).

The effects of circulating gonadal hormones upon sex differences in morphine antinociception have been examined with inconsistent results. Some studies reported

that adult ovariectomy blunted antinociceptive responses to morphine (Banerjee, Chatterjee and Ghosh, 1983; Kepler et al., 1989;) while some other found the opposite effect (Ali, Sharif and Elkadi, 1995; Kasson and George, 1984), or no effect (Baamonde, Hidalgo and Andres-Trelles, 1989; Cicero et al., 1996). Similarly, adult castration has been reported to decrease (Chatterjee, Das, Banerjee and Ghosh, 1982), increase (Ali, Sharif and Elkadi, 1995) or have no effect (Cicero et al., 1996; Kasson and George, 1984) on morphine antinociception in male rats. In trying to reconcile these conflicting results, Kest (Kest et al., 2000) suggested that the effects of adult gonadectomy may be nociceptive modality-specific and may also depend on the route of administration of morphine.

It should be noted that even when gonadectomy-related effects are found (e.g., Kepler et al., 1989), they are typically limited to only one dose, inconsistent and much too small to fully account for the marked sex differences in antinociceptive responses to morphine. In addition, significant sex effects in the responsivity to morphine were evident in very young sexually-immature mice, further suggesting that the activational effects of gonadal hormones are not necessary for expression of sex differences in morphine antinociception (Kavaliers and Innes, 1990).

Another line of evidence supporting the view that sex differences in morphine antinociception are not strongly dependent upon acute, activational effects of physiological levels of gonadal hormones is derived from the studies which investigate female sensitivity to morphine as a function of the estrous phase. These studies show that while the levels of female hormones fluctuate widely throughout the rat estrous cycle, the corresponding fluctuations in the magnitude and potency of morphine antinociception are

small and not consistently observed. For example, one study demonstrated slightly enhanced antinociception to centrally-administered morphine during proestrous (Kepler et al., 1989), while in two other studies, female rats were most sensitive to systemic effects of morphine during diestrous (Banerjee et al., 1983; Berglund and Simpkins, 1988). Still another study (Innes and Kavaliers, 1987) failed to discern any significant variations in the magnitude of morphine antinociception in mice as a function of the estrous phase. Collectively, these data generally suggest that the expression of sex differences in morphine antinociception is not strongly dependent on acute activational effects of steroid gonadal hormones. A comprehensive review of findings on the effects estrous phase and gonadal status upon opioid antinociception can be found in Kest et al.(2000).

The role of organizational effects of gonadal hormones in the mediation of sex differences in morphine antinociception has not been examined. In contrast to the activational effects of gonadal hormones that may be transient, typically occur later in development and are affected by adult gonadectomy and cyclic hormonal fluctuations, the organizational effects of gonadal hormones are typically permanent, occur early in development and can be altered by neonatal gonadectomy, but not by any hormonal changes or fluctuations occurring in adulthood (Phoenix et al.., 1959). Further, whereas the neural effects of the activational effects of gonadal hormones are believed to involve functional changes, such as changes in brain neurochemistry and electrical activity, the organizational effects are thought to reflect permanent changes in neural connections and pathways.

Several studies have failed to observe overall sex differences in morphine antinociception (Ali et al, 1995; Islam et al, 1993, Kanarek and Homolesky, 2000). The reason for these inconsistencies is not clear and methodological differences may account for some of the observed discrepancies. However, another plausible explanation is that the genetic background of the particular rodent population studied may interact with sex differences in morphine antinociception, with some strains displaying greater differential sex-dependent effects than others (Mogil, Kest, Sadowski and Belknap, 1996; Mogil et al., 2000). For example, a recent study (Mogil et al, 2000) showed that male Long-Evans and Sprague-Dawley rats displayed greater systemic morphine antinociception than their female counterparts. In contrast, morphine antinociception was equivalent in male and female rats of the Wistar Kyoto strain. Similar inter-strain differences in sex-related antinociceptive responses to morphine were also observed in different strains of mice (Mogil et al, 2000).

The age of the animal also appears to play an important role in shaping the expression of sex differences in morphine antinociception (Islam, Cooper and Bodnar, 1993). Aging male rats manifest increased antinociceptive sensitivity to morphine, while aging female rats show decreased age-dependent effects. Interestingly, the age-dependent decline in the female rat's sensitivity to morphine can be eliminated by adult gonadectomy performed at three months of age.

Environmental variables, such as diet and circadian rhythms may also interact with sex-related differences in morphine antinociception. For example, in one study (Kanarek and Homolesky, 2000), a sucrose-rich diet was found to potentiate morphine antinociception in both sexes but female rats appeared to be more sensitive to this effect

than male rats. Similarly, circadian rhythm variations differentially affect antinociception of male and female deer mice with females displaying reduced nocturnal responsiveness (Kavaliers and Innes, 1987). These findings underscore the importance of controlling for various genetic, organismic and environmental variables while testing for sex differences in morphine antinociception.

Sex Differences in Antinociception to Opioids Other than Morphine. While sex differences in morphine antinociception are relatively consistent, findings are somewhat more ambiguous with respect to whether such effects can be elicited by other opioid agonists. Sex differences in antinociception have been demonstrated following intraventricular administration of the mu receptor agonist DAMGO with intact male rats displaying significantly greater antinociception than intact female rats on the tail flick but not the jump test (Kepler et al, 1991). Greater antinociception in males than females was also observed following systemic administration of another potent mu agonist alfentanil (Cicero et al., 1997). In contrast, sex differences did not occur following the mu agonist fentanyl or buprenorphine (Bartok and Craft, 1997).

Antinociception elicited by the kappa agonist U50,488 has been found to be sensitive to sex differences in deer mice, with male animals displaying greater antinociception than females on the hot plate test (Kavaliers and Innes, 1987). In contrast, female rats have been found to be more sensitive than male rats to the antinociceptive effects of the systemic kappa agonist bremazocine on the tail withdrawal test (Bartok and Craft, 1997). Although female rats also displayed longer tail withdrawal latencies following systemic administration of the selective kappa 1 agonist U69, 593, this effect was time-dependent, and it did not alter the overall potency of the drug between males and females.

Sex differences in delta opioid antinociception have also been examined with generally negative results. For example, ventricular administration of the general delta receptor agonist DSLET failed to elicit significant sex differences on the tail flick or jump tests (Kepler et al., 1991). In another study (Bartok and Craft, 1997), ventricular administration of high doses of the selective delta 1 agonist DPDPE or the selective delta 2 deltorphin II significantly increased hot- plate latencies in male rats more than in female rats (Bartok and Craft, 1997). However, this effect was strongly dose-dependent and present only following very high doses of DPDPE or deltophrin, and the overall potencies of either agonist of males and females failed to differ.

In summary, significant sex differences in opioid antinociception have been reported for two out of the three main opioid families. Drugs with either mu or kappa receptor activity have been found to elicit sex differences in antinociception, although not all mu and kappa agonists examined displayed this effect. In a majority of cases, males showed greater antinociception than females, although one kappa agonist, bremazocine, produced the opposite effect. In contrast, delta agonists were generally ineffective in eliciting sex differences in antinociception, although some sex effects were evident at very high doses of DPDPE and deltorphin II, with males showing greater magnitudes of antinociception than females.

Sex differences in antinociceptive responses to nonopioid agents have also been examined. Males demonstrated greater antinociceptive responses to the alpha 2 noradrenergic agonist clonidine, the muscarinic agonist pilocarpine, and cocaine (Craft and Milholland, 1998; Kiefel and Bodnar, 1991). However, females were found to show enhanced antinociceptive sensitivity to nicotine (Craft and Milholland, 1998).

The effects of adult gonadectomy on antinociceptive responses produced but some, but not all, of these opioid and nonopioid agonists have been examined. Adult gonadectomy of either male or female rats failed to consistently influence antinociception elicited by opioid agonists DAMGO or DSLET (Kepler et al., 1991). In contrast, whereas adult castration had no effect on antinociception produced by either clonidine or pilocarpine, adult ovariectomy significantly decreased antinociceptive responses elicited by pilocarpine and significantly increased antinociceptive responses produced by clonidine (Kiefel and Bodnar, 1991). The following section summarizes the main points of this review and provides a rationale for the present study.

Rationale for the Present Study

The reviewed literature clearly demonstrates that significant sex differences exist in pain sensitivity and inhibition, and in responsiveness to various antinociceptive drugs. Evidence from both human and animal research suggests that females are more sensitive to many experimentally induced noxious stimuli, particularly electrical stimulation (Beatty and Beatty, 1970; Fessler and Beatty, 1976; Fillingim and Maixner, 1995; Kepler et al., 1991; Marks et al.,1972; Mogil, et al., 2000; Pare, 1969; Riley et al., 1998; Romero and Bodnar, 1986). Compared to males, female rodents typically show significantly lower magnitudes of antinociception in response to opioid- and nonopioid-mediated environmental stressors (Romero and Bodnar, 1986) while human females report more endogenous pain complaints and are more likely to suffer from painful medical conditions (Merskey and Bogduk, 1994; Unruh, 1996). Sex differences in antinociceptive sensitivity to opioid drugs have also been examined with extensive animal data consistently indicating greater antinociception in males and several human studies

suggesting the opposite effect (Cicero et al., 1996; Gear et al., 1996a; Gear et al., 1996b; Kepler et. al., 1989; Miaskowski and Levine, 1999; Mogil et al., 2000).

Since sex differences in antinociceptive responses to morphine and other opioids have important clinical and therapeutic implications, this dissertation research focused upon identification of potential neural sites and mechanisms which mediate sex-related effects in opioid antinociception. Previous studies have shown that antinociceptive responses to morphine following systemic and ventricular administration are sensitive to sex differences (Candido et al., 1992; Cicero et al, 1996; Kavaliers and Innes, 1987; Kepler et al., 1989). Male rodents typically display significantly greater magnitudes and potencies of morphine-induced antinociception than female rodents. These sex effects are evident across many different nociceptive tests and are not due to differences in the pharmacokinetics of morphine but rather appear to be related to intrinsic sex differences in brain sensitivity to this drug (Cicero et al., 1996; Cicero et al., 1997; Mogil et al., 2000). Adult gonadectomy may modulate the expression of sex differences in morphine antinociception, but this effect appears small and has not been consistently observed in all studies (see review: Kest et al., 2000).

Because most of the data on sex differences in opioid antinociception have been derived from the studies which utilized systemic or intraventricular administrations, the brain sites mediating these sex-specific effects have not been identified. The vlPAG has emerged as one of the sites which may be subject to sex differences in morphine antinociception due to its critical role in both pain inhibition and reproductive behavior. The vlPAG is a major part of the well-characterized pain inhibitory system, which mediates supraspinal antinociception primarily through mu opioid receptors (Basbaum

and Fields, 1984; Bodnar et al., 1988; Fang et al., 1986; Ling and Pasternak, 1983). The vlPAG has extensive connections with the RVM, another important structure in the pain inhibitory system, which was recently demonstrated to be sensitive to sex differences in morphine antinociception (Boyer et. al., 1998). The PAG also plays a crucial role in reproduction because it mediates sex-dependent and hormone-dependent reproductive behaviors, particularly through its interaction with estradiol-containing hypothalamic loci. Further, the PAG mediates interactions between sex hormones and opioid peptides by regulating the synthesis of preproenkephalin by estradiol (Lauber et al., 1990; Pfaff et. al., 1994; Pfaff, 1999; Romano et al., 1988; Sakuma and Pfaff, 1979a; Sakuma and Pfaff, 1979b).

Given this evidence, **the first aim of this study** was to evaluate the hypothesis that morphine antinociception elicited from the vlPAG is subject to sex differences. An additional aim also evaluated the effects of adult gonadectomy upon the expression of antinociceptive responses to morphine in the vlPAG. To this effect, dose-dependent and time-dependent antinociceptive responses induced by morphine administration into the vlPAG were examined and tested for sex differences and adult gonadectomy effects in sham-operated male rats, castrated male rats, sham-operated female rats, and ovariectomized female rats.

To explore the generalizability of these sex differences two different nociceptive tests were used: the tail-flick and the jump test. These tests were selected because they assess different aspects of pain control and may be mediated by different physiological mechanisms. The tail-flick test measures responsivity to heat while the jump test measures responsivity to electric shock. Although both these tests assess responsivity to

acute, phasic pain, the tail-flick test elicits a quick reflexive response which is less complex than the response measured by the jump test. An additional advantage of these two tests is that they can be used consecutively during a single testing session since tail-flick latency testing does not affect subsequent jump threshold testing. This reduces the number of testing sessions, and the total number of animals necessary for the paradigm. An important caveat is that both the tail-flick and the jump test require motor responses that can be affected by opioid agonists independently of their effects on antinociception. This is why locomotor activity and independent behavioral assessment measures were performed in some of the experiments.

Although estrous cycle effects failed to systematically influence morphine antinociception following intraventricular administration (Kepler et al., 1989), the present study still controlled this hormonal variable by consistently testing sham females only during the estrous phase of the cycle in all antinociceptive studies. This paradigm allowed to maximize the study of dose-response functions of morphine and other opioid agonists used in this study while minimizing the number of animals used.

While sex differences in morphine antinociception are well established, such differences have been less consistently observed following administration of other opioid agonists. Sex differences have been shown to occur in response to agonists with either mu or kappa receptor activity while delta opioid agonists at normal therapeutic doses were ineffective (Bartok and Craft, 1997; Kavaliers and Innes, 1987; Kepler et al., 1991). However, only mu and delta 2, but not kappa or delta 1, opioid agonists are effective in inducing antinociception from the vlPAG (Bodnar et al., 1988; Fang et al., 1986; Ling and Pasternak, 1983; Rossi et al., 1994). Collectively, these data appear to suggest that mu

opioid receptors in the vlPAG are involved in mediating sex differences in opioid antinociception elicited from this site. If activity of mu opioid receptors in the vlPAG is responsible for the observed sex differences in morphine antinociception, then it would be expected that other agonists with high affinity for mu opioid receptors should also be effective in inducing sex differences in antinociception following their microinjections into the vlPAG. Therefore, **the second aim of this research** was to evaluate the generalizability of sex differences in morphine-induced antinociception in the vlPAG. To this end, two endogenous opioid agonists, beta-endorphin and D-Pro2- Endomorphin-2, that act through mu opioid receptors in the vlPAG were examined for antinociceptive sex differences in this site. Intact and adult-gonadectomized male and female rats were tested for sex and gonadectomy effects on the tail flick and jump tests using a protocol which was identical to that employed in the first study. Since opioid-induced responses in the PAG, particularly those elicited by mu agonists, may also increase auxiliary motor behaviors (Jacquet, Carol and Russell, 1976; Jacquet and Lajtha, 1974), additional studies also examined whether these behaviors were related to any observed sex differences in antinociception. For this, intact male and female rats were tested for sex differences on activity and behavioral activation measures following administration of either beta-endorphin or D-Pro2-Endomorphin-2 in the vlPAG.

The underlying mechanism of action by which the sex differences in opioid antinociception might be expressed in the PAG is currently not well understood. However, the effects of gonadal hormones upon the neurons that mediate antinociception provide one plausible modus operandi by which such effects could occur (Cicero et al., 1996; Fillingim and Ness, 2000; Kepler, at al., 1989;). There are two ways in which

gonadal hormones could mediate sex differences in opioid antinociception: acute activational effects, which would be affected by adult gonadectomy, and long-term organizational effects, which would be affected by neonatal gonadectomy (Phoenix et al., 1959). Previous research has shown that acute, activational effects of gonadal hormones may modulate expression of morphine antinociception in either one or both sexes, but they are unlikely to fully account for marked sex differences in antinociceptive sensitivity to morphine and other opioid agents (Banerjee et al., 1983; Chatterjee et al., 1982; Cicero et al., 1996; Kepler et al.,1989; Kepler et al., 1991). The role of organizational effects of gonadal hormones upon morphine antinociception and expression of sex differences in morphine antinociception has not been examined. Therefore, **the third aim of this research** was to evaluate organizational effects of the gonadal hormones upon sex differences in morphine antinociception by systematically examining the effects of neonatal (Day 1 post-natal) gonadectomy upon the antinociceptive response to vlPAG morphine of male and female rats tested in adulthood on the tail flick and the jump tests.

CHAPTER 2

General Methods

Subjects

Male and female albino Sprague-Dawley rats (Charles River Laboratories, approximately 70 days of age) were used as subjects in all studies. Animals were housed individually in polyethylene cages in the Queens College Vivarium and maintained on a 12 h light/12 h dark cycle with water and rat chow available ad libitum.

Gonadectomy Procedures

Adult Castrations and Ovariectomies

Animals were anesthetized with a combination of chlorpromazine (3mg/kg, i.p.) and ketamine (120mg/kg, i.m.). Castrations of male rats were performed by removing the testes and epididymal fat following a single 1.5 cm midscrotal incision. Sham castrations were performed by exposing but not removing the testes. Ovariectomies were performed by removing the ovaries and ovarian fat following a single bilateral dorsal incision. (Kepler et al., 1989; Romero and Bodnar, 1986; Romero et al., 1987, 1988). Sham ovarectomies were performed by exposing but not removing the ovaries. Body weight of each animal was assessed before the gonadal surgery and approximately 30 days post-operatively to assess gonadectomy-induced weight changes.

Neonatal Castrations and Androgenization

Timed pregnant female rats, approximately 12-14 days into gestation, were obtained from Charles River Laboratories and monitored until delivery, approximately one week later. On the first day after birth, pups in each litter were sexed and randomly assigned to either experimental or sham condition. Each pup was briefly anesthetized

using Halothane inhalation. Surgical castrations of male pups were performed by removing testes and surrounding tissue following a bilateral 3 mm ventral incision. Sham castrations were performed by exposing but not removing the testes. Androgenization of female pups were performed by subcutaneous injection of testosterone proprionate (TP, 250 ug/kg). Sham-treated females received subcutaneous injection of the sesame oil vehicle. Following surgeries, all pups were returned to their mothers and allowed to recuperate. All rats were weighed at approximately 70 days of age to assess any gonadectomy-induced body weight changes. Nociceptive testing commenced a minimum 90 days postnatally to ensure the full expression of experimentally-induced hormonal changes.

Stereotaxic Surgery

Two weeks after gonadectomy, each animal was anesthetized with a combination of chlorpromazine HCL (3 mg/ml, IP) and ketamine HCL (100 mg/ml, IM). Anesthetized rats were implanted with a stainless steel guide cannula (26 gauge, Plastics One, Roanoke, VA) which was stereotaxically (Kopf Instruments) placed into the PAG using the following coordinates: incisor bar (-5 mm), 0.3-0.6 mm anterior to the lambda suture, 1.5-2.0 mm lateral to the sagittal suture, 6.8-7.0 mm from the top of the skull, and angled 12 degrees toward the sagittal plane. Cannulae were secured to anchor screws with dental acrylic and held patent with dummy cannulae. Animals were allowed a minimum of seven days to recuperate before commencing behavioral testing.

Estrous Phase Determination

Estrous phase in intact females was monitored by daily vaginal smears taken 1-4 hours prior to testing. Samples were collected with saline-dipped cotton tips and smeared on

glass microscope slides. The slides were stained with Cresyl violet and viewed with light microscopy under 4x magnification. Based on the evidence showing possible differences in antinociceptive response in female rats as a function of estrous phase (eg., Kepler et al., 1989), sham females were tested only during the estrous phase of their cycle.

Drugs and Injections

Morphine (Pennick Laboratories), B-endorphin (Peninsula Labs), and endomorphin-1 (synthesized by S. Wilk) were dissolved in normal saline. All intracerebral infusions were administered in 0.5-ul volumes at the rate of 0.1 ul every 10 sec through a stainless steel internal cannula (33-gauge, Plastics One) which was connected to a Hamilton microsyringe by polyethylene tubing. The internal cannula was held in place for at least 30 sec. to prevent efflux of the drug by suction. Testosterone proprionate (Peninsula Labs) for the neonatal androgenization protocol was dissolved in sesame oil and administered s.c.in 0.25 ml volumes at the rate of 0.05 ml every 5 sec through a standard 1 ml syringe. The syringe needle was held in place for at lest 30 sec. to prevent efflux of the drug.

Nociceptive Procedures

In a given session, each animal was tested on the tail-flick and jump tests in that order to minimize carry-over effects. A tail-flick analgesiometer (IITC Co., Woodland Hills, CA) provided a radiant heat source that was mounted 8 cm above a photocell upon which the rat=s tail was placed. Radiant heat was applied 3-9 cm proximal to the tip of the rat=s tail; removal of the tail activated the photocell and determined the latency (0.01 s accuracy). The thermal intensity of the radiant heat source was set to produce baseline tail-flick latencies between 2 and 3.5 s. Each session consisted of three latency

determinations at different points on the tail at 10-s intervals. To avoid tissue damage, a trial was automatically terminated if a response did not occur within 12 s.

Rats were tested on the jump test immediately after tail-flick testing. Electric shock was delivered to the feet of the rat by a shock generator (BRS/LVE) and shock scrambler (Campden Instruments). The jump threshold was defined in mA as the lowest of two consecutive ascending intensities in which the animal simultaneously removed both hindpaws from the grids. Each of six trials began with the animal receiving a 300-ms foot shock at a current intensity of 0.10 mA with subsequent shocks increased in 0.05 mA steps at 10-s intervals until the jump threshold was determined. A cut-off threshold of 1.2 mA was used for all jump threshold trials.

Baseline latencies and thresholds were determined for at least 4 days before experimental testing began to ensure stability of responding. All animals displayed consistent latencies and thresholds in baseline and vehicle testing that did not appear subject to desensitization.

Behavioral Tests

Locomotor Activity

Rats were acclimated to a testing room prior to all experimental procedures. In this room, groups of six rats each were testedin their individual home cages at 4 h into the light cycle. Food and water were removed during testing to prevent meal-related ingestive behaviors from being added into spontaneous activity. Each of the six polyethylene cages was placed in a Digiscan Micro Monitor (46 x 26 x 20 cm: Omnitech Electronics, Columbus, OH), which was equipped with a horizontal sensor panel of 16 infra-red light beams spaced 2.54 cm apart. Data from each of the six monitors was

accepted by a Digiscan Micro Analyzer (Omnitech Electronics, Columbus, OH), which recorded total activity count. Total activity included both the number of broken and released beams made in one direction as well as the number of times the same beam was broken and released repetitively. These data were in turn sent to a printer in 15 min or 30 min time blocks over the course of the particular experiment.

Behavioral Activation

Rats were assessed for behavioral activation in their home cages using the following four parameters: barrel rolls, explosive continuous running, seizures and grooming. Barrel rolls were defined as full axial rotation (flip) of the rat=s body and were scored on a 4-point scale: 0 (no barrel rolls), 1 (1-5 flips in less than 1 min), 2 (5-10 flips in 1-2 min) or 3 (>10 flips over 2 min). Explosive continuous running was scored on a 3-point scale: 0 (no running), 1 (running for less than 30 s), or 2 (running for more than 30 s). Seizures were scored on a 2-point scale: 0 (no seizures) or 1 (facial and/or forepaw convulsive behavior). Grooming was scored on a 3-point scale: 0 (no grooming), 1 (continuous grooming for less than 1min), or 2 (continuous grooming for 1-2 min). All behaviors were observed and scored by an observer uninformed about the drug condition and sex of the animal.

Histological Procedures

Determination of Cannula Placement

Following the completion of experimental testing, all rats were deeply anesthesized and received a transcardiac perfusion with 0.9% normal saline followed by 10% buffered formalin. Coronal (40 μm) brain sections were stained with Cresyl violet and examined

by light microscopy by an observer unfamiliar with the behavioral data. Only animals with confirmed cannula placements were included in the data analysis.

In all three experiments histological placements in four experimental groups were all localized within the vlPAG and were found as far rostral as the level of the III cranial nerve, and as far caudal as the dorsal raphe nucleus. However, due the possibility of diffusion, the drug aimed at the vlPAG at the level of the dorsal raphe nucleus could cover a larger brain area. Based on the 0.5 ug volume of each microinjection, the maximum diffusion of the drug might reach as far rostral as the vlPAG at the level of the III cranial nerve, as far caudal as the caudal dorsal raphe, as far ventral as the raphe linearis, as far dorsal as the cerebral aqueduct, and as far lateral as the central tegmental tract.

Post-mortem Examination of Accessory Sex Organs

After an overdose of anesthesia, the ventral prostates and seminal vesicles of castrated and sham-operated male rats were removed, blotted dry and weighted. The same procedure was followed with the uteri of ovariectomized and sham-operated females. Consistent with the results of previous studies (Kepler et al., 1989, 1991), the weights of the seminal vesicles in castrated males were approximately 20% of those of sham-operated males, while uterine weights in ovariectomized females were approximately 33% of these of sham-operated females, thereby confirming the completeness of the gonadectomy procedures. The differences in weight of gonadal organs of gonadectomized and sham-operated male and female rats were highly similar in all experiments.

CHAPTER 3

Experiment 1: Sex Differences and Adult Gonadectomy Effects on Morphine Antinociception Elicited from vlPAG

Background

Sex differences in antinociception are commonly observed following systemic and intraventricular morphine administration, with male rats displaying significantly greater magnitudes of morphine antinociception than female rats (Cicero et al., 1996; Kepler et al., 1989). These sex differences are evident on many nociceptive tests and do not appear to be due to differences in pharmokinetics of morphine (Cicero et al., 1997). Adult gonadectomy or changes in the female estrous cycle appear to have a modulatory effect upon sex differences in morphine antinociception but this effect appears to be small and has not been consistently observed (Ali et al., 1995; Banerjee et al., 1982; Chatterjee et al., 1982; Cicero et al, 1996; Kepler et al., 1989).

Because most of the data on sex differences in morphine antinociception have been derived from studies which utilized systemic or intraventricular administration, the brain site mediating these sex effects could not be identified. The vlPAG, however, has emerged as a strong candidate to mediate sex differences in morphine antinociception due to its critical role in both pain inhibition and reproductive behavior. The vlPAG is a major part of a well-characterized pain inhibitory system, which mediates supraspinal antinociception primarily through μ opioid receptors (Basbaum and Fields, 1984; Bodnar et al., 1988; Fang et al., 1986; Ling and Pasternak, 1983). The PAG has extensive connections with the RVM, another crucial structure in the inhibitory pain system, which was recently demonstrated to be sensitive to sex differences in morphine antinociception

(Boyer et al., 1998). The PAG also plays a crucial role in reproduction because it mediates sex-dependent and hormone-dependent reproductive behaviors, particularly through its interaction with estradiol-containing hypothalamic loci. Further, the PAG mediates interactions between sex hormones and opioid peptides by regulating the transcription of the preproenkephalin gene by estradiol (Lauber et al., 1990; Pfaff et al., 1994; Pfaff, 1999; Romano et al., 1988; Sakuma and Pfaff, 1979a; Sakuma and Pfaff, 1979b).

The primary purpose of Experiment 1 was to test the hypothesis that the vlPAG is one of the sites at which sex differences in morphine antinociception are observed . An additional aim was to examin the effects of adult gonadectomy upon the expression of antinociceptive responses to morphine. To this end, antinociceptive responses induced by morphine administration into the vlPAG were examined and tested for sex differences and gonadectomy effects in sham-operated male rats, castrated male rats, sham-operated female rats in estrous, and ovariectomized female rats on two nociceptive measures: the tail-flick and jump. Sham-operated females were tested only during the estrous phase of the cycle in order to maximize the study of dose-response function of morphine. The findings of this study have previously been published in Brain Research (Krzanowska & Bodnar, R.J., 1999).

Methods

Procedures and Protocol

Adult-castrated and sham-operated male rats and adult-ovariectomized and sham-operated female rats were stereotaxically implanted with a stainless steel guide in the vlPAG. Subgroups of 6-8 rats in the sham-operated male, castrated male, sham-operated

female, and ovariectomized female groups received morphine doses of 0, 1.0, 2.5 and 5.0 ug. Both groups of female rats additionally received a morphine dose of 10 ug while both groups of male rats additionally received a morphine dose of 1.75 ug. All doses were tested in a counterbalanced order. Treatment conditions were separated by at least one week to minimize possible tolerance effects. Testing always took place between 2 and 8 hours into the light cycle. Each rat was tested at 30, 60, 90, and 120 min after each microinjection on the tail flick and jump tests. Each rat received between 2 and 5 injections, including vehicle treatment.

Statistical Analyses

Overall summed latencies and thresholds were determined for each animal in each condition, and separate analyses of variance were performed at each dose of morphine to evaluate significant differences as functions of sex, gonadal status and vehicle/drug conditions. Tukey planned comparisons ($p < .05$) were used to discern significant effects relative to corresponding vehicle values. The potency of effects was evaluated by constructing log dose-response functions and performing linear regression analyses. The antinociceptive potency was defined as the ED50 for total antinociceptive effects for each nociceptive measure. Because baseline latencies and threshold were very similar across the four experimental groups, in this and the following experiments, the criterion for the ED50 was that minimal dose elicited an 8 s increase in total tail-flick latencies and a 0.40 mA increase in totall jump thresholds relative to total vehicle values for each nociceptive measure. This criterion was selected because it is commonly used in our laboratory since it has been found to provide reliable interpolations of the ED50 values.

Results

Body Weight

Consistent with previous findings (Kepler et al., 1989), gonadectomy accelerated weight gain in females and slowed weight gain in males in this, and in the following, experiments. Ovariectomized female rats increased their body weight by average of 47 g (18% increase) while sham-operated females gained 13 g (4% increase). Similarly, castrated male rats on average gained 60 g (18% increase) while sham-operated males gained 87 g (26% increase).

Morphine Antinociception

Significant differences were observed between vehicle and all tested morphine doses on both the tail-flick and jump tests: 1ug [tail flick: $F(1,46)= 28.79$, $p<.0001$; jump: $F(1,46)= 11.99$, $p<.0012$], 1.75 ug [tail flick: $F(1,23)= 8.45$, $p<.008$; jump: $F(1,23)= 12.27$, $p<.0019$], 2.5 ug [tail flick: $F(1,48)= 62.51$, $p<.0001$; jump: $F(1,48)= 53.21$, $p<.0001$], 5.0 ug [tail flick: $F(1,46)= 63.97$, $p<.0001$; jump: $F(1,46)=106.76$, $p<.0001$] and 10 ug [tail flick: $F(1,23)= 7.94$, $p<.0098$; jump: $F(1,23)= 9.89$, $p<.0045$].

Significant sex differences were observed following morphine doses of 1 [tail-flick: $F(1,46)= 6.08$, $p<.018$; jump: $F= 5.95$, $p<.019$], 2.5 [tail-flick: $F(1,48)= 14.94$, $p<.0003$; jump:$F= 16.34$, $p<.0002$] and 5 [tail-flick: $F(1,46)= 10.22$, $p<.0025$; jump: $F= 36.17$, $p<.0001$] ug with male rats displaying significantly higher latencies and thresholds following each dose of morphine than female rats. Gonadectomy failed to significantly alter latencies and thresholds following morphine doses of 1, 1.75 and 2.5 ug, but significantly increased thresholds in males and females following the 5 ug dose [$F(1,46)= 17.29$, $p<.0001$], and significantly increased latencies [$F(1,23)= 7.94$, $p<.0098$] and

Figure 1. Alterations in tail-flick latencies (s, mean, ±SEM) summed across the 120 min time course following vehicle and morphine microinjections into the ventrolateral periaqueductal gray (vlPAG) in male and female rats surgically treated 1 month earlier to sham or gonadal surgery. The asterisks (*) denote significant alterations in tail-flick latencies relative to corresponding vehicle control treatment. The crosses (+) denote those morphine doses in which male rats displayed significantly higher latencies or thresholds relative to female rats. The pound signs (#) denote those morphine doses in which gonadectomized animals of a given sex displayed significantly higher latencies or thresholds relative to corresponding sham-operated controls.

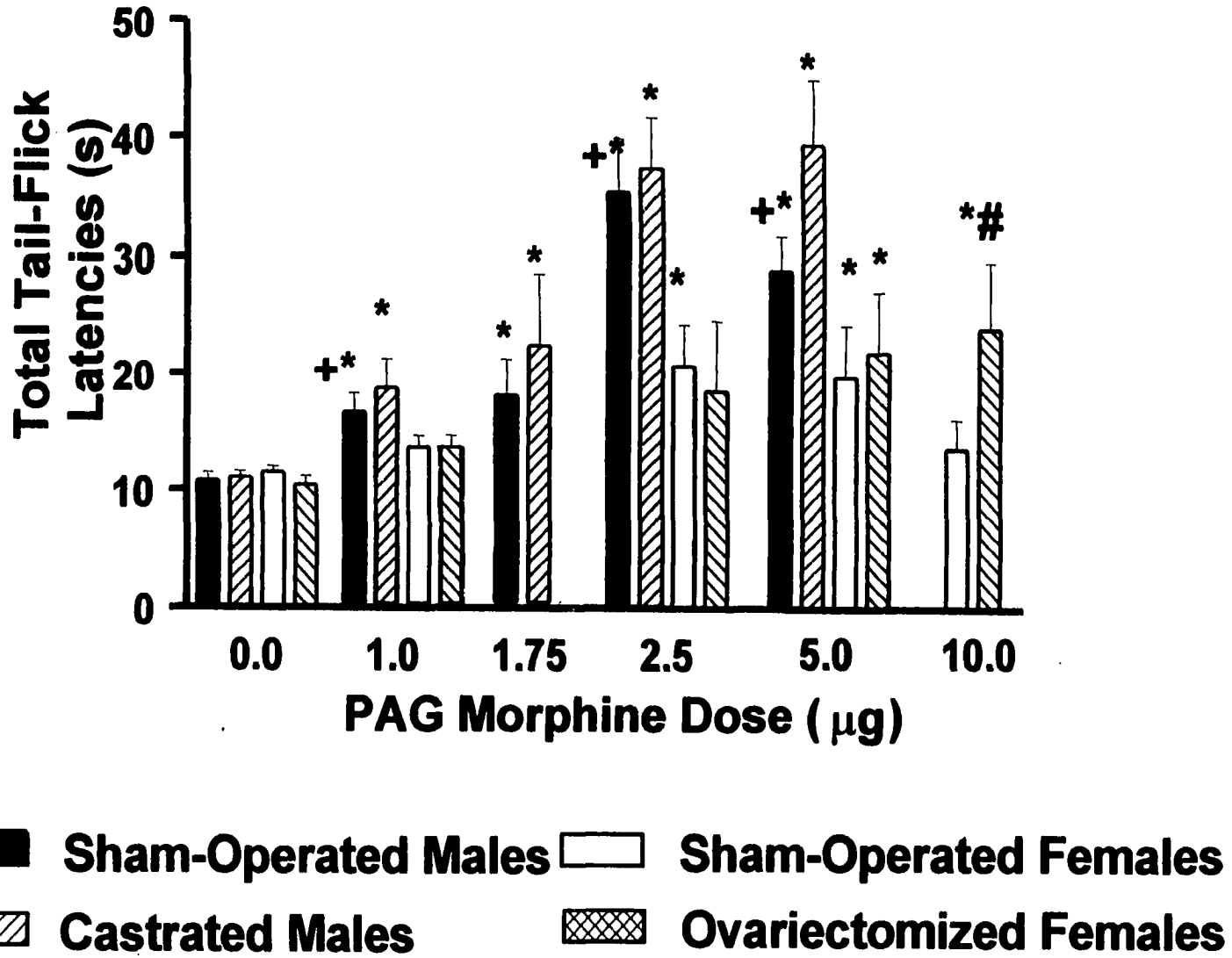
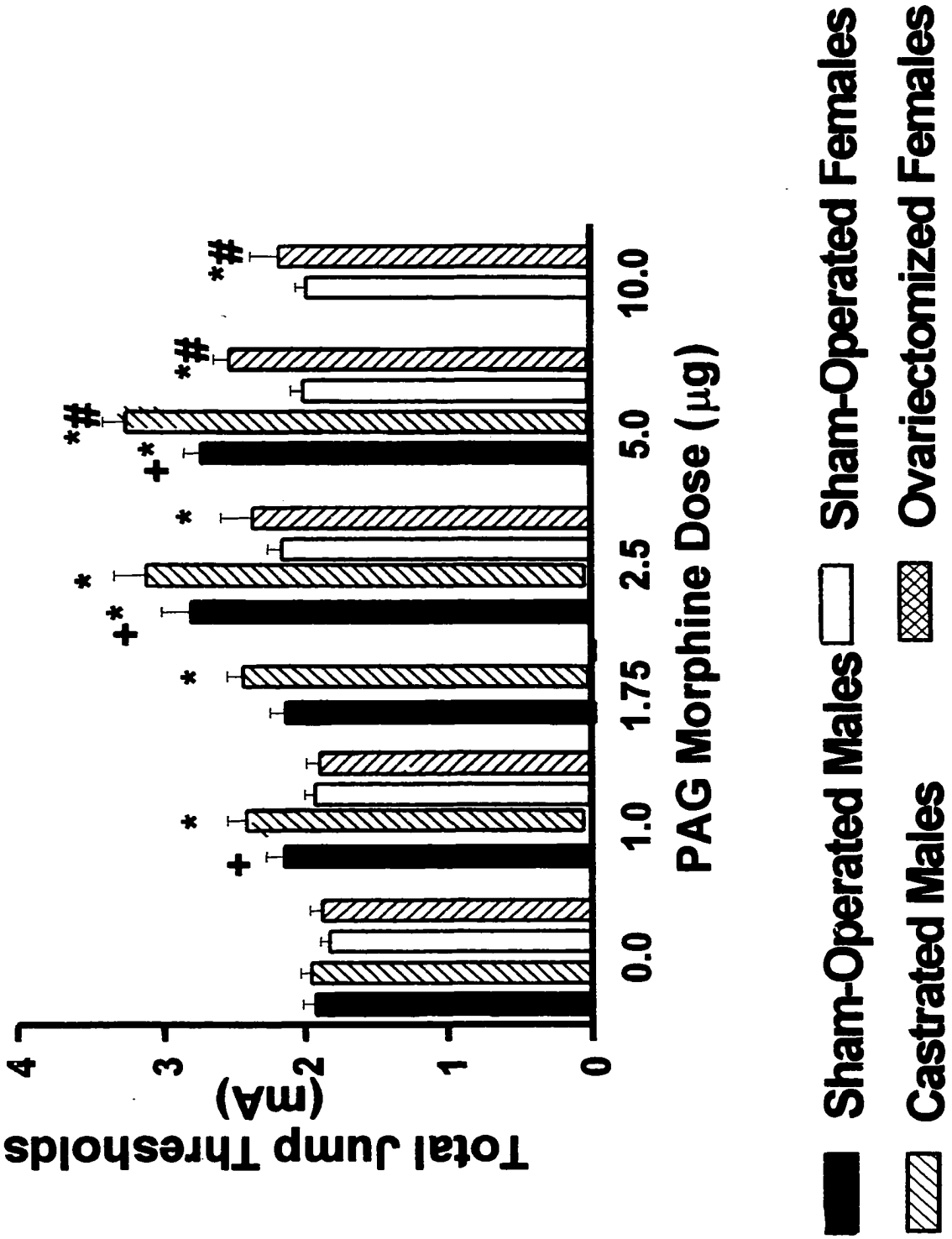


Figure 2. Alterations in jump thresholds (mA, mean, ±SEM) summed across the 120-min time course following vehicle and morphine microinjections into the vlPAG in male and female rats surgically treated 1 month earlier to sham or gonadal surgery. The asterisks (*) denote significant alterations in jump thresholds relative to corresponding vehicle control treatment. The crosses (+) denote those morphine doses in which male rats displayed significantly higher thresholds relative to female rats. The pound signs (#) denote those morphine doses in which gonadectomized animals of a given sex displayed significantly higher thresholds relative to corresponding sham-operated controls.



thresholds [F(1,23)= 9.89, p<.0045) following the 10 ug dose in females. Figures 1 and 2 illustrate the significant increases in morphine-induced antinociception on the tail-flick and jump tests respectively in male rats relative to female rats, and in gonadectomized relative to intact rats.

Regression analyses revealed significant differences in the potency of morphine-induced antinociception on the tail-flick (F(6,90)= 6.86, p<.00001) and jump (F= 12.68, p<.00001) tests.

The ED_{50} for morphine-induced antinociception was similar in all male rats on the tail-flick (intact males: 1.20 ug; castrated males: 1.08 ug) and jump (intact males: 1.60 ug; castrated males: 1.09 ug) tests. In contrast, the ED_{50} for morphine-induced antinociception on both tests was far higher than in males in intact sham-operated female rats (>50 ug) since the magnitude of the antinociceptive responses reached asymptote at doses higher than 2.5 ug. Ovariectomy partially reinstated the potency of morphine-induced antinociception since the ED_{50} values of ovariectomized females on the tail-flick (2.51 ug) and jump (1.98 ug) began approximate potency values observed in male rats.

Discussion

The first experiment clearly demonstrated that the vlPAG is an important site at which there are marked sex differences both in terms of the magnitude and potency of morphine-induced antinociception on two different measures of thermal and shock-induced nociceptive reactivity. Male rats displayed significantly greater magnitudes and potencies of morphine antinociception elicited from the vlPAG than female rats on both thermal and shock reactivity tests. The pattern of effects was quite similar to that observed following intraventricular morphine administration (Kepler et al., 1989; Cicero

et al, 1996) or following microinjections of morphine into the RVM (Boyer et al., 1998). In all of the above cases, the magnitude of morphine-induced antinociception in male rats increased towards cut-off values as the morphine doses increased from 1 to 5 μg. In contrast, the magnitude of morphine-induced antinociception at doses of 2.5-5.0 μg in each central placement in female rats was significantly higher than vehicle values, but significantly lower than corresponding values in male rats. As the morphine doses increased towards 10 μg, the antinociceptive magnitude in female rats reached asymptote. These findings clearly suggest large sex differences in the CNS sensitivity to morphine, with male rats displaying significantly greater antinociceptive effects than female rats.

It could be argued that one reason why male rats displayed greater antinociceptive responses than female rats is that morphine tolerance due to multiple microinjections may have developed differentially across the sexes. This interpretation is unlikely, however, because: 1) the order of dose was carefully counterbalanced across subjects in all four experimental groups; 2) treatment conditions were separated by at least one week, minimizing the possibility of tolerance effects (Yaksh et al., 1976); and, most importantly, 3) the direction of the observed sex differences in morphine antinociception was *opposite* to that predicted to be the result of differential tolerance, since previous research has shown that male rats develop tolerance *faster* than female rats and therefore would be expected to show *decreased rather than increased* antinociceptive responses relative to females in a repeated microinjection paradigm (Badillo-Martinez, Kirchgessner, Butler & Bodnar, 1984; Craft et al., 1999; Kasson & George (1984). Interestingly, no sex differences in morphine tolerance following chronic intracerebroventricular administration were observed in mice (Kest and Hopkins, 2001)

although female mice developed tolerance faster than male mice following chronic systemic morphine. These findings suggest that the rate of development of morphine tolerance in males and females may differ across species, and it may also depend on the route of administration of morphine.

The pattern of morphine-induced antinociception elicited from the vlPAG was largely unaffected by male gonadectomy, an effect similar to that observed following icv morphine administration (Kepler et al., 1989). This suggests that circulating and activational male sex steroid hormones are not crucial for the full expression of male morphine-induced antinociception elicited from the vlPAG. Since castration failed to alter the potency of morphine antinociception elicited from the vlPAG, the present study did not employ steroid replacement therapy. It should be noted that antinociceptive responses elicited by continuous cold-water swims are significantly lower in castrated relative to sham-operated male rats, and that testosterone replacement therapy reinstated the full antinociceptive potency in the former group (Bodnar et al., 1988).

Ovariectomy changed the pattern and potency of morphine-induced antinociception elicited from the vlPAG relative to sham-operated female rats tested during the estrous phase of their cycle. This is in contrast to the failure of ovariectomy to significantly shift the dose-response curve of morphine-induced antinociception following intraventricular administration (Kepler et al., 1989). Ovariectomized females displayed a greater magnitude of antinociception at higher, but not at lower, morphine doses in the vlPAG. Thus, there was a leftward shift in the potency of morphine antinociception in ovariectomized relative to sham-operated, estrous-tested females, although it was still less potent than in intact and castrated male animals. What potential mechanism(s) of

action might produce this pattern? Since ovariectomized animals are deficient in circulating gonadal hormones relative to sham-operated estrous-tested females, this suggests that individual or combined hormones in the latter group act as inhibitors of morphine-induced antinociception within the vlPAG. However these effects appear to be modulatory since ovariectomized and sham-operated, estrous-tested females display similar antinociceptive profiles at low morphine doses. Thus, two mechanisms are proposed. The first mechanism suggests an essential sex difference between male and female rats regardless of adult gonadal status, that may develop during gestation and perinatal life. The second is a gonadal effect of circulating sex hormones during adulthood in intact females that prevents the full antinociceptive expression of morphine, and thereby vitiates the magnitude of responses at high doses. Two circulating hormones, estradiol and progesterone, may participate in such actions.

The PAG mediates sex-dependent and hormone-dependent reproductive behaviors in female rodents, particularly through its interaction with estradiol-containing hypothalamic nuclei (Pfaff and Schwartz-Giblin, 1988). The PAG also mediates interactions between sex hormones and opioid peptides, particularly control of transcription of the PPE gene by estradiol (Lauber at al., 1990). In this regard, estradiol increases PPE mRNA in the hypothalamus at a rapid rate in female rats relative to male rats according to the different levels of estradiol across the estrous cycle (Romano et al., 1988; Romano et al., 1989).

Although estradiol-induced increases in PPE mRNA in female rats might logically suggest greater availability of endogenous opioids, and therefore greater magnitudes of antinociception, such sex steroid-induced modulatory effects may also produce changes

in responsivity to exogenously-applied opiates, particularly at high doses and in a site (the PAG) which would be maximally sensitive to such changes.

Progesterone itself has been implicated in antinociceptive processes in ovariectomized female rats (Frye and Duncan, 1994; McCarthy, Caba, Komisaruk and Beyer, 1990) and male mice (Kavaliers and Wiebe, 1987), since it increases nociceptive thresholds following acute administration. However, this effect is dose-dependent and biphasic since moderate (1 mg/kg) progesterone doses increased latencies, while higher (2-4 mg/kg) doses were without effect (Fry, Van Keuren, Rao and Erskine, 1996). Moreover, chronic progesterone pretreatment decreases antinociception elicited by sucrose exposure (Fry, Bock and Kanarek, 1992). Dawson-Basoa and Gintzler (1993) found that ovariectomized female rats displayed a pattern similar to pregnancy-induced antinociception only if progesterone and estrogen were simultaneously administered to mimic blood profile levels corresponding to late pregnancy and parturition. However, these effects did not occur if the hormones were given singly, indicating a synergistic interaction between the two hormones. In contrast, estrogen-primed ovariectomized rats displayed an enhanced antinociceptive response to vaginal stimulation which was blocked by concurrent progesterone administration (Crowley et al., 1976; Rothfield, Gross and Watkins, 1985). Furthermore, concurrent progesterone administration dampened the antinociceptive effects of the testosterone metabolite, 3alpha-androstanediol (Fry et al., 1996) and blocked the estrogen-induced facilitation of antinociception elicited by subthreshold doses of intrathecal muscimol (McCarthy et al., 1990). Therefore, in certain situations like pregnancy, progesterone synergizes with estrogen to facilitate antinociceptive processes, while in other situations like vaginal

stimulation, progesterone inhibits estrogen-mediated antinociceptive responses. The present study indicated that female rats in the estrous phase display a muted antinociceptive response to morphine elicited from the vlPAG, which strongly suggests that circulating levels of both gonadal hormones may produce mutually antagonistic effects. Further studies using steroid replacement of one and/or both of these hormones in ovariectomized rats are necessary to address these possibilities for morphine-induced antinociception elicited from the vlPAG.

Thus, sex differences in morphine-induced antinociception are observed in two of the most important nuclei mediating supraspinal controls in pain-inhibition: the vlPAG and the RVM. These sites have been shown to interact with each other in synergy studies using primarily mu receptor agonists (Rossi et al., 1993; Rossi et al., 1994), and the full expression of morphine-induced antinociception in the vlPAG is dependent upon the integrity of serotonergic, opioid, NMDA and cholinergic receptors within the RVM (Kiefel, Cooper and Bodnar, 1992a, 1992b; Kiefel, Rossi and Bodnar, 1993; Spinella, Cooper and Bodnar, 1996; Spinella, Schaefer and Bodnar, 1997). It would be of additional interest to determine whether spinally-mediated antinociception elicited by morphine is subject to sex differences. Further, although morphine appears to be the prototypical opioid agonist for which sex differences can be observed, one cannot generalize such effects to opioid agonists other than morphine since systemic and icv studies using other mu and delta and kappa agonists frequently report less marked effects (e.g. Bartok and Craft, 1997; Kepler et al., 1991). Hence, the following experiment evaluated the generalizability of sex differences in antinociception elicited from the

vlPAG by two other potent mu opioid agonists, beta-endorphin and D-Pro2-Endomorphin 2.

CHAPTER 4

Experiment 2: Sex Differences and Adult Gonadectomy Effects in Beta-endorphin and D-Pro2-Endomorphin-2 Antinociception and Locomotor Activity Elicited from the vlPAG

Background

While sex differences in morphine antinociception are relatively unambiguous, such differences are less consistently observed following administration of other opioid agonists. Sex differences have been shown to occur in response to agonists with either mu or kappa receptor activity while delta opioid agonists at normal therapeutic doses are generally ineffective (Kepler et al., 1991; Kavaliers and Innes, 1987; Bartok and Craft, 1997). However, only mu and delta 2 opioid agonists are effective in inducing antinociception from the vlPAG (Bodnar et al., 1988; Fang et al., 1986; Ling and Pasternak,1983; Rossi et al., 1994). Collectively, these data appear to suggest that mu opioid receptors in the vlPAG are most likely to be involved in mediating sex differences in opioid antinociception elicited from this site. If activity of mu opioid receptors in the PAG is responsible for the observed sex differences in morphine antinociception, then it might be expected that other agonists with high affinity for the mu opioid receptors should also be effective in inducing sex differences in antinociception following the microinjections into the vlPAG.

To establish the generalizability of sex differences in morphine-induced antinociception in the vlPAG, the present experiment examined whether two endogenous opioid agonists, beta endorphin and D-Pro2-Endomorphin-2, that act through mu opioid receptors in the vlPAG, would display antinociceptive sex differences in this site.

Because opioid-induced responses in the vlPAG, particularly elicited by mu agonists such as morphine, may also include increased auxiliary motor behaviors (Jacquet, Carol and Russell, 1976; Jacquet and Lajtha, 1974), additional protocols were employed to determine whether these behaviors were related to any observed sex differences in antinociception.

This experiment consisted of five protocols. Protocol 1 examined sex differences and adult gonadectomy effects in antinociceptive responses to vlPAG beta-endorphin on the tail-flick and jump test and have been published in the European Journal of Pharmacology (Krzanowska & Bodnar, 2000). Protocol 2 assessed potential sex differences in locomotor activity of male and female rats in response to vlPAG beta-endorphin across those dose and time courses employed in the nociceptive paradigm. The findings of this study has been published in Physiology and Behavior (Krzanowska & Bodnar, 2000). Protocols 3 and 4 respectively examined sex differences in antinociceptive and locomotor activity in response to vlPAG D-Pro2-Endomorphin-2 using identical measures and procedures to those employed in the first two protocols. Finally, Protocol 5 assessed sex differences in the effects of vlPAG D-Pro2-Endomorphin2 upon behavioral activation, defined as excessive grooming behavior, seizures, explosive running, and barrel rolls. The findings of Protocols 3-5 have been published in Peptides (Krzanowska, Znamensky, Wilk & Bodnar, 2000).

Protocol 1: Sex Differences and Adult Gonadectomy Effects in Beta-endorphin Antinociception Elicited from the vlPAG

Procedures

Adult-castrated and sham-operated male rats and adult-ovariectomized and sham-operated female rats were stereotaxically implanted with a stainless steel cannula in the vlPAG. Subgroups of 8-12 rats in the sham-operated male, castrated male, sham-operated female and ovariectomized female groups received beta-endorphin doses of 0, 5.2, 6.5,13.0, and 26 ug in a counterbalanced order. This dose range was chosen because it was equimolar to the dose range used for morphine in the previous experiment. Each dose was tested at 30, 60, 90, and 120 min after each microinjection on the tail-flick and jump tests. Treatment conditions were separated by at least one week to minimize possible tolerance effects. Testing always took place between 2 and 8 hours into the light cycle. Each rat received 2 to 4 doses of beta endorphin, plus the vehicle condition.

Statistical Analyses

Three-way analyses of variance were performed on each nociceptive measure separately, with sex and gonadal status as between-groups factors, and drug doses and test time as within-groups factors. Tukey planned comparisons ($p<.05$) were used to determine significant effects relative to corresponding vehicle values. The potency of effects was evaluated by constructing log dose response functions of difference scores values for each dose and performing linear regression analyses to derive ED_{50}s at which total latencies were increased by 8 sec.

Results

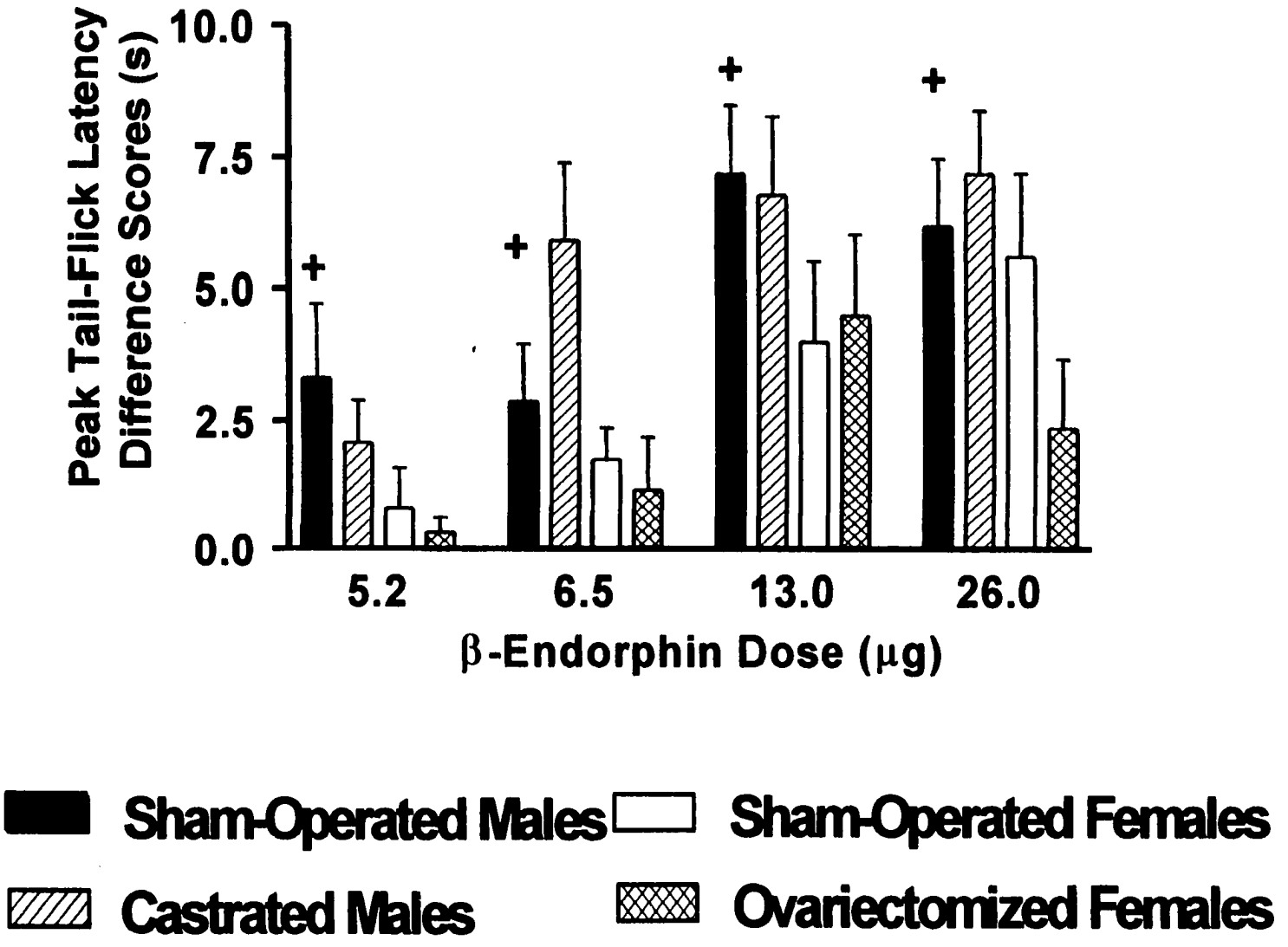
Tail-flick Test. Significant differences were observed in tail-flick latencies among sex and gonadectomy groups [$F(3,33)= 3.41$, $p<.029$], among beta-endorphin doses [$F(4,44)= 98.91$, $p<.0001$], across the 2 h time course [$F(3,33)= 92.69$, $p<.0001$], and for all interactions between and among these variables.

Latencies failed to differ among groups following vehicle treatment, indicating no sex or gonadectomy differences in baseline latencies. Beta-endorphin in the vlPAG significantly increased tail-flick latencies in sham-operated males following the 5.2 (30 min), 6.5 (30 min), 13 (30-120 min) and 26 (30-120 min) ug doses, in castrated males following the 6.5 (30-60 min), 13 (30-90 min) and 26 (30-120 min) ug doses, in sham-operated females following the 13 (30-90 min) and 26 (30-120 min) ug doses, and in ovariectomized females following the 13 (30-90 min) and 26 (30-120 min) ug doses.

Because peak effects in all groups occurred 30 min after beta-endorphin administration, specific sex and gonadectomy differences were analysed at this time point. Figure 3 illustrates peak antinociceptive effects of beta-endorphin in the vlPAG on the tail flick test, expressed in terms of difference scores. Significant differences in antinociceptive difference scores were observed between male and female rats [$F(1,7)= 40.32$, $P<0.0004$] and across beta-endorphin doses [$F(3,21)= 11.69$, $P<0.0001$], but not between sham-operated and gonadectomized animals [$F(1,7)= 0.02$, n.s.] or for any of the interaction terms. Male rats displayed significantly greater magnitudes of peak antinociception than female rats following the 5.2 [$F(1,7)= 8.03$, $P<0.025$], 6.5 [$F=10.43$, $P<0.015$], 13 [$F= 7.22$, $P<0.031$] and 26 [$F= 7.74$, $P<0.027$] ug doses of beta-endorphin administered into the vlPAG.

Regression analyses revealed significant differences in the potency of beta-endorphin antinociception on the tail-flick test [$F(6,117)= 2.40$, $p<.032$]. The ED_{50} for beta-endorphin antinociception was similar in sham-operated (17.78 ug) and castrated (13.25 ug) male rats, but significantly higher in sham-operated (35.81 ug) and ovariectomized (58.63 ug) female rats.

Figure 3. Alterations (s, mean, S.E.M.) in the magnitude of peak (30 min) beta-endorphin-induced antinociception on the tail-flick test following microinjections into the vlPAG of sham-operated male rats, castrated male rats, sham-operated female rats tested during the estrous phase of the estrous cycle and ovariectomized female rats. Peak antinociceptive difference scores for each beta-endorphin dose in each of the four groups were ascertained by subtracting each agonist score from its corresponding baseline score. The crosses (+) denote a significant difference between pooled sham-operated and castrated male rats relative to pooled sham-operated and ovariectomized female rats at each of the beta-endorphin doses. Sham-operated male rats and castrated male rats failed to differ from each other in beta-endorphin-induced antinociception. Similarly, sham-operated female rats and ovariectomized female rats failed to differ from each other in beta-endorphin-induced antinociception.



Jump Test. Significant differences were observed in jump thresholds among beta-endorphin doses [F= (4,44) =35.91, p<.0001], across the 2 h time course [F= 63.23, p<.0001), and for all 2-way interactions between and among these variables, but not among sex and gonadectomy groups [F= (3,33) 1.58, n.s]. Thresholds failed to differ among groups following vehicle treatment, indicating no sex or gonadectomy differences in baseline thresholds. Beta-endorphin in the vlPAG significantly increased jump thresholds in sham-operated males following the 13 (30-60 min) and 26 (30-120 min) ug doses, in castrated males following the 5.2 (30-90 min), 6.5 (30 min), 13 (30-90 min) and 26 (30-120 min) ug doses, in sham-operated females following the 5.2 (30-90 min), 6.5 (30 min), 13 (30-60 min) and 26 (30-120 min) ug doses, and in ovariectomized females following the 5.2 (60 min), 6.5 (60-90 min), 13 (30-60 min) and 26 (30-120 min) ug doses. No consistent sex or gonadectomy effects were observed in the magnitude of beta-endorphin antinociception on the jump test.

Protocol 2: Sex Differences in Beta-endorphin Locomotor Activity Elicited from the vlPAG

Procedures

This protocol was employed to evaluate whether the observed sex effects in beta-endorphin antinociception elicited from the vlPAG were related to sex differences in locomotor activity associated with the drug. Intact male (n=10) and female (n=6) rats received vlPAG microinjections of beta-endorphin at identical doses to those employed in the antinociceptive protocol (e.g., 0, 5.2, 6.5, 13.0, and 26 ug) and administered in a counterbalanced order. Immediately following microinjections, the rats were returned to their cages and their total locomotor activity was measured at 30 min time intervals for

up to 2 h to parallel the time course employed in the nociceptive study. Treatment conditions were separated by at least one week to minimize possible tolerance effects. Testing always took place between 2 and 8 hours into the light cycle. Each rat received 2 to 4 doses of beta endorphin, plus the vehicle condition.

Statistical Analyses

Three-way analysis of variance were performed on the total activity measure, with sex as a between-groups factor, and drug doses and test time as within-groups factors. Tukey planned comparisons ($p<.05$) were used to determine significant effects relative to corresponding vehicle values.

Results

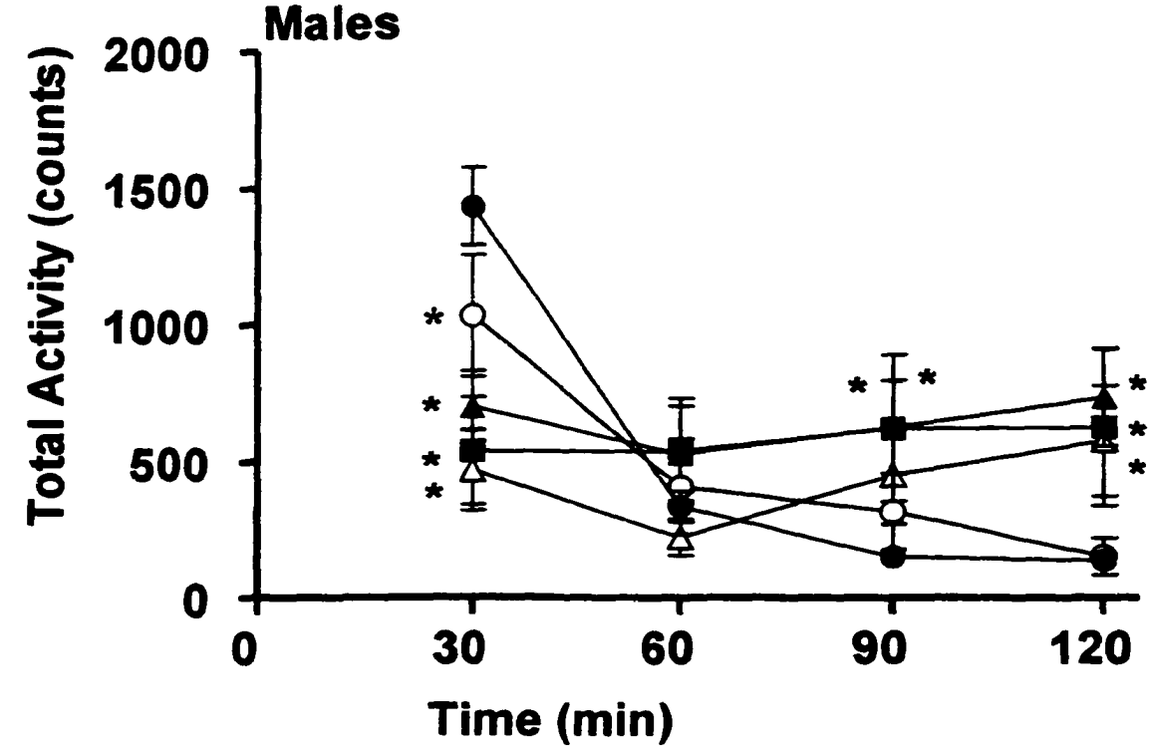
Significant differences in total activity were observed between males and females [$F(1,9)= 6.09$, $p<.036$] and across test times [$F(3,27)= 186.45$, $p<.0001$], but not across beta-endorphin doses [$F(4,36)= 1.19$, n.s.]. Highly significant interactions between doses and times, and among sex, dose and time variables were also observed. Significant initial reductions in total activity occurred 30 min following all doses in male rats (Figure 4A), and following the 6.5, 13 and 26 ug doses in female rats (Figure 4B). Significant subsequent increases in total activity occurred following the 6.5 (90-120 min), 13 (120 min) and 26 (90-120 min) ug doses in male rats, but not in female rats.

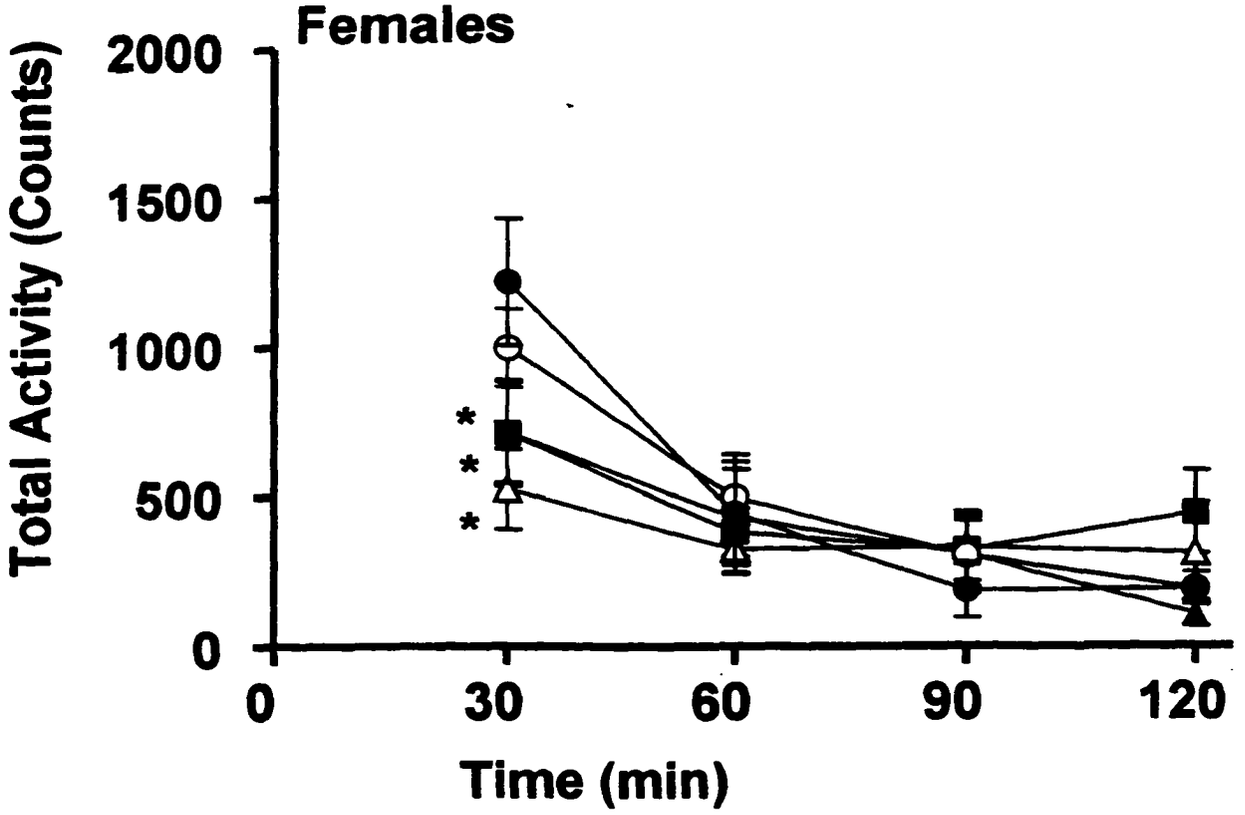
Protocol 3: Sex Differences and Adult Gonadectomy Effects in D-Pro2-Endomorphin2 Antinociception Elicited from the vlPAG

Procedures

Subgroups of 6 rats in the sham-operated male, castrated male, sham-operated female and ovariectomized female groups received D-Pro2-Endomorphin-2 doses of 0,

Figure 4. Alterations in total activity (mean, ±SEM) over a 120 min time course following beta-endorphin doses of 0 (●), 5.2 (○), 6.5 (▲), 13 (△), and 26 (■) ug microinjected into the vlPAG of male (upper panel) and female (lower panel) rats. The asteriks denote significant alterations in total activity following beta-endorphin treatment relative to vehicle treatment at each corresponding time point.





1, 10, 25, and 50.0 ug in the vlPAG in a counterbalanced order. Each dose was tested at 15, 30, 45, and 60 min on the tail-flick and jump tests. The one-hour time course was employed for D-Pro2-Endomorphin-2 because this peptide has a shorter half-life than morphine and beta-endorphin.

Statistical Analyses

Three-way analyses of variance were performed on each nociceptive measure separately, with sex and gonadal status as between-groups factors, and drug doses and test time as within-groups factors. Tukey planned comparisons ($p<.05$) were used to determine significant effects relative to corresponding vehicle values. The potency of effects was evaluated by constructing log dose response functions of difference scores values for each dose and performing linear regression analyses to derive ED_{50}s at which total latencies were increased by 8 sec.

Results

Tail-flick Test. Significant differences were observed in tail-flick latencies among sex and gonadectomy groups [$F(3,21)= 9.97$, $p<.0003$], among D-Pro^2-Endomorphin-2 doses [tail-flick: $F(4,28)= 38.49$, $p<.0001$], across the 1 h time course [$F(3,21)= 3.61$, $p<.03$], and for both interactions between groups and doses. Latencies failed to differ among groups following vehicle treatment, indicating no sex or gonadectomy differences in baseline latencies. D-Pro^2-Endomorphin-2 in the vlPAG significantly increased tail-flick latencies in sham-operated males following the 50 (30-60 min) ug dose, in sham-operated females following the 10 (15 min), 25 (15-60 min) and 50 (15-60 min) ug doses, and in ovariectomized females following the 10 (15 min), 25 (15-60 min) and 50 (15-60 min) ug doses, but failed to do so in castrated males.

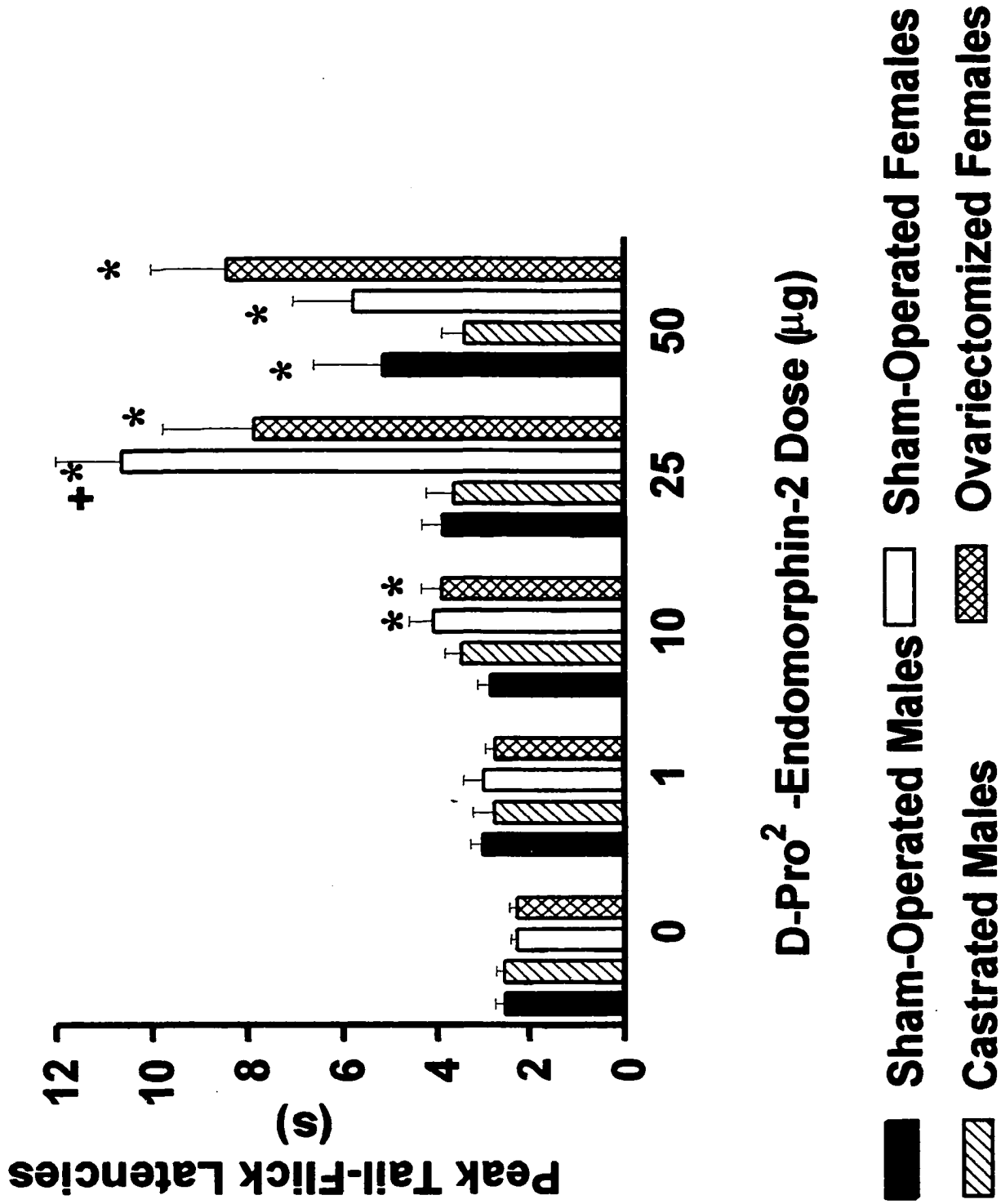
Because peak effects in all groups occurred 15 min after D-Pro2-Endomorphin-2 administration, specific sex and gonadectomy differences are illustrated at this time point. Figure 5 shows peak antinociceptive effects of D-Pro2-Endomorphin in the vlPAG on the tail flick test. Significant sex differences were observed in the peak magnitudes of D-Pro^2-Endomorphin-2 antinociception on the tail-flick test in that sham-operated female rats displayed significantly greater effects than sham-operated males following the 25 ug

No significant male gonadectomy differences were observed in the peak magnitudes of D-Pro^2-Endomorphin-2 antinociception on the tail-flick test. Significant female gonadectomy differences were observed in the peak magnitudes of D-Pro^2-Endomorphin-2 antinociception on the tail-flick test in that ovariectomized females displayed significantly lesser effects than sham-operated females following the 25 ug dose but significantly greater effects following the 50 ug dose.

Regression analyses revealed significant differences in the potency of D-Pro^2-Endomorphin-2 antinociception on the tail-flick test [$F(6,88= 4.50, p<.0005$]. The ED_{50} for D-Pro^2-Endomorphin-2 antinociception was similar in sham-operated (3.15 ug) and ovariectomized (4.61 ug) female rats, but significantly higher in sham-operated (31.4 ug) and castrated (>100 ug) male rats.

Jump Test. Significant differences were observed on the jump test among D-Pro^2-Endomorphin-2 doses [$F (4,28)= 31.97, p<.0001$), across the 1 h time course [$F= (3,21)=5.28, p<.007$), for both interactions between groups and doses, but not among sex and gonadectomy groups [$F= (3,21)=1.77$, n.s]. Thresholds failed to differ among groups following vehicle treatment, indicating no sex or gonadectomy differences in baseline thresholds. D-Pro^2-Endomorphin-2 in the vlPAG significantly increased jump thresholds

Figure 5. Alterations (s, mean, S.E.M.) in the magnitude of peak (15 min) D-Pro2-Endomorphin-2 -induced antinociception on the tail-flick test following microinjections into the vlPAG of sham-operated male rats, castrated male rats, sham-operated female rats tested during the estrous phase of the estrous cycle and ovariectomized female rats. The asterisks (*) denote significant alterations in tail-flick latencies relative to corresponding vehicle control treatment. The cross denotes a significant sex difference in tail-flick latencies between sham-operated male rats and sham-operated female rats.



in sham-operated males following the 10 (60 min), 25 (15-60 min) and 50 (15-60 min) ug doses, in castrated males following the 25 (15-60 min) and 50 (15-60 min) ug doses, in sham-operated females following the 25 (15-60 min) and 50 (15-60 min) ug doses, and in ovariectomized females following the 25 (30 min) and 50 (15-60 min) ug doses. Neither sex nor gonadectomy differences were observed in the magnitude of D-Pro^2-Endomorphin-2 antinociception on the jump test.

Protocol 4: Sex Differences in D-Pro2-Endomorphin 2 Locomotor Activity Elicited from the vlPAG

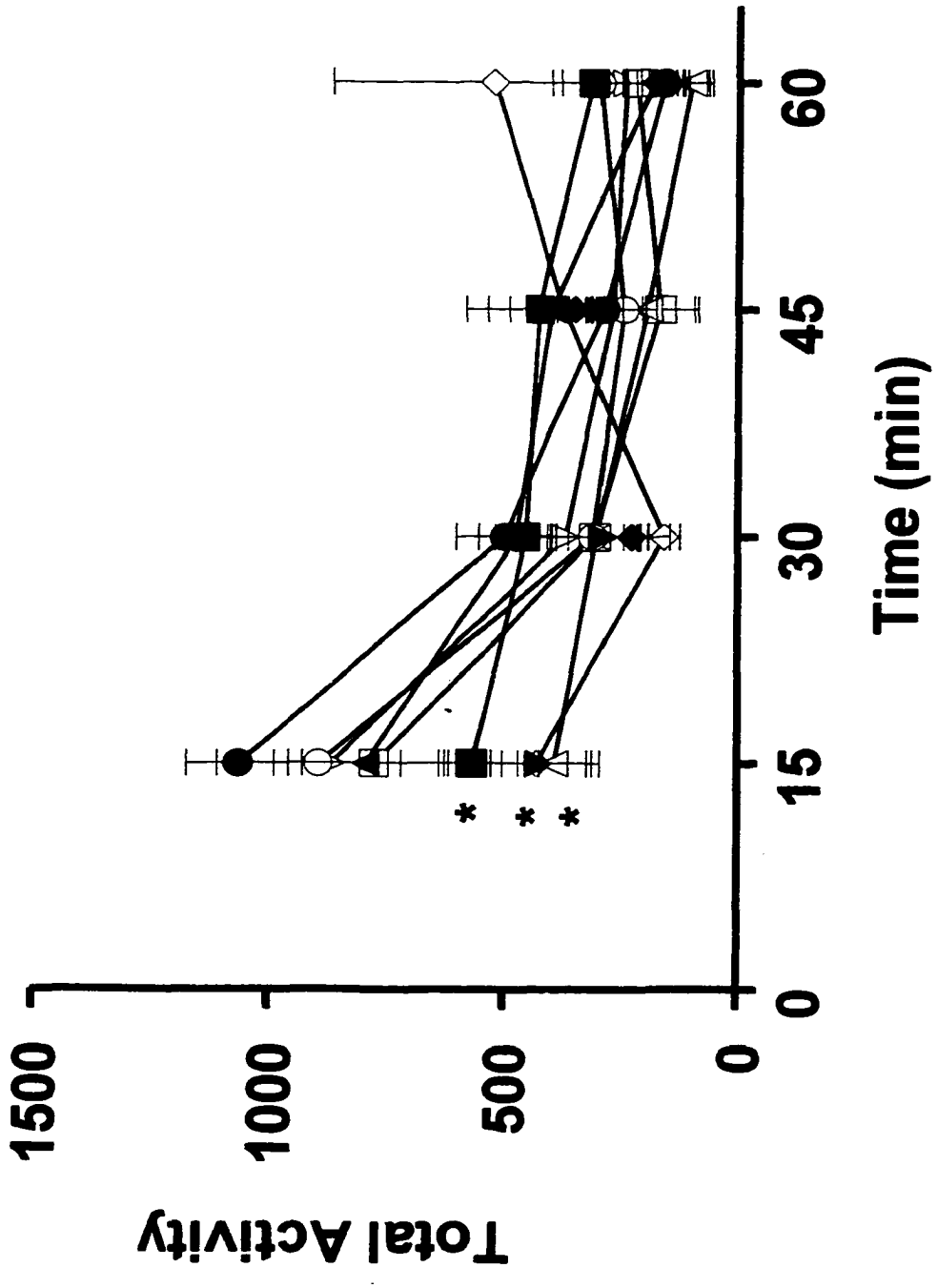
Procedures

This protocol was employed to evaluate whether the observed sex effects in D-Pro2-Endomorphin-2 antinociception elicited from the vlPAG were related to sex differences in locomotor activity associated with the drug. Intact male (n=6-13) and female rats (n= 6-9) rats received vlPAG microinjections of D-Pro2-Endomorphin-2 at doses identical to those employed in the antinociceptive protocol (e.g., 0, 10, 25 and 50 ug). Immediately following microinjections, the rats were returned to their cages, and total activity was measured at 15min time intervals for up to 1 h.

Statistical Analyses

Three-way analysis of variance were performed on the total activity measure, with sex as a between-groups factor, and drug doses and test time as within-groups factors. Tukey planned comparisons ($p<.05$) were used to determine significant effects relative to corresponding vehicle values.

Figure 6. Alterations in total activity (mean, ±SEM) over a 60 min time course in male (open symbols) and female (closed symbols) rats following D-Pro2-Endomorphin-2 doses of 0 (circles), 1 (squares), 10 (upside down triangles), 25 (diamonds), and 50 (triangles) ug microinjected into the vlPAG. The asteriks (*) denote significant alterations in total activity following D-Pro2-Endomorphin-2 treatment relative to vehicle treatment at each corresponding time point.



Results

Significant differences in total activity were observed across D-Pro^2-Endomorphin-2 doses ($F(4,48)= 4.50$, $p<.004$), across test times ($F(3,36)= 359.37$, $p<.0001$), and for all between-variable and among-variable interactions. Male and female rats failed to differ from each other in total activity ($F(1,12)= 1.91$, n.s.). Significant initial reductions in total activity occurred 15 min following the 10, 25 and 50 ug doses in male rats, and following the 1 and 25 ug doses in female rats (Figure 6).

Protocol 5: Sex Differences in D-pro2-Endomorphin-2 Behavioral Activation Elicited from the vlPAG

Procedures

This protocol was employed to directly assess sex differences in behavioral activation in response to vlPAG D-Pro2-Endomorphine-2. Six intact male rats and 6 intact female) rats received vlPAG microinjections of D-Pro^2-Endomorphin-2 at doses of 0 and 50 ug in counterbalanced order. Each of the rats was assessed for drug-induced behavioral activation in their home cages.

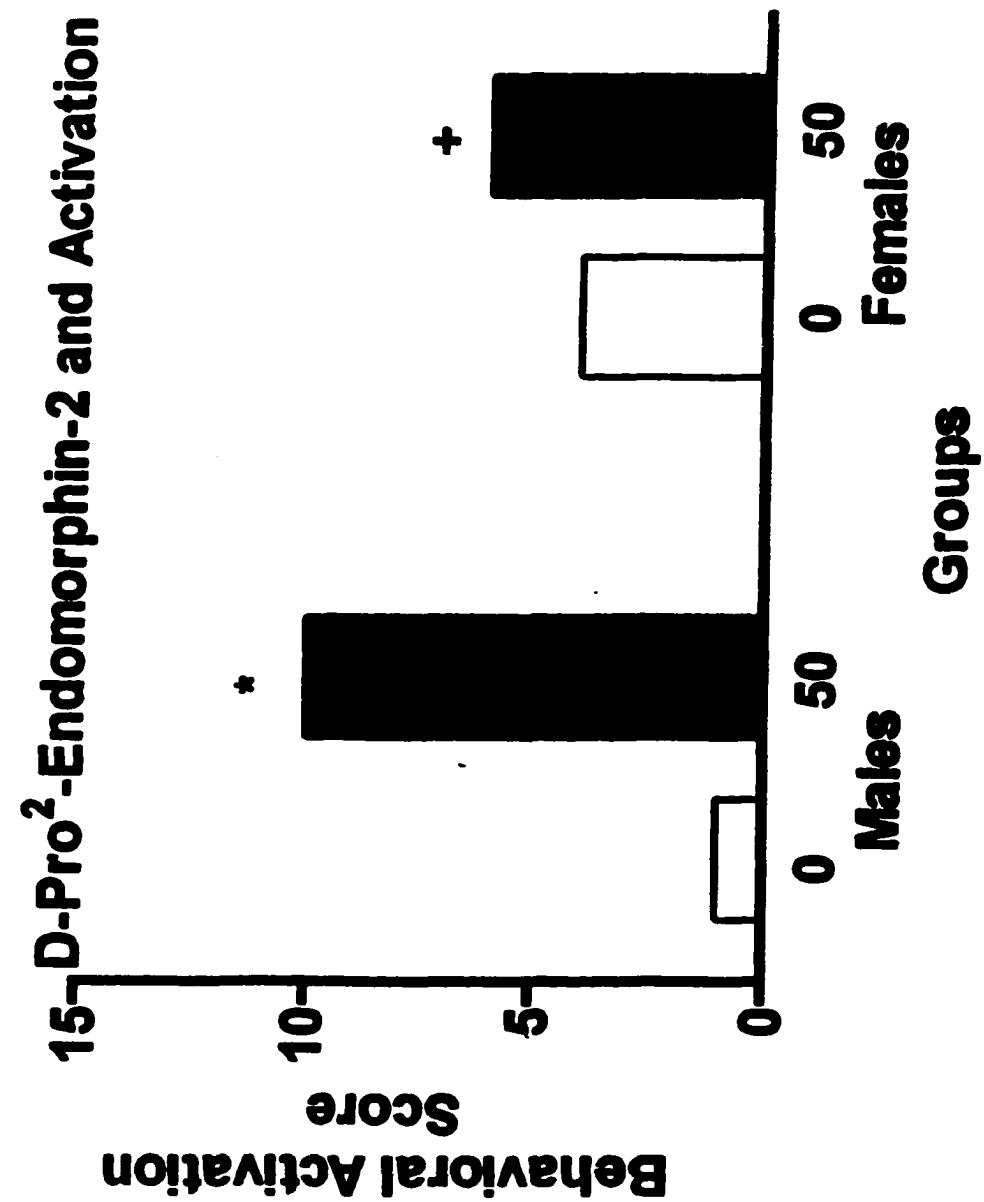
Statistical Analyses

The sum of the behavioral activation scores for each animal of each sex in each condition was calculated, and a non-parametric statistic, the Wilcoxin signed-rank for correlated groups, was performed to determine whether D-Pro2-Endomorphin-2 altered these measures in male and female animals.

Results

Figure 7 illustrates the ability of D-Pro^2-Endomorphin-2 (Activation Score = 10) to significantly increase the behavioral activation measures relative to vehicle treatment

Figure 7. Effects of vehicle (open bars) or D-Pro^2-Endomorphin-2 (50 μg, closed bars) administration into the vlPAG in male and female rats upon behavioral activation measures which consisted of a composite of blind-rated scores measuring the presence (Score =1) or absence (Score =0) of excessive grooming, explosive motor behavior, seizures and barrel rolls. The asterik (*) denotes significant alterations in behavioral activation following 50 ug dose of D-Pro2-Endomorphin-2 treatment relative to vehicle treatment



(Activation Score = 1) in male rats ($z = -2.42$, $p<.016$). In contrast, female rats failed to display significant changes ($z = -0.54$, n.s.) in behavioral activation between vehicle (Activation Score = 4) and D-Pro^2-Endomorphin-2 (Activation Score = 6).

Discussion

In the first experiment, significant sex differences were demonstrated in the antinociceptive responses to morphine following administration into the vlPAG with marked effects noted on two different measures of thermal and shock-induced nociceptive reactivity. Since morphine antinociception elicited from the vlPAG is mediated primarily through mu opioid receptors subtypes and since beta-endorphin and D-Pro2-Endomorphin-2 each appear to utilize some mu opioid receptor-mediated actions (Monroe et al., 1996; Smith et al., 1992; Tseng et al., 1998; Zadina et al., 1997 , the present experiment tested the generalizability of sex differences in these forms of antinociception . Two major findings emerged in comparing the effects of sex and adult gonadectomy upon antinociception elicited by administration of either beta-endorphin or D-Pro2-Endomorphin-2 into the vlPAG. First, beta-endorphin produced significant sex differences in antinociception elicited from the vlPAG on the tail-flick test with male rats displaying significantly greater peak antinociceptive effects than female rats over the entire dose range. Whereas male rats displayed near-maximum degrees of antinociception on the tail-flick test following vlPAG beta-endorphin doses as low 13 ug, female rats displayed only moderate degrees of antinociception across the beta-endorphin dose range. Since these doses were equimolar to those employed for morphine antinociception in the first experiment, they indicate a consistent pattern in the direction and magnitude of sex effects across these two agonists on the tail flick test. Adult

gonadectomy failed to significantly alter the magnitude of antinociception in male or female rats. This relative lack of gonadectomy effects differs from morphine antinociception elicited from the vlPAG in which ovariectomized females displayed significantly greater levels of morphine antinociception relative to sham-operated females tested during the estrous phase. Also in contrast to the marked sex differences in antinociception on the jump test following vlPAG morphine administration, vlPAG beta-endorphin antinociception on the jump test failed to display any sex differences. Parallel, test-specific sex differences in antinociception have been observed for ventricular administration of the selective mu opioid agonist, DAMGO which also produced potent sex differences on the tail-flick, but not the jump test (Kepler et al., 1991).

The second major finding was that D-Pro2-Endomorphin-2 antinociception elicited from the vlPAG displayed the opposite pattern of sex differences on the tail-flick test than that observed for vlPAG morphine and vlPAG beta-endorphin antinociception. Thus, D-Pro2Endomorphin-2 microinjected into vlPAG produced significantly greater magnitudes of antinociception on the tail-flick test in sham-operated and ovariectomized female rats than in sham-operated and castrated male rats. Both sham-operated and ovariectomized female rats displayed an earlier onset of antinociception on the tail-flick test at a lower (10 ug) D-Pro2-Endomorphin-2 dose, and displayed greater antinociception across the time course at the higher 25 dose of D-Pro2-Endomorphin-2 than sham-operated and castrated male rats. These sex differences in D-Pro2-Endomorphin-2-induced antinociception were test-specific since they were not observed on the jump test. Like beta-endorphin, D-Pro2-Endomorphin-2 in the vlPAG produced a consistent dose-dependent increase in jump thresholds in all test groups. As reviewed

earlier, although sex differences in opioid antinociception have been typically reported with male rats displaying greater effects, there have been several instances in which female rats showed greater antinociception. For example, Bartok and Craft (1997) found that systemic administration of the kappa agonist, bremazocine produced greater antinociception in female rats on the tail-withdrawal test, and that another kappa agonist, U69,593 produced earlier effects in female rats on that test. Further, nicotine-induced antinociception on the hot-plate was also higher in female than in male rats (Craft and Milholland, 1998). Thus, in marked contrast to prominent sex differences in morphine antinociception elicited from the vlPAG in which males displayed significantly greater effects on the tail-flick and jump tests than females, D-Pro^2-Endomorphin-2 elicited from the vlPAG produced greater antinociception on the tail-flick test in females relative to males and failed to exhibit sex differences in antinociceptive responses on the jump test. These data suggest that D-Pro^2-Endomorphin-2 antinociception elicited from the vlPAG may be mediated by different opioid receptors than morphine antinociception elicited from this site.

Another possible reason for as to male rats displayed a lesser antinociceptive responses on the tail-flick flick test following D-Pro2-Endomorphin-2 in the vlPAG might be an increase in auxiliary motor behaviors following microinjections. Male rats appeared to display greater agitation immediately after D-Pro2-Endomorphin-2 than female rats. In contrast, no such sex differences in locomotor activity were noted following beta-endorphin administration into vlPAG of male and female rats. Since these behaviors were not formally characterized during testing, we employed independent locomotor activity measures as a procedure to assess whether the observed

antinociceptive sex differences in response to beta-endorphin and D-Pro2-Endomorphin-2 were secondary to sex-selective and agonist-specific alterations in activity measures.

Beta-endorphin in the vlPAG produced a dose-dependent decrease in total activity 30 min after microinjection in both males and females, and subsequent increases in total in male rats at 90 and 120 min after injection. In contrast, beta-endorphin injected into the vlPAG failed to produce any subsequent increases in total activity in female rats, indicating the presence of a sex difference in this motor response. However, since no sex differences in motor activity occurred within the first 30 min following microinjection, when peak antinociceptive responses were observed, it appears that these sex-dependent antinociceptive and motor responses were not related.

D-Pro^2-Endomorphin-2 in the vlPAG produced significant initial reductions in total activity 15 min following microinjection, but failed to alter this measure thereafter, and the expectation that male rats should either display increases in activity or fail to display the same magnitude of decreased activity exhibited by females following D-Pro^2-Endomorphin-2 in the vlPAG was not confirmed. Although female, but not male rats displayed reductions in initial total activity following the 1 μg dose, male, but not female rats displayed this effect following the 10 μg dose. Indeed, at the 50μg dose, male rats displayed less total activity than females 15 min following microinjection. Thus, it does not appear that the sex differences in D-Pro^2-Endomorphin-2 antinociception were attributable to overall changes in total activity of the animals.

However, male rats displayed significantly higher behavioral activation responses, characterized by either excessive grooming, seizures, barrel rolls and/or explosive running behavior, following D-Pro^2-Endomorphin-2 in the vlPAG during the precise

periods of time when they were failing to display robust antinociceptive responses on the tail-flick test. In contrast, estrous-phase female rats displayed a pattern of activation responses following D-Pro^2-Endomorphin-2 in the vlPAG that failed to differ from vehicle treatment, and displayed normal, orderly, dose-dependent increases in tail-flick latencies following this agonist. Therefore, these data suggest that the sex differences in D-Pro^2-Endomorphin-2 antinociception in the vlPAG on the tail-flick test were due to behaviorally-disruptive activation responses which did not allow for a normal antinociceptive response to be observed in males. It is interesting to note that this agonist failed to possess such actions following icv administration (Shane et al., 1999). Indeed, icv treatment with D-Pro^2-Endomorphin-2 seemed to produce significantly greater antinociceptive responses at lower doses in male rats than microinjections in the vlPAG. The seminal studies by Jacquet and coworkers (Jacquet et al., 1976; Jacquet and Lajtha, 1974) found that the vlPAG elicited these activating behaviors at lower morphine doses than that observed in the lateral ventricles. Thus, any attributed sex differences in these responses following D-Pro^2-Endomorphin-2 in the vlPAG do not appear to affect nociceptive processing per se, but rather appear to be an epiphenomenon of behavioral activation.

It should be noted that the cannula placements in the vlPAG are adjacent to one of the major subdivisions of the PAG, the posterior lateral PAG (Beitz, 1985; Conti, Barbaresi and Fabri, 1988). This area has been implicated in forward avoidance behavior, tachycardia and increased blood pressure, especially following microinjections of excitatory amino acids into this site in male rats (Bandler, Carrive and Zhang, 1992). This is in contrast to the role of the lateral division of the PAG in the expression of lordotic

behavior in the female (Pfaff et al., 1988). In contrast, the vlPAG is associated with antinociception, immobility, decreased blood pressure and bradycardia (Bandler et al., 1992). One potential pharmacokinetic effect of D-Pro^2-Endomorphin-2 might be its greater diffusion to adjacent sites relative to either morphine or beta-endorphin, thereby eliciting the observed auxiliary motor behaviors.

CHAPTER 5

Experiment 3: Neonatal Gonadectomy Effects upon Sex Differences in Morphine Antinociception Elicited from the vlPAG

Background

The first experiment demonstrated that antinociceptive responses to morphine are sensitive to sex differences following administration into the vlPAG. Male rats displayed significantly greater magnitudes and potencies of morphine-induced antinociception than female rats. However, the marked sex differences in antinociception following vlPAG morphine administration were only minimally affected by adult gonadectomy, suggesting that the activational effects of gonadal hormones play a relatively minor role in this response. There are two mechanisms by which gonadal hormones could mediate sex differences in opioid antinociception: acute activational effects, which would be affected by adult gonadectomy, and long-term organizational effects, which would be affected by neonatal gonadectomy (Phoenix, Goy, Gerall and Young, 1959). The role of the organizational effects of gonadal hormones upon morphine antinociception and sex differences in morphine antinociception has not been examined. Therefore, the present study evaluated the effects of neonatal castration in male rat pups relative to sham-operated controls and the effects of systemic neonatal androgenization with testosterone proprionate in female rat pups relative to vehicle-treated controls upon morphine antinociception elicited from the vlPAG.

Methods

Procedures and Protocols

Neonatally-castrated and sham-operated male rats and neonatally-androgenized and sham-treated female rats were stereotaxically implanted with stainless steel cannulae in the vlPAG approximately 90 days following neonatal gonadal surgery. Subgroups of 10-12 rats in the neonatally sham-operated male, neonatally castrated male, neonatally sham-treated female, and neonatally androgenized female groups received morphine doses of 0, 1.0, 1.75, 2.5, and 5.0 ug. Subgroups of 7-10 rats in neonatally sham-treated female, neonatally-androgenized female, and neonatally-castrated male groups additionally received a morphine dose of 10 ug. All doses were tested in a counterbalanced order. Rats received between 4-6 microinjection conditions including control treatment. All nociceptive testing procedures and post-mortem histological verifications were identical to those described in Experiment 1.

Statistical Analyses

Two statistical approaches were utilized to analyze the data in terms of magnitude and potency of effect. To determine the magnitude of effects, three-way analyses of variance were performed on each nociceptive measure, with sex and gonadal status as a between-groups factor, drug doses as a within-groups factor, and test time as a within-groups factor. Tukey planned comparisons ($p<.05$) determined significant effects relative to corresponding vehicle values. The potency of effects was evaluated by constructing log dose response functions of difference scores values for each dose and performing linear regression analyses to derive ED_{50}s at which peak latencies were doubled and peak thresholds were increased by 0.10 mA.

Results

Body Weight

Significant differences in body weight were observed among groups [$F(3,36)= 60.15$, $p<0.0001$]. Regardless of gonadectomy condition, male rats were significantly heavier than female rats. Neonatally-operated sham males were significantly heavier (497 g) than neonatally-castrated male (426 g) rats but neonatally-androgenized females (309 g) and neonatally-treated sham females (317 g) failed to differ from each other.

Morphine Antinociception

Tail-flick Test. Differences in analgesia on the tail-flick test elicited by morphine in the vlPAG were observed as functions of sex and neonatal gonadal hormone manipulations. Significant differences were observed in latencies at the four lower commonly-tested morphine doses among the four groups ($F(3,33)= 14.63$, $p<.0001$), among doses ($F(4,44)= 43.53$, $p<.0001$), among times ($F(3,33)= 85.84$, $p<.0001$), and for virtually all 2-way and 3-way interactions. Significant differences were observed in latencies at the high (10 ug) morphine dose among the three tested groups ($F(2,22)= 13.32$, $p<.0001$), between vehicle control and morphine ($F(1,11)= 39.39$, $p<.0001$), among times ($F(3,33)= 20.50$, $p<.0001$), and for all 2-way and 3-way interactions. Figure 8 illustrates the peak analgesic action on the tail-flick test of the morphine doses across groups; Table 1 summarizes the time course of morphine doses across groups. Latencies failed to differ among the four groups following vehicle treatment, indicating that the neonatal gonadectomy treatment failed to alter baseline latencies. Significant sex differences in morphine analgesia were noted for both peak (Figure 8) and overall (Table 1) effects following the 2.5 and 5 ug doses with neonatally sham-operated males

Figure 8. Alterations in tail-flick latencies (s, mean, ±SEM) 30 min following either vehicle or morphine doses (1-10 ug) administered into the vlPAG of adult rats treated neonatally (within 24 h of birth) with either sham or castration surgeries as male pups, or with vehicle or testosterone proprionate (250 micrograms/kg, sc) as female pups. The asterisks (*) denote significant alterations in tail-flick latencies relative to corresponding vehicle control treatment. The crosses (+) denote significant alterations in tail-flick latencies in neonatally sham-operated male rats relative to corresponding neonatally vehicle-treated female rats. The pound signs (#) denote significant alterations in tail-flick latencies in neonatally-castrated male rats relative to neonatally sham-operated male rats, and in neonatally-androgenized female rats relative to corresponding neonatally vehicle-treated female rats.

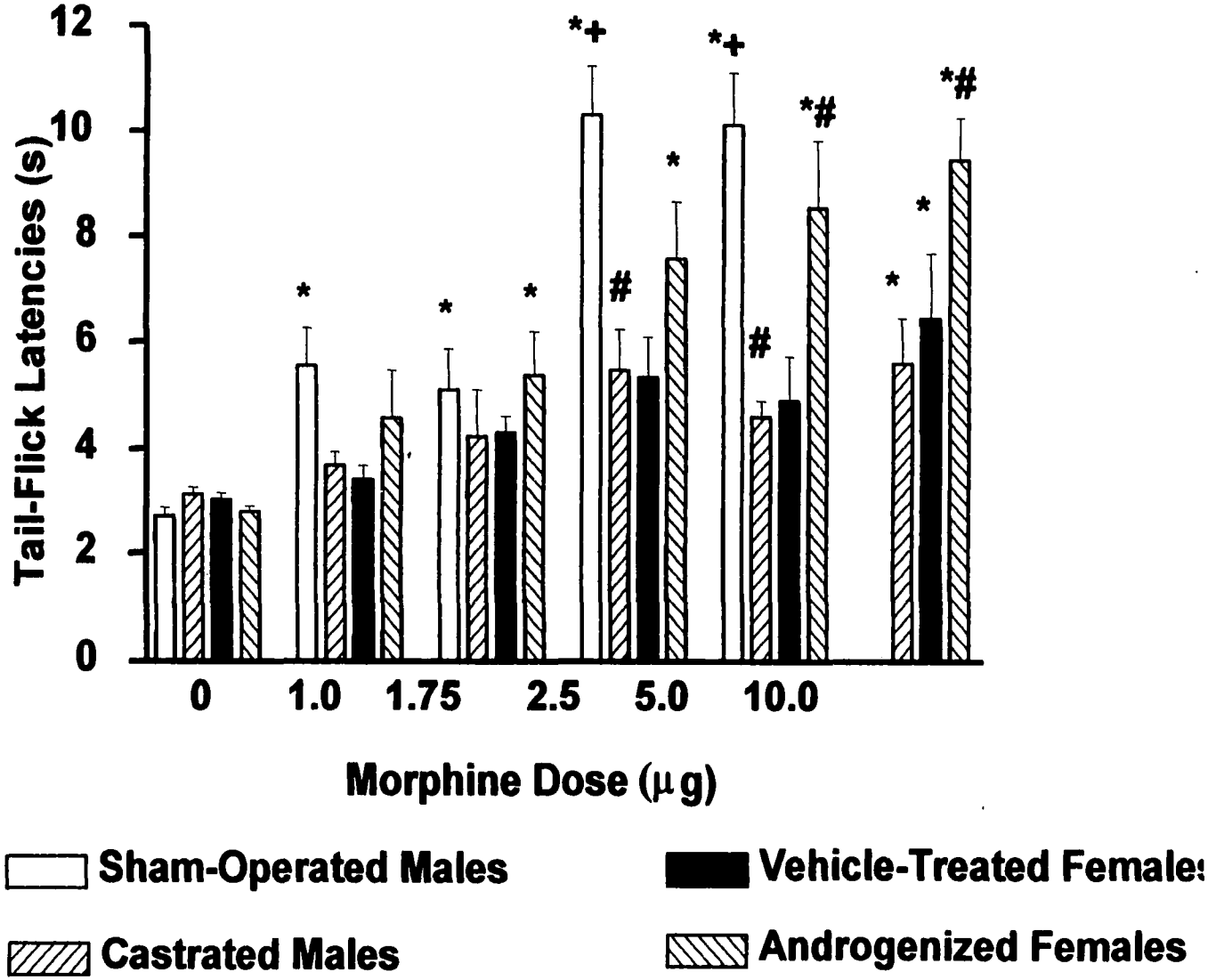


TABLE 1. Time course of alterations in tail-flick latencies (s, mean, ±SEM) following either vehicle or morphine doses (1-10 micrograms) administered into the ventrolateral periaqueductal gray (vlPAG) of adult rats treated neonatally (within 24 h of birth) with either sham or castration surgeries as male pups, or with vehicle or testosterone proprionate (TP, 250 micrograms/kg, sc) as female pups.

Group	0.00	1.00	1.75	2.50	5.00	10.0
60 min						
Sham Males	2.89 (0.19)	5.65*+ (1.29)	3.50 (0.27)	8.13*+ (1.14)	9.29*+ (1.14)	
Castrated Males	2.85 (0.13)	3.48 (0.14)	3.45 (0.16)	5.68*# (1.14)	5.19# (0.83)	5.56* (1.05)
Sham Females	3.14 (0.12)	2.75 (0.19)	4.29 (0.35)	4.52 (0.50)	4.46 (0.93)	5.11* (1.26)
TP Females	2.92 (0.15)	4.76 (0.24)	5.45* (0.81)	7.73*# (1.23)	9.92*# (1.27)	11.60*# (0.34)
90 min						
Sham Males	2.79 (0.14)	3.54 (0.50)	3.40 (0.35)	5.37* (1.02)	8.38*+ (1.25)	
Castrated Males	2.67 (0.11)	3.19 (0.26)	3.33 (0.30)	4.93 (1.19)	4.21# (0.38)	4.71* (0.90)
Sham Females	3.07 (0.10)	3.14 (0.21)	3.41 (0.30)	4.20 (0.84)	3.78 (0.27)	5.81* (1.36)
TP Females	2.85 (0.14)	3.87 (0.59)	3.43 (0.28)	6.96*# (1.38)	6.58*# (1.23)	8.82*# (1.28)
120 min						
Sham Males	2.80 (0.18)	2.59 (0.16)	3.21 (0.24)	3.28 (0.13)	6.40*+ (1.25)	
Castrated Males	3.08 (0.17)	3.13 (0.21)	2.97 (0.21)	2.95 (0.17)	3.76# (0.29)	3.14 (0.30)
Sham Females	3.03 (0.18)	2.76 (0.10)	2.99 (0.24)	3.54 (0.49)	2.89 (0.23)	5.01* (1.19)
TP Females	2.88 (0.18)	3.38 (0.24)	3.12 (0.22)	5.69* (1.05)	6.02*# (1.36)	4.98* (1.04)

The asterisks (*) in this and the subsequent table denote significant alterations in morphine analgesia relative to corresponding vehicle control treatment. The crosses (+) in this and the subsequent figure denote significant alterations in morphine analgesia in neonatally sham-operated male rats relative to corresponding neonatally vehicle-treated female rats. The pound signs (#) in this and the subsequent figure denote significant alterations in morphine analgesia in neonatally-castrated male rats relative to neonatally sham-operated male rats, and in neonatally-androgenized female rats relative to corresponding neonatally vehicle-treated female rats.

displaying significantly greater analgesia which approached cut-off values relative to neonatally vehicle-treated females. Neonatally-castrated males displayed significantly lower latencies than sham-operated males following the 2.5 and 5 ug doses of morphine, and displayed analgesic effects approximately equivalent to neonatally vehicle-treated females (Figure 8, Table 1). In contrast, neonatally-androgenized females displayed significantly higher latencies than neonatally vehicle-treated females following the 5 and 10 ug doses of morphine, and displayed analgesic effects approximately equivalent to neonatally sham-operated males (Figure 8, Table 1). Whereas androgenized females approached cut-off values at the highest dose, neonatally-castrated males and sham-operated females displayed only moderate (5.5-6.5 s) analgesia.

Regression analyses revealed significant differences in the potency of peak (30 min) morphine analgesia on the tail-flick test ($F(6,183)= 14.05$, $p<.0001$). The ED_{50} to double peak morphine analgesia on the tail-flick test was similar in neonataly sham-operated males (1.27 ug) and androgenized females (1.60 ug), but was far higher in neonatally-castrated males (19.19 ug) and sham-operated females (8.21 ug).

Jump Test. Differences in morphine analgesia elicited from the vlPAG were also observed on the jump test as functions of sex and neonatal gonadal hormone manipulations. Significant differences were observed in thresholds at the four lower commonly-tested morphine doses among groups ($F(3,33)= 3.69$, $p<.021$), among doses ($F(4,44)= 20.06$, $p<.0001$), among times ($F(3,33)= 73.62$, $p<.0001$), and for virtually all 2-way and 3-way interactions. Significant differences were observed in thresholds nociceptive responses at the high (10 ug) morphine dose between control and morphine ($F(1,11)= 68.81$, $p<.0001$), among times ($F(3,33)= 19.95$, $p<.0001$), and for all 2-way and

Figure 9. Alterations in jump thresholds (mA, mean, ±SEM) 30 min following either vehicle or morphine doses (1-10 ug) administered into the vlPAG of adult rats treated neonatally (within 24 h of birth) with either sham or castration surgeries as male pups, or with vehicle or testosterone proprionate (250 micrograms/kg, sc) as female pups. The asterisks (*) denote significant alterations in jump thresholds relative to corresponding vehicle control treatment. The crosses (+) denote significant alterations in jump thresholds in neonatally sham-operated male rats relative to corresponding neonatally vehicle-treated female rats. The pound signs (#) denote significant alterations in jump thresholds in neonatally-castrated male rats relative to neonatally sham-operated male rats, and in neonatally-androgenized female rats relative to corresponding neonatally vehicle-treated female rats.

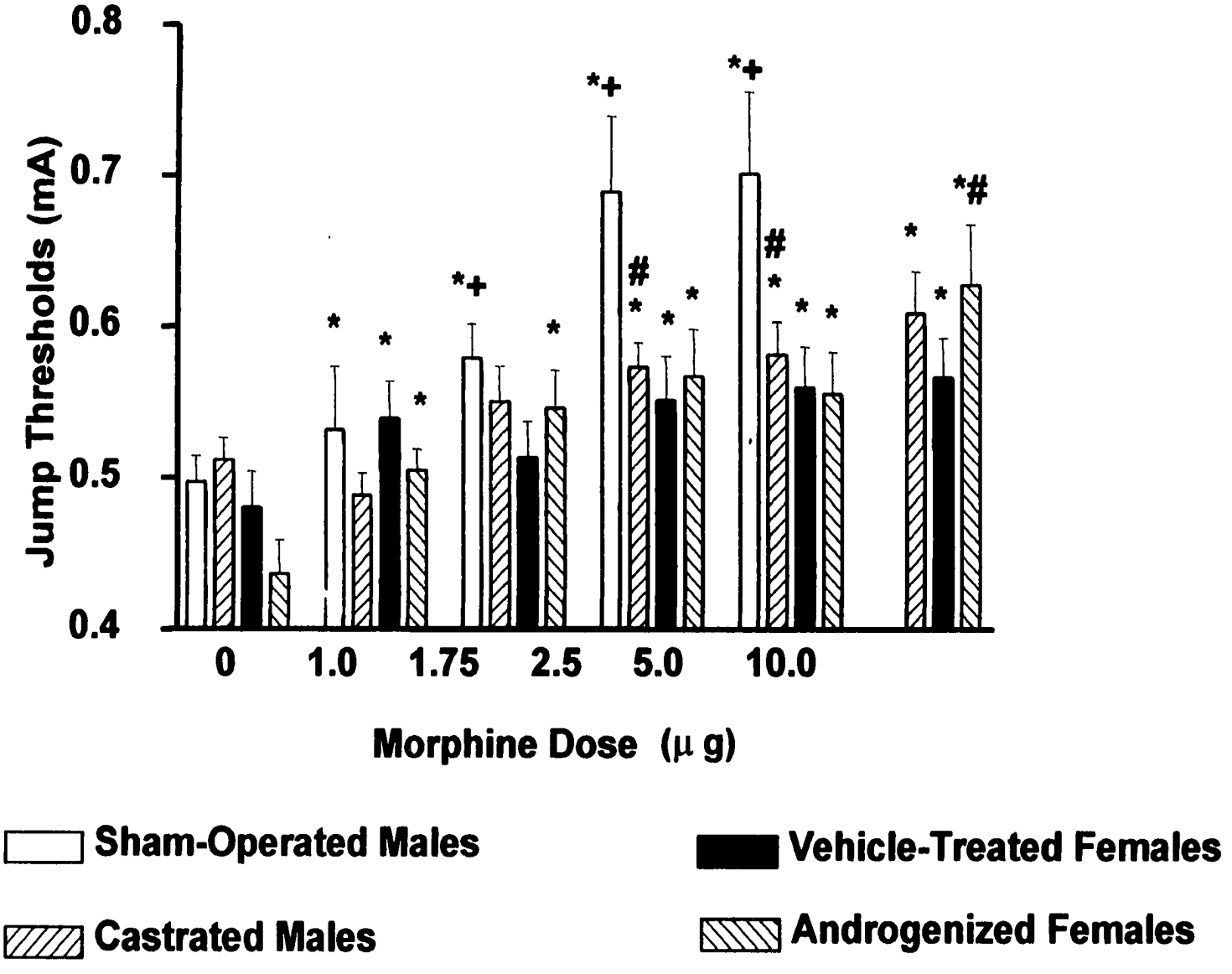


TABLE 2. Time course of alterations in jump thresholds (mA, mean, ±SEM) following either vehicle or morphine doses (1-10 micrograms) administered into the ventrolateral periaqueductal gray (vlPAG) of adult rats treated neonatally (within 24 h of birth) with either sham or castration surgeries as male pups, or with vehicle or testosterone proprionate (250 micrograms/kg, sc) as female pups.

Group	0.00	1.00	1.75	2.50	5.00	10.0
60 min						
Sham Males	.505 (.017)	.544 (.033)	.549 (.017)	.628*+ (.044)	.681*+ (.052)	
Castrated Males	.518 (.012)	.541 (.021)	.563 (.020)	.591* (.016)	.587*# (.024)	.591* (.028)
Sham Females	.487 (.021)	.535 (.029)	.510 (.020)	.550* (.021)	.538* (.021)	.576* (.031)
TP Females	.444 (.020)	.500* (.018)	.515* (.018)	.561* (.030)	.546* (.026)	.635*# (.045)
90 min						
Sham Males	.511 (.020)	.503 (.021)	.550 (.017)	.632*+ (.027)	.646*+ (.049)	
Castrated Males	.530 (.012)	.512 (.015)	.541 (.011)	.573# (.021)	.557# (.021)	.580* (.017)
Sham Females	.494 (.020)	.520 (.018)	.490 (.019)	.533 (.031)	.500 (.010)	.577* (.031)
TP Females	.448 (.022)	.486 (.017)	.506 (.018)	.546* (.023)	.513* (.031)	.598* (.030)
120 min						
Sham Males	.494 (.021)	.477 (.020)	.517 (.019)	.589*+ (.026)	.598*+ (.040)	
Castrated Males	.511 (.013)	.497 (.016)	.523 (.020)	.530# (.014)	.521# (.020)	.552 (.020)
Sham Females	.486 (.021)	.516 (.024)	.476 (.018)	.528 (.032)	.494 (.008)	.543* (.024)
TP Females	.453 (.021)	.469 (.013)	.484 (.018)	.493 (.023)	.494 (.026)	.526* (.028)

3-way interactions. Figure 9 illustrates the peak analgesic action on the jump test of the morphine doses across groups; Table 2 summarizes the time course of morphine doses across groups. Thresholds failed to differ among the four groups following vehicle treatment, indicating that the neonatal gonadectomy treatment failed to alter baseline thresholds. Significant sex differences in morphine analgesia were noted for both peak (Figure 9) and overall (Table 2) effects following the 1.75, 2.5 and 5 ug doses with neonatally sham-operated males displaying significantly greater analgesia relative to neonatally vehicle-treated females. Neonatally-castrated males displayed significantly lower thresholds than sham-operated males following the 2.5 and 5 ug doses of morphine, and displayed analgesic effects approximately equivalent to neonatally vehicle-treated females (Figure 9, Table 2). In contrast, neonatally-androgenized females displayed significantly higher thresholds than neonatally vehicle-treated females following the 10 ug dose of morphine (Figure 9, Table 2).

Regression analyses revealed significant differences in the potency of peak morphine analgesia on the jump test ($F(6,183)= 5.32$, $p<.00004$). The ED_{50} for peak morphine analgesia on the jump test was again similar in sham-operated males (1.69 ug) and androgenized females (2.15 ug), but was far higher in neonatally-castrated males (10.0 ug) and sham-operated females (25.12 ug).

Discussion

Following the demonstration in the first experiment of a minimal effect of adult gonadectomy upon the marked sex differences in morphine antinociception elicited from the vlPAG, the present experiment examined whether neonatal castration of male rats and

neonatal androgenization of female rats altered the pattern of the observed antinociceptive sex effects relative to sham-operated controls.

The results confirmed our previous findings of significantly greater antinociceptive responses to vlPAG morphine in sham-operated males than in sham-treated females tested during the estrous phase on both nociceptive measures. Neonatal gonadectomy had a profound influence upon the sex differences in morphine antinociception elicited from the vlPAG and essentially reversed the pattern of sex difference effects. Male rats castrated 1 day after birth and tested in adulthood displayed dramatic reductions in antinociceptive response to morphine, with almost a 15-fold rightward shift in the potency of morphine on the tail-flick test and a 6-fold shift on the jump test relative to sham-treated males. Indeed, their responses essentially mirrored those of sham-treated females. Conversely, androgenized female rats treated with testosterone proprionate 1 day after birth displayed dramatic increases in the antinociceptive responses to morphine on both tests relative to sham-treated females, with a 5-fold leftward shift in potency on the tail-flick test and a 12-fold shift on the jump test. Their antinociceptive responses approximated, but did not quite reach those observed in sham-treated males. The rank-order of potency of morphine on the tail flick test was as follows: neonatally-treated sham males>= neonatally-androgenized females>> neonatally sham-treated females>> neonatally-castrated males. The following rank-order of potency of morphine was observed on the jump test: neonatally-treated sham males>=neonatally androgenized females>>neonatally castrated males>> neonatally-treated sham females. Thus, neonatal gonadectomy reversed the commonly-observed pattern of sex differences in morphine antinociception on both nociceptive measures with neonatally-castrated males displaying

similar levels of antinociceptive responses to neonatally-treated sham females, and neonatally-androgenized females approaching the levels of antinociception displayed by neonatally-sham operated males. These findings are in marked contrast to the failure of adult gonadectomy to significantly alter the pattern of antinociceptive sex differences following morphine microinjections into the vlPAG, or following systemic (Cicero et al., 1996) or intraventricular (Kepler et al., 1989) administrations of morphine. Whereas in these previous studies adult male and female rats receiving sham surgeries displayed comparable levels of morphine antinociception to their gonadectomized counterparts, thus suggesting a minor role, at best, for activational effects of gonadal steroid hormones, the present potent differences between neonatally- castrated and sham-operated male rats and between neonatally-androgenized and sham-treated female rats strongly point to an **organizational** role of gonadal hormones in mediating sex differences in morphine antinociception.

One possible limitation of the present study is that an additional group of neonatally-castrated male rats receiving testosterone replacement therapy was not employed. This control group could test the possibility that their decreased antinociceptive responses might have been due to the disruption of time-dependent activational effects of testosterone rather than due to its early organizational effects. This possibility seems unlikely, however, because adult castration typically produces small and inconsistent changes in morphine antinociception regardless of the age of the animal at the time, and the latency between castration and nociceptive testing (see reviews Kest et al., 2000; Mogil et al., 2000; Islam et al., 1993). In addition, significant sex effects in the responsivity to morphine have been observed in very young sexually-

immature mice, further suggesting that the activational effects of gonadal hormones are not necessary for expression of sex differences in morphine antinociception (Kavaliers and Innes, 1990). However, the demonstration of decreased antinociception in neonatally-castrated rats receiving testosterone replacement therapy would further strengthen the validity of the organizational hypothesis. Therefore, it is important that the control group of neonatally-castrated male rats post-neonatally treated with testosterone be included in all future studies investigating the effects of neonatal hormone manipulations upon opioid antinociception.

The expression of many sex-typical reproductive and nonreproductive behaviors in rodents depends upon intact gonadal hormone action during critical periods of early development of the brain (Phoenix et al., 1959). Neonatal castration of male rats or neonatal treatment with testosterone of female rats result in dramatic alterations in reproductive and nonreproductive sex-dimorphic behaviors. Classic studies indicate that male rats deprived of testosterone by neonatal castration exhibit reduced male-typical sexual behaviors and increased female-typical sexual behaviors in adulthood while female rats treated neonatally with testosterone exhibit the opposite pattern of effects (Beach, 1974; Pfaff and Zigmond, 1971; Whalen and Edwards, 1967). Neonatal gonadectomy also alters an expression of a wide gamut of other sexually-dimorphic behaviors, including aggressiveness, rough and tumble play, avoidance learning, spatial learning, and anxiety (see Beatty, 1979, 1992 for review).

This study is, to our knowledge, the first demonstration of neonatal gonadectomy effects upon morphine antinociception and sex differences in morphine antinociception. Our results nicely complement the earlier findings of Kinsley and colleagues (Kinsley,

Mann and Bridges, 1988) who demonstrated a strikingly similar pattern to ours of reversal of sex differences in morphine antinociception in response to prenatal stress. Prenatally-stressed male rats exhibited significantly blunted antinociceptive responses to morphine on the tail-flick test relative to non-stressed male controls, while prenatally-stressed female rats displayed dramatically augmented antinociceptive responses relative to non-stressed females.

Prenatal stress administered to pregnant female rats between 15 and 21 days of gestation is known to disrupt sexual differentiation in the offspring (Ward, 1972). Male rats born to stressed mothers exhibit markedly altered reproductive and sexually-dimorphic nonreproductive behaviors, including demasculinization and feminization of copulatory behaviors (Crump and Chevins, 1989; Ward, 1972; Ward, 1977), changes in juvenile play patterns (Ward and Stehm, 1991) decreased aggression and infanticide (Kinsley and Svare, 1986, 1987; Miley, Frank & Hoxter, 1981), and increased paternal behaviors towards newborn pups (Kinsley and Bridges, 1988). These behavioral alterations are consistent with an attenuation of perinatal testosterone levels and are the result of the stress-induced lack of a prenatal testosterone surge on day 18 after conception, which is necessary for normal sexual differentiation of the male brain (Ward and Weisz, 1980). Female rats born to stressed mothers also display distinct changes in reproductive and nonreproductive behaviors which, although less striking, resemble those associated with neonatal androgenization (Herrenkohl and Scott, 1984). These behaviors include decreased fertility and fecundity (Herrenkohl, 1979), decreased nestbuilding and maternal aggression (Fride, Dan, Gavish and Weinstock, 1985), and decreased maternal behaviors towards newborn pups (Kinsley and Bridges, 1988).

The striking behavioral consequences of neonatal gonadectomy and prenatal stress are known to result from permanent alterations in brain morphology and neurochemistry due to the disruption of normal sexual differentiation of the central nervoussystem (Raisman and Field, 1973).

The process of sexual differentiation is very complex and consists of several overlapping, time-sensitive stages. (Bardin and Catterall, 1981; Haseltine and Ohno, 1981; Trbovich et al., 2001). The first stage involves the translation of chromosomal sex into gonadal sex , testes or ovaries, which develop from the gonadal ridge of the developing embryo. The main determinant of the direction of sexual differentiation at this stage is a single testis determining factor (TDF) gene, which is located on the Y chromosome and leads to the development of testes. The absence of TDF expression on the X chromosome leads to the development of ovaries. The second stage involves the formation of internal ducts and external genitalia, which determine phenotypic sex of the individual. This stage is controlled by embryonic testes, which secrete two hormones, Mullerian inhibitory hormone (MIH) which inhibits the development of the Mullerian duct, and testosterone which induces the differentiation of the Wolffian duct into the vas deferens, epididymis and seminal vesicles. Further, testosterone aromatized to dihydrotestosterone (DHT) induces the formation of external male genitalia. In the absence of MIH and testosterone (as in females), the Wolffian duct regresses and the Mullerian duct develops into the uterus, fallopian tubes and the anterior vagina. The third stage, which may be occurring concurrently with the second, involves sexual differentiation of the central nervous system. The male pattern of brain development

requires the hypothalamus to be exposed to testosterone aromatized to estradiol. In the absence of testosterone, the female brain develops.

Many parts of the rat brain are sexually dimorphic and are very sensitive to alterations of normal hormonal environment during critical periods of development. Neuroanatomical sex differences are particularly apparent in the medial preoptic area (mPOA) of the hypothalamus (Raisman and Field, 1973), the site which mediates coital behavior in the male and regulates phasic, endocrine-dependent reproductive behaviors in the female. The mPOA exhibits distinct sexual dimorphisms in cell number, size and packing density (Gorski, Gordon, Shryne and Southam, 1978), synaptic organization on dendritic spines (Raisman and Field, 1973), dendritic density patterns (Greenough, Carter Steerman and DeVoogd, 1977) and proportion of cells with dendritic bifurcation (Ayoub, Greenough and Juraska, 1983). The sexually dimorphic nucleus of the mPOA is much larger in males than females, and its volume can be altered in both sexes by neonatal gonadectomy or androgenization (Gorski et al., 1978). Evidence from in vitro studies supports the view that many of these sex differences in the mPOA can be attributed to the perinatal testosterone exposure since testosterone has been found to dramatically increase neuronal survival and neuronal outgrowth of cultured rat preoptic cells in tissue slice preparations (Kawashima and Takagi, 1994). Sexual dimorphisms have also been demonstrated in numerous brain structures outside the hypothalamus, including the amygdala, cortex, corpus callosum, hippocampus, and spinal cord (see review: Fitch and Denenberg, 1997). Many of these morphological sex differences are sensitive to perinatal hormone manipulations and can be reversed by neonatal castration of males or

testosterone treatment of females, suggesting the organizational role of gonadal hormones in their expression.

The ability of neonatal gonadectomy or prenatal stress to reverse the pattern of sex differences in morphine antinociception, together with the inability of adult gonadectomy to significantly alter these responses (Cicero, 1996; Kepler et al., 1989), strongly suggests that the development of the opioid pain inhibitory system may also be sexually dimorphic and dependent on testosterone for expression of the masculine pattern of antinociceptive responses. The absence of testosterone during critical periods of brain development may act to permanently alter this system – possibly by affecting the growth, migration, survival, and expression of opioid neurons and opioid receptors in the PAG, RVM and other sites which are critical for pain inhibition – resulting in the feminine pattern of antinociception.

There is substantial evidence to suggest that the development of the opioid system in the rat hypothalamus is sexually dimorphic. For example, mu opiate receptor binding in the mPOA is denser in females than in males and it shows distinct sex differences in regional distribution (Hammer, 1984, 1985, 1990). The distribution of Met-enkephalin fibers in the mPOA is also denser in females and is regulated by the actions of gonadal steroids, both during early development and in adulthood (Watson, Hoffman and Wiegand (1986). Sexual dimorphisms also exist in the distribution of enkephalinergic fibers in the anteroventral periventricular and medial preoptic nuclei (Hammer, 1985, 1990; Hijazi and Hammer, 1986;). Finally, neonatal treatment with gonadal hormones differentially affects levels of beta-endorphin in the pituitary of adult male and female rats (Forman and Estilow,1987). Further research is needed to determine whether

similar sex differences exist in the development of the opioid system in the vlPAG and other sites important for antinociception.

CHAPTER 6

General Discussion

The results of the present series of experiments confirmed our primary hypothesis that the vlPAG is a major site which mediates sex differences in opioid antinociception. Morphine microinjected into the vlPAG produced marked sex differences in antinociception with sham-operated male rats displaying significantly greater magnitudes and potencies of antinociceptive responses than sham-operated female rats on both the tail-flick and jump tests. Similarly, beta-endorphin microinjected into the vlPAG produced significantly greater magnitudes and potencies of antinociception in sham-operated male rats than in sham-operated female rats on the tail flick test. However, sex differences in beta-endorphin antinociception were test-specific since males and females displayed similar magnitudes of antinociceptive effects on the jump test. Significant sex differences in antinociception were also observed following the vlPAG microinjection of D-Pro2-endomorphin-2 but the pattern of sex effects was opposite to that observed for morphine and beta-endorphin as sham-operated female rats displayed greater magnitudes and potencies of D-Pro2-endomorphin-2 antinociception than sham-operated male rats on the tail-flick test. Like beta-endorphin, D-Pro2-Endomorphin-2 failed to elicit sex differences on the jump test.

Taken together, these data support the hypothesis that the vlPAG is involved in mediating sex differences in supraspinal opioid antinociception, presumably through interactions between estradiol-containing hypothalamic loci and transcription of the pre-pro-enkephalin gene. However, these data also suggest that the expression of sex differences in opioid-induced antinociception within the vlPAG is not uniform and may

depend upon the use of specific opioid agonist and the nociceptive measure. Thus, whereas morphine in the vlPAG elicited significant sex differences on both nociceptive measures, sex differences elicited by beta-endorphin and D-Pro2-endomorphin-2 in the vlPAG were only present on the tail- flick but not the jump test. Even more surprisingly, D-Pro2-endomorphin-2 in the vlPAG reversed the commonly observed pattern of sex differences as female rats displayed greater nociception on the tail-flick test than male rats. It should be noted, however, that although not predicted, neither of these two findings is unprecedented. Firstly, a number of earlier studies reported test-specific differences in antinociceptive responses to several other systematically and intraventricularly administered opioid agonists (Bartok and Craft, 1997; Cicero et al., 1996; Kepler et al., 1991), and the tail-flick test appeared to be the nociceptive measure in which sex differences were most often observed. We cannot at this point offer a definite explanation of why these test-specific effects are observed but it appears plausible that they may be due to the differences in either the circuitry mediating the nociceptive response or the circuitry underlying opioid modulation of the antinociceptive response. Secondly, although sex differences in opioid antinociception typically show a pattern of greater antinociception in males, there have been several reports of greater antinociceptive effects in females. As reviewed earlier, female rats were previously found to be more sensitive to the nociceptive effects of the kappa agonist, bremazocine, and they also manifest earlier antinociceptive effects in response to another kappa opioid agonist, U69,593 (Bartok and Craft, 1997). However, even more relevant in this respect is a recent study by Tershner et al (2000) which found that female rats are also more sensitive to the effects of DAMGO, following its administration into the vlPAG.

Collectively, this finding and the results of our study raise the possibility that antinociception elicited from the vlPAG by either DAMGO or D-Pro2-endomorphin-2, both highly selective mu opioid receptor agonists, may be mediated by different opioid receptors than morphine antinociception elicited from this site. However, this conclusion must be tempered by the fact that in our study D-Pro2-Endomorphin in the vlPAG also produced significant behavioral activation which was greater in male than female rats. Thus, the observed sex effects in antinociceptive responses elicited from the vlPAG by D-Pro2-endomorphin-2 were most likely secondary to sex-specific alterations in auxiliary motor behaviors.

In the present series of experiments, no differences in baseline tail-flick latencies or jump thresholds were observed between male and female rats. These results can be compared with those of some previous studies which demonstrated significant sex differences in nociceptive sensitivity. In particular, although a majority of these previous studies reported greater basal nociceptive sensitivity to electric shock in females (see review: Mogil et al., 2000) no significant sex differences in basal jump thresholds were found in any of our present studies. The reason for this discrepancy is not immediately evident but subtle methodological differences or differences in equipment used may possibly account for this finding. One important variable is acclimation to the testing procedures. Whereas many laboratories may use animals naïve to the testing procedures for baseline testing, our laboratory has historically tested the animals repeatedly under baseline conditions to insure stability of responding. Therefore, interestingly, sex differences in jump threshold were also not observed in previous studies conducted in this laboratory (Islam et al., 1993; Kepler et al, 1989). It should be noted that sex

differences in nociceptive sensitivity to thermal stimulation are typically not found, or found inconsistently (see review: Mogil et al., 2000). This agrees with our present finding of no significant sex effects in baseline latencies on the tail-flick test.

The second major aim of this study explored the mechanisms which mediate antinociceptive sex differences in the vlPAG by systematically evaluating the activational and organizational effects of gonadal steroid hormones upon the expression of opioid antinociception elicited from the vlPAG. Based on the results of previous studies demonstrating small and inconsistent effects of adult gonadectomy upon sex differences in antinociception elicited by systemic and intraventricular morphine administration (Cicero et al., 1996; Kepler et al., 1989), we hypothesized that activational effects of gonadal hormones do not play a major role in expression opioid antinociception elicited from the vlPAG. Further, we proposed that early organizational effects of gonadal hormones, which are affected by neonatal gonadectomy, are responsible for the observed sex differences in antinociceptive responses elicited from the vlPAG.

The results confirmed these hypotheses. The analysis of adult gonadectomy effects revealed that morphine antinociception elicited from the vlPAG was not altered by adult castration, as adult-castrated and sham-operated male rats displayed comparable levels of antinociception on both nociceptive measures. Although adult ovariectomy enhanced the overall potency of morphine analgesia relative to sham-operated females in estrous, this effect appeared to be modulatory as ovariectomized females displayed a grater magnitude of antinociception only at higher but not lower doses of morphine in the vlPAG. Further, adult gonadectomy of either male or female rats failed to consistently affect either beta-endorphin or D-Pro-2-endomorphin-2 antinociception elicited from the vlPAG.

In contrast, neonatal gonadectomy had a profound effect upon expression of morphine antinociception elicited from the vlPAG and essentially reversed the commonly observed pattern of antinociceptive sex differences. Neonatally-castrated male rats tested in adulthood displayed dramatic reductions in antinociceptive responses to morphine on both the tail-flick and the jump test relative to sham-treated males while neonatally-androgenized female rats displayed dramatic increases in morphine antinociception on both tests relative to sham-treated females. Taken together, the ability of neonatal gonadectomy to reverse the pattern of sex differences in morphine antinociception elicited from vlPAG and the inability of adult goadectomy to significantly alter these sex effects, strongly suggest that sex differences in opioid antinociception are mediated by early organizational effects of gonadal hormones. The activational effects of gonadal hormones acting later in life may have a modulatory influence upon the expression of sex differences in opioid antinociception but they are not capable of significantly altering these effects.

In view of this striking sexual dimorphism in expression of opioid antinociception, one is tempted to speculate on the possible evolutionary mechanisms that have shaped antinociceptive responses of males and females resulting in the present status quo of antinociceptive sex differences. It is plausible to assume that, like other sexually dimorphic behaviors, sex differences in antinociception have been subject to natural selection and are the result of evolutionary adaptation to sex-specific reproductive roles and environmental demands particular to each sex. It is for this reason that sex differences in antinociception are best understood in the broader context of sex differences in reproductive roles and behaviors.

One of the defining characteristics of the female rat reproductive system is periodicity. The female produces a limited number of gametes during relatively brief, distinct periods of receptivity and fertility which are highly dependent on a series of perfectly timed and synchronized phasic hormonal events (Naftolin, 1981). Likewise, gestation, parturition and maternal behavior are all periodic and predictable reproductive events which are dependent on the synchronized and integrated phasic actions of gonadal hormones (Rosenblatt, Siegel & Mayer, 1979). Aggressive behavior in the female rat is also phasic and is closely tied to her pattern of receptivity and hormonal status. Female rats are least aggressive on the night of proestrus, when mating normally occurs and levels of ovarian hormones are highest, and they are most aggressive on the night of estrus when they are no longer receptive and fertile, and hormone levels are at their lowest (Hood, 1984). This mechanism maximizes the chance that an unreceptive female will fend off advances of an inappropriately amorous male, thus preventing needless waste of resources and reducing the risk of predation.

In contrast, due to the tonic action of male gonadal hormones, the male rat is always sexually ready, fertile and aggressive (to fight off potential mating rivals), and constantly produces an unlimited number of gametes (Naftolin, 1981). This strategy assures the maximal reproductive success as mating needs to be adjusted to brief receptive periods of the female. Although the male system is rather prodigal with gametes, the excess aggression comes in handy in other life exigencies, such as intraspecies territorial disputes and interspecies encounters with prey and predators.

The antinociceptive patterns of males and females appear to have evolved to accommodate these sex-specific distinct reproductive strategies and demands. The female

antinociceptive system has evolved to maximize adaptation to periodic painful states related to reproduction. This system is relatively parsimonious, phasic, and maximally suited to cope with hormonally-signaled, periodic painful reproductive states, such as copulation and parturition. The best example of this type of response is pregnancy-induced antinociception (Gintzler, 1980), which has been documented in females of many mammalian species, including humans. This opioid-mediated antinociception is activated during the late stages of pregnancy in response to the rising levels of estradiol and progesterone, and is associated with the abrupt increase of pain thresholds in preparation for labor and delivery. Another form of female-specific antinociceptive response can be produced by stimulation of the vaginocervical area (Komisaruk and Whipple, 1986; Whipple and Komisaruk, 1985). This type of antinociception probably reflects an evolutionary adaptation to potentially aversive and painful copulatory stimuli and is designed to withstand repeated intromissions from the male, which are required for fertilization (Komisaruk and Whipple, 1986; Whipple and Komisaruk, 1985, 2000).

Likewise, the male pain inhibitory system has evolved to maximally facilitate adaptation to specific pain states resulting from unique aspects of male reproductive strategy and behavior, particularly intraspecies aggression. Aggressive encounters often result in bodily injury, pain and defeat. It appears that males have evolved a distinct pain inhibitory mechanism as a way of adaptation to these negative motivational states. Thus, in male mice aggressive encounters and the subsequent defeat have been shown to produce marked opioid-mediated antinociception that can be reversed by naloxone and is cross- tolerant with morphine (Miczek et al., 1982, 1985, 1988). Males may have also evolved a unique way of coping with other aversive or dangerous environmental stimuli

which present threat to preservation of species. For example, compared to females, male mice demonstrate significantly enhanced opioid antinociceptive responses when confronted with immobilization stress (Kavaliers and Innes, 1987), prolonged exposure to a predator (Kavaliers and Colvell, 1991) and exposure to biting flies (Kavaliers et al., 1998). Finally, increased antinociceptive responses are observed in male rats and other male rodents during copulation (Szechtman, Herskowitz and Simantov, 1981). This copulation-induced antinociception appears to be opioid-mediated since male rats subjected to sexual exhuastion by multiple copulations show a depletion of opioid peptides in the midbrain. In addition, naloxone significantly extends postejaculatory interval during which there is an increased responsiveness to noxious stimulation. It has been suggested that copulation-induced antinociception in males may reflect a biological mechanism designed to prevent genital stimulation from becoming too intense and thus aversive (Szechtman, Herskowitz and Simantov, 1981).

In summary, it appears that commonly observed sex differences in opioid antinociception may be the end points of evolutionary adaptation to the different types of painful stimulation that each sex is likely to experience while engaging in sex- and species-specific reproductive roles and behaviors. Whereas the female antinociceptive system appears to be best adapted to modulation of periodic pain states associated with reproductive behaviors, the male antinociceptive system has evolved to provide enhanced pain inhibition across a wide range of potentially aversive situations and reproductive behaviors designed to maximize sexual performance, reproductive success and preservation of species.

This study has clinical implications and utility as it clearly suggests that pain sensitivity and antinociceptive sensitivity to opioids should no longer be assumed to be equal in both sexes. This is particularly true for morphine which, in this, and in many other rodent studies, has been shown to elicit profound sex differences in antinociceptive responses across a range of different nociceptive measures. Since morphine is a prototypical analgesic in clinical use and a standard against which all new drugs are compared, this underscores the need of always including both sexes in animal and human experimental research and in clinical trials of morphine and novel opioid drugs.

As reviewed earlier, the results derived from human pain studies and animal models of nociception tend to present a somewhat contradictory picture of sex differences in sensitivity to morphine and other opioid agonists, with animal data generally reporting greater nociceptive effects in males (Cicero et al, 1996; Kepler et al, 1989) and human studies showing the opposite effect (e.g., DeKock and Scholtes, 1991; Gear et al., 1996). However, it should be kept in mind that, due to a number of methodological differences between human and animal studies, direct comparisons between rodent and human data are probably not valid and may be misleading. First, the majority of human data have been derived from studies which assessed opioid consumption whereas animal studies generally assess opioid antinociception. More importantly, human studies have been primarily concerned with sex differences in experience and modulation of *chronic pain* states whereas most animal studies evaluated *acute pain*. This is a very important difference since chronic pain, such as pain of cancer, is subject to learning whereas measures of acute pain (such as tail-flick and jump tests used in our study), have been shown not to be significantly affected by learning. Further, whereas measures of acute

pain primarily assess discriminative aspects of pain, the assessment of chronic, diffuse pain in humans is complicated by the fact that in addition to a discriminative component, these pain states invariably elicit the affective and cognitive aspects of pain (Melzack and Casey, 1972). Collectively, these differences suggest that the results of animal and human studies may be in fact less contradictory than they initially appear since they probably address somewhat different aspects of pain experience and antinociception.

There are several questions that are not answered by this study and might be addressed by future research. The first question concerns the possible sites and mechanisms by which organizational actions of gonadal hormones determine sex effects in antinociception. It appears that the VMH and mPOA are the most likely candidates to mediate these actions since they concentrate gonadal hormones, mediate reproductive behaviors via the link with the PAG, and express the estrogen-induced enkephalin gene and the gene for the delta opioid receptor (Pfaff, 2000). The mPOA is also one of the most sexually dimorphic areas of the brain, at which many striking neuroanatomical and neurochemical sex differences are expressed (Hammer, 1984, 1985; Raisman and Field, 1973). The possible mechanism by which the organizational effects of gonadal hormones are mediated in the VMH and mPOA might involve some molecular changes, such as alterations in the transcription rates of the enkephalin gene or the genes for the delta receptor. These alterations might in turn affect synapses in the vlPAG and produce the observed sex-effects in antinociception. This hypothesis is easily testable since one would expect that lesions of the VMH or/and mPOA would remove the link between gonadal hormones and their effects upon enkephalin gene regulation and the opioid

system expression in the PAG, resulting in the elimination or reduction of sex differences in opioid antinociception elicited from the vlPAG.

Some methodological issues should be addressed in future research. In particular, better grounds could be provided for distinguishing between time-dependent activational and organizational effects of gonadal hormones, by including additional control groups of animals to test for possible effects of post-neonatal testosterone in neonatally castrated male rats.

Another question that might be explored concerns the role of spinal mechanisms in mediating sex differences in opioid antinociception. As reviewed earlier, spinal opioid mechanisms have been implicated in pregnancy-induced antinociception in female rats (Gintzler, 1980). Since pregnancy-induced antinociception is produced by female gonadal hormones, it may be considered a female-specific, sexually-dimorphic form of pain inhibition. This conclusion has been recently reinforced by the finding that castrated male rats treated with hormonal regime which simulates the hormonal profile of pregnancy display antinociception that engages different populations of opioid receptors than pregnancy-induced antinociception of female rats (Liu and Gintzler, 2000). Whereas pregnancy-induced antinociception in females is mediated by delta and kappa opioid receptors, but not mu receptors, pregnancy-simulated antinociception in males is mediated by mu and delta, but not kappa receptors. These data suggest that antinociception elicited from the spinal cord may employ different opioid mechanisms in males than females, even under identical hormonal circumstances. In particular, it would appear that kappa receptors play a greater role in mediating spinal antinociception in females than in males. This hypothesis is worth exploring, especially in light of recent

reports of greater antinociceptive efficacy of kappa agonists in female rats and humans relative to their male counterparts (Bartok and Craft, 1997; Gear, Gordon, Heller, Paul, Miaskowski and Levine, 1996; Gear, Miaskowski, Gordon, Paul, Heller and Levine, 1996).

The role of other loci in mediating sex effects in opioid antinociception should also be explored, particularly that of the amygdala which mediates reproductive behaviors in the male and also plays an important role in supraspinal antinociception. Other potential sites might include the sites which are known to support antinociception, such as locus coeruleus, the pontine parabrachial area and medullary reticular nuclei.

In conclusion, our data confirm the results of a number of previous studies which reported pronounced sex differences in opioid antinociception. We also provide the conclusive evidence that these sex effects are centrally mediated in the vlPAG and are likely to be related to fundamental differences in the circuitry of pain inhibitory opioid pathways in male and female rats. These differences are determined and shaped by the organizational effects of gonadal hormones early in the development of the central nervous system and once established become permanent and relatively unresponsive to further hormonal influences.

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