Sex Differences in Systemic and Central Morphine Analgesia in Rats: Organizational-Activational Gonadal Hormone Interactions and Roles of Gonadal Hormone Accumulating Nuclei

by

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Abstract

Sex Differences in Systemic and Central Morphine Analgesia in Rats: Organizational-Activational Gonadal Hormone Interactions and Roles of Gonadal Hormone Accumulating Nuclei

by Giuseppe Cataldo

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Sex differences in morphine analgesia are commonly seen following systemic and intracerebral administration with male rats displaying greater analgesic magnitudes and potencies than females. The purpose of this dissertation research was to elucidate further possible neural mechanisms which elicit these differences. Due to its common roles in both antinociceptive and reproductive behaviors, we hypothesized that the ventrolateral periaqueductal gray (vlPAG) is subject to sex differences upon morphine analgesia sensitive to organizational-activational manipulations of gonadal hormones as well as lesions of hypothalamic estradiol-containing nuclei. Thus, the first experiment examined the organizational manipulation of gonadal hormones and effects of adult ovariectomy or estradiol replacement and systemic morphine analgesia. To assess the generalizability of these effects, the second experiment evaluated these differences upon morphine analgesia elicited from the vlPAG as well as the interaction between organizational and activational gonadal hormone manipulations. The third experiment then evaluated the ventromedial hypothalamus (VMH) and the medial preoptic area (MPOA) hypothalamic estradiol-containing nuclei's contribution to and their possible role by which female rats display a smaller opiate analgesic effect.

Adult ovariectomy minimally affected morphine analgesia in neonatal vehicle-treated females, while significantly reducing the magnitude but not the potency of morphine analgesia in neonatal androgenized female rats. This suggests a limited organizational-activational gonadal hormone interaction in the mediation of systemic morphine analgesia in female rats. In marked contrast, neonatal androgenized female rats displayed significantly greater magnitudes of vlPAG morphine analgesia than neonatal vehicle-treated female rats. Adult ovariectomy significantly enhanced the magnitude and potency of vlPAG morphine analgesia in female rats treated neonatally with either vehicle or testosterone with the latter effect suggesting a strong organizational-activational gonadal hormone interaction in the mediation of vlPAG morphine analgesia in female rats.

Lesions of the VMH and MPOA strongly suggest that they act to tonically inhibit endogenous pain-inhibitory circuits in the female, but not male brain, and that removal of circulating gonadal hormones by ovariectomy and/or excitotoxic destruction of these estrogen receptor accumulating nuclei disinhibit the female analgesic response to systemic morphine. Collectively, these results strongly implicate the vlPAG, organizational and activational effects of gonadal hormones as well as hypothalamic estradiol-containing nuclei in mediating sex differences in morphine analgesia in rats.

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Finally, I would like to thank my family, especially my parents Rosa and Giusto Cataldo for their love, nurturance and support. Mom and dad, I dedicate this dissertation to you.

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Glossary of Abbreviations

CCK-R - cholecystokinin receptors
CFA - complete Freund's adjuvant, an antigen solution used as an immunopotentiator
CGRP - calcitonin gene-related peptide
DAMGO - D-Ala2, N-Met-Phe4, Gly-ol5-enkephalin, mu agonist
DPDPE - D-Pen2, D-Pen5-enkephalin, delta agonist
EB – estradiol benzoate
ED_{50} – effective dose for a pharmacological effect in 50% of subjects
GABA – gamma-aminobutyric acid
GIRK-2 - G protein-coupled inwardly-rectifying potassium channel
GAD - glutamic acid decarboxylase
HCL - hydrochloride
Ibo – ibotenic acid
Il-1β - interleukin 1β, pro-inflammatory cytokine involved in immune defense against infection
IM – intramuscular injection
IP - intraperitoneal injection
MPE - mean percentage effect
MPOA - medial preoptic area
NMDA - N-methyl-D-aspartate
NRM - nucleus raphe magnus
ORL1 – orphanin-opioid receptor
Ovex - ovariectomy
PAG – periaqueductal gray
PGE2 - prostaglandin E2
PPE - preproenkephalin
ROD - relative optical density
RVM - rostral ventromedial medulla
SC – subcutaneous
TP – testosterone propionate
U50488H – kappa agonist
U69593 – kappa agonist
Veh – vehicle treated
vlPAG - ventrolateral periaqueductal gray
VMH - ventromedial hypothalamic nucleus

Chapter 1

Introduction and Specific Aims

Forty-five years after the paradigm-shifting Gate-Control Theory of pain (Melzack and Wall, 1965) was proposed, a supraspinal pain-inhibitory system in rodents and humans has been identified that is centered around the ventrolateral periaqueductal gray (vlPAG) and rostral ventromedial medulla (RVM) that mediates the systemic and central effects of morphine and other opioid drugs (see reviews: Fields and Basbaum, 1978; Basbaum and Fields, 1984; Akil et al., 1984). Of major theoretical and therapeutic importance was the subsequent finding that this opioid-mediated pain-inhibitory system is sensitive to sex differences in rodents such that male rats and mice display greater potencies and magnitudes of morphine analgesia following systemic and central administration than female rats and mice (see reviews: Berkley, 1997; Berkley et al., 2006; Bodnar et al., 2002; Craft, 2003; Craft et al., 2004; Greenspan et al., 2007). These sex differences in systemic and central opioid-mediated analgesia are mediated in part by the respective organizational and activational roles of gonadal hormones as determined by neonatal and adult gonadectomy studies and gonadal hormone replacement studies (see reviews: Bodnar et al., 2002; Bodnar and Kest, 2010; Craft et al., 2004; Loyd and Murphy, 2009). Some mechanisms of action by which gonadal hormones or sex status mediate the sex differences in morphine analgesia have been identified, including sex differences in: a) retrogradely-labeled PAG-RVM output neurons (Loyd and Murphy, 2006) onto physiologically-identified RVM ON and OFF cells (Morgan et al., 2008), b) Fos-induced activation levels in the PAG (Loyd et al., 2007), c) mu opioid receptor expression in the PAG (Loyd et al., 2008b), d) declines in the percentage of PAG-RVM output neurons activated by morphine during tolerance (Loyd et al., 2008a), and e) numbers of androgen receptor-immunoreactive neurons in the PAG that project to

the RVM (Loyd and Murphy, 2008). At least two mechanistic questions remain in the gonadal hormone mediation of sex differences in morphine analgesia, and these are addressed in the following two Specific Aims. The **First Specific Aim** of this dissertation was to evaluate the interaction between organizational and activational effects of gonadal hormones on morphine analgesia following systemic administration **(Specific Aim 1A)** and intracerebral administration into the vlPAG **(Specific Aim 1B)** in female rats receiving neonatal androgenization and/or adult ovariectomies and gonadal hormone replacement as compared to control male rats. The **Second Specific Aim** of this dissertation was to examine whether excitotoxic (ibotenic acid) chemical destruction of two gonadal hormone accumulating nuclei, the medial preoptic area (MPOA) or the ventromedial hypothalamic nucleus (VMH) will alter the dose-dependent, time-dependent and sex-dependent actions of systemic morphine analgesia in adult intact male, intact female and ovariectomized female rats. These studies have begun to establish the underlying interactive neurocircuitry between gonadal and analgesic systems mediating morphine analgesia.

The following background section provides an underlying conceptual basis and understanding for the proposed studies by covering the following research areas: 1) the endogenous opioid pain inhibitory system with emphasis on the vlPAG, RVM and the dorsal horn of the spinal cord; 2) sex differences in systemic opiate and opioid analgesia; 3) sex differences in central opiate and opioid analgesia; 4) gonadal hormonal modulation of sex differences including a) estrous cycle effects, b) adult gonadectomy effects, c) exogenous gonadal hormone effects d) neonatal gonadectomy effects; 5) and characterization of hypothalamic estradiol-containing nuclei: the VMH and the MPOA.

Background

The Endogenous Opioid Pain-Inhibitory System:

Following the initial observations that analgesia could be elicited following either electrical stimulation of or morphine microinjection into the vlPAG (Reynolds, 1969; Tsou and Jang, 1964; Jacquet and Lajtha, 1973), a great deal of research in the 1970's was concerned with the mapping of sites within the brain supporting opioid analgesia. Basbaum and Fields (Fields and Basbaum, 1978; Basbaum and Fields, 1984) initially proposed a descending pain-inhibitory system consisting of three tiers: (1) the vlPAG, (2) the rostral ventromedial medulla (RVM) and (3) the dorsal horn of the spinal cord. This conception was supported further by the description of a neurophysiological substrate for opioid analgesic actions in the RVM (see review: Fields et al., 1991). Off-cells cease activity immediately before the occurrence of nocifensive reflexes, and are activated by systemic or vlPAG administration of morphine, and are thus thought to provide pain-inhibitory output. On-cells increase their activity immediately before nocifensive responses and are thereby thought to exert net facilitatory effects on nociceptive processing. A role for spinal processing by opioid peptides and receptors was described, and separate yet interacting spinal and supraspinal analgesic systems were postulated (e.g., Yeung and Rudy, 1980; Yeung et al., 1977). Examination of agonists of mu, delta and kappa opioid receptors revealed that mu receptor agonists and antagonists were more effective in respectively eliciting and inhibiting supraspinal morphine analgesia, particularly within the vlPAG (e.g., Bodnar et al., 1988; Fang et al., 1986; Jensen and Yaksh, 1986a, 1986b, 1986c; Smith et al., 1988). Indeed, a supraspinal analgesic system consisting of the vlPAG, locus coeruleus and RVM were found to produce analgesic synergy when sub-analgesic doses of opiates were administered across pairs of sites (Rossi et al., 1993, 1994), and the amygdala and vlPAG displayed analgesic synergy as well

(Pavlovic and Bodnar, 1998). Moreover, morphine analgesia elicited from the vlPAG could be blocked by RVM pretreatment with serotonergic, cholinergic, excitatory amino acid and opioid antagonists (Kiefel et al., 1992, 1993; Spinella et al., 1996, 1997, 1999), and in turn, opioid antagonists administered into the vlPAG blocked morphine analgesia elicited from the amygdala (Pavlovic et al., 1996).

With seminal research almost exclusively performed in male animals, only recently have studies begun to include sex as an independent variable. Sex differences in morphine analgesia were first reported in the late 1980s with male rodents displaying greater analgesic responses to systemically-administered mu opioid agonists than female rodents (Kepler et al., 1989, 1991; Kavaliers and Innes, 1987). Recently, clinical studies in humans have also found sex differences in morphine analgesia (Unruh, 1996; Unruh et al., 1999) where human male subjects typically display a greater analgesic response than female subjects. Despite these sex differences, in the past decade, almost 80% of animal studies published in the journal *Pain* used only male subjects as compared to 8% using only females and only 4% explicitly designed to test for sex differences (Mogil and Chanda, 2005).

Sex Differences in Systemic Opiate and Opioid Analgesia:

Potent sex differences in analgesic processes have been described (e.g., see reviews: Bodnar et al., 2002; Craft et al., 2004), particularly in the magnitude of mu-opioid receptor agonist-induced analgesia with female rodents displaying significantly smaller responses than male rodents following systemic (e.g., Baamonde et al., 1989; Badillo-Martinez et al., 1984; Candido et al., 1992; Cook and Nickerson, 2005; Kavaliers and Innes, 1987) and ventricular (Kepler et al., 1989) administration. Such sex differences are sensitive to organismic variables and due putatively to pharmacodynamic opiate drug effects. Thus, sex differences in morphine

analgesia are sensitive to genotypic variance in both mice (Kest et al., 1999; Mogil et al., 2000) and rats (e.g., Terner et al., 2003a, 2003b). Three mouse strains (AKR/J, C57BL/6J, and SWR/J) displayed approximately 3.5- to 7.0-fold greater sensitivities to morphine analgesia in males relative to females, whereas CBA/J females were 5-fold more sensitive to morphine than males. The magnitude of kappa agonist (-)-pentazocine analgesia was greater in males in four rat strains with 2.5-fold differences noted in the F344 strain, but 11-fold noted in the Wistar strain. Further, the potency and pattern of sex differences also interact with the age of the animal such that older animals displayed greater maximal percentage effects than younger rats (Islam et al., 1993). Simple pharmacokinetic factors failed to explain the sex differences in the greater morphine analgesia in male relative to female rats (Cicero et al., 1996, 1997), but the potency of the particular mu opiate agonists was related to the presence of pronounced analgesic sex differences in that more potent mu-selective opioid agonists such as etorphine, DAMGO and beta-endorphine produced more pronounced analgesic sex differences in male animals than female animals than less potent mu-selective opioid agonists (Cicero et al., 1997; Cook et al., 2000; Craft and Bernal, 2001; Kepler et al., 1991; Krzanowska and Bodnar, 2000; Negus and Mello, 1999; Terner et al., 2003). Moreover, whereas some delta (e.g., [D-Pen^{2},^{5}] enkephalin (DPDPE) and deltorphin) and kappa (e.g., U50488H) opioid receptor agonists also produce greater analgesic responses in male rats, less potent agonists active at these receptor types do not cause sex differences in analgesia (Barrett et al., 2002a, 2002b; Bartok and Craft, 1997; Craft and Bernal, 2001; Kavaliers and Innes, 1990; Patrick et al., 1999). It has also been suggested that sex differences in male rodents and nonhuman primates display greater analgesic responses than females following mu and kappa opioids, particularly those that display low efficacy such that they appear to act as full agonists in males, but antagonists in females. These effects interact with

drug history, genotype and the modality, duration and intensity of the nociceptive stimulus (Barrett, 2006). Although opioids with diverse receptor selectivity such as morphine (mu), buprenorphine (ORL_1), butorphanol (mu) and spiradoline (U69593; kappa) each produce greater analgesic effects in male relative to female rats, the reduction in levels of the temporal summation of pain were equal in both sexes (Lomas and Picker, 2005). Relative to males, female Fischer rats show greater enhancement of contact hypersensitivity following the kappa opioid agonist, spiradoline, but both sexes showed comparable analgesic effects (Elliott et al., 2006a). Although female rats can display greater analgesia than males following treatment with the respective mu and kappa opioid receptor agonists oxycodone and U50488H, this may reflect the greater hyperalgesia evident in male rats following low oxycodone and U50488H doses (Holtman and Wala, 2006). The hyperalgesic effects of low doses of morphine were more pronounced in female relative to male rats with tolerance to this effect noted in both sexes. After tolerance, the efficacy of morphine analgesia was enhanced in females, and the sex differences in morphine analgesia were attenuated (Holtman and Wala, 2005). Furthermore, morphine, oxycodone and butorphanol were more potent in producing analgesia and anti-hyperalgesia in male than in female rats made arthritic with Freund's Adjuvant (complete form; Cook and Nickerson, 2005; Wang et al., 2006). Sex differences in the potency of anti-hyperalgesic effects of the kappa opioid agonists U50488 and U69593 (spiradoline) are observed following systemic treatment and at the site of inflammation against capsaicin-induced hyperalgesia in male relative to female rats. Yet, the mixed opioids butorphanol and nalbuphine, failed to display analgesic sex differences (Lomas et al., 2007). Importantly, the nature of the sex differences in these studies can be alternatively ascribed to sex-specific dose-dependent sensitivity in drug-induced analgesic responses, but also to asymptotic limitations in drug-induced analgesic effectiveness in

female animals relative to males. These collective data thereby underscore the importance for the development of opioid receptor-selective agonists that can produce maximal analgesia in both sexes, and the determination of effective dose ranges for each sex.

Both opioid and non-opioid drugs that modulate morphine analgesia also do so sex-dependently. For example, when ultra-low doses of naltrexone, which can induce analgesic responses, are paired with morphine, the combination respectively enhances and decreases morphine analgesia in mature female and male rats (Hamann et al., 2004). Further, sex differences in morphine were not accompanied by changes in opioid receptor number, binding affinity or opioid-stimulation of G-protein in whole brain, cortex, thalamus or spinal cord with the long-acting opioid antagonist, methocinnamox decreasing analgesia induced in males to a level observed in untreated females (Peckham et al., 2005). Similar sex-dependent effects on systemic morphine analgesia are reported for antagonists of the N-methyl-D-aspartate (NMDA) receptor. For example, whereas dextromethorphan, ketamine and MK-801 significantly enhance the magnitude and duration of morphine analgesia in female rats, only dextromethorphan modestly did so in males (Holtman et al., 2003). In addition, dextromethorphan increases morphine analgesia on the hot-plate test in males with greater potency than in females (Craft and Lee, 2005). In the same study, the ability of the NMDA antagonist LY235959 to potentiate morphine analgesia varied as a function of antagonist dose, nociceptive assay and sex. In mice, analgesia on the tail withdrawal test following low (but not high) morphine doses is potentiated only in males treated with dextromethorphan, dextorphan, and MK-801. The competitive NMDA antagonists, LY235959 and LY-701324 enhance analgesia in male, but not female mice across a morphine dose range (Nemmani et al., 2004). Finally, the more potent analgesic responses of morphine and clonidine in male mice failed to be observed in mice with a “knock-

out" of G protein-coupled inwardly-rectifying potassium channel subtype (GIRK-2), suggesting a role for this molecular modulator in the expression of sex differences (Mitrovic et al., 2003).

However, it should be noted that analgesic responses to delta-selective and kappa-selective opiate agonists display less consistent sex differences (Barrett et al., 2002a, 2002b; Bartok and Craft, 1997; Craft and Bernal, 2001; Kavaliers and Innes, 1990; Kepler et al., 1991; Patrick et al., 1999). For instance, the peak effects of U69593 on tail-withdrawal and DPDPE on hot plate tended to occur earlier in females than in males, and bremazocine produced greater tail-withdrawal antinociception in females than in males, whereas the highest doses of the two delta opioids produced greater hot-plate antinociception in males than in females. Further, whereas morphine, buprenorphine, butorphanol and spiradoline produced greater analgesic effects in male rats relative to females, their reductions in levels of temporal summation of pain were equal in both sexes (Lomas and Picker, 2005).

Sex Differences in Central Opiate and Opioid Analgesia:

The ventrolateral periaqueductal gray (vlPAG) is an ideal locus for opiate and gonadal steroid hormone interaction, particularly with respect to pain inhibition. Hypothalamic enkephalinergic neurons, sensitive to changes in sex hormone levels, express enkephalin gene products in females to a greater degree than males (Priest et al., 1995; Romano et al., 1988, 1989, 1990), and eventually project to estrogen-binding PAG neurons (Turcotte & Blaustein, 1999), a pathway that is eliminated by hypothalamic lesions (Hoffman et al., 1996). Mu opioid agonists facilitate excitation in vlPAG cells through interactions with NMDA receptors (Kow et al., 2002); the ultrastructural arrangement of mu opioid receptors with GABAergic PAG neurons or PAG projection neurons labeled retrogradely from the medulla indicate that mu opioid receptor ligands act to both inhibit the former and directly act on the latter (Commons et al., 2000).

Further, although male rats possess significantly greater numbers of androgen receptor-immunoreactive neurons in the PAG that project to the rostral ventromedial medulla (RVM) than females, the two sexes do not differ in amount of estrogen receptor alpha, suggesting specificity of which gonadal hormone mechanisms might mediate sex differences in central morphine analgesia (Loyd and Murphy, 2008).

As with mu-opioids delivered systemically, male rodents typically display greater analgesia following their administration into the lateral ventricle, the vlPAG or the RVM than female rodents (Boyer et al., 1998; Kepler et al., 1989, 1991; Krzanowska & Bodnar, 1999, 2000; Loyd & Murphy, 2006). Further, male rats display greater analgesia on a visceromotor pain test relative to females following systemic and intraventricular, but not intrathecal treatment with the mu opioid loperamide (Ji et al., 2006). Administration of the selective mu opioid antagonist, beta-funaltrexamine into the vlPAG causes significantly greater rightward shifts in systemic morphine's dose-response curve in female relative to male rats indicating sex differences in the central efficacy of mu-selective antagonists (Bernal et al., 2007). Loyd and Murphy (2006) recently found that males displayed greater morphine analgesia on an inflammatory pain test following vlPAG administration. Corresponding anatomical data showed that although females displayed significantly more retrogradely-labelled PAG-RVM output neurons than males, inflammatory pain activated more PAG-RVM cells in male than in female rats. Loyd and co-workers (2007) further demonstrated that Fos-induced activation levels in the PAG induced by inflammation was suppressed by systemic morphine in male, but not female rats, and morphine preferentially activated the PAG-RVM pathway in the male rat, providing a potential central mechanism of action for morphine analgesic sex differences. This group (Loyd et al., 2008b) further implicated the PAG as a locus underlying opioid analgesic sex differences

by demonstrating that males display greater mu opioid receptor expression in the PAG than females, and display significantly greater thermal hyperalgesia than females after intra-PAG morphine injection. Indeed, selective lesions of mu-opioid receptor-expressing PAG neurons blocked systemic morphine analgesia in males only. Thus, the PAG, an important focal point in the mediation of central morphine analgesia in seminal descriptions of pain-inhibitory pathways (see reviews: Basbaum and Fields, 1978; Fields and Basbaum, 1984), appears integral in the mediation of sex differences in morphine analgesia.

As indicated, mu-opiate agonists appear to produce the most potent analgesic effects when administered supraspinally, whereas delta and kappa agonists appear more effective following spinal administration. These data indicate how neurochemical interactions may minimize the sex differences observed for morphine analgesia and how the reduced analgesic response in females can be amplified. Given the prevalent role for mu, relative to delta and kappa, opioid agonists in eliciting analgesia from the vlPAG, and given a predominant role of the vlPAG in displaying sex differences, it would appear that any interaction between gonadal manipulations and opiate analgesia would center its effects within the vlPAG. The next section details the roles of gonadal hormones in mediating sex differences in opiate analgesia.

Gonadal Hormonal Modulation of Sex Differences in Opiate Analgesia:

Four different types of studies can be employed to assess gonadal hormone modulation of sex differences in opiate analgesia: a) differences in opiate-mediated analgesic responses across the different phases of the estrous cycle in female rats; b) the effects of adult gonadectomy upon opiate analgesia in male and female rats; c) the effects upon opiate analgesia of exogenous administration of gonadal hormones in intact or gonadectomized animals; and d) the effects of neonatal gonadectomy upon opiate analgesia in male and female rats.

Estrous Cycle Effects: The phase of the estrous cycle affects the basal and antinociceptive responses to nociceptive stimuli. Female rats display their greatest basal sensitivity to shock during the estrus phase (Drury and Gold, 1978), and longer tail-flick and hot plate latencies are noted during diestrus relative to proestrus or estrus (Frye et al., 1993; Stoffel et al., 2003). Whereas the magnitude of continuous cold-water swim induced antinociception was similar in female rats across the phases of the estrous cycle (Romero and Bodnar, 1986), females during the estrus phase were more sensitive to the antinociceptive effects of inescapable footshock (Ryan and Maier, 1988). Whereas evaluation of systemic morphine antinociception during the estrous cycle revealed greatest sensitivity during either the late diestrus phase (Banerjee et al., 1983), or the proestrus and diestrus phases (Stoffel et al., 2003), greater antinociceptive responses following intracerebroventricular morphine were observed during proestrus relative to the met-diestrus phases (Kepler et al., 1989). Further, ovariectomy decreased basal nociceptive thresholds, but increased systemic morphine and especially buprenorphine analgesia in female F344, Lewis, Long Evans and Wistar rats. During normal cycling, systemic morphine and buprenorphine were most sensitive in metestrus and proestrus and least potent in estrus (Terner et al., 2005). Morphine analgesia elicited from the vlPAG displayed estrous phase differences with greater and more potent effects observed in cycling female rats tested during proestrus and diestrus phases of the estrous cycle relative to the estrus phase of the cycle (Bernal et al., 2006; Shane et al., 2007). Thus, although the formal evaluation of estrous cycle effects is not considered to be the first step in evaluating a role for gonadal hormones in sex differences in opiate analgesia (Greenspan et al., 2007), it is clear that the most pronounced sex differences in morphine analgesia, particularly elicited from the vlPAG, are observed when female rats are tested during the estrus phase of the cycle.

Adult Gonadectomy Effects: Gonadectomy in adult male and female animals significantly reduced analgesic sex differences elicited by both opioid-mediated and nonopioid-mediated swim and shock stressors (e.g., Bodnar et al., 2002; Craft et al., 2004) such that castrated male rats displayed analgesic responses to swim stress and electric shock that was significantly lower than intact males, and similar to intact females. Adult ovariectomy significantly lowered analgesic responses to swim stress and electric shock relative to intact females (Romero et al., 1987, 1988; Ryan and Maier, 1988). Interestingly however, these manipulations minimally altered the magnitude or the potency of mu agonist-mediated analgesia following systemic and ventricular administration relative to intact animals (Ali et al., 1995; Banerjee et al., 1983; Cicero et al., 1996, 2002; Islam et al., 1993; Kasson and George, 1984; Kepler et al., 1989, 1991). For instance, castration produced small, but significant reductions in the magnitude of systemic morphine analgesia; the effective dose for a pharmacological effect in 50% of subjects (ED_{50}) of morphine analgesia, however, was not changed. Although female rats in either proestrus or estrus displayed significantly greater magnitude of analgesia than ovariectomized rats or rats in a combined met-/di-estrous phase at some doses, the ED_{50} of morphine analgesia was not significantly altered as functions of estrous phase or ovariectomy (Kepler et al. 1989). This suggests that the classically-described activational effects of gonadal hormones (e.g., Phoenix et al., 1959) may not be pivotal in the mediation of these analgesic responses, although adult gonadectomy does affect systemic μ agonist-mediated analgesia when using less potent agonists (Terner et al., 2002). Interestingly however, morphine analgesia elicited from the vlPAG was subject to sex differences with males displaying greater and more potent analgesia than females, adult gonadectomy differences with ovariectomized females displaying greater and more potent analgesia than intact females tested during the estrus phase

(Krzanowska and Bodnar, 1999; Loyd and Murphy, 2006). Moreover, female rats have more PAG-RVM output neurons than males, but males have more activated PAG-RVM cells than females during inflammatory pain. Systemic morphine significantly suppressed complete Freund's adjuvant CFA-induced Fos only in males (Loyd and Murphy, 2006).

Exogenous Gonadal Hormone Effects: Although progesterone increases nociceptive thresholds in ovariectomized female rats (Frye and Duncan, 1994; McCarthy et. al., 1990) and male mice (Kavaliers and Wiebe, 1987) following acute administration, its effect can be biphasic and greatly dependent upon specific doses such that moderate (1 mg/kg) progesterone doses increased latencies, while higher (2–4 mg/kg) doses were without effect (Frye et al., 1996). Moreover, chronic progesterone pretreatment decreases antinociception elicited by sucrose exposure (Frye et al., 1992). Dawson-Basoa and Gintzler (1993) found that “pseudo-pregnant” 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 (Rothfield et al., 1985). Furthermore, concurrent progesterone administration dampened the antinociceptive effects of the testosterone metabolite, 3α-androstanediol (Frye 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.

Krzanowska and Bodnar (1999) found that female rats in the estrous phase display a muted antinociceptive response to morphine elicited from the vlPAG which strongly suggests that high circulating levels of both gonadal hormones may produce mutually antagonistic effects. These studies are supplemented by other work demonstrating that estrogen and/or progesterone produced mixed facilitatory and inhibitory effects upon opioid-induced analgesia (Banerjee et al., 1983; Chatterjee et al., 1982; Negus and Mello, 1999, 2002; Nomikos et al., 1991; Ratka and Simpkins, 1991). Testosterone produces anti-hyperalgesic effects during development in male animals that is maintained during adulthood. Morphine analgesia inhibits inflammation-induced pain in adult gonadectomized, but not neonatally-gonadecomized animals (Borzan and Fuchs, 2006). Systemic morphine analgesia was also less potent in gonadectomized females receiving estradiol, progesterone or testosterone (Stoffel et al., 2003). Moreover, castration and testosterone replacement respectively decreased and increased mu agonist-induced analgesia in male rats, whereas ovariectomy and hormone replacement had more variable effects in female rats with estradiol decreasing mu agonist-induced analgesia (Stoffel et al., 2005). Therefore, both endogenous activation and exogenous administration of gonadal hormones can elicit highly specific changes in basal nociception and analgesic responses, by putatively acting on nuclei (predominantly in the hypothalamus) that specifically accumulate gonadal hormones through internalized receptor systems.

Neonatal Gonadectomy Effects: Further, classically-described organizational effects of gonadal hormones (e.g., Phoenix et al., 1959) appear important in the mediation of these responses given that morphine analgesia elicited from the vlPAG (Krzanowska et al., 2002) or following systemic administration (Cicero et al., 2002) was profoundly affected by neonatal gonadectomy. Thus, adult male rats neonatally castrated on Day 1 after birth displayed

magnitudes and potencies of either intracerebral or systemic morphine-induced analgesia that were significantly lower than sham-operated males, but similar to that of neonatal vehicle-treated females tested during the estrus phase. Correspondingly, adult female rats neonatally treated with testosterone propionate (TP) on Day 1 after birth displayed magnitudes and potencies of either intracerebral or systemic morphine-induced analgesia that were significantly higher than neonatal vehicle-treated females tested during the estrus phase, but similar to that of sham-operated males. However, neonatal androgenization in female rats is capable of producing an anovulatory syndrome that could change the adult hormonal milieu in female rats (e.g., Compann et al., 1993; Micevych et al., 1994; Seale et al., 2005; Stewart and Kolb, 1994), and can deplete the female of estrogen and/or progesterone, both of which produce mixed facilitatory and inhibitory effects upon opioid-induced analgesia (Banerjee et al., 1983; Chatterjee et al., 1982; Negus and Mello, 1999, 2002; Nomikos et al., 1991; Ratka and Simpkins, 1991). Thus, again, it appears that the vlPAG is the most sensitive supraspinal site for gonadal hormone-opiate analgesic interactions, and would appear to be the most viable candidate for determining further interactions between these two physiologically-relevant systems.

Hypothalamic Estradiol-Containing Nuclei:

The previous sections implicate the vlPAG as being maximally sensitive to changes in morphine analgesia as a function of sex differences, organizational and activational effects of gonadal hormones and estrous phase (Bernal et al., 2006; Krzanowska and Bodnar, 1999; Krzanowska et al., 2002; Loyd and Murphy, 2006; Shane et al., 2007). Importantly, hypothalamic enkephalinergic neurons sensitive to changes in sex hormone levels that turn on enkephalin genes in females to a greater degree than males (Priest et al., 1995; Romano et al., 1988, 1989, 1990) eventually project to estrogen-binding PAG neurons (Turcotte and Blaustein,

1999), and are absent following hypothalamic lesions (Hoffman et al., 1996). Mu opioids facilitate excitation in vlPAG cells via interactions with NMDA (Kow et al., 2002), and the ultrastructural arrangement of the mu opioid receptor with GABAergic PAG neurons or PAG projection neurons labeled retrogradely from the medulla indicate that mu opioid receptor ligands both act to inhibit the former, and act directly on the latter (Commons et al., 2000). The two major nuclei implicated in accumulation of gonadal hormones, involvement of gonadal-enkephalin interactions, and that project to the PAG are the MPOA and VMH; the following two sections briefly provide a background for the study of specific aim 2.

The Medial Pre-optic Area (MPOA): The MPOA is a sexually dimorphic hypothalamic nucleus (e.g., Bloch and Gorski, 1988; Gorski et al., 1980; Simerly et al., 1984) playing a crucial role in sexual behavior (e.g., Docke et al., 1984; Hansen et al., 1982; Lisk, 1966; Powers and Valenstein, 1972) and possessing androgen and estrogen mRNA-containing cells (Simerly et al., 1990). The MPOA has an orderly, reciprocally-connected and longitudinally-organized columnar organization projection that extends along the whole rostro-caudal axis of the PAG (Rizvi et al., 1992). These MPOA afferents innervate those PAG cells that subsequently project to the RVM as demonstrated by combined Fos and Phaseolus vulgaris tract tracing (Rizvi et al., 1996), provide gonadal steroid receptor innervation from the MPOA to the PAG (Murphy and Hoffman, 2001), and have been confirmed in neurophysiological studies (Jiang and Behbehani, 2001; Lumb and Morrison, 1986). Moreover, the MPOA connections with the pontine and medullary midline nuclei (Holstege, 1987; Veening et al., 1990), particularly the nucleus raphe magnus, have been shown to be direct and reciprocal (Murphy et al., 1999). Electrical stimulation of MPOA neurons suppresses spinal and medullary dorsal horn neuronal responses to cutaneous and visceral noxious input (Carstens et al., 1982; Lumb, 1990; Lumb and Cervero, 1989; Mokha

et al., 1987). Moreover, the prostaglandin E receptor EP3 subtype has been implicated in thermal hyperalgesia through its actions in the preoptic hypothalamus as well as the diagonal band of Broca (Hosoi et al., 1997). This hyperalgesic effect of prostaglandin E2 in the MPOA activates pain-modulating circuitry in the RVM by activating pain-facilitatory ON-cells, and concomitantly suppressing pain-inhibitory OFF-cells (Heinricher et al., 2004). Finally, a recent study (Zhang and Ennis, 2007) demonstrated that inactivation of the PAG with lidocaine attenuated the antinociception elicited by chemical stimulation with D,L homocysteate administered into the MPOA. These data collectively suggest a direct MPOA-PAG connection that is directly implicated in nociceptive and antinociceptive responses.

The Ventromedial Hypothalamus (VMH): Like the MPOA, the VMH is a sexually-dimorphic nucleus intimately involved in the initiation of the lordotic response in female rodents (Pfaff, 1980; Pfaff et al., 1994) acting through estrogen receptors found in the VMH nuclei (McCarthy et al., 1993; Ogawa et al., 1996). Therefore, inactivation of estrogen receptors in the VMH through gene knockdown techniques can disrupt the entire lordotic sequence even though all of the brainstem and spinal apparatus necessary for the muscular responses are intact. Further, such responses are sex-dependent in that sexual behaviors in the male are unaffected (Ogawa et al., 1997). There is considerable evidence for a gonadal-opiate interaction such that activation of mu opiate receptors in the VMH (Ono et al., 1980) inhibits lordosis in female rats (Acosta-Martines and Etgen, 2002). Like the MPOA, there are direct VMH-PAG connections involved in descending inhibitory control of nociception (Dostrovsky et al., 1983) as well as the VMH termination of an ascending nociceptive pathway, the spino (trigemino) parabrachiohypothalamic pathway (Bester et al., 1995). Whereas electrical stimulation of the VMH induces analgesia (Rhodes and Liebeskind, 1978; Culhane and Carstens, 1988), electrical destruction of the VMH

produces hyperalgesia (Vidal and Jocob, 1980). Intra-VMH microinjection of interleukin 1β (Il-1β) produces analgesia in rats which is blocked by a cyclooxygenase inhibitor, indicating the role of prostanoids synthesis (Oka et al., 1995). Il-1β increases the release of prostaglandin E2 (PGE2) from rat hypothalamic explants (Navarra et al., 1992), and PGE2 in the VMH has been shown to have antinociceptive effects through its actions on EP1 receptors in rats (Masako et al., 1999). Taken together, these properties of VMH neurons enable them to have direct or at least indirect influence on nociception and analgesic processes.

Rationale and Hypothesis for Specific Aims

The previous background section provided evidence for the demonstration that: a) a supraspinal pain-inhibitory system in rodents and humans was described centered around the vlPAG and RVM mediating the systemic and central effects of morphine; b) activation of this opioid-mediated pain-inhibitory system is greater both in terms of magnitude and potency in male relative to female rodents; c) the sex differences in systemic and central opioid-mediated analgesia are mediated in part by the respective activational and organizational roles of gonadal hormones; and d) mechanisms by which gonadal hormones or sex status mediate the sex differences in morphine analgesia include differences in retrogradely-labeled PAG-RVM output neurons, Fos-induced activation levels in the PAG, mu opioid receptor expression in the PAG, and numbers of androgen receptor-immunoreactive neurons in the PAG.

Two mechanistic questions have been identified in the gonadal hormone mediation of sex differences in morphine analgesia that are addressed in the following two Specific Aims.

The **First Specific Aim** of this dissertation was to evaluate the interaction between organizational and activational effects of gonadal hormones on morphine analgesia following systemic administration **(Specific Aim 1A)** and intracerebral administration into the vlPAG

(Specific Aim 1B) in female rats receiving neonatal androgenization and/or adult ovariectomies and gonadal hormone replacement as compared to control male rats. In these two studies, the following seven groups of rats are evaluated: a) female rats receiving vehicle treatment 1 day after birth and sham gonadal surgeries in adulthood and tested during the estrus phase (Veh-Sham Females); b) female rats receiving neonatal androgenization with TP one day after birth and sham gonadal surgeries in adulthood (TP-Sham Females); c) female rats receiving vehicle treatment 1 day after birth and ovariectomy in adulthood (Veh-Ovex Females); d) female rats receiving neonatal androgenization with TP one day after birth and ovariectomy in adulthood (TP-Ovex Females); e) Veh-Ovex female rats receiving estradiol benzoate (EB) replacement (Veh-Ovex-EB); f) TP-Ovex female rats receiving EB replacement (TP-Ovex-EB); and g) untreated male rats. Dose-dependent and time-dependent effects of morphine analgesia are monitored in two studies with Veh-Sham females tested during the estrus phase of the cycle: one using systemic administration of morphine (Study 1A) and one using intracerebral vlPAG administration of morphine (Study 1B).

The following hypotheses and inquiries are evaluated in the two studies:

1. Male rats will display significantly greater magnitudes and potencies of morphine analgesia than Veh-Sham Females, thereby confirming a sex difference.
2. TP-Sham Females will display significantly greater magnitudes and potencies of morphine analgesia than Veh-Sham Females, thereby confirming neonatal gonadectomy effects.
3. Veh-Ovex Females will display greater magnitudes and potencies of morphine analgesia than Veh-Sham Females, although to a lesser extent than TP-Sham female effects, thereby confirming occasionally-observed adult gonadectomy effects.

4. The relationship of neonatal and adult gonadectomy effects to further changes in morphine analgesia will be observed in the TP-Ovex female group relative to the TP-Sham, Veh-Ovex and Veh-Sham female groups, thereby examining interactions between organizational and activational effects of gonadal hormones.
5. The effectiveness of EB replacement therapy upon activational effects per se, and combined organizational-activational effects on morphine analgesia will be evaluated, thereby determining whether this replacement strategy reverses any of the observed adult gonadectomy effects.

The **Second Specific Aim** of this dissertation was to examine whether excitotoxic (ibotenic acid) chemical destruction of two gonadal hormone accumulating nuclei, the medial preoptic area (MPOA) or the ventromedial hypothalamic nucleus (VMH) would alter the dose-dependent, time-dependent and sex-dependent actions of systemic morphine analgesia in adult intact male, intact female and ovariectomized female rats. Both the MPOA and the VMH are estradiol, estrogen, androgen and progestin receptor-accumulating nuclei that have important sexually dimorphic characteristics. Second, the neurocircuitry of both have important connections with the PAG and other levels (e.g., RVM) of the pain-inhibitory axis. Third, both nuclei support close neurochemical interactions between gonadal steroid hormones on the one hand, and endogenous opioid systems on the other hand. Fourth, both nuclei also appear to be important in independently modulating both pain facilitatory and pain inhibitory actions. Therefore, the following groups of animals are assessed in this study: a) male rats receiving vehicle injections into the MPOA or VMH (Male-Veh MPOA, Male-Veh VMH); b) female rats receiving vehicle injections into the MPOA or VMH and tested during the estrus phase (Female-Veh MPOA, Female-Veh VMH); c) Female-Veh MPOA and Female-Veh VMH rats

subsequently receiving adult ovariectomy (Female-Veh-Ovex MPOA; Female-Veh-Ovex VMH), d) male rats receiving ibotenic acid excitotoxic lesions in the MPOA (Male-Ibo MPOA) or the VMH (Male-Ibo VMH), e) female rats receiving ibotenic acid excitotoxic lesions in the MPOA (Female-Ibo MPOA) or the VMH (Female-Ibo VMH), and f) female ovariectomized rats receiving ibotenic acid excitotoxic lesions in the MPOA (Female-Ovex-Ibo MPOA) or the VMH (Female-Ovex-Ibo VMH).

The following hypotheses were tested:

1. Male rats treated with vehicle will display significantly greater magnitudes and potencies of morphine analgesia than intact female rats treated with vehicle, thereby confirming a sex difference.

2. Veh-Ovex Females will display greater magnitudes and potencies of morphine analgesia than Veh-Sham Females, thereby confirming occasionally-observed adult gonadectomy effects.

3. The present data will reveal whether ibotenic acid-induced destruction of the MPOA and VMH alter baseline tail-flick latencies, and if so, are the changes in basal nociception observed in intact males alone, intact or Ovex females alone or in all groups?

4. Independent of any changes in basal nociception does ibotenic acid-induced destruction of the MPOA and VMH alter the magnitude and/or potency of systemic morphine-induced analgesia, and if so, are the changes in systemic morphine analgesia observed in intact males alone, intact or Ovex females alone or in all groups?

CHAPTER 2

General Methods

Subjects

Male and female albino Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA at approximately 70 days of age) were used as subjects and subsequently housed individually in wire mesh cages in the Queens College Vivarium. They were maintained on a 12 hour light/12 hour dark cycle with Purina rat chow and water available ad libitum.

Adult Female Gonadectomy Procedures

Animals were weighed and anesthetized with a combination of chlorpromazine hydrochloride (HCL) (5 mg/kg, IP) and Ketamine HCl (140 mg/ml, IM). Ovariectomies were performed by removing the ovaries and ovarian fat following a bilateral 1.0 cm dorsal incision (Kepler, et al., 1989; Romero and Bodnar, 1986; Romero et al., 1987, 1988). The ovarian artery was then tied and the skin stapled (Reflex Wound Clip System, Fine Scientific Tools, Foster City, CA). Sham ovariectomies were performed in the same manner except that the ovaries were only exposed and not removed.

Drugs and Injections (Systemic and Central)

Normal saline (0.9%) was used for all morphine vehicle injections. Morphine (Pennick Laboratories) was dissolved in normal saline. All subcutaneous injections of morphine were made through a standard 1 ml 25 gauge syringe into the loose skin between the shoulder blades. Intracerebral injections of morphine were administered in 1.0 µl volumes through a stainless steel internal cannula (33-gauge, Plastics One) that extended 0.5 mm past the guide cannula, and was connected to a Hamilton microliter syringe by polyethylene tubing. The internal cannula was left in place for at least 30 seconds to prevent loss of the drug by suction. Testosterone

Propionate (Sigma Chemical Co., St. Louis) was dissolved in sesame oil (Sigma Chemical Co., St. Louis) and administered subcutaneously through a standard 1 ml syringe with a 28 gauge needle to minimize the site of injection. The syringe needle was held in place for at least 30 seconds to prevent expulsion of the drug.

Estrous Phase Determination

Estrous cycle in intact females was assessed by daily vaginal smears taken 0-1 hours into the light cycle prior to testing which occurred 1-7 hours later. Samples were obtained using a transfer pipette to inject a small quantity (0.5-1.0 ml) of saline into the vagina and then evacuating it out and placing it on glass microscope slides. The slides were then coverslipped and viewed with light microscopy under 4.0x magnification. Sham females were only tested in the estrus phase of the estrous cycle based on the evidence showing possible differences in antinociceptive response in female rats as a function of estrous phase (e.g., Kepler et al., 1989). Although vaginal probing produces analgesia (e.g., Crowley et al., 1976), its time course of action completely dissipates within 2 min and the applied force to produce analgesia far exceeds the smear procedure.

Nociceptive Test

A tail-flick analgesiometer (IITC Co., Woodland Hills, CA) provided a radiant-heat source mounted 8 cm above a photocell upon which the rat's tail was placed. Radiant heat was applied 4 to10 cm proximal to the tip of the rat's tail; removal of the tail from the heat source 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 2.5 and 3.5 seconds. Each session consisted of three latency determinations at different points on the tail at 10 second intervals. To avoid tissue damage, a trial (and that session) was automatically terminated if a

response did not occur within 10 seconds. Baseline latencies were determined for at least 4 days before experimental testing to ensure stability of responding. All animals displayed consistent latencies in baseline and vehicle testing that did not appear subject to desensitization. The tail-flick test was chosen because of the substantial literature demonstrating sex differences in morphine and μ-opioid analgesia within labs, (e.g., Islam et al., 1993; Kepler et al., 1989; Kepler et al.1991; Krzanowska and Bodnar, 1999; Krzanowska and Bodnar, 2000; Krzanowska et al., 2002) across labs, (e.g., Barrett et al., 2002a; Barrett et al., 2002b; Bartok and Craft, 1997; Cook et al., 2000; Craft and Bernal, 2001; Craft et al., 2001; Kavaliers and Innes, 1990; Terner et al., 2002) and across species (e.g., Kest et al., 1999 and Mogil et al., 2000).

CHAPTER 3

Experiment 1 (Specific Aim 1A): Organizational Manipulation of Gonadal Hormones and Systemic Morphine Analgesia in Female Rats: Effects of Adult Ovariectomy and Estradiol Replacement

Background

Potent sex differences in analgesic processes have been described (e.g., see reviews: Bodnar et al., 2002; Craft et al., 2004), particularly in the magnitude of μ-opioid receptor agonist-induced analgesia with female rodents displaying significantly smaller responses than male rodents following systemic, ventricular or intracerebral injections (e.g., Baamonde et al., 1989; Badillo-Martinez et al., 1984; Boyer et al., 1998; Candido et al., 1992; Kaviliers and Innes, 1987; Kepler et al., 1989; Krzanowska and Bodnar, 1999, 2000). Genotype influences sex differences in morphine analgesia such that AKR/J, C57BL/6J and SWR/J murine strains show greater morphine analgesia in males, and the CBA/J strain shows greater morphine analgesia in females (e.g., Kest et al., 1999; Mogil et al., 2000). Moreover, sex differences in morphine analgesia are most potent in Wistar and Lewis rat strains, moderate in Sprague–Dawley and F344 strains, and least in Long Evans, Brown Norway and Holtzman strains (Terner et al., 2003a; 2003b). Age and sex interact in mediating these effects given significant age-related increases in female rats and decreases in male rats in the ED_{50} of morphine analgesia (Islam et al., 1993). Pharmacokinetic factors fail to explain the sex differences in the greater morphine analgesia in male relative to female rats (Cicero et al., 1996; 1997). It appears that more potent μ agonists (e.g., etorphine, DAMGO, hendorphin) produce more pronounced analgesic sex differences than less potent μ agonists (Cicero et al., 1997; Cook et al., 2003; Craft et al., 2001; Kepler et al., 1991; Krzanowska and Bodnar 2000; Negus and Mello, 1999; Terner et al., 2002).

Moreover, some (DPDPE, deltorphin), but not all δ agonists, and some (U50488H), but not all κ agonists produced greater analgesic responses in male rats (Barrett et al., 2002a; 2002b; Bartok and Kraft, 1997; Craft and Bernal, 2001; Kaviliers and Innes, 1990; Kepler et al., 1991; Patrick et al., 1999).

Whereas gonadectomy in adult male and female animals reduced analgesic sex differences elicited by both opioid-mediated and nonopioid-mediated swim stressors (e.g., see reviews: Bodnar et al., 2002; Craft et al., 2004), these manipulations minimally altered the magnitude or the potency of either morphine or DAMGO analgesia relative to intact males and females (Ali et al., 1995; Banerjee et al., 1983; Cicero et al., 1996; Cicero et al., 1997; Islam et al., 1993; Kasson and George, 1984; Kepler et al., 1989; Kepler et al., 1991; Krzanowska and Bodnar, 1999). This suggests that the classically-described activational effects of gonadal hormones (e.g., Phoenix et al., 1959) may not be pivotal in the mediation of these analgesic responses, although adult gonadectomy does affect μ agonist-mediated analgesia when using less potent agonists (Terner et al., 2002). Morphine analgesia was more potent in female rats during proestrus and diestrus than during estrus, but was less potent in gonadectomized females receiving estradiol, progesterone or testosterone (Kepler et al., 1989; Stoffel et al., 2003). In contrast, classically described organizational effects of gonadal hormones (e.g., Phoenix et al., 1959) may be important in the mediation of these responses given that morphine analgesia elicited from either the ventro-lateral periaqueductal gray (vlPAG: Krzanowska et al., 2002) or following systemic administration (Cicero et al., 2002) was profoundly affected by neonatal gonadectomy. Thus, adult male rats neonatally castrated on Day 1 after birth displayed magnitudes and potencies of either intracerebral or systemic morphine-induced analgesia that were significantly lower than sham-operated males, but similar to that of neonatal vehicle-treated

females tested during the estrus phase. Correspondingly, adult female rats neonatally treated with testosterone propionate (TP) on Day 1 after birth displayed magnitudes and potencies of either intracerebral or systemic morphine-induced analgesia that were significantly higher than neonatal vehicle-treated females tested during the estrus phase, but similar to that of sham operated males. Thus, these organizational manipulations may produce important changes in brain circuitry that influence gonadal hormone modulation of supraspinal (e.g., see reviews: (Bodnar et al., 2002; Craft et al., 2004; Pfaff, 1999) and spinal (Liu and Gintzler, 1999; 2000) sites relevant to analgesic processes.

Although these neonatal gonadectomy effects suggest a potential pure organizational role of gonadal hormones in mediating sex differences in morphine analgesia, this is not certain because neonatal androgenization in female rats produces an anovulatory syndrome that could change the adult hormonal milieu in female rats (see review: Pfaff, 1999). Several instances of such an interaction include the ability of adult ovariectomy to increase TP-induced aggressive behaviors in female mice treated neonatally with TP, but not vehicle (Compann et al., 1993) and to reverse the reductions in physiologically-elicited corticosterone parameters in female rats treated neonatally with TP (Seale et al., 2005). Combinations of neonatal TP and adult ovariectomy reversed the sex difference observed for the constitutive expression of preprocholecystokinin mRNA in the medial amygdala and bed nucleus of the stria terminalis with males showing higher expression than females (Micevych et al., 1994). However, adult ovariectomy failed to alter the increased dendritic branching in cortical pyramidal cells observed in female rats treated neonatally with TP (Stewart and Kolb, 1994). More detailed differentiation of the various causal routes of neonatal androgenization influences would also require exogenous steroid hormone treatment especially in the absence of female gonads. Estrogen alone or in

combination with progesterone has had mixed facilitatory and inhibitory effects upon opioid-induced analgesia per se (Banerjee et al., 1983; Chatterjee et al., 1982; Negus and Mello, 1999; 2002; Nomikos et al., 1991; Ratka and Simpkins, 1991), but exogenous gonadal hormone steroid replacement has not been tested for analgesic responses in female animals exposed to neonatal androgenization and/or adult gonadectomy. Therefore, the present study first examined whether adult ovariectomy altered the adult potency and magnitude of systemic morphine analgesia in neonatal androgenized female rats relative to female rats neonatally treated with vehicle. The present study then examined whether gonadal hormone steroid replacement with estradiol benzoate (EB) in neonatal androgenized and neonatal vehicle-treated females altered any adult ovariectomy-induced effects upon systemic morphine analgesia. To determine whether sex differences were observed, an additional group of male rats were tested as well. Both full dose–response (0, 1.0, 1.7, 2.5 and 5.0 mg/kg, SC) and time–response (30, 60, 90 and 120 min) functions of systemic morphine analgesia were assessed using the tail-flick test in all groups. The following hypotheses and inquiries were evaluated in the this study: male rats will display significantly greater magnitudes and potencies of morphine analgesia than Veh-Sham Females, thereby confirming a sex difference; TP-Sham Females will display significantly greater magnitudes and potencies of morphine analgesia than Veh-Sham Females, thereby confirming neonatal gonadectomy effects; Veh-Ovex Females will display greater magnitudes and potencies of morphine analgesia than Veh-Sham Females, although to a lesser extent than TP-Sham female effects, thereby confirming occasionally-observed adult gonadectomy effects; The relationship of neonatal and adult gonadectomy effects to further changes in morphine analgesia will be observed in the TP-Ovex female group relative to the TP-Sham, Veh-Ovex and Veh-Sham female groups, thereby examining interactions between organizational and activational effects of

gonadal hormones. This paper was published in Brain Research (1059: 13-19, 2005) with the candidate as senior author.

Methods

Procedure:

Timed pregnant female rats were obtained at approximately 12–14 days into gestation and were subsequently housed individually in polyethylene cages with animal bedding and monitored for delivery between days 20 and 22. With the date of birth denoted as day 0, pups were sexed and female rats randomly assigned to either experimental or sham condition and received either a vehicle injection of sesame oil (0.001 ml/g body weight, SC) or Testosterone Propionate (250 µg/kg in sesame oil, sc) on post-natal day 1. Female and male pups were weaned 3 weeks later and at 70 days of age, housed in individual wire mesh cages. Following the neonatal hormone manipulations, the female Veh and TP rats received either sham surgeries (Sham) or ovariectomy (Ovex). All animals were allowed a month to recover to allow gonadectomy-induced changes. At 100–110 days of age after baseline tail-flick testing, subgroups of Veh-Ovex and TP-Ovex females were chronically implanted with silastic tubing containing 75 µg of estradiol benzoate (EB: Veh-Ovex-EB or TP-Ovex-EB). A comparison group of untreated male animals from the same litters were raised as described above. These latter animals were not injected with oil as neonates, and given sham adult surgeries, as the female controls were. However, our laboratory has previously demonstrated that the analgesic time courses, magnitudes and potencies of morphine administered into the vlPAG were similar in untreated male rats (Krzanowska and Bodnar, 1999) and in male rats that received neonatal sham surgeries (Krzanowska et al., 2002). Therefore, seven groups of animals were tested in the behavioral procedures beginning at about 115 days of age. The Veh-Ovex-EB and TP-Ovex-EB

groups were tested under vehicle conditions twice, once before and once 4 days after EB implantation. The other five groups were tested under a single vehicle condition with tail-flick latencies assessed 30, 60, 90 and 120 min following each injection with all testing taking place 2–8 h into the light cycle. All groups then received ascending doses of morphine 1.0, 1.7, 2.5 and 5.0 mg/kg, subcutaneously at weekly intervals to minimize possible tolerance effects. The ascending regimen was employed to minimize the occurrence of going to cut-off values until later in the procedure. Although estrus phase does not markedly alter the magnitude of systemic morphine analgesia (Kepler et al., 1989; Stoffel et al., 2003), the present experiment still controlled for estrous phase by only testing the Veh-Sham group during the estrus phase of the cycle. On the test day, vaginal smears were taken 0–1 h into the light cycle with experimental testing occurring 1–7 h later. Although vaginal probing produces analgesia (e.g., Crowley et al., 1976), its time course of action completely dissipates within 2 min and the applied force to produce analgesia far exceeds the smear procedure. Latencies were determined 30, 60, 90 and 120 min following each morphine injection. This regimen samples the time course of peak systemic morphine action (e.g., Cicero et al., 1996; 1997; 2002; Islam et al., 1993).

Statistical Analysis:

Separate two-way repeated-measures analyses of variance were performed on vehicle-treatment tail-flick latencies with pre- and post-EB implantation as one variable and post-injection test times as the second variable for the Veh-Ovex-EB and TP-Ovex-EB groups. EB implantation failed to alter the vehicle-treatment latencies across the time course (data not shown), and the post-EB vehicle scores for each group served as the basis for vehicle treatment in the subsequent analyses. An initial three-way randomized-block analysis of variance was performed on tail-flick latencies with the seven groups serving as a between-subjects variable,

the vehicle and four morphine doses as one within-subject variable, and the four post-injection test times as the second within-subject variable. A subsequent three-way randomized-block analysis of variance was performed on the two peak time points (30 and 60 min) for each of the variables indicated above. Finally, to assess differences in analgesic magnitudes among groups and doses summed baseline latencies at 30 and 60 min were subtracted from peak (30 + 60 min) analgesic latencies for each dose across groups, and a two-way randomized-block analysis of variance was performed with groups as the between-subject variable and morphine doses as the within subject variable. For all analyses, Tukey planned two-tailed comparisons ($P < 0.05$) discerned significant effects relative to corresponding vehicle values within groups, and to corresponding dose and time conditions relative to other groups. Changes in the potency of systemic morphine analgesia across groups were evaluated by performing linear regression analyses for peak (30 + 60 min) analgesic difference score latencies, and calculating ED_{50} values as a doubling of latency difference scores (5.8 s) above the mean baseline value (2.9 s).

Results:

Significant differences in tail-flick latencies were observed among groups, across doses, across times and for all interactions (Table 1, left column). Baseline tail-flick latencies failed to differ among groups, indicating lack of baseline differences as functions of sex, neonatal manipulation, adult ovariectomy or gonadal hormone replacement. As expected, morphine dose-dependently increased tail-flick latencies relative to corresponding group vehicle values in the Veh-Sham [1.7 (60 min), 2.5 (60 min), 5 (30–120 min) mg/kg], Veh-Ovex [2.5 (30–60 min), 5 (30–120 min) mg/kg], Veh-Ovex-EB [1 (30 min), 1.7 (30–90 min), 2.5 (30–120min), 5 (30–120 min) mg/kg], TP-Sham [1 (30–60 min), 1.7 (30–60 min), 2.5 (30–90 min), 5 (30–120 min) mg/kg], TP-Ovex [1 (60 min), 1.7 (60–90 min), 2.5 (60–90 min), 5 (30–120 min) mg/kg], TP-

TABLE 1. Summary of significant statistical effects across analyses.

Factors	Time Course ANOVA	Peak Effect ANOVA	Peak Difference Score ANOVA
Groups	$F(6,60)=3.39, p<0.006$	$F(6,60)=8.76, p<0.0001$	$F(6,60)=8.13, p<0.0001$
Morphine Doses	$F(4,40)=752.15, p<0.0001$	$F(4,40)=443.75, p<0.0001$	$F(3,30)=392.04, p<0.0001$
Test Times	$F(3,30)=128.81, p<0.0001$	$F(1,10)=64.83, p<0.0001$	n/a
Group x Dose	$F(24,240)=13.92, p<0.0001$	$F(24,240)=9.062, p<0.013$	$F(18,180)=9.18, p<0.0001$
Group x Time	$F(18,180)=15.48, p<0.0001$	$F(6,60)=3.70, p<0.003$	n/a
Dose x Time	$F(12,120)=30.08, p<0.0001$	$F(4,40)=57.04, p<0.0001$	n/a
Group-Dose-Time	$F(72,720)=4.82, p<0.0001$	$F(24,240)=3.48, p<0.0001$	n/a

Ovex-EB [1 (30–60 min), 1.7 (30–60 min), 2.5 (30–120 min), 5 (30–120 min) mg/kg] and Males [1 (30–60 min), 1.7 (30–60 min), 2.5 (30–120 min), 5 (30–120 min) mg/kg] groups. Thus, peak morphine analgesia was most evident 30 and 60 min following injection, whereas the analgesic effects and differences among groups dissipated after 90 and 120 min. Therefore, subsequent analyses focused on peak analgesic effects, and confirmed that significant differences in tail-flick latencies were observed among groups, across doses, between the two times and for all interactions at these peak intervals (Table 1, middle column; Fig. 1: depicted by asterisks) as well as for the magnitude of combined peak morphine analgesia 30 and 60 min after injection after subtracting baseline values from each drug dose score (Table 1, right column). Significant sex differences in the magnitude of systemic morphine analgesia were observed with the Males group displaying significantly greater analgesic responses than the female Veh-Sham group after 30 and 60 min following the 1, 1.7 and 2.5 mg/kg doses, and after 30 min following the 5 mg/kg dose (Fig. 1: +). There was a corresponding 2.3-fold leftward shift in peak analgesic potency of the Male group (ED_{50} = 1.66 mg/kg) relative to the female Veh/Sham group (ED_{50} = 3.87 mg/kg) (Table 2). The Male group also displayed significantly greater analgesic responses than the female TP-Sham group following the 1.7 (30–60 min), 2.5 (30 min) and 5 (60min) mg/kg doses (Fig. 1: #) with an approximate 2-fold leftward shift in the potency of morphine analgesia in the Male relative to the TP/Sham (ED_{50} = 3.04 mg/kg) groups (Table 2). Although neonatal androgenization produced significant differences in the magnitude of systemic morphine analgesia with the female TP-Sham group displaying significantly greater analgesic responses than the female Veh-Sham group following the 1 (30–60 min) and 5 (30–60 min) mg/kg doses (Fig. 1: +), the potency of morphine analgesia was only shifted modestly leftward (20%) in the TP/Sham (3.04 mg/kg) relative to the Veh/Sham (3.87 mg/kg) groups

Figure 1. Alterations in tail-flick latencies (s, mean ±S.E.M.) 30 and 120 min following subcutaneous administration of vehicle or morphine at doses of 1 (Panel A), 1.7 (Panel B), 2.5 (Panel C) and 5 (Panel D) mg/kg in adult female rats neonatally (within 24 h of birth) treated with either vehicle (Veh: sesame oil, SC) or testosterone propionate (TP: 250 μg, SC), and then receiving either surgical control (Veh-Sham, TP-Sham) or ovariectomy (Veh-Ovex, TP-Ovex) procedures 70 days after birth. At 100-110 days after birth, subgroups of the ovariectomized animals were subcutaneously implanted with silastic tubing filled with estradiol benzoate (EB: 75 μg: Veh-Ovex-EB, TP-Ovex-EB), and were tested for baseline latency determinations before and following EB administration with no differences observed. A comparison group of male rats (Males) is depicted as well. The morphine doses were given in ascending order at weekly intervals. Latencies for the Veh-Sham (2.54-2.92 s; n=6), Veh-Ovex (2.81-2.89 s; n=6), Veh-Ovex-EB (2.52-2.65 s; n=6), TP-Sham (3.06-3.09 s; n=8), TP-Ovex (3.00-3.10 s; n=11), TP-Ovex-EB (2.98-3.00 s; n=8) and Male (2.94-3.10 s; n=6) groups observed 30 and 60 min following vehicle injection treatments failed to differ from one another, and the overall mean vehicle tail-flick latency of all groups at both times is depicted as a horizontal line in each figure panel to display analgesic magnitude for each morphine dose in each group. Significant differences (Tukey planned comparisons, $P<0.05$) in tail-flick latencies are respectively depicted between specific morphine doses and corresponding vehicle conditions for each group (*), between Veh-Sham females and other groups (+), between TP-Sham females and other groups (#), and between EB-induced changes in Ovex animals ($).

Figure 1

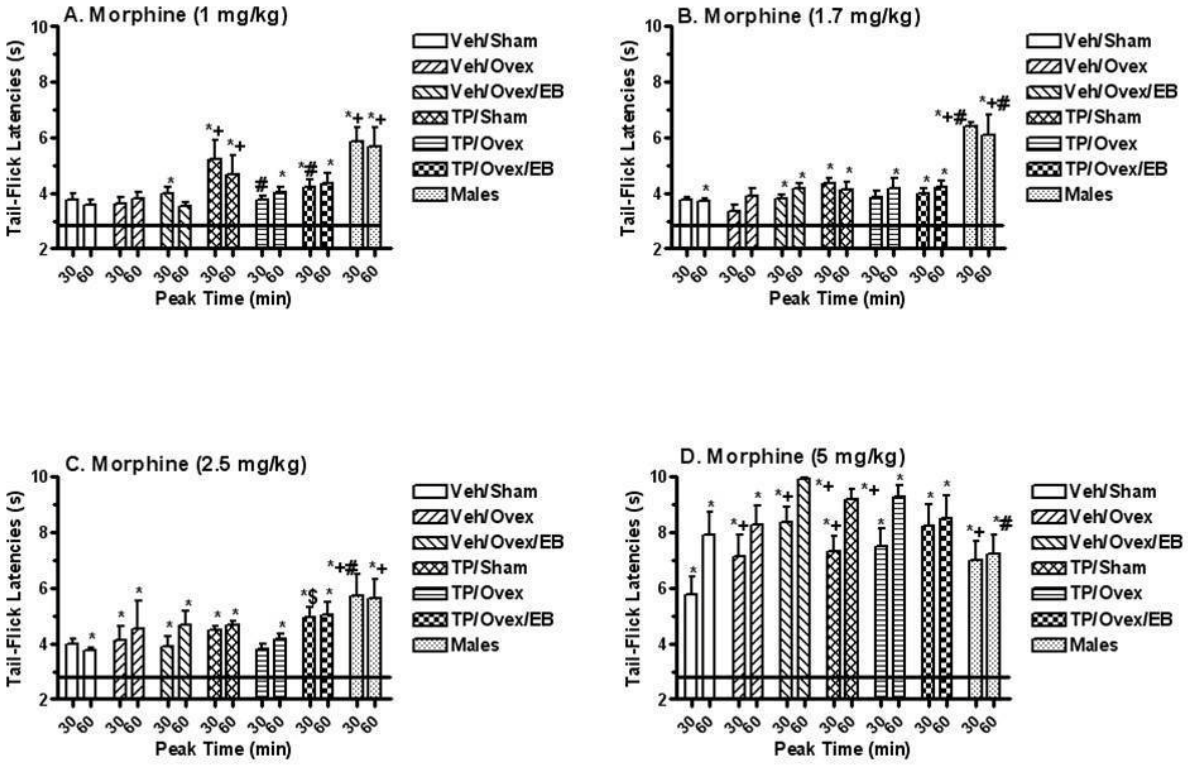

A. Morphine (1 mg/kg)
B. Morphine (1.7 mg/kg)
C. Morphine (2.5 mg/kg)
D. Morphine (5 mg/kg)
Tail-Flick Latencies (s)
Peak Time (min)
Veh/Sham
Veh/Ovex
Veh/Ovex/EB
TP/Sham
TP/Ovex
TP/Ovex/EB
Males

TABLE 2. Summary of ED_{50} values* for morphine analgesia across groups.

Group	**ED_{50} Value**
Veh-Sham Females	3.87 mg/kg
Veh-Ovex Females	3.38 mg/kg
Veh-Ovex-EB Females	2.69 mg/kg
TP-Sham Females	3.04 mg/kg
TP-Ovex Females	3.28 mg/kg
TP-Ovex-EB Females	2.95 mg/kg
Males	1.66 mg/kg

*ED_{50} values were calculated as a doubling of latency difference scores (5.8 s) above the mean baseline value (2.9 s).

(Table 2). Adult ovariectomy failed to alter the magnitude (Fig. 1) or potency (Table 2) of morphine analgesia in the female Veh-Ovex group (ED_{50} = 3.38 mg/kg). Although the female TP-Ovex (ED_{50} = 3.28 mg/kg) and TP-Ovex-EB (ED_{50} = 2.95 mg/kg) groups displayed significant reductions in morphine analgesia 30 min following the 1 mg/kg dose relative to the female TP-Sham group (Fig. 1: #), there was only a minimal 8% change in the potency of morphine analgesia (Table 2). However, EB replacement significantly increased the magnitude of morphine analgesia after 30 and 60 min at the highest 5 mg/kg dose in the female Veh-Ovex-EB group (ED_{50} = 2.69 mg/kg) as well as the potency of morphine analgesia relative to either the female Veh-Sham (30% leftward shift) or the Veh-Ovex (20% leftward shift) groups (Fig. 1: +/S; Table 2). EB replacement also significantly increased morphine analgesia after 30 min at the 2.5 mg/kg dose in the female TP-Ovex-EB group (Fig. 1: $; ED_{50} =2.95 mg/kg), but failed to alter the potency of morphine analgesia (Table 2).

Discussion

The following hypotheses and inquiries were evaluated in this study: male rats will display significantly greater magnitudes and potencies of morphine analgesia than Veh-Sham Females, thereby **confirming a sex difference**; TP-Sham Females will display significantly greater magnitudes and potencies of morphine analgesia than Veh-Sham Females, thereby **confirming neonatal gonadectomy effects**; Veh-Ovex Females will display greater magnitudes and potencies of morphine analgesia than Veh-Sham Females, although to a lesser extent than TP-Sham female effects, thereby **confirming occasionally-observed adult gonadectomy effects**; The relationship of neonatal and adult gonadectomy effects to further changes in morphine analgesia will be observed in the TP-Ovex female group relative to the TP-Sham, Veh-

Ovex and Veh-Sham female groups, thereby examining **interactions between organizational and activational effects of gonadal hormones**.

Sex Differences Neonatal and Adult Gonadectomy Effects: In the present study, intact female rats (Veh-Sham group) displayed significantly lower magnitudes and reduced potencies of systemic morphine analgesia than intact male rats (Males group). This is in keeping with the profound sex differences observed for μ-opioid receptor agonist-induced analgesia following systemic, ventricular or intracerebral injections in rats (e.g., Baamonde et al., 1989; Badillo-Martinez et al., 1984; Boyer et al., 1998; Candido et al., 1992; Cicero et al., 1996, 1997; Cook et al., 2000; Craft et al., 2001; Islam et al., 1993; Kasson and George, 1984; Kepler et al., 1989, 1991; Krzanowska and Bodnar, 1999, 2002; Negus and Mello, 1999; Terner et al., 2002) or inbred strains of mice (Kest et al., 1999; Mogil et al., 2000). Female rats neonatally androgenized with TP (TP-Sham group) displayed significantly greater magnitudes of systemic morphine analgesia than intact female rats (Veh-Sham group), although the shift in the potency of morphine analgesia was not as pronounced. TP/Sham female rats displayed lesser magnitudes and potencies of morphine analgesia relative to intact male rats, a finding generally consistent with previous findings for systemic (Cicero et al., 2002) and intracerebral vlPAG (Krzanowska et al., 2002) morphine analgesia. These data confirm an important role for the classically described organizational effects of gonadal hormones (e.g., Phoenix et al., 1959) in potentially modulating supraspinal (e.g., see: Bodnar et al., 2002; Craft et al., 2004; Pfaff, 1999) and spinal (Liu and Gintzler, 1999, 2000) sites relevant to analgesic processes. Adult ovariectomy produced minimal effects upon the magnitude or potency of morphine analgesia in female rats neonatally treated with vehicle, confirming the previously-described general inability of adult ovariectomy to alter morphine or DAMGO analgesia relative to intact females (Cicero et al., 1996, 2002;

Islam et al., 1993; Kepler et al., 1989, 1991; Krzanowska and Bodnar, 1999). This further suggests that the classically described activational effects of gonadal hormones (e.g., Phoenix et al., 1959) may not be very pivotal in the mediation of these analgesic responses in normal females.

Interactions Between Organizational and Activational Effects of Gonadal Hormones: Whereas ovariectomy decreased the magnitude of morphine analgesia in female rats treated neonatally with TP following the lowest dose of morphine, there were only minimal (8%) changes in the potency of morphine analgesia between the two groups. These data indicate that the interaction between manipulations of organizational (e.g., neonatal androgenization) and activational (e.g., adult ovariectomy) effects of gonadal hormones do not appear to add or detract from the ability of neonatal androgenization to increase the magnitude of morphine analgesia in female rats. This differs from other instances in which either neonatal androgenization affected adult ovariectomy-induced changes in function, or adult ovariectomy affected neonatal androgenization-induced changes in function. Thus, adult ovariectomy increased TP-induced aggressive behaviors in female mice treated neonatally with TP, but not vehicle (Compann et al., 1993). Adult ovariectomy also reversed the reductions in physiologically-elicited corticosterone parameters in female rats treated neonatally with TP (Seale et al., 2005). Combinations of neonatal TP and adult ovariectomy reversed the sex difference observed for the constitutive expression of preprocholecystokinin mRNA in the medial amygdala and bed nucleus of the stria terminalis with males showing higher expression than females (Micevych et al., 1994). However, adult ovariectomy failed to alter the increased dendritic branching in cortical pyramidal cells observed in female rats treated neonatally with TP (Stewart and Kolb, 1994). A major candidate for any activational modulation of organizational effects is estrogen given its previously

described actions upon morphine analgesia in intact animals (Banerjee et al., 1983; Chatterjee et al., 1982; Negus and Mello, 1999, 2002; Nomikos et al., 1991; Ratka and Simpkins, 1991). In this regard, opioid analgesia was facilitated (Negus and Mello, 1999, 2002; Nomikos et al., 1991), unaffected (Banerjee et al., 1983) or reduced (Chatterjee et al., 1982; Ratka and Simpkins, 1991) in ovariectomized female rats treated with EB alone or in combination with progesterone. The present study also found that EB treatment significantly increased the magnitude and potency (20–30%) of morphine analgesia, particularly at the highest morphine dose relative to Veh-Sham and Veh-Ovex female rats. In contrast, EB administered to neonatally androgenized females produced relatively minor changes in the magnitude and potency of morphine analgesia relative to intact and ovariectomized female rats receiving neonatal androgens. Thus, the circulating levels of EB appear to be more important in modulating morphine analgesia in normal and ovariectomized females than in females treated neonatally with androgens. Hence, the organizational effects of neonatal androgenization in female rats upon the magnitude and potency of morphine analgesia appear to be due more to intrinsic changes in that stage of development, and less affected by adult gonadal hormone manipulations.

CHAPTER 4

Experiment 2 (Specific Aim 1B): Organizational and Activational Gonadal Hormone Interactions upon Morphine Analgesia Elicited From the Ventrolateral Periaqueductal Gray in Female Rats

Background

The activational and organizational effects of gonadal hormones have been clearly differentiated (e.g., Phoenix et al., 1959), and can be manipulated experimentally in animals by adult and neonatal gonadectomy respectively. There are also clear instances of interactions between activational and organizational effects of gonadal hormones in female rodents in which either neonatal androgenization affected adult ovariectomy-induced changes in function, or adult ovariectomy affected neonatal testosterone propionate (TP) androgenization-induced changes in function. Thus, adult ovariectomy increased TP-induced aggressive behaviors in female mice treated neonatally with TP (Compann et al., 1993), and reversed the reductions in physiologically-elicited corticosterone parameters in female rats treated neonatally with TP (Seale et al. 2005). Neonatal androgenization and adult gonadectomy enhanced the amount of time spent in the open arms of a plus maze in female, but not male rats with the combined gonadal treatments produced the most marked effects (Zimmerberg and Farley, 1993). Adult gonadectomy blocked the neonatal gonadectomy-induced changes in electroencephalographic activity in both male and female rats (Corsi-Cabrera et al., 2000). Combinations of neonatal TP and adult ovariectomy reversed the sex difference observed for the constitutive expression of preprocholecystokinin mRNA in the medial amygdala and bed nucleus of the stria terminalis with males showing higher expression than females (Micevych et al., 1994).

Changes in nociceptive and analgesic responses are also observed as a result of activational and organizational gonadal hormone manipulations. In animals with lumbar radiculopathy, female rats display decreased thresholds to mechanical and thermal stimuli relative to males, an effect reversed in neonatally-androgenized females and in females receiving adult ovariectomy six weeks earlier (LaCroix-Fralish et al., 2005). Borzan and Fuchs (2006) demonstrated that male rats undergoing adult or neonatal gonadectomy displayed enhanced inflammation-induced sensitivity to mechanical, but not thermal stimulation with thresholds in the former, but not latter group reinstated by testosterone. A low morphine was less effective in inducing analgesia in control or inflamed animals receiving neonatal gonadectomy. Analgesic sex differences have been observed with female rodents displaying significantly less μ-opioid receptor agonist-induced analgesia than male rodents following systemic (e.g., Baamonde et al., 1989; Badillo-Martinez et al., 1984; Candido et al., 1992; Cicero et al., 1996; Kavaliers and Innes, 1987) and central (Boyer et al., 1998; Kepler et al., 1998, 1989, 1991; Krzanowska and Bodnar, 1999, 2000; Loyd and Murphy, 2006) administration, effects not due to pharmacokinetic factors, but sensitive to the potency of the μ agonists (Bartok and Craft, 1997; Cicero et al., 1997; Cook et al., 2000; Craft and Bernal, 2001; Kepler et al., 1989; Krzanowska and Bodnar, 2000; Negus and Mello, 1999; Terner et al., 2003a). Estrous phase differences were also noted in central morphine analgesia in female rats tested during proestrus and diestrus relative to estrus (Bernal et al., 2007; Kepler et al., 1989; Shane et al., 2007). Adult gonadectomy in male and female animals typically produce minimal alterations in the magnitude or the potency of μ agonist-mediated analgesia following systemic and ventricular administration relative to intact animals (e.g., Ali et al., 1995; Banerjee et al., 1983; Cicero et al., 1996, 2002; Islam et al., 1993; Kasson and George, 1984; Kepler et al., 1989, 1991), although adult gonadectomy reduces

analgesia in males and increases analgesia in females when using less potent μ agonists (Terner et al., 2003a). Moreover, systemic morphine analgesia was less potent in adult gonadectomized females receiving estradiol, progesterone or testosterone (Stoffel et al., 2003). Further, adult, ovariectomized females displayed greater and more potent morphine analgesia elicited from the ventrolateral periaqueductal gray (vlPAG) than intact females tested during the estrus phase (Krzanowska and Bodnar, 1999). Neonatal gonadectomy, affecting the organizational effects of gonadal hormones, mediates morphine analgesia elicited from the vlPAG (Krzanowska et al., 2002) or following systemic administration (Cicero et al., 2002). Thus, adult male rats neonatally castrated on Day 1 after birth displayed magnitudes and potencies of either intracerebral or systemic morphine-induced analgesia that were significantly lower than sham-operated males, but similar to that of neonatal vehicle-treated females tested during the estrus phase. Correspondingly, adult female rats neonatally treated with TP on Day 1 after birth displayed magnitudes and potencies of either intracerebral or systemic morphine-induced analgesia that were significantly higher than neonatal vehicle-treated females tested during the estrus phase, but similar to that of sham-operated males.

The present study was designed to examine organizational and activational gonadal hormone interactions in female rats in their analgesic response to morphine elicited from the vlPAG because this site appears maximally sensitive to changes in morphine analgesia as a function of sex differences, organizational and activational effects of gonadal hormones, and estrous phase (Bernal et al., 2007; Krzanowska and Bodnar, 1999; Krzanowska et al., 2002; Loyd and Murphy, 2006; Shane et al., 2007). Moreover, hypothalamic enkephalinergic neurons, sensitive to changes in sex hormone levels, turn on enkephalin genes in females to a greater degree than males (Priest et al., 1995; Romano et al., 1988, 1989, 1990), and eventually project

to estrogen-binding PAG neurons (Turcotte and Blaustein, 1999), a pathway absent following hypothalamic lesions (Hoffman et al., 1996). Mu opioids facilitate excitation in vlPAG cells via interactions with NMDA (Kow et al., 2002), and the ultrastructural arrangement of the mu opioid receptor with GABAergic PAG neurons or PAG projection neurons labeled retrogradely from the medulla indicate that mu opioid receptor ligands both act to inhibit the former, and act directly on the latter (Commons et al., 2000).

Therefore, the present study compared the analgesic effects of morphine analgesia elicited from the vlPAG across a dose range (1-5 μg) and time course (30-120 min) on the tail-flick test in females neonatally treated with either vehicle or TP and subjected to sham surgery or ovariectomy as compared to a group of male rats. The following hypotheses and inquiries were also evaluated in this study except for morphine effects within the vlPAG: male rats will display significantly greater magnitudes and potencies of morphine analgesia than Veh-Sham Females, thereby confirming a sex difference; TP-Sham Females will display significantly greater magnitudes and potencies of morphine analgesia than Veh-Sham Females, thereby confirming neonatal gonadectomy effects; Veh-Ovex Females will display greater magnitudes and potencies of morphine analgesia than Veh-Sham Females, although to a lesser extent than TP-Sham female effects, thereby confirming occasionally-observed adult gonadectomy effects; The relationship of neonatal and adult gonadectomy effects to further changes in morphine analgesia will be observed in the TP-Ovex female group relative to the TP-Sham, Veh-Ovex and Veh-Sham female groups, thereby examining interactions between organizational and activational effects of gonadal hormones. This paper was accepted for publication in the International Journal of Neuroscience scheduled for publication in 2010 with the candidate as senior author.

Methods

Procedure:

Timed pregnant female rats were obtained at approximately 12–14 days into gestation and subsequently housed individually in polyethylene cages with animal bedding were monitored for delivery between days 20 and 22. With the date of birth denoted as day 0, pups were sexed and female rats randomly assigned to either experimental or control condition and received either a vehicle injection of sesame oil (0.001 ml/g body weight, SC) or Testosterone Propionate (250 µg/kg in sesame oil, SC) on post-natal day 1. Female and male pups were weaned three weeks later and at 70 days of age, housed in individual wire mesh cages. Following the neonatal hormone manipulations, the female Veh and TP rats were weighed and anesthetized with a combination of chlorpromazine (5 mg/kg, IP) and Ketamine HCl (100 mg/ml, IM). These two groups of animals received either sham surgeries (Veh-Sham, TP-Sham) or ovariectomy (Veh-Ovex, TP-Ovex). All four groups of females and a group of anesthetized male rats (Males) treated post-natally with sesame oil were then anesthetized with a combination of chlorpromazine HCL (5 mg/ml, IP) and ketamine HCL (140 mg/ml, IM) and each animal was stereotaxically (Kopf Instruments 900 series) implanted with a guide cannula (26 ga., Plastics One Inc.) aimed at the vlPAG using the following coordinates: 0.3-0.5 mm anterior to the lambda suture, 1.5-1.7 mm lateral and angled 12^{o} towards the sagittal suture, and 6.8 mm from the top of the skull. The cannula was secured to the skull by three anchor screws with dental acrylic, and kept patent with a dummy cannula (Plastics One Inc). To allow full drug clearance, all animals were allowed at least one week to recover from stereotaxic surgery before any other procedure was performed. Therefore, five groups of animals were tested in the behavioral

procedures beginning at about 90 days of age: Veh-Sham (n=17), Veh-Ovex (n=15), TP-Sham (n=18), TP-Ovex (n=20) and Males (n=6).

All five groups were tested following a single vehicle microinjection (1.0 μl of 0.9% normal saline) delivered into the vlPAG. Tail-flick latencies were then assessed 30, 60, 90 and 120 min following each injection with all testing taking place 2-8 h into the light cycle. All female groups then received morphine at ascending doses of 1.0, 1.7, 2.5 and 5.0 μg at weekly intervals to minimize possible tolerance effects. The 1.0 μl injection produces a spread of approximately 1.0 mm^3 which should be noted, could possibly spread slightly beyond the intended target and into the cerebral aqueduct although the ED_{50} of morphine doses administered into the ventricular system has been shown to be 5-10x higher than central doses (Kepler et al., 1989). The Male group was only tested at the 1.0, 1.7 and 2.5 μg doses in the vlPAG as they previously reached peak analgesia at this dose range (see review: Bodnar et al., 2002). The ascending regimen was employed to minimize the occurrence of going to cut-off values until later in the procedure. The present experiment controlled for estrous phase given observed differences in vlPAG morphine analgesia (Bernal et al., 2007; Shane et al., 2007) by testing the Veh-Sham group only during the estrus phase of the cycle. On the test day, vaginal smears were taken 0-1 h into the light cycle with experimental testing occurring 1-7 h later. Latencies were determined 30, 60, 90 and 120 min since this regimen samples the time course of peak vlPAG morphine analgesic action (see review: Bodnar et al., 2002).

After the completion of testing, all animals received an overdose of anesthetic (Euthasol, Del Marva Laboratories, 390 mg/ml sodium pentobarbital; 50 mg/ml sodium phenytoin; 0.05 ml/kg, IP), and transcardiac perfusions with 0.9% normal saline followed by 10% buffered formalin. After removal, their brains were sectioned coronally at 40 μm and then stained with

Cresyl violet. They were then examined by light microscopy by an observer unfamiliar with the behavioral data and only animals with confirmed cannula placements were included in the data analysis.

Statistical Analysis:

A three-way randomized-block analysis of variance was performed on tail-flick latencies with the five groups serving as a between-subjects variable, the vehicle and three common (1, 1.7 and 2.5 μg) morphine doses as one within-subject variable, and the four post-injection test times as the second within-subject variable. A second three-way randomized-block analysis of variance was performed with the four female groups serving as the between-subject variable, the vehicle and high (5 μg) morphine dose as one within-subject variable, and the four post-injection test times as the second within-subject variable. For all analyses, Tukey comparisons ($P<0.05$) discerned significant effects relative to corresponding vehicle values within groups, and to corresponding dose and time conditions relative to other groups. Changes in the potency of vlPAG morphine analgesia across groups were evaluated by performing linear regression analyses on vlPAG analgesic difference score latencies which were derived by subtracting each of the vehicle latencies from each of the post-drug latencies, and summing those scores across the time course. ED_{50} values were defined as a tripling of latency difference scores above each mean baseline value.

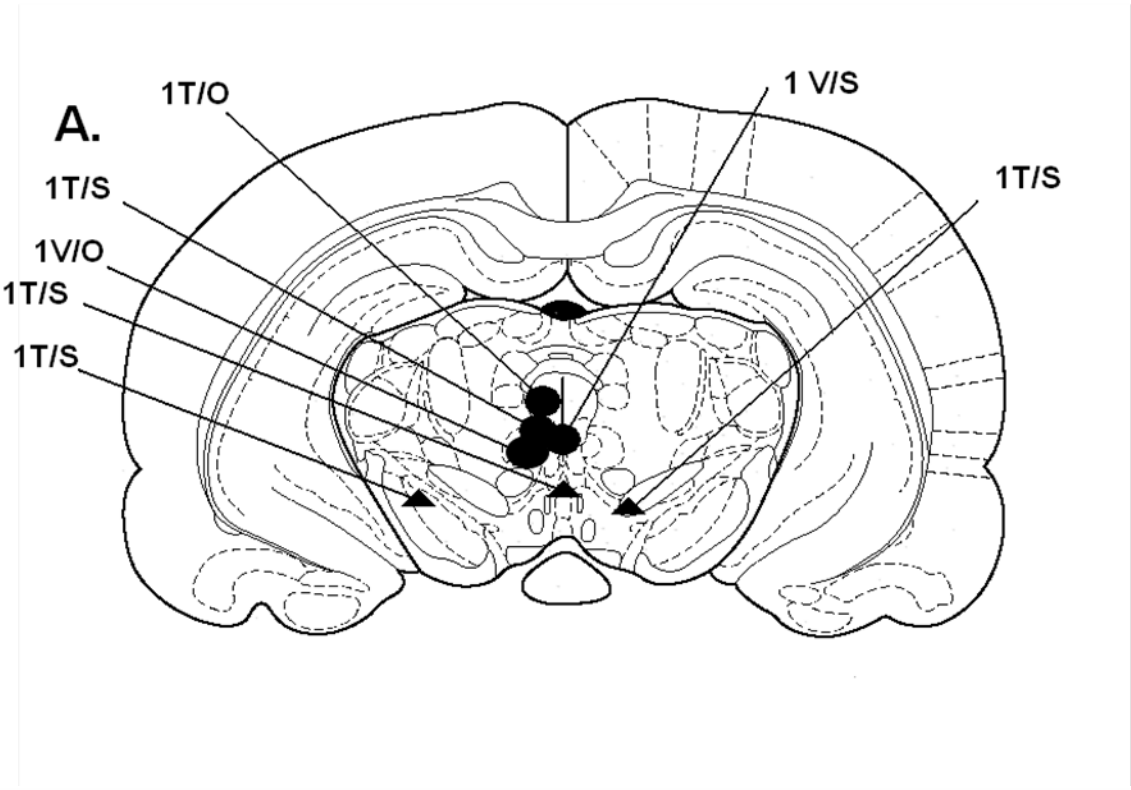
Results

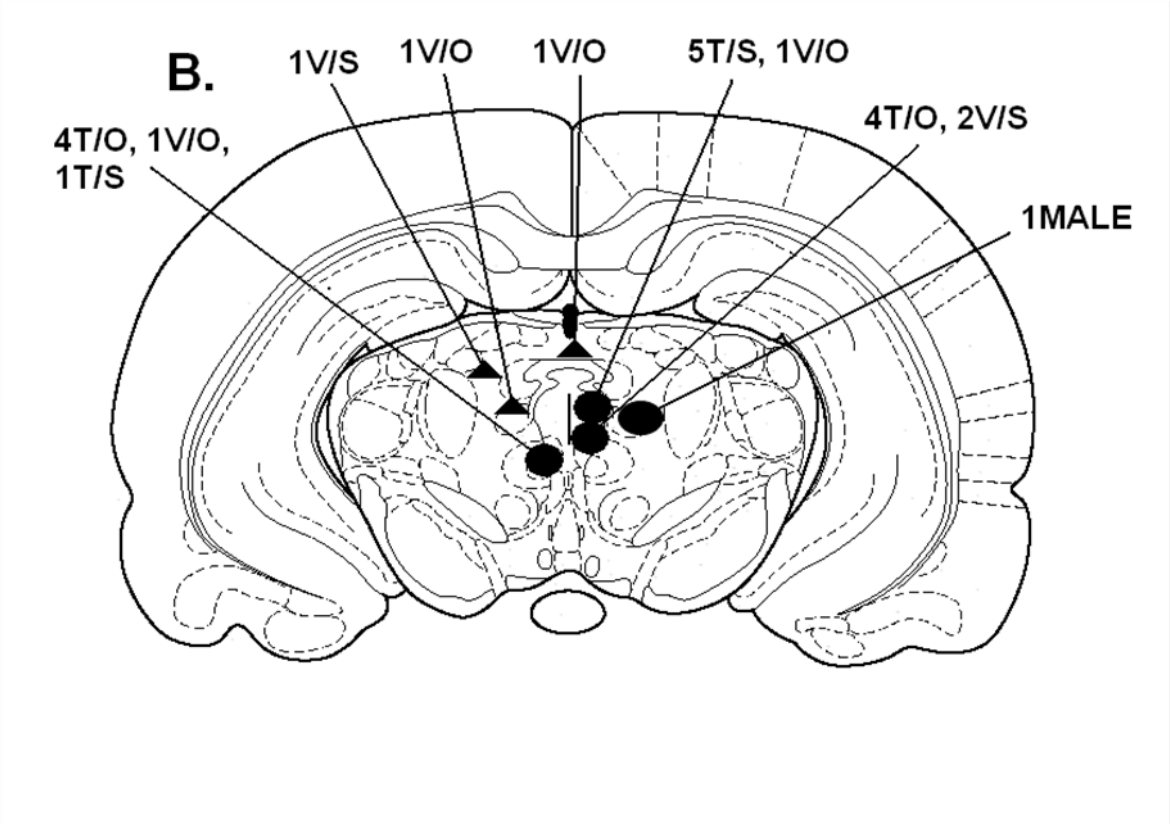
Histological Verification:

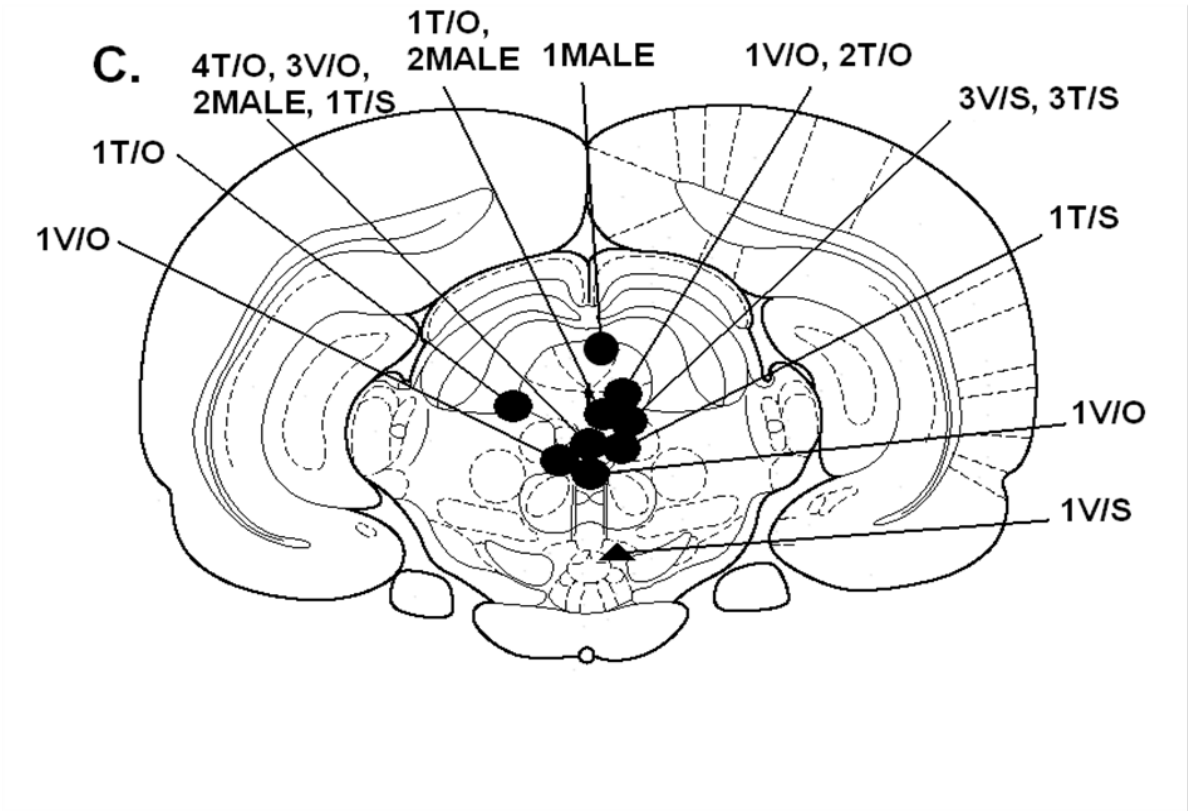
Figure 2 displays the cannula placements of the five groups of animals, Veh-Sham (V/S, n=17), Veh-Ovex (V/O, n=15), TP-Sham (T/S, n=18) and TP/Ovex (T/O, n=20) females as well as Males (n=6). Appropriate vlPAG placements were observed for 14 Veh-Sham, 13 Veh-Ovex,

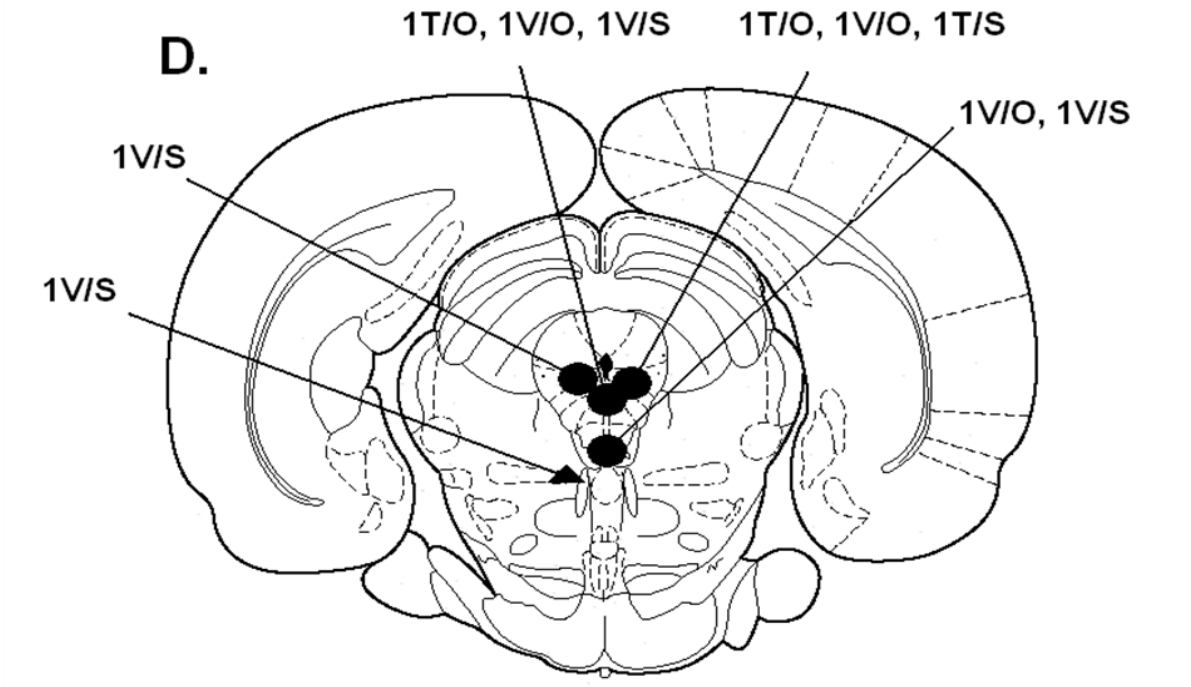
Figure 2. Representation of cannula sites successfully (closed circles) and unsuccessfully (closed triangles) aimed at the ventrolateral periaqueductal gray (vlPAG) region using Figures 38 (Panel A), 39 (Panel B), 43 (Panel C) and 46 (Panel D) of the stereotaxic atlas of Paxinos and Watson (2004) on the following three pages. Multiple animals had highly similar cannula placements, and the considerable overlap of placements is depicted on the panels among male (MALE) rats and female rats receiving neonatal vehicle (Veh: V) or testosterone (TP: T) treatment, and adult sham (S) or ovariectomy (Ovex: O) surgeries. The five groups of animals are represented: V/S (n=17), V/O (n=15), T/S (n=18) and T/O (n=20) females as well as Males (n=6). Appropriate (closed circles) vlPAG placements were observed for 14 V/S, 13 V/O, 15 T/S and 20 T/O females and for the 6 males, whereas misplaced cannulae (closed triangles) were observed for 3 V/S, 2 V/O and 3 T/S females.

Figure 2


A.
1T/O
1 V/S
1T/S
1T/S
1V/O
1T/S
1T/S


B.
1V/S
1V/O
1V/O
5T/S, 1V/O
4T/O, 2V/S
4T/O, 1V/O,
1T/S
1MALE


C.
4T/O, 3V/O, 2MALE, 1T/S
1T/O, 2MALE
1MALE
1V/O, 2T/O
3V/S, 3T/S
1T/O
1V/O
1T/S
1V/O
1V/S


D.
1T/O, 1V/O, 1V/S
1T/O, 1V/O, 1T/S
1V/O, 1V/S
1V/S
1V/S

15 TP-Sham and 20 TP-Ovex females and for the 6 males, whereas misplaced cannulae were observed for 3 Veh-Sham, 2 Veh-Ovex and 3 TP-Sham females. Multiple animals within groups had highly similar cannula placements, and the considerable overlap of placements among groups were noted for cannula placements located in the rostral vlPAG a the level of the third cranial nerve (Figure 2, Panels A and B) as well as the more caudal vlPAG at the level of the dorsal raphe nucleus (Figure 2, Panels C and D). The more rostral vlPAG placements were consistent with positive placements in many of our previous studies (see review: Bodnar et al, 2002). Only animals with appropriate vlPAG cannula placements were included in the subsequent analyses. It should be noted that morphine failed to elicit significant analgesia in those females with misplaced cannulae located dorsal, lateral, ventral and ventrolateral to the vlPAG.

Tail-Flick Test:

Morphine Analgesia and Effects of Sex, Neonatal Gonadectomy and Adult Gonadectomy

Evaluation of vlPAG morphine analgesia at the three (1, 1.7 and 2.5 μg) lower doses revealed significant differences in tail-flick latencies among groups ($F(4,76)= 32.31$, $p<0.0001$), across doses ($F(3,57)= 153.61$, $p<0.0001$), across times ($F(3,57)= 147.23.59$, $p<0.0001$) and for all two-way and three way interactions ($p<0.0001$). Evaluation of vlPAG morphine analgesia at the highest (5 μg) dose in the four female groups revealed significant differences in tail-flick latencies across doses ($F(1,18)= 216.60$, $p<0.0001$), across times ($F(3,54)= 64.92$, $p<0.0001$) and for the interactions between, doses and times ($F(3,54)= 65.32$, $p<0.0001$) and among groups, doses and times ($F(9,162)= 3.23$, $p<0.001$), but not among groups ($F(3,54)= 1.25$, ns) or for the interactions between groups and doses ($F(3,54)= 0.98$, ns) or groups and times ($F(9,162)= 1.65$, ns). Vehicle baseline tail-flick latencies failed to differ among groups or across test times,

indicating lack of baseline differences as functions of sex, neonatal manipulation and adult ovariectomy treatments. Therefore, Figure 3 depicts the mean vehicle baseline latency (3.06s) pooled among groups and test times to indicate this value relative to the drug effects across groups and doses. Morphine dose-dependently and time-dependently increased tail-flick latencies relative to vehicle values in their corresponding group for Veh-Sham females [1 (30-60 min), 2.5 (30-60 min), 5 (30-120 min) μg], Veh-Ovex females [1 (30-90 min), 1.7 (30-120 min), 2.5 (30-120 min), 5 (30-120 min) μg], TP-Sham [1 (30-120 min), 1.7 (60-90 min), 2.5 (30-120 min), 5 (30-120 min) μg], TP-Ovex [1 (30-120 min), 1.7 (30-90 min), 2.5 (30-120 min), 5 (30-120 min) μg], and Males [1 (30-90 min), 1.7 (30-120 min), 2.5 (30-120 min) μg].

Male rats displayed significantly greater magnitudes of vlPAG morphine analgesia either than Veh-Sham females [1 (30-90 min), 1.7 (30-90 min), 2.5 (30-120 min) μg] or TP-Sham females [1 (30 min), 1.7 (30-60 min), 2.5 (30-120 min) μg] (Figure 3), confirming the presence of sex differences. TP-Sham females displayed significantly greater magnitudes of vlPAG morphine analgesia than Veh-Sham females following the 1 (60 min), 1.7 (90 min) 2.5 (30-120 min) and 5 (120 min) μg doses (Figures 3 and 4), confirming the enhancement of central morphine analgesia following neonatal androgenization. Adult ovariectomy significantly increased the magnitude of vlPAG morphine analgesia in Veh-treated female rats following the 1 (30-60 min), 1.7 (30-120 min) and 2.5 (30-90) μg doses (Figures 3 and 4), confirming the enhancement of central morphine analgesia following adult gonadectomy. An organizational-activational gonadal hormone interaction was observed such that TP-Ovex female rats displayed significantly greater magnitudes of vlPAG morphine analgesia following the 1.7 (30-60, 120 min), 2.5 (30-120 min) and 5 (30 min) μg doses (Figures 3 and 4).

Figure 3. Alterations in tail-flick latencies (s, mean ±S.E.M.) across a 120 min time course following intracerebral vlPAG administration of vehicle or morphine at doses of 1 (Panel A) and 1.7 (Panel B) μg in adult female rats neonatally (within 24 h of birth) treated with vehicle (Veh: sesame oil, sc) and then receiving either surgical control (Veh-Sham) or ovariectomy (Veh-Ovex) as adults, in adult female rats neonatally (within 24 h of birth) treated with testosterone propionate (TP, 250 μg/kg, sc) and then receiving either surgical control (TP-Sham) or ovariectomy (TP-Ovex) as adults, and in adult male rats neonatally (within 24 h of birth) treated with vehicle (Males). In this and the subsequent figure, significant differences (Tukey planned comparisons, $P<0.05$) in tail-flick latencies are depicted between specific morphine doses and their corresponding vehicle conditions for each group (*), between males and either the Veh-Sham or TP-Sham female groups at specific morphine doses and times (+), between Veh-Sham and TP-Sham females ($), and between Veh-Sham and Veh-Ovex females on the one hand, and TP-Sham and TP-Ovex females on the other hand (#). Because vehicle baseline (BL) tail-flick latencies failed to differ among the five groups or across the four test times, the resultant mean (3.06 s) was calculated, and is represented on the four panels by a marked horizontal line.

Figure 3

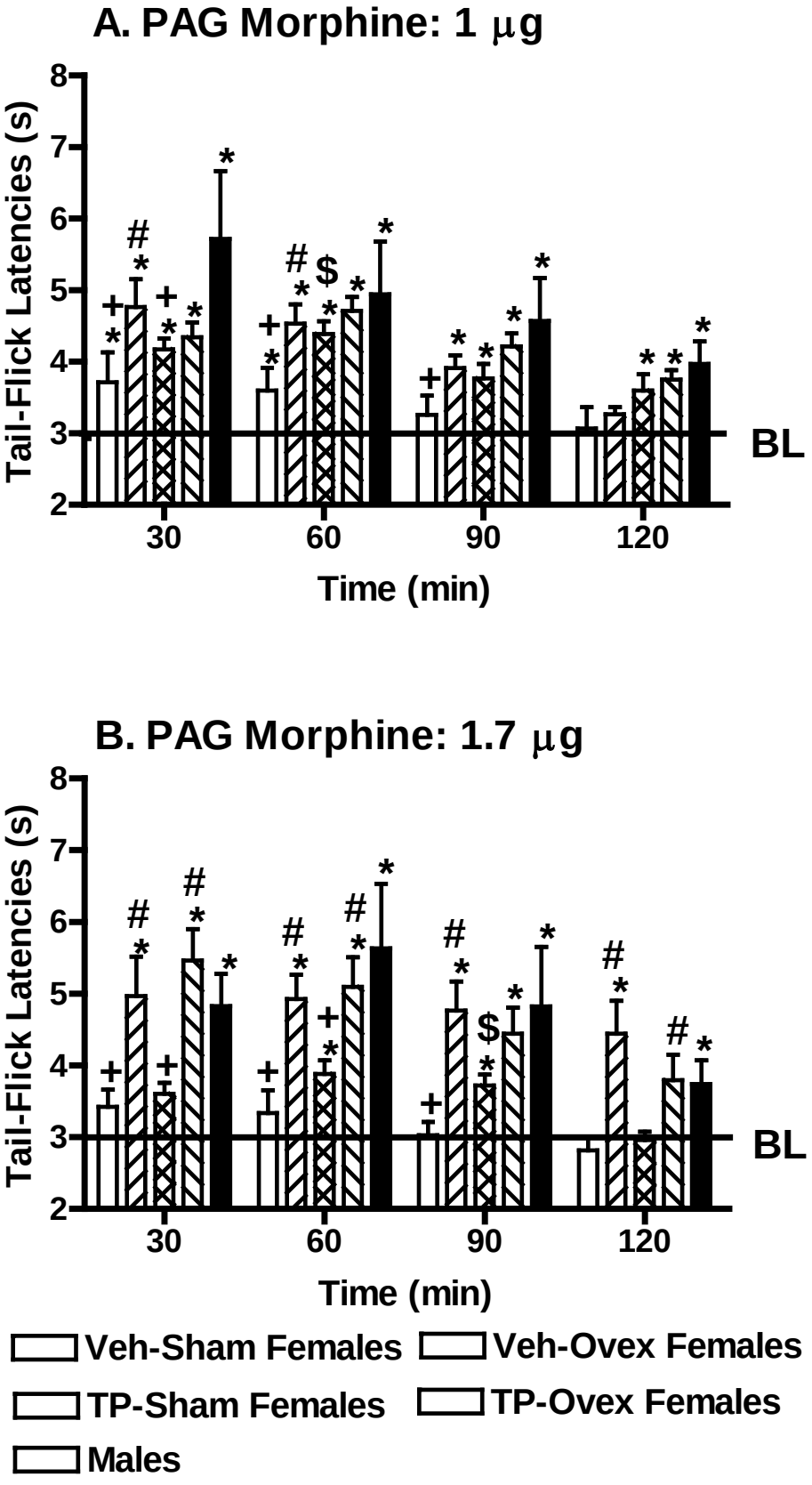
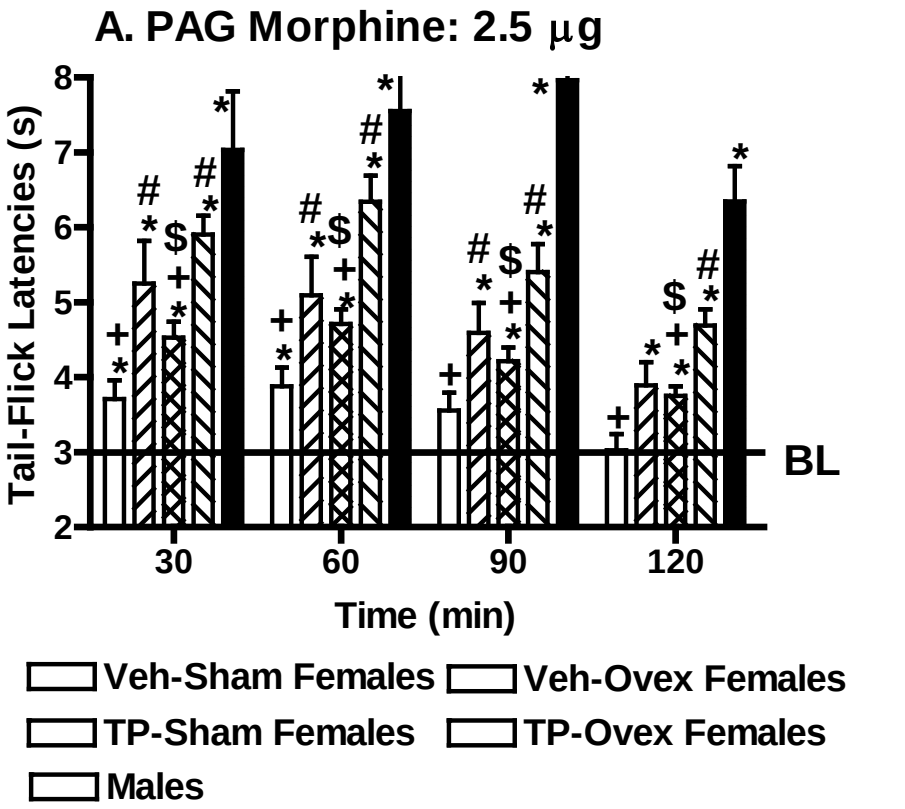
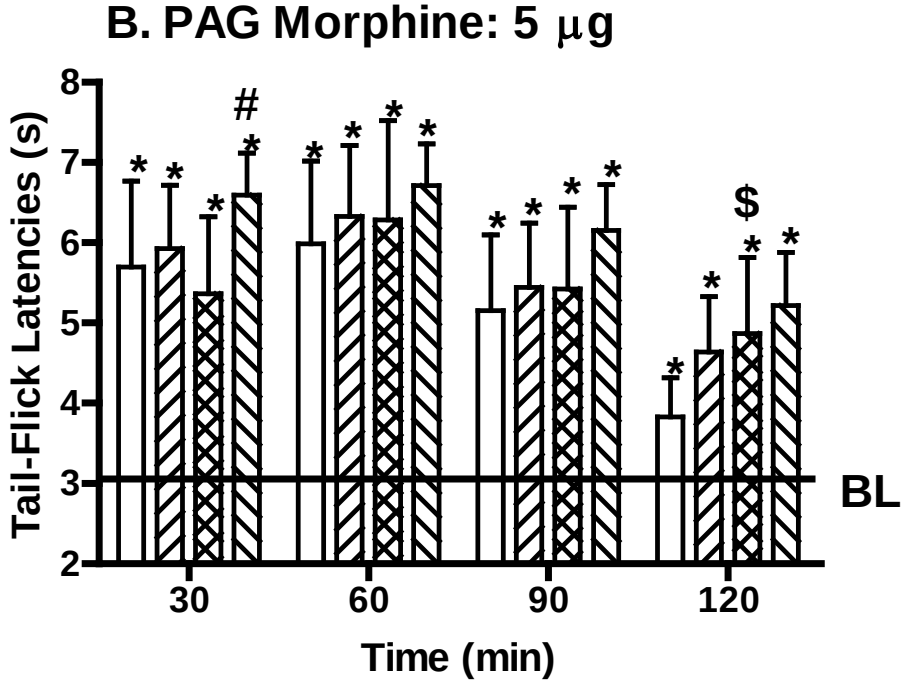

A. PAG Morphine: 1 μg
Tail-Flick Latencies (s)
8
7
6
5
4
3
2
BL
30
60
90
120
Time (min)
B. PAG Morphine: 1.7 μg
Tail-Flick Latencies (s)
8
7
6
5
4
3
2
BL
30
60
90
120
Time (min)
Veh-Sham Females
Veh-Ovex Females
TP-Sham Females
TP-Ovex Females
Males

Figure 4. Alterations in tail-flick latencies (s, mean ±S.E.M.) across a 120 min time course following intracerebral vlPAG administration of vehicle or morphine at doses of 2.5 (Panel A) and 5 (Panel B) μg in the Veh-Sham, Veh-Ovex, TP-Sham and TP-Ovex female groups as well as the Males group.

Figure 4


A. PAG Morphine: 2.5 μg
Tail-Flick Latencies (s)
8
7
6
5
4
3
2
BL
30
60
90
120
Time (min)
Veh-Sham Females
Veh-Ovex Females
TP-Sham Females
TP-Ovex Females
Males


B. PAG Morphine: 5 μg
Tail-Flick Latencies (s)
8
7
6
5
4
3
2
BL
30
60
90
120
Time (min)

Potency Differences in vlPAG Morphine Analgesia across Groups

Evaluation of the ED_{50} of vlPAG morphine analgesia necessary to elicit a tripling of latencies again revealed a sex difference such that males (ED_{50} = 0.64 μg) displayed respective 6.1-fold and 4.7-fold leftward shifts in the potency of vlPAG morphine analgesia relative to Veh/Sham (ED_{50} = 3.93 μg) and TP/Sham (ED_{50} = 3.02 μg) female groups. The neonatal androgenization effect was less pronounced (0.3-fold) in analgesic potency for the Veh-Sham and TP-Sham female groups. The alterations in activational effects of female gonadal hormones induced by adult ovariectomy (Veh-Ovex: ED_{50} = 1.56 μg) produced a 2.5-fold leftward shift in the potency of vlPAG morphine analgesia relative to Veh-Sham females (ED_{50} = 3.93 μg). Similarly, the alterations in activational-organizational interactions of female gonadal hormones observed in androgenized females receiving adult ovariectomy (TP-Ovex: ED_{50} = 1.38 μg) produced a 2.2-fold leftward shift in the potency of vlPAG morphine analgesia relative to TP-Sham females (ED_{50} = 3.02 μg).

Discussion

As hypothesized, the present study found organizational and activational gonadal hormone interactions in female rats in their analgesic response to morphine elicited from the vlPAG. The specific hypotheses included: male rats will display significantly greater magnitudes and potencies of morphine analgesia than Veh-Sham Females, thereby confirming a sex difference; TP-Sham Females will display significantly greater magnitudes and potencies of morphine analgesia than Veh-Sham Females, thereby confirming neonatal gonadectomy effects; Veh-Ovex Females will display greater magnitudes and potencies of morphine analgesia than Veh-Sham Females, although to a lesser extent than TP-Sham female effects, thereby confirming occasionally-observed adult gonadectomy effects; The relationship of neonatal and adult

gonadectomy effects to further changes in morphine analgesia will be observed in the TP-Ovex female group relative to the TP-Sham, Veh-Ovex and Veh-Sham female groups, thereby examining interactions between organizational and activational effects of gonadal hormones.

As previously described for morphine analgesia elicited following systemic (Cicero et al., 2002) and vlPAG (Krzanowska et al., 2002) administration, TP-Sham females displayed significantly greater magnitudes of vlPAG morphine analgesia than Veh-Sham females across a range of morphine doses, indicating an organizational effect of gonadal hormones in the mediation of this response in this brain site. As previously described for morphine analgesia elicited following vlPAG administration (Krzanowska and Bodnar, 1999), Veh-Ovex females displayed significantly greater magnitudes of vlPAG morphine analgesia than Veh-Sham females across a range of morphine doses, indicating an activational effect of gonadal hormones in the mediation of this response in this brain site. The central premise of a proposed organizational-activational gonadal hormone interaction in female rats was confirmed by the observation that TP-Ovex females displayed significantly greater magnitudes of vlPAG morphine analgesia than TP-Sham females across a range of morphine doses, thereby implicating the vlPAG as a central locus at which such interactions occur. The observed positive organizational and activational gonadal hormone interaction for morphine analgesia elicited from the vlPAG stands in contrast to previous failures to observe such consistent gonadal hormone interactions for systemic morphine analgesia. A potential reason for this apparent discrepancy might lie in the fact that although both systemic and vlPAG morphine analgesia are both sensitive manipulations altering the organizational effects of gonadal hormones (Cicero et al., 2002; Krzanowska et al., 2002), systemic morphine analgesia is typically insensitive to manipulations altering the activational effects of gonadal hormones (e.g., Ali et al., 1995; Banerjee et al., 1983; Cicero et al., 1996,

2002; Islam et al., 1993; Kasson and George, 1984) except if less potent μ agonists are employed (Terner et al., 2002).

These data confirm previous studies suggesting that the vlPAG may be a locus at which pharmacological (opiate) actions and gonadal steroid hormones interact, particularly with respect to pain inhibition. The vlPAG has been identified as sensitive to changes in morphine analgesia as a function of sex differences, organizational and activational effects of gonadal hormones, and estrous phase (Bernal et al., 2007; Krzanowska and Bodnar, 1999; Krzanowska et al., 2002; Loyd and Murphy, 2006; Shane et al., 2007). Moreover, hypothalamic enkephalinergic neurons, sensitive to changes in sex hormone levels, turn on enkephalin genes in females to a greater degree than males (Priest et al., 1995; Romano et al., 1988, 1989, 1990), and eventually project to estrogen-binding PAG neurons (Turcotte and Blaustein, 1999), a pathway absent following hypothalamic lesions (Hoffman et al., 1996). Mu opioids facilitate excitation in vlPAG cells via interactions with NMDA (Kow et al., 2002), and the ultrastructural arrangement of the mu opioid receptor with GABAergic PAG neurons or PAG projection neurons labeled retrogradely from the medulla indicate that mu opioid receptor ligands both act to inhibit the former, and act directly on the latter (Commons et al., 2000). This organizational-activational gonadal hormone interaction for vlPAG morphine analgesia is apparently independent of changes in basal pain thresholds. In contrast to observed sex differences and adult-neonatal gonadectomy differences in thermal and mechanical pain thresholds following lumbar radiculopathy (LaCroix-Fralish et al., 2005) or carrageenan-induced inflammation (Borzan and Fuchs, 2006), the present study failed to observe differences in vehicle baseline tail-flick latencies among the four female and one male group. Finally, the question as to how the vlPAG might modulate such sex and gonadectomy-induced differences in morphine analgesia has not been fully addressed. However,

Loyd and Murphy (2006) found sex differences in inflammatory pain and morphine's analgesic effects on this response following administration into the vlPAG. Using retrograde labeling, although females displayed significantly more PAG-RVM output neurons than males, inflammatory pain activated more PAG-RVM cells in males than in females. Further Fos-induced activation of the PAG by inflammation was suppressed by systemic morphine in males only. Thus, the organizational-activational interaction of gonadal hormones in centrally-mediated pain inhibition adds yet another behavioral component to the observation of such interactions in aggressive (Compann et al., 1993), stress-related (Seale et al., 2005), anxiety-related (Zimmerberg and Farley, 1993), electroencephalographic (Corsi-Cabrera et al., 2000), and anatomical (Micevych et al., 1994) responses.

Chapter 5

Experiment 3 (Specific Aim 2): Ventromedial and Medial Preoptic Hypothalamic Ibotenic Acid Lesions Potentiate Systemic Morphine Analgesia in Female, But Not Male Rats

Background

Female rodents display significantly smaller analgesic responses than male rodents following systemic (e.g., Baamonde et al., 1989; Badillo-Martinez et al., 1984; Candido et al., 1992; Kavaliers and Innes, 1987), ventricular (Kepler et al., 1989) or intracerebral (Bobeck et al., 2009; Boyer et al., 1998; Krzanowska and Bodnar, 1999, 2000; Loyd and Murphy, 2006) injections of mu-opiate drugs, particularly morphine. Gonadectomy in adult male and female animals minimally alters the magnitude or the potency of mu-agonist-mediated analgesia following systemic and ventricular administration relative to intact animals (Ali et al., 1995; Banerjee et al., 1983; Cicero et al., 1996, 2002; Islam et al., 1993; Kasson and George, 1984; Kepler et al., 1989, 1991; Stoffel et al., 2003), but ovariectomy of adult female rats produces greater and more potent morphine analgesia elicited from the ventrolateral periaqueductal gray (vlPAG: Krzanowska and Bodnar, 1999) than that of intact females tested during the estrus phase. Classically-described organizational effects of gonadal hormones (e.g., Phoenix et al., 1959) altered by neonatal gonadectomy profoundly affect morphine analgesia elicited from the vlPAG (Krzanowska et al., 2002) or following systemic administration (Cicero et al., 2002) with adult male rats postnatally-castrated displaying less morphine analgesia than sham-operated males, but similar to sham-treated females, and adult female rats postnatally treated with testosterone propionate (TP) displaying greater morphine analgesia than sham-treated females, but similar to sham-operated males. The mechanisms by which sex differences and

gonadectomy-induced differences in morphine analgesia are modulated by neuroanatomical circuits sensitive to sex-related hormones are not known.

One possible means by which the vlPAG may mediate sex differences in morphine analgesia is through its interaction with estradiol-containing hypothalamic loci (Pfaff and Schwartz-Giblin, 1988), that mediate in part interactions between sex hormones and opioid peptides, particularly control of transcription of the preproenkephalin (PPE) gene by estradiol (Pfaff et al., 1996). Importantly, hypothalamic enkephalinergic neurons, sensitive to changes in sex hormone levels, turn on enkephalin genes in females to a greater degree than males (Priest et al., 1995; Romano et al., 1988, 1989, 1990). These neurons eventually project to estrogen-binding PAG neurons (Turcotte and Blaustein, 1999), and such connections are absent following hypothalamic lesions (Hoffman et al., 1996). Mu opioid agonists facilitate excitation in vlPAG cells through interactions with NMDA receptors (Kow et al., 2002). The ultrastructural arrangement of the mu opioid receptor with GABAergic PAG neurons or PAG projection neurons labeled retrogradely from the medulla, indicate that mu opioid receptor ligands both act to inhibit the former, and act directly on the latter (Commons et al., 2000). Two estradiol receptor-containing hypothalamic nuclei which send dense enkephalinergic projections to the vlPAG are the ventromedial (VMH) and medial pre-optic (MPOA), and are therefore candidate links by which gonadal hormones differentially influence sex differences in opiate analgesia.

The MPOA is a sexually-dimorphic hypothalamic nucleus (e.g., Bloch and Gorski, 1988; Gorski et al., 1980; Simerly et al., 1984) that plays a crucial role in the mediation of sexual behavior (e.g., Docke et al., 1984; Hansen et al., 1982; Lisk, 1966; Powers and Valenstein, 1972) because of their androgen and estrogen mRNA-containing cells (Simerly et al., 1990). The MPOA has an orderly, reciprocally-connected and longitudinally-organized columnar

organization projection that extends along the whole rostro-caudal axis of the PAG (Rizvi et al., 1992). These MPOA afferents innervate those PAG cells that subsequently project to the rostro-ventral medulla (RVM) as demonstrated by combined Fos and Phaseolus vulgaris tract tracing (Rizvi et al., 1996), and thereby provide gonadal steroid receptor innervation from the MPOA to the PAG (Murphy and Hoffman, 2001). These anatomical connections have also been functionally confirmed in neurophysiological studies (Jiang and Behbehani, 2001; Lumb and Morrison, 1986). Moreover, the MPOA connections with the pontine and medullary midline nuclei (Holstege, 1987; Veening et al., 1990), particularly the nucleus raphe magnus (NRM), have been shown to be direct and reciprocal (Murphy et al., 1999). Electrical stimulation of MPOA neurons suppresses spinal and medullary dorsal horn neuronal responses to cutaneous and visceral noxious input (Carstens et al., 1982; Lumb, 1990; Lumb and Cervero, 1989; Mokha et al., 1987). Moreover, the prostaglandin E receptor EP3 subtype has been implicated in thermal hyperalgesia through its actions in the preoptic hypothalamus as well as the diagonal band of Broca (Hosoi et al., 1997). This hyperalgesic effect of prostaglandin E2 in the MPOA activates pain-modulating circuitry in the RVM by activating pain-facilitatory ON-cells, and concomitantly suppressing pain-inhibitory OFF-cells (Heinricher et al., 2004). Finally, a recent study (Zhang and Ennis, 2007) demonstrated that inactivation of the PAG with lidocaine attenuated the antinociception elicited by chemical stimulation with D,L-homocysteate administered into the MPOA. These data collectively suggest direct MPOA-PAG and MPOA-RVM connections that are directly implicated in nociceptive and antinociceptive responses, and potentially in the sex differences in antinociceptive responses.

Like the MPOA, the VMH is a sexually-dimorphic nucleus intimately involved in the initiation of the lordotic response in female rodents (Pfaff, 1980; Pfaff et al., 1994) acting

through estrogen receptors found in the VMH nuclei (McCarthy et al., 1993; Ogawa et al., 1996). Therefore, inactivation of estrogen receptors in the VMH through gene knockdown techniques can disrupt the entire lordotic sequence even though all of the brainstem and spinal apparatus necessary for the muscular responses are intact. Further, such responses are sex-dependent in that sexual behaviors in the male are unaffected (Ogawa et al., 1997). There is considerable evidence for a gonadal-opiate interaction such that activation of mu opiate receptors in the VMH (Ono et al., 1980) inhibits lordosis in female rats (Acosta-Martines and Etgen, 2002). Like the MPOA, there are direct VMH-PAG connections involved in descending inhibitory control of nociception (Dostrovsky et al., 1983) as well as the VMH termination of an ascending nociceptive pathway, the spino (trigemino) parabrachiohypothalamic pathway (Bester et al., 1995). Whereas electrical stimulation of the VMH induces analgesia (Rhodes and Liebeskind, 1978; Culhane and Carstens, 1988), electrical destruction of the VMH produces hyperalgesia (Vidal and Jacob, 1980). Intra-VMH microinjection of interleukin 1β produces analgesia in rats which is blocked by a cyclooxygenase inhibitor, indicating the role of prostanoids synthesis (Oka et al., 1995). Il-1β increases the release of prostaglandin E2 from rat hypothalamic explants (Navarra et al., 1992), and PGE2 in the VMH has been shown to have antinociceptive effects through its actions on EP1 receptors in rats (Masako et al., 1999). Taken together, these properties of VMH neurons enable them to have direct or at least indirect influence on nociception and analgesic processes, and potentially mediate the sex differences in analgesic responses.

Therefore, the present study respectively examined whether excitotoxic (ibotenic acid, IBO) chemical destruction of the MPOA or the VMH altered the dose-dependent and time-dependent actions of systemic morphine analgesia in sexually-intact male rats, sexually-intact female rats, and adult Ovex female rats to establish whether such lesions alter sex differences

and gonadectomy differences in this analgesic response. The experimental paradigm in this study examined whether IBO-induced destruction of the MPOA and VMH altered baseline tail-flick latencies and whether any changes in morphine-induced analgesia occurred independently of any changes in basal nociception. The experimental design allowed detailed analysis of changes in the temporal and peak magnitude and potency (ED_{50}) of morphine analgesia in intact males, intact females and Ovex females that would occur specifically in one group or occurred across all groups. The following hypotheses were tested: male rats treated with vehicle will display significantly greater magnitudes and potencies of morphine analgesia than intact female rats treated with vehicle, thereby confirming a sex difference; Veh-Ovex Females will display greater magnitudes and potencies of morphine analgesia than Veh-Sham Females, thereby confirming occasionally-observed adult gonadectomy effects; The present data will reveal whether ibotenic acid-induced destruction of the MPOA and VMH alter baseline tail-flick latencies, and if so, are the changes in basal nociception observed in intact males alone, intact or Ovex females alone or in all groups?; Independent of any changes in basal nociception, does ibotenic acid-induced destruction of the MPOA and VMH alter the magnitude and/or potency of systemic morphine-induced analgesia, and if so, are the changes in systemic morphine analgesia observed in intact males alone, intact or Ovex females alone or in all groups? This experiment has been submitted for publication in Behavioural Brain Research with the candidate as senior author.

Methods

Procedure:

Male and Female animals in the MPOA and VMH conditions were anesthetized employing a combination of chlorpromazine (5 mg/kg, IP) and ketamine hydrochloride (140 mg/kg, IM). Bilateral excitotoxic lesions of the MPOA were stereotaxically produced in groups of intact male, intact female and Ovex female animals with injections of 1.0 μg of ibotenic acid (1.0 μg/ μl in phosphate-buffered saline pH 7.2) at the following coordinates: 0.9 mm posterior, 2.2 mm lateral, 8.7 mm ventral from bregma and angled 10^{o} towards the sagittal suture. Three control groups received identical surgeries except that phosphate buffered saline vehicle (VEH) microinjections were administered. Bilateral excitotoxic lesions of the VMH were also stereotaxically produced in groups of intact male, intact female and Ovex female animals with injections of 1.0 μg of ibotenic acid (1.0 μg/ μl in phosphate-buffered saline pH 7.2) at the following coordinates: 2.5 mm posterior, 2.2 mm lateral, 9.3 mm ventral from bregma and angled 10^{o} towards the sagittal suture. Three control groups received identical surgeries except that VEH microinjections were administered. Each injection was made using a 1-μl Hamilton positive displacement syringe (7001 series, Hamilton, Reno, NV) placed into a Kopf microinjector attached to a Kopf Ultra Precise stereotaxic instrument (David Kopf Instruments, Tunjunga, CA). After each injection, the syringe was left in place for 5 minutes. Vehicle treated animals were done in the same manner except that phosphate buffered saline containing no ibotenic acid was injected. The three Ovex female groups were anesthetized approximately 10-14 days after stereotaxic surgery, and ovariectomies were performed. All groups began nociceptive testing approximately 1 month after surgeries.

There were nine experimental groups; intact male, intact female and Ovex female animals which received VEH, IBO MPOA lesions or IBO VMH lesions. All behavioral testing took place 2–8 h into the light cycle. All rats in all groups received a single vehicle injection with tail-flick latencies assessed 30, 60, 90 and 120 min thereafter. Following stable baseline determinations, all groups then received ascending doses (1.0, 2.5, 5.0, 7.5 and 10.0 mg/kg, SC) of morphine at 8-12 day intervals to minimize any possible tolerance and tissue damage effects. The ascending regimen was employed to minimize the occurrence of going to cut-off values until later in the procedure. Although estrous phase does not markedly alter the magnitude of systemic morphine analgesia (e.g., Kepler et al., 1989; Stoffel et al., 2003), the paradigm still controlled for estrous phase by only testing the intact female groups during the estrus phase of the cycle. In these groups, vaginal smears were taken and then latencies determined 30, 60, 90 and 120 min following each morphine injection. This dose regimen samples the time course of peak systemic morphine analgesic action (e.g., Cicero et al., 1996; Cicero et al., 1997; Cicero et al., 2002 and Islam et al., 1993).

After completion of testing, the uteri of Ovex and intact females were removed and excess fat dissected. They were then blotted dry and weighed as in previous studies (Kepler et al., 1989, 1991) to the nearest 0.0001g to confirm the completeness of the gonadectomy procedure. Consistent with the results of previous studies (Kepler et al., 1989, 1991) the uterine weights in ovariectomized females was approximately 13% of that of intact females thereby confirming the completeness of the gonadectomy procedure. All animals received an overdose of anesthetic (Euthasol, Del Marva Laboratories, 390 mg/ml sodium pentobarbital; 50 mg/ml sodium phenytoin; 0.05 ml/kg, IP), and transcardiac perfusions with 0.9% normal saline followed by 10% buffered formalin. After removal, their brains were placed in 30% sucrose

solution until they sank to the bottom (approximately 2 days). Coronal (40 μm) sections were cut through either the MPOA or VMH of the three groups receiving either VEH or IBO lesions. The sections were then stained with cresyl violet, and examined microscopically by observers uninformed about the experimental condition, experimental group or behavioral data. The extent of the lesions in each animal in each group was reconstructed on sections of the Paxinos and Watson (2004) atlas. In examining these reconstructions, all animals in each group were represented according to a "Min-Max" procedure on a central section of the Paxinos and Watson (2004) atlas. A lesion area common to all animals in the group was defined as the "Min" (dark area on atlas section), whereas any area in any animal(s) unique to that subgroup was defined as the "Max" (lighter area on atlas section). Representative actual bilateral lesion placements (at 1.25x magnification) were chosen as well as a representative actual unilateral lesion placement (at 4.0x magnification) chosen for both lesion and control placements for each group for each lesion site.

Photomicrograph and imaging protocol was then followed as previously described (Chen et al., 2009). Briefly, brightfield images were taken under a Microfire (Optronics) camera using the Neurolucida program (MBF Bioscience, Inc. Version 8.0). Image exposure, gain, contrast, brightness, color balance, and aperture size were optimized and held constant for the purpose of pre-standardizing the tissue background luminosity prior to densitometry analyses, similarly to previously described studies (Gazzaley et al., 1996; Kozorovitskiy et al., 2005). The absolute value of tissue background luminosity (area devoid of Nissl staining) was further confirmed by directly measuring the pixel intensity using the Neurolucida software (under luminosity measurements). Photomicrograph snapshots were converted to grayscale images (using Photoshop 7.0), in which pixel values representing the intensity of staining were expressed in a

grayscale (0-255). The overall luminosity and relative optical density (ROD) of VMH and MPOA were measured and assessed by an observer unaware of the experimental conditions using the software Densita (MBF Bioscience, Inc.).

Statistical Analyses:

Differences in mean baseline tail-flick latencies among the three sex conditions (intact male, intact female, Ovex female) and three groups (VEH, IBO VMH and MPOA lesion) across the time course (30, 60, 90, 120 min) were evaluated using a three-way randomized block analysis of variance. To assess group differences in the magnitude of morphine analgesia, a mean latency for the four latency determinations for vehicle and each morphine dose condition was calculated for each animal in each group at all vehicle and morphine doses. Then, an overall Mean Percentage Effect (MPE) was calculated for each animal using the following formula: (Experimental Latency – Vehicle Latency / Cut-off Latency (10 sec) – Vehicle Latency) *100. Two (one for the MPOA groups, one for the VMH groups) three-way randomized block analyses of variance were performed on the MPE scores with the three conditions, and two groups as between-subjects variables, and the five morphine doses (1.0, 2.5, 5.0, 7.5 and 10.0 mg/kg) as the within-subjects variable. Subsequent two-way completely randomized analyses of variance were performed for MPE values at each morphine dose for the three conditions and two groups. Moreover, two-way repeated-measures analyses of variance were performed for each condition and group with the vehicle and five morphine doses and four test times as the dependent measures. Tukey planned comparisons ($P<0.05$) were used to determine significant individual effects. Linear regression analyses were then performed for MPE scores for each condition and group to derive the ED_{50} of peak morphine analgesia. Analyses of accessory sex organ weights were performed using a completely randomized one-way analysis of variance evaluating intact

VEH-treated, Ovex VEH-treated, Ovex MPOA-lesioned, and Ovex VMH-lesioned females. Separate two-way analyses of variance were performed in three conditions and two groups for luminosity and ROD measures in the MPOA and VMH. Tukey planned comparisons ($P<0.05$) were used to determine significant individual effects.

Results

Accessory Sex Organ Analysis:

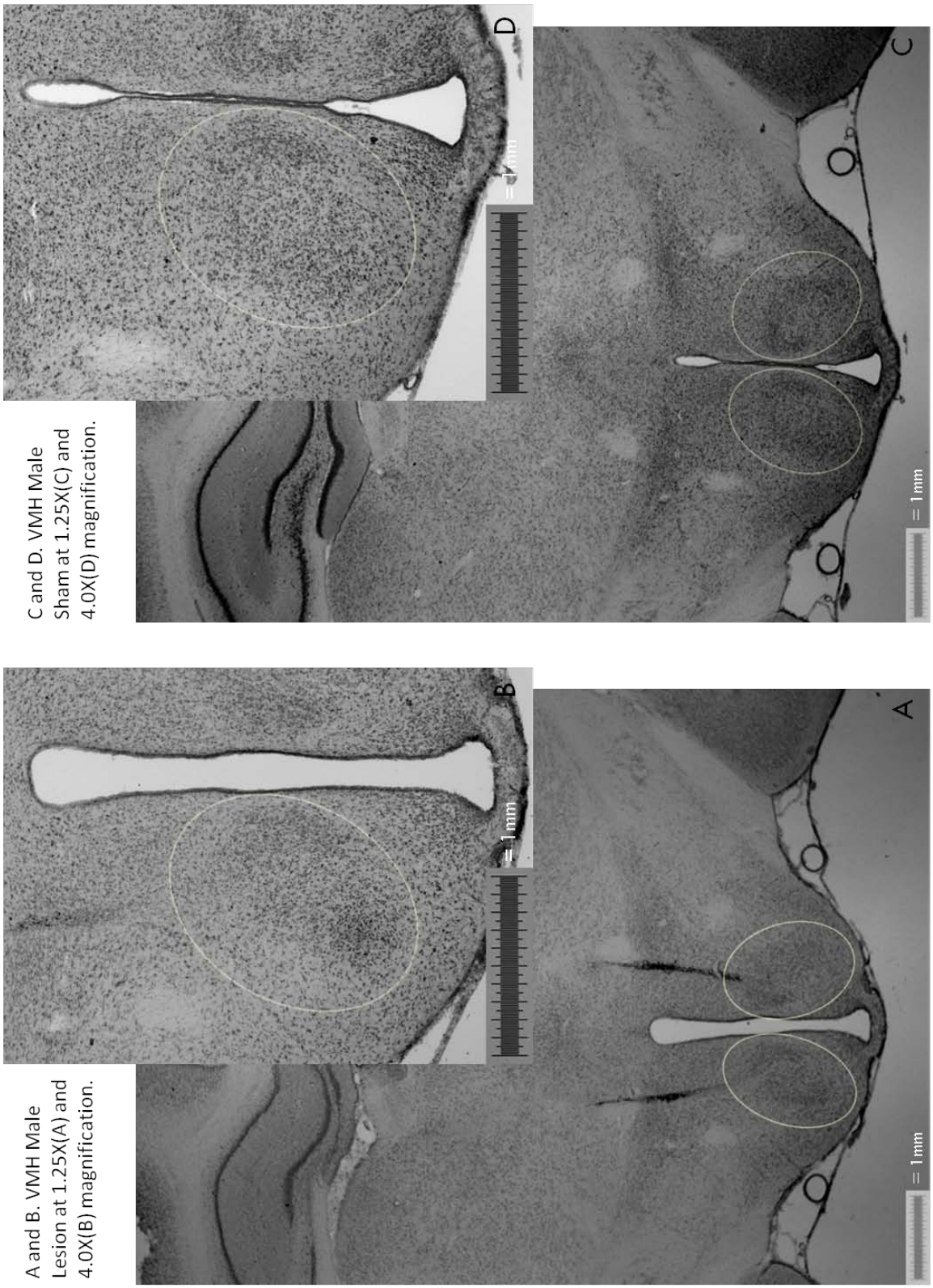
Significant differences in uterine weights were observed ($F(3,40)= 278.97$, $p<0.0001$) among the intact VEH-treated, Ovex VEH-treated, Ovex MPOA-lesioned, and Ovex VMH-lesioned females. The uterine weights of intact VEH-treated female rats (3.26 g) were significantly greater than Ovex female rats receiving VEH (0.29 g, 92% reduction), VMH IBO (0.38 g, 89% reduction) or MPOA IBO (0.55 g, 83% reduction) treatments. In turn, MPOA IBO- and VMH IBO-lesioned Ovex females had significantly higher uterine weights than VEH-treated Ovex females, and the MPOA IBO-lesioned group was significantly higher than the VMH IBO-lesioned group.

Histological Verification:

All bilateral injections aimed at the VMH (Figure 5) and MPOA (Figure 6) in males, intact females and Ovex females were localized within their respective desired areas. Minimum-maximum delineations appeared to be uniform in the intact male, intact female and Ovex female groups receiving VMH IBO lesions relative to the VEH-treated groups. Therefore, the three groups had highly-similar extents of VMH lesions. Moreover, whereas the VMH appeared either partially or completely destroyed in all animals in each of the three groups, the extent of the lesions appeared to be confined within the nucleus. Examination of representative lesion

Figure 5. Representative microinjection placements are displayed bilaterally using a 1.25x magnification for intact male (Figure 5A), intact female (Figure 5B) and Ovex female (Figure 5C) groups for animals treated with VMH IBO (A and B) relative to animals treated with VEH (C and D). Representative unilateral 4.0x (inset) magnification placements are depicted for the VMH VEH and IBO treatments for each of the three groups.

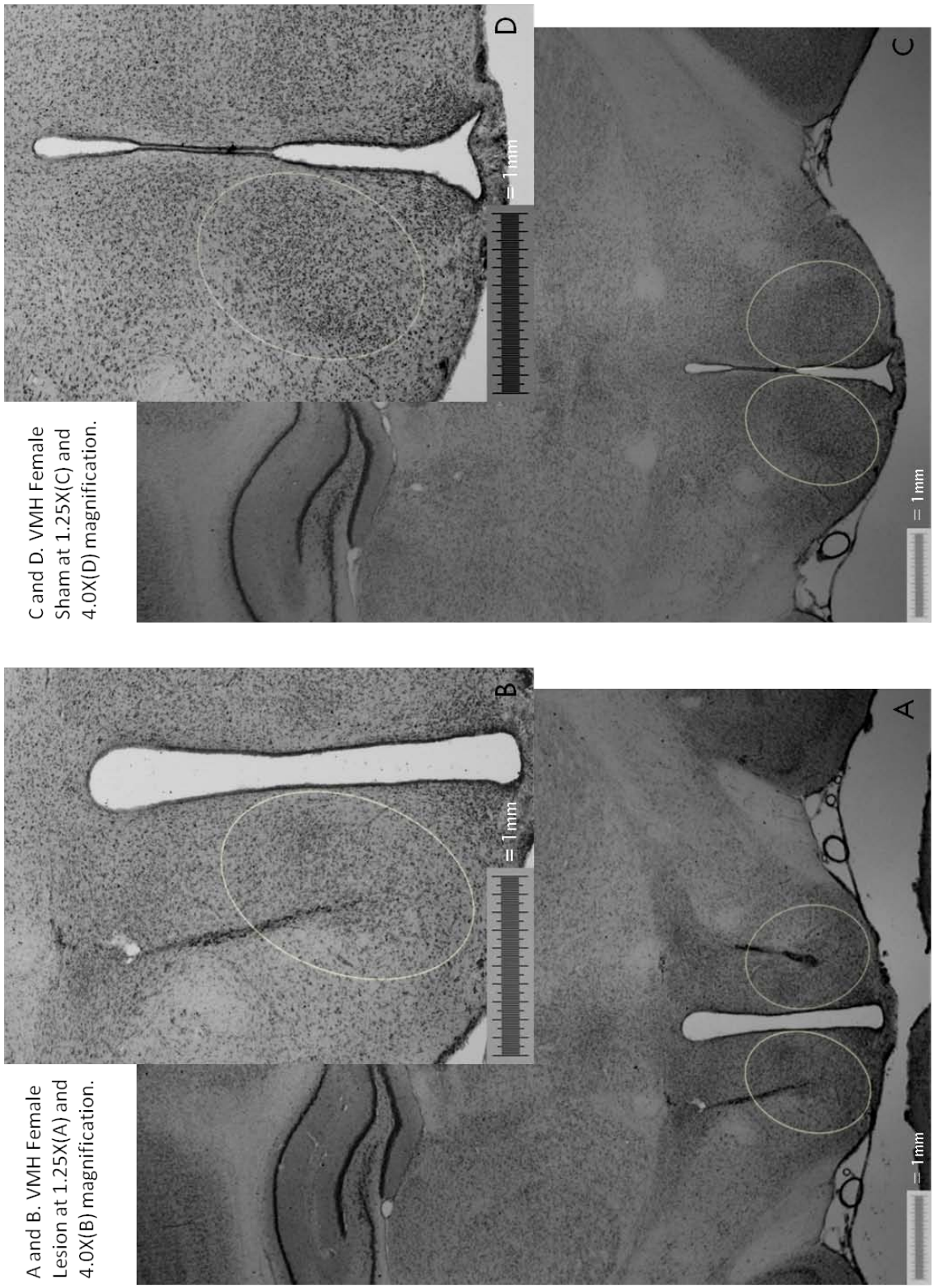
Figure 5A



A and B. VMH Male
Lesion at 1.25X(A) and
4.0X(B) magnification.

C and D. VMH Male
Sham at 1.25X(C) and
4.0X(D) magnification.

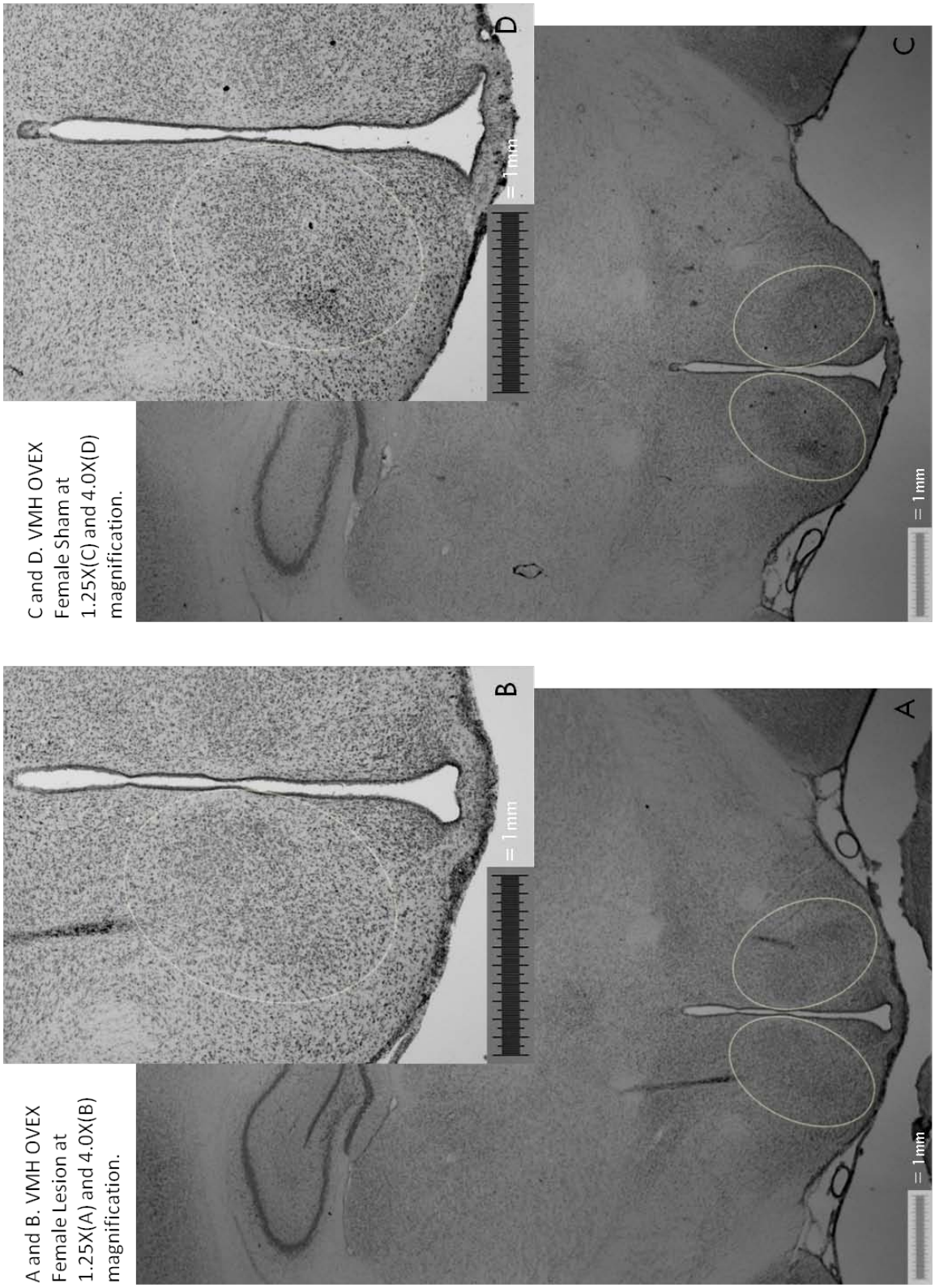
Figure 5B



A and B. VMH Female Lesion at 1.25X(A) and 4.0X(B) magnification.

C and D. VMH Female Sham at 1.25X(C) and 4.0X(D) magnification.

Figure 5C

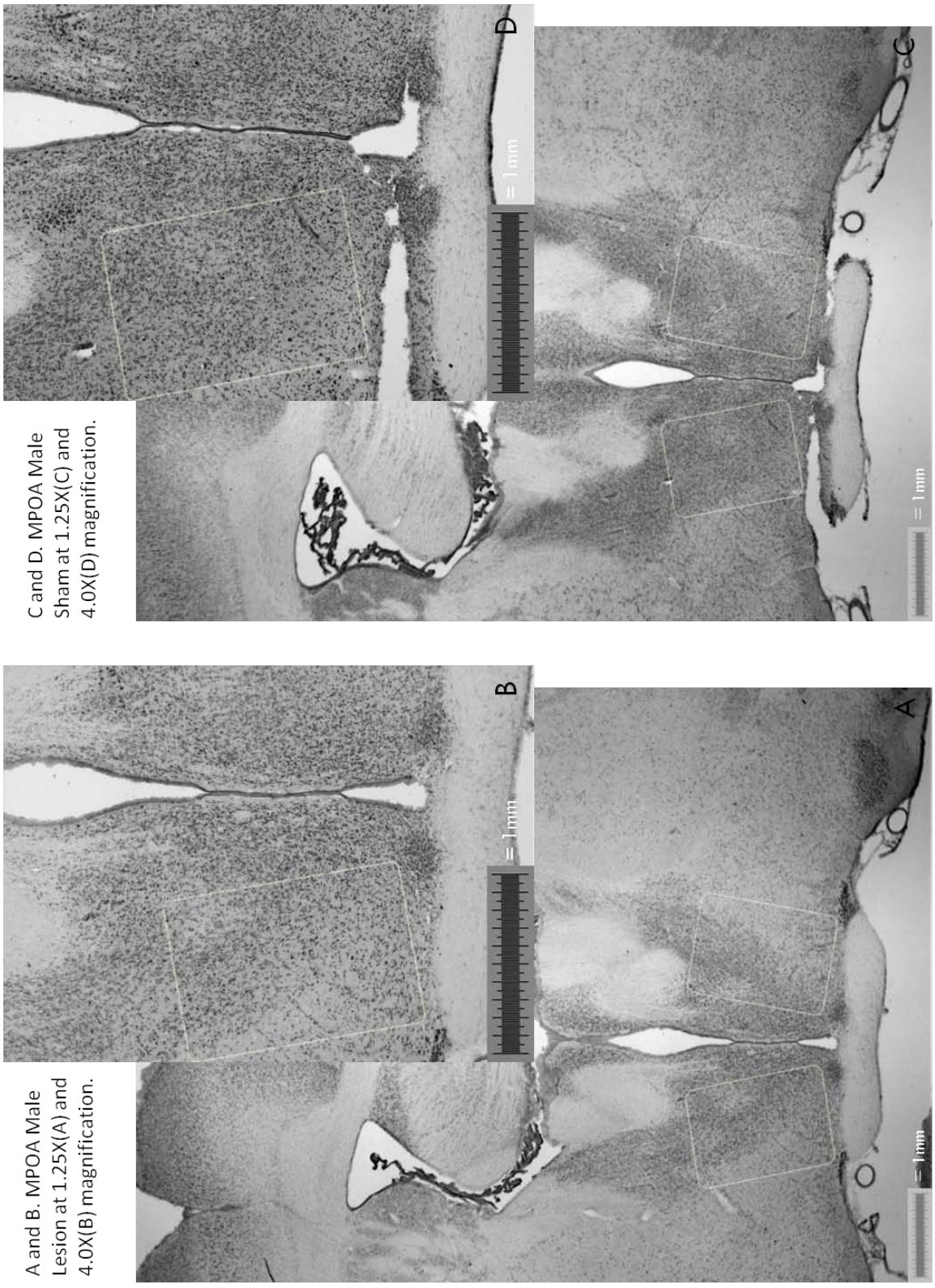


A and B. VMH OVEX Female Lesion at 1.25X(A) and 4.0X(B) magnification.

C and D. VMH OVEX Female Sham at 1.25X(C) and 4.0X(D) magnification.

Figure 6. Representative microinjection placements are displayed bilaterally using a 1.25x magnification for intact male (Figure 6A), intact female (Figure 6B) and Ovex female (Figure 6C) groups for animals treated with MPOA IBO (A and B) relative to animals treated with VEH (C and D). Representative unilateral 4.0x (inset) magnification placements are depicted for the MPOA VEH and IBO treatments for each of the three groups.

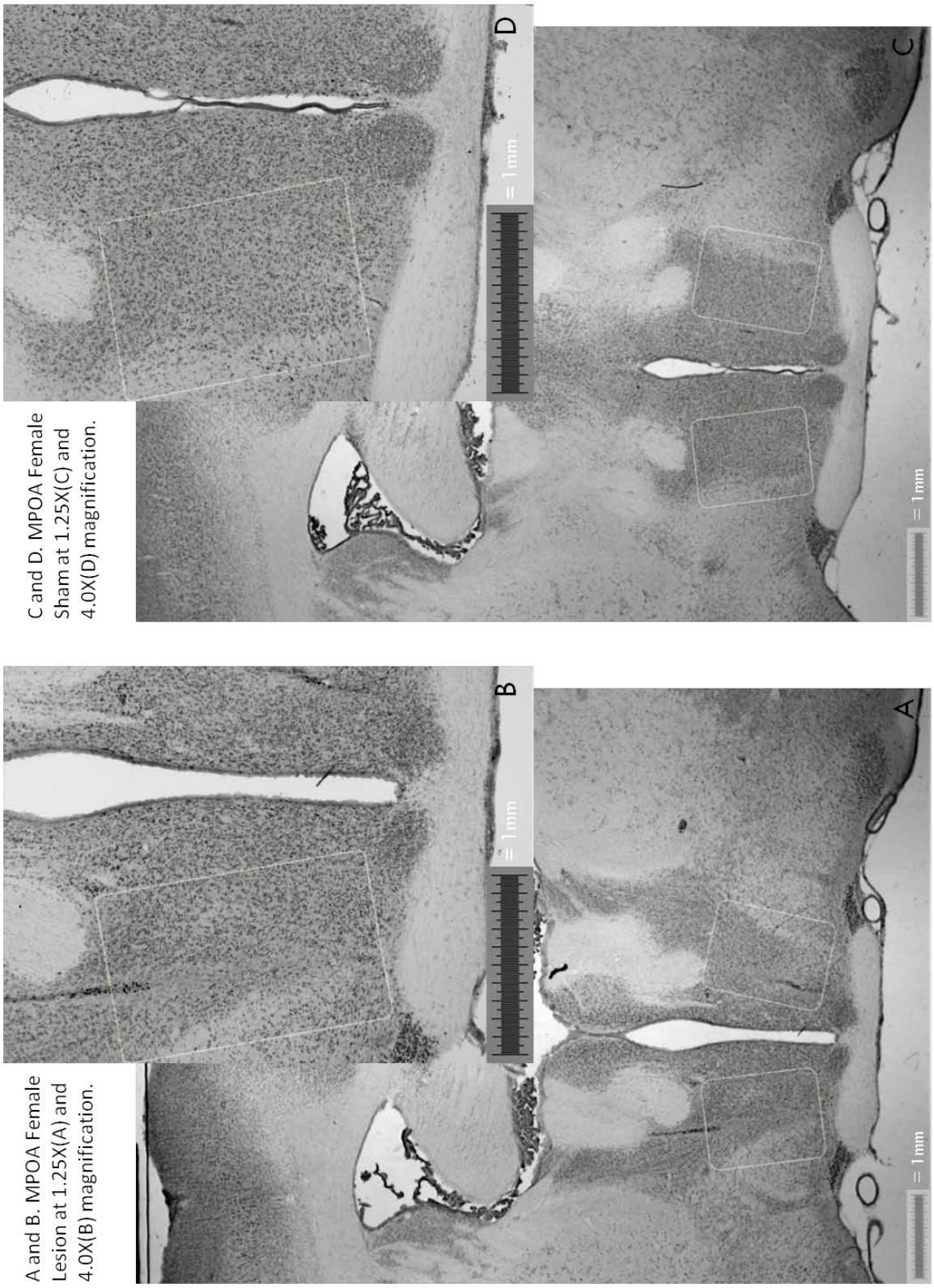
Figure 6A



A and B. MPOA Male Lesion at 1.25X(A) and 4.0X(B) magnification.

C and D. MPOA Male Sham at 1.25X(C) and 4.0X(D) magnification.

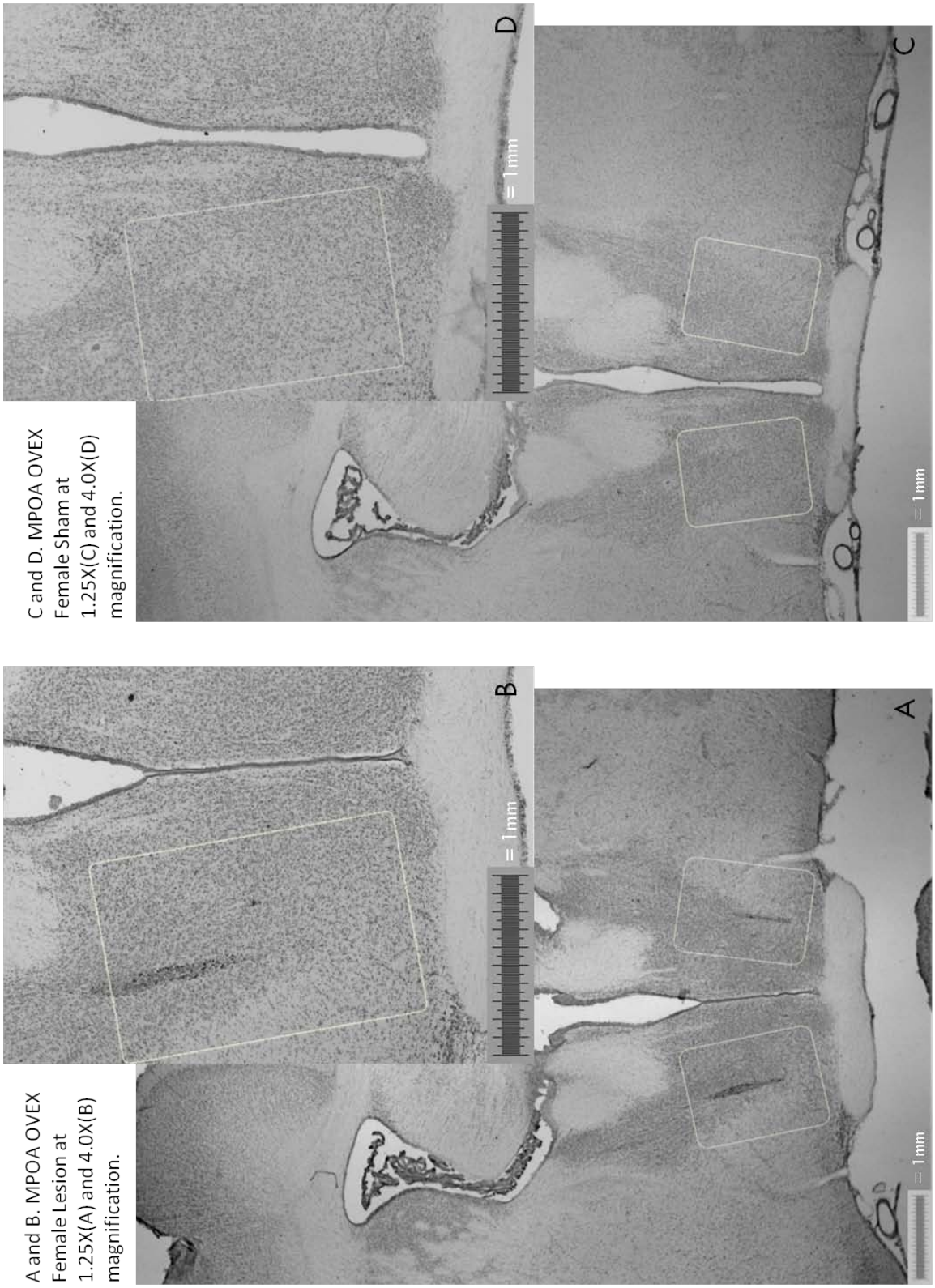
Figure 6B



A and B. MPOA Female Lesion at 1.25X(A) and 4.0X(B) magnification.

C and D. MPOA Female Sham at 1.25X(C) and 4.0X(D) magnification.

Figure 6C



A and B. MPOA OVEX Female Lesion at 1.25X(A) and 4.0X(B) magnification.

C and D. MPOA OVEX Female Sham at 1.25X(C) and 4.0X(D) magnification.

placements in the VMH (1.25x magnification) confirmed this premise for animals in the three groups, and this was further confirmed by examination of high-magnification (4.0x) representative placements.

Lesion damage was independently verified by obtaining the luminance and ROD of each section in each of the VEH and VMH IBO groups (Table 3). Significant differences in luminance were observed among the three conditions ($F(2,40)=18.09$, $p<0.0001$) and between the VEH and VMH IBO ($F(1,40)=19.18$, $p<0.0001$) groups. Moreover, significant differences in ROD were observed among the three conditions ($F(2,40)=19.26$, $p<0.0001$) and between the VEH and VMH IBO ($F(1,40)=18.82$, $p<0.0001$) groups. The reciprocal relationship between luminance and ROD was confirmed in these VMH groups. Thus, luminance and ROD of VMH IBO-lesioned animals were significantly higher and lower respectively than their respective VEH-treated counterparts in both intact males and intact females, but not in Ovex females. Further, tissue from Ovex VEH-treated females displayed significantly higher luminance and lower ROD relative to VEH-treated males (Table 3)

Minimum-maximum delineations appeared to be uniform in the intact male (Figure 6A), intact female (Figure 6B) and Ovex female (Figure 6C) groups receiving MPOA IBO lesions relative to the VEH-treated groups. Therefore, the three groups had highly-similar extents of MPOA lesions. Moreover, whereas the MPOA appeared either partially or completely destroyed in all animals in each of the three groups, unlike the VMH, the extent of the MPOA lesions appeared to diffuse cylindrically to immediately adjacent lateral and ventral structures bordering the nucleus. Examination of representative lesion placements in the MPOA (1.25x magnification) confirmed this description for animals in the three groups, and this was further

TABLE 3. VMH and MPOA Ibotenic Acid Lesions in Intact Male, Intact Female and Ovariectomized Female Rats: Luminance and Relative Optical Density (ROD) Measures.

Group	Control VMH	Lesion VMH	Control VMH	Lesion VMH	Control MPOA	Lesion MPOA	Control MPOA	Lesion MPOA
	Luminance	Luminance	ROD	ROD	Luminance	Luminance	ROD	ROD
Intact Male	156.97 (1.87)	165.06* (2.27)	0.21755 (.0055)	0.19407* (.0064)	162.79 (1.81)	171.94* (1.14)	0.20044 (.0052)	0.17475* (.0029)
Intact Female	163.46 (1.36)	171.65* (1.48)	0.19797 (.0039)	0.17523* (.0040)	167.99 (1.21)	173.12 (2.33)	0.18499 (.0033)	0.17087 (.0061)
Ovex Female	169.78+ (0.86)	169.68 (0.86)	0.17999+ (.0024)	0.18089 (.0032)	175.94+x (0.97)	171.423 (1.11)	0.16348+x (.0024)	0.17561 (.0031)

Significant Difference: * Control vs. Lesion; + Intact Males vs. Ovex Females; x: Intact Females vs. Ovex Females

confirmed by examination of high-magnification (4.0x) representative placements. Lesion damage was again independently verified by obtaining the luminance and ROD of each section in each of the VEH-treated and MPOA IBO groups as well (Table 3). Significant differences in luminance were observed among the three conditions ($F(2,40)=9.73$, $p<0.0004$) and between the VEH and MPOA IBO ($F(1,40)=7.74$, $p<0.0085$) groups. Correspondingly, significant differences in ROD were observed among the three conditions ($F(2,40)=10.95$, $p<0.0002$) and between the VEH and MPOA IBO ($F(1,40)=8.56$, $p<0.006$) groups. The reciprocal relationship between luminance and ROD was also confirmed in these MPOA groups. The luminance and ROD of MPOA IBO-lesioned animals was significantly higher and lower respectively than the VEH condition in intact males, but not in intact or Ovex females. VEH Ovex females had significantly higher luminance and lower ROD as compared to intact VEH males or females (Table 3).

Baseline Tail-Flick Latencies:

Significant differences in baseline tail-flick latencies were observed among the three groups ($F(2,22) = 93.93$, $p<0.0001$), among the three lesion conditions ($F(2,22) = 303.06$, $p<0.0001$), across the test times ($F(3,33) = 9.69$, $p<0.0001$), and for the interactions between groups and conditions ($F(4,44) = 5, 45$, $p<0.004$), between groups and times ($F(6,66) = 3.35$, $p<0.006$), between conditions and times ($F(6,66) = 2.18$, $p<0.05$), but not among groups, conditions and times ($F(12,132) = 1.63$, ns). Whereas VEH-treated intact males and females failed to differ in baseline tail-flick latencies, latencies of VEH-treated Ovex females were significantly higher than VEH-treated intact males or females (Table 4). Baseline tail-flick latencies of MPOA and VMH IBO- lesioned intact males, intact females and Ovex females were significantly higher than corresponding VEH-treated intact males, intact females and Ovex females (Table 4). Because of these small (~ 0.6-0.7 s) but significant differences in baseline

TABLE 4. Baseline Tail-Flick Latencies (Mean, ±SEM) of Intact Male, Intact Female and Ovarectomized (Ovex) Female Groups Receiving Sham, VMH or MPOA Ibotenic Acid Lesions.

Condition	**Group**	**30**	**60**	**90**	**120**
Intact Males	Sham	3.08 (0.1)	3.03 (0.1)	3.03 (0.1)	2.94 (0.5)
Intact Females	Sham	3.02 (0.1)	3.05 (0.1)	2.90 (0.1)	2.94 (0.1)
Ovex Females	Sham	3.41 (0.1)#$	3.35 (0.1)#$	3.40 (0.1)#$	3.34 (0.1)#$
Intact Males	VMH	3.54 (0.1)*	3.50 (0.1)*	3.52 (0.1)*+	3.50 (0.1)*
Intact Females	VMH	3.41 (0.1)*	3.42 (0.1)*	3.34 (0.1)*	3.36 (0.1)*
Ovex Females	VMH	3.90 (0.1)*	3.97 (0.1)*	3.95 (0.1)*	3.98 (0.1)*
Intact Males	MPOA	3.71 (0.1)*+	3.61 (0.1)*+	3.56 (0.1)*+	3.62 (0.1)*+
Intact Females	MPOA	3.42 (0.1)*	3.43 (0.1)*	3.37 (0.1)*	3.35 (0.1)*
Ovex Females	MPOA	4.00 (0.1)*	3.97 (0.1)*	3.94 (0.1)*	3.93 (0.1)*

*Significantly higher than corresponding sham-operated animals
+Significantly higher than corresponding intact females
#Significantly higher than Intact Males
$Significantly higher than Intact Females

tail-flick latencies, morphine's analgesic effects across doses and times are analyzed separately across the six groups and conditions. To assess differences among groups and among conditions, MPE differences in magnitude and potency (ED_{50}) are also examined.

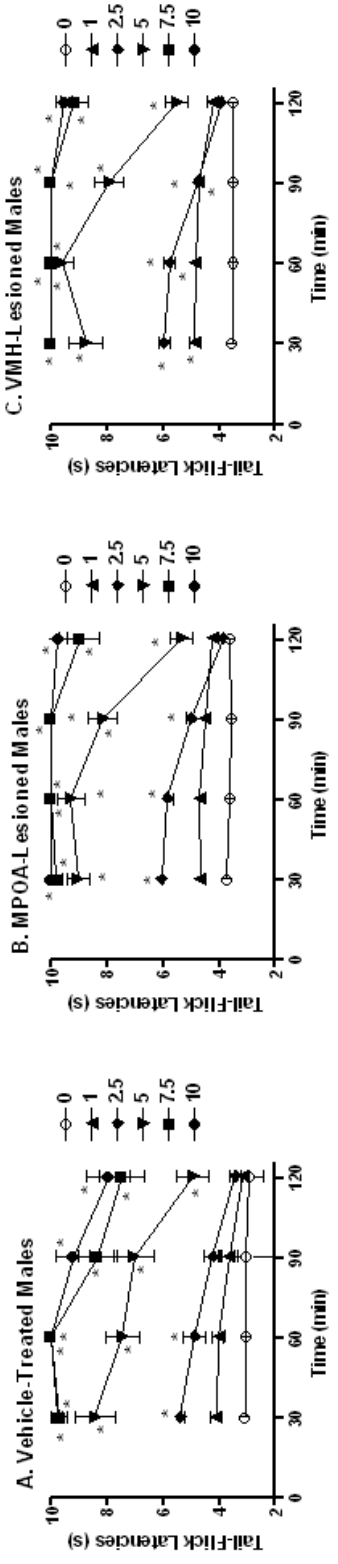
Morphine Analgesia in the Three Groups and Three Lesion Conditions:

Males: In the 10 **VEH-treated male** rats, significant differences in latencies were observed among doses ($F(5,48 = 73.57, p<0.0001$) across times ($F(3,162) = 37.03, p<0.0001$) and for the interaction between doses and times ($F(15,162) = 2.90, p<0.0004$). Morphine significantly increased latencies following the 2.5 (30-60 min), 5 (30-120 min), 7.5 (30-120 min) and 10 (30-120 min), but not the 1, mg/kg doses (Figure 7A). In the 8 **MPOA IBO-lesioned male** rats, significant differences in latencies were observed among doses ($F(5,42) = 266.29$, $p<0.0001$), across times ($F(3,126) = 48.34$, $p<0.0001$) and for the interaction between doses and times ($F(15,126) = 11.00$, $p<0.0001$). Morphine significantly increased latencies following the 2.5 (30-90 min), 5 (30-120 min), 7.5 (30-120 min) and 10 (30-120 min) but not the 1, mg/kg doses (Figure 7B). In the 8 **VMH IBO-lesioned male** rats, significant differences in latencies were observed among doses ($F(5,42) = 271.01$, $p<0.0001$), across times ($F(3,126) = 30.99$, $p<0.0001$) and for the interaction between doses and times ($F(15,126) = 5.18$ $p<0.0001$). Morphine significantly increased latencies following the 1 (30-90 min), 2.5 (30-90 min), 5 (30-120 min), 7.5 (30-120 min) and 10 (30-120 min) mg/kg doses (Figure 7C).

Intact Females: In the 9 **VEH-treated intact female** rats, significant differences in latencies were observed among doses ($F(5,44 = 119.64, p<0.0001$) across times ($F(3,144) = 74.73, p<0.0001$) and for the interaction between doses and times ($F(15,144) = 11.79$ $p<0.0001$). Morphine significantly increased latencies following the 2.5 (30 min), 5(30-90 min), 7.5 (30-120

Figure 7. Alterations in tail-flick latencies (s, mean +S.E.M.) across a 120 min time course following systemic administration of vehicle or morphine at doses of 1.0, 2.5, 5.0, 7.5 and 10.0 mg/kg in VEH-treated males (left panel), MPOA IBO-lesioned males (middle panel), and VMH IBO-lesioned males (right panel).

Figure 7


A. Vehicle-Treated Males
B. MPOA-Lesioned Males
C. VMH-Lesioned Males
Tail-Flick Latencies (s)
Time (min)
0
1
2.5
5
7.5
10

min) and 10 (30-120 min), but not the 1, mg/kg doses (Figure 8A). In the 8 **MPOA IBO-lesioned intact female** rats, significant differences in latencies were observed among doses ($F(5,36) = 189.18$, $p<0.0001$), across times ($F(3,108) = 53.24$, $p<0.0001$) and for the interaction between doses and times ($F(15,108) = 10.32$, $p<0.0001$). Morphine significantly increased latencies following the 1 (30-90 min) 2.5 (30-90 min), 5 (30-120 min) 7.5 (30-120 min) and 10 (30-120 min) mg/kg doses (Figure 8B). In the 8 **VMH IBO-lesioned intact female** rats, significant differences in latencies were observed among doses ($F(5,42) = 112.91$, $p<0.0001$), across times ($F(3,126) = 30.99$, $p<0.0001$) and for the interaction between doses and times ($F(15,126) = 5.18$, $p<0.0001$). Morphine significantly increased latencies following the 2.5 (30-90 min), 5 (30-120 min), 7.5 (30-120 min) and 10 (30-120 min) but not the 1, mg/kg dose (Figure 8C).

Ovex Females: In the 11 **VEH-treated Ovex female** rats, significant differences in latencies were observed among doses ($F(5,60) = 594.28$, $p <0.0001$, across times ($F(3,180) = 11.97$, $p<0.0001$) and for the interaction between doses and times ($F(15, 180) = 4.15$, $p <0.0001$). Morphine significantly increased latencies following the 1 (60-120 min), 2.5 (30-120 min), 5 (30-12- min), 7.5 (30-120 min) and 10 (30-120 min) mg/kg doses (Figure 9A). In the 11 **MPOA IBO-lesioned Ovex female** rats, significant differences in latencies were observed among doses ($F(5, 60) = 274.70$, $p< 0.0001$) across times ($F(3,180) = 14.70$, $p <0.0001$) and for the interaction between doses and times ($F(15,180) = 6.98$, $p< 0.0001$). Morphine significantly increased latencies across all doses and times (Figure 9B). In the 12 **VMH IBO-lesioned Ovex female** rats, significant differences in latencies were observed among doses ($F(5,66) = 336.32$, $p<0.0001$), across times ($F(3,198) = 21.85$, $p< 0.0001$) and for interaction between doses and

Figure 8. Alterations in tail-flick latencies (s, mean +S.E.M.) across a 120 min time course following systemic administration of vehicle or morphine at doses of 1.0, 2.5, 5.0, 7.5 and 10.0 mg/kg in VEH-treated females (left panel), MPOA IBO-lesioned females (middle panel), and VMH IBO-lesioned females (right panel) tested during the estrus phase of the estrous cycle.

Figure 8

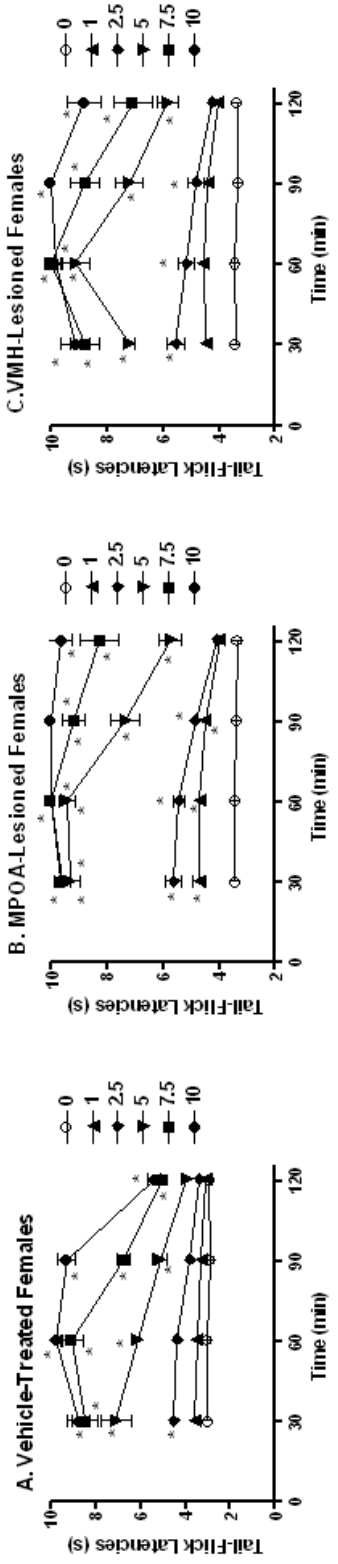
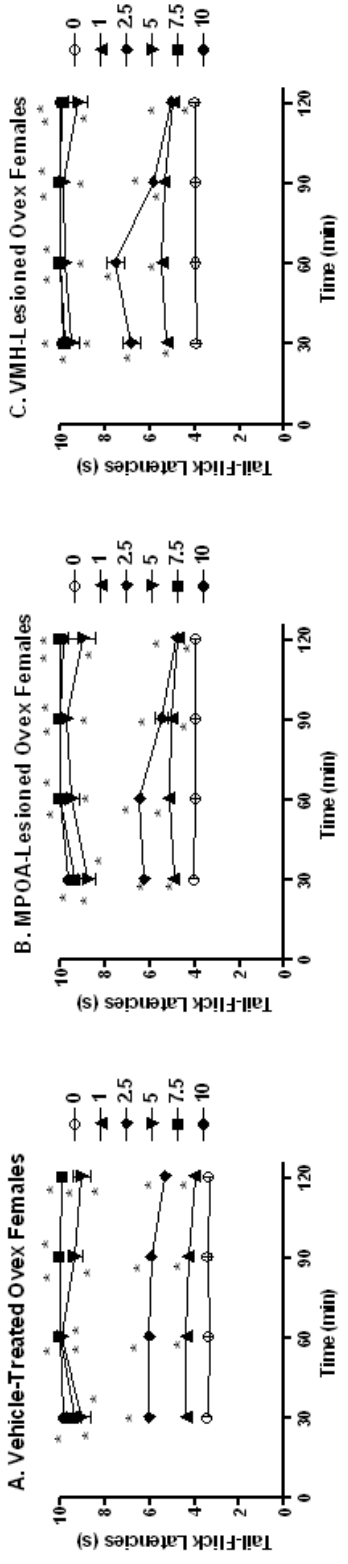

A. Vehicle-Treated Females
B. MPOA-Lesioned Females
C.VMH-Lesioned Females
Tail-Flick Latencies (s)
Time (min)
0
1
2.5
5
7.5
10

Figure 9. Alterations in tail-flick latencies (s, mean +S.E.M.) across a 120 min time course following systemic administration of vehicle or morphine at doses of 1.0, 2.5, 5.0, 7.5 and 10.0 mg/kg in VEH-treated Ovex females (left panel), MPOA IBO-lesioned Ovex females (middle panel), and VMH IBO-lesioned Ovex females (right panel).

Figure 9


A. Vehicle-Treated Ovex Females
B. MPOA-Lesioned Ovex Females
C. VMH-Lesioned Ovex Females
Tail-Flick Latencies (s)
Time (min)
0
1
2.5
5
7.5
10

times ($F(15,198) = 11.20$, $p<0.0001$). Morphine significantly increased latencies across all doses and times (Figure 9C).

Differences in the Magnitude (MPE) of Morphine Analgesia across Groups and Lesion Conditions:

Significant differences in the morphine MPE were observed among groups ($F(2,22)= 84.93$, $p<0.0001$), lesion conditions ($F(2,22)= 17.63$, $p<0.0001$) and doses ($F(4,44)= 2573$, $p<0.0001$), as well as for the interactions between groups and conditions ($F(4,44)= 12.48$, $p<0.0001$), groups and doses ($F(8,88)= 45.25$, $p<0.0001$), conditions and doses ($F(8,88)= 4.43$, $p<0.0001$), and among groups, conditions and doses ($F(16,176)= 2.45$, $p<0.002$). MPE effects were evaluated at each morphine dose.

Morphine (1 mg/kg): Significant differences in morphine (1 mg/kg) MPE were noted among groups ($F(2,75)= 8.42$, $p<0.0005$) and lesion conditions ($F(2,75)= 28.55$, $p<0.0001$) as well as for the interaction between groups and conditions ($F(4,75)= 2.57$, $p<0.04$). Whereas MPE effects failed to differ among lesion conditions in males, both MPOA, and VMH IBO-lesioned intact females displayed significantly higher MPE values than VEH-treated intact females (Table 5). Ovex VEH females displayed significantly higher MPE values than intact VEH females, indicating a gonadectomy-induced difference, and this effect was significantly increased further in VMH IBO-lesioned Ovex females (Table 5).

Morphine (2.5 mg/kg): Significant differences in morphine (2.5 mg/kg) MPE were noted among groups ($F(2,75)= 20.92$, $p<0.0001$), but not among lesion conditions ($F(2,75)= 2.01$, ns) or for the interaction between groups and conditions ($F(4,75)= 2.36$, ns). Whereas MPE effects failed to differ among lesion conditions in intact males and intact females, Ovex VEH

TABLE 5. Differences in the Magnitude (Maximum Percent Effect: MPE) of Morphine Analgesia in Intact Male, Intact Female and Ovariectomized (Ovex) Female Groups Receiving Sham, VMH or MPOA Ibotenic Acid Lesions.

Dose	Sham Intact Males	Sham Intact Females	Sham Ovex Females	MPOA Intact Males	MPOA Intact Females	MPOA Ovex Females	VMH Intact Males	VMH Intact Females	VMH Ovex Females
1.0	10.45	5.52	13.11+	14.09	16.42#	16.38	17.74	15.02#	21.35#+
2.5	21.15	14.85	37.09+$	24.52	24.27	29.56	17.74	23.64	38.94+
5.0	56.71*	36.93	89.56+$	67.87	69.06#	87.14	68.23	59.68	92.95
7.5	84.24*	61.60	97.26+	95.28	88.93#	96.56	96.62	79.69	98.70
10.0	89.07*	75.45	98.88+	98.94	97.00#	97.74	98.15	91.52#	99.25

*Significantly higher than Sham Intact Females (Sex Difference)
+Significantly higher than corresponding Females (Gonadectomy Difference)
#Significantly higher than corresponding sham-operated animals
$Significantly higher than corresponding Intact Males

females displayed significantly higher MPE values than both VEH-treated intact males and females, and VMH IBO-lesioned Ovex females displayed significantly higher MPE values than VMH IBO-lesioned intact females (Table 5).

Morphine (5 mg/kg): Significant differences in morphine (5 mg/kg) MPE were noted among groups ($F(2,75)= 37.53$, $p<0.0001$) and lesion conditions ($F(2,75)= 6.67$, $p<0.0002$) as well as for the interaction between groups and conditions ($F(4,75)= 2.94$, $p<0.026$). The significantly higher MPE values at this and subsequently higher doses in intact VEH-treated males relative to intact VEH-treated females indicated a sex difference (Table 5). Ovex VEH females again displayed significantly higher MPE values than VEH intact males, and both groups again displayed significantly higher MPE vales than VEH intact females (Table 5). Whereas MPE effects failed to differ among male and Ovex female IBO lesion conditions, MPOA IBO-lesioned intact females displayed significantly higher MPE values than VEH intact females (Table 5).

Morphine (7.5 mg/kg): Significant differences in morphine (7.5 mg/kg) MPE were noted among groups ($F(2,75)= 34.58$, $p<0.0001$) and lesion conditions ($F(2,75)= 13.65$, $p<0.0001$) as well as for the interaction between groups and conditions ($F(4,75)= 5.14$, $p<0.0001$). VEH-treated intact males and Ovex females displayed significantly higher MPE values than VEH-treated intact females (Table 5). Whereas MPE effects failed to differ among male and Ovex female IBO lesion conditions, both MPOA and VMH IBO-lesioned intact females displayed significantly higher MPE values than VEH intact females (Table 5).

Morphine (10 mg/kg): Significant differences in morphine (10 mg/kg) MPE were noted among groups ($F(2,75)= 15.26$, $p<0.0001$) and lesion conditions ($F(2,75)= 15.14$, $p<0.0001$) as well as for the interaction between groups and conditions ($F(4,75)= 5.80$, $p<0.0004$). VEH-

treated intact males and Ovex females displayed significantly higher MPE values than VEH-treated intact females (Table 5). Whereas MPE effects failed to differ among male and Ovex female IBO lesion conditions, both MPOA and VMH IBO-lesioned intact females displayed significantly higher MPE values than VEH intact females (Table 5).

Differences in the ED_{50} of Morphine Analgesia across Groups and Lesion Conditions:

The three groups of male animals displayed comparable ED_{50} values for morphine analgesia among the VEH (4.96 mg/kg), MPOA IBO-lesioned (4.23 mg/kg) and VMH IBO-lesioned (4.11 mg/kg) groups. VEH-treated intact females (6.57 mg/kg) displayed a 32% rightward shift in morphine's dose response curve relative to VEH-treated males, again indicating the presence of the expected gender difference, and a 90% rightward shift relative to Ovex VEH-treated females (3.46 mg/kg), indicating a gonadectomy difference. Whereas IBO lesions placed in the MPOA (3.63 mg/kg) or VMH (3.00 mg/kg) failed to appreciably shift the morphine dose response curve in Ovex females, the morphine dose response curve was shifted markedly to the left in MPOA (4.26 mg/kg) and VMH (4.77 mg/kg) IBO-lesioned intact females, eliminating the gender difference.

Discussion

The present study was designed to examine whether IBO-induced chemical destruction of the MPOA or the VMH altered the dose-dependent and time-dependent actions of systemic morphine analgesia in intact male rats, intact female rats tested during the estrus phase, and adult Ovex female rats to establish whether such lesions alter sex differences and gonadectomy differences in the magnitude and potency of systemic morphine's analgesic response as well as evaluate changes in baseline tail-flick latencies to establish whether any changes in morphine-induced analgesia occurred independently of any changes in basal nociception. The following

principal findings were observed: 1) sex and gonadectomy differences in morphine analgesia were observed with intact VEH-treated male rats displaying significantly greater morphine analgesia than intact VEH-treated female rats without concomitant changes in baseline latencies, and with Ovex VEH-treated female rats displaying significantly greater morphine analgesia than intact VEH-treated female rats with concomitant though small increases in baseline latencies; 2) the extent and pattern of IBO lesions placed in the VMH were confined to the nucleus and similar in the three groups of rats, whereas the extent and pattern of IBO lesions placed in the MPOA were similar in the three groups of rats, but extended cylindrically beyond the nuclear borders; 3) VMH and MPOA IBO-induced lesions significantly enhanced the magnitude and potency of morphine analgesia selectively in intact female rats, but not in either intact male rats or Ovex female rats. Implications of these data for a model elucidating sex and gonadectomy differences in morphine analgesia are described in Section 4.

Sex and Gonadectomy Differences in Morphine Analgesia: In the present study, intact VEH-treated female rats tested during the estrus phase displayed significantly lower magnitudes and reduced potencies of systemic morphine analgesia than intact VEH-treated male rats. These data are consistent with the profound sex differences observed for morphine-induced analgesia following systemic (e.g., Baamonde et al., 1989; Badillo-Martinez et al., 1984; Candido et al., 1992; Kavaliers and Innes, 1987), ventricular (Kepler et al., 1989) or intracerebral (Boyer et al., 1998; Krzanowska and Bodnar, 1999; Loyd and Murphy, 2006) injections.

Also in the present study, female adult gonadectomy effects were observed such that Ovex VEH-treated female rats displayed significantly greater magnitudes and potencies of systemic morphine analgesia than intact VEH-treated female rats tested during the estrus phase. These effects were accompanied by concomitant though small increases in baseline latencies

observed in the Ovex rats. The efficacy of the ovariectomy was confirmed in two ways. First, there was a profound 92% reduction in the size of accessory uterine sexual organs. Second, the luminosity and relative optical density measures in the MPOA of VEH-treated intact females were respectively significantly lower and higher than those of VEH-treated Ovex females; the pattern of these measures in the VMH were identical, but failed to achieve significance. These data strongly suggest that neural morphology of these sites is structurally decreased following adult ovariectomy. There is some evidence suggesting that there are functional deficits observed within the MPOA and VMH in Ovex as compared to intact females as demonstrated by decreased estrogen receptor immunoreactive cells in the MPOA and VMH (Ehret and Buckenmeier, 1978), decreased estrogen-binding neurons (Koch, 1990) and CCK-R binding (Popper et al., 1996) in the VMH, decreased beta-endorphin-like (Cheung et al., 1995) and galanin (Bloch et al., 1993) immunoreactivty in the MPOA and decreased firing to 17-beta estradiol application in the MPOA (Kelly et al., 1978), but no changes in GAD-induced modulation by estrogen (Herbison et al., 1992) or in CGRP immunoreactivity (Herbison, 1992). Our laboratory (Krzanowska et al., 1999) previously demonstrated that ovariectomy of adult female rats produces greater and more potent morphine analgesia elicited from the vlPAG than that of intact females tested during the estrus phase. The previously-reported minimal alterations in the magnitude or the potency of morphine analgesia following systemic administration in Ovex relative to intact female animals (Ali et al., 1995; Banerjee et al., 1983; Cicero et al., 1996, Islam et al., 1993; Kasson and George, 1984; Stoffel et al., 2003) might be attributed to their failure to compare Ovex females with intact females in the estrus phase of the estrous cycle since the latter condition induces the smallest magnitude of morphine analgesia elicited from the

vlPAG (Bernal et al., 2007; Shane et al.,2007), differences in nociceptive measures, or completeness of the ovariectomies.

Efficacy and Specificity of VMH and MPOA IBO-Induced Lesions: The extent and pattern of excitotoxic IBO lesions placed in the VMH were similar in intact males, intact female and Ovex female rats, and very importantly, largely confined to the nucleus. Some previous excitotoxic IBO-induced lesion studies employed variable methodologies that proved difficult to control lesion extent yet produce consistently complete lesions of the VMH (e.g., Challet et al., 1997; Hoffman et al., 1996; Pagani and Rosen, 2009). Yet these studies provided a framework by which we were able to maximally control such variability in pilot studies in which we chose effective concentrations (1 ul/side), pH (7.2), doses (1 ug/side), times (syringe left in place for 5 min), and bilateral microsyringe placements within rather than above the VMH. These pilot studies led to our subsequent studies in the three groups that resulted in selective and similar VMH cell damage across the three groups. The consistency of the VMH IBO lesion was confirmed histologically by observers uninformed about the behavioral data or experimental group. The efficacy of the VMH IBO lesions may also have been due to the shape of the target, as the destroyed area tended to create a cylindrical shape below and around the microsyringe tip. It is possible that the spread of these injections were contained by the capsule of lipid-rich fibers of the tuberal region of the lateral hypothalamus that is found lateral and dorsolaterally to the VMH nucleus; such a finding was observed in the containment of horseradish peroxidase (HRP) in tracing studies (e.g., Fahrbach et al., 1984, 1989). Significant independently-analyzed increases in luminance and corresponding decreases in ROD measures within the VMH were also observed in intact male and female groups. The failure to observe these effects in Ovex

females receiving VMH IBO lesions may have been attributed to “ceiling” and “floor” actions upon these measures observed in VEH-treated Ovex animals.

The extent and pattern of excitotoxic IBO acid lesions placed in the MPOA were also similar in intact males, intact female and Ovex female rats, but the confinement of the damage extended beyond this nucleus’ borders. Unlike the VMH, the MPOA is immediately bordered by a greater heterogeneous nuclear area with multiple cell populations found medially (the medial, central and lateral subdivisions of the medial preoptic nucleus and periventricular nucleus) and laterally (lateral preoptic area) (Paxinos and Watson, 2004). Previous excitotoxic IBO lesion studies aimed at the MPOA (e.g., Guarraci and Clark, 2006; Meerts and Clark, 2009; Yang and Clemens, 2000) employed doses (2-10 ug) that were sizably larger than our (1 ug) study, and produced far larger lesion extents in both rostro-caudal and medio-lateral extents that indeed damaged the MPOA, the medial preoptic nucleus and the lateral preoptic area. Our present IBO lesion patterns of the MPOA were both cylindrical in shape ventral to, but also lateral to the microsyringe track that diffused more laterally from the epicenter than our VMH lesions. However in contrast to these other studies, our damage was largely found within the MPOA itself, and minimally extended medially and laterally. However, any “conservative” interpretations about the effects of these MPOA lesions should be interpreted as damage to the MPOA “region” including many of the above-cited nuclei. Again, significant independently-analyzed increases in luminance and corresponding decreases in ROD measures within the MPOA were also observed in intact male and female groups. Again, the failure to observe these effects in Ovex females receiving MPOA lesions may have attributed to “ceiling” and “floor” actions upon these measures observed in VEH-treated Ovex animals.

VMH and MPOA IBO-Induced Lesions Selectively Enhance Morphine Analgesia in Intact Females, but not in Intact Males or Ovex Females: In the present study, VMH and MPOA IBO-induced lesions significantly enhanced the magnitude and potency of systemic morphine analgesia selectively in intact female rats tested during the estrus phase, but failed to alter either analgesic pattern in intact male rats or Ovex female rats. The failure of VMH or MPOA IBO lesions to significantly affect the magnitude or potency of systemic morphine analgesia in intact male rats suggests that accumulation of estradiol in these nuclei is neither necessary nor sufficient to explain the greater magnitude of analgesic effects in males. As indicated previously, the removal of circulating sex hormones (e.g., estrogen, progesterone) by adult gonadectomy in the VEH-treated Ovex female groups resulted in significantly greater magnitudes and potencies of systemic morphine analgesia relative to intact VEH-treated females tested during the estrus phase. This would suggest that the effects of high circulating levels of the sex hormones, estrogen and progesterone in the intact female rats tested during the estrus phase were important in suppressing systemic morphine's analgesic response. Yet VMH or MPOA IBO-induced lesions failed to significantly alter the enhanced magnitude or potency of systemic morphine analgesia noted in VEH-treated Ovex female rats, suggesting that loss of both the circulating hormones and their VMH or MPOA ERA receptors did not further accentuate the effectiveness of systemic morphine's analgesic effects relative to the loss of the circulating hormones per se.

The highly selective and specific enhancements of the potency and magnitude of systemic morphine analgesia following VMH or MPOA IBO lesions were thus limited to intact female rats tested during the estrus phase of the cycle, a point at which the effects of estrogen and progesterone levels are highest. Therefore, removal of the estradiol and progestin receptors

within the VMH or MPOA mitigated any effects of these heightened estrogen and progesterone levels. The enhanced systemic morphine analgesia coupled with the ineffectiveness of these circulating female gonadal hormones in female rats with VMH or MPOA IBO lesions thereby suggests that the sex difference in morphine analgesia is due to an inhibitory presence of these circulating sex hormones upon systemic morphine analgesia in the intact and cycling female rat. Thus, the significantly greater magnitude and potency of systemic morphine analgesia in intact females with MPOA or VMH IBO lesions suggest that the lesser degree of morphine analgesia in intact females during estrus is due to tonic inhibitory influences of gonadal hormones, particularly estrogen, acting on these estradiol-accumulating hypothalamic nuclei. Destruction of these nuclei with IBO lesions thereby produced a disinhibition of endogenous pain-inhibitory neural circuitry, and thereby greater analgesia in these intact estrous-cycling females because hypothalamic estrogen-binding neurons sensitive to changes in sex hormone levels project to PAG neurons (Turcotte and Blaustein, 1999), a pathway absent following hypothalamic lesions (Hoffman et al., 1996).

A Proposed Model of Hypothalamic-Brainstem Circuits Mediating Sex and Gonadectomy Differences in Morphine Analgesia:

In addition to the consistent observations that systemic morphine analgesia is significantly greater in male relative to female rats, the same pattern of effects is observed on acute models of nociception following microinjection of morphine into the vlPAG (e.g., Bobeck et al., 2009; Krzanowska and Bodnar, 1999) and RVM (Boyer et al., 1998). Male rats display significantly greater magnitudes of morphine analgesia elicited from the vlPAG on a chronic inflammatory model of pain (Loyd and Murphy, 2006). Physiological and anatomical studies provide further support for the vlPAG and RVM as a locus of sex differences in morphine

analgesia. First, mu opioid agonists facilitate excitation in vlPAG cells through interactions with NMDA receptors (Kow et al., 2002); the ultrastructural arrangement of mu opioid receptors with GABAergic PAG neurons or PAG projection neurons labeled retrogradely from the medulla indicate that mu opioid receptor ligands act to both inhibit the former and directly act on the latter (Commons et al., 2000). Second, corresponding anatomical data showed that although females displayed significantly more retrogradely-labeled PAG-RVM output neurons than males, inflammatory pain activated more PAG-RVM cells in male than in female rats (Loyd and Murphy, 2006). This PAG-RVM circuit includes physiologically-identified RVM ON and OFF cells that in turn send GABA projections to the spinal cord (Morgan et al., 2008). Third, Fos-induced activation levels in the PAG induced by inflammation was suppressed by systemic morphine in male, but not female rats, and morphine preferentially activated the PAG-RVM pathway in the male rat, providing a potential central mechanism of action for morphine analgesic sex differences (Loyd et al., 2007). Fourth, male rats displayed greater mu opioid receptor expression in the PAG than females, and displayed significantly greater thermal hyperalgesia than females after intra-PAG morphine injection. Indeed, selective lesions of mu-opioid receptor-expressing PAG neurons blocked systemic morphine analgesia in males only (Loyd et al., 2008b). Fifth, the greater decrease in analgesia following chronic morphine tolerance treatment in male relative to female rats is accompanied by a steady decline in the percentage of PAG-RVM output neurons activated by morphine in males, but not females (Loyd et al., 2008a). Finally, male rats possess significantly greater numbers of androgen receptor-immunoreactive neurons in the PAG that project to the RVM than females. However, the two sexes do not differ in amount of estrogen receptor alpha, suggesting specificity of which gonadal

hormone mechanisms might mediate sex differences in central morphine analgesia (Loyd and Murphy, 2008).

The present findings provide potential hypothalamic substrates by which sex hormone-accumulating nuclei (the VMH and MPOA) influence the ability of supraspinal morphine analgesia to be differentially expressed in male and female rats. Given that the chemical IBO destruction of VMH and MPOA cells bodies selectively enhanced systemic morphine analgesia in intact estrus-phase females, but not in intact males or Ovex females, this suggests that these sex hormone-accumulating VMH and MPOA outputs would normally act to directly or indirectly inhibit endogenous pain-inhibitory circuits. The two best candidates for such inhibition are the vlPAG and RVM. The MPOA has an orderly, reciprocally-connected and longitudinally-organized columnar organization projection that extends along the whole rostro-caudal axis of the PAG (Rizvi et al., 1992), innervating PAG cells that subsequently project to the RVM (Rizvi et al., 1996). The MPOA-PAG connection provides gonadal steroid receptor innervation especially for the male (Murphy and Hoffman, 2001). Direct reciprocal anatomical connections have also been observed between the MPOA and pontine and medullary midline nuclei (Holstege, 1987; Veening et al., 1990), particularly the nucleus raphe magnus (NRM) (Murphy et al., 1999). Similarly, a strong VMH-PAG anatomical connection has been described classically (Beitz, 1982; Dostrovsky et al., 1983; Mantyh, 1982; Saper et al., 1976). The anatomical connections especially between the MPOA and PAG have also been functionally confirmed in neurophysiological studies (Jiang and Behbehani, 2001; Lumb and Morrison, 1986; Carstens et al., 1982; Lumb, 1990; Lumb and Cervero, 1989; Mokha et al., 1987). Further, hypothalamic enkephalinergic neurons, sensitive to changes in sex hormone levels, express enkephalin gene products in females to a greater degree than males (Priest et al., 1995; Romano

et al., 1988, 1989, 1990), and eventually project to estrogen-binding PAG neurons (Turcotte & Blaustein, 1999), a pathway that is eliminated by hypothalamic lesions (Hoffman et al., 1996). Two further lines of research are necessary to conclude that these hypothalamic-midbrain-medullary circuits are involved in the mediation of sex differences in central morphine analgesia: a) demonstration that IBO lesions of the VMH and MPOA block the sex differences observed following morphine administered directly into the vlPAG and RVM in the same manner as systemic morphine, and b) demonstration that direct inactivation of sex hormone receptors in the VMH and MPOA mimic IBO lesion effects upon sex differences in systemic and central morphine analgesia.

Chapter 6

General Discussion

The **First Specific Aim** of this dissertation evaluated the interaction between organizational and activational effects of gonadal hormones on morphine analgesia following systemic administration **(Specific Aim 1A)** and intracerebral administration into the vlPAG **(Specific Aim 1B)** in female rats receiving neonatal androgenization and/or adult ovariectomies and gonadal hormone replacement as compared to control male rats. The **Second Specific Aim** of this dissertation examined whether excitotoxic (ibotenic acid) chemical destruction of two gonadal hormone accumulating nuclei, the medial preoptic area (MPOA) or the ventromedial hypothalamic nucleus (VMH) would alter the dose-dependent, time-dependent and sex-dependent actions of systemic morphine analgesia in adult intact male, intact female and ovariectomized female rats. Based on previous observations, the results of all three experiments confirmed our hypothesis that male rats would display significantly greater magnitudes and potencies of morphine analgesia than intact female rats tested during the estrus phase of the cycle, thereby confirming the presence of sex differences following systemic and intracerebral PAG administration of morphine. Based on previous observations, we also hypothesized that adult ovariectomy in female rats would result in greater magnitudes and potencies of morphine analgesia than cycling intact female rats tested during the estrus phase, with similar patterns of analgesic effects to that of male rats. Ovariectomy significantly increased the potency and magnitude of morphine analgesia elicited from the vlPAG in Study 1B, and increased the potency and magnitude of systemic morphine analgesia in Study 2, but not in Study 1A. In previous work, sex differences in morphine analgesia have been invariably consistent (see reviews: Bodnar et al., 2002; Craft, 2003; Craft et al., 2004; Greenspan et al., 2007), whereas

female gonadectomy effects have resulted in increased analgesic effects (Krzanowska and Bodnar, 1999; Loyd and Murphy, 2006) or a failure to observe effects (e.g., Cicero et al., 1996, 2002; Islam et al., 1993; Kepler et al, 1989, 1991). Although the dose ranges in Studies 1A and 2 were not identical, they certainly were overlapping, and could not explain the results between studies. It should be noted that nociceptive test, ascending regimen of doses, and time course of treatment were identical between studies, and thereby also could not account for the different outcomes. The hypothesis concerning the ability of perinatal treatment with TP in female pups to produce significantly greater magnitudes and potencies of morphine analgesia than pups treated with perinatal vehicle was confirmed for both systemic (Experiment 1A) and intracerebral vlPAG (Experiment 1B) treatments, and thereby replicated neonatal gonadectomy effects for systemic (Cicero et al., 2002) and intracerebral vlPAG (Krzanowska et al., 2002) morphine analgesia. Thus, the effects of three major manipulations, sex differences, adult gonadectomy differences and neonatal gonadectomy differences, which previously produced powerful changes in systemic and central morphine analgesia, were confirmed in the present series of studies.

One series of novel outcomes of the present experiments involved the examination of interactions between organizational and activational effects of gonadal hormones in their ability to modulate systemic (Study 1A) and central (Study 1B) morphine analgesia. These interactions differed as a function as to whether morphine was administered systemically or centrally. In Study 1A, whereas ovariectomy decreased the magnitude of systemic morphine analgesia in female rats treated neonatally with TP following the lowest dose of morphine, there were only minimal (8%) changes in the potency of systemic morphine analgesia between the two groups. These data indicated that the interaction between manipulations of organizational (e.g., neonatal

androgenization) and activational (e.g., adult ovariectomy) effects of gonadal hormones do not appear to add or detract from the ability of neonatal androgenization to increase the magnitude of systemic morphine analgesia in female rats. Moreover, the circulating levels of EB appeared to be more important in modulating systemic morphine analgesia in normal and ovariectomized females than in females treated neonatally with androgens. In Study 1B, the central premise of a proposed organizational-activational gonadal hormone interaction in female rats was confirmed by the observation that neonatally-androgenized and adult ovariectomized female rats displayed significantly greater magnitudes of vlPAG morphine analgesia than neonatally-androgenized but gonadally intact female rats across a range of morphine doses, thereby implicating the vlPAG as a central locus at which such interactions occur. The observed positive organizational and activational gonadal hormone interaction for morphine analgesia elicited from the vlPAG stands in contrast to previous failures to observe such consistent gonadal hormone interactions for systemic morphine analgesia. The failure to observe these interactions following systemic administration may be due to the multiple sites of action (spinal cord, medullary and pontine sites) at which morphine exerts analgesic actions, yet are not the direct recipient of sex steroid changes as in the vlPAG.

The second series of novel outcomes of the present experiments involved the examination of whether destruction of gonadal hormone-accumulating nuclei, specifically the MPOA and VMH, would alter the pattern of sex and gonadectomy differences in baseline nociceptive responses or systemic morphine analgesia. Whereas adult ovariectomy increased baseline latencies relative to intact males or females, MPOA and VMH lesions increased baseline latencies than the three groups of animals receiving sham surgeries. In terms of systemic morphine analgesia, VMH and MPOA lesions significantly enhanced the magnitude and potency

of morphine analgesia selectively in intact female rats, but failed to appreciably change the pattern or magnitude in either intact male rats or Ovex female rats.

Implications for Sex Differences and Mechanisms of Action in Animal Models:

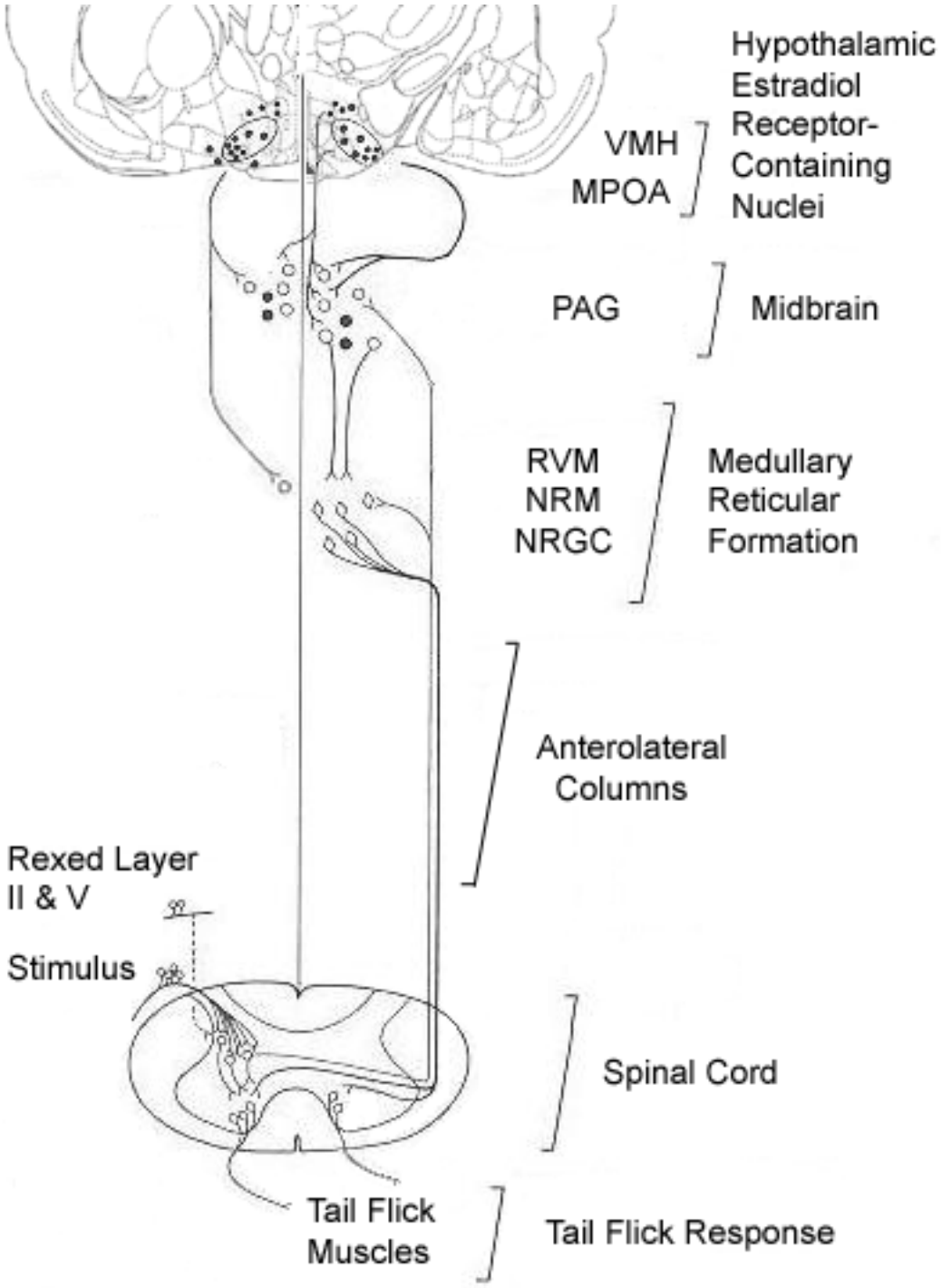
The present findings provide evidence for a novel model elucidating how hypothalamic substrates by which sex hormone-accumulating nuclei (the VMH and MPOA) influence supraspinal morphine analgesia differentially in male and female rats. Given that the chemical excitatory amino acid-induced destruction of VMH and MPOA cell bodies selectively enhanced systemic morphine analgesia in intact estrus-phase females, but not in intact males or Ovex females, this suggests that these sex hormone-accumulating VMH and MPOA outputs would normally act to directly or indirectly inhibit endogenous pain-inhibitory circuits of the vlPAG and RVM which in turn, inhibit the spinal cord (Figure 10).

The similar abilities of MPOA and VMH lesions to selectively enhance systemic morphine analgesia in intact females is quite unique given previous instances of MPOA and VMH functioning with MPOA neuronal physiology frequently opposing hypothalamic effects. For example, in reproduction, although estrogen increases the excitability of the medial hypothalamus and preoptic area which are responsible for lordosis and courtship behavior respectively, the concept that the physiological opposition between the two enforces a sequential performance of the two behaviors: courtship and only then lordosis (Pfaff, 1999).

The MPOA with its reciprocal projections innervating PAG cells that subsequently project to the RVM (Rizvi et al., 1996) provides gonadal steroid receptor innervation especially for the male (Murphy and Hoffman, 2001). The anatomical connections between the MPOA and PAG have been functionally confirmed in neurophysiological studies (Jiang and Behbehani, 2001; Lumb and Morrison, 1986; Carstens et al., 1982; Lumb, 1990; Lumb and Cervero, 1989;

Figure 10. Model elucidating how hypothalamic substrates by which sex hormone-accumulating nuclei influence the ability of supraspinal morphine analgesia differentially in male and female rats showing connections between the VMH and MPOA, the vlPAG, the RVM and the spinal cord.

Figure 10


Hypothalamic
Estradiol
Receptor-
Containing
Nuclei
VMH
MPOA
PAG
Midbrain
RVM
NRM
NRGC
Medullary
Reticular
Formation
Anterolateral
Columns
Rexed Layer
II & V
Stimulus
Spinal Cord
Tail Flick
Muscles
Tail Flick Response

Mokha et al., 1987). Direct reciprocal anatomical connections have also been observed between the MPOA and the nucleus raphe magnus (NRM) (Murphy et al., 1999). Similarly, a strong VMH-PAG anatomical connection has been described classically (Beitz, 1982; Dostrovsky et al., 1983; Mantyh, 1982; Saper et al., 1976). Further, hypothalamic enkephalinergic neurons, present in the MPOA and VMH and sensitive to changes in sex hormone levels, eventually project to estrogen-binding PAG neurons (Turcotte & Blaustein, 1999), a pathway that is eliminated by hypothalamic lesions (Hoffman et al., 1996).

It has been established that morphine administration produces greater antinociception in males compared to females as is consistent with our observations. Since the same pattern of effects has been observed on acute models of nociception following microinjection of morphine into the vlPAG (e.g., Bobeck et al., 2009; Krzanowska and Bodnar, 1999) and RVM (Boyer et al., 1998) as well as chronic inflammatory models of pain with male rats display significantly greater magnitudes of morphine analgesia elicited from the vlPAG (Loyd and Murphy, 2006), efforts to explain sex differences in morphine antinociception have concentrated on the vlPAG and the RVM. For example, Loyd and Murphy (2006) showed that females displayed significantly more retrogradely-labeled PAG-RVM output neurons than males the extent of which was greater by a third and that inflammatory pain preferentially activated more PAG-RVM cells in male than in female rats. Only half as many PAG-RVM output neurons were activated by inflammatory pain in females. This PAG-RVM circuit includes physiologically-identified RVM ON and OFF cells that in turn send GABA projections to the spinal cord (Morgan et al., 2008). Also, Fos-induced activation levels in the PAG induced by inflammation was shown to be suppressed by systemic morphine in male, but not female rats, and morphine preferentially activated the PAG-RVM pathway in the male rat, providing another potential

central mechanism of action for morphine analgesic sex differences (Loyd et al., 2007). Furthermore, male rats displayed greater mu opioid receptor expression in the PAG than females, and displayed significantly greater thermal hyperalgesia than females after intra-PAG morphine injection. Indeed, selective lesions of mu-opioid receptor-expressing PAG neurons blocked systemic morphine analgesia in males only (Loyd et al., 2008b). In addition, the greater decrease in analgesia following chronic morphine tolerance treatment in male relative to female rats is accompanied by a steady decline in the percentage of PAG-RVM output neurons activated by morphine in males, but not females (Loyd et al., 2008a). Finally, male rats possess significantly greater numbers of androgen receptor-immunoreactive neurons in the PAG that project to the RVM than females. However, the two sexes do not differ in amount of estrogen receptor alpha, suggesting specificity of which gonadal hormone mechanisms might mediate sex differences in central morphine analgesia (Loyd and Murphy, 2008).

Expression of the SRY gene on the Y chromosome eventually leads to the development of testes resulting in high levels of testosterone early in the life of the genetic male. The critical period for the rat brain being from birth until about 5 days after birth have at least two types of permanent effects on the developing male brain: first, masculinization or increased male-typical functions and second, defeminization which is a loss of female-typical behavior and neuroendocrine functions. Therefore, genetic male rats and mice displaying greater potencies and magnitudes of morphine analgesia following systemic and central administration than female rats and mice can be explained in terms of the presence or absence of the SRY gene. Thus, adult male rats neonatally castrated on Day 1 after birth essentially removed the effects of the SRY gene, displayed magnitudes and potencies of either intracerebral or systemic morphine-induced analgesia that were significantly lower than sham-operated males, but similar to that of neonatal

vehicle-treated females tested during the estrus phase. Correspondingly, adult female rats neonatally treated with testosterone propionate on Day 1 after birth essentially mimicked the presence of the SRY gene, and displayed magnitudes and potencies of either intracerebral or systemic morphine-induced analgesia that were significantly higher than neonatal vehicle-treated females tested during the estrus phase, but similar to that of sham-operated males.

Our model of morphine analgesia influenced by hypothalamic substrates is not mutually exclusive and does not necessarily conflict with other proposed models of analgesia. Rather, our model with the MPOA and VMH and their connections to the PAG and RVM further downstream on the way to the spinal cord make for our model to be effortlessly integrated into the whole that is morphine antinociception.

Implications for Human Sex Differences in Analgesia:

The study of sex differences in animals has clinical implications in humans as the data support previous findings that pain sensitivity and sensitivity to opioids should not be assumed to be equal in both sexes. This is especially true for morphine which, in many other rodent studies as well as this one, has been shown to elicit profound sex differences. Since morphine is the prototypical analgesic in clinical use and the gold standard to which all new analgesics are compared, the importance of evaluating sex as an independent variable by including both sexes in experimental pain research cannot be emphasized enough.

The findings from human pain studies and animal models of pain tend to be somewhat contradictory when it comes to sex differences in sensitivity of morphine and other opioids with animal models generally reporting greater effects in males (e.g., Cicero et al., 1996; Kepler et al., 1989; Krzanowska et al., 2002) and human studies showing the opposite. The majority of human data have been derived from studies which assessed opioid consumption whereas animal studies

generally assess opioid antinociception. One decade ago, Miaskowski and Levine (1999) reviewed all available studies (*n* =18, studies published from 1966 to 1998) on mu-opioid patient-controlled analgesia (PCA) for postoperative pain that listed data from men and women. Opioid consumption was higher in men than in women in ten studies, whereas the remainder found no sex differences. In recent prospective studies comparing PCA opioid consumption during the first postoperative days, higher opioid use in men was a consistent finding (Chia et al., 2002; Lehman et al., 2001). A major limiting factor in these reviewed PCA studies is that sex comparisons were not the primary focus of investigation, and there were inadequate controls for confounding variables such as underlying disease, age and opioid plasma concentrations. Moreover, they have been criticized for studying opioid consumption rather than analgesia. Opioid consumption may be affected by other factors than just postoperative pain such as baseline pain sensitivity, expectation, fear of addiction, and the occurrence of nausea/vomiting, which occurs with greater frequency in women. In addition, since onset times of opioids may differ between men and women (Chia et al., 2002), studies that assessed pain scores and opioid efficacy during the earlier postoperative hours would thereby yield different results from studies in which an extended study period is utilized. With this in mind, due to a number of methodological differences between human and rodent studies, comparisons between the two are probably not valid and can even be misleading and furthermore, human studies have been primarily concerned with sex differences in experience and modulation of chronic pain whereas most animal studies evaluated acute pain.

More recently, these concerns have been addressed in a number of experimental human and clinical studies that were designed to examine sex differences in opioid effect a priori. In studies using morphine, there were either no sex differences in analgesia assayed with several

experimental pain modalities (Bijur et al., 2008; Fillingim et al., 2005; Gordon et al., 1995) or such differences were reliant on the specific pain model used. In the latter instance, Zacny (2002) found sex differences in morphine analgesia in the cold pressure test, but not for pressure pain. However, Sarton and co-workers (2000) compared the analgesic effects of morphine in men and women and linked any effects to plasma concentrations using a pharmacokinetic-pharmacodynamic modeling design. Studying healthy volunteers using an experimental electrical pain model, they reported that morphine is more potent in women than men (as expressed by the C50 values), and that the onset/offset of morphine is slower in women than in men. Nonetheless, the plasma concentrations of morphine and its two major metabolites, morphine 6- and 3-glucuronide, were identical between sexes. Other mu-receptor opioids such as alfentanil and morphine-6-glucuronide, an active morphine metabolite, caused analgesic responses of similar magnitude in the two sexes in an electrical pain model (Olofsen et al., 2005; Romberg et al., 2004). However, women experienced more pain and required more morphine than men to achieve a similar degree of analgesia (Cepeda and Carr, 2003). Collectively, these data suggest that mu-receptor opioids are not subject to robust analgesic sex differences in humans. Opioid analgesics acting primarily through the kappa-opioid receptor such as nalbuphine, butorphanol and pentazocine have also been studied, and have been reported to produce more intense and prolonged pain relief in women than men after dental (molar extraction) surgery (Gear et al., 1996a, 1996b, 1999). Similar sex-dependent analgesic potency in that study were not similarly found using morphine. In contrast, two other studies (Fillingim et al., 2005; Mogil et al., 2003) showed that pentazocine produced significant analgesia of similar magnitude in men and women on a variety of experimental pain models. The discrepancy between studies is best explained by

differences in the pain models. Specifically, during and after dental surgery, other agents (e.g., benzodiazepines, nitrous oxide) may have contributed to the observed analgesic sex differences. Furthermore, dental pain has a strong inflammatory component that is absent in the acute experimental pain models (Fillingim et al., 2005).

Based on their previous mouse data, Mogil and co-workers (2003, 2005) demonstrated that kappa agonist-mediated analgesia is sex-dependently modulated by Mc1r, the gene that encodes melanocortin-1 receptors. In humans, variants of the Mc1r gene are associated with red hair, fair skin, freckles and high chance of melanoma (60% of red heads have at least two variant alleles of the Mc1r gene). Accordingly, women (but not men) in their study with two or more variant alleles of the Mc1r gene (and all with red hair) displayed significant greater antinociception from pentazocine than women without variants (or with just one variant) of the Mc1r gene. The authors suggest that melanocortin-1 receptor activation by endogenous neuromodulators (α-MSH but possibly also dynorphin) produces anti-opioid actions in females only.

The available animal and human data suggest that, under particular interacting conditions and circumstances, the magnitude of opioid effects upon analgesia, hyperalgesia, tolerance and dependence appears to be sex-dependent. These include conditions specific to the drug itself, including dose, pharmacology, and route and time of administration, those particular to the subject, such as species, type of pain, genetic background, age, and gonadal–hormonal status, and those related to the underlying endogenous pain-inhibitory system at both spinal and supraspinal levels. It is also important to consider the vast literature documenting sex differences in pain perception in animals and humans which may greatly impact analgesic efficacy due to factors which have little, if anything, to do with opioid actions per se. This is particularly true in

humans, and there is likely a geometric increase in confounding variables as one considers some of the variables of pain within a social context. Specifically, clinical sex differences in opioid use and efficacy may reflect sex differences in reporting pain and seeking pain relief, and by unwarranted psychogenic attributions made by health care providers regarding pain in one sex but not the other (Unruh, 1996; Yates et al., 1998). Stereotypical gender roles and associated expectations are also thought to contribute to males reporting less pain and exhibiting higher pain thresholds when the examiner is female, an effect that is increased when the examiner is an attractive female (Levine and De Simone, 1991). Interestingly, female subjects reported more pain and had lower nociceptive thresholds with attractive male examiners (Gijsbers and Nicholson, 2005). Such social biases are likely to contribute to the perception of pain after opioid treatment in the clinic (and experimental settings) as well. Unfortunately, the studies above most likely have identified only the most salient variables. Given the multitude of such variables and their putative interactions not only with the pharmacokinetic and/or pharmacodynamic actions of opioids but with each other as well, the conditions under which one is able to reasonably expect sex differences are few if any. This, then, remains the task of investigators; to more accurately elucidate the conditions under which opioid approaches to pain management are most effective in each sex.

Evolutionary Explanations for Sex Differences:

In view of the dimorphism in expression of opioid analgesia in males and females, one can speculate on the possible evolutionary mechanisms that have shaped antinociceptive responses resulting in the present state of antinociceptive sex differences. It is reasonable to assume that like other sexually dimorphic behaviors such as aggression, 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.

One of the major defining characteristics of the female reproductive system including that of rats is periodicity. Females produce a limited number of gametes during relatively brief and distinct periods of receptivity and fertility which are highly dependent on a series of perfectly timed and synchronized phasic hormonal events (Naftolin, 1981). Gestation, parturition and maternal behavior are all periodic reproductive events which are dependent on gonadal hormones. Aggressive behavior in the female rat is also phasic and is tied to her pattern of receptivity and hormonal status (Pfaff, 1999) in which female rats are least aggressive during proestrus, when mating normally occurs and levels of ovarian hormones are highest, and they are most aggressive during estrus when they are no longer receptive and fertile, and hormone levels are at their lowest (Hood, 1984). Small female rodents typically employed for laboratory use in nature are prey and contacts from a variety of other animals (including researchers) would signal immediate danger. Indeed, the primary behavioral reactions of sexually unreceptive female rats to somatosensory contacts, unless handled and soothed, frequently feature vigorous escape, immediate aggression and anguished vocalizations (Bodnar et al., 2002). This mechanism not only ensures that an unreceptive female will fend off the advances of a male, thus preventing needless waste of resources but also reduces her risk of predation.

Due to the tonic action of male gonadal hormones, the male rat is always sexually ready, fertile and aggressive, and constantly produces an unlimited number of gametes (Naftolin, 1981) This is in contrast to female rats and assures the maximal reproductive success as mating needs to be adjusted to brief receptive periods of female. Although the male system is rather prodigious with gametes, the excess aggression comes in handy in other life necessities, such as intraspecies territorial disputes in which the winner is rewarded with access to the female.

The antinociceptive pattern of males and females appears to have evolved to accommodate these sex-specific distinct reproductive strategies and demands. The female antinociceptive system has evolved to maximize adaption to periodic painful states related to reproduction. This system is phasic and maximally suited to cope with hormonally signaled painful reproductive states such as copulation and parturition. An example of this type of response is pregnancy-induced antinociception (Gintzler, 1980). This physiological gestation, as well as the simulation of the associated changes in estrogen and progesterone is associated with significant elevations in nociceptive response thresholds (Gintzler, 2001), 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. The presence of 'high gain' multiplicative spinal opioid antinociceptive pathways that can be activated by estrogen and progesterone has hyperalgesic implications as well, i.e. it could result in disproportionately increased pain responsiveness. This might explain in part findings that women are more prone to recurrent pain and pain of greater duration and intensity than men (Gintzler 2001). The underlying mechanisms of gestational antinociception could point the way to pain pharmacotherapies that are gender-based. Another form of a female-specific analgesic response can be exhibited by vaginocervical probing analgesia. Komisaruk and coworkers found that vaginocervical probing in rats as well as humans produces analgesia across a range of pain tests without producing anesthesia or motor dysfunction (Komisaruk and Whipple, 1986). They also found that opioid mediation of vaginocervical probing-induced analgesia was test specific with changes in tail-flick latencies and tail shock-induced vocalization respectively sensitive and insensitive to opioid antagonism.

This type of analgesia 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 to successfully take place (Komissaruk and Whipple, 1986).

The male pain inhibitory system has also evolved to maximally facilitate adaptation to specific pain states resulting from aspects of male reproduction and behavior, particularly intraspecies aggression. Aggressive encounters often result in injury, pain and defeat. It appears that males have evolved a distinct pain inhibitory mechanism as a way of adaptation to these states. Among different models of rodent aggression, the resident-intruder paradigm possesses a great deal of behavioral control because when an intruder rodent is placed into the home cage of another resident rodent, the resident typically wins despite weight and size differences (Bodnar et al., 2002). Defeat in an aggressive encounter produced analgesia with its degree increasing with the intensity of the encounter (Miczek, et al., 1982). Thus, male rodent aggressive encounters and the subsequent defeat have been shown to produce marked opioid-induced analgesia that can be reversed by naloxone and it cross-tolerant with morphine (Miczek et al., 1982). 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). Finally, increased antinociceptive responses are observed in male rats and other male rodents during copulation (Szechtman, et al., 1981). This copulation induced analgesia appears to be opioid-mediated since male rats subjected to sexual exhaustion by multiple copulations show a depletion of opioid peptides in the midbrain. In addition, naloxone significantly extends post-ejaculatory interval during which there is an increased responsiveness to noxious stimulation. It has been suggested that copulation-induced

analgesia in males may reflect a biological mechanism designed to prevent genital stimulation from becoming too intense and thus aversive (Szechtman et al., 1981).

In summary, it appears that observed sex differences in opioid analgesia may ultimately be the result 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 the 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.

Potential Future Lines of Inquiry:

There are several questions that have not been fully answered by this series of studies and could be addressed by future research. One question concerns other sites and possible mechanisms by which IBO lesions of the MPOA and VMH block the sex differences observed in morphine analgesia in female rats. To conclude that these hypothalamic-midbrain-medullary circuits are involved in the mediation of sex differences in central morphine analgesia, it would have to be demonstrated that IBO lesions of the MPOA and VMH block the sex differences observed following morphine administered directly into the vlPAG and RVM in the same manner as systemic morphine. Another question revolves around direct inactivation of sex hormone receptors in the MPOA and VMH. To further conclude that these hypothalamic-midbrain-medullary circuits are involved in the mediation of sex differences in central morphine analgesia, it would have to also be demonstrated that direct inactivation of sex hormone receptors in the MPOA and VMH mimic IBO lesion effects upon sex differences in systemic and

central morphine analgesia. This could be done elegantly and efficiently by employing an antisense oligonucleotide strategy or antineuronal immunotoxins to make selective neural lesions to inactivate estradiol receptors.

In conclusion, our data substantiates the results of a number of previous studies which established profound sex differences in morphine analgesia with male rats displaying greater analgesic magnitudes and potencies than females. We showed both systemically and centrally that these sex differences are not only determined and shaped by organizational and activational effects and their interactions but are subject to the influences of hypothalamic estradiol receptor containing nuclei, the MPOA and the VMH and that these sex differences are likely to be related to fundamental differences in the circuitry of pain inhibitory pathways in male and female rats.

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