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Central opioid antinociception in rats: Gender differences and gonadal effects

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City University of New York, 1991

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CENTRAL OPIOID ANTINOCICEPTION IN RATS : GENDER DIFFERENCES AND GONADAL EFFECTS

by

Karen L. Kepler

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

1991

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

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Abstract

CENTRAL ANTINOCICEPTION IN RATS

GENDER DIFFERENCES AND GONADAL INFLUENCES

by

Karen L. Kepler

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

Gender and gonadal function have been shown to influence the magnitude of analgesia following systemic morphine and opioid and non-opioid forms of stress-induced analgesia with male rats displaying greater analgesia than female rats and gonadectomy reducing analgesic magnitude in both genders. These effects have been presumed to be centrally mediated. The purpose of the present dissertation was to assess the influence of gender, gonadectomy and estrus phase variables in central antinociception. The first experiment evaluated the roles of gender, gonadectomy, and estrous phase upon dose-response and time response functions of antinociception following intracerebroventricular administration of morphine as measured by the spinally mediated tail-flick test and the supraspinally jump test. Sham-operated male rats displayed significantly greater magnitudes of peak

and total analgesia following central morphine than sham-operated female rats on both nociceptive measures. This striking effect was reflected both in terms of magnitude and potency. Gonadectomy, specifically castration produced small, but significant reductions in the magnitude of central morphine analgesia. Although female rats in either proestrous or estrous displayed significantly greater magnitudes of analgesia than ovariectomized rats or rats in the met/diestrous phase at some doses, potency of morphine analgesia was not effected by ovariectomy or estrous phase. Since mu and delta opioid receptor subtypes have been implicated in supraspinal analgesia, the second experiment evaluated the role of gender and gonadectomy following central administration of the mu-selective agonist, DAMGO, and the delta selective agonist, DSLET. Sham-operated male rats displayed significantly greater magnitudes of analgesia than sham-operated females on the tail-flick test following DAMGO, but not DSLET. Gonadectomy failed to consistently affect either DAMGO or DSLET analgesia. The interaction of opiate receptors and gonadal steroid receptors and differential metabolic rates of drug clearance are suggested as possible determinants of gender differences observed in antinociception following central administration.

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The study of endogenous mechanisms mediating pain-inhibition has centered upon its neuroanatomical, neurophysiological, neurochemical and neuropharmacological substrates. Recent work however, has identified organismic variables such as gender and gonadectomy in the mediation of pain-inhibition (Bodnar, Romero, and Kramer, 1988). Both gender and adult gonadectomy influence basal nociception; nociceptive thresholds are significantly lower in female and gonadectomized rats (Bodnar, et al., 1988a). The antinociception following acute exposure to either of two forms of environmental swim stress is also significantly lower in female and gonadectomized rats (Romero and Bodnar, 1986). These two forms of swim stress differ parametrically, and are capable of differentially activating opioid and nonopioid pain inhibitory systems (Bodnar, 1990). The gonadectomy-induced differences in opioid and nonopioid mediated swim stress antinociception are sensitive to steroid replacement therapy such that testosterone reinstates the antinociception in gonadectomized rats relative to sham-operated controls (Romero, Cooper, Komisaruk, and Bodnar, 1988). Thus, both opioid and nonopioid pain-inhibitory systems appear to be sensitive to gender differences. Antinociception induced by systemic morphine administration also appears similarly sensitive to gender and gonadectomy influences, (Chatterjee, 1982), but such effects may also be affected by pharmacokinetic factors such as differences in weight, dosage,

drug absorption and drug clearance. Further, following the initial discoveries of the opiate receptor and endogenous opioid ligands, subsequent work has identified multiple opioid receptor subtypes and multiple gene-related precursor peptide families. Given these considerations, the present research had three major aims. In the first experiment, a time-dependent and dose-dependent analysis of antinociception was performed following intracerebroventricular administration of morphine to determine the relative influence of gender, adult gonadectomy and estrous phase upon two measures of nociception, the spinally-mediated tail flick test and the supraspinally-mediated jump test. Since supraspinal antinociception appears to rely primarily upon mu and delta opioid receptor subtypes, the second and third experiments evaluated the time-dependent and dose-dependent effects upon antinociception induced by the mu-selective agonist, [D-ala2, Me-Phe4,Gly(ol)5] (DAMGO) and the delta selective agonist, [D-ser2,Leu5]-enkephalin-Thr6 (DSLET), respectively as functions of either gender and adult gonadectomy. These studies will evaluate a hypothesis linking gonadal steroids as modulators of specific opioid systems with respect to the latter's influence on activation of endogenous pain-inhibition.

To provide the underlying conceptual basis for the proposed experiments, the following background sections cover: a) the characterization of pain transmission and endogenous

opioid pain-inhibitory system(s), b) initial discovery of the opiate receptor and endogenous ligands, c) the identification of multiple endogenous opioid receptor subtypes, d) the identification of multiple endogenous opioid peptide families, e) antinociception and opioid receptor subtypes, f) the interaction between gender influences and endogenous and exogenous opioid function, g) the influence of gender differences in antinociception, and h) a rationale for the present experiments.

A. Mechanisms of pain transmission and inhibition

The considerable interest in elucidating mechanisms underlying pain transmission, pain inhibition and endogenous analgesic system derives from the potential to manipulate them for clinical treatment of pain. The variability in the human response to pain suggests a complexity of neural mechanisms which modulate both the transmission of pain and the organism's emotional or behavioral reaction to pain (Kelly, 1982). Similar to the investigation of other sensory systems, initial research focused on delineating a specific "pain receptor"; however, this approach proved to be problematic as painful stimuli appeared to excite free nerve endings which are also excited by other innocuous thermal and mechanical stimulation. Several types of nociceptive receptors appear to mediate pain, including mechanoreceptors which respond to intense mechanical stimulation, thermoreceptors which respond to heating, cooling, and

irritant chemicals, and wide dynamic range receptors, which are stimulated by a wide range of stimulus intensities. (Whitehorn and Burgess, 1973). The lack of receptor specificity is one of a number of factors which limits the effectiveness of direct intervention on the afferent system to relieve pain. Pain transmission is largely subserved by two types of peripheral afferent fibers, the small finely myelinated A delta fibers associated with sharp pain, and the small unmyelinated C fibers associated with long-lasting, burning pain (e.g., Fields and Basbaum, 1978). Historically, these fibers were thought to enter the spinal cord exclusively through the dorsal horn and synapse on neurons in lamina I, II, III, and V. Surgical treatment to relieve chronic pain, such as dorsal rhizotomies, capitalized on this presumed neuroanatomical organization. The finding that some unmyelinated afferent fibers actually enter the ventral horn and form an ipsilateral component of the spinothalamic tract provides an explanation for the failure of dorsal rhizotomizies to provide complete and/or lasting pain relief (Coggeshall, Applebaum, Frazen, Stubbs, and Sykes, 1975). The organization of second order afferent fibers at the level of the spinal cord is complex as fibers ascend polysynaptically in the contralateral and ipsilateral anterolateral funiculus, including largely the spinothalamic, spinoreticular, and spinotectal pathways. These pathways terminate at various sites including the tectum, periaqueductal grey, various

thalamic nuclei where the medial lemniscus system conveying proprioceptive information joins with the anterolateral pathway (Brodal, 1981). The complexity of nociception at the receptor level, the diversity of primary pathways which are polysynaptic in nature, and the variety of termination sites make the parsimonious pharmacological or surgical manipulation of the afferent pain system very problematic. As the interception of afferent pain messages was unlikely to have a major impact on the alleviation of pain, an emphasis on the elucidation and manipulation of a pain inhibitory system developed.

The seminal gate control theory of pain proposed by Melzack and Wall (1965) focused attention upon centrifugal, descending control of pain inhibition. This theory described "gate" cells in the spinal cord which received modulatory, collateral input from supraspinal loci to influence the sensory-discriminative aspects of pain perception. Although the anatomical and physiological substrates of "gate" cells were never identified, this theory had heuristic value in calling attention to descending influences which were most elaborately studied evaluating the antinociceptive properties of focal electrical stimulation of the brain (Mayer and Price, 1976) and intracerebral microinjection of morphine into the brain (Yaksh and Rudy, 1978). A model for the descending centrifugal opioid mechanism of pain inhibition described a system which originated in the periaqueductal grey and dorsal

raphe nucleus in the mesencephalon which projected to medullary nuclei (nucleus raphe magnus (NRM), nucleus reticularis gigantocellularis (NRGC)), which in turn sent bulbospinal projections which selectively terminated in the dorsal horn of the spinal cord to directly block nociceptive input (Fields and Basbaum, 1978; Basbaum and Fields, 1984). Following an initial report that electrical stimulation of the mesencephalic grey produced potent antinociception in rats (Reynolds, 1969), efficacy of stimulation in medial brainstem regions to produce antinociception has been noted in rat, cat, rhesus monkey, and man (Mayer and Price, 1976). Mayer and colleagues (1971) demonstrated that stimulation produced analgesia (SPA), which was dependent in magnitude and duration upon the intensity and frequency of stimulation pulses, produced a specific antinociceptive effect which was not a generalized sensory, motor, or motivational deficit.

Both central morphine antinociception and SPA appeared to share similar mechanisms of action as demonstrated by mapping studies which implicated the periaqueductal grey (PAG), dorsal raphe nucleus, NRM, locus coeruleus, and lateral hypothalamus in these effects (Mayer and Price, 1976). Cross-tolerance between SPA and morphine developed in that rats receiving repeated stimulation of PAG displayed analgesic tolerance similar to tolerance reported after repeated central and systemic injections of morphine (Mayer and Hayes, 1975). In addition, rats made tolerant to morphine showed decreased

responses to SPA and vice-versa.

Either stimulation or morphine microinjection into the PAG elicited antinociception by inhibition of the dorsal horn neurons (Liebeskind, Guilbaud, Besson, and Oliveras, 1973). These actions depended upon an intact DLF for their expression since selective transection of this pathway blocked the antinociceptive responses (Basbaum, Morley, O'Keefe, and Clanton, 1977). Further analysis of microinjection studies (Fields and Basbaum, 1978; Yaksh and Rudy, 1978) produced a working model of a pain inhibitory system which proposed that intracerebral opiate injection into PAG or systemic morphine injection produced an activation of excitatory connections between the PAG and the NRM. While the intrinsic circuitry of the PAG has not been entirely elucidated, opiate actions on target neurons are thought to be inhibitory. The PAG is known to receive input from the frontal and insular cortex, amygdala, hypothalamus, pontine reticular formation, locus coeruleus, and the spinal cord (Beitz, 1982). Injection of the opiate antagonist, naloxone, into these loci reverses the morphine antinociception (Tsou and Jang, 1964; Yeung and Rudy, 1978). Antagonism of descending serotonergic and noradrenergic projections, originating in the NRM and NRGC, projecting through the dorsolateral funiculus (DLF) and terminating in the dorsal horn of the spinal cord blocked both SPA and morphine analgesia (Basbaum, et al., 1977; Basbaum and Fields, 1984).

This proposed model of descending pain inhibition also implicates the dorsal horn of the spinal cord in supraspinal modulation of antinociception. Based upon the profound antinociceptive actions of opiates following intrathecal administration, a spinal antinociceptive system has been proposed (Yaksh, 1981). Both direct postsynaptic inhibition (Basbaum and Fields, 1984) and presynaptic inhibition upon these primary afferents have been proposed as functional mechanisms by which supraspinal and spinal opiate systems exert their effects (Atweh and Kuhar, 1977a,b,c). Additionally, Yeung and Rudy (1980) demonstrated a multiplicative interaction of narcotic agonism between spinal and supraspinal opiate sites following concurrent intrathecal and intracerebroventricular injection.

Another dimension of the pain-inhibitory system which has potential clinical relevance is the elucidation of non-opioid analgesic systems. Three lines of evidence support the existence of non-opioid analgesia. First, antinociception has been demonstrated following injection of a variety of non-opioid neurotransmitters and peptides including the muscarinic cholinergic agonist, pilocarpine, (Houser and Van Hart, 1973; Houser, 1976) and the alpha-2 noradrenergic agonist, clonidine (Fielding, Wilker, Hynes, Szewczak, Novick, and Lal, 1978; Paalzow and Paalzow, 1976; Kiefel, Paul, and Bodnar, 1989). Secondly, some sites which support stimulation produced analgesia (SPA) are insensitive to naloxone reversal (Terman

and Liebeskind, 1986). Evidence suggests that while analgesia elicited by stimulation of ventral periaqueductal sites was blocked by naloxone, analgesia elicited by stimulation of dorsal periaqueductal sites was not attenuated by the opiate antagonist naloxone (Cannon, Prieto, Lee, and Liebeskind, 1982). Lastly, acute exposure to environmental stressors elicits an antinociceptive response which appears to be due to the stressful consequences of the stimulus rather than the stimulus per se (Bodnar, Kelly, Brutus, and Glusman, 1980; Bodnar, 1986). Interestingly, parametric variation of the stressful stimulus can yield opioid-mediated and nonopioid-mediated antinociceptive responses. For instance, acute exposure to a continuous cold-water swim (CCWS) at 2oC for 3.5 min elicits antinociception which fails to display cross-tolerance with morphine or reversal by naloxone (Bodnar et al., 1978a,b). In contrast, acute exposure to an intermittent cold-water swim (ICWS) at 2oC in which rats are exposed to 10-sec swims and 10-sec recovery periods over a 6 min time period elicits antinociception which displays cross-tolerance with morphine and significant reductions following naloxone pretreatment (Girardot and Holloway, 1984a,b). Additionally, Lewis and co-workers demonstrated that parametric variation in shock delivery was responsible for differential opioid vs. non-opioid analgesic effects such that prolonged intermittent footshock analgesia was blocked by opiate receptor antagonism, but brief continuous footshock analgesia was not (Lewis,

Cannon, and Liebeskind, 1980). The delineation of these mechanisms of pain inhibition was strongly influenced by the discovery and elucidation of a number of opiate receptor subtypes and endogenous opioid compounds.

B. The Discovery of the Opiate Receptor and Endogenous Opioid Ligands.

The early study of endogenous opioids and multiple opiate receptors evolved from attempts to develop nonaddicting substitutes for opiate analgesics (Himmelsbach, 1939). The finding that morphine, a plant alkaloid, bound to receptors on neuronal membranes precipitated a search for endogenous opioid ligands. With the advent of radioimmunoassay techniques, Goldstein (1971) discovered that differential binding occurred in brain tissue as a function of stereospecific opiate isomers. In 1973, three laboratories discovered opioid binding to receptors in the brain by [3H]-labelled naloxone, an opiate antagonist (Pert, Pasternak, and Snyder, 1973), [3H]-dihydromorphine, an opiate agonist (Terenius, 1973), and [3H]-etorphine, a potent opiate agonist (Simon, Hiller, and Edelman, 1973). Subsequent studies revealed significant differences in levels of binding in a variety of brain structures. Early homogenate binding studies demonstrated high levels of opioid binding in limbic areas including the medial thalamus, amygdala, hippocampus, and caudate nucleus (Kuhar, Pert, and Snyder, 1973). Development of

autoradiographic techniques revealed additional distributions of binding sites not elucidated with homogenate binding techniques; these included dense binding sites in the striatum, the subcallosal streak ventral to the corpus callosum, the interpeduncular nucleus, the substantia gelatinosa of spinal cord, locus coeruleus, and zona compacta of the substantia nigra (Pert, Kuhar, and Snyder, 1975). In 1977, Atweh and Kuhar (1977 a,b,c) provided an extensive study of [3H]-diprenorphine binding throughout the CNS, and demonstrated a high correlation between these binding sites and the regions mediating opiate actions. Regions implicated in the expression of opioid analgesia included layers I and II of the spinal cord, periaqueductal and periventricular gray, raphe nuclei, intralaminar and medial thalamic nuclei. Additionally, high levels of opioid receptors were found in nucleus accumbens, lateral septal nucleus, hippocampus, olfactory tubercle, amygdala, and hypothalamic nuclei, inferior and superior colliculi, layers I and III of cortex, parabrachial nucleus, vestibular nucleus, dorsal cochlear nucleus, and optic accessory tract. Variables affecting the efficacy of radiolabelled agonists and antagonists to bind to the opiate receptor include enzymes, temperature, and cations like sodium. Sodium ions were found to discriminate between agonist and antagonist binding as sodium ions enhance antagonist binding and decrease agonist binding (Pert, et al., 1973; Pasternak, Snowman, and Snyder, 1975).

Following the discovery of the opiate receptor, much interest was focused upon the identification of endogenous ligands that interacted with this opiate binding site. Hughes and coworkers (1975a,b) identified two pentapeptide chains as highly interactive with the opiate receptor, methionine enkephalin, (Tyr-Gly-Gly-Phe-Met) and leucine enkephalin (Tyr-Gly-Gly-Phe-Leu) which differ in their C terminal amino acid. The N-terminal fragment is essential for opioid biochemical activity. In contrast, C terminal modification failed to preclude opioid activity. Another endogenous compound, Beta-endorphin, a 31 amino acid peptide with long-lasting analgesic properties, was found to be present as the C-terminal chain in Beta-lipotropin (B-LPH), a 91 amino acid peptide present in brain and pituitary (Kosterlitz and McKnight, 1981). Beta-endorphin (B-LPH 61-91) fragments with opioid activity include alpha-endorphin (B-LPH 61-76), gamma-endorphin (B-LPH 61-77) and Beta-endorphin 1-27 (B-LPH 61-87); all contain the Met-enkephalin sequence at the N-terminal (Guillemin, 1976; Bradbury, 1976). Using bioassay techniques of mouse vas deferens and guinea pig ileum, the presence of enkephalins and Beta-endorphin was confirmed with reversal of assay activity by opiate antagonism determining the presence of opioid bioactivity (Hughes, et al., 1975). This breakthrough proved to be a major impetus in the subsequent delineation of over 100 peptide fragments that have been identified as bioactive neuromodulators.

Beta-endorphin and Beta-LPH are present in high concentrations in the anterior and intermediate lobes of the pituitary where they are stored in the secretory granules of corticotrophin cells and are co-released with adrenocorticotrophic hormone (ACTH) during stress (Guillemin, et al., 1977). Additionally, the immunocytochemical distributions of ACTH, Beta-LPH, and Beta-endorphin are similar in the rat brain with cell bodies localized in the arcuate nucleus and periarcuate region of basal hypothalamus (Watson, Akil, Richard, and Barchas, 1978). Immunoreactive fibers were visualized in medial and lateral hypothalamus, stria terminalis, zona incerta, median eminence, paraventricular nucleus of thalamus, ventrolateral septum, nucleus accumbens, amygdala, reticular formation, periaqueductal grey, locus coeruleus, parabrachial nucleus of the pons, and nucleus solitarius (Watson and Barchas, 1979).

Originally, Beta-endorphin was thought to be the precursor of met-enkephalin. This hypothesis was discounted as the distribution of immunoreactive enkephalin differed from that of Beta-endorphin (Watson, et.al., 1978), and the peptide cleavage required to form met-enkephalin from Beta-endorphin was also found to be highly unusual. Thus, the beta-endorphin and met-enkephalin peptides were thought to derive from different peptide pools. Isolation and characterization of the third group of opioid peptides, the dynorphins, revealed potent binding to opioid receptors (Goldstein, 1981).

C. Multiple Opiate Receptor Subtypes

Martin and colleagues indicated that various opiates exerted differential actions suggestive of the existence of multiple opiate receptors, supporting their previous theoretical perspective of duplicity theory of opiate action. Pharmacologically-induced behavioral syndromes were critical in the differentiation of receptor subtypes. For example, cyclazocine, but not morphine produced ataxia, sleepiness, and feelings of intoxication in human subjects (Martin, Eades, Thompson, Huppler, and Gilbert, 1965). The following two sections will cite evidence for multiple receptor subtypes and multiple families of endogenous opioids.

The original studies which suggested the existence of multiple opioid systems derived from human clinical studies were extended to the spinally prepared dog. Comparing three prototypic opiate drugs which produced distinct behavioral effects, Martin and coworkers (1976) proposed three classes of opioid receptors, mu for morphine-like compounds, kappa for ketocyclazocine, and sigma for SKF 10,047. Martin found that tolerance development of one class did not produce tolerance to the other classes, suggestive of distinct opioid receptors. Martin cited four criteria to differentiate among various opiate receptor subtypes: 1) behavioral effects, 2) dependence, 3) biphasic dose response curves, and 4) naloxone antagonism of various agonists and antagonists. According to this model, these receptors exert effects on physiologic

systems through different, but converging pathways. Drugs interacting with opioid receptors could act as competitive antagonists, partial agonists, and strong agonists. It should be noted that the putative sigma agonist, SKF-10047 produced many behavioral and neurochemical actions which were not reversed by nalaxone. More recently, SKF-10047, unlike other opioid agonists, has been observed to displace [3H]-phencyclidine binding, indicating that the sigma receptor is not a true opiate receptor (Wood, 1981).

Using two pharmacological bioassays, the guinea pig ileum and the mouse vas deferens, and two additional assays to measure inhibition of the specific binding of [3H]-Leu-enkephalin and [3H]-nalaxone in brain homogenates, Lord and colleagues in 1977 concluded that opioid peptides interact at three potential opiate receptor sites: the mu, kappa, and delta receptors. Of the endogenous opioids, Beta-endorphin produces equipotent effects in both the guinea pig ileum and mouse vas deferens bioassays, and produces equipotent inhibition of binding of [3H]-Leu-enkephalin, [3H]-naloxone, and [3H]-naltrexone. In contrast, leu-enkephalin was 50 times more potent in the mouse vas deferens bioassay than in the guinea-pig ileum bioassay. Thus, in the guinea pig ileum assay, peptides bind largely to the mu receptor which mediates actions of morphine-like compounds. In contrast, in the mouse vas deferens, opioid peptides bind preferentially with delta receptors and secondarily with mu receptors. Additionally,

the guinea pig ileum has considerable numbers of kappa receptors, whereas the mouse vas deferens has significantly less (Lord, Waterfield, Hughes, and Kosterlitz, 1977). Further, the inhibition of [3H]-Leu-enkephalin binding was 25 times greater than the inhibition of [3H]-naltrexone binding in the presence of leu-enkephalin, indicating greater selectivity for delta receptors in brain. Receptor affinities in the brain for met-enkephalin appeared to be intermediate to that for Beta-endorphin. Met-enkephalin binds equally well with several opiate receptors, and Leu-Enkephalin, suggesting the existence of at least three classes of opiate receptors, with Leu-enkephalin presumably more specifically binding to the delta receptor subtype (Lord, et al., 1977). Additional evidence supporting the heterogeneity of opiate receptors demonstrated that binding at the delta binding site relative to the mu site is much less sensitive to naloxone or naltrexone reversal (Kosterlitz and McKnight, 1981).

Additional differentiation of receptor sites occurred with discovery of multiple mu binding sites, termed mu1 and mu2. Early studies (Pasternak and Snyder, 1975) using [3H]-dihydromorphine and [3H]-naloxone demonstrated a high and low affinity component, initially theorized to represent mu and delta sites. However, the long-acting and irreversible antagonist, naloxazone, inhibited the high affinity component of radiolabelled agonists of mu, kappa and delta receptor subtypes, including morphine, the enkephalins, Beta-endorphin,

and the dynorphins, as well as such antagonists as naloxone and naltrexone (see review: Pasternak and Wood, 1986). These data suggested the existence of a common, high-affinity binding site for each of these compounds. Using saturation and competition studies, the common high affinity or mu1 site was found to bind both opiates and enkephalins equally well. The lower affinity sites were found to correspond to the morphine-selective (mu2) and enkephalin selective (delta) sites. The functional significance of the high affinity, mu1 site was initially confirmed by observations that the antinociceptive, but not the respiratory depressant actions of morphine could be blocked by pretreatment with the mu1-selective antagonist, naloxazone (Pasternak, Childers, and Snyder, 1980; Wolozin and Pasternak, 1981).

Schultz (1981) proposed the existence of another subtype of opioid receptor, epsilon, thought to bind Beta-endorphin. Pasternak and colleagues (Houghten, Johnson, and Pasternak, 1984) theorized that Beta-endorphin labelled an epsilon site and the mu1 site with similar affinity. There is little functional confirmation of the epsilon binding site given the lack of specific beta-endorphin analogues or antagonists.

Autoradiographic studies (Goodman, 1980; Mansour, Khachaturian, Lewis, Akil, and Watson, 1987, 1988) demonstrate that the mu opioid receptor subtype is distributed primarily in supraspinal loci, but can also be found spinally. Mu binding is most dense in the layers I and IV of the neocortex,

caudate-putamen, nucleus accumbens, sub-callosal streak, thalamus, pyramidal layer of the hippocampus, amygdala, inferior and superior colliculi, nucleus tractus solitarius, spinal trigeminal nucleus, and dorsal horn. Moderate densities of mu receptors are found in the periaqueductal gray and raphe nuclei with significantly less binding visualized in the hypothalamus, preoptic area and globus pallidus. Delta receptors are more restricted in their supraspinal distribution, and predominate in the olfactory tubercle, Layers II, III, and V of the cortex, amygdala, pontine nuclei, and substantia gelatinosa of the spinal cord (Goodman, 1980). In contrast to mu receptor distribution, little or no delta binding is found in thalamus, hypothalamus or brainstem (Mansour, et al., 1988). Areas which demonstrate both mu and delta sites include the nucleus ambiguous, nucleus tractus solitarius, substantia gelatinosa of the spinal cord, trigeminal tract and layer IV of cortex. The relative concentration of mu and delta receptors correlates functionally with areas involved in antinociception (Basbaum and Fields, 1984).

Kappa opioid receptor binding is most dense in the caudate-putamen, nucleus accumbens, amygdala, hypothalamus, the posterior lobe of the pituitary gland, median eminence, and nucleus tractus solitarius. Moderate amounts of kappa binding sites are found in periaqueductal gray, raphe nuclei, spinal trigeminal nucleus and the dorsal horn of the spinal

cord. The distribution of kappa binding sites corresponds functionally with brain areas involved in the regulation of water balance, feeding, and pain perception (Mansour, et al., 1988).

Autoradiographic studies demonstrate that mu1 and mu2 binding sites have similar, but not identical distributions (Moskowitz and Goodman, 1985a,b; Goodman and Pasternak, 1985). Cortical mu1 binding is denser in the frontal lobe, whereas mu2 binding predominates in the parietal, occipital, and temporal cortices. Mu1 binding is found in the striatum, ventral pallidum, caudal nucleus accumbens, medial thalamus, interpeduncular nucleus and median raphe, whereas mu2 binding is found in the hippocampus and amygdaloid region. Mu1 binding is high in the ventral periaqueductal gray. In contrast, areas involved in control of respiration, such as the dorsal motor nucleus of the vagus and the nucleus tractus solitarius, contain few mu1 sites and many mu2 sites. The differential mu1 and mu2 receptor subtype distributions provide anatomical correlates for the respective roles of these binding sites in antinociceptive and respiratory depressant effects following opioid administration (Pasternak, Childers, and Snyder, 1980).

D. Multiple Endogenous Opioid Families

The opioid peptides appear to be derived from three precursor peptide families which were identified using mRNA

techniques, and which display homology suggestive of a common ancestral link (Akil, Watson, Young, Lewis, and Walker, 1984). The three families are: a) propriomelanocortin which eventually produces Beta-endorphin, b) proenkephalin which yields 4 major enkephalin peptides ([Met]-enkephalin, [Leu]-enkephalin, [Met]-enkephalin-Arg6-Phe7 and [Met]-enkephalin-Arg6-Phe7-Leu8); and c) prodynorphin which yields alpha and beta-neoendorphin and various dynorphin peptides (McDowell and Kitchen, 1987).

Propriomelanocortin (POMC) has been identified as the precursor of ACTH, B-LPH, Beta-endorphin, alpha-melanocyte-stimulating hormone (MSH) with duplication occurring in the MSH portion of the molecule (Roberts and Herbert, 1977). Bioactive fragments occur at the C-terminal and N-terminal. Labelling studies in pituitary tissue have shown initial cleavage at the C-terminal of ACTH, generating B-LPH which in turn contains the sequence of B-endorphin. Additional processing results in ACTH and B-endorphin (1-31) which can be processed further into alpha-MSH. The highest concentration of POMC is found in the anterior lobe of the pituitary where ACTH is produced and released to control corticosteroid release from the adrenal cortex. While B-LPH occurs in the anterior lobe, additional processing to form Beta-endorphin occurs in the intermediate lobe. The majority of neuronal POMC-containing cell bodies are located within the arcuate nucleus of the hypothalamus (Bloom, 1978) with additional cell

bodies in the nucleus tractus solitarius (Romagnano and Josephs, 1984). The projections from POMC-containing cell bodies in the hypothalamus correspond to the distribution cited earlier for beta-endorphin and extend to the periventricular thalamic nucleus, nucleus accumbens, ventral septum, medial amygdala, locus coeruleus, periaqueductal gray, and the reticular formation.

The proenkephalin precursor contains multiple copies of the enkephalin sequence, specifically six copies of met-enkephalin and one copy of leu-enkephalin. Many perikarya and fibers containing proenkephalin are present in the central and peripheral nervous system with most processes serving as interneurons. Large concentrations of cell bodies found in the reticular formation, hypothalamus, hippocampus, substantia nigra, amygdala, striatum, frontal, pyriform and endorhinal cortices, anterior olfactory nucleus, lateral septum, posterior lobe of the pituitary, and periaqueductal gray (Hokfelt, Elde, Johansson, Telenius, and Stein, 1977a,b; Khachaturian, Lewis, Schafer, and Watson, 1985, Watson, et al., 1977a, 1978a). Post-translational processing of proenkephalin produces [Met]-enkephalin, [Leu]-enkephalin, [Met]-enkephalyl-Arg6-Phe7, [Met]-enkephalyl-Arg6-Phe7-Leu8, Peptide E and Peptide F (McDowell and Kitchen, 1987).

The processing of the third precursor family, prodynorphin, appears to be simpler than either POMC or proenkephalin systems with fewer post-translational steps (see

review: Akil et al., 1984). Opioid compounds derived from prodynorphin include alpha and beta-neoendorphin, dynorphin A, dynorphin B and leu-enkephalin. Dynorphin A (1-17) which is subsequently processed to yield dynorphin (1-8), has been observed in fibers which are widespread throughout the brain and spinal cord with highest concentrations in the anterior hypothalamic nuclei projecting to the posterior lobe of the pituitary (Akil, et al., 1984, Khachaturian et al., 1982). Additional areas of high density include the anterior lobe of the pituitary, reticular formation, caudate, hippocampus, and periaqueductal gray. Dynorphin B is a 13 amino acid peptide processed from an arginine cleavage of prodynorphin (Cone, et al., 1983). Proenkephalin and prodynorphin precursors are often co-localized (i.e. PAG; Basbaum and Fields, 1984); however processing of these precursors utilize different chemical paths.

E. Antinociception and Opioid Receptor Subtypes.

A number of opioid receptor subtypes have been implicated in opiate analgesia. Mu receptors are mostly implicated in mediating supraspinal analgesia (Bodnar, Williams, Lee, and Pasternak, 1988; Jensen and Yaksh, 1986; Heyman, Williams, Burks, Mosberg, and Porreca, 1988). As indicated previously, the mu receptor can be subdivided to mu1 binding sites which possesses high affinity for both opiates and enkephalins, and mu2 binding sites which preferentially bind morphine and other

mu-selective agents with low affinity (Goodman and Pasternak, 1985; Moskowitz and Goodman, 1985; Pasternak and Wood, 1986, Zhang and Pasternak, 1980). Using long-acting and selective mu1 antagonists as naloxonazine, the mu1 receptor subtype has been implicated in some opiate actions, including supraspinal analgesia, but not others, like respiratory depression, inhibition of gastric motility, and physical dependence (Ling, Macleod, Lee, Lockhart, and Pasternak, 1984, Ling and Pasternak, 1983; Pasternak and Wood, 1986, Bodnar, et al., 1988; Paul, Bodnar, Gistrak, and Pasternak, 1989). Delta receptors have also been implicated in supraspinal analgesia (Heyman et al., 1987; Jensen and Yaksh, 1986; Porreca, Mosberg, Hurst, Hruby, and Burks, 1984, 1987). Two selective delta agonists, D-Ala2-D-Leu5-enkephalin, (DADL) and D-Ser2-Leu5-enkephalin-Thr6 (DSLET) each produce antinociception following intracerebral administration (Bodnar et al., 1988; Heyman et al., 1987, 1988; Jensen and Yaksh, 1986; Porreca et al., 1984). However, DADL and DSLET also bind with relatively high affinity to mu1 sites (Pasternak and Wood, 1986; Itzhak and Pasternak, 1987). Microinjection of morphine and DSLET produces significant supraspinal antinociception in the PAG, locus coeruleus (LC), NRM and NRGC which is blocked by the mu1 antagonist, naloxonazine (Bodnar et al., 1988). In contrast, another delta-selective peptide (D-Pen2- D-Pen5)-enkephalin (DPDPE) which does not show significant mu 1 binding (Mosberg et al., 1983; Clark et al., 1986) can be used to differentiate

mu from delta action. Although DPDPE produces antinociception following ventricular administration (Porreca et al., 1987), it fails to produce significant antinociception when administered directly into the PAG, LC, NRM, or NRGC (Bodnar et al., 1988). Beta-endorphin also produces supraspinal analgesia, but the receptor subtype involved has not been specifically delineated (Tseng, Cheng, and Fujimoto, 1983). Supraspinal analgesia elicited by selective kappa agonists has been shown to be weak, test-specific and/ or accompanied by motor dysfunction (Yaksh, 1984; Wood, et al., 1981, Ward and Takemori, 1983; Takemori, Larson, and Portoghese, 1981). Mu, delta and kappa agonists each produce spinal analgesia following intrathecal administration (Heyman et al., 1988; Porreca et al., 1984, 1987; Schmauss and Yaksh, 1984; Tung and Yaksh, 1982). However, it appears that the lower-affinity mu2 binding site is responsible for mu-related spinal antinociception (Heyman et al., 1988; Paul et al., 1989).

The previously cited evaluations of opiate analgesia have been done almost exclusively in the young adult male rat. Subject variables, such as age and gender of organism, have been shown to influence various parameters of opiate and environmental analgesia (Bodnar et al., 1988b). The next section will review gonadal and opioid interactions.

F. Gonadal and Opioid Interactions

Steroid hormones, produced by the adrenal cortex,

ovaries, and testes are synthesized from cholesterol. The study of steroid hormone action in the brain and pituitary involves identification and mapping of putative receptor sites, determination of their functional importance, and elucidation of their cellular mode of action (McEwen, Davis, Parsons, and Pfaff, 1979). The five major classes of brain and steroid hormone receptors include those that bind estrogen, androgen, progestin, glucocorticoids, and mineralocorticoids. The neuroanatomical distribution of steroid receptor systems shows distinct patterns for all classes of steroid with some degree of overlap in densely concentrated areas like the hypothalamus, pre-optic area and septum (Pfaff and Keiner, 1973).

Autoradiographic analysis of gonadal steroid receptors using [3H]-estradiol and [3H]-testosterone reveal steroid-containing cells in the medial preoptic area, anterior, ventromedial and arcuate hypothalamic areas, lateral septum, bed nucleus of the stria terminalis, medial and cortical amygdaloid nuclei and the mesencephalic grey (Morrell and Pfaff, 1981; Pfaff and Keiner, 1973). [3H]-estradiol labelled steroid receptors are largely concentrated in the preoptic area, the periventricular nucleus, ventromedial nucleus, premammillary nucleus, and arcuate nucleus of the hypothalamus, cortical and medial nucleus of the amygdala, and the anterior lobe of the pituitary, with smaller concentrations present in the septum, diagonal band of Broca

and the nucleus of the stria terminalis (Pfaff and Keiner, 1973). Analysis of 5-alpha dihydroxytestosterone binding revealed a large concentration of receptors in the lateral septum, specific distributions in the periventricular nucleus and premammillary areas of the hypoththalamus, and smaller concentrations in the nucleus of the stria terminalis, arcuate and ventromedial nucleus of the hypothalamus, medial nucleus of the amygdala, hippocampus, and anterior pituitary (Sar and Stumpf, 1977).

Gonadal steroids have been found to play a functional role in influencing the expression of analgesia (Bodnar, et al., 1988). The mechanisms of action by which gonadal hormones and opioid systems reciprocally interact with one another may involve direct or indirect interaction between central opiate receptors and central gonadal steroid receptors. Areas like the medial preoptic area of the hypothalamus, a sexually dimorphic nucleus, larger in males than females may play a role in gonadal steroid-opioid interaction (Gorski, Harlan, Jacobson, Shryre, and Southan, 1980). Several neurotransmitters involved in opiate analgesia display gender-specific patterns in the preoptic area. Serotonin fibers are most dense in the lateral part of the medial preoptic nucleus which is proportionally larger in females (Simerly, 1984). A greater density of tyrosine hydroxylase immunoreactive cells and fibers, but not dopamine beta hydoxylase immunoreactive cells and fibers, are found in

the anteroventral periventricular preoptic nucleus of female rats than male rats, suggesting a sexual dimorphism for dopamine (Simerly, Gorski, and Swanson, 1986). Similarly, met-enkephalin immunoreactivity is also much denser in the anteroventral periventricular nucleus of female rats which is regulated by ovariectomy and neonatal testosterone treatment (Watson, Hoffman, and Wiegand, 1986). An example of a possible site of either direct or indirect interaction between gonadal and opioid systems has been demonstrated by Simerly and colleagues (1988). They found that antisera directed against leucine-enkephalin produced denser immunoreactivity in females than in males, but that antisera directed against peptide E which does not cross-react with dynorphin, produces denser immunoreactivity in males than in females. This effect was sensitive to gonadectomy and selective in that antisera directed against either Beta-endorphin of dynorphin B displayed gender-insensitive immunoreactivity. These data indicate clear gender differences in the organization of opioid peptides in these areas. Whether gonadal steroid/gender differences occur in other opioid-containing loci which display sexual dimorphism remain to be clarified.

In addition to neuroanatomical substrates demonstrating gender differences, biochemical studies have shown this as well. Levels of pituitary met-enkephalin in male rats are twice that in the female rat (Hong, Yoshikawa, and Lamartinere, 1982). Additionally, female rats display lower

levels of pituitary Beta-endorphin, (Mueller, 1980), and dynorphin (Molineaux, Hassen, Rosenberger, and Cox, 1986). Several studies have investigated opioid effects upon gonadal hormone function. Leu-enkephalin stimulates luteinizing hormone while Met-enkephalin stimulates prolactin release (Leadem and Kalra, 1985). Beta-endorphin, dynorphin and such methionine enkephalin analogs as FK-33824 and DALAMID stimulate prolactin release and inhibit luteinizing hormone release.

Gonadal steroids have been proposed to modulate gender differences by acting in either an organizational or an activational mode (Phoenix, 1959). The organizational effects of gonadal steroids typically occur early in fetal development, in the late pre-natal, and/or in the perinatal periods, and appear to organize neural pathways that can potentially mediate steroid-sensitive behaviors. The means by which one would interfere with organizational effects would include invasive techniques, and/or pharmacological and steroid interventions during the pre- and peri-natal periods. The activational effects of gonadal steroids refer to behaviors directly mediated by gonadal steroids; these effects can be altered by adult gonadectomy and/or steroid administration. Since adult gonadectomy is one of the primary variables employed in the present proposal, a review of gonadectomy effects and steroid-sensitive effects (e.g., estrous phase) upon opioid function follows.

Gonadectomy reduced levels of met-enkephalin in male rats, but increased levels in female rats (Hong, et al., 1982; Dupont, Barden, Cusan, Merand, Labrie, and Vaudry, 1985). Beta-endorphin concentration in the midbrain of male rats is higher than in female rats, and castration reduces this gender difference (Lee, Panerai, Bellabarba, and Friesen, 1980). Gonadal steroids appear to affect hypothalamic content of Beta-endorphin and its release into hypophyseal-portal blood. Wardlaw (1988) found that both testosterone or estradiol treatment in castrated male rats produced significant decreases in hypothalamic Beta-endorphin and two other POMC derivatives, corticotropin-like intermediate lobe peptide (CLIP) and alpha-MSH. Castration of young male rats produces a 50-60% decrease in Dynorphin A and B three days after surgery and a subsequent increase to 2.5 times the levels of Dynorphin A and B in control males at one month after castration (Molineaux, et al., 1986). Ovariectomy also produced a significant increase in levels of immunoreactive Dynorphin A and B two weeks after surgery. Immunoreactive Beta-endorphin was significantly decreased, and luteinizing hormone was significantly increased in ovariectomized female rats at one month after surgery. These effects were reversed by steroid replacement with a single injection of estradiol benzoate within 48 hours. A similar decrease was observed in Beta-endorphin and leutinizing hormone of castrated males, and these effects were reversed by testosterone propionate

(Forman, Tingle, Estilow, and Cater, 1985), suggesting that gonadal steroids may mediate feedback relating to the release of Beta-endorphin from the pituitary. Further studies of ovariectomized female rats (Forman and Estilow, 1986) indicate that whereas anterior pituitary Beta-endorphin levels are regulated by alpha-adrenergic drugs, Beta-endorphin levels in the neurointermediate lobe of the pituitary are modulated by both alpha-adrenergic and dopaminergic drugs.

Estrous cyclicity in normal female rats also generates changes in opioid function. Differential changes in Beta-endorphin levels in different hypothalamic areas occur across the estrous cycle (Knuth, Sikand, Casanueva, Havlicek, and Friesen, 1983). Beta-endorphin concentration is highest in the arcuate nucleus and median eminence during proestrous, but it is significantly decreased during diestrous in the preoptic suprachiasmatic region.

Evidence of gonadal steroid-opioid interactions exist, but the underlying mechanism of action is unknown. Antinociception induced by systemic drug administration or environmental stressors appear to be sensitive to gender and gonadectomy which will be summarized in the following section.

G. Gender and Antinociception

Gender differences in antinociception have been observed in the organism's response to stress, basal nociceptive sensitivity, and responsivity to opiate analgesia. Shock

thresholds of female rats are significantly lower than of male rats (Pare, 1969; Beatty and Beatty, 1970; Marks, Fargason, and Hobbs, 1972). The ventromedial hypothalamus, a sexually dimorphic structure in the rat, has been implicated in the neural control of gender differences in shock reactivity (Matsumoto and Arai, 1986). Lesions placed in the ventromedial nucleus lower jump thresholds in male rats to levels observed in females without altering responsivity to shock in the females. Though some studies suggest that gender differences in shock thresholds may reflect gender differences in body weight (Pare, 1969; Marks and Hobbs, 1972), other studies demonstrate that adult gonadectomy fails to significantly influence shock thresholds, yet produces marked weight changes (Fessler and Beatty, 1976). These gender differences can be eliminated by neonatal androgenization of the female rat followed by steroid replacement therapy in adulthood or by neonatal castration of the male rat. Adult ovariectomy failed to produce similar effects on pain thresholds, suggesting an organizational role for gonadal hormones. Neonatal castration has been found to decrease pain threshold and can be reversed by testosterone administration in adulthood (Beatty and Fessler, 1976). Circulating ovarian hormones appear to influence sensitivity to shock as females display threshold fluctuations across the estrous cycle and exhibit their greatest basal sensitivity to shock during the estrous phase (Drury and Gold, 1978).

Gender and gonadal steroids appear to modulate opiate antinociception since systemic morphine produced smaller magnitudes of analgesia in female than in male rats (Badillo-Martinez, Kirchgessner, Butler, and Bodnar, 1984). Systemic morphine analgesia may be modulated by gonadal steroids as castration decreases morphine analgesia in males, and testosterone sensitizes females to the analgesic effects of morphine (Pinsky, Sheldon, and LaBella, 1975; Chatterjee, Das, Banerjee, and Ghosh, 1982). Estrous cyclicity also modulates systemic morphine analgesia with the greatest sensitivity observed during the late diestrous phase (Bannerjee, et al., 1983).

Gender differences have also been evaluated using opioid and non-opioid forms of stress induced analgesia. Both the nonopioid-mediated CCWS and the opioid-mediated ICWS forms of antinociception are sensitive to gender differences. Female rats display significantly less CCWS and ICWS antinociception on the supraspinally-mediated jump test relative to age-matched or weight-matched males (Romero and Bodnar, 1986). Adult gonadectomy reduced both CCWS and ICWS antinociception with a rank order potency of intact males >> intact females = castrated males >> ovariectomized females (Romero, et al., 1987). Steroid replacement therapy with testosterone proprionate or estradiol benzoate increased both CCWS and ICWS analgesia in castrated males and ovariectomized females to levels observed in intact animals without altering analgesic

magnitude in intact rats (Romero, et al., 1988). In contrast, estrous phase failed to produce any significant difference in cold water swim analgesia (Romero and Bodnar, 1986).

Two other forms of non-opioid antinociception were also found to be sensitive to gender differences. Antinociception can be elicited by the muscarinic receptor agonist, pilocarpine (Houser and VanHart, 1973, 1976) and by the alpha2-noradrenergic receptor agonist, clonidine (Fielding et al., 1978; Paalzow and Paalzow, 1976). Both pilocarpine and clonidine antinociception are sensitive to gender differences such that male rats show significantly greater degrees of analgesia than female rats (Kiefel et al., 1989). Further, gonadectomy produced small reductions in pilocarpine and clonidine antinociception and estrous phase failed to alter either response. The similar gender differences in nonopioid pilocarpine, clonidine and CCWS analgesia are interesting since CCWS analgesia is mediated in part by cholinergic (Sperber, Romero, and Bodnar, 1986) and alpha2-noradrenergic (Bodnar, Merrigan, and Sperber, 1983; Kepler and Bodnar, 1988) influences.

Thus, it appears that both opioid and nonopioid-mediated responses are sensitive to gender differences, with females displaying smaller magnitudes and/or durations of antinociception following these manipulations. In some cases, adult gonadectomy reduces the antinociceptive effects, indicating that gonadal steroids might act in an activational

role to modulate antinociception. This is supported by the further observation that steroid replacement therapy acts to increase antinociceptive levels in gonadectomized rather than intact animals. Finally, estrous phase plays a role in modulating some antinociceptive responses, but not others.

H. Rationale

Multiple endogenous opioid and non-opioid pain-inhibitory systems have been identified with the vast majority of research conducted in the male rodent. Recent research investigating the roles of gender and gonadal function has established a modulatory role for gonadal steroids in pain perception, opiate mediated analgesia and opioid and non-opioid pain inhibitory systems activated by environmental stressors. Male rats display significantly higher basal nociceptive thresholds than females (Pare, 1969; Beatty and Beatty, 1970; Marks, et al., 1972), and higher magnitudes of analgesia following systemic morphine and stress induced analgesia than females (Badillo-Martinez, et al., 1984; Romero and Bodnar, 1986). Gonadectomy reduces these forms of analgesia relative to same sex controls and steroid replacement therapy reverses these deficits (Romero and Bodnar, 1986; Romero, et al., 1987; 1988). Estrous phase is capable of altering some, but not all of these forms of analgesia (Romero and Bodnar, 1986; Bannerjee, et al., 1983).

The focus of this dissertation research was to evaluate central opioid substrates of antinociception and their relative sensitivity to gender, gonadectomy and estrous phase. The approach employed to answer these questions was used for the following reasons. First, although analgesia induced by environmental stressors displayed the most sensitive responses to gender differences, evaluation of these forms of analgesia could only be accomplished by either lesion and/or pharmacological antagonist studies. Any subsequent effects of these manipulations upon gender differences would be problematic in distinguishing between changes in either antinociceptive or stress-related responses. Second, morphine is the prototypical analgesic in clinical use, and a systematic evaluation of its central effects relative to gender, gonadectomy and estrous phase would potentially have clinical and therapeutic relevance. Third, the opioid system has been well-categorized with the clear identification of gene-related peptide families and opiate receptor subtypes. The evaluation of gender, gonadectomy and estrous phase differences in specific agonist effects of these opioid peptides may demonstrate either associations or dissociations which again might have implications for neuropharmacological interventions.

The site of these gender and gonadectomy effects upon analgesic processes is unknown but presumed to be centrally mediated. Therefore, the first study systematically evaluated

the relative importance of three gender-related variables, gender differences, gonadectomy differences, and estrous phase upon the central analgesic effects of morphine, the prototypical mu agonist, following intracerebroventricular administration. Though gender, gonadectomy and estrous differences have been observed for systemic morphine analgesia, the systemic route of administration is susceptible to differences in weight, metabolism, absorption, clearance, and other pharmacokinetic effects. Drug efficacy was evaluated in terms of magnitude of peak analgesic effects following central administration of the morphine, as well as total analgesic effects, represented as the sum of analgesic magnitude across the time course. Dose-response and time-response (across a two hour time course) actions of central morphine analgesia were evaluated in sham operated male rats, castrated male rats, ovariectomized female rats, and sham operated female rats during the estrous, proestrous, and combined met/diestrous phases of the cycle. Adult gonadectomies were performed to evaluate whether gender and gonadal differences observed following other analgesic manipulations were centrally mediated. Since responsivity to opiate analgesia might differentially affect the type of nociceptive measure, two tests were employed: the tail flick test which measures responsivity to heat (D'Amour and Smith, 1941) and the jump test, which measures responsivity to electric shock (Evans, 1961). These tests also assess

analgesic effects at different levels of the neuraxis as the jump test is supraspinally mediated and the tail flick test is mediated by both spinal and supraspinal influences. To decrease potential confounding by tolerance effects, each subject received a maximum of one vehicle and two drug treatments separated by at least a one week interval between conditions. Two treatment injections assessed at weekly intervals should not reduce the efficacy of the analgesic response (Bodnar, et al., 1988; Yaksh, et al., 1976). In contrast, evaluating the full dose range on each individual animal would be problematic as the development of tolerance effects and ventricular damage would likely confound the expression of analgesic effects. Additionally, multiple dosing of animals reduced the total number of subjects necessary for the paradigm.

The second series of studies further assessed the gender and gonadectomy effects upon central opioid analgesia by evaluating their effects following central administration of the mu-selective opioid receptor subtype agonist, DAMGO, and the delta-selective agonist, DSLET. Given the short-lived effects of peptide analogs, these compounds must be centrally administered to produce analgesic effects and their antinociceptive time course is limited to a one hour time period.

The use of a central intracerebroventricular route of administration was chosen to: a) decrease effects of

pharmacokinetic variables, b) increase the antinociceptive efficacy of the opioid receptor subtype agonists, DAMGO and DSLET, which produce antinociception only following central injection, and c) maximize the possibility of interaction between central antinociceptive-sensitive neurons and gonadal steroid neurons. Although gonadal steroids are present in the spinal cord, denser concentrations are found supraspinally in the medial preoptic area, anterior and ventromedial hypothalamus, arcuate nucleus, lateral septum, amygdala, and mesencephalic grey (Morrell and Pfaff, 1981; Pfaff and Keiner, 1973). In postulating sites of action for direct central effects between opiate and steroid receptors, the three leading candidates would be the arcuate nucleus, the amygdala, and the mesencephalic grey, with the latter among the most sensitive supraspinal sites supporting antinociception (see reviews, Akil, et al., 1984; Yaksh and Rudy, 1978). However, the evaluation of opioid antinociception following intracerebral injections such as the PAG as functions of gender, gonadectomy, estrous phase, agonist and dose becomes quite difficult methodologically given the inherent variability of the intended injection site as a function of stereotaxic placement. In intracerebral injection studies, a within-subject design is often used to control antinociceptive sensitivities between and within sites. Given that this research attempted to relate gender differences, gonadectomy differences, and estrous phase differences to changes in

antinociception induced by three different agonists with full dose-response curves, an intracerebral injection paradigm would be prohibitive though possibly more informative. Therefore, systematic analysis of the effects of intracerebroventricular administration presents the most efficient strategy to clarify the generalizability or specificity of gender differences.

GENERAL METHODS

A. Subjects

Male and female albino Sprague-Dawley rats were used as subjects. All animals were purchased from Charles River Breeding Laboratories (Wilmington, MA) and were housed singly in the Queens College Vivarium in individual flat bottomed plastic cages. They were maintained on a 12 h light/12 h dark cycle with ambient temperature ranges from 21 to 25 C. Purina rat chow and water were available ad libitum.

B. Surgical Procedures

1. Anesthesia: Each animal in all surgical procedures was pretreated with chlorpromazine HCL (3 mg/ml normal saline/ kg body weight, IP) 15 min prior to anesthesia with Ketamine HCL (Parke-Davis: 100 mg/ml sterile water/kg body weight, IM). Anesthetic viability was assessed by the lack of limb withdrawal to pinch or eyeblink to corneal touch. Recovery from anesthetic effects was determined by the return of the righting reflex.

2. Castration: After anesthetization, a midscrotal incision of 1.5 cm was made in male rats, and the testes and epididymal fat were removed. The testicular artery was tied to prevent bleeding and the incision was stitched (Marks and Hobbs, 1972). A minimum of four weeks was allowed for recovery.

3. Ovariectomy: Ovaries, together with ovarian fat, were removed from female rats via a single dorsal incision.

The ovarian artery was tied, with the muscle and skin sutured in layers. A minimum of four weeks was allowed for recovery.

4. Sham surgery: Animals were anesthetized with appropriate incisions made and subsequently closed. No organs were removed or disturbed. Gonadal and sham surgeries were performed 10-14 days prior to central surgery. Body weight was assessed before the gonadal surgery and at approximately one month after the initial surgery. Post-operative baseline testing occurred one month after gonadal surgery to allow for full expression of effects of the gonadectomy (Marks and Hobbs, 1972).

5. Intracerebroventricular (ICV) cannulation: A stainless steel 22 gauge guide cannula (Plastic Products) was stereotaxically implanted so that its tip was positioned 0.3 mm above the left lateral ventricle. With the incisor bar set at +5 mm, the coordinates were 0.5 mm anterior to the bregma suture, 1.3 mm lateral to the mid-sagittal suture and 3.6 mm from the top of the skull. Three stainless steel screws and dental acrylic secured the cannula to the skull. Animals were allowed a minimum of seven days to recover from surgery before beginning postoperative baseline testing of nociceptive thresholds.

C. Histological Procedures

1. Determination of cannula placement: Following experimental testing, animals were killed with an overdose of anesthesia (Euthanasia, No.5, H. Schein and Co.). As the

animals could not be perfused due to the need to analyze gonadal tissue, the unfixed brains were removed, blocked, and were visually inspected through the lateral ventricle to determine cannula placement; only the data from those animals with a properly placed cannula were included in the statistical analyses.

2. Post-mortem examination of accessory sex organs: After an overdose of anesthesia, the ventral prostates and seminal vesicles of castrated and sham-operated male animals were removed. Excess fat was dissected and the organs emptied of any secretion. The tissue was blotted dry and weighed to the nearest 0.01 g (Beyer, et.al., 1973). The same procedure was followed with the uteri of ovariectomized and sham-operated females (Beyer and Komisaruk, 1971).

D. Estrous Phase Determination.

Estrous phase was monitored in the sham-operated females using daily vaginal smears to determine the proestrous, estrous, or combined met/diestrous phase of the cycle. Smearing was done with a saline filled eyedropper. Slides were stained with Cresyl violet stain and were viewed with light microscopy under 4x magnification. Females were tested only within one phase of the estrous cycle, i.e., during either the estrous, proestrous, or combined met/diestrous phase. Vaginal smears were taken 0-1 h into the light cycle with experimental testing occurring between 1 and 7 h after smears. It should be noted that vaginal smears were taken on successive days

before the experimental procedure began to adapt animals to the potential stressful consequences of the procedure. Although vaginal probing produces analgesia (Komisaruk and Wallman, 1977) , its time course of action completely dissipates within 2 min and the applied force necessary to produce analgesia far exceeds the smear procedure.

E. Nociceptive Tests

1. Tail-flick test: This measure (D'Amour and Smith, 1941) utilizes a radiant heat source (IITC Company) mounted 8 cm dorsal and 4-10 cm proximal to the tip of the tail of a lightly restrained animal. The onset of the radiant heat stimulus activates a digital timer (accuracy: 0.01 sec) which is stopped by exposure of a photocell caused by the withdrawal of the animal's tail. The mean of three latency determinations, conducted at 10 sec intervals, constituted baseline latency in each session. The intensity of the thermal stimulus, which was identical for all animals, was set to produce baseline latencies between 2.5 and 3.5 sec. In order to avoid tissue damage, a trial was automatically terminated if the animal did not respond within 10 sec.

2. Jump Test: This measure of reactivity to shock (Evans, 1961) was assessed by placing the animal in a 30 cm by 24 cm plexiglass chamber with a floor consisting of 16 grids set 1.5 cm apart. Electric shock was delivered to the feet of the animal by a 60 Hz constant shock generator (BSE/LVE) through a shock scrambler (Campden Instruments). Each trial

began with the animal receiving a 300 msec shock at a current intensity of 0.1 mA. Subsequent shocks were increased in 0.05 mA steps at 10 sec intervals. For each trial, the jump threshold was defined as the lowest of two consecutive intensities at which the animal removed both hind paws simultaneously from the grids. Six trials were administered during each session with the jump threshold defined as the mean intensity of these six trials. Only ascending series of shocks were employed for ethical reasons and because suprathreshold intensities of electric shock produce analgesia (Watkins and Mayer, 1982). Further, our laboratory has repeatedly demonstrated that this method does not result in errors of anticipation or habituation. The order of tail-flick latency and jump threshold determinations yields minimal carry-over effects in baseline testing.

F. Statistical Analyses:

Two statistical approaches were utilized to analyze the data in terms of magnitude and potency. Split-plot analyses of variance, corrected for repeated measures, for each dependent variable (tail-flick latencies and jump thresholds), were employed to assess significant differences among vehicle and each individual agonist dose, among groups (sham and gonadectomized males and females), and across test times. Individual determinations of significant drug effects relative to corresponding vehicle conditions were assessed with Dunnett comparisons ($p < 0.05$). Analyses of significant drug effects

and interaction effects across groups at corresponding times and doses were assessed using differences scores which were derived by subtracting each postdrug effect from its corresponding vehicle value; Dunn comparisons ($p< 0.05$) were used to discern significant individual effects among groups. The potency of effects was evaluated by constructing log dose response functions and performing linear regression analyses. Analgesic potency was defined as the ED50 for peak and total analgesic effects for each nociceptive measure. The criterion for the ED50 was that minimal dose elicited a 50% increase relative to vehicle values for peak and total effects. Calculations from linear regression analyses allowed for determination of significant differences between slopes and intercepts across groups by evaluating confidence intervals (95%).

G. Drugs and Injections

All intracerebroventricular injections were made in a 5 ul volume of normal saline and infused by a Hamilton syringe and polyethylene tubing at a rate of 1 ul every 15 sec through a stainless steel internal cannula (28 gauge, Plastic Products) which protruded beyond the tip of the guide cannula into the lateral ventricle. Morphine sulfate (Pennick laboratories) was dissolved in normal saline (5 mg/ml normal saline). The opioid peptide agonists, DAMGO and DSLET (Peninsula Laboratories), were also dissolved in normal saline.

EXPERIMENT 1: Gender, Gonadectomy and Estrous Phase Effects upon Central Morphine Antinociception

As morphine is the prototypical analgesic in clinical use, a systematic evaluation of central effects of morphine relative to gender, gonadectomy, and estrous phase has clinical and therapeutic relevance. Although gender, gonadectomy, and estrous phase differences have been observed following systemic morphine administration (Badillo-Martinez, et al., 1984; Kavaliers and Innis, 1987; Banerjee, et al., 1982; Chatterjee, et al., 1983), the systemic route of administration may be sensitive to such pharmacokinetic variables as weight, metabolism, absorption and clearance. To minimize potential pharmacokinetic effects, the efficacy and duration of the analgesic responses following intracerebroventricular morphine administration were evaluated in six groups: sham-operated male rats, castrated male rats, ovariectomized female rats, and sham-operated female rats tested during either the estrous, proestrous, or combined met/diestrous phase of the estrous cycle. Central morphine analgesia was assessed using two nociceptive measures, the spinally-mediated tail flick test and the supraspinally-mediated jump test. This research has previously been published (Kepler, et al., 1989).

Protocol

One hundred and twenty animals were divided among the six treatment groups, sham-operated males, castrated males, sham-

operated females tested consistently in either the proestrous, estrous, or combined met/diestrous condition, and ovariectomized females. Morphine doses (Pennick laboratories) of 1, 5, 10, 20 and 40 ug, dissolved in normal saline, were employed to construct a dose-response curve; doses over 40 ug were not used because of their possible seizure effects (Urca, et al., 1977). All testing took place between 2 and 10 h into the light cycle to control for basal and opiate circadian oscillations (Kavaliers and Innis, 1987). All rats received a maximum of three injection conditions, a vehicle injection and one relatively high (e.g., 10-40 ug) and one relatively low (e.g., 1-5 ug) morphine dose. Treatment conditions were separated by at least one week to minimize possible tolerance effects (Yaksh et al., 1976). The order of dose was counterbalanced across subjects. All rats were tested at 30, 60, 90, and 120 min after each microinjection on the tail-flick and jump tests; this interval between tests yields stable baseline and vehicle data across the time course (Badillo-Martinez, et al., 1984; Romero and Bodnar, 1986; Romero, et al., 1987,1988). Rats comprising each of the six groups (n= 8-11 rats) were tested at various morphine doses to produce a full dose response curve. Estrous phase was monitored in sham-operated female rats using daily vaginal smears to determine the proestrous, estrous, or combined met/diestrous phases of the cycle as described in the General Methods.

RESULTS

Body Weight and Accessory Sexual Organs: Significant differences in body weight were observed between sham and gonadectomized males and females, $F(3,57)=29.76$, $p<0.0001$, between pre-and postoperative measures, $F(1,57)=55.33$, $p<0.0001$, and for the interaction between groups and times, $F(3,57)=2.86$, $p<0.045$. Table 1 indicates that ovariectomized and sham-operated female rats gained 53 g (17% increase) and 29 g (9% increase) respectively with gonadectomy significantly accelerating weight gain in female rats. Castrated and sham-operated male rats gained 68 g (14% increase) and 72 g (18% increase) respectively; this difference was not statistically significant. Significant differences in the weights of seminal vesicles were observed between sham-operated and castrated male rats, $F(1,17)=125.74$, $p<0.0001$. Significant differences in the uterine weights were observed in sham-operated and ovariectomized female rats, $F(1,42)=49.44$, $p<0.0001$. Castration and ovariectomy significantly reduced the weights of these accessory sexual organs by 84% and 67% respectively (Table 1).

Baseline Nociceptive Thresholds: Table 2 indicates the significant differences in baseline tail-flick latencies among groups, $F(5,64)=4.27$, $p<0.002$. Although sham-operated and castrated males failed to show basal differences in tail-flick latencies, ovariectomized females and sham-operated females in the proestrous phase displayed significantly longer tail-flick

TABLE 1

Alterations in Body Weight and Accessory Sexual Organ Weights following Sham or Gonadal Surgery

Group	Preoperative Body Weight (g. SEM)	Postoperative Body Weight (g. SEM)	Access. Sex. Organ Weight (mg. SEM)
Males (n):			
Sham (8)	409 (20)	483 (30)	940 (81.8)
Cast.(11)	455 (27)	523 (18)	152 (10.9)
% Change	-	-4%	-84%*
Females (n):			
Sham (33)	315 (9)	344 (10)	734 (38.5)
Ovar.(9)	309 (14)	362 (13)	248 (31.5)
% Change	-	+8%*	-67%*

Note: The asterisks denote significant differences between the sham and gonadectomy conditions ($p<0.05$). Percent weight gains were calculated for each group; the % change represents the difference between the sham and gonadectomy conditions. The accessory sexual organs measured were the seminal vesicles in males and the fallopian tubes in females.

TABLE 2

Basal Tail-Flick Latencies and Jump Thresholds (Mean, SEM) following Sham or Gonadal Surgery

Group	Jump Thresholds (sec)	Tail-flick Latency (mA)
Males (n):		
Sham (11)	3.25 (0.32)	0.460 (0.012)
Castrated (14)	3.03 (0.24)	0.435 (0.009)
Females (n):		
Proestrous (9)	3.46 (0.28)*	0.448 (0.016)
Estrous (11)	2.73 (0.12)	0.431 (0.014)
Met/Di-estrous (11)	2.72 (0.19)	0.430 (0.012)
Ovariectomy (13)	3.74 (0.27)*	0.458 (0.018)

Note: The asterisks denote significant differences in tail flick latencies in female rats relative to other phases of the estrous cycle.

latencies than sham-operated females in estrous or in the combined met/diestrous phase. In contrast, there were no significant differences between groups in baseline jump thresholds, $F(5,63)=1.06$.

Overall Analgesic Effects: Significant differences were observed between vehicle and the 1 ug dose [tail-flick: $F(1,25)=7.56$, $p<0.011$; jump: $F=9.49$, $p<0.005$], among groups [tail-flick: $F(5,25)=4.68$, $p<0.004$; jump: $F=4.79$, $p<0.003$], and across test times (jump: $F=5.49$, $p<0.002$). Significant differences were observed between vehicle and the 5 ug dose [tail-flick: $F(1,33)= 13.48$, $p<0.001$; jump: $F=60.28$, $p<0.001$], among groups [tail-flick: $F(5,33)=2.91$, $p<0.028$; jump: $F=5.93$, $p<0.001$], and across test times (jump: $F=11.53$, $p<0.001$). Significant differences were observed between vehicle and the 10 ug dose [tail-flick: $F(1,32)= 6.89$, $p<0.013$; jump: $F=72.29$, $p<0.001$], among groups [tail-flick: $F(5,32)=4.55$, $p<0.003$, jump: $F=4.35$, $p<0.004$] and across test times [tail-flick: $F(3,96)=6.05$, $p<0.001$; jump: $F=11.13$, $p<0.001$]. Significant differences were observed between the vehicle and the 20 ug dose, (jump: $F=29.51$, $p<0.001$), among groups [tail-flick: $F(3,21)=3.24$, $p<0.043$], and across test times (jump: $F=22.58$, $p<0.001$). Significant differences were observed between vehicle and the 40 ug dose [tail-flick: $F(1,28)=7.23$, $p<0.012$; jump: $F= 49.38$, $p<0.001$], and across test times [tail-flick: $F(3,84)=6.34$, $p<0.001$; jump: $F=10.36$, $p<0.001$].

Central Morphine Analgesia: Estrous and Gonadal Status in

FIGURE 1. Alterations in peak analgesia 60 min following intracerebroventricular administration of morphine on the tail-flick test (left panel) and jump (right panel) tests as functions of estrous phase and gonadectomy in female rats. In this and subsequent figures, the data are expressed as difference scores which were derived from subtracting each experimental score from its corresponding vehicle control score. The closed stars denote significant differences between the experimental and control values (Dunnett comparisons, $p<0.05$). The open stars denote significant differences among the different estrous phases relative to ovariectomized animals (Dunn comparisons, $p<0.05$). Separate groups of animals were tested at each dose point for each group: ovariectomy (n=5-11 rats); proestrous (n= 3-8 rats); estrous (n=4-9 rats); met/di-estrous (n=3-6 rats).

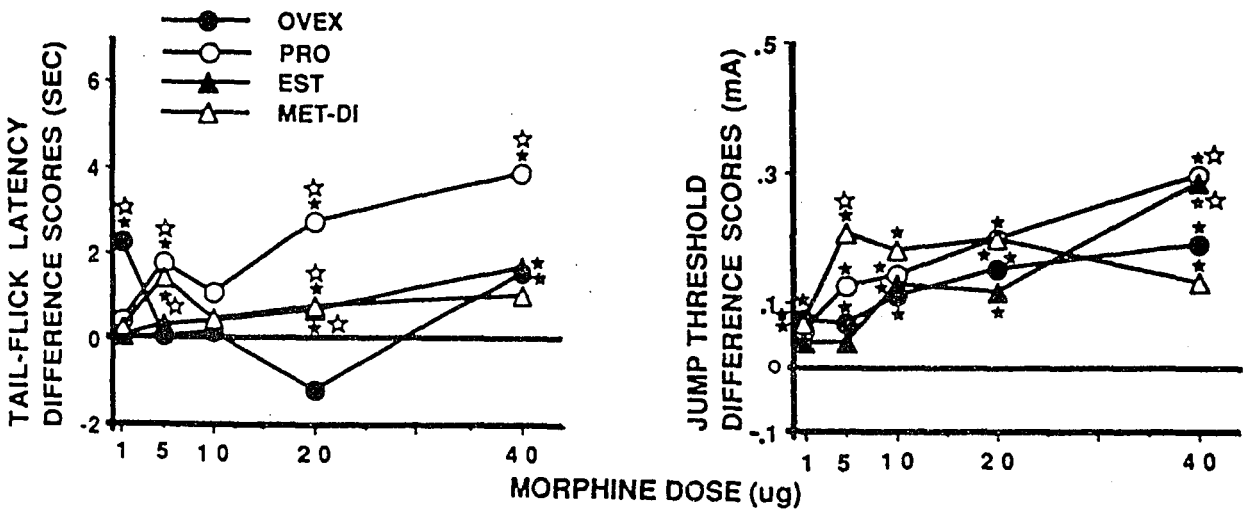

OVEX
PRO
EST
MET-DI
TAIL-FLICK LATENCY
DIFFERENCE SCORES (SEC)
6
4
2
0
-2
1 5 10 20 40
JUMP THRESHOLD
DIFFERENCE SCORES (mA)
.5
.3
.1
0
-.1
1 5 10 20 40
MORPHINE DOSE (ug)

FIGURE 2. Alterations in total analgesia following intracerebroventricular administration of morphine on the tail-flick and jump tests as functions of estrous phase and gonadectomy in female rats. Total analgesia was defined as the sum of the difference scores derived from the 30, 60, 90 and 120 min experimental time course. The closed stars denote significant differences between total experimental and control values (Dunnett comparisons, $p<0.05$). The open stars denote significant differences among the different estrous phases relative to ovariectomized animals (Dunn comparisons, $p<0.05$).

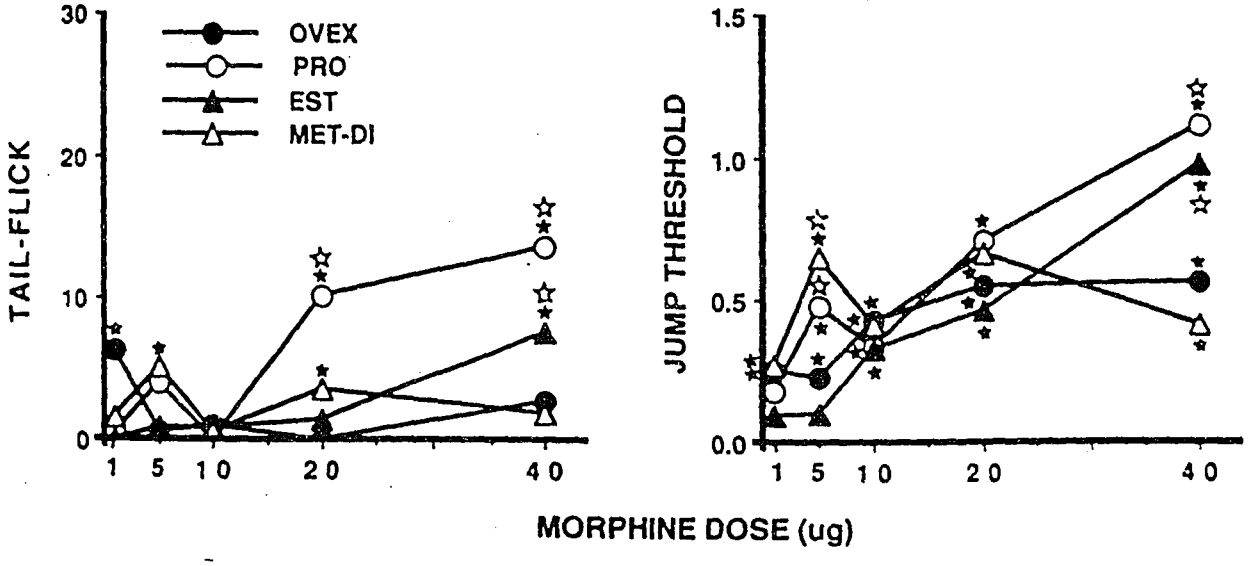

TAIL-FLICK
30
20
10
0
OVEX
PRO
EST
MET-DI
1 5 10 20 40
JUMP THRESHOLD
1.5
1.0
0.5
0.0
1 5 10 20 40
MORPHINE DOSE (ug)

TABLE 3

Regression Analysis of the Log Dose/Response Functions of Central Morphine Analgesia in Sham and Gonadectomized Male and Female Rats on the Tail-Flick Test

Group	ED50 (ug)	Slope	Intercept	Std. Error of Estimate
Peak Tail-Flick Latencies				
Males (n):				
Shams (11)	1.0	7.96	1.47	4.35
Castrated (14)	1.0	4.13	2.06	5.04
Females (n):				
Proestrous (9)	2.5	1.93	0.46	3.97
Estrous (11)	40.0	1.06+	0.31	1.66
Met/Diest.(11)	40.0	0.51+	0.24	1.01
Ovariectomy (13)	40.0	0.74+	1.39	1.69
Total Tail-Flick Latencies				
Males (n):				
Sham (11)	1.7	33.62	2.94	16.95
Castrated (14)	1.0	8.12	7.47	16.97
Females (n):				
Proestrous (9)	10.5	7.61	-1.05	13.17
Estrous (11)	40.0	3.99+	-1.53	8.12
Met/Diest. (11)	40.0	0.64+	1.97	2.94
Ovariectomy (13)	40.0	-2.52+	4.51	3.79

The ED50 is defined that minimal morphine dose which elicits a 50% increase in baseline latencies for peak (60 min) effects or for total (120 min time course) effects.
+ Significant difference relative to sham males (confidence intervals: 95%)

TABLE 4

Regression Analysis of the Log Dose/Response Functions of Central Morphine Analgesia in Sham and Gonadectomized Male and Female Rats on the Jump Test

Group	ED50 (ug)	Slope	Intercept	Std. Error of Estimate
Peak Jump Thresholds:				
Males (n):				
Sham (11)	2.1	0.536	-0.031	0.140
Castrated (14)	1.9	0.300	0.074	0.162
Females (n):				
Proestrous (9)	15.1	0.155+	0.022	0.156
Estrous (11)	25.1	0.146+	-0.008	0.126
Met/Diest.(11)	40.0	0.043+	0.113	0.129
Ovariectomy(13)	40.0	0.083+	0.043	0.102
Total Jump Thresholds:				
Males (n):				
Sham (11)	1.8	1.936	0.026	0.472
Castrated (14)	2.6	1.015	0.193	0.593
Females (n):				
Proestrous (9)	18.6	0.505+	0.141	0.610
Estrous (11)	40.0	0.541+	-0.098	0.401
Met/Di-est.(11)	40.0	0.186+	0.234	0.010
Ovariectomy(13)	40.0	0.243+	0.191	0.311

* The Ed50 is defined that minimal morphine dose which elicits a 50% increase in baseline thresholds for peak (60 min) effects or for total (the 120 min time course) effects.
+ Significant difference relative to sham males (confidence intervals: 95%).

Female Rats:

Figures 1 and Figure 2 display the differences in the respective magnitudes of peak (60 min) and total (2 hr time course) central morphine analgesia on the tail-flick (left panels) and jump (right panels) tests in ovariectomized female rats and in the female rats tested in the proestrus, estrous, and combined met/diestrous phases. Tables 3 and 4 summarize the potency of peak and total morphine analgesia on the tail-flick and jump tests, respectively, as determined by regression analyses for all groups. Although small, significant changes in central morphine analgesia occurred on both nociceptive tests as functions of estrous phase and female gonadectomy (Figures 1 and 2), the regression analyses indicated that neither estrous phase nor ovariectomy significantly altered the slope or the intercepts of the log dose response functions of central morphine analgesia (Tables 3 and 4).

Indeed, only rats tested in the proestrous phase displayed supracriterion values on the tail-flick and jump tests in which changes in analgesic magnitude exceeded 50% over baseline values. The following significant increases in central morphine analgesia relative to vehicle values were observed : a) ovariectomy (tail-flick: peak, 1 and 40 ug, 2-45%; total, 1 ug, 3-36%; jump: peak, 1-40 ug, 14-39%; total, 1-40 ug, 12-30%), b) met/di-estrous (tail-flick: peak, 1-40ug, 7-47%; total 1-40 ug, 5-38%; jump: peak, 1-40 ug, 16-49%;

total, 15-39%), c) proestrous (tail-flick: peak, 1-40 ug, 17-139%; total, 1-40 ug, 3-123%; jump: peak, 1-40 ug, 12-83%; total, 1-40 ug, 10-66%), d) estrous (tail-flick: peak, 1-40 ug, 1-50%; total, 1-40 ug, 1-64%; jump: peak, 1-40 ug, 8-67%; total, 4-57%). At the most effective analgesic dose of morphine (40 ug) in female rats, a rank order of peak and total analgesic effects across tests was: proestrous > estrous > ovariectomy = met/di-estrous. Significant differences between estrous phases failed to demonstrate a consistent pattern. To assess gender effects, the data from all of the estrous phases were combined in sham-operated females.

Central Morphine Analgesia: Gender and Gonadectomy Effects:

Figures 3 and 4 display the respective peak (60 min) and total analgesic effects of morphine on the tail-flick (left panels) and jump (right panels) tests following intracerebroventricular administration in sham-operated and gonadectomized male and female rats. Significant gender effects were observed both in terms of magnitude (Figures 3 and 4) and potency (Tables 3 and 4) for both nociceptive tests. At a commonly administered dose of 5 ug, the magnitude of morphine analgesia observed in sham-operated male rats relative to sham-operated female rats was seven-fold greater for total analgesia on the tail-flick test and three-fold greater for total analgesia on the jump test (Figure 4). Both

tail-flick latencies and jump thresholds of male sham-operated rats were either at, or close to cut-off values following the 5 ug dose of morphine. Indeed, the gender differences were still apparent if comparisons of analgesic magnitude involved the 5 ug dose for sham-operated males and the 40 ug dose for sham-operated females. Linear regression analyses revealed significant differences in the slope, but not in the intercepts of the log-dose response functions of sham-operated male rats relative to all female groups on the jump test (Table 4), and to all but the proestrous group on the tail-flick test (Table 3). Relative to sham-operated males, rightward shifts in the ED50 of peak and total analgesia were 20-40 fold for ovariectomized females and intact females during the met/di-estrous female rats, 12-40 fold for intact females during the estrous phase, and 2.5-20 fold for intact

FIGURE 3. Alterations in peak analgesia 60 min following intracerebroventricular administration of morphine on the tail-flick and jump tests as functions of gender and gonadectomy. The peak data of sham-operated females constituted the mean values of the proestrous, estrous, and combined met-/diestrous groups. The closed stars denote significant differences between the experimental and control values (Dunnett comparisons, $p<0.05$). The open stars and enclosed stars denote significant gonadectomy and gender differences respectively (Dunn comparisons, $p<0.05$). Separate groups of animals were tested at each dose point for each group: sham-operated males (n=4-8 rats); castrated males (n=3-10 rats); sham-operated females (n=11-22 rats); ovariectomized females (n=5-11 rats).

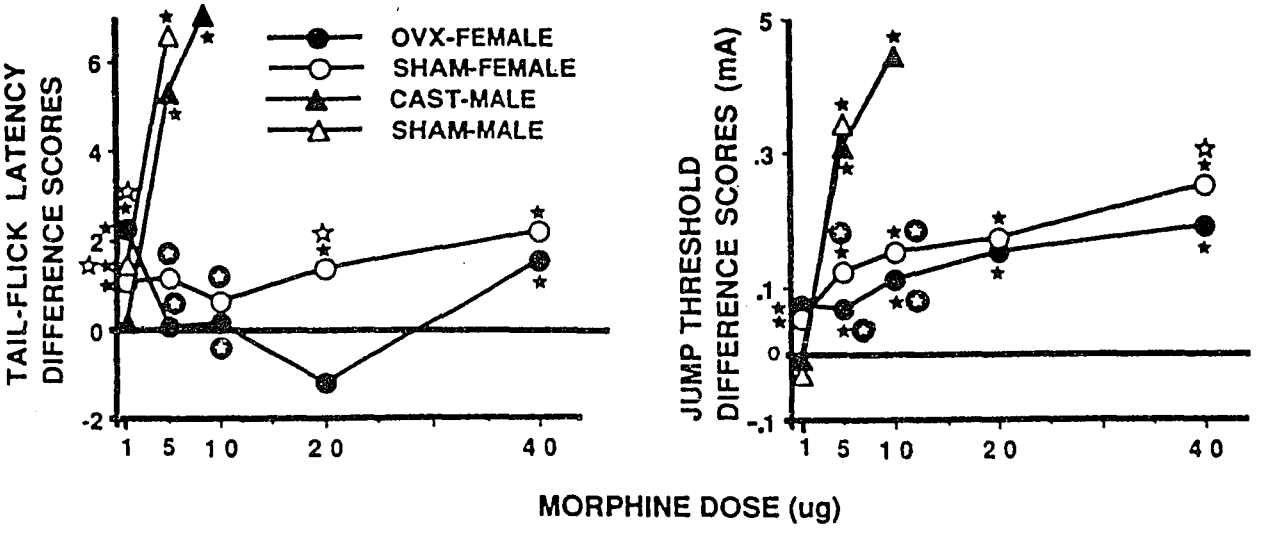
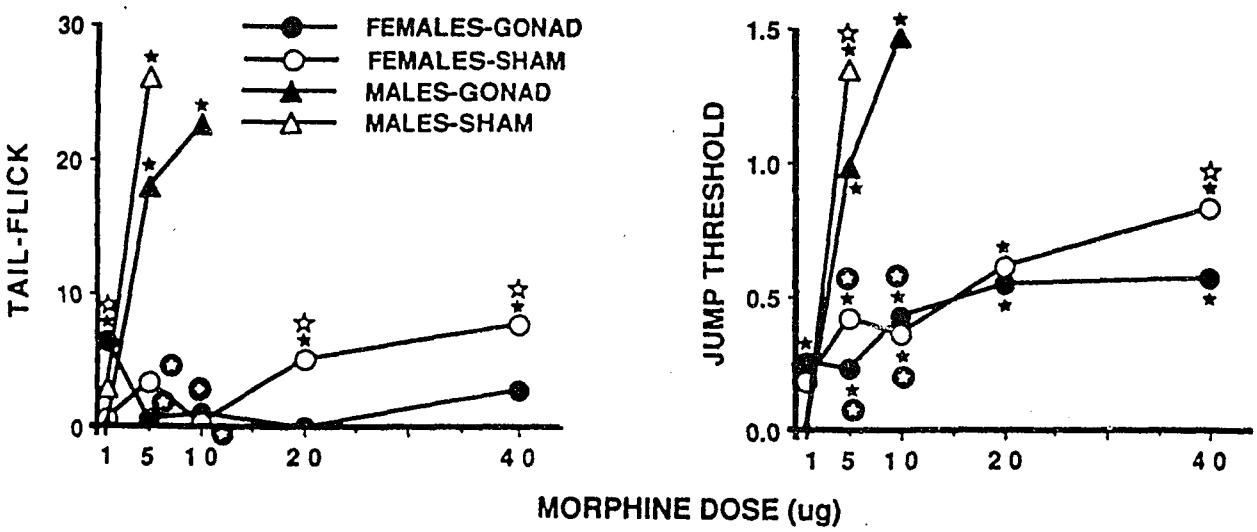

TAIL-FLICK LATENCY
DIFFERENCE SCORES
OVX-FEMALE
SHAM-FEMALE
CAST-MALE
SHAM-MALE
6
4
2
0
-2
1
5
10
20
40
JUMP THRESHOLD
DIFFERENCE SCORES (mA)
.5
.3
.1
0
-.1
MORPHINE DOSE (ug)

FIGURE 4. Alterations in total analgesia following intracerebroventricular administration of morphine on the tail-flick and jump tests as functions of gender and gonadectomy. The closed stars denote significant differences between the experimental and control values (Dunnett comparisons, $p<0.05$). The open stars and enclosed stars denote significant gonadectomy and gender differences respectively (Dunn comparisons, $p<0.05$).


FEMALES-GONAD
FEMALES-SHAM
MALES-GONAD
MALES-SHAM
TAIL-FLICK
30
20
10
0
1 5 10 20 40
JUMP THRESHOLD
1.5
1.0
0.5
0.0
1 5 10 20 40
MORPHINE DOSE (ug)

females during the proestrous phase.

Whereas marked gender differences were observed, the effects of male gonadectomy were not as striking. Sham-operated and castrated male rats failed to exhibit significant differences in the potency of peak or total morphine analgesia on either nociceptive measure (Tables 3 and 4). Significant differences in analgesic magnitude were observed following the 1 ug dose of morphine for peak analgesia on the tail-flick test (Figure 3), and following the 5 ug dose of morphine for total analgesia on the jump test (Figure 4). Comparable ranges (1-5 ug) of analgesic magnitude were observed for peak analgesia on the tail-flick (shams: 38-188%; castrates: 6-183%) and the jump (shams: 7-72%; castrates: 2-74%) tests and for total analgesia on the tail-flick (shams: 0-70%; castrates: 0-56%) tests. Thus, a rank-order potency of analgesic magnitude was sham-operated males> castrated males >> sham-operated females=> ovariectomized females.

Discussion

The first experiment evaluated the effects of gender, gonadal status, and estrous phase upon morphine analgesia following intracerebroventricular administration. Gender differences represented the most striking effects in the present data as sham- operated male rats displayed significantly greater analgesic effects both in terms of magnitude and potency on both nociceptive measures for both

peak and total analgesic effects than sham-operated female rats. A 5 ug dose of morphine produced a near-total analgesia in sham-operated males with a large percentage of animals approaching or attaining cut-off values; this dose range is consistent with effects previously observed (Yaksh, Yeung, and Rudy, 1976). Pronounced gender differences were observed since maximal peak and total effects observed in sham-operated males at a 5 ug dose was greater than those of sham-operated females at a 40 ug dose of morphine. Gender differences following central morphine analgesia are similar to previously-reported effects following systemic administration of morphine (Badillo-Martinez, et al., 1984; Kavaliers, et al., 1987) and suggest further that the observed gender differences following systemic administration were probably not due to such pharmacokinetic factors as differences in absorbance, storage, and release. Gender differences in central morphine analgesia are also consistent with the observed gender differences in both opioid and nonopioid forms of swim analgesia (Romero and Bodnar, 1986) such that adult female rats displayed significantly less analgesia following CCWS and ICWS than age-matched or weight-matched male rats. The gender-specific alterations following CCWS, ICWS, systemic morphine and central morphine analgesia occurred independently of gender differences in baseline pain thresholds (Romero and Bodnar, 1986).

Although significant changes in the magnitude of central

morphine analgesia occurred as functions of gonadectomy and estrous phase for some doses at some time points, regression analyses revealed that castrated and sham-operated male rats failed to differ significantly from each other in analgesic potency. This effect differs from reports indicating that castration reduced analgesia following systemic morphine, CCWS, and ICWS to levels observed in sham-operated females (Chatterjee, et al., 1982; Romero, et al., 1987). Although ovariectomized rats displayed significantly smaller magnitudes of central morphine analgesia than sham-operated female rats, regression analyses of gonadectomized and sham-operated females also failed to reveal significant differences in potency.

Estrous phase also induced small, significant, yet inconsistent changes in analgesic magnitude following central morphine. However, regression analyses again failed to reveal significant differences in analgesic potency. Female rats in the proestrous phase displayed greater magnitudes of peak and total analgesia on both the tail-flick and jump tests relative to females in other estrous phases at the higher (20 ug and 40 ug) morphine doses. These findings are in contrast to previous reports (Banerjee, et al., 1983) that indicated an increased sensitivity to systemic morphine analgesia during the diestrous phase. Comparisons with the present data are difficult given differences in injection route, nociceptive measure, and definitions of magnitude. Ryan and co-workers

(1985) found that an opioid form of shock-induced analgesia was greatest during the estrous phase, and that steroid replacement therapy with estradiol and progesterone enhanced this analgesia in ovariectomized females. In contrast, estrous phase failed to affect the magnitude of CCWS analgesia on either the tail-flick or jump test (Romero and Bodnar, 1986).

The marked gender differences as well as the smaller gonadectomy and estrous differences in central morphine analgesia could not be attributed to basal shifts in nociceptive reactivity since a) the difference score analyses factored out basal effects, b) basal effects were not observed for vehicle jump thresholds, and c) minimal latency differences of less than 1 sec were observed in females across the estrous phases. Additionally, the general inability of gonadectomy to significantly affect central morphine analgesia relative to the observed gonadectomy differences in opioid and non-opioid forms of stress-induced analgesia appear to be due to the analgesic procedure employed since the reductions in accessory gonadal tissue were comparable in both studies (Romero, et al., 1987). As a cautionary note, the analgesic effects following stress-induced analgesia are somewhat difficult to compare as gender, gonadectomy, and estrous phase may modulate the actual stress response, the pain-inhibitory response, or a combination of both output systems. The consistency of reported central morphine effects appear to

vary as a function of nociceptive test employed. Although both sham-operated and castrated male rats showed rapidly accelerating dose-response curves following central morphine analgesia on both nociceptive measures, sham-operated and ovariectomized female rats showed less variability in the dose-response functions on the jump test than on the tail-flick test. Given the intracerebroventricular route of administration, differential results across tests may reflect the supraspinal level of the neuraxis at which the drug is potentially acting.

The mechanism mediating gender differences in central morphine analgesia is unknown, but may be conceptualized as either direct or indirect interactions between central opiate receptors and central gonadal steroid receptors. Central morphine analgesia appears to be mediated through supraspinal mu receptors (Bodnar, et al., 1988; Jensen and Yaksh, 1986), implicating analgesia-sensitive areas with high concentrations of mu receptors such as the PAG, NRM, and NRGC. Autoradiographic localization of gonadal steroid receptors using 3H-estradiol and 3H-testosterone reveal steroid-containing cells in the medial preoptic area, anterior and ventromedial hypothalamic areas, arcuate nucleus, lateral septum, bed nucleus of the stria terminalis, medial and cortical amygdaloid nuclei, and the mesencephalic central gray (Morrell and Pfaff, 1981; Pfaff and Keiner, 1973). Potential sites of action for direct central effects between opiate and

steroid receptors would include the mesencephalic central gray, the arcuate nucleus and the amygdala. The leading candidate appears to be the mesencephalic central gray, which is among the most sensitive supraspinal sites to support opiate analgesia (Akil, et.al, 1984; Yaksh, et al., 1976). The arcuate nucleus contains cells of the medial-basal hypothalamic propriomelanocortocin system responsible for the synthesis of beta-endorphin which projects to mesencephalic, metencephalic and myelencephalic loci involved in antinociception (Akil, et al., 1984; Mansour, et al., 1987; Watson, et al., 1978). Lesions placed in this area reduce the analgesic responses following either electrical stimulation or footshock stress (Millan, Przewlocki, Jerlicz, Gramsch, Hollt, and Herz, 1981). Combined radioreceptor assays with 3H-estradiol and immunocytochemistry with beta-endorphin reveal cellular co-localization of gonadal target receptors on beta-containing cells (Jirikowski, Merchenthaler, Reiger, and Stumpf, 1986). A third potential site of action is the amygdala which is rich in both mu and delta opiate receptors (Akil, et al., 1984; Mansour, et al., 1987) and has been shown to support analgesia following microinjection of morphine (Rodgers, 1977). Steroid-opioid interactions may also occur through an indirect mechanism or second messenger system, such as cAMP, mRNA, or differential protein synthesis.

The actual mechanism by which gonadal steroids act to modulate endogenous opioid pain inhibition is also not known,

but interactions between gonadal steroids and endogenous opioids have been observed. Female rats display lower levels of beta-endorphin (Mueller, 1980), dynorphin (Molineaux, 1986), and Met-enkephalin (Hong, et al., 1982) than male rats. Concentrations of these endogenous opioids are altered by either gonadectomy or the phase of the estrous cycle (Hong, et al., 1982; Knuth, et al., 1983). Castration decreases the number of brain opioid receptors (Hahn, Norton, and Fishman, 1985), and reduces both beta-endorphin (Wardlaw, et al., 1982) and Met-enkephalin (Hong, et al., 1982) in the pituitary. Gender differences have also been observed in the distribution and amount of opiate receptor binding in cortex and whole brain (Messing and McGaugh, 1981) using naloxone as the radio-labelled ligand. Several neurotransmitters involved in opiate analgesia (Mayer and Price, 1976; Messing and Lytle, 1977) display gender-specific patterns in the medial preoptic area of the hypothalamus, a sexually dimorphic nucleus. For example, serotonin fibers are most dense in the lateral part of the medial preoptic area which is proportionately larger in females (Simerly, et al., 1984). A greater density of tyrosine hydroxylase immunoreactive cells and fibers, but not dopamine beta hydroxylase immunoreactive cells and fibers, are found in the anteroventral periventicular preoptic nucleus of female rats relative to male rats, implicating a sexual dimorphism for dopamine (Simerly, et al., 1985). Met-enkephalin immunoreactivity (Watson, et al., 1986) and leu-

enkephalin immunoreactivity (Simerly, et al., 1988) are also much denser in the anteroventral periventricular nucleus of females and are affected by gonadectomy and steroid replacement treatment. These data indicate clear gender differences in the organization of opioid peptides in these gonadal steroid-sensitive nuclei. Whether this is the site of action for the opioid-sensitive gender differences, and/or whether other opioid-containing loci display sexual dimorphism remain to be clarified. Central administration of various selective opiate receptor agonists may be one means to differentiate potential gender and/or gonadal status influences on opioid concentration and distribution. In the original delineation of opiate receptor heterogeneity, the mu receptor was defined in terms of its intrinsic binding characteristics with morphine (Martin, et al., 1976), however, morphine also binds to delta receptors, although with less affinity than the enkephalins (Lord, et al., 1977). Autoradiographic localization of mu and delta receptors reveal an extensive supraspinal (mu > delta) and spinal (delta > mu) distribution (Akil, et al., 1984; Mansour, et al., 1987), however, delta receptors have also been implicated in supraspinal analgesia (Heyman, et al., 1987; Jensen and Yaksh, 1986; Porreca, et al., 1984,1987). Hence, the following experiment evaluated the role of gonadal steroids as modulators of specific opioid subtypes with respect to central antinociception by assessing potential gender and gonadectomy

differences in central antinociception produced by the mu-selective agonist, DAMGO, and the delta-selective agonist, DSLET.

EXPERIMENT 2 Gender Differences and Gonadectomy Effects on Central DAMGO and DSLET Antinociception

Analgesic responses to systemic and central administration of morphine have been shown to be sensitive to gender differences. Whereas, gonadectomy minimally altered central morphine analgesia, analgesic responses were not systematically related to estrous phase. Since the mu and delta opioid receptor subtypes have been implicated in the mediation of supraspinal analgesia, the present experiment evaluated whether gender or gonadectomy systematically altered the central analgesic responses of the mu-selective agonist, DAMGO, and the delta-selective agonist, DSLET. Time-dependent and dose-dependent analgesic responses following central administration of either DAMGO or DSLET were assessed using two nociceptive measures, the spinally mediated tail-flick test and the supraspinally mediated jump test in four treatment groups: sham-operated males, castrated males, sham-operated females in estrous, and ovariectomized females. As estrous phase failed to systematically influence central morphine analgesia, this estrous variable was held constant in the present study to maximize the study of dose-response functions across agonists while minimizing the number of animals used. The sham female rats were assessed during the estrous phase only. This phase was selected to maximize potential hormonal differences between the ovariectomized females and the sham females. This research has recently been

accepted for publication (Kepler, Standifer, Paul, Kest, Pasternak, and Bodnar, 1991).

Protocol

One hundred and twenty rats, comprised four treatment groups: sham-operated males, castrated males, sham-operated females tested during estrous, and ovariectomized females. The rats received either the mu agonist, DAMGO (n=60) or the delta agonist, DSLET (n=60). Rats (n=7/8) comprising the four treatment groups were tested at each dose for both of the agonists producing full dose response curves. Both DAMGO and DSLET (Peninsula Laboratories) were dissolved in normal saline and the following doses were employed : 1, 5, 10, and 20 ug. All rats received a maximum of three injection conditions, including a vehicle injection; treatment conditions were separated by at least one week to minimize possible tolerance effects. Each rat received one relatively high (e.g., 10-20ug) and one relatively low (e.g., 1-5 ug) dose of a given agonist. The order of the doses were counterbalanced. All testing took place between 2 and 10 hours into the light cycle to control for basal and circadian oscillations. All rats were tested at 15, 30, 45, and 60 min after each microinjection on the tail flick and jump tests. The one hour time course was employed since the agonists have a shorter half-life than morphine. Estrous phase was monitored in sham-operated female rats using daily vaginal smears. All sham-

TABLE 5
Alterations in Body Weights and Accessory Sexual Organ Weights following Sham or Gonadal Surgery

Group	Pre-Operative Body Weight (g, SEM)	Post-Operative Body Weight (g, SEM)	Acc.Sex. Organ Wgt. (mg, SEM)
Males (n):			
Shams (29)	429 (12)	495 (12)	900 (34)
Castrated (33)	462 (16)	546 (15)	107 (32)*
Females (n):			
Sham (29)	307 (6)	346 (8)	762 (35)
Ovex (27)	315 (9)	334 (10)*	232 (21)*

NOTE: The asterisks denote significant differences between the sham and gonadectomy conditions ($p<.05$). The accessory sexual organs measured were in the seminal vesicles in males and the fallopian tubes in females.

operated females were tested during the estrous phase of the cycle.

Results

Accessory Sexual Organs and Body Weight: Table 5 summarizes the changes in accessory sexual organs and body weight in sham and gonadectomized animals. Castration significantly reduced the weight of the seminal vesicles by 88%, while ovariectomy significantly reduced uterine weight by 70%. Whereas sham and castrated male rats failed to differ from each other in post-operative body weight gain, ovariectomized females (18 g gain) gained significantly less weight than sham-operated female rats (38 g gain).

Basal Nociceptive Thresholds: Table 6 summarizes the baseline tail-flick latencies and jump thresholds in sham-operated and gonadectomized male and female rats. Baseline tail-flick latencies failed to differ among groups. Jump thresholds were significantly higher in male rats than female rats, and gonadectomy failed to alter this difference. The difference score analysis partialed out differences in basal thresholds.

Overall Analgesic Effects: Significant differences were observed for DAMGO analgesia between vehicle and the 1 ug dose [tail-flick: $F(1,24)= 5.32$, $p<0.030$; jump: $F=19.36$, $p<0.0002$], across test times [tail-flick: $F(3,72)=5.18$, $p<0.003$; jump: $F=13.84$, $p<0.000$], but not between groups [tail-flick: $F(1,24)=3.14$, jump: $F=2.37$]. Significant differences were

TABLE 6

Basal Tail-Flick Latencies and Jump Thresholds (Mean, SEM) following Sham or Gonadal Surgery

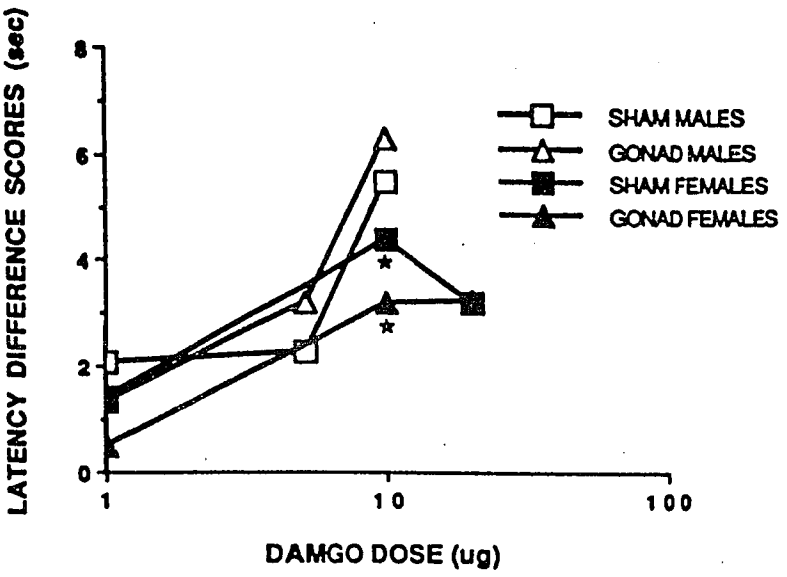
Group	Tail-Flick Latencies (sec)	Jump Thresholds (mA)
Males (n):		
Sham (28)	3.27 (0.14)	.459 (.006)*
Castrated (27)	3.49 (0.14)	.455 (.008)*
Females (n):		
Sham (26)	3.66 (0.14)	.410 (.007)
Ovariectomy (28)	3.45 (0.13)	.407 (.007)

Note: The asterisks denote significant differences in jump thresholds between male and female rats irrespective of sham or gonadal surgery. The mean values were derived by collapsing all of the vehicle value scores for a given animal in the DAMGO or DSLET protocols.

observed between vehicle and the 5 ug dose [tail-flick: $F(1,9)= 5.14$, $p<0.049$; jump: $F=18.95$, $p<0.002$], across test times [jump: $F(3,27)=7.49$, $p<0.008$], but not between groups [tail-flick: $F(1,9)=0.00$, jump: $F=0.12$]. Significant differences were observed between vehicle and the 10 ug dose [tail-flick: $F(1,25)= 37.87$, $p<0.000$; jump: $F=62.08$, $p<0.000$], across test times [tail-flick [$F(3,75)=4.91$, $p<0.003$; jump: $F=8.64$, $p<0.0001$], but not among groups [tail-flick: $F(1,25)=0.66$, jump: $F=2.62$]. Significant differences were observed between the vehicle and the 20 ug dose, [tail-flick: $F(1,10)=11.40$, $p<0.007$; jump: $F=18.87$, $p<0.001$], across test times [tail-flick: $F(3,30)=4.67$, $p<0.008$, jump: $F=13.01$, $p<0.000$], but not among groups [tail-flick: $F(1,10)=0.09$, jump: $F=0.08$].

DAMGO Analgesia: Figures 5 and 6 illustrate respectively the peak (15 minutes) and total analgesic effects following DAMGO on the tail-flick (upper panels) and jump (lower panels) tests in sham-operated and gonadectomized male and female rats. Significant increases in tail-flick latencies relative to vehicle values were observed in sham-operated and castrated male rats and in sham-operated female rats following all DAMGO doses (1-20 ug); ovariectomized female rats displayed significant increases in tail-flick latencies following the 5-20 ug doses of DAMGO. Significant gender differences were observed in the peak analgesic response to DAMGO on the tail-flick test at the 10 and 20 ug

FIGURE 5. Alterations in the peak (15 min) analgesia on the tail-flick (upper panel) and the jump (lower panel) tests following intracerebroventricular (icv) administration of DAMGO in sham and gonadectomized male and female rats. The data are expressed as difference scores which were derived from subtracting each experimental score from its corresponding vehicle control score. Significant reductions (Dunn comparisons, $p<.05$) in analgesic magnitude are represented by filled stars relative to groups showing the optimal response for that dose and condition.


LATENCY DIFFERENCE SCORES (sec)
8
6
4
2
0
1
10
100
DAMGO DOSE (ug)
SHAM MALES
GONAD MALES
SHAM FEMALES
GONAD FEMALES

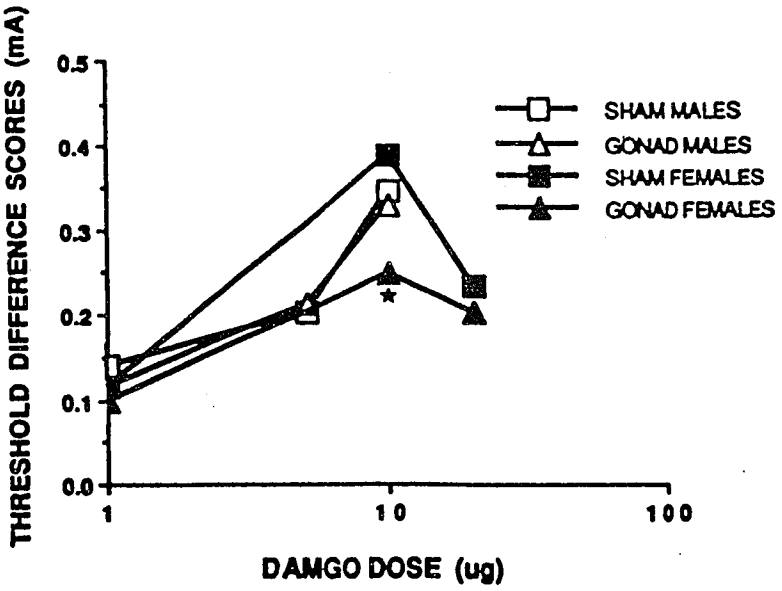
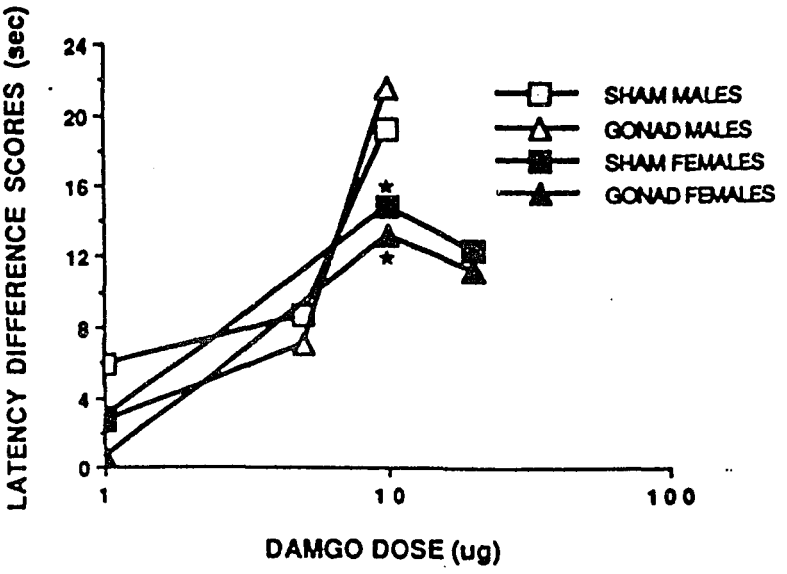
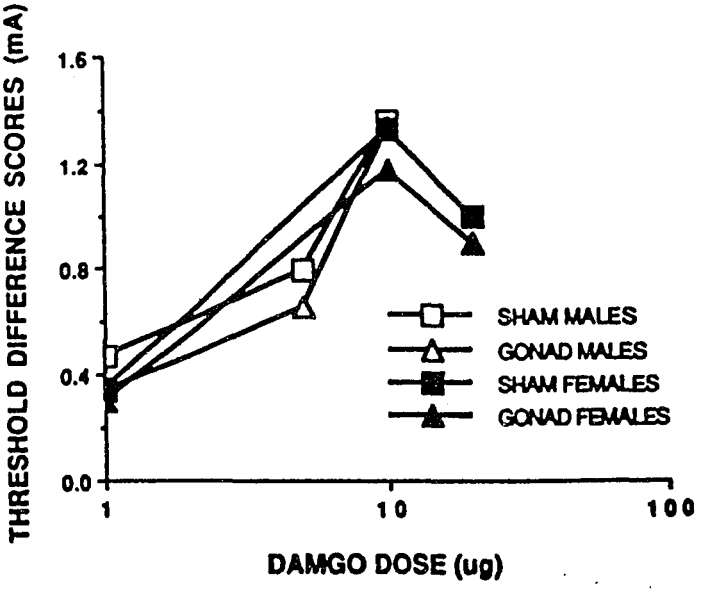

THRESHOLD DIFFERENCE SCORES (mA)
0.5
0.4
0.3
0.2
0.1
0.0
1
10
100
DAMGO DOSE (ug)
SHAM MALES
GONAD MALES
SHAM FEMALES
GONAD FEMALES

FIGURE 6. Alterations in total analgesia on the tail-flick (upper panel) and the jump (lower panel) tests following intracerebroventricular (icv) administration of DAMGO in sham and gonadectomized male and female rats.


LATENCY DIFFERENCE SCORES (sec)
24
20
16
12
8
4
0
1
10
100
DAMGO DOSE (ug)
SHAM MALES
GONAD MALES
SHAM FEMALES
GONAD FEMALES


THRESHOLD DIFFERENCE SCORES (mA)
1.6
1.2
0.8
0.4
0.0
1
10
100
DAMGO DOSE (ug)
SHAM MALES
GONAD MALES
SHAM FEMALES
GONAD FEMALES

doses. Both sham and castrated male rats displayed significantly greater peak and total DAMGO analgesia on the tail-flick test following the 10 ug dose relative to sham and ovariectomized females. As sham-operated and castrated male rats displayed maximal analgesia at the 10 ug dose of DAMGO, sham-operated and ovariectomized female rats displayed significantly smaller increases in analgesia than males at the 10 ug and 20 ug dose. Although female rats displayed respective 2.3 and 3.1-fold rightward shifts in the ED50 of peak and total DAMGO analgesia (Table 7), regression analyses failed to indicate significant changes in the dose-response functions of peak and total DAMGO analgesia on the tail-flick test. Gonadectomy failed to alter significantly either peak or total DAMGO analgesia on the tail-flick test.

Gender differences failed to occur for peak and total DAMGO analgesia on the jump test (Table 7). Whereas castration failed to significantly alter DAMGO analgesia on this measure, ovariectomy reduced peak analgesia following the 10 ug dose of DAMGO, and produced a rightward shift in the ED50 of peak and total analgesia by 3.3 and 1.5-fold, respectively (Table 7). Regression analyses failed to reveal significant changes in the dose-response function on the jump test.

Overall Analgesic Effects: Significant differences were observed for DSLET analgesia between vehicle and the 1 ug dose [tail-flick: $F(1,26)=26.42$, $p<0.001$; jump: $F=157.26$, $p<0.001$],

across test times [tail-flick: $F(3,78)=9.41$, $p<0.001$; jump: $F=42.53$, $p<0.001$), and among groups [tail-flick: $F(1,26)=13.17$, $p<0.001$; jump: $F=17.17$, $p< 0.001$]. Significant differences were observed between vehicle and the 5 ug dose [tail-flick: $F(1,20)= 19.86$, $p<0.001$; jump: $F=48.54$, $p<0.001$], across test times ([tail-flick : $F(3,60)=13.38$, $p<0.001$, jump: $F=33.76$, $p<0.001$), and among groups (jump: $F(1,20)=11.49$, $p<0.002$). Significant differences were observed between vehicle and the 10 ug dose [tail-flick: $F(1,25)= 42.40$, $p<0.001$; jump: $F=152.14$, $p<0.001$], across test times [tail-flick: $F(3,75)=9.04$, $p<0.001$; jump: $F=53.16$, $p<0.001$],

TABLE 7

Regression Analysis of Log Dose/Response of Central DAMGO Analgesia

Measure	Males		Females	
	Sham	Castrated	Sham	Ovex
DAMGO PEAK TAIL-FLICK				
ED50	3.9	3.2	8.9	18.6
Slope	2.95	4.63	1.97	2.27
Intercept	1.73	1.09	1.59	0.59
SE of Estimate	1.83	1.84	1.31	1.13
DAMGO TOTAL TAIL-FLICK				
ED50	5.8	5.8	18.2	22.9
Slope	11.88	17.22	8.96	9.28
Intercept	4.83	0.84	3.43	1.28
SE of Estimate	6.41	5.88	4.75	4.60
DAMGO PEAK JUMP				
ED50	3.5	4.2	2.9	9.5
Slope	.186	.198	.155	.099
Intercept	.129	.107	.138	.113
SE of Estimate	.087	.094	.073	.087
DAMGO TOTAL JUMP				
ED50	3.9	5.2	4.2	6.5
Slope	.829	.897	.664	.552
Intercept	.428	.270	.406	.371
SE of Estimate	.361	.348	.282	.294

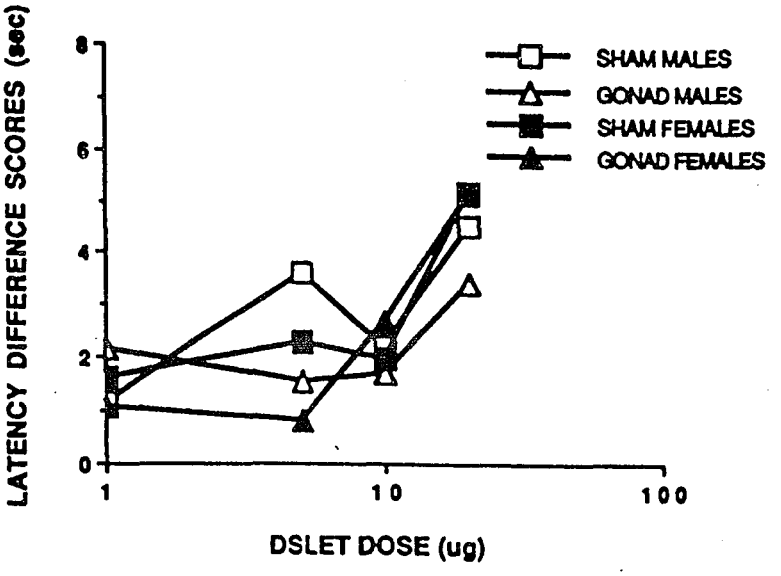
NOTE: The ED50 is defined as that minimal agonist dose which elicits a 100% rise in baseline tail-flick latencies or a 50% rise in baseline jump thresholds for peak (15 min) or for total (the 60 min time course) effects.

and among groups [tail-flick: $F(1,25)=6.13$, $p<0.020$, jump: $F=10.86$, $p<0.003$]. Significant differences were observed between the vehicle and the 20 ug dose, [tail-flick: $F(1,22)=59.11$, $p<0.001$; jump: $F=120.15$, $p<0.001$], across test times [tail-flick: $F(3,66)=22.77$, $p<0.001$, jump: $F=71.60$, $p<0.001$), and among groups [tail-flick: $F(1,22)=4.94$, $p<0.037$].

DSLET Analgesia: Figures 7 and 8 illustrate respectively the peak and total analgesic effects following DSLET on the tail-flick (upper panels) and jump (lower panels) tests across the four groups. Significant increases in tail-flick latencies were observed relative to vehicle values in all groups following all DSLET doses (1-20 ug). In contrast to effects following DAMGO, the peak analgesic effect of DSLET on the tail-flick test failed to differ across doses as functions of either gender or gonadectomy. Total DSLET analgesia on the tail-flick test was significantly less in sham-operated males relative to females following the 20 ug dose, and in gonadectomized rats relative to the sham males following the 5 ug dose.

Inconsistent differences in peak and total DSLET analgesia occurred on the jump test among groups. Intact males displayed significantly greater peak analgesia than intact females (5 ug dose) and castrated males (10 ug dose), but intact females displayed greater peak analgesia than intact males (1 ug dose) and ovariectomized females (1 ug

FIGURE 7. Alterations in peak (15 min) analgesia on the tail-flick (upper panel) and jump (lower panel) tests following icv administration of DSLET in sham and gonadectomized male and female rats.


LATENCY DIFFERENCE SCORES (sec)
8
6
4
2
0
1
10
100
DSLET DOSE (ug)
SHAM MALES
GONAD MALES
SHAM FEMALES
GONAD FEMALES

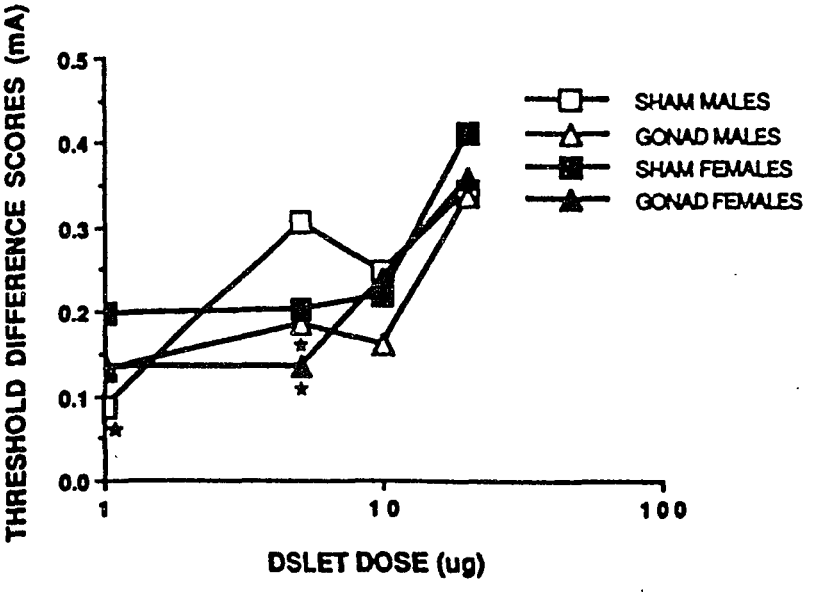
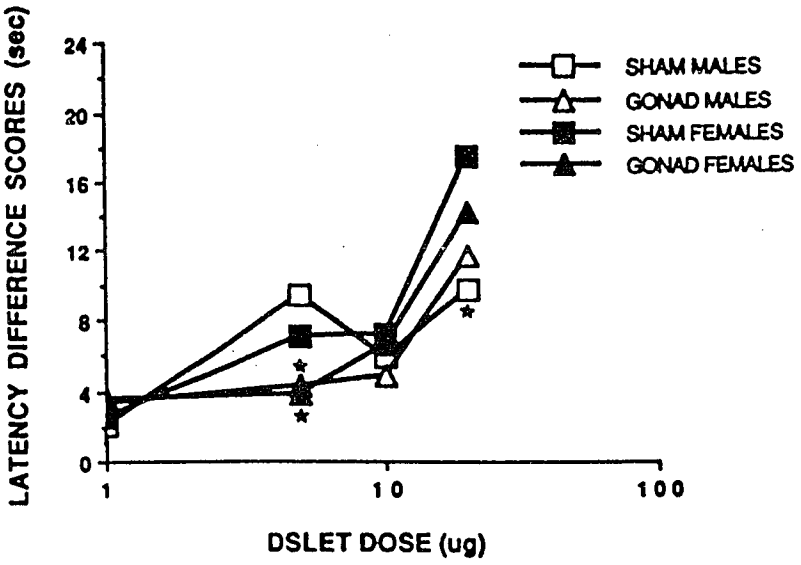
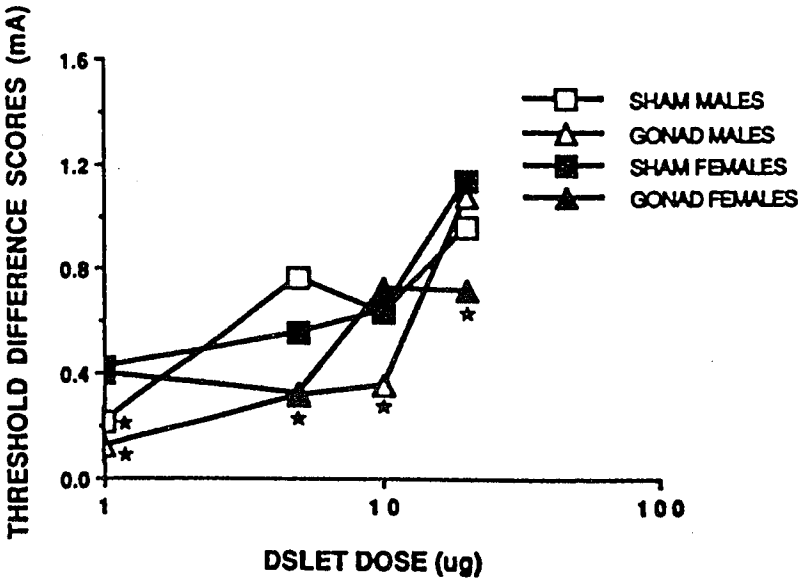

THRESHOLD DIFFERENCE SCORES (mA)
0.5
0.4
0.3
0.2
0.1
0.0
1
10
100
DSLET DOSE (ug)
SHAM MALES
GONAD MALES
SHAM FEMALES
GONAD FEMALES

FIGURE 8. Alterations in total analgesia on the tail-flick (upper panel) and jump (lower panel) tests following icv administration of DSLET in sham and gonadectomized male and female rats.


LATENCY DIFFERENCE SCORES (sec)
24
20
16
12
8
4
0
1
10
100
DSLET DOSE (ug)
SHAM MALES
GONAD MALES
SHAM FEMALES
GONAD FEMALES


THRESHOLD DIFFERENCE SCORES (mA)
1.6
1.2
0.8
0.4
0.0
1
10
100
DSLET DOSE (ug)
SHAM MALES
GONAD MALES
SHAM FEMALES
GONAD FEMALES

dose). Sham-operated females displayed significantly greater total DSLET analgesia on the jump test than sham-operated males (1 ug), castrated males (1, 5, and 10 ug) and ovariectomized females (20 ug). However, regression analyses failed to reveal significant changes in the dose-response functions of peak and total DSLET analgesia on either measure (Table 8).

Discussion

The second experiment evaluated whether gender and/or gonadectomy altered antinociception induced by intracerebroventricular administration of the selective mu-receptor agonist, DAMGO, or the selective delta-receptor agonist, DSLET on the tail-flick test and jump tests. The evaluation of potential gender and gonadectomy differences in analgesia following central administration of selective opioid receptor subtype agonists was prompted by the observation of marked gender differences (4-8 fold) in central morphine analgesia with males greater than females. Castration produced small, but significant reductions in the magnitude of central morphine analgesia. In the present experiment gender appeared to exert greater effects upon DAMGO and DSLET analgesia than gonadectomy. The more striking gender differences occurred for DAMGO analgesia than for DSLET analgesia. Further, gender differences occurred selectively on the tail-flick test, but not on the jump test, even though nociceptive assessments were made in the same animals at the

TABLE 8

Regression Analyses of the Log Dose/Response Functions of Central DSLET Analgesia in Sham and Gonadectomized Male and Female Rats on the Tail-Flick and Jump Tests

MEASURE	MALES		FEMALES	
	Sham	Castrated	Sham	Ovex
DSLET PEAK TAIL-FLICK				
ED50	8.7	>50	11.5	13.5
Slope	2.32	0.54	2.16	2.64
Intercept	1.30	1.79	1.17	0.48
SE of Estimate	1.10	1.03	1.16	0.78
DSLET TOTAL TAIL-FLICK				
ED50	>50	>50	19.5	>50
Slope	5.79	5.25	9.86	6.70
Intercept	2.99	2.21	1.19	2.06
SE of Estimate	2.43	2.19	2.74	2.57
DSLET PEAK JUMP				
ED50	4.4	6.9	2.2	4.9
Slope	.201	.154	.183	.152
Intercept	.101	.100	.146	.105
SE of Estimate	.059	.069	.069	.041
DSLET TOTAL JUMP				
ED50	19.5	33.9	8.7	>50
Slope	.522	.583	.464	.273
Intercept	.243	.020	.381	.347
SE of Estimate	.150	.157	.125	.081

-NOTE: The ED50 is defined as that minimal agonist dose which elicits a 100% rise in baseline tail-flick latencies or a 50 % rise in baseline jump thresholds for peak (15 mnin) or for total (the 60 min time course) effects.

same time.

Male rats displayed significantly greater magnitudes of peak and total analgesia on the tail-flick test following central administration of the mu-selective agonist, DAMGO. This effect appeared similar to the significantly greater magnitude of analgesia following central and systemic morphine (Badillo-Martinez, et al., 1984; Kavaliers and Innis, 1987), and both opioid and non-opioid forms of swim analgesia (Romero and Bodnar, 1986). Following central administration of DAMGO, male rats displayed peak analgesic effects on the tail-flick test that approached or reached cut-off values at the 10 ug dose. Comparable to the effects of central morphine analgesia, peak DAMGO analgesia on the tail-flick test asymptoted about 4 sec over baseline values in female rats, and maintained this level at doses as high as 20 ug of DAMGO. The duration of analgesia following the effective 10 ug dose of DAMGO also declined more quickly in female rats on the tail-flick test. In contrast to the marked gender differences in analgesia on the jump test following central morphine administration, DAMGO analgesia on the jump test failed to exhibit any gender differences. Failure to find gender differences on the jump test could be due to smaller analgesic effect following DAMGO in males relative to central morphine, and/or to a greater analgesic effect following DAMGO in females relative to central morphine. Male rats displayed similar magnitudes of analgesia on the jump test

(approximately 0.3 mA increase over vehicle values) following the 10 ug dose of DAMGO and the 5 ug dose of morphine. In contrast, female rats displayed a greater magnitude of analgesia (0.3 mA above vehicle values) following DAMGO (10 ug) than following morphine (5-40 ug: 0.1-0.2 mA above baseline values). Thus the ability of DAMGO to induce greater analgesia in females may account for failure to observe gender differences on the jump test. Gonadectomy failed to consistently alter DAMGO analgesia on either nociceptive measure relative to same-sex controls; this is consistent with findings observed for central morphine analgesia, but differs from the gonadectomy-sensitive analgesic effects following systemic morphine (Chatterjee, et al., 1982), as well as following opioid and non-opioid forms of swim analgesia (Romero, et al., 1987; Wong, 1987). Gonadectomy-induced differences in analgesic effect following environmental stressors may represent gonadal manipulation of the stress response as well as the pain-inhibitory response.

Another striking finding was the failure to observe consistent gender or gonadectomy effects following central administration of the delta-selective agonist, DSLET. Gender-selective or gonadectomy-selective effects following DSLET analgesia failed to occur consistently across doses or across nociceptive measures providing additional evidence that test-specific and agonist differences need to be addressed in considering the role of gender differences in different forms

of analgesia. Test-specific effects may reflect differential mediation of each pain test at various levels of the neuraxis. Additionally, the pain tests measure responsivity to different noxious stimuli, as the tail-flick test measures sensitivity to thermal stimulation and the jump test measures sensitivity to shock. The spinal mediation of the tail-flick test has been confirmed anatomically (Grossman, et.al., 1982) through the tracing of afferent and efferent connections. Further, spinally transected rats are capable of displaying this nociceptive reflex (Hayes, et al., 1978). Additional studies of supraspinal and spinal opiate effects confirm a multiplicative interaction as measured by the tail-flick test (Yeung and Rudy, 1980). In contrast, the jump test is mediated only by supraspinal mechanisms (Brodal, 1981; Evans, 1961). DAMGO displays differential analgesic actions as a function of the site of injection. Naloxonazine, a selective and irreversible antagonist of the mu1 binding site (Hahn, et al., 1982) significantly attenuates DAMGO analgesia following intracerebroventricular, but not intrathecal injection (Heyman, et al., 1988; Paul, et al., 1989). In contrast, the irreversible mu antagonist, beta-funaltrexamine (Portoghese, et al., 1980; Takemori, et al., 1981), but not the delta antagonist, ICI 174864, blocked intrathecal DAMGO analgesia, indicating a role for the mu2 binding site in this form of analgesia (Paul, et al., 1989).

If the gender-specific actions of DAMGO are mediated by

mu2 receptors, gender differences may exist in this form of binding assay. In a study done in collaboration with our lab (Kepler, et al., 1991) mu1, mu2, and delta binding assays were performed. Mu1 binding was assessed using 3[H]DADL upon either hypothalamic or cortical membrane homogenates in the presence of the highly-specific delta agonist, DPDPE. Saturation studies were carried out with 0.7-1.7 nM of 3[H]DADL for cortical tissue. Mu2 binding was assessed using 3[H]DAMGO upon either hypothalamic or cortical membrane homogenates in the presence of DSLET. DAMGO binds to both mu1 and mu2 sites, while DSLET binds to mu1 and delta sites. Saturation studies included 0.05-2.5 nM of 3[H]DAMGO for cortical tissue. 3[H]DPDPE binding was performed using either hypothalamic tissue or cortical homogenates to determine delta binding. The steroid-rich hypothalamus as well as the cortex failed to exhibit gender-selective differences in binding in either mu1, mu2, or delta assays. It is possible, however, that another site or sites may display gender differences at the receptor level, or that gender differences may be expressed in terms of modulation of endogenous opioid levels. Steroid modulation of endogenous opioids has been previously discussed in the discussion of Experiment 1. Whatever the mechanism of action by which gender differentially alters central opioid analgesia, it is increasingly clear that gender effects may represent fundamental differences in organismic responses to nociceptive stimuli as well as in the gender-

selective ability to promote and maintain an analgesic response.

General Discussion

The results of these experiments indicate that central administration of morphine produced striking gender differences in analgesia such that sham-operated male rats displayed significantly greater magnitudes of peak and total analgesia than sham-operated female rats on both nociceptive measures. This striking effect was reflected both in terms of magnitude and ED50; while males displayed a near-maximal analgesia at 5 ug dose of morphine, female rats displayed only moderate analgesia at doses as high as 40 ug. In comparison, intracerebroventricular administration of the mu-selective agonist, DAMGO, produced significantly greater magnitudes of peak and total analgesia in sham-operated males than sham-operated females on the spinally mediated tail-flick test, but failed to significantly alter jump thresholds. In contrast, gender differences were not observed following central administration of the delta-selective agonist, DSLET, on either of the nociceptive measures.

Gonadectomy effects following central administration of morphine revealed that castration produced small, but significant reductions in the magnitude, but not in the potency, of morphine analgesia. Gonadectomy failed to consistently affect either DAMGO or DSLET analgesia. Although female rats in either proestrous or estrous displayed significantly greater magnitudes of analgesia than ovariectomized rats or rats in a combined met/diestrous phase

at some doses, the potency of central morphine analgesia was not significantly altered as a function of estrous or ovariectomy.

The observed gender differences following intracerebroventricular morphine and to a lesser degree, intracerebroventricular DAMGO, a mu-selective agonist, are consistent with gender differences observed following systemic morphine analgesia (Badillo-Martinez, et al., 1984), both opioid and non-opioid mediated forms of stress-induced analgesia (Romero and Bodnar, 1989), and systemic administration of the muscarinic cholinergic agonist, pilocarpine, and the alpha2-noradrenergic agonist, clonidine (Kiefel, et al., 1989). Stress-induced analgesia and systemic route of drug administration appear to be susceptible to differences in body weight, metabolism, absorption and other pharmacokinetic effects. The observed gender effects following a central route of administration suggest a component of gonadal steroid-opioid interaction which is potentially centrally mediated.

The lack of significant gonadectomy effects following central morphine, DAMGO, DSLET analgesia contrasts with the reduction in opioid and non-opioid stress-induced analgesia following adult gonadectomy, suggesting that gonadal steroids play an activational role to modulate antinociception following environmental stressors. In contrast, analgesic effects following central opiate analgesia may be at least

partially modulated by early organizational gonadal hormone effects. Post-natal morphine treatment significantly increased the magnitude of morphine analgesia on both the tail-flick and jump tests in females, and significantly decreased the magnitude of morphine analgesia on both tests in males (Arjune and Bodnar, 1989) indicating that early post-natal morphine treatment exerts selective effects upon analgesic responses which are gender sensitive and which occur in the absence of disruption of other developmental processes. As evidence for activational influence of gonadal hormones on stress-induced analgesia, adult gonadectomy decreases both opioid and non-opioid forms of swim analgesia relative to same sex controls, such that castrated male rats demonstrated analgesic magnitude similar to intact females. Steroid replacement therapy with testosterone reversed these deficits (Romero, et al., 1986, 1987, 1988). The male gonadal steroid, testosterone, is hypothesized to be responsible for the increased analgesia observed in males as systemic morphine analgesia is reduced following castration (Chatterjee, et al., 1982; LaBella, 1975), and testosterone has been found to reinstate analgesic potency following systemic morphine in both gonadectomized male and female rats (Banerjee, et al., 1983). These gonadal effects may be partially mediated peripherally with modulation occurring at the hypophysial-adrenal axis since manipulations which alter this system affect various forms of stress-induced analgesia (Bodnar,

1986). Stress-induced analgesic manipulations are known to activate many neuroendocrine and behavioral systems, while pharmacologically induced central analgesia may activate a smaller subset of neuroregulatory systems.

Estrous phase appears to be capable of altering some, but not all forms of analgesia (Romero, et al., 1986; Banerjee, et al., 1983). The lack of significant estrus effects following central morphine administration contrasts with previous findings that circulating gonadal hormones modulate systemic morphine analgesia with greatest sensitivity during late diestrous (Banerjee, et al., 1983). Due to several methodological differences, it is difficult to compare our results with the previous study.

Perhaps the most striking finding that emerged from this series of experiments was the marked sensitivity of central morphine analgesia to gender differences relative to the insensitivity of DAMGO and DSLET analgesia to gender differences. Two potential explanations for the differential effects include: 1) differential receptor activity for the different receptor agonists and/or 2) differential metabolic breakdown and clearance rates for the different pharmacological agents with the latter hypothesis being the most viable option.

Mu receptors can be divided into two distinct subtypes: mu1 receptors which bind both opiates and most enkephalins with similar high affinity; and mu2 receptors which bind

morphine preferentially (Pasternak and Wood, 1986). Although morphine is relatively specific for mu receptors, it does retain activity at other receptors as well (Pasternak and Wood, 1986; Zukin and Zukin, 1981). In contrast, DAMGO and DSLET each possess little specificity for other opioid receptor subtypes (Handa, et al., 1981; Itzhak and Pasternak, 1987). Therefore, the observed gender differences for central morphine analgesia might reflect activation of multiple opioid receptor subtypes. This possibility must be tempered by the observations that supraspinal morphine, DAMGO, and DSLET analgesia are each blocked by the mu1 receptor antagonist, naloxonazine (Bodnar, et al., 1988; Paul, et al., 1989), suggesting a common mechanism of action. It is theoretically possible however, that the gender differences observed for DAMGO analgesia may be acting upon its mu2 receptor actions, rather than its mu1 receptor actions. Additionally, the inability of DSLET to display gender differences provides support for this hypothesis. While DSLET is a delta-selective compound, it also displays affinity for the mu1 binding site (Itzhak and Pasternak, 1987). The actions of DSLET analgesia elicited following supraspinal microinjections into the periaqueductal gray and locus coeruleus are mediated by the mu1 binding site since naloxonazine blocks this form of analgesia (Bodnar, et al., 1988). In contrast, the actions of DSLET analgesia following intrathecal microinjections appear to be mediated through delta receptors given the

ineffectiveness of naloxonazine to alter this form of analgesia (Paul, et al., 1989). Potentially, similar mu2 actions of morphine and DAMGO may be the common link by which gender differences and test-specific differences may be explained, however, a common mu1 site of action for morphine, DAMGO, and DSLET has also been found.

Perhaps the most viable alternative explanation for the pronounced gender differences following centrally administered morphine analgesia is qualitative and/or quantitative differences in the metabolism and decay of exogenous opiates vs. endogenous opioids. Gender differences may occur in the rate of disposition or quantity of metabolites produced . Whereas DAMGO and DSLET are enkephalin derivatives subject to eventual degradation by aminopeptidases, carboxypeptidases and endopeptidases (Roques and Fournie-Zaluski, 1986), morphine is eventually metabolized into morphine-3Beta-glucuronide and morphine-6Beta-glucuronide. Morphine-6Beta-glucuronide, but not morphine-3Beta-glucuronide possesses potent supraspinal analgesic activity which is dependent upon the mu1 binding site for its expression (Abbott and Palmour, 1988; Pasternak, et al., 1988; Paul, et al., 1989). The rate or occurrence of each metabolite might be subject to gender differences via differential metabolism. Males may potentially metabolize morphine into a relatively large concentration of Morphine-6Beta-glucuronide and relatively small concentration of Morphine-3Beta-glucuronide. In contrast, females may

demonstrate an increased concentration of Morphine-3Beta-glucuronide and decreased concentration of Morphine-6Beta-glucuronide relative to males. An alternative explanation suggests that the rate of breakdown of the morphine metabolite, Morphine-6Beta-glucuronide, is significantly slower in males resulting in accumulation of the potent analgesic, Morphine-6Beta-glucuronide, for a longer amount of time relative to females. Notably, Hahn (1985) found gonadal steroids differentially influence metabolic transformation, N-demethylation of morphine, in rat liver and brain as the male rat liver was found to be more effective in N-demethylation than females. This difference in enzymatic activity was influenced by testosterone. In contrast, the inverse relationship existed in the brain as females demonstrated greater N-demethylation than males. As brains of castrated and intact male rats have similar morphine content, testosterone appears to exert a differential influence at the level of the rat liver and brain implicating that the enzyme responsible for biotransformation of morphine in the liver differs from the enzyme in the brain (Hahn, 1985). Additionally, differential enzyme-gonadal steroid interactions may partially account for gender differences following central morphine administration.

There are both evolutionary implications as well as clinical implications which can be derived from the results of this series of studies. From an evolutionary perspective,

increased analgesic magnitude and potency in the male of the species are consistent with increased aggressive behaviors in the male of the species which are modulated by gonadal hormones. One model of rodent aggression which combines the study of aggression and analgesia is the defeat analgesia paradigm. Defeat in an aggressive encounter produces analgesia with the degree of analgesia positively correlated with the intensity of the encounter (Miczek, et al., 1982). Central opioid mechanisms have been implicated in defeat analgesia as naloxone administered into the periaqueductal grey or arcuate nucleus reduced defeat analgesia (Miczek, et al., 1985). The arcuate nucleus and/or periaqueductal grey may be hypothesized as the site of gonadal steroid-opioid interaction relative to this form of analgesia. Defeat analgesia alters the number, but not affinity of mu binding sites and reduces the immunoreactivity of Beta-endorphin in the periaqueductal grey (Miczek, et al., 1986; Kulling, et al., 1988).

While males may display increased analgesia relative to females across a variety of behaviors designed to enhance preservation of the species, females may also demonstrate increased analgesic responses during behaviors designed to specifically facilitate procreation. Vaginocervical probing produces analgesia in rats and humans across a variety of pain measures (Whipple and Komisaruk, 1985; Komisaruk and Whipple, 1986), and may reflect an evolutionary mechanism to insure

copulation. Another form of analgesia related to procreation of the species is opioid analgesia induced by pregnancy and labor (Gintzler, 1980; Sander and Gintzler, 1986; Sander, et al., 1989). This analgesia is thought to be spinally mediated and dependent on the kappa receptor for its expression (Sander and Gintzler, 1989). Additionally, Gintzler and Bohan (1990) suggest that pain thresholds are elevated during pseudopregnancy, a condition which mimics the hormone concentration in the bloodstream without actual conception. These results provide evidence that critical levels of peripheral circulating gonadal steroids rather than phase of estrous may be modulators of endogenous opioid systems which regulate antinociception.

The most salient implication for clinical treatment of pain states derived from this series of studies is the additional evidence which supports the need for considering gender when dosing morphine, the prototypical analgesic. These results support earlier work in our laboratory which provided evidence for significant effects of both gender and age on the expression of a variety of analgesic manipulations (Kramer, et al., 1985; Kramer and Bodnar, 1986a; Kramer and Bodnar, 1986b; Romero and Bodnar, 1986; Romero, et al., 1987; Romero, et al., 1988). It is important to note, however, that it is difficult to translate gender differences in antinociception in the rat to the human chronic pain state. First, our data assessed analgesic efficacy, which is

different from pain reactivity, often the variable studied in human pain research. There are reports of differential responsivity to analgesics in women as compared to men (Classen and Netter, 1985). Second, animal studies rely on pain measures which assess the sensory discriminative aspects of pain, i.e., the use of punctate stimuli, the spinally mediated tail-flick and the supraspinally mediated jump test. These measures have been shown not to be influenced by learning as basal thresholds do not significantly decrease with repetition. The nature of these nociceptive stimuli are short acting and focused, admittedly different from the long-lasting and diffuse pain present in human chronic pain states such as cancer pain or musculoskeletal pain. In addition to a sensory discriminative aspect of pain, human pain states invariably elicit both a motivational/affective component and a cognitive/learned component (Melzack and Casey, 1972). Third, our model is a model of antinociception following acute pain. While animal models of chronic pain exist, subjecting animals to chronic pain is difficult to justify in this era of heightened sensitivity to research ethics, when valuable information can be derived first from a model of acute pain. Therefore, we must be conservative in providing implications for human chronic pain. However, pain management which incorporates organismic variables such as gender, gonadal status and age would be efficacious. Additionally, further elucidation of the modulatory role of gonadal steroids may

provide neuropharmacological alternatives.

There are several future research directions that may significantly contribute to elucidate further the relationship among gonadal steroids, endogenous opioids, and exogenous opiates. From a neuroanatomical perspective, microinjection mapping studies could potentially delineate the potential site or sites of action for gonadal-opioid modulation. Potential microinjection sites of direct central interaction could include periaqueductal grey, arcuate nucleus and amygdala, in addition to various sites known to support analgesia such as NRM, and NRGC. A comparison of spinal and supraspinal sites could potentially yield information about the site of action in addition to activation of different receptor subtypes. The use of a variety of agonists which bind with varying affinities to different receptor populations would potentially yield data with important clinical implications. The correlation of analgesic responding with data derived from binding assays may also provide additional lines of evidence and would combine different levels of analysis. The assessment of antinociceptive responses of animals with adult gonadectomy vs. perinatal gonadectomy would enable a comparison of activational and organizational effects of gonadal status. Lastly, studies to elucidate the potential gender differences in the metabolic breakdown of opioids and the clearance rates of exogenous compounds like morphine would provide an important component to understanding the complexity

of gonadal steroid-opioid interactions.

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