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Steady-State Methadone Effect on Generalized Arousal in Male and Female Mice

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Abstract

Methadone is widely used in treatment of short-acting opiate addiction. The on-off effects of opioids have been documented to have profound differences from steady-state opioids. We hypothesize that opioids play important roles in either generalized arousal (GA) or aversive state of arousal during opioid withdrawal. Both male and female C57BL6 mice received steady-state methadone (SSM) through osmotic pumps at 10 or 20 mg/kg/day and GA was measured in voluntary motor activity, sensory responsivity, and contextual fear conditioning. SSM did not have any effect on those GA behaviors in either sex. Females had higher activity and less fear conditioning than males. The effects of SSM on stress responsive orexin gene expression in the lateral hypothalamus (LH) and medial hypothalamus (MH, including perifornical and dorsomedial areas) were measured after the behavioral tests. Females showed significantly lower basal LH (but not MH) orexin mRNA levels than males. A panel of GA stressors increased LH orexin mRNA levels in females only; these increases were blunted by SSM at 20 mg/kg. In summary, SSM had no effect on GA behaviors. In females, SSM blunted the GA stress-induced LH orexin gene expression.

Keywords

arousal; methadone; gender; orexin; contextual fear

1. INTRODUCTION

Recent evidence has begun to link mechanisms of reward and addiction with changes in mechanisms governing CNS arousal and importantly also changes in orexin neurons that may be crucial for arousal. For example, Harris et al. demonstrated activation of Fos-immunoreactivity in orexin neurons due to experimental manipulations related to reward (Harris et al., 2005). Recent work from one of our laboratories showed increased orexin mRNA levels during withdrawal from chronic morphine, thus linking arousal to the aversive effect of addiction (Zhou et al., 2006). In the present study, we examined potential relations

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between methadone (a pharmacotherapy for opioid addiction) and any possible changes in arousal in both females and males by using a novel assay of CNS generalized arousal (GA) in mice during steady-state methadone administration by pumps. We have developed this novel assay in mice which measures (i) sensory responsiveness to olfactory, tactile and vestibular stimuli; (ii) horizontal, vertical and ambulatory motor activity in the home cage daily throughout the entire experiment; and (iii) contextual fear conditioning.

Important mechanisms affecting behavioral arousal involve orexins (Easton et al., 2006), which are produced exclusively in the lateral hypothalamus (LH), perifornical area (PFA) and dorsomedial hypothalamus (PFA-DMH) (de Lecea et al., 1998; Sakurai et al., 1998) with extensive CNS projections. They have been implicated in the regulation of sleep-wakefulness, feeding, neuroendocrine and autonomic functions (Ferguson & Samson, 2003; Sakurai, 2002; Saper et al., 2005; Winsky-Sommerer et al., 2005). Recent studies showing orexins' involvement in reward processing and addiction have emerged. For example, animal behavioral studies suggest that the interaction between orexins and their receptors may contribute to motivated behaviors induced by morphine, cocaine or food (Borgland et al., 2006; Boutrel et al., 2005; Harris et al., 2005). In the present study, therefore, we also determined whether a panel of GA stressors with or without steady-state methadone would affect orexin mRNA expression in the LH or medial hypothalamus (including PFA-DMH) of both male and female mice.

Pro-opiomelanocortin (POMC)-derived beta-endorphin exerts inhibitory effects on the hypothalamic-pituitary-adrenal (HPA) axis in both humans (Schluger et al., 1998; Volavka et al., 1979) and rodents (e.g., Eisenberg, 1980; Nikolarakis et al., 1987; Zhou et al., 2005). Beta-endorphin immunoreactive (ir) fibers and corticotropin-releasing hormone (CRH)-ir perikarya are colocalized in the paraventricular nucleus of the hypothalamus (e.g., Pilcher & Joseph, 1984). To exert a tonic inhibition on CRH neuronal activity, it has been suggested that beta-endorphin acts primarily at the μ -opioid receptor (see recent review Koob & Kreek, 2007). In the present study, therefore, we use methadone (a μ -opioid receptor agonist), which in humans is long-acting and widely used in treatment of short-acting opiate (primarily heroin) addiction (Dole et al., 1966). In the present mouse study, due to methadone's short half-life in rodents (Ling et al., 1981), methadone was delivered through osmotic pumps to mimic steady-state methadone maintenance in humans (Inturrisi & Verebely, 1972; Kreek, 1973b). We examined the effects of steady-state methadone on the HPA axis and on orexin and POMC mRNA levels, while comparing females and males. Primarily, we were testing the hypothesis that steady-state methadone (SSM) would not significantly affect arousal in both sexes.

2. MATERIALS AND METHODS

2.1. Animals

A total of 58 male and 58 female intact C57BL6 mice from Taconic Farms (Germantown, NY) were studied in these experiments. At the start of experiments, mice were 12 weeks of age and mean body weights were 25 ± 0.5 g for females and 27 ± 0.5 g for males. Mice were housed individually and were maintained on a 12:12 light/dark cycle with lights on at 18:00. All animals had food and water available *ad libitum* and were cared for in accordance with a protocol approved by the Rockefeller University Institutional Animal Care and Use Committee.

2.2. Treatment and experimental design

After seven days of adjustment to our facility, osmotic minipumps (Alzet model 2ML2, 0.5 μ l per hour for 14 days, Durect Corporation, Cupertino, CA) were implanted subcutaneously

in the nape of the neck of the animals under isoflurane sodium anesthesia. The minipumps were filled with saline or methadone, 100 or 200 mg/ml dissolved in saline. The contents were delivered at a constant rate of 1 μ l/h resulting in a dose of methadone of 0, 10 or 20 mg/kg/day.

Half of the animals completed the behavioral testing protocol, conducted as follows: home cage motor activity, sensory testing and contextual fear conditioning. Sensory testing occurred during the light phase of the light/dark cycle, contextual fear conditioning was conducted during the dark phase of light/dark cycle and home cage activity was measured throughout a 24-h period. In home cage, motor activity was monitored for 10 days. At the end of the 10th day, during the light phase of light/dark cycle, mice were given only one sensory test. After the sensory test animals were tested for contextual fear conditioning on the 11th, 12th and 13th days. In order to assess neurochemical and hormonal changes associated with GA and SSM, all mice were briefly exposed to CO₂ (15 sec) and sacrificed by decapitation at 14:00 on the 14th day (one day after the last behavioral testing).

Half of the animals serving as controls were implanted with pumps at 0 or 20 mg/kg/day methadone without the behavioral testing. At 14:00 hours on the 14th day (the identical time point as in the behavioral groups), all mice were sacrificed for neurochemical and hormonal assays.

2.3. Generalized Arousal (GA) Assay

2.3.1. Home Cage Motor Activity—After receiving osmotic pump implants, mice were transferred into a computer-regulated behavior monitoring system (Accuscan Instruments, Trabue, OH). Each animal's cage was surrounded with a set of infrared photobeams. Disruption of a beam was recorded as an activity count. Data was collected with a PC using Versamax software (Accuscan Instruments). We allowed mice to acclimate to this environment for up to 5 days and data for analyses was taken from the last 5 days of total of 10 days observed for home cage motor activity. During motor activity testing, total distance traveled (TD), horizontal activity (HA) and vertical activity (VA) were measured.

2.3.2. Sensory testing—On the last day of home cage motor activity monitoring, mice were exposed to a series of three types of sensory stimuli in the following order: olfactory, tactile and vestibular. All stimuli were presented when mice were in a “resting state”, which was when there was no home cage activity detected by the computer (i.e., zero TD, HA or VA) for at least 5 min. During the home cage activity assay, disruption of a beam was recorded and data collected for TD, HA and VA. The stimuli consisted of brief changes in sensory input to the mouse. First, an olfactory stimulus of the odor from 100% benzaldehyde (Sigma, St. Louis, MO) was applied for a 20-sec duration. The change in the mouse's home cage activity was measured until the animal reached a resting state again. Second, the tactile stimulus consisted of a 2-sec air puff supplied by a compressed air can (Ernest Fullam, Latham, NY) at a distance of 10cm. Third, the vestibular stimulus consisted of moving the cage in a circular motion about its vertical axis on an orbital shaker (Barnstead International, Dubuque, IA) for an 8-sec period at 90 rpm.

2.3.3. Contextual fear conditioning—In order to evaluate the effect of steady-state methadone administration on GA, the last test was a classical contextual fear-conditioning paradigm, which consisted of one introductory, one training and one testing session. Each step was done in this order on three different days. Both the training and testing occurred during the dark phase of the light/dark cycle. On the first day, each mouse was introduced to the conditioning chamber, a sound-attenuating, transparent Plexiglas chamber equipped with a metal shock grid floor. The mouse was allowed to explore it for 5 min, then placed back

into its home cage and returned to the colony room. Between each testing, the chamber was cleaned with 70% ethanol. Twenty-four hours later, each mouse was placed in the same conditioning chamber for 3 min and then received a series of three foot shocks (0.5mA for 1 sec), unconditioned stimuli. Training and testing of all mice occurred in a sound-attenuating cabinet in an isolated testing room. On the last day, each animal was placed back into the same chamber and its behavior was recorded for a 5-min period. The freezing behavior (absence of all movement) was scored in 5-sec bins.

2.4. Neurochemical Assays

2.4.1. Preparation of RNA extracts—Each brain was removed from the skull and placed in a chilled mouse brain matrix (ASI Instruments, Houston, Texas). Coronal slices containing the brain regions of interest were removed from the matrix and placed on a chilled petri dish. Dissection was carried out under a dissecting microscope using razor blades and forceps. The brain regions of interest were identified according to the Mouse Brain in Stereotaxic Coordinates (Franklin & Paxinos, 1997). Three brain and pituitary regions, including the anterior pituitary, lateral hypothalamus (LH), and medial portion of the hypothalamus (MH, including the PFA, DMH, PVN and arcuate nucleus) were dissected on ice, homogenized in guanidinium thiocyanate buffer and extracted with acidic phenol and chloroform as previously described (Chomczynski & Sacchi, 1987). After the final ethanol precipitation step, each extract was resuspended in diethylpyrocarbonate (DEPC) -treated H₂O and stored at -80 °C.

2.4.2. Plasmids—A 531 bp fragment from the rat hypocretin (or orexin) cDNA (GenBank accession number AF019565, from 38 to 569 nucleotide) was cloned into the polylinker region of pBC SK+ (Stratagene, La Jolla, CA). A 476 bp fragment from the rat pro-opiomelanocortin (POMC) cDNA (GenBank accession number J00759, from 258 to 734 nucleotide) was cloned into the polylinker region of pSP64 or SP65 in both the sense and antisense orientations. The plasmid pS/E (a pSP65 derivative) was used to synthesize riboprobe for the 18S rRNA for determination of total RNA. ³³P-labeled cRNA antisense probes and unlabeled cRNA sense standards were synthesized using an SP6, T3 or T7 transcription system. A denaturing agarose gel containing 1.0 M formaldehyde showed that a single full-length transcript had been synthesized from each plasmid (this method was validated earlier [Zhou *et al*, 2006]).

2.4.3. Solution hybridization ribonuclease (RNase) protection-trichloroacetic acid (TCA) precipitation assay—The solution hybridization RNase protection-TCA precipitation protocol has been described in detail in earlier reports (Branch *et al.*, 1992; Zhou *et al.*, 1996). RNA extracts were dried in 1.5 ml Eppendorf tubes and resuspended in 30 µl of 2 × TESS (10 mM N-Tris[hydroxy-methyl]methyl-2-aminoethane sulfonic acid, pH 7.4; 10 mM ethylenediaminetetraacetic acid [EDTA]; 0.3 M NaCl; 0.5% sodium dodecyl sulfate [SDS]) that contained 150,000 to 300,000 cpm of a probe. Samples were covered with mineral oil and hybridized overnight at 75 °C. For RNase treatment, 250 µl of a buffer that contained 0.3 M NaCl; 5 mM EDTA; 10 mM Tris-HCl (pH 7.5), 40 µg/ml RNase A (Worthington Biochemicals, Lakewood, NJ) and 2 µg/ml RNase T1 (Calbiochem, San Diego, CA) were added and each sample was incubated at 30 °C for 1 hour. TCA precipitation was effected by the addition of 1 ml of a solution that contained 5% TCA and 0.75% sodium pyrophosphate. Precipitates were collected onto a filter in sets of 24 using a cell harvester (Brandel, Gaithersburg, MD) and were measured in a scintillation counter with liquid scintillant (Beckman Instruments, Palo Alto, CA).

The procedure to measure mRNA levels involved comparison of values obtained from experimental samples (brain extracts) to those obtained for a set of calibration standards.

The calibration standards had known amounts of an *in vitro* sense transcript, the concentration of which was determined by optical absorbance at 260 nm. The set of calibration standards included those with no added sense transcript and those that contained between 1.25 and 80 pg of the sense transcript. To determine the total picograms of each mRNA in each extract, the amounts calculated from the standard curves were multiplied by 2.3 for POMC or 1.3 for orexin to correct for the difference in length between the sense transcript (476 or 531 bases for the POMC or orexin respectively) and the full-length mRNA (1.1 or 0.7 k base for the POMC or orexin). A new standard curve was generated each time experimental samples were analyzed and all extracts of a particular tissue were assayed for each mRNA as a group in a single assay.

Total cellular RNA concentrations were measured by hybridization of diluted extracts to a ^{33}P -labeled probe complementary to 18S rRNA at 75 °C. The calibration standards for this curve contained 10 µg of *E. coli* tRNA plus either 0.0, or from 2.5 to 40 ng of total RNA from rat brain, the concentration of which was determined by optical absorbance at 260 nm.

2.4.4. Radioimmunoassays—At the time of decapitation, blood from each mouse was collected in tubes, placed on ice, and spun in a refrigerated centrifuge. Plasma was separated and stored at - 40 °C for later hormonal measurement by radioimmunoassay. Corticosterone levels were assayed using a mouse corticosterone ^{125}I kit from MP Biomedicals (Costa Mesa, CA). Plasma estrogen concentration in female mice was measured using Estradiol EIA kit (Cayman Chemical, Ann Arbor, MI). All values were determined in duplicate in a single assay.

2.5. Drugs

Methadone HCL was dissolved in physiological saline. Animals were randomly assigned to three different methadone groups (n=10-12) and implanted with osmotic minipumps. In humans, a dose of 100 mg/day results in an average daily plasma concentration of about 240 ng/ml (Kreek, 1973a, b, 1979). In rats, it has been demonstrated that 10 mg/kg/day results in a mean plasma level of 123 ng/ml (range: 100-150 ng/ml) (Zhou et al., 1996). Thus, in this mouse experiment, we used methadone doses (i.e., 10-20 mg/kg/day) corresponding to typical therapeutic ranges (Dole, 1988).

2.6. Data analysis and statistics

Data were analyzed using “STATISTICA” (StatSoft, Inc. Tulsa, OK), and graphs were prepared using “GraphPad PRISM” (GraphPad Software, Inc. San Diego, CA) software. For behavioral data, two-way analyses of variance (ANOVA) [gender: male, female; and methadone: saline control, 10mg/kg SSM, 20mg/kg SSM] were used, followed by planned comparisons. To evaluate differences in gene expression, three-way ANOVA [gender: male, female; methadone: saline control, SSM; and stress: non-stress control, GA stress] were used followed by Newman-Keuls *post hoc* tests or planned comparisons when appropriate. The accepted level of significance was $p < 0.05$.

3. RESULTS

3.1. Sensory test

During the sensory test, mice were subjected to three types of stimuli: tactile, vestibular and olfactory. Horizontal activity (HA), total distance traveled (TD) and vertical activity (VA) were measured during each test. Data for behavioral analyses were collected from the moment when stimuli were delivered until the mouse went back to the resting state (approximately 10min). During data analysis, the data from one mouse (male at 10mg/kg SSM) were removed because its result was more than three standard deviations above the

mean of all mice. In this sensory test, there was no difference between treatments or across sexes, except when a tactile stimulus was presented. In response to tactile stimuli there was a major gender difference across all behavioral measurements taken: TD [$F(1, 60)=10.08$, $p<0.001$, Figure 1a], HA [$F(1, 60)=8.67$, $p<0.005$, Figure 1b] and VA [$F(1, 60)=9.18$, $p<0.005$, Figure 1c]. Further evaluation of SSM effect on gender differences showed that, only at the 20mg/kg dose of SSM, females were significantly more sensitive to tactile stimuli than males: TD [$F(1, 60)=7.3$, $p<0.01$, Figure 1a], HA [$F(1, 60)=8.7$, $p<0.005$, Figure 1b] and VA [$F(1, 60)=5.6$, $p<0.01$, Figure 1c].

3.2. Home cage motor activity

Two-way ANOVA showed a significant gender effect in home cage motor activity: TD [$F(1,62)=11.97$, $p<0.001$, Figure 2a], HA [$F(1,62)=20.28$, $p<0.0001$, Figure 2b] and VA [$F(1,62)=14.84$, $p<0.0001$, Figure 2c]. The results were further analyzed to evaluate gender differences due to different doses of SSM. At the highest dose only (20mg/kg), home cage motor activity was significantly greater in females than males (planned comparisons): TD [$F(1,62)=11.2$, $p<0.001$, Figure 2a], HA [$F(1,62)=26.3$, $p<0.0001$, Figure 2b] and VA [$F(1,62)=9.9$, $p<0.001$, Figure 2c].

3.3. Contextual fear conditioning

In line with sensory and motor activity tests, contextual fear conditioning also showed a statistically significant gender effect [two-way ANOVA, $F(1, 59)=7.9$, $p<0.005$, Figure 3] in freezing behavior, which was clear at all doses. There was an apparently greater response in males at the 20mg/kg dose, but this failed to be significant [$F(1, 59)=3.2$, $p=0.076$].

3.4. Effects of GA stress with SSM at 20 mg/kg/day on orexin mRNA levels in the LH and MH

In the LH, three-way ANOVA showed a significant Gender $\times$ SSM $\times$ Stress interaction [$F(1,51) = 4.60$, $p < 0.05$], with a marginally significant main effect of Stress [$F(1,51) = 2.92$, $p = 0.08$] (Figure 4). In males (Figure 4A), the GA stress alone or in combination with SSM had no effect on orexin mRNA levels. In females (Figure 4B), orexin mRNA levels after GA stress were significantly higher than controls (Newman-Keuls *post hoc* tests, $p < 0.05$). In females, compared to GA stress alone, there was a significantly lower orexin mRNA levels after GA stress with 20 mg/kg/day SSM [planned comparison, $F(1,51) = 4.11$, $p < 0.05$].

Since our recent study showed a decreased contextual fear conditioning after orexin cell deletion in the mouse LH (Easton et al. 2006), and in the present study, control female mice (SSM at 0 mg/kg) displayed less contextual fear conditioning than control male mice (see above [Section 3.3.](#)), a planned comparison was carried out to examine the gender difference in basal orexin mRNA level in control animals. We found a significantly lower orexin mRNA level in the LH of the females [$F(1,51) = 6.90$, $p < 0.05$] (Figure 4A and 4B).

In the MH, the GA stress alone or in combination with SSM had no effect on orexin mRNA levels in this region in either gender (Table 1).

3.5. Effects of GA stress with SSM at 20 mg/kg/day on POMC mRNA levels in the medial hypothalamus

The GA stress alone or in combination with SSM had no effect on POMC mRNA levels in this region, in either gender (Table 1).

3.6. Effects of GA stress with SSM at 20 mg/kg/day on POMC mRNA levels in the anterior pituitary

Three-way ANOVA showed a significant main effect of SSM [$F(1,79) = 4.57$, $p < 0.05$] (Figure 5). In males, the GA stress alone or in combination with SSM had no effect on anterior pituitary POMC mRNA levels. In females, POMC mRNA levels after GA stress showed an apparent, but not statistically significant, increase (Newman-Keuls *post hoc* tests, $p=0.074$). In females, compared to GA stress alone, there was a significantly lower POMC mRNA level after GA stress with 20 mg/kg/day SSM [planned comparison, $F(1,79) = 9.39$, $p < 0.005$].

3.7. Effects of GA stress with SSM at 20 mg/kg/day on plasma corticosterone levels in female or male mice

The GA stress alone or in combination with SSM had no effect on plasma corticosterone levels in either gender (Table 1).

3.8. Effects of GA stress with SSM on plasma estrogen levels in female mice

All three groups tested in the GA paradigm maintained similar estrogen levels in plasma; in the control group (0 mg/kg methadone, $n=8$), the average amount detected was 47.1 ± 9.2 pg/ml, at the 10mg/kg dose of SSM 44.3 ± 12.0 pg/ml ($n=7$) and at the 20mg/kg dose of SSM 46.1 ± 18.0 pg/ml ($n=7$).

4. DISCUSSION

Arousal or the activation of brain and behavior occurs first in any chain of behavioral responses. Thus, its alterations can be causal to later behavioral alterations. Generalized arousal (GA) is significant medically in that resultant disorders in humans may be disastrous, ranging from conditions in which it is blunted, such as comatose, vegetative, and fatigue states, through other conditions in which it is exaggerated, such as attention deficit disorders, and also problems with vigilance and mood. We have proposed an operational definition of GA that is precise and complete and leads to quantitative, physical measures (Pfaff, 2005). This operational definition states that a more aroused animal or human (higher GA) (i) is more responsive to stimuli in all sensory modalities; (ii) emits more voluntary motor activity; and (iii) is more emotionally reactive.

An objective of the current study was to compare males and females in measures of GA. A surprising result was that males showed greater contextual fear responsiveness than females. While of no known medical relevance, this result may have some ethological significance. Male mice are well known to show much higher levels of aggression than females and thus to suffer noxious stimulation and wounding. Ordinarily, the pain would interfere with ongoing aggressive behavior. Although long-term occupation of μ -opioid receptors by an agonist might be speculated to dampen the effects of these insults on the male animal's subsequent aggressive behavior, thus allowing the male to maintain high levels of emotional responsivity, including fear, no alteration of the level of fear responsivity was found in this study with the steady-state methadone doses used.

In females but not males, stress of GA testing led to significant increases in orexin mRNA in the LH. It was interesting that in such females methadone suppressed that effect on orexin mRNA. Thus, our current data are in line with a large literature showing greater effects of stress in female rodents, and may also be related to the Easton et al. (2006) findings quoted below, in which females were used to show the importance of orexin neurons in GA.

The current study showed that there were significant male/female differences in several aspects of the GA assay results. Whenever there was a sex difference, females were greater than males in measures of motor activity or sensory responsiveness, never the reverse. In terms of expectations from reasoning about biologically adaptive animal behavior, since female mice spend a remarkably high percentage of their adult lives either pregnant or defending a nest full of pups, a greater sensitivity to unexpected stimuli might be understandable in the light of these biological responsibilities.

One of the most interesting questions of these experiments was to investigate the effects of GA alone or with SSM on two arousal/stress responsive systems in both males and females: the orexin in the LH and the HPA axis. In the females, SSM alone did not alter orexin mRNA levels in the LH. In contrast, we found that GA was associated with an increase in the orexin mRNA levels, which were found to be nearly two times higher than levels observed in controls. The increase was found primarily in the LH, whereas that in PFA-DMH was not affected, suggesting that LH orexin gene expression is involved in GA stress in a region-specific manner. This effect was also prevented by SSM; we noted no significant increases in orexin mRNA levels in the female animals after GA with SSM. Furthermore, this effect was gender-specific, since we found no effect of GA in males, who showed higher basal orexin mRNA levels in the LH than females. The increase in the LH orexin mRNA levels during GA suggests an enhanced orexin biosynthesis, though it cannot be determined from assays of mRNA levels alone which steps (orexin gene transcription, processing, and/or degradation of mRNA) are affected. Although the stimulatory factors influencing elevation of orexin mRNA levels are not yet fully elucidated, it is possible that an increased orexin release is responsible for the increase in orexin mRNA to compensate for GA-induced peptide depletion.

Recent work from one of our laboratories has demonstrated a decreased fear conditioning after orexin cell deletion in the LH of female mice, providing the first evidence of an involvement of the LH orexin in GA (Easton et al., 2006). It has also been found that aversive and arousal states during stress or in acute withdrawal from chronic morphine are associated with an increase in orexinergic activity and LH orexin gene expression in male rats (Boutrel et al., 2005; Zhou et al., 2006). In line with these findings, we found in this study reported here that female mice, showing lower basal orexin mRNA levels in the LH (with no difference in the MH), tended to have lower levels of fear conditioning than males. We further found that in females, SSM was able to completely block the orexin mRNA increase induced by GA, suggesting that the GA-induced stimulation of LH orexin gene expression is prevented by constant μ -opioid receptor occupation. In the LH, around 50% of orexin neurons express μ -opioid receptor (Georgescu et al., 2003). This observed functional interaction between the opioidergic and orexinergic systems may be fundamental to arousal-induced behavioral consequences, like fear conditioning.

Our findings of gender differences in basal level of and in responses of the orexin gene expression to GA stressors and steady-state methadone are in line with the growing body of evidence that males and females respond differently to stress or exogenous pharmacological agents, including methadone. In rats, gender differences have been shown to be involved in regulation of pain (Islam et al., 1993; Loyd & Murphy, 2006). In humans, gender differences have been found to be involved in cardiovascular and corticoadrenal response to both stress and drug cues in cocaine dependent individuals studied in laboratory setting (Fox et al., 2006). In rodents, studies have suggested that estrogen may be involved in the regulation of the opioid systems: (1) there was an increase in μ -opioid receptor mRNA levels in the arcuate nucleus and ventromedial nucleus of the hypothalamus of gonadectomized female rats after 48 h of estradiol injection (10 ug) (Quinones-Jenab et al., 1997); (2) an increase in DAMGO-stimulated GTP γ S binding was found in the medial preoptic area and caudate

putamen of gonadectomized female rats after 2 days of estradiol injections (2 ug/injection) (Acosta-Martinez & Etgen, 2002); and (3) estradiol capsules (180 ug, sc) for 8 days decreased POMC mRNA levels in the arcuate nucleus of young (3-4 months) gonadectomized female rats (Weiland et al., 1992). A potential mechanism by which GA stressors change orexin mRNA levels in female mice may involve changes in the plasma levels of the ovarian hormones, which could be interesting for future study. The effects of estrogen, which regulate many cellular and biological functions, are now thought to be primarily mediated through its nucleus receptors, and the targets of its action may include orexins and their receptors (Russell et al., 2001; Johren et al., 2003; Porkka-Heiskanen et al., 2004). It is also well known that the activation of estrogen receptors which acts as liganddependent transcription factors, generally modulates transcription of target genes by stabilizing transcription protein complexes (see review by Pfaff et al. in 2002). Thus, the alterations in plasma estrogen levels during the GA stress, in turn, could conceivably transform the estrogen receptors to bind to steroid response elements present in the orexin or its receptor genes to modulate their expression. Future work is needed to identify whether estrogen plays a critical role in the effects of steady-state methadone on the orexin and POMC gene expression induced by the GA stress in mice, using gonadectomized female with hormone replacement.

In former heroin addicts, the responsivity of the HPA axis is normalized in steady-state chronic methadone-maintained patients: their HPA axis responses to metyrapone-induced stress are no different from those of healthy volunteer subjects (Kreek et al., 1984; Schluger et al., 2001). Our previous rat studies using methadone pump infusion confirmed that steady-state methadone does not alter HPA responsivity in rodent models (Zhou et al., 1996; Leri et al., 2006). In the present study, plasma corticosterone levels did not show difference from controls in either males or females after SSM. In females after GA stress, there was an apparent, but not statistically significant, increase in POMC mRNA levels after GA stress in the anterior pituitary, which was completely prevented by SSM. These results further suggest that SSM may modulate corticotrope responsivity to hypothalamic CRF and/or arginine vasopressin input through the reduction of pituitary POMC gene expression.

In summary, the current paper can be considered in the light of medical experience with patients. In several respects these results confirm previous studies in revealing some of the ways in which methadone can be viewed as a safe medication. SSM did not disturb the sensory responsiveness and motor activity assay components in our GA assay. GA stress increased LH orexin mRNA levels in females only; these increases were blunted by SSM. Orexin mRNA data suggest that possible gender differences may be important variables in the effect of methadone. The mechanism of this orexin effect is unclear; future research is needed to help explain documented sex differences.

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Figure 1.

Effect of steady-state methadone on TD, HA and VA in response to a tactile stimulus is shown. In response to a tactile stimulus, there was a large gender difference across all behavioral measurements. Further evaluation of SSM effect on gender differences showed that only at the 20mg/kg dose of SSM, females were significantly more sensitive to tactile stimuli than males. The data are presented as mean $\pm$ SEM and significance was set at $p < 0.05$. * $p < 0.05$, ** $p < 0.01$, vs. females.

Homecage motor activity

Figure 2.

Effect of steady-state methadone on home cage motor activity. Two-way ANOVA showed a significant main effect of gender, with no effect of methadone. Further, only at highest dose (20 mg/kg), home cage motor activity was significantly greater in females than males (planned comparisons). The data are presented as mean $\pm$ SEM and significance was set at $p < 0.05$. *** $p < 0.001$ or **** $p < 0.0001$ vs. females.

Figure 3.

Total numbers of times that freezing occurred in contextual fear conditioning. Contextual fear conditioning showed statistically significant effect of gender ($p < 0.005$). There was an apparently greater response in males at the 20mg/kg dose, but this failed to be significant ($p = 0.078$). The data is presented as total numbers of times that freezing occurred.

Figure 4.

Effect of steady-state methadone (SSM) and generalized arousal (GA) on orexin mRNA levels in the lateral hypothalamus (LH) in male (A) and female (B) mice. In Figure 4A, the dashed line represents basal orexin mRNA level in female mice. There was a significantly higher orexin mRNA level in the LH of the males than females. In Figure 4B, GA stressors increased LH orexin mRNA levels in females; these increases were blunted by SSM at 20 mg/kg. The data are presented as mean $\pm$ SEM and significance was set at $p < 0.05$. # $p < 0.05$ vs. female; * $p < 0.05$ vs. GA.

Figure 5.

Effect of steady-state methadone (SSM) and generalized arousal (GA) on proopiomelanocortin (POMC) mRNA levels in the anterior pituitary (AP) in male (A) and female (B) mice. In Figure 5B, there was an apparent, but not statistically significant, increase in AP POMC mRNA levels after GA stress in females ($p=0.074$); this increase was blunted by SSM at 20 mg/kg. The data are presented as mean $\pm$ SEM and significance was set at $p<0.05$. ** $p<0.005$ vs. GA.

Table 1

Effect of steady-state methadone (SSM) and generalized arousal (GA) on orexin and pro-opiomelanocortin (POMC) mRNA levels in the medial hypothalamus (MH), and plasma corticosterone (B) levels in male and female mice.

		SSM (0 mg/kg/day)		SSM (20 mg/kg/day)	
		Non GA	GA	Non GA	GA
Orexin in the MH	Male	0.83 ± 0.07	0.83 ± 0.09	0.91 ± 0.08	0.84 ± 0.04
	Female	0.81 ± 0.14	0.94 ± 0.15	0.95 ± 0.10	0.91 ± 0.11
POMC in the MH	Male	3.1 ± 0.48	2.9 ± 0.56	2.9 ± 0.69	3.4 ± 0.64
	Female	3.4 ± 0.86	2.8 ± 0.55	3.3 ± 0.53	3.1 ± 0.67
Plasma B	Male	14.3 ± 6.1	12.0 ± 3.1	15.2 ± 3.5	19.6 ± 3.5
	Female	20.0 ± 8.1	12.1 ± 4.4	13.6 ± 3.6	12.0 ± 2.9