Sex Differences in μ-Opioid Receptor Regulation of the Rat Locus Coeruleus and Their Cognitive Consequences

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Stress-related neuropsychiatric pathologies are more prevalent in females compared with males. An important component of the stress response is activation of the locus coeruleus (LC)–norepinephrine system. Because LC activation is tempered by endogenous opioid release during stress, the magnitude of opioid regulation of the LC could determine stress vulnerability. Here we report convergent evidence for decreased μ-opioid receptor (MOR) function in the female rat LC. The selective MOR agonist, DAMGO (10 pg), completely inhibited LC discharge of male but not female rats and DAMGO (30 pg) produced no further inhibition of female LC neurons. Consistent with a decreased maximum DAMGO response, MOR protein and mRNA expression were decreased in female compared with male LC. These molecular and cellular sex differences were associated with sexually distinct effects of LC-MOR activation on cognitive processing in an operant strategy-shifting task. Although DAMGO (10 pg intra-LC) increased the number of trials to reach criterion for both sexes, it increased the duration to complete the task and the total number of errors selectively in males. Specifically, DAMGO increased premature responses, regressive errors, and random errors in males and perseverative errors in females. The sexually distinct cognitive consequences of activating LC-MOR may contribute to sex differences in opioid abuse patterns and may guide sex-specific therapies. Finally, given evidence that endogenous opioids restrain stress-induced LC activation and promote recovery of activity to pre-stress levels, decreased MOR function in the female LC could contribute to LC-NE overactivity that underlies the hyperarousal symptoms of stress-related psychiatric diseases.

INTRODUCTION

Many neuropsychiatric diseases, such as post-traumatic stress disorder (PTSD), and depression, are nearly two times more prevalent in females compared with males (Kessler *et al*, 1995; Kessler *et al*, 1994). These diseases have been associated with stress, suggesting that sex differences in prevalence arise from sex differences in stress response systems. In addition to sharing an association with stress, these disorders share symptoms of hyperarousal, implicating a common defect in arousal systems. The locus coeruleus (LC)-norepinephrine (NE) system is a major brain arousal system that is activated by stressors and LC hyperactivity has been implicated in the altered arousal that characterizes stress-related psychiatric disorders (Wong *et al*, 2000). Therefore, the LC-NE system is a site at which sex differences could be translated to differential vulnerability to stress-related psychiatric disorders.

The LC is the principal source of norepinephrine in many forebrain regions that underlie cognition, such as the cortex and the hippocampus (Swanson, 1976). During acute stress, LC neurons are activated by the stress-related neuropeptide, corticotropin-releasing factor (CRF), and this is associated with enhanced arousal and cognitive flexibility (Snyder *et al*, 2012; Valentino and Van Bockstaele, 2008). Interestingly, CRF antagonist administration prior to acute stress not only prevents LC activation but also reveals an underlying inhibition of LC neurons that is opioid-mediated (Curtis *et al*, 2001; Curtis *et al*, 2012).

Axon terminals containing the endogenous opioid, enkephalin, densely innervate the LC and LC neurons express μ-opioid receptors (MOR; Drolet *et al*, 1992; Pert *et al*, 1976). MOR activation robustly inhibits LC discharge (Williams and North, 1984). Under basal conditions, opioid antagonists do not alter LC discharge, indicating that endogenous opioids are not tonically released into the LC. However, when administered prior to acute stress, opioid antagonists increase LC activation and prolong recovery time after stressor termination, suggesting that endogenous opioids in the LC function to restrain stress-induced activation and to promote recovery of neuronal firing to baseline levels when the stressor is terminated (Abercrombie and Jacobs, 1988; Curtis *et al*, 2001; Curtis *et al*, 2012).

Given the role of CRF in mediating stress-induced LC activation, sex differences in CRF signaling and trafficking in LC neurons (Bangasser *et al*, 2010) have been proposed as

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one mechanism underlying LC-NE dysregulation that contributes to female vulnerability to stress-related psychiatric disorders. An additional mechanism by which the LC-NE system could become dysregulated is through decreased opioid inhibition. Consistent with this, analgesic studies in both humans and rodents provide evidence for decreased opioid sensitivity of females (Craft, 2003; Kest et al, 2000). On the basis of these observations, this study was designed to compare the neuronal and behavioral consequences of MOR activation in the LC of male and female rats. In addition, MOR protein and mRNA expression in the LC were compared.

MATERIALS AND METHODS

Subjects

Age-matched adult male and female Sprague Dawley rats (Charles River, Wilmington, MA) were shipped from the vendor at ~70 days of age. Experiments were conducted 1 week after arrival. Rats were singly housed in a climate-controlled room with a 12-h light–dark cycle (lights on at 0700 hours). Food and water were freely available except as noted for behavioral experiments. Female rats were intact. Animal use and care was approved by the institutional animal care and use committee of the Children's Hospital of Philadelphia.

Electrophysiological Studies

Surgical and electrophysiological recording protocols were similar to those described previously (Curtis et al, 1997; Supplementary Information). Rats were anesthetized with isofluorane and surgically prepared for recording LC single-unit discharge. Double-barrel glass micropipettes were used for simultaneous recording and microinfusion of DAMGO ([D-Ala2, N-MePhe4, Gly-ol]-enkephalin; Abcam, Cambridge, MA), a synthetic opioid peptide with high MOR specificity. LC activity was recorded before and after DAMGO administration (Supplementary Information). The effects of different DAMGO doses were compared between sexes by a two-way repeated measure ANOVA with time as the repeated measure. In addition, the area under the time-effect curve (0–300 s after injection) for the 10 pg dose was calculated and compared between sexes by a Student's t-test (two tailed).

Western Blotting

Male and female rats were decapitated and brains quickly removed and frozen. Thick (1000 μm) coronal sections containing the LC were cut on a cryostat and LC tissue punches were taken from these sections using a trephine. The tissue was processed for protein analysis by western blot as described (Curtis et al, 2006) (Supplementary Information). Membranes were probed for MOR and β-actin (1:1000 rabbit anti-MOR, Invitrogen and 1:5000 mouse anti-β-actin, Sigma) as previously described (Bangasser et al, 2010; Supplementary Information). The ratio of target protein (MOR) to loading control (β-actin) was calculated and the mean ratios were statistically compared using an ANOVA. Characterization and

specificity of the rabbit MOR antiserum have been described (Cheng et al, 1996; Surratt et al, 1994; Van Bockstaele et al, 1996). In addition, MOR antibody specificity was tested by probing rat heart lysates, which do not express MOR (Peng et al, 2012; Ventura et al, 1989) (Supplementary Figure S1).

Quantitative PCR Analysis of MOR mRNA

LC punches were collected as described above. LC tissue was lysed and homogenized according to manufacturer's instructions in the PureLink RNA mini kit (AMBION, Life Technologies; Supplementary Information). Real-time PCR was performed using TaqMan gene expression assays with TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA). Assays utilized were MOR (Oprm1, Rn01430371_m1) and GAPDH (Gapdh, Rn01775763_g1). Gene expression analysis was performed using the comparative CT (cycle threshold) method as described (Schmittgen and Livak, 2008). An ANOVA was used for sex comparisons.

Operant Strategy Shifting Assay

Male and female rats were implanted with a dual cannula guide (Plastic One Inc, Roanoke VA) for bilateral LC injections as previously described (Snyder et al, 2012). At least 4 days after surgery and 3 days prior to the start of training, rats were food restricted to 85% of their weight. Rats were trained and tested in an Operant Strategy Set Shifting Task (OSST) that was a modification from Floresco et al (2008) as previously described (Snyder et al, 2015; Supplementary Information). Rats were first trained to press one of two levers for food reinforcement on the first training day and the opposite lever on the following day. On the third training day, stimulus lights that were located above both levers were illuminated for 15 s during which levers were randomly selected to deliver reward and over many trials both levers were equally likely to deliver a reward. Tests were conducted the following day during which rats received bilateral intra-LC microinfusions of either ACSF or DAMGO (3, 10 pg in 200 nl) delivered by a syringe pump 10 min prior to behavioral testing. The OSST has three stages that involve different forms of learning, a simple discrimination (SD), reversal learning (REV), and strategy shifting (SHIFT). Animals proceeded from one stage of the task to the next stage after achieving a criterion of eight consecutive correct presses, provided that at least 30 trials had been attempted. The minimum of 30 trials requirement was added to ensure that each animal experienced sufficient trials for the transition from one type of discrimination to the next to be cognitively meaningful. During testing for all stages, only one of the stimulus lights above the levers was randomly illuminated. During the SD stage, reward was contingent on lever presses on the side opposite the animal's side bias (determined during training) and the location of the stimulus light was unrelated to the contingency. During the REV stage, reward was contingent on lever presses on the opposite lever. During the SHIFT stage, the correct lever was designated as the lever underneath the illuminated stimulus light. Upon reaching the criterion of eight consecutive correct presses in the SHIFT stage, the test ended and the
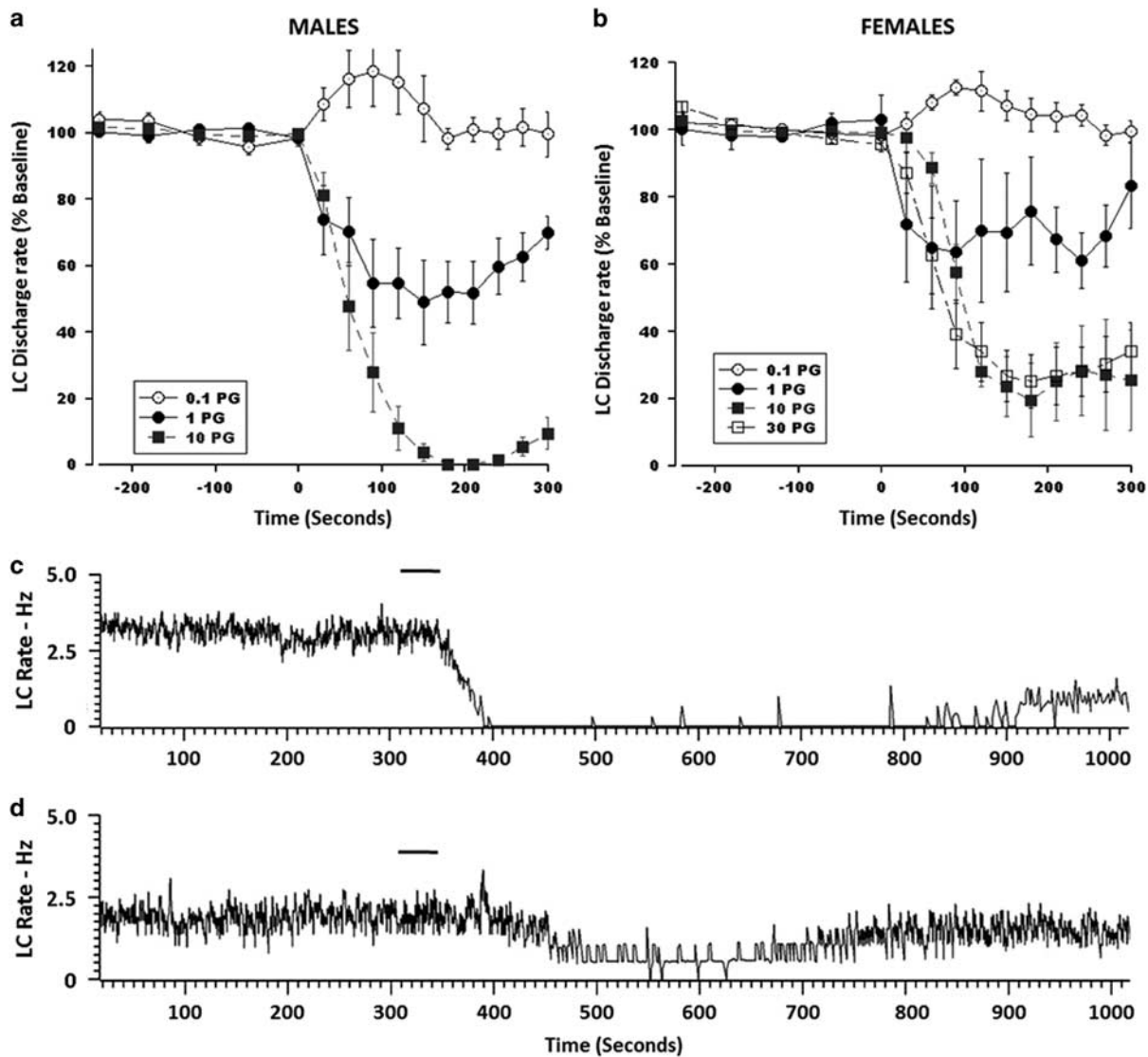
Figure 1 Dose-related inhibition of locus coeruleus (LC) neuronal discharge rate by DAMGO (D-Ala2, N-MePhe4, Gly-ol]-enkephalin) in male and female rats. (a and b) Line graphs show the time course of DAMGO effects on LC discharge rate. The abscissae indicate time (s) before and after DAMGO, which was administered at time = 0. The ordinates indicate LC discharge rate expressed as a percentage of the baseline rate before DAMGO. For 0.1 pg: males (*n* = 6 cells/3 rats), females (*n* = 3 cells/3 rats); for 1 pg: males (*n* = 7 cells/6 rats), females (*n* = 6 cells/4 rats); for 10 pg: males (*n* = 7 cells/5 rats), females (*n* = 7 cells/5 rats); for 30 pg females (*n* = 10 cells/6 rats). (c and d) Representative ratemeter records from a single locus coeruleus neuron of a (c) male and (d) female rat before and after DAMGO 10 pg microinfusion into the LC (indicated by the bars above the traces).

animal was removed from the testing chamber. Dye was infused through the cannulae for histological identification of injection sites. Trials to criterion were recorded during each stage of the OSST. Error types within the shift to light stage of the OSST were characterized using logistic regression in order to determine whether treatments had an effect on the perseveration of the previous rule or the acquisition and maintenance of the new rule (Snyder *et al*, 2015; Supplementary Information). Errors of omission and premature responses that occurred during the intertrial interval were also calculated and the number of all total errors (perseverative, regressive, random, omission, and premature responses) were compared between groups. In addition, the duration to complete the strategy shifting stage and mean correct and incorrect response latencies

for rats administered ACSF or DAMGO (10 pg) were compared.

OSST Statistical Analysis

OSST data (trials to reach criterion) were first analyzed using a three-factor repeated measures ANOVA with dose and sex as between factors and stage as the repeated measure. Each stage was then analyzed separately by a two-factor ANOVA to determine effects of dose, sex and dose X sex interactions for each individual stage (SD, REV, and SHIFT). The Tukey's HSD test was used *post hoc* to determine statistically significant differences between individual sex/dose groups. Total errors, error type, duration to complete the strategy shifting stage, and correct and incorrect response latencies

were analyzed by a two-factor ANOVA with dose and sex as factors. To analyze regional specificity, the effect of accurate *vs* inaccurate DAMGO (3 pg) injections and injections of ACSF on each stage of the set shifting task were analyzed for each sex individually by one way ANOVAs with Tukey's HSD *post hoc* for individual comparisons. An alpha level of $p < 0.05$ was the maximum threshold for statistical significance. The 3 pg dose was chosen for this analysis rather than the 10 pg dose because there were an insufficient number of inaccurate injections in the 10 pg group to provide sufficient power for the statistical comparison of that dose group.

Estrous Cycle Monitoring

It was not the goal of this study to determine the role of gonadal hormones in MOR regulation of the LC. However, for electrophysiological studies, qPCR and behavioral studies estrous cycle status was monitored by vaginal cytology as previously described (Bangasser and Shors, 2008). For these studies females were subdivided into those in relatively high (proestrus) or relatively low (estrus and diestrus pooled) estrus states and the DAMGO effect determined in these specific groups.

RESULTS

Decreased Sensitivity of Female LC Neurons to MOR–mediated Inhibition

LC spontaneous discharge rates were comparable between males (2.10 ± 0.2 Hz, $n = 20$ cells/14 rats) and females (1.74 ± 0.22 Hz, $n = 26$ cells/18 rats; $F(1, 44) = 1.4$, $p > 0.05$). Figures 1a and b shows the time-course of the mean LC activity (expressed as a percentage of the baseline rate) before, during, and after DAMGO (0.1, 1, and 10 pg) microinfusion. No sex differences were found in the response to DAMGO (0.1 pg; $F(1, 68) = 0.27$, $p = 0.60$) or DAMGO (1 pg; $F(1, 99) = 0.49$, $p = 0.49$). Notably, the 10 pg DAMGO dose completely suppressed LC firing in male but not female rats (Figures 1c and d). The mean inhibition of LC discharge rate produced by this DAMGO dose was different in males and females ($F(1, 12) = 15.281$, $p < 0.002$). To determine whether a higher dose of DAMGO could completely inhibit LC neurons of female rats, (30 pg, 10 cells/6 rats) DAMGO was tested. The magnitude of inhibition produced by this dose in females was similar to that produced by the 10 pg DAMGO dose in females ($F(1, 15) = 0.088$, $p < 0.77$) and less than that produced by the 10 pg DAMGO dose in males ($F(1, 15) = 4.30$, $p < 0.047$; Figure 1b). The sex difference was also apparent as a decreased area under the curve describing the effect over time (males: $23\,017 \pm 1105$ *vs* females: $15\,837 \pm 1541$; $p < 0.005$, Student's *t*-test two tailed). The decreased effect of high doses of DAMGO on LC neuronal activity was true for both female rats in diestrus/estrus and those in proestrus (Supplementary Figure S2). For this comparison, because most cells tested with DAMGO (10 pg) came from female rats in proestrus, data from these were pooled with cells from female rats tested with 30 pg DAMGO which had a similar effect as 10 pg DAMGO in females and remained less than that produced by 10 pg in males (Supplementary Figure S2).
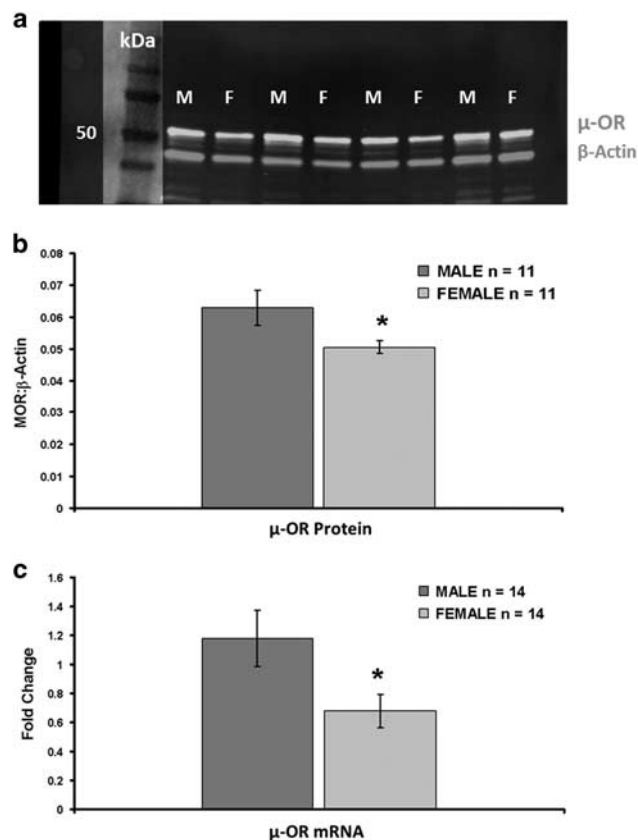
Figure 2 Sex differences in locus coeruleus-μ-opioid receptor (LC-MOR) protein and mRNA. (a) Blots represent the MOR protein band (green) and β-actin band (red) as a loading control of LC-tissue punches from male (M) and female (F) rats. Note that the contrast was increased selectively around the molecular weight ladder to be able to visualize it. (b) Bars indicate the mean ratio of the integrated intensity of each band of MOR protein to the corresponding band of β-Actin as loading control from the same samples ($n = 11$, each group). (c) Bars indicate relative quantification (RQ) of the *MOR* gene in the LC. Data are represented as the mean $\pm$ SEM; ($n = 14$, each group). GAPDH was used as an endogenous control. *$p < 0.05$. A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

Decreased MOR Expression in Female LC

The decreased maximum inhibition of LC activity produced by DAMGO in females suggested differences in MOR expression. Consistent with this, quantification of MOR protein in the LC using Western blot indicated lower levels in females (Figure 2). Figure 2a shows a representative western blot of MOR (green) and β-actin (red). The mean MOR:β-actin ratio was greater in male rats when compared with female rats ($F(1, 20) = 4.5$, $p = 0.045$; each group $n = 11$; Figure 2b).

To determine whether sex differences in LC-MOR levels were related to differences in LC-MOR transcription, qPCR was used to quantify and compare MOR mRNA in the LC. Consistent with the Western blot analysis, the qPCR analysis revealed decreased levels of LC-MOR transcripts in females when compared with males ($F(1, 26) = 4.87$, $p = 0.036$; $n = 14$ both groups; Figure 2c). Levels of transcripts were comparable in females that were in an estrus cycle stage of high estrogen (proestrus, 0.62 ± 0.17) or relatively low estrogen (diestrus or estrus pooled, 0.72 ± 0.17, $p = 0.71$; Supplementary Figure S3).
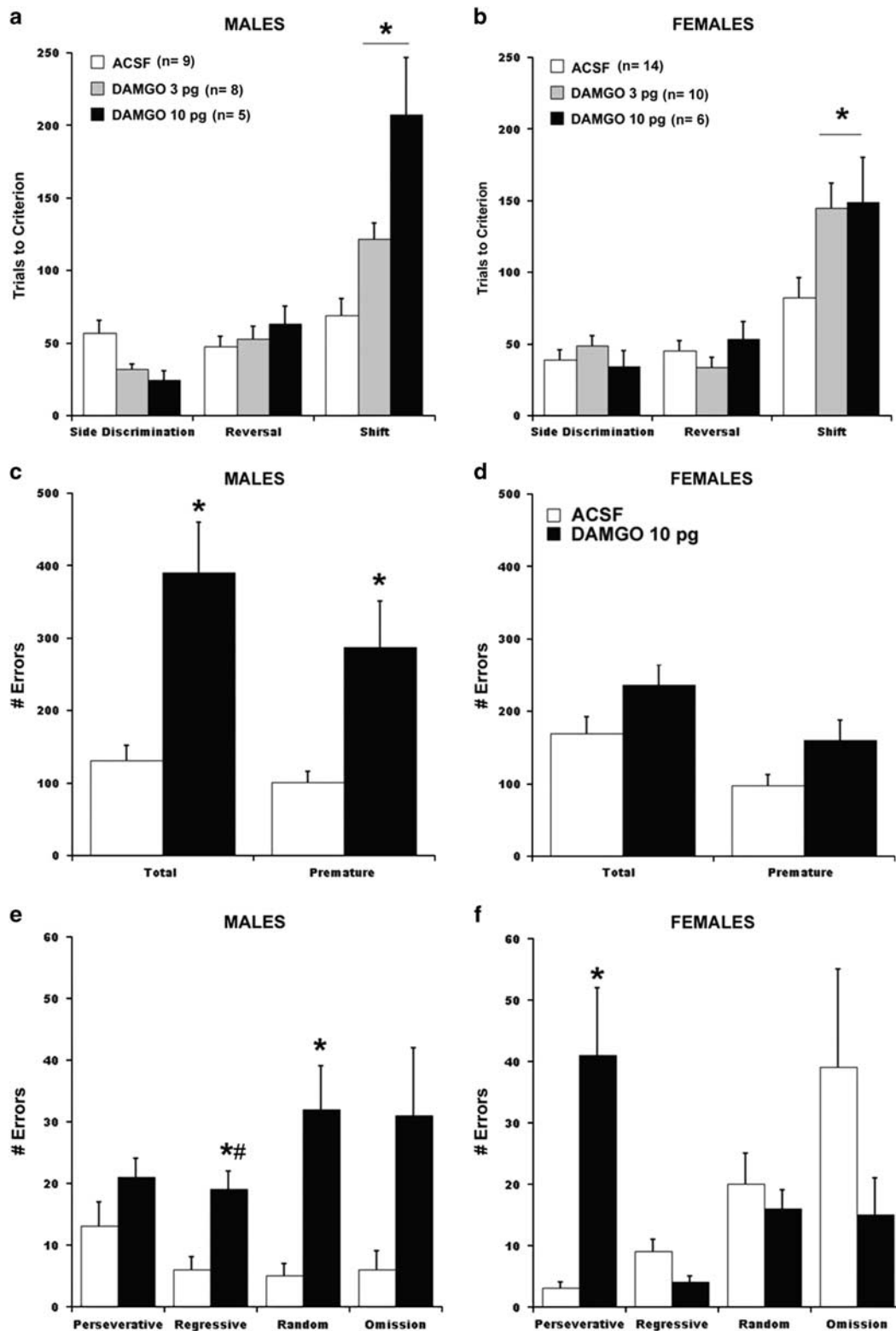
Figure 3 Sex differences in behavioral consequences of activating μ-opioid receptor (MOR) in the locus coeruleus (LC). (a and b) Effects of ACSF and DAMGO (D-Ala2, N-MePhe4, Gly-ol]-enkephalin; 3 and 10 pg) bilaterally infused into the LC of male (a) and female (b) rats on performance in the operant strategy set-shifting task. The bars represent the mean number of trials necessary to reach the criterion for side discrimination, side reversal and shift to light stages of the task. Vertical lines represent SEM. The number of subjects is indicated in the graph legend. Asterisks above the bars indicate that both DAMGO doses were associated with increased trials to reach criterion compared to ACSF ($p < 0.05$). (c and d) The bars represent the mean number of total errors and mean number of premature responses in male (c) and female (d) rats administered ACSF or DAMGO (10 pg). Asterisks indicate an effect of DAMGO over ACSF for the same sex ($p < 0.05$, Tukey's HSD). (e and f) Analysis of error types in the shift stage in male (e) and female (f) rats. The bars indicate the mean number of each error type. Vertical lines represent SEM. Asterisks indicate a significant effect of DAMGO compared with ACSF for the same sex group ($p < 0.05$, Tukey's HSD). [#]$p < 0.05$ (Tukey's HSD) compared with effect of DAMGO in females.

Sex Differences in the Behavioral Consequences of MOR Activation in the LC

A total of 52 rats were implanted with dual intra-LC cannula and completed all stages of the OSST. Figure 3 shows the mean number of trials to reach criterion for each stage of the task for males (Figure 3a) and females (Figure 3b) administered DAMGO or vehicle. A three-factor ANOVA to test for main effects of dose and sex with stage as the within-subject factor revealed a main effect of the whole model (F(5, 46) = 5.3, $p < 0.0006$), an effect of dose (F(2, 46) = 11.4, $p < 0.001$), an effect of stage (F (2, 45) = 46.5, $p < 0.0001$), dose X stage interaction (F(4, 90) = 6.9, $p < 0.001$) and a trend for stage X sex X dose interaction (F(4, 90) = 2.4, $p = 0.055$) for trials to reach criterion (Figures 3a and b). Further analysis of individual stages revealed a trend for a main effect in SD (F(5, 51) = 2.03, $p = 0.09$) and a trend for a sex X dose interaction (F = 3.2, $p = 0.051$) such that DAMGO tended to facilitate SD performance in male rats. However, Tukey's HSD post-hoc test did not indicate group differences. There was no main effect on REV (F(5,51) = 1.18, $p = 0.33$). Analysis of behavior during the SHIFT stage revealed a main effect of the whole model (F(5, 51) = 6.03, $p < 0.0002$) and an effect of dose (F = 13.5, $p < 0.0001$) such that 3 and 10 pg DAMGO increased the number of trials to reach criterion. There was no significant sex X dose interaction (F = 2.1, $p = 0.14$). An examination of behavioral results broken down into proestrus and estrus/diestrus groups suggested that results were comparable regardless of estrus status (Supplementary Figure S4).

Although there was no sex difference in the number of trials to reach criterion for the strategy-shifting task, male rats took significantly longer to complete this stage after DAMGO (10 pg) (Table 1). Specifically, there was a significant main effect (F(3, 33) = 3.97, $p = 0.017$), an effect of dose (F = 8.4, $p < 0.006$) and sex X dose interaction (F = 4.8, $p < 0.03$; $p < 0.05$, Tukey's HSD). This was not likely the result of decreased motivation because there was no sex difference in the duration to complete the reversal stage. Females administered ACSF or DAMGO (10 pg) took 1135 ± 162 s and 1334 ± 247 s, respectively to complete the REV stage and males took 1050 ± 202 s and 1202 ± 271 s after ACSF and DAMGO (10 pg), respectively (F(3,33) = 0.28, $p = 0.8$). The increased duration for males to complete the

task was also not due to increased response latency as this was unaffected by dose and was comparable between sexes (Table 1). Rather, males made more total errors (F(3,33) = 6.7, $p < 0.005$; Figures 3c and d). There was an effect of dose on total errors (F(1,33) = 15.8, $p < 0.0005$) and a sex X dose interaction (F(1,33) = 5.5, $p < 0.02$) indicating that DAMGO (10 pg) increased total errors selectively for males. A detailed analysis of error type indicated that the number of omitted trials was comparable between males and females (F(3,33) = 1.3, $p = 0.29$; Figures 3e and f). DAMGO (10 pg) increased premature responding (Main effect: F(3,33) = 5.9, $p < 0.005$, dose effect: F(1,33) = 13.5, $p < 0.001$) and there was a trend for a sex X dose interaction: F(1,33) = 3.3, $p = 0.07$). A Tukey's HSD post-hoc test indicated that compared with vehicle, DAMGO (10 pg) increased premature responses in males and not females ($p < 0.05$ Tukey's HSD; Figures 3c and d). DAMGO (10 pg) also promoted different error types during the SHIFT trials depending on sex. For males, DAMGO (10 pg) increased regressive and random errors (Figure 3e). There was a main effect of treatment for regressive errors (F(3, 33) = 4.7 $p < 0.01$) and a sex × dose interaction (F(1,33)11.4, $p < 0.005$). Likewise, for random errors there was a main effect of treatment (F(3, 33) = 3.3 $p < 0.05$) and a sex x dose interaction (F = 6.7, $p < 0.05$) such that DAMGO increased regressive and random errors selectively in male rats when compared with vehicle control ($p < 0.05$, Tukey's HSD, both error types). In contrast, DAMGO (10 pg) increased perseverative errors in females (Figure 3f). There was a significant main effect of treatment (F(3, 33) = 8.7 $p = 0.0003$) and a sex X dose interaction (F = 6.8, $p < 0.05$) such that 10 pg DAMGO increased perseverative errors selectively in female rats when compared with vehicle control ($p < 0.05$, Tukey's HSD).

Regional Specificity

The behavioral effects of DAMGO were regionally localized to the LC. Figure 4a shows representative histology of an accurate bilateral injection into the LC in a rat that was administered 3 pg DAMGO. Because most of the 10 pg DAMGO injections were accurate (Supplementary Figure S5), only the effects of accurate *vs* inaccurate injections of the 3 pg dose were statistically compared (Figures 4b–d). DAMGO injections outside of the LC had no effect on performance in any task stage for either males or females (Figures 4c and d). For males there was an effect of placement on SD performance (F(2, 21) = 6.9, $p < 0.01$) and SHIFT performance (F(2,21) = 8.5, $p < 0.005$). *Post hoc* comparisons revealed that only rats that received accurate injections were impaired compared with the ACSF group ($p < 0.05$, Tukey's HSD; Figure 4c). Likewise, only female rats administered DAMGO (3 pg) into the LC required a greater number of trials to reach criterion during the SHIFT stage compared with females administered ACSF (F(2,28) = 4.2, $p < 0.05$, $p < 0.05$ Tukey's HSD test; Figure 4d).

DISCUSSION

The present study provided convergent cellular, molecular, and behavioral evidence for decreased MOR function in the LC of female compared with male rats. At a cellular level,
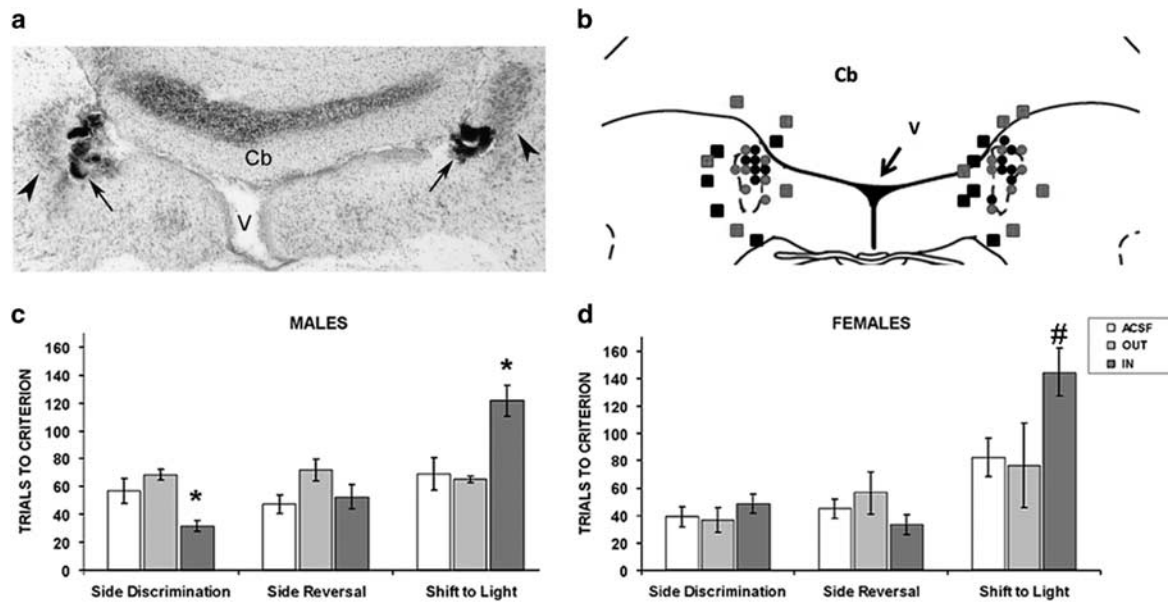
Table 1 Effects of DAMGO on the Duration to Complete the SHIFT Stage of the Task and Response Latencies

Sex	Treatment	Duration (s)	Correct response latency (s)	Incorrect response latency (s)
Male (9)	ACSF	1358 ± 206	4.5 ± 0.5	3.7 ± 0.4
Male (5)	DAMGO	**4112 ± 859***	4.3 ± 0.3	3.1 ± 0.2
Female (14)	ACSF	2444 ± 468	4.7 ± 0.3	3.9 ± 0.3
Female (6)	DAMGO	2834 ± 458	4.4 ± 0.3	3.4 ± 0.2

For stage duration: main effect: F(3,33) = 4.0, $p < 0.02$, Treatment effect: F(1,33) = 8.4, $p < 0.01$, sex × treatment interaction: F(1,33) = 4.8, *$p < 0.05$; $p < 0.05$ Tukey's HSD Male DAMGO > Male ACSF.
For correct response latency: main effect: F(3,33) = 0.15, $p = 0.93$.
For incorrect response latency: main effect: F(3,33) = 0.68, $p = 0.57$.
The bold value highlights that this is significantly different from the ACSF control.

Figure 4 Regional specificity of DAMGO (D-Ala2, N-MePhe4, Gly-ol]-enkephalin) effects on strategy shifting. (a) Photomicrograph of a Neutral Red counterstained section through the LC showing histological verification of the injection site from a representative animal that was injected with DAMGO. The arrowhead points to the LC and the arrow points to the dye, which is localized to the LC. Cb, cerebellum; V, ventricle. (b) Plots of accurate (circles) and missed (squares) injection sites for DAMGO (3 pg) for males (black) and females (red). DAMGO effects from these cases were used for the graphs in c and d. (c) Comparison of the effects of DAMGO (3 pg) microinfused into the LC of male rats (in, $n = 8$), outside of the LC (out, $n = 5$) and ACSF ($n = 9$) on performance in different components of the OSST. The bars indicate the number of trials necessary to reach the criterion for each stage. Vertical lines represent SEM. Asterisks indicate a significant difference compared with both the ACSF and DAMGO out groups ($p < 0.05$). (d) Comparison of the effects of DAMGO (3 pg) microinfused into the LC of female rats (in, $n = 10$), outside of the locus coeruleus (LC; out, $n = 5$) and ACSF ($n = 14$) on performance in different components of the OSST. The bars indicate the number of trials necessary to reach the criterion for each stage. Vertical lines represent SEM. $^{\#}p < 0.05$ compared with ACSF. A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

postsynaptic responses of LC neurons to relatively high DAMGO doses were attenuated in female compared with male rats. Consistent with a decreased maximum response to a MOR agonist, quantification of MOR protein and mRNA in LC tissue indicated decreased MOR expression in female compared with male LC tissue. Notably, these molecular and cellular sex differences were associated with sexually distinct behavioral consequences of LC-MOR activation. Thus, a DAMGO dose that completely inhibited LC neuronal activity of male but not female rats significantly increased the time to complete the strategy shifting stage selectively in males as a result of increasing the total number of errors. DAMGO produced sexually distinct cognitive effects that were expressed as differences in error types during strategy shifting performance. Sex differences in the effects of LC-MOR activation on cognitive processing may be relevant for sex differences in opioid abuse. The findings agree with evidence for decreased MOR function in females from analgesia and receptor signaling studies (Craft, 2003; Kest et al, 2000; Wang et al, 2014). Given evidence for an inhibitory influence of endogenous opioids in the LC that restrains stress-induced LC activation and promotes recovery of LC activity to pre-stress levels, the decreased MOR function in the female LC could contribute to LC-NE overactivity that underlies hyperarousal symptoms of stress-related psychiatric diseases (Gold and Chrousos, 2002; Wong et al, 2000). This may have a role in the greater prevalence of stress-related psychiatric disorders in females.

Sex Differences in LC Neuronal Responses to MOR Activation

Anatomical and physiological evidence implicate the enkephalin-MOR system as an important inhibitory regulator of LC activity (Drolet et al, 1992; Pert et al, 1976; Williams and North, 1984). This regulation is not tonically active because opioid antagonists do not alter LC spontaneous discharge rates (Abercrombie and Jacobs, 1988; Valentino and Wehby, 1989). Rather, it becomes engaged during acute stress where it functions to counter LC activation and promote recovery with stressor termination (Curtis et al, 2001; Curtis et al, 2012). Identification of individual differences in MOR regulation of the LC is therefore important as this can determine the magnitude and duration of the LC-NE response to stress.

Human and rodent studies report a decreased sensitivity of females to opioid-induced analgesia, behavioral suppression, tolerance, and dependence (Craft, 2003; Kest et al, 2000). This has been attributed in part to decreased MOR expression and MOR-G-protein coupling (Loyd et al, 2008; Murphy et al, 2009; Wang et al, 2014). However, these sex differences are regionally specific and there have been no studies of sex differences in MOR in the LC.

As previously reported, there were no sex differences in baseline LC spontaneous discharge rate (Curtis et al, 2006). Although DAMGO produced the characteristic LC inhibition in both sexes, the maximum magnitude of inhibition was significantly less in females, even when the dose was increased beyond that which completely inhibited male LC

neurons. The decreased maximal effect suggested decreased LC-MOR levels in the female rather than a decrease in agonist affinity.

Molecular Basis for Sex Differences in LC Postsynaptic Responses to a MOR Agonist

Protein quantification by western blot confirmed decreased MOR protein in the LC of female compared with male rats. The interpretation of this finding relies on the specificity of the MOR antibody. Characterization and specificity of the rabbit antiserum against the MOR have been described previously (Cheng *et al*, 1996; Surratt *et al*, 1994; Van Bockstaele *et al*, 1996). In addition, antibody specificity was tested using heart tissue, which does not express MOR protein (Peng *et al*, 2012; Ventura *et al*, 1989) (Supplementary Figure S1). Decreased MOR expression in female compared with male rats has been documented in other brain regions including the periaqueductal gray (Loyd *et al*, 2008) and the rat anterior pituitary gland (Carretero *et al*, 2004). Quantitative PCR corroborated the interpretations of the western blot studies and suggested that decreased MOR transcription underlies decreased MOR protein.

Sex Differences in Behavioral/Cognitive Endpoints of MOR Activation in the LC

Importantly, sex differences in MOR expression and cellular function in the LC were reflected as differences in the behavioral consequences of LC-MOR activation. The LC regulates cognitive flexibility through its projections to the prefrontal cortex (PFC). The relationship between LC activity and PFC function is hypothesized to resemble an inverted U-shaped curve whereby PFC function is optimal at moderate levels of LC activity. However, excessive LC drive impairs cognitive flexibility as a result of the interaction of higher levels of norepinephrine with lower-affinity adrenergic receptors (Arnsten, 2011). CRF, at doses that produce a moderate activation of LC neurons, facilitates cognitive flexibility in an attentional set shifting task. However, this effect reverses as the CRF dose is increased (Snyder *et al*, 2012). Because stress also engages enkephalin release in the LC that could counter the effects of CRF, it is important to understand how this could impact cognitive flexibility.

Our electrophysiological and molecular findings predicted an enhanced behavioral response to relatively high doses of DAMGO in the LC in males compared with females, particularly in the strategy shifting stage. Although the highest DAMGO dose had similar effects on the number of trials to reach criterion in this stage, males took significantly more time to complete this stage. The increased duration could not be attributed to an increase in response latency once a trial started or solely to trial omissions. Rather this was due to an increase in total errors made by males and particularly premature responses, which are indicative of impulsive behavior. This is consistent with reports of opioid-elicited impulsive behavior in rodents in behavioral tasks such as the 5-choice serial response time task (5-CSRTT) and the response inhibition task and the decrease in motor impulsivity in MOR-knockout mice (Mahoney *et al*, 2013;

Olmstead *et al*, 2009; Pattij *et al*, 2009). As these previous studies examined only males, the present findings suggest that this may be a male-biased effect that involves the LC-norepinephrine system.

In addition to increases in premature responding, DAMGO (10 pg) also increased regressive and random errors selectively in males, indicative of an inability to acquire and maintain the new strategy (Floresco *et al*, 2008). In contrast, females administered DAMGO made more perseverative errors, which are indicative of an impaired ability to shift from a previously learned rule. Taken with evidence for decreased LC-MOR receptor expression and physiological function, the data suggest that that the ability to shift from a previously learned rule may be more sensitive to disruption by LC-MOR activation than facilitation of impulsive behavior or impairment in the ability to learn a new strategy, which may require greater LC-MOR occupancy and LC inhibition.

Implications

The present findings suggest that the ability of endogenous opioids to buffer LC activation during stress and to promote recovery would be less effective in females. A decreased opioid influence in the LC in females would converge with increased CRF receptor signaling to produce an enhanced arousal response to stressors that could contribute to a greater prevalence of stress-related psychiatric disorders in females. Several studies provide evidence that the effects of endogenous opioids released during stress are attenuated in females compared with males. For example, stress-induced opioid-mediated analgesia (Kavaliers and Innes, 1987; Mogil *et al*, 1993; Romero and Bodnar, 1986), naloxone-induced freezing after stress (Klein *et al*, 1998) and naloxone-precipitated withdrawal in rats with a history of stress (Klein *et al*, 1997) are all greater in males compared with females. The results also have implications for the treatment of post-traumatic stress disorder (PTSD). Morphine administration during trauma care is associated with a decreased incidence of PTSD, particularly the arousal symptom cluster (Bryant *et al*, 2009; Holbrook *et al*, 2010; Stoddard *et al*, 2009). The present findings predict that this course of treatment would be less effective in females.

Finally, sex-specific effects of LC-MOR activation on cognitive processing are relevant for understanding sex differences in opioid abuse and for designing sex-specific treatments. The greater promotion of impulsive behavior in males may facilitate an earlier onset of abuse. The high rate of perseverative responding in females suggests that once the cycle of opioids abuse begins it may be more difficult reverse in females. This is consistent with the finding that once initiated, substance abuse accelerates at a faster pace in females compared with males, craving is more severe, and it is more difficult to quit (Back *et al*, 2011; Becker and Hu, 2008). The present findings underscore the potential for sex-specific treatment of opioid abuse based on pharmacological and/or cognitive therapies that target different cognitive dimensions.

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