SEX DIFFERENCES IN μ-OPIOID REGULATION OF THE RAT LOCUS COERULEUS

Herminio Manuel Guajardo

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Supervisor of Dissertation

_____

Dr. Rita J Valentino
Professor of Anesthesiology and Critical Care,
The Children's Hospital of Philadelphia/
University of Pennsylvania

Graduate Group Chairperson

_____

Dr. Joshua I. Gold,
Professor of Neuroscience,
Perelman School of Medicine at the University of Pennsylvania

Dissertation Committee

Dr. Marc Schmidt, Associate Professor of Biology, University of Pennsylvania (Chair)

Dr. Seema Bhatnagar, Associate Professor of Anesthesiology and Critical Care
The Children's Hospital of Philadelphia/ University of Pennsylvania

Dr. Heath Schmidt, Assistant Professor of Psychiatry, Perelman School of Medicine, University of Pennsylvania.

Dr. Alexxai Kravitz, Principal Investigator, NIDDK, NIDA, National Institutes of Health.

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DEDICATION

For my God, for my mother Flora Elia Reyes,
for her love, dedication, prayers and guidance,
and for my family that supported me through this hard
but amazing experience.

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ABSTRACT

SEX DIFFERENCES IN μ-OPIOID REGULATION OF THE RAT LOCUS COERULEUS

Herminio Manuel Guajardo

Rita J. Valentino

There are sex differences in disease susceptibility, time of onset of symptoms, and drug responses. Notably, sex differences are particularly prominent in pain and opioid analgesic responses, with females being less sensitive to opioid analgesia. A major site of action of opioids in the brain is the locus coeruleus (LC)-norepinephrine (NE) system. LC neurons express mu-opiate receptors (MOR), and MOR-agonists potently inhibit LC neuronal activity. Evidence suggests that endogenous opioids are released during stress, to restrain LC activation and to facilitate LC recovery when the stressor ends. On the basis of these observations, this dissertation tested the hypothesis that the opioid regulation of the LC is decreased in females relative to males. By implementing electrophysiological, biochemical, and behavioral approaches, sex differences in MOR regulation of the LC-NE system were examined. MOR mRNA was greater in male compared to female LC as indicated by quantitative PCR. This translated to an increased level of MOR protein in male compared to female LC tissue as detected by Western blot analysis. Consistent with sex differences in MOR expression in the LC, recordings of single unit LC activity in anesthetized rats demonstrated that the maximal magnitude of inhibition produced by intracoerulear injection of a MOR agonist, DAMGO, was greater in males. The decreased response of female LC neurons to MOR activation was expressed as a diminished response in upstream targets. Thus, intra-LC DAMGO

increased synchronization of local field potential activity in male but not female medial prefrontal cortex (mPFC). Notably, the LC-NE system affects cognitive function through its projections to the mPFC. The molecular and cellular sex differences in MOR regulation of the LC were associated with sexually distinct effects on cognitive processing in an operant strategy-shifting task. Intra-LC DAMGO increased the duration to complete the task and the total number of errors, selectively in males. DAMGO increased premature responses, regressive and random errors in males, and perseverative errors in females. The sex-specific effects of LC-MOR activation on cognitive processing may contribute to an early onset of opioid abuse in males, and susceptibility to opioid relapse in females. Ultimately, given the role of endogenous opioids in restraining the stress response of the LC system, decreased opioid sensitivity in females could enhance female vulnerability to stress.

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CHAPTER 1: INTRODUCTION

The goal of this dissertation is to determine the neural basis for sex bias in stress-related neuropsychiatric diseases by studying sex differences in the regulation of a major brain stress response system by endogenous opioids.

Sex Differences in Disease

Sex differences in brain function can arise from an assortment of genetic and hormonal events that occur at early stages of development. These changes continue throughout the lifespan of the organism and dictate the many physiological differences in disease susceptibility, manifestation, and treatment between the sexes. In fact, it is well known that there are differences between the sexes in disease susceptibility, time of onset of symptoms, and drug response in conditions such as coronary heart disease, cancer, obesity, autoimmune diseases, and mental health conditions, among others (Becker *et al*, 2005). Therefore, there is a general calling within the basic and clinical scientific community to identify the biological bases for differences between males and females in the development of disease, signs and symptoms of pathophysiology, and response to therapy. Notably, the FDA has implemented regulations and guidance to ensure that both sexes are represented in all phases of clinical trials, and medical products are labeled to alert physicians and patients regarding sex-differences in drug responses (Soldin *et al*, 2011). This knowledge will advance our ability to target treatments for specific individuals. Moreover, on a basic science level, this knowledge will help elucidate the pathophysiology of diseases that exhibit sex differences.

Sex Differences in Stress-related Psychiatric Disorders

Sex differences are particularly prominent in psychiatric diseases. For example, women are diagnosed with anxiety and mood-related pathology at higher rates than men, with many epidemiological studies indicating that the female-to-male ratio for these disorders is approximately 2:1 (Gater *et al*, 1998; Weissman and Klerman, 1977). Many psychiatric diseases that are more prevalent in females are associated with stress. For example, stress may exacerbate or precipitate symptoms and the disorders are often associated with dysfunctional stress responses (Gold and Chrousos, 2002; Heim and Nemeroff, 2001; Wong *et al*, 2000). This has led to the hypothesis that higher rates of certain psychiatric diseases in women are due to sex differences in stress response systems. The primary mediators of stress response are corticotropin-releasing factor (CRF) and glucocorticoids. In response to stress, CRF initiates the endocrine cascade that culminates in glucocorticoid release (Vale *et al*, 1981). CRF also acts as a brain neurotransmitter to elicit behavioral and autonomic responses to stress (Li *et al*, 1996). Sex differences in CRF receptors have been identified. For example, CRF binding is significantly greater in the basolateral and posteroventral nuclei of the amygdala of female rats compared to male rats (Weathington and Cooke, 2012). This sex difference could translate into increased anxiety following stressful events in females, which if true in humans, would increase the predisposition of women to anxiety disorders such as PTSD. In addition, our laboratory has identified sex differences in CRF_1 receptor (CRF_1) signaling and trafficking in rat cortex and Locus Coeruleus (LC) neurons. For example, CRF_1-Gs association is greater in female cortex compared to males, and stress-induced CRF_1 association with β-arrestin 2, a step that is critical for CRF_1 internalization is

decreased in female compared to male rats. Consistent with this, stress induces CRF_1 internalization in locus coeruleus (LC) neurons of male rats, but not female rats. This makes CRF-receptive neurons of females more sensitive to low levels of CRF, and less adaptable to high levels of CRF (Bangasser *et al*, 2010). Taken together, these experimental data suggest that sex differences in CRF receptors render females into a dysregulated state of stress reactivity that could be linked to the development of mood and anxiety disorders.

The Locus Coeruleus –Norepinephrine System

The LC-norepinephrine (NE) system is a major stress response system in the brain that is important for arousal and cognitive aspects of stress response. The LC is a small nucleus of neurons located in the pons just lateral to the wall of the fourth ventricle (Foote *et al*, 1983). The LC is one of seven noradrenergic subgroups (the A1-A7 groups) in the rat brainstem (A6, in the nomenclature of Dahlstroem and Fuxe, 1964). All LC neurons synthesize norepinephrine (NE) (Dahlstroem *et al*, 1964), and it is the primary source of NE in the brain (Swanson, 1976; Swanson and Hartman, 1975). There are two types of neurons observed within the LC; large multipolar cells (~35 mm) located in the dorsal part of the LC and smaller fusiform cells (~20mm) located mainly in the ventral LC (Grzanna and Molliver, 1980; Swanson, 1976). The LC projects to the spinal cord, brainstem, cerebellum, hypothalamus, thalamus, basal telencephalon, and the entire isocortex via highly collateralized projections (Dahlstroem *et al*, 1964; Moore and Bloom, 1979). Notably, LC is the sole source of NE in many forebrain regions that have been implicated in cognition such as cortex and hippocampus (Waterhouse *et al*, 1983).

In addition to NE, experimental evidence suggests that several neuropeptides are expressed within subsets of LC neurons. For example, galanin (Gal) is expressed in up to 80% of LC neurons (Holets *et al*, 1988). Gal modulates many behaviors in the brain such as wake/sleep states, nociception, feeding, and parental behavior (Lang *et al*, 2015). LC neurons co-expressing NE and Gal are found throughout the LC, but are most densely localized to the dorsal and central LC (Holets *et al*, 1988). Another neuropeptide co-expressed in LC neurons is Neuropeptide Y (NPY), which is present in a smaller population of LC neurons (~20 %) in the dorsal portion of the LC (Holets *et al*, 1988). Many other neuropeptides such as acetylcholinesterase, neurotensin, and vasoactive intestinal protein have been detected in small subsets of neurons in the LC (Sutin and Jacobowitz, 1991).

The LC receives inputs from several brain regions that promote diversity of LC function during different behaviors. Specifically, the LC receives multiple varied inputs, which all influence LC firing to different extents. In the majority of instances, the neurotransmitter involved in these inputs to the LC is known; however, some neurotransmitters in these synaptic inputs remain unidentified. Major brain structures, for example, areas of the neocortex, amygdala, hypothalamus, brainstem, and spinal cord project to the LC (Figure 1 adapted from Schwarz and Luo (2015)).

Neocortex. The parietal, temporal, infralimbic, insular, and frontal cortices project and provide limited input to the LC (Arnsten and Goldman-Rakic, 1984; Cedarbaum and Aghajanian, 1978; Luppi *et al*, 1995). Moreover, there is a strong reciprocal connection between the LC and the prefrontal cortex (PFC) (Jodo and Aston-Jones, 1997; Jodo *et al*, 1998; Singewald and Philippu, 1998). The projection from PFC

to the LC is suggested to provide tonic activation of the LC (Jodo *et al*, 1998). Although the neurotransmitter responsible for this activation is unclear, glutamate maybe involved given that NMDA receptors are expressed in LC neurons (Samuels and Szabadi, 2008).

Amygdala. The LC receives an input from the central nucleus of the amygdala (CNA) (Cedarbaum *et al*, 1978; Singewald *et al*, 1998; Wallace *et al*, 1989). The projection from CNA to the LC is suggested to be involved in the observed increase in LC activity in response to stressful stimuli (Berridge and Waterhouse, 2003). For example, neurons containing CRF in the CNA, project to the LC and activate these cells in response to stress (Van Bockstaele *et al*, 1998).

Hypothalamus. The ventrolateral preoptic area of the hypothalamus sends an inhibitory projection to the LC via its GABAergic neurons (Cedarbaum *et al*, 1978; Lee *et al*, 2005; Simson, 2001; Steininger *et al*, 2001). Furthermore, the paraventricular nucleus (PVN) of the hypothalamus, projects to the LC (Aston-Jones *et al*, 1986; Cedarbaum *et al*, 1978; Luiten *et al*, 1985; Luppi *et al*, 1995; Reyes *et al*, 2005; Simson, 2001; Swanson and Sawchenko, 1980). CRF has been suggested as the primary neurotransmitter in the projection to the LC, since excitatory CRF immunoreactive fibers in the PVN have been found to project to, and increase the activity of the LC (Reyes *et al*, 2005). The lateral hypothalamic/perifornical area projects to the LC (Cedarbaum *et al*, 1978; Lee *et al*, 2005) with fibers that contain orexin peptides (Date *et al*, 1999; Espana *et al*, 2005; Horvath *et al*, 1999; Peyron *et al*, 1998). Administration of orexin into the LC has been found to increase cell firing (Date *et al*, 1999; Hagan *et al*, 1999), suppress REM sleep, and increase wakefulness (Bourgin *et al*, 2000). The tuberomamillary nucleus (TMN), which is mainly composed of histaminergic neurons, has been found to

project to the LC (Iwase *et al*, 1993; Lee *et al*, 2005). TMN neurons are known to promote wakefulness, and histamine H_3 receptors have been identified on the cell bodies of LC neurons, where they inhibit NE release (Haas and Panula, 2003; Pieribone *et al*, 1994).

Brainstem. The ventral tegmental area (VTA) neurons project to the LC (Beckstead *et al*, 1979; Deutch *et al*, 1986; Oades and Halliday, 1987; Ornstein *et al*, 1987; Simon *et al*, 1979; Swanson, 1982). These VTA neurons are dopaminergic and may contribute to the maintenance of arousal (Samuels *et al*, 2006, 2007). Different areas of the raphe nuclei project to the LC. Strong evidence suggests that the serotonergic neurons of the dorsal raphe project to the LC (Cedarbaum *et al*, 1978; Kim *et al*, 2004; Luppi *et al*, 1995; Pasquier *et al*, 1977; Sim and Joseph, 1993; Simson, 2001; Vertes and Kocsis, 1994), and this input is likely related to the wakefulness-promoting roles of the dorsal raphe. The raphe magnus neurons have been reported to project to the LC (Sim and Joseph, 1992), and this connection is thought to be related to the modulation of nociception (Sim *et al*, 1992). Cholinergic neurons from pedunculopontine (PPT) and laterodorsal tegmental nuclei (LDT) have been found to project to the LC (Jones and Yang, 1985). PPT and LDT neurons are known to be active during wakefulness or REM sleep (el Mansari *et al*, 1989; Jones, 2005; Kayama *et al*, 1992). Local microinfusion of acetylcholine or acetylcholine receptor agonists into the LC increase the firing of LC neurons (Egan and North, 1985; Engberg and Svensson, 1980), suggesting an excitatory role for the PPT and LDT projection to the LC. LC receives an input from neurons of the periaqueductal grey matter (PAG) in the midbrain (Cedarbaum *et al*, 1978; Lee *et al*, 2005; Luppi *et al*, 1995; Simson, 2001), particularly from the dorsolateral cell column of

the PAG (Cameron *et al*, 1995). PAG neurons that project to the LC are diverse in neurotransmitter content; ventral PAG neurons are dopaminergic (Lu *et al*, 2006), and are suggested to be involved in the activation of LC neurons during wakefulness. In addition, the ventral and ventrolateral PAG are suggested to be involved in the regulation of sleep-wakefulness state *via* inhibitory glycinergic projections to the LC (Rampon *et al*, 1999). Tract-tracing and electrophysiology studies have revealed that major inputs to the LC are found in two structures, the nucleus paragigantocellularis (PGi) and the perifascicular area of the nucleus prepositus hypoglossi (PrH), both located in the rostral medulla (Aston-Jones *et al*, 1991; Luppi *et al*, 1995). The projection from the PrH to the LC contains GABAergic neurons, and is thus inhibitory to LC neuronal activity (Ennis and Aston-Jones, 1989a, b). This GABAergic projection is likely to be involved in the inhibition of LC activity during REM sleep (Verret *et al*, 2006). On the other hand, PGi projections to the LC are excitatory *via* the release of glutamate (Simson, 2001). In addition to the GABAergic and glutamatergic projections to the LC from the rostral medulla, both the PrH and the PGi innervate the LC with fibers containing the endogenous opiate enkephalin (Drolet *et al*, 1992; Johnson *et al*, 2002; Van Bockstaele, 1998). These projections activate opiate receptors found in high concentrations in the LC to inhibit cell firing (Toyama *et al*, 1974; Van Bockstaele, 1998), and the administration of endogenous opioids or opiate agonists inhibits spontaneous firing of the LC (Illes and Norenberg, 1990; Korf *et al*, 1974; Mansour *et al*, 1994; Pepper and Henderson, 1980; Pert and Snyder, 1976b; Valentino and Wehby, 1988c; Williams and North, 1984).

Spinal cord. The LC receives projections from the dorsal horn of the spinal cord (Cedarbaum *et al*, 1978; Craig, 1992). It has been suggested that this pathway may

communicate information relating to the detection of nociceptive and/or thermal stimuli from sensory spinal nuclei (Craig, 1992). However, it is unclear the neurotransmitter responsible for this communication (Samuels *et al*, 2008).

Electrophysiological studies on LC neuronal activity have determined that LC neurons exhibit two distinct modes of activity: tonic and phasic (Aston-Jones and Cohen, 2005). The tonic rate of LC neuronal discharge has been correlated with behavioral arousal (Aston-Jones and Bloom, 1981a; Foote *et al*, 1980). Additionally, pharmacological manipulation of tonic LC neuronal activity has been shown to impact electroencephalographic indices of arousal (Berridge and Foote, 1991; Berridge *et al*, 1993). LC neurons respond phasically to a broad range of sensory stimuli (Aston-Jones and Bloom, 1981b; Foote *et al*, 1980). The phasic response precedes the orientation towards the stimulus, and may be a signal to redirect behavior towards salient stimuli. The phasic pattern of discharge is characterized by a brief excitatory component followed by a longer duration of inhibition. This is consistent with evidence for a role of excitatory amino acid (i.e., glutamate) neurotransmission in LC sensory responses (Ennis *et al*, 1992), as these agents produce a robust, albeit brief, activation of LC neurons. The extensive distribution of the axonal network of the LC provides tonic and phasic arousal-related signals to the forebrain. These signals have been suggested to direct attention toward behaviorally relevant sensory information (Aston-Jones *et al*, 2005). It was originally hypothesized that the primary role of the LC-NE system was to regulate arousal and sleep-wake cycles (Berridge *et al*, 2012); however, the two distinct modes of LC activity (i.e. tonic and phasic) indicate a more specific role in the cognitive processing of relevant sensory information (Aston-Jones *et al*, 2005). For example, phasic firing of

LC neurons, specifically in response to behavioral tasks that are relevant for sensory stimuli, mediates focused attention and optimal task performance; yet, when rewards associated with task performance based on the current attentional strategy cease, LC activity switches to a high tonic mode until a more favorable strategy can be determined. The shift from phasic to high tonic LC discharge has been suggested to promote behavioral flexibility, disengaging animals from attention to specific stimuli and ongoing behaviors and favoring scanning the environment for stimuli that promote alternate, more rewarding behaviors (Aston-Jones *et al*, 2005). Notably, the prefrontal cortex (PFC) mediates executive functions such as cognitive flexibility, a function thought to be influenced by the LC-NE projections that target PFC neurons (Arnsten, 2011). Pharmacological studies suggest a relationship between LC and PFC activity which resembles an inverted U-shaped function, such that the PFC function requires an optimal level of LC input, beyond which increases in LC drive negatively impact PFC function as a result of the interaction of higher levels of NE with lower-affinity adrenergic receptors (Arnsten, 2011).

The Locus Coeruleus –Norepinephrine System, Stress, and CRF

Several studies suggest that stressors that trigger the initiation of the hypothalamic-pituitary-adrenal (HPA) response to stress, activate in parallel the LC-NE system. The parallel engagement of these two systems helps to coordinate the endocrine and cognitive limbs of the stress response (Valentino and Van Bockstaele, 2008). LC-NE activation by stressors is mediated by CRF release in the LC because LC-NE activation is blocked by administration of CRF antagonists into the LC (Valentino and Wehby, 1988b). CRF was initially characterized as the paraventricular hypothalamic

neurohormone that initiates anterior pituitary adrenocorticotropin secretion in response to stressors (Vale *et al*, 1981). The neuromodulatory actions of CRF are mediated through two distinct receptors, CRF_1 and CRF2, differentially distributed throughout the brain (Primus *et al*, 1997; Van Pett *et al*, 2000). CRF_1 activation, which has been clearly associated with promotion of stress-response behaviors and activation of CRF_2, originally thought to dampen stress sensitivity, may actually function in other stress-related responses, including stress-associated learning (Bale and Vale, 2004; Hauger *et al*, 2009). Electron microscopic studies have shown that CRF-immunoreactive axon terminals form synaptic specializations with LC dendrites in the core and peri-LC, with a majority of these synapses being asymmetric, and thus excitatory in function (Van Bockstaele *et al*, 1996c). Many CRF axon terminals in the LC co-localize glutamate, while fewer co-localize enkephalin and GABA (Tjoumakaris *et al*, 2003; Valentino *et al*, 2001). CRF axon terminals are also found next to unlabeled terminals that form synaptic specializations with LC dendrites, providing a mechanism for indirect presynaptic modulation of LC activity (Van Bockstaele *et al*, 1996c). During periods of stress, CRF is released from a number of brain regions. Notably, CRF afferent to LC dendrites in the peri-LC derive from the CNA and the PVN of the hypothalamus (Reyes *et al*, 2005; Valentino *et al*, 1992; Van Bockstaele *et al*, 1998; Van Bockstaele *et al*, 1999), whereas those afferent to the nuclear LC include the nucleus paragigantocellularis, Barrington's nucleus and the paraventricular hypothalamic nucleus (Reyes *et al*, 2005; Valentino *et al*, 1996; Valentino *et al*, 1992). Hypothalamic CRF neurons that project to the LC are a distinct population from those that project to the median eminence to regulate adrenocorticotropin release (Reyes *et al*, 2005).

CRF increases spontaneous LC discharge rates when locally administered into the LC, either *in vivo* or *in vitro* (Curtis *et al*, 1997; Jedema and Grace, 2004). CRF in the LC interacts with G_s-protein coupled CRF receptor 1 (Reyes *et al*, 2007). This stimulates adenylate cyclase (Bale *et al*, 2004), ultimately leading to depolarization of LC neurons through a cyclic AMP-dependent reduction in potassium conductance (Jedema *et al*, 2004; Schulz *et al*, 1996). LC activation by CRF is associated with c-fos expression by LC neurons and norepinephrine release in terminal fields (Page and Abercrombie, 1999; Rassnick *et al*, 1998). Notably, *in vivo* studies have shown that intracerebroventricular or direct CRF administration into the LC can mimic the effects of stressors on LC neuronal activity (Valentino *et al*, 1988b). LC activation by CRF is translated to activation of cortical electroencephalographic (EEG) activity, indicative of increased arousal (Curtis *et al*, 1997) and enhanced behavioral flexibility in a rat attentional set-shifting task (Snyder *et al*, 2012). In addition to increasing spontaneous or tonic LC discharge rates, CRF attenuates phasic sensory-evoked LC activity (Valentino and Foote, 1987, 1988a). This may occur through presynaptic inhibition as suggested by electron microscopic studies that show that CRF axon terminals also synapse with synaptic specializations in LC dendrites, possibly providing a mechanism for the indirect presynaptic modulation of LC activity (Van Bockstaele *et al*, 1996c). Given the evidence for co-localization of CRF and glutamate in axon terminals in the LC, and convergence onto common LC dendrites, there are multiple potential mechanisms for this interaction. The net effect of CRF on LC neurons is to shift the mode of LC discharge to a high tonic-low phasic state (Valentino *et al*, 1987, 1988a; Valentino *et al*, 2008). This mode of firing has been associated with high

arousal (Butler *et al*, 1990), decreased focused attention, and increased behavioral flexibility or going off-task in a search for optimal outcomes (Aston-Jones *et al*, 2005).

Under normal physiological conditions, the LC-NE system is not under tonic regulation by endogenous CRF because CRF antagonists have no effect on either LC discharge rates or norepinephrine release in LC upstream targets (Curtis *et al*, 1994; Page *et al*, 1999). However, there is important evidence suggesting that acute stressors elicit the release of endogenous CRF within the LC to activate LC neurons during acute stress. For example, hypotensive challenge, which activates the HPA axis, mimics the effects of CRF on tonic and phasic LC discharge (Valentino *et al*, 1988b). In addition, stressors such as non-noxious visceral stimuli (colon distention) increase LC discharge rates, and cortical EEG activity by a CRF-dependent mechanism (Lechner *et al*, 1997). Taken together, the anatomical and electrophysiological evidence supports a model whereby acute stress engages CRF inputs to the LC to bias activity towards a high tonic state that would favor increased arousal and behavioral flexibility.

Locus Coeruleus Co-regulation during Acute Stress: CRF and Endogenous Opioids

Anatomical and electrophysiological evidence suggests that LC neurons are co-regulated by CRF and the endogenous opioid, enkephalin during acute stress. The LC expresses μ, δ and κ-opioid receptors. Notably, μ-opioid receptors (MOR) are localized post-synaptically, while δ and κ are mainly expressed pre-synaptically in the LC (Kreibich *et al*, 2008; Reyes *et al*, 2009; Van Bockstaele *et al*, 1995; Van Bockstaele and Chan, 1997; Van Bockstaele *et al*, 1996a). MORs are highly expressed by LC neurons to potently inhibit activity (Mansour *et al*, 1994; Pert *et al*, 1976b; Williams *et al*, 1984). The LC receives projections from the endogenous opioid ENK system that

innervates both the LC core and pericoeruleular region (Van Bockstaele *et al*, 1995; Van Bockstaele *et al*, 1997). ENK projections arise from the rostral medullary nuclei including the PGi and PrH (Aston-Jones *et al*, 1986; Drolet *et al*, 1992). ENK axon terminals co-localizes with GABA (Van Bockstaele, 1998; Van Bockstaele *et al*, 1997) and glutamate axon terminals (Barr and Van Bockstaele, 2005; Van Bockstaele *et al*, 2000) on LC dendrites. The endogenous opioid dynorphin (DYN) has afferents to the LC that arise from the CNA (Reyes *et al*, 2008), which project onto κ-opioid receptors (KORs) that are located pre-synaptically in axon terminals of the LC containing glutamate transporter or CRF (Kreibich *et al*, 2008). Electrophysiological evidence suggests that endogenous opioids are not tonically released onto LC under normal physiologic conditions, because opioid antagonist administration has no effect on LC baseline activity (Valentino and Wehby, 1989). In general, opioid receptor agonists decrease LC firing rates. However, receptor specificity exists in how these responses are changed. MOR-agonists are inhibitory on tonic LC neuronal discharge *in vitro*, as well as *in vivo* (Aghajanian and Wang, 1987; Valentino *et al*, 1988c; Williams *et al*, 1984). For example, the local application of morphine or enkephalin (ENK) inhibits the spontaneous activity of LC neurons through activation of MORs and hyperpolarization with increased potassium conductance (Aghajanian *et al*, 1987; Bird and Kuhar, 1977; North and Williams, 1985). Moreover, phasic responses are facilitated, increasing the signal to noise ratio of LC evoked activity (Valentino *et al*, 1989). In contrast, KOR agonist administration diminishes phasic LC activity, as well as stress-mediated CRF activation of the LC (Kreibich *et al*, 2008).

LC dendrites receive convergent input from CRF and enkephalin-containing axon terminals and co-localize MOR and CRF_1 (Reyes *et al*, 2007; Tjoumakaris *et al*, 2003; Xu *et al*, 2004). Recordings of LC neuronal activity during acute stress indicate that stressors release both CRF and enkephalin to co-regulate LC activity in opposing manners. The net overall effect is a CRF-mediated excitation. However, removal of this influence using a CRF antagonist reveals a naloxone-mediated inhibition. This inhibition restrains the CRF activation and also facilitates a return towards baseline activity when the stressor is terminated (Curtis *et al*, 2001; Curtis *et al*, 2012). These CRF-opioid interactions adjust the activity and reactivity of LC neurons so that the level of arousal and processing of sensory stimuli are optimized to facilitate adaptive behavioral responses to stressors (Figure 2 adapted from (Valentino and Van Bockstaele, 2015)).

Sex Differences in Response to Opioids

Sex differences in opioid sensitivity have been reported in laboratory animals and humans, with females being less sensitive to morphine-induced analgesia and more sensitive to experimentally-induced pain compared to males (Kest *et al*, 2000a). For example, of 50 analgesic assay comparisons, male rodents exhibited more robust analgesia than females in 28 (56%) assays, females showed greater analgesia in 2 (4%) assays, and there were no sex differences in 20 (40%) assays (Kest *et al*, 2000b). In addition, male animals typically have a greater analgesic response to opioids compared to females (Craft, 2003). Consistent with data from studies in rodents, studies in humans determined that women exhibit higher pain intensity after surgery and have larger weight–adjusted morphine requirements than men, to achieve a similar degree of analgesia(Cepeda and Carr, 2003). The higher requirement of morphine for females to

improve pain suggests less sensitivity to the drug. Taken as a whole, these findings suggest a decreased sensitivity of females to the analgesic effects of opiates.

Sex Differences in Opioid Regulation of the LC: a Potential Determinant of Stress Vulnerability

Given the role of endogenous opioids in restraining the stress response of the LC system, excessive or insufficient opioid influence in the LC may have pathological implications. Individual differences in either enkephalin expression or MOR sensitivity are potential determinants of stress vulnerability or vulnerability to pathology. For example, decreased MOR function may predispose to hyperarousal symptoms of stress related neuropsychiatric disorders because of a decreased ability to temper CRF effects in the LC. On the other hand, greater MOR sensitivity could be predicted to protect from hyperarousal symptoms in stress-related pathologies. Importantly, sex and hormonal status can affect expression, sensitivity, and trafficking of MORs, as well as enkephalin expression (Craft, 2008; Gonzales *et al*, 2011; Milner *et al*, 2013). However, these relationships are not well understood and may be dependent on the species, brain region, or endpoint. Notably, it is well documented that females are less responsive to opiates (Ji *et al*, 2006; Kepler *et al*, 1991; Wang *et al*, 2006) and this would be consistent with reports that stress-related diseases characterized by hyperarousal are more prevalent in females (Breslau, 2002; Kessler *et al*, 1994; Kessler *et al*, 1995). Given the role of endogenous opioids in restraining the stress response of the LC system, decreased opioid sensitivity in females could increase the stress-sensitivity of this system, and enhance female vulnerability to stress.

Conclusion

Given that there are sex differences in stress-related diseases characterized by hyperarousal symptoms that are more prevalent in females compared with males (Breslau, 2002; Kessler *et al*, 1994; Kessler *et al*, 1995), observations indicating that females are less responsive to opiates (Ji *et al*, 2006; Kepler *et al*, 1991; Wang *et al*, 2006), and the important role of endogenous opioids in restraining stress-related activation of LC neurons, Chapter 2 investigates sex differences in MOR expression in the LC, and the neuronal and behavioral consequences of MOR activation in the LC of male and female rats. Chapter 3 further explores how molecular and cellular sex differences of the LC-MOR system translates to changes in neuronal activity in PFC regions that guide cognitive strategies. Ultimately, the studies in Chapters 2 and 3, which examine sex differences in opioid regulation of the LC may have a significant impact on the field by helping to elucidate a molecular mechanism that explains why there are higher rates of females being diagnosed with stress-related psychiatric disorders.

Figures and Legends

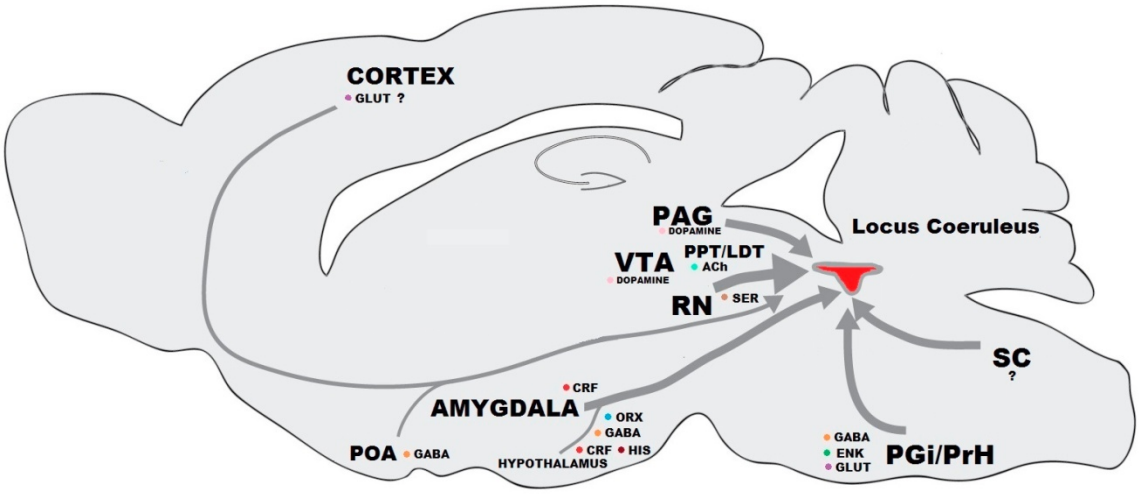

Figure 1. Afferent projections to the LC-NE system.

A saggital schematic illustrating the LC in the brainstem (red), and brain regions that provide the largest fraction of direct input to the LC-NE neurons (dark gray arrows) and their respective neurotransmitters. Abbreviations: PAG, periaqueductal gray; VTA, ventral tegmental area; RN, raphe nucleus; POA, preoptic area; PPT, pedunculopontine tegmental nuclei; LDT, laterodorsal tegmental nuclei; PGi, nucleus paragigantocellularis; PrH, nucleus prepositus hypoglossi; SC, spinal cord; ORX, orexin; HIS, histamine; ENK, enkephalin; GLUT, glutamate; CRF, corticotropin releasing factor; Ach, acetylcholine; GABA, gamma-Aminobutyric acid; SER, serotonin. Figure adapted from Schwarz *et al* (2015).

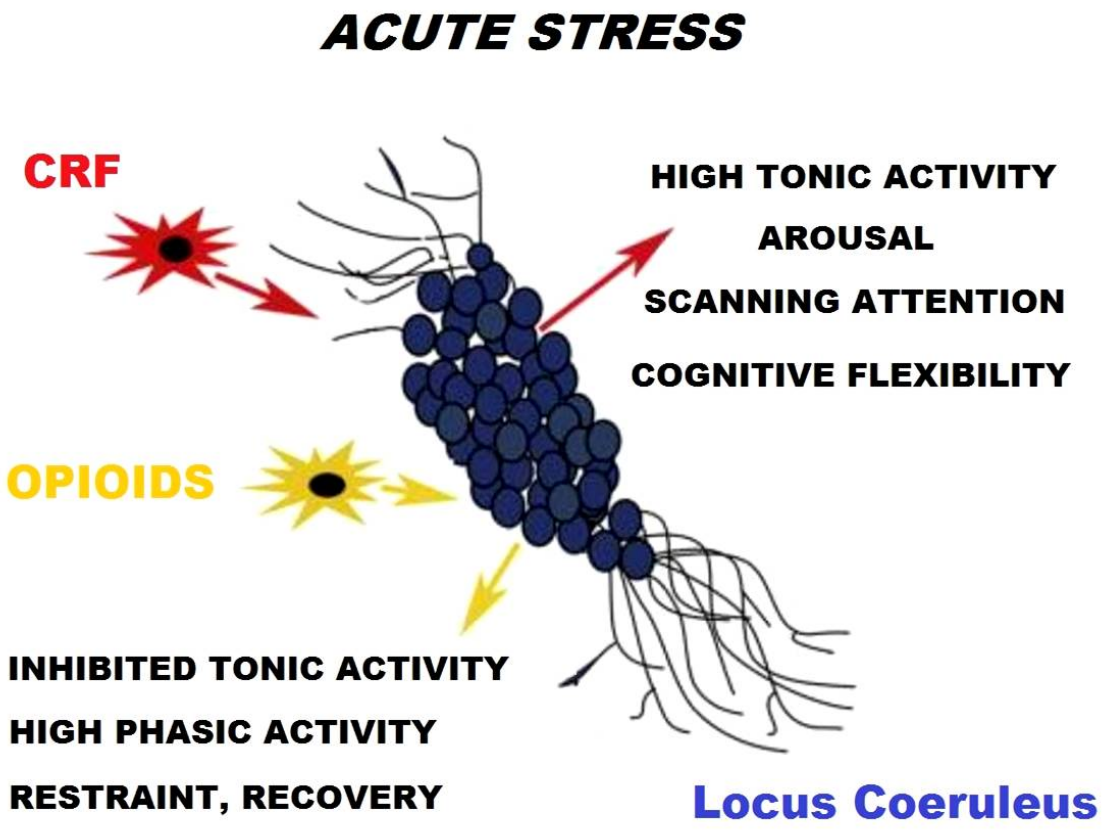

Figure 2. The opposing regulation model of LC activity during acute stress. During acute stress, both CRF (red) and endogenous opioids (yellow) afferents to the LC are engaged. The net effect is a shift of LC activity towards high tonic activity that is associated with increased arousal, scanning attention, and behavioral flexibility. Endogenous opioids act as a restraint and facilitate recovery of neuronal activity to pre-stress levels after the stress is terminated. Figure adapted from Valentino et al (2015).

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CHAPTER 2: SEX DIFFERENCES IN μ-OPIOID RECEPTOR OF THE RAT LOCUS COERULEUS AND THEIR COGNITIVE CONSEQUENCES

Abstract

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 over activity 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 twice as prevalent in females compared to males (Kessler *et al*, 1994; Kessler *et al*, 1995). 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-norepinephrine (LC-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 *et al*, 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*, 1976a). MOR activation robustly inhibits LC discharge (Williams *et al*, 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, 1988a; 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) has been proposed as one mechanism underlying LC-NE dysregulation, and 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*, 2000b). 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 using a two-way repeated measure ANOVA with time as the repeated measure. In addition, the area under the time-effect curve (0–300s after injection) for the 10 pg dose was calculated and compared between sexes using a Student's t-test (two tailed).

Western Blotting. Male and female rats were decapitated and brains quickly removed and frozen. Thick (1000μm) 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*, 1996a). 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 7).

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 of 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 seconds 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 minutes 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 ensured that each animal experienced sufficient trials for the transition from one type of discrimination to the next, and to ensure it was 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 of 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 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 inter-

trial interval were also calculated and the numbers 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 (10pg) 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 using one way ANOVAs with Tukey's HSD post hoc for individual comparisons. An alpha level of po0.05 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 10pg 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±0.2Hz, n=20cells/14 rats) and females (1.74±0.22Hz, n=26 cells/18 rats; $F(1, 44) = 1.4$, $p > 0.05$). Figures 3a and 3b show 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 3c and 3d). 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 3b). The sex difference was also apparent as a decreased area under the curve describing the effect over time (males: 23017±1105 vs females: 15837±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 8). 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 8).

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 4). Figure 4a 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 to female rats ($F(1, 20) = 4.5$, $p=0.045$; each group n=11; Figure 4b). 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 4c). 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 9).

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 5 shows the mean number of trials to reach the criterion for each stage of the task for males (Figure 5a) and females (Figure 5b) 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 5a and 5b). 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 pg 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 10).

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 sec and 1334 ± 247

sec, respectively to complete the REV stage and males took 1050 ± 202 sec and 1202 ± 271 sec 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 5c and 5d). 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 5e and 5f). 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 5c and 5d). 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 5e). There was a main effect of treatment for regressive errors ($F(3, 33) = 4.7$ $p < 0.01$), and a sex X 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 5f). 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 6a 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 11), only the effects of accurate vs inaccurate injections of the 3 pg dose were statistically compared (Figures 6b–6d). DAMGO injections outside of the LC had no effect on performance in any task stage for either males or females (Figures 6c and 6d). 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 6c). 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 6d).

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, 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*, 2000b; Wang *et al*, 2014). Given the 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 over activity that underlies hyperarousal symptoms of stress related psychiatric diseases (Gold *et al*, 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*, 1976a; Williams *et al*, 1984). This regulation is not tonically active because opioid antagonists do not alter LC spontaneous discharge rates (Abercrombie *et al*, 1988a; Valentino *et al*, 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*, 2000b). 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 suggests decreased LC-MOR levels in the female rather than a decrease in agonist affinity.

Molecular Basis for Sex Differences in LC Post-synaptic 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 protein have been described previously (Cheng *et al*, 1996; Surratt *et al*, 1994; Van Bockstaele *et al*, 1996b). 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 7). 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, which 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), 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 together with evidence for decreased LC-MOR receptor expression and physiological function, the data suggests 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 to reverse in females. This is consistent with the findings 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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Figures and Legends

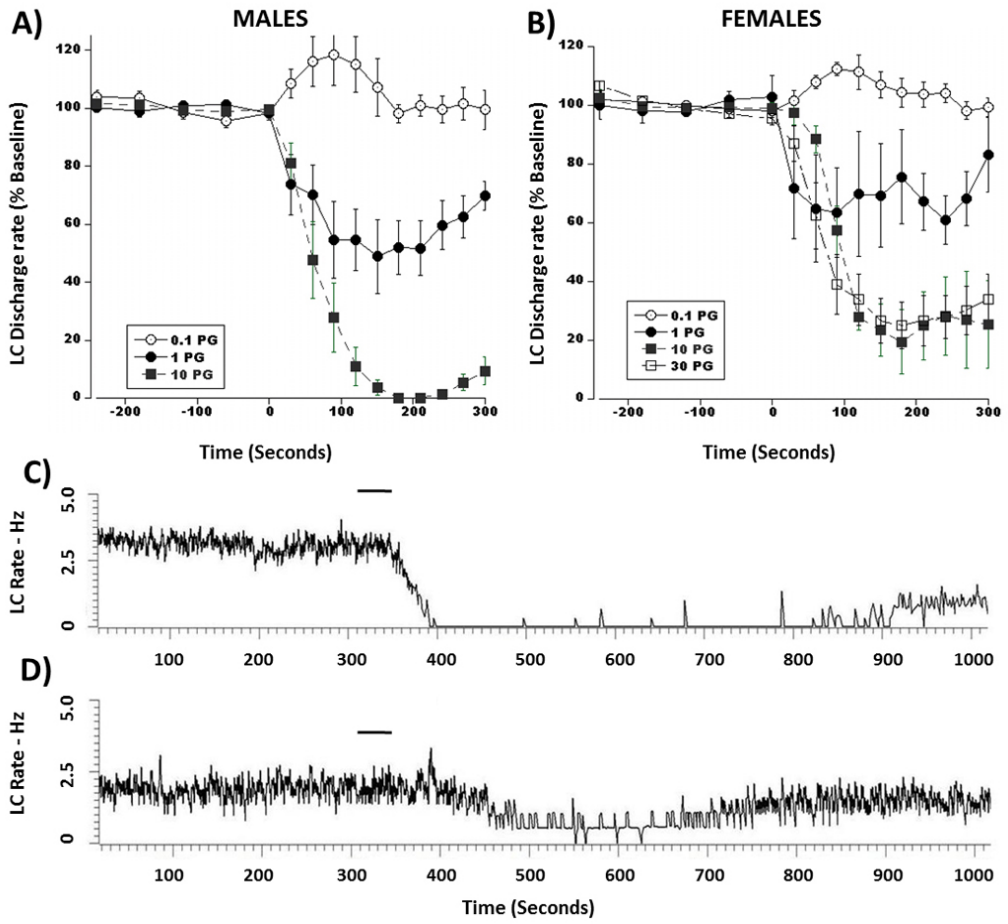

Figure 3. Dose-related inhibition of locus coeruleus (LC) neuronal discharge rate by DAMGO (D-Ala2, N-MePhe4, Gly-ol]-enkephalin) in male and female rats.

(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).

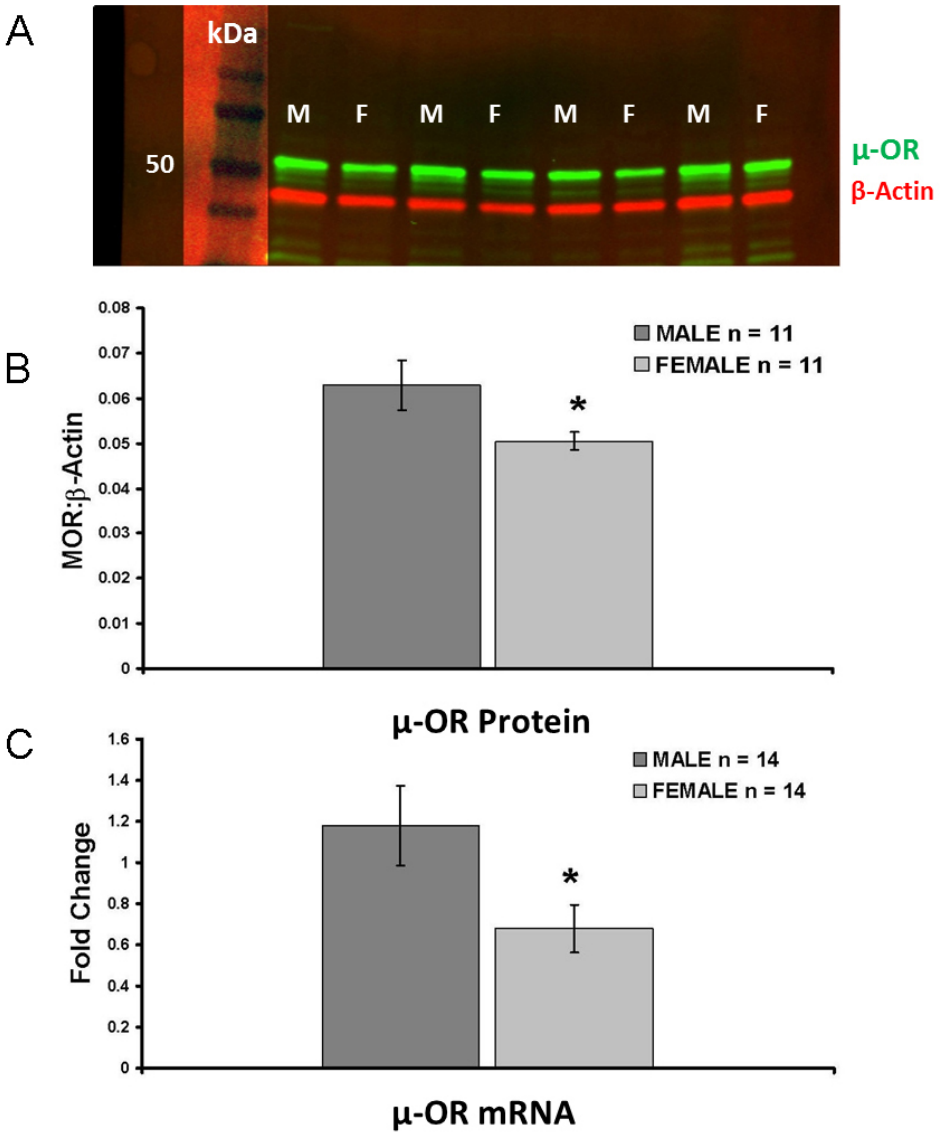

Figure 4. Sex differences in locus coeruleus-μ-opioid receptor (LC-MOR) protein and mRNA.

(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 ± SEM ;(n=14, each group). GAPDH was used as an endogenous control. *$p<0.05$.

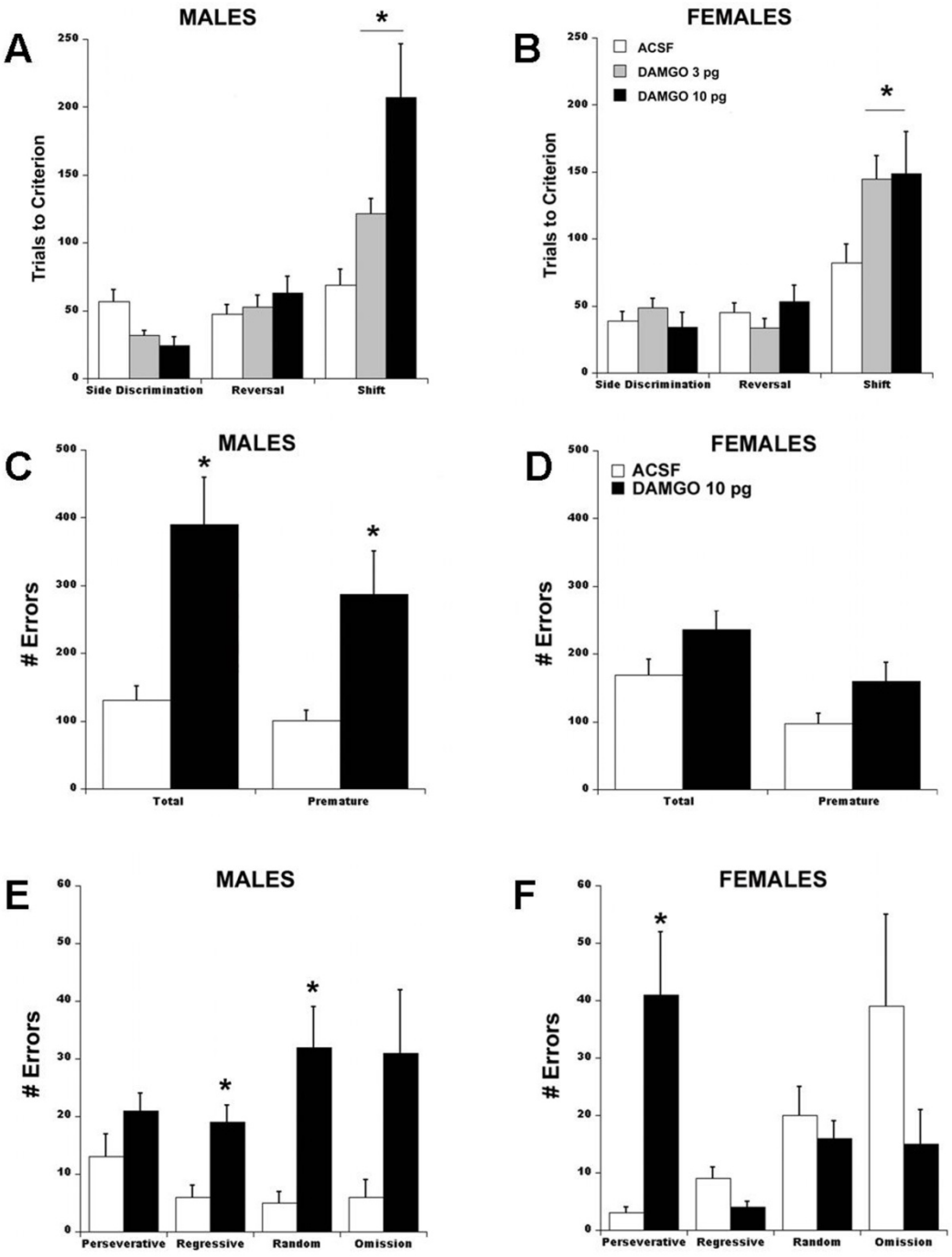

Figure 5. Sex differences in behavioral consequences of activating μ-opioid receptor (MOR) in the locus coeruleus (LC).

(A and B) Effects of ACSF and DAMGO (D-Ala2, N-MePhe4, Gly-ol]-enkephalin; 3 pg 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.

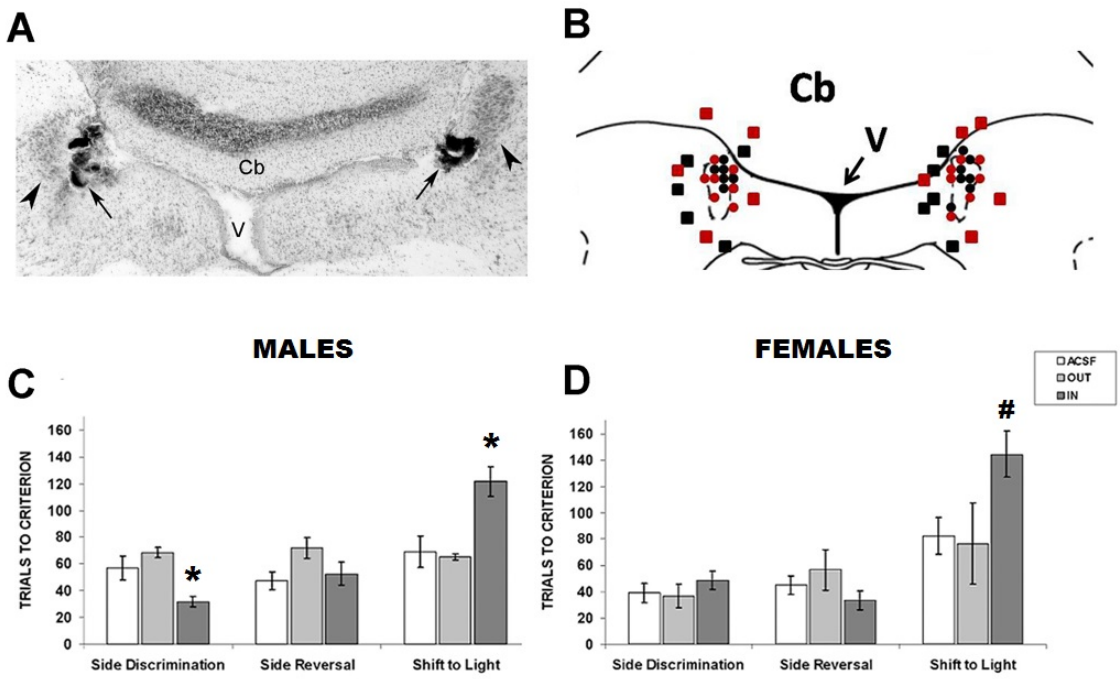

Figure 6. Regional specificity of DAMGO (D-Ala2, N-MePhe4, Gly-ol]-enkephalin) effects on strategy shifting.

(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.

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+0.36
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 X 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.

Supplemental Information

Supplemental Methods

Electrophysiological Studies. Rats were anesthetized with a 2% isoflurane-in-air mixture administered through a nose cone and positioned in a stereotaxic instrument. Anesthesia was maintained at 1.0-1.5% throughout the experiment and body temperature was maintained at 37.5 C by a feedback controlled heating pad. Animals were positioned in a sterotaxic instrument and a craniotomy was performed 1.2 mm lateral to the midline and 3.6 mm caudal to lambda. The recording glass micropipette (2-4 um diameter tip, 4-7 Ohm) was filled with 0.5 M sodium acetate buffer saturated with 2% pontamine sky blue dye (PSB). The microinfusion pipette (60-90 um diameter tip) was angled at 30-45 degrees with the tip of the pipette 130-150 um dorsal to the tip of the recording pipette. Both pipettes were glued using a photo-polymerizing resin (Silux, 3M Dental Products, St. Paul, MN). DAMGO intra-LC infusions were made by applying pulses of pressure to the microinfusion pipette (15-25 psi, duration 10-30 ms) at a frequency of 0.2-1.0 Hz to deliver a total microinfusion volume of 30 nl. Neuronal signals were amplified, filtered, and monitored with an oscilloscope and a loudspeaker for localization of the LC. LC neurons were tentatively identified by their spontaneous discharge rates (0.5-5.0 Hz). LC single unit activity was recorded before, during, and after DAMGO microinfusion [0.1, 1.0, or 10.0 pg]. Only female rats were tested at [30.0 pg]. Only one dose of DAMGO was tested on a single cell in an individual rat. The recording site was marked by iontophoresis of PBS (-15 μA, 15 min) at the end of the experiment. Brains were removed, and frozen 30 μm-thick coronal sections were cut on a cryostat before being

mounted on gelatinized glass slides. The sections were stained with neutral red dye for localization of the PSB spot.

Western Blotting. LC tissue punches were homogenized and centrifuged. Protein content was determined using the BCA method. Protein extracts (10 μg) were subjected to SDS-PAGE gel electrophoresis and then transferred to polyvinylidene fluoride membranes (Immobilon-FL) as described (Curtis *et al*, 2006). Membranes were blocked with Odyssey buffer (diluted in PBS 1:1) and incubated with specific primary antibody overnight at 4^{o} C to detect MOR (1:1000, rabbit anti-MOR, Invitrogen). Following rinsing, membranes were incubated with infrared fluorescent secondary antibodies for one hour (1:5000, donkey anti rabbit IRDye 800CW, LiCor, Lincoln, NE). Membranes were scanned, and proteins were detected using the Odyssey Infrared Imaging System (LiCor). Following quantification of the target protein (MOR), membranes were incubated with the β-actin antibody (1:5000, mouse anti-β-actin, Sigma) for 90 minutes at room temperature and a fluorescent secondary antibody with a different infrared wavelength for one hour (1:5000 donkey anti-mouse IRDye 680CW, LiCor). Molecular weights were determined using the Biorad Precision Plus Protein Standards.

Quantitative Polymerase Chain Reaction (PCR) Analysis of MOR mRNA. *LC tissue punches lyses and homogenizations*. Samples were homogenized with mortar and pestle in a Lysis buffer and a series of washing buffers (AMBION, Life Technologies). DNA treatment was performed using the final RNA product plus DNAse 1 buffer and DNAse1 (AMBION, Life Technologies). Following RNA purification, integrity of the samples was measured by spectrometry. All samples had A260/280 ratios between 1.8-2.0. cDNA synthesis was performed using a High capacity cDNA reverse

transcription kit (Applied Biosystems, Foster City, CA). The cDNA samples were then diluted to a concentration of 20 ng/µl and stored for real-time PCR.

Real Time PCR. Each well consisted of a total volume of 20 µl reaction mixture containing 10 µl of universal master mix, one µl of gene expression assay, 2 µl of cDNA and 7 µl of RNAse-free H2O (QIAGEN, Germany). Each sample was loaded onto 96-well plates and run in triplicates. Reactions were performed using an AB1 7500 Real-time PCR System (Applied Biosystems, Foster city CA) under the manufacturer's recommended settings.

Operant Chamber. Each box was equipped with a fan to provide air ventilation and block out potentially distracting outside noises. Each chamber contained two levers on either side of a food receptacle where grain-based food pellet rewards (45 mg; BioServ, Frenchtown, NJ, USA) could be delivered. A stimulus light was positioned above each lever, and a house light was positioned top-center on the wall opposite the levers. Data was recorded and stored onto a PC computer via an interface module.

OSST Training. Rats were first trained to press one of two levers for food reinforcement with one press resulting in food presentation. On the following day, rats were trained to press the lever opposite to the one from the first day for food reinforcement. On the third training day, rats were introduced to the trial structure of the task, under conditions with no discernable rule. On each trial, the house light and both stimulus lights were illuminated for 15 seconds during which rats could press one of the two levers for food reward. One of the two levers was randomly selected to deliver reward one, three, or five trials in a row, such that over many trials both levers were equally likely to deliver a reward. This was done to encourage animals to press both

levers during training while not allowing them to use spatial or light cues to reliably predict which lever would deliver a reward. If the correct lever was pressed within 15 seconds of trial initiation, a single reward pellet was delivered and all lights remained illuminated for 3 seconds followed by darkness for a 5-seconds timeout before initiation of the next trial. If the incorrect lever was pressed within 15 seconds of trial initiation, no reward was delivered and all lights were immediately shut off for a 10 second timeout before initiation of the next trial. If neither lever was pressed within 15 seconds of trial initiation, all lights were shut off for a 5-seconds timeout before initiation of the next trial. Additionally, if either lever was pressed during a dark timeout period, the initiation of the following trial would be reset to occur 5 seconds after the time of this lever press. Trials continued until an animal achieved 50 correct trials. Each animal's side bias was determined to be toward the lever on the side that the animal pressed on the majority of trials. The following day was the testing day.

MOR antibody specificity controls. The MOR antibody (1:1000, rabbit anti-MOR, Invitrogen) used for protein quantification was tested for specificity using cortical and heart tissue. The MOR protein is not expressed in the rat heart (Wittert *et al*, 1996) whereas, it is abundantly expressed in cortex.

Supplemental Figures and Legends

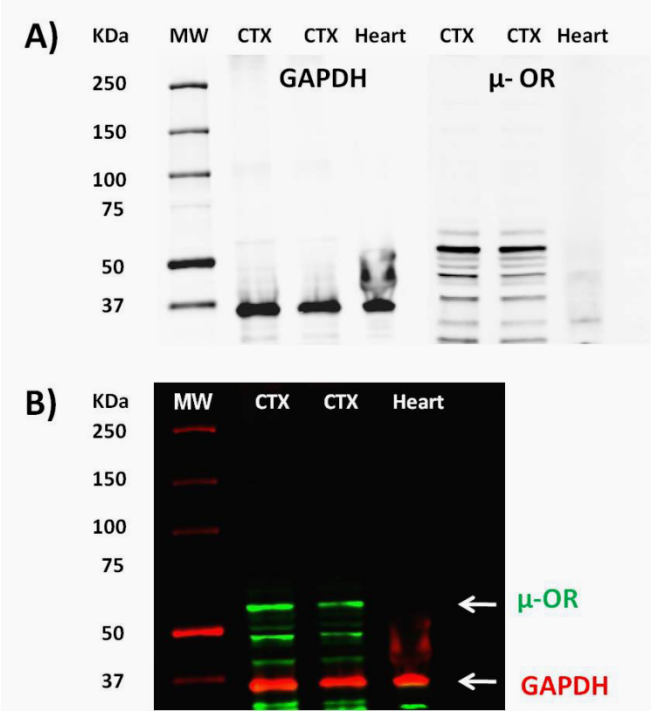

Figure 7. MOR antibody specificity control.

A Representative blot, A) Black and white, and B) Odyssey Infrared Imaging System (LiCor) showing MOR protein (green) and GAPDH (red) immunoreactive bands (52 and 37 KDa, respectively) from cortical and heart tissue. The antibody against MOR detected the protein in cortex but not in heart lysates. The same blots were probed to detect GAPDH to confirm that the protein was loaded in the cortical and heart lanes. GAPDH was detected in cortical and heart lysates of the same blot.

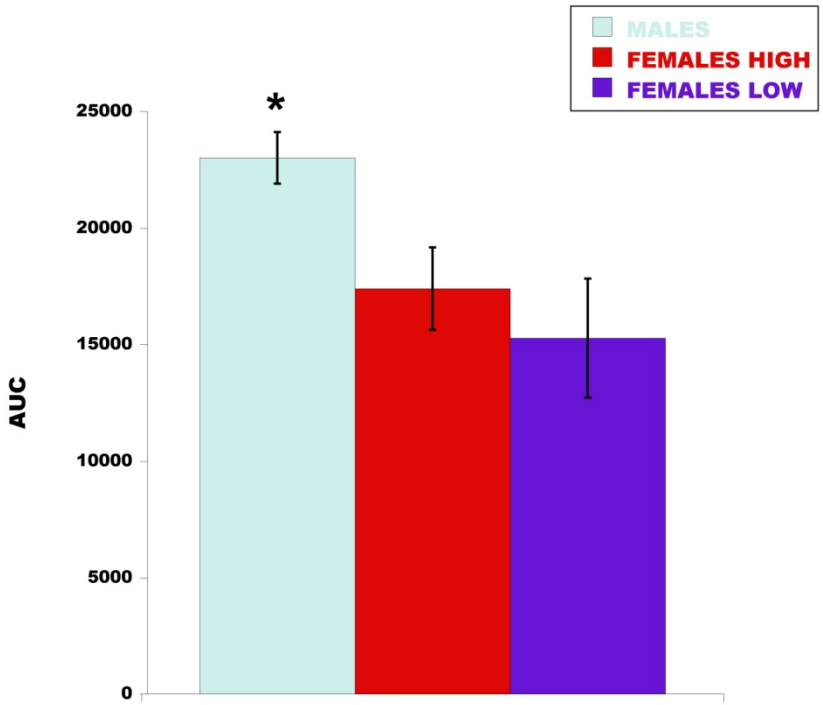

Figure 8. Effect of intra-LC DAMGO on LC firing rate in male rats and female rats at different estrogen statuses.

Bar graphs show the magnitude of the effect of DAMGO on LC discharge rate represented as the area under the curve of the percent change from baseline after DAMGO microinfusion into the LC. Males were administered 10 pg DAMGO (7 cells/5 rats). Because all but 2 female rats that were administered 10 pg DAMGO were proestrus, the effects of the 10 pg and 30 pg doses were pooled. Red bars are the mean of 11 cells/6 rats in proestrus. Five of the cells were exposed to 10 pg and 6 were exposed to 30 pg. Blue bars are the mean of 6 cells/5 rats in diestrus/estrus. Two of these cells were exposed to 10 pg and 4 were exposed to 30 pg. The mean AUC was greater in males when compared to female groups at either high or low estrogen (One Way ANOVA: $F(2, 23) = 3.88$ $p=0.036$, $*p<0.05$ compared to both female groups, Student's t-test two tailed). The inhibition produced by DAMGO in males was greater than that produced in females

regardless of estrogen status and even though many of the female neurons were exposed to a higher concentration of DAMGO.

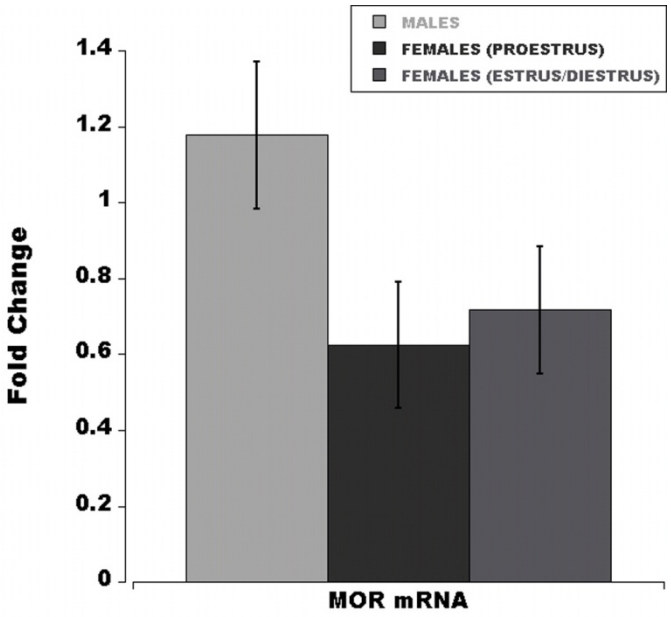

Figure 9. LC-MOR mRNA levels in males and females at relatively different estrogen levels.

Bars indicate relative quantification (RQ) of the MOR gene in the LC. Data are represented as the mean (SEM). GAPDH was used as a loading control. Levels of transcripts were comparable in females in an estrus cycle stage of high estrogen (proestrus, 0.62$\pm$0.17, n=6), or relatively low estrogen (diestrus or estrus pooled, 0.72$\pm$0.17, p=0.71, n=8). For males, n=14.

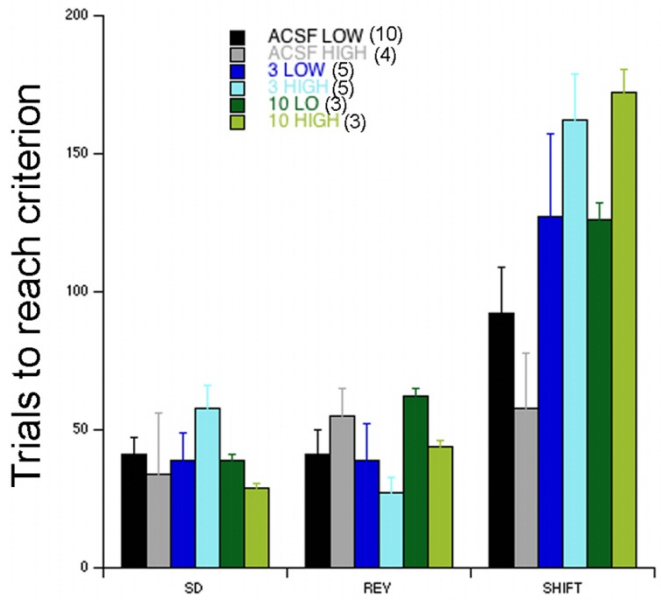

Figure 10. Effects of DAMGO on performance in the operant strategy set-shifting task (OSST) in females at different estrogen levels.

Effects of ACSF and DAMGO (3 pg and 10 pg) bilaterally infused into the LC on performance in the OSST in females. Female groups were broken down into relative HIGH (proestrus) and LOW (diestrus/estrus pooled) estrogen levels. The bars indicate the mean number of trials necessary to reach the criterion for side discrimination (SD), side reversal (REV), and shift to light (SHIFT) stages of the task. Vertical lines represent SEM. The number of rats for each group is shown in the graph legend.

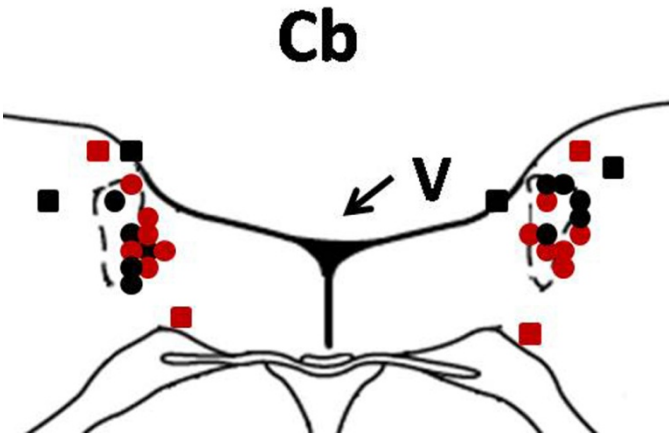

Figure 11. Location of 10 pg DAMGO infusions in and outside the LC.

The location of infusions was reconstructed onto figure 60 from Paxinos (1986). Black filled circles represent infusions within the LC in males. Black filled squares represent infusions outside of the LC in males. Red filled circles represent infusions within the LC of females. Red filled squares represent infusions outside of the LC in females (Cb, cerebellum; V, ventricle).

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CHAPTER 3: μ-OPIOID RECEPTOR ACTIVATION IN THE LOCUS COERULEUS INCREASES SYNCHRONIZATION OF THE MALE, BUT NOT FEMALE MEDIAL PREFRONTAL CORTEX

Abstract

Stress-related neuropsychiatric pathologies are more prevalent in females compared with males. Activation of the locus coeruleus (LC)-norepinephrine (NE) system is a component of the stress response that is thought to affect cognition. Evidence suggests that endogenous opioid neuropeptides are released during stress to restrain LC activation, and to facilitate a return to baseline activity when the stressor ends. Sex differences in this opioid influence could be a basis for sex differences in stress vulnerability. We previously demonstrated a decrease in μ-opioid receptor (MOR) mRNA and protein in the LC of female compared to male rats. As a result, LC neurons of female rats were less sensitive to inhibition by the μ-opioid receptor (MOR) agonist, DAMGO. Given that the LC-NE system affects cognitive function through its projections to the medial prefrontal cortex (mPFC), the present study determined whether LC-MOR activation translates to changes in mPFC neural activity, and whether there are sex differences in this effect. Local field potential (LFPs) were recorded from the mPFC of freely behaving male (n=4) and female (n=4) rats before and following local LC microinjection of DAMGO (10 pg). LFPs were analyzed as power spectral density plots and the power at different frequency bands (delta 2-4 Hz, theta 4-8 Hz, alpha 8-12 Hz, and beta 12-20 Hz) was analyzed and compared between sexes. Intra-LC DAMGO resulted in a time-dependent synchronization of mPFC activity in male but not female rats. Specifically, DAMGO increased synchronization in delta and alpha frequency bands. LC microinfusion of

ACSF had no effect on mPFC activity in either male or female rats. Together, the results are consistent with previous evidence for decreased MOR function in the LC of female rats and demonstrate that this translated to a diminished effect on cortical activity. Decreased LC-MOR function in females could contribute to greater stress-induced activation of the LC, and increased vulnerability of females to hyperarousal symptoms of stress-related neuropsychiatric pathologies.

Introduction

Stress-related neuropsychiatric pathologies are more prevalent in females relative to males. For example, post-traumatic stress disorder (PTSD), and depression are nearly two times more prevalent in females when compared with males (Kessler *et al*, 1994; Kessler *et al*, 1995). These neuropsychiatric diseases are strongly associated with stress and symptoms of hyperarousal (Wong *et al*, 2000). It is hypothesized that sex differences in the prevalence of these stress-related neuropsychiatric diseases may be related to differences in brain arousal systems and their regulation by stress. The major brain norepinephrine (NE) nucleus, locus coeruleus (LC), is involved with arousal and cognitive responses to stress and it is thought that the LC-NE system is dysregulated in these stress-related psychiatric diseases. The LC is the principal site for NE synthesis in the brain, and is the only source of NE in regions that are important in cognition such as the cortex and hippocampus (Abercrombie *et al*, 1988b; Morrison *et al*, 1978; Waterhouse *et al*, 1983). The LC neuronal discharge rate is positively correlated with arousal state (Aston-Jones *et al*, 1981a; Berridge *et al*, 1991; Berridge *et al*, 1993) and LC hyperactivity is thought to underlie the core feature of hyperarousal that characterizes

stress-related neuropsychiatric diseases (Gold and Chrousos, 1999; Gold *et al*, 1996; Koob, 1999; O'Donnell *et al*, 2004).

Electrophysiological studies have demonstrated that during acute stress, LC neurons are co-regulated in an opposing manner by the stress-related neuropeptide corticotropin releasing factor (CRF), which mediates excitation of LC neurons, and endogenous opioids, which inhibit LC neurons, with CRF excitation predominating (Curtis *et al*, 2001; Curtis *et al*, 2012). LC activation produced by CRF during stress has been associated with increased arousal and cognitive flexibility (Snyder *et al*, 2012; Valentino *et al*, 2008). On the other hand, endogenous opioids during stress, temper CRF-mediated activation of the LC via the μ-opioid receptors (MOR) that are expressed in LC neurons (Pert *et al*, 1976a), and promotes LC recovery when the stressor ends. Sex differences could be expressed through differences in sensitivity to either CRF or to endogenous opioids. Any sex differences in the opposing regulation of the LC-NE system could be translated to differences in stress-sensitivity, and vulnerability to stress-related psychiatric disorders. In a prior study, it was established that sex differences in CRF signaling and trafficking in LC neurons of females (Bangasser *et al*, 2010) render females into a dysregulated state of stress reactivity and could be linked to the development of stress-related psychiatric disorders. In addition to this mechanism, decreased opioid inhibition in the LC-NE system can contribute to vulnerability to stress-related illnesses.

We previously demonstrated that female rat LC neurons express less μ-opioid receptor (MOR) mRNA and protein compared to males. Because of these molecular changes in the LC, female rats are less sensitive to inhibition by the μ-opioid receptor (MOR) agonist, DAMGO(Guajardo *et al*, 2017). Notably, the LC-NE system affects

cognitive function through its projections to the medial prefrontal cortex (mPFC) (Loughlin *et al*, 1986; Swanson *et al*, 1975), and stress-related dysfunctions in this pathway are hypothesized to impair cognitive flexibility(Arnsten, 2009; Birnbaum *et al*, 1999). The molecular and cellular sex differences found in the LC were associated with sexually distinct effects of LC-MOR activation on performance and cognitive processing in an operant strategy-shifting task. On the basis of these observations, this study was designed to determine how these molecular and cellular sex differences of the LC-MOR system translates to changes in neural activity in PFC regions that guide cognitive strategies.

Materials and Methods

Animals

Adult male (n=4) and female (n=4) 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. 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.

Surgery

Male and female rats were implanted with a single cannula guide into the LC (26 gauge, Plastics One, Roanoke, VA). Rats were anesthetized with isofluorane (2%) and positioned in a stereotaxic instrument with the head tilted at a 15º angle to the horizontal plane (nose down). A hole was drilled centered at LC coordinates relative to lambda: AP -3.6 mm, ML ±1.1 mm, and the guide cannula was lowered to 5.0 mm below brain

surface. The guide cannula was affixed to skull and skull screws with cranioplastic cement. An obdurator was inserted into the guide cannula to prevent occlusion. During the same surgery, a depth electrode (100 μm) (Microprobes for life science, Gaithersburg, MD) was implanted in the mPFC (+3.2 AP, -0.6 ML, 3.0 DV) ipsilateral to the LC-cannula guide for recording local field potentials (LFP). A ground wire was attached to the skull and skull screws. Animals were allowed 5 days to recover before experimental manipulations.

Drugs for intra-LC microinjections

Drugs used for intra-LC microinfusion were DAMGO ([D-Ala2, N-MePhe4, Gly-ol]-enkephalin; Abcam, Cambridge, MA), a synthetic opioid peptide with high MOR specificity and clonidine HCl (Sigma, St Louis, MO). These compounds were aliquoted and concentrated using a Speed Vac and stored at -20^{o}C before experimental procedures. On the day of the experiments, drugs were dissolved in artificial cerebrospinal fluid (ACSF). The doses that were microinfused into the LC were DAMGO (10 pg in 200 nl) and clonidine (50 ng in 200 nl). The dose of DAMGO is one that has been demonstrated to produce sexually distinct effects on LC activity and on cognitive endpoints when microinfused into the LC (Guajardo *et al*, 2017). The dose of clonidine has been demonstrated to produce a long lasting cessation of LC spontaneous discharge without altering baseline electroencephalograph (EEG) activity (Page *et al*, 1993).

Electrophysiological recordings

Experiments began at least 5 days after surgery. All mPFC recordings were performed in the unanesthetized state. For each rat in the study, cables connected the head-stage to a data acquisition system (AlphaLab; Alpha Omega; Nazareth Israel).

Cannula (26 gauge, Plastics One, Roanoke, VA) were inserted into the guides and secured using a connector that screws around the cannula guide when inserted. Tubing connecting the cannula to the syringe pump was threaded through a flexible coil that allowed free movement of the rat, yet maintained stability of the cannula in the guide. For all animals in the study, pre-injection LFP recordings were taken for 60 minutes. This allowed the rat to undergo multiple sleep/wake cycles that could be used to validate the mPFC LFP recording. LFP activity was recorded and amplified at a gain of 5000 Hz, bandwidth of 1-350 Hz. Following the pre-injection recording period, ACSF (200 nl), DAMGO (10 pg in 200 nl), or clonidine (50 ng in 200 nl) was microinfused into the LC. Recordings continued for 60 minutes after drug administration. ACSF and DAMGO were administered to the same subjects on different days with treatments being 7 days apart.

Histology

After the experiment rats were anesthetized with isofluorane, pontamine sky blue dye (PSB, 200 nl) was injected through the LC cannula to verify placement. Brains were removed, and frozen 30 μm-thick coronal sections were cut on a cryostat and mounted on pre-cleaned plus slides. The sections were stained with neutral red dye for localization of the PSB spot (Figure 12A).

Data Analysis

LFP raw traces were time stamped in Spike2 to remove noise and converted to Power Spectra Density (PSD) raw plots indicating the power in 128 bins from 0 to 20 Hz using Neuroexplorer (Nex Technologies, Madison, AL). The power in different frequency bands (delta, 2-4 Hz; theta, 4-8 Hz; alpha, 8-12 Hz; and beta, 12-20 Hz) was calculated for each rat. A two-way repeated measures analysis of variance (rmANOVA)

with sex as the between factor and time with respect to injection as the repeated measure for each individual frequency band. The Tukey's HSD was used for post hoc comparisons between means. An alpha level of $p<0.05$ was the maximum threshold for statistical significance. Post hoc tests were only performed if an interaction is indicated.

Results

Effects of intra-LC DAMGO on cortical activity

DAMGO was microinfused into the LC of 4 male and 4 female rats. Figure 12 shows an example of histological verification of the injection site into the LC and the location of injections in all rats. The local LC injection of DAMGO increased mPFC synchronization in male rats. Representative raw LFP traces and their corresponding spectrograms displaying power in different frequencies over time show an increase in amplitude within 100 s after the injection that is consistently long lasting in the male rat. In contrast, the LFP trace and spectrogram of the female rat suggests a less consistent and shorter acting effect (Figure 13A and 13B). LFPs were analyzed as PSD plots and the power in different frequency bands at different time blocks were analyzed and compared between sexes (Figure 13C-13F). A two way repeated measures (rm) ANOVA indicated an effect of Time ($F(3, 4) = 21.50$, $p<0.006$), and a Sex X Time interaction for power in the delta frequency band ($F(3, 6) = 28.4$, $p<0.003$). There was a trend for effect of Sex ($F(1, 6) = 5.34$, $p=0.06$) and a trend for a Sex X Time interaction for power in the theta frequency band ($F(3, 4) = 6.2$, $p=0.05$). Analysis of power in the alpha frequency band indicated an effect of Sex ($F(1, 6) = 11.20$, $p<0.01$), and a Sex X Time interaction ($F(3, 4) = 6.93$, $p<0.04$). There was no Sex X Time interaction for power in the beta frequency band ($F(3, 4) = 4.75$, $p<0.08$).

Further analysis within specific frequency bands at different time blocks after DAMGO administration (0 to 10 minutes, 10 to 15 minutes and 45 to 60 minutes) revealed a time-dependent synchronization of mPFC activity such that there was no significant effect of DAMGO in any frequency band at 0-10 minutes after injection (Figure 13C and 1D). Notably, DAMGO significantly increased power in the delta and alpha frequency bands at 10-15 minutes after administration in males only (delta, $p<0.05$; alpha, $p<0.05$, Tukey's HSD) (Figure 13C and 13D). At this time period, there was also a trend for DAMGO to increase power in the theta frequency band selectively in male rats ($p=0.05$). DAMGO effects recovered by 45-60 min post-injection (Figure 13E and 13F).

Lack of effect of intra-LC ACSF on mPFC network activity

In contrast to DAMGO, local ACSF injection into the LC did not affect mPFC activity of the same rats (Figure 14). A two way ANOVA comparing power in different frequency bands at different post-injection times revealed an effect of Sex ($F(1, 6) = 7.5$, $p=0.03$), and no Sex X Time interaction for power in the delta ($F(3, 4) =5.21$, $p=0.07$). No Sex X Time interaction for theta ($F(3, 4) = 1.39$, $p=0.4$), alpha ($F(3, 4) = 2.61$, $p=0.2$) or beta ($F(3, 4) = 3.30$, $p=0.14$) frequency bands (Figure 14).

Comparable effects of intra-LC clonidine on cortical activity of male and female rats

Like DAMGO, clonidine inhibits LC discharge rate, and intra-LC injection increases cortical electroencephalographic synchrony of anesthetized rats (Berridge *et al*, 1993). Consistent with this, intra-LC microinfusion of clonidine (50 ng) increased the LFP amplitude in rats not anesthetized. Notably, this effect appeared comparable in a male and female rat (Figure 15A-15D).

Discussion

The present results are consistent with previous findings of decreased MOR function in the LC of female compared to male rats(Guajardo *et al*, 2017). Decreased MOR mRNA expression and protein levels in the LC of female rats result in a decreased efficacy of DAMGO to inhibit LC neuronal activity. This sex difference was particularly apparent at doses (10 pg and 30 pg) that maximally inhibited male LC neurons. Notably, these sex differences in LC-MOR function translated to a sexually distinct impairment of performance of a prefrontal cortex-mediated cognitive task (Guajardo *et al*, 2017). The current demonstration that LC-MOR activation by a similar DAMGO dose selectively affects prefrontal cortical network activity of male rats links the previous molecular and behavioral findings. Taken together, the results suggest that decreased MOR expression in the LC of females relative to males, translates to an attenuation of the cortical response to LC-MOR activation, and this can account for sexually distinct cognitive consequences of LC-MOR activation. These sex-specific cognitive consequences are relevant for understanding sex differences in opioid abuse. Additionally, given the evidence that MOR activation in LC neurons may counteract stress effects on this system (Valentino *et al*, 2008), the present findings suggest that sex differences in MOR expression contribute to the increased vulnerability of females to the hyperarousal components of stress-related disorders (Gold *et al*, 2002; Koob, 1999; Wong *et al*, 2000).

Relationship to previous studies

The LC regulates executive function and cognitive flexibility through its widespread projections to the prefrontal cortex (Loughlin *et al*, 1986; Swanson *et al*, 1975). Previous studies demonstrated that selective pharmacological manipulations of LC

neuronal activity are sufficient to affect cortical activity. For example, in anesthetized rats, compounds that inhibited LC discharge rate such as the α_2-agonist, clonidine, shifted the power spectrum of cortical EEG towards a high-amplitude, low frequency state that was similar to that seen during slow wave sleep (Aston-Jones *et al*, 1981a; Berridge *et al*, 1993). The effects of intra-LC DAMGO on cortical EEG activity recorded from screws inserted into the frontoparietal and fronto occipital regions showed a similar effect (Bagetta *et al*, 1990). Conversely, compounds that increased LC discharge rate such as the cholinergic agonist bethanechol, induced cortical EEG desynchronization characterized by low amplitude, high frequency activity (Berridge *et al*, 1991). Likewise, intra-LC microinfusion of CRF increases LC discharge rate, and produces cortical EEG desynchronization (Curtis *et al*, 1997). Notably, LC activation produced by CRF shifts the mode of LC discharge towards a high tonic state that is thought to facilitate behavioral flexibility (Curtis *et al*, 1997; Valentino *et al*, 1987, 1988a). Consistent with this, relatively low doses of CRF injected into the LC improve cognitive flexibility in an attentional set-shifting task mediated by the mPFC, and increase c-fos (an indicator of neuronal activation) in mPFC neurons (Snyder *et al*, 2012). Taken together, these results suggest that alterations in LC neuronal activity are sufficient to alter mPFC activity and impact mPFC-mediated cognitive functions. Although MORs are highly expressed in LC neurons, and engaging MOR in the LC impairs a mPFC-mediated cognitive task, its effects on mPFC activity have not been documented previously. Importantly, decreased MOR function in the LC of females relative to males resulted in sexually distinct consequences in mPFC-mediated cognitive tasks, implying sex differences in the impact of activating LC-MOR on mPFC neuronal activity. Therefore, in this study we quantified

and compared the effects of activating LC-MOR on mPFC neuronal activity between male and female rats.

Effects of LC-MOR activation on prefrontal cortex activity

Field potential measurements provide an excellent tool for the exploration of network activity in mPFC. Previous studies of cortical network activity demonstrated that inhibition of LC discharge by clonidine induced high-amplitude, low frequency cortical oscillations (Berridge *et al*, 1993; de Sarro *et al*, 1988). Like clonidine, a DAMGO dose that completely inhibited LC activity, increased high-amplitude, low frequency oscillations in the mPFC. However, this effect was selective to male rats. The effects on the mPFC network activity were not artifacts of injection, as these effects were not reproduced with intra-LC infusion of ACSF. The effects of DAMGO on mPFC network activity were also time-dependent in that they peaked at 10-15 min, and recovered by 45-60 min. Although for females there was a trend for an immediate effect of DAMGO on mPFC activity, this did not achieve statistical significance and was not apparent at later times after the injection. The lack of effect of intra-LC DAMGO (10 pg) in females is consistent with the reduced effect of the same dose on LC activity previously described, and attributed to decreased MOR expression in the LC (Guajardo *et al*, 2017). Finally, the observation that intra-LC clonidine produces comparable effects on mPFC activity in males and females supports the notion that the sex differences in the DAMGO response are due to differences at the level of MOR expression in the LC.

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

Microinfusion of DAMGO into the LC produced sex-specific effects on a strategy-shifting task thought to be mediated by the mPFC (Guajardo et al., 2017). For

males, DAMGO significantly increased the time to complete the task, in part because it increased premature responding. Overall, male rats administered DAMGO made more errors during the task than females, particularly the number of regressive errors, which is indicative of an inability to acquire and maintain the new strategy. Consequently, sex-differences in LC-MOR expression that result in an enhanced ability of DAMGO to regulate mPFC activity, translated to impairments in learning to shift strategies selectively in males. Interestingly, female rats administered DAMGO into the LC committed more perseverative errors than male rats, which is indicative of an impaired ability to shift from a previously learned rule. Perseverative responding has been attributed to brain regions other than the prelimbic region, which was the site of recordings in the present study, including the infralimbic mPFC (Baran *et al*, 2010). This may explain why DAMGO in the female LC can have behavioral effects in the absence of electrophysiological effects in the prelimbic mPFC. A prior study using a place recognition task demonstrated that female rats were more sensitive to disruption of the infralimbic cortex as indicated by increased perseverative responding (Baran *et al*, 2010). Taken with the present results, this suggests that DAMGO in the LC of females may sufficiently affect activity in cortical regions, other than the prelimbic mPFC, that regulate other cognitive processes. In this way engaging MOR receptors in the LC can have sexually distinct effects on cognitive processing.

mPFC activity and strategy-shifting are physiological and behavioral endpoints of LC-MOR activation, respectively. Because for both endpoints, the same intra-LC dose of DAMGO was tested and the experimental time-courses were comparable, the temporal relationship between cortical activity and behavior can be assessed. The time during

which DAMGO had a maximal effect on cortical activity in male rats (10-15 min post injection) corresponded to performance of the simple discrimination stage of the task. DAMGO (10 pg) tended to enhance side discrimination performance when compared to vehicle control in males only, suggesting that mPFC synchronization may protect against distraction and is optimal for learning simple tasks.

At the time point corresponding to strategy-shifting stage of the task (45 min), mPFC network activity in males partially recovered while effects on behavioral performance were apparent. This suggests an enduring effect on mPFC neuronal function that may not be expressed as changes in network activity. Alternatively, the discrepancy in the temporal correlation between physiological and behavioral endpoints may be a function of not recording both simultaneously in the same subject.

Conclusion

Taken with our previous findings, the current findings demonstrate that the consequences of activating LC-MOR on mPFC activity and function are greatly diminished in females as a result of decreased MOR expression. MOR in the LC serves to counter stress-elicited excitation that is mediated by CRF and promotes recovery. The impact of reduced LC-MOR influence during stress coupled with increased CRF receptor signaling in females (Bangasser *et al*, 2010) would be predicted to result in a prolonged hyperactivity of the LC-NE system in response to stress and hyperarousal symptoms that characterize stress-related disorders in females(Gold *et al*, 2002; Koob, 1999; Wong *et al*, 2000).

Sex differences in LC-MOR function also have implications for sex differences in opioid abuse. In male rats, repeated social stress causes an imbalance between

endogenous opioids, and CRF which favors opioid regulation (Chaijale *et al*, 2013). The increased opioid influence in the LC would be predicted to promote premature responding, which is indicative of impulsive behavior (Pattij *et al*, 2009) in males only (Guajardo *et al*, 2017). Impulsivity is a key feature associated with opioid abuse (Baldacchino *et al*, 2015).These cellular alterations in LC after chronic stressors, coupled with the cognitive consequences of LC-MOR activation may predispose males to opiate abuse.The present study underscores how sex differences at the molecular level can translate to sex differences in network activities that govern behavior and cognition.

Figures and Legends

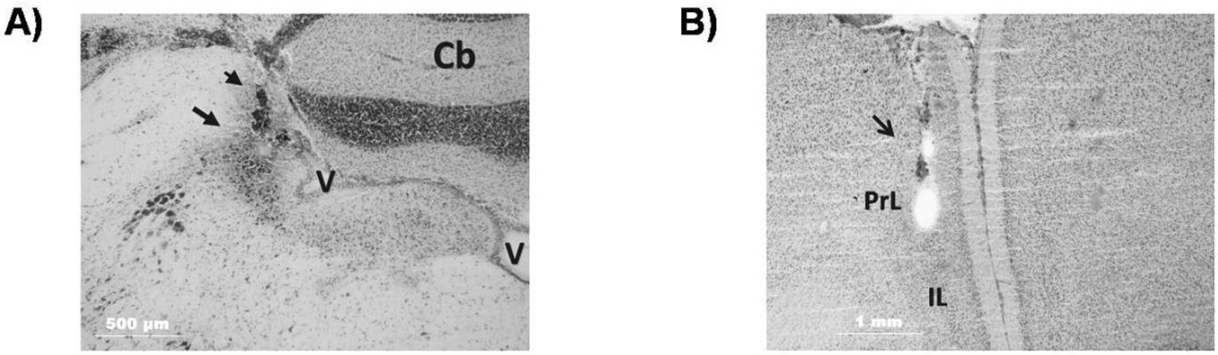

Figure 12. Histological verification of intra-LC injection and mPFC electrode placement.

Brightfield photomicrograph of a neutral red counterstained section through the LC showing histological verification of the single injection site. (A) Arrow points to the LC and the arrowhead points to the dye, which is localized to the LC (Cb, cerebellum; V ventricle). (B) Neutral red counterstained section through the prelimbic mPFC (PrL) showing histological verification of the recording electrode placement. Arrow points to the electrode track (IL, infralimbic mPFC).

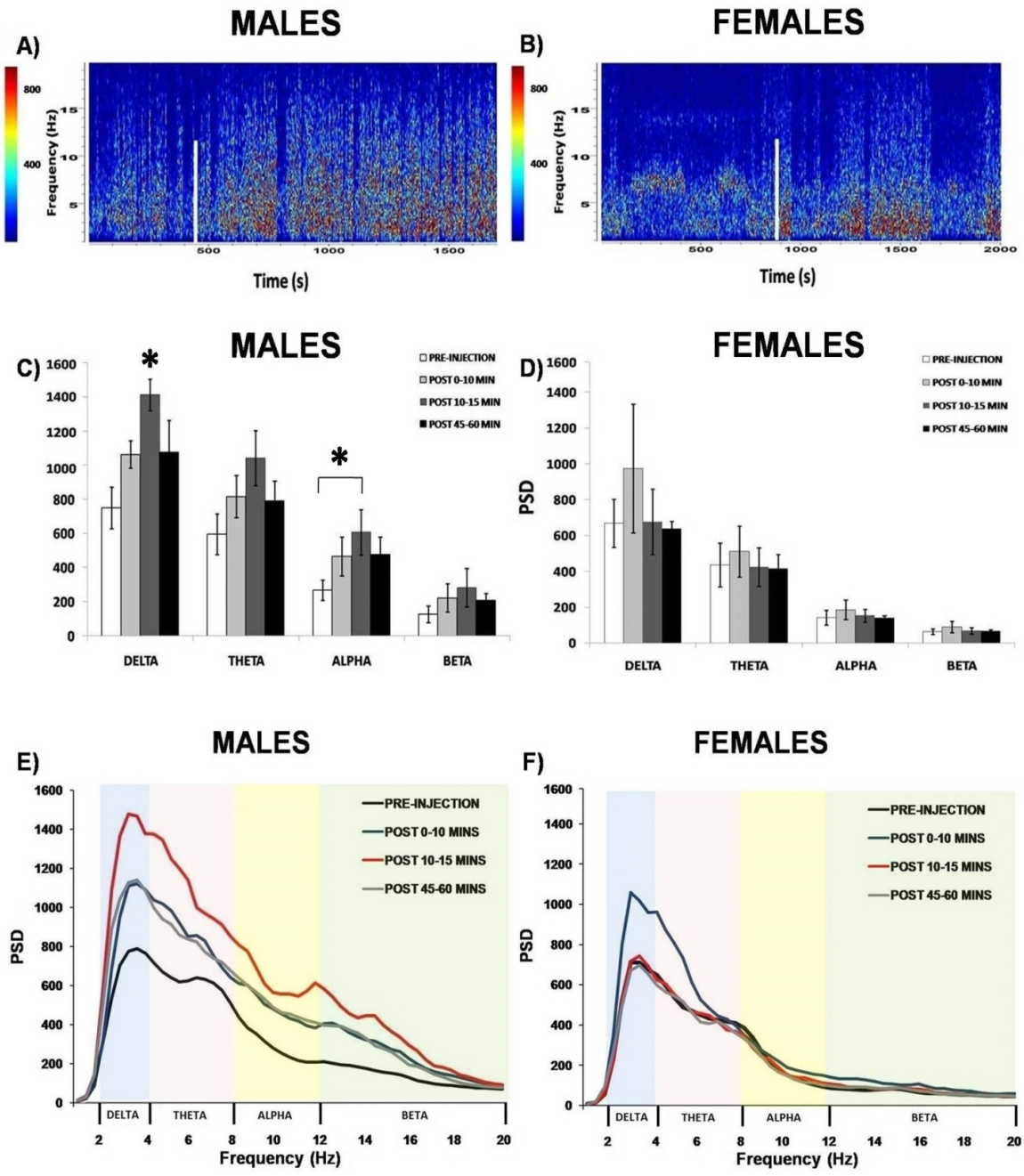

Figure 13. Intra-LC DAMGO resulted in a time-dependent synchronization of mPFC activity in male but not female rats.

Representative time-frequency spectrograms of mPFC LFP activity generated from a male and female rat. (A and B) Power in different frequencies (ordinate) is indicated as color (hotter colors being greater). DAMGO (10 pg in 200 nl) was microinfused into the

LC at white vertical line. (C and D) The bar graphs represent the mean raw power spectral density values at different frequency bands (delta 2-4 Hz, theta 4-8 Hz, alpha 8-12 Hz and beta 12-20 Hz) in male (n=4) and female (n=4) rats, respectively, determined 10 min before (pre-injection), 0-10 min, 10-15 min, and 45-60 min after intra-LC DAMGO on mPFC network activity. Error bars represent ± SEM. *$p<0.05$. (E and F) The line graphs are power spectral density (PSD) plots indicating the raw power of the mPFC LFP at different frequencies. These line graphs were generated from the same subjects as shown in A and B, 10 min before (pre-injection), 0-10 min, 10-15 min, and 45-60 min after intra-LC DAMGO on mPFC network activity in males and females.

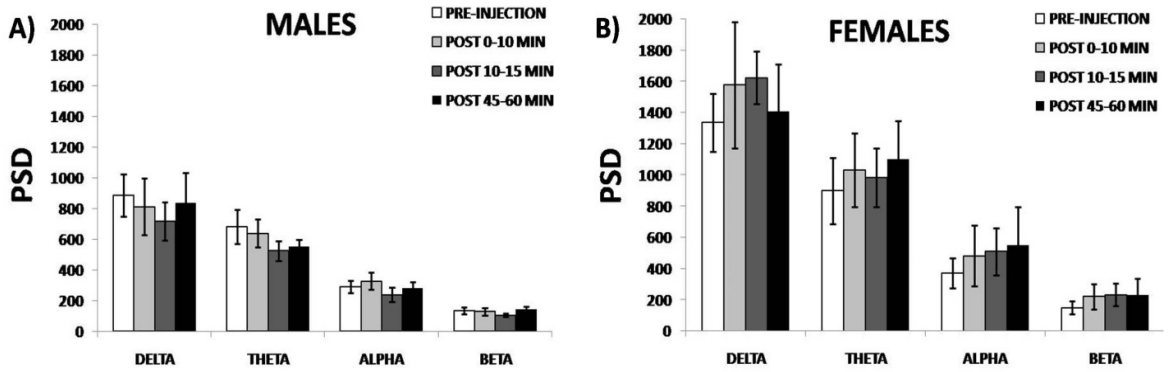

Figure 14. Lack of effect of intra-LC ACSF on mPFC network activity.

Bar graphs represent the mean raw power in different frequency bands at different times before and after intra-LC ACSF (200 nl) in the same male and female rats indicated in Figure 13 above. A) Males and B) Females at different time blocks (0-10, 10-15, 45-60 minutes after Intra-LC DAMGO injection). Error bars represent ± SEM.

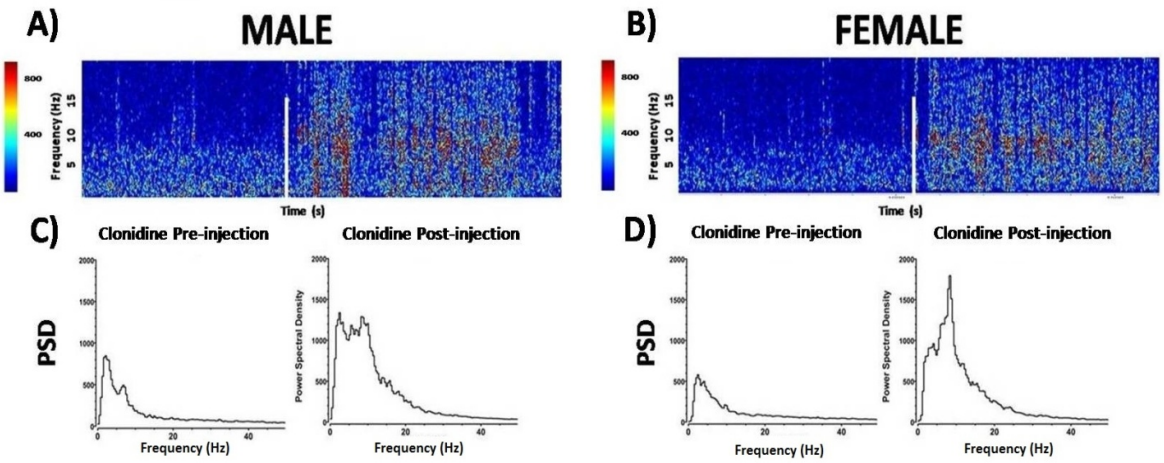

Figure 15. Similar effects of intra-LC clonidine on mPFC network activity.

Time-frequency spectrograms of mPFC LFP activity from a male and female rat before and after (A and B) clonidine (50 ng in 200 nl). Power in different frequencies (ordinate) is indicated as color. The vertical white line indicates the time of microinfusion. (C and D) Power spectral density plots corresponding to their respective time-frequency spectrograms shown above.

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CHAPTER 4: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Sex differences are increasingly recognized as important in the prevalence and severity of neurological and psychiatric diseases. This dissertation explored the role of sex in a major stress response system in the brain, the Locus Coeruleus (LC)-Norepinephrine (NE) system, as a factor of stress vulnerability. By implementing electrophysiological, biochemical, and behavioral approaches, sex differences in μ-opiate receptor (MOR) function in the LC that could underlie stress vulnerability were examined. In the first set of experiments in Chapter 2, it was determined that female rat LC neurons express less MOR mRNA and protein compared to males. Because of these molecular differences, female rat LC neurons are less sensitive to inhibition by the selective MOR agonist, DAMGO. Notably, the LC-NE system affects cognitive function through its projections to the medial prefrontal cortex (mPFC) (Loughlin *et al*, 1986; Swanson *et al*, 1975), and stress-related dysfunctions in this pathway are hypothesized to impair cognitive flexibility (Arnsten, 2009; Birnbaum *et al*, 1999). The molecular and cellular sex differences found in the LC were associated with sexually distinct effects of LC-MOR activation on performance and cognitive processing in an operant strategy-shifting task. In Chapter 3, the molecular and cellular sex differences in LC-MOR were further investigated through the activation of LC-MOR on PFC network activity that guides cognitive strategies. The decreased efficacy of DAMGO on LC activity translated to a diminished effect on mPFC network activity in females, and was consistent with less disruption of an mPFC-mediated operant task in females. Taken together, the results suggest that decreased MOR expression in the female rat LC is sufficient to result in a decreased ability to inhibit LC neurons and to affect PFC network activity, and this

translates to sexually distinct cognitive effects. Sexually distinct cognitive effects of MOR activation in the LC may play a role in sex differences in vulnerability to aspects of opioid abuse. Additionally, given the role of opioids in tempering LC activation during stress, sex differences in opioid inhibition of LC neurons could contribute to female vulnerability to stress-related psychiatric disorders.

Sex Differences in MOR Expression

The MOR is highly expressed in the brain, and has been demonstrated in studies showing high correspondence between the MOR mRNA and receptor binding distributions in many brain regions including the LC (Mansour *et al*, 1994; Mansour *et al*, 1987). MORs are highly expressed by LC neurons, and MOR agonists potently inhibit LC neuronal activity (Mansour *et al*, 1994; Williams *et al*, 1984). Notably, sex differences in MOR expression have been documented in the brain. For example, in the anterior pituitary gland, MOR expression in female rats is decreased relative to males (Carretero *et al*, 2004). In addition, female rats express significantly less MOR in the periaqueductal gray relative to males (Loyd *et al*, 2008). Notably, Estrogen status in females appears to play a role in the modulation of MOR expression in the periaqueductal gray. Consistent with these findings, in Chapter 2 it was determined that females expressed less MOR in the LC compared with males. Western blot analysis revealed decreased MOR protein in female LC relative to male LC. This result was corroborated with mRNA studies indicating decreased MOR mRNA transcripts in female LC when compared with male LC. Importantly, sex differences in MOR expression in the LC were independent of female estrogen status as determined by measuring mRNA transcript levels in the LC of females at different stages of the estrous cycle. This suggests that sex

differences arise from the organizational effects of female hormones early in development.

Sex Differences in MOR Activation on Neuronal Activity

MORs are highly distributed within the LC (Mansour *et al*, 1994; Williams *et al*, 1984). The MOR is prominently localized postsynaptically within noradrenergic somato dendritic processes (Van Bockstaele *et al*, 1996a; Van Bockstaele *et al*, 1996b). Potent inhibitory effects of MOR activation on LC neurons have been well documented. MOR activation inhibits the formation of cyclic AMP and hyperpolarizes LC neurons through an increase in potassium conductance (Aghajanian *et al*, 1987; Williams *et al*, 1984).

The electrophysiological studies conducted in Chapter 2 were the first to examine and compare sex differences in LC postsynaptic responses to MOR agonists. Although there are reports of sex differences in opioid-induced analgesia and behavioral suppression elicited by opioids (Craft, 2003; Kest *et al*, 2000a), there are no reports on sex differences characterizing the electrophysiological effects of activation of MOR in the brain. Few studies have investigated functional sex differences in other receptors in the LC. One study found that female LC neurons are more sensitive to exogenous CRF relative to males, and this was attributed to differences in CRF receptor coupling to the GTP-binding protein, Gs (Bangasser *et al*, 2010; Curtis *et al*, 2006).

In contrast to the aforementioned sex difference in CRF receptor sensitivity, the present study identified sex differences in the maximal effect of MOR activation. Based on the electrophysiological assessment of MORs in the LC indicated in Chapter 2, the maximum magnitude of inhibition produced by the MOR agonist was significantly less in females, even when the dose was increased beyond that which completely inhibited male

LC (10 pg). Thus, sex differences in LC MOR expression are sufficient to result in a decreased efficacy of MOR agonists in inhibiting LC neuronal activity.

LC Regulation of Cortical Activity

If the sex differences in MOR expression in the LC that were observed in Chapter 2 are relevant, they should be reflected as sex differences in LC modulation of its postsynaptic neuronal targets. The LC has widespread projections to the prefrontal cortex, and through its modulation of cortical neuronal activity it can regulate executive function and cognitive flexibility (Loughlin *et al*, 1986; Robbins and Arnsten, 2009; Swanson *et al*, 1975). Therefore, sex differences in LC-MOR expression and in the ability of intra-LC DAMGO to inhibit LC neurons should be expressed as differences in LC modulation of cortical activity and its behavioral consequences.

Previous studies have demonstrated that selective pharmacological manipulations of LC neuronal activity are sufficient to affect cortical activity. For example, in anesthetized rats, compounds that inhibited LC discharge rate such as the α_2-agonist, clonidine, shifted the power spectrum of cortical EEG towards a high-amplitude, low frequency state that was similar to that seen during slow wave sleep (Aston-Jones *et al*, 1981a; Berridge *et al*, 1993). The effects of intra-LC DAMGO on cortical EEG activity recorded from screws inserted into the frontoparietal and fronto occipital regions showed a similar effect (Bagetta *et al*, 1990). Conversely, compounds that increased LC discharge rate such as the cholinergic agonist bethanechol, induced cortical EEG desynchronization even in anesthetized rats (Berridge *et al*, 1991). Likewise, intra-LC microinfusion of CRF increases LC discharge rate and produces cortical EEG desynchronization (Curtis *et al*, 1997). Together, these studies provided strong evidence

that regionally selective manipulation of LC discharge is sufficient to alter cortical activity. LC activity is also necessary for cortical activation by stressors. For example, cortical activation by hypotensive stress is prevented by selectively silencing the LC with the α_2-agonist, clonidine (Page *et al*, 1993).

The experiments conducted in Chapter 3 examined how molecular and cellular sex differences of the LC-MOR system found in Chapter 2, affected LC modulation of mPFC network activity that guides cognitive strategies. To our knowledge, these studies were the first to systematically quantify the impact of the effects of MOR activation in the LC on mPFC activity in both male and female rodents. A novel sex difference was found in the effects of the MOR agonist, DAMGO, in the LC on cortical network activity. Notably, administration of DAMGO at a dose that completely inhibits LC activity increased cortical synchronization of in males only. The effects of intra-LC DAMGO on mPFC network in males were time-dependent. Although there was a trend for an immediate effect of DAMGO on mPFC activity in females, this did not achieve statistical significance, and was not apparent at later times after treatment. These results are consistent with a previous study demonstrating that intra-LC DAMGO increases cortical synchrony of male unanesthetized rats (Bagetta *et al*, 1990). However, the present studies were a refinement in demonstrating that much lower doses (10 pg in the current study vs. 15 ng in the previous study) increase the LFP amplitude, specifically in the mPFC. Most importantly, it was demonstrated that this effect is sex specific. As the LFP recorded in the mPFC is modulated by LC-MOR activation, the results confirmed the sex difference in LC-MOR expression, and the functional activity revealed in Chapter 2.

LC Regulation of Cognitive Function

A quantifiable endpoint of mPFC function is cognitive flexibility. The LC regulates cognitive flexibility through its projections to the mPFC (Loughlin *et al*, 1986; Swanson *et al*, 1975). Lesions of LC-cortical projections impair cognitive flexibility (Tait *et al*, 2007). 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, just as removal of LC-NE drive impairs cognitive flexibility, so can excessive LC drive as a result of the interaction of higher levels of norepinephrine with lower-affinity adrenergic receptors, including α1- and β-adrenergic receptors (Arnsten, 2011). CRF, microinfused into the LC 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).

Sex differences in the effects of LC-MOR activation on mPFC network activity should translate to sex differences in endpoints of cognitive flexibility. The behavioral studies in Chapter 2 were unique in testing the impact of intra-LC administration of a MOR agonist on the performance of mPFC-mediated cognitive tasks in both male and female rats. Two important discoveries arose from this experiment. First, males were more impaired in strategy shifting after activation of LC-MOR, as indicated by an increased number of errors. This is in line with the enhanced effect of intra-LC DAMGO on mPFC network activity in males. An unexpected finding was that there were sex differences in the error type. Although females made fewer errors than males when LC-MORs were engaged, the types of error were different. One explanation for this is that

activating MOR in the LC affects different targets in male and female rats, with a bias towards impairment in the prelimbic mPFC in males, and perhaps the infralimbic PFC or orbitofrontal cortex in the female. LFP recordings in these latter LC targets during intra-LC DAMGO infusion could test this hypothesis.

Sex Differences in Opioid Regulation of the LC-NE System and its Implications for Vulnerability to Stress-Related Psychiatric Disorders

Stress-related neuropsychiatric pathologies are more prevalent in females relative to males. For example, post-traumatic stress disorder (PTSD) and depression are nearly two times more prevalent in females compared with males (Kessler *et al*, 1994; Kessler *et al*, 1995). Given that PTSD and depression are strongly associated with stress, and stress is thought to exacerbate neurological and psychiatric diseases, it has been hypothesized that higher rates of psychiatric diseases in females are due to sex differences in stress reactivity. Notably, a vast number of neuropsychiatric conditions such as depression and post-traumatic stress disorder (PTSD) are strongly associated with dysfunctional stress responses and symptoms of hyperarousal (Gold *et al*, 2002; Heim *et al*, 2001; Wong *et al*, 2000). Given its role in arousal, sex differences in the LC-NE system could underlie female vulnerability to neuropsychiatric disorders that are characterized by hyperarousal (Aston-Jones *et al*, 2005; Berridge *et al*, 2003). During acute stress, LC neurons are activated by CRF, and this is associated with enhanced arousal and cognitive flexibility (Snyder *et al*, 2012; Valentino *et al*, 2008). CRF mediates stress-induced LC activation, and sex differences in CRF signaling and trafficking in LC neurons have been reported. For example, CRF_1-Gs association is greater in females compared to males, and stress-induced CRF_1 association with β-arrestin 2, a step that is critical for CRF_1 internalization

is decreased in female relative to male rats. Consistent with this, stress induces CRF_1 internalization in LC neurons of male rats, but not female rats (Bangasser *et al*, 2010). Sex differences in CRF signaling have been proposed as one mechanism underlying LC-NE dysregulation that contributes to female vulnerability to stress-related psychiatric disorders.

In addition to CRF release in the LC, endogenous opioids are released, and these serve to temper stress-elicited excitation that is mediated by CRF and promote recovery of LC neurons (Curtis *et al*, 2012). The findings in this dissertation suggest that the ability of endogenous opioids to temper LC activation during stress, and to promote LC neuron recovery would be less effective in females. The impact of reduced LC-MOR influence during stress coupled with increased CRF receptor signaling in females (Bangasser *et al*, 2010) would be predicted to result in a prolonged hyperactivity of the LC-NE system in response to stress and hyperarousal symptoms that characterize stress-related disorders in females (Gold *et al*, 2002; Koob, 1999; Wong *et al*, 2000).

Sex Differences in Opioid Regulation, Cognitive Processing and Vulnerability to Opiate Abuse

In drug abuse, opioids produce their reinforcing effects by increasing the activity of the mesolimbic dopaminergic (DA) system (Di Chiara and Imperato, 1988; Koob and Bloom, 1988; Wise and Rompre, 1989), which projects to extensive areas of the PFC (Thierry *et al*, 1973). Chronic abuse of opioids alters opioid receptor functions in the PFC (Mansour *et al*, 1988) and produces adaptive alterations in the cellular and synaptic function of DA system (Nestler, 2001). Sex differences are present in all of the phases of drug abuse, for example in the initiation, escalation of use, addiction, and relapse. In

general, males have higher rates of use or dependence relative to females (Brady and Randall, 1999). However, females may be more susceptible to craving (Fox *et al*, 2014; Hitschfeld *et al*, 2015; Kennedy *et al*, 2013; Robbins *et al*, 1999) and relapse (Kippin *et al*, 2005; Rubonis *et al*, 1994). Sex differences in LC-MOR function may have implications for sex differences in opioid abuse. The sex-specific effects of LC-MOR activation on cognitive processing may be relevant for understanding sex differences in opioid abuse and for sex-specific design of treatment for opioid addiction.

The activation of the LC-MOR promoted premature responding in males only, a response that is indicative of impulsive behavior (Dalley et al, 2011; Pattij et al, 2009). Impulsive behavior, for which a simple definition is the tendency to act prematurely without foresight, is associated with most forms of drug taking. It is often considered to be a product of impaired cognitive control, and could potentially affect several aspects of the addictive process. Notably, impulsivity is a key feature associated with opioid abuse (Baldacchino *et al*, 2015). Previous studies have shown that opioids elicit impulsive behavior in rodents in behavioral tasks such as the 5-choice serial response time task (5-CSRTT) and the response inhibition task, and a decrease in motor impulsivity in MOR-knockout mice (Mahoney *et al*, 2013; Olmstead *et al*, 2009; Pattij *et al*, 2009).

Evidence in the literature suggests that PFC hypoactivity results in deficit of functions such as inhibitory control, attention, planning, and risk-taking (Aron *et al*, 2004; Gazzaley and Nobre, 2012; Nee and Jonides, 2008), and lead to greater cognitive and motor impulsivity (Dalley *et al*, 2011). Notably, lesions to the PFC produce profiles of impulsivity (Brennan and Arnsten, 2008). Our electrophysiological studies revealed that when MORs in the LC are engaged after agonist treatment, upstream targets such as

PFC activity is disrupted. Decreased PFC function produced by the engagement of MOR in the LC of males may account for the greater promotion of impulsive behavior during the cognitive task. This sex specific-cognitive consequence produced by the LC-MOR, when activated, may facilitate opioid taking and perhaps contribute to the higher rates of opiate abuse in males.

Interestingly, in male rats, repeated social stress causes an imbalance between endogenous opioids, and CRF that favors opioid regulation (Chaijale et al, 2013). After a single social stress exposure, CRF and enkephalin afferents to the LC are engaged; however, LC activation is predominant (Reyes et al, 2015). After repeated social stress, CRF receptors become downregulated and MOR becomes upregulated, particularly in rats with an active coping strategy. The increased opioid influence in the LC would be predicted to promote impulsive behavior. These cellular alterations in LC after chronic stressors, coupled with the cognitive consequences of LC-MOR activation, may predispose males to opiate abuse. Our recent studies in females indicate that acute social stress has similar effects as in males. However, with repeated social stress, MOR becomes downregulated in the female LC, thus this compensatory mechanism may be impaired. This would make females more vulnerable to hyperarousal symptoms of stress.

Studies show that females are more vulnerable to some phases of drug abuse such as drug craving and relapse (Fox *et al*, 2014; Hitschfeld *et al*, 2015; Kennedy *et al*, 2013; Kippin *et al*, 2005; Robbins *et al*, 1999; Rubonis *et al*, 1994) relative to males. Previous studies suggest 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 *et al*, 2008). Notably, the engagement of LC-MOR selectively produced

perseverative behavior in females in the cognitive task. Studies in rats have shown that inhibition of the thalamo-cortical circuitry selectively increases perseverative behaviors (Block *et al*, 2007). Additionally, disruption of the orbitofrontal cortex produces perseveration and resistance to extinction of reward-associated behaviors in laboratory animals (Butter *et al*, 1963; Johnson and Rosvold, 1971).

Consistent with animal studies, human studies suggest that disruption of the thalamo-orbitofrontal circuit results in the perseverative behavior in addicted subjects, and the exaggerated motivation to procure and administer the drug regardless of its adverse consequences. This idea is supported by imaging studies showing disruption of the thalamic and orbitofrontal brain regions in drug abusers (Volkow *et al*, 1996). The thalamic and orbitofrontal regions are direct upstream targets of the LC-NE system (Chandler *et al*, 2014; Samuels *et al*, 2008). The sex specific-consequence of LC-MOR activation may lead to dysfunction of the thalamic-orbitofrontal circuit, resulting in perseverative behavior that may predispose females to opioid craving and relapse. These findings underscore the potential for sex-specific treatments of opioid abuse based on pharmacological and/or cognitive therapies that target different cognitive dimensions.

Future Directions

The results described in this dissertation were interpreted on the basis of reasonable assumptions and some of these results could be validated by further experimentation. Several follow-up experiments could be performed to further support or extend the conclusions drawn from this dissertation. For example, sex differences in the response to DAMGO in the LC could result in part from MOR signaling. Examining and comparing MOR coupling to its GTP-binding protein, Gi, using the GTPγS method in

both sexes, could test this. This technique measures the level of G protein activation following DAMGO occupation of MOR in the LC. The [^{35}S] GTPγS binding assay replaces endogenous GTP, and binds to the G-α subunit following activation of the receptor to form a Gα-[^{35}S] GTPγS species. Since the γ-thiophosphate bond is resistant to hydrolysis by the GTPase of Gα, G-protein is prevented from reforming as a heterotrimer and thus [^{35}S] GTPγS labeled Gα-subunits accumulate, and can be measured by counting the amount of [^{35}S]-label incorporated (Harrison and Traynor, 2003).

To determine whether there are sex differences in intracellular signaling pathways initiated by MOR, Designer receptors exclusively activated by designer drugs (DREADD) technology could be used. The MOR is linked to G*i* and selective engagement of G*i*-signaling in the LC can be accomplished using DREADDs. LC neurons can be transduced to express DREADDs coupled to G*i*. Administration of the DREADD ligand, clozapine-N-oxide (CNO), would bypass MOR to then engage G*i* signaling. A follow-up study would be to record local field potentials in mPFC in animals expressing the inhibitory G*i*-coupled DREADDs in the LC while CNO is administered. This follow-up experiment would provide a direct comparison of the mPFC network activity while LC-NE neurons are inhibited via G*i*-coupled DREADDs, with the mPFC network activity results obtained in Chapter 3, when LC neurons were inhibited with DAMGO in both sexes. Sex differences in the DREADD response to CNO would suggest that there is a general sex difference in the Gi-intracellular signaling pathway, on the other hand, no differences between the sexes in the DREADD response to CNO would suggest that the difference is at the level of the MOR exclusively.

Other experiments could be performed to answer questions that arose from the results of this dissertation. For example, in Chapter 3, the discrepancy in the temporal correlation between physiological and behavioral endpoints of LC-MOR activation arise from not recording mPFC network activity during the performance of the mPFC-mediated cognitive task simultaneously in the same subject. Recording mPFC network activity during the performance of the mPFC-mediated cognitive task could assess this correlation. This study would provide a real-time picture of circuit dynamics of the mPFC network activity during task performance between the sexes.

Additionally, further experiments can be conducted in order to characterize the effects of intra-LC DAMGO on LC neuronal activity and mPFC network activity during the OSST performance. Recording LC unit activity and LC-LFPs after intra-LC DAMGO administration while the animals are performing the cognitive task, and at the same time recording mPFC-LFPs, would dissect the specific role of the LC-NE system on the sex-specific cognitive consequences produced in the cognitive task. Previous electrophysiological studies suggest that application of opiates in the LC inhibits tonic LC-neuronal discharge (Valentino *et al*, 1988c). Based on this observation coupled with the results in the anesthetized LC-single unit study, we predict that intra-LC DAMGO at relative high doses would produce an enhanced inhibitory effect in males relative to females on tonic LC-neuronal discharge rate. This study would provide a characterization of LC unit activity under the influence of DAMGO that underlies correct trials, premature responses, and different error types.

Furthermore, by recording mPFC network activity in the same animal, coherence analysis between the LC and mPFC can be achieved. Coherence analysis would reveal

the strength of communication between these two brain regions during task performance. We predict that LC-mPFC network communication will be decreased in male and female animals treated with DAMGO during the strategy-shifting (SHIFT) component. However, we expect that male coherence would be greatly disrupted, relative to females, during the SHIFT component of the task, and that coherence will be increased in males during the simple discrimination task. Our predictions on coherence are based on the behavioral results during the performance of males at the simple discrimination stage, in which DAMGO, at a high dose, improves performance relative to control and greatly impaired performance during the SHIFT component of the OSST.

Moreover, this study would reveal the strength in LC-mPFC communication during correct trials, premature responses, and different error types. Although, this experimental procedure appears technically challenging, considering the size of the LC, it would require a microwire bundle and a cannula being implanted chronically in the LC and an electrode in mPFC. The best way to achieve this experimental procedure would be using inhibitory Gi-coupled DREADDs to selectively manipulate LC neuronal activity; however, as discussed earlier in this section, it must first be determined whether the G*i*-coupled DREADDs in the LC turns on the same intracellular pathways activated by MOR in the LC.

A set of experiments can be conducted in order to assess the impact of sex differences in cognitive processing in opioid taking and relapse. The premature responding produced by the engagement of the LC-MOR in males indicates impulsive behavior. Impulsivity is a key feature associated with opioid abuse (Baldacchino *et al*, 2015). Previous studies suggest that PFC disruption elicits impulsive behaviors (Brennan

et al, 2008; Dalley *et al*, 2011). Consistent with this, the highest dose of intra-LC DAMGO in our studies produced PFC network activity disruption and impulsivity in the cognitive task. Based on these results, we hypothesize that impulsivity elicited by the engagement of LC-MOR in males would facilitate opioid taking. Administering intra-LC DAMGO or vehicle control in both sexes, and implementing an operant conditioning paradigm underlying acquisition/initiation of opiate drugs could test this hypothesis. We expect that males, after DAMGO treatment, will be more vulnerable than females to acquisition/initiation of opioids under the conditions of this experimental procedure. On the other hand, intra-LC DAMGO produced perseveration in females.

It is hypothesized that disruption of the thalamo-orbitofrontal cortex (OFC) circuit underlies perseverative behavior in drug abusers (Volkow *et al*, 1996). Although, none of our studies tested the effects of intra-LC DAMGO in thalamus and OFC, both brain areas are upstream targets of LC (Chandler *et al*, 2014; Samuels *et al*, 2008). A set of experiments can be performed in order to test the effects of the engagement of MOR in the LC on these areas that govern perseveration and on relapse. A first set of experiments would be testing the effects of intra-LC DAMGO on thalamic and OFC neuronal activity. Based on the results of the cognitive task in which intra-LC DAMGO produced perseverative behavior, we hypothesize that the engagement of LC-MOR disrupts neuronal activity in thalamus and OFC in females only. To test this idea, thalamic and OFC network activity can be recorded while DAMGO is microinfused into the LC. Moreover, recording thalamic and OFC network activity during OSST task engagement in animals treated with DAMGO in the LC would provide a real-time picture of circuit dynamics in these brain regions underlying perseverative behavior.

Second would be assessing how sex differences in perseveration produced by the engagement of LC-MOR impacts relapse in opiate addiction. We hypothesize that the sex-specific effects of intra-LC DAMGO on cognitive processing in females that produced perseveration will render females more vulnerable to relapse. In order to test this hypothesis, reinstatement procedures used to model relapse following a period of abstinence can be implemented. Reinstatement procedures require previous exposure to opioids that may alter LC-MOR function; therefore, blocking LC-MOR antagonists would be appropriate to test this question. After a period of abstinence, application of the selective MOR antagonist CTAP can be microinfused into the LC in both sexes, and reinstatement can be tested. CTAP is a potent MOR antagonist (Chieng *et al* 1996). We hypothesize that blockage of LC-MOR by CTAP will prevent reinstatement in females.

Our studies focused on the effects of the local activation of MOR in the LC and its consequences on electrophysiological and cognitive endpoints. In general, the main route of administration of drugs of abuse is systemically. Conducting experiments evaluating sex differences in response to systemically delivered opioids on cortical activity and behavior relevant to opioid abuse would expand the findings presented in this dissertation. Notably, systemic administration of opioid agonists produces mPFC hypofunction.

Previous studies in rats suggest that systemic and local administration of opioid agonists decrease excitatory neurotransmission in mPFC. Specifically, systemic administration of opioid agonists diminishes cellular response to excitatory activation of three major mPFC afferents such as thalamus, basolateral amygdala, and hippocampus (Giacchino and Henriksen, 1998). Consistent with these findings, our studies suggest that

in males, local administration of opioid agonists in the LC decreases mPFC function. Further experiments can be conducted in order to dissect the specific role of the LC-MOR system in the modulation of PFC function when opioid drugs are systemically delivered to the brain, and whether sex differences in cognitive processing produced by a local administration of opioid agonists in the LC are produced by systemic administration of opioids.

To examine the specific role of the LC-MOR system on PFC modulation during systemic administration of opioids, mPFC network activity can be recorded while the animals receive a dose of systemic opioids at the same time as application of the selective MOR antagonist CTAP intra-LC. CTAP is a potent MOR antagonist (Chieng *et al*, 1996). Blockage of MORs in the LC will remove the LC-MOR influence to mPFC when opioid agonists are delivered systemically and take effect on the brain. Previous studies in male rodents show that systemic morphine administration induced cortical EEG slow wave synchronization in naive animals (Lukas *et al*, 1982).

Our electrophysiological studies showed a similar effect when DAMGO was microinfused intra-LC in males only. Based on this observations, we hypothesize that blockage of LC-MOR will translate to decreased cortical network activity synchronization at lower frequency bands, when systemically delivered opioid agonists take effect on mPFC network activity of males. We also hypothesize that cortical network activity in females will be unaffected by systemic delivered opioid agonists, a similar effect produced by intra-LC DAMGO.

A major finding in this dissertation is that intra-LC DAMGO at a high dose produced sex differences in cognitive procession during the SHIFT stage of the cognitive

task. It would be pertinent to further investigate the cognitive processing produced by the engagement of LC-MOR through the local administration of DAMGO in the LC, and evaluate if systemic administration of opioid agonists produce sex differences in cognitive processing during performance of the OSST. To answer this question, animals would be tested on the different components of the OSST under the effects of systemically delivered opioid agonists. One possible outcome is that the systemic injection of opioid agonists may produce the same sex-specific cognitive consequences produced by intra-LC DAMGO. In that case, it would be important to corroborate if these sex-specific behaviors are produced by the MORs engagement in LC projections to PFC or other brain structures. One way to achieve this is by antagonizing MORs in LC while the animals are performing the different stages of the OSST under the influence of systemic delivered opioid agonists.

Finally, it is well documented that chronic opioid administration (i.e. morphine) leads to a wide range of neuroadaptations at different levels, including receptors, intracellular signaling pathways, and synaptic morphology and plasticity (Christie, 2008). Previous studies revealed that chronic morphine shifts PFC network activity state progressively to an apparent normal functional state, despite the continuous presence of morphine. Specifically, the acute systemic administration of morphine produces increased PFC synchronization; however, repeated administration of morphine by day 3 leads to a complete reversal of these "abnormal" oscillatory network activities and produces an apparent normalization of the activity in PFC (Dejean *et al*, 2013). Moreover, treatment with the opioid antagonist naloxone promotes PFC synchronization and withdrawal signs in morphine dependent rats (Dejean *et al*, 2013). These data

suggests that chronic exposure to opioids promotes a new equilibrium in PFC that requires the presence of morphine to endure, and to perform normal cognitive processing.

All the studies in this dissertation show results from an acute exposure to intra-LC DAMGO. Given the different experimental conditions, it would be important to test how these opioid-induced neuroadaptations after chronic exposure impact cognitive function in both sexes. Furthermore, how changes in MOR expression levels, MOR receptor density, and intracellular pathways activated by MOR promoted by chronic exposure to opioids affects the LC-NE system, and whether there are sex differences at any of these levels should be determined. Many of these questions lie beyond the scope of this dissertation but inspire much future research.

Conclusion

These studies have provided concrete evidence for sex differences in opioid regulation of the LC-NE system, revealed that female LC neurons are less sensitive to opioid inhibition relative to male LC neurons, and evaluated sex differences in physiological and behavioral correlates of LC-MOR activation. The findings from this investigation have advanced our understanding of stress-related psychiatric disorders, and may lead to improved treatment of patients suffering from stress-related psychiatric disorders.

Figures and Legends

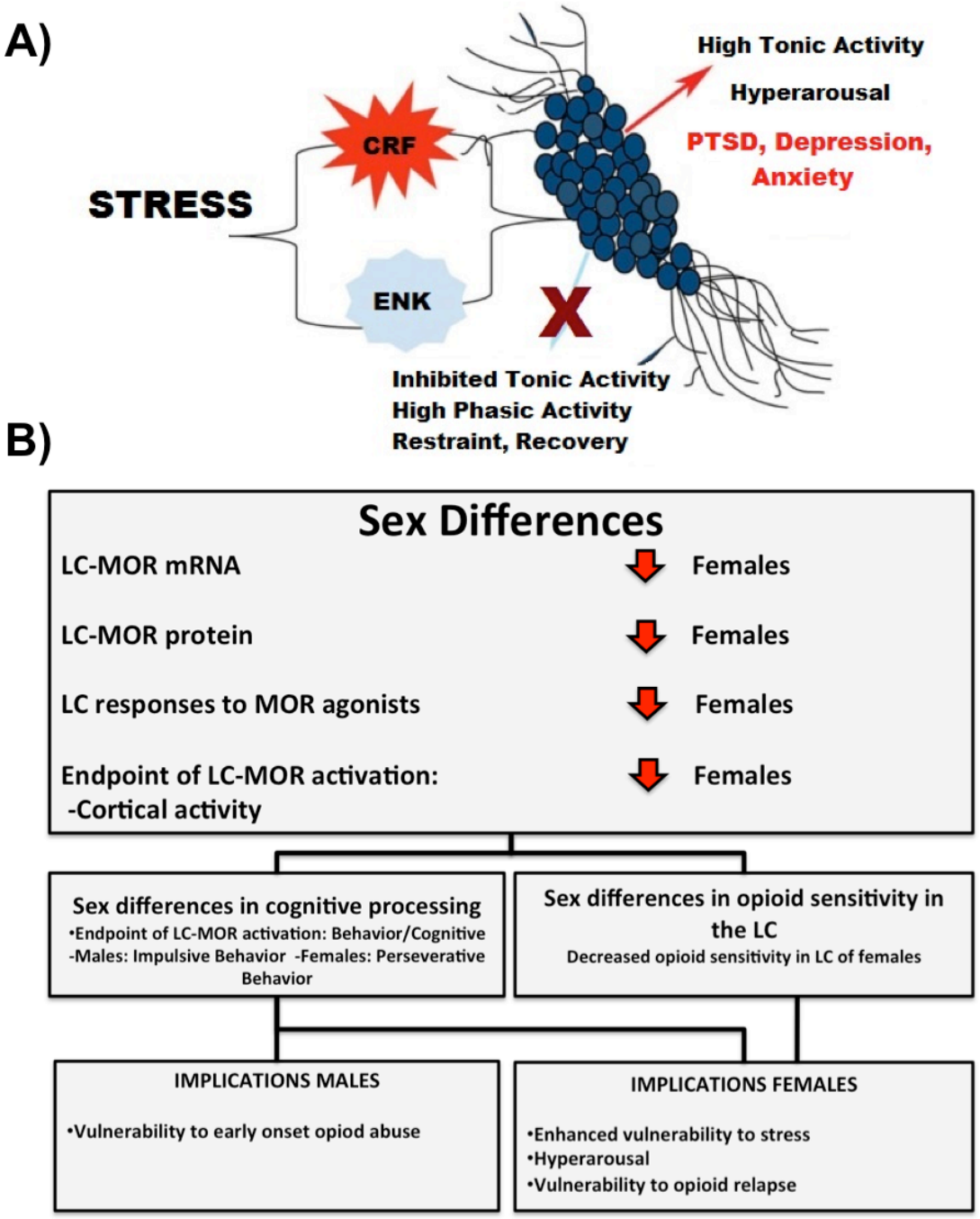

Figure 16. Sex differences in MOR regulation of the LC-NE system and its pathological consequences.

A) The opposing regulation model of LC activity during acute stress. Stress engages both CRF and enkephalin (ENK) inputs that converge on LC neurons. CRF increases tonic

activity, and this is associated with increased arousal. Activation of MORs on LC neurons inhibits LC tonic discharge, and facilitates recovery of LC neurons after the stressor is terminated. An imbalance in this opposing regulation model favoring CRF may increase vulnerability to stress-related disorders characterized by hyperarousal. Figure adapted from Valentino et al (2015). B) Schematic diagram showing the potential physiological and cognitive consequences of sex differences in LC-MOR regulation in the LC and the pathological implications. Abbreviations: PTSD, post-traumatic stress disorder.

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