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Long-Term Impacts of Acute Stressor Exposure on Locus Coeruleus Function and Anxiety-Like Behavior in Rats

Olga Borodovitsyna
Rowan University

LONG-TERM IMPACTS OF ACUTE STRESSOR EXPOSURE ON LOCUS COERULEUS FUNCTION AND ANXIETY-LIKE BEHAVIOR IN RATS

Olga Borodovitsyna, MD

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2 Abstract

Stress is a physiological state characterized by behavioral arousal that occurs during exposure to harmful or threatening stimuli, and usually facilitates an adaptive behavioral response. The persistence of stress sometimes causes it to become maladaptive, potentially contributing to disease development, including physiological complications with altered neuroendocrine signaling and impaired function of organ systems, and psychological conditions including depression and anxiety. Anxiety disorders in particular are associated with a history of stress and are the most common class of mental disorders, with a lifetime prevalence of 33.7% in the general population. The locus coeruleus (LC) is a major node in the stress response, which integrates input from multiple stress responsive neural circuits and releases norepinephrine throughout the central nervous system (CNS) to promote vigilance and anxiety. Stress-induced adaptations in LC may lead to impaired noradrenergic transmission throughout the CNS and is thought to contribute to mood disorder pathogenesis. Although immediate cellular actions of stress on LC have been extensively studied, the long-term changes in LC are poorly described.

It is well-established that acute stress causes the release of corticotropin-releasing factor (CRF) in the CNS, including in LC, where it increases tonic firing to promote increased norepinephrine in target brain areas, thereby promoting anxiety-like behavior. However, LC morphology and stress responsiveness varies among males and females and changes throughout development. In addition, CRF activity on the LC is counteracted by endogenous opioid neurotransmission. The endogenous opioid

peptides Leu- and Met-enkephalins act through d and m opioid receptors, which decrease cAMP and increase potassium conductance through a Gi-coupled mechanism, leading to cell hyperpolarization and decreased firing. Therefore, we hypothesized that acute stressor exposure would have variable impacts on LC physiology and anxiety-like behavior in rats of different ages in both sexes. We further postulated that dysregulation of LC following stress is associated with altered function of opioid receptors in LC. To test this, we used a combination of whole-cell patch clamp recordings of LC neurons, rodent assays of anxiety-like behavior, and analysis of gene and protein expression. Finally, by using a viral-genetic method, we assessed the impact of overexpression of δ-opioid receptors in LC on stress responsiveness.

Here we report that acute intense stressor exposure results in opposing changes in anxiety-like behavior and LC physiological properties in adolescent male and female rats. No such changes were observed in adult animals. We also found that the same stressor during adolescence in males is associated with impaired δ opioid receptor expression and function, which may contribute to a reduced capacity to terminate the stress response. These results suggest that endogenous opioid signaling in LC, particularly in adolescent males, is a key target, and regulator of, the stress response, and may have critical implications for the development and treatment of various stress-associated pathological conditions.

3 Chapter I: Introduction

Stress is a physiological state that occurs in response to harmful or threatening stimuli, and is characterized by a number of endocrine and behavioral changes that aid in maintaining homeostasis and promoting survival. Based on the nature of the stimulus, stressors can be psychological or physiological. The stress response functions to reinstate homeostasis, and if an organism is not able to accommodate to the conditions, stress converts from an adaptive physiological response into a maladaptive pathological condition (Selye 1975). Despite the variable nature of stressors organisms are faced with, the response is universal and begins with an activation of the hypothalamic pituitary adrenal (HPA) axis. In response to a stressor, the hypothalamus releases CRF and stimulates the pituitary gland to secrete adrenocorticotropic hormone into the vasculature, ultimately leading to release of glucocorticoids from the adrenal cortex into the bloodstream in order to modify energy metabolism. This response also engages circuits which promote a fight-or-flight response through sympathetic nervous system activation and release of epinephrine and norepinephrine (NE) by the adrenal medulla.

Levels of the glucocorticoid stress hormone corticosterone peak in rodents about 30-40 min after the beginning of stressor exposure and then slowly return to baseline levels due to negative feedback regulation. Negative regulation of the HPA axis is an essential mechanism for stress termination. However, in response to chronic or traumatic stress, this negative feedback loop may become impaired and lead to abnormal stress circuit function and predisposition for different psychiatric

conditions. For example, negative regulation is decreased in depression, while in post-traumatic stress disorder (PTSD), the HPA axis is hypersensitive to negative regulation (Yehuda, Southwick et al. 1993, Liberzon and Young 1997).

In addition to physiological responses, stress leads to psychological changes characterized by behavioral vigilance, agitation, anxiety and shifts in attention (Kogler, Muller et al. 2015). These behavioral changes during stressor exposure are adaptive as they facilitate survival; however, persistent anxiety-like behavior when a stressor is no longer present is maladaptive and may manifest as a number of mood disorders in humans. Pathological stress had been classified by the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD10) into acute stress disorder, PTSD and adjustment disorder (F43) (World Health Organization 1992). Despite different primary causes of stress, psychotherapy and antidepressant medications are the most commonly used forms of treatment and management (American Psychiatric Association 2013). However, the development of new diagnostics and therapeutics should be pursued for several reasons. First, recent advances in the understanding of the structure and function of stress-related circuits in the CNS makes this area ripe for investigation. Second, stress associated disorders present a large socio-economic burden, accounting for 42 billion dollars spent annually on medical visits including exacerbated systemic disorders, and it has a negative impact on job performance. Third, there is high comorbidity between stress-associated disorders and other related pathological conditions, such as addiction disorder (Fareed, Eilender et al. 2013, Bernardy, Lund et al. 2014, Patel, Elmaadawi

et al. 2017). This comorbidity may reflect attempts to self-medicate to alleviate the symptoms of PTSD (Brady, Killeen et al. 2000). Critically, anxiety disorder is the most common type of mental disorder and a history of stress is a primary factor in its development (Kalia 2002).

It has been demonstrated that NE release is tightly associated with both physiological and behavioral aspects of stress response. While the adrenal medulla is the source of systemic NE, the LC is the main source of NE in the CNS. The role of LC in stress has been the subject of study since 1970 , when karyometric studies demonstrated an increase in nuclear size during stress in sleep resistant rabbits (Korf, Aghajanian et al. 1973). A critical study by Valentino et al. demonstrated that LC activation during stress occurs primarily through the actions of CRF produced by the central nucleus of amygdala (CeA) (R J Valentino, Foote et. al. 1993), which led to many new studies investigating a role of LC as an extra-hypothalamic node in the stress response. Despite the high volume of studies in this area, there are conflicting reports on how LC responds to different types of stress. For example, Pavcovitch et al. describe a decreased number of active cells in LC after acute immobilization stress, while after 7 days of chronic immobilization NE transmission was enhanced (Pavcovich, Cancela et al. 1990). Conversely, another study of a single prolonged stress reported decreased spontaneous LC activity with increased evoked responses (George, Knox et al. 2013). Such disparities warrant a more detailed analysis of stress-induced LC adaptations.

3.1 LC anatomy and physiology

LC is a bilateral nucleus located in the pons of the brainstem adjacent to the fourth ventricle. It comprises 10-15,000 cells in humans and 1500 in the rat (Berridge and Waterhouse 2003). The name "Locus coeruleus" is derived from Latin and means "blue spot" due to heavily pigmented melanin granules which give it a blue color (Foster F.D. (1891-1893), Russell 1955, Amaral and Sinnamon 1977). Most LC neurons are medium-sized polar and fusiform neurons (Benarroch 2018), though multi-polar, spindle or fusiform, and round have been described in caudal, ventrolateral and subcoeruleus regions that project primarily to the brainstem and spinal cord (Westlund and Coulter 1980, Benarroch 2018). LC is the largest of several noradrenergic nuclei and is the primary source of NE to both the brain and the spinal cord (Lindvall and Bjorklund 1974, Swanson and Hartman 1975, Foote, Bloom et al. 1983). LC is a neuromodulatory nucleus and it regulates the function of its efferent targets through widespread projections in the CNS (Foote, Bloom et al. 1983, Berridge and Waterhouse 2003, Aston-Jones and Cohen 2005) . LC has been reported to be a source of several neuropeptides including galanin and Neuropeptide Y (Holets, Hokfelt et al. 1988), which are thought to have a variety of functions in cognition, feeding, sleep and parental behaviors (Mechenthaler 2008). However, NE is the main neurotransmitter synthesized and released by all LC neurons. NE synthesis starts with the conversion of tyrosine into DOPA by tyrosine hydroxylase, the rate-limiting step in NE synthesis. NE is then synthesized from dopamine by dopamine-β-hydroxylase. NE is stored in presynaptic vesicles and Ca^{2+} influx triggers

its release during cell firing. The amount of NE released by LC is linearly correlated with LC neuronal tonic firing rate (Florin-Lechner, Druhan et al. 1996, Berridge and Abercrombie 1999). NE interacts with its receptors on pre or postsynaptic membranes (Maletic, Eramo et al. 2017). There are three superfamilies of NE receptors: α1, α2, and β. All adrenoreceptors (AR) are G-protein coupled receptors but they vary in their affinity for NE (Arnsten 1998, Arnsten 2007, Arnsten 2011, Atzori, Cuevas-Olguin et al. 2016), and different receptors promote physiological actions and influence on different forms of plasticity and learning (Marzo, Bai et al. 2010, Schutsky, Ouyang et al. 2011, Hansen and Manahan-Vaughan 2015). The receptors with the highest affinity for NE are the α2 ARs (Ramos and Arnsten 2007). They are located both pre- and post-synaptically and are inhibitory Gi-coupled such that their activation leads to decreased levels of intracellular cAMP. Low to moderate concentrations of NE engage the high-affinity α2 ARs, particularly in the prefrontal cortex, which promotes working memory, sustained attention, and other cognitive functions (Arnsten 2011, Amemiya, Noji et al. 2014, Berridge and Spencer 2016). Notably, α2 ARs are also located presynaptically on LC neurons and their activation through collateral release of NE leads to negative feedback regulation of LC neurons and decreases firing (Samuels and Szabadi 2008). The a1 ARs are Gq protein-coupled receptors and have slightly lower receptor affinity for NE. They are localized postsynaptically and their activation leads to phospholipase C activation. The β-ARs are Gs coupled and their activation leads to increased levels of intracellular cAMP. b-ARs have the lowest affinity for the NE and are located postsynaptically. The availability of several functionally distinct receptors with varying affinity for a single neurotransmitter

allows for generation of a range of responses in the target brain areas depending on the state of LC activity which contributes to a multitude of functions in both normal and pathological conditions.

3.2 LC function during stress

LC contributes to several major CNS functions, including waking, arousal, attention, sensory discrimination, and cognition. LC induces and maintains forebrain activity associated with waking and also facilitates the processing of salient sensory stimuli (Berridge and Waterhouse 2003). However, perhaps the context in which LC has been most extensively studied is its role in regulation of stress. As such, the LC/NE system has been recognized as a major brain system involved in the modulation of the central stress response. During stressor exposure, CRF is released onto the LC by the CeA and other CRF-containing stress responsive structures, such as the bed nucleus of stria terminalis, Barrington's nucleus, and PVN (Valentino, Page et al. 1992, Valentino, Rudoy et al. 2001, Reyes, Valentino et al. 2005, Bangasser, Zhang et al. 2011, Reyes, Zitnik et al. 2015, Schwarz, Miyamichi et al. 2015) which increases its tonic discharge (Valentino, Foote et al. 1983, Jedema and Grace 2004, Curtis, Leiser et al. 2012, Snyder, Wang et al. 2012, Borodovitsyna, Flamini et al. 2018) and promotes NE release in the forebrain (Valentino, Foote et al. 1983, Valentino and Foote 1988, Swinny, O'Farrell et al. 2010, Curtis, Leiser et al. 2012, Su, Tanaka et al. 2015) and other target brain areas. Accordingly, stress promotes noradrenergic volume transmission in brain areas such as the amygdala,

hippocampus, and PFC (Lemon, Aydin-Abidin et al. 2009, Marzo, Bai et al. 2010, Hansen and Manahan-Vaughan 2015, Salgado, Trevino et al. 2016).

CRF circuits activating LC can be stressor-specific. For example, hypotensive stress implicates CeA (Rouzade-Dominguez, Curtis et al. 2001, Curtis, Bello et al. 2002), while during colonic distention Barrington's nucleus activates LC (Rouzade-Dominguez, Curtis et al. 2001). CeA is a primary source of CRF for LC activation after psychological stress and is involved in anxiety-like behavior during stress (McCall, Al-Hasani et al. 2015). Importantly, a positive feedback activation loop was recently described from LC to basolateral amygdala (BLA) (McCall, Siuda et al. 2017), which indicates tight reciprocal control between LC and amygdala.

During stress, CRF causes increased tonic discharge which compromises the ability of LC to respond to salient sensory stimuli with phasic firing. This leads to impairments in sensory signal discrimination, several aspects of cognition, and a generally anxious state (Valentino, Foote et al. 1993, Arnsten 2000, Berridge and Waterhouse 2003, Aston-Jones and Cohen 2005, Bangasser and Valentino 2014, McCall, Siuda et al. 2017). While this might seem generally maladaptive, a consequence of short-term stress-induced LC activation is to promote behaviors that increase the likelihood of survival in a threatening situation (Arnsten 2000, Snyder, Wang et al. 2012). By increasing tonic LC discharge (Valentino, Foote et al. 1983, Curtis, Leiser et al. 2012, Snyder, Wang et al. 2012, Borodovitsyna, Flamini et al. 2018) and therefore forebrain NE release (Mana and Grace 1997, Arnsten 1998, Bouchez, Millan et al. 2012, Patki, Atrooz et al. 2015, Rajbhandari, Baldo et al.

2015), prefrontal cortical operations are inhibited (Arnsten 1998, Arnsten 2011) promoting a behavioral phenotype characterized by broad scanning attention and vigilance (Arnsten, Mathew et al. 1999, Arnsten 2000, Snyder, Wang et al. 2012, Snyder, Wang et al. 2012, Patki, Atrooz et al. 2015). It might be advantageous due to the increased responsiveness to a different potential stressors which might appear in this situation and were not encountered before by an adolescent animals.

CRF application increases tonic LC firing (Curtis, Lechner et al. 1997), by decreasing hyperpolarization through inhibition of potassium conductance (Jedema and Grace 2004). CRF actions are blocked by a CRFR1 antagonist as well as a cAMP/PKA antagonist (RP-cAMP-S), but not PKA inhibitor (H89). Therefore, it is thought that the effects of CRF on LC neurons are mediated through cAMP signaling and independently on PKA (Jedema and Grace 2004).

LC firing has two modes: phasic and tonic. Phasic firing is a rapid discharge with 2-3 very high frequency action potentials in a burst in response to behaviorally relevant stimuli followed by a quiescent period of decreased firing (Aston-Jones and Bloom 1981). Phasic responses to salient stimuli accordingly elevate levels of NE in efferent targets. Conversely, tonic firing is spontaneous activity characterized by a regular discharge rate with the frequency from 0 to 5 Hz. Tonic activity varies according to the waking state. For example, during active waking the discharge is >2Hz, during quiet waking the discharge is <2Hz, and during slow-wave sleep <1 Hz (Aston-Jones and Bloom 1981, Berridge and Waterhouse 2003). Tonic firing is spontaneous rather than evoked and may occur with or without electrotonic coupling

between neurons, while phasic firing is induced by the release of excitatory neurotransmitters such as glutamate, from presynaptic terminals, and is thought to occur in a coupled fashion among LC neurons through gap junctions (Ishimatsu and Williams 1996, Rash, Olson et al. 2007). While tonic activity is either low or very high, phasic firing is diminished, but with moderate levels of LC activity the phasic firing is high, leading a robust signal to noise ratio (Aston-Jones and Cohen 2005). The balance in tonic and phasic firing relates directly to behavioral outcomes and follows a Yerkes-Dodson-like inverted U relationship (Aston-Jones and Cohen 2005) (Figure 1).

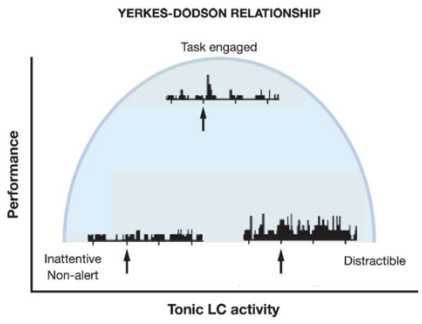


Figure 1. The proposed relationship between tonic LC activity and behavioral performance (Adapted from Aston-Jones and Cohen 2005).

LC activity regulates cortical arousal and seems to be indirectly involved in behavior modulation. It has been shown that the tonic firing of LC neurons within a range of 1-3 Hz promotes goal-directed behavior, while increased tonic firing impairs it while promoting avoidance and hyperarousal (Howells, Stein et al. 2012). These observations are corroborated by multiple studies. For example, elevated LC tonic

firing through optogenetic release of CRF from CeA has been shown to causally stimulate aversion and anxiety-like behavior (McCall, Al-Hasani et al. 2015).

Because stress alters LC discharge (Valentino and Foote 1988, Swinny, O'Farrell et al. 2010, Snyder, Wang et al. 2012, Borodovitsyna, Flamini et al. 2018) and NE release (Mana and Grace 1997, Arnsten 1998, Bouchez, Millan et al. 2012, Patki, Atrooz et al. 2015, Rajbhandari, Baldo et al. 2015) different adrenergic receptors might become engaged during and after stressor exposure. High NE concentration as occurs in response to stress causes engagement of the α1 and β adrenergic receptors. The α1 receptor promotes long term depression (LTD) of prefrontal synapses (Marzo, Bai et al. 2010) and inhibition of prefrontal-dependent cognitive functions such as working memory and sustained attention (Arnsten, Mathew et al. 1999, Arnsten 2000). Indeed, enhanced α1 signaling in PFC is associated with increased behavioral flexibility (Nicholls, Alarcon et al. 2008). Furthermore, stressor exposure has been shown to increase tonic LC discharge and promote scanning attention and behavioral flexibility (Curtis, Leiser et al. 2012, Snyder, Wang et al. 2012). It has been proposed that such a change would permit lower-order sensorimotor regions to guide behavior with little modulation by prefrontal circuitry, allowing disengagement from specific stimuli and goal-oriented behaviors to instead promote rapid impulsive responses (Arnsten 2000). Such a stress-induced shift might be beneficial when an animal is facing a threatening stimulus and a quick escape must be made. Additionally, engagement of the β receptor promotes hippocampal plasticity and encoding and recall of contextual fear

memory (Murchison, Zhang et al. 2004, Schutsky, Ouyang et al. 2011, Ouyang, Young et al. 2012). Therefore, persistent stress-induced changes in LC function would elevate NE concentration in both prefrontal cortex and hippocampus, enhancing plasticity in these areas through signaling at α1 and β receptors to synergistically promote encoding and recall of fear memories, impaired cognition, hypervigilance, and behaviors that allows the generation of an appropriate behavioral response. Therefore, an inverted U relationship between LC firing and arousal/behavioral performance model has been proposed (Arnsten 2000, Berridge and Waterhouse 2003, Arnsten 2007) (Figure 1), with maximal cognitive function corresponding to "ideal" levels of LC tonic firing (Howells, Stein et al. 2012) and hyperarousal and vigilance corresponding with excessive levels of discharge.

LC is known to receive input from CRF-containing terminals, (Van Bockstaele, Colago et al. 1998) and CRF application on LC increases tonic LC firing. Also, as noted above, it has been demonstrated that optogenetically increased LC firing promotes anxiety-like behavior (McCall, Al-Hasani et al. 2015), and stress itself promotes anxiety-like behavior. Relatedly, LC ablation has been shown in several studies to have an anxiolytic effect in behaving animals (Lapiz, Mateo et al. 2001, Itoi 2008). Treatment of rats with neurotoxin DSP-4, which selectively denervates noradrenergic projections, had an anxiolytic effect in the elevated plus maze (EPM) (Lapiz, Mateo et al. 2001). Similarly, mice which received immunotoxin-dependent bilateral LC-ablations spent more time in the open arms of EPM and traveled longer distances in open arms compared to control mice (Itoi 2008)

without an effect on neuroendocrine responses (Itoi 2007). In contrast, others have reported increased neophobia during the complex exploratory test in DSP-4-treated female rats (Harro, Oreland et al. 1995). This sex-dependence suggests that LC plays a different role in anxiety and exploration between males and females. In addition, LC structure, function, and stress-responsiveness also change with age, so it is important to consider the interaction between LC function and stress outcomes with development. Particularly interesting is the potential susceptibility of LC to stress during adolescence, when the neurobehavioral transition and CNS maturation affects cognition and stress reactivity (Spear 2000), and if this susceptibility persists into adulthood when the LC/NE system is fully developed and into the period of age-related changes in aged animals (Buechel, Popovic et al. 2014). Adolescence itself is not a homogenous period and can be conditionally divided into early adolescence (27-34PND), middle adolescence (34-46 PND) which corresponds to puberty, and late adolescence (47-59 PND) (Wulsin, Wick-Carlson et al. 2016). Additionally, female puberty occurs earlier (33-36 PND) than male puberty (42 PND) (Toledo-Rodriguez and Sandi 2011). Each period is characterized by milestones of neuroendocrine development that likely affects stress responses.

3.3 LC sexual dimorphism

In addition to age-related changes in the LC, it is known that LC is sexually dimorphic in both morphology and stress responsiveness (Bangasser, Wiersielis et al. 2016). Sex-differential expression of more than 100 genes has been identified in adult mouse LC (Mulvey, Bhatti et al. 2018). It has also been reported that rat female LC

has more neurons compared to male LC (Luque, de Blas et al. 1992), which may be explained by continuing LC neurogenesis during puberty in females (Pinos, Collado et al. 2001). Furthermore, female LC dendrites are generally longer and expand more in the pericerulear region than in males (Bangasser, Zhang et al. 2011), which may increase afferent connectivity. The female sex hormone estrogen also presynaptically modulates NE release and decreases the degradation of NE (Parvez, Ismahan et al. 1978, Bangasser, Wiersielis et al. 2016), which was demonstrated by increased postsynaptic NE release in LC terminal fields (Selmanoff, Pramik-Holdaway et al. 1976, Alfinito, Chen et al. 2009). As a compensatory mechanism for such increased NE release in LC it was proposed that estrogen decreases the function of postsynaptic NE receptors in the target brain areas (Wagner and Davies 1980, Meitzen, Perry et al. 2013). These factors contribute to the sexually dimorphic LC/NE system and may relate to the increased prevalence of stress-associated psychiatric disorders in women and the higher incidence of anxiety and hyperarousal symptoms in female patients (Bandelow and Michaelis 2015).

There are also differences in stress-regulation between male and female LC neurons. For example, CRF receptors internalize more slowly in females, potentially leading to prolonged activation in the presence of ligand (Bangasser, Wiersielis et al. 2016). The evidence for denser afferentation by limbic areas such as CeA and BNST (Van Bockstaele, Bajic et al. 2001) in the female LC might also contribute to increased CRF input to LC neurons. It has also been shown that female LC neurons are both hyperresponsive to hypotensive stress challenge, as well as hyperresponsive

to intracoerulear CRF infusion compared to male LC neurons, without any baseline differences (Curtis, Bethea et al. 2006). The same study demonstrated that CRF receptor protein levels were higher in ovariectomized females compared to males. Notably, all the sex differences described by Curtis et al. (Curtis, Bethea et al. 2006) were observed in adult LC neurons and were independent from adult hormonal status. A lack of available data for adolescent females and adult females in different stages of estrus warrants further investigation of the effects of sex differences in LC stress responsiveness.

3.4 Stress-endogenous opioid interactions

The HPA axis is one of the most important systems activated during stress. Its activation occurs in response to a stressor and begins with the release of CRF by the paraventricular nucleus of the hypothalamus (PVN), which stimulates the production of both adrenocorticotropic hormone (ACTH) and β-endorphin by the pituitary gland (Castro and Morrison 1997, Trumbach, Graf et al. 2010). While ACTH is responsible for activation of fight-or-flight responses, β-endorphin is thought to decrease pain (Fareed, Eilender et al. 2013). Other extra-hypothalamic sources of endogenous opioids are also recruited during the stress response. Therefore, during stress response, pro-stress effectors such as CRF and ACTH act hand-in-hand with endogenous opioid systems to maintain homeostasis. Dysregulation of these systems in response to chronic or traumatic stress may therefore contribute to various disease states. For example, decreased production of

both ACTH and endorphin are thought to contribute to depressive and addiction withdrawal symptoms (Fareed, Eilender et al. 2013, Karin, Raz et al. 2020).

Both endogenous opioid peptides and their receptors are ubiquitous in the brain and have long been recognized to have a critical role in pain regulation (Corder, Castro et al. 2018). In addition to their anti-nociceptive function, opioid receptors have been described to have roles in reward, motivated behavior, and cognition (Barbano and Cador 2007, Jacobson, Wulf et al. 2018, Nummenmaa and Tuominen 2018). Of particular note are their roles in the stress response (Valentino and Van Bockstaele 2015). Accumulating evidence for anxiolytic properties of endogenous opioids (Henry, Gendron et al. 2017) has sparked an interest in their effects beyond their classic analgesic function. Multiple studies indicate the involvement of endogenous opioids in the stress response in general, as well as emotional responses in particular (Filliol, Ghozland et al. 2000, Erbs, Faget et al. 2015).

Several studies have indicated a role for enkephalins and their receptors in pain processing, memory formation, emotional behaviors (including fear and stress responses) and motivation (Chu Sin Chung, Keyworth et al. 2015, Henry, Bisht et al. 2018). We will focus on the enkephalinergic regulation of mood and affect, with a particular focus on enkephalins' contribution to stress-induced anxiety.

Enkephalins are a class of endogenous opioid peptides including Leu-enkephalin and Met-enkephalin, which were synthesized and characterized soon after their identification (Hughes, Smith et al. 1975, Pasternak, Goodman et al. 1975, Pasternak, Simantov et al. 1976). Numerous anatomical experiments have

demonstrated broad expression of enkephalin throughout the brain and spinal cord (Hokfelt, Elfvin et al. 1977, Simantov, Childers et al. 1977, Finley, Maderdrut et al. 1981, Merchenthaler, Maderdrut et al. 1986). Enkephalins interact with m opioid receptor (MOR) and δ opioid receptor (DOR) with higher specificity toward DOR (Al-Hasani and Bruchas 2011). Opioids have long been recognized as key regulators of pain processing, but also play a role in parental behaviors, respiration and gastrointestinal function. More recently additional roles of opioid receptors in reward, behavior formation, cognition and, importantly, participation in stress response have also been identified.

All opioid receptors are seven transmembrane domain inhibitory G-protein coupled receptors with an N-terminal glycosylated domain extracellularly and C-terminal intracellular domain, where phosphorylation occurs. When a ligand interacts with the N-terminus it leads to conformational changes and Gα and Gβγ subunits dissociation. In LC neurons after activation of opioid receptors, the Gα subunit directly activates $K_{ir}3$ inwardly rectifying potassium channels (Williams, North et al. 1988). This leads to cellular hyperpolarization and decreased cell firing as a result. The Gα subunit deactivates Ca conductance through N and P/Q-type channels by directly binding to the α1 subunit and stabilizing them in the closed state. This therefore prevents Ca channel opening (Zamponi and Snutch 1998) and Ca influx inside the cell, interfering with depolarization and fusion of synaptic vesicles and transmitter release. Additionally, opioid receptor activation suppresses cAMP

synthesis and dependent signaling by inhibition of adenylate cyclase (Minneman and Iversen 1976).

The phosphorylation of the C-terminal end leads to binding by arrestin and subsequently receptor internalization. The nature of the receptor agonist determines the dynamics of arrestin recruitment and receptor internalization, as biased agonism has been demonstrated at both MORs and DORs (Cheng, Yu et al. 1998, Pradhan, Walwyn et al. 2010). Because arrestins are more efficiently recruited by DORs than MORs, each receptor exhibits different dynamics of desensitization in response to binding enkephalin, and DORs will likely desensitize and internalize on a shorter time course than MORs in response to enkephalin binding (Lowe and Bailey 2015),while MORs, in contrast, rapidly recycle back to the plasma membrane (Pan and Pasternak 2010). Although for each opioid receptor type only one single gene has been identified, MORs have multiple splice variants and more than a thousand single nucleotide polymorphisms (SNPs) (Pan and Pasternak 2010). However, splice variants of the C-terminus affect the plasma membrane recycling of MOR (Tanowitz, Hislop et al. 2008). These MOR variations are also thought to be associated with variability in opioid tolerance susceptibility and general efficacy of different agonists used in the clinic or in laboratory settings (Hoehe, Kopke et al. 2000, Pan and Pasternak 2010).

The DOR gene oprd1 is similar between mouse, rat and human and and codes for a single receptor protein with only two SNPs and no alternative splicing sites. One SNP is a silent C for T exchange which affects codon 307 (Gly307Gly) and another is

(G80T) which results in a Cys-Phe substitution (Gendron, Cahill et al. 2016) and affects maturation and ligand-independent trafficking of the receptor. This leads to a gain-of-function phenotype and affects regulation of receptor expression at the cell surface (Leskela, Markkanen et al. 2009). Another modification to DORs is an isomerization between the active and inactive conformations of DOR (Gendron, Cahill et al. 2016), which may lead to constitutive receptor activity. This has also been described for other types of G-protein coupled receptors, where it stabilizes their active confirmation (Kenakin 2004).

Opioid receptors have a distinct pattern of distribution (Mansour, Fox et al. 1994), which may contribute to their distinct effects on physiology and behavior, which have been described in a number of experiments. Conditional knockout of DORs in the frontal cortex, CeA (Poulin, Berube et al. 2013) and hippocampus all produce anxiolytic effects (Chu Sin Chung, Keyworth et al. 2015), making enkephalinergic neurotransmission anxiogenic in those areas. Conversely, activation of endogenous opioid system in BLA has been found to be anxiolytic (Berube, Poulin et al. 2014). Because of the innervation of LC by enkephalinergic afferents and its powerful role in regulating behavioral state, the impact of enkephalin within LC should be explored.

An anxiolytic role for enkephalin was demonstrated in several animal studies (Henry, Bisht et al. 2018). In female mice, genetic knockout of the preproenkephalin gene (PPE-KO), whose protein product leads to production of both leu- and met-enkephalin, led to increased freezing (as a passive coping strategy) in response to foot

shock stress during the open field test (OFT). Preproenkephalin knockout mice also spent less time in the center zone of OFT, which corresponds to increased anxiety-like behavior (Ragnauth, Schuller et al. 2001). In a different study, male PPE-KO mice showed increased anxiety-like behavior based on the number of entries and the time in the center zone of the OFT following footshock stress (Kung, Chen et al. 2010). These data indicate that the endogenous enkephalin system reduces stress-induced anxiety-like behavior and may contribute to stress coping strategies in both males and females.

Further studies have helped to reveal opioid receptor-specific effects. Animal studies demonstrate that DOR activation decreases anxiety and depressive-like behavior and MOR activation has a rewarding effect on behavior (Valentino and Volkow 2018). DOR KO mice show increased anxiety-like behavior in the EPM, as well as aversion to the lit chamber of a light-dark box. Conversely, MOR KO animals demonstrate increased anxiolytic-like behavior, suggesting that these two opioid receptors may play opposing roles in mediating affect. Locomotion was also differentially affected by genetic knockout of these two receptors, with DOR KO mice demonstrating hyperlocomotion and MOR KO mice showing hypolocomotion (Filliol, Ghozland et al. 2000). A similar effect of MOR function on locomotion in the OFT was described in another study in which the MOR agonist DAMGO was injected into the VTA. In this study, the authors ascribed this effect to the "enhanced emotionality" (Calenco-Choukroun, Dauge et al. 1991). The DOR agonist SNC80 has been shown to reduce anxiety-like behavior in the EPM in wild type SD rats, as well

as in the defensive shock probe burial task. A role for DORs in anxiety-like behavior is further corroborated by reports that the specific DOR antagonist naltrindole increases anxiety-like behavior as demonstrated by time spent in the open arms of EPM (Marin, Marco et al. 2003, Saitoh, Yoshikawa et al. 2005, Perrine, Hoshaw et al. 2006) and increased maximal corticosterone response to the mild stress of EPM exposure (Marin, Marco et al. 2003, Saitoh, Yoshikawa et al. 2005). A more localized approach utilizing intracerebroventricular injection of endomorphin 1 (a selective agonist of MOR) increased the preference for the open arms of the EPM in mice (Asakawa, Inui et al. 1998), indicating that MOR agonists may also have anxiolytic potential.

The inhibition of enkephalin degradation might also affect anxiety-like behavior (Waksman, Hamel et al. 1986, Noble and Roques 2007). It has been shown that inhibition of enkephalin degradation by injection of RB101 in MOR KO mice increased both the time spent in open arms and number of visits to the open arms of the EPM, but did not restore normal antinociception in a hot plate and tail immersion test. These findings indicate an anxiolytic role for DORs that is independent of MORs (Nieto, Guen et al. 2005).

A small body of clinical data also suggests a potential role of opioid receptors in stress responsiveness. A study on healthy volunteers demonstrated that the specific MOR agonist hydromorphone decreased the cortisol response to stress and decreased subjective appraisal on how difficult participants found the stress (Bershad, Miller et al. 2018) compared to placebo. Similar data were also observed with buprenorphine

(partial MOR and KOR agonist), such that after a standardized psychosocial stress situation (Trier social stress test) in healthy individuals buprenorphine decreased subjective appraisal of how stressful the test was rated based on questionnaires and decreased saliva corticosterone response. However, it did not have any effect on anxiety levels in healthy individuals (Bershad, Jaffe et al. 2015). Despite the body of evidence supporting a role for endogenous opioids and their receptors in contributing to stress responsiveness and affect, there is relatively little data available on endogenous opioidergic neurotransmission in LC in this regard. LC is innervated by enkephalin-containing terminals from nucleus paragigantocellularis and nucleus prepositus hypoglossi. In addition, it is known that opioid release in LC contributed to the termination of the stress response by decreasing LC firing (Curtis, Bello et al. 2001). Several studies have shown that animals which are more behaviorally resilient to social defeat stress have greater activation of the enkephalinergic input to LC (Reyes, Zitnik et al. 2015, Reyes, Zhang et al. 2019), Therefore, opioids may not just have anxiolytic potential but also help to cope with stressful situations. It is clear that LC and endogenous opioidergic neurotransmission are key regulators of the stress response and emotional behaviors. However, as noted above, LC properties and stress responsiveness vary with both age and sex. In addition, there is a lack of data available on how endogenous opioid receptors within LC specifically react to stress and contribute to the stress response. Therefore, this dissertation aims to determine how an acute traumatic stressor alters LC electrophysiological properties and anxiety-like behavior in adolescent and adult males and females, and the role of endogenous opioid receptors in these changes. The findings presented here indicate that

adolescence is a key period of susceptibility to stressor exposure and that endogenous opioid receptors in LC may become dysregulated by stress so as to promote anxiety-like behavior.

4 Chapter 2

4.1 Introduction: Chapter 2

Stress allows an appropriate physiological and behavioral response to a stressor. If an organism is not able to adapt to stress, it converts from an adaptive physiological response into a maladaptive pathological condition (Selye 1976). During periods of stress, the peptide transmitter CRF is released onto the brainstem nucleus LC, depolarizing its neurons and promoting NE release in the forebrain (Valentino, Foote et al. 1983, Valentino and Foote 1988, Swinny, O'Farrell et al. 2010, Curtis, Leiser et al. 2012, Su, Tanaka et al. 2015). The LC is the largest and primary source of NE to the CNS. The numerous afferent and efferent connections with functionally diverse brain areas explain the wide range of effects of LC stimulation on brain function and behavior, including arousal, regulation of memory consolidation through interactions with amygdala, regulation of synaptic plasticity in the hippocampus, and sensory discrimination through modulation of thalamic and cortical primary sensory neurons (Devilbiss, Page et al. 2006). Because of the wide array of functions modulated by LC, interest in LC function as it relates to diseases, including stress-related conditions, has risen. Particularly interesting are long term changes in LC after a single intense stressful episode, which could be a potential physiological basis for many symptoms of PTSD, anxiety disorders, and acute stress disorder through increased arousal level, hypervigilance and anxiety.

Studies of the relationship between behavioral outcomes and LC firing have shown that there are two firing modes: phasic, when spontaneous discharge is

generally less frequent with bursts of activity in response to behaviorally relevant stimuli, and tonic, which is characterized by a highly regular discharge rate. LC tonic activity levels correlate highly with behavioral indices of arousal, such that it discharges in a slow regular fashion during quiet rest and becomes hyperactive during periods of stress (Berridge and Waterhouse 2003, Swinny, O'Farrell et al. 2010, Curtis, Leiser et al. 2012, Bangasser, Reyes et al. 2013). Increased LC discharge and elevated NE levels in the forebrain leads to increased arousal and hypervigilant behavioral states characterized by impulsivity, impaired cognition and anxiety (Arnsten 1998, Arnsten 2011), traits that are characteristic of a number of neuropsychiatric diseases. Despite many different primary causes of stress, psychotherapy and antidepressant medications are most commonly used for its treatment (American Psychiatric 2013). While many psychoactive drugs target the noradrenergic transmitter system (for example, monoamine oxidase inhibitors (MAOIs), serotonin and norepinephrine reuptake inhibitors (SNRIs), noradrenergic antagonists (Mirtazapin), tricyclic and tetracyclic antidepressants), the long-term impact of stress on the function of NE-containing cell bodies is not fully elucidated. Studies of stress-induced adaptations by the noradrenergic system in humans are limited to functional imaging (fMRI), postmortem studies, and body fluid analysis (Newport, Heim et al. 2003, Lucassen, Pruessner et al. 2014). In this context, animal models are of great value because they allow a more thorough investigation of various aspects of stress pathophysiology.

Both *in vivo* and *in vitro* studies have shown that stress and CRF activate LC through a CRF receptor 1/cAMP dependent mechanism (Valentino, Foote et al. 1983, Valentino and Foote 1987, Jedema and Grace 2004, Curtis, Leiser et al. 2012) It has also recently been shown that in addition to its effects on LC discharge, CRF dose-dependently modulates spontaneous excitatory synaptic transmission (Prouty, Waterhouse et al. 2017), suggesting that stressor exposure and CRF fundamentally alter the way in which LC neurons are able to respond to and transmit incoming excitatory information. Both pre-clinical animal studies and clinical observations have revealed changes in LC itself and in its terminal field regions following stress. LC neurons undergo neuroplastic changes following stress in the neonatal period (Swinny and Valentino 2006, Swinny, O'Farrell et al. 2010) and physiological changes in response to social stress in adolescence (Bingham, McFadden et al. 2011). Furthermore, the LC in females, who are at higher risk for anxiety and mood disorders, is more dendritically complex and stress-susceptible than that of males (Bangasser, Zhang et al. 2011, Bangasser, Reyes et al. 2013, Bangasser, Wiersielis et al. 2016).

fMRI studies of trauma patients have demonstrated increased activity of the right PFC, which is associated with negative emotions, and the amygdala immediately after trauma (Reynaud, Guedj et al. 2015). Furthermore, functional imaging studies of patients with PTSD reveal changes in hippocampal volume, and altered function in amygdala, hippocampus, medial prefrontal, orbitofrontal, anterior cingulate, and insular cortices (Liberzon and Phan 2003), all of which are innervated

and modulated by the LC/NE system. Additionally, studies of patients with PTSD demonstrate that administration of yohimbine, an α_{2A} receptor antagonist which increases presynaptic NE release and forebrain NE concentration, produced panic attacks in up to 70% of patients, and in 89% of patients with PTSD and comorbid panic disorder (Fullerton, Herberman Mash et al. 2015). The nonselective β-receptor antagonist propranolol is also considered a potential therapeutic option for prevention and treatment of PTSD due to the role of NE in memory consolidation and formation of emotions (Taylor and Cahill 2002, Strawn and Geracioti 2008)

Despite a large number of studies, data on the role of LC in different types of stress at different developmental time points are not consistent. For example, chronic social stress has been shown to increase spontaneous LC discharge rate in early adolescent rats, but not adult rats (Bingham, McFadden et al. 2011). Additionally, a decreased number of active cells in LC after acute immobilization stress has been reported, while after 7 days of chronic immobilization, NE transmission is enhanced (Pavcovich, Cancela et al. 1990). Another study of single prolonged stress describes decreased spontaneous LC activity with increased evoked responses (George, Knox et al. 2013). Given the importance of LC and norepinephrine in stress disorders, conflicting reports on the effects of varying types and durations of stressors exposure on LC function, and a lack of direct electrophysiological description of LC neurons in humans, we sought to investigate whether the properties of LC neurons are modified in an animal model of acute stress.

4.2 Rationale: Chapter 2

Activation of LC neurons during stress and accumulating clinical data about LC involvement in the pathogenesis of various stress disorders point to persistent changes in LC in response to acute stress, especially in the adolescent brain due to its increased susceptibility to the negative effects of stress. Of particularly interest are the potential effects of a single traumatic stressor exposure due to the fact that in humans a single episode of intense stress is capable of causing acute stress disorder, adjustment stress disorder or PTSD. Acute stress is frequently overlooked and during adolescence it might produce maladaptive neuroadaptations and contribute to psychiatric diseases in the future.

Stress has been found to have long lasting effects on both behavioral and endocrine function. Van Dijken et al. reported that a single foot shock can cause long lasting behavioral changes (van Dijken, de Goeij et al. 1993), and it was subsequently demonstrated that acute stress in adult rats causes a desensitization of the HPA axis to homotypic stressors 17 days later (Belda, Márquez et al. 2004). Therefore, we hypothesized that acute stress is capable of producing long-term changes in LC neurons of adolescent male rats which may affect anxiety-like behavior.

The goal of these studies is to characterize the effect of acute stress on LC neuronal physiology and anxiety-like behavior immediately and one week later in adolescent male rats. This may provide a physiological basis for the LC involvement in stress-disorders pathogenesis and help to design future therapies based on that knowledge. We chose a multi-modal form of acute stress which combines both

predator odor and physical restraint (Burton, Chatterjee et al. 2007, Gilabert-Juan, Castillo-Gomez et al. 2011, George, Knox et al. 2013) to induce a highly traumatic and stressful state (Brady, Killeen et al. 2000, Bisson 2007, Zhang and Davidson 2007, Lucassen, Pruessner et al. 2014). Rats were subject to fifteen minutes of combined physical restraint and predator odor, and whole-cell patch clamp recordings of LC neurons were performed immediately or one week after stressor exposure. Rats were also tested for anxiety at both time points. These experiments show that acute stress promotes immediate changes in LC neuronal function which persist for at least a week post-stress that correlate with increased indices of anxiety-like behavior.

4.3 Material and Methods: Chapter 2

4.3.1 Subjects

Adolescent male (30 PND) Sprague Dawley rats (Taconic Farms) were housed two to three per cage on a 12 h reverse light schedule (lights on at 9:00pm) with access to standard rat chow and water *ad libitum*. Animal protocols were approved by the Rowan University Institutional Animal Care and Use Committee and were conducted in accordance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

4.3.2 Stressor Exposure

Rats were handled by the experimenter for 5-10 minutes per day for a week prior to experimentation to habituate them to handling. To induce acute stress, rats were placed in a rodent restrainer (Harvard Apparatus) for 15 minutes which was

placed inside of a sealed anesthesia induction chamber connected by silicone tubing to an aquarium pump. A small cylindrical piece of plastic was positioned in-line with the tubing. A 1" x 1" piece of filter paper was placed inside of the chamber and saturated with 100µL predator odor (2,4,5-trimethylthiazole, TMT, Sigma-Alrdich). Odor delivery was achieved by turning on the aquarium pump so that the air forced through the tubing carried the odor into the sealed odor exposure chamber.

4.3.3 Behavioral tests

Elevated plus maze. Immediately after exposure to stress or control conditions, rats were placed in the center of an EPM. The EPM consisted of a plus shaped black plexiglass apparatus elevated 76 cm off the ground with two sets of opposing arms (each arm = 40cm in length) meeting in a central 10cm x 10cm area. Two opposing arms have vertical walls extending 30cm from the floor of the maze, while the other two arms do not have walls. Rats were allowed to explore the maze for 10 minutes, during which their activity was filmed with an infrared camera situated above the maze connected to a Lenovo ThinkCentre M700 PC. At the conclusion of each test, rats were either returned to their home cage for a week, or sacrificed for electrophysiological recordings. The maze was cleaned with 10% bleach between each test. Behavior was scored using AnyMaze behavioral tracking software (Stoelting). Rats were tested in different mazes at the two time points to eliminate the possibility of habituation to any one test confounding anxiety-like behavior.

Open field test. One week after testing in the EPM, rats were placed in the center of an OFT. The OFT consisted of a 90cm x 90cm x 30cm black plexiglass box. Rats were allowed to explore the apparatus for 10 minutes, during which their activity was filmed with an infrared camera situated above the maze connected to a Lenovo ThinkCentre M700 PC. At the conclusion of each test, rats were sacrificed for electrophysiological recordings. The maze was cleaned with 10% bleach between each test. Behavior was scored using AnyMaze behavioral tracking software (Stoelting). Rats were tested in different mazes at the two time points to eliminate the possibility of habituation to any one test confounding anxiety-like behavior.

4.3.4 Electrophysiology

Brain slice preparation. Rats were deeply anesthetized with an intraperitoneal injection of Euthasol (100mg/kg, Virbac) and transcardially perfused with 60mL ice cold oxygenated artificial cerebrospinal fluid (aCSF) of the following composition, in mM: NaCl 126, KCl 2.5, $CaCl_2$ 2.4, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $NaHCO_3$ 25, D-glucose 11. Rats were then rapidly decapitated and the skull was removed so that gross coronal cuts could be made at the level of the medulla and the pineal gland; the resulting block of brain tissue was then extracted from the skull and transferred to 30mL of ice cold oxygenated sucrose-aCSF of the following composition, in mM: sucrose 58.4, NaCl 85, KCl 2.5, $CaCl_2$ 2.4, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $NaHCO_3$ 25. The brain remained in the sucrose-aCSF for 1-2 minutes after which it was transferred to a piece of filter paper saturated with ice cold oxygenated sucrose aCSF, and the lateral edges of the brain were trimmed off. The dorsal aspect of the brain

was then glued to the stage of a Compresstome VF-300-0Z tissue slicer, embedded in agarose, submerged in ice cold oxygenated sucrose aCSF and 200μM thick horizontal sections were cut at a speed of 0.1mm/s with an amplitude of 1.0mm. Sections containing LC (typically, 3 to 4 per animal) were transferred to a holding incubator containing ~300mL aCSF continuously bubbled with 95% O_2/5% CO_2 maintained at 35.5°C and supported by nylon mesh for 1 h. After 1 h, the holding incubator was maintained at room temperature.

Electrophysiological recordings. Slices were individually transferred to a recording chamber which was continuously superfused at 1.5-2mL/min with oxygenated aCSF maintained at 37°C by a Warner Instrument Corporation in-line heater (model 60-01013). LC was visualized as a semi-translucent crescent-shaped region located lateral to the fourth ventricle at 5X magnification using an Olympus BX51WI fixed-stage upright microscope with differential interference contrast and an infrared filter. Individual LC neurons were visualized with a 40X immersion lens and QImaging Rolera Bolt camera connected to a Lenovo ThinkCentre M700 desktop computer using QCapture Pro software. Neurons were approached with sharp glass electrodes (resistance = 5-10MΩ) controlled with Sutter MPC-200 manipulators. For current clamp recordings, electrodes were filled with intracellular solution of the following composition, in mM: KCl 20, K-gluconate 120, $MgCl_2$ 2, EGTA 0.2, HEPES 10, Na_2ATP 2. For voltage clamp recordings, 50μM picrotoxin was added to the aCSF, and electrodes were filled with intracellular solution of the following composition, in mM: Cs-methanesulfonate 145, tetraethylammonium-Cl 5, EGTA 2,

HEPES 10, MgATP 5. After a GΩ seal was established between the pipette and neuronal membrane, the membrane was ruptured and whole-cell recordings were obtained through a MultiClamp 700B amplifier, Digidata 1550B digitizer equipped with two HumSilencer channels, and ClampEx 10.2 software. To assess membrane properties in current clamp mode, spontaneous activity was recorded for 60s without any input and the average firing rate was calculated. They were then subject to a series of increasing current steps from -250pA to 300pA with 50pA intervals between sweeps, and the input resistance and number of action potentials fired in response to each level of current was determined. To assess excitatory synaptic transmission in voltage clamp mode, neurons were clamped at -70mV and spontaneous excitatory post synaptic currents (sEPSCs) were recorded for 6 minutes. sEPSCs were detected in each 6-minute trace using an automated template-matching protocol in ClampFit 10.2 software. Mean sEPSC parameters were calculated across the whole 6 minute recording session.

4.3.5 Data analysis

Electrophysiological data were recorded using Molecular Devices ClampEx 10.2 and MultiClamp 700B acquisition software and analyzed with ClampFit 10.2. Behavioral data were acquired and analyzed using AnyMaze behavioral tracking software (Stoelting). Statistical analyses were performed with R-studio Version 1.1.453. Data were tested for normality using Shapiro-Wilk test. Normally distributed data were tested for equal variances of distribution. Data with equal variances underwent two-tailed t-tests, while those with unequal variances underwent

t-tests with Welch modifications. Wilcoxon Rank Sum test or Mann Whitney U tests were used for non-normally distributed data sets. Statistical significance was set with alpha = 0.05. Error bars in all figures are presented as mean ± SEM unless otherwise is indicated.

4.4 Experimental results: Chapter 2

c-Fos expression in LC cells. To confirm the activation of LC neurons by stress, 7 stressed and 6 control rats were sacrificed two hours after stressor exposure. Coronal brain sections of LC were prepared and immunostained for dopamine β hydroxylase and the immediate early gene c-Fos. c-Fos immunoreactive neurons in the LC were counted in ImageJ. An unpaired Student's t-test ($t[11]=-5.336$, $p<0.001$) showed that stressor exposure significantly increased c-Fos expression in LC (Figure 2).

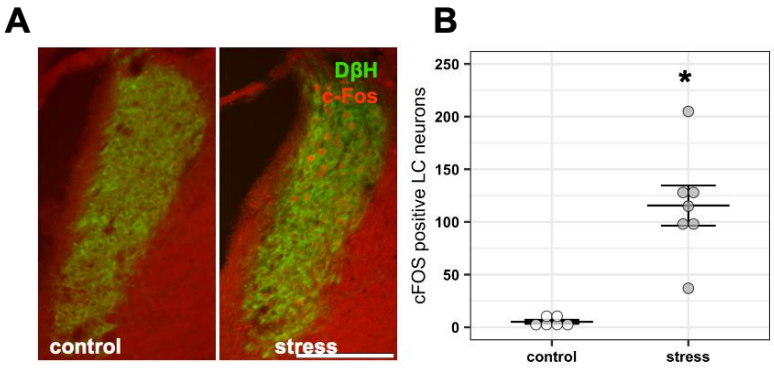


Figure 2. Acute stress increases c-Fos expression in LC neurons. (A) Representative photomicrographs of LC from control (left) and stressed (right) rats show increased c-Fos expression in a subset of LC neurons from stressed rats. (B) The total number of c-Fos immunoreactive cells was significantly greater in stressed

(n=7) than control (n=6) rats. Each dot represents a total number of cFos positive cells from a single animal. Scale bar = 200μm.

Anxiety-like behavior in the EPM. Twenty control and twenty stressed rats were tested in the elevated plus maze for 10 minutes immediately after exposure to stressful or control conditions (Figure 3). Mann Whitney U tests revealed that stressor exposure significantly decreased the number of open arm entries ($U=21.5$, $p<0.001$, Figure 3A), percent time spent in the open arms ($U=23$, $p<0.001$, Figure 3B), increased percent total freezing time ($U=84$, $p=0.002$, Figure 3C) and significantly decreased the total distance traveled in the maze ($U=34$, $p<0.000$, Figure 3D). Mean heat maps for each condition are shown in Figure 3E.

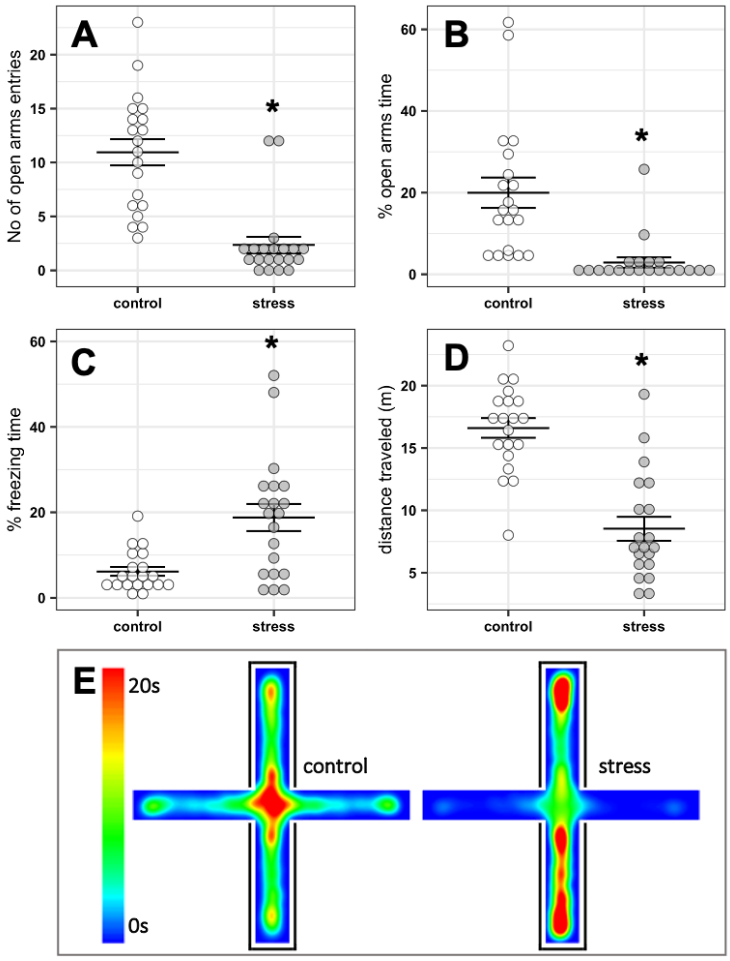


Figure 3. Acute stress immediately increases anxiety-like behavior in the EPM. Immediately after exposure to control or stressful conditions, rats were tested in the EPM. Stressed rats (n=20) entered and spent significantly less time in the open arms than control (n=20) rats (A&B). Additionally, they froze significantly more and traveled significantly less distance than control rats (C&D). Mean heat maps for both conditions are shown in E.

LC discharge properties and excitatory synaptic transmission immediately after acute stress. To determine if increased anxiety was associated with acute changes in LC neuronal physiology, rats were sacrificed for whole-cell electrophysiological recordings immediately after testing in the EPM (Figure 4). A

Mann Whitney U test showed increased spontaneous discharge ($U=106$, $p=0.0012$, Figure 4A) in LC cells from stressed animals (n=25 cells from 5 rats) relative to control (n=16 cells from 3 rats). Input resistance was not found to be significantly different between groups ($t[39]=0.385$, $p=0.702$, Figure 4B). Likewise, a 2 x 7 ANOVA comparing the effects of stress and current injection into LC neurons on the number of action potentials generated revealed a significant main effect of current ($F[6,34]=63.765$, $p<0.001$) , but not of treatment ($F[1,39]=0.066$, $p=0.798$), or the interaction effect of these two variables ($F[6,34]=1.162$, $p=0.349$). Firing in response to current injection didn't differ between stress and control groups (Figure 4D). Representative traces of spontaneous firing action potentials are shown in Figure 4C, while representative traces of action potentials generated in response to 300pA current injection are shown in Figure 4 E.

These changes were accompanied by effects of stress on excitatory synaptic transmission in LC (Figure 5). A Mann Whitney U test ($U=58$, $p=0.04$) showed that mean sEPSC amplitude (Figure 5A) was significantly smaller in LC cells from stressed rats (n=14 cells from 3 rats) than control (n=15 cells from 3 rats). This finding was corroborated by a Kolmogorov-Smirnov test performed on cumulative probability distributions for all sEPSCs amplitudes pooled according to treatment ($Z=18.433$, $p<0.001$, Figure 5B). Charge transfer was also found to be significantly decreased in LC cells from stressed rats ($t[27]=2.573$, $p=0.016$, Figure 5C). The frequency of sEPSCs was not found to be significantly affected by stress ($t[27]=-$

0.001, p=1.000, Figure 5D). Representative traces of sEPSCs from control and stressed rats are shown in Figure 5E.

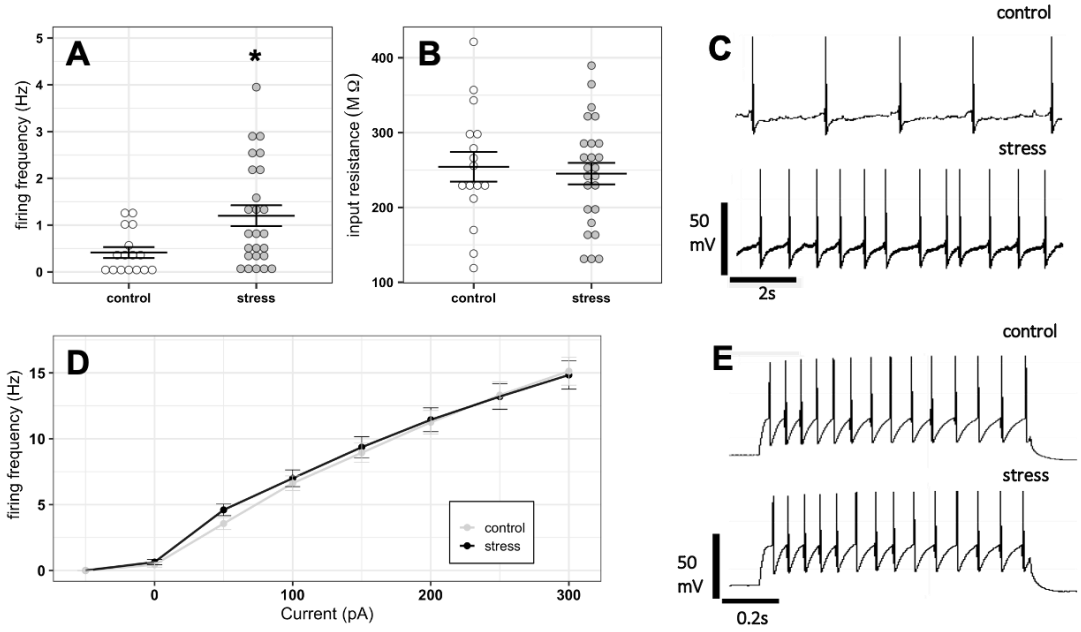


Figure 4. Acute stress increases LC spontaneous discharge. Immediately after stressor exposure, LC cells from stressed rats (n=25 cells from 5 rats) showed a significant increase in spontaneous discharge rate (A) relative to control (n=16 cells from 3 rats). Input resistance did not differ between groups (B). Representative traces of spontaneous activity are shown in (C). The number of action potentials fired in response to increasing levels of current also did not differ as a function of treatment (D). Representative traces of action potentials generated in response to 300pA current injection are shown in (E).

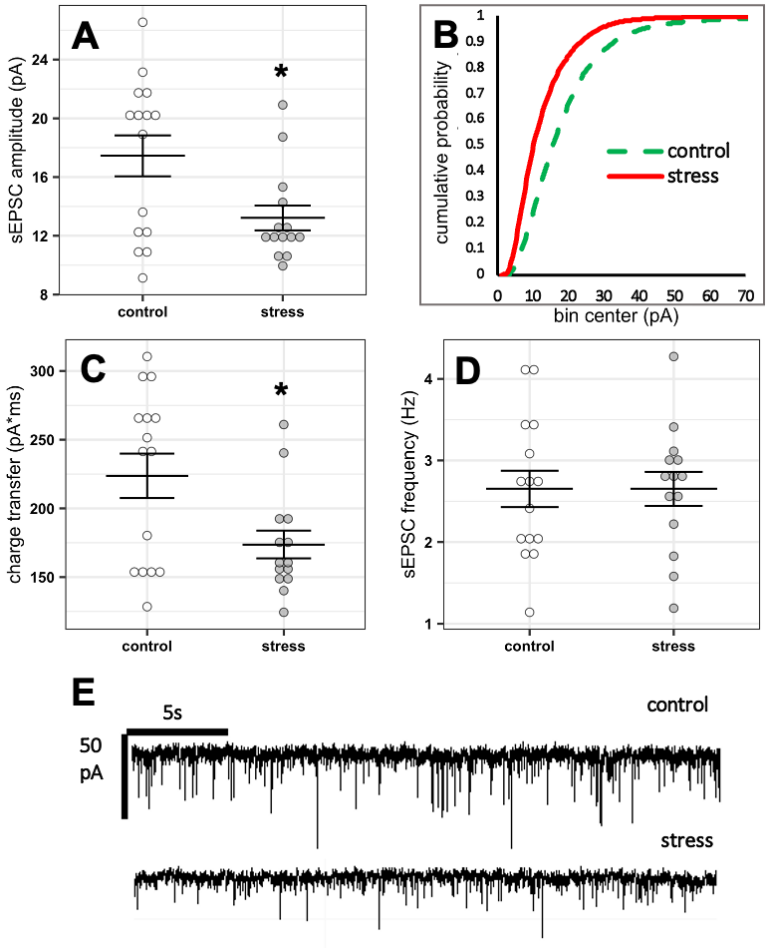


Figure 5. Acute stress immediately decreases LC sensitivity to excitatory synaptic transmission. Immediately after stressor exposure, recordings of LC neurons clamped at -70mV showed that mean sEPSC amplitude (A) was significantly lower in cells from stressed (n=14 cells from 3 rats) than control (n=15 cells from 3 rats) rats. Kolmogorov-Smirnov Z-tests performed on the cumulative probability histogram of all sEPSCs pooled according to condition also showed a significant effect of stressor exposure on amplitude (B). Charge transfer was likewise decreased in cells from stressed rats (C). Frequency was unaffected by stressor exposure (D). Representative traces of sEPSCs from control and stressed rats are shown in E.

Anxiety-like behavior in the open field test a week after stress. To determine if exposure to an acute stressor chronically alters anxiety-like behaviors,

twenty control and twenty stressed rats were tested in the PFT one week after treatment (Figure 6). Mann Whitney U tests showed that the number of entries to the center region ($U=40.5$, $p<0.001$, Figure 6A) and the percent total time spent in the center region ($U=40$, $p<0.001$, Figure 6B) were both significantly decreased in stressed rats. Percent freezing time was significantly increased by stressor exposure ($t[38]=5.916$, $p<0.001$, Figure 6C) and total distance traveled was significantly decreased ($t[38]=5.907$, $p<0.001$, Figure 6D. Mean heat maps for each condition are shown in Figure 6E.

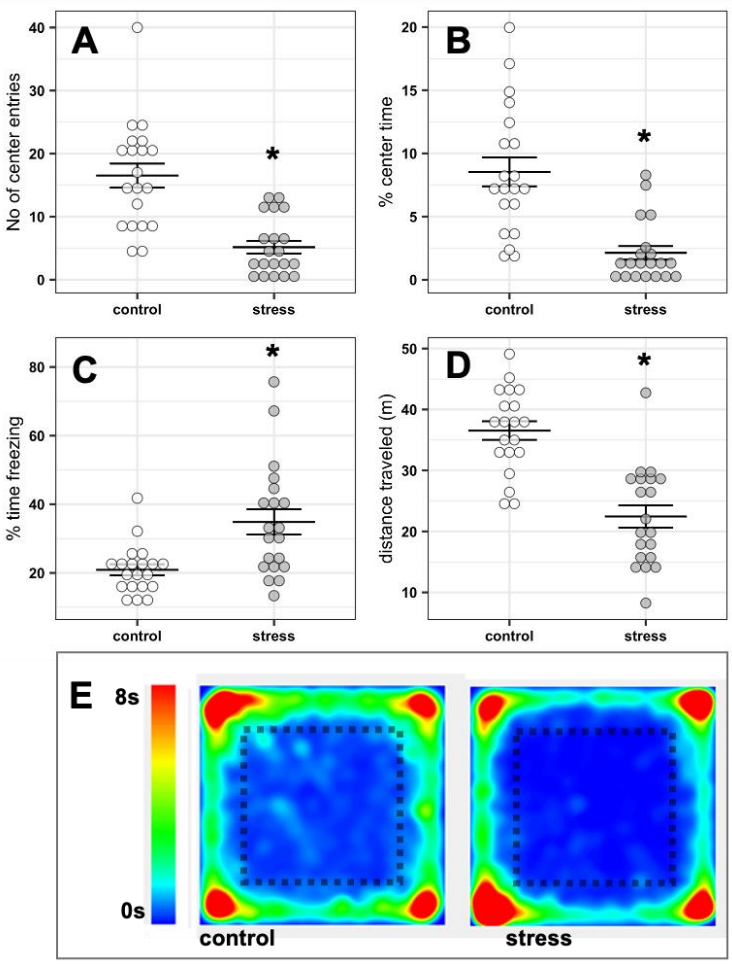


Figure 6. Acute stress produces persistent increases in anxiety-like behavior in the OFT. One week after stressor exposure, rats underwent anxiety testing in the OFT. Stressed rats (n=20) entered and spent significantly less time in the center of the maze than control (n=20) rats (A&B). Additionally, they froze significantly more and traveled significantly less distance than control rats (C&D). Mean heat maps for both conditions are shown in E.

LC discharge properties and excitatory synaptic transmission a week after stress. To determine if increased anxiety during the OFT was associated with chronic changes in LC neuronal physiology, rats were sacrificed for whole-cell electrophysiological recordings immediately after behavioral testing (Figure 7). An

unpaired Student's t-test showed increased spontaneous discharge ($t[35]=2.619$, $p=0.0013$, Figure 7A) in LC cells from stressed animals (n=19 cells from 5 rats) relative to control (n=18 cells from 5 rats). Unlike in the acute study, a Mann Whitney U test revealed a significant effect of stress on LC neuronal input resistance ($U=91$, $p=0.0015$, Figure 7B). Similarly, a 2 x 7 ANOVA comparing the effects of stress and current injection into LC neurons on the number of action potentials revealed main effects of both treatment ($F[1,35]=7.664$, $p=0.009$) and current ($F[6,30]=84.454$, $p<0.001$) Figure 7D).

These changes were also accompanied by effects of stress on excitatory synaptic transmission in LC (Figure 8). An unpaired Student's t test ($t[27]=-2.688$, $p=0.012$) showed that mean sEPSC amplitude (Figure 8A) was significantly smaller in LC cells from stressed rats (n=13 cells from 5 rats) than control (n=16 cells from 4 rats). This finding was corroborated by a Kolmogorov-Smirnov test performed on cumulative probability distributions for all sEPSCs amplitudes pooled according to treatment ($Z=21.292$, $p<0.001$, Figure 8B). Charge transfer was also found to be significantly decreased in LC cells from stressed rats ($t[27]=2.852$, $p=0.008$, Figure 8C). The frequency of sEPSCs was not found to be significantly affected by stress (Figure 8D; $t[27]=-0.401$, $p=691$).

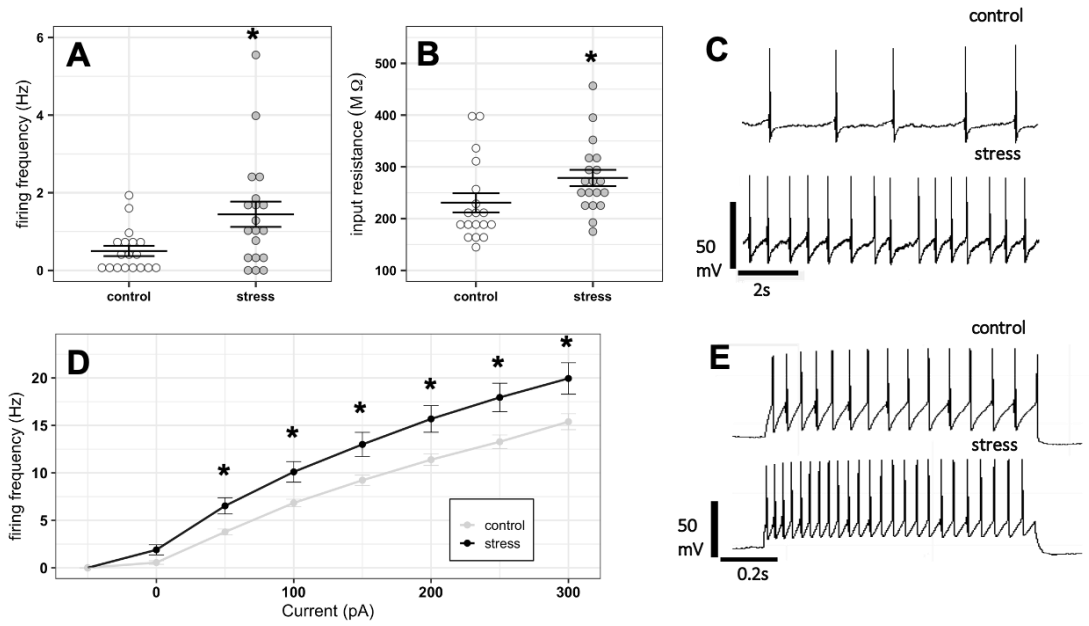


Figure 7. Acute stress persistently increases LC spontaneous discharge and excitability. One week after stressor exposure, LC cells from stressed rats (n=19 cells from 5 rats) showed a significant increase in spontaneous discharge rate (A) relative to control (n=18 cells from 5 rats). Input resistance was also significantly increased in stressed rats (B). Representative traces of spontaneous activity are shown in C. A 2x7 ANOVA revealed significant main effects of both stress and current injected on the number of action potentials generated in response to increasing levels of current (D). The interaction effect of these two variables did not reach statistical significance. Representative traces of action potentials generated in response to 300pA current injection are shown in E.

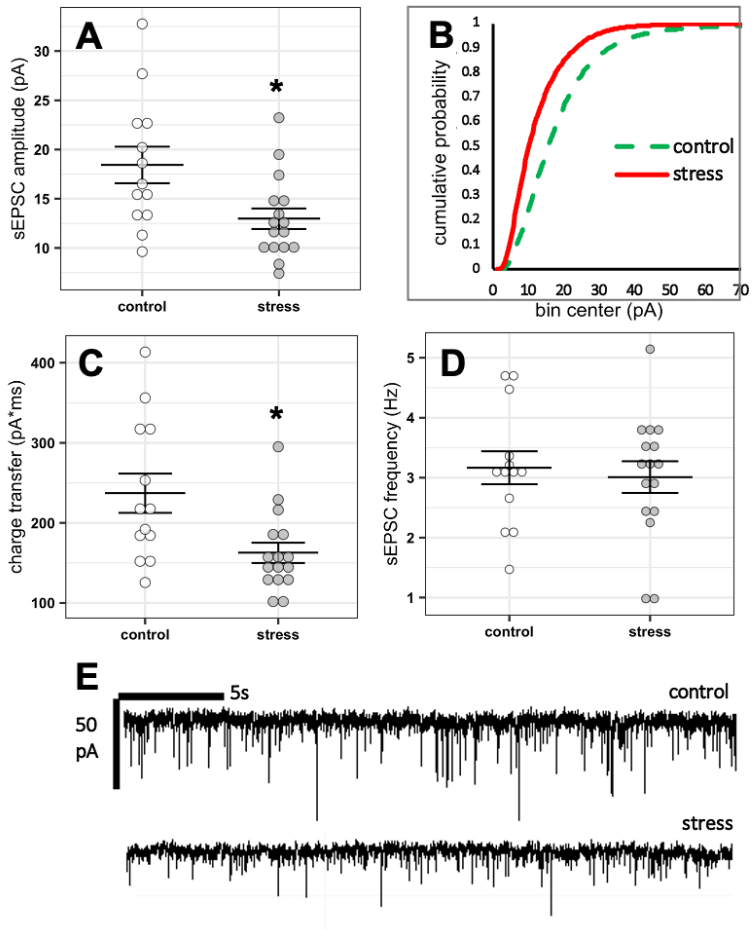


Figure 8. Acute stress chronically decreases LC sensitivity to excitatory synaptic transmission. One week after stressor exposure, recordings of LC neurons clamped at -70mV showed that mean sEPSC amplitude (A) was significantly lower in cells from stressed (n=13 cells from 5 rats) than control (n=16 cells from 4 rats) rats. Kolmogorov-Smirnov Z-tests performed on the cumulative probability histogram of all sEPSCs pooled according to condition also showed a significant effect of stressor exposure on amplitude (B). Charge transfer was likewise decreased in cells from stressed rats (C). Frequency was unaffected by stressor exposure (D). Representative traces of sEPSCs from control and stressed rats are shown in E.

Basis of increased spontaneous discharge at rest varies between acute and one-week time points. Because we found that LC cells from stressed animals were

spontaneously more active at both time points, but more excitable at the chronic (one-week) time point, but not acute time point, we sought to determine if the activation gap (the amount of voltage required to move from resting membrane potential to threshold) varied not only according to treatment, but also to the time of recording. A 2 (treatment) × 2 (time) ANOVA performed on activation gap (Figure 9A) revealed a significant main effect of treatment (stress versus control; $F[1,77] = 17.093$, $p < 0.001$), but the main effect of time (acute versus chronic), and the interaction of these two variables, were not significant ($p > 0.05$). To determine if the decreased activation gap in stressed animals at both time points was the result of alterations in resting membrane potential or action potential threshold, two-way ANOVAs were performed on these variables. The main effect of treatment on resting membrane potential (Figure 9C) was found to be highly significant ($F[1,77] = 10.979$, $p = 0.001$), but the main effect of time, and the interaction effect, were both found to be not significant ($p > 0.05$). Interestingly, the main effect of treatment on threshold (Figure 9B) was not found to be significant, but the main effect of time ($F[1,77] = 23.047$, $p < 0.001$) and the interaction effect ($F[1,77] = 8.316$, $p = 0.005$) were both found to be highly significant.

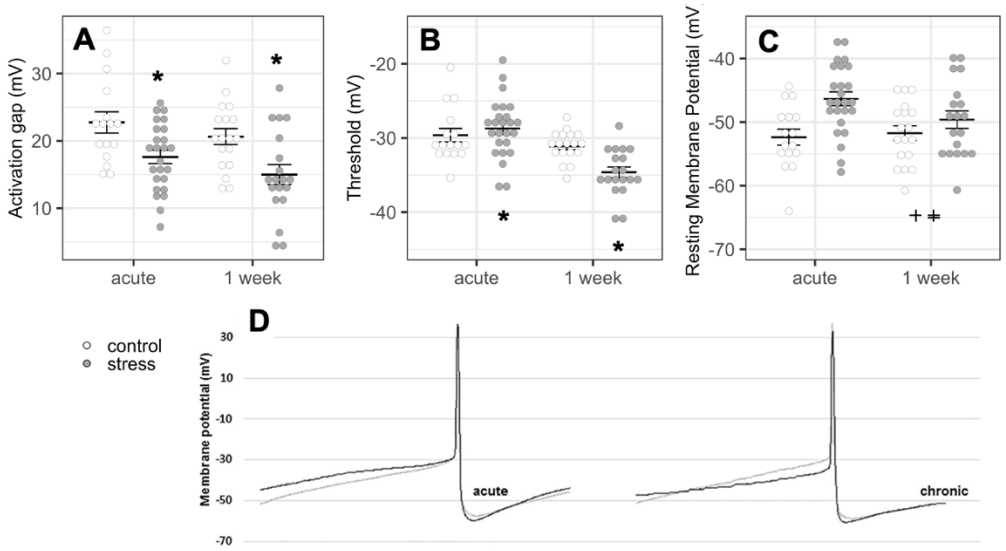


Figure 9. Hyperpolarized threshold contributes to decreased activation gap in LC cells from stressed rats at the chronic, but not acute, time point. (A) A 2 × 2 ANOVA showed that LC cells from stressed rats have a decreased activation gap, or require less voltage to reach threshold from rest, both immediately and one week after stressor exposure. The main effect of time and the interaction effects were not significant. (B) A 2 × 2 ANOVA revealed a significant main effect of time, and a significant interaction of time and treatment, on action potential threshold. The main effect of treatment was not significant. (C) A 2 × 2 ANOVA showed that resting membrane potential is significantly depolarized in LC cells from stressed rats at both time points, but no effect of time or interaction of these variables. (D) Representative action potentials from control (gray) and stressed (black) animals. Action potentials are aligned at their time of initiation to show differences in resting membrane potential, but not threshold, at the acute time point, and differences in both resting membrane potential and threshold at the chronic time point. *Main effect of treatment ($p < 0.05$). †Main effect of time ($p < 0.05$). ‡Interaction effect of treatment and time ($p < 0.05$).

4.5 Conclusions: Chapter 2

Here we have shown that exposure to a single stressful event is capable of precipitating immediate changes in LC function that are accompanied by anxiety like behaviors, both of which persist for at least a week after stressor exposure. While other groups have reported on the use of predator odor, TMT specifically, as a stressor, varying degrees of post-exposure anxiety-like behavior have been reported (Curtis, Leiser et al. 2012, Hacquemand, Choffat et al. 2013, Janitzky, D'Hanis et al. 2015, Rosen, Asok et al. 2015, Asok, Schulkin et al. 2016). Therefore, we chose a multi-modal form of acute stress which combined both predator odor and physical restraint, another well-documented form of stress (Burton, Chatterjee et al. 2007, Gilabert-Juan, Castillo-Gomez et al. 2011, George, Knox et al. 2013) to induce a highly traumatic and stressful state, which is known to contribute to diseases such as PTSD in humans (Brady, Killeen et al. 2000, Bisson 2007, Zhang and Davidson 2007, Lucassen, Pruessner et al. 2014). Our results confirm alterations of LC firing mode in response to stress that have been documented previously. Interestingly, we also found that only a subset of LC cells expresses c-Fos in response to acute stressor exposure (Figure 2), potentially suggesting that only a subset of LC cells is stress responsive. There have been prior reports that the function of LC cells varies on the basis of their efferent connectivity (Chandler, Gao et al. 2014), and others have recently reported that LC cells which project to the amygdala are preferentially activated and required for encoding of fearful memories (Uematsu, Tan et al. 2017). It is therefore possible that the cells which were immunoreactive for c-Fos after stress preferentially innervate the amygdala to facilitate fear memory encoding.

Accordingly, it is important to acknowledge that our electrophysiological recordings were performed on LC cells without regard for their terminal field innervation patterns. Because earlier studies show that LC electrophysiological properties depend on where they project (Chandler, Gao et al. 2014), and reports that circumstances such as fear conditioning (Matsuo, Reijmers et al. 2008, Descalzi, Li et al. 2012) and drug administration (Koya, Cruz et al. 2012, Li, Rubio et al. 2015) produce long-lasting molecular and electrophysiological changes preferentially within neurons that express c-Fos, it is possible that our observed electrophysiological effects would have been stronger if only those stress-responsive neurons had been recorded.

Our observations that both spontaneous LC discharge and markers of anxiety are increased at both time points agree with recent findings which show that high tonic activation of LC is aversive and anxiogenic in animals (McCall, Al-Hasani et al. 2015). Additionally, the results we report here were obtained from adolescent male rats. The increased anxiety and spontaneous LC discharge they displayed is in line with previous reports of elevated stress susceptibility that occurs during adolescence (Bingham, McFadden et al. 2011). Furthermore, the increased LC discharge we have demonstrated immediately after stressor exposure matches well with previous findings from *in vivo* electrophysiological recordings that have shown that local CRF application increases tonic activity of LC neurons and reduces stimulus-driven phasic discharge (Valentino and Foote 1988, Snyder, Wang et al. 2012). This phenomenon may be related to our observation that excitatory synaptic transmission is diminished in LC cells from stressed rats. Because sEPSC amplitude and charge transfer were

both decreased by stressor exposure at both time points, sensory-evoked glutamate release in LC would convey less excitatory current to LC cell bodies during exposure to a stimulus, thus reducing the likelihood of generation of phasic action potentials in response. Therefore, these data may reflect a basis for how a single transmitter (CRF) is capable to increase the likelihood of one type of action potential (spontaneous/tonic) yet decrease the likelihood of another (evoked/phasic). It has been previously shown that these effects are dependent upon CRF receptor 1 and cAMP (Jedema and Grace 2004, Prouty, Waterhouse et al. 2017) The fact that acute stress produced immediate electrophysiological changes such as increased spontaneous discharge, and decreased sEPSC amplitude and charge transfer suggests that LC is capable of adapting to stress in a relatively short time frame. It is likely that these short term changes are the result of CRF signaling in LC which increases tonic discharge by decreasing potassium conductance (Jedema and Grace 2004) and decreased sEPSC amplitude through a CRF receptor 1 dependent mechanism that occurs within a matter of minutes (Prouty, Waterhouse et al. 2017). One potential explanation for decreased sEPSC amplitude immediately after stress is receptor trafficking: it has previously been reported that stress and CRF causes internalization of CRF receptor 1 by LC on a relatively short time scale (Holmes, Babwah et al. 2006, Reyes, Valentino et al. 2008), and thus it is possible that there is mutual deactivation of AMPA and CRF receptors.

At extended time points, permanent molecular changes, such as altered gene expression, could take place. Previous studies have in fact shown that the expression

of a number of genes by LC are sensitive to various stressors (Mamalaki, Kvetnansky et al. 1992, Rusnak, Kvetnansky et al. 2001, Salim, Hite et al. 2007, Fan, Chen et al. 2013, George, Knox et al. 2013). Furthermore, increased expression of a voltage gated sodium channel subunit is associated with elevated spontaneous and evoked activity (Chandler, Gao et al. 2014). Therefore, it is possible that at extended time points, increased expression of voltage gated channels has made them more spontaneously active and excitable. Other molecular adaptations by LC might also contribute to chronically elevated LC discharge and anxiety-like behaviors. It was recently reported that LC from male rats express opioid receptors at greater levels than those from females (Guajardo, Snyder et al. 2017), whose LC is more spontaneously active and stress-responsive than in males (Bangasser, Reyes et al. 2013, Bangasser, Wiersielis et al. 2016). Endogenous opioid transmitter systems in LC counteract the effects of CRF after stressor termination to facilitate a return to a non-anxious state (Curtis, Bello et al. 2001, Van Bockstaele, Reyes et al. 2010, Curtis, Leiser et al. 2012, Chaijale, Curtis et al. 2013). Based on these observations, it is possible that expression of opioid receptors by LC is decreased in stressed animals, leading to increased LC discharge which cannot be appropriately turned down by opioid signaling after stressor termination. This would contribute to a chronically active LC and a generally anxious and hypervigilant behavioral state as we have shown here. Furthermore, the appearance of increased excitability and evoked discharge in stressed rats a week after stressor exposure, but not immediately after, suggests that a molecular change may have taken place over the seven days before recordings were performed. Changes in expression of genes for several ion

channels could further contribute to membrane depolarization in addition to the acute actions of CRF on potassium conductance (Jedema 2004), as well as lead to increased input resistance (Randall, Booth et al. 2012). Such changes would not have had time to take place immediately after stressor exposure, accounting for the presence of these effects at the extended, but not acute time point.

It is also important to note that in addition to generally increased intrinsic excitability in stressed rats, we found evidence for decreased excitatory synaptic transmission in LC at both time points. This is generally in agreement with previous reports which showed that *in vitro* application of CRF to LC cells causes a decrease in sEPSC amplitude and charge transfer, but not frequency, suggesting a post-synaptic adaptation by LC neurons (Prouty, Waterhouse et al. 2017). Of particular interest at the extended time point, however, is the possibility that decreased expression of AMPA receptor subunits might contribute to our observation of diminished sEPSC amplitude and charge transfer. It has previously been reported that decreased expression of glutamate receptor subunits in LC cells are associated with diminished excitatory synaptic transmission (Chandler, Gao et al. 2014). Our finding that AMPA receptor-dependent currents are decreased following stress might also have implications for plasticity of the noradrenergic system. Specifically, by decreasing the amplitude of sEPSCs, stress could scale down synaptic plasticity (Turrigiano, Leslie et al. 1998, Turrigiano 2008). Additionally, LC neurons are characterized by the presence of inward rectifying potassium currents (Torrecilla, Marker et al. 2002, Shirasaki, Abe et al. 2004, Torrecilla, Quillinan et al. 2008),

which have been shown to weaken long-term potentiation through dephosphorylation of AMPA receptors (Chung, Ge et al. 2009). It has also been shown that CRF itself promotes dendritic arborization in LC cells from neonatal rats through a MAP kinase and Rho GTPase dependent mechanism (Swinny and Valentino 2006, Swinny, O'Farrell et al. 2010). Rho GTPases are known to interact with surface-expressed AMPA receptors to regulate cytoskeletal dynamics and synaptic plasticity (Kang, Guo et al. 2009). Therefore, internalization or decreased expression of AMPA and/or CRF receptors could result in neuroplastic adaptations by LC. Identifying the link between CRF signaling during stress and AMPA receptor function therefore represents a potential mechanism to modulate LC plasticity, which becomes perturbed during stress, as well as in a number of pathological conditions (Gesi, Soldani et al. 2000, Swinny and Valentino 2006, Weinshenker 2008, Swinny, O'Farrell et al. 2010, McMillan, White et al. 2011)

Behavioral testing in both the EPM and OFT indicate increased levels of anxiety at both time points. We demonstrated that stressed animals avoided the open arms of the EPM and the center of the OFT, reflecting a fearful behavioral state and aversion toward open spaces. Stressed animals also showed a greatly increased amount of freezing in both mazes; however, this effect was accompanied by a large decrease in total distance traveled (Figures 2 & 5). Although it cannot be ruled out that stressor exposure impaired locomotor function as evidenced by a decreased total distance traveled relative to controls, it is probable that this effect is the result of an increase in freezing behavior, which is widely used as an indicator for fearfulness and

anxiety (Graeff 1994, Soya, Shoji et al. 2013, McCall, Al-Hasani et al. 2015). Importantly, these findings match well with recent reports that high tonic activation of LC is aversive to animals and promotes conditioned place aversion (McCall, Al-Hasani et al. 2015). Although recorded *in vitro* after stressor exposure and behavioral testing, our findings of increased spontaneous discharge by LC neurons from stressed rats support these observations. Collectively, these findings show that even a single stressful episode is able to produce long-lasting changes in the function of a broadly projecting transmitter system that would promote chronically elevated forebrain levels of NE to generate an anxious behavioral phenotype and facilitate encoding of fearful memories by limbic structures (Lemon, Aydin-Abidin et al. 2009, Uematsu, Tan et al. 2015, Uematsu, Tan et al. 2017). Such a behavioral state is characterized by hypervigilance, impaired cognition, anxiety and motivated behavior (Arnsten 2006, Arnsten 2007, Arnsten 2011) reminiscent of that seen in several disease states including PTSD and other anxiety disorders (Arnsten 2007, Zhang and Davidson 2007, Bangasser and Valentino 2014, Janitzky, D'Hanis et al. 2015), whose symptoms can be alleviated or exacerbated by drugs that limit (Nugent, Christopher et al. 2010, Cohen, Kaplan et al. 2011, Giustino, Fitzgerald et al. 2016) or potentiate (Bremner, Innis et al. 1997, Rasmusson, Hauger et al. 2000, Wangelin, Powers et al. 2013) noradrenergic signaling, respectively.

5 Chapter 3

5.1 Introduction: Chapter 3

An important consideration in the study of stress responsiveness are both the age and the sex of the animals used. In the previous Chapter all rats were adolescent males. However, LC is known to undergo developmental changes with age (Olpe and Steinmann 1982) and displays a fair degree of sexual dimorphism. This is of particular importance because adolescence is an important period of transition between childhood and adulthood and is a sensitive period for stressor exposure (van Dijken, de Goeij et al. 1993). Neuroendocrine development in adolescence is characterized by maturation of the CNS and development of HPA axis. A surge of sexual hormones during puberty makes the adolescent HPA axis more susceptible to negative effects of stress. For example, it takes almost twice as long for adolescent rats to return to the normal baseline level of the stress hormone corticosterone, compared to adults, during recovery after stress (Romeo, Lee et al. 2004, Romeo, Lee et al. 2004). Adolescence and pubertal maturation in particular are associated with increased reactivity of the HPA axis to stress and increased baseline corticosterone (Adam 2006, Gunnar, Wewerka et al. 2009). Important sex differences in stress responsiveness are also evident. Female rats have been shown to have higher baseline corticosterone levels and increased responsiveness to stressors compared to males (Kitay 1961, Weinberg, Gunnar et al. 1982, Kirschbaum, Wust et al. 1992). A human study has also demonstrated higher levels of cortisol in males as a result of

stress anticipation, while females had decreased levels of cortisol during stress anticipation (Kirschbaum, Wust et al. 1992).

Behaviorally, adolescence in humans is characterized by a pursuit of independence, changing social status, and increased risk-taking behavior. These changes are paralleled in rodents by increased play behavior and social interactions, and greater exploration of novel environments (Spear 2000). It has been reported that peripubertal stress leads to increased risk-taking behavior and decreased anxiety-like behavior in late adolescence (Toledo-Rodriguez and Sandi 2011). Increased behavioral and emotional susceptibility to stress and risk-taking behavior in adolescence could be explained in part by faster development of limbic structures, including the CeA, compared to PFC (Arain, Haque et al. 2013). This disparity in the rate of development of limbic structures could make PFC even more vulnerable to the negative effects of stress (Arnsten 2009) during adolescence. There have also been inconsistent reports of the effects of stress on behavior, cognitive function, and HPA axis responsiveness depending on the gender, sex, and type of stress used (McCormick, Mathews et al. 2010). Rarely has more than one treatment group been included in studies, so additional research is necessary to compare the stress effects across different ages and sexes.

As noted in earlier chapters, the LC/NE system is intimately involved in the generation of behavioral responses to stress. It is activated by stress through release of CRF which interacts with CRFR1. This increases tonic firing of LC neurons and promotes forebrain release of NE (Valentino, Foote et al. 1983, Valentino, Page et al.

1992, Jedema and Grace 2004). As we have shown in Chapter 2, simultaneous physical restraint and predator odor exposure elicits long-term behavioral and LC electrophysiological changes in the adolescent male LC. However, LC is known to be sexually dimorphic. Additionally, the transition from adolescence into adulthood is characterized by a number of changes in the LC/NE system: decreased transporter content in PFC (Bradshaw, Agster et al. 2016), age-related decline in LC spontaneous cell firing (Olpe and Steinmann 1982) and increased capacity for the recovery and adaptation in response to social stress (Zitnik, Curtis et al. 2016). Accordingly, it is worthwhile to investigate if stress-induced changes in LC physiology and behavior might differ between male and female animals of different ages. Indeed, studies have shown that behavioral and physiological changes occur in adulthood after adolescent stress (Yohn and Blendy 2017), but the impact of stressor exposure during adulthood with regards to LC function and dependent behaviors specifically are less clear. Postnatal environmental changes are also known to impact the developing brain (Kolb and Gibb 2011) and thus stressor exposure in adolescence may lead to neuroadaptations that are distinct from those that occur as a result of stressor exposure in adults.

As described in Chapter Two, a single acute stressor exposure (fifteen minutes combined predator odor and physical restraint) is sufficient to produce behavioral and LC electrophysiological changes a week after stress (Borodovitsyna, Flamini et al. 2018) in adolescent male rats. Despite these findings, those studies did not take into account animal age or sex in how stressor exposure affects LC function and anxiety-

like behavior. As noted above, LC physiology changes with age, and is sexually dimorphic in both morphology and stress responsiveness (Bangasser, Wiersielis et al. 2016). Specifically, there is evidence for denser afferentation from limbic areas in female LC, prolonged activity of CRF receptor activation on LC neurons as a result of slower receptor internalization, and presynaptic modulation of NE release with increased estrogen-dependent release and decreased degradation of NE (Bangasser, Wiersielis et al. 2016). Sex-differential expression of more than 100 genes was also identified in adult mouse LC (Mulvey, Bhatti et al. 2018). Based on these findings, a more comprehensive analysis of how age, sex, and stress interact to modulate anxiety-like behavior is necessary. Therefore, in this study, both adolescent males and females, as well as adult male and female rats are included. Here we describe electrophysiological changes of LC neurons and anxiety-like behavior in response to stress in multiple assays at different time points. We further assess the HPA axis response based on serum corticosterone levels. Together endocrine, physiological and behavioral responses to stressor exposure among animals of both sexes and different ages will help to create a more refined picture of the effects of acute stressor exposure on the LC/NE system.

5.2 Rationale: Chapter 3

There have been inconsistent reports of the effects of stress on behavior, cognitive function, and HPA axis responsiveness depending on the gender, age and type of stress used (McCormick, Mathews et al. 2010). After finding the effect of stress on LC physiology and behavior in adolescent male rats we sought to test the

effect of the same stress on rats of different ages and sexes. Along with behavior and electrophysiology we also sought to analyze the corticosterone response in all groups to access the HPA axis responsiveness to a particular stressor. We hypothesized that animals of different ages and sexes would respond differently to the same stressor, with higher susceptibility to the negative effects of stress occurring in adolescent animals.

5.3 Material and Methods: Chapter 3

5.3.1 Subjects

Adolescent male (30 PND), adolescent female (30 PND), adult male (77 PND) and adult female (77 PND) Sprague Dawley rats (Taconic Farms), were housed two to three per cage on a 12 h reverse light schedule (lights on at 9:00pm) with access to standard rat chow and water *ad libitum*. Animal protocols were approved by the Rowan University Institutional Animal Care and Use Committee and were conducted in accordance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

5.3.2 Stressor Exposure

Rats were handled by the experimenter for 5-10 minutes per day for a week prior to experimentation to habituate them to handling. To induce acute stress, rats were placed in a rodent restrainer (Harvard Apparatus) for 15 minutes which was placed inside of a sealed anesthesia induction chamber connected by silicone tubing to an aquarium pump. A small cylindrical piece of plastic was positioned in-line with

the tubing. A 1" x 1" piece of filter paper was placed inside of the chamber and saturated with 100μL predator odor (2,4,5-trimethylthiazole, TMT, Sigma-Alrdich). Odor delivery was achieved by turning on the aquarium pump so that the air forced through the tubing carried the odor into the sealed odor exposure chamber.

5.3.3 Behavioral tests

Elevated plus maze. Immediately after exposure to stress or control conditions, rats were placed in the center of an EPM. The EPM consisted of a plus shaped black plexiglass apparatus elevated 76 cm off the ground with two sets of opposing arms (each arm = 40cm in length) meeting in a central 10cm x 10cm area. Two opposing arms have vertical walls extending 30cm from the floor of the maze, while the other two arms do not have walls. Rats were allowed to explore the maze for 10 minutes, during which their activity was filmed with an infrared camera situated above the maze connected to a Lenovo ThinkCentre M700 PC. At the conclusion of each test, rats were either returned to their home cage for a week or sacrificed for electrophysiological recordings. The maze was cleaned with 10% bleach between each test. Behavior was scored using AnyMaze behavioral tracking software (Stoelting). Rats were tested in different mazes at the two time points to eliminate the possibility of habituation to any one test confounding anxiety-like behavior.

Open field test. One week after testing in the EPM, rats were placed in the center of an OFT. The OFT consisted of a 90cm x 90cm x 30cm black plexiglass box. Rats were allowed to explore the apparatus for 10 minutes, during which their activity was

filmed with an infrared camera situated above the maze connected to a Lenovo ThinkCentre M700 PC. At the conclusion of each test, rats were sacrificed for electrophysiological recordings. The maze was cleaned with 10% bleach between each test. Behavior was scored using AnyMaze behavioral tracking software (Stoelting). Rats were tested in different mazes at the two time points to eliminate the possibility of habituation to any one test confounding anxiety-like behavior.

5.3.4 Electrophysiology

Brain slice preparation. Rats were deeply anesthetized with an intraperitoneal injection of Euthasol (100mg/kg, Virbac) and transcardially perfused with 60mL ice cold oxygenated artificial cerebrospinal fluid (aCSF) of the following composition, in mM: NaCl 126, KCl 2.5, $CaCl_2$ 2.4, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $NaHCO_3$ 25, D-glucose 11. Rats were then rapidly decapitated and the skull was removed so that gross coronal cuts could be made at the level of the medulla and the pineal gland; the resulting block of brain tissue was then extracted from the skull and transferred to 30mL of ice cold oxygenated sucrose-aCSF of the following composition, in mM: sucrose 58.4, NaCl 85, KCl 2.5, $CaCl_2$ 2.4, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $NaHCO_3$ 25. The brain remained in the sucrose-aCSF for 1-2 minutes after which it was transferred to a piece of filter paper saturated with ice cold oxygenated sucrose aCSF, and the lateral edges of the brain were trimmed off. The dorsal aspect of the brain was then glued to the stage of a Compresstome VF-300-0Z tissue slicer, embedded in agarose, submerged in ice cold oxygenated sucrose aCSF and 200μM thick horizontal sections were cut at a speed of 0.1mm/s with an amplitude of 1.0mm. Sections

containing LC (typically, 3 to 4 per animal) were transferred to a holding incubator containing ~300mL aCSF continuously bubbled with 95% O_2/5% CO_2 maintained at 35.5°C and supported by nylon mesh for 1 h. After 1 h, the holding incubator was maintained at room temperature.

Electrophysiological recordings. Slices were individually transferred to a recording chamber which was continuously superfused at 1.5-2mL/min with oxygenated aCSF maintained at 37°C by a Warner Instrument Corporation in-line heater (model 60-01013). LC was visualized as a semi-translucent crescent-shaped region located lateral to the fourth ventricle at 5X magnification using an Olympus BX51WI fixed-stage upright microscope with differential interference contrast and an infrared filter. Individual LC neurons were visualized with a 40X immersion lens and QImaging Rolera Bolt camera connected to a Lenovo ThinkCentre M700 desktop computer using QCapture Pro software. Neurons were approached with sharp glass electrodes (resistance = 5-10MΩ) controlled with Sutter MPC-200 manipulators. For current clamp recordings, electrodes were filled with intracellular solution of the following composition, in mM: KCl 20, K-gluconate 120, $MgCl_2$ 2, EGTA 0.2, HEPES 10, Na_2ATP 2. After a GΩ seal was established between the pipette and neuronal membrane, the membrane was ruptured and whole-cell recordings were obtained through a MultiClamp 700B amplifier, Digidata 1550B digitizer equipped with two HumSilencer channels, and ClampEx 10.2 software. To assess membrane properties in current clamp mode, spontaneous activity was recorded for 60s without any input and the average firing rate was calculated. They were then subject to a

series of increasing current steps from -250pA to 300pA with 50pA intervals between sweeps, and the input resistance and number of action potentials fired in response to each level of current was determined.

5.3.5 Serum corticosterone assessment

In some of the animals used for behavioral and electrophysiological experiments, blood (0.2-0.4 mL) was collected from the saphenous vein immediately before the onset of stress or control conditions (labeled as "baseline") and 35 min after the onset (labeled as "35 min"). In some subjects who were studied for long-term effects, an additional blood sample (0.2-0.4 mL) was collected from the saphenous vein after behavioral testing one week after control or stress conditions and was labeled as "1 week". In all cases, blood was collected according to a standard protocol for frequent blood collection from lateral saphenous vein in unanesthetized animals (Beeton, Garcia, and Chandy 2007; Parasuraman, Raveendran, and Kesavan 2010). Blood was collected into 1.5mL Eppendorf tubes and left for at least 10 min to coagulate, and then centrifuged at 3000 r.p.m. for 4 min. Serum was kept at -80°C prior to analysis using a Corticosterone Enzyme Immunoassay kit from Enzo (ADI-900-097). Results were calculated using Four Parameter Logistic Curve fit in R-Studio.

5.3.6 Data analysis

Electrophysiological data were recorded using Molecular Devices ClampEx 10.2 and MultiClamp 700B acquisition software and analyzed with ClampFit 10.2.

Behavioral data were acquired and analyzed using AnyMaze behavioral tracking software (Stoelting). Statistical analyses were performed with R-studio Version 1.1.453. Data were tested for normality using Shapiro-Wilk test. Normally distributed data were tested for equal variances of distribution. Data with equal variances underwent two-tailed t-tests, while those with unequal variances underwent t-tests with Welch modifications. A Wilcoxon Rank Sum test or Mann Whitney U tests were used for non-normally distributed data sets. Pearson correlation coefficients were also calculated. Statistical significance was set with alpha = 0.05. Error bars in all figures are presented as mean ± SEM unless otherwise is indicated.

5.4 Experimental results: Chapter 3

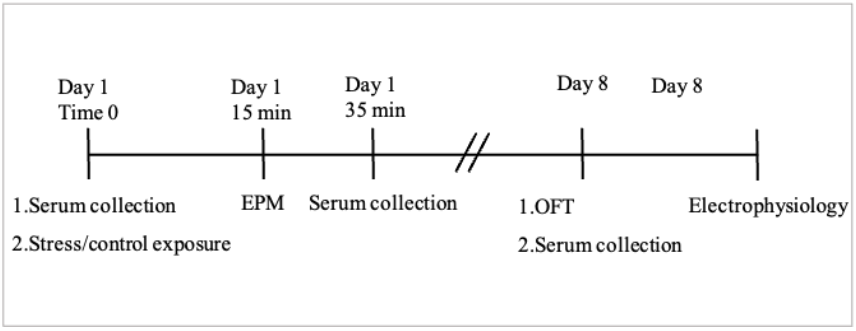


Figure 10. Experimental timeline.

Adolescent females. To determine the effects of acute stress on adolescent female rats the study was performed according to the experimental timeline shown in Figure 10. Serum corticosterone levels from female rats are shown in Figure 11A. Acute exposure to TMT and restraint stress significantly increased corticosterone levels 35 min after the beginning of stress exposure compared to control animals ($t = -4.4089$, $df = 11$, $p = 0.001$). No significant differences in corticosterone levels

between stress and control rats were identified at either baseline or one week measurements.

Anxiety-like behavior was assessed immediately after (Figure 11B) and one week after (Figure 11C) stress in the EPM and OFT, respectively. Percent time spent in the open arms and percent of time spent in the center of open field were used as measurements of anxiety-like behavior. We did not observe any statistical difference in time spent in the open arms in the EPM between control and stress rats. However, stressed female rats spent significantly more time in the center of the OFT a week after stress ($t = -2.6908$, $df = 15.181$, $p = 0.017$).

There was a trend towards, but no significant decrease, in the spontaneous firing rate of female LC neurons a week after stress compared to controls ($W = 1242.5$, $p = 0.08225$; Figure 11D). Input resistance didn't differ between stress and control groups (Figure 11E). There was a significant decrease in evoked LC firing in response to current injection for all current steps beyond 50 pA in stressed adolescent females compared to controls (Figure11F): 50 pA ($W = 1308.5$, $p = 0.03659$), 100 pA ($W = 1353$, $p = 0.01484$), 150 pA ($W = 1303.5$, $p = 0.04159$), 200pA ($W = 1328$, $p = 0.02584$), 250pA ($W = 1343$, $p = 0.01905$), 300 pA current injection ($W = 1368$, $p = 0.01108$).

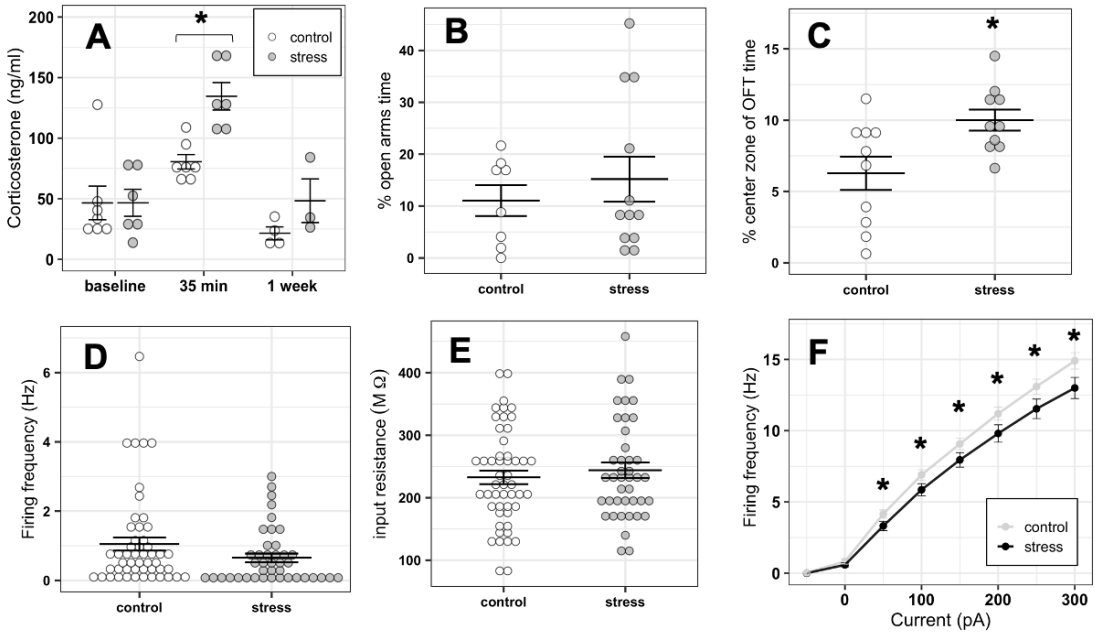


Figure 11. In adolescent female rats stress activates HPA axis and causes increased exploratory activity a week after stress. (A) Serum corticosterone prior to (control=7 animals, stress=6 animals), 35 min after (control=7 animals, stress=6 animals), and 1 week (control=4 animals, stress=3 animals) after stress or control conditions. (B) Percent time spent in open arms of the EPM immediately after stress (n=12) or control (n=8). (C) Percent time spent in the center zone of the open field test a week after stress (n=10) or control (n=10). (D) Spontaneous firing of LC neurons a week after stress (n=41 cells from 7 animals) or control (n=50 cells from 8 animals). (E) Input resistance of LC neurons a week after stress (n=41 cells from 7 animals) or control (n=50 cells from 8 animals). (F) Induced firing in response to current injection with 50 pA steps from -50pA to 300 pA.

Adolescent males. Adolescent male rats were subject to the same experimental timeline as adolescent females (Figure 10). Corticosterone values were significantly higher in animals exposed to the stressor at the 35 min time point compared to controls ($t = -2.6893$, $df = 27$, $p = 0.012$ (Figure 12A). There were no differences between groups at the baseline or one-week time points.

We did not observe a difference in the behavior in EPM immediately after stress (Figure 12B). However, consistent with data reported in Chapter Two, stressed adolescent male rats spent significantly less time in the center of the OFT a week after stress (Figure 12C, $W = 141.5$, $p = 0.009$). LC neurons from stressed adolescent male rats fired also more frequently compared to those from control animals (Figure 12D, $W = 620$, $p = 0.031$). There was no difference in input resistance between stress and control groups (Figure 12E). The induced firing of LC neurons in response to current injection was significantly different between groups at 50 pA ($W = 558.5$, $p = 0.006155$), 100pA ($W = 586.5$, $p = 0.01356$), 150pA ($W = 590.5$, $p = 0.0152$) and 250 pA current injection ($W = 624.5$, $p = 0.04877$, Figure 12F). In contrast to adolescent female rats, neurons from stressed animals fired more frequently in response to current injection compared to those from control animals (Figure 11F).

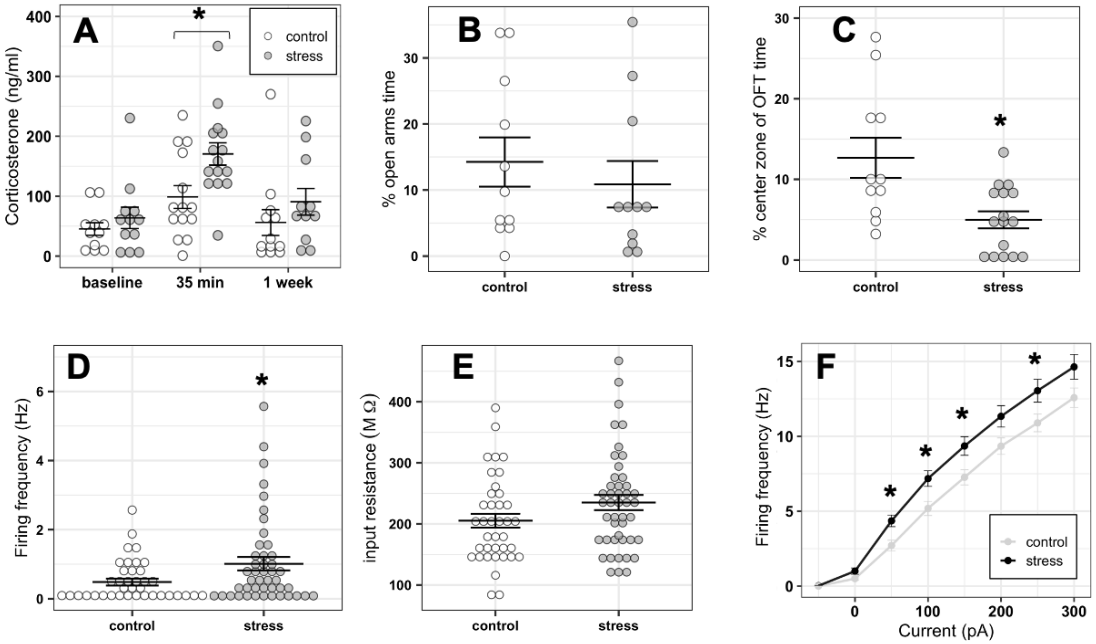


Figure 12. In adolescent male rats stress activates HPA axis and causes increased anxiety-like behavior a week after stress. Stress responses in adolescent male rats. (A) Serum corticosterone prior to (control=11 animals, stress=12 animals), 35 min (control=14 animals, stress=15 animals), and 1 week (control=12 animals, stress=11 animals) after stress or control conditions. (B) Percent time spent in open arms of the EPM immediately after stress (n=11) or control (n=11 animals). (C) Percent time spent in the center zone of the OFT a week after stress (n=16), control (n=11). (D) Spontaneous firing of LC neurons a week after stress (n=45 cells from 8 animals), or control (n=38 cells from 8 animals). (E) Input resistance of LC neurons a week after stress (n=45 cells from 8 animals) or control (n=38 cells from 8 animals). (F) Induced firing in response to current injection with 50 pA steps from -50pA to 300 pA.

Adult males. Adult male rats were subject to the same experimental timeline as the other groups (Figure 10). Corticosterone levels were significantly higher in the stressor-exposed rats at the 35 min time point relative to controls (t = -2.1101, df =

20, p = 0.048 Figure 13A). No such differences were observed between groups at the baseline or one week time points.

Anxiety-like behavior of adult male rats was unaffected by stressor exposure in both the EPM at the acute time point and the OFT at the one-week time point (Figures 13B&C). Similarly, spontaneous and induced LC neuronal firing rates did not differ between stress and control groups a week after stress (Figures 13D&F). Also input resistance didn't differ between stress and control groups (Figure 13E).

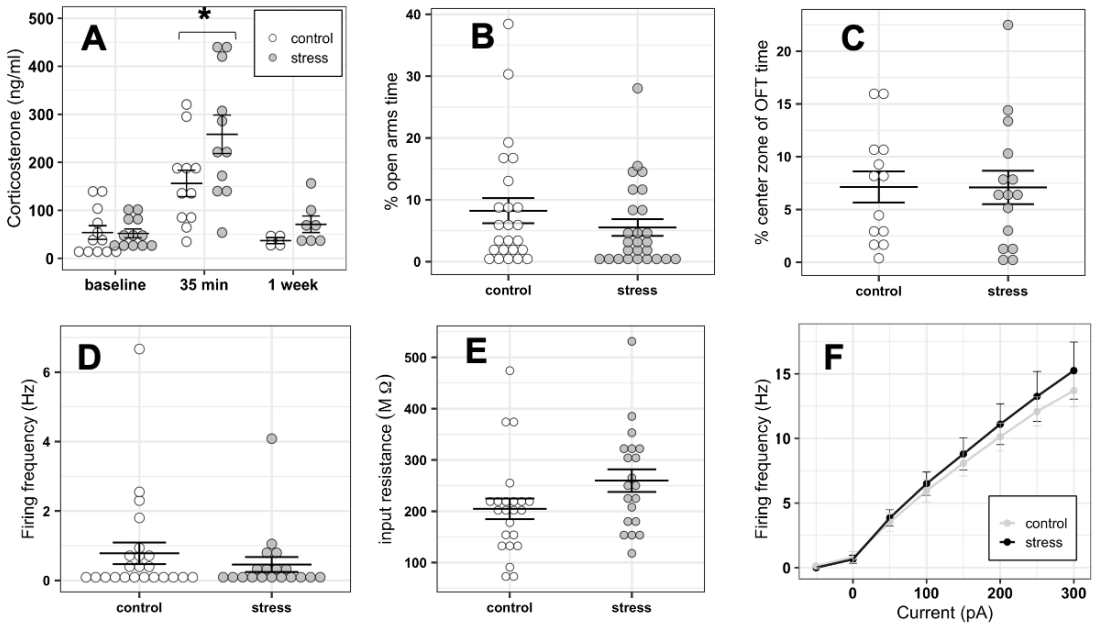


Figure 13. Stress response in adult male rats. (A) Serum corticosterone prior to (control=12 animals, stress=12 animals), 35 min (control=11 animals, stress=11 animals), and 1 week (control=4 animals, stress=7 animals) after stress or control conditions. (B) Percent time spent in open arms of elevated plus maze immediately after stress (n=26) or control (n=24). (C) Percent time spent in the center zone of open field test a week after stress (n=12) or control (n=16). (D) Spontaneous firing of LC neurons a week after stress (n=20 cells from 3 animals) or control (n=23 cells

from 3 animals). (E) Input resistance of LC neurons a week after stress (n=20 cells from 3 animals) or control (n=23 cells from 3 animals). (F) Induced firing in response to current injection with 50 pA steps from -50pA to 300 pA.

Adult females. Adult female rats were subject to the same experimental timeline as the other groups (Figure 10). Corticosterone levels were unaffected in the stressor-exposed rats at the 35 min time point relative to controls.

Anxiety-like behavior of adult female rats was unaffected by stressor exposure in both the EPM at the acute time point and the OFT at the one-week time point (Figures 14B&C). Similarly, spontaneous and induced LC neuronal firing rates did not differ between stress and control groups a week after stress (Figures 14D&F). All the females were in diestrus period during stress.

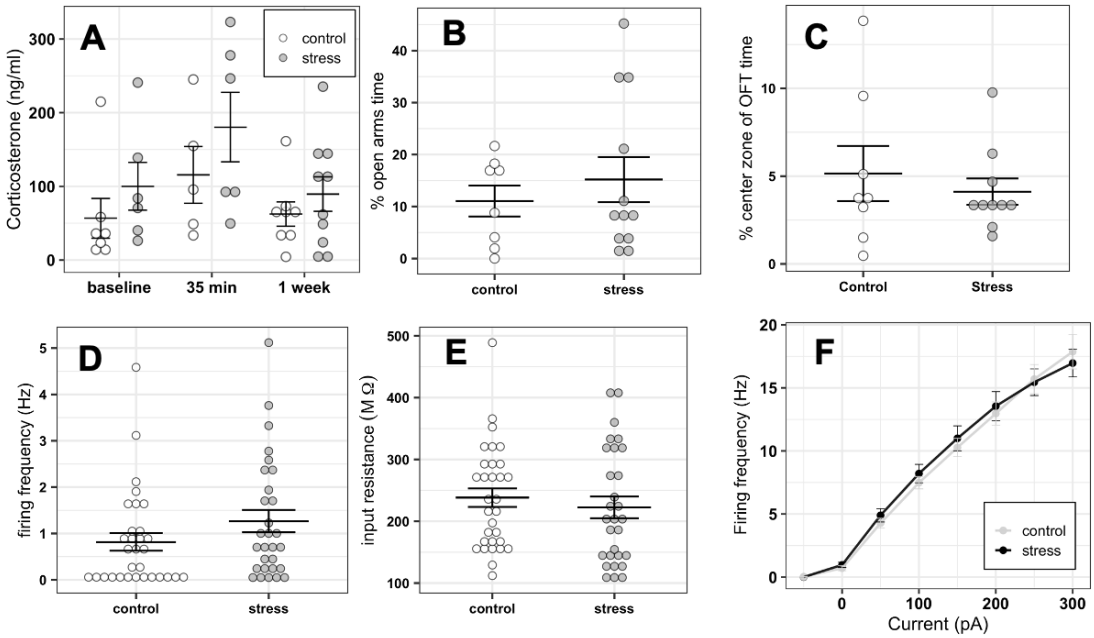


Figure 14. Stress response in adult female rats. (A) Serum corticosterone prior to (control=7 animals, stress=6 animals), 35 min after (control=5 animals, stress=6 animals), and 1 week (control=8 animals, stress=10 animals) after stress or control conditions. (B) Percent time spent in open arms of elevated plus maze immediately after stress (n=12) or control (n=8). (C) Percent time spent in the center zone of open field test a week after stress (n=10) or control (n=8). (D) Spontaneous firing of LC neurons a week after stress (n=29 cells from 8 animals) or control (n=31 cells from 6 animals). (E) Input resistance of LC neurons a week after stress (n=29 cells from 8 animals) or control (n=31 cells from 6 animals). (F) Induced firing in response to current injection with 50 pA steps from -50pA to 300 pA.

Correlation analysis. Because adolescent male rats show a clear relationship between stressor exposure and anxiety-like behavior, we sought to explore how corticosterone levels at the 35 min and one-week timepoints in this group related to behavior in both the EPM and OFT. Correlation mapping of data from all adolescent males (both stress and control) shows that both time spent in the open arms of EPM

(R^2=0.2832, p=0.003, Figure 15B) and distance traveled in the EPM (R^2=0.42, p= 0.0001, Figure 15A) are significantly negatively correlated with corticosterone levels at the 35 min time point. Similarly, considering all adolescent male rats regardless of treatment, there were significant negative correlations between corticosterone at the one-week time point and distance traveled in the OFT (R^2= 0.3054, p=0.006, Figure 15C) and corticosterone at the one-week time point and number of entries to the center of the OFT (R^2=0.2144, p=0.026, Figure 15D). No correlations between firing frequency and behavior were identified in adolescent males. No such significant correlations were identified in adolescent females or adult males and females.

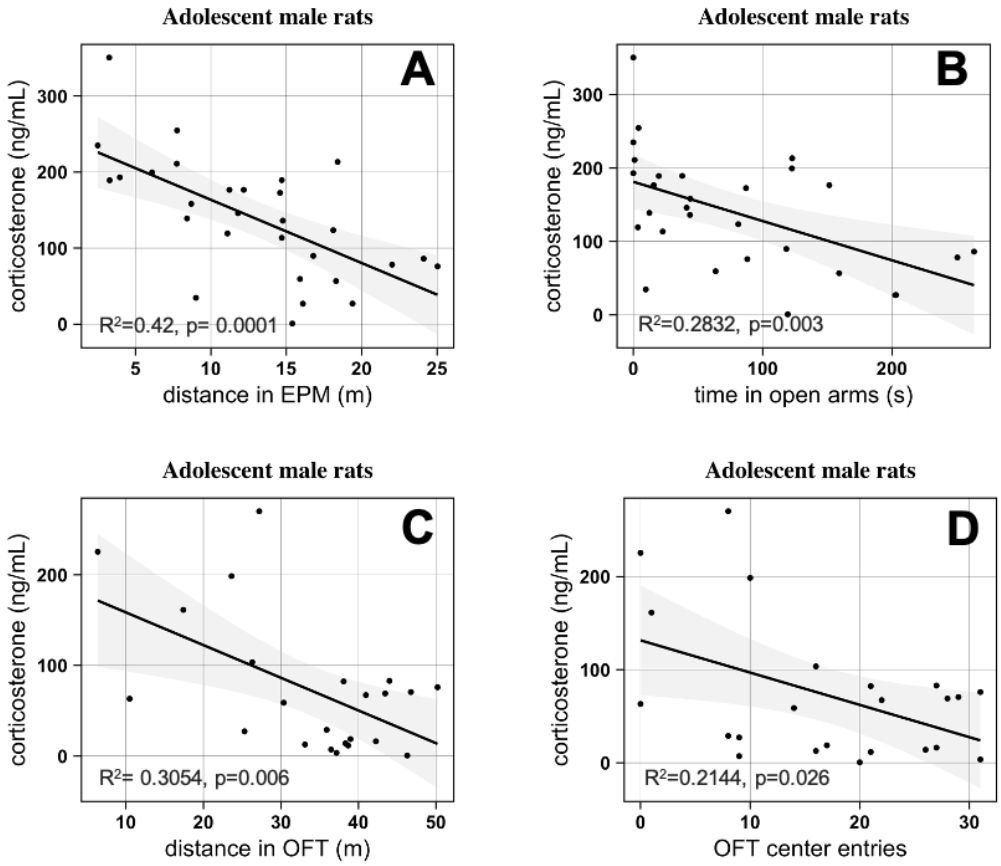


Figure 15. Anxiety-like behavior significantly correlates with corticosterone levels in adolescent male rats. Correlations between corticosterone levels immediately after stress/control exposure and distance travelled in EPM (A), between corticosterone levels immediately after stress/control exposure and time spent in open arms of EPM (B), between corticosterone levels one week after stress/control exposure and distance travelled in the OFT (C), and between corticosterone levels one week after stress/control exposure and number of entrances to the center zone of OFT (D). Shaded regions correspond to 95% confidence interval (CI).

5.5 Conclusions: Chapter 3

In this study we demonstrated how acute stress differentially affects hormonal, electrophysiological and behavioral responses in both male and female rats. In Chapter Two, we showed the effect of combined restraint and predator odor stress on adolescent male LC physiology and anxiety-like behavior (Borodovitsyna, Flamini et al. 2018). Here, we extend these findings to show how the same stressor affects HPA axis activation, LC physiology and behavior in adolescent and adult rats of both sexes.

The HPA axis is the central regulator of the stress response. Its activation results in the release of steroidal hormones by the adrenal glands, which leads to energy metabolism mobilization in order to adapt to an adverse situation (Selye 1975). We observed significantly elevated levels of corticosterone 35 min after stress that also were similar in adolescent males and females and adult males (Figures 11A, 12A& 13A). We observed only a trend for increased corticosterone in adult females in response to stress. This is likely due to small sample size, so we are planning additional experiments to further assess the corticosterone response to stress in a larger group of females. These results confirm that the stressor used in this study was sufficient to produce an endocrine response in most animal groups. The slight but statistically non-significant increase in corticosterone in control animals at 35 min can likely be attributed to the brief restraint and pain associated with unanesthetized blood drawing. A week after stress, corticosterone concentration returned to the baseline levels in all groups, regardless of the treatment (Figures 11A, 12A, 13A& 14A),

which indicates that the stressor does not result in chronic dysregulation of the HPA axis.

HPA axis activation starts with the release of CRF by the PVN of hypothalamus. While it is known that there is a CRF-containing projection from hypothalamus to LC (Reyes, Valentino et al. 2005), it is unclear if the changes in LC physiology we identified in adolescent males and females relate directly to activation of the HPA axis. This is due to the fact that we did not detect a direct relationship between serum corticosterone levels and LC firing rates and only were able to find correlations between HPA axis activation and behavior (Figure 15). Although the HPA axis and LC/NE systems are both activated during stress, observations from this study suggest that they may operate independently of one another and not in a sequential or ordered fashion, despite the known role of CRF in LC activation (Valentino, Foote et al. 1983, Valentino, Page et al. 1992, Jedema and Grace 2004). It is also interesting to note that although the hormonal response to stress is similar in adolescent males, adolescent females and adult males, the adolescent male LC is particularly susceptible to stress-induced physiological changes, and that behavioral adaptations are different in response to stress among these groups. Specifically, adolescent female rats showed a significant stress-induced increase in time spent in the center of the OFT, while males showed a significant decrease. These findings indicate that there are behavioral long-term adaptations in response to stress, but they vary according to the sex of the animal. It is difficult to assess whether this corresponds directly to a stress-induced pro-exploratory and/or anxiolytic effect in

females, or if anxiety-like behavior in the OFT manifests differently in males and females. Interestingly, however, these findings parallel the stress-induced physiological changes we identified in LC. Specifically, females showed increased time in the center of the OFT and decreased neuronal excitability, while males showed decreased time in the center of the open field and increased spontaneous and evoked LC discharge. Given the well-established relationship between LC discharge rates and anxiety-like behavior, it can at least be postulated that stressor exposure produces an anxiolytic effect, or perhaps reduced fear in response to a novel environment, in females one week later. The described changes in adolescent male behavior and LC physiology might have an evolutionary adaptive effect due to the fact that after encountering the stressor for the first time during adolescence those animals would avoid the stressor in the future which would be adaptive. This is why adults have less plastic nervous system and less sensitive to the acute stressor.

These data match well with findings from other studies which have also shown that adolescent female and male rats differ in their stress responses. For example, female Wistar rats have been shown to display increased exploratory behavior and reduced anxiety-like behavior later in adulthood after either lipopolysaccharide-induced inflammatory stress or acute restraint stress during adolescence, while no changes were observed in male rats (Ariza Traslavina, de Oliveira et al. 2014). Adolescent female Long-Evans rats were also shown to have decreased anxiety-like behavior after social stress, whereas males were unchanged (McCormick, Smith et al. 2008). On the other hand, it has been reported that

peripubertal stress leads to increased risk-taking behavior and novelty seeking in Wistar Han male and female rats with a more pronounced effect in females (Toledo-Rodriguez and Sandi 2011). Our findings from adolescent female and male rats are also consistent with another recent publication (Lovelock and Deak 2019), which used acute footshock stress during adolescence (30 PND) and reported long-term increased anxiety-like behavior in males but decreased anxiety-like behavior in females in adulthood (70 PND).

The fact that there is a disparity in reports of sex differences in stress responses may be explained in part by the nature and the duration of the stressor (Ariza Traslavina, de Oliveira et al. 2014). While the existing body of literature on this topic reviews the behavioral effects of stress, there are few studies of sex differences in stress responsiveness of the LC/NE system in adolescent rats, despite its sexually dimorphic nature and clear role in mediating arousal, vigilance, and anxiety-like behavior. Therefore, we assessed electrophysiological characteristics of LC neurons using whole-cell patch clamp electrophysiology. We found that LC neurons from stressed adolescent females have decreased evoked activity compared to control LC neurons. This was a surprising finding because the female LC/NE system is known to be more susceptible to CRF and has been demonstrated to be more sensitive to both acute hypotensive and swim stress (Curtis, Bethea et al. 2006, Bangasser, Curtis et al. 2010, Bangasser, Wiersielis et al. 2016). Such decreased excitability of female LC neurons may be due to possible long-term effects of CRF signaling which alters gene expression and changes in ion channel trafficking

(Borodovitsyna, Joshi et al. 2018). Additionally, a recent genetic analysis (Mulvey, Bhatti et al. 2018) demonstrated sex-differential expression of multiple genes in LC including elevated Prostaglandin E2 (PGE2) receptor Ptger3 (EP3) in female mice. The same study demonstrated that activation of EP3 with the agonist sulprostone led to decreased anxiety-like behavior in female mice after restraint stress and decreased firing in female LC neurons compared to males. Similar mechanisms might contribute to the long-term effects observed in our study.

Another relevant question that this study raises is why adolescent female rats show opposing changes to males in anxiety-like behavior and LC neuronal physiology in response to stress, whereas human females are generally more susceptible to stressor exposure and have almost twice as high an incidence of psychiatric conditions such as PTSD (Kessler, Sonnega et al. 1995), anxiety disorder (Bandelow and Michaelis 2015) and depressive disorder (Angst, Gamma et al. 2002, Accortt, Freeman et al. 2008). It has been demonstrated that repeated restrained stress decreases BLA neuronal firing and causes neuronal atrophy in the lateral nucleus of the amygdala in females, while males show opposite changes in morphology and physiology (Blume, Padival et al. 2019). These data parallel our findings, and sex-specific changes in amygdala function may contribute to the sex-specific stress-induced changes in LC physiology we have identified in adolescent rats. As noted above, it remains to be determined if the increased time in the center of the OFT we identified in stressed females is an anxiolytic effect, or if coping strategies differ between males and females. For example, avoidant coping might contribute to

peritraumatic and posttraumatic dissociation syndrome, and peritraumatic distress is described more frequently among female patients (Boisclair Demarble, Fortin et al. 2020). Therefore, impaired BLA function in response to repeated stress that has been reported in female rats (Blume, Padival et al. 2019) combined with the disrupted LC neuronal activity we observed in response to acute stress in female rats, might contribute to poor behavioral long-term adaptations in response to stress and higher risk of stress-associated psychiatric disorders in females. The amygdala is a major source of CRF for LC, and this pathway is responsible for LC activation and anxiety-like behavior formation during stress (McCall, Al-Hasani et al. 2015). A positive feedback activation loop was recently described from LC to BLA that contributes to anxiety-like behavior (McCall, Siuda et al. 2017), indicating tight reciprocal control between these structures. Therefore, sexually dimorphic stress responses in one region may compound sexually dimorphic changes in the other.

While we observed opposing stress-induced changes in LC physiology and anxiety-like behavior between adolescent male and female rats, any such changes in adult animals regardless of sex were notably absent. This is of particular interest because in humans, adolescence is a critical period of development when many psychiatric disorders first emerge due to structural and functional synaptic changes in the CNS (Paus, Keshavan et al. 2008). Our data confirm that susceptibility of the adolescent LC/NE system to stress may contribute to the development of aberrant anxiety-like behavior. This is in contrast to the developed brain and LC/NE system observed in adults. Therefore, it is possible that during the transition from

adolescence to adulthood, adaptations may occur in LC which confer resilience to stressor exposure at both the behavioral and physiological levels. While this study investigated stress-induced changes in LC physiology, and anxiety-like behavior occurred within groups of rats of specific ages and sexes, it did not explore any such differences between groups. This was done in order to preserve statistical power to detect effects within groups; however, others (Olpe and Steinmann 1982) have reported that there is an age-related decline in LC firing rate. Therefore, the normal developmental trajectory for LC neurons as they progress from and adolescence to adulthood may represent a critical period when this development could be perturbed by stress. Our results are also consistent with findings from recent *in vivo* electrophysiology recordings after social stress (Zitnik, Curtis et al. 2016), where adolescent male rats had elevated spontaneous firing of LC neurons but adult rats showed decreased firing. Another study similarly showed that social stress in early adolescence significantly increases LC spontaneous discharge relative to adult rats, and adult rats did not differ in LC tonic firing properties in response to social stress (Bingham, McFadden et al. 2011).

The findings from these studies indicate that behavioral and LC neuronal responses to stressor exposure differ according to age and sex. These findings highlight the need to consider age and sex as important sources of variation when designing and performing experiments which investigate stress, behavior, and LC physiology. There are multiple factors involved in the development of psychiatric disorders such as anxiety disorders or PTSD, which include different brain areas and

neurotransmitter systems, genetic predispositions and genetic polymorphisms, and emotional and behavioral coping strategies. While LC is only one of many systems that contributes to behavioral state and the stress response, these findings help to clarify how it contributes to these phenomena among rats of different ages and sexes. These disparities may further point to important differences between male and female humans of different ages in the prevalence, progression, and pathophysiology of various clinical psychiatric conditions.

6 Chapter 4

6.1 Introduction: Chapter 4

In Chapter One, the involvement of LC in the stress response was reviewed. Here we will explore the role of enkephalins and their receptors in stress-related LC changes. LC is innervated by Leu- and Met- enkephalin-containing terminals, primarily arising in nucleus paragigantocellularis (PGi) and nucleus prepositus hypoglossus (PrH) (Drolet, Van Bockstaele et al. 1992). Enkephalins are nonselective DOR agonists but also activate MORs. The effects of endogenous opioid receptor stimulation have previously been shown by electrophysiological recordings during hypotensive stress (Curtis, Bello et al. 2001). LC firing rate increased during stress exposure with a subsequent decrease in firing below baseline after stress termination (Curtis, Bello et al. 2001). The increased firing during stress was attributed to CRF activation and was abolished by a CRFR1-antagonist, while the decrease of firing after stress termination was abolished by naloxone. Naloxone is a competitive non-selective opioid antagonist with MOR>DOR>KOR affinity (Newman, Wallace et al. 2000, Johansson, Hirvonen et al. 2019). Therefore, enkephalins and their receptors regulate LC activity during stress and restore baseline LC activity to enable an adaptive response to stress. Activation of MOR and DOR acts in opposition to CRF and leads to decreased cAMP production, hyperpolarizing LC neurons through activation of rectifying potassium channels and blockade of Ca^{2+} channels (Nestler, Alreja et al. 1994, Travagli, Dunwiddie et al. 1995). This crosstalk between opioid and CRF pathways at the level of cAMP may lead to long-term adaptations in nuclear

gene expression (Borodovitsyna, Joshi et al. 2018). The interaction of CRF and opioids within LC seems to be restricted to the duration of stressor exposure, as CRF and opioid antagonists have no effect on LC firing outside of stress activation (Abercrombie and Jacobs 1988, Valentino and Wehby 1989), confirming the absence of tonic CRF and opioid release in LC. This highlights the particular importance of CRF and opioid innervation of LC during stress and their interaction as key reciprocal regulators of the LC stress response.

Histological studies demonstrate the colocalization of MOR and CRFR1 around LC (Van Bockstaele, Colago et al. 1996, Reyes, Glaser et al. 2007), while functional studies demonstrate the inhibitory effects of MOR activation on LC neurons (Aghajanian and Wang 1987, Valentino and Wehby 1988). This colocalization might lead to opioid tolerance in response to stress (Reyes, Glaser et al. 2007), due to the stress-induced mutual receptor internalization and further desensitization. The receptor colocalization may also indicate a concomitant release of enkephalins and CRF.

Opioidergic transmission at MORs in LC produces its effect through post-synaptic mechanisms (Nestler, Alreja et al. 1994). Electron microscopy has confirmed localization of MORs extrasynaptically in the plasma membrane of LC somata and dendrites with infrequent distribution in axons and axon terminals. MOR dendrites are contacted by axon terminals with heterogeneous types of synaptic vesicles and form asymmetric synapses (Van Bockstaele, Colago et al. 1996). The effects of enkephalins on MOR seem to be location-dependent. For example, when

Met-enkephalin was applied close to the cell, it caused an outward current that reversed polarity near the potassium equilibrium potential. When Met-enkephalin was applied at a distance from the cell body (>200 pm), it led to an outward current that did not reverse polarity even at strongly negative potentials (Travagli, Dunwiddie et al. 1995).

The ability of MORs to be internalized and deactivated is important due to the variety of effects different opioid analgesics/agonists have and their potential for tolerance and abuse. Deactivation of the MOR occurs through phosphorylation and subsequent arrestin-dependent internalization and receptor desensitization. It was demonstrated that robust internalization happens after interaction with full agonists like etorphine or endogenous ligands (Van Bockstaele and Commons 2001) and at least 70% internalization is necessary for receptor desensitization (Alvarez, Arttamangkul et al. 2002). While morphine as a partial agonist does not produce acute internalization, chronic morphine exposure modifies the function of LC MORs, such that acute withdrawal precipitated by naltrexone leads to internalization of MOR in LC dendrites (Scavone and Van Bockstaele 2009). Based on this mechanism, chronic exposure to opiates promotes opioid tolerance and inability to control LC firing in response to stress.

Importantly, MOR function in LC is sexually dimorphic (Guajardo, Snyder et al. 2017). Female rats have been shown to express lower levels of MOR mRNA and MOR protein in LC compared to males, and female LC neurons show reduced LC responsiveness to the MOR agonist DAMGO compared to male LC neurons. This

may contribute to increased susceptibility of the female LC to the effects of stress, which would agree with other sex differences (Wagner and Davies 1980, Meitzen, Perry et al. 2013, Bandelow and Michaelis 2015, Bangasser, Wiersielis et al. 2016).

Despite the abundance of data on MOR function in LC neurons, research about the role of DORs in LC is relatively sparse. Immunoperoxidase labeling demonstrated a presynaptic location of DORs around the LC (van Bockstaele, Commons et al. 1997). Based on their localization in terminals containing various neurotransmitters, DORs were shown to form both excitatory and inhibitory synapses. The same study suggests that DORs also autoregulate Met-enkephalin release (van Bockstaele, Commons et al. 1997). Assuming a presynaptic location of DORs relative to LC neurons, it was proposed that activation of presynaptic DORs around LC activates a subpopulation of spinally-projecting LC neurons through the inhibition of GABA release, which plays a role in the analgesia through NE-mediated inhibition of dorsal horn neurons (Pan, Li et al. 2002).

Another study has demonstrated a postsynaptic location of DORs in the mouse LC (Pierre, Ugur et al. 2019), with chronic morphine exposure increasing DOR density. The same study also showed increased density of DOR/MOR heteromers in LC in morphine-dependent mice after intraperitoneal injections, which persisted even in abstinent animals. Though the percent of neurons co-expressing DORs and MORs was only 10% among LC neurons (Pierre, Ugur et al. 2019), the potential dual localization of DORs to both pre- and postsynaptic sites in LC neurons, may diversify its physiological effects, hyperpolarizing LC neurons as well as

modifying the release of other neurotransmitters from presynaptic terminals, including enkephalin.

6.2 Rationale: Chapter 4

It has been demonstrated that upon stressor termination, the endogenous opioid enkephalin is released onto LC. Enkephalins are inhibitory neuropeptides and interact with their DORs and MORs on LC neurons, leading to hyperpolarization and reduced LC firing and therefore a reduction in LC-dependent anxiety-like behavior. Based on the findings presented in Chapters Two and Three, we therefore hypothesized that acute stress modifies endogenous opioid signaling in LC. The goal of these studies was to explore how acute stressor exposure affects opioids receptor expression and sensitivity in LC. We then tested whether LC-specific DOR overexpression would affect LC stress responsiveness and anxiety-like behavior in adolescent male rats. These studies confirm that DOR expression is both affected by and regulates the stress response in adolescent male rats.

6.3 Material and Methods: Chapter 4

6.3.1 Subjects

Adolescent male (30 PND), Sprague Dawley rats (Taconic Farms), were housed two to three per cage on a 12 h reverse light schedule (lights on at 9:00pm) with access to standard rat chow and water *ad libitum*. Animal protocols were approved by the Rowan University Institutional Animal Care and Use Committee and

were conducted in accordance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

6.3.2 Stressor Exposure

Rats were handled by the experimenter for 5-10 minutes per day for a week prior to experimentation to habituate them to handling. To induce acute stress, rats were placed in a rodent restrainer (Harvard Apparatus) for 15 minutes which was placed inside of a sealed anesthesia induction chamber connected by silicone tubing to an aquarium pump. A small cylindrical piece of plastic was positioned in-line with the tubing. A 1" x 1" piece of filter paper was placed inside of the chamber and saturated with 100μL predator odor (2,4,5-trimethylthiazole, TMT, Sigma-Aldrich). Odor delivery was achieved by turning on the aquarium pump so that the air forced through the tubing carried the odor into the sealed odor exposure chamber.

6.3.3 Behavioral tests

Elevated plus maze. Immediately after exposure to stress or control conditions, rats were placed in the center of an EPM. The EPM consisted of a plus shaped black plexiglass apparatus elevated 76 cm off the ground with two sets of opposing arms (each arm = 40cm in length) meeting in a central 10cm x 10cm area. Two opposing arms have vertical walls extending 30cm from the floor of the maze, while the other two arms do not have walls. Rats were allowed to explore the maze for 10 minutes, during which their activity was filmed with an infrared camera situated above the maze connected to a Lenovo ThinkCentre M700 PC. At the

conclusion of each test, rats were either returned to their home cage for a week, or sacrificed for electrophysiological recordings. The maze was cleaned with 10% bleach between each test. Behavior was scored using AnyMaze behavioral tracking software (Stoelting). Rats were tested in different mazes at the two time points to eliminate the possibility of habituation to any one test confounding anxiety-like behavior.

Open field test. One week after testing in the EPM, rats were placed in the center of an OFT. The OFT consisted of a 90cm x 90cm x 30cm black plexiglass box. Rats were allowed to explore the apparatus for 10 minutes, during which their activity was filmed with an infrared camera situated above the maze connected to a Lenovo ThinkCentre M700 PC. At the conclusion of each test, rats were sacrificed for electrophysiological recordings. The maze was cleaned with 10% bleach between each test. Behavior was scored using AnyMaze behavioral tracking software (Stoelting). Rats were tested in different mazes at the two time points to eliminate the possibility of habituation to any one test confounding anxiety-like behavior.

6.3.4 Electrophysiology

Brain slice preparation. Rats were deeply anesthetized with an intraperitoneal injection of Euthasol (100mg/kg, Virbac) and transcardially perfused with 60mL ice cold oxygenated artificial cerebrospinal fluid (aCSF) of the following composition, in mM: NaCl 126, KCl 2.5, $CaCl_2$ 2.4, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $NaHCO_3$ 25, D-glucose 11. Rats were then rapidly decapitated and the skull was removed so that gross coronal cuts could be made at the level of the medulla and the pineal gland; the

resulting block of brain tissue was then extracted from the skull and transferred to 30mL of ice cold oxygenated sucrose-aCSF of the following composition, in mM: sucrose 58.4, NaCl 85, KCl 2.5, $CaCl_2$ 2.4, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $NaHCO_3$ 25. The brain remained in the sucrose-aCSF for 1-2 minutes after which it was transferred to a piece of filter paper saturated with ice cold oxygenated sucrose aCSF, and the lateral edges of the brain were trimmed off. The dorsal aspect of the brain was then glued to the stage of a Compresstome VF-300-0Z tissue slicer, embedded in agarose, submerged in ice cold oxygenated sucrose aCSF and 200μM thick horizontal sections were cut at a speed of 0.1mm/s with an amplitude of 1.0mm. Sections containing LC (typically, 3 to 4 per animal) were transferred to a holding incubator containing ~300mL aCSF continuously bubbled with 95% O_2/5% CO_2 maintained at 35.5°C and supported by nylon mesh for 1 h. After 1 h, the holding incubator was maintained at room temperature.

Electrophysiological recordings. Slices were individually transferred to a recording chamber which was continuously superfused at 1.5-2mL/min with oxygenated aCSF maintained at 37°C by a Warner Instrument Corporation in-line heater (model 60-01013). LC was visualized as a semi-translucent crescent-shaped region located lateral to the fourth ventricle at 5X magnification using an Olympus BX51WI fixed-stage upright microscope with differential interference contrast and an infrared filter. Individual LC neurons were visualized with a 40X immersion lens and QImaging Rolera Bolt camera connected to a Lenovo ThinkCentre M700 desktop computer using QCapture Pro software. Neurons were approached with sharp glass

electrodes (resistance = 5-10MΩ) controlled with Sutter MPC-200 manipulators. For current clamp recordings, electrodes were filled with intracellular solution of the following composition, in mM: KCl 20, K-gluconate 120, $MgCl_2$ 2, EGTA 0.2, HEPES 10, Na_2ATP 2. After a GΩ seal was established between the pipette and neuronal membrane, the membrane was ruptured and whole-cell recordings were obtained through a MultiClamp 700B amplifier, Digidata 1550B digitizer equipped with two HumSilencer channels, and ClampEx 10.2 software. To assess membrane properties in current clamp mode, spontaneous activity was recorded for 60s without any input and the average firing rate was calculated. They were then subject to a series of increasing current steps from -250pA to 300pA with 50pA intervals between sweeps, and the input resistance and number of action potentials fired in response to each level of current was determined. For recordings with opioid receptor agonists spontaneous firing was recorded for 10 min per cell. Two minutes were recorded in the absence of drug followed by eight minutes of drug-inclusive recordings. To prevent carry-over effects from drugs not being washed out, only the last cell per slice was recorded in the presence of drug.

6.3.5 Serum corticosterone assessment

In some of the animals used for behavioral and electrophysiological experiments, blood (0.2-0.4 mL) was collected from the saphenous vein immediately before the onset of stress or control conditions (labeled as "baseline") and 35 min after the onset (labeled as "35 min"). In some subjects who were studied for long-term effects, an additional blood sample (0.2-0.4 mL) was collected from the

saphenous vein after behavioral testing one week after control or stress conditions and was labeled as "1 week". In all cases, blood was collected according to a standard protocol for frequent blood collection from lateral saphenous vein in unanesthetized animals (Beeton, Garcia, and Chandy 2007; Parasuraman, Raveendran, and Kesavan 2010). Blood was collected into 1.5mL Eppendorf tubes and left for at least 10 min to coagulate, and then centrifuged at 3000 r.p.m. for 4 min. Serum was kept at -80°C prior to analysis using a Corticosterone Enzyme Immunoassay kit from Enzo (ADI-900-097). Results were calculated using Four Parameter Logistic Curve fit in R-Studio.

6.3.6 Viral injection surgeries

Early adolescent male Sprague-Dawley rats (Taconic, 60g) were deeply anesthetized through isoflurane inhalation (4%) and placed in a stereotaxic frame. Coordinates for injection of the DOR overexpression vector (AAV-PRSx8-oprd1) or control virus (AAV-PRSx8-mCherry) from bregma were as follows: LC: AP = -1.2 mm; ML = ±2.9 mm; DV = -5.3 mm @ 15° from the dura. All animals (n=40) received bilateral pressure injections of virus (0.3µl) via a 1.0µl Hamilton syringe fitted in a stereotaxic syringe pump at a flow rate of 50 nl/min. The syringe remained in place for 10 minutes before removal. Craniotomies were filled with sterile bone wax, and the incision was closed with wound clips. Upon recovery after surgery animals were exposed to stress or control conditions.

6.3.7 Polymerase Chain Reaction (PCR)

Tissue Processing and LC punches. All tools, materials and instruments were autoclaved and treated with RNAse Zap (Invitrogen) prior to use to prevent degradation of RNA. Rats were deeply anesthetized with 4% isoflurane and rapidly decapitated. Brains were extracted and blocked coronally to a piece of tissue containing cerebellum and pons < 0.5 mm in length. This piece of tissue was placed in 1.8 ml RNALater (Invitrogen) at 4° C for 24 h, then placed in a new dry vial followed by long term storage at -20° C. Brain blocks containing LC were attached to a cryostat mounting block with tissue freezing medium (Triangle Biomedical Sciences; Durham NC) at -30°C and placed in a cryostat. The brain was trimmed to approximately 1.5mm in length to contain the full rostrocaudal extent of LC. A 1 mm trephine was then used to collect bilateral punches of the area directly lateral to the fourth ventricle to collect LC. LC punches were collected in 350 µL RLT lysis buffer (Qiagen) and homogenized using a glass Dounce homogenizer.

RNA Extraction, cDNA Synthesis and RT-PCR. Total RNA was extracted from each LC tissue punch using a Qiagen RNeasy Micro Kit according to manufacturer instructions to produce 14µl samples (n=5/group). RNA concentration and purity within each sample was assessed by using 1µl from each sample in a NanoDrop spectrophotometer (Thermo Scientific). A TaqMan Reverse Transcription Reagent kit (Invitrogen) was used according to manufacturer instructions to produce and amplify ten 50µl samples of cDNA for each animal in a BioRad DNA Engine. Samples were stored at -20°C until further use in RT-PCR experiments. GAPDH was used as a housekeeping gene as a control. In each experiment individual wells contained 10µl reaction mixture (consisting of 10µl

TaqMan 2X Master Mix, 4µl DEPC water, 1µl 20x primers, plus 5µl cDNA per sample). Individual ΔΔCt experiments were carried out using software in conjunction with RT-PCR at Mastercycler ep *realplex* (Eppendorf) to quantify relative expression of each mRNA. Fluorescence baselines and thresholds were manually set for each experiment. Threshold cycle (Ct) values were measured from each sample, and a mean Ct value was calculated from all samples. This same mean value was then subtracted from each individual ΔCt value obtained from each sample to produce an individual ΔΔCt value for each sample. These values were then used to generate relative quantity = $2^{-\Delta\Delta Ct}$ for each sample. This relative quantity for each sample was then divided by the mean control population relative quantity such that the mean of the control group was equal to 1. Therefore, each relative quantity for each sample represents a fold-change from the mean control population.

6.3.8 Western Blotting

Rats were deeply anesthetized with 4% isoflurane and rapidly decapitated. Brains were extracted and blocked coronally to a piece of tissue containing cerebellum and pons < 0.5 mm in length. This piece of tissue was dried frozen in -80° C freezer. Brain blocks containing LC were attached to a cryostat mounting block with tissue freezing medium (Triangle Biomedical Sciences; Durham NC) at -30°C and placed in a cryostat. The brain was trimmed to approximately 1.5mm in length to contain the full rostrocaudal extent of LC. A 1 mm trephine was then used to collect bilateral punches of the area directly lateral to the fourth ventricle to collect LC.

LC tissue punches were placed in a lysis buffer containing (per mL): 50 mL Hepes 10mM, 4 mL EDTA 2mM, 33 mL EGTA 2mM, 10 mL 1% Triton X, 10 mL 1x protein inhibitor I (Calbiochem, Lot 2847204), 10 mL 1x protein inhibitor II (Phosphatase Inhibitor Cocktail Set II, Millipore), 883 mL water. The vials were kept on ice and LC tissue punches were homogenized using an ultrasonic homogenizer (Qsonica Sonicators) for several seconds. Protein concentration was measured using a Bio-Rad *DC* protein assay according to a standard protocol on a microplate reader at 750 nm wavelength. 10 mg of protein per lane were mixed with 4x dye (XT Sample buffer Bio-Rad) and 20x reducing agent. The mix was heated to 95°C for 5 min and centrifuged. Premade gels (Criterion XT Precast Gel 4-12% Bis-Tris #3450125) were used for electrophoresis. The gel cassette was inserted in the chamber and the integral chamber was filled with running buffer (40mL MOPS+ 760 H_20). Each lane was washed with running buffer to ensure potency. 10 µL of a standard ladder (Precision Plus Protein Standard, BioRad) was loaded with 5 mL of fluorescent dye (MagicMark XP Western Standard, Invitrogen) to one lane and samples were then loaded. and run at 200V for 40 min. After the run was complete the gel was removed, washed with water and left in the transfer buffer for 15 min.

For blotting, the membrane was cut to the size of the gel and soaked in methanol for 20 sec, rinsed with water and soaked in the transfer buffer for 15 min. Transfer cassettes were assembled with a semi-dry method horizontally and run at 100V for 45 min. After transfer was complete, the membrane was blocked in 5% BSA-TBSt overnight at 4°C. The next day rabbit polyclonal DOR primary antibodies (Novus Biologicals NB110-79879) and rabbit polyclonal MOR primary antibodies

(abcam, ab17934), mouse polyclonal anti-actin antibodies were diluted in 1%BSA-TBSt and the membrane was incubated for 2 hours in primary antibodies (MOR 1:1000, DOR 1:1000 and actin 1:2000). After the wash the membrane was incubated in secondary antibodies: anti-rabbit 1:5000 and anti-mouse 1:5000. Imaging was performed on Azure biosystems c400 imaging system.

6.3.9 Data analysis

Electrophysiological data were recorded using Molecular Devices ClampEx 10.2 and MultiClamp 700B acquisition software and analyzed with ClampFit 10.2. Behavioral data were acquired and analyzed using AnyMaze behavioral tracking software (Stoelting). Statistical analyses were performed with R-studio Version 1.1.453. Data were tested for normality using the Shapiro-Wilk test. Normally distributed data were tested for equal variances. Data sets that met this requirement underwent testing with a two-tailed t-test. If variances were unequal, t-tests with Welch modifications were conducted. Wilcoxon Rank Sum test or Mann Whitney U tests were used for non-normally distributed data sets. Pearson correlation coefficient were also calculated. Statistical significance was set with alpha = 0.05. Error bars in all figures are presented as mean ± SEM unless otherwise indicated.

6.4 Experimental results: Chapter 4

Effect of stress on mRNA expression of MOR and DOR in adolescent male LC. 9 control and 10 stressed rats were tested for MOR and DOR mRNA expression. Statistical tests revealed that stress significantly decreased mRNA

expression of both MOR (t = 2.353, df = 11.752, p = 0.03692) and DOR (W = 70, p = 0.04347) compared to control (Figure 16).

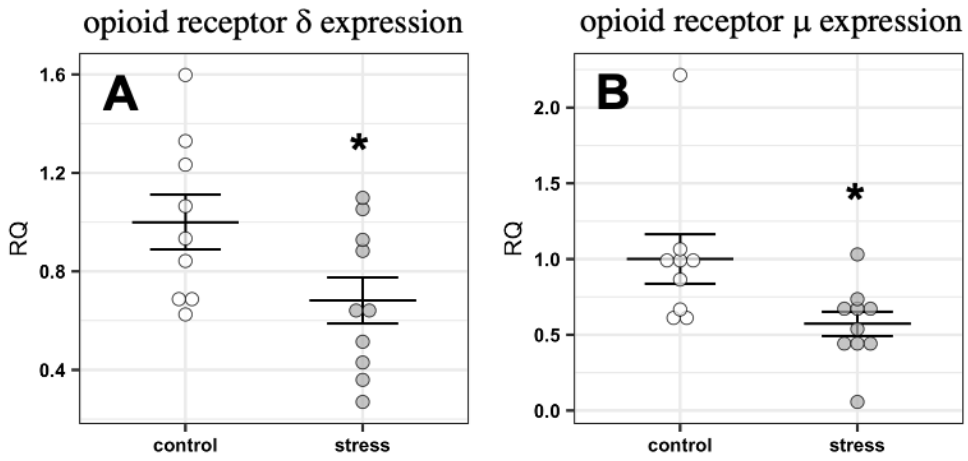
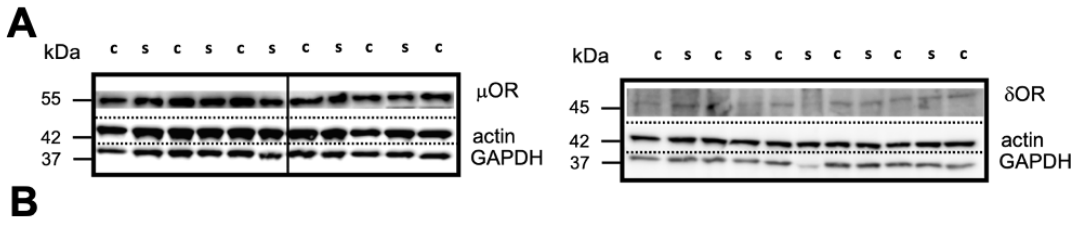


Figure 16. mRNA expression of DOR (A) and MOR (B) was significantly lower in stressed (n=10) rats compare to control (n=9).

Effect of stress on total protein expression of MOR and DOR in adolescent male LC neurons. Total protein expression was not significantly affected by stressor exposure neither for MOR (t=1.6749, df = 7.6597, p-value = 0.1342) nor for DOR (t = -2.6634, df = 3.1448, p-value = 0.0708) (Figure 17).



condition	c	s	c	s	c	s	c	s	c	s	c
MOR relative expression	0.88	0.90	1.16	0.87	1.00	1.00	1.15	0.95	1.17	1.03	0.94
DOR relative expression	0.65	1.36	N/A	1.2	0.69	N/A	0.78	0.68	0.71	1.27	0.75

Figure 17. Representative figures for western blot for MOR and DOR protein expression by LC neurons one-week after stress and control exposure (A). There was no significant effect of stressor exposure on protein expression of either receptor. Relative expression values quantified (B).

Correlation between DOR mRNA expression and anxiety-like behavior.

We found a significant Pearson correlation (R=0.47, p=0.043) between DOR expression and time spent in the center of OFT as an anxiety-like behavioral measure one week after stress or control exposure (Figure 18). This indicates that animals with higher levels of DOR mRNA expression show reduced indices of anxiety-like behavior. Additionally, stressed and control animals form clusters into distinct groups according to DOR levels.

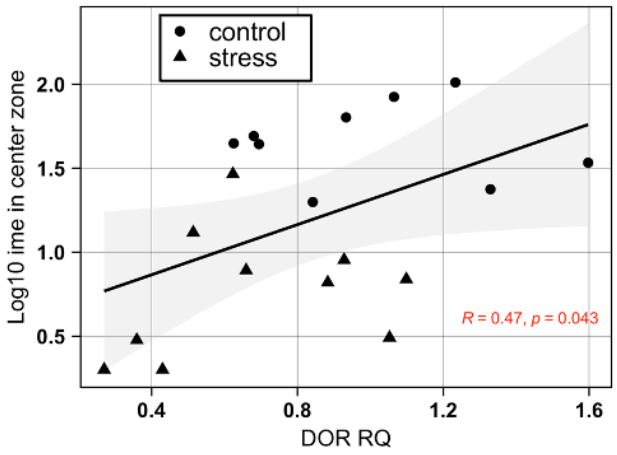


Figure 18. Correlations between DOR mRNA levels and time spent in the center zone of OFT a week after stress/control exposure.

Effect of opioid agonists on LC electrophysiological properties ex vivo one week after stressor exposure. After determining that stress modified mRNA transcription we sought to test how different opioid agonists would affect LC neuronal firing. In these studies, spontaneous LC activity was recorded for ten minutes, with infusion of various opioidergic agents into the recording chamber beginning after two minutes. The number of action potentials in each 30s bin was quantified and percent firing rate in each 30s bin was compared to the baseline firing rate in the first 30s bin. Leu-enkephalin, a DOR and partial MOR agonist, potently abolished the firing of LC neurons shortly after the onset of infusion at both 0.5mM and 5mM concentrations regardless of treatment (Figure 19). Only the 5mM concentration is shown in Figure 19. Similar results were found with the MOR agonist DAMGO at both 1mM and 10mM concentrations (Figure 20). In contrast, infusion of the selective DOR agonist SNC-162 potently inhibited LC neuronal firing

from control animals but had a much less robust effect on cells from stressed animals (Figure 21).

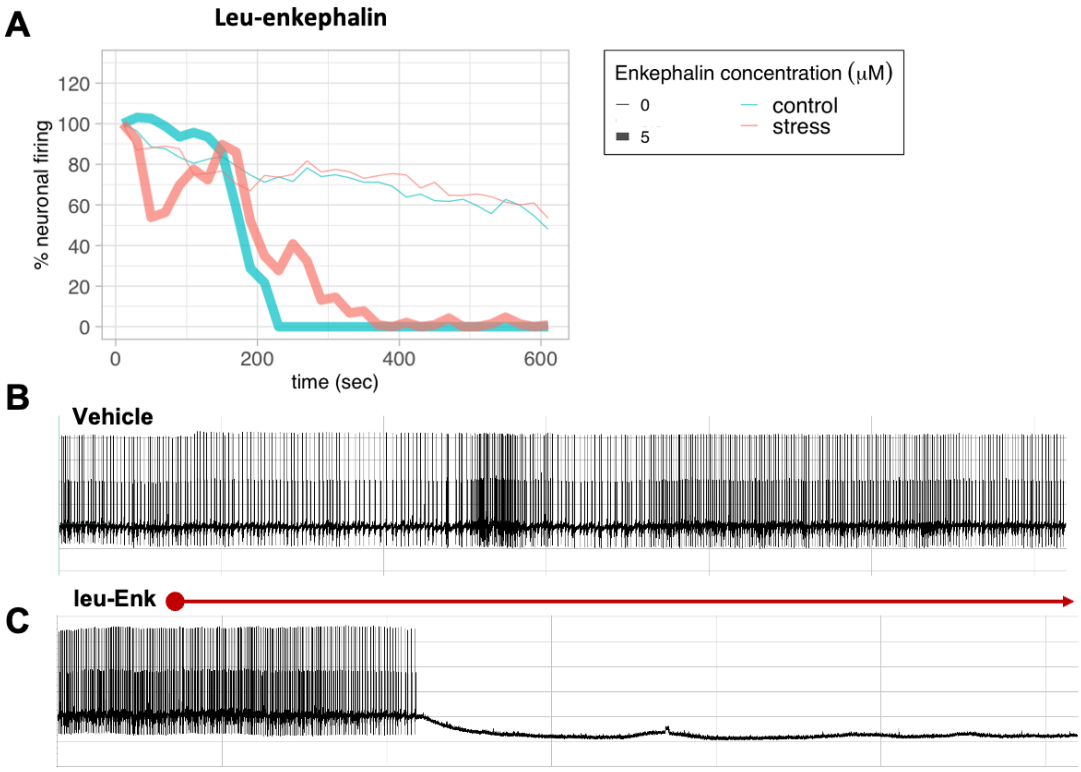


Figure 19. Spontaneous firing of LC neurons with and without Leu-Enkephalin (A) Percent baseline spontaneous neuronal firing over 10 min recording time (vehicle: stress = 13 cells, control = 16 cells; Leu-Enkephalin: stress = 4 cells, control = 3 cells). (B) Representative trace of spontaneous activity in the absence of drug, (C) and in the presence of leu-Enkephalin starting 2 min after the beginning of the recording.

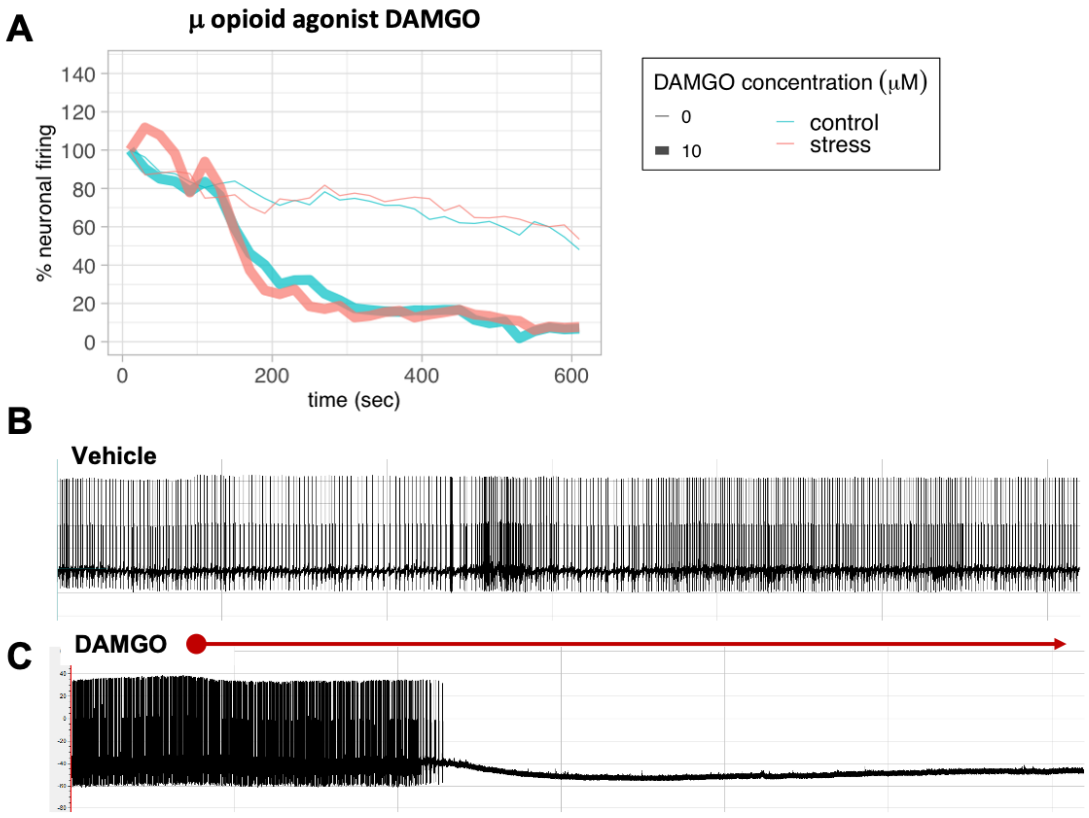


Figure 20. Spontaneous firing of LC neurons with and without DAMGO (A) Percent baseline spontaneous neuronal firing over 10 min recording time (vehicle: stress = 13 cells, control = 16 cells; DAMGO: stress = 10 cells, control = 12 cells). (B) Representative trace of spontaneous activity in the absence of drug, (C) and in the presence of DAMGO starting 2 min after the beginning of the recording.

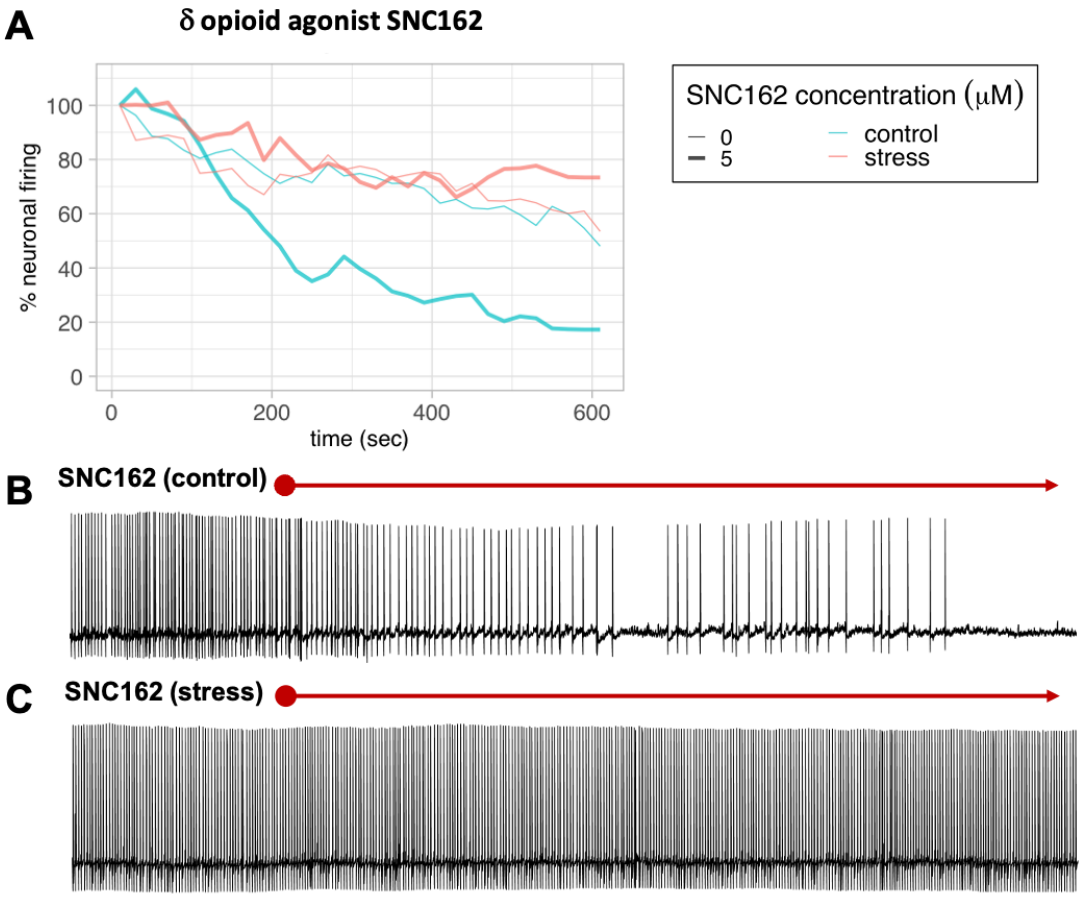


Figure 21. Spontaneous firing of LC neurons with and without SNC162. (A) Percent baseline spontaneous neuronal firing over 10 min recording time (vehicle: stress = 7 cells, control = 10 cells; SNC162: stress = 15 cells, control = 11 cells). (B) Representative trace of a recording from a control animal showing the response to SNC-162. (C) Representative trace of a recording from a stressed animal showing the lack of effect of SNC162.

Long-term effects of DOR overexpression on behavior and physiology after stress. After finding modified levels of mRNA expression for DORs, and that LC neurons from stressed animals were desensitized to a DOR agonist, we sought to test how artificially increasing DOR expression specifically in LC would affect behavioral and electrophysiological adaptations that occur in response to stress. Rats

underwent a stereotaxic survival surgery as described above to receive bilateral infusions of the pAAV-PRSx8-OPRD1 overexpression vector in LC. AAV-PRSx8-mCherry was used as a vector control. The PRSx8 promoter restricts expression to catecholaminergic neurons. Two weeks later, rats underwent stress or control conditions as described above. Behavior was then tested immediately after in the EPM and one week later in the OFT. Some rats were sacrificed to confirm overexpression with RT-PCR as described above. Others were sacrificed for electrophysiological recordings of LC neurons. Blood samples were also collected at both timepoints to assess the effects of DOR overexpression on HPA axis function. RT-PCR confirmed that viral injection leads to increased DOR mRNA expression (Figure 22A).

DOR overexpression in LC did not have a significant effect on stress-induced corticosterone levels 35 min after the beginning of control/stressor exposure in naïve animals (control=103 ng/mL, stress= 173 ng/mL) vs DOR overexpression (c control=110 ng/mL, stress= 185 ng/mL; Figure 22B).

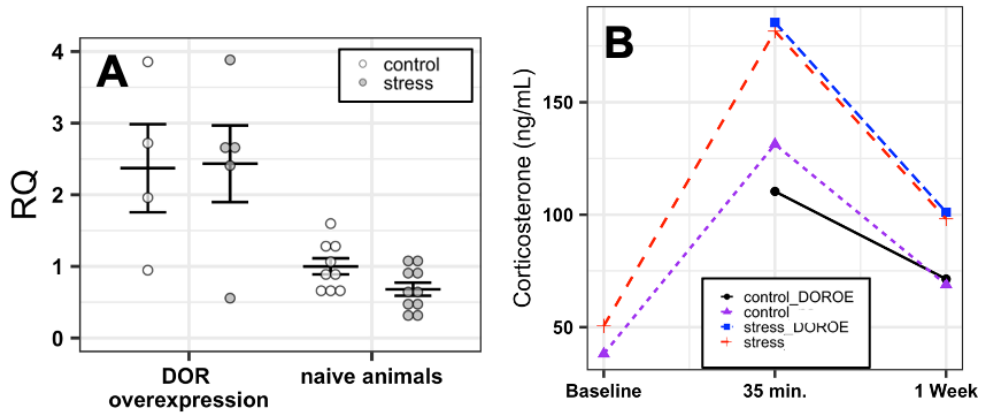


Figure 22. (A) DOR mRNA overexpression results from injections of AAV-PRSx8-oprd1 bilaterally into LC vs. LC from naïve animals, (B) Corticosterone levels prior to (baseline), 35 min after, and 1 week after control or stressor exposure in naïve animals vs. DOR-overexpressing animals in LC.

Notably, there were no differences in firing frequency between control and stress groups after pAAV-PRSx8-OPRD1 injection, despite a significant effect of stress on LC firing in sham virus-injected animals (pAAV-PRSx8-mCherry) (W = 134.5, p-value = 6.701e-05) (Figure 23A).

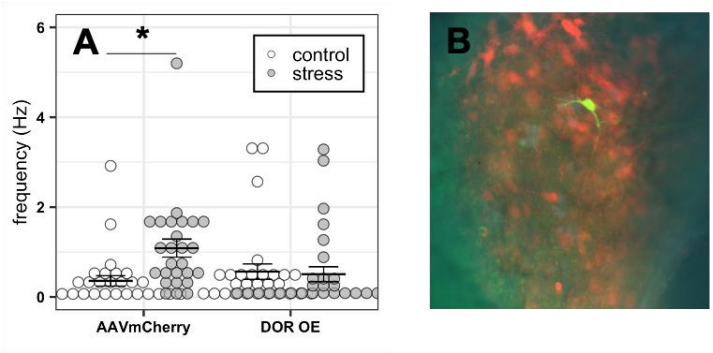


Figure 23. (A) Firing frequency of LC neurons from control and stressed animals injected with AAV-PRSx8-mCherry (left) vs. those injected with AAV-PRSx8-oprd1 (right) (B) Representative image of a recorded LC neuron (green) from LC-overexpressing DOR.

Consistent with these electrophysiological results, we did not observe any difference in anxiety-like behavior in the OFT between control and stressed animals in the AAV-PRSx8-oprd1 injected animals, while there was a significant difference in the time spent in center zone of OFT ($t = 4.8807$, $df = 17$, p-value = 0.0001408) (Figure 24C) and number of entries to the center zone of OFT ($t = 3.2153$, $df = 17$, p-value = 0.005077) (Figure 24D) between control and stressed animals that received the AAV-PRSx8-mCherry control vector. No significant changes were observed in the behavior immediately after stress and correlated with the data observed previously in adolescent male (Figure 12).

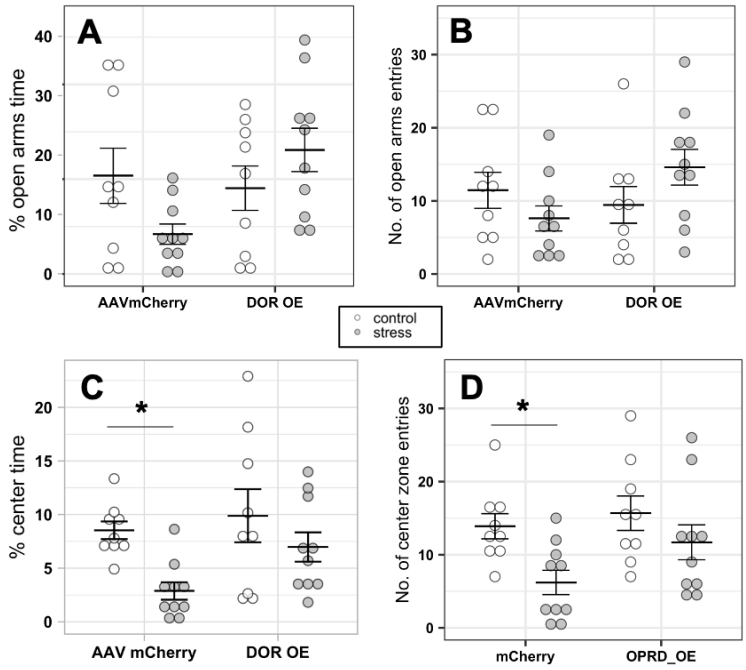


Figure 24. Acute and long-term behavior of animals with and without DOR overexpression. (A) Percent time spent in open arms of the EPM immediately after stress (n=10) or control (n=9) in AAV-PRSx8-mCherry-injected animals and immediately after stress (n=10) or control (n=9) in AAV-PRSx8-oprd1-injected animals. (B) Number of entries to open arms of the EPM immediately after stress. (C) Percent time spent in the center zone of the OFT. (D) Number of entries to the center zone of OFT.

6.5 Conclusions: Chapter 4

In this chapter we demonstrated that acute stress alters opioid receptor gene expression and function in adolescent male rats. Specifically, stress decreases mRNA expression of DORs and MORs a week after stress. Conversely, we did not detect any significant differences between control and stressed animals in opioid receptor total protein expression. This may be due to the fact that we used the whole cell lysate, while receptors can be in either an active membrane-bound state or in inactive

internalized cytoplasmic state. Further experiments analyzing expression levels within different cellular compartments would help to elucidate stress-induced opioid receptor protein changes according to the active and inactive receptor states.

Interestingly, there was a significant correlation between DOR mRNA expression and anxiety-like behavior, which is consistent with a role of LC in behavior formation and its regulation by DOR. DOR expression levels may be a good predictor of stress resilience and explain lower levels of anxiety-like behavior in some animals. Decreased opioid receptor expression could explain the inability of LC neurons to return to their baseline firing after the stress termination. Based on that we proceeded with functional testing of LC electrophysiology properties in response to different opioid agonists. We found that the MOR sensitivity was not altered in LC neurons from stressed rats as determined by the response to 1 and 10uM bath-applied DAMGO. Despite alterations in MOR mRNA levels, and the documented role of MOR signaling in LC during stress termination (Curtis, Bello et al. 2001), it is possible that the concentrations used were high enough to promote an effect even in the cells with decreased receptor levels. Additional experiments with lower DAMGO concentrations are necessary to explore this possibility. Alternatively, posttranslational modifications may negate the mRNA decrease and final protein levels are indistinguishable from control to stress animals . This would explain the absence of the difference of protein expression found with Western Blots. Similarly, stress did not precipiate any changes in LC responsiveness to Leu-enkephalin, which

is primarily a DOR, but also partial MOR agonist, at either 0.5 or 5uM. This lack of effect might also be explained by saturating concentrations for MOR receptors.

Finally, the non-peptide DOR agonist SNC162 revealed a desensitization of DORs one week after stressor exposure. This functional decrease in LC sensitivity to SNC162 together with PCR data showing decreased DOR mRNA expression indicates that DORs play a significant role in LC physiology and stress responsiveness. DORs therefore may be a key regulator of stress susceptibility and the development of stress-related disorders. These findings suggest that artificially increased LC-specific DOR expression might confer stress resilience to animals. Rats that were injected with the DOR overexpression virus pAAV-PRSx8-oprd1 in LC and were subjects to stressor exposure two weeks later did not show any of the stress-induced behavioral or electrophysiological adaptations that occurred in stressed rats that received the AAV-PRSx8-mCherry sham virus. This indicates that increased DOR expression by LC neurons is sufficient to provide a protective effect to LC physiology and behavior after stress. These results are in general agreement with another study (Reyes, Zitnik et al. 2015), which demonstrated that stress-resilient rats show increased activity of enkephalin neurons that project from PGi to LC.

Interestingly, pAAV-PRSx8-oprd1 injection did not affect the HPA axis function and hormonal stress response was indistinguishable from naïve animals. This indicates that even if an organism responds to stress with classical HPA axis recruitment, DOR overexpression reduces the risk for long-term behavioral changes and as a result the risk for the development of stress-associated disorders. Therefore,

behavioral and electrophysiological results are in parallel with endocrine changes indicating that despite the normal hormonal stress response, LC neurons don't undergo long-term increases in firing, and, accordingly, there is no parallel increase in anxiety-like behavior.

Notably, CRF and enkephalin-containing terminals in LC are derived from different afferent sources but converge on LC and both participate in its regulation in response to stress. As discussed earlier, enkephalinergic effects are in direct opposition to the CRFergic effects on LC activity. Specifically, CRF promotes cell depolarization and increased firing, while enkephalins promote cell hyperpolarization and decreased firing. Additionally, regulation of LC by both enkephalins and CRF occurs exclusively during stress, indicating that there is no tonic release of these peptides in LC in the absence of a stressor. An acute traumatic stressor during a susceptible adolescent age impairs DOR function in LC, which may contribute to long-term behavioral changes and stress-related disorders. The fact that LC-specific DOR overexpression precludes the effects of stress indicates that it may have significant translational value as a target for the treatment of stress-associated pathologies.

7 Chapter 5: Discussion, conclusions and future directions

Here we have shown that stress affects LC function and anxiety-like behavior, and the way that it becomes dysregulated varies according to the age and sex of the animal. Furthermore, we have demonstrated that the effects of acute stress in adolescent males are associated with dysregulation of LC-expressed DORs, and LC-specific DOR overexpression precludes the effects of stress on physiology and behavior. Notably, adolescent female rats showed opposing behavioral and physiological responses to stress from those seen in adolescent males. It is important for future experiments to determine if LC-expressed DORs in females are also similarly dysregulated in a fashion opposite to what occurs in adolescent males. Likewise, adult males and females, which showed neither behavioral nor physiological adaptations in response to stress, should also be investigated for changes, or a lack thereof, in DOR function in LC. Such findings would reinforce the idea that LC-expressed DORs are key regulators of the stress response and important therapeutic targets for the treatment of stress-associated disorders. One of the most notable findings of these studies was that physiological and behavioral adaptations in adolescent males and females persisted for at least one week after stressor exposure. Another important future direction is to test how far into the future an acute stressor might affect the adolescent brain. A pilot study showed that even one month after stressor exposure, some behavioral (Figure 25) and electrophysiological changes in adolescent males are still present (Figure 26).

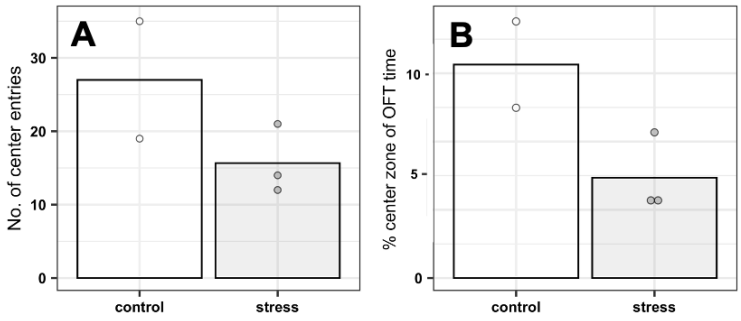


Figure 25. Increased anxiety-like behavior one month after adolescent rat stressor exposure. (A) Rats that experienced stress one month earlier show a trend for a decreased number of entries to the center zone of OFT, (B) and a trend for decreased total time spent in the center zone of OFT relative to controls. SEM values are not shown due to the small group size.

Despite a small sample size, there was a clear trend for rats that had experienced combined restraint and predator odor exposure one month earlier to avoid the center of the OFT.

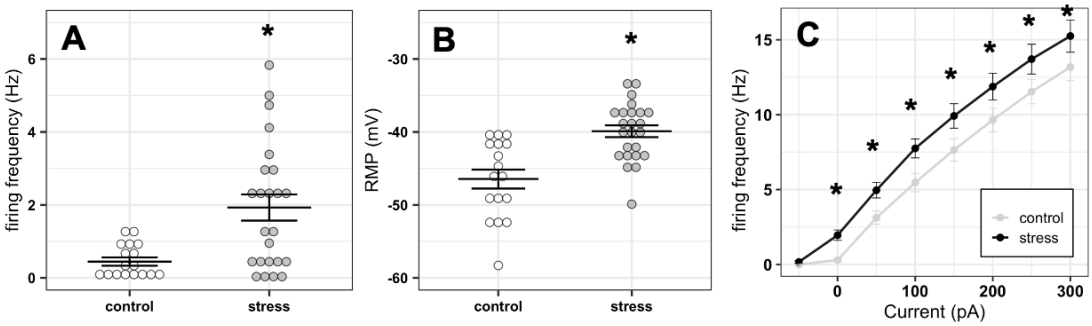


Figure 26. Increased spontaneous firing of LC neurons one month after adolescent rat stressor exposure. (A) One month after stressor exposure, LC cells show increased spontaneous firing frequency relative to controls. (B) Resting membrane potential of LC neurons is depolarized one month after stressor exposure relative to controls. (C) Induced firing in response to current injection is also increased one month after stressor exposure.

Electrophysiologically, firing frequency was significantly higher in stressed animals relative to control animals (t= -3.3935, df = 39, p-value = 0.001596). Additionally, RMP was significantly more depolarized in stressed animals (t = -4.4964, df = 39, p-value = 6.045e-05), indicating that less voltage is required for LC cells in those animals to reach threshold. Electrical excitability as indicated by firing induced by positive current injection was also significantly higher in stressed animals than controls. Importantly, these observations suggest that although animals exposed to stress as adults do not undergo adaptations that lead to increased anxiety-like behavior or LC hyperactivity, those that are exposed as adolescents may be subject to these changes well into their adulthood.

As noted above, the prominent LC dysregulation that occurred in adolescent males was also associated with both transcriptional (decreased mRNA expression) and functional (decreased sensitivity to a DOR agonist during *ex vivo* electrophysiology recordings) changes in LC-expressed DORs. We further found that viral-genetic overexpression of DORs is sufficient to occlude some of the effects associated with the stress at the behavioral and physiological levels. These findings are in general agreement with other recent studies that showed that resilience to repeated social stress (as defined by a long latency to defeat) is associated with activation of PGi-enkephalin afferents to LC, while stress-susceptible animals with short latency to defeat had stronger activation of CRF afferents from CeA (Reyes, Zitnik et al. 2015, Reyes, Zhang et al. 2019). These data highlight the importance of enkephalin innervation of LC neurons in the generation of coping strategies in response to stress and may indicate differential regulation of anxiety-like behavior

through CRF/enkephalin system interaction on LC neurons. Based on these findings, future experiments can be developed to test the hypothesis that enkephalin signaling in LC has both anxiolytic and reinforcing effects. Because adolescent male rats show LC hyperactivity in response to stress that is associated with persistent anxiety-like behavior, and LC hyperactivity is known to be anxiogenic and aversive (McCall, Al-Hasani et al. 2015), enhanced enkephalin signaling in these animals may have rewarding properties in these animals by reducing LC hyperactivity and the negative effects associated with it.

In order to test for an anxiolytic and rewarding role of opioid neurotransmission on LC, we carried out a pilot experiment in which we first implanted bilateral microinfusion cannulae in LC of adolescent male rats (n=20) (Figures 27&28).

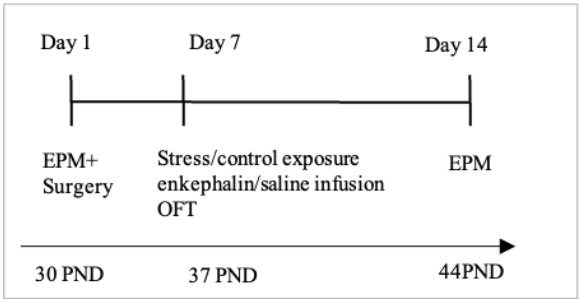


Figure 27. Timeline of a pilot experiment designed to test for an anxiolytic role for exogenously applied intra-LC enkephalin.

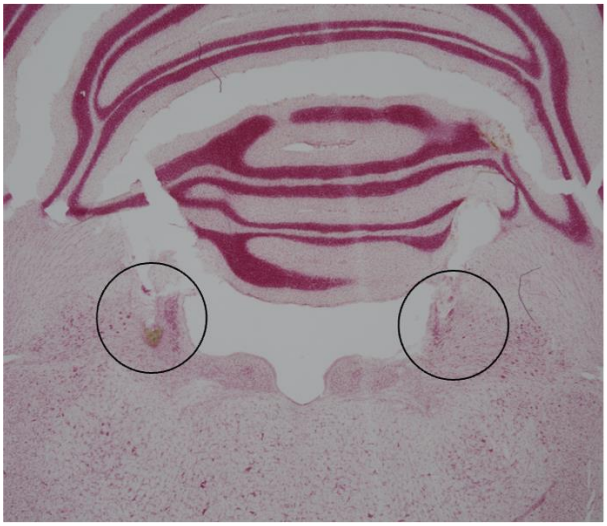


Figure 28. Representative image of bilateral cannulae tracks after implantation into the LC. Black circles highlight the cannulae tracks and LC.

After recovery from surgery, they were exposed to either control or stress conditions and immediately infused with either 1mL saline or enkephalin (36nM) bilaterally through the cannulae. Anxiety-like behavior was then tested in the OFT. We found a statistically significant interaction effect of stressor exposure and enkephalin infusion on freezing behavior in the OFT ($p=0.04$) using a 2x2 ANOVA, with a trend for decreased freezing time in the stressed rats that received enkephalin compared to stressed rats who were infused with saline. These observations suggest that enkephalin signaling in LC may influence passive stress coping strategies such as freezing behavior (Figure 29).

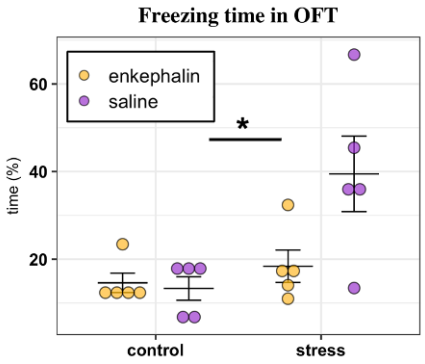


Figure 29. Effect of enkephalin and saline infusion on freezing behavior in the open field. Rats that underwent stressor exposure and received intra-LC enkephalin infusions showed a trend for decreased freezing time relative to stressed rats that received saline infusions.

In another experiment to test if enkephalin signaling in LC may exert rewarding effects, 6 rats underwent bilateral implantation of microinfusion cannulae adjacent to LC. Following recovery, rats were tested in a conditioned place preference task (CPP). Each day for 8 days, rats received bilateral sham infusions prior to being confined to one of two discriminable sides of the CPP apparatus for 30 min. 2 h later, rats received bilateral 1 µL infusions of either saline alone, or saline containing 10pg Leu-enkephalin, and were then confined to the second context for 30 minutes. 1 d after the final day of conditioning, rats were placed in the neutral center chamber of the CPP apparatus and allowed to freely explore the three chambers for 20 min. Preference was calculated as the difference in the amount of time spent in the sham-conditioned vs. saline/enkephalin-conditioned chamber. Enkephalin-treated rats exhibited a strong and significant preference for the conditioned chamber relative to saline-treated rats (Figure 30). Additionally, the latency to enter the conditioned

chamber was significantly lower for the rats that received enkephalin infusions than saline. Collectively, these preliminary observations build upon our earlier findings that stress increases LC activity and anxiety-like behavior and that LC-specific overexpression of DORs promote stress resilience and also suggest that intra-LC enkephalin signaling is both anxiolytic and a rewarding effect in a CPP paradigm.

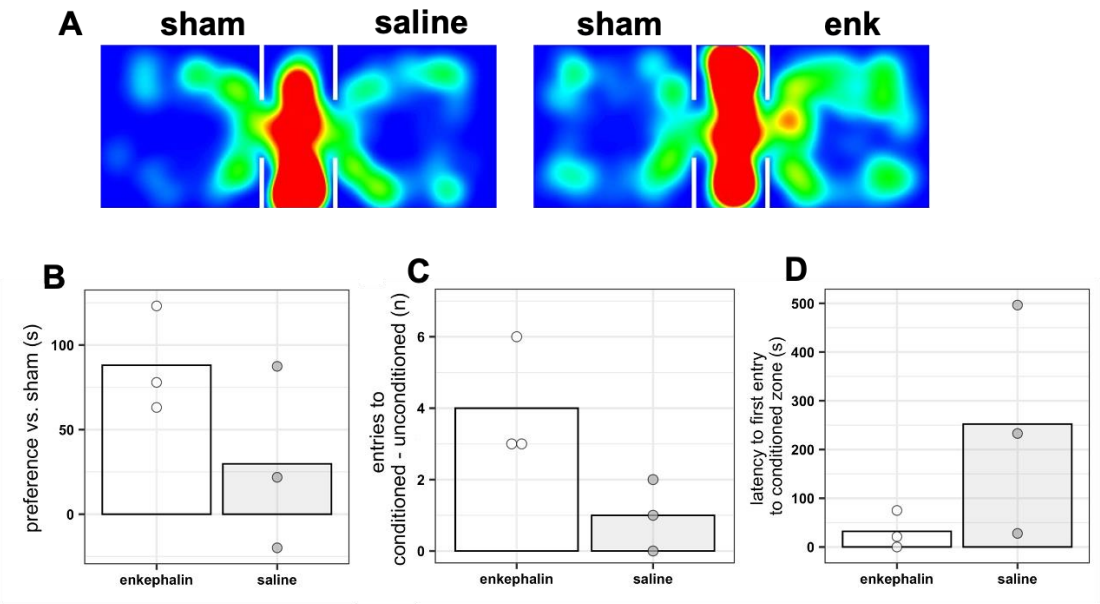


Figure 30. Intra-LC enkephalin promotes conditioned place preference. (A) Average heat maps for sham-saline and sham-enkephalin conditioned rats. (B) Preference was calculated as the difference in time spent in the conditioned (saline or enkephalin) versus sham-treated side. (C) Enkephalin-conditioned rats also showed a trend to enter the conditioned side more frequently than saline-conditioned rats, and also entered the conditioned chamber significantly earlier than the saline-conditioned rats (D). SEM values are not shown due to the small group size.

While the results of these studies are intriguing, they rely on the use of exogenously applied enkephalin and are uninformative of the function of a native enkephalinergic projection to LC. As noted above, there is evidence for the

enkephalinergic projection from PGi to LC in mediating resilience to social defeat stress. These experiments however were strictly observational and did not explore if manipulation of this circuit could directly impact behavior. A direct demonstration that activation or inhibition of this pathway has clear effects on anxiety-like and reward would greatly strengthen the notion that it, and intra-LC opioid signaling in particular, are key regulators of motivated behavior outside of the classical mesolimbic dopamine system. This would open the door to an abundance of new experiments that help reveal the neurobiological bases of various forms of behavior in both normal brains and animal models of disease. We have therefore recently begun to characterize a novel AAV construct that enables optical control of enkephalin release *in vivo* (AAV-PENK-ChR2). This vector drives expression of channelrhodopsin-eYFP in enkephalinergic neurons through the use of the preproenkephalin (PENK) promoter. To characterize the specificity and function of this vector, rats received bilateral infusions of AAV-PENK-ChR2-eYFP in PGi. Two weeks later, they were sacrificed for immunofluorescent staining against enkephalin or whole-cell recordings of LC neurons. Vector specificity was determined by calculating the percentage of eYFP-positive neurons that co-expressed enkephalin. From a cohort of five rats, injections resulted in 498.2 ± 87.63 labeled neurons, of which an average of 480.20 ± 87.39 (96.4%) co-expressed enkephalin (Figure 31). During whole-cell recordings of LC spontaneous activity, illumination of the recording chamber with 473nm blue light (10Hz, pulse width = 50msec) rapidly and robustly suppressed LC spontaneous activity and hyperpolarized its neurons (Figure 32). Therefore, this novel optogenetic probe will enable future experiments to

investigate how the enkephalinergic PGi → LC projection contributes to stress-induced anxiety-like behavior and motivated behavior in both stressed and unstressed animals.

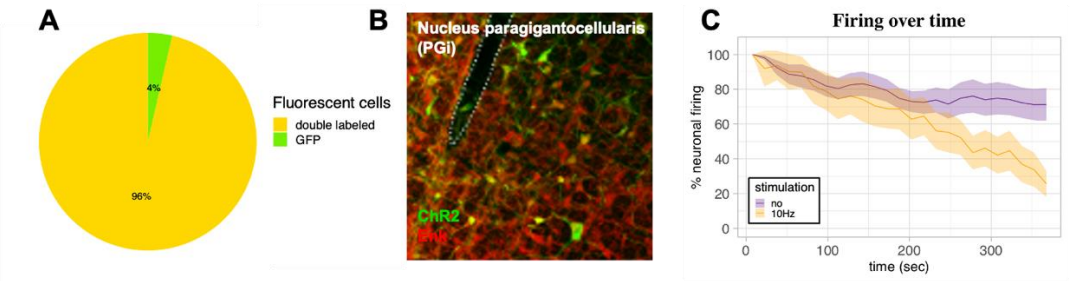


Figure 31. Transduction of PGi by injection of AAV-PENK-ChR2 (A) Quantification of single and double labeled cells expressing ChR2 (green) and enkephalin (red) shows that transgene expression is 96% restricted to enkephalin-containing neurons. (B) Representative photomicrograph of PGi with the injection track shown within the dotted line. Green neurons expressChr2-eYFP and red neurons express enkephalin. Double labeled neurons express both AAV-PENK-Chr2 and enkephalin. C) LC neuronal firing upon optogenetic stimulation (n=11 cells, yellow line) or no stimulation (n=16 cells, purple line). Shaded regions correspond to 95% CI.

As noted in Chapter One, a Yerkes-Dodson-like relationship between arousal and cognition has been proposed. In light of a role for LC in arousal and affect, high levels of arousal may be aversive (van Steenbergen, Eikemo et al. 2019). Aversive arousal could affect behavior and bias it towards avoidance and aversion. While it has been proposed that opioids may counteract the negative effect of aversive arousal by enhancing positive effect (van Steenbergen, Eikemo et al. 2019) from this perspective opioids may also have the capacity to modify cognition and bear positively

motivating effects through LC regulation. Though the effect of opioids on LC neurons is not rewarding by virtue of positive reinforcement, it may provide rewarding properties and improve cognition by reducing aversion and negative affect associated with LC hyperactivity. As a result, a patient whose LC is in a hyperactive state as a result of chronic or traumatic stress may be more likely to misuse or become dependent upon opioids prescribed for a therapeutic purpose.

Stress and opioid addiction have common pathophysiological mechanisms which involve different brain areas (Fareed, Eilender et al. 2013). In has been reported that the use of opioid analgesics is higher among patients with PTSD (Schwartz, Bradley et al. 2006). However, the relationship between PTSD and opioid dependence is also bi-directional, such that patients with PTSD are prescribed opioid analgesics more frequently due to objective pain with a physical cause or the perception of subjective pain without any physical basis (Phifer, Skelton et al. 2011). Additionally, opioids are reported to reduce PTSD symptoms. Conversely, patients with PTSD have higher risk of opioid dependence (Bremner, Southwick et al. 1996, Lejuez, Paulson et al. 2006). Based on the present findings, this may be explained in part by the influence of opioids on LC-dependent behaviors.

A multitude of brain substrates are involved in the interplay between opioid dependence and stress disorders, including mPFC, hippocampus, and anterior cingulate cortex (Liberzon, Taylor et al. 1999, Semple, Goyer et al. 2000, Pitman, Shin et al. 2001, Fareed, Eilender et al. 2013). Based on the results of these studies, the LC/NE system may also be a possible link between these comorbid conditions.

This is further supported by the prevalence of hyperarousal symptoms in patients with PTSD and analgesic use (Schwartz, Bradley et al. 2006). Another study found a positive relationship between symptoms of hyperarousal and heroin dependence (Tull, Gratz et al. 2010). Notably, LC hyperactivity is associated with hyperarousal, hypervigilance and sleep disturbances (Bremner, Krystal et al. 1996, Bremner, Krystal et al. 1996, Borodovitsyna, Joshi et al. 2018), all of which correspond to PTSD symptoms. Therefore, sedative drugs such as opioids might reduce some of these symptoms in PTSD patients, increasing their risk for abuse within this clinical population.

A significant body of literature has shown that stress causes CRF-dependent activation of LC neurons, which leads to increased release of NE in target brain areas such as mPFC and hippocampus, promoting symptoms of hyperarousal, hypervigilance, anxiety and scanning attention. Concomitantly, stress activates the release of endogenous opioids throughout the CNS, including but not limited to enkephalin release onto LC by PGi. In LC, enkephalin causes neuronal hyperpolarization, leading to decreased LC tonic firing and NE release throughout the CNS and reducing symptoms of LC hyperactivity. Stress-induced adaptations in LC may lead to increased sensitivity of LC neurons to CRF and reduce sensitivity to enkephalin due to the altered postsynaptic receptor kinetics (Valentino and Van Bockstaele 2001). This may contribute to LC hyperactivity and negative affect, increasing the reinforcing value for abused opioids by way of their ability to potently suppress LC function. The new observations we report here extend these findings by

showing not only that the effects of stress on LC function and behavior are age- and sex-dependent, but also that LC-expressed DORs are sensitive to stressor exposure and key regulators of the stress response. As such, further exploration of the role of these receptors in LC, which is not well defined in the existing body of literature, may have significant translational value in the treatment of both opioid use and stress disorders.

8 References

Accortt, E. E., M. P. Freeman and J. J. Allen (2008). "Women and major depressive disorder: clinical perspectives on causal pathways." J Womens Health (Larchmt) **17**(10): 1583-1590.

Adam, E. K. (2006). "Transactions among adolescent trait and state emotion and diurnal and momentary cortisol activity in naturalistic settings." Psychoneuroendocrinology **31**(5): 664-679.

Al-Hasani, R. and M. R. Bruchas (2011). "Molecular mechanisms of opioid receptor-dependent signaling and behavior." Anesthesiology **115**(6): 1363-1381.

Alfinito, P. D., X. Chen, R. Mastroeni, A. C. Pawlyk and D. C. Deecher (2009). "Estradiol increases catecholamine levels in the hypothalamus of ovariectomized rats during the dark-phase." Eur J Pharmacol **616**(1-3): 334-339.

Amemiya, S., T. Noji, N. Kubota, T. Nishijima and I. Kita (2014). "Noradrenergic modulation of vicarious trial-and-error behavior during a spatial decision-making task in rats." Neuroscience **265**: 291-301.

American Psychiatric, A. (2013). Diagnostic and Statistical Manual of Mental Disorders.

Angst, J., A. Gamma, M. Gastpar, J. P. Lepine, J. Mendlewicz, A. Tylee and S. Depression Research in European Society (2002). "Gender differences in depression. Epidemiological findings from the European DEPRES I and II studies." Eur Arch Psychiatry Clin Neurosci **252**(5): 201-209.

Arain, M., M. Haque, L. Johal, P. Mathur, W. Nel, A. Rais, R. Sandhu and S. Sharma (2013). "Maturation of the adolescent brain." Neuropsychiatr Dis Treat **9**: 449-461.

Ariza Traslavina, G. A., F. L. de Oliveira and C. R. Franci (2014). "Early adolescent stress alters behavior and the HPA axis response in male and female adult rats: the relevance of the nature and duration of the stressor." Physiol Behav **133**: 178-189.

Arnsten, A. F. (1998). "Catecholamine modulation of prefrontal cortical cognitive function." Trends Cogn Sci **2**(11): 436-447.

Arnsten, A. F. (2006). "Fundamentals of attention-deficit/hyperactivity disorder: circuits and pathways." J Clin Psychiatry **67 Suppl 8**: 7-12.

Arnsten, A. F. (2007). "Catecholamine and second messenger influences on prefrontal cortical networks of "representational knowledge": a rational bridge between genetics and the symptoms of mental illness." Cereb Cortex **17 Suppl 1**: i6-15.

Arnsten, A. F. (2009). "Stress signalling pathways that impair prefrontal cortex structure and function." Nat Rev Neurosci **10**(6): 410-422.

Arnsten, A. F. (2011). "Prefrontal cortical network connections: key site of vulnerability in stress and schizophrenia." Int J Dev Neurosci **29**(3): 215-223.

Asok, A., J. Schulkin and J. B. Rosen (2016). "Corticotropin releasing factor type-1 receptor antagonism in the dorsolateral bed nucleus of the stria terminalis disrupts contextually conditioned fear, but not unconditioned fear to a predator odor." Psychoneuroendocrinology **70**: 17-24.

Aston-Jones, G. and J. D. Cohen (2005). "An integrative theory of locus coeruleus-norepinephrine function: adaptive gain and optimal performance." Annu Rev Neurosci **28**: 403-450.

Atzori, M., R. Cuevas-Olguin, E. Esquivel-Rendon, F. Garcia-Oscos, R. C. Salgado-Delgado, N. Saderi, M. Miranda-Morales, M. Trevino, J. C. Pineda and H. Salgado (2016). "Locus Ceruleus Norepinephrine Release: A Central Regulator of CNS Spatio-Temporal Activation?" Front Synaptic Neurosci **8**: 25.

Bandelow, B. and S. Michaelis (2015). "Epidemiology of anxiety disorders in the 21st century." Dialogues Clin Neurosci **17**(3): 327-335.

Bangasser, D. A., A. Curtis, B. A. Reyes, T. T. Bethea, I. Parastatidis, H. Ischiropoulos, E. J. Van Bockstaele and R. J. Valentino (2010). "Sex differences in corticotropin-releasing factor receptor signaling and trafficking: potential role in female vulnerability to stress-related psychopathology." Mol Psychiatry **15**(9): 877, 896-904.

Bangasser, D. A., B. A. Reyes, D. Piel, V. Garachh, X. Y. Zhang, Z. M. Plona, E. J. Van Bockstaele, S. G. Beck and R. J. Valentino (2013). "Increased vulnerability of the brain norepinephrine system of females to corticotropin-releasing factor overexpression." Mol Psychiatry **18**(2): 166-173.

Bangasser, D. A. and R. J. Valentino (2014). "Sex differences in stress-related psychiatric disorders: neurobiological perspectives." Front Neuroendocrinol **35**(3): 303-319.

Bangasser, D. A., K. R. Wiersielis and S. Khantsis (2016). "Sex differences in the locus coeruleus-norepinephrine system and its regulation by stress." Brain Res **1641**(Pt B): 177-188.

Bangasser, D. A., X. Zhang, V. Garachh, E. Hanhauser and R. J. Valentino (2011). "Sexual dimorphism in locus coeruleus dendritic morphology: a structural basis for sex differences in emotional arousal." Physiol Behav **103**(3-4): 342-351.

Barbano, M. F. and M. Cador (2007). "Opioids for hedonic experience and dopamine to get ready for it." Psychopharmacology (Berl) **191**(3): 497-506.

Benarroch, E. E. (2018). "Locus coeruleus." Cell Tissue Res **373**(1): 221-232.

Bernardy, N. C., B. C. Lund, B. Alexander and M. J. Friedman (2014). "Increased polysedative use in veterans with posttraumatic stress disorder." Pain Med **15**(7): 1083-1090.

Berridge, C. W. and E. D. Abercrombie (1999). "Relationship between locus coeruleus discharge rates and rates of norepinephrine release within neocortex as assessed by in vivo microdialysis." Neuroscience **93**(4): 1263-1270.

Berridge, C. W. and R. C. Spencer (2016). "Differential cognitive actions of norepinephrine a2 and a1 receptor signaling in the prefrontal cortex." Brain Res **1641**(Pt B): 189-196.

Berridge, C. W. and B. D. Waterhouse (2003). "The locus coeruleus-noradrenergic system: modulation of behavioral state and state-dependent cognitive processes." Brain Res Brain Res Rev **42**(1): 33-84.

Bershad, A. K., J. H. Jaffe, E. Childs and H. de Wit (2015). "Opioid partial agonist buprenorphine dampens responses to psychosocial stress in humans." Psychoneuroendocrinology **52**: 281-288.

Bershad, A. K., M. A. Miller, G. J. Norman and H. de Wit (2018). "Effects of opioid- and non-opioid analgesics on responses to psychosocial stress in humans." Horm Behav **102**: 41-47.

Berube, P., J. F. Poulin, S. Laforest and G. Drolet (2014). "Enkephalin knockdown in the basolateral amygdala reproduces vulnerable anxiety-like responses to chronic unpredictable stress." Neuropsychopharmacology **39**(5): 1159-1168.

Bingham, B., K. McFadden, X. Zhang, S. Bhatnagar, S. Beck and R. Valentino (2011). "Early adolescence as a critical window during which social stress distinctly alters behavior and brain norepinephrine activity." Neuropsychopharmacology **36**(4): 896-909.

Bisson, J. I. (2007). "Post-traumatic stress disorder." Occupational medicine (Oxford, England) **57**(6): 399-403.

Blume, S. R., M. Padival, J. H. Urban and J. A. Rosenkranz (2019). "Disruptive effects of repeated stress on basolateral amygdala neurons and fear behavior across the estrous cycle in rats." Sci Rep **9**(1): 12292.

Boisclair Demarble, J., C. Fortin, B. D'Antono and S. Guay (2020). "Gender Differences in the Prediction of Acute Stress Disorder From Peritraumatic Dissociation and Distress Among Victims of Violent Crimes." J Interpers Violence **35**(5-6): 1229-1250.

Borodovitsyna, O., M. D. Flamini and D. J. Chandler (2018). "Acute Stress Persistently Alters Locus Coeruleus Function and Anxiety-like Behavior in Adolescent Rats." Neuroscience **373**: 7-19.

Borodovitsyna, O., N. Joshi and D. Chandler (2018). "Persistent Stress-Induced Neuroplastic Changes in the Locus Coeruleus/Norepinephrine System." Neural Plast **2018**: 1892570.

Bradshaw, S. E., K. L. Agster, B. D. Waterhouse and J. A. McGaughy (2016). "Age-related changes in prefrontal norepinephrine transporter density: The basis for improved cognitive flexibility after low doses of atomoxetine in adolescent rats." Brain Res **1641**(Pt B): 245-257.

Brady, K. T., T. K. Killeen, T. Brewerton and S. Lucerini (2000). "Comorbidity of psychiatric disorders and posttraumatic stress disorder." J Clin Psychiatry **61 Suppl 7**: 22-32.

Brady, K. T., T. K. Killeen, T. Brewerton and S. Lucerini (2000). "Comorbidity of psychiatric disorders and posttraumatic stress disorder." The Journal of clinical psychiatry **61 Suppl 7**: 22-32.

Bremner, J. D., R. B. Innis, C. K. Ng, L. H. Staib, R. M. Salomon, R. A. Bronen, J. Duncan, S. M. Southwick, J. H. Krystal, D. Rich, G. Zubal, H. Dey, R. Soufer and D. S. Charney (1997). "Positron emission tomography measurement of cerebral

metabolic correlates of yohimbine administration in combat-related posttraumatic stress disorder." Arch Gen Psychiatry **54**(3): 246-254.

Bremner, J. D., J. H. Krystal, S. M. Southwick and D. S. Charney (1996). "Noradrenergic mechanisms in stress and anxiety: I. Preclinical studies." Synapse **23**(1): 28-38.

Bremner, J. D., J. H. Krystal, S. M. Southwick and D. S. Charney (1996). "Noradrenergic mechanisms in stress and anxiety: II. Clinical studies." Synapse **23**(1): 39-51.

Bremner, J. D., S. M. Southwick, A. Darnell and D. S. Charney (1996). "Chronic PTSD in Vietnam combat veterans: course of illness and substance abuse." Am J Psychiatry **153**(3): 369-375.

Buechel, H. M., J. Popovic, K. Staggs, K. L. Anderson, O. Thibault and E. M. Blalock (2014). "Aged rats are hypo-responsive to acute restraint: implications for psychosocial stress in aging." Front Aging Neurosci **6**: 13.

Burton, C. L., D. Chatterjee, M. Chatterjee-Chakraborty, V. Lovic, S. L. Grella, M. Steiner and A. S. Fleming (2007). "Prenatal restraint stress and motherless rearing disrupts expression of plasticity markers and stress-induced corticosterone release in adult female Sprague-Dawley rats." Brain Res **1158**: 28-38.

Calenco-Choukroun, G., V. Dauge, G. Gacel, J. Feger and B. P. Roques (1991). "Opioid delta agonists and endogenous enkephalins induce different emotional reactivity than mu agonists after injection in the rat ventral tegmental area." Psychopharmacology (Berl) **103**(4): 493-502.

Castro, M. G. and E. Morrison (1997). "Post-translational processing of proopiomelanocortin in the pituitary and in the brain." Crit Rev Neurobiol **11**(1): 35-57.

Chaijale, N. N., A. L. Curtis, S. K. Wood, X. Y. Zhang, S. Bhatnagar, B. A. Reyes, E. J. Van Bockstaele and R. J. Valentino (2013). "Social stress engages opioid regulation of locus coeruleus norepinephrine neurons and induces a state of cellular and physical opiate dependence." Neuropsychopharmacology **38**(10): 1833-1843.

Chandler, D. J., W. J. Gao and B. D. Waterhouse (2014). "Heterogeneous organization of the locus coeruleus projections to prefrontal and motor cortices." Proc Natl Acad Sci U S A **111**(18): 6816-6821.

Cheng, Z. J., Q. M. Yu, Y. L. Wu, L. Ma and G. Pei (1998). "Selective interference of beta-arrestin 1 with kappa and delta but not mu opioid receptor/G protein coupling." J Biol Chem **273**(38): 24328-24333.

Chu Sin Chung, P., H. L. Keyworth, E. Martin-Garcia, P. Charbogne, E. Darcq, A. Bailey, D. Filliol, A. Matifas, G. Scherrer, A. M. Ouagazzal, C. Gaveriaux-Ruff, K. Befort, R. Maldonado, I. Kitchen and B. L. Kieffer (2015). "A novel anxiogenic role for the delta opioid receptor expressed in GABAergic forebrain neurons." Biol Psychiatry **77**(4): 404-415.

Chung, H. J., W.-P. Ge, X. Qian, O. Wiser, Y. N. Jan and L. Y. Jan (2009). "G protein-activated inwardly rectifying potassium channels mediate depotentiation of long-term potentiation." Proceedings of the National Academy of Sciences of the United States of America **106**(2): 635-640.

Cohen, H., Z. Kaplan, O. Koresh, M. A. Matar, A. B. Geva and J. Zohar (2011). "Early post-stressor intervention with propranolol is ineffective in preventing posttraumatic stress responses in an animal model for PTSD." Eur Neuropsychopharmacol **21**(3): 230-240.

Corder, G., D. C. Castro, M. R. Bruchas and G. Scherrer (2018). "Endogenous and Exogenous Opioids in Pain." Annu Rev Neurosci **41**: 453-473.

Curtis, A. L., N. T. Bello and R. J. Valentino (2001). "Evidence for functional release of endogenous opioids in the locus ceruleus during stress termination." J Neurosci **21**(13): RC152.

Curtis, A. L., T. Bethea and R. J. Valentino (2006). "Sexually dimorphic responses of the brain norepinephrine system to stress and corticotropin-releasing factor." Neuropsychopharmacology **31**(3): 544-554.

Curtis, A. L., S. M. Lechner, L. A. Pavcovich and R. J. Valentino (1997). "Activation of the locus coeruleus noradrenergic system by intracoerulear microinfusion of corticotropin-releasing factor: effects on discharge rate, cortical norepinephrine levels and cortical electroencephalographic activity." J Pharmacol Exp Ther **281**(1): 163-172.

Curtis, A. L., S. C. Leiser, K. Snyder and R. J. Valentino (2012). "Predator stress engages corticotropin-releasing factor and opioid systems to alter the operating mode of locus coeruleus norepinephrine neurons." Neuropharmacology **62**(4): 1737-1745.

Descalzi, G., X. Y. Li, T. Chen, V. Mercaldo, K. Koga and M. Zhuo (2012). "Rapid synaptic potentiation within the anterior cingulate cortex mediates trace fear learning." Mol Brain **5**: 6.

Devilbiss, D. M., M. E. Page and B. D. Waterhouse (2006). "Locus ceruleus regulates sensory encoding by neurons and networks in waking animals." J Neurosci **26**(39): 9860-9872.

Drolet, G., E. J. Van Bockstaele and G. Aston-Jones (1992). "Robust enkephalin innervation of the locus coeruleus from the rostral medulla." J Neurosci **12**(8): 3162-3174.

Fan, Y., P. Chen, Y. Li and M. Y. Zhu (2013). "Effects of chronic social defeat on expression of dopamine beta-hydroxylase in rat brains." Synapse **67**(6): 300-312.

Fareed, A., P. Eilender, M. Haber, J. Bremner, N. Whitfield and K. Drexler (2013). "Comorbid posttraumatic stress disorder and opiate addiction: a literature review." J Addict Dis **32**(2): 168-179.

Filliol, D., S. Ghozland, J. Chluba, M. Martin, H. W. Matthes, F. Simonin, K. Befort, C. Gaveriaux-Ruff, A. Dierich, M. LeMeur, O. Valverde, R. Maldonado and B. L. Kieffer (2000). "Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses." Nat Genet **25**(2): 195-200.

Finley, J. C., J. L. Maderdrut, L. J. Roger and P. Petrusz (1981). "The immunocytochemical localization of somatostatin-containing neurons in the rat central nervous system." Neuroscience **6**(11): 2173-2192.

Florin-Lechner, S. M., J. P. Druhan, G. Aston-Jones and R. J. Valentino (1996). "Enhanced norepinephrine release in prefrontal cortex with burst stimulation of the locus coeruleus." Brain Res **742**(1-2): 89-97.

Foote, S. L., F. E. Bloom and G. Aston-Jones (1983). "Nucleus locus ceruleus: new evidence of anatomical and physiological specificity." Physiol Rev **63**(3): 844-914.

Fullerton, C. S., H. B. Herberman Mash, K. N. Benevides, J. C. Morganstein and R. J. Ursano (2015). "Distress of Routine Activities and Perceived Safety Associated with Post-Traumatic Stress, Depression, and Alcohol Use: 2002 Washington, DC, Sniper Attacks." Disaster Med Public Health Prep **9**(5): 509-515.

Gendron, L., C. M. Cahill, M. von Zastrow, P. W. Schiller and G. Pineyro (2016). "Molecular Pharmacology of delta-Opioid Receptors." Pharmacol Rev **68**(3): 631-700.

George, S. A., D. Knox, A. L. Curtis, J. W. Aldridge, R. J. Valentino and I. Liberzon (2013). "Altered locus coeruleus-norepinephrine function following single prolonged stress." Eur J Neurosci **37**(6): 901-909.

Gesi, M., P. Soldani, F. S. Giorgi, A. Santinami, I. Bonaccorsi and F. Fornai (2000). "The role of the locus coeruleus in the development of Parkinson's disease." Neurosci Biobehav Rev **24**(6): 655-668.

Gilabert-Juan, J., E. Castillo-Gomez, M. Perez-Rando, M. D. Molto and J. Nacher (2011). "Chronic stress induces changes in the structure of interneurons and in the expression of molecules related to neuronal structural plasticity and inhibitory neurotransmission in the amygdala of adult mice." Exp Neurol **232**(1): 33-40.

Giustino, T. F., P. J. Fitzgerald and S. Maren (2016). "Revisiting propranolol and PTSD: Memory erasure or extinction enhancement?" Neurobiol Learn Mem **130**: 26-33.

Graeff, F. G. (1994). "Neuroanatomy and neurotransmitter regulation of defensive behaviors and related emotions in mammals." Braz J Med Biol Res **27**(4): 811-829.

Guajardo, H. M., K. Snyder, A. Ho and R. J. Valentino (2017). "Sex Differences in mu-Opioid Receptor Regulation of the Rat Locus Coeruleus and Their Cognitive Consequences." Neuropsychopharmacology **42**(6): 1295-1304.

Gunnar, M. R., S. Wewerka, K. Frenn, J. D. Long and C. Griggs (2009). "Developmental changes in hypothalamus-pituitary-adrenal activity over the transition to adolescence: normative changes and associations with puberty." Dev Psychopathol **21**(1): 69-85.

Hacquemand, R., N. Choffat, L. Jacquot and G. Brand (2013). "Comparison between low doses of TMT and cat odor exposure in anxiety- and fear-related behaviors in mice." Behav Brain Res **238**: 227-231.

Hansen, N. and D. Manahan-Vaughan (2015). "Hippocampal long-term potentiation that is elicited by perforant path stimulation or that occurs in conjunction with spatial learning is tightly controlled by beta-adrenoreceptors and the locus coeruleus." Hippocampus **25**(11): 1285-1298.

Harro, J., L. Oreland, E. Vasar and J. Bradwejn (1995). "Impaired exploratory behaviour after DSP-4 treatment in rats: implications for the increased anxiety after noradrenergic denervation." Eur Neuropsychopharmacol **5**(4): 447-455.

Henry, M. S., K. Bisht, N. Vernoux, L. Gendron, A. Torres-Berrio, G. Drolet and M. E. Tremblay (2018). "Delta Opioid Receptor Signaling Promotes Resilience to Stress Under the Repeated Social Defeat Paradigm in Mice." Front Mol Neurosci **11**: 100.

Henry, M. S., L. Gendron, M. E. Tremblay and G. Drolet (2017). "Enkephalins: Endogenous Analgesics with an Emerging Role in Stress Resilience." Neural Plast **2017**: 1546125.

Hokfelt, T., L. G. Elfvin, R. Elde, M. Schultzberg, M. Goldstein and R. Luft (1977). "Occurrence of somatostatin-like immunoreactivity in some peripheral sympathetic noradrenergic neurons." Proc Natl Acad Sci U S A **74**(8): 3587-3591.

Holets, V. R., T. Hokfelt, A. Rokaeus, L. Terenius and M. Goldstein (1988). "Locus coeruleus neurons in the rat containing neuropeptide Y, tyrosine hydroxylase or galanin and their efferent projections to the spinal cord, cerebral cortex and hypothalamus." Neuroscience **24**(3): 893-906.

Holmes, K. D., A. V. Babwah, L. B. Dale, M. O. Poulter and S. S. Ferguson (2006). "Differential regulation of corticotropin releasing factor 1alpha receptor endocytosis and trafficking by beta-arrestins and Rab GTPases." J Neurochem **96**(4): 934-949.

Hughes, J., T. W. Smith, H. W. Kosterlitz, L. A. Fothergill, B. A. Morgan and H. R. Morris (1975). "Identification of two related pentapeptides from the brain with potent opiate agonist activity." Nature **258**(5536): 577-580.

Itoi, K. (2008). "Ablation of the central noradrenergic neurons for unraveling their roles in stress and anxiety." Ann N Y Acad Sci **1129**: 47-54.

Jacobson, M. L., H. A. Wulf, C. A. Browne and I. Lucki (2018). "Opioid modulation of cognitive impairment in depression." Prog Brain Res **239**: 1-48.

Janitzky, K., W. D'Hanis, A. Krober and H. Schwegler (2015). "TMT predator odor activated neural circuit in C57BL/6J mice indicates TMT-stress as a suitable model for uncontrollable intense stress." Brain Res **1599**: 1-8.

Jedema, H. P. (2004). "Corticotropin-Releasing Hormone Directly Activates Noradrenergic Neurons of the Locus Ceruleus Recorded In Vitro." Journal of Neuroscience **24**(43): 9703-9713.

Jedema, H. P. and A. A. Grace (2004). "Corticotropin-releasing hormone directly activates noradrenergic neurons of the locus ceruleus recorded in vitro." J Neurosci **24**(43): 9703-9713.

Johansson, J., J. Hirvonen, Z. Lovro, L. Ekblad, V. Kaasinen, O. Rajasilta, S. Helin, J. Tuisku, S. Siren, M. Pennanen, A. Agrawal, R. Crystal, P. J. Vainio, H. Alho and M. Scheinin (2019). "Intranasal naloxone rapidly occupies brain mu-opioid receptors in human subjects." Neuropsychopharmacology **44**(9): 1667-1673.

Kalia, M. (2002). "Assessing the economic impact of stress--the modern day hidden epidemic." Metabolism **51**(6 Suppl 1): 49-53.

Kang, M. G., Y. Guo and R. L. Huganir (2009). "AMPA receptor and GEF-H1/Lfc complex regulates dendritic spine development through RhoA signaling cascade." Proc Natl Acad Sci U S A **106**(9): 3549-3554.

Karin, O., M. Raz, A. Tendler, A. Bar, Y. Korem Kohanim, T. Milo and U. Alon (2020). "A new model for the HPA axis explains dysregulation of stress hormones on the timescale of weeks." Mol Syst Biol **16**(7): e9510.

Kenakin, T. (2004). "Principles: receptor theory in pharmacology." Trends Pharmacol Sci **25**(4): 186-192.

Kessler, R. C., A. Sonnega, E. Bromet, M. Hughes and C. B. Nelson (1995). "Posttraumatic stress disorder in the National Comorbidity Survey." Arch Gen Psychiatry **52**(12): 1048-1060.

Kirschbaum, C., S. Wust and D. Hellhammer (1992). "Consistent sex differences in cortisol responses to psychological stress." Psychosom Med **54**(6): 648-657.

Kitay, J. I. (1961). "Sex differences in adrenal cortical secretion in the rat." Endocrinology **68**: 818-824.

Kogler, L., V. I. Muller, A. Chang, S. B. Eickhoff, P. T. Fox, R. C. Gur and B. Derntl (2015). "Psychosocial versus physiological stress - Meta-analyses on deactivations and activations of the neural correlates of stress reactions." Neuroimage **119**: 235-251.

Kolb, B. and R. Gibb (2011). "Brain plasticity and behaviour in the developing brain." J Can Acad Child Adolesc Psychiatry **20**(4): 265-276.

Koya, E., F. C. Cruz, R. Ator, S. A. Golden, A. F. Hoffman, C. R. Lupica and B. T. Hope (2012). "Silent synapses in selectively activated nucleus accumbens neurons following cocaine sensitization." Nat Neurosci **15**(11): 1556-1562.

Lapiz, M. D., Y. Mateo, S. Durkin, T. Parker and C. A. Marsden (2001). "Effects of central noradrenaline depletion by the selective neurotoxin DSP-4 on the behaviour of the isolated rat in the elevated plus maze and water maze." Psychopharmacology (Berl) **155**(3): 251-259.

Lejuez, C. W., A. Paulson, S. B. Daughters, M. A. Bornovalova and M. J. Zvolensky (2006). "The association between heroin use and anxiety sensitivity among inner-city individuals in residential drug use treatment." Behav Res Ther **44**(5): 667-677.

Lemon, N., S. Aydin-Abidin, K. Funke and D. Manahan-Vaughan (2009). "Locus coeruleus activation facilitates memory encoding and induces hippocampal LTD that depends on ??-Adrenergic receptor activation." Cerebral Cortex **19**(12): 2827-2837.

Leskela, T. T., P. M. Markkanen, I. A. Alahuhta, J. T. Tuusa and U. E. Petaja-Repo (2009). "Phe27Cys polymorphism alters the maturation and subcellular localization of the human delta opioid receptor." Traffic **10**(1): 116-129.

Li, X., F. J. Rubio, T. Zeric, J. M. Bossert, S. Kambhampati, H. M. Cates, P. J. Kennedy, Q. R. Liu, R. Cimbro, B. T. Hope, E. J. Nestler and Y. Shaham (2015). "Incubation of methamphetamine craving is associated with selective increases in expression of Bdnf and trkb, glutamate receptors, and epigenetic enzymes in cue-activated fos-expressing dorsal striatal neurons." J Neurosci **35**(21): 8232-8244.

Liberzon, I. and K. L. Phan (2003). "Brain-imaging studies of posttraumatic stress disorder." CNS Spectr **8**(9): 641-650.

Liberzon, I., S. F. Taylor, R. Amdur, T. D. Jung, K. R. Chamberlain, S. Minoshima, R. A. Koeppe and L. M. Fig (1999). "Brain activation in PTSD in response to trauma-related stimuli." Biol Psychiatry **45**(7): 817-826.

Liberzon, I. and E. A. Young (1997). "Effects of stress and glucocorticoids on CNS oxytocin receptor binding." Psychoneuroendocrinology **22**(6): 411-422.

Lindvall, O. and A. Bjorklund (1974). "The organization of the ascending catecholamine neuron systems in the rat brain as revealed by the glyoxylic acid fluorescence method." Acta Physiol Scand Suppl **412**: 1-48.

Lovelock, D. F. and T. Deak (2019). "Acute stress imposed during adolescence yields heightened anxiety in Sprague Dawley rats that persists into adulthood: Sex differences and potential involvement of the Medial Amygdala." Brain Res **1723**: 146392.

Lowe, J. D. and C. P. Bailey (2015). "Functional selectivity and time-dependence of mu-opioid receptor desensitization at nerve terminals in the mouse ventral tegmental area." Br J Pharmacol **172**(2): 469-481.

Lucassen, P. J., J. Pruessner, N. Sousa, O. F. X. Almeida, A. M. Van Dam, G. Rajkowska, D. F. Swaab and B. Czéh (2014). Neuropathology of stress. **127:** 109-135.

Luque, J. M., M. R. de Blas, S. Segovia and A. Guillamon (1992). "Sexual dimorphism of the dopamine-beta-hydroxylase-immunoreactive neurons in the rat locus ceruleus." Brain Res Dev Brain Res **67**(2): 211-215.

Maletic, V., A. Eramo, K. Gwin, S. J. Offord and R. A. Duffy (2017). "The Role of Norepinephrine and Its alpha-Adrenergic Receptors in the Pathophysiology and Treatment of Major Depressive Disorder and Schizophrenia: A Systematic Review." Front Psychiatry **8**: 42.

Mamalaki, E., R. Kvetnansky, L. S. Brady, P. W. Gold and M. Herkenham (1992). "Repeated immobilization stress alters tyrosine hydroxylase, corticotropin-releasing hormone and corticosteroid receptor messenger ribonucleic Acid levels in rat brain." J Neuroendocrinol **4**(6): 689-699.

Marzo, A., J. Bai, J. Caboche, P. Vanhoutte and S. Otani (2010). "Cellular mechanisms of long-term depression induced by noradrenaline in rat prefrontal neurons." Neuroscience **169**(1): 74-86.

Matsuo, N., L. Reijmers and M. Mayford (2008). "Spine-type-specific recruitment of newly synthesized AMPA receptors with learning." Science **319**(5866): 1104-1107.

McCall, J. G., R. Al-Hasani, E. R. Siuda, D. Y. Hong, A. J. Norris, C. P. Ford and M. R. Bruchas (2015). "CRH Engagement of the Locus Coeruleus Noradrenergic System Mediates Stress-Induced Anxiety." Neuron **87**(3): 605-620.

McCall, J. G., E. R. Siuda, D. L. Bhatti, L. A. Lawson, Z. A. McElligott, G. D. Stuber and M. R. Bruchas (2017). "Locus coeruleus to basolateral amygdala noradrenergic projections promote anxiety-like behavior." Elife **6**.

McCormick, C. M., I. Z. Mathews, C. Thomas and P. Waters (2010). "Investigations of HPA function and the enduring consequences of stressors in adolescence in animal models." Brain Cogn **72**(1): 73-85.

McCormick, C. M., C. Smith and I. Z. Mathews (2008). "Effects of chronic social stress in adolescence on anxiety and neuroendocrine response to mild stress in male and female rats." Behav Brain Res **187**(2): 228-238.

McMillan, P. J., S. S. White, A. Franklin, J. L. Greenup, J. B. Leverenz, M. A. Raskind and P. Szot (2011). "Differential response of the central noradrenergic nervous system to the loss of locus coeruleus neurons in Parkinson's disease and Alzheimer's disease." Brain Res **1373**: 240-252.

Mechenthaler, I. (2008). "Galanin and the neuroendocrine axes." Cell Mol Life Sci **65**(12): 1826-1835.

Meitzen, J., A. N. Perry, C. Westenbroek, V. L. Hedges, J. B. Becker and P. G. Mermelstein (2013). "Enhanced striatal beta1-adrenergic receptor expression following hormone loss in adulthood is programmed by both early sexual differentiation and puberty: a study of humans and rats." Endocrinology **154**(5): 1820-1831.

Merchenthaler, I., J. L. Maderdrut, R. A. Altschuler and P. Petrusz (1986). "Immunocytochemical localization of proenkephalin-derived peptides in the central nervous system of the rat." Neuroscience **17**(2): 325-348.

Minneman, K. P. and I. L. Iversen (1976). "Enkephalin and opiate narcotics increase cyclic GMP accumulation in slices of rat neostriatum." Nature **262**(5566): 313-314.

Mulvey, B., D. L. Bhatti, S. Gyawali, A. M. Lake, S. Kriaucionis, C. P. Ford, M. R. Bruchas, N. Heintz and J. D. Dougherty (2018). "Molecular and Functional Sex Differences of Noradrenergic Neurons in the Mouse Locus Coeruleus." Cell Rep **23**(8): 2225-2235.

Nestler, E. J., M. Alreja and G. K. Aghajanian (1994). "Molecular and cellular mechanisms of opiate action: studies in the rat locus coeruleus." Brain Res Bull **35**(5-6): 521-528.

Newman, L. C., D. R. Wallace and C. W. Stevens (2000). "Selective opioid agonist and antagonist competition for [3H]-naloxone binding in amphibian spinal cord." Brain Res **884**(1--2): 184-191.

Newport, D. J., C. Heim, M. J. Owens, J. C. Ritchie, C. H. Ramsey, R. Bonsall, A. H. Miller and C. B. Nemeroff (2003). "Cerebrospinal Fluid Corticotropin-Releasing Factor (CRF) and Vasopressin Concentrations Predict Pituitary Response in the CRF Stimulation Test: A Multiple Regression Analysis." Neuropsychopharmacology **28**(3): 569-576.

Nieto, M. M., S. L. Guen, B. L. Kieffer, B. P. Roques and F. Noble (2005). "Physiological control of emotion-related behaviors by endogenous enkephalins involves essentially the delta opioid receptors." Neuroscience **135**(2): 305-313.

Noble, F. and B. P. Roques (2007). "Protection of endogenous enkephalin catabolism as natural approach to novel analgesic and antidepressant drugs." Expert Opin Ther Targets **11**(2): 145-159.

Nugent, N. R., N. C. Christopher, J. P. Crow, L. Browne, S. Ostrowski and D. L. Delahanty (2010). "The efficacy of early propranolol administration at reducing PTSD symptoms in pediatric injury patients: a pilot study." J Trauma Stress **23**(2): 282-287.

Nummenmaa, L. and L. Tuominen (2018). "Opioid system and human emotions." Br J Pharmacol **175**(14): 2737-2749.

Olpe, H. R. and M. W. Steinmann (1982). "Age-related decline in the activity of noradrenergic neurons of the rat locus coeruleus." Brain Res **251**(1): 174-176.

Parvez, S., G. Ismahan, A. Raza-Bukhari and M. B. Youdim (1978). "Activity of catechol-o-methyltransferase in brain regions and adrenal gland during the oestrus cycle." J Neural Transm **42**(4): 305-312.

Pasternak, G. W., R. Goodman and S. H. Snyder (1975). "An endogenous morphine-like factor in mammalian brain." Life Sci **16**(12): 1765-1769.

Pasternak, G. W., R. Simantov and S. H. Snyder (1976). "Characterization of an endogenous morphine-like factor(enkephalin) in mammalian brain." Mol Pharmacol **12**(3): 504-513.

Patel, R. S., A. Elmaadawi, S. Nasr and J. Haskin (2017). "Comorbid Post-Traumatic Stress Disorder and Opioid Dependence." Cureus **9**(9): e1647.

Paus, T., M. Keshavan and J. N. Giedd (2008). "Why do many psychiatric disorders emerge during adolescence?" Nat Rev Neurosci **9**(12): 947-957.

Pavcovich, L. A., L. M. Cancela, M. Volosin, V. A. Molina and O. A. Ramirez (1990). "Chronic stress-induced changes in locus coeruleus neuronal activity." Brain Research Bulletin **24**(2): 293-296.

Phifer, J., K. Skelton, T. Weiss, A. C. Schwartz, A. Wingo, C. F. Gillespie, L. A. Sands, S. Sayyar, B. Bradley, T. Jovanovic and K. J. Ressler (2011). "Pain symptomatology and pain medication use in civilian PTSD." Pain **152**(10): 2233-2240.

Pierre, F., M. Ugur, F. Faivre, S. Doridot, P. Veinante and D. Massotte (2019). "Morphine-dependent and abstinent mice are characterized by a broader distribution of the neurons co-expressing mu and delta opioid receptors." Neuropharmacology **152**: 30-41.

Pinos, H., P. Collado, M. Rodriguez-Zafra, C. Rodriguez, S. Segovia and A. Guillamon (2001). "The development of sex differences in the locus coeruleus of the rat." Brain Res Bull **56**(1): 73-78.

Pitman, R. K., L. M. Shin and S. L. Rauch (2001). "Investigating the pathogenesis of posttraumatic stress disorder with neuroimaging." J Clin Psychiatry **62 Suppl 17**: 47-54.

Poulin, J. F., P. Berube, S. Laforest and G. Drolet (2013). "Enkephalin knockdown in the central amygdala nucleus reduces unconditioned fear and anxiety." Eur J Neurosci **37**(8): 1357-1367.

Pradhan, A. A., W. Walwyn, C. Nozaki, D. Filliol, E. Erbs, A. Matifas, C. Evans and B. L. Kieffer (2010). "Ligand-directed trafficking of the delta-opioid receptor in vivo: two paths toward analgesic tolerance." J Neurosci **30**(49): 16459-16468.

Prouty, E. W., B. D. Waterhouse and D. J. Chandler (2017). "Corticotropin releasing factor dose-dependently modulates excitatory synaptic transmission in the noradrenergic nucleus locus coeruleus." Eur J Neurosci **45**(5): 712-722.

Ramos, B. P. and A. F. Arnsten (2007). "Adrenergic pharmacology and cognition: focus on the prefrontal cortex." Pharmacol Ther **113**(3): 523-536.

Randall, A. D., C. Booth and J. T. Brown (2012). "Age-related changes to Na+ channel gating contribute to modified intrinsic neuronal excitability." Neurobiol Aging **33**(11): 2715-2720.

Rasmusson, A. M., R. L. Hauger, C. A. Morgan, J. D. Bremner, D. S. Charney and S. M. Southwick (2000). "Low baseline and yohimbine-stimulated plasma neuropeptide Y (NPY) levels in combat-related PTSD." Biol Psychiatry **47**(6): 526-539.

Reyes, B. A., J. D. Glaser and E. J. Van Bockstaele (2007). "Ultrastructural evidence for co-localization of corticotropin-releasing factor receptor and mu-opioid receptor in the rat nucleus locus coeruleus." Neurosci Lett **413**(3): 216-221.

Reyes, B. A., R. J. Valentino and E. J. Van Bockstaele (2008). "Stress-induced intracellular trafficking of corticotropin-releasing factor receptors in rat locus coeruleus neurons." Endocrinology **149**(1): 122-130.

Reyes, B. A., R. J. Valentino, G. Xu and E. J. Van Bockstaele (2005). "Hypothalamic projections to locus coeruleus neurons in rat brain." Eur J Neurosci **22**(1): 93-106.

Reyes, B. A., G. Zitnik, C. Foster, E. J. Van Bockstaele and R. J. Valentino (2015). "Social Stress Engages Neurochemically-Distinct Afferents to the Rat Locus Coeruleus Depending on Coping Strategy." eNeuro **2**(6).

Reyes, B. A. S., X. Y. Zhang, E. C. Dufourt, S. Bhatnagar, R. J. Valentino and E. J. Van Bockstaele (2019). "Neurochemically distinct circuitry regulates locus coeruleus activity during female social stress depending on coping style." Brain Struct Funct **224**(4): 1429-1446.

Reynaud, E., E. Guedj, M. Trousselard, M. El Khoury-Malhame, X. Zendjidjian, E. Fakra, M. Souville, B. Nazarian, O. Blin, F. Canini and S. Khalfa (2015). "Acute stress disorder modifies cerebral activity of amygdala and prefrontal cortex." Cogn Neurosci **6**(1): 39-43.

Romeo, R. D., S. J. Lee, N. Chhua, C. R. McPherson and B. S. McEwen (2004). "Testosterone cannot activate an adult-like stress response in prepubertal male rats." Neuroendocrinology **79**(3): 125-132.

Romeo, R. D., S. J. Lee and B. S. McEwen (2004). "Differential stress reactivity in intact and ovariectomized prepubertal and adult female rats." Neuroendocrinology **80**(6): 387-393.

Rosen, J. B., A. Asok and T. Chakraborty (2015). "The smell of fear: innate threat of 2,5-dihydro-2,4,5-trimethylthiazoline, a single molecule component of a predator odor." Front Neurosci **9**: 292.

Rusnak, M., R. Kvetnansky, J. Jelokova and M. Palkovits (2001). "Effect of novel stressors on gene expression of tyrosine hydroxylase and monoamine transporters in brainstem noradrenergic neurons of long-term repeatedly immobilized rats." Brain Res **899**(1-2): 20-35.

Salim, S., B. Hite and D. C. Eikenburg (2007). "Activation of the CRF(1) receptor causes ERK1/2 mediated increase in GRK3 expression in CATH.a cells." FEBS Lett **581**(17): 3204-3210.

Samuels, E. R. and E. Szabadi (2008). "Functional neuroanatomy of the noradrenergic locus coeruleus: its roles in the regulation of arousal and autonomic function part II: physiological and pharmacological manipulations and pathological alterations of locus coeruleus activity in humans." Curr Neuropharmacol **6**(3): 254-285.

Schutsky, K., M. Ouyang, C. B. Castelino, L. Zhang and S. A. Thomas (2011). "Stress and glucocorticoids impair memory retrieval via beta2-adrenergic, Gi/o-coupled suppression of cAMP signaling." J Neurosci **31**(40): 14172-14181.

Schwartz, A. C., R. Bradley, K. M. Penza, M. Sexton, D. Jay, P. J. Haggard, S. J. Garlow and K. J. Ressler (2006). "Pain medication use among patients with posttraumatic stress disorder." Psychosomatics **47**(2): 136-142.

Selmanoff, M. K., M. J. Pramik-Holdaway and R. I. Weiner (1976). "Concentrations of dopamine and norepinephrine in discrete hypothalamic nuclei during the rat estrous cycle." Endocrinology **99**(1): 326-329.

Selye, H. (1975). "Stress and distress." Compr Ther **1**(8): 9-13.

Selye, H. (1976). Stress without Distress**:** 137-146.

Semple, W. E., P. F. Goyer, R. McCormick, B. Donovan, R. F. Muzic, Jr., L. Rugle, K. McCutcheon, C. Lewis, D. Liebling, S. Kowaliw, K. Vapenik, M. A. Semple, C. R. Flener and S. C. Schulz (2000). "Higher brain blood flow at amygdala and lower frontal cortex blood flow in PTSD patients with comorbid cocaine and alcohol abuse compared with normals." Psychiatry **63**(1): 65-74.

Shirasaki, T., K. Abe, F. Soeda and K. Takahama (2004). "delta-Opioid receptor antagonists inhibit GIRK channel currents in acutely dissociated brainstem neurons of rat." Brain Res **1006**(2): 190-197.

Simantov, R., S. R. Childers and S. H. Snyder (1977). "Opioid peptides: differentiation by radioimmunoassay and radioreceptor assay." Brain Res **135**(2): 358-367.

Snyder, K., W. W. Wang, R. Han, K. McFadden and R. J. Valentino (2012). "Corticotropin-releasing factor in the norepinephrine nucleus, locus coeruleus, facilitates behavioral flexibility." Neuropsychopharmacology **37**(2): 520-530.

Soya, S., H. Shoji, E. Hasegawa, M. Hondo, T. Miyakawa, M. Yanagisawa, M. Mieda and T. Sakurai (2013). "Orexin Receptor-1 in the Locus Coeruleus Plays an Important Role in Cue-Dependent Fear Memory Consolidation." Journal of Neuroscience **33**(36): 14549-14557.

Spear, L. P. (2000). "The adolescent brain and age-related behavioral manifestations." Neurosci Biobehav Rev **24**(4): 417-463.

Strawn, J. R. and T. D. Geracioti (2008). Noradrenergic dysfunction and the psychopharmacology of posttraumatic stress disorder. **25:** 260-271.

Su, J., Y. Tanaka, T. Muratsubaki, M. Kano, M. Kanazawa and S. Fukudo (2015). "Injection of corticotropin-releasing hormone into the amygdala aggravates visceral nociception and induces noradrenaline release in rats." Neurogastroenterol Motil **27**(1): 30-39.

Swanson, L. W. and B. K. Hartman (1975). "The central adrenergic system. An immunofluorescence study of the location of cell bodies and their efferent connections in the rat utilizing dopamine-beta-hydroxylase as a marker." J Comp Neurol **163**(4): 467-505.

Swinny, J. D., E. O'Farrell, B. C. Bingham, D. A. Piel, R. J. Valentino and S. G. Beck (2010). "Neonatal rearing conditions distinctly shape locus coeruleus neuronal activity, dendritic arborization, and sensitivity to corticotrophin-releasing factor." Int J Neuropsychopharmacol **13**(4): 515-525.

Swinny, J. D. and R. J. Valentino (2006). "Corticotropin-releasing factor promotes growth of brain norepinephrine neuronal processes through Rho GTPase regulators of the actin cytoskeleton in rat." Eur J Neurosci **24**(9): 2481-2490.

Tanowitz, M., J. N. Hislop and M. von Zastrow (2008). "Alternative splicing determines the post-endocytic sorting fate of G-protein-coupled receptors." J Biol Chem **283**(51): 35614-35621.

Taylor, F. and L. Cahill (2002). "Propranolol for reemergent posttraumatic stress disorder following an event of retraumatization: A case study." Journal of Traumatic Stress **15**(5): 433-437.

Toledo-Rodriguez, M. and C. Sandi (2011). "Stress during Adolescence Increases Novelty Seeking and Risk-Taking Behavior in Male and Female Rats." Front Behav Neurosci **5**: 17.

Torrecilla, M., C. L. Marker, S. C. Cintora, M. Stoffel, J. T. Williams and K. Wickman (2002). "G-protein-gated potassium channels containing Kir3.2 and Kir3.3 subunits mediate the acute inhibitory effects of opioids on locus ceruleus neurons." J Neurosci **22**(11): 4328-4334.

Torrecilla, M., N. Quillinan, J. T. Williams and K. Wickman (2008). "Pre- and postsynaptic regulation of locus coeruleus neurons after chronic morphine treatment: a study of GIRK-knockout mice." Eur J Neurosci **28**(3): 618-624.

Travagli, R. A., T. V. Dunwiddie and J. T. Williams (1995). "Opioid inhibition in locus coeruleus." J Neurophysiol **74**(2): 519-528.

Trumbach, D., C. Graf, B. Putz, C. Kuhne, M. Panhuysen, P. Weber, F. Holsboer, W. Wurst, G. Welzl and J. M. Deussing (2010). "Deducing corticotropin-releasing hormone receptor type 1 signaling networks from gene expression data by usage of genetic algorithms and graphical Gaussian models." BMC Syst Biol **4**: 159.

Tull, M. T., K. L. Gratz, W. M. Aklin and C. W. Lejuez (2010). "A preliminary examination of the relationships between posttraumatic stress symptoms and crack/cocaine, heroin, and alcohol dependence." J Anxiety Disord **24**(1): 55-62.

Turrigiano, G. G. (2008). The Self-Tuning Neuron: Synaptic Scaling of Excitatory Synapses. **135:** 422-435.

Turrigiano, G. G., K. R. Leslie, N. S. Desai, L. C. Rutherford and S. B. Nelson (1998). "Activity-dependent scaling of quantal amplitude in neocortical neurons." Nature **391**(6670): 892-896.

Uematsu, A., B. Z. Tan and J. P. Johansen (2015). "Projection specificity in heterogeneous locus coeruleus cell populations: implications for learning and memory." Learn Mem **22**(9): 444-451.

Uematsu, A., B. Z. Tan, E. A. Ycu, J. S. Cuevas, J. Koivumaa, F. Junyent, E. J. Kremer, I. B. Witten, K. Deisseroth and J. P. Johansen (2017). "Modular organization

of the brainstem noradrenaline system coordinates opposing learning states." Nat Neurosci.

Valentino, R. J. and S. L. Foote (1987). "Corticotropin-releasing factor disrupts sensory responses of brain noradrenergic neurons." Neuroendocrinology **45**(1): 28-36.

Valentino, R. J. and S. L. Foote (1988). "Corticotropin-releasing hormone increases tonic but not sensory-evoked activity of noradrenergic locus coeruleus neurons in unanesthetized rats." J Neurosci **8**(3): 1016-1025.

Valentino, R. J., S. L. Foote and G. Aston-Jones (1983). "Corticotropin-releasing factor activates noradrenergic neurons of the locus coeruleus." Brain Res **270**(2): 363-367.

Valentino, R. J., M. Page, E. Van Bockstaele and G. Aston-Jones (1992). "Corticotropin-releasing factor innervation of the locus coeruleus region: distribution of fibers and sources of input." Neuroscience **48**(3): 689-705.

Valentino, R. J. and E. Van Bockstaele (2001). "Opposing regulation of the locus coeruleus by corticotropin-releasing factor and opioids. Potential for reciprocal interactions between stress and opioid sensitivity." Psychopharmacology (Berl) **158**(4): 331-342.

Valentino, R. J. and E. Van Bockstaele (2015). "Endogenous Opioids: The Downside of Opposing Stress." Neurobiol Stress **1**: 23-32.

Van Bockstaele, E. J., D. Bajic, H. Proudfit and R. J. Valentino (2001). "Topographic architecture of stress-related pathways targeting the noradrenergic locus coeruleus." Physiol Behav **73**(3): 273-283.

Van Bockstaele, E. J., E. E. Colago, P. Cheng, A. Moriwaki, G. R. Uhl and V. M. Pickel (1996). "Ultrastructural evidence for prominent distribution of the mu-opioid receptor at extrasynaptic sites on noradrenergic dendrites in the rat nucleus locus coeruleus." J Neurosci **16**(16): 5037-5048.

Van Bockstaele, E. J., E. E. Colago and R. J. Valentino (1998). "Amygdaloid corticotropin-releasing factor targets locus coeruleus dendrites: substrate for the co-ordination of emotional and cognitive limbs of the stress response." J Neuroendocrinol **10**(10): 743-757.

van Bockstaele, E. J., K. Commons and V. M. Pickel (1997). "Delta-opioid receptor is present in presynaptic axon terminals in the rat nucleus locus coeruleus: relationships with methionine5-enkephalin." J Comp Neurol **388**(4): 575-586.

Van Bockstaele, E. J., B. A. Reyes and R. J. Valentino (2010). "The locus coeruleus: A key nucleus where stress and opioids intersect to mediate vulnerability to opiate abuse." Brain Res **1314**: 162-174.

van Dijken, H. H., D. C. de Goeij, W. Sutanto, J. Mos, E. R. de Kloet and F. J. Tilders (1993). "Short inescapable stress produces long-lasting changes in the brain-pituitary-adrenal axis of adult male rats." Neuroendocrinology **58**(1): 57-64.

van Steenbergen, H., M. Eikemo and S. Leknes (2019). "The role of the opioid system in decision making and cognitive control: A review." Cogn Affect Behav Neurosci **19**(3): 435-458.

Wagner, H. R. and J. N. Davies (1980). "Decreased beta-adrenergic responses in the female rat brain are eliminated by ovariectomy: correlation of [3H]dihydroalprenolol binding and catecholamine stimulated cyclic AMP levels." Brain Res **201**(1): 235-239.

Waksman, G., E. Hamel, M. C. Fournie-Zaluski and B. P. Roques (1986). "Autoradiographic comparison of the distribution of the neutral endopeptidase "enkephalinase" and of mu and delta opioid receptors in rat brain." Proc Natl Acad Sci U S A **83**(5): 1523-1527.

Wangelin, B. C., M. B. Powers, J. A. Smits and P. W. Tuerk (2013). "Enhancing exposure therapy for PTSD with yohimbine HCL: protocol for a double-blind, randomized controlled study implementing subjective and objective measures of treatment outcome." Contemp Clin Trials **36**(2): 319-326.

Weinberg, J., M. R. Gunnar, L. P. Brett, C. A. Gonzalez and S. Levine (1982). "Sex differences in biobehavioral responses to conflict in a taste aversion paradigm." Physiol Behav **29**(2): 201-210.

Weinshenker, D. (2008). "Functional consequences of locus coeruleus degeneration in Alzheimer's disease." Curr Alzheimer Res **5**(3): 342-345.

Westlund, K. N. and J. D. Coulter (1980). "Descending projections of the locus coeruleus and subcoeruleus/medial parabrachial nuclei in monkey: axonal transport studies and dopamine-beta-hydroxylase immunocytochemistry." Brain Res **2**(3): 235-264.

Williams, J. T., R. A. North and T. Tokimasa (1988). "Inward rectification of resting and opiate-activated potassium currents in rat locus coeruleus neurons." J Neurosci **8**(11): 4299-4306.

Wulsin, A. C., D. Wick-Carlson, B. A. Packard, R. Morano and J. P. Herman (2016). "Adolescent chronic stress causes hypothalamo-pituitary-adrenocortical hypo-responsiveness and depression-like behavior in adult female rats." Psychoneuroendocrinology **65**: 109-117.

Yehuda, R., S. M. Southwick, J. H. Krystal, D. Bremner, D. S. Charney and J. W. Mason (1993). "Enhanced suppression of cortisol following dexamethasone administration in posttraumatic stress disorder." Am J Psychiatry **150**(1): 83-86.

Yohn, N. L. and J. A. Blendy (2017). "Adolescent Chronic Unpredictable Stress Exposure Is a Sensitive Window for Long-Term Changes in Adult Behavior in Mice." Neuropsychopharmacology **42**(8): 1670-1678.

Zamponi, G. W. and T. P. Snutch (1998). "Modulation of voltage-dependent calcium channels by G proteins." Curr Opin Neurobiol **8**(3): 351-356.

Zhang, W. and J. R. Davidson (2007). "Post-traumatic stress disorder: an evaluation of existing pharmacotherapies and new strategies." Expert Opin Pharmacother **8**(12): 1861-1870.

Zitnik, G. A., A. L. Curtis, S. K. Wood, J. Arner and R. J. Valentino (2016). "Adolescent Social Stress Produces an Enduring Activation of the Rat Locus Coeruleus and Alters its Coherence with the Prefrontal Cortex." Neuropsychopharmacology **41**(5): 1376-1385.

9 Abbreviations

LC - locus coeruleus
HPA axis - hypothalamo-pituitary adrenal axis
CNS - central nervous system
PFC - prefrontal cortex
LC/NE - locus coeruleus/norepinephrine
CRFR1 - corticotropin releasing factor receptor 1
CRF - corticotropin releasing factor
PND – postnatal days
OFT - open field test
EPM - elevated plus maze
aCSF - artificial cerebrospinal fluid
PVN - paraventricular nucleus of hypothalamus
CeA - central nucleus of the amygdala
PGi -nucleus paragigantocellularis
BLA - basolateral amygdala
PTSD – post-traumatic stress disorder
TMT - 2,4,5-trimethylthiazole
PGE2 - Prostaglandin E_2
CI – confidence interval
SD – standard deviation
SEM – standard error of the mean

LPS – lipopolysaccharide

DOR - delta opioid receptor

MOR - mu opioid receptor

CPP – condition place preference

ICV – intracerebroventricular

fMRI - Functional magnetic resonance imaging

10 Attributes