ELUCIDATING MECHANISMS THAT LEAD TO PERSISTENT ANXIETY-LIKE BEHAVIOR IN RATS FOLLOWING REPEATED ACTIVATION OF CORTICOTROPIN-RELEASING FACTOR RECEPTORS IN THE BASOLATERAL AMYGDALA

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ABSTRACT

Denise Gaskins

ELUCIDATING MECHANISIMS THAT LEAD TO PERSISTENT ANXIETY-LIKE BEHAVIOR IN RATS FOLLOWING REPEATED ACTIVATION OF CORTICOTROPIN-RELEASING FACTOR RECEPTORS IN THE BASOLATERAL AMYGDALA

Anxiety disorders are estimated to impact 1 in 4 individuals within their lifetime. For some individuals, repeated episodes of the stress response leads to pathological anxiety and depression. The stress response is linked to increased levels of corticotropin-releasing factor (CRF) in the basolateral nucleus of the amygdala (BLA), a putative site for regulating anxiety and associative processes related to aversive emotional memories, and activation of CRF receptors in the BLA of rats produces anxiety-like behavior. Mimicking repeated episodes of the stress response, sub-anxiogenic doses of urocortin 1 (Ucn1), a CRF receptor agonist, are microinjected into the BLA of rats for five consecutive days, a procedure called priming. This results in 1) behavioral sensitization, such that a previously non-efficacious dose of Ucn1 will elicit anxiety-like response after the 3rd injection and 2) the development of a persistent anxiety-like phenotype that lasts at least five weeks after the last injection without any further treatment. Therefore, the purpose of this thesis was to identify mechanisms involved in the Ucn1-priming-induced anxiogenesis.

The first a set of experiments revealed that the anxiety-like behavior was not due to aversive conditioning to the context or partner cues of the testing environment. Next, Ucn1-priming-induced gene expression changes in the BLA

were identified: mRNA expression for Sst2, Sst4, Chrna4, Chrma4, and Gabrr1 was significantly reduced in Ucn1-primed compared to Vehicle-primed rats. Of these, Sst2 emerged as the primary receptor of interest. Subsequent studies found that antagonizing the Sstr2 resulted in anxiety-like behavior and activation of Sstr2 blocked acute Ucn1-induced anxiety-like responses. Furthermore, pretreatment with a Sstr2 agonist delayed the behavioral sensitization observed in Ucn1-induced priming but did not stop the development of persistent anxiety-like behavior or the Ucn1-priming-induced decrease in the Sstr2 mRNA. These results suggest that the decrease in Sstr2 mRNA is associated with the expression of persistent anxiety-like behavior but dissociated from the mechanisms causing the behavioral sensitization. Pharmacological studies confirmed that a reduced Sstr2 mediated effect in the BLA is likely to play a role in persistent anxiety and should be investigated further.

Anantha Shekhar, M.D., PhD.

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INTRODUCTION

1.1 Stress and Anxiety Overview

All living organisms strive toward stability (or homeostasis). When this stability is threatened by real or perceived threat (stressors), anxiety, fear and/or stress responses are elicited in an attempt to preserve homeostasis and enhance survival through coordinated behavioral, autonomic, immune, and endocrine responses (Selye, 1976; Chrousos and Gold, 1992; Habib et al., 2001; Carrasco and Van de Kar, 2003; McEwen, 2007). Considerable evidence has shown that during stress, attention is enhanced and the brain focuses on the perceived threat to appraise the potential harm of the stressor (Davis and Whalen, 2001; Bishop et al., 2004; Bishop, 2008). Cardiac output and respiration are increased (Chrousos and Gold, 1992; Tao and Li, 2005; Abelson et al., 2008), catabolism is enhanced and blood flow is redirected to provide energy to the aroused brain, heart and muscles (Chrousos and Gold, 1992). The elicited response diminishes when the threat is over (Chrousos and Gold, 1992; Habib et al., 2001).

For some individuals, chronic stress leads to psychopathology such as depression and anxiety disorders (Arborelius et al., 1999; Haller, 2001; van Praag, 2004; Rosenkranz et al., 2010). Anxiety disorders alone impact almost 30% of individuals within their lifetime (Kessler et al., 2005) and are associated with impaired workplace performance and huge economic costs (Greenberg et al., 1999) as well as increased risk of cardiovascular morbidity and mortality (Kawachi et al., 1994; Albert et al., 2005; Smoller et al., 2007). Anxiety disorders are subdivided into several classifications based on their clinical presentation,

course and treatment responses. The classifications include general anxiety disorders, panic disorder, obsessive compulsive disorder, phobias, and posttraumatic stress disorder (American Psychiatric Association, 1994). Although evidence suggests that chronic stress can lead to a persistent anxiety disorder (Arborelius et al., 1999; Haller, 2001; van Praag, 2004; Rosenkranz et al., 2010), the mechanism(s) involved remain mostly unknown.

1.1.1 Neuroanotomy overview of the stress/anxiety response

Many brain regions involved in the response to stressors are also implicated in anxiety. The stress/anxiety response is regulated by feedback loops between the brain regions involved. The orbitofrontal cortex (OFC) is proposed to evaluate the situation (Cousens and Otto, 2003; Pickens et al., 2003; Schoenbaum et al., 2003; Winstanley et al., 2004; Schoenbaum and Roesch, 2005) then coordinate with the ventral medial prefrontal cortex (PFC) which, through its coordination with the hippocampus (Quirk and Mueller, 2007) and input into the baslolateral amygdala (BLA), modulates the organism's response to the incoming sensory cues (Rosenkranz and Grace, 1999; 2001; Grace and Rosenkranz, 2002; Rosenkranz and Grace, 2002; Rosenkranz et al., 2003; Rosenkranz et al., 2010).

Sensory information converges onto the lateral and basolateral nuclei of the amygdala (BLA complex) and the BLA is thought to integrate the sensory and memory information to encode the appropriate emotional valence to this information (Turner and Herkenham, 1991; Pitkänen, 2000, LeDoux, 2003). The BLA then relays this information to efferent connections such as the central

nucleus of the amygdala (CE), medial nucleus of the amygdala (ME), the bed nucleus of the stria terminalis (BNST), periaqueductal gray (PAG), and higher cortical centers such as the PFC, OFC and hippocampus (Krettek and Price, 1978b; Amaral et al., 1992; Graeff et al., 1993; Goldstein et al., 1996; Herman and Cullinan, 1997; LeDoux, 2000; Pitkänen, 2000; Akirav and Richter-Levin, 2002; Winstanley et al., 2004) to coordinate the appropriate behavioral response (Graeff et al., 1993; Goldstein et al., 1996; LeDoux, 2000). The BLA also influences other functions such as memory formation (Akirav and Richter-Levin, 2002; Roozendaal et al., 2002; Maroun and Richter-Levin, 2003; Richter-Levin and Maroun, 2010) and stimulus coding (Schoenbaum, 2003; Schoenbaum et al., 2005).

Perceived threats elicit stress circuits in the brain and a cue is perceived as threatening either because of novelty or based on prior experiences. The amygdala plays a role in emotional memories (LeDoux, 2000; Ghashghaei and Barbas, 2002) and modulates memory formation in the hippocampus (Akirav and Richter-Levin, 2002; Benes et al., 2004; Kim et al., 2005) and the PFC (Maroun and Richter-Levin, 2003; Richter-Levin and Maroun, 2010). The PFC guides behaviors, thoughts, and feelings by virtue of its role in modulating long-term or working memory stores (Arnsten and Li, 2005; Sierra-Mercado et al., 2006). The hippocampus is involved in spatial, episodic, and contextual memory formation (Ergorul and Eichenbaum, 2004; Eichenbaum, 2006). Furthermore, the hippocampus inhibits (Herman and Cullinan, 1997) and the amygdala [(Ce, ME), Gray et al., 1989; Gray, 1993; Herman and Cullinan, 1997] stimulates the

paraventricular nucleus of the hypothalamus (PVN) thereby influencing the initiation, propagation and termination of the neuroendocrine aspect of the stress response through the hypothalamic-pituitary-adrenocortical (HPA) axis (Gray et al., 1989; Chrousos and Gold, 1992; Gray, 1993; Herman and Cullinan, 1997; Chrousos, 1998; Carrasco and Van de Kar, 2003). The BNST also directly innervates the PVN (Davis, 1998) to activate the stress response.

The BNST and CE both send projections to areas involved in the behavioral and autonomic aspect of the stress/anxiety response (Davis and Whalen, 2001) such as 1) the lateral hypothalamus (LH) whose projections activate the sympathetic autonomic nervous system which leads to an increase in heart rate, blood pressure and respiration (LeDoux et al., 1988; Davis and Whalen, 2001), 2) the dorsomedial hypothalamus [(DMH), Pitkänen, 2000; Horiuchi et al., 2006] whose projections also activate the sympathetic autonomic nervous system (Shekhar et al., 1993; DiMicco et al., 2002; Horiuchi et al., 2006) as well as regulate anxiety-like behavior (Shekhar, 1993) and 3) the parabrachial nucleus (PBN) in the brain stem whose activation increases respiration (Krettek and Price, 1978a, Amaral et al., 1992). Whereas the CE can respond to conditioned stimuli (Campeau and Davis, 1995), the BNST guides responses to anxiety producing, unconditioned stimuli (Lee and Davis, 1997; Walker and Davis, 1997). The PAG is also a key subcortical region involved in behavioral and physiological responses to threatening stimuli (Graeff et al., 1993; Herman and Cullinan, 1997) and directs behavioral arousal, such as cessation of

movement (Bouton and Bolles, 1980; LeDoux et al., 1988; Graeff et al., 1993; Kim et al., 1993a).

Other areas that have important regulatory roles in the stress/anxiety response include the brain stem centers the ventral tegmental area [(VTA), Wallace et al., 1992; Goldstein et al., 1996], the raphé nuclei [(RN), Graeff et al., 1993; Rainnie, 1999; Rainnie et al., 2004; Spiga et al., 2006; Muller et al., 2007b], basal forebrain [(BF), Semba, 2000; Sarter et al., 2006] and the locus coeruleus [(LC), Butler et al., 1990; Koob, 1999; Koob and Heinrichs, 1999].

1.1.2 Neurochemical overview of the stress/anxiety response

The stress response involves, among others, the activation of the hypothalamic-pituitary-adrenocortical (HPA) axis and the parallel activation of the LC-norepinephrine response (Conti and Foote, 1995; Valentino and Van Bockstaele, 2008). Exposure to stressors leads to the release of corticotropin-releasing factor (CRF) and vasopressin into the portal circulation from the parvicellular neurons (PVN) leading to the release of adrenocorticotropin (ACTH) from the pituitaries. Minutes later, glucocorticoid levels increase in the bloodstream (see Chrousos, 1998; Habib et al., 2001; Tsigos and Chrousos, 2002; Carrasco and Van de Kar, 2003). The released glucocorticoids have an inhibitory influence on the HPA axis (Dallman et al., 1992; Carrasco and Van de Kar, 2003; de Kloet et al., 2005).

The release of CRF also leads to the increased release of the catecholamines [epinephrine and norepinephrine (NE)] from the adrenal medulla and the LC (Valentino et al., 2001; Koob and Heinrichs, 1999). Increased release

of CRF and NE also leads to autonomic and behavioral responses to stressors (Arborelius et al., 1999; Koob and Heinrichs, 1999; Bale and Vale, 2004; Valentino and Van Bockstaele, 2008). Adaptive behavioral changes of the stress response influenced by CRF systems (Koob and Heinrichs, 1999; Bittencourt and Sawchenko, 2000) involve extra-hypothalamic structures such as the CE (Makino et al., 2002), BNST (Lee et al., 2008), and LC (Butler et al., 1990; Koob and Heinrichs, 1999; Valentino et al., 2001).

Although a central component of the stress response involves the release of CRF from the PVN, thereby activating the endocrine aspect of this response (Selye, 1976; Chrousos, 1998; Habib et al., 2001; Tsigos and Chrousos, 2002; Carrasco and Van de Kar, 2003), the interplay of several neurotransmitter systems such as somatostatin [(SST), Tsigos and Carrasco, 2002] and the mesocortical (Deutch and Roth, 1990; Goldstein et al., 1996; Chrousos, 1998), GABAergic (Chrousos and Gold, 1992; Herman and Cullinan, 1997; Habib et al., 2001; Makino et al., 2002), serotonergic and sympathetic and non-sympathetic cholinergic systems (Aghajanian and VanderMaele, 1982; Chrousos, 1998; Semba, 2000; Habib et al., 2001; Carrasco and Van de Kar, 2003) are also involved.

In addition to their influence on the stress response dopaminergic [(VTA), Rosenkranz and Grace, 1999; Grace and Rosenkranz, 2002], serotonergic [(dorsal RN), Graeff et al., 1997; Abrams et al., 2005], cholinergic [(BF), Helmstetter and Bellgowan, 1994; Roozendaal et al., 1996; Calandreau et al., 2006] and/or GABAergic (gamma-aminobutyric acid) neurotransmitter [(BLA),

Sanders et al., 1995; Sanders and Shekhar, 1995] systems have all been associated with the regulation of anxiety states through BLA modulation. Furthermore, medications that modulate the neurotransmission of these transmitters are widely used to treat pathological anxiety (Bodnoff et al., 1989; Andrews and Stephens, 1990; Carrasco et al., 2006). For example, the benzodiazepine (BZ) group of anxiolytics modulate the GABAergic system activity in the BLA (Niehoff and Kuhar, 1983; Sanders et al., 1995) and are the most common treatment for anxiety.

The neuropeptides CRF and SST also have roles in regulating states of anxiety (Bale and Vale, 2004; Truitt et al., 2007; Viollet et al., 2008). For example, somatostatin receptor 2 (Sstr2) knock-out mice demonstrated anxiety-like behavior in a number of behavioral paradigms (Viollet et al., 2000). Moreover, lesioning a specific subset of GABAergic interneurons in the BLA that contain SST led to long-term anxiety-like behavior (Truitt et al., 2007). Numerous studies have shown that CRF receptor activation leads to anxiety-like behavior (Yehuda, 1997; Sajdyk et al., 1999; Steckler and Holsboer, 1999; Sajdyk and Gehlert, 2000; Heinrichs and Koob, 2004; Rainnie et al., 2004; Gehlert et al., 2005).

1.2 Corticotrophin-Releasing Factor (CRF)

The stress response involves the coordination of the endocrine, autonomic, behavioral and immune responses (Selye, 1976; Habib et al., 2001; Carrasco and Van de Kar, 2003; McEwen, 2007). In addition to its role in regulating the release of ACTH from the pituitaries thereby activating the HPA

axis (Vale et al., 1981; Rivier and Plotsky, 1986), the 41 amino acid neuropeptide, CRF, has a role in coordinating the stress response by acting as a neuropeptide in stress responsive nuclei including the amygdala (Makino et al., 2002), dorsal RN (Uryu et al., 1992; Lowry et al., 2000), hippocampus (Herman and Cullinan, 1997) and LC (Butler et al., 1990; Koob and Heinrichs, 1999; Valentino et al., 2001). The psychological component of stress stimulates the release of CRF into the amygdala (Makino et al., 2002) leading to increased defensive responses such as fear and anxiety (Campeau and Davis, 1995; Lee and Davis, 1997; Walker and Davis, 1997; Sajdyket al., 1999) and activation of the autonomic nervous system (Krettek and Price, 1978a; Davis, 1992; Pitkänen, 2000) through its efferent projections to areas such as the LH, DMH, RN, and PBN. Moreover, amygdala activation can possibly stimulate the HPA axis directly (Makino et al., 2002) and indirectly through the BNST (Krettek and Price, 1978a; Herman and Davis, 1998) whereas hippocampal activation can lead to dampening of the HPA axis of the stress response (Herman and Cullinan, 1997). CRF release into the LC promotes arousal and leads to PFC influence on the saliency to sensory information coming into the BLA (Valentino and Bockstaele, 2008). Thus, CRF integrates the stress response across multiple brain areas mediating appropriate responses (also see Vale et al., 1981; Dunn and Berridge, 1990; De Souza, 1995; Carrasco and Van de Kar, 2003; Muller et al., 2003b; Heinrichs and Koob, 2004) as well as the consolidation of long-lasting memories of emotionally significant experiences influenced by stressful conditions (McGaugh, 2002; Roozendaal et al., 2002; Hubbard et al., 2007).

CRF is a member of the peptide family that includes urocortin I [(Ucn1), Vaughan et al., 1995], urocortin II [(UcnII), Reyes et al., 2000], and urocortin III [(UcnIII), Lewis et al., 2001]. These peptides produce their biological response through two known G-protein coupled receptors; corticotropin-releasing factor 1 (CRF1) and corticotropin-releasing factor 2 (CRF2). While CRF has a higher affinity for CRF1 rather than CRF2, Ucn1 has an equally high affinity for both receptors. UcnII and UcnIII have greater affinity for CRF2 (Dautzenberg and Hauger, 2002; Bale and Vale, 2004; Hauger et al., 2006). Activation of both CRF receptors can stimulate adenylate cyclase activity (Chen et al., 1986; Dunn and Berridge, 1990; Dautzenberg et al., 2001) and the intracellular accumulation of cyclic adenosine monophosphate [(cAMP), Giguere et al., 1982; Dunn and Berridge, 1990; Sananbenesi et al., 2003] through coupling to the α subunit of G_s protein ($G\alpha_{s;}$ stimulatory) and the protein kinase A (PKA) pathway. Both CRF receptors are also involved in the regulation of the extracellularly regulated kinase 1/2 in AtT20 cells [(ERK1/2), Sananbenesi et al., 2003; Brar et al., 2004] through the PKA pathway. Independent of cAMP and the PKA pathway, CRF1 activation can lead to inhibition of $Ca_v3.2$ T-type calcium channels through $G\alpha_s$-dependant G protein βɣ (Gβɣ) subunits (Tao et al., 2008). CRF1 and CRF2 receptors can also signal through, a protein kinase C (PKC) mediated intracellular calcium mobilization and IP3 formation as observed in human embryonic kidney 293 (HEK293) cells, possibly by coupling to $G\alpha_q$ protein. However, this last response appears to be cell type specific because intracellular

calcium mobilization was not observed in SK-N-MC neuroblastomas (Dautzenberg et al., 2004; Wietfeld et al., 2004).

Dysfunction of the CRF-mediated circuits has been implicated in the pathophysiology of several psychiatric disorders such as anxiety, panic disorder, posttraumatic stress disorder and depression (Bremner et al., 1997; Yehuda, 1997; Arborelius et al., 1999; Steckler and Holsboer, 1999; Yehuda et al., 2001; Heinrichs and Koob, 2004). Behaviorally, transgenic mice that overproduce CRF exhibit increased stress/anxiety related behaviors that are reversed by central administration of CRF antagonist α-helical CRF 9-41 (Stenzel-Poore et al., 1994). Central administration of CRF agonists induced anxiety-like responses in several animal tests such as open field (Sutton et al., 1982; Liang and Lee, 1988), elevated plus maze (Baldwin et al., 1991; Rainnie et al., 2004), conflict test (Britton et al., 1985), social interaction (Dunn and File, 1987; Sajdyk et al., 1999; Rainnie et al., 2004; Gehlert et al., 2005), acoustic startle (Swerdlow et al., 1986; Lee and Davis, 1997), and aversive conditioning (Cole and Koob, 1988; Cador et al., 1992; Heinrichs and Joppa, 2001; Hubbard et al., 2007; Sherrin et al., 2008; Sherrin et al., 2009). Moreover, CRF receptor antagonist alone attenuates many behavioral effects of stress, supporting the role of endogenous CRF in mediating many stress-induced behaviors (Heinrichs et al., 1995; Habib et al., 2000).

Evidence suggests that the anxiogenic behaviors induced by CRF are likely due to activation of CRF1 (Heinrichs et al., 1997; Timpl et al., 1998; Muller et al., 2003b; Gehlert et al., 2005). For example, mice lacking a functional CRF1 had a higher percentage of entries into the lit compartment of a light-dark box

when compared to wild-type, indicating reduced anxiety-like behavior (Timpl et al., 1998; Habib et al., 2000). Furthermore, an intracranial (i.c.) pretreatment with a CRF1 antagonist prior to an anxiogenic-like dose of Ucn1 or restraint stress prevented an anxiety-like response in the social interaction test (Gehlert et al., 2005). Moreover, in a double blind, placebo-controlled study of monkeys exposed to intense social stress, the oral administration of the CRF1 antagonist, antalarmin, inhibited a repertoire of behaviors associated with anxiety and fear as well as diminish the increase of CRF in the cerebrospinal fluid and reduced the pituitary-adrenal, sympathetic, and adrenal medullary responses to the stress (Habib et al., 2000).

The role of the CRF2 receptor in the anxiety/stress responses is not well understood. CRF2 activation can reduce anxiety-like behavior induced by CRF1 activation (Reul and Holsboer, 2002; Bale and Vale, 2004) but can also induce anxiety-like behavior (Radulovic et al., 1999; Takahashi, 2001). Interestingly, a recent study found that the two CRF receptors have opposing influences on information-processing mechanisms that regulate responses to stressors (Risbrough et al., 2004). It has been suggested that CRF1 may be more involved in the cognitive aspects of behavior whereas CRF2 may be more important for motivation types of behavior essential for survival (Dautzenberg et al., 2001). Although the role of the CRF2 receptor in anxiogenesis can't be completely ruled out, based on the aforementioned information, it is highly likely that stress leads to persistent pathological anxiety through CRF's repeated activation of the CRF1 receptor.

1.3 Amygdala and Anxiety

The amygdala has a high expression level of both CRF receptors (De Souza et al., 1985; Reul and Holsboer, 2002; Bale and Vale, 2004). CRF is released into the amygdala during stress (Koob and Heinrichs, 1999; Makino et al., 2002; Cook, 2004) and stress-induced behavioral changes have been postulated to result from amygdala CRF receptor activation (Dunn and Berridge, 1990; Lee and Davis, 1997; Sajdyk et al., 1999; Sajdyk and Gehlert, 2000; Rainnie et al., 2004). Neurobiological studies of fear and anxiety have identified the amygdala as a central component in the processing of threat and in mediating an individual's emotional response to the perceived threat in both people and experimental animals (Adolphs et al., 1994; Adolphs et al., 1995; LeDoux, 2000; Adolphs et al., 2002; Rauch et al., 2003; Etkin et al., 2004; Phelps and LeDoux, 2005; Shekhar et al., 2005).

Located in the temporal lobe of the forebrain, the amygdala's key role in emotion is due largely to a study by Kluver and Bucy (1937) on the behavioral consequences of temporal lobe lesions in primates. It was found that lesioning of the temporal lobe altered the animals emotional response. For example, the lesioned animals lost their fear for snakes and people. However, it was Weiskrantz's (1956) primate study that implicated the amygdala as a key structure in emotion. Through the use of avoidance conditioning, Weiskrantz (1956) found that amygdala lesions produced emotional changes and proposed, based on this and other results, that amygdala lesions dissociate the emotional properties of the stimuli from their sensory representations. In humans,

individuals with bilateral degeneration of the amygdala exhibit disrupted emotional responses, especially fear and anxiety (Adolphs et al., 1994; Adolphs et al., 1995).

Both animal (Hilton and Zbrozyna, 1963; Kapp et al., 1982; Davis, 1992; Sanders et al., 1995; Sanders and Shekhar, 1995; Sajdyk and Shekhar, 1997; Rainnie et al., 2004) and human studies (Furmark et al., 1997; Adolphs et al., 2002 , Critchley et al., 2002; Anand and Shekhar, 2003; Zald, 2003) have shown that activation of the amygdala results in behavioral and physiological responses associated with anxiety. Electrical stimulation of the amygdala in animals can alter heart rate and blood pressure (Hilton and Zbrozyna, 1963; Stock et al., 1981; Kapp et al., 1982), cardiovascular responses consistent with an anxiety/fear reaction. Electrical stimulation of the amygdala in humans has been shown to induce feelings of anxiety/fear as well as autonomic reactions indicative of fear (Chapman et al., 1954; Feindel and Penfield, 1954). Furthermore, glucocorticoids are increased in the blood stream following electrical stimulation of the amygdala of animals (Matheson et al., 1971; Dunn and Whitener, 1986) indicating that the amygdala has an excitatory influence the HPA axis.

Amygdala activation can be measured using functional magnetic resonance imaging (fMRI) or positron emission tomography [(PET), see Anand and Shekhar, 2003; Rauch et al., 2003; Zald, 2003]. PET and fMRI yield maps of regional brain activity and amygdala activation was observed following invoked anxiety or fear. Amygdala activation can be invoked through multiple sensory modalities such as olfaction (Zald and Pardo, 1997; Zald and Pardo, 2000),

auditory (Morris et al., 2001; Zald and Pardo, 2002) and vision (Adolphs et al., 2002; Whalen et al., 2002; van Marle et al., 2009).

The amygdala was found to have a role in different components of emotion associated with fear and anxiety using neuroimaging. These components include the fear response (Adolphs et al., 1995; Whalen, 1998; Whalen et al., 2002), vigilance, emotional valence or arousal (Whalen, 1998; Yang et al., 2002; Etkin et al., 2004; van Marle et al., 2009), phobia's (Larson et al., 2006), novelty detection (Blackford et al., 2010) and aversive learning (Büchel and Dolan, 2000; Critchley et al., 2002; Bishop et al., 2004; Knight et al., 2005).

Futher evidence supporting amygdala's role in emotion is that anxiolytic drugs, such as the serotonin reuptake inhibitor (SRI) antidepressants (Abrams et al., 2005; Norbury et al., 2009; Sim et al., 2010) and BZ, seem to target the amygdala; particularly the BLA (Scheel-Krüger and Petersen, 1982; Petersen et al., 1985; Hodges et al., 1987; Sanders and Shekhar, 1995).

The combined evidence that 1) the amygdala contains CRF receptors (De Souza et al., 1985; Reul and Holsboer, 2002; Bale and Vale, 2004), 2) CRF is released into the amygdala during stress (Koob and Heinrichs, 1999; Makino et al., 2002; Cook, 2004), 3) the amygdala is the central component in the evaluation of threatening stimuli and activates behavioral and physiological responses associated with emotion such as anxiety (LeDoux, 2000; Adolphs et al., 2002; Rauch et al., 2003; Etkin et al., 2004; Phelps and LeDoux, 2005; Shekhar et al., 2005) and 4) activation of the amygdala leads to activation of the

HPA axis (Matheson et al., 1971; Dunn and Whitener, 1986) suggests that the amygdala is where CRF links stress and anxiety.

1.3.1 Subnuclei of the amygdala

The amygdala is divided into multiple subnuclei that have different functions including the basolateral (anterior (BLA) and posterior (BLP)), lateral (LA), medial (ME), and central (CE) amygdala. The LA and basolateral subnuclei are the major receiving areas and the ME and CE are major outflow areas (Amaral et al., 1992; Killcross et al., 1997; Pitkänen et al., 1997; see **Fig. 1**). The LA and BLA/P funnel and integrate the incoming sensory and cognitive information and the CE and ME are involved in modulating the behavioral, autonomic, and/or endocrine responses (Silverman et al., 1981; Gray et al., 1989; Davis et al., 1994; Campeau and Davis, 1995; LeDoux, 2000; Carrasco and Van de Kar, 2003).

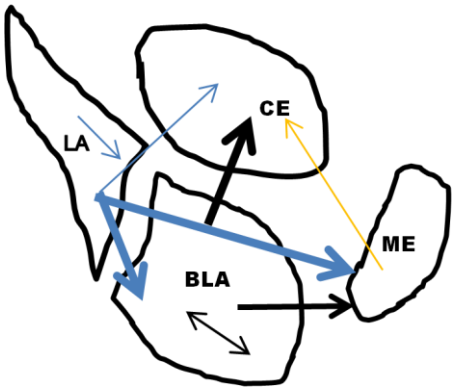


Figure 1. Schematic representation of the major intra-amygdala connections. Arrows represent projection direction with the thicker arrows being heavier projections. Blue arrows represent LA projections, black arrows represent BLA projections, and the orange arrow represents a ME projection. Abbreviations: LA, lateral; BLA, basolateral; CE, central; ME, medial. (Adapted from Sanders, 2001).

The separation of the specific nuclei of the amygdala involved in the generation of anxiety/fear responses was facilitated by studying the development of conditioned fear responses (Davis, 1992; Davis, 2000; LeDoux, 2000). Fear or aversive conditioning involves learning that certain environmental stimuli can predict aversive events. Thus, a previously neutral conditioned stimulus (CS) such as a light elicits a fear response after being associated with an aversive event such as a foot shock [unconditioned stimulus (US)]. After pairing, the CS then elicits a constellation of responses typically used to define a state of anxiety or fear such as defensive behaviors, autonomic and endocrine response as well as alterations in reflex expression like fear-potentiated startle (Davis, 1992; Davis, 2000). The aversive association can occur to the contextual stimuli of the pairing environment as well as the explicit CS (Selden et al., 1991; Helmstetter and Bellgowan, 1994; Muller et al., 1997; Maren, 2003; Huff and Rudy, 2004; Yaniv et al., 2004).

Using a fear conditioning paradigm, the BLA was found to have a key role in the development of associative memories and the subsequent expression of defensive behavior (Helmstetter and Bellgowan, 1994; Campeau and Davis, 1995; Maren et al., 1996; Muller et al., 1997; Cousens and Otto, 2003; Huff and Rudy, 2004). For example, Campeau and Davis (1995) paired both auditory and visual stimuli (CSs) to a foot shock (US) and used the acoustic startle reflex as the behavioral index of conditioning. Pre- and post-training (pairing) electrolytic or N-methyl-D-aspartate (NMDA)-induced lesions of the BLA (and part of the LA) disrupted fear-potentiated startle to both the auditory and visual CSs (Campeau

and Davis, 1995). Chemical inhibition of the BLA with muscimol pre- or post-conditioning training or testing also showed an attenuated fear response to the CS and training context (Muller, Corodimas et al., 1997).

1.3.2 Basolateral amygdala (BLA) and anxiety

Many lines of evidence support the BLA as key in the expression of stress/anxiety related behaviors (Sanders and Shekhar, 1991; Muller et al., 1997; Sajdyk and Shekhar, 1997; Sajdyk et al., 1999; Thielen and Shekhar, 2002; Rainnie et al., 2004; Sajdyk et al., 2006) as well as the acquisition of associative memories that influence these defensive behaviors (Helmstetter and Bellgowan, 1994; Campeau and Davis, 1995; Maren, 1996; Muller et al., 1997; Cousens and Otto, 2003; Huff and Rudy, 2004). For example, rats that received four daily intraBLA microinjections of an anxiogenic-like dose of the CRF receptor agonist, urocortin 1 [(Ucn1), 100 fmoles/100 nl/side], paired with a specific type of floor in a two floor choice chamber (context), exhibited a dose dependant aversion to the floor paired with the Ucn1 injection in subsequent testing (Sajdyk et al., 2006). Moreover, repeatedly blocking the inhibitory gamma-aminobutyric acid ($GABA_A$) receptor in the BLA of rats once a day for five days with a subthreshold dose of the $GABA_A$ receptor antagonist bicuculline methiodide (BMI), when paired with a distinct environment, results in avoidance of the treatment-paired environment (Thielen and Shekhar, 2002). These experiments demonstrate that BLA excitation can be involved in the association of an aversive motivational state with a distinct environment.

In general, inhibition of the BLA results in the reduction of fear or anxiety-like responses, while excitation of the BLA leads to the expression of such responses. For example, inhibition of glutamatergic transmission in the BLA blocks the acquisition and expression of conditioned fear and leads to changes in basal levels of anxiety (Campeau et al., 1992; Kim et al., 1993a; Sajdyk and Shekhar, 1997). Moreover, non-specific lesioning of the BLA of rats blocks the expression of aversive conditioning (Campeau and Davis, 1995) and lesioning a specific portion of the BLA interneuronal population results in long-lasting anxiety-like behavior (Truitt et al., 2007; Truitt et al., 2009). Conversely, chemical activation of the BLA with BMI, presumably via disinhibition of a glutamate signaling (Rainnie et al., 1991b, a), results in characteristics of a defense reaction such as increased heart rate and blood pressure (al Maskati and Zbrozyna, 1989; Sanders and Shekhar, 1991) as well as anxiety-like behavior in SI, conflict tests, and conditioned place avoidance (Sanders and Shekhar, 1995; Thielen and Shekhar, 2002).

Activation of the BLA with a threshold dose of Ucn1 (25 or 100 fmoles) results in an anxiety-like response in the social interaction (SI) and elevated plus maze (EPM) tests (Sajdyk et al., 1999; Sajdyk and Gehlert, 2000; Rainnie et al., 2004; Gehlert et al., 2005). Furthermore, repeated microinjections of a subthreshold dose of Ucn1 (6 fmoles/100 nl/side) once a day for five consecutive days, a procedure called priming, results in long-term anxiety-like behavior (Sajdyk et al., 1999; Sajdyk and Gehlert, 2000; Rainnie et al., 2004; Gehlert et al., 2005). The fact that direct chemical application into the BLA results in

changes in anxiety-type responses suggests that the cell bodies within the area, and not the fibers of passage, are involved (Davis, 1992).

Experimental evidence suggests that the BLA may be the primary site for mediating the anxiolytic effects of benzodiazepines (BZ). Benzodiazepines work by enhancing the inhibitory actions of the $GABA_A$ receptor. The BLA contains one of the highest concentrations of GABA-benzodiazepine receptor sites (Niehoff and Kuhar, 1983). Direct intraBLA injections of BZ into rats elicits anticonflict effects (Scheel-Krüger and Petersen, 1982; Petersen et al., 1985; Hodges et al., 1987; Sanders et al., 1995) and anxiolytic-like effects in social interaction (Sanders et al., 1995). Microinjection of the $GABA_A$ agonist, muscimol, into the BLA also has anticonflict effects (Petersen et al., 1985). Moreover, intraBLA injections of the BZ antagonist, flumazenil, or the $GABA_A$ antagonist, BMI, block the anxiolytic-like effects of peripheral administration of the benzodiazepine, chlordiazepoxide (Sanders et al., 1995).

Taken together, it appears that the BLA has a critical role in modulating 'anxiety' and that repeated activation of the BLA leads to persistent pathological anxiety.

1.3.3 Anatomical connections of the BLA

1.3.3.1 *Afferent connections to the BLA*

The lateral and basolateral nuclei (BLA complex) receive information from all sensory and association cortices as well as directly from the sensory thalamus (Ottersen and Ben-Ari, 1979; LeDoux et al., 1990a; Amaral et al., 1992; Maren, 1996). Other afferents to the BLA complex include the PFC (Davis et al., 1994;

McDonald, 1998; LeDoux, 2000), hippocampus (Phillips and LeDoux, 1992; Ikegaya et al., 1995; Richardson et al., 2004), BNST (Krettek and Price, 1978a; Pitkänen, 2000), and brainstem areas such as the parabrachial nuclei, raphé nuclei (RN), VTA, and LC (Krettek and Price, 1978a; Amaral et al., 1992; Fallon and Ciofi, 1992).

1.3.3.2 *Efferent connections from the BLA*

Efferents from the BLA project onto the OFC (McDonald, 1998; Pitkänen, 2000; Schoenbaum et al., 2003), hippocampus (Ikegaya et al., 1995; Krettek and Price, 1978b; Pitkänen, 2000), thalamus (LeDoux et al., 1990b; Turner and Herkenham, 1991) and BNST (Krettek and Price, 1978a; Davis and Whalen, 2001).

From the BNST projections innervate the PVN and brain stem area (Davis and Whalen, 2001). CE projections terminate onto the PAG (Loughlin and Fallon, 1983; Graeff et al., 1993), PBN (Krettek and Price, 1978a; Amaral et al., 1992), LH (LeDoux et al., 1988), PVN (Gray et al., 1989), thalamus (Pitkänen, 2000), VTA, and LC (Krettek and Price, 1978a; Amaral et al., 1992; Fallon and Ciofi, 1992). The ME projects to the PVN and DMH (Amaral et al., 1992; Pitkänen, 2000).

Thus the BLA is well situated to coordinate the stress/anxiety response to incoming threats. The BLA assesses the environmental situation based on the sensory and association memories that converge onto it (LeDoux et al., 1990a; LeDoux, 2000) then BLA projections to the CE, ME, and the BNST regulate the behavioral, autonomic, and endocrine responses influenced by stressors

(LeDoux et al., 1988; Davis, 1998; Davis and Whalen, 2001) as described in section 1.1.1.

1.3.4 Internal circuitry of the BLA

The BLA is a cortex-like structure consisting of two major classes of neurons, large glutamatergic pyramidal cells (projection neurons) and smaller GABAergic nonpyramidal cells (interneurons). The output of the BLA is via pyramidal cell axons. In the BLA the nonpyramidal cells impinge on the pyramidal cells and modulate the activity of these projection neurons (McDonald, 1982; 1992).

The excitatory amino acid ionotrophic receptors of N-methyl-D-aspartate (NMDA) and non-NMDA type are found on pyramidal as well as nonpyramidal cells of the BLA (McDonald, 1994). The BLA contains both intrinsic and extrinsic excitatory amino acid projections (Krettek and Price, 1978b; Amaral et al., 1992; Pitkänen, 2000). Afferent activation of pyramidal cells from connections such as the LA and the stria terminalis results in the expression of both a fast-excitatory postsynaptic potential (f-EPSP), mediated by a non-NMDA glutamate receptor, and a slow-EPSP (s-EPSP) that is NMDA receptor mediated (Rainnie et al., 1991a). A concurrently activated inhibitory postsynaptic potential (IPSP) temporally overlaps the NMDA-mediated s-EPSP and is sensitive to BMI (Rainnie et al., 1991b). Local application of BMI elicited bursts of firing from the BLA pyramidal cells suggesting that tonic GABAergic inhibition determines the activity of BLA neurons (Rainnie et al., 1991b).

The pyramidal cells appear to have both $GABA_A$ and $GABA_B$ receptors that elicit fast- and slow-IPSP's (f- and s-IPSP), respectively (Rainnie et al., 1991b). Most of the nonpyramidal cells in the BLA contain the neurotransmitter GABA as well as neuropeptides (McDonald and Pearson, 1989). The basal neuronal network responses within the BLA are maintained by a balanced interaction between GABAergic inhibition and glutamatergic excitation (Rainnie et al., 1991b, a; McDonald et al., 1996).

1.3.5 BLA inhibitory interneurons exist as a network

Electrophysiological studies have demonstrated that the firing of the projection neurons of the BLA is tonically inhibited by GABAergic interneurons of the BLA (Rainnie et al., 1991b, a; Gean and Chang, 1992). There are currently at least four distinct classes of non-pyramidal GABAergic interneurons recognized within the BLA based on calcium binding protein and neuropeptide content; 1) parvalbumin/calbindin positive neurons; 2) somatostatin/calbindin positive neurons; 3) small, bipolar and bitufted internuerons that are positive for vasoactive intestinal peptide, calretinin, and cholecystokinin; and 4) large, multipolar cholecystokinin positive neurons that often contain calbindin (McDonald and Betette, 2001; McDonald and Mascagni, 2001; McDonald and Mascagni, 2002; Mascagni and McDonald, 2003; Muller et al., 2003a; 2007a; Muller et al., 2007b).

Deficits in distinct classes or sub-classes of these interneurons have been demonstrated to have profound effects on behaviors. For example, loss of SST interneurons in the BLA increases susceptibility to epileptic seizures (Tuunanen

et al., 1997) and selective ablation of neurokinin (substance P)-1 receptor (NK-1r) positive cells, which are part of a larger population of SST containing interneurons, results in increased anxiety-like behaviors (Truitt et al., 2007). In addition to their unique combination of protein and neuropeptide content, these interneurons appear to be functionally distinct (McDonald and Mascagni, 2001; Mascagni and McDonald, 2003; Muller et al., 2003a; Mascagni and McDonald, 2007; Truitt et al., 2007; Mascagni and McDonald, 2009). Furthermore, each of these interneurons can be differentiated by their efferent and afferent connectivity (McDonald, 1992; McDonald and Mascagni, 1996; Muller et al., 2003a; Muller et al., 2007b).

1.3.6 Summary of BLA role in anxiety

Overall, the neuroanatomical and functional data obtained suggest that dysfunction of information processing through the BLA could result in inappropriate modulation of stress control that leads to persistent pathological anxiety; 1) Many lines of evidence support the BLA as key in the expression of stress/anxiety related behaviors (Sanders and Shekhar, 1991; Sanders et al., 1995; Sajdyk and Shekhar, 1997; Sajdyk et al., 1999; Sajdyk and Shekhar, 2000; Thielen and Shekhar, 2002; Rainnie et al., 2004; Sajdyk et al., 2006) as well as the acquisition of associative memories that influence these defensive behaviors (Helmstetter and Bellgowan, 1994; Campeau and Davis, 1995; Maren, 1996; Muller et al., 1997; Cousens and Otto, 2003; Huff and Rudy, 2004); 2) Inhibition of the BLA results in the reduction of fear or anxiety-like responses (Campeau et al., 1992; Kim et al., 1993b; Sajdyk and Shekhar, 1997) while excitation of the

BLA leads to the expression of such responses (al Maskati and Zbrozyna, 1989; Sanders and Shekhar, 1991; Sanders and Shekhar, 1995; Sajdyk et al., 1999; Sajdyk and Shekhar, 2000; Rainnie et al., 2004; Gehlert et al., 2005); 3) Deficits in distinct classes or sub-classes of BLA interneurons have been demonstrated to have profound effects on behaviors (Tuunanen et al., 1997; Truitt et al., 2007); 4) The BLA may be the primary site for mediating the anxiolytic effects of benzodiazepine group of anxiolytics (Scheel-Krüger and Petersen, 1982; Niehoff and Kuhar, 1983; Petersen et al., 1985; Hodges et al., 1987; Sanders and Shekhar, 1995); and 5) The BLA is well situated to regulate various components of the stress/anxiety response (LeDoux et al., 1990a; LeDoux, 2000; Davis and Whalen, 2001; Shekhar et al., 2005).

1.4 Priming of the BLA with Urocortin 1 (Ucn1) as a Model of Persistent Pathological Anxiety

As mentioned earlier, chronic stress can lead to pathological anxiety (Arborelius et al., 1999; Haller, 2001; van Praag, 2004; Rosenkranz et al., 2010) however the central mechanisms involved remain largely unknown. Animal models of psycho-pathological conditions may be used as simulations to study the mechanisms or possible etiologies of the disorder the animal model mimics (Willner, 1990; Willner and Mitchell, 2002). However, for an animal model to be useful it must have face, predictive, and construct validity (Willner and Mitchell, 2002). Predictive validity suggests that the behavior of the animal model mimics the clinical behavior, face validity refers to the symptom similarities between the

disorder and the model, and construct validity addresses biochemical/ neurochemical changes as well as the anatomic area involved.

Neuroimaging studies suggest an increase in amygdala activation associated with anxiety (Anand and Shekhar, 2003; Rauch et al., 2003). Both the BLA and CRF are key components involved in the regulation of the of the stress/anxiety response (Goldstein et al., 1996; Bale and Vale, 2004; Yaniv et al., 2004). Previous studies have shown that an acute bilateral microinjection of a threshold dose of the CRF agonist Ucn1 (100 fmole/100 nl/side), into the BLA of male Wistar rats results in the expression of anxiety-like behavior as measured in the SI test (Sajdyk et al., 1999; Rainnie et al., 2004; Gehlert et al., 2005). Furthermore, when a sub-threshold dose of Ucn1 (6 fmole/100 nl/side), a dose that does not produce an anxiety-like response when given acutely, is given repeatedly into the BLA for five consecutive days (D1-D5) two things occur; 1) behavioral sensitization in that there is a significant decrease in social interaction on injection D3 as compared to baseline (Sajdyk et al., 1999; Rainnie et al., 2004) and 2) a persistent anxiety-like phenotype develops that can last at least five weeks after the last Ucn1 injection(Sajdyk et al., 1999; Sajdyk and Gehlert, 2000; Rainnie et al., 2004; Gehlert et al., 2005). Moreover, the persistent changes in anxiety-like behavior induced by Ucn1-priming are associated with an increase in BLA network excitability (Rainnie et al., 2004).

The microinjection of a subthreshold dose of Ucn1 (6 fmoles) into the BLA once a day for five consecutive days (D1-D5) is termed Ucn1-priming. Priming

the amygdala with Ucn1 produces an animal model that has predictive, face and construct validity for pathological anxiety.

The animal model of pathological anxiety induced by Ucn1 priming has face validity in that 1) the Ucn1-primed animals develop a persistent anxiety-like behavior and 2) the persistent change in anxiety-like behavior induced by Ucn1-priming is associated with an increase in BLA network excitability (Rainnie et al., 2004) indicative of increased amygdala activation.

The construct validity of the Ucn1-primed animal model of pathological anxiety is evident in that both CRF and the BLA are key components involved in the regulation of the of the stress/anxiety response (Goldstein et al., 1996; Bale and Vale, 2004; Yaniv et al., 2004) and Ucn1, a CRF agonist, was infused into the BLA of the rat to induce the development of the persistent anxiety-like behavior.

The animal model mimicks clinical behavior in that repeated microinjections of Ucn1 into the BLA, mimicking repeated exposure to stress, leads to the development of a persistent anxiety-like phenotype (Sajdyk et al., 1999; Sajdyk and Gehlert, 2000; Rainnie et al., 2004; Gehlert et al., 2005). Moreover, the persistent anxiety-like phenotype that develops responds to the anxiolytic drug, alprazolam, a potent benzodiazepine agonist (Shekhar et al., 2003). Furthermore, the physiological symptoms of anxiety include increased heart rate, blood pressure, and respiratory rate (Habib et al., 2001; Carrasco and Van de Kar, 2003). Animals primed with Ucn1 also demonstrate increased heart rate and respiration following intravenous sodium lactate, a panicogenic agent in

susceptible individuals (Sajdyk and Shekhar, 2000). These physiological symptoms also increase the face validity of the Ucn1-primed animal of pathological anxiety.

Thus, the rats that develop behavioral sensitization during and a persistent anxiety-like phenotype following repeated CRF1 activation in the BLA could be a mechanistic model of the neurobiological changes that occur during the development of anxiety disorders.

1.5 Specific Aims, Rationale, and Working Hypotheses

For some individuals, repeated episodes of the stress response lead to pathological anxiety and depression (Gispen-de Wied and Jansen, 2002; van Praag, 2004) however the central mechanisms that lead to anxiety disorders remain largely unknown. As described earlier, CRF and the BLA have been identified as important mediators of stress and anxiety responses (Vale et al., 1981; Koob and Heinrichs, 1999; Adolphs et al., 2002; Shekhar et al., 2005) as well as for associative memories of aversive events (Goldstein et al., 1996; LeDoux, 2000; McGaugh, 2002; Roozendaal et al., 2002; McGaugh, 2004) and dysfunction in CRF-mediated circuits has been implicated in the pathophysiology of anxiety (Bremner et al., 1997; Yehuda, 1997; Arborelius et al., 1999; Steckler and Holsboer, 1999; Heinrichs and Koob, 2004). Mimicking repeated episodes of the stress response, a sub-anxiogenic dose of Ucn1 (6 fmoles) is microinjected into the BLA of rats once a day for five consecutive days (priming). This results in 1) behavioral sensitization, such that a previously non-efficacious dose of Ucn1 will elicit anxiety-like response after the third injection and 2) the development of

a persistent anxiety-like phenotype that lasts at least five weeks after the last injection without any further treatment. Therefore, the **overall objective** of this thesis is to identify mechanisms involved in the Ucn1-priming-induced anxiogenic-like effects. The **central hypothesis** is *that Ucn1-priming involves intrinsic changes within BLA neurotransmitter/neuropeptide systems induced by repeated stimulation of the BLA CRF receptors that leads to the expression of persistent anxiety-like behavior.* The central hypothesis will be tested with the following specific aims:

1.5.1 Specific Aim 1

Determine the extent to which the persistent anxiety-like phenotype of rats observed following Ucn1-priming is the result of aversive conditioning to the SI arena (context) and/or the presence of a novel partner.

1.5.1.1 *Working Hypothesis 1*

If the persistent anxiety-like phenotype induced by Ucn1-priming is due to intrinsic changes in the BLA from repeated stimulation of the CRF receptors then priming without exposure to the SI arena and/or a novel partner will result in a persistent anxiety-like phenotype post-priming.

1.5.1.2 *Rationale 1*

To date, the development of a persistent anxiety-like state/phenotype induced by Ucn1-priming in rats has been investigated using the following priming protocol: Male Wistar rats, fitted with chronic bilateral guide cannulae targeting the BLA, receive a daily bilateral i.c. microinjection with either vehicle [Veh, 1% bovine serum albumin/100 nl/side] or a sub-anxiogenic dose of

Urocortin 1 [(Ucn1), 6 fmoles/100 nl/side] for five consecutive days (Sajdyk et al., 1999; Rainnie et al., 2004). The animals undergo SI testing at baseline (D0) and priming days 1 (D1), 3 (D3), and 5 [(D5), see **Fig. 2**] with post-priming SI tests anywhere from one to five weeks later carried out without any further treatment (Shekhar et al., 2003; Rainnie et al., 2004; Truitt et al., 2007). By priming D3 Ucn1-primed animals display a significant decrease in SI compared to baseline and veh-primed rats and the anxiety-like behavior continues post-priming.

Considering that CRF receptor activation facilitates aversive conditioning (Heinrichs and Joppa, 2001; Sherrin et al., 2008; Sherrin et al., 2009), that conditioning can occur to a testing apparatus following BLA pharmacological manipulation (Helmstetter and Bellgowan, 1994; Thielen and Shekhar, 2002; Sajdyk et al., 2006) and confirmation of a persistent anxiety-like phenotype occurs under the same conditions as were used during the priming injections, it is possible that the persistent anxiety-like phenotype that develops is a result of aversive conditioning to the SI testing procedure (introduction of a novel partner) or the arena itself.

1.5.1.3 *Objective 1*

To investigate the role of the testing environment on the development of persistent anxiety-like behavior following Ucn1-priming, adult rats underwent repeated pharmacological manipulation of the BLA with Ucn1 then anxiety-like behavior was assessed with the social interaction (SI) test. The contextual cues tested were 1) the familiar open field SI arena and 2) the presence of a novel partner.

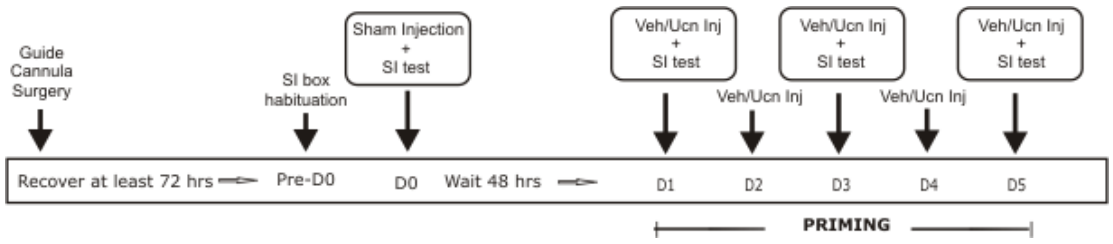


Figure 2. Timeline for a standard priming experiment. Male Wistar rats fitted were with bilateral guide cannulae targeting the BLA then habituated to the SI arena after recovering from surgery. Forty-eight hours following a baseline SI test, rats were microinjected once a day for 5 consecutive days with either vehicle (Veh) or Urocortin 1 (Ucn). Under standard priming conditions rats are placed in SI 30 minutes after microinjection on D1, D3, & D5 of priming.

1.5.2 Specific Aim 2

Determine the potential intrinsic neurotransmitter/ neuropeptide system(s) changes involved in Ucn1-priming that induce a persistent anxiety-like phenotype.

1.5.2.1 *Working Hypothesis 2*

If Ucn1-priming induces changes in the gene expression of the neurotransmitter/neuropeptide receptor(s) within the BLA then changes in the transcription of the neurotransmitter/neuropeptide receptors in the BLA likely involved in the expression of the persistent anxiety-like phenotype will occur and the likely candidates are the neurotransmitter/neuropeptide receptor(s) within the BLA previously shown to have a role in anxiety-like behavior.

1.5.2.2 *Rationale 2*

Normal responding of rats in SI appears to be regulated by a balanced interaction between GABAergic inhibition and glutamatergic excitation which maintains the basal neuronal network response within the BLA (Rainnie et al., 1991b, a; McDonald and Mascagni, 1996). The persistent anxiety-like behavior observed after Ucn1-priming correlates with an increase in network excitability in the BLA of Ucn1-primed rats (Rainnie et al., 2004). The activity of the projection neurons is modulated by the GABAergic interneurons (McDonald, 1982; 1992) therefore this change in the network properties observed following Ucn1-priming is likely due to a change in the inhibitory neurotransmission.

Currently, there are at least four subclasses of GABAergic interneurons recognized within the BLA that are functionally distinct and contain a unique distribution of neuropeptides (McDonald, 1982; 1992; Muller et al., 2003a; Truitt et al., 2009). Many neurotransmitters and neuropeptides located within the BLA have been implicated in anxiety (Washburn and Moises, 1992a; Washburn and Moises, 1992b; Washburn and Moises, 1992c; Heilig et al., 1994; Helmstetter and Bellgowan, 1994; Sanders et al., 1995; Sanders and Shekhar, 1995; Rainnie, 1999; Viollet et al., 2000; Grace and Rosenkranz, 2002; Millan, 2003; Sajdyk et al., 2004; Abrams et al., 2005; Viollet et al., 2008; Engin and Treit, 2009; Rostkowski et al., 2009). A long-term change could be occurring within any of the neurotransmitters/ neuropeptides systems within the BLA that leads to persistent changes in the neuronal response of the BLA associated with the persistent anxiety-like phenotype.

Regulation of gene expression is one mechanism that can lead to relatively stable changes within neurons (Nestler et al., 1993). Therefore changes in the gene expression of neurotransmitter/neuropeptide receptors, and regulators thereof, within the BLA were further investigated to determine what neurotransmitter/neuropeptide systems are likely involved in the persistent anxiety-like behavior observed post Ucn1-priming. The mechanisms of the suggested network change are likely complex.

1.5.2.3 *Objective 2*

To investigate what neurotransmitter/neuropeptide systems within the BLA are possibly involved in the development of the persistent anxiety-like phenotype observed after Ucn1-priming, adult rats underwent repeated pharmacological manipulation of the BLA with Ucn1 for five consecutive days. SI was used to verify the development of the persistent anxiety-like phenotype. Changes in mRNA between Ucn1- and Veh-primed rats were assessed using a RT-PCR array system composed of 84 neuroscience related genes.

1.5.3 Specific Aim 3

Further test the role of the identified neurotransmitter/neuropeptide receptor system(s) identified in Specific Aim 2 in the expression of anxiety-like behavior utilizing pharmacological manipulations in the BLA.

1.5.3.1 *Working Hypotheses 3a*

If decreased receptor mRNA expression in the BLA observed following Ucn1-priming is causally linked to the expression of persistent anxiety-like behavior then reducing the receptor activity with the appropriate antagonist will increase anxiety-like behavior and vice versa.

1.5.3.2 *Working Hypotheses 3b*

If decreased somatostatin regulated inhibition through the Sstr2 receptors in the BLA is causally linked to the expression of persistent anxiety-like behavior following Ucn1-priming then increasing the Sstr2 receptor activity in the BLA with the appropriate agonist prior to the Ucn1-priming injection will block the development of 1) behavioral sensitization and/or 2) the persistent anxiety-like phenotype.

1.5.3.3 *Rationale 3*

In this current study, results from the Neurotransmitter Receptors and Regulators RT²*Profiler*™ PCR Array system showed a unique profile of changes with a significant decrease in only five mRNAs in the Ucn1-primed group of tissue as compared to the veh-primed; they are somatostatin receptors 2 and 4 (Sstr2 and Sstr4), cholinergic nicotinic receptor alpha4 (Chrna4), cholinergic muscarinc receptor 4 (Chrm4) and GABA receptor rho1 (Gabrr1).

At the time that Experiment 2 was completed the tools available for studying these receptors varied by receptor. Antagonists were available for Sstr2 (Feniuk et al., 2000; Nunn et al., 2003) and Gabrr1 (Chebib, 2004). A specific Sstr4 receptor antagonist did not exist and antagonists for muscarinic receptors

lacked specificity for individual receptor subtypes (Degroot and Nomikos, 2006). Antagonists and agonists for Chrna4 were indirectly available in that nAchR were developed for specific combinations of α and β subunits or α7 (Andersen and Arneric, 1994; Xiao et al., 1998; Dhar et al., 2000). Specific agonists were available for Sstr2 (Halloway et al., 1996; Way et al., 1996; Cescato et al., 2006), Sstr4 (Ankersen et al., 1998; Smith et al., 2001), Gabrr1 (Chebib, 2004) and Chrma4 (Ishii and Kurachi, 2006).

Reviewing the literature did not reveal a role for Sstr4 or Gabrr1 containing receptors, $GABA_C$, in anxiety but the remaining three genes did. Chrm4 knock-out mice displayed anxiolytic-like behavior by decreased shock-probe burying in the shock-probe burying model of anxiety (Degroot and Nomikos, 2006). Sstr2 (Viollet et al., 2000) and Chrna4 (Ross et al., 2000) knock-out mice displayed anxiety-like behavior in the EPM. Of these genes, Sstr2 and Chrna4 mRNA showed the most significant decrease in mRNA for Ucn1-primed compared to Veh-primed rats. However, in the amygdala, cholinergic receptors (both nicotinic and muscarinic) are involved in memory consolidation of emotional learning tasks (Ohno et al., 1993; Introini-Collison et al., 1996; Addy et al., 2003; Boccia et al., 2009). Sstr2's role in anxiety is more specific to the BLA.

SST increases inward rectifying K+ current of amygdala projection neurons in amygdala slice preparations through Sstr2 (Meis et al., 2005) suggesting that Sstr2 activation would lead to a decrease in neuronal excitability and eventually inhibit spontaneous firing of the projection neurons (Meis et al., 2005). Furthermore, targeted lesions of neurokinin 1 receptor (NK-1r) positive

cells which contain about half of the SST-GABA interneurons in the BLA resulted in persistent anxiety-like behavior (Truitt et al., 2007).

1.5.3.4 *Objective 3*

To determine the role of Chrna4 or Sstr2 in anxiety, adult rats underwent pharmacological manipulation of the BLA with the appropriate antagonists 1) nicotinic acetylcholine (nAch) antagonists; Mecamylamine Hydrochloride or Dihydro-beta-erythroidine hydrobromide (DHβE) or 2) the Sstr2 antagonist CYN-154806.

To determine the effect of an agonist pretreatment during priming on the expression and development of anxiety-like behavior, the Sstr2 agonist BIM-23027 was co-administered with Ucn1 during priming. SI was used to assess the expression of anxiety-like behavior during and post priming.

1.5.4 Specific Aim 4

Determine if a reduction in Sstr2 mRNA is associated with the behavioral sensitization.

1.5.4.1 *Working Hypotheses 4*

The quantity of Sstr2 mRNA will be reduced in rats expressing anxiety-like behavior following three Ucn1-priming injections as compared to those rats that do not.

1.5.4.2 *Rationale 4*

Ucn1-priming results in the development of behavioral sensitization and a persistent anxiety-like phenotype post priming (Sajdyk et al., 1999; Rainnie et al., 2004). Findings from experiments within this thesis revealed a significant

reduction in the Sstr2 mRNA post Ucn1-priming of the BLA. Pretreatment with a Sstr2 agonists during priming delayed the development of behavioral sensitization but did not prevent the development of the persistent anxiety-like phenotype.

Sstr2 activation can lead to a decrease in neuronal excitability (Meis et al., 2005) and Ucn1-priming leads to an increase in neuronal excitability (Rainnie et al., 2004). If the decrease in SI is associated with the Sstr2 mRNA content then there will be less Sstr2 mRNA for the Ucn1-primed group pretreated with a vehicle during priming as compared to the Ucn1-primed rats pretreated with the Sstr2 agonist BIM-23027.

1.5.4.3 *Objective 4*

Experiments were designed to investigate the effect pretreatment with the Sstr2 agonist BIM-23027 had on the Sstr2 mRNA quantity. Adult rats underwent repeated pharmacological manipulation of the BLA with co-administration of BIM-23027and Ucn1. Changes in mRNA were assessed on priming D3 and post priming with qRT-PCR (standard curve).

METHODS AND MATERIALS

The overall objective of this thesis was to elucidate mechanisms involved in the persistent anxiety-like behavior/phenotype that develops after urocortin 1 (Ucn1)-priming. Methods and procedures outlined below are well established. The general methods used throughout the thesis will be described first then the specific protocols for each experiment will follow. Each protocol contains details of the procedures used for that particular experiment and the type of statistics used.

2.1 General Methods

2.1.1 Animals

All experiments were conducted with male Wistar rats (275 - 300 g) obtained from Harlan Laboratories (Indianapolis, IN). Upon arrival the animals are individually housed, given food and water *ad libitum,* and maintained at standard environmental conditions (72 ˚F; 12 - 12 hour light/dark schedule; lights on at 7:00 A.M.). Rats were acclimated to the animal care facility for 5 - 10 days prior to any surgery/procedure. Animal care procedures were conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and the Indiana University-Purdue University Indianapolis Institutional Animal Care and Use Committee.

2.1.2 Cannulae placement surgery procedure

All drugs/compounds used for this thesis were introduced into the basolateral amygdala (BLA) by intracranial (i.c.) microinjections. Guide cannulae were implanted in the brain to provide specific access to the BLA for the

microinjection. Before guide cannulae placement surgery, rats were anesthetized in a closed Plexiglas box which was connected to an isoflurane system (MGX Research Machine; Vetamic, Rossville, IN, USA). The anesthetized rat was placed in a stereotaxic instrument (Kopf Instruments, Tujunga, CA) with the incisor bar set at -3.3 mm. Isoflurane was continually administered via a nose cone on the incisor bar permitting rats to remain under anesthesia for the remainder of the surgery. Two sterilized stainless steel guide cannulae (26 gauge, 10 mm length; Plastics One, Roanoke, VA) were fixed onto the stereotaxic arms used to locate amygdala coordinates (anterior/posterior (AP): -2.1 and medial/lateral (ML): $\pm$5.0 from bregma) according to the Paxinos and Watson (1986) rat brain atlas. Two small holes were bored into the skull so that the cannula guide could be lowered into the BLA to the dorsal/ventral (DV) coordinate -8.0 from the top of the skull (Paxinos and Watson, 1986). This depth placed guide cannulae just above the BLA. The guide cannulae were placed in this position so that the microinjectors, which extend 1 mm below the guide cannulae, target the proper place of the BLA for the i.c. microinfusion. The guide cannulae were secured into place using three 2.4-mm stainless steel screws attached to the skull, Loc-tite adhesive, and cranioplastic cement layered on top of the dried adhesive to stabilize the adhesive cap. The guide cannulae were sealed with removable stylets (Plastics One, Roanoke, VA) that also extended 1 mm below the guide cannulae to ensure patency. After surgery, rats were returned to their home cage and allowed to recover for at least 72 hours before behavior testing.

2.1.3 Intracranial drug microinjections

All compounds were dissolved in a 1% Bovine Serum Albumin [(BSA), Sigma; St. Louis, MO] vehicle and delivered bilaterally into the BLA through 33 gauge injection cannulae (Plastics One, Roanoke, VA) each connected to a 10 µl Hamilton syringe via polyethylene tubing (PE 50; Fisher Scientific, Pittsburgh, PA). Drugs were microinfused at a volume of 100 nl delivered over 30 seconds via a syringe pump (Havard Apparatus, Holliston, MA, Model PHD 2000). The cannulae were left in place for one minute following the microinjection to maximize diffusion away from the tip. To insure drug delivery, smooth flow from the cannulae tip was verified before and after each microinjection (Sajdyk et al., 1999).

2.1.4 Experimental drugs/compounds

Intracerebral injections were carried out using the following compounds: Urocortin 1 (Ucn1), Mecamylamine Hydrochloride, and Dihydro-beta-erythroidine hydrobromide [(DHβE), Sigma; St. Louis, MO], BIM-23027 (American Peptide Company, Inc; Sunnyvale, CA); and CYN-154806 [Ac-4NO2-Phe-c(DCys-Tyr-DTrp-Lys-Thr-Cys)-D/LTyr-NH2; [(Tocris Cookson, Inc; Ellisville, MO), **Table 1**]. The dose given was specific to the designed experiment and outlined within the relevant experimental protocol.

Table 1. Drugs/Compounds utilized for the experiments for this thesis

Drugs/Compounds	Abbreviation	Activity
Bovine Serum Albumin (BSA)	Veh	vehicle
Urocortin 1	Ucn1	CRF receptor agonist
BIM-23027	BIM-23027	Sstr2 agonist
Ac-4NO2-Phe-c(DCYs-Tyr-DTrp-Lys-Thr-Cys) -D/L Tyr-NH2	CYN-154806	Sstr2 antagonist
Mecamylamine Hydrochloride		α3β4nAch antagonist
Dihydro-beta-erythroidine hydrobromide	DHβE	α4β2nAch antagonist

2.1.5 Placement verification

Rats were anesthetized with isoflurane, then immediately sacrificed. Only data from animals with bilateral cannulation of the BLA by comparison with the Paxinos and Watson (1986; 2005) atlas were included in the data analysis. To verify placement, rats were sacrificed using a guillotine. The brains were quickly removed from the skull and "flash" frozen in 4-methylbutane (Fisher Scientific) on dry ice (approximately -50 ˚C). The brains were stored at -80 ˚C until they were sliced with a cryostat to verify placement of injectors and/or for tissue analysis of RNA.

If the brain tissue was going to be collected for RNA analysis; the brains were sectioned with a cryostat into 300 micron thick sections. Cannulae guide placement was verified visually, and the microinjector tip location was immediately marked on an amygdala template copied from Paxinos and Watson (2005) to represent the section of the brain where the tip was located. If the brain tissue was not needed for RNA analysis, the brains were sliced into 40 micron sections, mounted onto a positive charged microscope slide then stained to

facilitate verification of the cannulae guide placement. (The neutral red and cresyl violet tissue staining protocols were equally effective.)

Identifying the amygdala was discerned using Paxinos and Watson (2005) as a guide. Markers such as the fiber tracts of the external capsule, stria terminalis and the optic tract as well as the size and location of the lateral ventricles and the hippocampal formation were used to facilitate amygdala identification. A dissecting microscope was used to examine the coronal sections mounted on microscope slides.

2.1.6 Social interaction (SI) test

The social interaction (SI) test is a fully validated test for anxiety-like behavior (File and Hyde, 1978; File, 1980; File and Seth, 2003). A modified version of the SI test was used to measure anxiety-like behavior for this thesis (Guy and Gardner, 1985; Sanders and Shekhar, 1995; Sajdyk et al., 1999; Rainnie et al., 2004). The SI time was defined as the time the experimental animal spent engaging in SI in a five minute test. Social interaction is defined as the non-aggressive contact of the experimental animal with the partner animal such as sniffing, crawling over, following, and tail pulling. A decrease in SI time is considered an increase in anxiety-like behavior and vice-versa. Rats have higher baseline SI times in low-lighted, familiar areas (File and Hyde, 1978; File, 1980). Therefore SI experiments were carried out under low light conditions (40 watt red light) and in a familiar SI testing arena to facilitate detection of SI reductions influenced by treatment conditions (anxiogenic-like).

The SI testing arena was a 36”L x 36”W x 12”H wooden box with an open top. On the floor of the SI arena, three rows of three 12” x 12” square cells were outlined. The following interactions were also quantified from the SI session to measure mobility: (1) *vertical exploration or rearing;* measured as the number of times the rat reared onto hind legs and (2) *locomotion*; measured as the number of times at least the upper half of the body of the treated rat advanced from one floor square to the next. All behavior testing was performed between 8:00 a.m. and 1:00 p.m. All sessions were recorded via a camera mounted above the testing arena and scored later.

To familiarize the rats with the testing conditions, both the experimental and the partner rats were *habituated* to the behavior room and SI apparatus at least 24 hours, but no more than 48 hours, before the SI test. Habituation involved placing the animals in the behavior room under low light conditions (40 watt red light) for at least a half an hour before placing them into the SI arena. Only one rat was habituated to the SI apparatus at a time. The rat was released into a corner of the SI apparatus and allowed to explore for 5 minutes and 30 seconds.

The SI protocol involves simultaneously releasing the treated experimental rat and a novel untreated partner into the SI arena from opposing corners. All partners were of the same sex and similar weight, had been housed under identical conditions, and had no previous contact with the treated animal. The experimental rat was placed in the testing room immediately after the i.c. microinjection and allowed to acclimate to the testing room for 30 minutes before

the SI test. All partner rats were placed in the room at least 30 minutes prior to testing. To ensure that a full five minutes of the test were captured on tape, the SI test was recorded for five minutes and 30 seconds with only the first five minutes of the session scored from the videotape. The SI testing arena was thoroughly cleaned after each individual test session. SI tests were separated by 48 hours.

2.1.7 Exclusion criteria data analysis

Animal data were excluded from analysis for a number of reasons. These reasons included loss of guide cannulae cap, blockage of a guide cannula preventing microinjection, infection, illness, loss of data point due to incomplete recording of SI session (tape stopped), expiration, outlier, guide cannulae placement outside of amygdala or only unilateral, unable to verify cannulae placement, brain deformity or low N. A minimum of N=3 per treatment group was required for analysis.

2.2 Experimental Protocols

2.2.1 Experiment 1 Protocol: Determine if the persistent anxiety-like phenotype of rats observed following Ucn1-priming is the result of adverse conditioning to the SI arena (context) and/or the presence of a novel partner

2.2.1.1 *Hypothesis 1*

If the persistent anxiety-like behavior observed after Ucn1-priming is a result of aversive conditioning to the SI arena and/or the novel partner of the SI test then priming without exposure to the SI arena and/or a novel partner

following the Ucn1 priming injection will not lead to the development of the persistent anxiety-like phenotype.

Under standard Ucn1-priming protocol, the animals underwent SI testing at baseline (D0) and priming days 1 (D1), 3 (D3), and 5 [(D5), see **Fig. 3a**] with post-priming SI tests anywhere from one to five weeks later carried out without any further treatment (Shekhar et al., 2003; Rainnie et al., 2004; Truitt et al., 2007). Previous studies show that CRF receptor activation can facilitate aversive conditioning (Heinrichs and Joppa, 2001; Sherrin et al., 2008; Sherrin et al., 2009) and that conditioning can occur to a testing apparatus following BLA pharmacological manipulation (Helmstetter and Bellgowan, 1994; Thielen and Shekhar, 2002; Sajdyk et al., 2006). Considering that confirmation of a persistent anxiety-like phenotype occurs under the same conditions as were used during the priming injections, it is possible that the persistent anxiety-like phenotype that develops following Ucn1-priming is a result of aversive conditioning to the SI testing procedure (introduction of a novel partner) or the arena itself. To determine the extent to which conditioning during BLA priming affected the development of the persistent anxiety-like behavior observed in Ucn1-primed rats, rats were fitted with bilateral cannulae guides targeting the BLA. On D1, D3, and D5 of priming, rats were exposed to one of three priming conditions; 1) home cage (HC) condition, where the rat was immediately returned to his home cage following the priming injection, 2) no partner (NP) condition, where the rat was placed alone in the SI testing arena (without a partner) 30 minutes following the priming injection and 3) partner (P) condition; where the rat was placed into the

SI testing arena with a novel partner 30 minutes after the priming injection. On D7 (48 hours after priming was completed) all rats were re-exposed (re-habituated) for a five minute period to the SI chamber they were habituated to then baselined in previously. Seventy two hours after priming was completed (D8) all primed rats were placed in the familiar SI test arena with a novel untreated partner rat for a five minute SI test to determine if a persistent anxiety-like phenotype developed (**Fig. 3b**). In addition to testing the different conditioning paradigms, the affects of Ucn1- versus veh-priming was also assessed. There was an N=6 per testing group and the six testing groups were; 1) Veh.HC, veh-primed, home cage condition, 2) Veh.NP, veh-primed, no partner condition, 3) Veh.P, veh-primed, partner condition, 4) Ucn.HC, Ucn1-primed, home cage condition, 5) Ucn.NP, Ucn1-primed, no partner condition and 6) Ucn.P, Ucn1-primed, partner condition.

Vertical exploration and *locomotion* were also quantified from the SI session to measure mobility. Rats were sacrificed 48 hours after the last SI test [(D10); five days after priming ceased] following light anesthesia with isoflurane. Cannulae guide placement was verified and brain tissue was micropunched for RNA analysis.

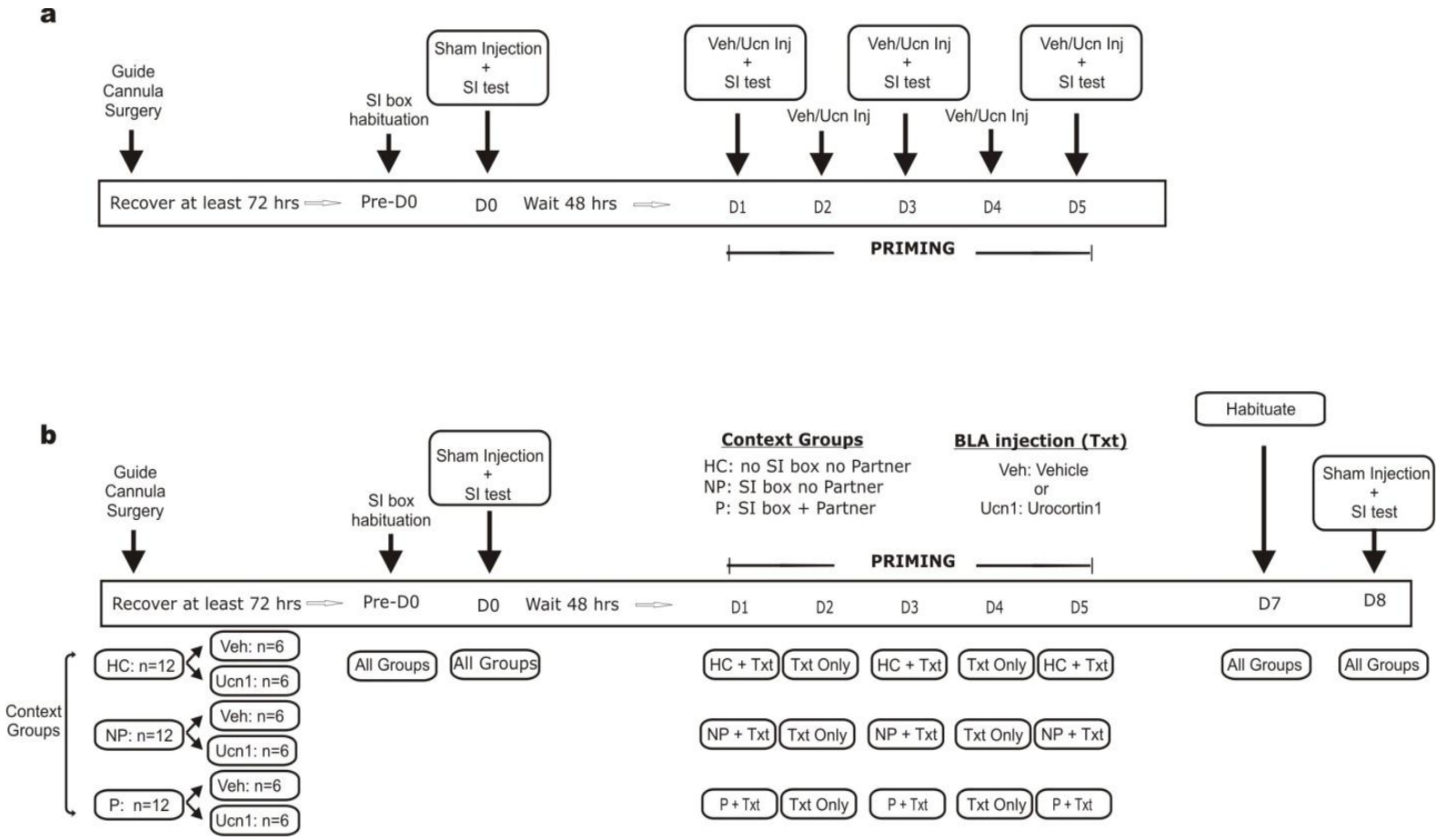


Figure 3. Timelines for (a) a standard priming experiment versus (b) Experiment 1 design

Figure 3. Timelines for (a) a standard priming experiment versus (b) Experiment 1 design; an experiment designed to test the influence of the SI arena (context) and/or partner on the development of a persistent anxiety-like phenotype. a. & b. Male Wistar rats were fitted with bilateral guide cannulae targeting the BLA then were habituated to the SI arena after recovering from surgery. 48 hours following a baseline SI test, rats were microinjected once a day for 5 consecutive days with either vehicle (Veh) or Urocortin 1 (Ucn). **a**. Under standard priming conditions rats are placed in SI 30 minutes after microinjection on D1, D3, & D5 of priming. **b.** To test the influence of context (SI arena) or SI procedure (partner) during priming on the post-priming SI, rats were prepared for priming as described for standard priming, however, on D1, D3, & D5 rats were exposed to one of three different priming contexts 30 minutes after a Veh or Ucn1 priming injection; 1) home cage (HC) condition, where the rat was immediately returned to his home cage following the priming injection, 2) no partner (NP) condition, where the rat in placed in the SI testing arena without a partner present 30 minutes following the priming injection and 3) partner (P) condition (standard priming) where the rat was placed in the SI arena with a partner 30 minutes following a priming injection.

2.2.1.2 *Data analysis*

Only data from rats with bilateral cannulation of the BLA were included in the analysis (Paxinos and Watson, 1998; see **Fig. 4**). Using SPSS analysis software, social interaction, locomotion, and vertical exploration were analyzed using the two way analysis of variance (ANOVA) with repeated measure where context and drug treatment were the independent variables and time [SI (secs)], square advances, and rearing frequency were dependant variable(s), and day of testing was the repeated measure. Significance was set at $p<0.05$. When appropriate, post hoc tests were performed with a Dunnett's used for comparison of SI times back to baseline within group and a Fisher's Least Square Difference (LSD) compared SI times between groups. The Bonferroni pairwise (Graphpad PRISM) test was used to compare replicate means back to baseline for the SI tests of the different priming conditions.

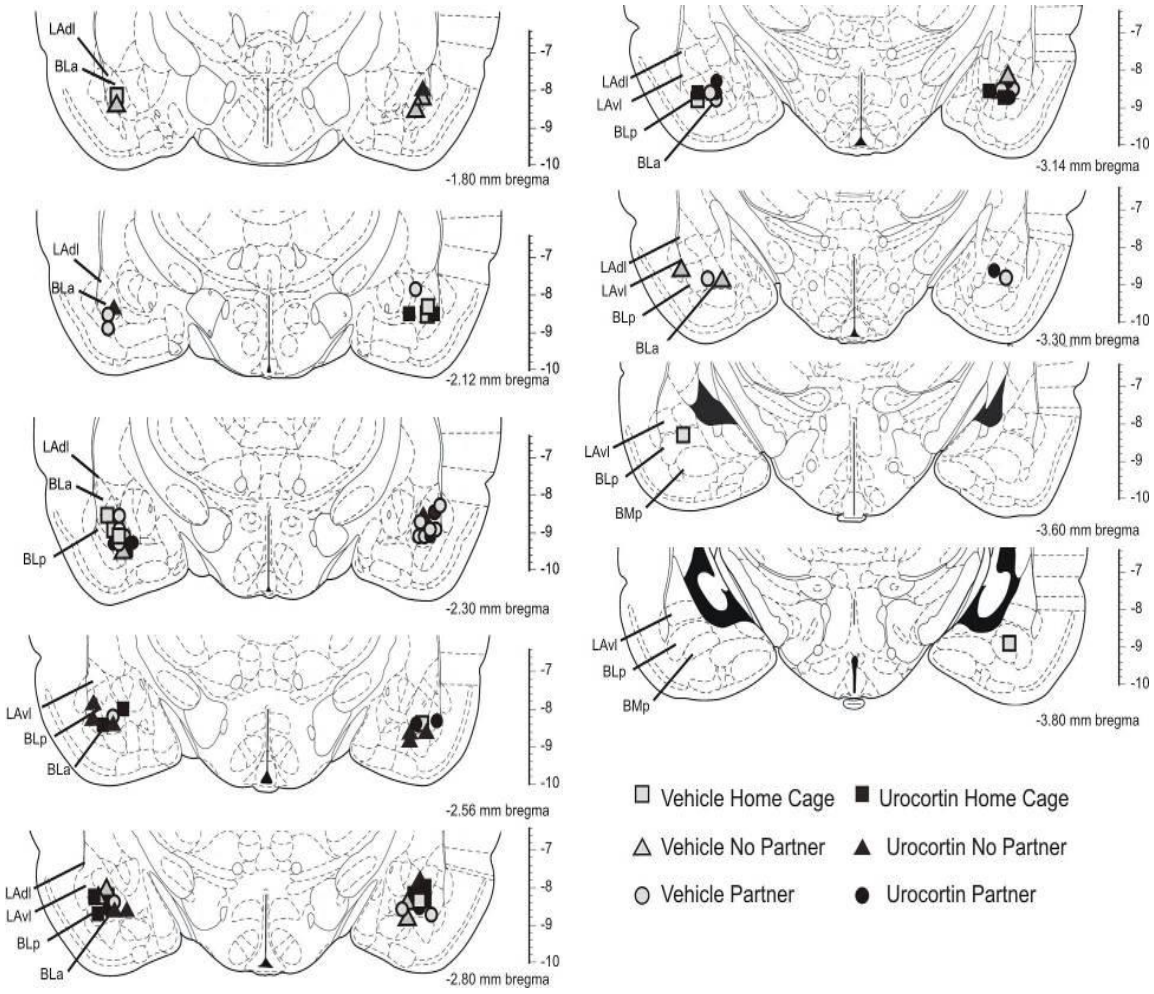


Figure 4. Schematic representation of the bilateral injection sites of rats used to determine the effect of the the SI arena and/or the partner rat on persistent anxiety-like behavior. Section templates represent amygdala sections from the atlas of Paxinos and Watson (1998). Numbers on the lower right indicate distance (mm) posterior from bregma. The scale on the right represents distance (mm) ventral to bregma. Symbols represent: open square, veh-primed home cage condition; open triangle, veh-primed, no partner condition; open circle, veh-primed, partner condition; closed square, Ucn1-primed, home cage condition; Ucn1-primed, no partner condition; Ucn1-primed, partner condition. Some sites may be obscured due to overlapping symbols. Abbreviations: LAdl - dorsal lateral amygdala; LAv l- ventral lateral amygdala; BLp - posterior basolateral amygdala; BLa - anterior basolateral amygdala.

2.2.2 Experiment 2 Protocol: Determine if Ucn1-priming induces changes in gene expression in neurotransmitter/neuropeptide receptors within the BLA

2.2.2.1 *Hypothesis 2*

If Ucn1-priming induces changes in gene expression in neurotransmitter/neuropeptide receptors in the BLA then changes in transcription of neurotransmitter/neuropeptide receptors will occur in the BLA of the rats primed with Ucn1.

Regulation of gene expression is one mechanism that can lead to relatively stable changes within neurons (Nestler et al., 1993). Therefore changes in gene expression for receptors within the BLA previously associated with anxiety were further investigated to determine what neurotransmitter/ neuropeptide receptor systems are likely involved in the expression of the persistent anxiety-like behavior observed post Ucn1-priming. The mRNA expression from the BLA of veh- and Ucn1-primed animals was analyzed with relative quantitative real-time polymerase chain reaction (qRT-PCR) using a predesigned panel of 84 neuroscience genes referred to as "Neurotransmitter Receptors and Regulators RT²*Profiler*™ PCR Array" (**Table 2**, SABioscience). Tissue samples were collected five days after priming ceased (D10) from a subset of rats from Experiment 1[vehicle (N=6), Ucn1-primed (N=6), partner rats as untreated controls (N=6)]. The rats were sacrificed by decapitation, the brains promptly removed, quickly frozen then stored at -80 ˚C until they were sliced with a cryostat and micro-punched to obtain tissue samples for qRT-PCR. The total

Table 2. Gene panel for the Neurotransmitter Receptors and Regulators RT²*Profiler*™ PCR Array

Class	Receptor / rec. subunit genes	Biosynthesis, catabolism, anchoring, transport genes
Inhibitory amino acids	*GABA*: Gabra1, Gabra2, Gabra3, Gabra4, Gabra5, Gabra6, Gabrb2, Gabrb3, Gabrd, Gabre, Gabrg1, Gabrg2, Gabrp, Gabrq, Gabrr1, Gabrr2, *Glycine*: Glra1, Glra2, Glra3, Glrb	Gad1, Gad2, Abat
Catecholamines	*Dopamine*: Drd1a, Drd2, Drd3, Drd4, Drd5 *Serotonin*: Htr3a	Comt, Maoa, Th
Cholinergic	*Nicotinic*: Chrna1, Chrna2, Chrna3, Chrna4, Chrna5, Chrna6, Chrnb1, Chrnb2, Chrnb3, Chrnb4, Chrnd, Chrne, Chrng *Musarinic*: Chrm1, Chrm2, Chrm3, Chrm4, Chrm5	Chat, Prima1, Anxa9
Neuropeptides	*CCK*: Ccka, Cckb *Galanin*: Galr1, Galr2, Galr3 *neuropeptide FF*: Npffr1, Npffr2 *NPY*: Npy1r, Npy2r, Npy5r, Ppyr1, Gpr83, Prokr1, Prokr2 *Somatostatin*: Sstr1, Sstr2, Sstr3, Sstr4, Sstr5 *Tachykinin*: Tacr1, Tacr2, Tacr3 *Other* *(26RFa*, Adrenocorticotropin, Bombesin, Neuromedin U, prolactin releasing hormone): Gpr103 (Qrfpr), Mc2r, Brs3, Grpr Nmur1, Nmur2	

RNA was extracted from these tissue samples and converted to cDNA. The cDNA conversion was confirmed prior to relative qRT-PCR analysis.

2.2.2.2 *Micropunch to collect BLA tissue for RNA analysis*

To obtain BLA tissue sections for RNA analysis, frozen tissue samples were punched from 300 micron sections containing the BLA using a punch (Vibratome) with a diameter of 0.96 mm. Two samples per side were collected using the coordinates from Paxinos and Watson (1986) -2.0 and -2.4 from bregma. All four samples were placed in the same sterilized microtube (one 1.5 microtube per animal) containing 75 µl RNA*later* (a RNA stabilization reagent from RNeasy Micro Kits, QIAGEN, Valencia, CA). The microtube was spun briefly with a mini centrifuge to ensure the collected tissue went into the RNA*later,* promptly placed on dry ice, then stored at -80 ˚C till isolation/ extraction.

2.2.2.3 *RNA isolation*

Total RNA was isolated from the collected BLA tissue samples with RNeasy Micro Kits (QIAGEN, Valencia, CA) according to the manufacturer's protocol with slight modification to enhance RNA elution. Since the abundance of tissue in each sample was relatively low, 20 ng of a carrier RNA was added to the lysate before homogenization to increase extraction efficiency. The Buffer RPE (washing buffer for membrane bound RNA to remove contaminants) was allowed to incubate for at least 2 minutes before centrifugation (modification to step 11 of the RNeasy Micro Handbook 04/2003) and, in the final RNA elution step, the supplied RNase-free water was warmed to 50 ˚C in a water bath then

allowed it to sit on the silica-gel membrane for at least 2 minutes. Total RNA was assessed using the ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE) by determining the light absorbance at ultraviolet light wavelength 260 nm (indicates nucleic acid concentration) and monitoring the 260/280 and 260/230 ratio(s) [indicators of sample purity, (NanoDrop Technologies Inc., 2007)].

2.2.2.4 *Reverse transcription (Convert RNA to cDNA)*

To convert RNA to cDNA for mRNA quantification, 100 ng of total RNA from each sample in a 20 µl reaction was reverse transcribed using Reaction Ready™ First Strand cDNA Synthesis Kit (SuperArray Biosciences, Frederick, MD) according to manufacturer's protocol. The reverse transcription conditions were 37 ˚C for 60 minutes then 95 ˚C for 5 minutes. The cDNA was diluted to a final volume of 1 ng/µl with RNase-free water and stored at -20 ˚C. One µl of cDNA was removed so that the reverse transcription could be confirmed by amplification of the endogenous control gene beta-Actin.

2.2.2.5 *Confirmation of cDNA*

cDNA conversion was confirmed with the GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA). Beta-Actin (an endogenous control gene) was amplified in each test sample using the beta-Actin forward and reverse primer shown in **Table 3**. The 25 µl PCR reaction contained 1.5 mM $MgCl_2$, 0.3 µM of each primer, and 0.2 mM of each of the four deoxynucleotide triphosphates. PCR cycling conditions were 95 ˚C for 10 minutes then 40 cycles of 95 ˚C for 30 seconds, 64 ˚C for 30 seconds, and 72 ˚C for 1 minute, followed by 72 ˚C for 5 minute using a GeneAmp® PCR System 2700 (Applied

Biosystems). The PCR product was run out on a 2% agarose gel in a 1XTAE buffer. A DNA fragment a little over 200 base pairs (bp) was expected.

Table 3. Oligonucleotide primers used for cDNA confirmation

Name	Primer	Sequence	Product length
Beta-Actin	Forward	5'-GAAGATCAAGATCATTGCTCCTCC-3'	approx. 200 bp
	Reverse	5'-TTTTCTGCGCAAGTTAGGTTTTGTC-3'	

2.2.2.6 *Relative quantitative real-time polymerase chain reaction (qRT-PCR)*

A master mix containing RT^2 Real-Time^{TM} SYBR Green/ROX PCR Master Mix (SuperArray Bioscience,now SABioscience, Frederick, MD), and the equivalent of 1 ng/µl of mRNA per well was prepared for each 96-well plate of the Neurotransmitter Receptors and Regulators RT²*Profiler*™ PCR Array (SuperArray Bioscience,now SABioscience, Frederick, MD). An aliquot of 25 µl of this master mix was added to each well. The cycling conditions were 50 ˚C for 2 minutes, 95 ˚C for 10 minutes then 40 cycles of 95 ˚C for 15 seconds, 65 ˚C for 15 seconds, and 72 ˚C for 1 minute in an Eppendorf Mastercycler® ep *realplex* instrument (Eppendorf; Westbury, NY).

2.2.2.7 *Data normalization and analysis for relative qRT-PCR*

SuperArray Bioscience, now SABioscience, provided pre-programmed EXCEL spreadsheets to analyze the data for this experiment. The analysis program utilized the relative quantification based on the comparative C_t (threshold cycle) method as described by Livak et al. (2001). The fold change in

gene expression is defined by $2^{-\Delta\Delta Ct}$ and the method for getting the $2^{-\Delta\Delta Ct}$ is described below.

Five “house-keeping” or reference genes [ribosomal protein, large, P1 (Rplp1), ribosomal protein L13A (Rpl13a), beta-Actin (Actb), hypoxanthine guanine phosphoribosyl transferase (Hprt), and lactate dehydrogenase A (Ldha)] were included as control genes on the assay plate to use for normalization. To normalize the data, the average threshold cycle (C_t) of the reference genes that were unchanged by the treatments used in the experiment was subtracted from the average C_t for each sample on that plate to obtain the ΔC_t for each gene. All sample groups; the Ucn1-primed, vehicle-primed, and untreated controls, were normalized to their respective reference controls. $\Delta\Delta C_t$ was defined as, $\Delta\Delta C_T = \Delta C_t$ (treated) - ΔC_t (control). Quantification of the PCR Array was based on the C_t number. Any $C_t \geq 35$ was considered background and automatically changed to null for the analysis.

A list of differentially expressed genes was identified using a 2-tailed t-test with the criteria of a p value less than 0.05. A mean difference equal to or greater than an absolute 1.5 fold change from the control group was a priori threshold to classify a gene as a gene of interest. The statistical calculation was based on ΔC_t. The fold change of mRNA expression for those genes with significant reduction comparing Ucn1- to veh-primed were converted to $\log_{10}$ for graphical presentation.

2.2.2.8 *Selecting gene candidates for further study*

Selection of gene candidates for further study was based on a review of the literature, the availability of tools necessary for further study, the number of genes affected per system and the confidence intervals (highest to lowest for likely candidates per literature review and available tools). To begin, the role of individual genes candidates in the expression of anxiety-like behavior was explored with a receptor specific antagonist based on the hypothesis that if decreased mRNA expression of the receptor following Ucn1 priming is causally linked to the expression of persistent anxiety-like behavior then blocking the receptor function should acutely increase anxiety-like behavior.

In this study, results from the Neurotransmitter Receptors and Regulators RT²*Profiler*™ PCR Array system showed a unique profile of changes with only five mRNAs; somatostatin receptor subtype 2 (Sstr2), somatostatin receptor subtype 4 (Sstr4), cholinergic nicotinic receptor alpha4 (Chrna4), cholinergic muscarinic receptor 4 (Chrm4), and GABA receptor rho1 (Gabrr1). Of the five, Sstr2 and Chrna4 mRNA showed the most significant decrease and were the best-fit targets based on the a priori criteria.

2.2.3 Experiment 3a Protocol: Determine the extent Sstr2 receptors regulate anxiety-like behavior

2.2.3.1 *Hypothesis 3*

If decreased Sstr2 mRNA expression in the BLA observed with Ucn1-priming is causally linked to the expression of persistent anxiety-like behavior, then antagonizing this receptor should increase anxiety-like behavior.

Sstr2 has previously been linked to anxiety-like behavior as Sstr2 knock-out mice display anxiety-like behavior in the elevated plus-maze [(EPM), Viollet et al., 2000]. To determine if blocking the Sstr2 function in the BLA increases anxiety-like behavior, 28 male Wistar rats underwent chronic bilateral cannulae guide placement surgery targeting the BLA. After recovery from surgery, the rats were habituated to the social interaction arena. SI time was accessed following intraBLA injections of one of six doses of the selective Sstr2 antagonist, CYN-154806 [(Feniuk et al., 2000), 0.1, 1, 10, 30, 90 and 180 pmoles]. Each rat received up to 3 doses and testing sessions. To avoid any type of priming effect, rats were tested in SI following BLA injections of vehicle and only 1 or 2 doses of CYN-154806 into the BLA (see **Table 4**). Rats were tested in SI 30 minutes after microinjection of the compound and received only one microinjection per testing day. SI testing sessions were conducted 48 hours apart. Upon completion of the experiment, rats were sacrificed by decapitation. The brains were quickly removed, quick frozen then stored at -80 ˚C until cannulae guide placements were verified.

Table 4. Microinjection sequence for Sstr2 antagonist CYN-254806

1st dose	2nd dose	3rd dose
CYN 10 pmole	Veh	Veh
CYN 10 pmole	Veh	CYN 1 pmole
CYN 30 pmole	Veh	CYN 1 pmole
CYN 30 pmole	Veh	CYN 180 pmole
CYN 90 pmole	Veh	Veh
CYN 90 pmole	Veh	CYN 1 pmole
Veh	CYN 10 pmole	CYN 180 pmole
Veh	CYN 10 pmole	Veh
Veh	CYN 30 pmole	Veh
Veh	CYN 30 pmole	CYN 180 pmole
Veh	CYN 90 pmole	CYN 180 pmole
Veh	CYN 90 pmole	CYN 1 pmole
Veh	CYN 100 fmole	N/A
Veh	CYN 100 fmole	N/A
Veh	CYN 100 fmole	N/A
CYN 100 fmole	Veh	N/A
CYN 100 fmole	Veh	N/A
CYN 100 fmole	Veh	N/A
Veh	CYN 1 pmole	CYN 180 pmole
Veh	CYN 1 pmole	CYN 180 pmole
CYN 1 pmole	Veh	CYN 180 pmole
CYN 1 pmole	Veh	CYN 180 pmole

2.2.3.2 *Data analysis*

In order to compare all rats back to their relevant vehicle injection, SI data were converted to percent of vehicle SI time. One way ANOVA was used to analyze the data and a $p<0.05$ was considered significant. When appropriate, post-hoc tests were performed with a Dunnett's for comparison of converted SI times back to baseline within groups and a Tukey's test for comparison of converted SI times between groups.

2.2.4 Experiment 3b Protocol: Determine the role of the BLA cholinergic nicotinic receptor α4 subunit (Chrna4) function in the expression of anxiety-like behaviors

2.2.4.1 *Hypothesis 4*

If decreased Chrna4 receptor subunit mRNA expression in the BLA observed with Ucn1-priming is causally linked to the expression of persistent anxiety-like behavior, then antagonizing this receptor should acutely increase anxiety-like behavior.

Ross and colleagues (2000) found that mice with the Chrna4 subunit knocked-out displayed anxiety-like behavior in the EPM. To determine the extent Chrna4 in the BLA regulate anxiety, a total of six rats underwent chronic bilateral guide cannulation of the BLA for this experiment. Following recovery from surgery the rats were habituated to the SI apparatus and baseline SI scores were obtained 24 hours later.

The nicotinic acetylcholine receptor (nAchR) antagonists' doses were selected after a review of the literature (Dhar et al., 2000; Addy et al., 2003; Jonkman and Markou, 2006). Nicotinic receptors are composed of a combination of two or more α and β subunits. The available selective nicotinic antagonists block either specific combinations of α and β subunits or α7. The α4β2 receptor subtype is the most predominant nicotinic acetylcholine receptor (nAchR) in the brain (Whiting et al., 1991; Flores et al., 1992). Dihydro-β-erythrodinie (DHβE), the cholinergic nicotinic receptor α4β2 subunit antagonist, was used to determine the extent to which anxiety-like behaviors could be induced by antagonizing the

α4β2nAchR receptor in the BLA. However, DHβE has some affinity for the α3β4 subunit as well (Xiao et al., 1998). To discern specificity of action at α4β2 compared to the α3β4 subunit, the anxiety-like effects of BLA injections of mecamylamine, the predominant cholinergic nicotinic receptor α3β4 subunit antagonist (Xiao et al., 1998; Dhar et al., 2000), was also included.

DHβE (3 or 15 μg/100 nl/side), mecamylamine (1mM/100 nl/side) or vehicle (100 nl/side) was microinjected 15 minutes prior to SI testing. SI testing was repeated a minimum of 48 hours later with the injection of a different drug/compound in a counterbalanced design to prevent a day effect. After the initial counterbalanced microinjections of vehicle, DHβE (3 μg), and mecamylamine a second vehicle injection was randomized with DHβE (15 μg) to verify that the prior treatments were not effecting a change in the neuronal activity (see **Table 5**). Upon completion of the experiment, rats were sacrificed by decapitation. The brains were quickly removed, quick frozen then stored at -80 ˚C until cannulae guide placements were verified.

2.2.4.2 *Data analysis*

Only bilateral BLA cannulated rats were included in the data analysis. Data were analyzed with a one way ANOVA. SI data are reported as mean ± SEM. A $p<0.05$ was considered significant. When appropriate, post hoc tests were performed.

Table 5. Nicotinic acetylcholine receptor antagonists microinjection sequence

Animal ID	Txt1	Txt2	Txt3	Txt4	Final Txt
ACH01	Veh	DHbE 3	Mecamyl	Veh	DHbE15
ACH02	Veh	Mecamyl	DHbE 3	DHbE15	Veh
ACH03	DHbE 3	Veh	Mecamyl	Veh	DHbE15
ACH04	DHbE 3	Mecamyl	Veh	DHbE15	Veh
ACH05	Mecamyl	Veh	DHbE 3	Veh	DHbE15
ACH06	Mecamyl	DHbE 3	Veh	DHbE15	Veh

A within subject counterbalanced design was used for the first 3 injections. Injections were given every 2-3 days. Abbreviations: vehicle (Veh), dihydro-β-erythrodinie (DHbE; 3 or 15 µg), mecamylamine (Mecamyl).

2.2.5 Experiment 4a Protocol: Determine the effect Sstr2 activation in the BLA has on basal SI time

2.2.5.1 Hypothesis 5

If antagonizing Sstr2 in the BLA induces anxiety-like behavior then activating BLA-Sstr2 will induce anxiolytic-like behavior.

To determine the effect Sstr2 activation in the BLA had on basal SI time, 29 male Wistar rats underwent chronic bilateral cannulae placement surgery targeting the BLA. Fourty-eight hours after baseline SI time was accessed, one of six doses of the Sstr2 agonist, BIM-23027; was microinjected into the BLA bilaterally followed by the SI test 30 minutes later. The doses of BIM-23027 tested were 1 or 100 fmole, 1, 10, 30, or 90 pmoles/100 nl/side. Upon completion of the experiment, all rats were sacrificed by decapitation the brains quickly removed, quick frozen then stored at -80 ˚C until cannulae guide placements were verified.

2.2.5.2 *Data Analysis*

In order to compare all rats back to their relevant baseline, SI data were converted to percent of baseline SI time. Data were analyzed using one way ANOVA. Significance was set at $p<0.05$. Only animals with bilateral cannulation were included in the data analysis. The Grubb's test was utilized to identify outliers.

2.2.6 Experiment 4b Protocol: Determine if the microinfusion of a Sstr2 agonist (BIM-23027) can override an anxiogenic-like dose of Ucn1

2.2.6.1 Hypothesis 6

If Sstr2 receptors in the BLA have a role in counteracting anxiogenic stimuli then microinfusing a Sstr2 receptor agonist into the BLA prior to infusion of a threshold (anxiogenic-like) dose of Ucn1 into the BLA will block the expression of the Ucn1-induced anxiety-like behavior.

It is possible that the role of BLA Sstr2 is to counteract anxiogenic stimuli in the BLA. Previous studies have shown that a threshold dose of Ucn1, 100 fmoles (in 100nl/ side) microinjected bilaterally into the BLA will induce an anxiety-like response in the SI test (Sajdyk et al., 1999; Spiga et al., 2006). To determine the anti-anxiety-like effect of Sstr2 activation, 10 male Wistar rats underwent bilateral cannulation of the BLA. Forty-eight hours following a baseline SI, either vehicle or one of two different doses of BIM-23027 (30 or 90 pmoles/100 nl/side; BIM30preUcn and BIM90preUcn respectively), was microinjected into the BLA 30 minutes prior to a threshold dose of Ucn1. The control was Ucn1 (100 fmoles) pretreated with vehicle 30 minutes prior to the

Ucn1 injections (Veh.Ucn). SI was accessed 30 minutes following the 2nd injection. Upon completion of the experiment, rats were sacrificed by decapitation. The brains were quickly removed, quick frozen then stored at -80 ˚C until cannulae guide placements were verified.

2.2.6.2 *Data analysis*

The SI data were analyzed with a one-way ANOVA. When appropriate, a Dunnett's post-hoc test was used for comparisons of test day SI times back to baseline within group. Significance was established at $p<0.05$.

2.2.7 Experiment 5a Protocol: Determine if microinfusion of Sstr2 agonist, BIM-23027, into the BLA 30 minutes prior to a Ucn1 priming injection can prevent the development of a persistent anxiety-like phenotype

2.2.7.1 *Hypothesis 7*

If Sstr2 receptor activation in the BLA can prevent an Ucn1-induced anxiety-like response acutely then pretreatment with a Sstr2 agonist (BIM-23027) into the BLA during Ucn1-priming can prevent the development of a persistent anxiety-like phenotype.

To determine if activating Sstr2 receptors in the BLA 30 minutes prior to Ucn1 priming injections could prevent the development of the persistent anxiety-like phenotype, 20 male Wistar rats underwent chronic bilateral cannulae guide placement surgery targeting the BLA. BIM-23027 (90 pmoles; BIM.Ucn; N=10) or Veh (Veh.Ucn; N=10) was microinjected into the BLA 30 minutes prior to the sub-threshold dose of Ucn1 (6 fmole) on D1 through D5 of priming. SI was assessed at baseline (D0), D1, D3, and D5 30 minutes after the 2nd injection, and 72 hours

after priming ceased [(D8), see **Fig. 5**]. Veh.Ucn was the control for the BIM.Ucn data set. Rats were sacrificed five days after priming ceased (D10) by decapitation following light anesthesia with isoflurane. The brains were quickly removed, flash frozen, then kept at -80 ˚C until sliced with a cryostat to verify placement and to collect tissue samples from the BLA for RNA analysis.

2.2.7.2 *Data analysis*

The post-priming SI was compared to baseline with a repeated measures ANOVA followed by a Dunnett's test post-hoc for within group comparisons of SI times back to baseline, when appropriate. Data representing the effect of BIM-23027 pretreatment during priming on D1, D3, and D5 were analyzed with a two way repeated measures ANOVA. The independent variable was the unique drug pretreatment and treatment combinations, the dependant variable was the SI time, and the SI testing day was the repeated measures. The Fisher's Least Significant Difference (LSD) comparing SI times between groups was used post-hoc when appropriate. Significance was set at $p<0.05$. Only data from bilateral cannulation was included in the analysis.

2.2.8 Experiment 5b Protocol: Determine the effect of repeated Sstr2 activation with the Sstr2 agonist, BIM-23027, on basal levels of social interaction

2.2.8.1 *Hypothesis 8*

If the role of Sstr2 receptors in anxiety is to counteract anxiogenic stimuli then repeated activation of the Sstr2 receptor under basal conditions will not alter basal SI time.

To determine if repeated microinjections with BIM-23027 would induce a change in basal SI scores, 19 male Wistar rats underwent chronic bilateral cannulae guide placement surgery targeting the BLA. BIM-23027 (90 pmoles) or veh was microinjected into the BLA 30 minutes prior to a 2nd injection of veh [BIM.Veh (N=10) and Veh.Veh (N=9), respectively] on D1 through D5. SI was assessed at baseline (D0), D1, D3, and D5 30 minutes after the 2nd injection (veh), and three days after priming ceased [(D8), see **Fig. 5**]. Rats were sacrificed five days after priming ceased (D10) by decapitation following light anesthesia with isoflurane. The brains were quickly removed, flash frozen, then kept at -80 ˚C until sliced with a cryostat to verify placement and to collect tissue samples from the BLA for RNA analysis.

2.2.8.2 *Data analysis*

Two way repeated measures ANOVA was used to compare SI data between rats with repeated Sstr2 activation (BIM.Veh) and the vehicle control (Veh.Veh). Only data from rats with bilateral cannulation of the BLA were included in the analysis. Post hoc tests were run when appropriate.

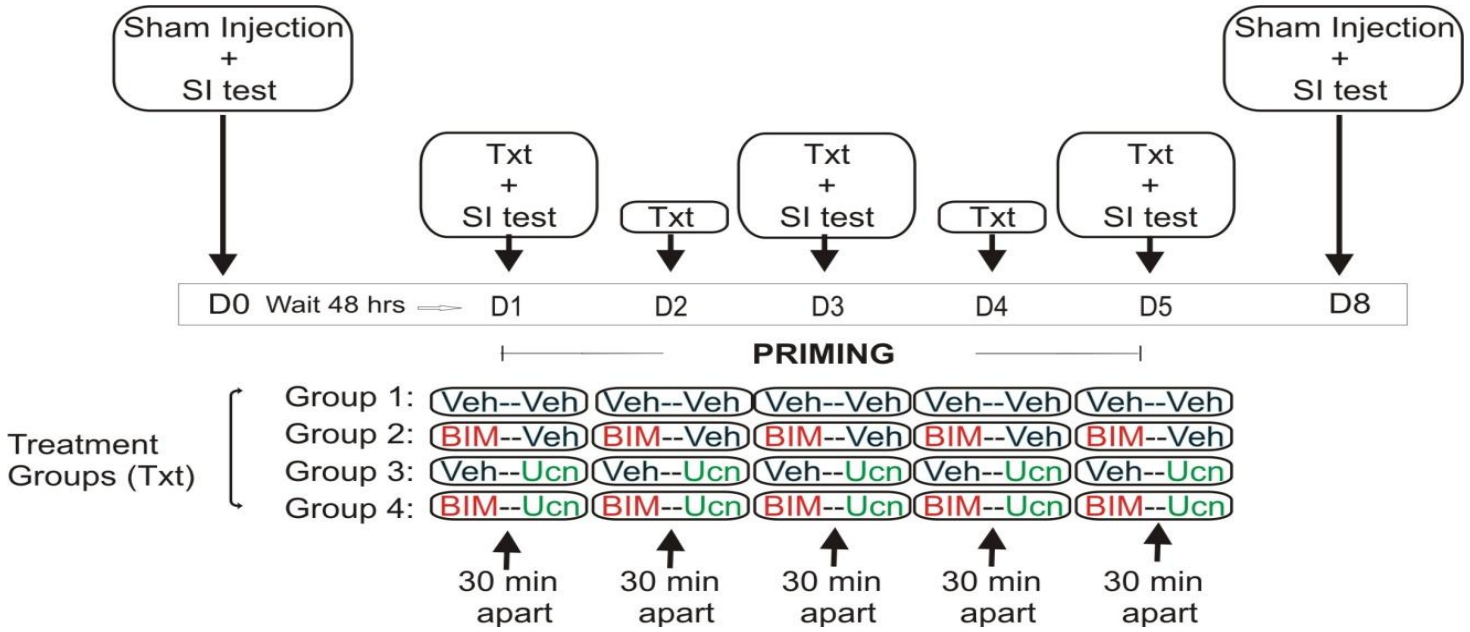


Figure 5. **Timeline for investigation of Sstr2 agonist, BIM-23027 pretreatment effect on Ucn1 priming.** Experiment 5 timeline. To test if the Sstr2 agonist BIM-23027 will block Ucn1 priming, male Wistar rats were fitted with bilateral cannulae targeting the BLA. Rats were habituated to the SI apparatus after recovering from surgery. Fourty-eight hours following a baseline SI test, rats were microinjected once a day for 5 consecutive days (D1 - D5) with either Veh or BIM (90pmole/100 nl/side) 30 minutes before either Veh (1%BSA) or Ucn (6 fmoles/100 nl/side). On injection days D1, D3, and D5 rats were placed in SI 30 minutes after the second microinjection. Rats were tested in SI again 72 hrs after priming ended (D8). Abbreviations: vehicle (Veh), urocortin1 (Ucn), BIM-23027 (BIM), treatment group injections (Txt), social interaction (SI).

2.2.9 Experiment 6a Protocol: Determine the effect BIM-23207 pretreatment in the BLA 30 minutes before the Ucn1-priming injection has on the Sstr2 mRNA expression on D3 of priming as compared to Ucn1-priming without BIM-23027 pretreatment

2.2.9.1 *Hypothesis 9*

If the expression of behavioral sensitization is associated with the level of Sstr2 mRNA in the BLA then the level of Sstr2 mRNA will be higher in the BLA on priming D3 in rats pretreated with the Sstr2 agonist, BIM-23027, in the BLA prior to each Ucn1-priming injection as compared to Ucn1-primed rats pretreated with vehicle.

To investigate, 12 male Wistar rats underwent chronic bilateral guide cannulation of the BLA. Following recovery from surgery rats received either the Sstr2 agonist, BIM-23027 (90 pmole) or a vehicle microinjection into the BLA 30 minutes prior to a Ucn1 (6 fmole) priming injection (BIM.Ucn and Veh.Ucn, respectively). SI time was ascertained at baseline, D1, and D3. The rats were sacrificed immediately after the SI test on D3, the brains quickly removed, flash frozen, then kept at -80 ˚C until they were processed for RT-PCR as described in Experiment 2, with a few exceptions. Total RNA was isolated from the tissue as described in Experiment 2 however a different cDNA synthesis kit was used to convert RNA to cDNA. Products from the same company were used to generate the cDNA as well as analyze the mRNA expression difference between the treatment groups for Experiment 2.

2.2.9.2 *Reverse transcription of RNA for animals sacrificed on day three of priming (D3)*

For this experiment, the GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA) was used to convert RNA to cDNA. For mRNA quantification 40 ng of total RNA from each sample was reverse transcribed in a 40 µl reaction The reaction components included 2.5 mM $MgCl_2$, 0.25 mM of the deoxynucleotide, 1.25 µM of oligodeoxythymidylic acid primer, 0.5 U/µl of RNase inhibitor, and 0.45 U/µl MultiScribe reverse transcriptase. The reaction conditions were 24 ˚C for 10 minutes, 42 ˚C for 60 minutes, 68 ˚C for 10 minutes, and 95 ˚C for 5 minutes. The cDNA was diluted to a final volume of 1 ng/5 µl with Nuclease-free water and stored at -20 ˚C. cDNA conversion was verified as described in Experiment 2.

2.2.9.3 *Generating a standard curve*

The copy number of Sstr2 present in the BLA tissue was determined by a standard curve generated using clones made in the laboratory. The endogenous control gene beta-Actin was used to normalize the data. The primers designed for Sstr2 and beta-Actin are listed in **Table 6**. PCR product was generated using the designed primers for Sstr2 or beta-Actin then cloned into a pCR®4-TOPO vector. qRT-PCR was used to quantify Sstr2 in the BLA based on the standard curve generated from the clones.

Table 6. Designed real-time primers used for cloning Somatostatin 2 receptor (Sstr2) or beta-Actin

Name	Primer	Sequence	Product length
beta Actin	Forward	5’-GAAGATCAAGATCATTGCTCCTCC-3’	approx. 200 bp
	Reverse	5’-TTTTCTGCGCAAGTTAGGTTTTGTC-3’	
Somatostatin 2 receptor	Forward	5'-TATCCTCACCTACGCCAACAGCT-3’	approx. 180 bp
	Reverse	5'-CTCTGGGTCTCCGTGGTCTCATT-3’	

2.2.9.4 *PCR: Amplify desired gene fragment*

To amplify the desired gene fragment for cloning the reaction components in a 50 µl reaction contained 0.8 mM deoxynucleotide triphosphates, 0.04 ng of rat brain BD ™ Marathon-Ready cDNA (BD biosciences), 0.6 µM of each primer, and 0.1U/µl of Cloned *Pfu* DNA polymerase. Cycling conditions were 95 ˚C for 4 minute and 35 cycles of 95 ˚C for 30 seconds, 65 ˚C for 30 seconds, and 72 ˚C for 1 minute on a GeneAmp® PCR System 2700 (Applied Biosystems). Ten µl of the PCR product was run out on a 2% agarose gel in 1xTAE buffer for product confirmation.

2.2.9.5 *Cloning with TOPO plasmid kit for the standard curve*

The PCR product was cloned into a pCR®4-TOPO vector according to the handbook using a TOPO® TA Cloning™ Kit for sequencing (Invitrogen). Briefly plasmids were chemically transformed into competent Mach1-T1 cells. Colonies were grown overnight on agar plates then a colony was picked to grow in 4 milliliters (mls) of LB (containing 25 mg/ml of Kanamyacin) overnight at 37 ˚C. The following morning, DNA was isolated for further screening from 3 of the 4 mls using the Quantum Prep® Plasmid Miniprep Kit (Bio-RAD). The plasmid

insert was further analyzed by restriction analysis using *Eco*RI. After confirmation, a sample was sequenced using T3 and T7 primers to make sure the sequence was accurate. After sequence confirmation, the remaining refrigerated culture (1 ml) was used to inoculate 100 mls of LB, containing 25 mg/ml of Kanamyacin, overnight. The QIAGEN Plasmid Maxi Kit (QIAGEN) was used to purify the plasmid according to the handbook.

After purification, the plasmid DNA concentration was measured using a ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). The mass of a single plasmid molecule was determined by multiplying the plasmid concentration by the total number of base pairs contained in the plasmid. The mass of plasmid DNA needed for the specific PCR product was calculated (300,000 copies for Sstr2, 3,000,000 for beta-Actin) and serial dilutions for the standard curve were prepared accordingly.

2.2.9.6 *Absolute quatitative real-time PCR (qRT-PCR)*

To quantify Sstr2 (or beta-Actin) the PCR reaction components were 3 mM $MgCl_2$, 1 mM of the deoxynucleotide from a SYBR Green Kit (Applied Biosystems) plus 0.4 µM of each primer. The cDNA equivalent to 1 ng of RNA was added for priming D3 samples. The cycling conditions were 50 ˚C for 2 minutes, 95 ˚C for 10 minute then 40 cycles of 95 ˚C for 15 seconds, 65 ˚C for 15 seconds, and 72 ˚C for 1 minute in an Eppendorf Mastercycler® ep *realplex* instrument (Eppendorf, Westbury, NY). The melting curve function was utilized to check for nonspecific amplification. The copy number present in the tissue was determined by a standard curve that was generated using clones made in the

laboratory. Briefly, the C_t value of the samples was used to calculate the log input amount based on the slope and y-intercept of the standard curve line. The copy number for each sample was ascertained by taking 10 to the calculated log input amount ($=10^{[\text{log input amount}]}$).

2.2.9.7 *Data normalization and analysis*

All cDNA samples were analyzed in triplicate for samples from priming D3 (3 samples per animal). The C_t number was used to calculate the copy number based on a standard curve (Applied Biosystems, 2001). Beta-Actin was used as the endogenous control gene to normalize the data. To determine relative values for the Sstr2, the copy numbers for Sstr2 and beta-Actin were first averaged for each animal. Next, these averaged copy numbers for Sstr2 and beta-Actin were averaged per treatment group then normalized by dividing the treatment group average for Sstr2 by the treatment group average for beta-Actin. The normalized Sstr2 was analyzed with a 2-tailed t-test and a confidence interval of $p<0.05$. Only rats with bilateral cannulae guide placement were considered for analysis.

2.2.10 Experiment 6b Protocol: Determine the effect of BIM-23207 pretreatment in the BLA during Ucn1-priming has on the Sstr2 mRNA expression level post-priming

2.2.10.1 *Hypothesis 10*

If the reduction of Sstr2 mRNA is associated with the persistent anxiety-like phenotype then BIM-23027 pretreatment during priming will not stop the Ucn1 priming-induced reduction of Sstr2 mRNA observed post-priming.

To determine the effect of BIM-23207 pretreatment during Ucn1-priming on the Sstr2 mRNA expression, the BLAs from a subset of animals used in Experiment 5 were collected five days after priming ceased (D10) and processed for qRT-PCR. These samples included rats from each of the four treatment groups: Ucn1-primed with and without BIM-23027 (90 pmole) microinjections into the BLA 30 minutes prior to each Ucn1 (6 fmole) priming injection [BIM.Ucn (N=5) and Veh.Ucn (N=4) respectively] as well as a vehicle control where the vehicle was microinjected into the BLA 30 minutes prior to 2nd vehicle injection [Veh.Veh, (N=3)] and [BIM.Veh (N=4)] where BIM-23027 (90 pmole) was microinjected 30 minutes prior to the vehicle injection. Five days following the last injection rats were sacrificed by decapitation, and BLA tissue was processed for RT-PCR as described in Experiment 2. However the RNA was converted to cDNA as described next (in section 2.2.10.2).

2.2.10.2 *Reverse transcription of RNA (to cDNA) for samples collected post-priming*

To convert the RNA to cDNA for RNA analysis, 80 ng of total RNA from each sample in a 20 µl reaction was reverse transcribed using Reaction Ready™ First Strand cDNA Synthesis Kit (SuperArray Biosciences; Frederick, MD) according to manufacturer's protocol. The cycling conditions were 42 ˚C for 15 minutes then 95 ˚C for 5 minutes. The cDNA was diluted to a final volume of 2 ng/5 µl with Nuclease-free water and stored at -20 ˚C. The conversion to cDNA was verified as described in Experiment 2.

2.2.10.3 *Absolute quatitative real-time PCR (qRT-PCR) for samples collected post-priming*

Sstr2 and beta-Actin were quantified as described in Experiment 6a however cDNA equivalent to 2 ng of RNA was added to each 25 µl reaction for D10 samples instead of the 1 ng of RNA used for D3 samples.

2.2.10.4 *Data normalization and analysis*

All cDNA samples were analyzed as described in Experiment 6a. There were replicates of four per animal for the experimental D10 samples. The Grubb's test was used to identify outliers. If the standard deviation of the C_t between each replicate per animal was greater than 0.4 then the reading was considered invalid and the animal's data were excluded from analysis. A normalized Sstr2 value per animal was analyzed with a one way ANOVA to determine if the gene was differentially expressed between pretreatment conditions. When appropriate, a Tukey's post-hoc test separated the effects of

the pretreatment conditions on mRNA expression. Significance was set at $p<0.05$.

RESULTS

3.1 Experiment 1: Priming without exposure to the SI arena and/or a novel partner did not stop the development of a persistent anxiety-like phenotype

Three different contextual conditions were utilized during priming, 1) Home cage (HC) condition; rats were immediately placed back in their home cage following priming injections so there was no exposure to the SI arena or partner rat, 2) no partner (NP) condition; rats were placed in the SI arena without a partner rat following priming injections on days 1, 3 and 5 and 3) partner (P) condition; rats were placed in the SI arena with a novel partner rat following priming injections on days 1, 3 and 5. In addition to testing the different conditioning paradigms, the affects of Ucn1- versus veh-priming was also assessed.

The conditioning paradigm did not make a significant contribution to the development of a persistent anxiety-like phenotype as all Ucn1-primed rats, regardless of the priming condition, had a significant decrease in SI as compared to their baseline three days (D8) after priming ceased. A significant main drug effect was observed, with lower SI times in Ucn1-primed rats compared to veh-primed rats [$F_{1,30}$=17.59; p<0.0001] but there was not a main context-condition effect [F(2,30)=2.817; p=0.076] or drug x context-condition interaction [F(2,30)=0.494; p=0.615; **Fig. 6**]. Post-hoc comparisons of the SI changes within priming condition (D0 vs D8) with the Bonferroni pairwise test revealed a significant decrease in SI for each of the Ucn1 primed groups but not for the veh-primed groups.[Ucn HC (t_5=3.35; p<0.05; **Fig. 6A**), Ucn NP (t_5=4.087; p<0.01;

Fig. 6B), and Ucn P (t_5=5.873; p<0.001; **Fig. 6C**); Veh HC (t_5=0.98; p>0.05; **Fig. 6A**), Veh NP (t_5=0.25; p>0.05; **Fig. 6B**), and Veh P (t_5=2.301; p>0.05; **Fig. 6C**)]. To assure that the decrease in SI observed in Ucn1-primed rats was not the result of motor impairment, locomotion (square advances) and rearing were also measured during the SI test. Ucn1-priming did not cause a significant change in locomotion ($F_{1,30}$=0.330; p=0.570; **Fig. 6D, E, & F**) or in the number of rearing episodes ($F_{1,30}$=0.218; p=0.644; data not shown). Collectively, these data suggest that the development of a persistent anxiety-like phenotype induced by Ucn1-priming is an effect of Ucn1 microinjections into the BLA and not a result of aversive conditioning to the partner rat or the SI arena.

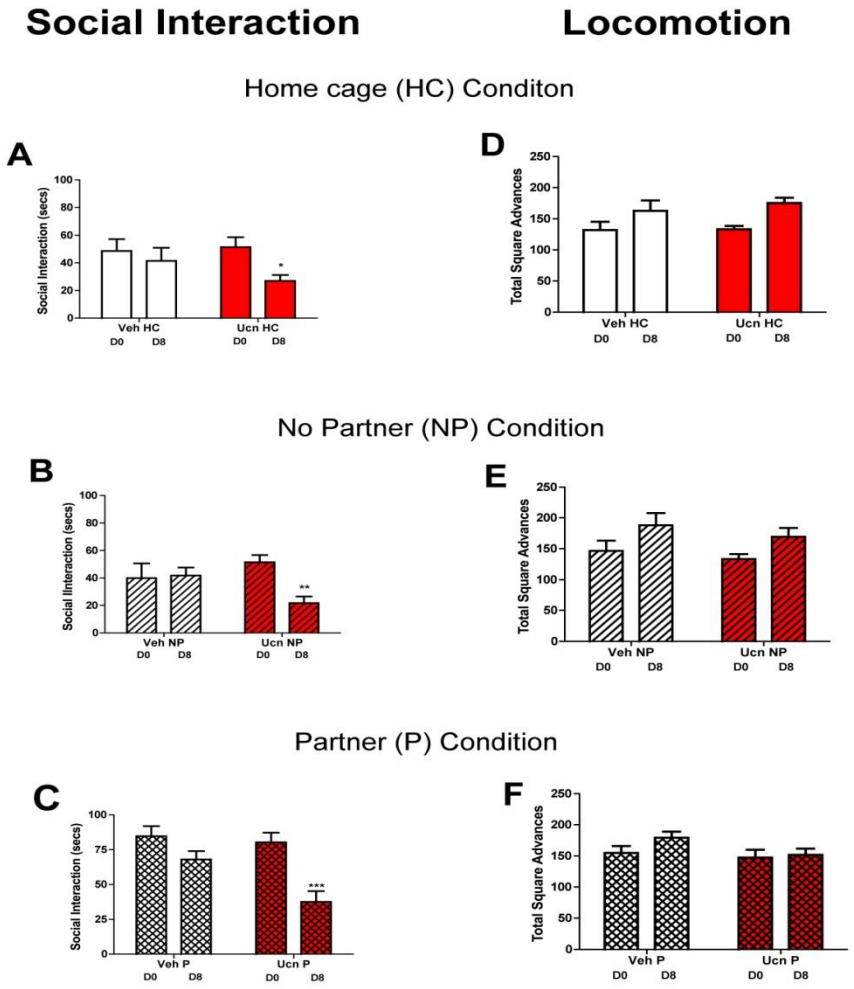


Figure 6. A persistent anxiety-like phenotype develops without exposure to the SI arena and/or a novel partner following the Ucn1 priming injection. Graphs reflect effects of priming [vehicle (Veh) or urocortin 1 (Ucn)] and contextual conditions during priming (HC, NP, P) on social interaction (A, B, C) and mobility (locomotion; D, E, F) three days after the last priming injection. Significant difference: *($p<0.05$), **($p<0.01$), ***($p<0.001$) by Bonferroni pairwise analysis. Abbreviations: (HC) no exposure to SI arena or partner during priming, (NP) SI arena without partner rat during priming, or (P) standard priming condition where rat is place in the SI arena with a novel partner rat during priming. Data are presented as mean $\pm$ SEM.

3.2 Experiment 2: Ucn1-priming produces a persistent anxiety-like phenotype and lasting reductions in neurotransmitter receptor gene expression in the BLA

Regulation of gene expression is one mechanism that can lead to relatively stable changes within neurons (Nestler et al., 1993). The mRNA expression from the BLA of veh- and Ucn1-primed animals was analyzed with qRT-PCR using a predesigned panel of 84 neuroscience genes. Tissue samples were collected five days after the last priming injection (D10) from a subset of animals from Experiment 1. Since the priming context did not cause a significant difference in SI behavior, tissues were grouped by treatment regardless of priming context (Veh N=6; Ucn1-primed N=6; untreated control N=6). Some of the collected samples could not be used because the extracted total RNA was not enough to convert to cDNA. The final sample size for this experiment was Ucn1-primed (N=5), veh-primed (N=4), untreated control (N=4). Anxiety-like behavior was assessed with the SI test prior to (baseline) and 72 hours after cessation of priming injections. SI time of rats primed with Ucn1, but not vehicle, was significantly reduced 72hr post-priming (repeated measures ANOVA test time by treatment interaction $F_{1,7}$=23.71; p=0.001; **Fig. 7A**).

Expression of each gene was normalized to the average of four endogenous control genes (Actb, Ldha, Rpbp1 and Rpl13a). The reference gene Hprt was excluded as a reference because there was a significant decrease in the mRNA for this gene between the vehicle and the untreated control samples. The data from the four untreated control animals were not considered for any further analysis for the thesis. The veh-primed cohort groups were the controls to

the Ucn1-primed group. A main effect of drug on overall gene expression was observed ($F_{1,83}$=4.42, p=0.036). Of the 84 target genes screened, difference in mRNA levels for Ucn1-compared to veh-primed rats reached significance for five genes (p<0.05, **Fig. 7B**). The five genes were somatostatin receptors 2 and 4 (Sstr2 and Sstr4; $t_{7,2}$=4.485; p=0.0028 and $t_{7,2}$=2.93; p=0.022, respectively), cholinergic nicotinic receptor alpha4 (Chrna4; $t_{7,2}$=3.536; p=0.0095), cholinergic muscarinic receptor 4 (Chrm4; $t_{7,2}$=2.439; p=0.0448) and GABA receptor rho1 (Gabrr1; $t_{7,2}$=2.940; p=0.0217). All of these genes had a minimum of 1.5 fold reduction in mRNA levels of Ucn1-induced primed compared to veh-primed. Relative quantization (RQ) of gene expression was determined using delta delta Ct method. For linear comparisons RQ values were converted to Log base 10, such that values of ± 0.3, 0.7 or 1 represents a ± 2, 5 or 10 fold change (**Fig. 7C**).

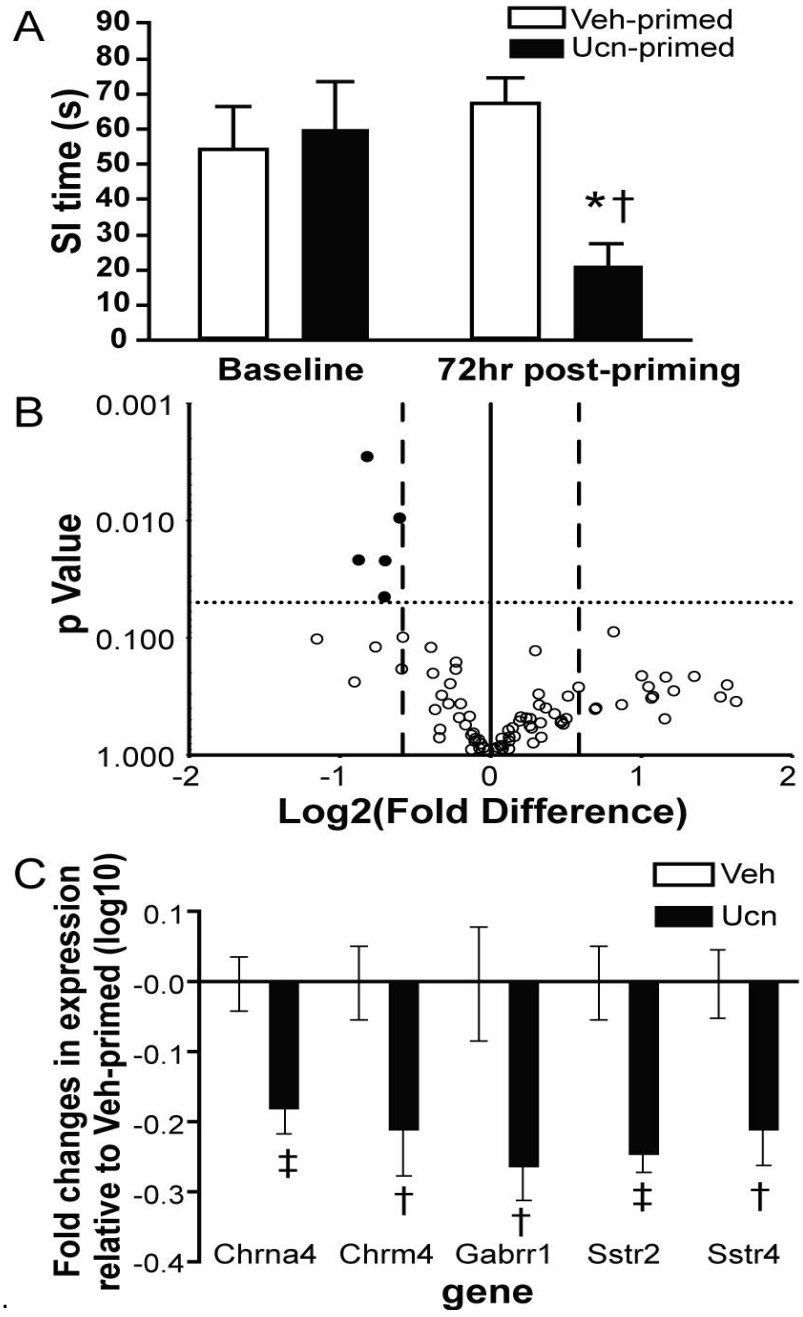


Figure 7. Ucn1-priming induces persistent anxiety-like behavior and selective reduction of gene expression in the BLA. Graphs represent **A.** Changes in SI following Ucn1- verses veh-priming compared to their respective baselines (mean ± SEM). **B.** Volcano plot of gene expression from the Neurotransmitter Receptors and Regulators RT²*Profiler*™ PCR Array. Data presented are mean BLA mRNA levels, for each of the 84 target genes, of Ucn1-primed rats relative to veh-primed rats expressed as ± fold changes (log2) plotted against level of significance (p-values). Horizontal dotted line represents the assigned cut-off for significance (p value of 0.05), thus values above the dotted

line are significantly different from vehicle values. The dashed vertical lines represent a 1.5 fold change in expression, thus values laterally to the left dashed line represent a greater than 1.5 fold reduction in gene expression while values lateral to the right dashed line represent values with a greater than 1.5 fold increase in relative expression. Closed circles are genes that reached significance and open circles are genes that were not found to have significantly different expression compared to veh-primed rats. Data presented in **C.** are mean ± SEM, for Ucn1- and veh-primed rats, relative expression of the genes found to be significantly different in B. Here data are plotted as $\log_{10}$(RQ) where RQ is the fold change in gene expression relative to vehicle primed rats determined by the delta delta Ct method; Ucn1-primed (N=5), Veh-primed (N=4). Abreviations: Sstr2, somatostatin receptor 2; Chrna4, nicotinic receptor alpha4; Gabrr1, GABA r receptor rho1; Sstr4, somatostatin receptor 4; Chrm4, muscarinc receptor 4; Ucn, urocortin 1; Veh, vehicle. Significance, * indicates significantly different from baseline $p<0.05$; † indicates significantly different from vehicle $p<0.05$ and ‡ indicates significantly different from vehicle $p<0.01$.

3.3 Experiment 3a: Blocking Sstr2 function in the BLA leads to the expression of anxiety-like behavior

To determine if blocking the Sstr2 receptors in the BLA increases anxiety-like behavior, the effects on SI behavior following intraBLA injections of six doses of the selective Sstr2 antagonist, CYN-154806 were investigated. Data from seven rats were excluded and one data point in the 90 pmole group was considered an outlier as determined by Grubbs' test (alpha=0.05). Therefore, two doses (30 and 90 pmole) were excluded from analysis due to resulting low N's. The SI time was analyzed for the remaining four CYN-154806 doses [0.1 (N=3), 1 (N=5), 10 (N=3), 180 (N=7) pmoles]. In order to compare all rats back to their relevant vehicle injection, SI data are converted to percent of vehicle SI time. A biphasic dose response was observed with the lowest and highest dose of CYN-154806 (0.1 and 180 pmol) producing no-change in SI behavior. The two intermediate doses of CYN-154806 (1 and 10 pmol) significantly reduced SI

times compared to vehicle injections and the lowest dose of CYN-154806 [0.1 pmol), $F_{4,27}$ =6.660; p=0.001, Dunnett's p<0.05; Tukey's p<0.01; **Fig. 8**]. These data suggest that acute antagonism of Sstr2 in the BLA induces anxiety-like behavior. This is consistent with the hypothesis that reduced Sstr2 mRNA expression in the BLA following Ucn1-induced priming is at least in part involved with the development of the persistent anxiety-like behavior.

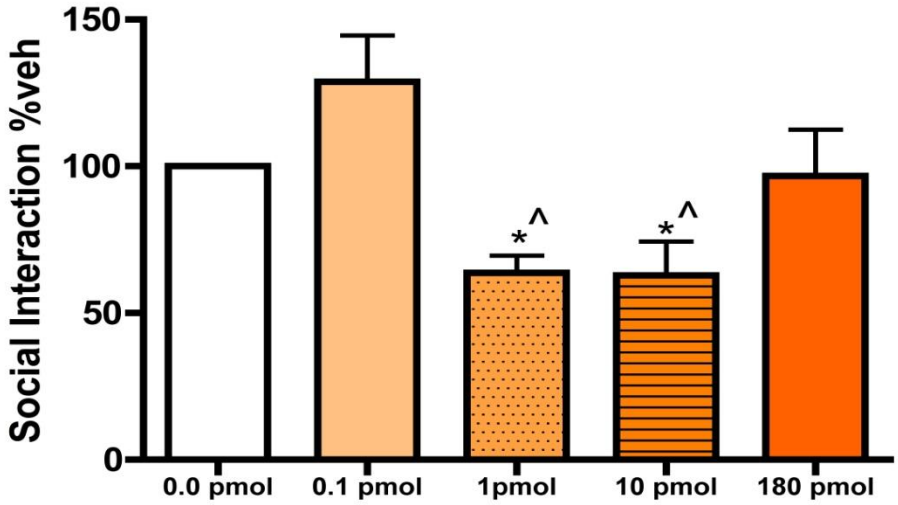


Figure 8. Blocking Sstr2 function in the BLA leads to the expression of anxiety-like behavior. Graph represents a dose response curve for somatostatin receptor 2 (Sstr2) antagonist, CYN-154806. Social interaction data are presented as percent of vehicle SI time and measured in seconds (mean ± SEM). Significance: * indicates significantly different from vehicle ($p<0.05$) by Dunnett's; ^ indicates significantly different from CYN-154806 dose 0.1 pmol ($p<0.01$) by Tukey's.

3.4 Experiment 3b: Role of the BLA cholinergic nicotinic receptor α4 subunit (Chrna4) function in the expression of anxiety-like behaviors

If decreased Chrna4 expression in the BLA observed with Ucn1-priming is causally linked to the expression of persistent anxiety-like behavior, then antagonizing this receptor should acutely increase anxiety-like behavior. Six rats were tested in this paradigm however, cannula placement was outside of the BLA for two rats leaving an N=4 for analysis. Compared to vehicle, SI times were not significantly reduced following microinjections of DHβE (either 3 or 15 μg), mecamylamine or the second vehicle injection (repeated measures ANOVA: $F_{4,12}$=1.294; p=0.3267; N=4, **Fig. 9**). These results suggest that antagonism of cholinergic nicotinic receptor containing the α4 or α3 subunit in the BLA did not result in increased anxiety-like behaviors (antagonists DHβE and mecamylamine, respectively). The expression of persistent anxiety-like behavior following Ucn1-priming is unlikely to be simply a consequence of reduced Chrna4 expression alone. If the reduction in Chrna4 is related to the expression of the behavior it is likely to be one part of a complex of events.

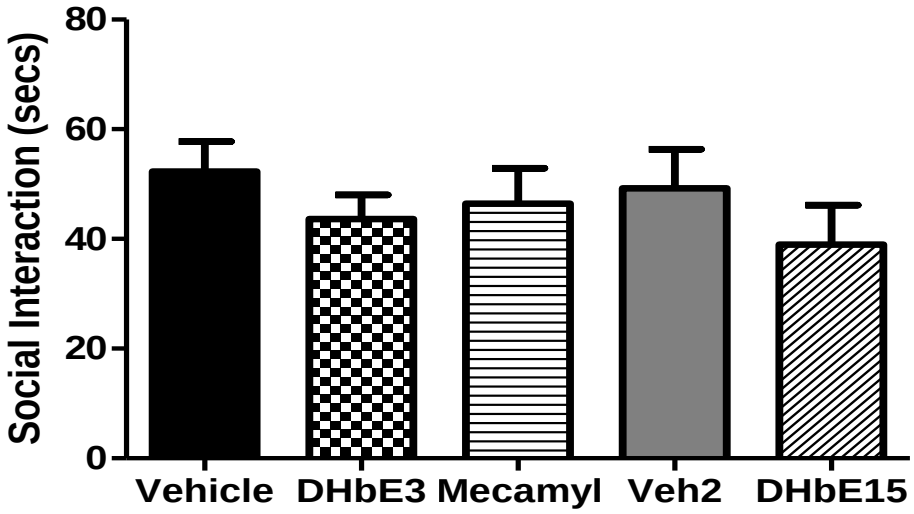


Figure 9. Blocking BLA nicotinic acetylcholinergic receptors containing Chrna4 or the α3β4 subunit does not lead to the expression of anxiety-like behavior. Graph represents the effect of nicotinic receptor antagonists on social interaction. DHβE is the antagonist for the nAchR containing the α4β2 subunit, however, DHβE has some affinity for the α3β4 subunit as well (Xiao et al., 1998). To discern specificity of action at α4β2 compared to the α3β4 subunit, the anxiety-like effects of BLA injections of mecamylamine, the predominant α3β4nAchR antagonist (Xiao et al., 1998; Dhar et al., 2000), was also included. Abbreviations: DHbE3, Dihydro-beta-erythroidine hydrobromide 3 μg dose; Mecamyl, Mecamylamine Hydrochloride; DHbE15, Dihydro-beta-erythroidine hydrobromide 15 μg dose; Veh2, second Vehicle test. Data are represented as mean ± SEM and N=4.

3.5 Experiment 4: Microinjection of Sstr2 agonist by itself does not increase SI time but it prevents the expression of an anxiety-like response in SI if microinjected into the BLA of rats 30 minutes before microinjection of a threshold dose of Ucn1

3.5.1 Experiment 4a

Thus far Sstr2 mRNA expression in the BLA has been shown to be reduced in rats that display persistent anxiety-like behavior and antagonizing Sstr2 in the BLA produces acute increases in anxiety-like behavior. The effect of BLA Sstr2 activation on anxiety-measures was investigated by injecting the Sstr2 agonist, BIM-23027 [1 and 100 fmole, 1, 10, 30, and 90 pmole (in 100 nl/side)], bilaterally into the BLA and assessing SI times. Data from 10 animals were excluded from analysis. The final number per dose were N=3 per group except for the 1 pmole dose where the N was 4. The 100 fmole dose was excluded from analysis because one data point was considered an outlier as determined by Grubbs' test (alpha=0.05). It was found that activation of the Sstr2 with BIM-23027 does not increase SI time compared to baseline (one-way ANOVA, $F_{5,14}$=0.1502, p=0.9766; **Fig. 10**).

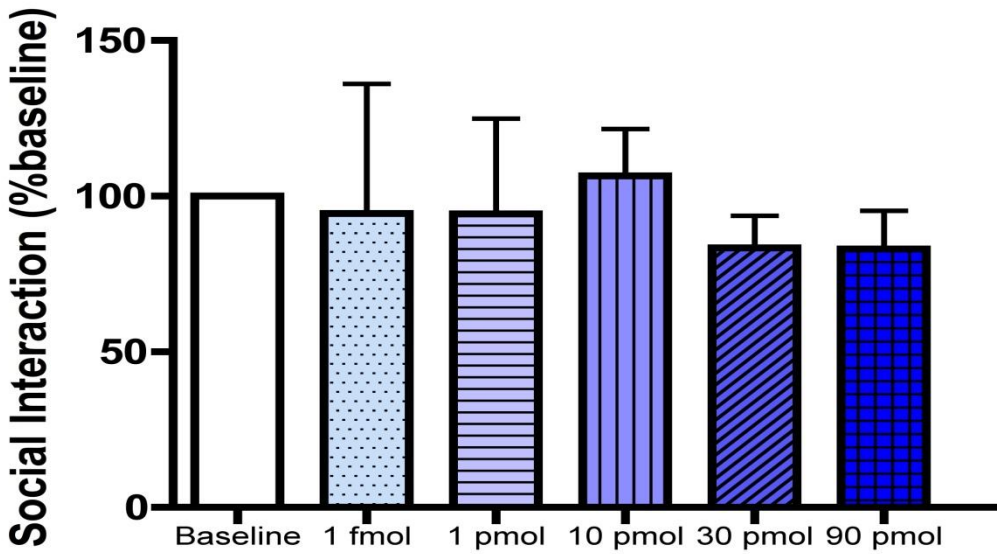


Figure 10. BLA Sstr2 activation does not change SI from baseline SI times. Graph represents the dose response for the effect of Sstr2 agonist, BIM-23027, on baseline SI time. SI is presented as percent of baseline (mean ± SEM). N=3 per group except the 1 pmole dose had N=4.

3.5.2 Experiment 4b

A threshold dose of Ucn1, 100 fmoles (in 100nl/side) microinjected bilaterally into the BLA will induce an anxiety-like response in the SI test (Sajdyk et al., 1999; Spiga et al., 2006). The anti-anxiety-like effects of BLA-Sstr2 activation were investigated by microinjecting BIM-23027 (30 or 90 pmoles/100 nl; BIM30preUcn and BIM90preUcn, respectively) into the BLA of rats 30 minutes prior to a threshold dose of Ucn1 (100 fmoles). Three data points were lost from the Veh.Ucn group however, the SI data for the "BIM-23027 30 minutes prior to Ucn1" test from these three rats were still included in the data analysis because it has been established repeatedly in other experiments that an acute dose of 100 fmoles of Ucn1 will cause a significant decrease in SI (Sajdyk et al., 1999; Spiga et al., 2006; and unpublished observations).

Ucn1 pretreated with vehicle (VehpreUcn) significantly reduced SI time and pretreatment with BIM-23027 (90 pmol) blocked this effect [one-way ANOVA, $F_{2,23}$=4.746; p=0.0102; Dunnett's, (VehpreUcn), p<0.01; (BIM30preUcn), p<0.05; (BIM90preUcn), p>0.05; **Fig. 11**]. Data from Experiments 4a and 4b suggest that the role of Sstr2 may be to counteract anxiogenic stimuli in the BLA.

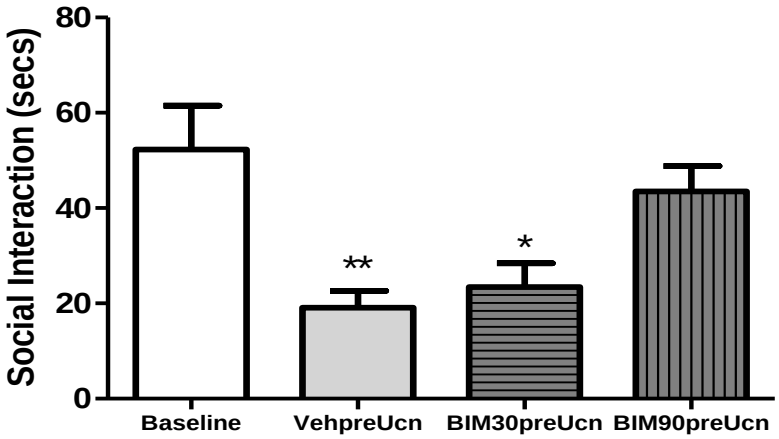


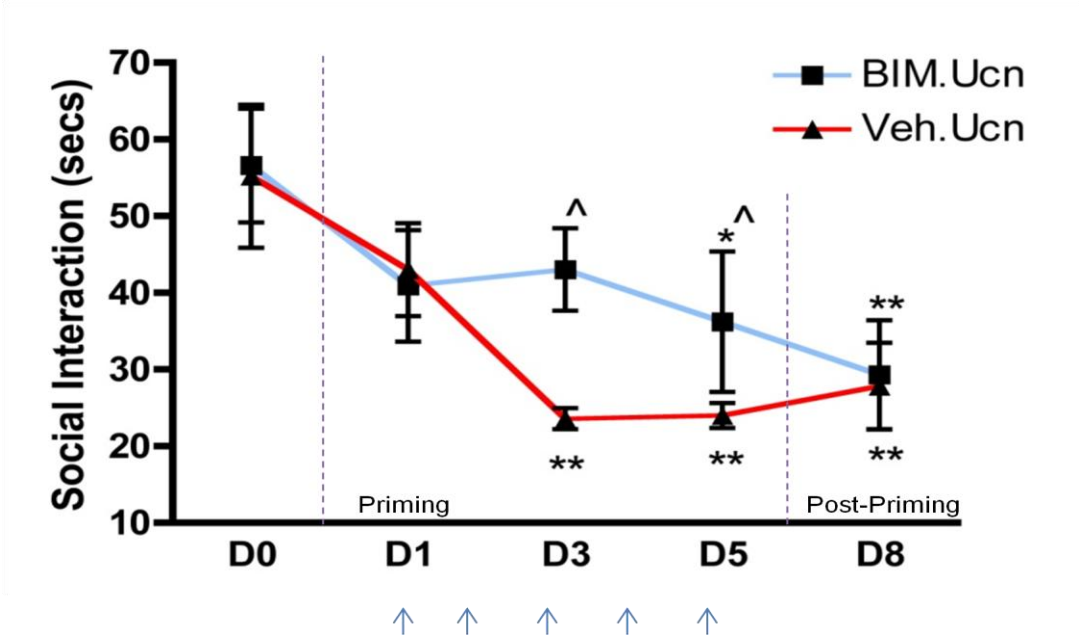
Figure 11. Sstr2 activation in the BLA prior to a threshold dose of Ucn1 blocks the expression of anxiety-like behavior. Graph represents the effect BIM-23027 injected prior to a threshold dose of Ucn1 has on social interaction (mean ± SEM). Significance: Following a one-way ANOVA, ** indicates significantly different from baseline ($p<0.01$) by Dunnett's and * indicates significantly different from baseline ($p<0.05$) by Dunnett's. Abbreviations: BIM30preUcn, 30 pmole BIM-23027 microinjection prior to Ucn1; BIM90preUcn, 90 pmole BIM-23027 microinjection prior toUcn1; VehpreUcn, vehicle microinjection prior to Ucn1. The numbers per group were baseline (N=10), VehpreUcn (N=7), BIM30preUcn (N=5) and BIM90preUcn (N=5).

3.6 Experiment 5: Activating Sstr2 receptors in the BLA of rats 30 minutes prior to an Ucn1 priming injection delays behavioral sensitization but does not stop the development of the persistent anxiety-like phenotype

3.6.1 Experiment 5a

Four data points were lost from both the Veh.Ucn and BIM.Ucn treatment groups. Data analyzed included Veh.Ucn (N=6) and BIM.Ucn (N=6). As expected, Ucn1-priming, when preceded by vehicle, showed significant reduction in SI time during priming D1, D3, and D5 as well as persistent anxiety-like behavior post-priming (D8) [repeated measures ANOVA (Veh.Ucn), $F_{2,2}$=6.524; p=0.0016; Dunnett's (D3, D5, & D8), p<0.01; N=6; **Fig. 12A**]. However, when Ucn1-priming microinjections are preceded by BIM-23027 the behavioral sensitization is delayed compared to the Ucn1-primed rats pretreated with vehicle (Veh.Ucn; D1, D3, & D5). Furthermore, BIM-23027 pretreated rats had significantly higher SI times on D3 and D5 when compared to rats pretreated with vehicle during priming [(BIM.Ucn vs Veh.Ucn), two-way repeated measure ANOVA, drug x time, $F_{2,20}$=3.574; p=0.0471; Fisher's LSD, (D3 & D5) p=0.026; N=6; **Fig. 12A**]. Although the behavioral sensitization is delayed the persistent anxiety-like phenotype still develops [repeated measures ANOVA (BIM.Ucn), $F_{4,20}$=4.140, p=0.0133; Dunnett's, (D5) p<0.05 and (D8) p<0.01; N=6; **Fig. 12A**].

A.



B.

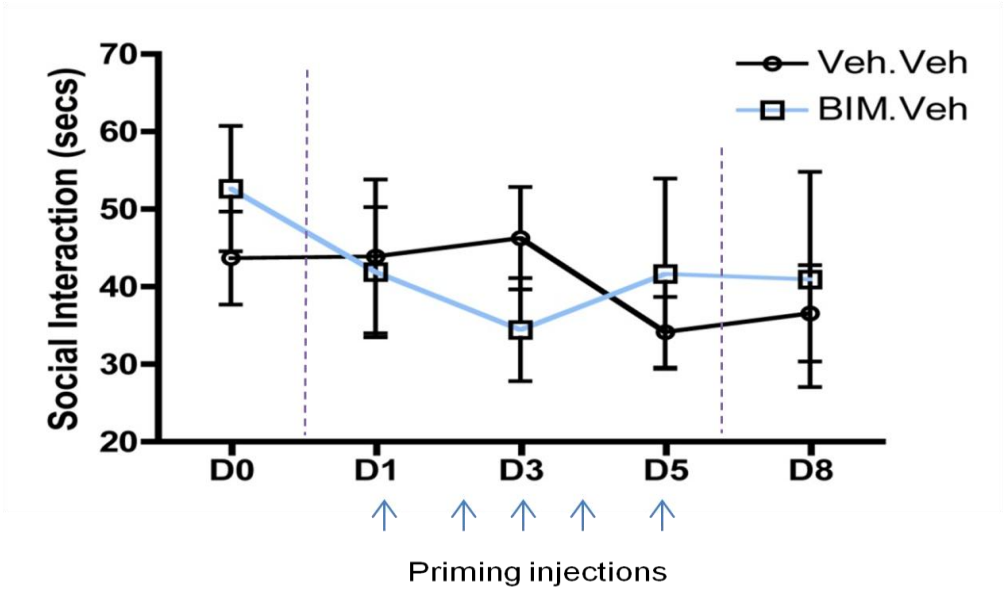


Figure 12. Pretreatment with BIM-23027 during priming does not prevent the development of a persistent anxiety-like phenotype, however, it does attenuate behavioral sensitization without effecting a change in SI alone. Data reflect social interaction times (mean ± SEM). The arrows indicate the days of injection (daily on D1 through D5). The graph area between the vertical dash

lines represents the SI following the priming injections. Laterally to the left of the dashed line is the baseline SI and laterally to the right of the dashed line is the post-priming SI. **A.** The blue line with closed squares represent Ucn1-priming with BIM-23027 pretreatment (N=6). The red line with triangles represents Ucn1-priming with Veh pretreatment (N=6). Graph reflects effect of BIM-23027 pretreatment during priming on social interaction. In graph **B.** the blue line with open squares represent BIM-23027 injection before Veh (N=6) and the black lines with the open circle represent the vehicle control (N=8). Graph reflects the effect of BIM-23027 alone on SI. Significance: ^ indicates significantly different from Veh.Ucn during priming ($p=0.026$) by Fisher's LSD; * indicates significantly different from baseline ($p<0.05$) by Dunnett's; ** indicates significantly different from baseline ($p<0.01$) by Dunnett's. Abbreviations: BIM.Ucn, BIM-23027 microinjected prior to Ucn1 priming injecition; Veh.Ucn, Vehicle microinjected prior to Ucn1 priming injection; Veh.Veh, vehicle microinjection before 2nd injection of vehicle; BIM.Veh, BIM-23027 microinjection before 2nd vehicle injection.

3.6.2 Experiment 5b

Four data points were excluded from the BIM.Ucn treatment group and the Veh.Veh group lost one. It was found that repeated injections with BIM-23027 did not significantly change SI time [two-way repeated ANOVA BIM.Veh (N=6) vs Veh.Veh (N=8), $F_{1,48} = 0.05074$, $p=0.8256$; repeated measures ANOVA (BIM.VEH), $F_{4,20}=0.9167$; $p=0.4735$; N=6; **Fig. 12B**]. As expected, repeated vehicle injections did not significantly change SI behavior either [(Veh.Veh), repeated measures ANOVA, $F_{4,20}=1.47$; $p=0.2487$; N=6; **Fig. 12B**].

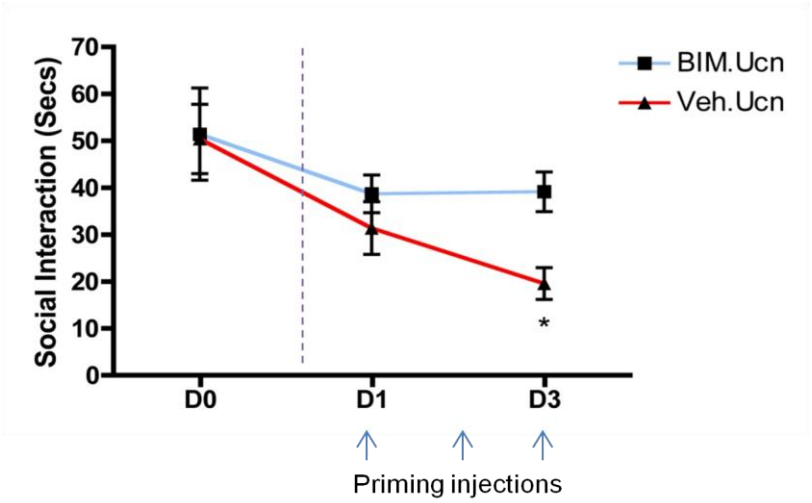
Combined, these data suggest that Sstr2 activation does not prevent the development of persistent anxiety-like behavior/phenotype. Furthermore, pretreatment with BIM-23027 delays behavioral sensitization induced by Ucn1-priming but does not change baseline SI behavior when microinjected into the BLA alone thereby offering support to an earlier conclusion from Experiment 4 suggesting that the role of Sstr2 is to counteract anxiogenic stimuli in the BLA.

3.7 Experiment 6: Pretreatment with BIM-23027, 30 minutes prior to a BLA Ucn1 priming injection does not attenuate the reduction of Sstr2 mRNA expression in the BLA

3.7.1 Experiment 6a

Up to this point it has been shown that Sstr2 mRNA is reduced five days after cession of Ucn1-priming (D10, Experiment 2) and that activating Sstr2 during priming delayed the development of the behavioral sensitization (Experiment 5). To investigate if there is a difference in mRNA on D3 with or without prior Sstr2 activation during priming, rats received either BIM-23027 [90 pmole, (BIM.Ucn)] or a vehicle (Veh.Ucn) microinjections into the BLA 30 minutes prior to a Ucn1 (6 fmole) priming injection. To verify behavioral sensitization, rats were placed in SI at baseline (D0), D1, and D3, and sacrificed immediately after the SI test on D3 of priming. The BLAs were processed for qRT-PCR and the Sstr2 gene was normalized to the average of the endogenous control gene beta-Actin. As expected, behavioral sensitization occurred by D3 during Ucn1-priming but not if Sst2 receptors were activated during priming [repeated measure ANOVA (Veh.Ucn), $F_{2,6}$=6.847; p=0.0283; Dunnett's ($p<0.05$); N=4; and repeated measure-ANOVA (BIM.Ucn), $F_{2,8}$=1.209; p=0.3477; N=5; **Fig. 13A**]. However, Sstr2 mRNA expression was virtually equal on D3 following Ucn1-priming with or without prior Sstr2 activation [(BIM.Ucn and Veh.Ucn, respectively), t-test, t(8)=0.1379; p=0.8937, N=5; **Fig. 13B**].

A.



B.

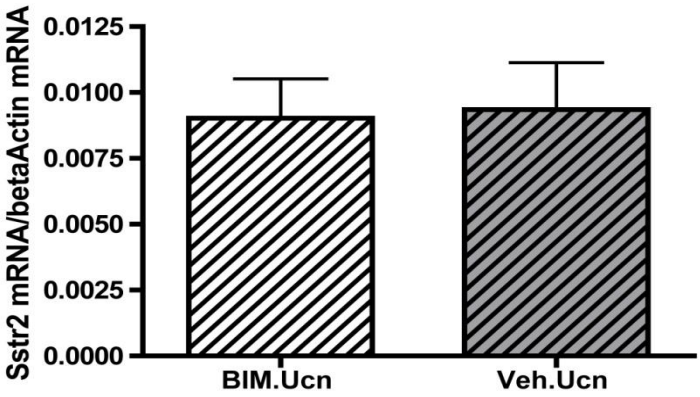


Figure 13. Sstr2 agonist, BIM-23027, masks Ucn1-priming induced behavioral sensitization. Sstr2 mRNA expression is virtually the same with or without Sstr2 activation during priming. A. Graph represents effect of Sstr2 activation on the expression of behavioral sensitization during priming. The three arrows below the graph represent the days of injection (D1, D2, & D3). The SI data lateral to the dash vertical line represent the baseline SI and the SI scores to the right of the vertical dashed line represent the SI 30 minutes post the Ucn1-priming injection (pretreatment was with BIM or Veh). The blue line with closed square reflects directional changes in BIM.Ucn SI (N=5) and the red line with the closed triangle reflects directional changes in Veh.Ucn SI (N=5). **B.** Graph represents normalized Sstr2 mRNA expression on priming D3. Significance: *Significantly different from baseline ($p<0.5$) by Dunnett's. Abbreviations: BIM.Ucn, BIM-23207 microinjected prior to Ucn1-priming injection; Veh.Ucn, vehicle microinjected prior to Ucn1 priming injection.

3.7.2 Experiment 6b

Sstr2 mRNA was found to be reduced five days after cessation of Ucn1 priming microinjections (D10, Experiment 2). Furthermore, activating Sstr2 during Ucn1-priming delayed the development of the behavioral sensitization of priming but did not stop the development of the persistent anxiety-like phenotype after Ucn1-priming (Experiment 5a). BLA tissue from a subset of animals used in Experiment 5 were collected five days after priming ceased (D10) and processed for qRT-PCR. Two of the collected samples from the BIM.Veh group could not be used because the extracted total RNA was not enough to convert to cDNA. This left an N=2 in the group therefore excluding it from further analysis. One sample was excluded from the Veh.Ucn group because the replicate StDev was>4 leaving (N=3). Two data points in the BIM.Ucn treatment group were identified as outliers as determined by the Grubbs' test (alpha=0.05) so they were excluded from analysis leaving BIM.Ucn with an N=3. The Veh.Veh cohort had an N=3.

The data show that both Ucn1 treated groups, but not vehicle, had a significant reduction in Sstr2 mRNA following Ucn1-priming regardless of Sstr2 activation during priming [one-way ANOVA, $F_{2,6}$=7.205; p=0.0254;Tukey's, (Veh.Veh vs BIM.Ucn) and (Veh.Veh vs Veh.Ucn), $p<0.05$; N=3; **Fig.14**]. These data confirm the previous finding from Experiment 2 that Sstr2 mRNA expression in the BLA is reduced following Ucn1-priming and further suggest that Sstr2 activation during Ucn1-priming does not prevent the Sstr2 mRNA expression decrease.

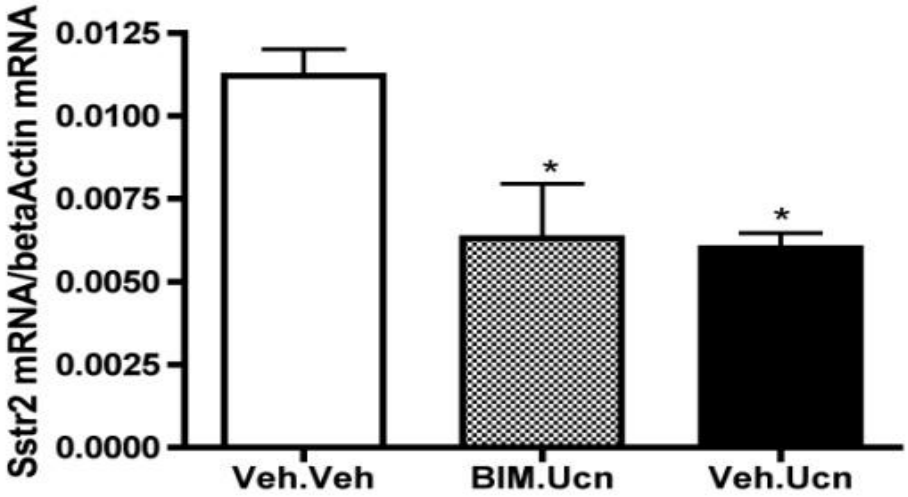


Figure 14. Sstr2 activation does not prevent the reduction of Sstr2 mRNA expression observed following Ucn1-priming. Graph represents changes in Sstr2 mRNA post-priming with and with Sstr2 activation during Ucn1 priming. Data are normalized to the mRNA of beta actin and presented as mean ± SEM; N=3 per group. Significance: * indicates significantly different from Veh.Veh ($p<0.05$) by Tukey's. Abbreviations: Veh.Veh, vehicle microinjected prior to 2nd vehicle injection; BIM.Ucn, BIM-23027 microinjected prior to Ucn1priming injection; Veh.Ucn, vehicle microinjected prior to Ucn1priming injection; Sstr2, somatostatin receptor 2.

The Sstr2 mRNA expression on D3 is not different between BLAs of rats primed with or without Sstr2 activation during Ucn1-induced priming. Data from 6a and 6b combined suggests that although BIM-23027 pretreatment during Ucn1-priming overrides the anxiogenic effect of Ucn1 during priming on priming D3 it does not attenuate the reduction of Sstr2 mRNA.

DISCUSSION

The goal of this research was to further elucidate mechanism(s) involved in the persistent anxiety-like phenotype that develops following Ucn1-priming of the BLA. It was predicted that repeated stimulation of the CRF receptors in the BLA by Ucn1-priming, leads to the development of the persistent anxiety-like phenotype because of intrinsic changes within of the BLA neural network. Because of the BLA's potential to influence behavior in response to a context (Helmstetter and Bellgowan, 1994; Campeau and Davis, 1995; Maren et al., 1996; Muller et al., 1997; Cousens and Otto, 1998; Huff and Rudy, 2004), Experiment 1 was run to determine the extent conditioning to the SI testing arena or partner rat during BLA priming affected the expression of the persistent anxiety-like behavior observed in rats post-priming.

Although other studies demonstrate that BLA excitation can lead to the association of an aversive motivational state with a distinct environment such as a testing context (Thielen and Shekhar, 2002; Sajdyk et al., 2006), the results from Experiment 1 demonstrate that the persistent anxiety-like phenotype following Ucn1-priming develops regardless of exposure to the SI arena and/or a novel partner rat during priming. Therefore, the expression of persistent anxiety-like behavior following Ucn1-priming does not appear to be due to aversive conditioning to the testing arena or the novel partner rat. Thus suggesting that repeated stimulation of CRF receptors in the BLA are causing intrinsic changes within the neuronal circuitry of the BLA that lead to the development of a persistent anxiety-like phenotype.

Consistent with this conclusion, using the same BLA-Ucn1 priming strategy as used in the current study, whole-cell patch-clamp recordings suggest that Ucn1-priming results in a reduction of inhibitory control in the BLA local network leading to hyperexcitability in the BLA output neurons (Rainnie et al., 2004). These recordings were taken weeks after the last Ucn1-priming injection. Furthermore, a subset of Ucn1-primed animals were re-tested in SI once a week for five consecutive weeks without any further Ucn1 injections and continued to display anxiety-like behavior without any evidence of extinction as would be expected if the effect were a result of aversive conditioning (Rainnie et al., 2004).

Another interesting finding from this study was that both $GABA_A$ mediated spontaneous and stimulation-evoked IPSPs from the projection neurons in the Ucn1-primed, compared to veh-primed tissue slices were reduced (Rainnie et al., 2004). Although the $GABA_A$ receptor-mediated inhibitory response was deficient, the $GABA_A$ receptor functioning appeared to be unaltered (Rainnie et al., 2004), suggesting that perhaps CRF1 receptor activation is activating some mechanism that regulates GABA release or possibly a change has occurred in the regulation of $GABA_A$ receptor function.

Consistent with intact $GABA_A$ receptor functioning, data from the current work revealed that the mRNA for the $GABA_A$ receptor subunits from the BLA of Unc1-primed tissue was unchanged compared to veh-primed BLA tissue. However, lasting reductions in the expression of five other neurotransmitter receptor within the BLA were significantly decreased compared to veh-primed controls; Chrna4, Chrm4, Gabrr1, Sstr2, and Sstr4. Knock-out mice studies

indicated that Chrna4, Chrm4, and Sstr2 play a role in anxiety (Ross et al., 2000; Degroot and Nomikos, 2006; Viollet et al., 2000, respectively) but Sstr2's role in anxiety is more specific to the BLA (Meis et al., 2005; Truitt et al., 2007).

Somatostatin is known to potentiate $GABA_A$ receptor responses (Gardette et al., 1995; Moneta et al., 2002; Cammalleri et al., 2006; Momiyama and Zaborszky, 2006) and the mRNA of two SST receptors, were significantly reduced in the Ucn1-primed compared to veh-primed rats. Subsequent experiments support the role of Sstr2 in the regulation of anxiety and suggest that the state of Sstr2 is may be involved in the expression of persistent anxiety-like behavior induced by Ucn1-priming.

Terminals of SST containing interneurons are found in close proximity to the dendrites of BLA projection neurons as well as a few interneurons showing that they are in a position to regulate both the GABA activity of select populations of BLA interneurons as well as neuronal excitability of BLA projection neurons (Muller et al., 2003a; 2007a; Muller et al., 2007b). The SST-GABA interneurons in the BLA, form synapses with $GABA_A$ receptors on distal dendrites of the BLA projection neurons and are juxtaposed to excitatory glutamatergic inputs (Muller et al., 2003a). These inhibitory inputs are capable of regulating excitatory inputs onto the dendritic pyramidal cells by blocking the generation of calcium dependent postsynaptic potentials in the dendrites and ultimately shunt activation of the pyramidal cells (Muller et al., 2003a). Whole cell patch-clamp recordings show that a dampening of cell excitability could result from an inwardly rectifying K+ current in amygdala neurons induced by SST (Meis et al., 2005). Loss of

neuronal regulation by SST has the potential to lead to increased network excitability resulting in the expression of anxiety-like behavior as observed following Ucn1-priming (Rainnie et al., 2004).

Sstr2 mRNA was reduced in the BLA following Ucn1-priming. To determine if reduced Sstr2 mRNA expression in the BLA may explain the development of persistent anxiety-like behavior following Ucn1-priming, the acute effect of blocking Sstr2 receptor function on anxiety-like behaviors was investigated. Data from this study showed that antagonizing Sstr2 receptor functioning in the BLA was sufficient to increase anxiety-like behavior. Consistent with this finding, loss of Sstr2 receptors has been associated with anxiety-like behavior in a study characterizing Sstr2 knock-out mice (Viollet et al., 2000). Moreover, lesioning approximately half of the SST-GABA interneurons by targeting neurokinin 1 receptor (NK-1r) containing cells in the BLA, led to persistent anxiety-like behavior in a SI paradigm (Truitt et al., 2007).

While the persistent anxiety-like phenotype induced by Ucn1-priming is marked by a reduction in Sstr2 mRNA and, acute blockade of Sstr2 receptors induced anxiety-like behavior, it is still unclear if Sstr2 receptor activation is sufficient to reduce anxiety-like behaviors. To determine the effect of Sstr2 activation on basal anxiety as measured by the SI test, rats were tested with a range of doses of a Sstr2 agonist. The basal levels of SI were not modified by any of the doses of the Sstr2 receptor agonist tested in the BLA. These results are in contrast to other studies where rats that received intracerebroventricular (i.c.v.) microinfusion of SST displayed anxiolytic-like effects in the EPM test of

anxiety (Engin et al., 2008) through the Sstr2 receptor activation (Engin and Treit, 2009). However, since these studies were carried out through i.c.v. microinfusions the location of the Sstr2 receptors mediating the anxiolytic-like effect is unknown.

In previous studies within the BLA, the $GABA_A$ receptor antagonist BMI led to the expression of anxiety-like behavior and the $GABA_A$ agonist muscimol did not change the basal SI behavior from baseline (Sanders and Shekhar, 1995), even though muscimol has been shown to alleviate anxiety (Muller et al., 1997), thus suggesting that the inhibitory tone of BLA is at maximal (Sanders and Shekhar, 1995). Therefore, discovering that a Sstr2 receptor agonist does not modify basal SI behavior is not unexpected.

Despite the observation that activation of the Sstr2 receptors in the BLA was not anxiolytic, it is still possible that the role of Sstr2 receptors in the BLA may be to block stress-induced anxiogenesis. To determine if Sstr2 activation in the BLA can override induction of anxiety-like behavior, rats were microinfused with a Sstr2 agonist into the BLA 30 minutes before a microinfusion of an anxiogenic-like dose of Ucn1 into the BLA. Activating Sstr2 receptors in the BLA with an agonist prior to an anxiogenic-like dose of Ucn1 prevented the expression of an anxiety-like response thus suggesting that the role of Sstr2 may be to counteract anxiogenic stimuli in the BLA. Consistent with this conclusion, rats exposed to predator stress displayed an increase in Sstr2 mRNA expression three hours after exposure to a ferret, a natural predator of the rat (Nanda et al.,

2008). Moreover, an analog of SST has been successfully used to treat panic-like attacks (Abelson et al., 1990).

Since Sstr2 receptor activation in the BLA can override an anxiogenic-like dose of Ucn1, it is possible that activation of Sstr2 receptors in the BLA during Ucn1-priming could prevent the development of the persistent anxiety-like phenotype. To determine if Sstr2's ability to counteract the acute anxiogenic-like effect of Ucn1 will carry over into blocking the Ucn1 priming-induced expression of behavior sensitization and/or persistent anxiety-like behavior, rats underwent pretreatment with a Sstr2 agonist into the BLA 30 minutes prior to each Ucn1-priming injection. With Sstr2 agonist pretreatment during Ucn1-priming, the anxiety-like behavior usually observed on D3 in the Ucn1-primed group, as compared to veh-primed or the within-group baseline, is not evident until priming D5. Pre-activation of Sstr2 receptors in the BLA during Ucn1-priming appears to delay the expression of the behavioral sensitization but does not prevent the development of the persistent anxiety-like phenotype. Consistent with the lack of blocking the expression of persistent anxiety-like behavior, Sstr2 agonist pretreatment also did not block the Ucn1-priming induced reduction in Sstr2 mRNA. Although pretreatment with a Sstr2 agonist during priming did not prevent the Ucn1 priming-induced reduction of Sstr2 mRNA observed post-priming, it is possible that Sstr2's ability to delay the expression of behavioral sensitization on D3 of priming was due to attenuation in the reduction of Sstr2 mRNA in the Ucn1-primed cohort pretreated with a Sstr2 agonist prior to the Ucn1-priming injection compared to the Ucn1-primed cohort pretreated with vehicle. To

determine if there is a difference in the level of Sstr2 mRNA on D3 of priming between the Ucn1-primed rats pretreated with or without a Sstr2 agonist during priming the rats were divided into two groups. One group of rats received a microinfusion of a Sstr2 agonist into the BLA 30 minutes prior to each Ucn1-priming injection; the other group were pretreated with vehicle. Brains were collected on priming D3 immediately following the SI test. Although the Ucn1-primed rats pretreated with a Sstr2 agonist during priming did not display anxiety-like behavior as compared to the rats who went through Ucn1-priming without prior Sstr2 receptor activation, the Sstr2 mRNA expression in the BLA was virtually equal between the two Ucn1-primed cohorts on priming D3. Sstr2's ability to delay the expression of behavioral sensitization does not translate into a delayed reduction of Sstr2 mRNA as compared to Ucn1-primed rats primed without a Sstr2 agonist pretreatment. Therefore, the reduced state of Sstr2 mRNA on post-priming D8 may be one factor behind the expression of a persistent anxiety-like behavior but not the development of the persistent anxiety-like phenotype.

Sstr2's ability to mask the behavioral sensitization but not stop the development of the persistent anxiety-like phenotype induced by Ucn1-priming suggests that different mechanisms are involved in these two effects. Although behavioral sensitization is expressed by D3 of Ucn1-priming, if the priming injections are stopped after D3, rats return to pre-priming levels of anxiety (baseline levels). However, if the same rats are challenged with another subthreshold dose of Ucn1 up to six weeks later, the anxiety-like behavior is

reinstated on priming D1, unlike Ucn1 naïve rats, suggesting some type of plasticity is occurring during Ucn1-priming (Sajdyk et al., 2004).

Both NMDA and calcium calmodulin-dependent kinase II (CaMKII) are strongly implicated in the mediation of synaptic plasticity (Bliss and Collingridge, 1993; Colbran and Brown, 2004). Previous studies have shown that the persistent anxiety-like behavior induced by Ucn1-priming injections can be blocked by either co-administration of the active form of the NMDA receptor antagonist DL-AP-5 with Ucn1 (Rainnie et al., 2004) or by pretreatment with the CaMKII inhibitor, KN-62, prior to each consecutive Ucn1 microinjection (Shekhar et al., 2003; Rainnie et al., 2004). The persistent anxiety-like phenotype following Ucn1-priming appears to be dependent upon activation of a NMDA receptor-mediated CaMKII-dependent second messenger cascade (Shekhar et al., 2003; Rainnie et al., 2004).

NMDA receptor potentiation can be mediated by direct phosphorylation of serine/threonine kinases PKA and PKC (Leonard and Hell, 1997; Dautzenberg et al., 2004). Microinjections of Ucn1 into the BLA appear to induce anxiogenic-like behaviors via activation of CRF1 G-protein coupled receptors (Sajdyk and Gehlert, 2000; Gehlert et al., 2005). Both PKA and PKC pathways are linked to CRF1 receptor activation (**Fig. 15**; Dautzenberg and Hauger, 2002; Hauger et al., 2006). Further study is needed to determine if Ucn1-priming induces long-term synaptic facilitation through either the PKA or PKC pathway or a combination of both.

Data from the experiments outlined in this thesis, support the role of Sstr2 in the regulation of anxiety and suggest that the state of Sstr2 is may be involved in the expression of persistent anxiety-like behavior induced by Ucn1-priming in that pretreatment with a Sstr2 agonist during Ucn1-priming delays the expression of behavioral sensitization but did not stop the development of the persistent anxiety-like phenotype. Furthermore, pretreatment with a Sstr2 agonist can override an anxiogenic-like dose of Ucn1. One signal transduction pathway initiated through ligand activation of the CRF1 receptor is the adenylyl cyclase-cAMP-PKA pathway via G_s protein (Giguere et al., 1982; Chen et al., 1986; De Souza, 1995; Spiess et al., 1998; Perrin and Vale, 1999; Dautzenberg and Hauger, 2002). It is highly possible that the induction of acute anxiety by Ucn1 is through this signaling pathway.

Stimulation of adenylate cyclase (Chen et al., 1986; Dunn and Berridge, 1990; Dautzenberg et al., 2001) leads to the intracellular accumulation of cAMP (Giguere et al., 1982; Dunn and Berridge, 1990; Sananbenesi et al., 2003) then couples to the PKA pathway. PKA could then potentiate NMDA receptor activity. Sstr2 receptors may be counteracting the acute Ucn1-induced anxiety-like behavior through its inhibition of the adenylyl cyclase- cAMP-PKA pathway via G_i protein (G_i, inhibitory; **Fig. 15**) which is recognized as Sstr2's predominant mode of action (see Csaba and Dournaud, 2001).

Another mechanisms potentially involved in Sstr2's ability to override the acute anxiety-like behavior induced by Ucn1, may be through its regulation of calcium (Ca2+) signaling pathways. Activation of K+ channels leads to the

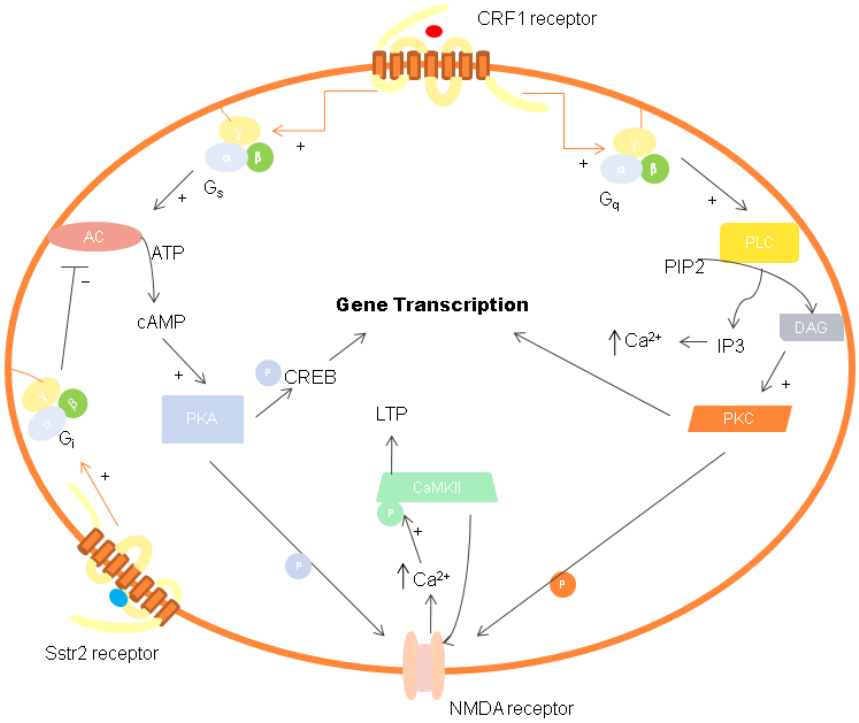


Figure 15. Possible pathways involved in the expression of anxiety-like behavior and/or the development of the persistent anxiety-like phenotype induced by Ucn1-priming. Ucn1 binding to CRF1 receptors activate G-proteins that in turn activate enzymes leading to the activation of kinases that regulate different cellular events through different pathways. For example kinases PKA and PKC can both potentiate the function of the NMDA receptor through one pathway and initiate gene transcription through a different pathway. NMDA activity can lead to BLA excitability. Moreover, activation of the NMDA receptor leads to the increase of calcium within the cell that activate the CaMKII enzyme eventually leading to LTP. This pathway is implicated in the development of the persistent anxiety-like phenotype induced by Ucn1-priming. Sstr2 binding to its receptor can possibly over-ride the effect of Ucn1 activation of the CRF1 receptor in that Ucn1 leads to the activation of AC and Sstr2 inhibits AC. Abbreviations: Ucn1, urocortin 1; Sstr2, somatostatin 2 receptor; AC, adenylyl cyclase; NMDA, N-methyl D-aspartate; Ca2+, calcium; LTP, long-term potentiation; CREB, cAMP response element-binding; PKA, protein kinase A; PKC, protein kinase C; CaMKII, calcium-calmodulin dependant protein kinase II; Gi, inhibitory G-protein subunit; Gs, stimulation G-protein; Gq, other G-protein subunit; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5-triphosphate; DAG, diacyl glycerol; β, beta subunit of G-protein complex; ɣ, gamma subunit of G-protein complex; P, phosphorylates.

reduction of intracellular Ca2+ concentrations indirectly through inhibition of voltage-dependant Ca2+ channels (Csaba and Dournaud, 2001; Yang et al., 2007).

Five genes were significantly down regulated in the BLA tissue of the Ucn1-primed, compared to veh-primed rats. Changes in gene expression can occur through CRF1 activation of serine/threonin kinases PKC (Dautzenberg and Hauger, 2002; Gutknecht et al., 2010), MAPK (Dautzenberg and Hauger; 2002; Sananbenesi et al., 2003; Brar et al., 2004; Hauger et al., 2006), and PKA (Chen et al., 1986; De Souza, 1995; Spiess et al., 1998; Perrin and Vale, 1999; Dautzenberg and Hauger, 2002). These kinases can work individually or together on the same effector to organize cellular machinery leading to changes in gene expression (Calkhoven and Ab, 1996; Pearson et al., 2001). Gene expression regulation can occur through transcription activation leading to mRNA production (Hansen et al., 1999; Kovalovsky et al., 2002; Parham et al., 2004), repression of transcription (Servillo et al., 2002), and/or translation that leads to new protein (Servillo et al., 2002). PKC, MAPK, and PKA kinases are also involved in modifying proteins directly or indirectly through transcription activation. Production of new protein or phosphorylation of existing effectors can lead to protein degradation through ubiquitin pathways (Upadhya et al., 2004), receptor internalization (Chapell et al., 1998; Elberg et al., 2002), modulation of receptor activity (Kawaguchi and Hirano, 2002; Sananbenesi et al., 2003; Gerdin et al., 2004; Bayer et al., 2006), and modulation of ion channels (Tao and Li, 2005; Tao et al., 2008). These are a few of the cellular events that can lead to changes

associated with learning (Huang et al., 2000; Sánchez-Muñoz et al., 2010) and/or behavioral responses (Selcher et al., 2002; Sananbenesi et al., 2003; Sherrin et al., 2009). The interaction of these signaling pathways could play a role in Ucn1's priming-induced development of a persistent anxiety-like phenotype.Specific enzyme inhibitors could be used as the next step to further explore potential mechanism(s) involved in the changes of gene expression induced by Ucn1-priming.

The complex mechanisms involved in the development of the persistent anxiety-like phenotype may also involve the cellular signaling machinery activated through Sstr4, Chrna4, Chrm4, and Gabbr1. These genes were also down regulated in the Ucn1-primed, compared to the veh-primed, tissue. As part of this research the role of Chrna4 mRNA in anxiety was also investigated.

Post-priming reduction of the BLA Chrna4 mRNA from Ucn1-primed rats raises the possibility that a decrease in Chrna4 containing receptors played a role in the persistent anxiety-like behavior observed in Ucn1-primed rats. Support for this premise comes from a study that found Chrna4 knock-out mice displayed an anxiety-like profile in the elevated plus maze (Ross et al., 2000). To determine if the reduction in Chrna4 in the BLA could explain increases in anxiety-like behavior, the acute affects of blocking Chrna4 activity was investigated. Since nicotinic receptors are composed of a combination of two or more α and β subunits the role Chrna4 played in basal levels of anxiety was sought by blocking the function of the predominant nAchR receptor subtype in the brain, α4β2.

Antagonizing α4β2 in the BLA did not lead to anxiety-like behavior in the SI test suggesting that Chrna4 does not play a role in the expression of persistent anxiety-like behavior. However, based on limitations of the antagonist used, the influence of BLA Chrna4 type receptors on anxiety-like behavior can't be completely ruled out. First, the specific BLA Chrna4 receptor subtype potentially involved in the persistent anxiety-like phenotype is unknown therefore the α4β2 subtype may not be the Chrna4 receptor subtype involved. Furthermore, the doses for the nicotinic antagonists used for this study were based on reports by Bancroft and Levin (2000), Jonkman and Markou (2006) and Addy and associates (2003). None of these studies involved an anxiety type response but a couple of the studies were carried out in the amygdala (Addy et al., 2003; Jonkman and Markou, 2006). Each of these investigators used a different range of doses for their respective studies but there was some crossover of doses between studies. The doses of DHβE for their studies ranged from (0.2 μg - 20 μg) and the efficacy of similar doses of DHβE varied depending on the experimental paradigm and target area (Bancroft and Levin, 2000; Addy et al., 2003; Jonkman and Markou, 2006). To test the role of the α4β2 nicotinic receptor on the expression of anxiety-like behavior in the BLA, one low and one high dose of DHβE (3 and 15 μg) was selected from the doses previously found to be effective (Bancroft and Levin, 2000; Addy et al., 2003; Jonkman and Markou, 2006). More testing is needed to determine the role of Chrna4 in the BLA in the expression of persistent anxiety-like behavior.

CONCLUSION

5.1 Summary

Although the mechanisms involved in BLA Ucn1-priming are complex, one result is a change in the $GABA_A$ receptor-mediated inhibitory transmission. This deficit then contributes to BLA hyperexcitability that leads to the development of a persistent anxiety-like phenotype (Rainnie et al., 2004). Data from the current study demonstrate that the persistent anxiety-like phenotype was not the result of aversive conditioning to the SI testing arena or procedure but was marked by another change; reduction of mRNA expression of Sstr2, Sstr4, Chrna4, Chrm4, and Gabrr1 mRNA. Further investigation into Sstr2 receptor's role in anxiety-like behavior suggests that the role of Sstr2 receptors in the BLA may be to counteract anxiogenic stimuli or stave off inappropriate anxiety responses since blocking Sstr2 receptor function leads to the expression of anxiety-like behavior, Sstr2 activation can override an acute anxiogenic stimulus, and basal levels of SI do not change with Sstr2 receptor activation.

Furthermore, Sstr2 activation delays the expression of behavioral sensitization induced by Ucn1-priming but does not stop the development of a persistent anxiety-like phenotype. It appears that a reduction in Sstr2 receptor function may be one factor behind the expression of a persistent anxiety-like phenotype but not the development of the persistent anxiety-like phenotype.

The expression of persistent anxiety-like behavior may be the result of inefficient regulation of incoming sensory information. The SST-GABA interneurons are part of the local interneuronal circuitry of the BLA complex. The

BLA complex is a cortex-like structure with glutamatergic projection neurons that are regulated by local networks of GABAergic interneurons (McDonald, 2003) which are unique in cellular phenotype, function, (McDonald and Mascagni, 2001; Muller et al., 2003a; 2007a; Muller et al., 2007b; Truitt et al., 2009) and connection profile (McDonald, 1992; McDonald et al., 1996; Muller et al., 2003a; 2007a; Muller et al., 2007b). Glutamatergic afferents of the cortical sensory association area are capable of blunting EPSPs in BLA complex pyramidal cells by activating interneurons in the BLA complex (Rosenkranz and Grace, 1999; Grace and Rosenkranz, 2002; Rosenkranz and Grace, 2002; Rosenkranz et al., 2003; Rosenkranz et al., 2010). However, the exact phenotype of these interneurons remains unknown.

SST-GABA interneurons contain about half of the NK-1r interneurons and recent anatomical data suggests that NK-1r-IR cells may be one of the targets of the PFC inputs (Truitt et al., 2007). The SST-GABA interneurons in the BLA are capable of regulating excitatory inputs to the pyramidal cells thereby shunting activation of pyramidal cells (Muller et al., 2003a; 2007a) and SST-GABA interneuronal regulation of pyramidal cells occurs through synapses on distal dendrites of pyramidal neurons (Muller et al., 2003a; 2007a). The Sstr2 receptors are predominantly on the distal dendritic terminals (Way et al., 1996). Therefore, Sstr2 receptors have the potential to play a role in assigning emotional significance to incoming sensory information. Loss of cellular mechanisms involved in assigning saliency to incoming sensory information initiated by Sstr2

receptor function could result in increased anxiety-like responses to non-anxiogenic cues.

The results from this study provide potential targets for studying the mechanisms behind aberrant plasticity that leads to psychopathology. This may have broader implications beyond the development of anxiety in that the basolateral amygdala plays a role in other stress related disorders such as depression (Arborelius et al., 1999; Steckler and Holsboer, 1999; Heinrichs and Koob, 2004). Investigations from the current study suggest that signaling pathways regulating Sstr2 receptor expression are involved. Moreover, the mRNA of Sstr4, Chrna4, Chrm4, and Gabbr1 was also down regulated in the Ucn1-primed, compared to the veh-primed, tissue. The role of these genes in anxiety-like behavior warrants further investigation.

Second, Sstr2 receptors signaling pathways in the BLA provide a specific target for the development of specific pharmacotherapy. Somatostatin has a wide range of effects in both the CNS and the periphery, some of which may not be desirable. These effects, combined with its relatively short plasma half-life and lack of receptor selectivity, make it an undesirable candidate for clinical use (Pintér et al., 2006). The more stable somatostatin analogs such as octreotide and lanreotide have been used in clinical settings for conditions such as pancreatitis, growth, and tumor formation (Hofland et al., 1992; Uhl et al., 1999; Pintér et al., 2006; de Jong et al., 2009). These analogs do bind with high affinity to Sstr2 receptors (Pintér et al., 2006) but do not show high receptor selectivity (Pawlikowski and Melen-Mucha, 2003). Preclinical exploration for therapeutic

targets has expanded into targeting components of signaling transduction pathways (see Mathew et al., 2008). Investigation into the effectiveness of treating targets in the signaling pathway of Sstr2 receptors has potential to yield safe and effective therapeutics for the treatment of pharmacotherapy for treating stress related disorders such as anxiety and depression (Engin and Treit, 2009).

Lastly, Sstr2 receptors provide targets for investigation into the unique phenotype of the local interneuronal circuitry involved in assigning saliency to incoming sensory information (Muller et al., 2003a; 2007a; Truitt et al., 2009). Sstr2 receptors are in position to modulate excitability of BLA pyramidal projection neurons (Way et al., 1996). This not only has implications for anxiety or aversive pathways, but also for associative processes involved in reward (Moller et al., 1997; Schoenbaum et al., 2003; Schoenbaum and Roesch, 2005; Tye and Janak, 2007; Morrison and Salzman, 2010).

5.2 Conclusion

In conclusion, repeated stimulation of CRF receptors in the BLA are causing intrinsic changes within the neuronal circuitry of the BLA that lead to the development of a persistent anxiety-like phenotype. The gene expression for five receptors was reduced in animals expressing persistent anxiety-like behavior revealing Sstr2, Sstr4, Chrna4, Chrm4, and Gabrr1 as potential candidates involved in the development and/or expression of this phenotype during Ucn1-priming. The Sstr2 receptors appear to regulate anxiety in the BLA and the state of Sstr2 mRNA may be one of the factors behind the expression of the persistent

anxiety-like behavior but not the development of the persistent anxiety-like phenotype.

REFERENCES

Abelson JL, Mark SK, Giardino LN (2008) Respiratory irregularity and stress hormones in panic disorder: exploring potential linkages. Depression and Anxiety 25(10):885-887.

Abelson JL, Nesse R, Vink A (1990) Treatment of panic-like attacks with a long-acting analogue of somatostatin. Journal of Clinical Psychopharmacology 10:128-132.

Abrams JK, Johnson PL, Hay-Schmidt A, Mikkelsen JD, Shekhar A, Lowry CA (2005) Serotonergic systems associated with arousal and vigilance behaviors following administration of anxiogenic drugs. Neuroscience 133:983-997.

Addy NA, Nakijama A, Levin ED (2003) Nicotinic mechanisms of memory: effects of acute local DH[beta]E and MLA infusions in the basolateral amygdala. Cognitive Brain Research 16:51-57.

Adolphs R, Baron-Cohen S, Tranel D (2002) Impaired Recognition of Social Emotions following Amygdala Damage. Journal of Cognitive Neuroscience 14:1264-1274.

Adolphs R, Tranel D, Damasio H, Damasio A (1994) Impaired recognition of emotion in facial expressions following bilateral damage to the human amygdala. Nature 372:669-672.

Adolphs R, Tranel D, Damasio H, Damasio A (1995) Fear and the human amygdala. J Neurosci 15:5879-5891.

Aghajanian GK, VanderMaele CP (1982) Intracellular identification of central noradrenergic and serotonergic neurons by a new double labeling procedure. J Neuroscience 2:1786-1792.

Akirav I, Richter-Levin G (2002) Mechanisms of Amygdala Modulation of Hippocampal Plasticity. J Neurosci 22:9912-9921.

al Maskati HA, Zbrozyna AW (1989) Cardiovascular and motor components of the defence reaction elicited in rats by electrical and chemical stimulation in amygdala. J Auton Nerv Syst 28:127-131.

Albert CM, Chae CU, Rexrode KM, Manson JE, Kawachi I (2005) Phobic Anxiety and Risk of Coronary Heart Disease and Sudden Cardiac Death Among Women. [Article]. Circulation 111:480-487.

Amaral DG, Price JL, Pitkanen A, Carmichael ST (1992) Anatomical organization of the primate amygdaloid complex. In: The Amygdala: Neurobiological aspects of emotion, memory, and mental dysfunction (Aggleton, J. P., ed), pp 1-66 New York: Wiley-Liss, Inc.

American Psychiatric Association AP (1994) Diagnostic and Statistical Manual of Mental Disorders. Washington, D.C.

Applied Biosystems. 2001. ABI Prism 7700 sequence detection system user bulletin 2: relative quantitation of gene expression. Applied Biosystems, Foster City, Calif. http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf

Anand A, Shekhar A (2003) Brain Imaging Studies in Mood and Anxiety Disorders. Annals of the New York Academy of Sciences 985:370-388.

Andrews JS, Stephens DN (1990) Drug discrimination models in anxiety and depression. Pharmacology & Therapeutics 47:267-280.
Arborelius L, Owens MJ, Plotsky PM, Nemeroff CB (1999) The role of corticotropin-releasing factor in depression and anxiety disorders. J Endocrinol 160:1-12.
Arnsten AFT, Li B-M (2005) Neurobiology of Executive Functions: Catecholamine Influences on Prefrontal Cortical Functions. Biological Psychiatry 57:1377-1384.
Baldwin HA, Rassnick S, Rivier J, Koob GF, Britton KT (1991) CRF antagonist reverses the "anxiogenic" response to ethanol withdrawal in the rat. Psychopharmacology 103:227-232.
Bale TL, Vale WW (2004) CRF AND CRF RECEPTORS: Role in Stress Responsivity and Other Behaviors. Annual Review of Pharmacology and Toxicology 44:525-557.
Bancroft A, Levin ED (2000). Ventral hippocampal [alpha]4[beta]2 nicotinic receptors and chronic nicotine effects on memory. Neuropharmacology 39: 2770-2778.
Bayer KU, LeBel E, McDonald GL, O'Leary H, Schulman H, De Koninck P (2006) Transition from Reversible to Persistent Binding of CaMKII to Postsynaptic Sites and NR2B. J Neurosci 26:1164-1174.
Benes FM, Burke RE, Walsh J, Berretta S, Matzilevich D, Minns M, Konradi C (2004) Acute amygdalar activation induces an upregulation of multiple monoamine G protein coupled pathways in rat hippocampus. Mol Psychiatry 9:932-945.
Bishop SJ (2008) Neural Mechanisms Underlying Selective Attention to Threat. Annals of the New York Academy of Sciences 1129:141-152.
Bishop SJ, Duncan J, Lawrence AD (2004) State Anxiety Modulation of the Amygdala Response to Unattended Threat-Related Stimuli. J Neurosci 24:10364-10368.
Bittencourt JC, Sawchenko PE (2000) Do Centrally Administered Neuropeptides Access Cognate Receptors?: An Analysis in the Central Corticotropin-Releasing Factor System. J Neurosci 20(3):1142-1156.
Blackford JU, Buckholtz JW, Avery SN, Zald DH (2010) A unique role for the human amygdala in novelty detection. NeuroImage 50:1188-1193.
Bliss T, Collingridge G (1993) A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361:31-39.
Boccia MM, Blake MG, Baratti CM, McGaugh JL (2009) Involvement of the basolateral amygdala in muscarinic cholinergic modulation of extinction memory consolidation. Neurobiology of Learning and Memory 91:93-97.
Bodnoff SR, Suranyi-Cadotte B, Quirion R, Meaney MJ (1989) A comparison of the effects of diazepam versus several typical and atypical anti-depressant drugs in an animal model of anxiety. Psychopharmacology 97:277-279.
Bouton ME, Bolles RC (1980) Conditioned fear assessed by freezing and by the suppression of three different baselines. Anim Learn Behav 8:429-434.

Brar BK, Chen A, Perrin MH, Vale W (2004) Specificity and Regulation of Extracellularly Regulated Kinase1/2 Phosphorylation through Corticotropin-Releasing Factor (CRF) Receptors 1 and 2{beta} by the CRF/Urocortin Family of Peptides. Endocrinology 145:1718-1729.

Bremner JD, Licinio J, Darnell A, Krystal JH, Owens MJ, Southwick SM, Nemeroff CB, Charney DS (1997) Elevated CSF corticotropin-releasing factor concentrations in posttraumatic stress disorder. Am J Psychiatry 154:624-629.

Britton KT, Morgan J, Rivier J, Vale W, Koob GF (1985) Chlordiazepoxide attenuates response suppression induced by corticotropin-releasing factor in the conflict test. Psychopharmacology 86:170-174.

Büchel C, Dolan RJ (2000) Classical fear conditioning in functional neuroimaging. Current Opinion in Neurobiology 10:219-223.

Butler PD, Weiss JM, Stout JC, Nemeroff CB (1990) Corticotropin-releasing factor produces fear-enhancing and behavioral activating effects following infusion into the locus coeruleus. J Neurosci 10:176-183.

Cador M, Ahmed SH, Koob GF, Le Moal M, Stinus L (1992) Corticotropin-releasing factor induces a place aversion independent of its neuroendocrine role. Brain Research 597:304-309.

Calandreau L, Trifilieff P, Mons N, Costes L, Marien M, Marighetto A, Micheau J, Jaffard R, Desmedt A (2006) Extracellular Hippocampal Acetylcholine Level Controls Amygdala Function and Promotes Adaptive Conditioned Emotional Response. J Neurosci 26:13556-13566.

Calkhoven CF, Ab G (1996) Multiple steps in the regulation of transcription-factor level and activity. Biochem J 317:329-342.

Cammalleri M, Cervia D, Monte MD, Martini D, Langenegger D, Fehlmann D, Feuerbach D, Pavan B, Hoyer D, Bagnoli P (2006) Compensatory changes in the hippocampus of somatostatin knockout mice: upregulation of somatostatin receptor 2 and its function in the control of bursting activity and synaptic transmission. European Journal of Neuroscience 23:2404-2422.

Campeau S, Davis M (1995) Involvement of the central nucleus and basolateral complex of the amygdala in fear conditioning measured with fear-potentiated startle in rats trained concurrently with auditory and visual conditioned stimuli. J Neurosci 15:2301-2311.

Campeau S, Miserendino MJD, Davis M (1992) Intra-amygdala infusion of the N-Methyl-D-Aspartate receptor antagonist AP5 blocks acquisition but not expression of fear-potentiated startle to an auditory conditioned stimulus. Behavioral Neuroscience 106:569-574.

Carrasco GA, Van de Kar LD (2003) Neuroendocrine pharmacology of stress. European Journal of Pharmacology 463:235-272.

Carrasco MC, Vicens P, Vidal J, Redolat R (2006) Effects of co-administration of bupropion and nicotinic agonists on the elevated plus-maze test in mice. Progress in Neuro-Psychopharmacology and Biological Psychiatry 30:455-462.

Chapell R, Bueno OF, Alvarez-Hernandez X, Robinson LC, Leidenheimer NJ (1998) Activation of Protein Kinase C Induces gamma-Aminobutyric Acid Type A Receptor Internalization in Xenopus Oocytes. J Biol Chem 273:32595-32601.

Chapman WP, Schroeder JR, Guyer G, Brazier MAB, Fager C, Poppen JL, Solomon HC, Yakolev PI (1954) Physiological evidence concerning the importance of the amygdaloid nuclear region in the integration of circulating function and emotion in man. Science 129:949-950.

Chen FM, Bilezikjian LM, Perrin MH, Rivier J, Vale W (1986) Corticotropin releasing factor receptor-mediated stimulation of adenylate cyclase activity in the rat brain. Brain Research 381:49-57.

Chrousos GP (1998) Stressors, Stress, and Neuroendocrine Integration of the Adaptive Response: The 1997 Hans Selye Memorial Lecture. Annals of the New York Academy of Sciences 851:311-335.

Chrousos GP, Gold PW (1992) The Concepts of Stress and Stress System Disorders: Overview of Physical and Behavioral Homeostasis. JAMA 267:1244-1252.

Colbran RJ, Brown AM (2004) Calcium/calmodulin-dependent protein kinase II and synaptic plasticity. Current Opinion in Neurobiology 14:318-327.

Cole BJ, Koob GF (1988) Propranolol antagonizes the enhanced conditioned fear produced by corticotropin releasing factor. Journal of Pharmacology and Experimental Therapeutics 247:902-910.

Conti LH, Foote SL (1995) Effects of pretreatment with corticotropin-releasing factor on the electrophysiological responsivity of the locus coeruleus to subsequent corticotropin-releasing factor challenge. Neuroscience 69:209-219.

Cook CJ (2004) Stress induces CRF release in the paraventricular nucleus, and both CRF and GABA release in the amygdala. Physiology & Behavior 82:751-762.

Cousens GA, Otto T (2003) Neural Substrates of Olfactory Discrimination Learning with Auditory Secondary Reinforcement. I. Contributions of the Basolateral Amygdaloid Complex and Orbitofrontal Cortex. Integrative Physiological & Behavioral Science 38:272-294.

Critchley HD, Mathias CJ, Dolan RJ (2002) Fear Conditioning in Humans: The Influence of Awareness and Autonomic Arousal on Functional Neuroanatomy. Neuron 33:653-663.

Csaba Z, Dournaud P (2001) Cellular biology of somatostatin receptors. Neuropeptides 35:1-23.

Dallman MF, Akana SF, Scribner KA, Bradbury MJ, Walker C, Strack AM, Cascia CS (1992) Stress, feedback and facilitation in the hypothalamo-pituitary-adrenal axis. J Neuroendocrinology 4:517-526.

Dautzenberg FM, Gutknecht E, Linden IVd, Olivares-Reyes JA, Dürrenberger F, Hauger RL (2004) Cell-type specific calcium signaling by corticotropin-releasing factor type 1 (CRF1) and 2a (CRF2(a)) receptors: phospholipase C-mediated responses in human embryonic kidney 293 but not SK-N-MC neuroblastoma cells. Biochemical Pharmacology 68:1833-1844.

Dautzenberg FM, Hauger RL (2002) The CRF peptide family and their receptors: yet more partners discovered. Trends in Pharmacological Sciences 23:71-77.
Dautzenberg FM, Kilpatrick GJ, Hauger RL, Moreau J-L (2001) Molecular biology of the CRH receptors-- in the mood. Peptides 22:753-760.
Davis M (1992) The Role of the Amygdala in Fear and Anxiety. Annual Review of Neuroscience 15:353-375.
Davis M (1998) Are different parts of the extended amygdala involved in fear versus anxiety? Biological Psychiatry 44:1239-1247.
Davis M (2000) The role of the amydala in conditioned and unconditioned fear and anxiety. In: The Amygdala: A functional analysis(Aggleton, J. P., ed), pp 213-287 New York: Oxford University Press.
Davis M, Rainnie D, Cassell M (1994) Neurotransmission in the rat amygdala related to fear and anxiety. Trends in Neurosciences 17:208-214.
Davis M, Whalen PJ (2001) The amygdala: vigilance and emotion. Mol Psychiatry 6:13-34.
de Jong M, Breeman WAP, Kwekkeboom DJ, Valkema R, Krenning EP (2009) Tumor Imaging and Therapy Using Radiolabeled Somatostatin Analogues. Accounts of Chemical Research 42:873-880.
de Kloet ER, Joels M, Holsboer F (2005) Stress and the brain: from adaptation to disease. Nat Rev Neurosci 6:463-475.
De Souza EB (1995) Corticotropin-releasing factor receptors: Physiology, pharmacology, biochemistry and role in central nervous system and immune disorders. Psychoneuroendocrinology 20:789-819.
De Souza EB, Insel TR, Perrin MH, Rivier J, Vale WW, Kuhar MJ (1985) Corticotropin-releasing factor receptors are widely distributed within the rat central nervous system: an autoradiographic study. J Neurosci 5:3189-3203.
Deutch AY, Roth RH (1990) The determinants of stress-induced activation of the prefrontal cortical dopamine system. Prog Brain Res 85:367-402.
Dhar S, Nagy F, McIntosh JM, Sapru HN (2000) Receptor subtypes mediating depressor responses to microinjections of nicotine into medial NTS of the rat. Am J Physiol Regul Integr Comp Physiol 279:R132-140.
DiMicco JA, Samuels BC, Zaretskaia MV, Zaretsky DV (2002) The dorsomedial hypothalamus and the response to stress: Part renaissance, part revolution. Pharmacology Biochemistry and Behavior 71:469-480.
Dunn AJ, Berridge D (1990) Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? Brain Res Rev 15:71-100.
Dunn AJ, File SE (1987) Corticotropin-releasing factor has an anxiogenic action in the social interaction test. Hormones and Behavior 21:193-202.
Dunn JD, Whitener J, (1986) Plasma corticosterone responses to electrical stimulation of the amygdaloid complex: cytoarchitectural specificity. Neuroendocrinology 42:211-217.
Eichenbaum H (2006) Remembering: Functional Organization of the Declarative Memory System. Current Biology 16:R643-R645.

Elberg G, Hipkin RW, Schonbrunn A (2002) Homologous and Heterologous Regulation of Somatostatin Receptor 2. Mol Endocrinol 16:2502-2514.
Engin E, Stellbrink J, Treit D, Dickson CT (2008) Anxiolytic and antidepressant effects of intracerebroventricularly administered somatostatin: Behavioral and neurophysiological evidence. Neuroscience 157:666-676.
Engin E, Treit D (2009) Anxiolytic and antidepressant actions of somatostatin: the role of sst2 and sst3 receptors. Psychopharmacology 206:281-289.
Ergorul C, Eichenbaum H (2004) The hippocamups and memory for "what," "when," and "where". Learn Mem 11:397-405.
Etkin A, Klemenhagen KC, Dudman JT, Rogan MT, Hen R, Kandel ER, Hirsch J (2004) Individual Differences in Trait Anxiety Predict the Response of the Basolateral Amygdala to Unconsciously Processed Fearful Faces. Neuron 44:1043-1055.
Fallon JH, Ciofi P (1992) Distribution of monoamines within the amygdala. In: The Amygdala: Neurobiological Aspects of Emotion, Memory and Mental Dysfunction(Aggleton, J. P., ed), pp 97-114 New York: Weily-Liss, Inc.
Feindel W, Penfield W (1954) localization of discharge in temporal lobe automatism. Neurol Psych 72:603-605.
Feniuk W, Jarvie E, Luo J, Humphrey PPA (2000) Selective somatostatin sst2 receptor blockade with the novel cyclic octapeptide, CYN-154806. Neuropharmacology 39:1443-1450.
File SE (1980) The use of social interaction as a method for detecting anxiolytic activity of chlordiazepoxide-like drugs. Journal of Neuroscience Methods 2:219-238.
File SE, Hyde JR (1978) Can social interaction be used to measure anxiety? Br J Pharmacol 62:19-24.
File SE, Seth P (2003) A review of 25 years of the social interaction test. European Journal of Pharmacology 463:35-53.
Flores CM, Rogers SW, Pabreza LA, Wolfe BB, Kellar KJ (1992) A subtype of nicotinic cholinergic receptor in rat brain is composed of alpha 4 and beta 2 subunits and is up-regulated by chronic nicotine treatment. Molecular Pharmacology 41:31-37.
Furmark T, Fischer H, Wik G, Larsson M, Fredrikson M (1997) The amygdala and individual differences in human fear conditioning. NeuroReport 8:3957-3960.
Gardette R, Faivre-Bauman A, Loudes C, Kordon C, Epelbaum J (1995) Modulation by somatostatin of glutamate sensitivity during development of mouse hypothalamic neurons in vitro. Brain Res Dev Brain Res 86:123-133.
Gean P-W, Chang F-C (1992) Pharamacological characterization of excitaory synaptic potentials in rat basolateral amygdaloid neurons. Synapse 11:1-9.
Gehlert DR, Shekhar A, Morin SM, Hipskind PA, Zink C, Gackenheimer SL, Shaw J, Fitz SD, Sajdyk TJ (2005) Stress and central Urocortin increase anxiety-like behavior in the social interaction test via the CRF1 receptor. European Journal of Pharmacology 509:145-153.

Gerdin MJ, Masana MI, Dubocovich ML (2004) Melatonin-mediated regulation of human MT1 melatonin receptors expressed in mammalian cells. Biochemical Pharmacology 67:2023-2030.
Ghashghaei HT, Barbas H (2002) Pathways for emotion: interactions of prefrontal and anterior temporal pathways in the amygdala of the rhesus monkey. Neuroscience 115:1261-1279.
Giguere V, Labrie F, Cote J, Coy DH, Sueiras-Diaz J, Schally AV (1982) Simulation of cyclic AMP accumulation and corticotropin release by synthetic ovine corticotropin-releasing factor in rat anterior pituitary cells: site of glucocorticoid action. Proceedings of the National Academy of Sciences of the United States of America 79:3466-3469.
Gispen-de Wied CC, Jansen LM (2002) The stress-vulnerability hypothesis in psychotic disorders: focus on the stress response systems. Current Psychiatry Rep 4:166-170.
Goldstein LE, Rasmusson AM, Bunney BS, Roth RH (1996) Role of the Amygdala in the Coordination of Behavioral, Neuroendocrine, and Prefrontal Cortical Monoamine Responses to Psychological Stress in the Rat. J Neurosci 16:4787-4798.
Grace AA, Rosenkranz JA (2002) Regulation of conditioned responses of basolateral amygdala neurons. Physiology & Behavior 77:489-493.
Graeff FG, Silveira MCL, Nogueira RL, Audi EA, Oliveira RMW (1993) Role of the amygdala and periaqueductal gray in anxiety and panic. Behavioural Brain Research 58:123-131.
Graeff FG, Viana MB, Mora PO (1997) Dual role of 5-HT in defense and anxiety. Neuroscience & Biobehavioral Reviews 21:791-799.
Gray TS (1993) Amygdaloid CRF Pathways: Role in Autonomic, Neuroendocrine, and Behavioral Responses to Stress. Annals of the New York Academy of Sciences 697:53-60.
Gray TS, Carney ME, Magnuson DJ (1989) Direct projections from the central amygdaloid nucleus to the hypothalamic paraventricular nucleus: possible role in stress-induced adrenocorticotropin release. Neuroendocrinology 50:433-446.
Greenberg PE, Sisitsky T, Kessler RC, Finkelstein SN, Berndt ER, Davidson JRT, Ballenger JC, Fyer AJ (1999) The Economic Burden of Anxiety Disorders in the 1990s. The Journal of Clincal Psychiatry 60:427-435.
Gutknecht E, Vauquelin G, Dautzenberg FM (2010) Corticotropin-releasing factor receptors induce calcium mobilization through cross-talk with Gq-coupled receptors. European Journal of Pharmacology 642:1-9.
Guy AP, Gardner CR (1985) Pharmacological characterisation of a modified social interaction model of anxiety in the rat. Neurospsychobiology 13:194-200.
Habib KE, Gold PW, Chrousos GP (2001) Neuroendocrinology of stress. Endocrinol Metab Clin North Am 30:695-728; vii-viii.

Habib KE, Weld KP, Rice KC, Pushkas J, Champoux M, Listwak S, Webster EL, Atkinson AJ, Schulkin J, Contoreggi C, Chrousos GP, McCann SM, Suomi SJ, Higley JD, Gold PW (2000) Oral administration of a corticotropin-releasing hormone receptor antagonist significantly attenuates behavioral, neuroendocrine, and autonomic responses to stress in primates. Prodeedings of the National Academy of Sciences of the United States of America 97:6079-6084.

Haller J (2001) The link between stress and the efficacy of anxiolytics. A new avenue of research. Physiology & Behavior 73:337-342.

Hansen TvO, Rehfeld JF, Nielsen FC (1999) Mitogen-Activated Protein Kinase and Protein Kinase A Signaling Pathways Stimulate Cholecystokinin Transcription via Activation of Cyclic Adenosine 3',5'-Monophosphate Response Element-Binding Protein. Mol Endocrinol 13:466-475.

Hauger RL, Risbrough V, Brauns O, Dautzenberg FM (2006) Corticotropin Releasing Factor (CRF) Receptor Signaling in the Central Nervous System: New Molecular Targets. CNS & Neurological Disorders - Drug Targets 5:453-479.

Heilig M, Koob GF, Ekman R, Britton KT (1994) Corticotropin-releasing factor and neuropeptide y: role in emotional integration. Trends in Neurosciences 17:80-85.

Heinrichs SC, Joppa M (2001) Dissociation of arousal-like from anxiogenic-like actions of brain corticotropin-releasing factor receptor ligands in rats. Behavioural Brain Research 122:43-50.

Heinrichs SC, Koob GF (2004) Corticotropin-Releasing Factor in Brain: A Role in Activation, Arousal, and Affect Regulation. Journal of Pharmacology and Experimental Therapeutics 311:427-440.

Heinrichs SC, Lapsansky J, Lovenberg TW, De Souza EB, Chalmers DT (1997) Corticotropin-releasing factor CRF1, but not CRF2, receptors mediate anxiogenic-like behavior. Regulatory Peptides 71:15-21.

Heinrichs SC, Menzaghi F, Pich EM, Britton KT, Koob GF (1995) The Role of CRF in Behavioral Aspects of Stress. Annals of the New York Academy of Sciences 771:92-104.

Heinrichs SC, Joppa M (2001) Dissociation of arousal-like from anxiogenic-like actions of brain corticotropin-releasing factor receptor ligands in rats. Behavioural Brain Research 122:43-50.

Helmstetter FJ, Bellgowan PS (1994) Effects of muscimol applied to the basolateral amygdala on acquisition and expression of contextual fear conditioning in rats. Behavioral Neuroscience 108:1005-1009.

Herman JP, Cullinan WE (1997) Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. Trends in Neurosciences 20:78-84.

Hilton SM, Zbrozyna AW (1963) Amygdaloid region for defence reaction and its efferent pathway to the brainstem. Journal of Physiology 165:160-173.

Hodges H, Green S, Glenn B (1987) Evidence that the amygdala is involved in benzodiazepine and serotonergic effects on punished responding but not on discrimination. Psychopharmacology 92:491-504.

Hofland L, van Koetsveld P, Wouters N, Waaijers M, Reubi J, Lamberts S (1992) Dissociation of antiproliferative and antihormonal effects of the somatostatin analog octreotide on 7315b pituitary tumor cells. Endocrinology 131:571-577.
Horiuchi J, McDowall LM, Dampney RAL (2006) Differential control of cardiac and sympathetic vasomotor activity from the dorsomedial hypothalamus. Clinical and Experimental Pharmacology and Physiology 33:1265-1268.
Huang Y-Y, Martin KC, Kandel ER (2000) Both Protein Kinase A and Mitogen-Activated Protein Kinase Are Required in the Amygdala for the Macromolecular Synthesis-Dependent Late Phase of Long-Term Potentiation. J Neurosci 20:6317-6325.
Hubbard DT, Nakashima BR, Lee I, Takahashi LK (2007) Activation of basolateral amygdala corticotropin-releasing factor 1 receptors modulates the consolidation of contextual fear. Neuroscience 150:818-828.
Huff NC, Rudy JW (2004) The amygdala modulates hippocampus-dependent context memory formation and stores cue-shock associations. Behavioral Neuroscience 118:53-62.
Ikegaya Y, Saito H, Abe K (1995) High-frequency stimulation of the basolateral amygdala facilitates the induction of long-term potentiation in the dentate gyrus in vivo. Neuroscience Research 22:203-207.
Introini-Collison IB, Dalmaz C, McGaugh JL (1996) Amygdala [beta]-Noradrenergic Influences on Memory Storage Involve Cholinergic Activation. Neurobiology of Learning and Memory 65:57-64.
Jonkman S, Markou A (2006) Blockade of nicotinic acetylcholine or dopamine D1-like receptors in the central nucleus of the amygdala or the bed nucleus of the stria terminalis does not precipitate nicotine withdrawal in nicotine-dependent rats. Neuroscience Letters 400:140-145.
Kapp BS, Gallagher M, Underwood MD, McNall CL, Whitehorn D (1982) Cardiovascular responses elicited by electrical stimulation of the amygdala central nucleus in the rabbit. Brain Research 234:251-262.
Kawachi I, Colditz GA, Ascherio A, Rimm EB, Giovannucci E, Stampfer MJ, Willett WC (1994) Coronary Heart Disease/Myocardial Infarction: Prospective Study of Phobic Anxiety and Risk of Coronary Heart Disease in Men. [Report]. Circulation May 89:1992-1997.
Kawaguchi S-y, Hirano T (2002) Signaling Cascade Regulating Long-Term Potentiation of GABAA Receptor Responsiveness in Cerebellar Purkinje Neurons. J Neurosci 22:3969-3976.
Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR, Walters EE (2005) Lifetime Prevalence and Age-of-Onset Distributions of DSM-IV Disorders in the National Comorbidity Survey Replication. Arch Gen Psychiatry 62:593-602.
Killcross S, Robbins TW, Everitt BJ (1997) Different types of fear-conditioned behaviour mediated by separate nuclei within amygdala. Nature 388:377-380.

Kim JJ, Koo JW, Lee HJ, Han J-S (2005) Amygdalar Inactivation Blocks Stress-Induced Impairments in Hippocampal Long-Term Potentiation and Spatial Memory. J Neurosci 25:1532-1539.
Kim JJ, Rison RA, Fanselow MS (1993a) Effects of amygdala, hippocampus, and periaqueductal gray lesions on contextual fear. Behav Neurosci 107:1093-1098.
Kim M, Campeau S, Falls WA, Davis M (1993b) Infusion of the non-NMDA receptor antagonist CNQX into the amygdala blocks the expression of fear-potentiated startle. Behav Neural Biol 59:5-8.
Kluver H, Bucy P (1937) 'Psychic blindness' and other symptoms following bilateral temporal lobectome in rhesus monkeys. American Journal of Physiology 352-353.
Knight DC, Nguyen HT, Bandettini PA (2005) The role of the human amygdala in the production of conditioned fear responses. NeuroImage 26:1193-1200.
Koob GF (1999) Corticotropin-releasing factor, norepinephrine, and stress. Biological Psychiatry 46:1167-1180.
Koob GF, Heinrichs SC (1999) A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. Brain Research 848:141-152.
Kovalovsky D, Refojo D, Liberman AC, Hochbaum D, Pereda MP, Coso OA, Stalla GK, Holsboer F, Arzt E (2002) Activation and Induction of NUR77/NURR1 in Corticotrophs by CRH/cAMP: Involvement of Calcium, Protein Kinase A, and MAPK Pathways. Mol Endocrinol 16:1638-1651.
Krettek JE, Price JL (1978a) Amygdaloid projections to subcortical structures within the basal forebrain and brainstem in the rat and cat. Journal of Comparative Neurology 178:225-254.
Krettek JE, Price JL (1978b) A description of the amygdaloid complex in the rat and cat with observations on intra-amygdaloid axonal connections. Journal of Comparative Neurology 178:255-279.
Larson CL, Schaefer HS, Siegle GJ, Jackson CAB, Anderle MJ, Davidson RJ (2006) Fear Is Fast in Phobic Individuals: Amygdala Activation in Response to Fear-Relevant Stimuli. Biological Psychiatry 60:410-417.
LeDoux J, Cicchetti P, Xagoraris A, Romanski L (1990a) The lateral amygdaloid nucleus: sensory interface of the amygdala in fear conditioning. J Neurosci 10:1062-1069.
LeDoux J, Farb C, Ruggiero D (1990b) Topographic organization of neurons in the acoustic thalamus that project to the amygdala. J Neurosci 10:1043-1054.
LeDoux J, Iwata J, Cicchetti P, Reis D (1988) Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. J Neurosci 8:2517-2529.
LeDoux JE (2000) Emotion Circuits in the Brain. Annual Review of Neuroscience 23:155-184.
LeDoux J (2003) The Emotional Brain, Fear, and the Amygdala. Cellular and Molecular Neurobiology 23:727-738.

Lee Y, Davis M (1997) Role of the Hippocampus, the Bed Nucleus of the Stria Terminalis, and the Amygdala in the Excitatory Effect of Corticotropin-Releasing Hormone on the Acoustic Startle Reflex. J Neurosci 17:6434-6446.

Lee Y, Fitz S, Johnson PL, Shekhar A (2008) Repeated Stimulation of CRF Receptors in the BNST of Rats Selectively Induces Social but not Panic-Like Anxiety. Neuropsychopharmacology.

Leonard AS, Hell JW (1997) Cyclic AMP-dependent Protein Kinase and Protein Kinase C Phosphorylate N-Methyl-d-aspartate Receptors at Different Sites. Journal of Biological Chemistry 272:12107-12115.

Lewis K, Li C, Perrin MH, Blount A, Kunitake K, Donaldson C, Vaughan J, Reyes TM, Gulyas J, Fischer W, Bilezikjian L, Rivier J, Sawchenko PE, Vale WW (2001) Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. Proceedings of the National Academy of Sciences of the United States of America 98:7570-7575.

Liang KC, Lee EHY (1988) Intra-amygdala injections of corticotropin releasing factor facilitate inhibitory avoidance learning and reduce exploratory behavior in rats. Psycopharmacology 96:232-236.

Livak KJ, Schmittgen TD (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Loughlin SE, Fallon JH (1983) Dopaminergic and non-dopaminergic projections to amygdala from substantia nigra and ventral tegmental area. Brain Research 262:334-338.

Lowry CA, Rodda JE, Lightman SL, Ingram CD (2000) Corticotropin-Releasing Factor Increases In Vitro Firing Rates of Serotonergic Neurons in the Rat Dorsal Raphe Nucleus: Evidence for Activation of a Topographically Organized Mesolimbocortical Serotonergic System. J Neurosci 20:7728-7736.

Makino S, Hashimoto K, Gold PW (2002) Multiple feedback mechanisms activating corticotropin-releasing hormone system in the brain during stress. Pharmacology Biochemistry and Behavior 73:147-158.

Maren S (1996) Synaptic transmission and plasticity in the amygdala. Molecular Neurobiology 13:1-22.

Maren S (2003) What the Amygdala Does and Doesn't Do in Aversive Learning. Learning & Memory 10:306-308.

Maren S, Aharonov G, Fanselow MS (1996) Retrograde abolition of conditional fear after excitotoxic lesions in the basolateral amygdala of rats: absence of a temporal gradient. Behavioral Neuroscience 110:718-726.

Maroun M, Richter-Levin G (2003) Exposure to Acute Stress Blocks the Induction of Long-Term Potentiation of the Amygdala-Prefrontal Cortex Pathway In Vivo. J Neurosci 23:4406-4409.

Mascagni F, McDonald AJ (2003) Immunohistochemical characterization of cholecystokinin containing neurons in the rat basolateral amygdala. Brain Research 976:171-184.

Mascagni F, McDonald AJ (2007) A novel subpopulation of 5-HT type 3A receptor subunit immunoreactive interneurons in the rat basolateral amygdala. Neuroscience 144:1015-1024.
Mascagni F, McDonald AJ (2009) Parvalbumin-immunoreactive neurons and GABAergic neurons of the basal forebrain project to the rat basolateral amygdala. Neuroscience 160:805-812.
Matheson GK, Branch BJ, Taylor AN (1971) Effects of amygdaloid stimulation on pituitary-adrenal activity in conscious cats. Brain Research 32:151-167.
Mathew SJ, Manji HK, Charney DS (2008) Novel Drugs and Therapeutic Targets for Severe Mood Disorders. Neuropsychopharmacology 33:2080-2092.
McDonald AJ (1982) Neurons of the lateral and basolateral amygdaloid nuclei: a Golgi study in the rat. Journal of Comparative Neurology 211:293-312.
McDonald AJ (1992) Cell types and intrinsic connections of the amygdala. In: The Amygdala: Neurobiological Aspects of Emotion, Memory, and Mental Dysfunction(Aggleton, J. P., ed), pp 67-96 New York: Wiley-Liss, Inc.
McDonald AJ (1994) Neuronal localization of glutamate receptor subunits in the basolateral amygdala. Neuroreport 6:13.
McDonald AJ (1998) Cortical pathways to the mammalian amygdala. Progress in Neurobiology 55:257-332.
McDonald AJ, Betette RL (2001) Parvalbumin-containing neurons in the rat basolateral amygdala: morphology and co-localization of Calbindin-D28k. Neuroscience 102:413-425.
McDonald AJ, Mascagni F (1996) Immunohistochemical localization of the [beta]2 and [beta]3 subunits of the GABAA receptor in the basolateral amygdala of the rat and monkey. Neuroscience 75:407-419.
McDonald AJ, Mascagni F (2001) Colocalization of calcium-binding proteins and GABA in neurons of the rat basolateral amygdala. Neuroscience 105:681-693.
McDonald AJ, Mascagni F (2002) Immunohistochemical characterization of somatostatin containing interneurons in the rat basolateral amygdala. Brain Research 943:237-244.
McDonald AJ, Mascagni F, Guo L (1996) Projections of the medial and lateral prefrontal cortices to the amygdala: a Phaseolus vulgaris leucoagglutinin study in the rat. Neuroscience 71:55-75.
McDonald AJ, Pearson JC (1989) Coexistence of GABA and peptide immunoreactivity in non-pyramidal neurons of the basolateral amygdala. Neuroscience Letters 100:53-58.
McEwen BS (2007) Physiology and Neurobiology of Stress and Adaptation: Central Role of the Brain. Physiol Rev 87:873-904.
McGaugh JL (2002) Memory consolidation and the amygdala: a systems perspective. Trends in Neurosciences 25:456-461.
McGaugh JL (2004) The amygdala modulates the consolidation of memories of emotionally arousing experiences. Annual Review of Neuroscience 27:1-28.

Meis S, Sosulina L, Schulz S, Hollt V, Pape H-C (2005) Mechanisms of somatostatin-evoked responses in neurons of the rat lateral amygdala. European Journal of Neuroscience 21:755-762.
Millan MJ (2003) The neurobiology and control of anxious states. Progress in Neurobiology 70:83-244.
Moller C, Wiklund L, Sommer W, Thorsell A, Heilig M (1997) Decreased experimental anxiety and voluntary ethanol consumption in rats following central but not basolateral amygdala lesions. Brain Research 760:94-101.
Momiyama T, Zaborszky L (2006) Somatostatin Presynaptically Inhibits Both GABA and Glutamate Release Onto Rat Basal Forebrain Cholinergic Neurons. J Neurophysiol 96:686-694.
Moneta D, Richichi C, Aliprandi M, Dournaud P, Dutar P, Billard JM, Carlo AS, Viollet C, Hannon JP, Fehlmann D, Nunn C, Hoyer D, Epelbaum J, Vezzani A (2002) Somatostatin receptor subtypes 2 and 4 affect seizure susceptibility and hippocampal excitatory neurotransmission in mice. European Journal of Neuroscience 16:843-849.
Morris JS, Buchel C, Dolan RJ (2001) Parallel Neural Responses in Amygdala Subregions and Sensory Cortex during Implicit Fear Conditioning. NeuroImage 13:1044-1052.
Morrison SE, Salzman CD (2010) Re-valuing the amygdala. Current Opinion in Neurobiology 20:221-230.
Muller J, Corodimas KP, Fridel Z, LeDoux JE (1997) Functional inactivation of the lateral and basal nuclei of the amygdala by muscimol infusion prevents fear conditioning to an explicit conditioned stimulus and to contextual stimuli. Behavioral Neuroscience 111:683-691.
Muller JF, Mascagni F, Mcdonald AJ (2003a) Synaptic connections of distinct interneuronal subpopulations in the rat basolateral amygdalar nucleus. The Journal of Comparative Neurology 456:217-236.
Muller JF, Mascagni F, McDonald AJ (2007a) Postsynaptic targets of somatostatin-containing interneurons in the rat basolateral amygdala. The Journal of Comparative Neurology 500:513-529.
Muller JF, Mascagni F, McDonald AJ (2007b) Serotonin-immunoreactive axon terminals innervate pyramidal cells and interneurons in the rat basolateral amygdala. The Journal of Comparative Neurology 505:314-335.
Muller MB, Zimmermann S, Sillaber I, Hagemeyer TP, Deussing JM, Timpl P, Kormann MSD, Droste SK, Kuhn R, Reul JMHM, Holsboer F, Wurst W (2003b) Limbic corticotropin-releasing hormone receptor 1 mediates anxiety-related behavior and hormonal adaptation to stress. Nat Neurosci 6:1100-1107.
Nanda SA, Qi C, Roseboom PH, Kalin NH (2008) Predator stress induces behavioral inhibition and amygdala somatostatin receptor 2 gene expression. Genes, Brain and Behavior 7:639-648.

NanoDrop Technologies Inc. 2007. Technical Support Bulletin 2009 260/280 Ratios NanoDrop® ND-1000 and ND-8000 8-Sample Spectrophotometers. NanoDrop Technologies Inc., Wilmington, DE http://www.biolabanalytical.com.au/analytical/lifescience/nanodrop/pdf/T009-ND-1000-&-ND-8000-Nucleic-Acid-Purity-Ratios.pdf

Nestler EJ, Hope BT, Widnell KL (1993) Drug addiction: A model for the molecular basis of neural plasticity. Neuron 11:995-1006.

Niehoff D, Kuhar M (1983) Benzodiazepine receptors: localization in rat amygdala. J Neurosci 3:2091-2097.

Norbury R, Taylor M, Selvaraj S, Murphy S, Harmer C, Cowen P (2009) Short-term antidepressant treatment modulates amygdala response to happy faces. Psychopharmacology 206:197-204.

Ohno M, Yamamoto T, Watanabe S (1993) Amygdaloid NMDA and muscarinic receptors involved in working memory performance of rats. Physiology & Behavior 54:993-997.

Ottersen O, Ben-Ari Y (1979) Afferent connections to the amygdaloid complex of the rat and cat. I. Projections from the thalamus. J Comp Neurology 187:401-424.

Parham KL, Zervou S, Karteris E, Catalano RD, Old RW, Hillhouse EW (2004) Promoter Analysis of Human Corticotropin-Releasing Factor (CRF) Type 1 Receptor and Regulation by CRF and Urocortin. Endocrinology 145:3971-3983.

Pawlikowski M, Melen-Mucha G (2003) Perspectives of new potential therapeutic applications of somatostatin analogs. Neuro Endocrinol Lett 24:21-27.

Paxinos S, Watson G (eds.) (1986) The Rat Brain in Steriotaxic Coordinates. San Diego: Academic Press.

Paxinos S, Watson G (eds.) (1990) The Rat Brain in Stereotaxic Coordinates. San Diego: Elsevier Academic Press.

Paxinos S, Watson G (eds.) (1998) The Rat Brain in Stereotaxic Coordinates. San Diego: Academic Press.

Pearson G, Robinson F, Beers Gibson T, Xu B-e, Karandikar M, Berman K, Cobb MH (2001) Mitogen-Activated Protein (MAP) Kinase Pathways: Regulation and Physiological Functions. Endocr Rev 22:153-183.

Perrin MH, Vale WW (1999) Corticotropin Releasing Factor Receptors and Their Ligand Family. Annals of the New York Academy of Sciences 885:312-328.

Petersen EN, Braestrup C, Scheel-Krüger J (1985) Evidence that the anticonflict effect of midazolam in amygdala is mediated by the specific benzodiazepine receptors. Neuroscience Letters 53:285-288.

Phelps EA, LeDoux JE (2005) Contributions of the Amygdala to Emotion Processing: From Animal Models to Human Behavior. Neuron 48:175-187.

Phillips RG, LeDoux JE (1992) Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behavioral Neuroscience 106:274-285.

Pickens CL, Saddoris MP, Setlow B, Gallagher M, Holland PC, Schoenbaum G (2003) Different Roles for Orbitofrontal Cortex and Basolateral Amygdala in a Reinforcer Devaluation Task. J Neurosci 23:11078-11084.
Pintér E, Helyes Z, Szolcsányi J (2006) Inhibitory effect of somatostatin on inflammation and nociception. Pharmacology & Therapeutics 112:440-456.
Pitkänen A (2000) Connectivity of the rat amygdaloid complex. In: The Amygdala: A functional analysis(Aggleton, J. P., ed), pp 31-115 New York: Oxford University Press.
Pitkänen A, Savander V, LeDoux JE (1997) Organization of intra-amygdaloid circuitries in the rat: an emerging framework for understanding functions of the amygdala. Trends in Neurosciences 20:517-523.
Quirk GJ, Mueller D (2007) Neural Mechanisms of Extinction Learning and Retrieval. Neuropsychopharmacology 33:56-72.
Radulovic J, Ruhmann A, Liepold T, Spiess J (1999) Modulation of Learning and Anxiety by Corticotropin-Releasing Factor (CRF) and Stress: Differential Roles of CRF Receptors 1 and 2. J Neurosci 19:5016-5025.
Rainnie DG (1999) Serotonergic Modulation of Neurotransmission in the Rat Basolateral Amygdala. J Neurophysiol 82:69-85.
Rainnie DG, Asprodini EK, Shinnick-Gallagher P (1991a) Excitatory transmission in the basolateral amygdala. J Neurophysiol 66:986-998.
Rainnie DG, Asprodini EK, Shinnick-Gallagher P (1991b) Inhibitory transmission in the basolateral amygdala. J Neurophysiol 66:999-1009.
Rainnie DG, Bergeron R, Sajdyk TJ, Patil M, Gehlert DR, Shekhar A (2004) Corticotrophin Releasing Factor-Induced Synaptic Plasticity in the Amygdala Translates Stress into Emotional Disorders. J Neurosci 24:3471-3479.
Rauch SL, Shin LM, Wright CI (2003) Neuroimaging Studies of Amygdala Function in Anxiety Disorders. Annals of the New York Academy of Sciences 985:389-410.
Reul JMHM, Holsboer F (2002) Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. Current Opinion in Pharmacology 2:23-33.
Reyes TM, Lewis K, Perrin MH, Kunitake KS, Vaughan J, Arias CA, Hogenesch JB, Gulyas J, Rivier J, Vale WW, Sawchenko PE (2000) Urocortin II: A member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. Proceedings of the National Academy of Sciences of the United States of America 98:2843-2848.
Richardson MP, Strange BA, Dolan RJ (2004) Encoding of emotional memories depends on amygdala and hippocampus and their interactions. Nat Neurosci 7:278-285.
Richter-Levin G, Maroun M (2010) Stress and Amygdala Suppression of Metaplasticity in the Medial Prefrontal Cortex. Cereb Cortex bhp311.

Risbrough VB, Hauger RL, Roberts AL, Vale WW, Geyer MA (2004) Corticotropin-Releasing Factor Receptors CRF1 and CRF2 Exert Both Additive and Opposing Influences on Defensive Startle Behavior. J Neurosci 24:6545-6552.

Rivier CL, Plotsky PM (1986) Mediation by Corticotropin Releasing Factor (CRF) of Adenohypophysial Hormone Secretion. Annual Review of Physiology 48:475-494.

Roozendaal B, Brunson KL, Holloway BL, McGaugh JL, Baram TZ (2002) Involvement of stress-released corticotropin-releasing hormone in the basolateral amygdala in regulating memory consolidation. Proceedings of the National Academy of Sciences of the United States of America 99:13908-13913.

Roozendaal B, Van der Zee EA, Hensbroek RA, Maat H, Luiten PGM, Koolhaas JM, Bohus B (1996) Muscarinic acetylcholine receptor immunoreactivity in the amygdala--II. Fear-induced plasticity. Neuroscience 76:75-83.

Rosenkranz JA, Grace AA (1999) Modulation of Basolateral Amygdala Neuronal Firing and Afferent Drive by Dopamine Receptor Activation In Vivo. J Neurosci 19:11027-11039.

Rosenkranz JA, Grace AA (2001) Dopamine Attenuates Prefrontal Cortical Suppression of Sensory Inputs to the Basolateral Amygdala of Rats. J Neurosci 21:4090-4103.

Rosenkranz JA, Grace AA (2002) Cellular Mechanisms of Infralimbic and Prelimbic Prefrontal Cortical Inhibition and Dopaminergic Modulation of Basolateral Amygdala Neurons In Vivo. J Neurosci 22:324-337.

Rosenkranz JA, Moore H, Grace AA (2003) The Prefrontal Cortex Regulates Lateral Amygdala Neuronal Plasticity and Responses to Previously Conditioned Stimuli. J Neurosci 23:11054-11064.

Rosenkranz JA, Venheim ER, Padival M (2010) Chronic Stress Causes Amygdala Hyperexcitability in Rodents. Biological Psychiatry 67:1128-1136.

Ross SA, Wong JYF, Clifford JJ, Kinsella A, Massalas JS, Horne MK, Scheffer IE, Kola I, Waddington JL, Berkovic SF, Drago J (2000) Phenotypic Characterization of an alpha 4 Neuronal Nicotinic Acetylcholine Receptor Subunit Knock-Out Mouse. J Neurosci 20:6431-6441.

Rostkowski AB, Teppen TL, Peterson DA, Urban JH (2009) Cell-specific expression of neuropeptide Y Y1 receptor immunoreactivity in the rat basolateral amygdala. The Journal of Comparative Neurology 517:166-176.

Sajdyk TJ, Fitz SD, Shekhar A (2006) The role of neuropeptide Y in the amygdala on corticotropin-releasing factor receptor-mediated behavioral stress responses in the rat. Stress: The International Journal on the Biology of Stress 9:21-28.

Sajdyk TJ, Gehlert DR (2000) Astressin, a corticotropin releasing factor antagonist, reverses the anxiogenic effects of urocortin when administered into the basolateral amygdala. Brain Research 877:226-234.

Sajdyk TJ, Schober DA, Gehlert DR, Shekhar A (1999) Role of corticotropin-releasing factor and urocortin within the basolateral amygdala of rats in anxiety and panic responses. Behavioural Brain Research 100:207-215.
Sajdyk TJ, Shekhar A (1997) Excitatory amino acid receptors in the basolateral amygdala regulate anxiety responses in the social interaction test. Brain Research 764:262-264.
Sajdyk TJ, Shekhar A (2000) Sodium lactate elicits anxiety in rats after repeated GABA receptor blockade in the basolateral amygdala. European Journal of Pharmacology 394:265-273.
Sajdyk TJ, Shekhar A, Gehlert DR (2004) Interactions between NPY and CRF in the amygdala to regulate emotionality. Neuropeptides 38:225-234.
Sajdyk TJ, Fitz SD, Shekhar A (2006) The role of neuropeptide Y in the amygdala on corticotropin-releasing factor receptor-mediated behavioral stress responses in the rat. Stress: The International Journal on the Biology of Stress 9:21-28.
Sananbenesi F, Fischer A, Schrick C, Spiess J, Radulovic J (2003) Mitogen-Activated Protein Kinase Signaling in the Hippocampus and Its Modulation by Corticotropin-Releasing Factor Receptor 2: A Possible Link between Stress and Fear Memory. J Neurosci 23:11436-11443.
Sánchez-Muñoz I, Sánchez-Franco F, Vallejo M, Fernández A, Palacios N, Fernández M, Cacicedo L (2010) Activity-dependent somatostatin gene expression is regulated by cAMP-dependent protein kinase and Ca2+-calmodulin kinase pathways. Journal of Neuroscience Research 88:825-836.
Sanders SK, Morzorati SL, Shekhar A (1995) Priming of experimental anxiety by repeated subthreshold GABA blockade in the rat amygdala. Brain Research 699:250-259.
Sanders SK, Shekhar A (1991) Blockade of GABAA receptors in the region of the anterior basolateral amygdala of rats elicits increases in heart rate and blood pressure. Brain Research 567:101-110.
Sanders SK, Shekhar A (1995) Regulation of anxiety by GABAA receptors in the rat amygdala. Pharmacology Biochemistry and Behavior 52:701-706.
Sanders (2001) Cardiovascular and behavioral effects of GABA manipulation in the region of the anterior basolateral amygdala of rats. Indiana University Medical Neuroscience program PhD dissertation pg. 12.
Sarter M, Gehring WJ, Kozak R (2006) More attention must be paid: The neurobiology of attentional effort. Brain Research Reviews 51:145-160.
Scheel-Krüger J, Petersen eN (1982) Anticonflict effect of the benzodiazepines mediated by a gabaergic mechanism in the amygdala. European Journal of Pharmacology 82:115-116.
Schoenbaum G, Roesch M (2005) Orbitofrontal Cortex, Associative Learning, and Expectancies. Neuron 47:633-636.
Schoenbaum G, Setlow B, Saddoris MP, Gallagher M (2003) Encoding Predicted Outcome and Acquired Value in Orbitofrontal Cortex during Cue Sampling Depends upon Input from Basolateral Amygdala. Neuron 39:855-867.

Selcher JC, Weeber EJ, Varga AW, Sweatt JD, Swank M (2002) Book Review: Protein Kinase Signal Transduction Cascades in Mammalian Associative Conditioning. The Neuroscientist 8:122-131.
Selden NRW, Everitt BJ, Jarrard LE, Robbins TW (1991) Complementary roles for the amygdala and hippocampus in aversive conditioning to explicit and contextual cues. Neuroscience 42:335-350.
Selye H (1976) Fourty years of stress research: principal remaining problems and misconceptions. Canadian Medical Association Journal 115:53-56.
Semba K (2000) Multiple output pathways of the basal forebrain: organization, chemical heterogeneity, and roles in vigilance. Behav Brain Res 115:117-141.
Servillo G, Della Fazia MA, Sassone-Corsi P (2002) Coupling cAMP Signaling to Transcription in the Liver: Pivotal Role of CREB and CREM. Experimental Cell Research 275:143-154.
Shekhar A (1993) GABA receptors in the region of the dorsomedial hypothalamus of rats regulate anxiety in the elevated plus-maze test. I. Behavioral measures. Brain Research 627:9-16.
Shekhar A, Sims LS, Bowsher RR (1993) GABA receptors in the region of the dorsomedial hypothalamus of rats regulate anxiety in the elevated plus-maze test. II. Physiological measures. Brain Research 627:17-24.
Shekhar A, Sajdyk TJ, Gehlert DR, Rainnie DG (2003) The Amygdala, Panic Disorder, and Cardiovascular Responses. Ann NY Acad Sci 985:308-325.
Shekhar A, Truitt W, Rainnie D, Sajdyk T (2005) Role of stress, corticotrophin releasing factor (CRF) and amygdala plasticity in chronic anxiety. Stress: The International Journal on the Biology of Stress 8:209-219.
Sherrin T, Blank T, Saravana R, Rayner M, Spiess J, Todorovic C (2009) Region specific gene expression profile in mouse brain after chronic corticotropin releasing factor receptor 1 activation: the novel role for diazepam binding inhibitor in contextual fear conditioning. Neuroscience 162:14-22.
Sherrin T, Todorovic C, Zeyda T, Tan CH, Wong PTH, Zhu YZ, Spiess J (2008) Chronic stimulation of corticotropin-releasing factor receptor 1 enhances the anxiogenic response of the cholecystokinin system. Mol Psychiatry 14:1143-1143.
Sierra-Mercado DJ, Corcoran KA, Lebrón-Milad K, Quirk GJ (2006) Inactivation of the ventromedial prefrontal cortex reduces expression of conditioned fear and impairs subsequent recall of extinction. European Journal of Neuroscience 24:1751-1758.
Silverman AJ, Hoffman DL, Zimmerman EA (1981) The descending afferent connections of the paraventricular nucleus of the hypothalamus (PVN). Brain Research Bulletin 6:47-61.
Sim H-B, Kang E-H, Yu B-H (2010) Changes in cerebral cortex and limbic brain functions after short-term paroxetine treatment in panic disorder: an [18F] FDG-PET pilot study. Psychiatry Investigation 7:215-219.

Smoller JW, Pollack MH, Wassertheil-Smoller S, Jackson RD, Oberman A, Wong ND, Sheps D (2007) Panic Attacks and Risk of Incident Cardiovascular Events Among Postmenopausal Women in the Women's Health Initiative Observational Study. Arch Gen Psychiatry 64:1153-1160.

Spiess J, Dautzenberg FM, Sydow S, Hauger RL, Rühmann A, Blank T, Radulovic J (1998) Molecular Properties of the CRF Receptor. Trends in Endocrinology and Metabolism 9:140-145.

Spiga F, Lightman SL, Shekhar A, Lowry CA (2006) Injections of urocortin 1 into the basolateral amygdala induce anxiety-like behavior and c-Fos expression in brainstem serotonergic neurons. Neuroscience 138:1265-1276.

Steckler T, Holsboer F (1999) Corticotropin-releasing hormone receptor subtypes and emotion. Biological Psychiatry 46:1480-1508.

Stenzel-Poore M, Heinrichs S, Rivest S, Koob G, Vale W (1994) Overproduction of corticotropin-releasing factor in transgenic mice: a genetic model of anxiogenic behavior. J Neurosci 14:2579-2584.

Stock G, Rupprecht U, Stumpf H, Schlor KH (1981) Cardiovascular changes during arousal elicited by stimulation of amygdala, hypothalamus and locus coeruleus. J Auton Nerv Syst 3:503-510.

Sutton RE, Koob GF, Le Moal M, Rivier J, Vale W (1982) Corticotropin releasing factor produces behavioural activation in rats. Nature 297:331-333.

Swerdlow NR, Geyer MA, Vale WW, Koob GF (1986) Coricotropin-releasing factor potentiates acoustic startle in rats: Blockade by chlordiazepoxide. Psychopharmacology 88:147-152.

Takahashi LK (2001) Role of CRF1 and CRF2 receptors in fear and anxiety. Neuroscience and Biobehavioral Reviews 25:627-636.

Tao J, Hildebrand ME, Liao P, Liang MC, Tan G, Li S, Snutch TP, Soong TW (2008) Activation of corticotropin-releasing factor receptor 1 selectively inhibits Cav3.2 T-type calcium channels. Molecular Pharmacology 73:1596-1609.

Tao J, Li S (2005) Effects of urocortin via ion mechanisms or CRF receptors? Biochemical and Biophysical Research Communications 336:731-736.

Thielen SK, Shekhar A (2002) Amygdala priming results in conditioned place avoidance. Pharmacology Biochemistry and Behavior 71:401-406.

Timpl P, Spanagel R, Sillaber I, Kresse A, Reul JMHM, Stalla GK, Blanquet V, Steckler T, Holsboer F, Wurst W (1998) Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. Nat Genet 19:162-166.

Truitt W, Sajdyk T, Dietrich A, Oberlin B, McDougle C, Shekhar A (2007) From anxiety to autism: spectrum of abnormal social behaviors modeled by progressive disruption of inhibitory neuronal function in the basolateral amygdala in Wistar rats. Psychopharmacology 191:107-118.

Truitt WA, Johnson PL, Dietrich AD, Fitz SD, Shekhar A (2009) Anxiety-like behavior is modulated by a discrete subpopulation of interneurons in the basolateral amygdala. Neuroscience 160:284-294.

Tsigos C, Chrousos GP (2002) Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. Journal of Psychosomatic Research 53:865-871.
Turner BH, Herkenham M (1991) Thalamoamygdaloid projections in the rat: A test of the amygdala's role in sensory processing. The Journal of Comparative Neurology 313:295-325.
Tuunanen J, Halonen T, Pitkänen A (1997) Decrease in somatostatin-immunoreactive neurons in the rat amygdaloid complex in a kindling model of temporal lobe epilepsy. Epilepsy Research 26:315-327.
Tye KM, Janak PH (2007) Amygdala Neurons Differentially Encode Motivation and Reinforcement. J Neurosci 27:3937-3945.
Uhl W, Anghelacopoulos SE, Friess H, Büchler MW (1999) The Role of Octreotide and Somatostatin in Acute and Chronic Pancreatitis. Digestion 60:23-30.
Upadhya SC, Smith TK, Hegde AN (2004) Ubiquitin-proteasome-mediated CREB repressor degradation during induction of long-term facilitation. Journal of Neurochemistry 91:210-219.
Uryu K, Okumura T, Shibasaki T, Sakanaka M (1992) Fine structure and possible origins of nerve fibers with corticotropin-releasing factor-like immunoreactivity in the rat central amygdaloid nucleus. Brain Research 577:175-179.
Vale W, Spiess J, Rivier C, Rivier J (1981) Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science 13:1394-1397.
Valentino RJ, Rudoy C, Saunders A, Liu XB, Van Bockstaele EJ (2001) Corticotropin-releasing factor is preferentially colocalized with excitatory rather than inhibitory amino acids in axon terminals in the peri-locus coeruleus region. Neuroscience 106:375-384.
Valentino RJ, Van Bockstaele E (2008) Convergent regulation of locus coeruleus activity as an adaptive response to stress. European Journal of Pharmacology 583:194-203.
van Marle HJF, Hermans EJ, Qin S, Fernández G (2009) From Specificity to Sensitivity: How Acute Stress Affects Amygdala Processing of Biologically Salient Stimuli. Biological Psychiatry 66:649-655.
van Praag HM (2004) Can stress cause depression? Progress in Neuro-Psychopharmacology and Biological Psychiatry 28:891-907.
Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, Chan R, Turnbull AV, Lovejoy D, Rivier C, Rivier J, Sawchenko PE, Vale W (1995) Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. Nature 378:287-292.
Viollet C, Lepousez G, Loudes C, Videau C, Simon A, Epelbaum J (2008) Somatostatinergic systems in brain: Networks and functions. Molecular and Cellular Endocrinology 286:75-87.

Viollet C, Vaillend C, Videau C, Bluet-Pajot MT, Ungerer A, L'Heritier A, Kopp C, Potier B, Billard JM, Schaeffer J, Smith RG, Rohrer SP, Wilkinson H, Zheng H, Epelbaum J (2000) Involvement of sst2 somatostatin receptor in locomotor, exploratory activity and emotional reactivity in mice. European Journal of Neuroscience 12:3761-3770.

Walker DL, Davis M (1997) Double Dissociation between the Involvement of the Bed Nucleus of the Stria Terminalis and the Central Nucleus of the Amygdala in Startle Increases Produced by Conditioned versus Unconditioned Fear. J Neurosci 17:9375-9383.

Wallace DM, Magnuson DJ, Gray TS (1992) Organization of amygdaloid projections to brainstem dopaminergic, noradrenergic, and adrenergic cell groups in the rat. Brain Research Bulletin 28:447-454.

Washburn M, Moises H (1992a) Electrophysiological and morphological properties of rat basolateral amygdaloid neurons in vitro. J Neurosci 12:4066-4079.

Washburn MS, Moises HC (1992b) Inhibitory responses of rat basolateral amygdaloid neurons recorded in vitro. Neuroscience 50:811-830.

Washburn MS, Moises HC (1992c) Muscarinic Responses of Rat Basolateral Amygdaloid Neurons Recorded in vitro. Journal of Physiology 449:121-154.

Way SH, Feniuk W, Kidd EJ, Humphrey PPA (1996) A quantitative autoradiographical study on the distribution of somatostatin sst2 receptors in the rat central nervous system using [125I]-BIM-23027. Neuropharmacology 35:1109-1120.

Weiskrantz L (1956) Behavioral changes associated with ablation of the amygdaloid complex in monkeys. J Comp Physiol Psychol 4:381-391.

Whalen PJ (1998) Fear, Vigilance, and Ambiguity: Initial Neuroimaging Studies of the Human Amygdala. Current Directions in Psychological Science (Wiley-Blackwell) 7:177-188.

Whalen PJ, Shin LM, Somerville LH, McLean AA, Kim H (2002) Functional neuroimaging studies of the amygdala in depression. Semin Clin Neuropsychiatry 7:234-242.

Whiting P, Schoepfer R, Lindstrom J, Priestley T (1991) Structural and pharmacological characterization of the major brain nicotinic acetylcholine receptor subtype stably expressed in mouse fibroblasts. Molecular Pharmacology 40:463-472.

Wietfeld D, Heinrich N, Furkert J, Fechner K, Beyermann M, Bienert M, Berger H (2004) Regulation of the coupling to different G proteins of rat corticotropin-releasing factor receptor type 1 in human embryonic kidney 293 cells. J Biol Chem 279:38386-38394.

Willner P (1990) Animal models of depression: An overview. Pharmacology & Therapeutics 45:425-455.

Willner P, Mitchell PJ (2002) The validity of animal models of predisposition to depression. [Review]. Behavioural Pharmacology 13:169-188.

Winstanley CA, Theobald DEH, Cardinal RN, Robbins TW (2004) Contrasting Roles of Basolateral Amygdala and Orbitofrontal Cortex in Impulsive Choice. J Neurosci 24:4718-4722.
Xiao Y, Meyer EL, Thompson JM, Surin A, Wroblewski J, Kellar KJ (1998) Rat α3/β4 Subtype of Neuronal Nicotinic Acetylcholine Receptor Stably Expressed in a Transfected Cell Line: Pharmacology of Ligand Binding and Function. Molecular Pharmacology 54:322-333.
Yang S-K, Parkington HC, Epelbaum J, Keating DJ, Chen C (2007) Somatostatin decreases voltage-gated Ca2+ currents in GH3 cells through activation of somatostatin receptor 2. Am J Physiol Endocrinol Metab 292:E1863-1870.
Yang TT, Menon V, Eliez S, Blasey C, White CD, Reid AJ, Gotlib IH, Reiss AL (2002) Amygdalar activation associated with positive and negative facial expressions. Neuroreport 13:1737-1741.
Yaniv D, Desmedt A, Jaffard R, Richter-Levin G (2004) The amygdala and appraisal processes: stimulus and response complexity as an organizing factor. Brain Research Reviews 44:179-186.
Yehuda R (1997) Sensitization of the Hypothalamic-Pituitary-Adrenal Axis in Posttraumatic Stress Disorder. Annals of the New York Academy of Sciences 821:57-75.
Yehuda R, Halligan SL, Bierer LM (2001) Relationship of parental trauma exposure and PTSD to PTSD, depressive and anxiety disorders in offspring. Journal of Psychiatric Research 35:261-270.
Zald DH (2003) The human amygdala and the emotional evaluation of sensory stimuli. Brain Research Reviews 41:88-123.
Zald DH, Pardo JV (1997) Emotion, olfaction, and the human amygdala: Amygdala activation during aversive olfactory stimulation. Proceedings of the National Academy of Sciences of the United States of America 94:4119-4124.
Zald DH, Pardo JV (2000) Functional neuroimaging of the olfactory system in humans. International Journal of Psychophysiology 36:165-181.
Zald DH, Pardo JV (2002) The Neural Correlates of Aversive Auditory Stimulation. NeuroImage 16:746-753.

CURRICULUM VITAE

Denise Gaskins

EDUCATION:

2011 Indiana University, Indianapolis, IN
Master of Science
Medical Neuroscience

1980 Rhode Island College, North Providence, RI
Bachelor of Science
Major: Psychobiology; Minor: Chemistry
Graduated Cum laude

RESEARCH INTERESTS:

A: Mechanisms behind mood disorders at the molecular and cellular level
B: Glia motility

RESEARCH EXPERIENCE:

Fall 2006 - Spring 2011 Indiana University, Indianapolis, IN

Thesis project: Elucidating mechanism(s) involved in the persistent anxiety-like phenotype following activation of corticotropin-releasing factor receptors in the basolateral amygdala.

January 2004 - Fall 2006 Indiana University, Indianapolis, IN

Project 1: Develop an animal model of bipolar disorder by looking at glutamatergic dysfunction in the amygdala as a model for cycling in bipolar disorder.

Project 2: Study the role of astrocytes in mood disorder.

Project 3: Role of CRF receptors in the Bed Nucleus of the stria terminalis as they pertain to anxiety disorder

PUBLICATIONS:

Peer Review Original Article:

Lee Y, **Gaskins D**, Anand A, Shekhar A (2007) Glia mechanisms in mood regulation: a novel model of mood disorders. Psychopharmacology 191:55-65.

Ray B, **Gaskins DL**, Sajdyk T, Spence J, Fitz S, Shekhar A, Lahiri DK (2011) Restraint stress and repeated CRF receptor activation in the amygdala both increase amyloid β precursor protein (APP) and amyloid-β (Aβ) peptide but have divergent effects on BDNF and pre-synaptic proteins in the prefrontal cortex of rats. Neuroscience 184:139-150.

Conference Abstracts:

D.L. Gaskins, P.L. Johnson, P. Kelly, A. Dietrich, W. A. Truitt, A. Shekhar. "Somatostatin-2 receptors in the basolateral amygdala of rats modulate behavioral priming and anxiety-like response." *The Society for Neuroscience 38th Annual Meeting*, Washington, DC, November 2008.

B. Ray, **D.L. Gaskins**, J.M. Long, D.K. Lahari. "Acute restraint stress decreases neurotrophic factors (BDNF & GDNF) and presynaptic protein Syntaxin in rat cortex and its relevance in neurodegeneration." *The Society for Neuroscience 38th Annual Meeting*, Washington, DC, November 2008.

D.L. Gaskins, A. Dietrich, A. Shekhar. "Decreased Social Interaction Observed with Urocortin Priming of the Amygdala is an Unconditioned Response." *The Society for Neuroscience 37th Annual Meeting*, San Diego, CA, November 2007.

D.L. Gaskins, A. Shekhar. "Effects of Disrupting the Glutamate Reuptake Within the Amygdala on Social and Diurnal Activity." *The Society for Neuroscience 35th Annual Meeting*, Washington, DC, November 2005.

D.L. Gaskins, Y. Lee, A. Shekhar. "BEHAVIORAL EFFECTS RESULTING FROM THE DISRUPTION OF GLIAL FUNCTIONING IN THE BASOLATERAL AMYGDALA." *The International Behavioral Neuroscience Society***.** Santa Fe, NM, June 2005.

D.L. Gaskins, Y. Lee, A. Shekhar. "Behavioral Effects Resulting From the Disruption of Glia/Neuronal Communication in the Basolateral Amygdala". Gordon Research Conference (GRC), *Glial Biology: Functional Interactions Among Glia & Neurons*. Ventura, CA, March 2005.

D.L. Gaskins, T.J. Sajdyk, D.R. Gehlert, A. Shekhar. "Microinjections of Urocortin into the Bed Nucleus of the Stria Terminalis (BNST) and its Relationship to the Stress Response. *5th World Congress on Stress*. London, England, June 2004.

Invited Speaker:

Indiana University School of Medicine, Indianapolis, IN, Institute of Psychiatric Research Seminar Series, April 2004, "Novel Animal Model for Affective Disorder"

Manuscripts in Progress:

Gaskins DL, Johnson PL, Dietrich A, Sajdyk T, Shekhar A. Persistent anxiety-like behavior following repeated activation of corticotropin-releasing factor (CRF) receptors of the basolateral amygdala (BLA) in rats is independent of the priming context conditions.

Gaskins DL, Dietrich A, Minick P, Sonal S, Shekhar A, Truitt WA. Role of Somatostatin 2 receptor's in the development of persistent anxiety-like behavior following repeated activation of basolateral amygdala (BLA) corticotropin-releasing factor (CRF) receptors in rats.

AWARDS AND SOCIETIES:

2006 Poster award. *Indianapolis Society for Neuroscience*, Indianapolis, IN
2005 Scottish Rite Fellowship
2005 IUSM Graduate Student Travel Award
2005 Hingtgen Travel Award

TECHNIQUES ACQUIRED

Surgeries:Telemetry probe implant with arterial catheterizations, Stereotaxic surgeries, Mini-osmotic pump implants

Procedures: Site-specific delivery of pharmacological agents to select brain regions (microinjections), Perfusions, Micropunches, Cell counts, Immunohistochemistry, PCR

Behavior Tests: Social Interaction, Locomotion, Open Field, Forced Swim, Startle, and Elevated Plus Maze

Data Analysis Tools: SAS, SPSS, Graphpad Prism