ROLES OF NOREPINEPHRINE AND CORTICOTROPIN-RELEASING FACTOR IN THE MODULATION OF SENSORIMOTOR GATING

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A dissertation submitted in partial fulfillment of the requirement for the degree of

Doctor of Philosophy

(Neuroscience)

At the University of Wisconsin-Madison

2013

Date of final oral exam: 05/15/2013

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ABSTRACT

ROLES OF NOREPINEPHRINE AND CORTICOTROPIN-RELEASING FACTOR IN THE MODULATION OF SENSORIMOTOR GATING

Abha Karki Rajbhandari

Under the supervision of Dr. Vaishali Bakshi at the University of Wisconsin-Madison

Prepulse inhibition of the startle response (PPI), a measure of pre-attentional information-processing mechanisms like sensorimotor gating, refers to the ability of a weak stimulus to reduce the magnitude of the startle response to a subsequent startling stimulus. Sensorimotor gating is deficient in disorders like posttraumatic-stress (PTSD) and schizophrenia, and thought to contribute to cognitive fragmentation via entry of potentially irrelevant stimuli into the cognitive milieu. Repeated or intense stress exacerbates these illnesses and activates the corticotropin-releasing factor (CRF) and norepinephrine (NE) systems in forebrain regions like the amygdala. Yet, the neural substrates for stress-related exacerbation of these disorders are not understood. Therefore, the experiments in this thesis were designed to systematically investigate whether NE, CRF, and stress-induced plasticity between these systems in the amygdala leads to startle and PPI abnormalities similar to those as observed in PTSD and schizophrenia. The findings illustrate that acute or repeated pharmacological

stimulation of NE receptors in amygdala sub-regions including the basolateral amygdala (BLA) and bed nucleus of the stria terminalis (BNST) disrupts PPI, while in the central amygdala (CeA) it does not, indicating a site-specific effect of NE in regulating PPI. Acute CRF receptor stimulation in these brains regions did not affect PPI. Yet, repeated CRF receptor activation or psychogenic stress (predator exposure) led to a long-lasting PPI disruption and startle enhancement with subsequent low-dose NE infusion in the BLA. CRF1 receptor antagonism in the BLA prior to each predator stress prevented this effect, but interestingly, blocking CRF1 receptors after stress or right before the low-dose NE challenge did not, indicating that the NE receptor hypersensitivity begins with the activation of CRF1 receptors. These findings indicate that BLA could be a neural substrate that modulates a long-lasting stress-induced deterioration of basic information-processing like PPI. Lastly, to understand possible cellular mechanisms behind this NE receptor hypersensitivity, this thesis for the first time illustrated co-localization of CRF1 and alpha1 NE receptors on neuronal cells of the BLA that innervate the nucleus accumbens (NAcc), but not with presumptive GABA interneurons or glial cells. Therefore, intense psychological-trauma-induced NE receptor hypersensitivity in the BLA could modulate PPI via altered signaling to NAcc. Taken together, this thesis generated several novel findings regarding CRF and NE interactions in modulating sensorimotor gating and startle, both of which are compromised in disorders like PTSD and schizophrenia. Understanding the neuronal mechanisms that govern these functions could help develop better treatments for these patients.

THANKS AND ACKNOWLEDGEMENTS

First of all, I would like to thank my mentor Dr. Vaishali Bakshi for not just being a wonderful mentor, but also a good friend and a source of inspiration. Throughout my graduate studies, she provided me with invaluable skills for doing research, offered me great advice, relentless guidance, positively guided my critical thinking abilities, and taught me many valuable life lessons that I will cherish throughout my scientific career. In the lab, she gave me independence to do research and develop ideas, but was always available to talk about projects, my progress and any other scientific/non-scientific issues. Overall, she has helped me excel and become a better scientist in every single way.

I also want to thank Dr. Brian Baldo for being more or less a co-mentor to me and for often taking the time out of his busy schedule to provide support and advice when I needed. I want to especially thank him for advising and guiding me with my anatomy project (his keen eye for analyzing anatomical structures is inspirational to me!), and also for sitting down with me, hours at a time, during my application process to post-doctoral labs.

I also deeply thank my thesis committee members Dr. Craig Berridge, Dr. Lingjun Li, Dr. Elizabeth Meyerand and Dr. Patrick Roseboom for invaluable feedback during my preliminary exam, committee meetings, and, in general, providing me with excellent guidance for my dissertation work, for positively challenging my scientific thoughts, shaping my critical thinking abilities and helping me strive for higher standards to become better scientist.

I also thank all the members from Bakshi and Baldo labs including Karen, Jesus, Ryan, Sarah Newman, Ken and Sarah Baisley, and also Rothem for always being willing to lend helping hands, reading my papers, for allowing to share thoughts, stories, ideas, and problems from both science and the outside world.

I also want to deeply thank the members of the Neuroscience Training Program, especially Dr. Tom Yin, Jenny, Jessica, Allison and Tera for the help, support, timely reminders and many more throughout my time in the program.

My parents have been a great source of inspiration to me. I thank them for all the sacrifices they made so that I could achieve highest level of education, and for instilling in me the value of hard work and persistence, which I have always tried my best to follow. I also thank them for regular SKYPE conversations, always providing me with words of encouragement and listening patiently to everything I had to share.

Last but definitely not the least, I cannot even start to describe how much the support, care and love of my husband Prashant Rajbhandari has meant to me throughout my scientific endeavors. He was the one to instill in me the desire to pursue graduate school and has been my greatest source of inspiration. Despite his own stresses of graduate school, always gave me encouragement at every step and motivated me to do my best during up and down times.

Thus, I dedicate the work in this thesis to all the people who have inspired and helped me, especially to my mentor Dr. Bakshi, my parents and my dear husband.

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CHAPTER 1

INTRODUCTION

BACKGROUND AND SIGNIFICANCE

1. Sensorimotor gating in neuropsychiatric disorders

Schizophrenia and post-traumatic stress disorder (PTSD) are disorders of the thought process that combined affect about 1% to 12% of the United States population according to the National Institute of Health. Alarmingly, the paucity of reliable medications for PTSD combined with the increasing numbers of war veterans returning home from recent wars creates urgency for developing new treatments for this devastating illness (Callahan 2010). Although schizophrenic and PTSD patients suffer from disparate symptoms that presumably involve different neurological mechanisms, in both disorders patients have a compromised ability to parse out potentially extraneous stimuli from their internal and external milieu and cannot attend to the most salient environmental features (McGhie and Chapman 1961; Braff and Geyer 1990; Geyer, Krebs-Thomson et al. 2001). For instance, schizophrenic patients are not able to ignore irrelevant thoughts and display a breakdown of the thought process; while in PTSD patients, an inability to disregard innocuous stimuli promotes dysfunctional affective reactions. It is thought that the resulting unfiltered plethora of cognitive and sensory information in turn may lead to cognitive fragmentation and sensory inundation (Braff, Geyer et al. 2001). In fact, schizophrenic and PTSD patients demonstrate dysfunctions in the realm of cognition, attention and information-processing functions that result in increased distractibility (Callaway and Naghdi 1982; Grillon, Courchesne et al. 1990; Braff 1993; Gold and Harvey 1993; Uddo, Vasterling et al. 1993; Nuechterlein, Dawson et al. 1994; Perry and Braff 1994; Vasterling, Duke et al. 2002; Luck and Gold 2008). These multimodal dysfunctions are collectively modulated by neural processes of sensorimotor gating, a central filtering mechanism that permits an optimal information-processing (Braff 1993; Perry, Geyer et al. 1999).

Sensorimotor gating is a form of pre-attentional information-filtering that putatively 'protects' the organism from sensory/cognitive overload by preventing potential irrelevant information from reaching the conscious information-processing realm (Geyer, Krebs-Thomson et al. 2001; Braff and Light 2004). Healthy cognitive function requires information-processing to be intact in pre-attentional domains such as sensorimotor gating (involuntary) and attentional domains such as decision-making (voluntary), as these mechanisms are thought to lie in a continuum and are interrelated; alteration in any one domain can affect the entire cognitive function of an organism (Graham 1975; Braff and Light 2004). Compromised sensorimotor gating leading to disruption of cognition and behavioral disorganization is typically observed in psychopathological conditions including schizophrenia, PTSD, Tourette syndrome, attention-deficit-hyperactivity disorder (ADHD), obsessive compulsive disorder (OCD) and Huntington's disease, with breakdown of information-filtering in the cognitive, sensory or motor domains (Swerdlow, Paulsen et al. 1995; Castellanos, Fine et al. 1996; Geyer, Krebs-Thomson et al. 2001; Hawk, Yartz et al. 2003; Ahmari, Risbrough et al. 2012; Holstein, Vollenweider et al. 2013). Together, these ranges of neuropsychiatric disorders have been referred to as a family of 'gating disorders' (Braff, Geyer et al. 2001). The fact that sensorimotor gating dysfunctions are present in these wide-ranging illnesses implies that the intactness of such a filtering process is significant for normal cognitive functions. Hence, as genetic and environmental factors can also influence sensorimotor gating, it is imperative to understand the neurobiological mechanisms that govern it in order to derive crucial information for developing novel therapeutics for these disorders.

2. Prepulse inhibition of the startle response

Sensorimotor gating can be measured using a laboratory paradigm known as prepulse inhibition (PPI) of the startle response. PPI is a naturally-occurring phenomenon in which a weak stimulus (prepulse) (20 msec long; 3, 9 or 15 dB above a 65 dB background noise), that by itself cannot elicit a startle response, produces a subsequent inhibition in the magnitude of a startle response when presented 30-500 milliseconds (msec) before a startling stimulus (pulse) (40 msec long; 120 dB) (Hoffman and Searle 1965). The duration between prepulse and pulse is considered too short to elicit any voluntary responses; therefore, PPI is thought to index pre-attentional information processing mechanisms (Graham 1975; Swerdlow, Braff et al. 2000). It is thought that there is brief period of decreased sensitivity to incoming stimuli immediately after the prepulse, during which information regarding the prepulse is processed by the brain and any other information is filtered out (Braff, Geyer et al. 2001). Therefore, PPI is considered an operational measure of sensorimotor gating and a form of plasticity of the startle response in which a sensory event (prepulse) recruits inhibitory processes in the brain to gate motor (startle) responses (Swerdlow, Braff et al. 2000). The 'gating disorders' described above, that are thought to comprise a breakdown in sensorimotor gating, demonstrate deficiencies in measures of PPI (Swerdlow, Paulsen et al. 1995; Castellanos, Fine et al. 1996; Grillon, Morgan et al. 1996).

Normally PPI is calculated as a percent score; smaller percent PPI represents deficient gating due to a lesser reduction of the startle response in prepulse+pulse trials (as observed, for example in PTSD and schizophrenic patients), but an absolute reduction in startle can also be calculated as a difference score between pulse-only units and prepulse+pulse units (Fig. 1)

(Swerdlow, Braff et al. 2000). When measuring PPI, it is important to also analyze changes in pulse-alone trials (baseline startle) because occasionally changes in startle magnitude by some manipulation can concomitantly alter PPI in terms of floor or ceiling effects (Sandner and Canal 2007). PPI can be measured using identical parameters in humans and animals, typically using an auditory stimulus, which elicits a whole-body startle response in rodents or an eye-blink response in humans; however, tactile and visual stimuli can also be used to elicit PPI (Geyer 2006). An important feature of PPI is that psychotomimetic drugs such as phencyclidine (PCP) can disrupt PPI, while antipsychotic medications used in neuropsychiatric disorders can normalize this dysfunction, making PPI also a useful tool to investigate the antipsychotic potentials of drugs (Swerdlow, Braff et al. 1994). Lastly, repeated exposure to a prepulse does not lead to the development of learning, habituation, or tolerance (Geyer and Swerdlow 2001). Overall, while it is very difficult to represent all the elements of a disorder in an animal model, PPI has emerged as a reliable and quantifiable cross-species phenomenon and an endophenotype for understanding abnormal sensorimotor gating (Braff and Geyer 1990).

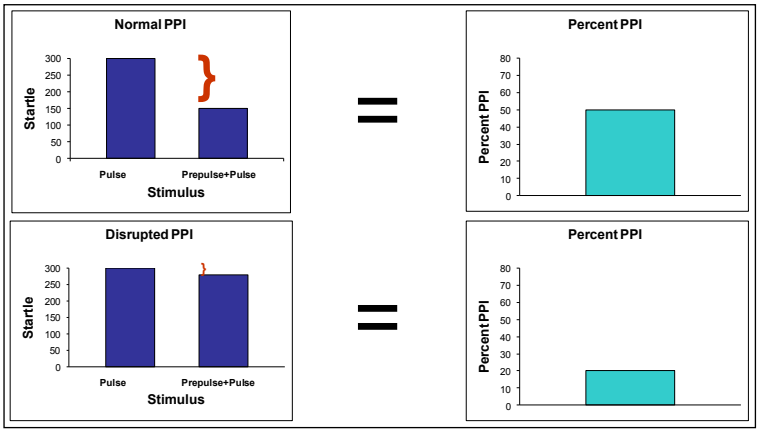

Figure 1: Top right panel represents high % PPI due to strong inhibition of startle response by the prepulse (top left panel) in normal circumstances. Bottom right panel represents low % PPI due to weak inhibition of the startle response by the prepulse (bottom left panel), as observed in schizophrenia.

3. PPI in psychopathology

As described previously, disorders like schizophrenia, PTSD and others that are correlated with abnormal sensorimotor gating display PPI deficits, which may also underlie symptoms such as anxiety, distractibility and cognitive impairments in these patients (Perry and Braff 1994; Castellanos, Fine et al. 1996; Grillon, Morgan et al. 1996; Karper, Freeman et al. 1996; Grillon, Morgan et al. 1998; Perry, Geyer et al. 1999; Ludewig, Geyer et al. 2003). Importantly, decreased PPI is not found universally in neuropsychiatric illnesses per se because, in disorders like unipolar depression that do not involve dysfunctions in sensorimotor gating, disturbances are not associated with PPI deficits (Swerdlow, Paulsen et al. 1995; Castellanos, Fine et al. 1996; Ludewig, Geyer et al. 2003).

Lesion, pharmacological, developmental and other studies using animal models of deficient PPI have been crucial for understanding the neurobiological underpinnings of sensorimotor gating. As PPI is measured in humans and rodents with identical parameters that yield comparable features, these models provide *face validity,* and because it is possible to measure psychophysical, neurochemical and neurodevelopmental alterations that contribute to PPI deficits, these models carry *construct validity* (Swerdlow, Braff et al. 2000). Furthermore, these models also hold *predictive validity* as drug effects for reversing PPI deficits can be predicted from them. Therefore, animal models of PPI are valuable and reliable resources with face, construct and predictive validity for evaluating psychiatric disorders involving deficient sensorimotor gating.

4. Neural circuitry and forebrain regions that modulate PPI

Understanding the neural circuitry behind PPI regulation provides a foundation for identifying neuroanatomical substrates for targeted therapies that may ameliorate sensorimotor deficits. The basic PPI circuit involves inhibitory projections from the pedunculopontine tegmental nucleus (PPTg) to the pontine reticular nucleus (PnC), which is an essential part of the primary startle circuit (Fig. 2) (Davis, Gendelman et al. 1982; Koch 1999; Fendt, Li et al. 2001). The primary startle circuit itself is a relatively short circuit consisting of only a few synapses, which has been well-characterized using anatomical tracing, electrical stimulation and lesion; activation of this circuit leads to activation of the spinal cord motor neurons eliciting the startle response (Fig. 2) (Davis, Gendelman et al. 1982; Koch, Lingenhohl et al. 1992; Yeomans and Frankland 1995; Lee, Lopez et al. 1996; Koch 1999). Activation of PPTg by a prepulse therefore leads to an inhibition of the startle response to pulse, but the extent of inhibition depends on the basal tone of the PPTg activation/inhibition, which in turn is set by its afferent projections from the cortico-striato-pallido-thalamic regions (CSPT) that impinge on the PPTg. Hence, although PPI is mediated by a simple brainstem circuit, it is modulated by higher-order forebrain sites such as the prefrontal cortex (PFC), nucleus accumbens (NAcc), ventral pallidum (VP) and thalamus. Therefore, manipulations of neurotransmitter systems within the CSPT that alter neuronal activation of these sites (eg. stimulation of glutamate

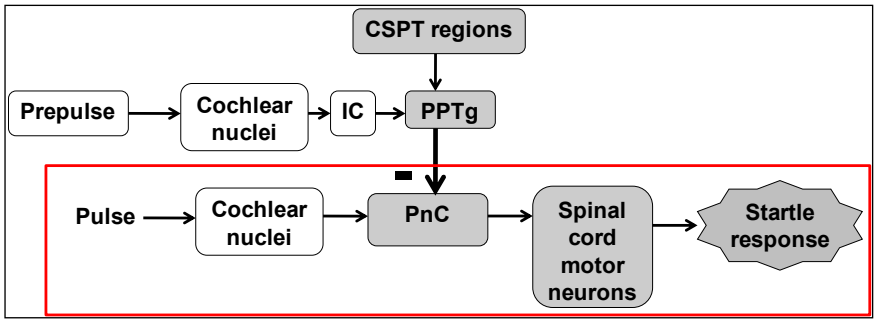

Figure 2: A circuit model representing PPI modulating circuit impinging on the primary startle circuit. Red box=primary startle circuit. IC= inferior colliculus, PPTg = pedunculopontine tegmental, PnC = pontine reticular and CSPT= Cortico-striato-pallido-thalamic.

receptors in the nucleus accumbens (NAcc) can modify PPI levels by ultimately impacting PPTg's sensitivity to a prepulse (Fig. 3) (Geyer, Krebs-Thomson et al. 2001; Swerdlow, Geyer et al. 2001).

Sites within the CSPT including the medial prefrontal cortex (mPFC), hippocampus, nucleus accumbens (NAcc), mediodorsal (MD) thalamus and basolateral amygdala (BLA) have been shown to modulate PPI through manipulations of various neurotransmitters within these structures including dopamine (DA), serotonin (5HT) and glutamate (Fig. 3) (Weinberger 1987; Kumari, Gray et al. 2003). Structures like the mPFC, hippocampus and BLA are implicated in schizophrenia and PTSD, perhaps through alterations in PPI as mediated by the DA system (Bogerts, Ashtari et al. 1990; Swerdlow, Caine et al. 1992; Weinberger, Berman et al. 1992; Csernansky and Bardgett 1998; Broersen, Feldon et al. 1999; St Jacques, Botzung et al. 2011). In the mPFC, decreasing DA or blocking GABA transmission disrupts PPI, consistent with the theory of decreased DA transmission in the PFC of schizophrenics, and these effects can be blocked by haloperidol, a typical antipsychotic that primarily inhibits D2 receptors (increases D1 activation) (Csernansky, Murphy et al. 1991; Koch and Bubser 1994; Ellenbroek, Budde et al. 1996; Japha and Koch 1999). Decreasing glutamate transmission by dizocilpine (a

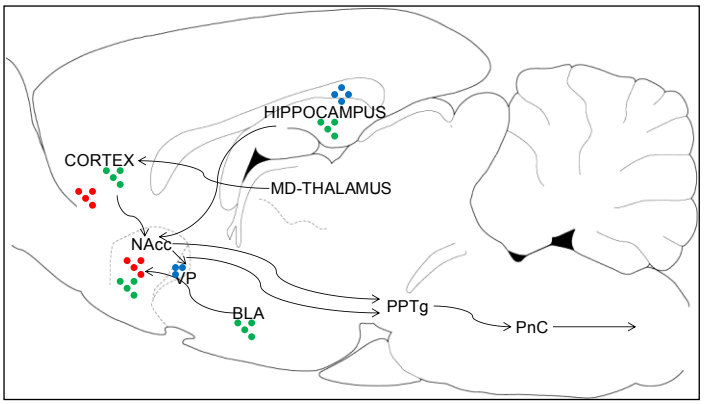

Figure 3: Schematic representation of forebrain regions along the CSPT (cortico-striato-pallido-thalamic) circuit and neurotransmitters in those regions that have been shown to modulate PPI. ●= dopamine, ●=glutamate and ●=serotonin. NAcc=nucleus accumbens, BLA=basolateral amygdala, PPTg = pedunculopontine tegmental and PnC=pontine reticular.

psychotomimetic) or serotonin transmission in certain sub-regions of the hippocampus disrupts PPI (Bakshi and Geyer 1998; Adams, Kusljic et al. 2008). In the NAcc, increasing DA or glutamate transmission disrupts PPI (Wan, Geyer et al. 1995; Swerdlow, Geyer et al. 2001). However, intra-MD-thalamus infusion of a selective direct agonist of DA D2 receptor, quinpirole, does not disrupt PPI, suggesting that the DA system may not modulate PPI through this brain region (Swerdlow, Pitcher et al. 2002). Intra-ventral pallidum (VP) serotonin agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) (hallucinogenic) disrupts PPI (Sipes and Geyer 1997). Kindling or lesioning the BLA, or decreasing GABA or glutamate transmission in this brain region disrupts PPI, and some of these effects are reversed by haloperidol (Wan and Swerdlow 1997; Koch and Ebert 1998; Fendt, Schwienbacher et al. 2000; Shoemaker, Pitcher et al. 2003). Taken together, these results reinforce the fact that PPI can be modulated by manipulations of various neurotransmitter systems within certain forebrain regions.

5. Pharmacology of PPI

As mentioned previously, both brain regions within the CSPT and neurotransmitters (including DA, 5HT and glutamate) modulate PPI (Hagan and Jones 2005). DA manipulations for understanding PPI modulation were primarily based on the 'DA hyperactivity' hypothesis of schizophrenia, which states that hyperactivation of DA-dependent neuronal functions leads to symptoms of schizophrenia (Meltzer and Stahl 1976). In general, treatments that increased DA transmission through systemic administration of direct DA agonists or indirect agonists such as amphetamine (psychotomimetic) disrupted PPI and these effects could be prevented by haloperidol, corroborating the idea that DA dysregulation could be involved in PPI deficits in

schizophrenia and providing a predictive validity for determining antipsychotic efficacies (Swerdlow, Braff et al. 1986; Mansbach, Geyer et al. 1988). However, a major drawback of the DA model is that the PPI-disruptive effects of dopaminergic agents can only be blocked by compounds that are specific for the D2 receptors, limiting the use of this model for the development of newer compounds that could act on other systems, which, as will be described throughout this thesis, are also crucial for PPI modulation. Another drawback of the DA-model-based antipsychotics is that they are not very effective at treating an important aspect of schizophrenia psychopathology; cognitive dysfunction (Geyer, Krebs-Thomson et al. 2001; Kumari and Sharma 2002).

Another well-studied neurotransmitter system in PPI modulation is the 5HT system, and stimulation of various receptors of this system modulates PPI. Drugs with hallucinogenic properties that increase 5-HT transmission like 3, 4-methylenedioxy-*N*-methylamphetamine (MDMA) or DOI disrupts PPI, which can be prevented by a pretreatment with 5-HT reuptake inhibitor or haloperidol (Mansbach, Braff et al. 1989; Sipes and Geyer 1994; Kehne, Ketteler et al. 1996; Padich, McCloskey et al. 1996; Martinez and Geyer 1997). However, there are several contradictory findings in rats, mice and humans regarding PPI modulation via 5HT compounds, weakening its predictive validity (Vollenweider, Remensberger et al. 1999; Dulawa, Gross et al. 2000; Liechti, Baumann et al. 2000). Partly based on a proposed glutamatergic deficiency in schizophrenic patients, the glutamate system has also been studied for understanding PPI modulation. It has been shown that drugs with psychotomimetic properties that act on glutamate receptors such as phencyclidine (PCP), a NMDA receptor antagonist, disrupt PPI and these effects can be reversed by atypical antipsychotics (such as clozapine) that act on the

norepinephrine system (among others), but not by DA antagonists (Garey 1979; Mansbach and Geyer 1989; Bakshi, Swerdlow et al. 1994). PCP-induced PPI deficits are found to be robustly consistent across rat strains and testing conditions; therefore, the glutamatergic model of PPI modulation offers predictive validity and allows for testing of newer antipsychotics or for differentiating typical versus atypical antipsychotic properties, but this model may not be useful for primarily screening antipsychotics (Geyer, Krebs-Thomson et al. 2001).

Overall, despite certain weaknesses associated with pharmacological approaches of studying neurotransmitter modulation of PPI, these studies implicate various neurochemical pathways in PPI modulation and provide important information for screening and for developing better treatments for disorders that involve sensorimotor gating dysfunctions. It is, however, worth noticing that one neurotransmitter system appears to have been overlooked for understanding PPI modulation; the norepinephrine (NE) system, known to play a vital role in various cognitive functions that are compromised in a range of disorders involving PPI deficits. Therefore, a major focus of this thesis has been to understand the role of the NE system in PPI regulation via certain forebrain regions.

6. NE system and PPI modulation

As alluded to above, there is a general gap in knowledge regarding regulation of sensorimotor gating by the NE system and its role in regulating PPI. This is quite perplexing because NE and its pathways are crucial for modulating arousal, vigilance, cognition, and sleep (Aston-Jones, Chen et al. 2001). Functions like arousal, vigilance, and cognition (at least at a

theoretical level) are related to sensorimotor gating; as such, NE abnormalities are found in disorders including PTSD, schizophrenia and ADHD (Farley, Price et al. 1978; Breier, Wolkowitz et al. 1990; Biederman and Spencer 1999; Strawn and Geracioti 2008).

6.1. ***NE pathways*****:** NE pathways are wide-spread in the brain, originating mainly from a small brainstem structure called the locus coeruleus (LC) (A6 region), which contains noradrenergic cell bodies that project to several forebrain structures along the CSPT circuit (Fig. 4) (Foote, Bloom et al. 1983; Waterhouse, Lin et al. 1983). However, NE transmission to a few forebrain areas also originates from other brainstem regions including the nucleus of the solitary tract (NST) (A2 region) and medullary nucleus (A1 region). Although LC-NE projections appear uniform, heterogeneities exist based on the topographic organization and density of NE efferents that can differentially modulate neural and behavioral functions (Waterhouse, Lin et al. 1983). For instance, neurons that project to the thalamocortical regions (eg. mPFC, BLA) lie in the dorsal aspect and receive extensive NE projections, whereas projections to ventral forebrain regions (e.g. central amygdala (CeA) lie in the ventral aspect and receive sparser projections (Amaral and Sinnamon 1977). Interestingly, animals with LC dorsal efferent bundle lesions display behaviors such as distractibility and inability to attend to relevant environmental stimuli-

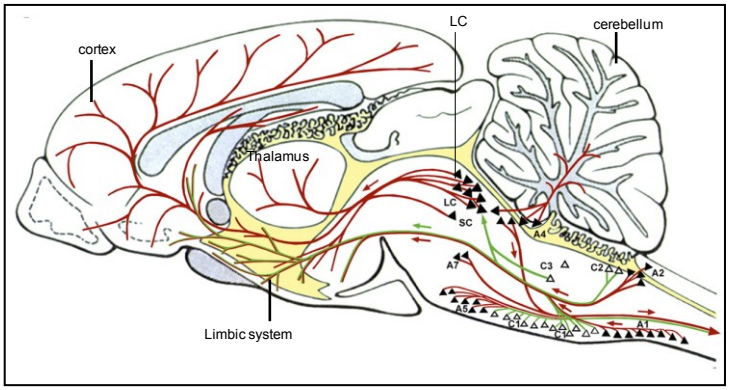

Figure 4: A schematic of a sagittal section of the rat brain depicting NE pathways from the LC to various forebrain regions. LC=locus coeruleus. Adapted and modified from Kvetnansky R et al., 2009.

behavioral features that match problems observed with deficient sensorimotor gating as seen in schizophrenia, also highlighting that LC projections appear to be important for sensorimotor gating modulation (Hornykiewicz 1986; Selden, Robbins et al. 1990; O'Donnell, Hegadoren et al. 2004). Thus, brain NE projections are positioned to control diverse neuronal activities and behavioral functions.

6.2. ***NE in physiological and behavioral functions*****:** Among the first functions to be attributed to LC-derived NE was the regulation of arousal (Hobson, McCarley et al. 1975). Depending upon the stages of sleep or arousal, LC-NE neurons display two types of discharge properties- tonic and phasic. Tonic activity is spontaneous and ongoing during waking, varying with behavioral state and found to be highest during waking, low during slow-wave sleep, grooming and food/water consumption, and virtually silent during sleep (Aston-Jones, Rajkowski et al. 2000; Aston-Jones and Cohen 2005). During a moderate tonic activity, LC-NE neurons can be transiently activated by stimuli that require attention, disrupting an ongoing activity and displaying phasic activation (selective attention) to produce an orienting behavioral response. Thus, LC neurons display an inverted U-shape for arousal, with poor task performance at very low or very high levels of tonic discharge and best task performance during moderate tonic activity (Aston-Jones, Chiang et al. 1991; Aston-Jones, Rajkowski et al. 2000). Increased LC tonic activity that releases elevated levels of NE in terminal brain regions is associated with poor cognitive functions in schizophrenic patients, presumably because of poor information-processing and heightened distractibility (Friedman, Stewart et al. 2004). NE phasic activity (enhanced arousal) permits processing of sensory stimuli by enhancing signal-to-noise ratio in cortical and thalamic neurons, with increased NE release that shifts the neuronal activation

pattern from burst (slow wave sleep with poor fidelity sensory transmission) to single spike (waking with efficient sensory transmission) mode, potentially permitting the diversion of attention to salient stimuli (Waterhouse, Moises et al. 1980; Waterhouse, Moises et al. 1984; Hopkins and Johnston 1988; Waterhouse, Sessler et al. 1988; Aston-Jones, Chiang et al. 1991; Florin-Lechner, Druhan et al. 1996; Berridge and Abercrombie 1999). In addition, NE release in the PFC has been shown to regulate working memory (Arnsten and Goldman-Rakic 1985; Arnsten, Cai et al. 1988; Arnsten and Contant 1992; Robbins TW 1995).

6.3. ***NE receptors*****:** NE transmission is carried out by three main types of NE receptors: α1, α2 and β. Alpha1 and β receptors are mostly postsynaptic heteroreceptors, whereas α2 receptors can be present on pre-or postsynaptic neurons; presynaptic α2 receptors are also known as autoreceptors because their activation decreases NE release (Graham, Perez et al. 1996; Wozniak M 2000). All noradrenergic receptors belong to subfamilies of guanine nucleotide regulatory protein coupled receptors (GPCRs) connected to distinct second messenger pathways (Wozniak M 2000). Each receptor is classified into receptor subtypes (α1a, α1b, α1d, α2a, α2b, α2c, β1, β2, β3), based upon their structural differences and distinct affinities for the endogenous ligand NE and for agonists/antagonists (Bylund, Eikenberg et al. 1994; Wozniak M 2000). Alpha1 receptors belong to the $G_q/_{11}$ type of GPCRs, which activate phospholipase C (PLC) and/or diacylglycerol (DAG) to mobilize calcium (Ca^{2+}) (Minneman, Theroux et al. 1994; Ruffolo and Hieble 1994; Graham, Perez et al. 1996). These receptors can also act on the cyclic adenosine monophosphate (cAMP) linked pathways. Alpha2 receptors, on the other hand, mediate their effects through the Gi/Go family, which inhibits adenyl cyclase (AC), leading to decreased cAMP signaling, reduction in Ca^{2+}, and a decrease in NE release (Jones, Halenda et al. 1991; Eason, Kurose et al.

1992; Pupo and Minneman 2001). Beta receptors belong to the G_s types that activate the AC-cAMP-protein kinase A (PKA) pathway (Bylund, Eikenberg et al. 1994; Barnes 1995; Wozniak M 2000). Depending upon the receptor subtypes and brain regions, NE receptors are distributed in the olfactory nucleus, cortex, septum, thalamus, hippocampus, striatum and amygdala (Palacios and Kuhar 1982; Rainbow, Parsons et al. 1984; Aoki, Joh et al. 1987; McCune, Voigt et al. 1993; Nicholas, Pieribone et al. 1993; Bylund, Eikenberg et al. 1994; Pieribone, Nicholas et al. 1994; Scheinin, Lomasney et al. 1994; Nicholas, Hokfelt et al. 1996; Wang, Macmillan et al. 1996). Taken together, activation of NE receptors, with their distinct molecular pathways, can lead to diverse central nervous system (CNS) functions.

6.4. ***NE and PPI*****:** Enhanced NE signaling that decreases arousal is also associated with reduced cognitive processing, producing symptoms such as hyperarousal, symptom re-experiencing and deficits in PPI, as observed in schizophrenia and PTSD (Farley, Price et al. 1978; van Kammen and Antelman 1984; Kosten, Mason et al. 1987; Breier, Wolkowitz et al. 1990; Yehuda, Southwick et al. 1992; Southwick, Krystal et al. 1993; Grillon, Morgan et al. 1996; Geracioti, Baker et al. 2001). Hence, a major interest of our lab has been to understand the role of the NE system in PPI modulation. Our lab was the first to discover that pharmacological stimulation of the LC disrupts PPI, and that NE release in certain brain regions along the CSPT circuit (such as PFC and NAcc) modulates PPI via stimulation of alpha1 and beta receptors (Alsene and Bakshi 2011; Alsene, Rajbhandari et al. 2011). This finding corroborates with several studies that have shown the role of the NE system in modulation of PPI via systemic manipulations (Carasso, Bakshi et al. 1998; Bakshi and Geyer 1999; Lahdesmaki, Sallinen et al. 2004; Shilling, Melendez et al. 2004; Swerdlow, Bongiovanni et al. 2006), yet we also primarily showed that stimulation

of central but not peripheral NE receptor stimulation leads to PPI disruption (Alsene, Carasso et al. 2006). Moreover, clinically it had been shown that administration of amphetamine, a psychotomimetic that disrupts PPI in humans, was related to dysregulation of the DA system (Kokkinidis and Anisman 1981; Kumari, Mulligan et al. 1998); however, studies in our lab demonstrated that the PPI deficit induced by amphetamine could be mediated via the NAcc NE (Alsene, Fallace et al. 2010). Hence, a better understanding of PPI modulation by NE could provide further insights into the neuronal mechanisms of disorders that involve PPI deficits. Illnesses like schizophrenia and PTSD that display enhanced NE signaling, worsen from drugs that increase NE transmission and benefit from NE-reducing drugs, further underscoring the clinical importance of studies examining the role of the NE system in PPI modulation (Southwick, Bremner et al. 1999; Southwick, Morgan et al. 1999; Friedman, Adler et al. 2001; Pitman, Sanders et al. 2002; Raskind, Thompson et al. 2002; Friedman, Stewart et al. 2004; Boehnlein and Kinzie 2007; Remington, Agid et al. 2013). Thus, understanding NE's role in PPI regulation may help in refining treatments for disorders like schizophrenia and PTSD.

6.5. ***Stress and NE system***

The NE system is also highly involved in the modulation of stress and anxiety, as this system is activated by stress to trigger a 'flight or fight' response (Morilak, Barrera et al. 2005). Normal stress responses are natural, adaptive reactions to threatening stimuli and are important for survival; however, extreme or chronic stress can disrupt biological homeostasis leading to maladaptation. Stress enhances LC-NE tonic firing and increases NE turnover in LC terminal regions, which (as previously mentioned) can lead to distractibility and impaired behavioral

performance, effects attenuated by anxiolytics (Tanaka, Kohno et al. 1983; Ida, Tanaka et al. 1985; Levine, Litto et al. 1990; Pavcovich, Cancela et al. 1990; Aston-Jones, Chiang et al. 1991; Arnsten 1998). The role of the NE system in modulating cognitive functions under stress is evidenced by a reversal of stress-induced working memory deficits by alpha1 receptor antagonists in the PFC (Birnbaum, Gobeske et al. 1999; Arnsten 2004). Furthermore, the NE system plays a role in sensitization of the stress response following prolonged stress (Irwin, Ahluwalia et al. 1986; Morilak, Barrera et al. 2005).

In turn, stress plays a prominent role in psychopathology, as it is now clear that intense/chronic stress can exacerbate clinical conditions or trigger symptom relapse in schizophrenia and PTSD, while currently available treatments are not effective at ameliorating stress-related cognitive dysfunctions in these disorders (Brown and Birley 1968; Norman and Malla 1993; Nuechterlein, Dawson et al. 1994; Walker and Diforio 1997; Yehuda 1999; Gispen-de Wied 2000; Yehuda 2001; Horan, Ventura et al. 2005; Keane, Marshall et al. 2006; Ulrich-Lai and Herman 2009). In schizophrenia, stressful life events cause symptom relapse, while in PTSD, prior exposure to extremely stressful (traumatic) experience can lead to behavioral changes manifesting at later time-points as enhanced startle or disrupted PPI (Lukoff, Snyder et al. 1984; Grillon, Morgan et al. 1996; Grillon, Morgan et al. 1998; Pitman, Orr et al. 1999; Yehuda 2004). Moreover, although positive symptoms of schizophrenia, such as delusions, are co-related with dysfunctions of the DA system, negative symptoms, such as anxiety, are co-related with the dysfunctions of the NE system, and in addition, drugs that reduce NE transmission improve cognition (Mason 1981; Friedman, Adler et al. 2001; Taylor and Raskind 2002). In a general sense, the NE system modulates cognition associated with both arousal and

affective states, and hence, understanding the role of this system in various functions could help develop novel treatments for schizophrenia or PTSD.

7. Corticotropin-releasing factor (CRF) system

Stress also activates the corticotropin-releasing factor (CRF) system. CRF is well-studied 41-amino acid signaling peptide activated in response to highly arousing or stressful stimuli (Bale and Vale 2004) and known to be a putative neurotransmitter that is involved in the expression of stress response such as increased blood pressure. Through its actions on the hypothalamic-pituitary-adrenal (HPA) axis, it releases cortisol, a glucocorticoid whose chronic release can have negative effects on the biological functioning of an organism (Munck and Guyre 1986)**.** CRF also exerts its effects via extra-hypothalamic sites such as the amygdala (Lupien, de Leon et al. 1998). Hence, intense or chronic stress acting via hypothalamic or extra-hypothalamic regions can produce detrimental effects in the biological and functional processes of an organism.

7.1. ***CRF extra-hypothalamic pathways***: Similar to the NE system, the CRF system is widely distributed throughout the CNS (Figure 5). Although the paraventricular nucleus of hypothalamus (PVN) is known to be a principal source of CRF in the brain, both the CeA and BNST contain CRF cell bodies that innervate brainstem autonomic structures such as the midbrain central gray, parabrachial nucleus, NST and the LC, with reciprocal connections that modulate diverse autonomic and behavioral responses to stress (Hopkins 1975; Schwaber, Kapp et al. 1982; Swanson, Sawchenko et al. 1983; Holstege, Meiners et al. 1985; Petrusz,

Merchenthaler et al. 1985; Brown 1986; Sakanaka, Shibasaki et al. 1986; Gray 1993; Sawchenko, Imaki et al. 1993; Van Bockstaele, Colago et al. 1998). Therefore, CRF projections from the CeA and BNST are well distributed to modulate a variety of stress-related behaviors.

7.2. *CRF receptors*: CRF transmission is modulated by its receptors, which have two main subtypes, CRF1 and CRF2 (CRF2α and CRF2β); like the NE system, these receptors belong to a family of GPCRs (Bale and Vale 2004). CRF1 and CRF2 receptors are 70% homologous to each other at the amino acid level, with CRF1 found mostly on postsynaptic neurons and CRF2 on pre-or-postynaptic neurons (Bale and Vale 2004). CRF1 has the highest affinity for CRF while CRF2 has higher affinity for the CRF-like peptide, urocortin (UCN) (Lovenberg, Liaw et al. 1995). Both receptors are coupled to the AC-cAMP-PKA pathway (Abou-Samra, Harwood et al. 1987; Battaglia, Webster et al. 1987; Grammatopoulos and Chrousos 2002; Grammatopoulos 2012). CRF1 and CRF2α receptors have distinct yet overlapping distributions in the BLA, BNST, NAcc, hippocampus, LC, NST, olfactory bulb, lateral septum, and hypothalamus, while CRF2βs have a peripheral distribution (Fig. 5) (De Souza, Perrin et al. 1984; De Souza, Insel et al. 1985; De Souza 1987; Potter, Sutton et al. 1994; Chalmers, Lovenberg et al. 1995; Lovenberg, Liaw et al. 1995; Primus, Yevich et al. 1997; Radulovic, Sydow et al. 1998). Activation of CRF1 receptors

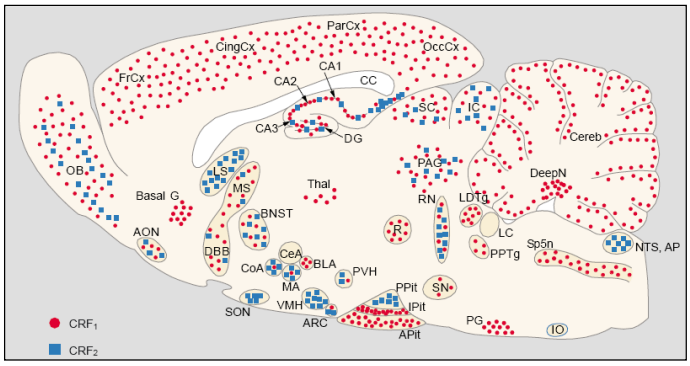

Figure 5: A schematic of a sagittal section of the rat brain depicting CRF1 (red) and CRF2 (blue) receptors. Adapted and modified from **Reul & Holsboer 2002**

produces anxiety-like responses and conversely, blocking these receptors reduces stress/anxiety responses (Smith, Aubry et al. 1998; Timpl, Spanagel et al. 1998; Gehlert, Shekhar et al. 2005; Arzt and Holsboer 2006; Walker, Yang et al. 2009; Adamec, Fougere et al. 2010). The role of CRF2 receptors in anxiety, however, is controversial; effects of acute CRF2 activation are similar to CRF1 activation, but transgenics that lack CRF2 receptors have an enhanced anxious phenotype, leading some to suggest that CRF2 may be important for coping with stress (Coste, Kesterson et al. 2000; Kishimoto, Radulovic et al. 2000; Bakshi, Newman et al. 2007). However, developmental manipulations can have compensatory changes, such as increased CRF levels in the amygdala, which could activate CRF1 receptors to produce the phenotype.

7.3. *CRF in physiological and behavioral functions*: Activation of CRF receptors either by stress or experimental manipulations produces behavioral and biological changes that can be reversed by CRF1 antagonists (Fisher, Rivier et al. 1982; Britton, Lee et al. 1986; Krahn, Gosnell et al. 1986; Berridge and Dunn 1987; Sherman and Kalin 1988; Swerdlow, Britton et al. 1989; Swiergiel, Takahashi et al. 1993; Heinrichs, Menzaghi et al. 1994; Eckart, Radulovic et al. 1999; Arborelius, Skelton et al. 2000; Zorrilla, Valdez et al. 2002; Adamec, Fougere et al. 2010). CRF plays an important role in modulating learning, feeding, locomotion, and other behaviors (Morley and Levine 1982; Dunn and Berridge 1990; Heinrichs, Menzaghi et al. 1995; Jochman, Newman et al. 2005).

CRF is important in schizophrenia and PTSD psychopathology, as patients with these disorders display overactive CRF neurotransmission (Banki, Bissette et al. 1987; Forman,

Bissette et al. 1994; Bremner, Licinio et al. 1997; Baker, West et al. 1999; Claes 2004). Interestingly, withdrawal from antipsychotic treatment increases cerebrospinal fluid (CSF) levels of CRF in chronic schizophrenic patients (Forman, Bissette et al. 1994). A recent study has shown that polymorphisms of CRF1 receptors can cause susceptibility for PTSD, and there is indication that CRF1 antagonists may be important prophylactic agents in this disorder (Amstadter, Nugent et al. 2011; Hauger, Olivares-Reyes et al. 2012). As both schizophrenia and PTSD involve deficits in PPI, it can be postulated that dysregulation of the CRF system (in addition to the NE system) could also contribute to PPI alterations; several lines of evidences support the role of the CRF system in PPI modulation.

7.4. *CRF & PPI*: Studies have shown that activation of CRF receptors by stress or through direct manipulations disrupts PPI, and this effect can be reversed by CRF1 or CRF2 receptor antagonists (Conti, Murry et al. 2002; Conti 2005; Sutherland and Conti 2011). Additionally, CRF over-expression in mice reduces PPI and is reversed by haloperidol (Dirks, Groenink et al. 2002; Dirks, Groenink et al. 2003; Risbrough, Hauger et al. 2004). It is, however, important to point out that strain differences can differentially impact CRF's effect on PPI; for instance, Brown Norway rats show lower baseline PPI than Wistar-Kyoto rats (Conti, Murry et al. 2002; Conti 2005). Data from our lab shows that both central administration of CRF and exposure to a predator stress paradigm (that presumably activates CRF) leads to a long-lasting disruption in PPI, evident 24 hrs after an initial CRF infusion or predator stress (Bakshi, Alsene et al. 2011). The long-lasting nature of the PPI deficit by acute CRF infusion as well as predator stress suggests that PPI alterations could occur due to intense activation of CRF receptors. Surprisingly, little is known about the role of repeated stimulation of CRF receptors in

modulating PPI, particularly in forebrain regions such as the amygdala that are enriched in this system. A recent study showed that acute intra-BLA CRF had no effect on PPI, but repeated (5 days) infusions caused PPI disruptions (Bijlsma, van Leeuwen et al. 2010). Thus, another focus of this thesis is to understand the role of CRF on PPI via various forebrain regions.

Given that dysregulation of CRF and NE systems are involved in neuropsychiatric disorders such as schizophrenia and PTSD, that these systems modulate PPI independently, and both of these systems are enriched in various forebrain regions, another major question for this thesis is whether these two systems interact with each other in specific brain areas such as the amygdala to modulate PPI.

8. Interactions between CRF and NE systems

There are several lines of evidence supporting the role of CRF and NE interactions in various functions. CRF arising from the CeA or BNST innervates the LC and modulates LC-NE activity, which in turn can lead to 'feed-forward' interactions between the NE and CRF systems in LC terminal brain regions such as the amygdala (Butler, Weiss et al. 1990; Van Bockstaele, Colago et al. 1996). CRF1 antagonists can prevent stress-or-CRF-induced NE release from the LC in terminal regions (Melia and Duman 1991; Page and Abercrombie 1999). Under stressful situations, CRF shifts LC firing into a high tonic state without altering phasic discharge properties via CRF1 receptors (Valentino and Van Bockstaele 2008). With long-term stress, the LC's sensitivity to CRF increases, leading to sensitization to lower levels of CRF; this can be prevented by administering intra-LC CRF1 antagonists (Curtis, Pavcovich et al. 1999; Xu, Van

Bockstaele et al. 2004). CRF also decreases sensory responses to evoked noxius stimuli in LC-NE by reducing signal-to-noise ratio (Ehlers 1986; Valentino and Foote 1987). Lastly, alpha1 and beta receptor antagonists reduce behavioral alterations to predator stress or cold water stresses, implicating interactions between NE and CRF systems during stress (Tan, Gan et al. 2003; Adamec, Muir et al. 2007; Howard, Carr et al. 2008).

Stress- and central CRF-evoked behavioral changes thus involve activation of the LC-NE system and NE release in brain regions such as the amygdala that also contain CRF, potentially allowing for interactions between these two systems in LC terminal regions. Studies show that both stress and CRF increase NE and its metabolites (and vice versa) in the amygdala, and stress-induced NE release in the amygdala can be blocked by CRF receptor antagonists (Beaulieu, Pelletier et al. 1989; Emoto, Koga et al. 1993; Pacak, McCarty et al. 1995; Raber, Koob et al. 1995; Smagin, Zhou et al. 1997; Li, Takeda et al. 1998; Lorrain, Baccei et al. 2005; Ma and Morilak 2005). Interestingly, it has also been shown that intra-amygdala CRF increases plasma NE levels, in some cases at a higher level than by CRF injection into the third ventricle, indicating that CRF acting in this brain region can have profound physiological effects (Brown 1986). Lastly, interactions of the CRF and NE system in the BLA facilitate aversive memory (Roozendaal, Castello et al. 2008). Thus, a substantial amount of evidences indicates that the NE and CRF systems interact with each other to modulate various biological and behavioral functions via amygdalar subregions; as these two systems independently modulate PPI, this thesis will explore whether these interact in the amygdala to regulate PPI.

9. The Amygdala

Even though the amygdala in general appears important for interactions between the NE and CRF systems, activation of these systems can have differential effects via amygdalar sub-regions that contain differential distributions of NE or CRF receptors. The amygdala, as a unitary structure, is located in the medial temporal lobe and is crucial for providing affective salience to sensory stimuli to modulate autonomic and behavioral functions, including fear, feeding, learning, attention and memory via diverse inputs and outputs (Liang, McGaugh et al. 1982; Davis 1992; Kim and Fanselow 1992; Gray 1993; Maren and Fanselow 1996; LeDoux 2003; Phelps and LeDoux 2005). Clinically, dysregulation of the amygdala is observed in PTSD and schizophrenia, with PTSD patients showing enhanced amygdala activation to fearful stimuli and schizophrenics showing increased amygdalar responses to the presentation of neutral faces and decreased responses to negative faces (Liberzon, Taylor et al. 1999; Rauch, Whalen et al. 2000; Hall, Whalley et al. 2008; Rasetti, Mattay et al. 2009). As such, damage to the amygdala is shown to reduce fear and anxiety (Bechara, Tranel et al. 1995; LaBar and LeDoux 1996).

The two major subregions of the amygdala are the aforementioned central (CeA) and basolateral (BLA) nuclei, while the BNST is considered part of the extended amygdala; all of which differ from each other in structure, number of cells, receptors, inputs and outputs, and functions (Alheid, Beltramino et al. 1998; Davis 1998; LeDoux 2007; Pitkanen, Jolkkonen et al. 2000). Each of these sites expresses high levels of NE receptors (Swanson and Hartman 1975; Young and Kuhar 1980; Rainbow, Parsons et al. 1984; Farb, Chang et al. 2010), but the expression of CRF1 receptors differs, with highest density of expression found in the BLA,

followed by the BNST and a sparse distribution in the CeA (De Souza, Insel et al. 1985; De Souza 1987). Nonetheless, both the CeA and the BNST are major sources of CRF to the PFC, thalamus, hypothalamus, BLA and brainstem regions such as the PnC and LC, yet play complementary roles in various functions (Price and Amaral 1981; Rosen, Hitchcock et al. 1991; Van Bockstaele, Colago et al. 1998; Van Bockstaele, Peoples et al. 1999; Walker, Miles et al. 2009). These amygdalar sub-regions participate in high-arousal states associated with affectively-valenced stimuli, including fear/anxiety-like states and appetitively motivated behaviors (Davis, Falls et al. 1993; Davis, Walker et al. 1997; Baldwin, Holahan et al. 2000; Berridge and Waterhouse 2003). More specifically, the BNST and BLA are considered significant modulators of anxiety, while CeA controls responses to fear (Hitchcock and Davis 1991; Aggleton 1993; Canteras, Resstel et al. 2010). The BLA is perfectly situated to integrate sensory information, as it sends and receives mostly glutamatergic auditory, taste, visual, and somatosensory inputs from cortical and subcortical regions, along with some GABAergic interneurons (LeDoux, Cicchetti et al. 1990; Amaral and Insausti 1992; Romanski, Clugnet et al. 1993; Wright, Beijer et al. 1996; McDonald and Mascagni 1997; Swanson and Petrovich 1998; Pikkarainen and Pitkanen 2001). Clinically, enhanced BLA activation, as would occur with high stress, is hypothesized to be an important contributor of schizophrenia pathophysiology (Benes 2010).

9.1. ***Role of NE in the amygdala****:* NE acts differentially in amygdalar sub-regions to modulate various behaviors depending on the type and level of NE afferents to these structures and receptors. Even though BLA, BNST and CeA possess similar distribution of NE receptors, NE projections to the BLA arise exclusively from the LC, while the CeA and BNST receive NE

from the LC, NST and medulla (Riche, De Pommery et al. 1990; Forray, Gysling et al. 2000). NE in the CeA and BNST is important for drug seeking behaviors, stress-induced hypoalgesia and memory formation (Liang, Chen et al. 2001; Leri, Flores et al. 2002; Chen, Chen et al. 2004; Ortiz, Close et al. 2008). NE also has distinct roles in the CeA and BNST, as it modulates stress-induced anxiety via the BNST, whereas CeA NE regulates social interaction (but not anxiety) (Cecchi, Khoshbouei et al. 2002). NE plays a major role in modulating consolidation and expression of fear memory, fear potentiated startle (FPS), and anxiety via the BLA (Liang, Chen et al. 1995; Ferry, Roozendaal et al. 1999). In summary, amygdala sub-regions are important for modulating various behavioral states through the NE system.

9.2. ***Role of CRF in the amygdala****:* CRF, acting via CRF1 receptors, also has distinct effects in amygdalar subregions important in modulating stress-related behaviors, learning, and memory (Eckart, Radulovic et al. 1999; Pitts, Todorovic et al. 2009). In the CeA, CRF mainly regulates fear behaviors such as freezing and fear conditioning via CRF1 receptors (LeDoux, Iwata et al. 1988; Heinrichs, Pich et al. 1992; Swiergiel, Takahashi et al. 1993; Bale and Vale 2004; Asan, Yilmazer-Hanke et al. 2005). In both the BNST and BLA, CRF plays a significant role in modulating startle responses also via CRF1 receptors (Lee and Davis 1997; Gewirtz, McNish et al. 1998). This function of CRF via the BLA and BNST on startle is thought to demonstrate sustained fear (anxiety) (Walker and Davis 2008; Walker, Miles et al. 2009). CRF is also important for regulating aversive memory processes via the BNST and BLA (Liang, Chen et al. 2001; Roozendaal, Schelling et al. 2008). In addition, chronic stress or repeated activation of CRF1 receptors in the BNST or BLA causes plasticity in behavioral responses of anxiety (Sajdyk, Schober et al. 1999; Rainnie, Bergeron et al. 2004; Lee, Fitz et al. 2008). BLA is also an

important substrate for predator stress effects via CRF1 receptors, as this paradigm increases behavioral indices of stress such as increased grooming and anorexia, which can reversed by CRF1 receptor antagonism (Jochman, Newman et al. 2005).

9.3. ***Amygdala and PPI*****:** As described above, amygdalar sub-regions can differentially modulate a variety of stress-related behaviors, but despite the fact the NE and CRF systems modulate PPI, their roles in PPI modulation via amygdala subregions have not been explored. Pharmacological, lesion and kindling studies show that the amygdala, especially the BLA, is important for modulating PPI (Vinkers, Bijlsma et al. ; Decker, Curzon et al. 1995; Wan and Swerdlow 1997; Shoemaker, Pitcher et al. 2003; Stevenson and Gratton 2004; Howland, Hannesson et al. 2007). Lesion, PCP, and DOI –induced PPI deficits via the BLA can be reversed, in most cases, by second generation antipsychotics and, in some cases, by haloperidol (Bakshi and Geyer 1998; Bakshi and Geyer 1999; Bakshi, Tricklebank et al. 1999; Fendt, Schwienbacher et al. 2000; Shoemaker, Pitcher et al. 2003). The role of DA in PPI modulation within the amygdala is complicated, as direct infusion of DA or of the D2 antagonist raclopride into the BLA disrupts PPI, as does the D2 agonist quinpirole, but the DA antagonist SCH23390 in the BLA enhances PPI, accompanied by dose-dependent decreases in startle levels; this could be one reason for the differential actions of DA receptor activation or antagonism on PPI (Swerdlow, Caine et al. 1992; Stevenson and Gratton 2004; Salum, Issy et al. 2010). Thus, the amygdala appears to play an important role in PPI modulation. Taken together, it is evident that the NE, CRF and amygdalar sub-regions regulate various stress/anxiety-related behaviors and PPI, but it remains to be explored whether the NE and CRF systems interact in amygdala sub-regions to regulate PPI.

10. Synthesis and theme

Previous evidences indicate that CNS NE and CRF disrupt PPI, that stress can elevate NE and CRF transmission and disrupt PPI, and that amygdalar sub-regions are primary sites through which stress alters NE and CRF functions. Thus, this thesis seeks to characterize NE and CRF regulation of PPI via amygdalar sub-regions. Given that schizophrenia and PTSD are associated with PPI deficits and symptoms in these disorders are elicited or exacerbated by stress, studies in this thesis have potential clinical relevance for identifying underlying mechanisms that could be involved in stress-induced disruption of sensorimotor gating. The overall work emanating from this thesis is divided into four chapters:

Chapter 2: Discrete forebrain neuronal networks supporting noradrenergic regulation of sensorimotor gating

Chapter 3: Noradrenergic neuroadaptations in the basolateral amygdala after psychological stress or corticotropin-releasing factor promotes enduring PTSD-like information processing deficits

Chapter 4: Cellular co-localization of alpha1 noradrenergic receptors and CRF1 receptors on projection neurons within the rat basolateral amygdala

Chapter 5: Sensitization of corticotropin-releasing factor receptors after repeated norepinephrine receptor stimulation in the bed nucleus of the stria terminalis leads to PTSD-like information processing deficits in rats

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CHAPTER 2

DISCRETE FOREBRAIN NEURONAL NETWORKS SUPPORTING NORADRENERGIC REGULATION OF SENSORIMOTOR GATING

The experiments in the following chapter were completed by myself. Technical assistance was provided by Summer Rozzi and Sarah Newman. General experimental design, data analysis and conclusions were developed with the help of my thesis advisor, Dr. Vaishali Bakshi.

Alsene KM*, **Rajbhandari AK***, Ramaker MJ, and Bakshi VP. 2011. Discrete Forebrain Neuronal Networks Supporting Noradrenergic Regulation of Sensorimotor Gating, *Neuropsychopharmacology.* ***=co-first authors**. 36(5):1003-14

ABSTRACT

Prepulse inhibition (PPI) refers to the reduction in the startle response when a startling stimulus is preceded by a weak prestimulus, and is an endophenotype of deficient sensorimotor gating in several neuropsychiatric disorders. Emerging evidence suggests that norepinephrine (NE) regulates PPI, however, the circuitry involved is unknown. We found recently that stimulation of the locus coeruleus (LC), the primary source of NE to the forebrain, induces a PPI deficit that is a result of downstream NE release. Hence, this study sought to identify LC-innervated forebrain regions that mediate this effect. Separate groups of male Sprague–Dawley rats received a cocktail solution of the α1-NE receptor agonist phenylephrine plus the β-receptor agonist isoproterenol (equal parts of each; 0, 3, 10, and 30 μg) into subregions of the medial prefrontal cortex (mPFC), nucleus accumbens (NAcc), extended amygdala, mediodorsal thalamus (MD-thalamus), or the dorsal hippocampus (DH) before PPI testing. NE agonist infusion into the posterior mPFC, NAcc shell, bed nucleus of the stria terminalis, basolateral amygdala, and the MD-thalamus disrupted PPI, with particularly strong effects in MD-thalamus. Sites in which NE receptor stimulation did not disrupt PPI (anterior mPFC, NAcc core, central amygdala, and DH) did support PPI disruptions with the dopamine D2 receptor agonist quinpirole (0, 10 μg). This pattern reveals new pathways in the regulation of PPI, and suggests that NE transmission within distinct thalamocortical and ventral forebrain networks may subserve the sensorimotor gating deficits that are seen in disorders such as schizophrenia, Tourette syndrome, and post-traumatic stress disorder.

INTRODUCTION

Sensorimotor gating is a fundamental form of information-processing permitting organisms to filter information from external and internal domains, and is presumed deficient in multiple psychiatric disorders in which there is a failure to filter cognitive, sensory, or motor information (Braff et al, 2008; Geyer, 2008; Swerdlow et al, 2008). Prepulse inhibition (PPI), referring to the inhibition by a weak pre-stimulus (prepulse) of the startle response to a subsequent intense stimulus (pulse), measures sensorimotor gating, and patients with disorders involving deficient sensorimotor gating show deficits in PPI (Braff et al, 2001). Thus, studying PPI in animal models provides a useful tool for delineating the neural circuitry underlying clinically observed deficits in sensorimotor gating, and may help to identify the neural substrates for information-processing disturbances in a number of psychiatric illnesses (Geyer et al, 2001; Swerdlow et al, 2001).

Noradrenergic (NE) substrates of PPI are not well characterized, which is surprising given the proposed role of NE in regulating attention, arousal, and cognition (Arnsten, 2004; Aston-Jones and Cohen, 2005; Berridge and Waterhouse, 2003; Sara, 2009). Recent findings indicate that NE receptors regulate PPI (Alsene et al, 2006; Carasso et al, 1998; Lahdesmaki et al, 2002, 2004; Sallinen et al, 1998), and that stimulation of the locus coeruleus (LC), the major source of NE to forebrain (Foote et al, 1983), produces an anatomically and behaviorally specific deficit in PPI that is independent of dopamine (DA) or serotonin transmission, is reversed preferentially by second-generation antipsychotics, and is mediated by downstream NE release (Bakshi and Alsene, 2010). The specific NE-innervated terminal fields through which these effects are produced, however, remain unknown.

Sites throughout the neuraxis receive NE input; many of these regions previously have been found through lesion or pharmacological studies to regulate PPI, but to what extent NE is involved is unknown (Berridge and Waterhouse, 2003; Schwabe and Koch, 2004; Swerdlow et al, 2001). Among these are portions of the medial prefrontal cortex (mPFC), ventral striatum, extended amygdala, hippocampus, and thalamus, all of which contain NE-synthesizing nerve terminals and significant concentrations of the main postsynaptic adrenoceptors and NE heteroreceptors (α1 and β) (Bylund et al, 1994; Foote et al, 1983; McCune et al, 1993; Nicholas et al, 1996; Palacios and Kuhar, 1982; Pieribone et al, 1994; Pupo and Minneman, 2001; Rainbow et al, 1984). Hence, this study was designed to map the forebrain sites at which infusions of an α1+β-adrenoceptor cocktail (stimulating non-autoreceptor NE receptors) disrupted PPI. This investigation provides a crucial step in delineating the substrates through which NE transmission regulates PPI and as such has the potential to identify novel pathways underlying the sensorimotor gating deficits seen in numerous psychiatric illnesses in which NE signaling abnormalities are hypothesized (Braff et al, 2001; Daly et al, 2005; Dvorakova et al, 2007; Hornykiewicz, 1982; Southwick et al, 1993).

SUBJECTS AND METHODS

Animals: A total of 134 male Sprague–Dawley rats (Harlan Laboratories, Madison, WI) weighing 300–400 g were housed in pairs in clear cages with *ad libitum* access to food and water in a light- and temperature-controlled vivarium, and were maintained under a 12-h light–dark cycle (lights on at 0700 hours). All facilities and procedures were in accordance with the guidelines regarding animal use and care from the NIH of the United States, and

were supervised and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin.

Surgery: Rats were anesthetized with ketamine/xylazine (80mg/12mg per ml; Phoenix Scientific, St Joseph, MO), and secured in a stereotaxic frame (Kopf Instruments, Tujunga, CA). Stainless steel cannulae (23-gauge, Small Parts, Miami Lakes, FL) were implanted and affixed to the skull with dental cement (Lang Dental Mfg, Wheeling, IL) and anchoring skull screws (Plastics One, Roanoke, VA) and were aimed bilaterally at sites using the atlas of Paxinos and Watson (1998). Final coordinates in mm from bregma were: anterior mPFC (+3.0 AP, ±0.75 LM, −4.1 DV); posterior mPFC (+2.0 AP, ±0.8 LM, −4.0); nucleus accumbens shell (NAccSh, +1.7 AP, ±0.8 LM, −7.5 DV); nucleus accumbens core (NAccCo, +1.3 AP, ±2.0 LM, −7.1 DV); mediodorsal thalamus (MD-thalamus, −3.0 AP, ±0.8 LM, −5.6 DV); bed nucleus of the stria terminalis (BNST, at an 11° angle from the midline, −0.8 AP, ±2.7 LM, −6.7 DV); dorsal hippocampus (DH, −3.3 AP, ±2.0 LM, −3.6 DV); central nucleus of amygdala (CeA, −2.1 AP, ±3.85, −7.9 DV); and basolateral amygdala (BLA, −3.0 AP, ±4.8 LM, −8.3 DV). Wire stylets were placed in the cannulae to prevent blockage, and rats recovered for a week before testing.

Drugs and Microinfusions: All drugs ((±)-phenylephrine hydrochloride, (±)-isoproterenol hydrochloride, and (−)-quinpirole hydrochloride) were obtained from Sigma (St Louis, MO) and dissolved in sterile isotonic saline. Doses were calculated as salts based on previous studies (Berridge *et al*, 2003), and the infusion volume for all experiments was 0.5µl per side.

For microinfusions, stylets were removed and cannulae were cleaned with a dental broach (Henry Schein, Melville, NY); stainless steel injectors (30-gauge, Small Parts) were lowered to extend 2.0mm past the tip of the cannula (DV coordinates reflect distance from skull surface to the injector tip after insertion). Injectors were attached with polyethylene tubing (PE-10, Becton Dickinson, Sparks, MD) to 10-µl glass Hamilton syringes (Hamilton, Reno, NV) mounted on a microdrive pump (Harvard Apparatus, Holliston, MA). After infusions, injectors were left in place for 1min to allow for absorption of the solution before replacement of stylets.

Startle Chambers: Startle chambers (San Diego Instruments, San Diego, CA) contained a nonrestrictive Plexiglas cylinder resting inside a ventilated and illuminated sound-attenuating cabinet, with a high-frequency loudspeaker to produce all acoustic stimuli. As described previously (Mansbach *et al*, 1988), the whole-body startle response of the animal caused vibrations, which were then converted into analog signals by a piezoelectric unit attached to the platform. These signals were digitized and stored by a microcomputer and interface unit. Monthly calibrations were performed on the chambers to ensure accuracy of the sound levels and measurements. Sound levels were measured using the dB(A) scale.

Startle and PPI Testing: The test session consisted of a background noise (65dB) that was presented alone for 5min and remained on for the length of the session, followed by presentation (in a pseudo-random order) of pulse-alone trials (40-ms, 120-dB broadband bursts), prepulse+pulse trials (20-ms noises that were 3, 9, or 15dB above the background noise and were presented 100ms before the onset of the 120-dB pulse), and no stimulus trials (only the background noise). The session was divided into two equivalent parts that each

contained 40 trials (8 each of the 3-, 9-, and 15-dB prepulse+pulse trials, 8 pulse-alone trials and 8 no stimulus trials). Four pulse-alone trials were presented at the beginning and the end of the session to ensure that startle magnitude was stable during the portion of the session when PPI was measured, as the most rapid habituation of the startle response occurs within the first several presentations (Geyer *et al*, 1990); these pulse-alone trials were excluded from the calculations of startle and %PPI. During the week before the drug testing began, all rats underwent one exposure to the startle test session per day on 3 separate days with sham infusions preceding the last test to familiarize rats with the testing and microinfusion procedures before the commencement of drug testing.

Experimental Design: Each experiment tested the effects of microinfusions of a mixed NE receptor agonist cocktail (α1 and β) in a specific brain region, using separate sets of experimentally naïve rats for each site. Rats were given microinfusions of an equal parts cocktail of the α1-receptor agonist phenylephrine plus the β-receptor agonist isoproterenol (0, 3, 10, or 30µg/0.5µl) into either the anterior mPFC (*N*=10), posterior mPFC (*N*=8), NAccSh (*N*=10), NAccCo (*N*=8), MD-thalamus (*N*=7), BNST (*N*=6), DH (*N*=8), CeA (*N*=12), or the BLA (*N*=13) immediately before testing in the startle chambers. For each experiment, doses were administered in a counterbalanced order with at least 2 days separating consecutive tests. For all of the sites in which no effect of the NE receptor agonists was found, an additional study was done to determine if the DA D2 receptor agonist quinpirole would produce any effect. Thus, separate groups of experimentally naïve rats received either saline or quinpirole (10µg/0.5µl) into either the anterior mPFC (*N*=13), NAccCo (*N*=16), DH

(*N*=8), and CeA (*N*=15) immediately before testing in startle chambers; the treatments were given in a counterbalanced order over 2 test days that were separated by 72h.

Histology: At the end of all experiments, rats were perfused transcardially with 10% formalin in phosphate buffer (Sigma Diagnostics, St Louis, MO), 60-μm brain sections were taken through the injection site and stained with cresyl violet, and placements were verified by an experimenter blind to the behavioral data; rats with missed placements were excluded from subsequent behavioral analyses. Sample sizes for each experiment reflect this final adjusted number.

Data Analysis: The startle response to the onset of the 120-dB burst was recorded for 100 ms for each pulse-alone, prepulse+pulse, and from the onset of each no stimulus trial. Two measurements (startle magnitude and PPI) were calculated from these values for each rat for each of the different treatment conditions. Startle magnitude was the average of the startle responses to all pulse-alone trials. PPI was calculated as a percent score for each prepulse+pulse trial type: % PPI=100−((startle response for prepulse+pulse trial)/(startle response for pulse-alone trial)). *Post hoc* analyses were done using Newman–Keuls tests. The α-level for significance was set at 0.05.

RESULTS

For every experiment, a significant main effect of prepulse intensity was seen on analyzing %PPI data (F-ratios ≥35, *P*-values <0.001); this is a standard parametric feature of PPI in which increasing prepulse intensities elicit higher levels of PPI (Braff *et al*, 2001). For the sake of brevity, this main effect is not repeated throughout the Results section, as there also

were no significant interactions between the prepulse intensity and drug factors for any experiment.

Stimulation of *α*1+*β*-NE Receptors in Discrete Forebrain Regions Disrupts PPI

Medial prefrontal cortex

ANOVA revealed a significant main effect of the *α*1+*β*-NE receptor agonist cocktail on PPI in the posterior mPFC ($F(3, 21)=6.3$, $P<0.003$), but not the anterior mPFC ($F(3, 27)=0.6$, NS). *Post hoc* analyses indicated that the highest dose reduced PPI levels significantly below vehicle levels at each prepulse intensity in the posterior mPFC ($P<0.05$) (Figure 1).

Nucleus accumbens

Differential effects on PPI with NE receptor stimulation were also obtained across NAcc subregions (Figure 2), with a significant main effect of NE agonist treatment in NAccSh ($F(3, 27)=2.9$, $P<0.048$), but not in NAccCo ($F(3, 21)=0.6$, NS). Subsequent analyses confirmed that in the NAccSh, the highest dose of the cocktail reduced PPI at the 9-dB ($P<0.05$) and 15-dB ($P<0.01$) prepulse intensities.

BNST

A significant main effect of drug was seen in the BNST ($F(3, 15)=6.4$, $P<0.006$), (Figure 3), with reduction of PPI at each prepulse intensity after administration of the highest dose of the cocktail ($P<0.05-P<0.01$).

Amygdala

Heterogeneity in the response to NE receptor stimulation also was seen across subregions of the amygdala (Figure 4), with significant main effects of drug treatment in the BLA ($F(3, 36)=3.3$, $P<0.030$), but not in the CeA ($F(3, 33)=0.3$, NS). In the BLA, the highest dose reduced PPI at the lower two prepulse intensities ($P<0.05$); at the 9-dB intensity, the 10-µg dose in BLA also reduced PPI ($P<0.05$). For these sites, a number of rats had injector placements that fell just outside of either amygdala subregion; these subjects were thus not included in the above groups. Instead, their behavioral data were analyzed separately in order to determine if NE receptor agonist infusions immediately outside of the BLA and CeA affected PPI. Neither the 'missed-BLA' group ($N=7$) nor the 'missed-CeA' group ($N=4$) showed any significant drug treatment effect (F ratios for main effects or interactions ≤0.5, P-values ≥0.7), indicating that the PPI-disruptive effect that was observed following NE agonist infusion into the BLA was highly anatomically specific.

Thalamus and hippocampus

The $\alpha 1+\beta$-receptor cocktail produced a significant main effect of drug treatment in the MD-thalamus ($F(3, 18)=12.6$, $P<0.001$), but not in the DH ($F(3, 21)=0.5$, NS). *Post hoc* analyses indicated that in MD-thalamus, both the 10-µg ($P<0.05$) and the 30-µg ($P<0.01$) doses reduced PPI at all three prepulse intensities; this effect in MD-thalamus was the strongest of all sites examined (Figure 5). In particular, comparison of the efficacy of the middle dose to disrupt PPI indicated that in MD-thalamus, this dose produced a 30% reduction in composite PPI from vehicle values, which was nearly double the PPI reduction produced by this dose in the BLA (the only other site in which the 10-µg dose had any effect at all). Thus, both in

terms of magnitude of the effect size as well as sensitivity to lower doses, the MD-thalamus showed a greater effect than any other site tested.

There was some variability in the non-drug (vehicle) levels of PPI and startle across brain regions, which either could be due to the differential patterns of tissue damage that would be incurred by targeting the different sites or that simply reflects the regular variability in baseline values from one cohort of rats to another. Nevertheless, because a within-subjects design was used for each study, this small variability in baseline values across experiments would not affect the experimental outcomes, because drug and vehicle values were obtained within the same rat.

Sites Unresponsive to NE Receptor Agonists do Support DA D2 Agonist-Induced PPI Disruption

As a positive control in sites where the NE agonist cocktail did not disrupt PPI, the D2 agonist quinpirole was tested. ANOVA indicated a significant main effect of quinpirole treatment in each of these regions: anterior mPFC ($F(1,12)=4.7$, $P<0.049$); NAccCo ($F(1,15)=8.9$, $P<0.009$); DH ($F(1, 7)=18.6$, $P<0.004$); CeA ($F(1, 14)=6.6$, $P<0.023$). *Post hoc* analyses confirmed that quinpirole significantly lowered PPI at multiple prepulse intensities in each site ($P<0.05-P<0.001$). Thus, the failure of anterior mPFC, NAccCo, DH, or CeA to display NE receptor-mediated PPI deficits is not because of imperviousness to PPI modulation in these regions; rather, it indicates that in these sites, stimulation of DA but not NE receptors disrupts PPI (Figure 6).

Effects on PPI are Dissociable from Changes in Baseline Startle

Table 1 displays the effects of the phenylephrine (α1)/isoproterenol (β) cocktail on baseline startle values (responses to the pulse-alone trials) in each site; Table 2 gives this information for the quinpirole tests. ANOVA indicated a significant main effect of the NE receptor cocktail on startle responses after infusion into the posterior mPFC ($F(3, 21)=4.8$, $P<0.01$), BLA ($F(3, 36)=5.9$, $P<0.003$), BNST ($F(3, 15)=3.6$, $P<0.04$), and CeA ($F(3, 33)=2.8$, $P<0.05$); *post hoc* analyses revealed that the highest dose reduced startle compared with the vehicle condition ($P<0.05$) in each of these sites. No significant main effect of the NE receptor cocktail was seen with infusion into any other site (all F ratios ≤ 1.8 and *P*-values ≥ 0.13). A significant main effect of quinpirole treatment was seen after infusion into the anterior mPFC ($F(1, 12)=10.7$, $P<0.007$), NAccCo ($F(1, 15)=18.9$, $P<0.001$), and DH ($F(1, 7)=13.1$, $P<0.009$), with quinpirole lowering startle in these sites ($P<0.05$), but not in the CeA.

Therefore, PPI reduction was seen without concomitant startle changes (ie, with cocktail infusion into NAccSh or MD-thalamus or quinpirole infusion into CeA), and in other cases, startle reduction was seen without accompanying alterations in PPI (ie, with cocktail infusion into CeA). Thus, alterations in baseline startle are neither necessary nor sufficient to produce PPI deficits. In order to further confirm this dissociation between startle and PPI effects, additional analyses were conducted for all experiments using a median-split method to divide rats within each experiment into two groups based on the degree of startle suppression induced by the drug treatment relative to the vehicle condition (the 30-µg dose of the cocktail, or the quinpirole for the D2 agonist studies). Thus, half of the rats in each

experiment were assigned to the 'high suppression' group, and the other half were in the 'low suppression' group. This grouping designation was then included as an additional factor in the original ANOVAs to examine whether the high and low groups showed differential PPI responses with drug treatment. No significant interactions on PPI were seen for the startle suppression grouping factor and the drug treatment factor for any experiment, indicating that the drug-induced PPI deficits were independent of any effect on baseline startle magnitude.

DISCUSSION

The goal of this study was to identify the forebrain NE terminal fields in which stimulation of NE receptors (excluding autoreceptors) disrupts PPI. Infusion of an equal parts cocktail of the α1-NE receptor agonist phenylephrine and the β-receptor agonist isoproterenol into posterior mPFC, NAccSh, BNST, BLA, or MD-thalamus caused a significant disruption of PPI, demonstrating that direct stimulation of NE receptors in these sites reproduces the deficit in information processing that is seen in multiple psychiatric illnesses including schizophrenia (Braff et al, 2001). These PPI deficits occurred with or without concomitant reductions in the startle response to the pulse-alone, indicating that simple alterations in baseline startle reactivity are not sufficient or necessary for NE receptor-mediated effects; instead, these effects reflect a veritable reduction in sensorimotor gating. Moreover, the α1+β-receptor-induced PPI deficit exhibited specificity for several discrete cortical and subcortical targets, rather than being promiscuously expressed in all sites tested; thus, for each site in which the agonist cocktail produced an effect, there was an adjacent control site in which the same infusion parameters had no behavioral effect. Nevertheless, in each of the 'negative' sites tested (anterior mPFC, NAccCo, CeA, and DH), stimulation of DA D2

receptors did disrupt PPI. This positive control manipulation reveals that the lack of NE effect in those sites was not because of a lack of functional significance for PPI modulation, but rather that there is a mechanistic difference between NE and DA signaling within those sites that underlies the dissociable effects of these two systems on PPI. In summary, the present results reveal a set of specific anatomical substrates that mediate the disruptions of PPI seen with systemic administration of NE receptor agonists (Alsene et al, 2006; Carasso et al, 1998; Swerdlow et al, 2006; Varty et al, 1999). Moreover, as LC stimulation disrupts PPI through downstream release of NE (Bakshi and Alsene, 2010), these findings provide the first demonstration of the specific NE terminal regions through which this effect might occur. Overall, these findings indicate novel pathways for the regulation of PPI that may be distinct from previously characterized DA-sensitive substrates.

Previous studies of DβH immunoreactivity (the enzyme for NE synthesis used to identify NE-containing neurons) indicated that in every site in which the α1+β-NE receptor agonist cocktail disrupted PPI, there was an abundance of NE-containing fibers (terminals) (Baldo et al, 2003; Berridge et al, 1997; Delfs et al, 1998; Swanson and Hartman, 1975). Among the 'PPI-negative' regions for the cocktail infusion, only NAccCo lacked DβH immunoreactivity; all other sites (anterior mPFC, DH, and CeA) displayed intense labeling. This observation confirms that the sites identified with the present direct-agonist mapping approach have physiological relevance with regard to understanding the behavioral effects of endogenous NE transmission. Moreover, high concentrations of both α1+β-NE receptors are found in many of the sites (anterior mPFC, DH, and CeA) in which the agonist cocktail had no effect on PPI (Pupo and Minneman, 2001; Rainbow et al, 1984; Swanson and Hartman,

1975; Young and Kuhar, 1980), and the dose range employed in the present study was sufficiently high to alter other behaviors in these 'PPI-null' sites (Azami et al, 2010; Ferry et al, 1999; Kerfoot et al, 2008). Hence, our negative results are not because of the use of behaviorally inactive doses. The most parsimonious explanation is that there is a veritable functional segregation across anatomical regions with regard to NE modulation of PPI.

The specific structures that were found in the present studies to support NE-mediated PPI deficits likely reflect nodes within a distinct network or networks that ultimately impinge on the pedunculopontine tegmental nucleus, which functions as a midbrain interface between forebrain cognitive processing and the brainstem startle circuit (Fendt et al, 2001; Koch et al, 1993; Swerdlow et al, 2001), and is regarded as the obligatory 'final pathway' for forebrain regulation of PPI. Two primary considerations are relevant to understanding these putative NE-sensitive networks. First, does NE signaling elicit differential effects on neuron firing, local circuit dynamics, or other relevant parameters in 'PPI-responsive' vs 'PPI-null sites' that could explain the differential NE agonist effects in these sites? Recent information regarding the subcellular localization of α1-receptors in NAccSh suggests specific substrates through which NE signaling might shift excitatory and DA neurotransmission in sites that regulate PPI (Rommelfanger et al, 2009; Verheij and Cools, 2009). Although not all the regions examined in this study have been tested with regard to such substrates, there are some independent findings that hint at possible mechanisms for the differential effects of NE receptor agonists across these regions. For example, measuring short-term potentiation in amygdala slices, it was found that β-receptor stimulation with isoproterenol increases synaptic plasticity in the medial portions of this structure such as CeA, but decreases it in

BLA (Watanabe et al, 1996). Similarly, in certain regions such as basal forebrain and amygdala, α1- and β-NE receptors produce additive effects or work in concert to support behavioral functions (Berridge, 2008; Ferry et al, 1999), but in cortical areas, these receptor subtypes can have opposing actions (Kobayashi, 2007). Thus, the net result of co-stimulating α1- and β-receptors (as would occur with endogenous enhancement of NE transmission) can vary across brain regions, and functional neuropharmacological differences such as these could represent the basis for the divergent efficacy of our NE direct agonist cocktail across the brain sites tested. Nevertheless, such factors cannot entirely account for the anatomical profile obtained in the present studies, as sites such as anterior vs posterior mPFC likely share similar α1- and β-receptor dynamics.

Thus, a second mechanism that could explain the divergent efficacy across sites of the α1+β-receptor cocktail is the differing input–output circuitry of these regions. All of the 'PPI-positive' sites fall into one of two putative anatomical circuits, a ventral forebrain network (with NAccSh and BNST), or a thalamocortical network (with posterior mPFC, BLA, and MD-thalamus). These two networks can be distinguished based on the sources of their afferent NE innervation as well as the magnitude of PPI disruption that was produced by NE receptor stimulation. One pattern that emerges is that sites subserving a more modest NE-based PPI disruption (ie, effects seen only with the highest dose and only at some prepulse intensities) receive NE innervation primarily from the A1/A2 cell bodies (nucleus of the solitary tract) with far lesser contributions from the A6 cell bodies of the LC (Berridge et al, 1997; Delfs et al, 1998; Terenzi and Ingram, 1995). This pattern might suggest that the types of stimuli that drive A1/A2 nuclei (largely related to ascending visceral and autonomic

information) do not exert as strong an effect on sensorimotor gating through these ascending NE projections.

In contrast, sites within our proposed thalamocortical circuit (BLA, posterior mPFC, and MD-thalamus) receive all NE innervation from the LC (Foote et al, 1983; Swanson and Hartman, 1975). Of particular interest in this regard is the MD-thalamus. We found that NE agonists produce a profound PPI disruption in this site; indeed, this effect was the strongest and most dose-dependent out of all the sites tested. Interestingly, the BLA and posterior mPFC both connect reciprocally with MD-thalamus; robust NE agonist-induced PPI deficits were found in these sites, but not in the DH, which does not connect to MD-thalamus (Gabbott et al, 2005; Giguere and Goldman-Rakic, 1988; Hoover and Vertes, 2007; Kuroda, 1998; Miyashita et al, 2007; Montagnese et al, 2003; Vertes et al, 2007). Moreover, the anterior mPFC, which was a 'null' site in this study, has proportionately less abundant reciprocal connections with MD-thalamus, compared with the posterior mPFC, which was a 'PPI-active' site (Gabbott et al, 2005; Hoover and Vertes, 2007). Hence, the BLA, posterior mPFC, and MD-thalamus may represent nodes within a reciprocally interconnected network by which affectively valenced processing in frontotemporal structures gains access to a thalamic relay for PPI, the MD-thalamus. Accordingly, when NE receptors are stimulated in sites within this thalamocortical network, a larger PPI disruption is seen than with the ventral forebrain regions, with multiple doses of the NE agonist cocktail being effective at all prepulse intensities in the BLA, posterior mPFC, and, in particular, the MD-thalamus. The magnitude of the PPI deficit with NE receptor stimulation in MD-thalamus is similar to that seen with LC stimulation, which disrupts PPI via downstream release of NE (Bakshi and

Alsene, 2010). Thus, a reciprocally interacting thalamocortical network with a strong NE projection from LC to MD-thalamus could be recruited by LC stimulation, and produce profound deficits in PPI because of connections with other neocortical sites such as BLA and posterior mPFC.

Interestingly, lesions or pharmacological inactivation of MD-thalamus disrupt PPI, but intra-MD-thalamus infusions of DA agonists do not, suggesting that this site regulates PPI independently of the DA system (Kodsi and Swerdlow, 1997; Swerdlow et al, 2002). The present results for the first time indicate a specific neurotransmitter system (NE) through which MD-thalamus regulation of PPI occurs, and raise the question of whether NE receptor stimulation in MD-thalamus disrupts PPI by functionally inactivating this nucleus. Although thalamus is known to contain particularly high levels of α1-receptors (McCune et al, 1993; Pieribone et al, 1994; Strazielle et al, 1999), the cellular and ultrastructural localization of NE receptors specifically within the MD nucleus is not known, thus it is unclear whether these receptors are positioned to shift the balance of excitatory vs inhibitory transmission within this site as they have been hypothesized to do in ventral striatum (Rommelfanger et al, 2009). Nevertheless, there is some evidence to suggest that in some thalamic regions, NE receptor stimulation can depress the firing rate of up to 90% of responsive neurons (Grasso et al, 2006). Whether these mechanisms also apply to the MD-thalamus remains to be determined, as the nature of NE-mediated electrophysiological responses in thalamus is exquisitely dependent on local transmitter concentration as well as cell type (McCormick et al, 1991; Moxon et al, 2007), but the notion that the present PPI disruption is due to a functional reduction of MD-thalamus activity is certainly a plausible hypothesis that merits further

investigation. MD-thalamus does receive dense NE-containing projections that are distributed throughout the nucleus, and are even more abundant than the DA innervation of this site (Melchitzky and Lewis, 2001), so there is an additional anatomical basis for hypothesizing that within the MD-thalamus, NE transmission may be a crucial modulator of sensorimotor gating.

Cellular and neurotransmission abnormalities have been found in schizophrenia patients in several of the brain regions identified presently to mediate NE-based sensorimotor gating deficits, including MD-thalamus (Alelu-Paz and Gimenez-Amaya, 2008; Byne et al, 2009; Lewis, 2009; Pakkenberg et al, 2009), and analysis of MD-thalamus-PFC innervation at the ultrastructural level has led to the suggestion that this site is well positioned to 'gate' information processing within PFC (Rotaru et al, 2005), a process that may be intimately connected to PPI. Hence, the MD-thalamus may represent an important site of action for second-generation ('atypical') antipsychotic drugs that act in part through DA-independent mechanisms such as α1-NE receptors (Bymaster et al, 1996), since second-generation antipsychotics are particularly effective in normalizing NE-based PPI deficits that are induced by stimulation of the LC (Bakshi and Alsene, 2010).

α1- and β-receptors in multiple forebrain sites have long been known to participate in higher-order cognitive tasks such as working memory and attentional set-shifting via mPFC (Arnsten et al, 1999, 1998; Lapiz and Morilak, 2006; Ramos et al, 2005, 2006), and learning and memory processes via hippocampus and amygdala (Ferry et al, 1999; Miranda et al, 2007; Roozendaal et al, 2004, 2008). The present results considerably broaden this view of the functional role of NE receptors in cognition by providing for the first time a systematic

delineation of the extended neuronal network through which NE signaling can regulate pre-attentional forms of information processing such as PPI. Given the conceptualization of sensorimotor gating as a breakdown in information filtering that can result in the processing of potentially irrelevant stimuli (Braff et al, 2008; Swerdlow et al, 2008), it is tempting to speculate that NE receptor-mediated deficits in PPI might contribute to impairments in volitional attention-based tasks such as delayed alternation paradigms by allowing too much (potentially irrelevant) information to gain access and compete with task-relevant information held online in frontotemporal networks. The present results are also consistent with theories that altered forebrain NE transmission contributes to the cognitive deficits seen in schizophrenia patients (Robbins and Arnsten, 2009).

These findings have implications for all psychiatric diseases that involve deficient sensorimotor gating. Schizophrenia is often considered the prime example of these diseases; however, Tourette syndrome and post-traumatic stress disorder (PTSD) are also reliably associated with disrupted PPI (Braff et al, 2001; Castellanos et al, 1996; Grillon et al, 1996; Swerdlow, 2001). These disorders are well known to be responsive to NE-based drugs and potentially involve dysregulation of the NE system (Bhidayasiri, 2005; Breier et al, 1990; Gay et al, 1989; Gomes et al, 1980; Leckman et al, 1995; Sandyk, 1986). Therefore, it is possible that increased NE signaling at α1- and β-receptors in regions such as posterior mPFC, NAccSh, BNST, BLA, and MD-thalamus could contribute to the pathophysiology of these psychiatric disorders. Indeed, the α1-NE receptor antagonist prazosin and drugs such as guanfacine and clonidine that reduce NE transmission via stimulation of α2-NE autoreceptors completely block NE-based PPI deficits (Alsene et al, 2006; Carasso et al, 1998; Swerdlow et

al, 2006) and are effective in treating PTSD and Tourette syndrome (Daly et al, 2005; Leckman et al, 1991), leading to the suggestion that NE-based models of PPI deficits may be particularly relevant for these illnesses (Swerdlow et al, 2006). Thus, normalization of NE signaling at α1- and β-receptors within nodes of these frontotemporal PPI networks could have beneficial effects on sensorimotor gating deficits in a number of psychiatric illnesses in which deficient PPI is considered a core endophenotype.

ACKNOWLEDGMENTS

This work was supported by T32GM007507, R01-MH075980 (VPB) and F31-MH077465 (KMA). We thank Dr Brian Baldo for his helpful comments on the paper.

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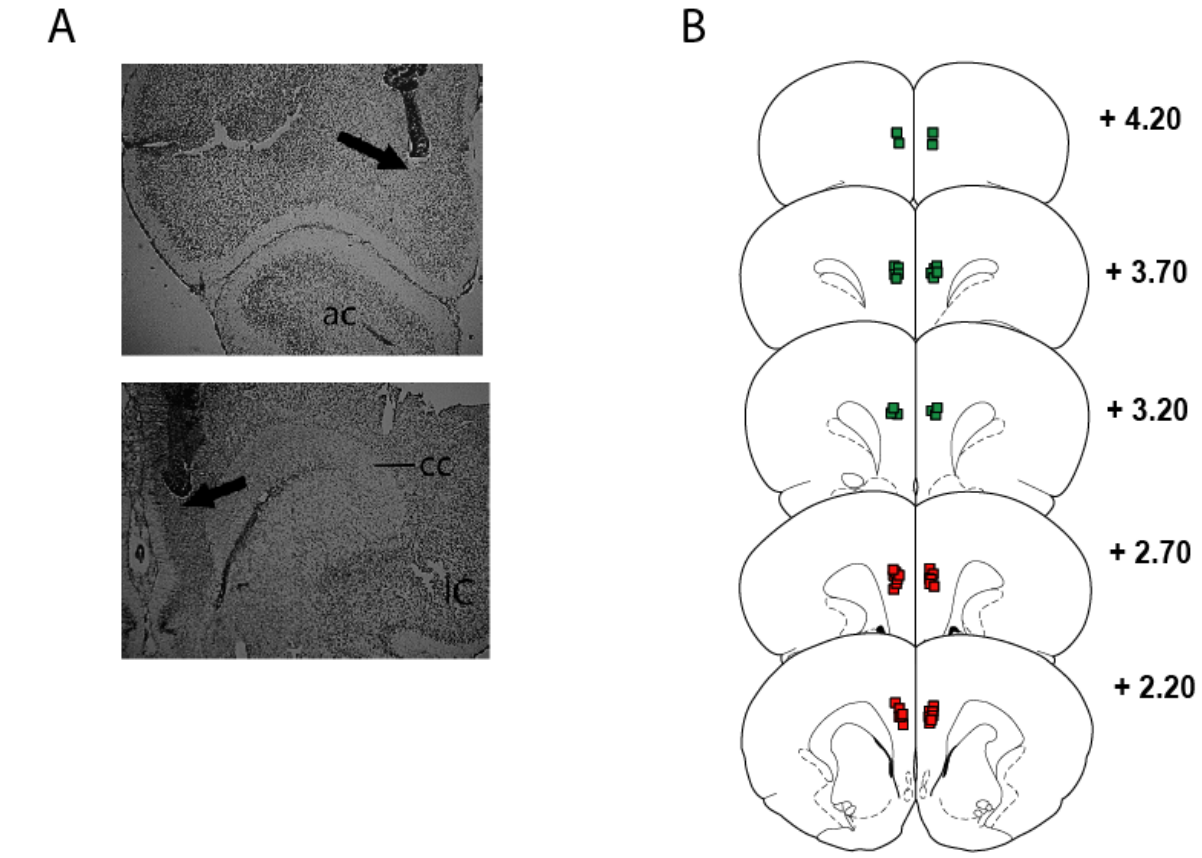
Fig. 1(A) Representative injector tip locations within the anterior (top panel) and posterior (bottom panel) medial prefrontal cortex (mPFC), indicated by the arrows. ac, anterior commissure; IC, insular cortex; cc, corpus callosum. (B) Chartings depicting the locations in which infusions of the α1+β-NE receptor agonist cocktail did (red) and did not (green) disrupt % prepulse inhibition (PPI). Distances are in mm from the bregma. (C) Effects on % PPI of the phenylephrine+isoproterenol cocktail in the anterior and posterior mPFC. Values represent means±SEM for each dose. Doses are in μg/0.5 μl. Prepulse intensity indicates decibels above the background noise level. ***** $P<0.05$, relative to VEH (vehicle) condition.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Anterior mPFC
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100
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Prepulse intensity (dB)
Posterior mPFC
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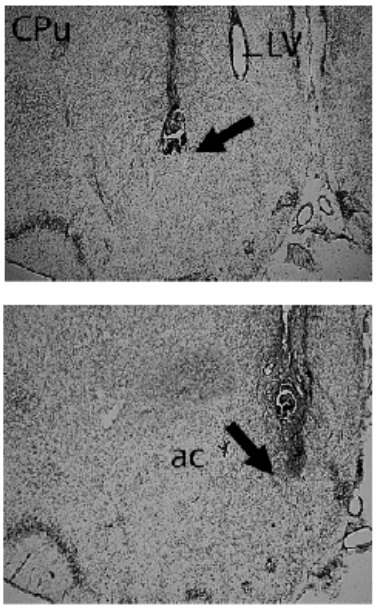
Fig.2 (A) Representative injector tip locations within the nucleus accumbens core (NAccCo) (top panel) and shell (NAccSh) (bottom panel), indicated by the arrows. ac, anterior commissure; CPu, caudate putamen; LV, lateral ventricle. (B) Chartings depicting the locations in which infusions of the α1+β-NE receptor agonist cocktail did (red) and did not (green) disrupt % prepulse inhibition (PPI). Distances are in mm from the bregma. (C) Effects on % PPI of the phenylephrine+isoproterenol cocktail in the NAccCo and NAccSh. Values represent means±SEM for each dose. Doses are in µg/0.5 µl. Prepulse intensity indicates decibels above the background noise level. ***** $P<0.05$, ****** $P<0.01$ relative to VEH (vehicle) condition.

A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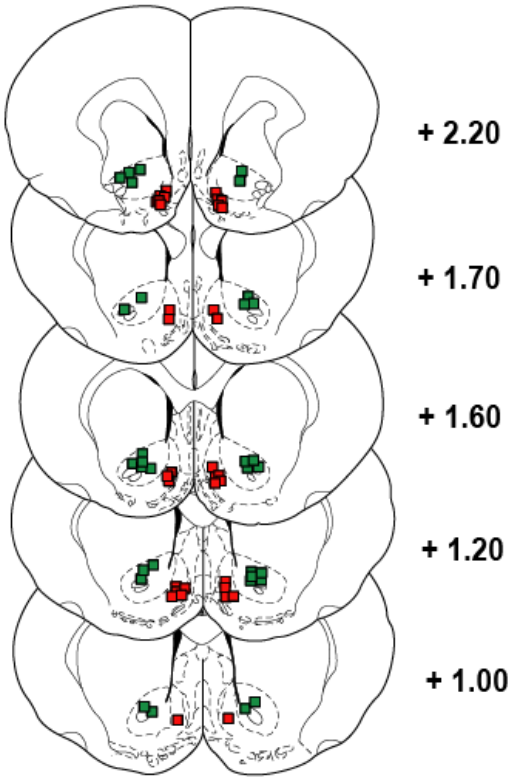
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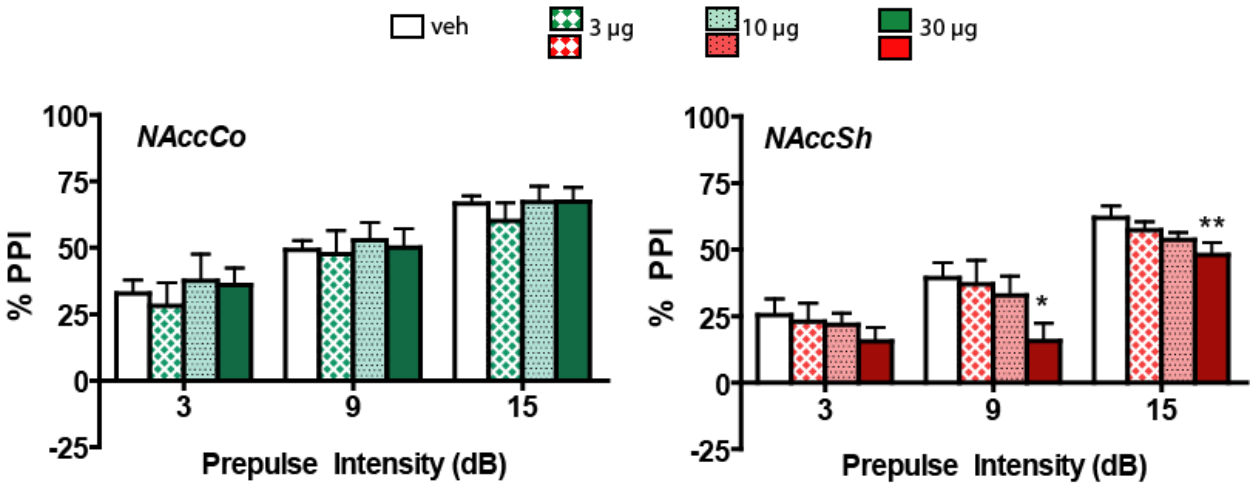
C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50
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-25
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Prepulse Intensity (dB)
*
**

Fig. 3 (A) Representative injector tip location within the bed nucleus of the stria terminalis (BNST), indicated by the arrow. LV, lateral ventricle; LGP, lateral globus pallidus. (B) Charting depicting the locations in which infusions of the α1+β-NE receptor agonist cocktail disrupted % prepulse inhibition (PPI). Distance is in mm from the bregma. (C) Effects on % PPI of the phenylephrine+isoproterenol cocktail in the BNST. Values represent means±SEM for each dose. Doses are in µg/0.5 µl. Prepulse intensity indicates decibels above the background noise level. ***** $P<0.05$, ******* $P<0.01$ relative to VEH (vehicle) condition.

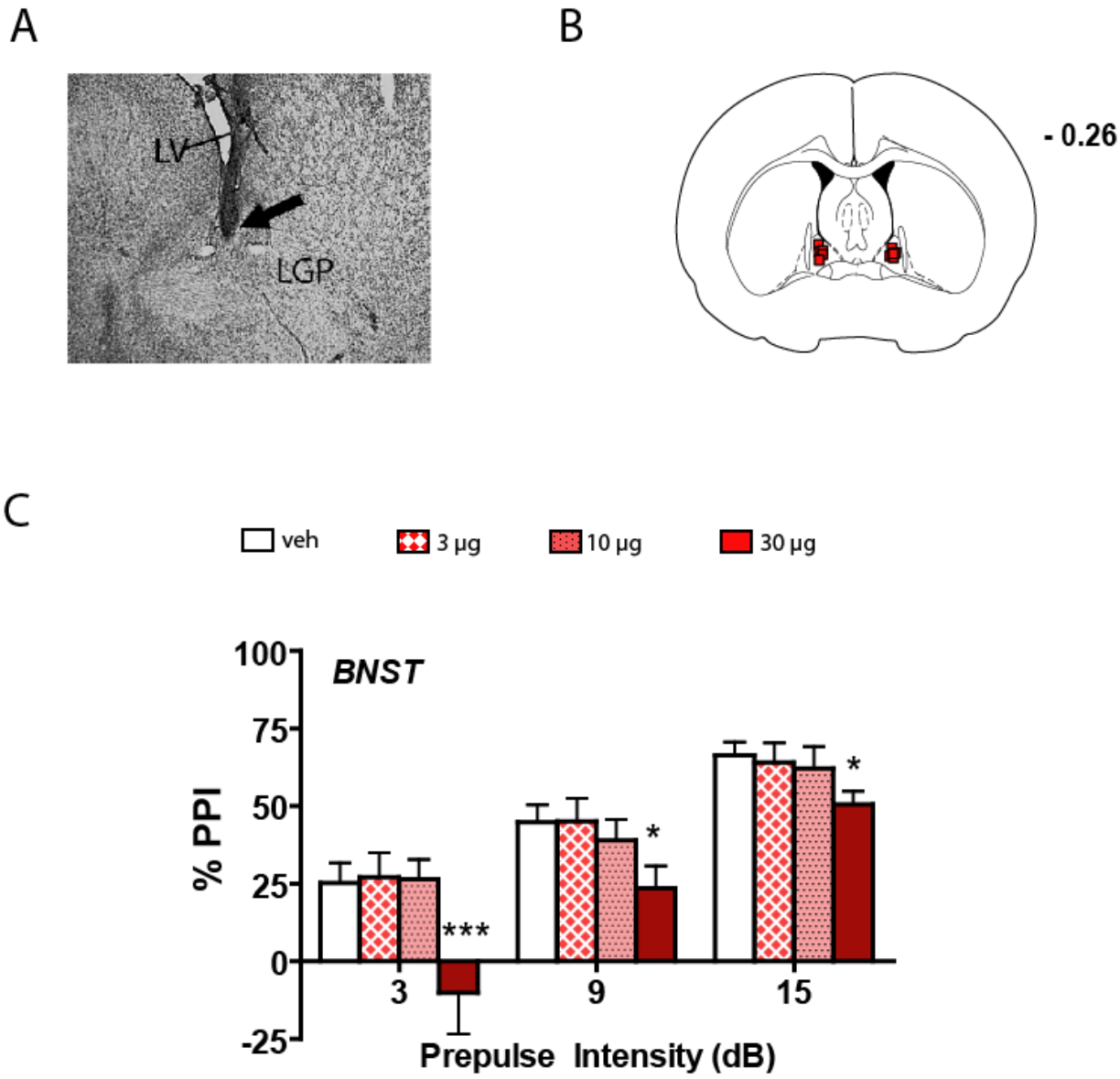


Fig. 4 (A) Representative injector tip locations within the central amygdala (CeA) (top panel), and the basolateral amygdala (BLA) (bottom panel), indicated by the arrows. PaMP, paraventricular hypothalamic nucleus (medial part); opt, optic tract; VM, ventromedial thalamic nucleus. (B) Chartings depicting the locations in which infusions of the α1+β-NE receptor agonist cocktail did (red) and did not (green) disrupt % prepulse inhibition (PPI). Distances are in mm from the bregma. (C) Effects on % PPI of the

phenylephrine+isoproterenol cocktail in the CeA and the BLA. Values represent means±SEM for each dose. Doses are in µg/0.5 µl. Prepulse intensity indicates decibels above the background noise level. * $P<0.05$, relative to VEH (vehicle) condition.

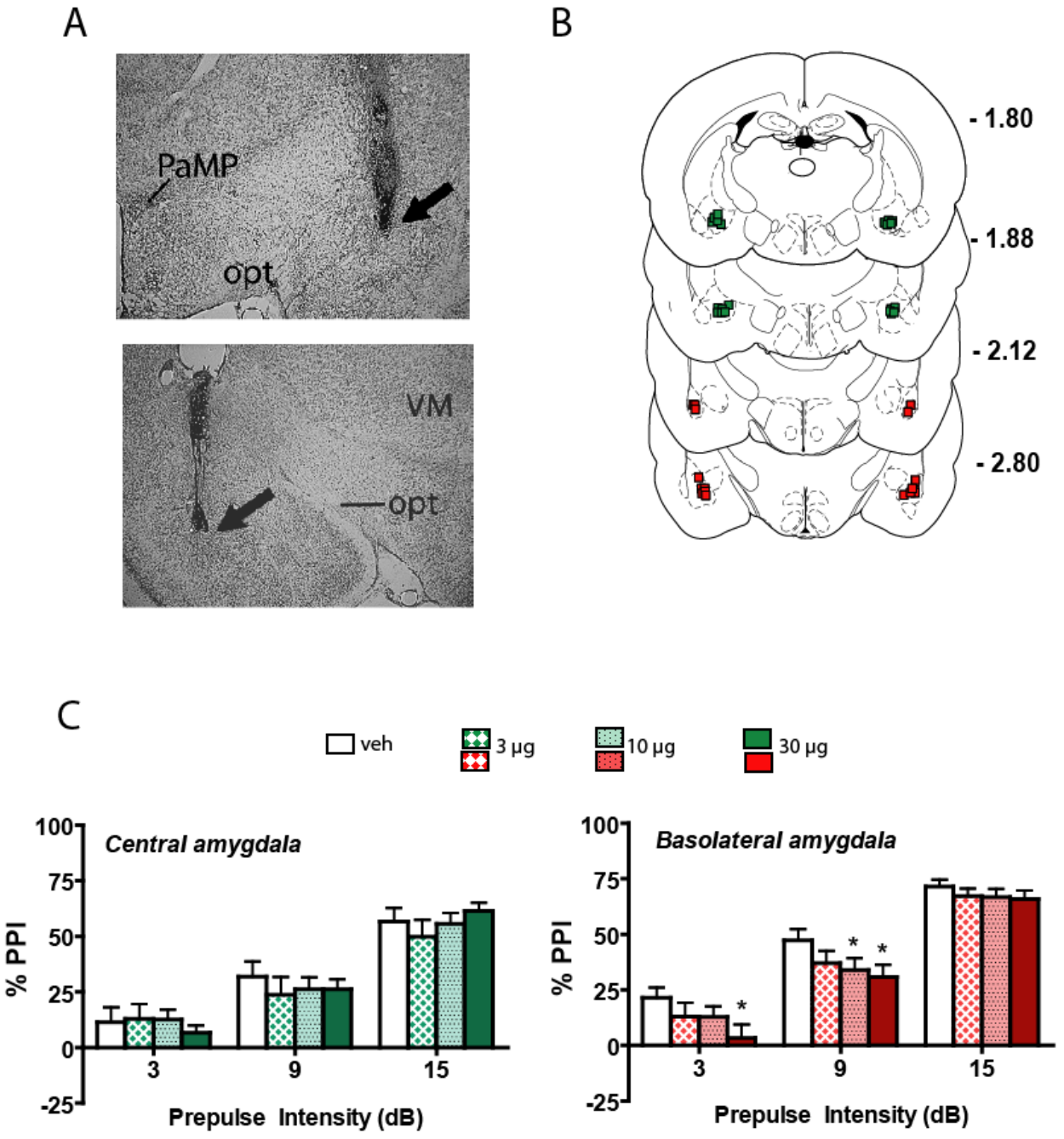
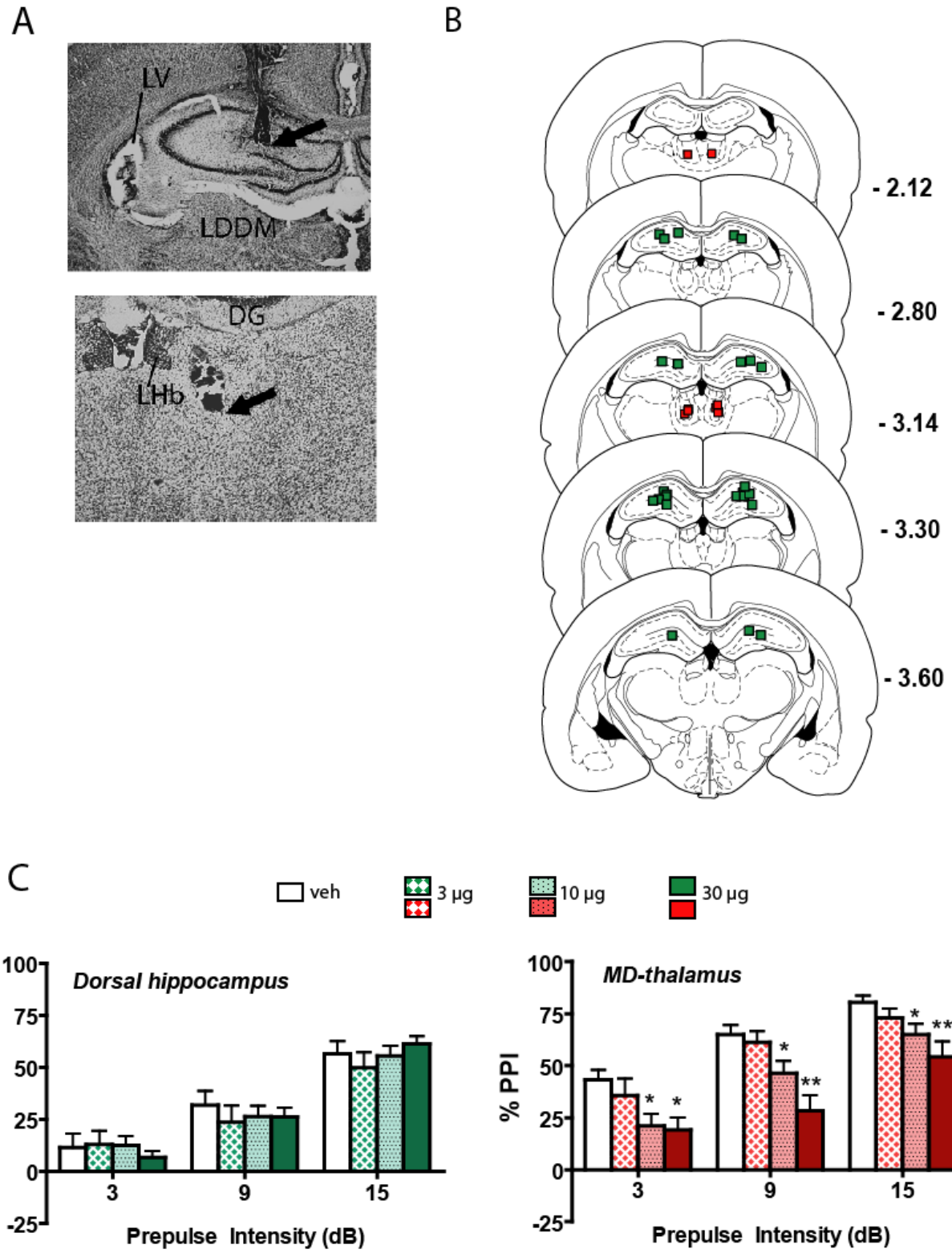


Fig. 5 (A) Representative injector tip locations within the dorsal hippocampus (DH) (top panel) and the mediodorsal (MD) thalamus (bottom panel), indicated by the arrows. LV, lateral ventricle; LDDM, laterodorsal thalamic nucleus; DG, dentate gyrus; LHb, lateral habenular nucleus. (B) Chartings depicting the locations in which infusions of the α1+β-NE receptor agonist cocktail did (red) and did not (green) disrupt % prepulse inhibition (PPI). Distances are in mm from the bregma. (C) Effects on % PPI of the phenylephrine+isoproterenol cocktail in the DH and the MD- thalamus. Values represent means±SEM for each dose. Doses are in μg/0.5 μl. Prepulse intensity indicates decibels above the background noise level. ***** P<0.05, ****** P<0.001, relative to VEH (vehicle) condition.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DG
LHb
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- 2.80
- 3.14
- 3.30
- 3.60
C
veh
3 µg
10 µg
30 µg
Dorsal hippocampus
MD-thalamus
% PPI
100
75
50
25
0
-25
3
9
15
Prepulse Intensity (dB)
*
**

Fig. 6 Effects on % prepulse inhibition (PPI) of treatment with the dopamine receptor agonist quinpirole in the (A) anterior mPFC, (B) nucleus accumbens core, (C) central amygdala, and (D) dorsal hippocampus. Values represent means±SEM for each drug condition. White bars are vehicle values and black bars are values for quinpirole (10 μg/0.5 μl). Prepulse intensity indicates decibels above the background noise level. **P*<0.05, ***P*<0.01, ****P*<0.001 relative to vehicle condition.

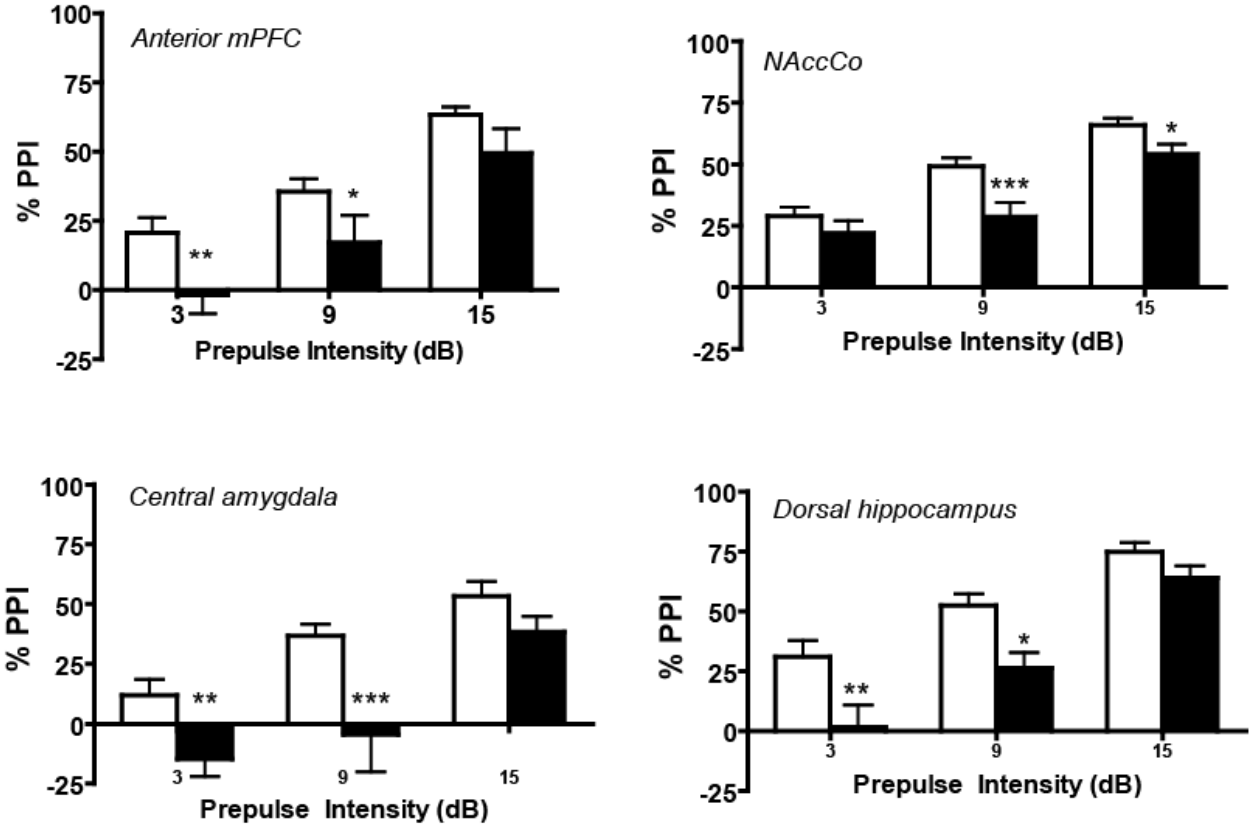

Table 1 Effects on Baseline Startle Magnitude (Responses to the 120-dB Pulse-Alone Trials) of the Cocktail Solution of the α1-Receptor Agonist Phenylephrine Plus the β-Receptor Agonist

Isoproterenol

	VEHICLE	3 µg Cocktail	10 µg Cocktail	30 µg Cocktail
Anterior mPFC	*294±43*	*337±24*	*275±43*	*261±40*
Posterior mPFC	*177±20*	*196±19*	*169±33*	*123±21**
NAccSh	*280 ± 30*	*243 ± 53*	*229 ± 38*	*177 ± 26*
NAccCo	*380 ± 42*	*388 ± 43*	*346 ± 32*	*361 ± 47*
BNST	*416 ± 92*	*393 ± 89*	*298 ± 62*	*208 ± 59**
Central amygdala	*418 ± 66*	*373 ± 57*	*355 ± 50*	*288 ± 42**
Basolateral amygdala	*409 ±41*	*390 ± 37*	*368 ± 50*	*299 ± 46**
MD-thalamus	*512 ± 199*	*303 ± 54*	*235 ± 37*	*231 ± 40*
Dorsal hippocampus	*326±55*	*341±60*	*350±61*	*326±71*

Table 2 Effects on Baseline Startle Magnitude (Responses to the 120-dB Pulse-Alone Trials) of the Dopamine D2 Receptor Agonist Quinpirole (10 mg/0.5 ml)

	VEHICLE	10 µg Quinpirole
Anterior mPFC	*315±35*	*217±31**
NAccCo	*408±49*	*163±38**
Central amygdala	*394 ± 53*	*309 ± 44*
Dorsal hippocampus	*254±40*	*164±21**

CHAPTER 3

NORADRENERGIC NEUROADAPTATIONS IN THE BASOLATERAL AMYGDALA AFTER PSYCHOLOGICAL STRESS OR CORTICOTROPIN-RELEASING FACTOR PROMOTES ENDURING PTSD-LIKE INFORMATION-PROCESSING DEFICITS

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The experiments in the following chapter were completed by myself. Technical assistance was provided by Sarah Newman and Jesus Mena. General experimental design, data analysis and conclusions were developed with the help of my thesis advisor, Dr. Vaishali Bakshi. These data are ready for submission to the journal of PNAS.

ABSTRACT

Prepulse inhibition (PPI), a measure of pre-attentional information-filtering, is deficient in posttraumatic-stress-disorder (PTSD) and schizophrenia, and may contribute to cognitive deficits stemming from an information-processing overload. Stress exacerbates these illnesses and activates corticotropin-releasing factor (CRF) and norepinephrine (NE) systems in the amygdala. It is presently unknown whether repeated stimulation of one of these stress-responsive systems produces plastic changes in the sensitivity of the other in modulating PPI. We tested the hypothesis that plasticity of these systems might occur with psychogenic stress, and contribute to subsequent PPI abnormalities. Separate groups of male Sprague Dawley rats received repeated infusions of CRF (200ng/0.5µl), NE (20µg/0.5µl), or vehicle into the basolateral (BLA) or central amygdala (CeA); CRF-treated rats were then challenged with a sub-threshold dose of NE (0.3µg/0.5µl) and NE-treated rats were challenged with CRF; PPI was measured after each infusion. A separate group of rats received 3 exposures to predator stress and were challenged with the low-dose intra-BLA NE. Initial or repeated CRF infusions had no effect on their own, but sensitized NE receptors such that a sub-threshold dose of NE in BLA now disrupted PPI. An identical profile of NE receptor sensitization in BLA was seen after repeated predator stress, and persisted even 18d later, when baseline startle was also increased by low-dose NE. The NE receptor-sensitization-induced PPI deficit could be prevented by intra-BLA CRF1 antagonist given prior to each predator stress exposure, but interestingly, not by this antagonist given after each stress or right before the NE challenge, showing that initiation of the NE receptor sensitization requires activation of CRF1 receptors in the BLA. We also found that activation of both alpha1 and beta receptors in the BLA are involved in sensitization to PPI-disruptive effects, as agonist challenges of either of these receptors after repeated predator stress disrupted PPI. No

effects were seen in CeA. Therefore, repeated exposure to intense psychological stress or repeated CRF1 receptor stimulation causes neuroadaptations of NE receptors within the BLA that result in hypersensitivity for PPI disruption and startle elevation from even low-level NE activation. These results indicate that BLA may be a critical modulator of underlying pathological information-processing deficits in response to mildly arousing stimuli, as seen in PTSD and schizophrenia.

INTRODUCTION

A deficit in information-filtering mechanisms such as sensorimotor gating is presumed to contribute to cognitive and affective impairment in neuropsychiatric disorders like schizophrenia and post-traumatic-stress-disorder (PTSD); these deficits can be quantified using prepulse inhibition (PPI), in which startle responses are inhibited by presentation of a weak prestimulus immediately prior to the startling event (Hoffman and Ison 1980; Grillon, Morgan et al. 1996; Braff, Geyer et al. 2001). PPI deficits are observed in both illnesses, and stress is thought to exacerbate or precipitate symptomatology in them, possibly through a long-lasting hypersensitivity to perceived threat from environmental stimuli (Brown and Birley 1968; Norman and Malla 1993; Walker and Diforio 1997). Among the most widely implicated systems in stress-induced psychopathology are corticotropin-releasing factor (CRF) and norepinephrine (NE), both of which are activated by stress and dysfunctional in PTSD and schizophrenia (Farley, Price et al. 1978; Breier, Wolkowitz et al. 1990; Southwick, Krystal et al. 1993; Baker, West et al. 1999; Strawn and Geracioti 2008). Accordingly, stress, CRF, or NE receptor stimulation can disrupt PPI (Conti, Murry et al. 2002; Risbrough, Hauger et al. 2004; Groenink,

Dirks et al. 2008; Alsene, Rajbhandari et al. 2011; Bakshi, Alsene et al. 2011; Sutherland and Conti 2011).

CRF and NE systems are well known to regulate each other (Valentino and Foote 1988; Emoto, Koga et al. 1993; Curtis, Lechner et al. 1997), but the extent to which they interact in modulating PPI remains unclear (Risbrough, Hauger et al. 2004; Gresack and Risbrough 2011). The amygdala, which contains high levels of CRF, NE, and their receptors, and recently found to regulate PPI via NE receptor stimulation (Rainbow, Parsons et al. 1984; De Souza, Insel et al. 1985; Pieribone, Nicholas et al. 1994; Alsene, Rajbhandari et al. 2011), is important for stress-related processes including fear and anxiety, in part through CRF and NE signaling (Davis 1992; Schulz, Fendt et al. 2002; Sandi, Cordero et al. 2008). Moreover, there is evidence for priming or plasticity in amygdala with repeated stimulation (Rainnie, Bergeron et al. 2004; Shekhar, Truitt et al. 2005). Thus, one mechanism by which exposure to repeated stress could lead to the enduring sensorimotor gating deficits seen in schizophrenia and PTSD is through stress-induced neuroadaptations in amygdala CRF and NE systems.

We hypothesized that plasticity between these two systems within subregions of the amygdala could promote deficient PPI such that previously ineffective manipulations would now disrupt PPI, and thereby model the phenomenon of a mild or innocuous stimulus triggering a pathological response when applied to a system that has been altered by repeated psychological trauma-like stress. To test this hypothesis, we repeatedly stimulated NE or CRF receptors within anatomically and functionally distinct subregions of the amygdala, the basolateral nucleus (BLA) and the central nucleus (CeA) (Davis 1998; LeDoux 2007), and tested whether this altered PPI

responses to sub-threshold doses of CRF or NE. In addition, we determined if repeated exposure to an animal model of traumatic psychological stress (live predator exposure) would produce similar neuroadaptations, and also tested whether such neuroadaptations require activation of CRF1 receptors within the BLA. NE or CRF neuroadaptations could potentially contribute to exaggerated responses to environmental stimuli long after the history of trauma has ended, similar to the enduring sensorimotor gating abnormalities seen in stress-sensitive disorders such as schizophrenia and PTSD (Grillon, Morgan et al. 1996). Our findings showed that repeated CRF or predator stress sensitizes the NE receptors within the BLA, such that a previously ineffective dose of NE leads to long-lasting PPI disruptions, effect that can be prevented by blockade of CRF1 receptors prior to each predator stress exposure.

METHODS

Subjects: 89 adult male Sprague Dawley rats (Harlan Laboratories, Madison WI) weighing 300-325g were pair-housed in clear polycarbonate cages in a light- and temperature-controlled vivarium (lights on at 0700 for 12h). Experiments were conducted between 1000 and 1500, food and water were available *ad libitum*, and rats were handled daily. All facilities and procedures were in accordance with the guidelines regarding animal use and care put forth by the National Institutes of Health of the United States, and were supervised and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin.

Surgeries: Rats were anesthetized via inhalation of an isofluorane/oxygen mixture and secured in a stereotaxic frame (Kopf instruments, Tujunga CA). Stainless steel cannulae (23-gauge, Small Parts Inc, Miami Lakes, FL) were implanted bilaterally using coordinates (in mm relative to bregma) based on the atlas of Paxinos and Watson (1998): BLA, AP= -3.0, LM= ±4.8 from

midline, DV= -5.8 from skull surface; CeA, AP= -2.1, LM= ±3.85, DV= -5.4. The cannulae were affixed to the skull with dental cement (Lang Dental Mfg Co, Wheeling, IL) and anchoring screws (Plastics One, Roanoke, VA) and wire stylets were inserted to prevent blockage. Rats recovered for one week before testing (with daily health checks and handling).

Predator Stress: In Experiment 3, rats were placed individually in a protective metal wire cage (7" x 8" x 9") that was secured to the floor of the homecage of a ferret, a natural predator of rats, for 5 min. This procedure allowed animals to see, hear, and smell each other, but did not permit physical contact.

Drugs and Microinfusions: Corticotropin-releasing factor (CRF) (rat, human) was from Bachem/Peninsula Labs (Torrance, CA) and Phoenix Pharmaceuticals (Burlingame, CA) and was dissolved in sterile distilled water. L-(-)-Norepinephrine bitartrate (NE) was from Sigma Aldrich (St Louis, MO) and was dissolved in a solution of 0.01 % ascorbic acid in isotonic saline. CRF1 antagonist NBI 27914-hydrochloride was from Tocris bioscience (Bristol, UK) and was dissolved in a solution of 5% DMSO+5% cremaphor+90% dH_2O. Yohimbine, (−)-Isoproterenol hydrochloride and (R)-(−)-Phenylephrine hydrochloride were from Sigma Aldrich (St Louis, MO) and were dissolved in saline. For microinfusions, stainless-steel injectors (30-gauge, Small Parts Inc., Miami Lakes, FL) were lowered to extend past cannulae tips by 2.5 mm (final DV coordinates for BLA and CeA were 8.3 and 7.9 mm below skull surface, respectively). Injectors were attached with polyethylene tubing (Becton Dickinson, Sparks, MD) to a 10-μl Hamilton syringe (Hamilton Co., Reno, NV) mounted on a microdrive pump (Harvard Apparatus, Holliston, MA), and 0.5-μl infusions were delivered over 1’33”. Injectors were left in

place for an additional minute to allow for absorption of the solution before stylets were replaced.

Startle Chambers: Startle chambers (San Diego Instruments, San Diego, CA) contained a nonrestrictive Plexiglas cylinder resting inside a ventilated and illuminated sound-attenuating cabinet, with a high-frequency loudspeaker to produce all acoustic stimuli. The whole-body startle response caused vibrations, which were converted into analog signals by a piezoelectric unit attached to the platform (Mansbach, Geyer et al. 1988), and were digitized and stored by a microcomputer and interface unit. Monthly calibrations were performed to ensure accuracy of the sound levels (using the dB (A) scale) and measurements.

Startle and PPI Testing: The test session consisted of a 65-dB background noise presented alone for 5 min and remaining on for the length of the session, followed by (in a pseudo-random order, 16 of each) pulse-alone trials (40-ms, 120-dB broadband bursts), prepulse+pulse trials (20-ms noises that were 3, 9, or 15 dB above the background noise and were presented 100 ms before the onset of the 120-dB pulse), and no stimulus trials. Four pulse-alone trials were also presented at the beginning of the session to ensure that startle magnitude was stable during the portion when PPI was measured, as the most rapid habituation of the startle response occurs within the first several presentations (Geyer, Swerdlow et al. 1990); these pulse-alone trials were excluded from calculations of startle and % PPI. Before experiments began, all rats underwent this test session once/day on 3 separate days with sham infusions preceding the last test to familiarize them with the testing and microinfusion procedures. The final baseline test occurred 2-3 days before the experiments, and PPI and startle values from this day were used to create equally matched treatment groups for each experiment.

Experimental Design: Experiment 1: BLA. Several infusion/test days were carried out in 3 separate groups of rats with 2-3 days separating consecutive tests. The sequence of tests was: a mock infusion (injectors lowered but nothing infused; 'mock1'); three drug infusions of either repeated CRF (200ng, N=7), NE (20μg, N=12), or vehicle (half the rats getting the CRF vehicle and the other half getting the NE vehicle, N=8 total); another mock infusion; a crossover drug challenge with NE (0.3μg) given to the repeated CRF and vehicle rats and CRF (200ng) given to the repeated NE rats; a final mock infusion. PPI was measured immediately after the infusion on each of the test days.

Experiment 2: CeA. The design of this experiment was identical, except that infusions of CRF (N=9), NE (N=12), or vehicle (N=8) were made into the CeA. The dose for the NE challenge in this experiment was 20μg.

Experiment 3: Predator Stress. To determine if the sensitization to the PPI-disruptive effects of NE that was seen after repeated CRF in the BLA would also occur with repeated stress exposure, one additional experiment was done examining sensitivity to a sub-threshold dose of intra-BLA NE in rats with a history of stress. Because the intent was to model in rats the intense psychogenic stress that is hypothesized to trigger cognitive deterioration in schizophrenia and PTSD (Walker and Diforio 1997; Yehuda 1999), a predator stress paradigm was utilized. This paradigm is considered to capture many of the precipitating features of psychological trauma and is used to study PTSD-like effects in animal models (Blanchard, Nikulina et al. 1998; Cohen and Zohar 2004; Adamec, Holmes et al. 2008). Thus, rats (N=8) with BLA cannulae underwent the same experimental protocol as Experiment 1, but instead of the repeated drug infusions received 3 exposures to a ferret (1/test day). Post-stress NE challenges (0.3μg) were done 4, 11 and 18 d after stress termination, with accompanying mock

infusions. Yohimbine, which is known to enhance startle responses in PTSD patients (Southwick, Morgan et al. 1999), was injected systemically 28 days after the last ferret exposure at a dose that has no effects in normal control rats (veh= 39±3; 1 mg/kg yohimbine= 40±6; P>0.05).

Experiment 4: CRF1 receptor antagonist. With this experiment we sought to determine whether CRF1 receptors are involved in the predator-stress-induced neuroplasticity via the BLA. Previous studies have shown that blockade of CRF1 receptors within the BLA prevents predator-stress-induced increases in grooming and decreases in feeding behavior (Jochman, Newman et al. 2005). Therefore, three separate groups of rats that had cannulae implanted in the BLA received CRF1 antagonist, NBI 27914 (1000 ng) either prior to each ferret exposure (N=8), or 30 minutes after each ferret exposure (N=8) or right before the NE-challenge (N=11). All the other experimental procedures and time-line were similar to Experiment 3.

Experiment 5: Isoproterenol or phenylephrine challenge. With this experiment we wanted to determine whether repeated ferret stress exposure-induced alterations in NE receptor sensitivity involves alpha1 and/or beta receptors in the BLA. Hence, two groups of rats with cannulae implanted into the BLA underwent the repeated ferret exposure as above, and subsequently were challenged with intra-BLA alpha1 receptor agonist, phenylephrine (PHEN) (30 µg, N=8) or beta receptor agonist, isoproterenol (ISO) (30 µg, N=9). These doses of PHEN and ISO when given separately into the BLA of non-stressed rats do not disrupt PPI [(veh=41±5; phen=32±4; P>0.05) (veh=41±5; iso=35±3; P>0.05)], but do disrupt PPI when given simultaneously (Alsene, Rajbhandari et al. 2011).

Histology for verifying cannulae placements: Rats were perfused transcardially with 10% formalin (Sigma Diagnostics, St Louis, MO), 60-μm sections were taken through the injection site and stained with cresyl violet, and placements were verified by an experimenter blind to the behavioral data; rats with missed placements were excluded from subsequent behavioral analyses. Sample sizes reflect this final adjusted number.

Data analysis: The startle response to the onset of the 120-dB burst was recorded for 100 ms for each pulse-alone and prepulse+pulse trial. Two measurements were calculated from these values for each rat: Startle magnitude was the average of the startle responses to all pulse-alone trials; PPI was a percent score for each prepulse+pulse trial type: % PPI=100−((startle response for prepulse+pulse trial)/(startle response for pulse-alone trial)). Because no significant interactions were seen with prepulse intensity and any other factor in any experiment, a single composite % PPI score (average PPI collapsed across all 3 prepulse intensities) was calculated for each subject, and this was used for all analyses. For each experiment, separate ANOVAs were run on startle and PPI data to analyze each repeated treatment regimen, with test day as a within subjects variable ('mock1' and the repeated drug infusion/stress days were the four levels of this factor). Significant main effects were analyzed further with Bonferroni-corrected t-tests. No differences were found in any experiment between the two mock infusions that 'bracketed' the drug challenge, so data from these two days were averaged and designated as 'mock2'. Planned contrasts between 'mock2' versus the drug challenge day were used to assess effects of the crossover treatment in each group; comparison of 'mock1' (the mock infusion occurring before the repeated drug infusion regimens) versus 'mock2' provided an evaluation of conditioned or non-specific carry-over effects from the repeated regimens.

RESULTS

Experiment 1: BLA

Prepulse Inhibition: ANOVA revealed no significant main effect of repeated CRF treatment on PPI [$F(3,18)=0.8$, NS], showing that none of the CRF infusions altered PPI in BLA (Fig. 1A). Repeated NE treatment, however, *did* have a significant main effect [$F(3,33)=5.4$, $P<0.004$] (Fig. 1B). *Posthoc* comparisons showed that NE infusions significantly lowered PPI compared to the pre-drug mock infusion ('mock1') ($P<0.05$). The size of this NE-induced PPI deficit shrank with repeated infusions, as PPI values for the 3rd NE infusion were significantly higher than those for the first ($P<0.05$); a similar trend was nearly significant for the 2nd NE infusion versus the 1st ($P<0.06$). Thus, it appeared that tolerance to the PPI-disruptive effects of NE developed with repeated NE infusions into BLA. Repeated vehicle infusion had no effect, indicating that a repeated treatment regimen is not in and of itself sufficient to alter PPI [$F(3,21)=0.5$, NS]. Thus, repeated NE, but not repeated CRF, into the BLA disrupted PPI.

There were no differences between the 'mock1' and 'mock2' test days, indicating that there were no conditioned or carry-over effects following repeated CRF, NE, or vehicle treatments. When the effects of drug challenges were evaluated by comparing 'mock2' versus 'drug challenge' days in each group, a marked reduction in PPI ($P<0.05$) was seen when NE was given to rats that had received repeated CRF infusions into BLA (Fig. 1A). Notably, this was a low dose of NE (0.3μg) that had *no* effect when given to the repeated vehicle group (Fig. 1C), indicating that this dose does not disrupt PPI in rats without a history of intra-BLA CRF infusions. In contrast, CRF challenge in the NE-treated rats did not have an effect on PPI (Fig. 1B). Thus, repeated CRF receptor stimulation in the BLA produces cross-sensitization to NE, with a sub-threshold dose of

NE significantly disrupting PPI after repeated CRF treatments. Startle: NE challenge in repeated CRF treated group significantly enhanced baseline startle ($P<0.05$). There were no effects on startle magnitude in any group (Table 1; all F-ratios <0.8, and P-values >0.5).

Experiment 2: CeA

Prepulse Inhibition: ANOVAs revealed no significant main effect of repeated CRF [$F(3,24)=0.687$, NS], repeated NE [$F(3,33)=1.8$, NS] or repeated vehicle [$F(3,21)=0.04$, NS] on PPI, suggesting that none of the treatments altered PPI in CeA on any test day (Fig.2A-C). Similarly, no significant differences were found between the different mock infusion days or between 'mock2' and 'drug challenge' days in any group. Startle: There were no effects on startle magnitude in any group (all F ratios <1.6, and P-values >0.2).

Experiment 3: Predator stress

Prepulse Inhibition: Similar to the CRF/BLA (Experiment 1), ANOVA revealed no significant main effect of repeated ferret exposure [$F(3,21)=1.8$, NS], showing that predator stress on its own did not alter PPI (Fig. 4). There were no differences between the 'mock1' and 'mock2' test days, indicating that there were no conditioned effects following repeated ferret exposure, but comparison of 'mock2' versus the 'NE challenge' days revealed a profound (nearly 50%) reduction in PPI ($P<0.01$) in response to a low dose of NE (0.3 μg) that had *no* effect when given to rats without a history of stress (i.e., Fig. 1C). Comparison of 'mock3' and the 2nd 'NE challenge' revealed a similar PPI deficit ($P<0.05$), and indicated that this hypersensitivity to low-dose NE persisted even 11d after the end of the stress regimen. Comparison of 'mock4' and the 3rd 'NE challenge' (18d post-stress) also revealed PPI deficit ($P<0.05$). Systemic challenge with a sub-threshold dose of the alpha2 receptor antagonist, yohimbine (28d post-stress) caused PPI

disruption (P=0.05). Thus, repeated stress that may represent psychological trauma (live predator exposure) causes an *enduring* sensitization of NE systems in the BLA such that a mild/sub-threshold NE dose now results in a marked PPI deficit. This profile exactly mirrors that elicited by repeated CRF infusions into the BLA (see above). Startle: A main effect of repeated ferret exposure was seen [$F(3,21)=5.3$, $P<0.007$]; startle values on the 3rd ferret day were slightly lower than those on 'mock1' ($P<0.05$). Importantly, startle values for the 2nd and 3rd 'NE challenge' were significantly higher than those following the corresponding mock infusion ('mock3' and 'mock4' respectively) ($P<0.01$), indicating that startle hyperreactivity in response to low-dose NE in the BLA also emerges long after a history of repeated psychological trauma.

Experiment 4: CRF1 receptor antagonist

Prepulse Inhibition: ANOVA revealed no significant main effect of repeated ferret exposure in any of the groups: CRF1 antagonist prior to ferret exposures [$F(3,21)= 0.184$, NS]; CRF1 antagonist after each ferret exposure [$F(3,21)= 0.933$, NS]; CRF1 antagonist before the NE challenge [$F(3,30)=0.409$, NS], showing that predator stress on its own did not alter PPI acutely (Fig. 5). There were no differences between the 'mock1' and 'mock2' test days in any group, indicating that there were no conditioned effects following repeated ferret exposure either with or without CRF1 antagonist. Comparison of 'mock2' versus the 'NE challenge' days revealed a reduction in PPI ($P<0.02$) in response to a low dose of NE (0.3 μg) in the group that received CRF1 antagonist after each ferret exposure or right before the NE challenge (i.e., Fig. 5B and 5C). However, 'mock2' versus 'NE challenge' comparison in rats that received CRF1 antagonist prior to each ferret revealed no significant difference. These results show that intra-BLA NBI 27914 before each ferret exposure completely prevented the

ability of a subsequent sub-threshold NE challenge to disrupt PPI, indicating that CRF1 receptors are necessary for the development of NE neuroplasticity in the BLA due to repeated stress, but that CRF1 receptors do not seem to participate in the consolidation of stress effects on PPI or the acute expression of the NE-induced PPI deficits. Startle: No main effect of repeated ferret exposure was seen in any CRF1 treatment groups [$F(3,21)=2.287$ (before each stress); $F(3,21)=0.821$ (after each stress)]; $F(3,30)=0.670$ (before the NE challenge). When startle values for 'mock2' and 'NE challenge' were compared, in the group that received NBI before the 'NE challenge', startle values were significantly higher than values for'mock2' ($P<0.02$).

Experiment 5: Isoproterenol or phenylephrine challenge

Prepulse Inhibition: ANOVA revealed no significant main effect of repeated ferret exposure for either the PHEN and ISO challenge group [$F(3,21)=0.386$, NS] and [$F(3,24)=0.379$, NS], showing that predator stress on its own did not alter PPI acutely (Fig. 6). There were no differences between the 'mock1' and 'mock2' test days, indicating that there were no conditioned effects following repeated ferret exposure, but comparison of 'mock2' versus the 'alpha1 or beta receptor agonist challenge' days revealed a significant reduction in PPI in both groups ($P<0.05$) in response to ISO or PHEN challenge. Notably, these doses of PHEN/ISO had *no* effect on PPI when given into BLA of rats without a history of stress. This hypersensitivity to intra-BLA alpha1 or beta receptor agonist challenge in rats with a history of exposure to repeated ferret stress matches the profile of the sub-threshold NE challenge on PPI elicited by repeated ferret stress (see above). Startle: No main effect of repeated ferret exposure was seen in either group (all F ratios <2.271, and P-values >0.8). Startle values for the 'ISO

challenge' were significantly lower than those 'mock2' ($P<0.01$), while no differences were observed between 'PHEN challenge' and 'mock 2'.

DISCUSSION

Several new results regarding NE and CRF regulation of sensorimotor gating were obtained from the studies described in this chapter. First, neither a single (day1) nor repeated infusions (3 days) of CRF into any amygdala subregions affected PPI acutely. In contrast, single and repeated NE infusion in the BLA (but not CeA) produced a disruption of PPI. Cross challenge with low-dose NE in rats that had a history of CRF infusions in the BLA led to a significant disruption of PPI. A history of ferret exposure also produced a long-lasting (evident even 18 days after last ferret exposure) disruption of PPI and startle enhancement to a cross challenge of sub-threshold dose of NE in the BLA. These effects could be prevented by antagonism of CRF1 receptors prior to each ferret exposure, but not by antagonizing these receptors after each ferret exposure or right before the NE challenge, showing that stress-induced NE receptor hypersensitivity for PPI begins with activation of CRF1 receptors within the BLA. In addition, we found that activation of either BLA alpha1 or beta receptor is sufficient to produce PPI-disruptive effects after repeated predator stress exposure, whereas in rats without stress exposure, simultaneous activation of both receptors is required (Alsene, Rajbhandari et al. 2011). Overall, our findings were shown using an ethologically relevant predator stress that produced certain elements of abnormalities that are seen in PTSD, including disrupted PPI and increased startle with sub-threshold levels of NE receptor stimulation.

Our finding that BLA is a brain substrate that undergoes neuroadaptations with repeated stress or CRF receptor stimulation is consistent with previous studies that have shown that repeated activation of CRF receptors in the BLA enhances neuronal excitation and leads to long-lasting plasticity of anxiety-like behavioral responses (Sajdyk, Schober et al. 1999; Rainnie, Bergeron et al. 2004; Shekhar, Truitt et al. 2005). Yet, our studies are unique because we have identified the role of a purely psychological (predator) stress acting through CRF1 receptors that triggers long-lasting pathological changes of NE receptors in the BLA and that ultimately leads to a much lowered threshold for NE-induced PPI deficits. While studies have used predator stress paradigms to understand the roles of BLA, NE, or CRF system in stress- and anxiety-like, these studies have not conducted a detailed analysis of the long-term effects of predator stress on both NE and CRF systems in the BLA (Blanchard, Nikulina et al. 1998; Adamec, Muir et al. 2007; Adamec, Fougere et al. 2010; Adamec, Hebert et al. 2012; Curtis, Leiser et al. 2012). The effect of repeated predator exposure in our studies that led to long-lasting startle and PPI abnormalities may be analogous to the enhanced startle reactivity and disrupted PPI that have been reported in combat veterans with PTSD long after traumatic experiences have ceased to occur (Grillon, Morgan et al. 1996; Morgan, Grillon et al. 1996). It is possible that alterations in NE signaling in the BLA of these patients potentially contribute to the exaggerated responses to environmental stimuli. Furthermore, our findings that repeated predator stress causes NE receptor hypersensitivity is also significant given that drugs that reduce NE transmission (e.g. clonidine) or block alpha1 or beta NE receptors (e.g. prazosin and propranolol) ameliorate symptoms in PTSD and schizophrenia (Freedman, Kirch et al. 1982; van Stegeren, Everaerd et al. 1998; Pitman, Sanders et al. 2002; Raskind, Thompson et al. 2002; Taylor and Raskind 2002; Vaiva, Ducrocq et al. 2003; Oranje and Glenthoj 2013; Remington, Agid et al. 2013).

We found that activation of CRF1 receptors is necessary for the stress-induced NE receptor hypersensitivity in the BLA, corroborating previous findings that show that CRF1 receptors are important for behavioral and physiological responses to stress (Jochman, Newman et al. 2005; Ugolini, Sokal et al. 2008). Antagonizing these receptors reverses increases in NE release (Isogawa, Akiyoshi et al. 2000; Claustre, Rouquier et al. 2006), anxiety-like responses (Walker, Miles et al. 2009) and initiation/consolidation of acute predator stress effects in behavioral measures of startle and memory (Adamec, Fougere et al. 2010). We demonstrated for the first time that activation of CRF1 receptors are required for repeated stress to alter NE signaling within the BLA to produce startle and PPI hyperreactivity. Given that our results show that blocking CRF1 receptors before each stress exposure prevents NE receptor hypersensitivity but blocking them after the stress or right before the NE sub-threshold challenge does not, we could hypothesize that initial activation of CRF1 receptors is necessary for the development of this NE receptor hypersensitivity, but not for the consolidation of stress effects or for acute expression of the NE-induced PPI deficits. Hence, our findings add to the notion that CRF1 antagonists could be an important prophylactic agent for normalizing stress-induced neuronal and behavioral alterations (Ugolini, Sokal et al. 2008; Adamec, Fougere et al. 2010).

A question that arises from our results is why intra-BLA CRF infusions or ferret exposures, which were effective at establishing hypersensitivity to NE, did not themselves produce an acute disruption of PPI. A previous study also shows that acute intra-BLA CRF has no effects on PPI if given for 3 days, as we did; 5 days of infusions decreases PPI but PPI returns to normal after 7 days of CRF infusion in BLA (Bijlsma, van Leeuwen et al. 2010). Thus,

it is possible that there may be a narrow window of time for repeated CRF infusions in the BLA to alter PPI that may not have been captured by our studies. Our findings may at first appear inconsistent with reports that have shown that intracerebroventricular (ICV) infusion of CRF disrupts PPI and that these effects can be reversed by CRF antagonism (Conti, Murry et al. 2002; Risbrough, Hauger et al. 2004; Gresack and Risbrough 2011). Nevertheless, it is probable that centrally-administered CRF mediates its acute effects on PPI via other brain regions besides the BLA or CeA. The lack of effect of CRF on PPI in our studies cannot be attributed to the use of too low a dose, as the same dose of CRF used in our study robustly increases grooming and anorexia when infused into BLA (Jochman, Newman et al. 2005).

The mechanisms for CRF1-induced neuroplasticity of NE receptors could be many: first, it could be that the neuroadaptation underlying NE hypersensitivity has a delayed onset after initial ferret exposure, akin to a kindling-like phenomenon. Our previous reports demonstrate that a high dose of ICV CRF exposure produces delayed-onset (24 hours later) of PPI disruption that is enduring (persistent for 9 days), indicating that intense stress or CRF receptor stimulation may fundamentally alter neuronal mechanisms or produce neuroadaptations (Bakshi, Alsene et al. 2011). Stress or CRF has been shown to cause internalization of CRF1 receptors in the locus coeruleus; therefore it is tempting to speculate that repeated stress/CRF may produce similar neuroadaptive responses of these receptors in the BLA (Reyes, Fox et al. 2006; Reyes, Valentino et al. 2008; Bangasser, Curtis et al. 2010). CRF1 internalization, in turn, may enhance expression or sensitivity of NE receptors via linking intracellular mechanisms ultimately resulting in the behavioral hypersensitivity that we saw in the PPI paradigm with sub-threshold NE challenge in BLA. Moreover, CRF1 receptor-induced NE plasticity is specific to the BLA as neither acute nor repeated CRF in the neighboring CeA produced PPI alterations to

subsequent drug challenges. The lack of effect of CRF in the CeA could be due to a sparse distribution of CRF1 receptors in this brain region, as it is known that this site although an extra-hypothalamic source of CRF, contains very few CRF1 receptors (De Souza, Insel et al. 1985; Radulovic, Sydow et al. 1998; Arzt and Holsboer 2006). Nevertheless, there are high numbers of NE receptors in CeA, so this receptor paucity mechanism cannot explain the lack of effects with NE challenge in CeA.

We found that in the absence of any stress or CRF infusions, acute high-dose intra-BLA NE disrupted PPI, while in the CeA this had no significant effects on PPI, in agreement with our previous reports that have shown PPI disruption via co-stimulation of alpha1 and beta NE receptors in the BLA, but not the CeA (Alsene, Rajbhandari et al. 2011). In the BLA, repeated NE also disrupted PPI, but the effect on PPI diminished in potency with each subsequent NE infusion, thereby suggesting a tolerance-like phenomenon. One possibility for this effect could be due to repeated NE-induced receptor down-regulation in the BLA leading to decreased effects on PPI (Price, Morris et al. 2002; Morris, Price et al. 2004; Swaminath, Xiang et al. 2004). While repeated NE produces tolerance to the PPI-disruptive effects, on the contrary, repeated CRF or stresses appear to sensitize NE receptors such that a sub-threshold, low-dose of NE can now disrupt PPI. Consistent with our results is the finding that repeated stress can increase NE-induced electrical activity of BLA neurons (Buffalari and Grace 2009). Also, our finding that individual agonists of NE alpha1 or beta receptors was sufficient to disrupt PPI after a history of repeated stress is interesting as other reports show that, in absence of stress, co-stimulation these receptors is necessary to alter PPI or other behavioral functions via BLA (Ferry, Roozendaal et al. 1999; Alsene, Rajbhandari et al. 2011). Hence, our current findings indicate that alpha1 and

beta receptors may undergo stress-induced functional uncoupling to produce PPI disruptions even with low-level NE activation in the after-math of repeated intense psychological stress.

Alterations in PPI by NE in the BLA were found to be independent of alterations in baseline startle. However, we found that the second (11d) and third (18d) NE challenges post predator-stress elevated baseline startle levels. This finding was interesting in the light of the fact that PTSD patients show exaggerated startle responses to low-level stimulation after traumatic experiences have ended (Orr, Lasko et al. 1995; Morgan, Grillon et al. 1996; Grillon, Morgan et al. 1998; Shalev, Peri et al. 2000). Hence, our behavioral model potentially recapitulates not only trauma-induced disruptions in PPI but also startle hyperreactivity that can be seen in PTSD patients (Grillon, Morgan et al. 1996; Grillon, Morgan et al. 1998).

Taken together, our findings are extremely relevant clinically to symptoms observed in PTSD patients who display deficient PPI long after traumatic events have ended, pointing to the fact that there may be a pathological sensitization of systems that regulate PPI in these patients. Indeed, our findings are consistent with studies that have shown that a challenge with a mild stimulus long after traumatic experiences have ended leads to deterioration of PPI and enhanced startle (Grillon, Morgan et al. 1996; Grillon, Morgan et al. 1998; Grillon, Baas et al. 2004). Our findings may also be relevant for understanding stress-induced triggering of symptoms in schizophrenia given that deficient PPI is a prominent feature of schizophrenia (Brown and Birley 1968; Lukoff, Snyder et al. 1984; Walker and Diforio 1997; Horan, Ventura et al. 2005; Betensky, Robinson et al. 2008). Our studies for the first time indicate that the BLA may be a crucial site for CRF/NE sensitization under intense stress, leading to abnormally sensitive

triggering of PPI and startle abnormalities (Kosten, Mason et al. 1987; Breier, Wolkowitz et al. 1990; Yehuda, Southwick et al. 1992; Forman, Bissette et al. 1994; Bremner, Licinio et al. 1997; Baker, West et al. 1999; Liberzon, Taylor et al. 1999; Rauch, Whalen et al. 2000; Shin, Rauch et al. 2006; Benes 2010). At a cellular level, CRF1 and NE receptors modulate common intracellular substrates through stimulatory G-protein coupled adenylate cyclase-protein kinase A or phospholipase C transduction system, that in turn could be involved in the NE receptor hypersensitivity that was seen here (Wozniak M 2000). Unknown, however, is whether CRF and NE receptors are localized on the same BLA neurons. While the involvement of such cellular and molecular mechanisms will need to be explored to further understand the basis of NE receptor plasticity in the BLA after intense stress, our present findings have set up a foundation to begin to parse out such mechanisms. Hence, our findings provide crucial new information to understand mechanisms of stress-induced information processing deficits in PTSD and/or schizophrenia patients.

ACKNOWLEDGEMENTS

This work was supported by R01 MH075980 (VPB), T32 GM007507, and a Young Investigator Award from the National Alliance for Research on Schizophrenia and Depression (NARSAD) to VPB. We are grateful to Dr. Brian Baldo for his comments on the manuscript.

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Fig. 1) Effects on % PPI of repeated CRF (200ng/ 0.5 µl), NE (20 µg/ 0.5 µl) and saline, and crossover challenge with NE, CRF and NE respectively in the BLA (Figures A, B & C respectively). Values represent means ± sem for each dose. Each repeated infusion day was compared to mock1 and challenge with mock2. **A)** NE crossover is 0.3 µg/ 0.5 µl. * P<0.05, relative to mock2 infusion day. **B)** CRF crossover is 200ng/ 0.5 µl. * P<0.05, relative to mock1 infusion day. $ P< 0.05, relative to NE day1 and + p<0.06 relative to NE day1 **C)** NE crossover is 0.3 µg/0.5 µL.

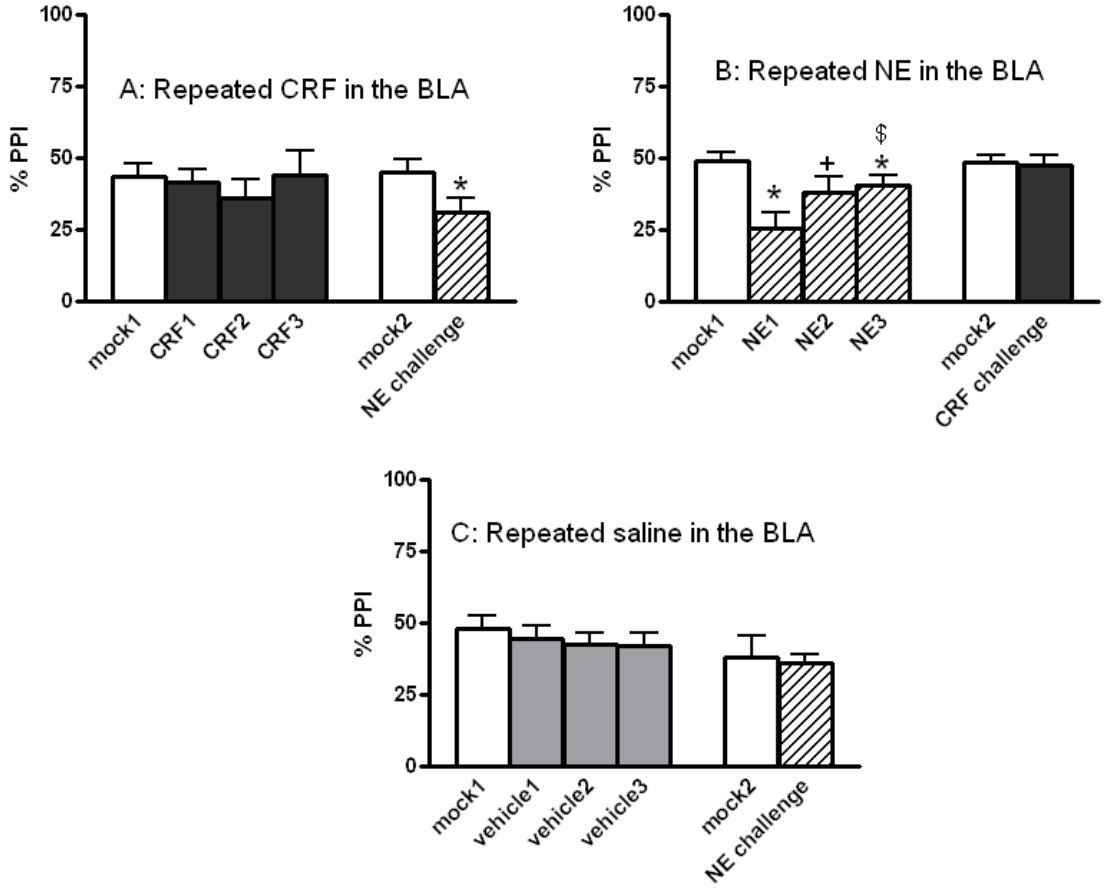


Fig. 2) Effects on % PPI of repeated CRF (200ng/ 0.5 µl), NE (20 µg/ 0.5 µl) and saline, and crossover challenge with NE, CRF and NE respectively in the CeA (Figures A, B & C respectively). Values represent means ± sem for each dose. Each repeated infusion day was compared to mock1 and challenge with mock2. **A)** NE crossover is 20 µg/ 0.5 µl. **B)** CRF crossover is 200ng/ 0.5 µl.

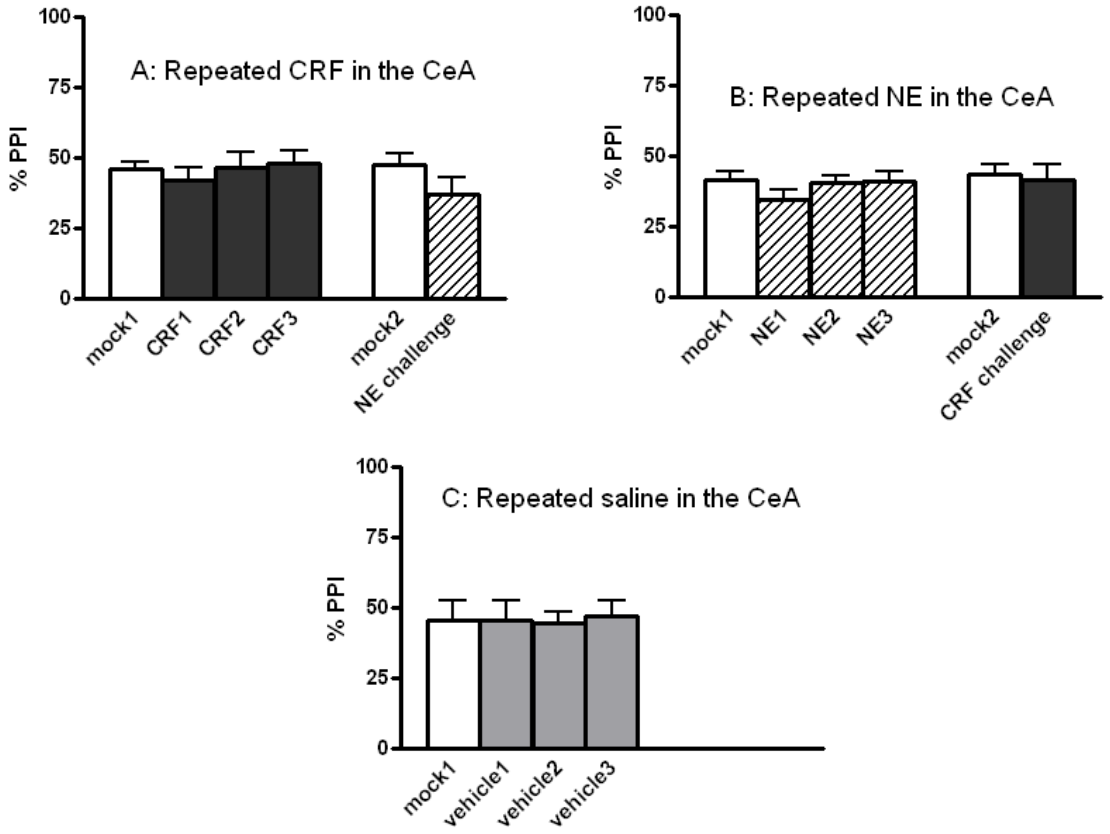
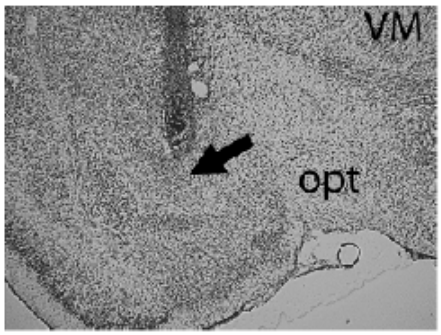


Fig. 3) Representative injector tip locations within the CeA and BLA (Figures A and C respectively) (left panel), indicated by the arrows; and chartings depicting the locations in which infusions of CRF (squares), NE (triangles) or saline (circles) (Figures B and D, respectively) (right panel) **A)** opt= *optic tract*, VM= *ventromedial thalamic nucleus* **C)** VM= *ventromedial thalamic nuclei*, Pe= *periventricular hypothalamus*

A



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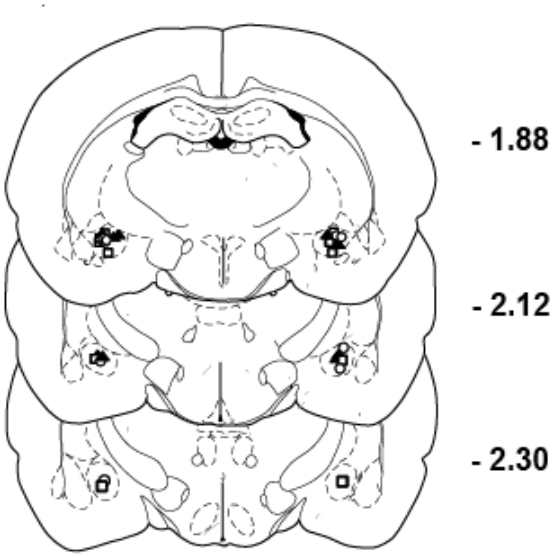

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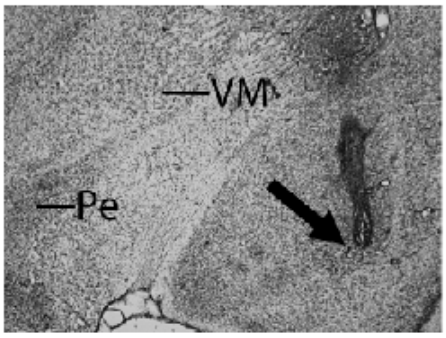

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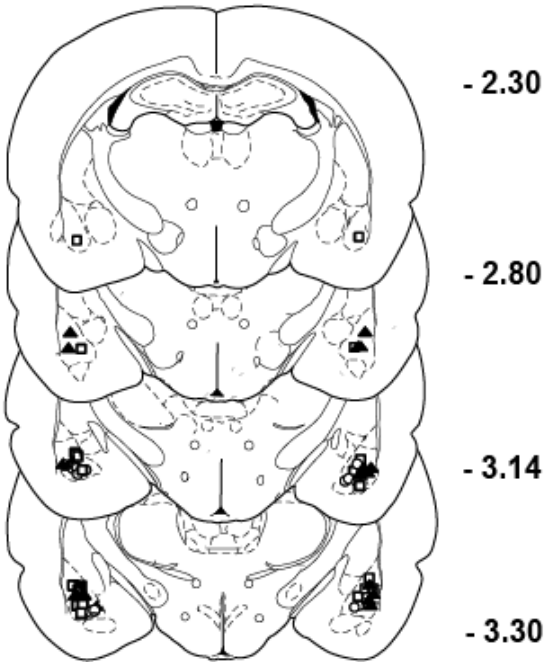

Fig. 4) Effects on % PPI of repeated ferret and crossover challenge with NE in the BLA. Values represent means ± sem for each dose. Each repeated test day was compared to mock1 and NE challenges with their respective mocks (mock 2, 3 and 4 respectively). Yohimbine challenge was compared against mock 4. NE crossover is 0.3 µg/ 0.5 µl and yohimbine challenge is 1mg/kg.

$^{\#}P<0.05$, relative to mock3 and mock4 infusion days, respectively. $^{\#\#}P<0.05$, relative to mock2 and NE challenge (18 days).

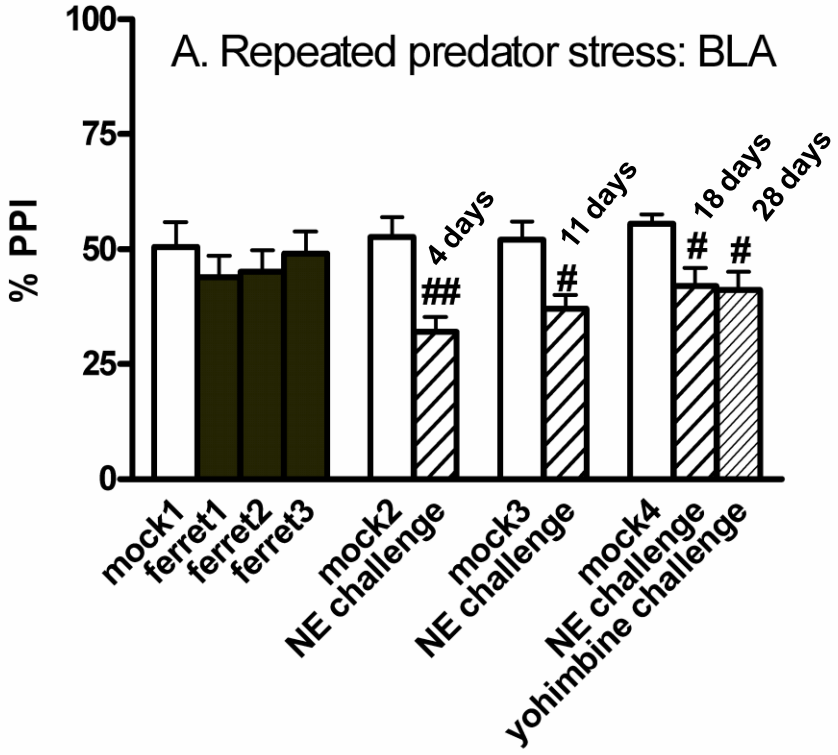

Fig. 5) Effects on % PPI of CRF1 antagonist into the BLA before or after each ferret exposure, or before the NE challenge (Figures A, B and C). Values represent means ± sem for each dose. Each repeated infusion day was compared to mock1 and NE challenge with mock 2. **A)** NBI 27914 before each ferret exposure. **B)** NBI 27914 after each ferret exposure. ***** P<0.05, relative to mock2. **C)** NBI 27914 before NE challenge. ***** P<0.05, relative to mock2 NBI 27914 is 1000ng/0.5µL and NE crossover is 0.3 µg/ 0.5 µl.

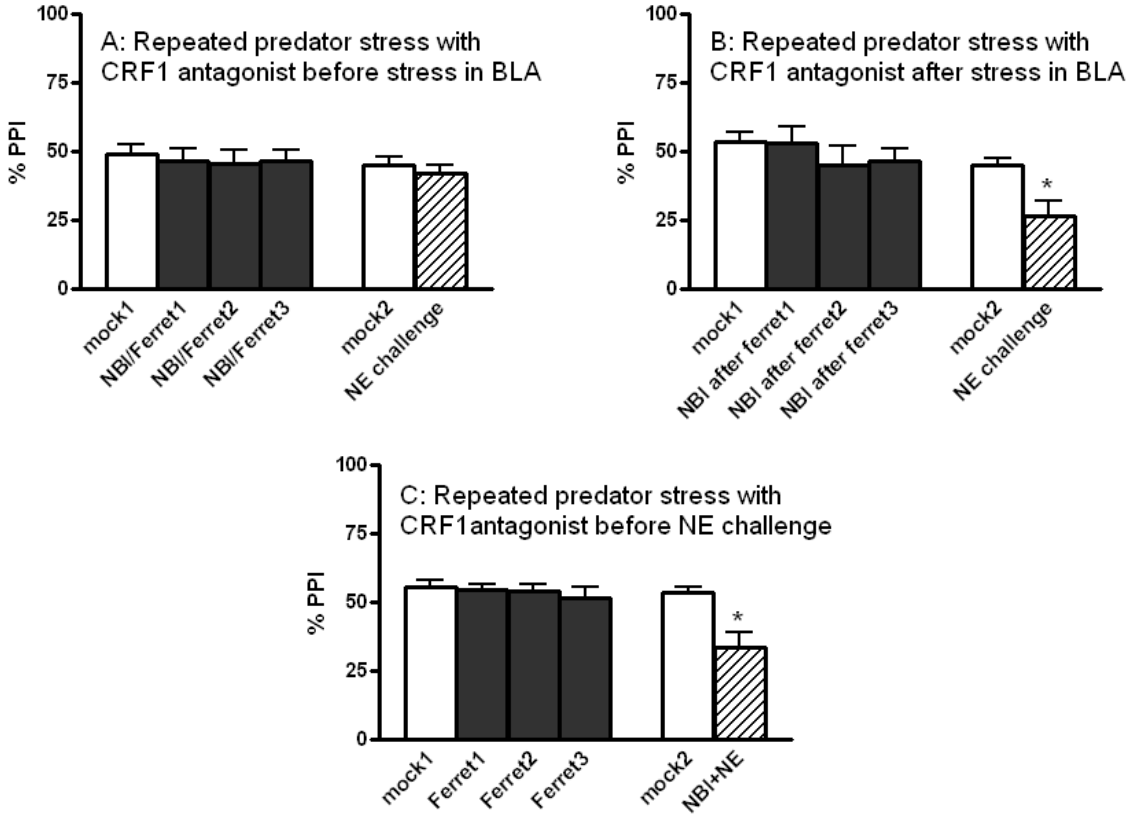

Fig. 6) Effects on % PPI of repeated ferret and challenge with alpha1 receptor agonist or beta receptor agonist in the BLA (Figures A and B). Values represent means ± sem for each dose. Each repeated infusion day was compared to mock1 and agonist challenges with mock 2. **A)** Phenylephrine (PHEN) challenge after repeated ferret. ***** P<0.05, relative to mock2. **B)** Isoproterenol (ISO) challenge after repeated ferret. ***** P<0.05, relative to mock2. PHEN and ISO are 30µg/0.5µL each.

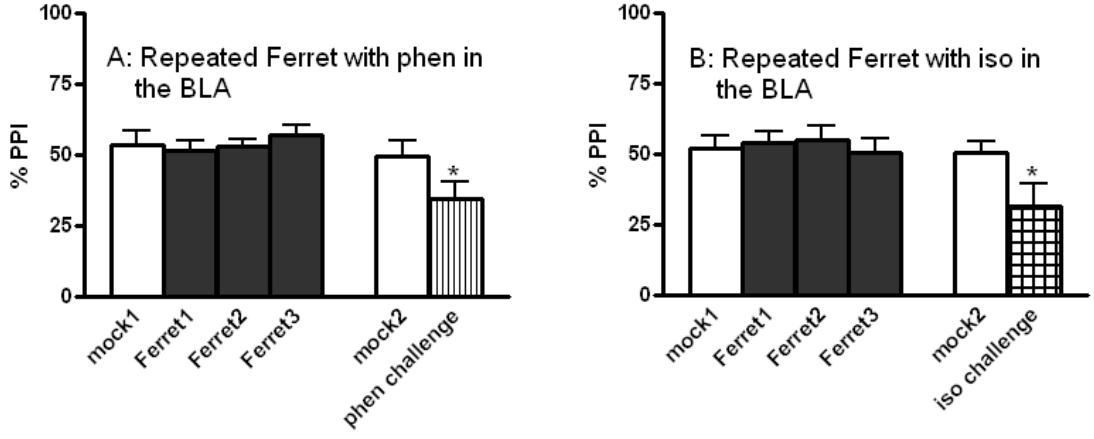

Table 1: Effects on startle of repeated CRF (200ng/ 0.5 µl), NE (20 µg/ 0.5 µl) and saline, and crossover challenge with NE, CRF and NE respectively in the BLA and CeA; Effects of repeated ferret alone or along with CRF1 antagonist (before, after each ferret stress and before NE challenge) and with alpha1 and beta receptors agonists in the BLA. Values represent means ± sem for each dose. Each repeated infusion day was compared to mock1 and challenge with mock2. $^{\#}P<0.05$, relative to mock2 infusion day (BLA); $^{\#}P<0.05$, relative to mock1, mock3, mock4 infusion days, respectively (Repeated ferret).

	BLA										
	MOCK1	DAY2	DAY3	DAY4	MOCK2	CROSSOVER					
CRF	452±64	422±69	432±71	428±46	442±66	525±86# (NE 0.3µg)					
NE	392±97	358±51	364±47	329±32	324±35	349±31 (CRF 200ng)					
SALINE	367±49	386±45	377±42	424±47	366±42	430±52 (NE 0.3µg)					
	BLA (REPEATED FERRET)										
	MOCK1	DAY2	DAY3	DAY4	MOCK2	CROSSOVER	MOCK3	NE 2	MOCK4	NE3	Yohimbine
REP. FERRET	481±70	402±40	359±51	352±51#	419±41	421±50 (NE 0.3µg)	351±50	561±70# (NE 0.3µg)	489±59	621±68# (NE 0.3µg)	560±91
	BLA (CRF1 ANTAGONIST)										
	MOCK1	DAY2	DAY3	DAY4	MOCK2	CROSSOVER					
NBI/BEFORE FERRET	292±45	276±51	241±26	326±59	295±31	309±35# (NE 0.3µg)					
NBI/AFTER FERRET	371±54	318±53	312±27	304±34	303±34	315±34 (NE 0.3µg)					
NBI/BEFORE NE	360±55	319±33	319±37	311±33	343±38	419±40 (NE 0.3µg)					
	BLA (ALPHA1 OR BETA AGONIST)										
	MOCK1	DAY2	DAY3	DAY4	MOCK2	CROSSOVER					
REP. FERRET/PHEN	452±74	411±58	409±57	456±56	431±58	427±57 (phen 30µg)					
REP. FERRET/ISO	266±26	331±42	334±39	304±31	336±30	259±28# (iso 30µg)					
	CeA										
	MOCK1	DAY2	DAY3	DAY4	MOCK2	CROSSOVER					
CRF	268±52	248±29	292±50	298±49	260±32	274±50 (NE 20µg)					
NE	398±71	360±35	409±35	402±47	392±55	430±54 (CRF 200ng)					
SALINE	543±55	549±62	559±76	586±77							

CHAPTER 4

CELLULAR CO-LOCALIZATION OF ALPHA1 NORADRENERGIC RECEPTORS AND CRF1 RECEPTORS ON OUTPUT PROJECTION NEURONS WITHIN THE RAT BASOLATERAL AMYGDALA

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The experiments in the following chapter were completed by myself. General experimental design, data analysis and conclusions were developed with the help of my thesis advisor, Dr. Vaishali Bakshi, and Dr. Brian Baldo. These results along with data in Chapter 3 are ready for submission to the journal of PNAS.

ABSTRACT

Stress-induced activation of CRF1 receptors within the BLA was in previous chapter shown to sensitize the NE receptors in BLA, as assessed by deficits in a measure of sensorimotor gating known as prepulse inhibition of the startle response (PPI). One mechanism through which CRF1 receptor activation could alter sensitivity of NE receptors could be through modulation of common intracellular signaling pathways, contingent upon the finding that these receptors are co-localized on the same neurons in the BLA. It is surprising that even though NE and CRF systems are known to modulate each other and that NE and CRF receptors are expressed in the BLA, their sub-cellular relationship with each other is currently unknown. It is known that BLA contains glutamatergic output neurons and GABAergic interneurons, but there is a paucity of knowledge regarding the specific presence of NE or CRF1 receptors on excitatory or inhibitory neurons of the BLA. Understanding the sub-cellular relationship between NE and CRF1 receptors and their specific sites of action (glutamatergic or GABAergic neurons) could provide a mechanistic framework for explaining interactions between the NE and CRF systems in the BLA. Using immunolabeling for CRF1 receptors and NE alpha1 receptors, this study demonstrated that these two receptors are highly co-localized on neuronal cells (not glial) in the BLA. Also, immunolabeling for glutamic acid decarboxylase (GAD) and alpha1 receptors showed that majority of alpha1 receptors do not co-localize on GABAergic neurons. Furthermore, using a combination of retrograde labeling and immunolabeling techniques, it was further characterized that the CRF1 receptors are present on glutamatergic projection neurons that innervate the nucleus accumbens (NAcc). Taken together, the results of this study provide an anatomical framework regarding CRF1 and NE alpha1 receptor interactions in the BLA and indicate that CRF1 and alpha1 receptors in the BLA are co-localized and positioned to modulate

excitatory output activity to the NAcc. These results, to our knowledge, are the first illustration of sub-cellular relationships between CRF1 and alpha1 receptors in the BLA. Given that CRF and NE systems are involved in modulating various stress-related functions, these findings have profound implications for a variety of behavioral processes mediated by BLA.

INTRODUCTION

Basolateral nucleus of the amygdala (BLA) is an important brain structure that modulates various stress- and anxiety-related behaviors via integration of sensory information that it receives through neuronal inputs from cortical and subcortical structures (Amaral and Insausti 1992; Swanson and Petrovich 1998; LeDoux 2007). Among these inputs, norepinephrine (NE) and corticotropin releasing factor (CRF) signaling play crucial roles in the BLA in modulating stress- and anxiety- related behaviors (Jochman, Newman et al. 2005; Buffalari and Grace 2007; Roozendaal, Schelling et al. 2008). Evidence also supports the role of interactions between these systems in modulating stress-related functions (Emoto, Koga et al. 1993; Raber, Koob et al. 1995; Li, Takeda et al. 1998). Our previous studies demonstrated that repeated CRF- or predator stress-induced activation of CRF1 receptors in the BLA leads to NE receptor hypersensitivity in the disruption of prepulse inhibition of the startle response (PPI) (Chapter 3).

BLA is known to contain abundant NE and CRF1 receptors (Palacios and Kuhar 1982; De Souza, Perrin et al. 1984; Rainbow, Parsons et al. 1984; Chalmers, Lovenberg et al. 1995; Nicholas, Hokfelt et al. 1996; Radulovic, Sydow et al. 1998). However despite the fact that these systems interact with each other, like stimulating CRF receptors alters NE transmission and vice versa (Tanaka, Yokoo et al. 1991; Valentino, Foote et al. 1993; Raber, Koob et al. 1995), it is

surprising that the sub-cellular relationship between these receptors is currently unknown. In order to better understand the anatomical basis for putative interactions between the NE and CRF systems, it is crucial to determine the cellular localization of their receptors in relation to each other. Determination of this localization could also help to test the involvement of various intracellular substrates that could be modulated in common by CRF1 and NE receptors; mechanisms that could be relevant for understanding our previous findings that repeated CRF1 activation leads to NE receptor hypersensitivity to NE in the BLA.

The inhibitory and excitatory neuronal properties of BLA are modulated by GABAergic interneurons and glutamatergic output projection neurons and excitatory versus inhibitory properties of BLA neurons are modulated in part by CRF and NE receptors, activation of which in turn is dependent upon stress and anxiety-like states (Cassell, Chittick et al. 1989; Rainnie, Asprodini et al. 1991; Rainnie, Asprodini et al. 1991; McDonald 1992; Sajdyk, Schober et al. 1999; Braga, Aroniadou-Anderjaska et al. 2004; McDonald and Mascagni 2004; Rainnie, Bergeron et al. 2004; Buffalari and Grace 2007; Buffalari and Grace 2009). Yet, it is unknown whether NE or CRF1 receptors are actually present on glutamatergic or GABAergic neurons in the BLA. Therefore, using immunohistochemical and retrograde labeling techniques, the present studies sought to examine the cellular distribution of NE alpha1 and CRF1 receptors in relation to each other, and their localization on GABAergic, glutamatergic and glial cells in rat brain slices containing the BLA. Understanding the anatomical localization of these receptors could help identify neuronal loci for interactions between these systems.

METHODS

Initially, when we started with our experimental design to determine cellular localization of NE and CRF1 receptors, we carried out experiments to test the effectiveness of antibodies of either NE alpha1 or beta receptors. However, after several parametric experimental manipulations, we found that only the alpha1 receptor antibody produced a clean and consistent labeling and that it would take significant additional time and resources to trouble-shoot the beta receptor labeling. Given that our previous behavioral experiments showed that activation of either alpha1 or beta receptor is sufficient to alter PPI, for the experiments here, we chose to focus on characterizing the cellular relationship between alpha1 and CRF1 receptors.

Subjects: 4 adult male Sprague-Dawley rats (Harlan, Madison, WI), each weighing 300- 325 g, were used in this study. Rats were housed together in a clear polycarbonate cage in a light- and temperature-controlled vivarium on a 12h light cycle with lights on at 0700. Food and water were available *ad libitum*. All facilities and procedures were in accordance with the guidelines regarding animal use and care put forth by the National Institutes of Health of the United States, and were supervised and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin.

Tissue preparation: Animals were deeply anesthetized with isofluorane and perfused transcardially with heparinized saline (1 unit of heparin/ml of 0.9% saline; heparin was obtained from APP Pharmaceuticals, LLC, Schaumburg, IL), followed immediately by 500 ml of 4% paraformaldehyde in 0.01 M phosphate buffer. The brains were post-fixed in the paraformaldehyde solution overnight and subsequently taken through graded sucrose solutions (10%–20% sucrose in 0.01 M phosphate-buffered saline [PBS], pH 7.3) at 4°C until the brains

sank in the sucrose solutions (96–120 hours). The brains were then frozen and 40µm coronal sections were taken through the brains on a cryostat microtome. Every second section was placed into an individual well containing 0.1 M PBS with 0.1% sodium azide (pH 7.3) and stored at 4°C for at least 24 hours.

Immunohistochemistry with peroxidase labeling: For immunohistochemical labeling, sections were washed four times (10 minutes each time) in 0.01 M PBS. Endogenous peroxidase activity was inhibited by incubating slides in a quenching solution containing 10% methanol and 0.75% hydrogen peroxide in 0.01 M PBS for 10 minutes at room temperature. Sections were then washed again and incubated at 4°C with primary rabbit anti-alpha1 antibody PA1-047 (1:500, Thermo scientific) for 72 hours or anti-CRF1/2 antibody SC-1757 (1:500, Santa Cruz biotechnology, Inc) for 48 hours in an antibody dilution buffer (0.1% Triton X-100, 0.1% casein, and 0.1% sodium azide in 0.1 M PBS, pH 7.2). After incubation, tissue was rinsed with 0.01 M PBS, and then exposed to a goat anti-rabbit biotinylated secondary antibody or rabbit anti-goat biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 2 hours. Tissue was rinsed with 0.01 M PBS, exposed to an avidin-biotin-peroxidase complex (ABC complex, Vector Laboratories) for 1 hour, rinsed again with 0.01 M PBS, and stained with diaminobenzidine (DAB, Vector Laboratories) to yield a brown precipitate or with nickel enhancement to yield black precipitate in some cases. Sections were mounted on Fisher Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), air-dried for 24 hours, taken through graded alcohols (95–100%), cleared in xylene for 1 hour, and cover-slipped with Permount (Fisher Scientific) mounting medium.

For double labeling of alpha1 receptors and glutamic acid decarboxylase (GAD), sections were taken through the steps described above except that the sections were incubated for 24 hours at 4°C in primary antibody for labeling GAD MAB5406 (1:1000, Millipore) and horse anti-mouse secondary antibody was used. After completing the DAB staining for GAD, sections were washed and taken through the steps described above using the alpha1 receptor antibody, except that the quenching step was omitted and a different stain was used. Tissues were exposed to the Vector SG chromagen (Vector Laboratories), which stained alpha1 receptors as a blue-gray precipitate.

Immunohistochemistry with fluorescence labeling: For double immunofluorescence labeling of alpha1 and CRF1 receptors, slices were washed, incubated in 5 % donkey serum for 30 minutes to block unspecific binding and then incubated in alpha1 primary antibody (1:500, 72 h) and then incubated for 2 hours in Alexa 488 donkey anti-rabbit secondary antibody (Life technologies) (1:200, 2 h). The sections were then washed and incubated in primary antibody (CRF1-1:500, 48 hours) and incubated for 2 hours in Alexa 594 donkey anti-goat secondary antibody (Life technologies) (1:200, 2h). After washing, these sections were counterstained with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Life technologies) (300nM in PBS) for 30 minutes, washed and mounted with Prolong Anti-fade medium (Life technologies). For labeling alpha1 receptors and GAD, same steps and primary antibodies as previously described above were used except that Alexa 594 donkey anti-mouse (Life technologies) secondary antibody was used for labeling GAD. For double labeling alpha1 receptors and NeuN, mouse anti-NeuN MAB377 (1:1000, Millipore) primary antibody and Alexa 594 donkey anti-mouse secondary antibody were used.

For control sections (alpha1 and CRF1), antibodies were pre-absorbed with their respective peptides (alpha1 receptor peptide, PEP-216, Thermo scientific; CRF1/2 receptor peptide, sc-1757p, Santa Cruz Biotechnology, Inc) and taken through the steps described above for immunoperoxidase or immunofluorescence labeling. For the secondary alone controls, the primary antibody was omitted and all the other steps were same as above.

Retrograde labeling with latex microspheres: For retrograde labeling of BLA to NAcc neurons, a rat was anesthetized via inhalation of an isofluorane/oxygen mixture and secured in a stereotaxic frame (Kopf instruments, Tujunga CA), and a unilateral hole (left side) was drilled above the Nacc using coordinates (in mm relative to bregma) based on the atlas of Paxinos and Watson (1998): Nacc, AP= +1.6, LM= +1.4, DV= -7. TransFluoSpheres Microspheres T8870 (0.4 μm-diameter, Life technologies) linked to Far Red fluorescent dye and diluted at 1:1 were loaded into a 10-μl Hamilton syringe (Hamilton Co., Reno, NV) mounted on a microdrive pump (Harvard Apparatus, Holliston, MA) and stainless steel injector (30-gauge, Small Parts Inc., Miami Lakes, FL) was attached with polyethylene tubing (Becton Dickinson, Sparks, MD) to the syringe. For microinfusions, stainless-steel injectors (30-gauge, Small Parts Inc., Miami Lakes, FL) were lowered into the brain regions. A total of 0.2-μl infusion was delivered over 2 minutes, and injectors were left in place for additional 10 minutes to allow for absorption of the solution (based on volumes and duration used in (Meloni, Gerety et al. 2006)). Following the infusion, and a survival time of 9 days, rat brain was extracted and slices were prepared with the procedure described above, and taken through immunofluorescence labeling steps for CRF1 receptors as described above.

Experimental design: Three sets of experiments were conducted:

Experiment 1: Anatomical localization of CRF1 and NE alpha1 receptors in the BLA. Given that localization of CRF1 and NE receptors in relation to each other has not been previously determined, with this experiment we explored the co-localization of these receptors on individual cells within the BLA using immunofluorescence and immunoperoxidase labeling. Although, the CRF receptor antibody used in this study recognizes both CRF1 and CRF2 receptors, BLA is known to contain virtually no CRF2 receptors (Chalmers, Lovenberg et al. 1995); therefore this antibody was expected to exclusively label CRF1 receptors in this brain region. We also carried out other immunolabeling for NeuN (neuronal nuclei) and alpha1 receptors to verify whether these receptors are present on neuronal cells, as opposed to glial cells in the BLA.

Experiment 2: Possible localization of alpha1 receptors on GABA-containing neurons in the BLA. BLA is known to contain inhibitory GABA interneurons that modulate local neural circuitry (McDonald and Mascagni 2004; Aroniadou-Anderjaska, Qashu et al. 2007). Therefore, with this experiment we explored whether alpha1 receptors are present on GABA-containing cells. Slices containing the BLA were taken through double immunoperoxidase or immunofluorescence labeling steps for GAD and alpha1 receptors.

Experiment 3: Determination of CRF1 receptor localization on BLA neurons projecting to the NAcc. BLA contains mainly glutamatergic projection neurons that innervate numerous forebrain regions including the NAcc; hence, neuronal alterations in the BLA could affect the outputs to the NAcc. These studies used retrograde labeling technique along with immunofluorescence labeling in BLA-containing slices to determine whether CRF1 receptors are present on projection neurons of the BLA that innervate the NAcc.

Light and confocal microscopy and image analysis: Bright-field images were captured with a Leica DMI 600B microscope, using the Image-Pro AMS 6.0 software. Under bright-field microscopy, alpha1 receptors (stained with the Vector SG chromogen) appeared blue–gray, whereas GAD containing processes (stained with DAB) appeared brown. In all the other cases, peroxidase staining was black due to nickel enhancement. All fluorescence images were captured using Nikon A1 confocal microscope at the W.M. Keck laboratory for biological imaging and at the Waisman Center Cellular and Molecular Neuroscience Core of UW-Madison. Alexa Fluor 488, Alexa Fluor 594 and TransFluoSpheres were excited with filters BP 500-550 nm, BP 570-620 nm or BP 663-738 nm to elicit Green, Red and Far Red Fluorophores (pseudo-colored blue for the purposes of this experiment), respectively.

RESULTS

Experiment 1: CRF1 and NE alpha1 receptors are highly co-localized on neuronal cells in the BLA. CRF1 and NE alpha1 receptors were found to be highly co-localized within the BLA and cell-count quantification revealed almost 96% co-localization (Fig. 1A-A5). In addition, labeling for NeuN and alpha1 receptors showed exclusive localization of alpha1 receptors on NeuN-containing cells, indicating that these receptors are localized on neuronal cells (as opposed to glial cells) (Fig 1B-B2). The pre-absorbed and secondary alone controls showed absence of labeling for alpha1 and CRF1 receptors (Fig. 3), indicating that staining represented specific labeling of receptors and not non-specific artifact.

Experiment 2: Alpha1 receptors are *not* present on GABA containing neurons. Double-labeling with alpha1 and GAD 67 antibody revealed that alpha1 receptors are not present on GAD-containing neurons, indicating that these receptors do not co-localize with GABA containing neurons in the BLA (Fig. 2A-A1 and Fig. 2B-B1). Interestingly, we found that alpha1 receptors do receive a substantial number of synapse-like varicosities that were immunolabeled for GAD.

Experiment 3: CRF1 receptors are present on projection neurons of the BLA that innervate the NAcc. Verification of the injection site showed that the microsphere deposition was restricted to the NAcc and did not spread to neighboring structures (Fig. 4A). Visualization of BLA-containing tissue showed microsphere localization in these sections, indicating that the microspheres were taken up by the neurons in the NAcc and transported back to the BLA (Fig. 4 E). This finding shows that BLA neurons project to the NAcc. In addition, immunolabeling for CRF1 receptors in the BLA slices revealed that these receptors co-localized with the microbeads, indicating that these receptors are present on the projection neurons of BLA that innervate the NAcc (Fig. 4 D, E, F). Although the microspheres were infused unilaterally in the NAcc, we found bilateral labeling in the BLA, but with higher degree of labeling on the ipsilateral rather than the contralateral BLA.

DISCUSSION

The present study provides previously uncharacterized information regarding the sub-cellular distribution of CRF1 and alpha1 receptors within the BLA by revealing that these two receptors are highly (almost completely) co-localized on neuronal cells in this brain region. In

addition, it was found that the alpha1 receptors do not co-localize with GABA-containing neurons in the BLA. CRF1 receptors were found to be present on presumptive glutamatergic projection neurons of the BLA that innervate the NAcc. These results together indicate that alpha1 and CRF1 receptors are positioned to modulate excitatory output activity of the BLA, and alterations of alpha1 or CRF1 receptor function due to stress (as seen in chapter 3) could affect PPI via changes in the activity of BLA neurons that project to the NAcc. Overall, the anatomical characterization of alpha1 and CRF1 receptors in the BLA in relation to each other and to GABAergic, glutamatergic and glial cells provides a new mechanistic framework to understand the interactions between NE and CRF systems in the BLA. Given that NE and CRF in BLA regulate a wide array of behaviors related to stress-like states, learning and drug abuse (Sajdyk, Schober et al. 1999; Shekhar, Truitt et al. 2005; Roozendaal, Castello et al. 2008; Roozendaal, Schelling et al. 2008; Zorrilla, Wee et al. 2012), the present anatomical findings have potential to profoundly influence our understanding of these processes.

The present findings showing a co-localization of CRF1 and NE alpha1 receptors on neuronal cells in the BLA provides cellular evidence that these receptors have a discrete substrate through which to interact with each other in the BLA. The verification that these receptors are exclusively localized on neuronal cells but not glial cells indicates that these receptors regulate neuronal functions in this brain region. While BLA was previously known to possess CRF1 and alpha1 receptors (Rainbow, Parsons et al. 1984; De Souza, Insel et al. 1985; Radulovic, Sydow et al. 1998; Wozniak M 2000), and interactions between NE and CRF systems were known to be involved in modulating various stress- and anxiety-related functions (Butler, Weiss et al. 1990; Melia and Duman 1991; Shekhar, Truitt et al. 2005; Roozendaal, Schelling et

al. 2008; Gresack and Risbrough 2011), our anatomical studies are to our knowledge the first detailed documentation of the sub-cellular relationship between CRF1 and NE alpha1 receptors in this site. The anatomical evidence of alpha1 and CRF1 co-localization is particularly significant for our previous finding that showed that repeated activation of CRF1 receptors in the BLA leads to hypersensitive NE receptors with regard to PPI disruptions (chapter 3), indicating that these receptors can regulate function of the same neurons. The high-level of CRF1 and alpha1 receptor co-localization (almost 100%) implies that an alteration in one of these receptors (for instance by intense stress) could potentially alter the function of the other receptors and thereby possibly lead to functional modifications in BLA neuronal activity. Moreover, the finding that the neurons that co-express alpha1/CRF1 receptors are presumptive glutamatergic output projection neurons indicates that in BLA, CRF/NE interactions may be particularly powerful in influencing larger neuron network.

Our double-immunolabeling studies for alpha1 receptors and GAD, an enzyme that converts glutamate into GABA and therefore labels GABA-containing neurons, revealed that alpha1 receptors are not present on GABAergic neurons. Interestingly though, we noticed that alpha1 receptors received a large volume of synapse-like varicosities from GABAergic cells, indicating that presumptive GABA interneurons could influence the neurons on which alpha1 receptors were found. BLA is known to be comprised of GABAergic interneurons that modulate the inhibitory neuronal properties (McDonald 1985; Rainnie, Asprodini et al. 1991; McDonald and Mascagni 2004) and these neuronal properties are regulated by noradrenergic and CRF receptors (Sanders, Morzorati et al. 1995; Buffalari and Grace 2007; Kaneko, Tamamaki et al. 2008). In addition, it has been shown that stress acting through alpha1 receptors alters

GABAergic inhibition and miniature inhibitory postsynaptic potentials via the BLA, a measure of neurotransmitter release (Braga, Aroniadou-Anderjaska et al. 2004), suggesting that these receptors could be present on GABAergic neurons. Also although, we found that vast majority of alpha1 receptors are not present on GABAergic cells, we cannot completely rule out the possibility that some alpha1 receptors may be present on a sub-population of GABA-containing neurons in the BLA. Hence, in order to draw definitive conclusions regarding the localization of alpha1 receptors in relation to GABAergic neurons future studies will need to conduct ultrastructural characterization using electron microscopy.

Given that the majority of non-GABA neurons in the BLA are glutamatergic and that alpha1/CRF1 receptors are not found on GABA-containing cells, it is reasonable to hypothesize that alpha1/CRF1 receptors are located on presumptive glutamatergic output neurons; importantly, previous studies indicate that CRF1 and alpha1 NE receptors do modulate BLA neuronal activity (Rainnie, Bergeron et al. 2004; Buffalari and Grace 2007). In addition, the glutamatergic neurons of the BLA are major output neurons that innervate cortical and sub-cortical structures such as the NAcc (Kelley, Domesick et al. 1982; Cassell, Chittick et al. 1989; McDonald 1992; Stuber, Sparta et al. 2011). Therefore, using a combination of retrograde and immunofluorescence labeling, we determined that CRF1 receptors are present on BLA output neurons that innervate the NAcc, confirming our hypothesis regarding the localization of these receptors on BLA output projection neurons. The finding that CRF1 receptors are present on glutamatergic neurons in the BLA, and thus could modulate excitatory neuronal properties of this brain region is consistent with the notion that stress enhances BLA excitation via CRF1 receptors (Rodriguez Manzanares, Isoardi et al. 2005; Shekhar, Truitt et al. 2005; Aroniadou-

Anderjaska, Qashu et al. 2007). The finding that NAcc is a downstream structure that receives projection neurons from the BLA neurons that express CRF1/alpha1 receptors indicates that alterations in the functioning of BLA CRF1/alpha1 receptors ultimately could alter the neuronal function of the NAcc, consistent with electrophysiological studies that show that NAcc neuronal properties are dependent upon BLA activation/inactivation state (Floresco, Blaha et al. 2001; McGinty and Grace 2009; Jones, Day et al. 2010; Stuber, Sparta et al. 2011; Papp, Borhegyi et al. 2012). Our findings for the first time provide a concrete anatomical mechanism for such possible effects.

The anatomical results from the present study have particular relevance to our previous behavioral findings that showed that stress-induced CRF1 activation in BLA leads to NE receptor hypersensitivity that contributes to sensorimotor gating deficits and enhances startle. Given the high degree of co-localization between CRF1 and alpha1 receptors in the BLA and the expression of these receptors on glutamatergic output neurons, it would be reasonable to hypothesize that stress-induced alpha1 receptor hypersensitivity via CRF1 receptors could ultimately alter sensorimotor gating via downstream effects on NAcc. This hypothesis is consistent with the role of NAcc in modulating sensorimotor gating (Kodsi and Swerdlow 1994; Kodsi and Swerdlow 1995; Wan and Swerdlow 1996; Alsene, Rajbhandari et al. 2011). Hence, stress could alter the BLA-NAcc circuitry to produce sensorimotor gating abnormalities as observed in disorders such as schizophrenia and PTSD (Grillon, Morgan et al. 1996; Swerdlow, Light et al. 2006).

In conclusion, our anatomical findings regarding the sub-cellular localization of CRF1 and alpha1 receptor in the BLA has implications for further probing the roles of molecular substrates that are linked to both of these receptors, such as extracellular signal-related kinase (ERK) that could be modulated via CRF1 and alpha1 receptor signaling through adenyl cyclase or phospholipase C pathways respectively (Wozniak M 2000; Grammatopoulos 2012). ERK has been shown to be activated by stress in the BLA, and hence, studying the role of this substrate in CRF and NE interactions could shed further light regarding the underlying molecular mechanisms involved in the CRF/NE system neuroadaptations that we found in chapter 3 (Schafe, Atkins et al. 2000; Grissom and Bhatnagar 2011). Overall, our studies provide a concrete anatomical framework for understanding the roles of CRF, NE, and BLA in stress-induced neuroadaptations that may be relevant to psychiatric illnesses with demonstrated PPI deficits (Shekhar, Truitt et al. 2005).

ACKNOWLEDGEMENTS

This work was supported by R01 MH075980 (VPB), T32 GM007507, and a Young Investigator Award from the National Alliance for Research on Schizophrenia and Depression (NARSAD) to VPB. We are grateful to Dr. Brian Baldo for his comments on the manuscript.

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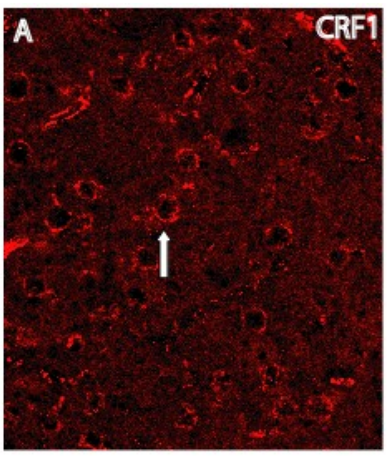
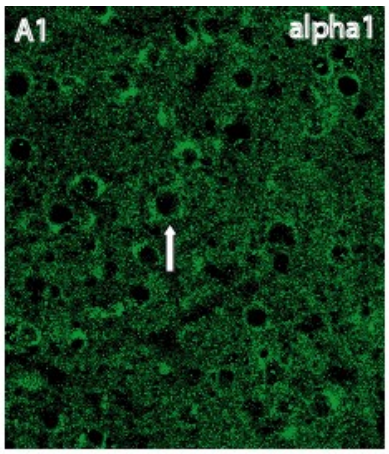
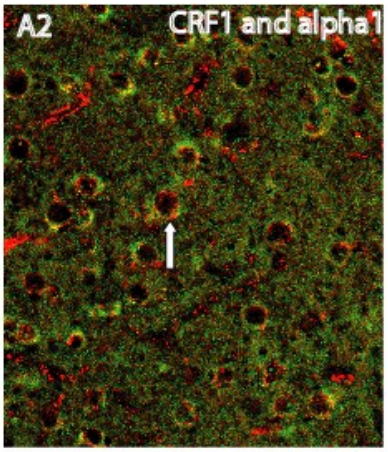
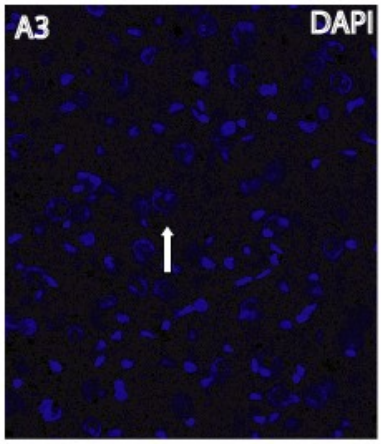
Fig. 1 A-A5) Immunofluorescence labeling showing CRF1 receptors (red) (A), NE α1 receptors (green) (A1) and DAPI-labeled cells (blue) (A3) in the BLA. CRF1 and alpha1 immunoreactivity were detected using antibodies coupled to Alexa Flour 594 and 488, respectively. Arrows point to CRF1, alpha1, CRF1+alpha1 and DAPI- labeled processes respectively. **A2)** Photomicrograph showing merged images of A1 and A2 with CRF1 (red) and alpha1 (green). **A4)** Photomicrograph showing merged images of A, A1 and A3 with CRF1 (red), alpha1 (green) and DAPI (blue). **A5)** Inset showing two cells from A4 that are triple-labeled for CRF1, alpha1 and DAPI. **A6)** Table showing cell-count analysis of co-localized CRF1 and alpha1 receptors in the left and right BLA.

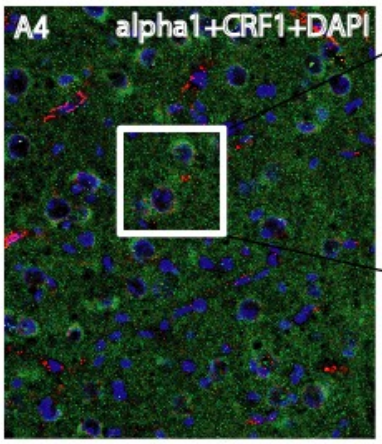
Fig. 1 B-B2) Double-immunofluorescence labeling in the BLA showing alpha1 receptor (green) (B) and NeuN (red) (B1). Alpha1 and NeuN immunoreactivity were detected using Alexa Flour 488 and Alexa 594 secondary antibodies respectively. Arrows indicate alpha1, NeuN and alpha1+NeuN labeled processes respectively. B2) Photomicrograph showing merged images of B and B1 with alpha1 and NeuN co-localization in the BLA.

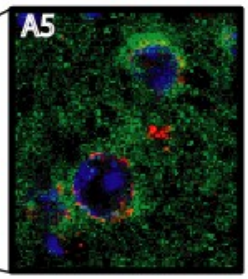






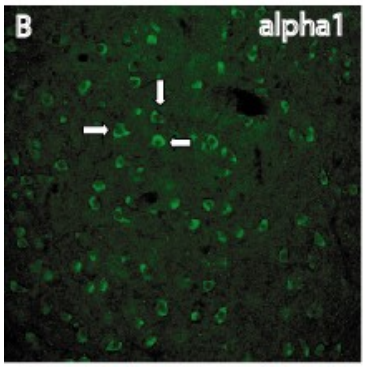


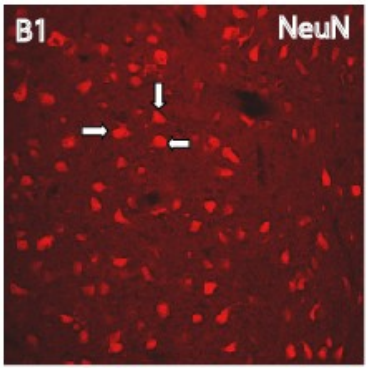




A6

	alpha1 alone	CRF1 alone	total alpha1 and CRF	alpha1 with CRF1	CRF1 with alpha1
left BLA	99	98	197	95/99	95/98
right BLA	111	113	224	106/111	106/113





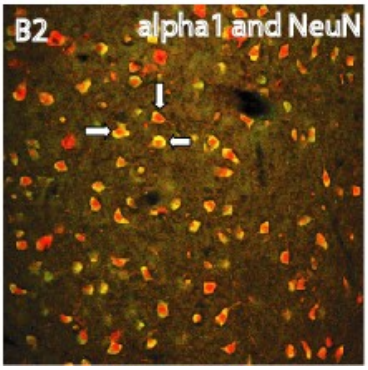
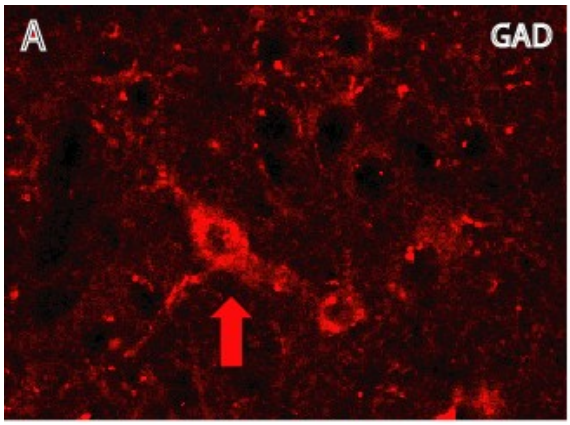
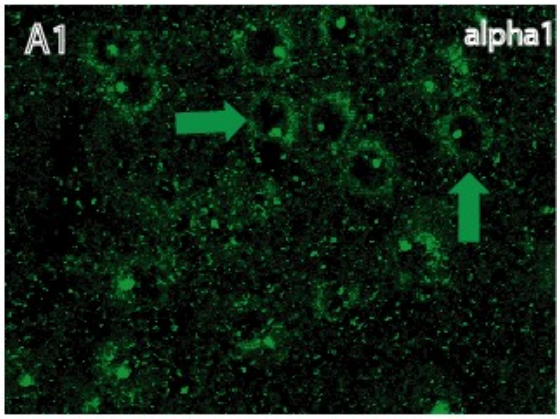
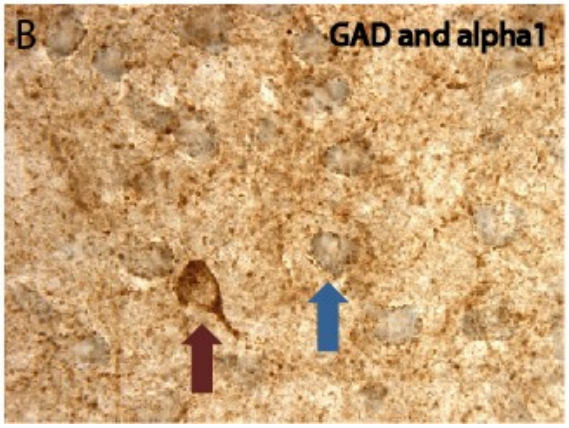


Fig. 2 A and A1) Double-immunofluorescence labeling showing GAD (red) (A) and NE α1 receptor (green) (A1) in the BLA. GAD and alpha1 immunoreactivity were detected using Alexa Flour 594 and Alexa 488 secondary antibodies respectively. Red arrow points to a GAD-labeled cell and green arrows point to alpha1 receptor-labeled cells. **B and B1)** Immunoperoxidase-labeling showing GAD (brown) and α1 receptors (blue) in the BLA. Brown arrows point to GAD-labeled cells and blue arrows point to alpha1-receptor labeled cells. Note that GAD and alpha1 staining of neural elements is non-overlapping.







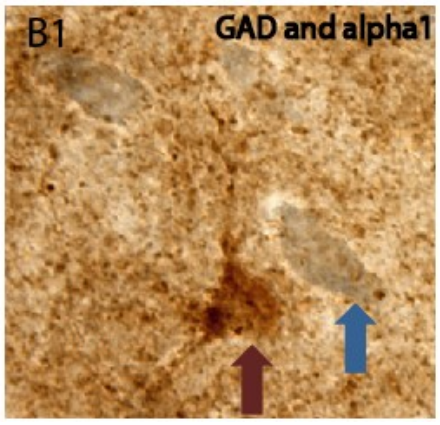


Fig. 3 A-A2) Immunofluorescence labeling of alpha1 receptors, and pre-absorbed alpha-receptor peptide and secondary alone controls respectively in the BLA. **A3-A5)** Immunoperoxidase labeling of alpha1 receptors, and pre-absorbed alpha1-receptor peptide and secondary alone controls respectively in the BLA. **B-B2)** Immunofluorescence labeling of CRF1 receptors, and pre-absorbed CRF1-receptor peptide and secondary alone controls respectively in the BLA. **B3-B5)** Immunoperoxidase labeling of CRF1 receptors, and pre-absorbed CRF1-receptor peptide and secondary alone controls respectively in the BLA.

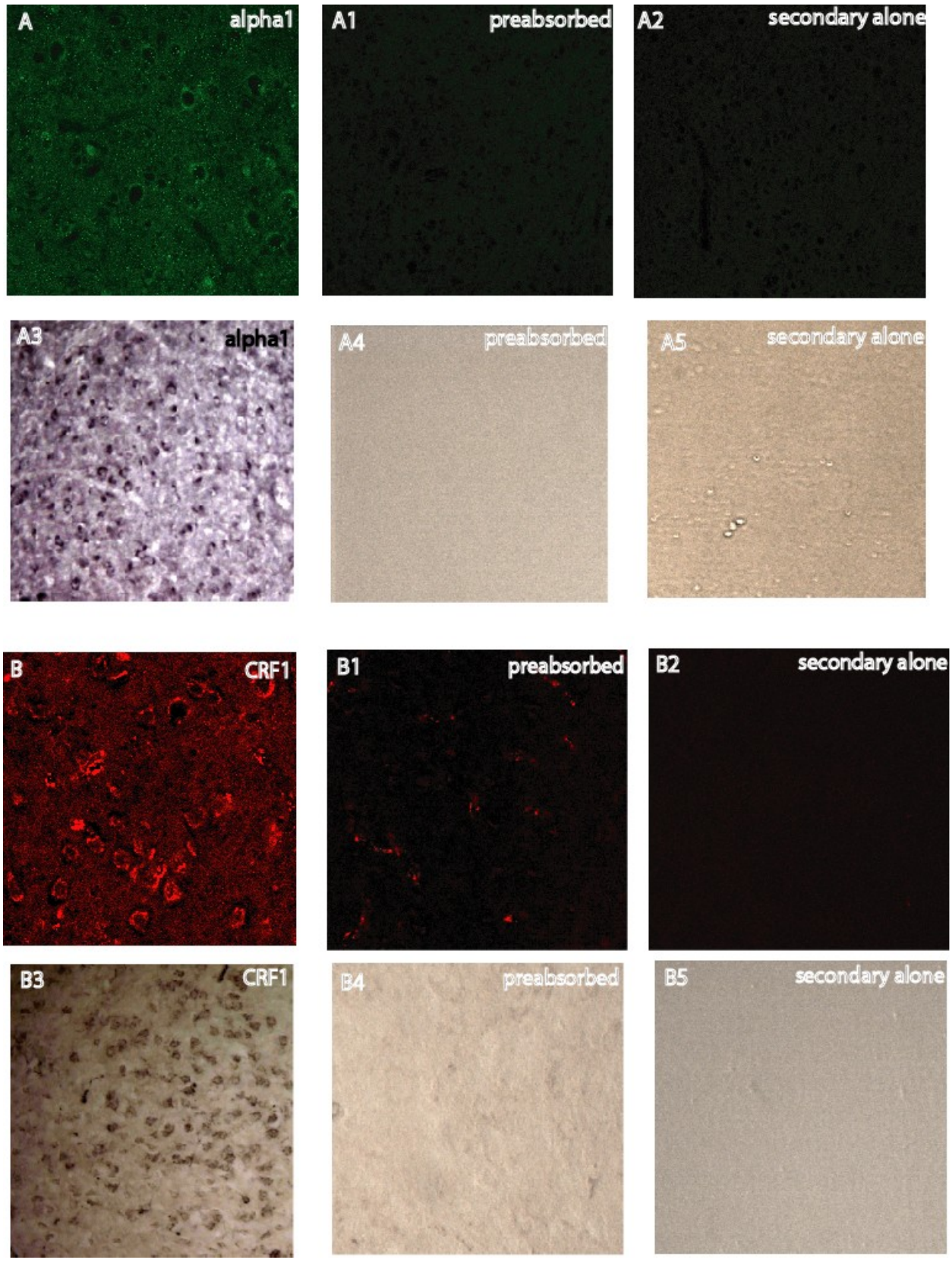
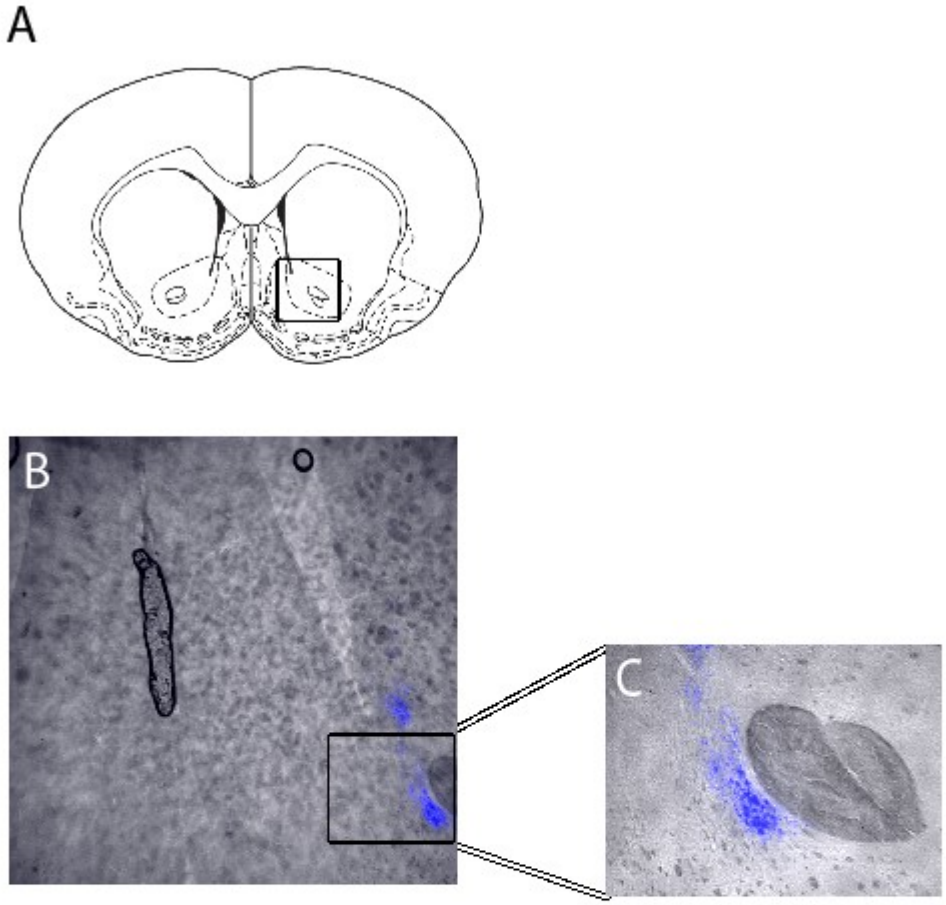
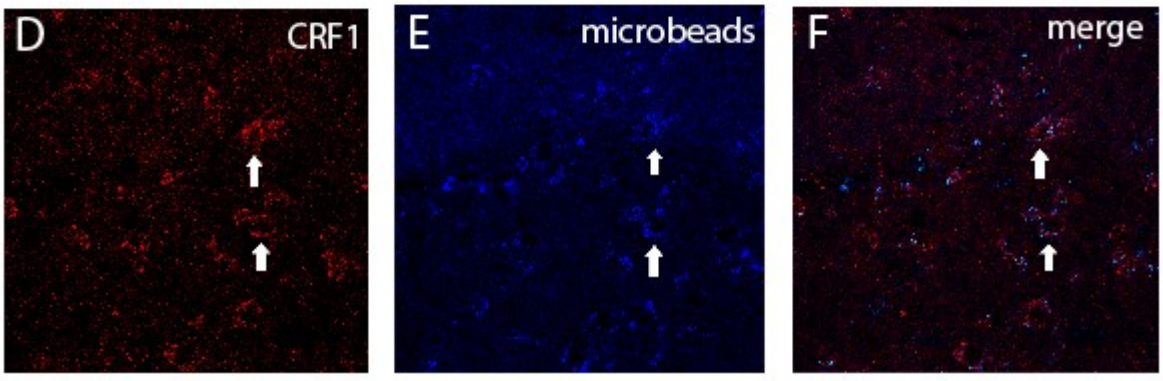

A
alpha1
A1
preabsorbed
A2
secondary alone
A3
alpha1
A4
preabsorbed
A5
secondary alone
B
CRF1
B1
preabsorbed
B2
secondary alone
B3
CRF1
B4
preabsorbed
B5
secondary alone

Fig. 4 A) Line drawing of coronal brain slice depicting the area of fluorescence-labeled microsphere infusion into the NAcc (square box). **B)** Fluorescent-image overlaid on a transmitted-light image from the same section showing deposition of retrograde tracer (0.04 μm Far red TransFluoSpheres; pseudo-colored blue). **C)** Inset showing restricted deposition of retrograde tracer beads around the NAcc. **D)** Immunofluorescence labeling in the coronal section of the BLA for CRF1 receptors, detected using the Alexafluor 594 secondary antibody (red). **E)** Cells with fluorescence-labeled microspheres (blue) in the BLA section. **F)** Merged image of D and E showing CRF1 receptors co-labeled with cells filled with fluorescence microspheres in the BLA. Arrows point to a representative cells with CRF1 labeling, cells filled with fluorescence microspheres, and CRF1 cells co-labeled with microspheres respectively.


A
B
C


D
CRF1
E
microbeads
F
merge

CHAPTER 5

SENSITIZATION OF CORTICOTROPIN-RELEASING FACTOR RECEPTORS AFTER REPEATED NOREPINEPHRINE RECEPTOR STIMULATION IN THE BED NUCLEUS OF THE STRIA TERMINALIS LEADS TO PTSD-LIKE INFORMATION PROCESSING DEFICITS IN RATS

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The experiments in the following chapter were completed by myself. Technical assistance was provided by Katie Fallace and Lindsay Pascal. General experimental design, data analysis and conclusions were developed with the help of my thesis advisor, Dr. Vaishali Bakshi. These results are ready for submission to the journal of Neuropsychopharmacology.

ABSTRACT

Patients with disorders such as post-traumatic stress (PTSD) and schizophrenia are vulnerable to stress, as intense stress exposure can precipitate or exacerbate these conditions. These patients also display compromised prepulse inhibition of the startle response (PPI), which refers to the reduction in startle when a weak pre-stimulus is presented immediately before a startling stimulus. PPI is an operational measure of sensorimotor gating, which is presumed to reflect a reduced ability to gate out potentially irrelevant information. Stress promotes the release of norepinephrine (NE) and/or corticotrophin releasing factor (CRF) within the brain. Both CRF and NE systems have also been shown to be involved in modulation of PPI, with our recent data showing that repeated stress-induced CRF receptor stimulation within the basolateral amygdala (BLA) results in long-lasting hypersensitivity of the NE receptors leading to disruptions in PPI. Part of the extended amygdala that is also important for modulating stress related information is the bed nucleus of the stria terminalis (BNST), which also regulates PPI and is enriched in CRF and NE. The present studies thus explored whether neuroadaptations of NE or CRF systems after repeated stimulation of the receptors of these systems in the BNST would alter PPI. Separate groups of male Sprague Dawley rats received either CRF (200ng/0.5µl), NE (20µg/0.5µl), or vehicle into the BNST, once/day for 3 days with PPI testing after each infusion. Following this treatment regimen, repeated CRF-or vehicle-treated rats were challenged with a sub-threshold dose of NE (0.3µg/0.5µl) while the NE-treated rats were challenged with CRF (200ng/0.5 µl), and PPI was measured. Initial/repeated CRF or vehicle in the BNST had no effects on PPI. In contrast, initial and repeated NE produced a disruption of PPI. Notably, the CRF challenge in rats that had received repeated intra-BNST NE produced a significant disruption of PPI, but challenge with a sub-threshold dose of NE did not produce a significant disruption of PPI in

either repeated CRF or repeated vehicle treated rats. Therefore, our results indicate that in the BNST, a dose of CRF that on its own does not disrupt PPI, leads to PPI disruptions after a history of repeated NE receptor stimulation in this site. These findings could indicate that the BNST may be an important brain region in which neuroadaptations of the CRF and NE systems takes place to modulate PPI, as might occur with the release of these neurotransmitters under repeated stress exposure like for example in PTSD populations.

INTRODUCTION

Post-traumatic stress disorder (PTSD) is a debilitating disorder triggered by intense or chronic stress, and is characterized by exaggerated and pathological responses to innocuous stimuli long after the traumatic or stressful experiences have ended (Yehuda, McFarlane et al. 1998; Pitman, Orr et al. 1999; Pitman, Rasmusson et al. 2012). Intense stress also triggers psychosis and worsens symptoms in schizophrenia (Walker and Diforio 1997). PTSD and schizophrenia patients also exhibit an abnormality in sensorimotor gating, which is a form of a pre-attentional information processing that allows organisms to filter out unwanted information from their internal and external milieu (Braff and Geyer 1990; Grillon, Morgan et al. 1996; Swerdlow, Light et al. 2006). PTSD patients display an increased arousal threshold for innocuous stimuli while schizophrenic patients suffer from cognitive fragmentation (Grillon, Courchesne et al. 1990; Braff 1993), symptoms that can possibly be attributed to deficient sensorimotor gating.

An operational measure of sensorimotor gating is a paradigm known as prepulse inhibition of the startle response (PPI), which is deficient in several neuropsychiatric disorders

including schizophrenia and PTSD (Grillon, Morgan et al. 1996; Grillon, Morgan et al. 1998; Braff, Geyer et al. 2001). PPI is a natural phenomenon in which a weak stimulus when presented right before a stronger startling stimulus produces a diminution of the startle response (Geyer and Swerdlow 2001). Years of research has now validated PPI to be a reliable cross-species phenomenon for measuring alterations in sensorimotor gating and therefore studying PPI deficits in animal models provides a strong translational benefit in order to develop newer and better treatment options for patients suffering from disorders that involve a breakdown in this type of central filtering mechanism (Hoffman and Ison 1980; Swerdlow, Braff et al. 2000; Geyer, Krebs-Thomson et al. 2001).

Intense stress can worsen the conditions of schizophrenia and PTSD by over-activating two candidate neurochemical pathways in the brain, including the corticotrophin releasing factor (CRF) and norepinephrine (NE) systems that are found to be dysfunctional in schizophrenia and PTSD (Southwick, Krystal et al. 1993; Baker, West et al. 1999; Newport and Nemeroff 2000; Bale and Vale 2004). Stimulation of CRF and NE receptors in the brain can disrupt PPI (Conti, Murry et al. 2002; Conti 2005; Conti, Costill et al. 2005; Alsene, Rajbhandari et al. 2011), and previous studies show that exposure to psychological stress produces an enduring disruption of PPI (Bakshi, Alsene et al. 2011), a behavioral dysfunction that likely could involve activation of CRF and NE receptors within the amygdala and the extended amygdala.

Recently, we demonstrated that repeated activation of CRF receptors in the basolateral amygdala leads to hypersensitivity of the NE receptors. Bed nucleus of the stria terminalis (BNST) is part of the extended amygdala and is enriched in NE and CRF receptors, which play

an important role in modulating emotional affective states such as anxiety (Cummings, Elde et al. 1983; Rainbow, Parsons et al. 1984; Gray 1990; Pieribone, Nicholas et al. 1994; Chalmers, Lovenberg et al. 1995; Davis 1998; Liang, Chen et al. 2001). Although it has been shown that CRF and NE receptors interact in modulating startle, that pharmacological stimulation of NE receptors within the BNST disrupts PPI, and CRF modulates startle via the BNST, little is known about interactions between CRF and NE systems in modulating PPI via the BNST (Lee and Davis 1997; Davis 1998; Walker, Miles et al. 2009). Given that both the NE and CRF systems are activated under stressful conditions, one mechanism by which exposure to repeated stress could alter PPI in schizophrenia and PTSD disorders is through hypersensitivity of these systems, to ultimately modulate core behavioral functions like PPI. It is also important to note that, principally in PTSD, evidence points to the notion that due to repeated stress there is priming or plasticity of the CRF or NE systems such that even an innocuous stimulus can trigger pathological reactions long after traumatic events have ended which could be manifested as disrupted PPI (Southwick, Krystal et al. 1993; Bremner, Licinio et al. 1997; Baker, West et al. 1999). Therefore we hypothesized that pharmacological stimulation of CRF or NE receptors within the BNST could lead to neuroadaptations of these systems which in turn could affect PPI. Findings from this study could shed light on the neuronal mechanisms that may be involved in stress-induced sensorimotor gating abnormalities in disorders such as PTSD and schizophrenia.

METHODS

Subjects: A total of 25 adult male Sprague Dawley rats (Harlan Laboratories, Madison WI) weighing 300- 325 g were housed in pairs in clear polycarbonate cages in a light- and temperature-controlled vivarium on a 12h light cycle with lights on at 0700. Experiments were

conducted between 1000 and 1500 hrs, food and water were available *ad libitum*, and rats were handled daily. All facilities and procedures were in accordance with the guidelines regarding animal use and care put forth by the National Institutes of Health of the United States, and were supervised and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin.

Surgeries: Rats were anesthetized via inhalation of an isofluorane/oxygen mixture and secured in a stereotaxic frame (Kopf instruments, Tujunga CA). Stainless steel cannulae (23-gauge, Small Parts Inc, Miami Lakes, FL) were implanted bilaterally into the BNST using coordinates (in mm relative to bregma) based on the atlas of Paxinos and Watson (1998): Cannulae were implanted at an 11^{o} angle to the midline with the coordinates, AP: -0.8 mm from Bregma, LM: +2.7 mm or -2.7 mm from midline, DV: -3.7mm from skull surface. The cannulae were affixed to the skull with dental cement (Lang Dental Mfg Co, Wheeling, IL) and anchoring skull screws (Plastics One, Roanoke, VA) and wire stylets were inserted to prevent blockage. Rats recovered for one week before testing (with daily health checks and handling).

Drugs and Microinfusions: Corticotropin-releasing factor (CRF) (rat, human) was from Bachem/Peninsula Labs (Torrance, CA) and Phoenix Pharmaceuticals Inc. (Burlingame, CA) and was dissolved in sterile distilled water. L-(-)-Norepinephrine bitartrate (NE) was from Sigma Aldrich (St Louis, MO) and was dissolved in a solution of 0.01 % ascorbic acid in isotonic saline. For microinfusions, stylets were removed and cannulae were cleaned using a dental broach (Henry Schein, Melville, NY) and stainless-steel injectors (30-gauge, Small Parts Inc., Miami Lakes, FL) were lowered to extend past cannulae tips by 3 mm in the BNST. Thus, the final DV coordinates was 6.7 mm below skull surface, respectively. Injectors were attached with

polyethylene tubing (PE-10, Becton Dickinson and Co., Sparks, MD) to a 10-µl glass Hamilton syringe (Hamilton Co., Reno, NV) mounted on a microdrive pump (Harvard Apparatus, Holliston, MA). After infusions, injectors were left in place for 1 min to allow for absorption of the solution before stylets were replaced.

Startle Chambers: Startle chambers (San Diego Instruments, San Diego, CA) contained a nonrestrictive Plexiglas cylinder resting inside a ventilated and illuminated sound-attenuating cabinet, with a high-frequency loudspeaker to produce all acoustic stimuli. As described previously (Mansbach, Geyer et al. 1988), the whole-body startle response of the animal caused vibrations, which were then converted into analog signals by a piezoelectric unit attached to the platform. These signals were digitized and stored by a microcomputer and interface unit. Monthly calibrations were performed on the chambers to ensure accuracy of the sound levels and measurements. Sound levels were measured using the dB (A) scale.

Startle and PPI Testing: The test session consisted of a background noise (65 dB) that was presented alone for 5 min and remained on for the length of the session, followed by presentation (in a pseudo-random order) of pulse-alone trials (40-ms, 120-dB broadband bursts), prepulse+pulse trials (20-ms noises that were 3, 9, or 15 dB above the background noise and were presented 100 ms before the onset of the 120-dB pulse), and no stimulus trials (only the background noise). Sixteen presentations of each trial type were made. Four pulse-alone trials were also presented at the beginning and the end of the session to ensure that startle magnitude was stable during the portion of the session when PPI was measured, as the most rapid habituation of the startle response occurs within the first several presentations (Geyer, Swerdlow et al. 1990); these pulse-alone trials were excluded from the calculations of startle and % PPI.

During the week before the drug testing began, all rats were exposed to the startle test session once per day on 3 separate days with sham infusions preceding the last test to familiarize them with the testing and microinfusion procedures before the commencement of drug testing. The final baseline test occurred 2-3 days before the experiments, and PPI and startle values from this last baseline day were used to create equally matched treatment groups for subsequent drug testing in each of the experiments.

Experimental Design: Several infusion/test days were carried out in 3 separate groups of rats with 2-3 days separating consecutive tests. The sequence of tests was: a mock infusion (injectors lowered but nothing infused; 'mock1'); three drug infusions of either repeated CRF (200ng/0.5μl, N=9), NE (20 μg/0.5μl, N=12), or vehicle (half the rats getting the CRF vehicle and the other half getting the NE vehicle, N=8 total); another mock infusion; a crossover drug challenge with NE (0.3 μg/0.5μl) given to the repeated CRF rats and CRF (200ng/0.5μl) given to the repeated NE rats; a final mock infusion. PPI was measured immediately after the infusion on each of the test days.

Histology: At the end of all experiments, rats were perfused transcardially with 10% formalin in phosphate buffer (Sigma Diagnostics, St Louis, MO), 60-μm brain sections were taken through the injection site and stained with cresyl violet, and placements were verified by an experimenter blind to the behavioral data; rats with missed placements were excluded from subsequent behavioral analyses. Sample sizes for each experiment reflect this final adjusted number.

Data analysis: The startle response to the onset of the 120-dB burst was recorded for 100 ms for each pulse-alone and prepulse+pulse trial. Two measurements were calculated from these values

for each rat: Startle magnitude was the average of the startle responses to all pulse-alone trials; PPI was a percent score for each prepulse+pulse trial type: % PPI=100− ((startle response for prepulse+pulse trial)/ (startle response for pulse-alone trial)). Because no significant interactions were seen with prepulse intensity and any other factor in any experiment, a single composite % PPI score (average PPI collapsed across all 3 prepulse intensities) was calculated for each subject, and this was used for all analyses. For each experiment, separate ANOVAs were run on startle and PPI data to analyze each repeated treatment regimen, with test day as a within subjects variable (mock1 and the repeated drug infusion days were the four levels of this factor). Significant main effects were analyzed further with Bonferroni-corrected paired t-tests. No differences were found in any experiment between the two mock infusions that 'bracketed' the drug challenge, so data from these two days were averaged and designated as 'mock2'. Planned contrasts between 'mock2' versus the drug challenge day were used to assess effects of the crossover treatment in each group; comparison of 'mock1' (the mock infusion occurring before the repeated drug infusion regimens) versus 'mock2' provided an evaluation of conditioned or non-specific carry-over effects due to the repeated drug regimens.

RESULTS

Prepulse inhibition

Repeated CRF

ANOVA revealed no significant main effect of repeated CRF treatment on PPI in the BNST [$F(3, 24) = .945$, NS], showing that none of the CRF infusions altered PPI in this brain region (Fig. 1A). NE challenge in repeated CRF treated rats did not produce a significant effect on PPI ($P>0.05$).

Repeated NE

Repeated NE treatment, however, *did* have a significant main effect [F (3, 24) =8.598, p<0.001] (Fig. 1B). *Posthoc* comparisons showed that each NE infusion significantly lowered PPI compared to the pre-drug mock infusion ('mock1') (P<0.05). The size of this NE-induced PPI deficit seemed to diminish with repeated infusions, as PPI values for the 3rd NE infusion tended to be higher than those for the first and the second (P=0.09 and P=0.05, respectively). Thus, it appeared that a tolerance-like trend to the PPI-disruptive effects of NE developed with repeated NE infusions into BNST. Interestingly, CRF challenge in the repeated NE treated rats produced a significant disruption of PPI (P=0.05).

Repeated vehicle

Repeated vehicle infusion had no effect, indicating that a repeated infusion treatment regimen is not in and of itself sufficient to alter PPI [F (3, 18) =1.963, NS] (Fig. 1C). No effect of NE challenge was found in the repeated vehicle treated rats, indicating that in the BNST 0.3 μg NE is a sub-threshold dose for PPI.

Startle

Table 1 displays the effects of the various treatments and challenges on baseline startle. In some cases, significant effects were observed in baseline startle. Repeated CRF [F (3, 24) = 11.898, P<0.01] and repeated NE [F (3, 24) = 11.259, p<0.01] had main effects. *Post hoc* comparison showed that each repeated CRF treatment decreased startle (p<0.05) and each NE treatment reduced startle (p<0.01). Surprisingly, challenge with NE significantly enhanced startle whereas challenge while CRF challenge significantly decreased startle (P<0.05).

DISCUSSION

We have established several novel results regarding the roles of the NE and CRF systems in modulating PPI via the BNST. First, neither a single (day1) nor repeated infusions (3 days) of CRF into the BNST affected PPI. In contrast, single or repeated NE infusion in the BNST led to a disruption of PPI. Cross challenge with a sub-threshold dose of NE in rats that had previously received repeated CRF infusions in the BNST did not lead to a significant disruption of PPI. However, a dose of CRF that on its own had no effects led to a significant disruption of PPI after prior repeated NE infusions in the BNST. Taken together, these findings indicate that the BNST may be a potential substrate that undergoes neuroadaptations of the CRF and NE systems that lead to PPI abnormalities, such that repeated NE treatment in this brain region increases vulnerability for PPI deficits via CRF receptor hypersensitivity, which could theoretically contribute to the sensorimotor gating abnormalities after stress in schizophrenia and PTSD.

The lack of effect of CRF on PPI via the BNST may at first appear inconsistent with the studies that have shown that CRF disrupts PPI (Conti, Murry et al. 2002; Risbrough, Hauger et al. 2003; Risbrough, Hauger et al. 2004; Conti 2005). Nonetheless, these studies used different strains of rats than the ones used in the current study and also used intracerebroventricular (ICV) infusions of CRF. Hence, it may be that other central nervous system sites besides that BNST are responsible for these previously reported effects. A simple explanation for the lack of effect of CRF on PPI via the BNST could be because CRF modulates PPI via other brain regions besides the BNST under normal conditions. A recent transgenic study shows that over-expression of CRF in the BNST decreases CRF1 receptor density (Sink, Walker et al. 2013), indicating that the

effect of CRF may be reduced after repeated exposure to this peptide. Other studies showing that ICV CRF disrupts PPI indicate that this effect is observed only two hours after infusion and evident only at certain inter-stimulus intervals between the prepulse and pulse onset (Risbrough, Hauger et al. 2004; Gresack and Risbrough 2011), to some extent indicating that the time-period of our PPI testing (which was immediately after BNST infusion) may not have captured the maximum efficacy of CRF for PPI changes. Nevertheless, it is unlikely that the absence of effect of CRF on PPI was due to using too low a dose of CRF because the dose of CRF administered in our studies produces potent behavioral changes such as increased grooming and anorexia when infused into the extended amygdala (Jochman, Newman et al. 2005).

Repeated NE in this brain region before the CRF challenge may, however, alter the later dynamics of CRF on PPI, causing an ineffective dose of CRF to now disrupt PPI. Interestingly, we observed reductions in baseline startle after each CRF infusion, which is somewhat surprising given that previous reports have shown that CRF in the BNST enhances startle and that ICV CRF- enhanced startle can be blocked by BNST lesion or CRF1 antagonist in this brain region (Lee and Davis 1997; Walker, Miles et al. 2009). In our studies, even the CRF challenge in the BNST in repeated NE-treated rats reduced startle levels. However, there are several important methodological and conceptual differences between these previous studies and our study that could explain why our CRF startle findings differ. Firstly, a better comparison of our test session versus the ones in the previous studies shows some important differences: our test sessions are designed to measure PPI (reduction in startle due to a prepulse) and therefore utilize a high pulse intensity (120 dB), but in the previously described studies that report CRF-enhanced startle, the sessions are designed to specifically detect startle increases, and use trials

with much lower startle intensities (90 dB, 105 dB) than ours (Lee and Davis 1997; Risbrough, Hauger et al. 2003; Gresack and Risbrough 2011). In addition, a closer look at the results of these studies shows that startle enhancement is most prominent 1-2 hours post-infusion of CRF. These findings indicate that either our test sessions do not capture startle enhancements in the same way as these studies, or that testing immediately after CRF infusion (as done in our studies) may not be an appropriate time-point that captures the maximum efficacy of CRF to increase startle. There is also indication from some studies that startle is augmented by CRF when rodents are tested during their dark cycle, which may have been a confounding variable in our studies as our behaviors were conducted in their light cycle (Risbrough, Hauger et al. 2003; Gresack and Risbrough 2011). Nonetheless, it is unlikely that baseline startle reactivity changes contributed to alterations in PPI because startle was reduced whilst PPI remained unaffected (i.e. the repeated CRF days or CRF challenge day).

While repeated CRF in the BNST did not alter PPI, repeated NE in this brain region did produce a disruption of PPI that was also independent of changes in startle. This finding is consistent with our previous report that has shown that acute stimulation of alpha1 and beta NE receptors in the BNST disrupts PPI (Alsene, Rajbhandari et al. 2011). Repeated NE also led to a hypersensitivity of CRF receptors in BNST, as evidenced by PPI disruption to a challenge with a dose of CRF that on its own had no effect on PPI. These results indicate that a long-term repeated release of high levels of NE within the BNST, as might occur endogenously with repeated stress in disorders like PTSD could make this brain region subsequently vulnerable to levels of CRF that normally have no effect in modulating sensorimotor gating.

Overall our studies show a functional interaction between NE and CRF systems in the BNST, but the underlying mechanisms through which repeated NE leads to CRF receptor hypersensitivity is currently unknown. Some studies do hint at a few possible mechanisms for the interaction between these systems. NE and CRF both modulate glutamate release in the BNST (Dumont and Williams 2004; Egli, Kash et al. 2005; Kash, Nobis et al. 2008; McElligott and Winder 2008; Shields, Wang et al. 2009; McElligott, Klug et al. 2010; Silberman, Matthews et al. 2013), but the modulation via NE receptor activation also requires simultaneous activation of CRF1 receptors (Nobis, Kash et al. 2011; Flavin and Winder 2013). Thus, perhaps repeated activation of NE receptors could alter CRF1 receptor signaling dynamics (for example, perhaps shifting CRF1-bearing neurons into a different electrophysiological firing pattern). This could be a possible mechanism that may be involved in the hypersensitivity of CRF receptors in our studies. In addition, given that anatomical evidence shows that CRF1 receptors are localized on glutamatergic terminals in the BNST to modulate excitatory transmission, and NE receptors also modulate glutamatergic transmission in the BNST, it may be that they are co-localized on these terminals. It might then be predicted that repeated activation of these NE receptors may cause changes in glutamate release dynamics in the BNST via subsequent CRF1 receptor signaling to regulate behavioral functions such as PPI (Forray, Bustos et al. 1999; Egli, Kash et al. 2005; Jaferi and Pickel 2009). It has been shown that the NE terminals in the BNST make synapses on the dendrites of CRF-containing neurons, and that the CRF neurons in turn synapse on locus coeruleus (LC)-NE neurons which in turn could project to BNST (Phelix, Liposits et al. 1994; Van Bockstaele, Colago et al. 1998; Van Bockstaele, Bajic et al. 2001). If it were shown for example, that NE and CRF1 receptors were co-localized on these CRF-synthesizing neurons of BNST then we might begin to hypothesize a feed-forward loop with CRF and NE. This could

explain how repeated activation of NE receptors could alter CRF signaling in a way that could modulate NE transmission in BNST or other forebrain regions ultimately modulating PPI (Alsene, Rajbhandari et al. 2011).

It has been shown that stress enhances NE levels and alters excitatory transmission in the BNST (Pacak, McCarty et al. 1995; McElligott, Klug et al. 2010). Our finding that repeated activation of NE receptors enhances CRF signaling is consistent with studies that show that long-term activation of NE receptors leads to decreased NE signaling but enhances CRF signaling in the BNST (Davis, Shields et al. 2008; McElligott and Winder 2008; McElligott, Klug et al. 2010). Perhaps the interactions between CRF and NE systems involve activation of downstream molecular pathways associated with these receptors through the AC-cAMP mediated activation of the cAMP response element binding protein (CREB) in the nucleus that could alter gene transcription and CRF receptor sensitivity. Supporting this theory, it has been shown that CREB is activated in the BNST by stress (Curtis, Bello et al. 2002). Further studies will be required to understand whether stress causes CRF1 receptor hypersensitivity in the BNST and if so, various intracellular molecules will need to be probed to understand the mechanisms of such neuroplasticity.

Taken together, our finding that there is a cross-sensitization between the CRF and NE systems within the BNST in modulating PPI is significant in various ways. Foremost, this is the first study to elucidate the effects of the repeated stimulation of NE or CRF systems on modification of PPI within the BNST. Secondly, the current findings showing that the repeated NE receptor stimulation-induced CRF receptor plasticity leads to PPI changes is distinct from

our previous finding that showed an opposite profile in the BLA, suggesting that the BSNT may be a another crucial structure that that undergoes neuroplastic changes under stress in a manner distinct from BLA. Our findings are also relevant for understanding the pathophysiology of PTSD and schizophrenia in which PPI deficits are seen and in which symptoms are triggered by stress (Grillon, Morgan et al. 1996; Grillon, Morgan et al. 1998). There is some evidence for abnormalities of the NE and CRF systems in these illnesses (Kosten, Mason et al. 1987; Southwick, Krystal et al. 1993; Baker, West et al. 1999). Thus, alterations in CRF receptor activity may represent a pathological adaptation to enhanced NE signaling during repeated stress in the BNST, and hence the present findings illustrate a putative mechanism through which to further study NE and CRF system abnormalities that could contribute to deficient sensorimotor gating in schizophrenia and PTSD patients.

ACKNOWLEDGEMENTS

This work was supported by RO1 MH075980 (VPB), NARSAD young investigator award to VPB and T32 GM007507 (AKR).

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Fig. 1) Effects on % PPI of repeated CRF (200ng/ 0.5 µl), NE (20 µg/ 0.5 µl) and saline, and crossover challenge with NE, CRF and NE respectively in the BNST (Figures A, B & C respectively). Values represent means ± sem for each dose. Each repeated infusion day was compared to mock1 and challenge with mock2. **A)** NE crossover is 0.3 µg/ 0.5 µl. **B)** CRF crossover is 200ng/ 0.5 µl. ***** P<0.05, relative to mock1 and mock2 infusion days respectively. $^{+}$ p<0.06 relative to NE2. **C)** NE crossover challenge is 0.3 µg/0.5 µL.

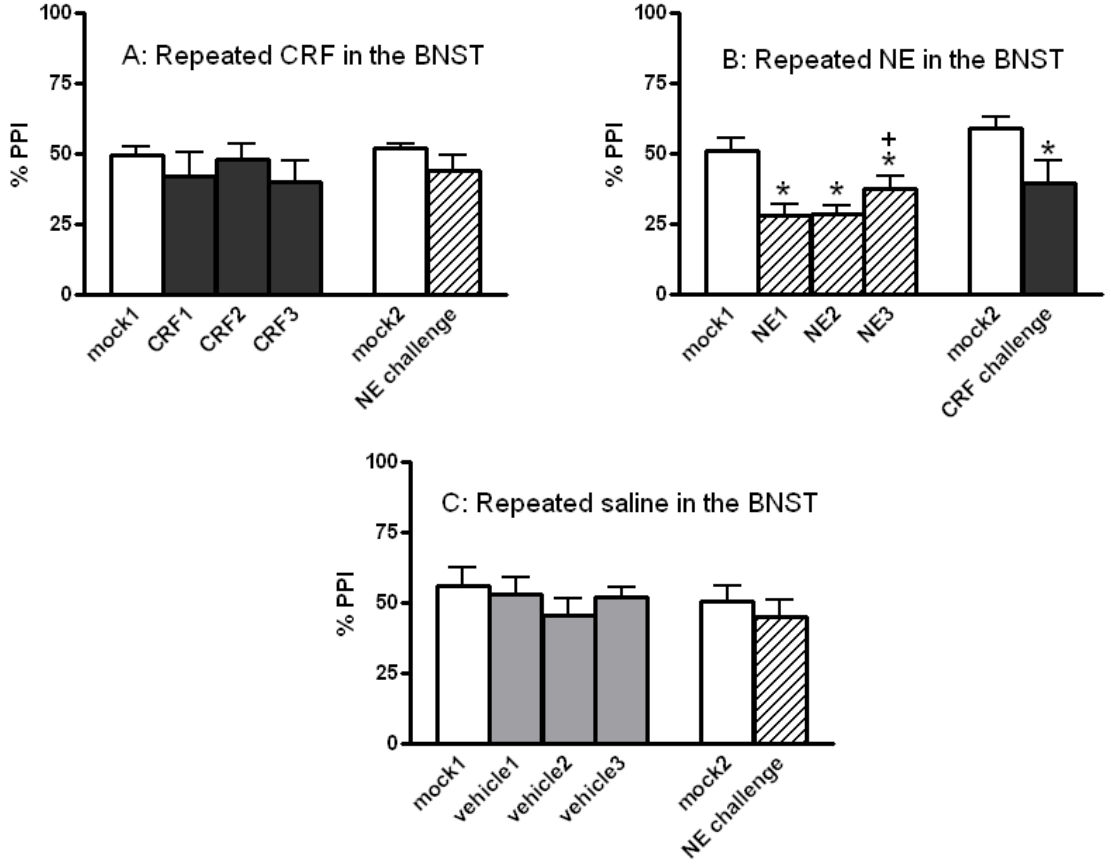

Fig. 2) Representative injector tip locations within the BNST (left panel), indicated by the arrows; and chartings depicting the locations in which infusions of CRF (squares), NE (triangles) or saline (circles) (right panel) **A)** CPu= *caudate putamen*, MnPO= *median preoptic*

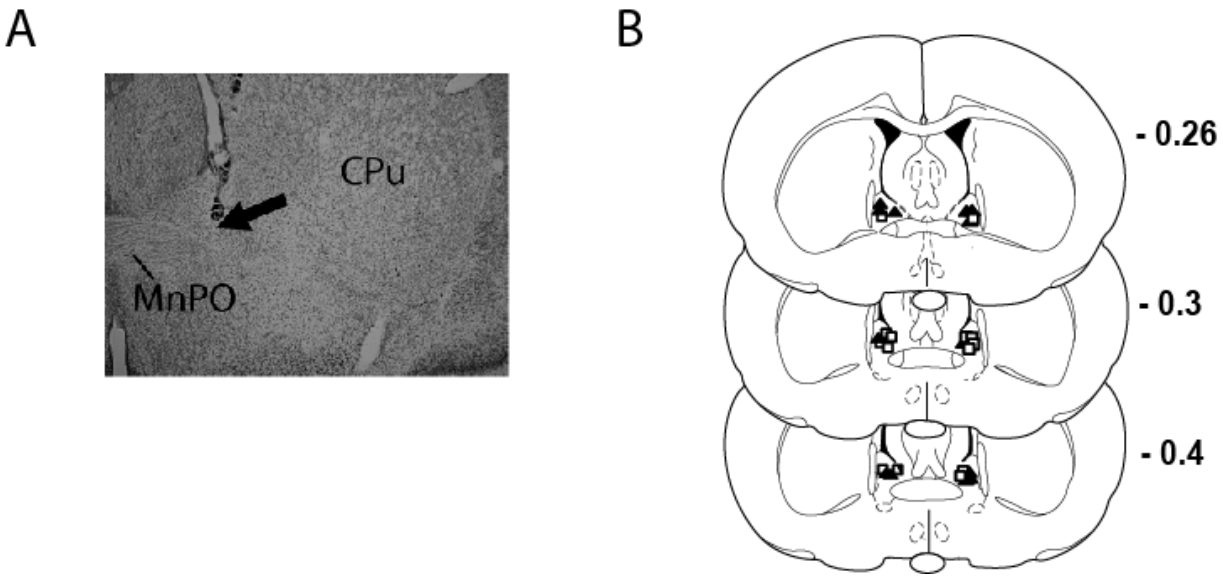

Table 1

Effects on startle of repeated CRF (200ng/ 0.5 µl), NE (20 µg/ 0.5 µl) and saline, and crossover challenge with NE, CRF and NE respectively in the BNST. Values represent means ± sem for each dose. Each repeated infusion day was compared to mock1 and challenge with mock2. ***** P<0.05, relative to mock1 infusion day. # P<0.05, relative to mock2 infusion day.

	MOCK1	DAY2	DAY3	DAY4	MOCK2	CROSSOVER
	BNST					
CRF	*398±65*	*219±37**	*287±37**	*223±28**	*314±53*	*383±57# (NE 0.3 µg)*
NE	*388±40*	*250±33**	*205±25**	*249±33**	*275±29*	*198±27#(CRF 200ng)*
SALINE	*514±184*	*416±114*	*362±87*	*428±84*	*377±70*	*440±66 (NE 0.3 µg)*

GENERAL DISCUSSION

Summary of all findings

Chapter 2 of this thesis demonstrated that acute co-stimulation of NE alpha1 and beta receptors within brain regions that receive NE projections from the LC, such as the BLA, BNST and MD-thalamus, disrupts PPI; no effect on PPI via the CeA and dorsal hippocampus was seen even though these two sites also receive NE projections from the LC. Nevertheless, in the CeA and dorsal hippocampus, stimulation of DA receptors disrupted PPI, indicating that NE transmission has a discrete, site-specific role in regulating PPI that may differ from the profile of sites affiliated with DA-modulation of PPI. These findings reveal that NE signaling in certain anatomical substrates that are downstream of the LC modulates sensorimotor gating deficits.

Studies described in chapter 3 revealed several important findings regarding CRF and NE system interactions in regulating PPI and how these systems may act with stressor presentation. Acute/repeated stimulation of CRF1 receptors within the BLA had no effect on PPI, but repeated CRF1 receptor stimulation led to a hypersensitivity of NE receptors to agonist stimulation such that a subsequent sub-threshold dose of NE produced a disruption of PPI when infused into the BLA. Acute or repeated intra-BLA NE infusion was shown to disrupt PPI, but this effect diminished with repeated NE infusions and did not lead to cross-sensitization to a CRF challenge for modulating PPI. In the CeA, neither acute nor repeated NE or CRF led to PPI disruptions. The profile of effects of NE on PPI via the BLA or CeA matched the results of chapter 2, which had shown that acute stimulation of NE alpha1 and beta receptors in the BLA produces PPI deficits while the same treatment in CeA does not. In order to test the physiological relevance of repeated CRF1 receptor activation in the BLA and

to determine whether stress would sensitize the NE receptors in a manner similar to repeated CRF, this chapter also tested the effects of repeated stress exposure using a predator stress paradigm on BLA NE receptor sensitivity with regards to PPI. Results showed that repeated (3 exposures) ferret stress produced the same profile as repeated intra-BLA CRF infusions, with rats showing disrupted PPI when given a sub-threshold NE challenge in the BLA 5, 11 or 18 days after the last ferret exposure. In addition, a challenge with a sub-threshold dose of systemic yohimbine almost a month after the last ferret exposure also significantly disrupted PPI in predator-exposed rats. Interestingly, it was found that the NE challenges also enhanced startle, consistent with startle abnormalities found in PTSD patients after traumatic experiences have ceased to occur (Grillon, Morgan et al. 1998). It was also shown that the repeated-stress-induced enhanced sensitivity of NE receptors occurs through activation of NE alpha1 or beta receptors, as challenges with agonists of either of these receptors in the BLA after repeated ferret stress also led to PPI disruption. This finding was particularly intriguing because there is indication that under normal circumstances (no stress), alpha1 and beta receptors are coupled to each other and individual stimulation of these receptors does not produce PPI deficits; simultaneous stimulation of both is required to disrupt PPI (Ferry, Roozendaal et al. 1999; Alsene, Rajbhandari et al. 2011). Hence, repeated ferret stress may 'uncouple' these receptors such that stimulation of either receptor is sufficient to alter PPI after repeated CRF1 receptor activation. Another crucial finding from this chapter was that the enhanced NE receptor sensitivity could be prevented by antagonism of CRF1 receptors in the BLA prior to each ferret exposure, but not 30 minutes after each ferret exposure or right before the NE challenge, suggesting that CRF1 receptor activation is necessary for the repeated ferret-induced NE neuroplasticity in the BLA.

Taken together, data in chapters 2 and 3 show that, at a behavioral level, NE and CRF systems interact within the BLA to modulate PPI, but it remained to be explored whether these systems interacted at an anatomical level. Thus, chapter 4 sought to characterize the distribution of NE receptors (alpha1) and CRF1 receptors in relation to each other and to excitatory/inhibitory neurons in the BLA. Using immunohistochemical procedures, it was shown that NE alpha1 receptors and CRF1 receptors highly co-localize on neurons within the BLA, providing a possible cellular basis for their interactions in regulating PPI via this site. Furthermore, it was also shown that alpha1 receptors do not co-localize with GABAergic neurons within the BLA, suggesting a possibility that these receptors could instead be present on glutamatergic projection neurons. Retrograde labeling with fluorescent-labeled microspheres confirmed that neurons that bear CRF1/alpha1 receptors are in fact projection neurons from the BLA that innervate the NAcc. Overall, these anatomical results provide an important, and novel, detailed characterization of the relationship between NE alpha1 and CRF1 receptors within the BLA. These findings thus provide crucial new information for understanding repeated stress/CRF-induced NE receptor sensitization and behavioral dysfunctions, including compromised sensorimotor gating.

Chapter 5 of this thesis sought to characterize whether the BNST could be another brain region besides the BLA that undergoes neuroplastic changes as a result of repeated activation of NE or CRF receptors to modulate PPI. The results of this chapter demonstrated that intra-BNST acute/repeated NE produced a disruption of PPI, but, unlike the BLA profile, repeated NE in the BNST also led a previously ineffective dose of CRF to subsequently disrupt PPI. However, neither acute/repeated CRF in the BNST nor a sub-threshold NE

challenge after repeated CRF led to PPI deficits. These results indicate that repeated NE receptor stimulation in the BNST may have led to enhanced sensitivity of CRF receptors in this brain region, behaviorally indexed by deficits in PPI. Therefore, these findings indicate that the BNST may be another putative substrate for the relapse of sensorimotor gating abnormalities due to stress-induced NE and CRF interactions. However, it remains to be determined whether stress exposure leads to plasticity of CRF receptors in a similar manner in the BNST. Why this profile in BNST (where repeated NE sensitized CRF receptors) is seemingly opposite to that seen in BLA (repeated CRF sensitizing NE receptors) is unclear.

Integrated discussion of results across all data chapters

When we began with our experimental hypothesis—that the amygdala may undergo neuroadaptations of the NE and CRF systems under stress—we had several reasons to believe that NE and CRF1 receptors may be involved in PPI modulation via amygdalar sub-regions, as evidence indicated that sub-regions of the amygdala such as the BLA, BNST and CeA are enriched in NE and CRF receptors (Fig. 1). These systems and the amygdala independently modulate PPI, and NE and CRF systems reciprocally regulate each other (Rainbow, Parsons et al. 1984; De Souza 1987; Emoto, Tanaka et al. 1993; Pacak, McCarty et al. 1995; Alsene, Carasso et al. 2006; Howland, Hannesson et al. 2007; Alsene, Rajbhandari et al. 2011; Bakshi, Alsene et al. 2012). This theory had not been tested

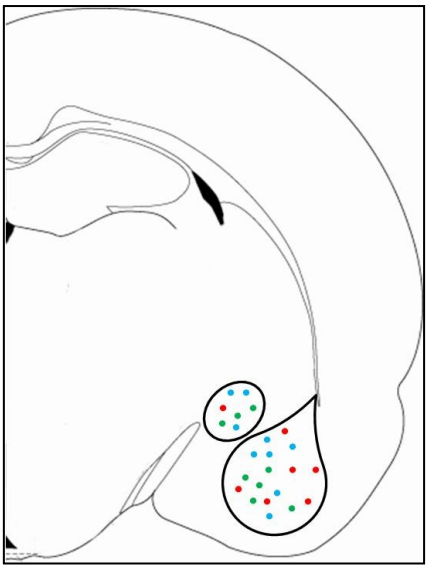

Figure 1: Schematic representation of distribution of NE alpha1 and beta, and CRF1 receptors in the BLA and CeA. ●=alpha1, ●=beta, ●=CRF1.

directly, however. Our findings showed that acute stimulation of NE receptors (alpha1+beta) with combined direct agonists of these receptors or through NE infusion in the BLA or BNST disrupts PPI, while the same treatment in the CeA does not (Chapter 2, 3 and 5), showing divergent actions of stimulating NE receptors in specific sub-regions of the amygdala (Fig. 2). We also showed that acute stimulation of CRF1 receptors in either BLA, BNST, or CeA had no effect on PPI, which corroborates earlier findings that PPI is not altered immediately after acute CRF administration (Bijlsma, van Leeuwen et al. 2010; Bakshi, Alsene et al. 2012). Delayed deficits in PPI after a single ICV CRF infusion have been reported (Risbrough, Hauger et al. 2003; Conti 2005; Bakshi, Alsene et al. 2012).

We then asked if there might be an interaction between the NE and CRF systems in modulating PPI and discovered that repeated stimulation of CRF1 receptors in the BLA led to subsequent PPI disruption with low-levels of NE receptor stimulation, while an opposite profile of enhanced CRF1 receptor sensitivity on PPI modulation via the BNST was found. PPI was not altered even with repeated NE or CRF in the CeA (Fig. 2). Our findings are also consistent with ideas that regions of the extended amygdala modulate stress and anxiety-like signals (Alheid, Beltramino et al. 1998; Davis 1998; Walker and Davis 2008) and that NE and CRF in these brain areas are also important for modulating aversive learning

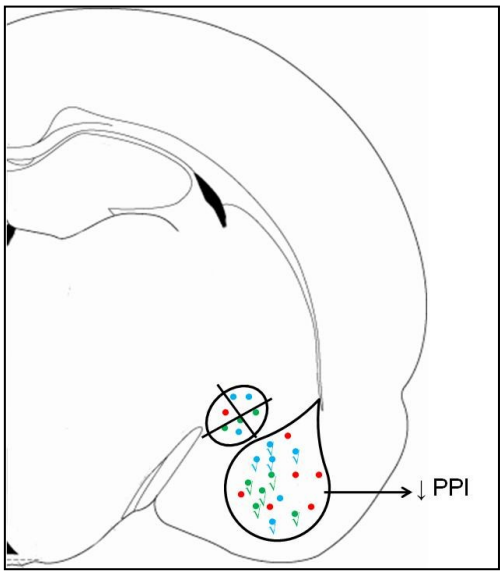

Figure 2: Schematic representation showing PPI modulation through acute or repeated stimulation of NE (not CRF1) receptors in the BLA, but not the CeA. ●√=alpha1, ●√=beta, ●=CRF1.

and memory (Liang, Chen et al. 2001; Roozendaal, Castello et al. 2008; Roozendaal, Schelling et al. 2008). Nonetheless, even though we found plasticity of the NE and CRF systems in the BNST and BLA, these effects were different in 'direction' across these brain regions. One reason for the divergent plasticity profile via these brain regions could be due to different distribution patterns of NE and CRF receptors on GABA interneurons versus glutamatergic projection neurons, which could determine whether the net effect in that brain region would be inhibitory or excitatory. Thus anatomical information has yet to be characterized. In vitro electrophysiological studies indicate that NE receptor stimulation may have differential effects on neuronal firing properties in BNST versus BLA and that these effects are altered by long-term activation of NE receptors via stress or other manipulations that increase NE transmission in these regions (Braga, Aroniadou-Anderjaska et al. 2004; Egli, Kash et al. 2005; Kaneko, Tamamaki et al. 2008; McElligott and Winder 2008). This differential profile of excitation and inhibition by NE or CRF and modification by stress or repeated receptor stimulation could thus potentially explain differential profiles seen in BLA versus BNST here.

Our next question then was whether exposure to stress would produce a similar profile to what we saw with repeated CRF infusions in the BLA. We discovered that repeated exposure to a predator stress paradigm leads to a PPI-disruptive effect by a low-dose NE in a manner similar to repeated CRF infusions in this brain region. The hypersensitivity to low-dose of NE after stress was long-lasting, as evidenced by PPI disruptions in response to almost a month after the last stress exposure. Furthermore, we were able to prevent stress-induced NE receptor hypersensitivity with a prior blockade of CRF1 receptors in the BLA,

implicating that the NE receptor sensitization begins with CRF1 receptor activation in BLA. We also found evidence to support that separate activation of either alpha1 or beta receptors in the BLA by sub-threshold agonist doses is sufficient to produce PPI deficits after repeated stress exposure, whereas without a history of stress, coincident activation of alpha1 and beta receptors is required to disrupt PPI (Alsene, Rajbhandari et al. 2011). Thus, our findings for the first time indicate that the BLA could be a crucial substrate that is vulnerable to stress and undergoes neuroadaptations in the CRF and NE systems that lead to alterations in information processing mechanisms like PPI. These results are consistent with previous reports that BLA regulation of anxiety-like responses also undergoes neuroplastic changes as a result of repeated activation of CRF receptors (Sajdyk, Schober et al. 1999; Rainnie, Bergeron et al. 2004; Ugolini, Sokal et al. 2008). Our findings importantly extend this information, as they outline a specific neuronal substrate that may be involved in cross-sensitization between CRF and NE in the BLA.

Working model for CRF and NE interactions in BLA for PPI modulation

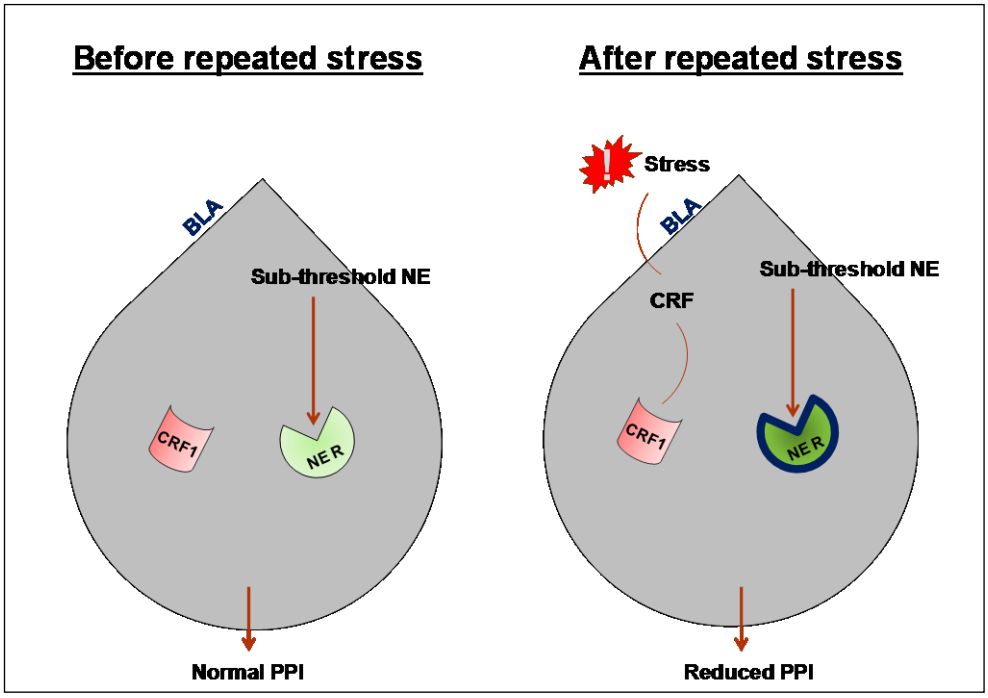

Figure 3: CRF and NE modulation of PPI via BLA

1. Before stress, low-dose NE in BLA had no effect on PPI
2. After repeated stress, same low-dose potently disrupted PPI
3. Repeated CRF1 receptor stimulation produced same profile

What is the cellular localization of CRF1 and alpha1 receptors in BLA?

Despite the fact that both NE and CRF receptors are present in the BLA—and it is well-established that these systems regulate stress-related functions and interact with each other (Swanson, Sawchenko et al. 1983; Rainbow, Parsons et al. 1984; De Souza, Insel et al. 1985; Gray 1990; Emoto, Koga et al. 1993; Pacak, McCarty et al. 1995; Berridge, Stratford et al. 1997; Davis, Walker et al. 1997; Smagin, Zhou et al. 1997; Sajdyk, Schober et al. 1999; Baldo, Daniel et al. 2003; Rainnie, Bergeron et al. 2004; Shekhar, Truitt et al. 2005; Ugolini, Sokal et al. 2008)—it has not been determined if these receptors are co-localized, nor well-characterized what types of cells express them. Therefore, using immunohistochemical

labeling techniques, we sought to characterize the distribution of NE and CRF1 receptors in relation to each other and to GABAergic neurons in the BLA.

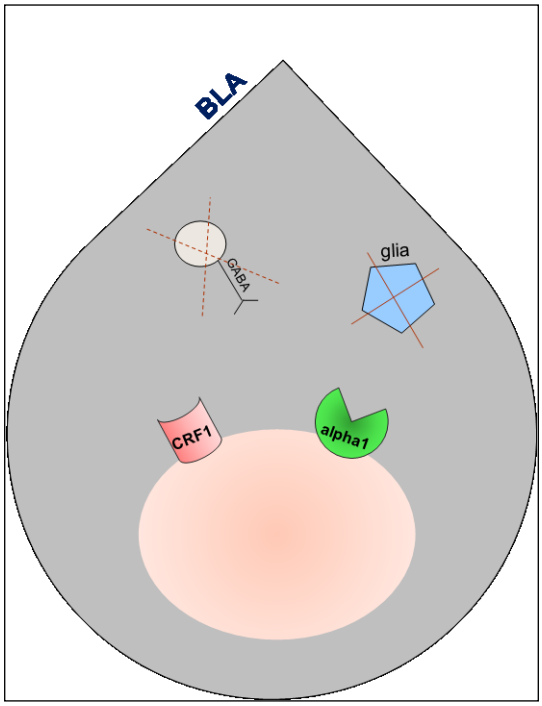

Figure 4: Our findings in reference to cellular localization of CRF1 and alpha1 receptors in BLA
1. CRF1 and alpha1 receptors are 96% co-localized in BLA
2. The cells expressing these receptors are not glia, but are neuronal cells (NeuN labeling)
3. The cells expressing these receptors are also not GABA-containing cells (GAD 67 labeling)

Are CRF1/alpha1-expressing neurons output projection cells of BLA?

As shown in Fig. 4 and its caption, we found high co-expression of CRF1 and alpha1 receptors on BLA neurons (not glia). We also found that these receptors are not present on GABA-containing neurons (Fig. 4), but did find that alpha1 receptors receive synapse-like varicosities from potential GABA neurons. Even though the population of GABA neurons is low in the BLA and our anatomical results demonstrated that the majority of alpha1 receptors are not present on GABA containing cells, we cannot completely exclude the possibility that some alpha1 receptors may be present on a sub-population of GABA interneurons that in turn could modulate BLA activation state (Rainnie, Asprodini et al. 1991; McDonald 1992; Washburn and Moises 1992; Rosenkranz and Grace 2002).

Nevertheless, the anatomical results of this thesis also provide another level of crucial information regarding potential modulation of excitatory signaling via CRF1 receptors in the BLA. Using retrograde fluorescent-microsphere infusion in known output projection targets of the BLA, such as the NAcc, we examined whether these microspheres were co-expressed with CRF1 receptors in the BLA. Our results showed that infusion of microspheres in the NAcc retrogradely labeled CRF1 containing neurons in the BLA. This final experiment confirmed our hypothesis that CRF1 and alpha1 receptors actually co-localize on BLA projection neurons that innervate the NAcc (Fig. 5).

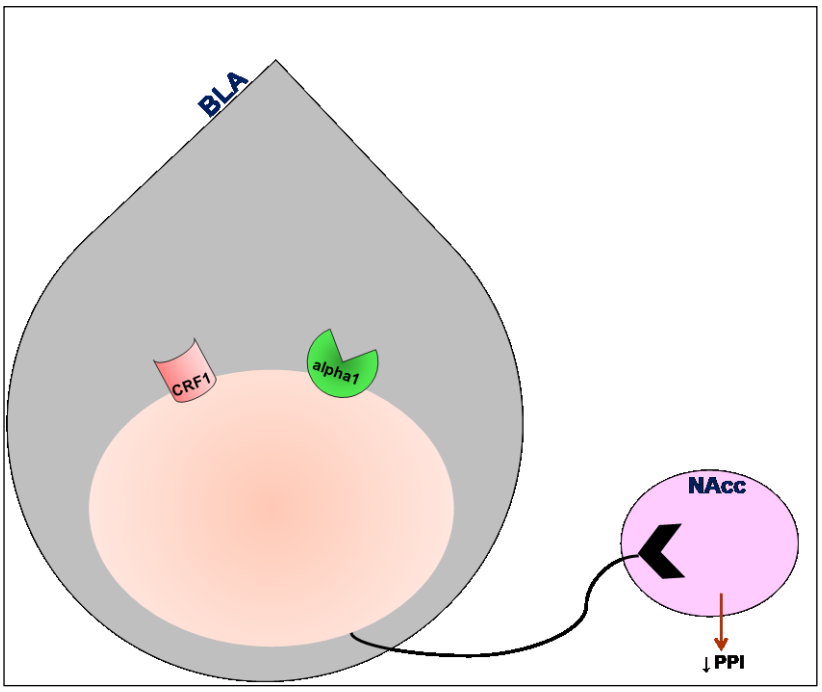

Figure 5: Our PPI findings explained in reference to anatomical model. CRF1 and alpha1 receptors co-localize on BLA projection neurons that innervate the NAcc, which in turn may modulate PPI.

Possible PPI modulation via BLA-NAcc projections

Therefore, with our anatomical studies we characterized cellular substrates that could be involved in CRF1 and alpha1 receptor interactions in the BLA. Based upon the findings that these receptors are present on BLA-NAcc projection neurons (likely glutamatergic) we can construct a plausible hypothesis regarding how PPI modulation could occur via this pathway. With repeated stress (that causes stimulation of BLA CRF1 receptors), sensitization

of co-localized alpha1 receptors appears to take place such that low-dose NE or alpha1 receptor agonists in the BLA in the aftermath of stress now disrupt PPI, possibly by altering the activity of BLA projection neurons. Other evidence in the literature supports our anatomical findings regarding modulation of BLA output electrophysiological activity. Activation of CRF1 receptors is known to modulate excitatory potentials in the BLA and, in stress-free conditions, the BLA is under a tonic GABAergic inhibition; chronic stress decreases GABAergic inhibition and enhances glutamatergic excitation in BLA thereby 'shifting' BLA into a different activity state and leading to anxiety-like behavioral states (Sanders and Shekhar 1995; Braga, Aroniadou-Anderjaska et al. 2004; Shekhar, Truitt et al. 2005; Aroniadou-Anderjaska, Qashu et al. 2007; Sandi, Cordero et al. 2008; Ugolini, Sokal et al. 2008). It is particularly interesting that in normal situations CRF facilitates while NE depresses BLA neuronal activity, but after chronic stress these patterns are switched, such that CRF-induced BLA-facilitation decreases whereas NE enhances neuronal excitation (Sandi, Cordero et al. 2008; Buffalari and Grace 2009), indicating that the *sensitivity to CRF decreases* while *that to NE increases* after stress. These observations are consistent with our behavioral findings, which showed that a low-dose of NE was sufficient to disrupt PPI after prior repeated stress exposure, indicative of functional hypersensitivity of NE in BLA. The resulting BLA activity after stress could be due to a summation of excitation and inhibition with the balance more toward excitation (Braga, Aroniadou-Anderjaska et al. 2004; Buffalari and Grace 2007).

Our finding that the NAcc is a downstream target of BLA projection neurons that contain CRF1/alpha1 receptors (Fig. 5), suggest that stress-induced alterations in CRF1 and

alpha1 functions in the BLA could ultimately modulate PPI via the NAcc. This theory is consistent with reports that demonstrate that NAcc neuronal activity is governed by the excitatory (glutamatergic) transmission received from the BLA (Floresco, Blaha et al. 2001; McGinty and Grace 2009; Jones, Day et al. 2010; Stuber, Sparta et al. 2011; Papp, Borhegyi et al. 2012). Interestingly, recent electron microscopic evidence shows that alpha1 receptors are present on glutamatergic neurons in the NAcc (Mitrano, Schroeder et al. 2012), but it is not known whether these receptors are on glutamatergic neurons that originate in the BLA. Nevertheless, alterations in NAcc neuronal properties could alter PPI, as evidence shows that manipulations in this brain region modulate PPI (Wan, Geyer et al. 1994; Alsene, Rajbhandari et al. 2011). BLA-NAcc projections could modulate PPI via downstream projections of the NAcc to the ventral pallidum (VP), a site that receives NAcc GABAergic projections and independently modulates PPI (Swerdlow, Braff et al. 1990; Kodsi and Swerdlow 1994; Kodsi and Swerdlow 1995; Kodsi and Swerdlow 1997). However, it is important to note that the BLA also modulates PPI independent of the NAcc via direct projections to the VP, with BLA inactivation shown to disrupt PPI due to VP disinhibition (Forcelli, West et al. 2012). Nonetheless, this mechanism of PPI modulation occurs due to GABA agonist-induced BLA inactivation. Therefore under stress, which is known to decrease GABAergic inhibition of BLA (Braga, Aroniadou-Anderjaska et al. 2004; Shekhar, Truitt et al. 2005; Aroniadou-Anderjaska, Qashu et al. 2007), the direct BLA-VP pathway would potentially be more active and affect PPI in the opposite direction, so this direct BLA-VP pathway may not be implicated in our results. Additional double-cannulation experiments will be needed to explore these questions directly.

Clinical relevance of findings

We have established that in the BLA, CRF1 and alpha1 receptors are co-localized on output projection neurons and that exposure to intense psychological stress causes long-lasting functional hyperreactivity of NE receptors to low-dose NE and subsequent PPI disruption, and that this process begins initially with CRF1 receptor activation. These findings are potentially clinically relevant, as the enhanced sensitivity to low-level NE stimulation in the aftermath of stress could provide a plausible substrate for long-lasting PPI deficits that are observed in disorders like schizophrenia and PTSD, both of which are exacerbated by stress, hypothesized to involve alterations in NE signaling, and are associated with deficits in PPI (Grillon, Morgan et al. 1996; Walker and Diforio 1997; Yehuda 2001). We showed that an ethologically relevant predator stress produced certain elements of PTSD-like pathology in our rodent model, including a lowered threshold for disrupted PPI, enhanced startle, and altered NE and CRF system interactions in a certain amygdalar sub-region. These findings are relevant for understanding symptoms of these disorders, which involve a loss of inhibitory control over the filtering of irrelevant/innocuous internal or external sensory stimuli, and are associated with diminished sensorimotor gating long after the traumatic stress has ended. The present results indicate that the BLA may be one candidate substrate through which these pathological effects could take place, and which may undergo stress-induced neuroadaptations of the NE and CRF systems. Our studies also indicate that the BNST may be another potential site that undergoes neuroplastic alterations of the NE and CRF systems, with repeated stimulation of those systems' receptors, as might also occur with repeated stress. Also, given that drugs that reduce NE transmission are beneficial for treating PTSD, our finding that stress-induced CRF1 activation leads to NE receptor hypersensitivity in the BLA is extremely relevant for

understanding the stress-related pathophysiology of these disorders (Pitman, Sanders et al. 2002; Raskind, Thompson et al. 2002; Vaiva, Ducrocq et al. 2003; Remington, Agid et al. 2013). Our anatomical result showing co-localization of NE alpha1 and CRF1 receptors in the BLA indicates that these receptors may modulate common intracellular substrates that could eventually be examined for therapeutic actions. Therefore, this thesis provides crucial information that could help in the development of novel pharmacological therapies for ameliorating stress-induced dysfunctions of sensorimotor gating in PTSD and/or schizophrenia.

Future directions

A crucial remaining question for our studies is how NE receptor hyperreactivity to low-dose NE and subsequent PPI deficits are instantiated after repeated CRF1 receptor activation in the BLA. One possibility is through alterations in common signal transduction pathways of CRF1 and alpha1 receptors which involve the adenylate cyclase (AC)-cAMP-PKA and PLC –DAG/IP3 pathways respectively (Fig. 6) (Battaglia, Webster et al. 1987; Graham, Perez et al. 1996; Gutkind 1998; Rossant, Pinnock et al. 1999; Wozniak M 2000; Hillhouse and Grammatopoulos 2006; Grammatopoulos 2012). These two intracellular pathways intersect at the level of mitogen-activated kinases (MAPK) (Fig. 6), a family of serine/threonine-specific kinases consisting of extracellular signal-related kinases (ERKs) which are distributed throughout the CNS and regulate a diverse array of cellular functions including gene expression, cell proliferation and synaptic plasticity (Seger and Krebs 1995; Flood, Finn et al. 1998; Waltereit and Weller 2003; Thomas and Huganir 2004). In agreement with the notion that ERK could be a downstream molecule activated via CRF1

receptors under stress, studies show that stress-exposure alters ERK-phosphorylation in the BLA (Schafe, Atkins et al. 2000; Shen, Tsimberg et al. 2004; Duvarci, Nader et al. 2005; Refojo, Echenique et al. 2005; Wu, Hsu et al. 2008; Ilin and Richter-Levin 2009; Grissom and Bhatnagar 2011). In our studies, repeated stress could cause functional changes in activity of one receptor (alpha1) by the other (CRF1) perhaps via altered ERK functions (Fig. 6). Evidence suggests that alteration in ERK could occur via receptor trafficking. CRF1 receptors have been shown to undergo internalization in other brain regions and internalization of this receptor has been shown to be necessary for ERK activation (Chalothorn, McCune et al. 2002; Reyes, Fox et al. 2006; Reyes, Valentino et al. 2008). It will be intriguing to see whether these pathways are involved in PPI changes that we observed here.

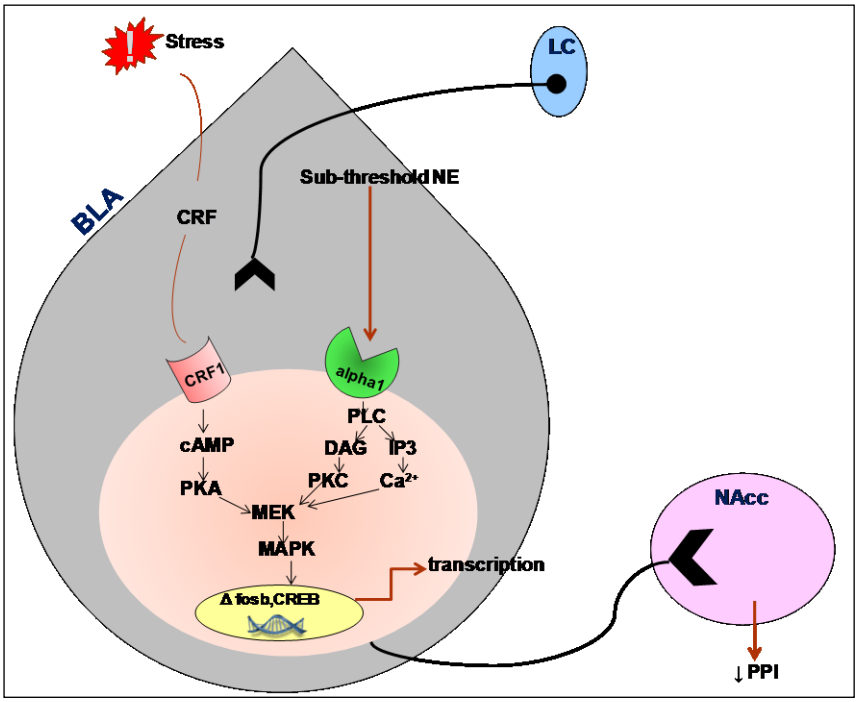

Figure 6: Working model based on our findings and known molecular substrates associated with CRF1 and alpha1 receptors could modulate PPI.

1) NE released from the LC could activate alpha1 receptors in the BLA
2) Given that CRF1 and alpha1 receptors are co-localized, they could act on common molecular substrate such as MAPK
3) MAPK activates transcription factors like Δfosb/CREB
4) Alterations in CRF1/alpha1 receptor functions could alter neuronal properties of BLA output neurons that have been shown to innervate the NAcc to modulate PPI.

Another potential future direction of the studies of this thesis will be to determine the localization of beta receptors in relation to alpha1/CRF1 receptors in the BLA, as previous behavioral studies (chapter 3) show that activation of either alpha1 or beta receptors is sufficient to alter PPI after prior repeated stress exposure. Evidence shows that beta receptors are present on glutamatergic, GABAergic, and glial cells in BLA (Farb, Chang et al. 2010). For this thesis, we focused on alpha1 receptors as the beta receptor antibody was problematic, but once the effectiveness of this receptor antibody is optimized, then the determination of anatomical localization of beta receptors in the BLA in relation to CRF1/alpha1 receptors will help to further refine our working model.

Lastly, future studies will also need to explore the involvement of other systems and their receptors in the BLA, as another possible mechanism that could affect BLA neuronal activity could be through these other systems regulating the neurons on which CRF1/alpha1 receptors are expressed. For instance, one of those systems could be neuropeptide Y (NPY). In this respect it is interesting that CRF and NPY are known to have opposing effects in modulating stress- and anxiety-like responses via the BLA (Sajdyk, Fitz et al. 2006; Giesbrecht, Mackay et al. 2010). While the sub-cellular localization of receptors of other systems like NPY in the BLA remain to be determined, it will be interesting to see what other receptors are expressed on neurons that contain CRF1/alpha1 receptors and whether ligands of these neurotransmitters alter the CRF1-mediated NE receptor hypersensitivity in the BLA. For example, NPY administration with stress presentation might prevent NE receptor hypersensitivity from occurring.

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