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Neuropeptide Y and Cholecystokinin Modulation of the Expression and Extinction of Fear-Potentiated Startle

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By

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B.S., The Pennsylvania State University, 1999

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Abstract

Neuropeptide Y and Cholecystokinin Modulation of the Expression and Extinction of Fear-Potentiated Startle
By Alisa R. Gutman

Neuropeptides are a promising target for novel treatments for anxiety and other psychiatric disorders. Two major candidates are neuropeptide Y (NPY) and cholecystokinin (CCK) and, for this reason, we focus on these peptides and their role in the expression and extinction of conditioned fear. We found that intracerebroventricular (i.c.v.) administration of NPY inhibits both baseline acoustic startle and the expression of fear-potentiated startle. Infusion of NPY (10 pmol/side) into the basolateral, but not the medial, nucleus of the amygdala reproduced the i.c.v. effect. Central administration of NPY (10 μg) also enhanced within-session extinction of fear-potentiated startle. This finding, coupled with the growing body of literature correlating NPY with resilience in humans, led us to the hypothesis that NPY may enhance the extinction of conditioned fear. When NPY (10 μg) is administered i.c.v. prior to extinction training, extinction retention for both the contextual and cued components of conditioned fear is enhanced when tested 48 hours later off drug. Additionally, we found that intra-basolateral amygdala administration of the NPY Y_1 receptor antagonist BIBO 3304 (200 pmol/side) prior to extinction training led to a profound deficit in extinction retention. We believe that the role of NPY in the extinction of conditioned fear may, at least in part, explain the mechanism underlying the association between NPY and psychobiological resilience in humans. Conversely, central infusion of pentagastrin, a CCK_2 receptor agonist, prior to extinction training yields impaired extinction retention. Anatomical studies have shown overlap between the CCK and endocannabinoid systems, and genetic and pharmacological studies indicate that CB1 receptors are involved in extinction. Based on this, we performed a series of experiments assessing interactions between the

endocannabinoid and CCK systems. These studies indicate that both systemic (3 mg/kg) and intra-basolateral amygdala (1 μg) administration of the CCK_2 antagonist CR2945 prior to extinction training reverses the blockade of extinction that we find following i.p. injection of the CB1 receptor antagonist SR141716A (5 mg/kg). Overall, these results suggest that enhancement of the NPY system and blockade of the CCK system may be beneficial for individuals with post-traumatic stress disorder and other anxiety disorders.

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Chapter 1

INTRODUCTION

Fear Conditioning, Startle, and the Amygdala

Fear, perhaps the best understood basic emotion, is an ideal model system from which to garner a deeper understanding of emotional learning and its relationship to psychopathology. Studies employing fear conditioning have the unique potential to glimpse underlying molecular, anatomical, and behavioral systems underlying human psychiatric disorders. In particular, we are interested in disorders that are rooted in dysregulation of fear learning circuits such as post-traumatic stress disorder (PTSD) and other anxiety disorders.

Advances in neuroscience over the last several decades have expanded the field of biological psychiatry and validated the premise that most, if not all, psychiatric diseases are physiologically based and pharmacologically treatable. In the case of anxiety disorders, understanding the neurobiology of fear conditioning and extinction processes has proven to be particularly useful. This strategy has revealed not only some of the mechanisms underlying human psychiatric conditions, but the promise of treatment via manipulation of the fear and extinction learning systems. Still, even with the most recent advances in pharmacotherapy, our current armamentarium remains inadequate. Current research is aimed at a better understanding of underlying pathophysiology and the subsequent identification of alternative treatments for affective disorders.

Fear Conditioning

Pavlovian, or classical, conditioning is accomplished by forming an association between a previously neutral conditioned stimulus (CS), such as a tone or a light, and an unconditioned stimulus (US) that leads to an

unconditioned response (UR). In the case of fear conditioning, the unconditioned response is fear. After an association has been made between the CS and US, presentations of the CS alone will lead to a conditioned response (CR) akin to the original UR. If then, for example, a light is paired with a shock, subsequent presentations of the light will lead to a CR of fear.

While fear conditioning is, by definition, a model for fear, it is also used to study anxiety and anxiety-like behavior in animals. For clarity, we will use the term *fear* when referring to a stimulus specific state (i.e. specific phobia in humans) and the term *anxiety* for a more generalized heightened state of awareness (i.e. generalized anxiety disorder in humans). Here, we define the stages of fear conditioning with the following nomenclature: 1) Processes that occur during training of the animal with presentations of CS and US are referred to as **acquisition** of conditioned fear. 2) Physiological or metabolic changes that occur following training, presumably to create either a short term or long term memory of training, are referred to as **consolidation**. 3) Behavioral or physiological responses to testing of the animal with presentations of the CS are termed the **expression** of conditioned fear.

Fear-Potentiated Startle

In our laboratory, we assay emotional learning using the fear-potentiated startle paradigm. Fear-potentiated startle, originally introduced by Brown and colleagues (Brown et al., 1951), occurs when the amplitude of the startle reflex is modulated by the affective state of the individual via connections between the

amygdala and the startle circuit. Acoustic startle is a relatively simple trisynaptic reflex in which a signal flows from the 1) cochlear root neurons to 2) the nucleus reticularis pontis caudalis (PnC) to 3) the spinal cord (or facial motor nucleus), causing the animal to jump (or blink its eyes) in response to a loud noise (Davis, 1998). Startle is potentiated by a light (CS) when the light and shock (US) are explicitly paired, and not when lights and shocks are presented in an unpaired or random fashion, indicating that prior Pavlovian fear conditioning is essential for the fear-potentiated startle effect to occur (Davis and Astrachan, 1978). When the light is presented repeatedly in the absence of footshock, it no longer increases startle amplitude, indicating that extinction of fear-potentiated startle occurs as for other fear conditioning paradigms (Falls et al., 1992).

There is a direct projection from the central nucleus of the amygdala to the PnC, and lesions along this pathway were found to block the expression of fear-potentiated startle, thereby implicating the central nucleus as the entry point for emotional information into the startle circuit (Hitchcock and Davis, 1991; Rosen et al., 1991). More recently, pharmacological and lesion studies have indicated that an important relay from the amygdala to the deep layers of the superior colliculus/deep mesencephalic nucleus (deep SC/DpMe) to the PnC is essential for the expression of fear-potentiated startle and that this is mediated by non-NMDA glutamategic receptors in the deep SC/DpMe (Frankland and Yeomans, 1995; Meloni and Davis, 1999; Zhao and Davis, 2004).

Fear-potentiated startle has several advantages over other fear conditioning paradigms. First, fear-potentiated startle has a non-zero baseline which enables the observation of both decreases and increases in the level of fear. Additionally, this non-zero baseline provides the potential to distinguish between treatment effects that modify fear vs. motor or other effects on the response measurement. For example, a treatment effect that blocks freezing may either effect fear or simply impede an animal's ability to stand still (Davis, 1998). Furthermore, since the anatomical underpinnings of acoustic startle and fear-potentiated startle have been delineated, it is possible to determine the locus in the brain where observed effects occur. These advantages are best appreciated when compared to other fear behaviors, such as freezing, which have complicated circuitry and a binary output (i.e. freezing/not freezing). Lastly, the automated nature of both eliciting acoustic startle and measuring the amplitude of startle provide the advantage of objectivity, whereas many other behavioral models are administered and scored subjectively. One particularly powerful feature of fear-potentiated startle is that the same basic paradigm can be used with rats, monkeys, and humans, thereby facilitating translational research.

Amygdala anatomy

The amygdala has long been considered a key locus in the circuitry mediating fear and fear learning, as well as an important site for control of stress and anxiety systems (McGaugh et al., 1990; LeDoux, 1993; Davis, 1997). Anatomically, the amygdala can be divided into two major components based on cell type and connectivity: 1) the cortical and basolateral nuclei (BLA, composed

of the basal and lateral amygdala nuclei), characterized by glutamatergic principal neurons that are morphologically similar to neurons of the cerebral cortex, and 2) the central and medial nuclei and the bed nucleus of the stria terminalis (BNST), characterized by GABAergic principal neurons that are anatomically and neurochemically similar to the striatopallidal system (McDonald, 2003).

Visual, auditory, and somatosensory information enter the basolateral amygdala via cortical projection cascades and thalamic input, whereas olfactory information is largely transmitted to the cortical nuclei and undergoes less cortical processing (McDonald, 1998). Information from different sensory modalities is then shared among the cortical and basolateral nuclei via extensive internuclear connections, supporting a role for this portion of the amygdala as an association complex for different sensory stimuli. These anatomical findings support a role for the amygdala in assigning behavioral significance to various sensory input (Pitkanen et al., 1997; McDonald, 2003). Downstream from the BLA are the two major output nuclei, the central nucleus of the amygdala and the BNST, which have parallel projections to a variety of hypothalamic and brain stem effector sites that mediate behavioral and physiological signs of fear and anxiety (Davis, 2000).

Fear Anatomy and Pharmacology

During fear training, neurons of the central nucleus of the amygdala show altered characteristics in response to the CS that correlate with the magnitude of

conditioned changes in heart rate, supporting a role for the amygdala in control of cardiovascular regulation following emotionally relevant stimuli (Pascoe and Kapp, 1985). Fear conditioning, but not unpaired presentations of the CS and US, leads to increased synaptic strength in a thalamic/amygdala pathway and within the lateral amygdala (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997). A great deal is known about the cellular events in the amygdala, particularly regarding synaptic changes in the lateral nucleus following fear conditioning using a tone paired with a shock (Sigurdsson et al., 2007).

As for other measures of fear, the amygdala is integral for both the acquisition and expression of fear-potentiated startle. Post-training electrolytic or ibotenic acid lesions of the central nucleus of the amygdala block the expression of fear-potentiated startle using either a visual or auditory CS (Hitchcock and Davis, 1987; Campeau and Davis, 1995). NMDA induced excitotoxic lesions of the basolateral amygdala cause a complete blockade of fear-potentiated startle when administered either before or after training to a visual (Sananes and Davis, 1992) or auditory CS (Campeau and Davis, 1995). Furthermore, both NMDA and AMPA glutamate receptors have been implicated in the amygdala as essential to the acquisition and expression, respectively, of fear-potentiated startle.

Pretraining pharmacological inactivation of the basolateral amygdala with an NMDA receptor antagonist blocks fear-potentiated startle to a visual (Miserendino et al., 1990), auditory (Campeau et al., 1992), or olfactory cue

(Walker et al., 2005). Pretest infusions of AMPA receptor antagonists into either the central nucleus or the basolateral amygdala completely block the expression of fear-potentiated startle to visual (Kim et al., 1993; Walker and Davis, 1997), auditory (Kim et al., 1993), and olfactory cues (Walker and Davis, 2002; Walker et al., 2005). These results are consistent with findings from other models indicating that the basolateral complex of the amygdala plays a key role in relaying sensory information involving fear conditioning.

Neuropeptides as a Novel Approach to Treatment for Affective Disorders

Neuropeptides appear to be a promising target for novel treatments for anxiety disorders and depression. This has been, perhaps, best demonstrated in the CRF system where clinical trials using the CRF_1 receptor antagonist NBI-30775 have shown efficacy in depressed patients with minimal side effects and no effects on general neuroendocrine measures (Zobel et al., 2000; Kunzel et al., 2003). The evolving CRF story is a fine example of the type of intelligent drug design that the junction between preclinical and clinical neuroscience seeks to deliver, and it highlights the potential importance of neuropeptides as more specific sites for the treatment of psychiatric disorders than are targeted with our current treatments.

Studies have shown that several gut-related peptides have a major role in modulation of anxiety. Two major players are cholecystokinin and neuropeptide Y and, for this reason, we have chosen to focus on these peptides and their role in

the expression and extinction of conditioned fear.

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Chapter 2

The Role of Neuropeptide Y in the Expression of Fear-Potentiated Startle

An evaluation of the expression of baseline and fear-potentiated startle following central and intra-amygdala administration of neuropeptide Y and a Y_1 receptor antagonist

Introduction:

The Neuropeptide Y System

Neuropeptide Y (NPY) is a 36 amino acid peptide isolated in 1982 from porcine brain extracts by Tatemoto and Mutt using the presence of a C-terminal amide (NH_2) group to isolate peptides from brain extracts (Tatemoto et al., 1982). NPY was found to have structural similarities to peptide YY and pancreatic polypeptide, but a novel amino acid sequence, thereby identifying it as a unique neuropeptide (Tatemoto, 1982). Using radioimmunoassay and immunocytochemistry, high concentrations of NPY were observed in human brain tissue at levels exceeding those of cholecystokinin and somatostatin, which had previously been considered the most abundant neuropeptides in the brain. Since its discovery, NPY has been implicated in several systems including feeding (Beck, 2006), circadian rhythms (Yannielli and Harrington, 2001), epilepsy (Baraban, 2004), anxiety (Heilig, 2004), addiction (Thiele et al., 2004), reproduction (Kalra and Kalra, 2004), immune regulation (Groneberg et al., 2004), and neuroprotection (Silva et al., 2005).

High levels of NPY-immunoreactivity are observed in the hypothalamus, nucleus accumbens, septum, and locus coeruleus, and more moderate levels are found in the hippocampus, cerebral cortex, basal ganglia, amygdala, and thalamus (Adrian et al., 1983; Allen et al., 1983; Chronwall et al., 1985). Double labeling studies have shown that almost all cortical NPY-immunoreactive neurons are also positive for GABA and glutamic acid decarboxylase (GAD) and

comprise a subpopulation of cortical GABAergic neurons (Hendry et al., 1984; Demeulemeester et al., 1988; Aoki and Pickel, 1990). NPY is particularly abundant in the hypothalamus; in the adult rat brain, NPY cell bodies in the hypothalamus are largely restricted to the arcuate nucleus (Bai et al., 1985; Chronwall et al., 1985; Grove and Smith, 2003), which reflects its importance in feeding behaviors. Additionally, widespread distribution of NPY-immunoreactive cells have been identified in the amygdala of rat (Chronwall et al., 1985; Gustafson et al., 1986), cat (Marcos et al., 1999), monkey (McDonald et al., 1995), and humans (Walter et al., 1990; Caberlotto et al., 2000).

A significant proportion of GABA containing cells in the basolateral amygdala coexpress one or more neuropeptides. This is true for NPY, which colocalizes with GABA in 87-89% of lateral nucleus cells and 77-83% of basolateral nucleus cells (McDonald and Pearson, 1989). Most GABA-positive and peptide-positive cells in the basolateral amygdala are local circuit neurons (McDonald and Pearson, 1989). Of note, the principle projection neurons of the basolateral amygdala are glutamatergic and NPY likely exerts an inhibitory control on these projection cells. Furthermore, NPY colocalizes with somatostatin in neurons of the rat amygdala, with the greatest number of double labeled cells in the medial nucleus, lateral nucleus, and the intra-amygdaloid portion of the bed nucleus of the stria terminalis. In the basolateral amygdala, there is extensive colocalization of NPY with somatostatin in non-pyramidal medium-sized bitufted and multipolar neurons that appear morphologically similar to non-pyramidal cortical neurons (McDonald, 1989).

NPY Receptors

To date, six classes of G-protein coupled NPY receptor subtypes have been identified and are referred to as NPY Y_1, Y_2, Y_3, Y_4, Y_5 and y_6 receptors (Michel et al., 1998). NPY Y_1 receptor (Y_1R) was originally cloned as an orphan receptor and later identified as having pharmacology indicative of the histologically identified Y_1 receptor (Eva et al., 1990; Krause et al., 1992). NPY Y_1 and Y_2 receptors are the predominant brain receptors, whereas NPY Y_4 receptors preferentially bind pancreatic polypeptide (PP) and have a restricted brain distribution. NPY Y_2 receptors are believed to be an autoreceptor that can decrease the endogenous release of NPY (Chen et al., 1997; Caberlotto et al., 2000). The NPY Y_3 receptor, characterized pharmacologically, has not yet been cloned. The NPY y_6 receptor subtype was first cloned in the mouse, but is not present in the rat and its human analogue is truncated and non-functional (Weinberg et al., 1996; Rose et al., 1997). NPY receptors belong to the G-protein coupled receptors superfamily and generally couple to pertussis toxin-sensitive G-proteins (G_i/G_o), although some responses have been found to be pertussis insensitive (Silva et al., 2002).

Receptor autoradiography evaluating the distribution of [^{125}I][Leu^{31},Pro^{34}]PYY and [^{125}I]PYY_{3-36} binding sites for NPY Y_1 and NPY Y_2 receptors, respectively, indicates different distributions for these receptors in the rat brain. NPY Y_1 receptor binding sites were particularly abundant in the cortex, olfactory tubercle, islands of Calleja, tenia tecta, molecular layer of the dentate gyrus, several thalamic nuclei, and the posterior part of the medial mammaliary

nucleus. NPY Y_2 receptor binding sites were most prevalent in the lateral septum, piriform cortex, triangular septal nucleus, bed nucleus of the stria terminalis, oriens layer and stratum radiatum of the dorsal hippocampus, ventral tegmental area, substantia nigra, dorsal raphe nucleus, and the granular cell part of the cerebellum. Significant amounts of both were found in the anterior olfactory nuclei, oriens layer and stratum radiatum of the ventral hippocampus, nucleus tractus solitarius, area postrema, and inferior olive (Dumont et al., 1996).

Coexpression of all four functional Y-receptor subtypes has been observed with *in situ* hybridization in the limbic system; of particular interest for our studies is the presence of NPY Y_1, Y_2, Y_4, and Y_5 receptor mRNA in several amygdala nuclei including the basolateral amygdala. In contrast, the central amygdala only expresses NPY Y_1 and Y_5 receptor mRNA (Parker and Herzog, 1999). In a study evaluating the comparative distribution of NPY Y_1 and Y_5 receptors, both receptor subtypes were found in the cerebral cortex, hippocampus, hypothalamus, amygdala, and brainstem. Differences in subtype distribution were found within the amygdala, where double-label immunocytochemistry revealed that while only NPY Y_1 receptor-immunoreactivity was observed in the central amygdala, both NPY Y_1- and Y_5-immunoreactive cells and fibers are present in the basolateral amygdala (Wolak et al., 2003). Double-label immunocytochemistry indicates that there is colocalization of NPY Y_1 and Y_5 receptors in the basolateral amygdala (Teppen, 2003).

NPY and Anxiety-Like Behavior

Changes in NPY levels following stress support a role for endogenous NPY in anxiety-like behavior. Following exposure to the conditioned context in a conditioned emotional response paradigm, rats exhibited increased NPY fiber staining in the hippocampus and basolateral amygdala as compared to control animals (Teppen, 2003). Acute restraint stress significantly decreased NPY mRNA expression in the amygdala (Thorsell et al., 1998) and increased NPY expression in the arcuate nucleus and the hilar region of the hippocampus (Conrad and McEwen, 2000). Decreased expression in the amygdala was associated with a modest reduction in NPY peptide levels that returned to normal within 4 hours. Repeated restraint stress (1hr/day for 9-10 days) leads to increased expression of prepro-NPY mRNA and NPY peptide in the amygdala, which the authors suggest may reflect a compensatory mechanism to cope with chronic stress (Thorsell et al., 1999).

Genetic manipulations of NPY further demonstrate that endogenous NPY levels correlate with behavioral measures of anxiety. Transgenic rats overexpressing NPY in the hippocampus exhibited attenuated responses to stress, as measured by the absence of normally anxiogenic effects of restraint stress on the elevated plus-maze (Thorsell et al., 2000). Viral overexpression of NPY in the amygdala reduced anxiety-like behavior in the elevated plus maze as compared with NPY-antisense viral vector (Primeaux et al., 2005). In contrast, NPY knockout mice display a mild anxiogenic phenotype including increased acoustic startle and less center activity in an open field (Bannon et al., 2000). In

addition, NPY Y_2 receptor knockout mice have reduced anxiety in the open field and elevated plus-maze (Redrobe et al., 2003; Tschenett et al., 2003), which is consistent with an autoreceptor function for Y_2 receptors in inhibiting NPY anxiolytic effects under normal conditions.

Central administration of NPY leads to an anxiolytic behavioral profile in several animal models, including conflict tests, social interaction, elevated plus-maze, and fear-potentiated startle. Broqua and colleagues found that several neuropeptides related to NPY, including NPY_{2-36}, [Leu^{31},Pro^{34}]-NPY, and PYY, reduced fear-potentiated startle and increased preference for the open arms of the plus-maze. A Y_2 agonist, NPY_{13-36}, had no effect on fear-potentiated startle or the plus-maze (Broqua et al., 1995). Central infusion of NPY increases punished responding in an operant conflict test, an effect attributed to an anxiolytic-like response (Heilig et al., 1992). Furthermore, the NPY Y_1 receptor agonists [Leu^{31},Pro^{34}]-NPY and [Gly^6, Glu^{26}, Lys^{26}, Pro^{34}]-NPY also increases punished responding in a conflict test, supporting a role for Y_1 in the anxiolytic effect of NPY administration. This effect was not blocked by either the benzodiazepine antagonist flumazenil or the picrotoxin receptor ligand isopropylbicyclophosphate (Britton et al., 1997). These data are supported by electrophysiological evidence that application of both NPY and NPY Y_1 agonists produce effects similar to anxiolytics, such as benzodiazepines, as measured by changes in electroencephalographic activity in cortex and amygdala (Ehlers et al., 1997).

There is evidence that the anxiogenic effects of NPY may be taking place at the level of the amygdala. For example, NPY injection into the amygdala and caudal hippocampus impaired memory retention for footshock avoidance in a T-maze, whereas injection into the rostral hippocampus and septum improved retention (Flood et al., 1989). Infusion of NPY in the amygdala leads to an anxiolytic profile in the elevated plus-maze (Heilig, 1995). Infusion of NPY into the basolateral amygdala increases social interaction time (Sajdyk et al., 1999). In contrast, infusion of a Y_2 receptor agonist into the basolateral amygdala dose-dependently decreased social interaction time and is reversed following intraperitoneal alprazolam, suggesting an anxiogenic effect of Y2 receptor activation in this region (Sajdyk et al., 2002b). Furthermore, administration of NPY and [Leu(31)Pro(34)]-NPY in the basolateral amygdala increased open arm time and entries in the elevated-plus maze (Kokare et al., 2005).

Studies using antisense inhibition and subtype specific NPY receptor antagonists have largely implicated the Y_1 receptor in mediating the anxiolytic effects of NPY. Antisense inhibition of Y_1 blocks the anxiolytic action of NPY on the elevated plus-maze (Heilig, 1995). BIBP3226 (N^2-(diphenylacetyl)-N-[(4-hydroxy-phenyl)methyl]-D-arginine amide), a nonpeptide Y_1 receptor antagonist, displayed an NPY Y_1 receptor specificity in *in vitro* tests, exhibiting a subnanomolar affinity for the human Y_1 receptor (K_i = 0.47 ± 0.07 nM); functional antagonism was exhibited by the ability of BIBP3226 to suppress both NPY induced Ca^{2+} mobilization and NPY mediated inhibition of cAMP synthesis (Wieland et al., 1995). Central administration of BIBP3226 produced a

conditioned place aversion at doses that had no effect on locomotor activity (Kask et al., 1999).

Co-administration of BIBO 3304 blocks the increase in social interaction normally observed following infusion of NPY in the basolateral amygdala (Sajdyk et al., 1999). Bilateral administration of the nonpeptide Y_1 receptor antagonist BIBP3226 into the amygdala results in increased anxiety-related behavior in the elevated plus maze (Primeaux et al., 2005). These data support an important role for Y_1 receptors in the basolateral amygdala in mediating the anxiolytic sequelae of NPY administration.

While a lack of pharmacological agents that can be used in humans has thus far hindered data about the effects of NPY in people, a variety of clinical data has shown an association between NPY levels and affective states. Individuals with a recent suicide attempt had decreased plasma NPY compared to healthy controls (Westrin et al., 1999). NPY concentrations in frontal cortex and caudate nucleus were decreased in the postmortem tissue of suicide victims as compared with age-matched controls and this decrease seemed to correlate with a history of depression (Widdowson et al., 1992). Patients with treatment refractory unipolar depression had a significant reduction in cerebrospinal fluid NPY levels, but not other putative CSF markers such as somatostatin or monoamine metabolites, as compared with volunteers without psychiatric diagnoses. Furthermore, the study found an association between two NPY gene polymorphisms and risk for depression (Heilig et al., 2004).

Of further functional significance is the overlap of NPY with several other systems that are important in anxiety modulation. Using a transgenic model with an NPY Y_1 receptor/LacZ fusion construct, Ferrara and colleagues have shown that chronic treatment with progesterone or allopregnanalone induces increased expression of NPY Y_1 receptor in the medial amygdala (Ferrara et al., 2001). Allopregnanalone, a 5α-reduced metabolite of progesterone, is a potent modulator of $GABA_A$ receptors thereby suggesting a link between $GABA_A$ receptor function and NPY. Another study showed enhanced NPY Y_1 receptor gene expression in the medial amygdala following chronic treatment with benzodiazepine agonists (Oberto et al., 2000), which are positive allosteric modulators of the $GABA_A$ receptor. This has been corroborated in studies of pregnant rats, where the physiological fluctuations in neuroactive steroids during pregnancy were associated with increased NPY Y_1 receptor expression in the medial amygdala (Oberto et al., 2002).

It has long been known that CRF and related peptides are involved in anxiety-like behaviors. Moreover, anatomical and behavioral data support the notion that CRF and NPY play complementary roles in anxiety-related behaviors. In the arcuate nucleus of the hypothalamus, $CRFR_1$ colocalizes with NPY cell bodies (Campbell et al., 2003). Interestingly, the regional distribution of NPY and NPY Y_1 R-immunoreactivity in the septum is similar to that of CRF binding sites, with a high density of both NPY-ir and NPY Y_1 receptor-ir in the dorsocaudal lateral septum (Kask et al., 2001). Behavioral evidence further supports this relationship; NPY injection into the basolateral nucleus of the

amygdala reverses the normally anxiogenic effect of the CRF agonist urocortin I (Sajdyk et al., 2006).

A functional interaction has also been found between NPY and the melanocortin system that may be important for anxiety systems in the amygdala. Anatomical evidence suggests a relationship between NPY and α-melanocyte stimulating hormone, a pro-opiomelanocortin (POMC) derivative. Synaptic contacts have been observed between NPY-immunoreactive nerve terminals and ACTH-immunoreactive neurons in the arcuate nucleus of the hypothalamus, as demonstrated by double immunolabeling (Csiffary et al., 1990). POMC gene expression significantly decreased in the rat arcuate nucleus following i.c.v. infusion of NPY and NPY_{13-36} (Garcia de Yebenes et al., 1995). A recent study demonstrated that intra-amygdala pretreatment with α-MSH blocked the anxiolytic effects of NPY on the elevated plus-maze (Kokare et al., 2005). While an interaction has been known to exist between α-MSH and NPY in feeding, the extension of this functional antagonism to anxiety is exciting and promising for a better understanding of the network of neuropeptides involved in the control of anxiety.

Rationale

Extensive anatomical, behavioral, and clinical evidence implicate the NPY system as promising targets for novel anxiety treatments. Based on the overwhelming evidence that NPY activation is anxiolytic and human literature showing that the presence of NPY is associated with psychobiological resilience

see Chapter 3), we hypothesized that NPY may enhance the extinction of conditioned fear. Because the majority of studies have examined the effects of NPY on anxiety and not fear, we first needed to characterize the effects of NPY on cued fear conditioning before embarking on studies to determine the effects of NPY on extinction. Experiments in Chapter 2 demonstrate that central NPY suppresses the expression of fear-potentiated startle, localize this effect to the basolateral amygdala, and attempt to identify the receptor subtype involved in the response. Thereafter in Chapter 3, we determine that central administration of NPY modulates extinction of conditioned fear, localize this effect to the basolateral amygdala, and implicate the NPY Y_1 receptor in our observed effects.

Materials and Methods:

Animals: The procedures used were approved by the Institutional Animal Care and Use Committee of Emory University and in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Adult male Sprague-Dawley rats (Charles River, Raleigh, NC) weighing between 350 and 500 grams were used. Animals were housed in groups of four in a temperature-controlled (24°C) animal colony, with ad libitum access to food and water. They were maintained on a 12 hr light/dark cycle with lights on at 8:00 A.M, with all behavioral procedures performed during the rats' light cycle.

Surgery: For studies employing intracerebroventricular (i.c.v.) drug administration, 22-gauge stainless-steel guide cannulae (Plastics One, Roanoke, VA) were implanted under ketamine/xylazine anesthesia, and secured using

dental cement and 1/8" cap screws (coordinates: AP:0, ML:-1.6, DV:-5.0; nosebar:+5.0). Animals were allowed 7-10 days recovery before habituation to the testing context and subsequent behavioral testing. Similar procedures were used to implant bilateral cannulae aimed at the basolateral complex of the amygdala (22-gauge guide cannulae, AP: -3.1, ML: +/- 5.4, DV: -8.4 from bregma; nosebar: -3.6) and medial nucleus of the amygdala (22-gauge guide cannulae, AP: -2.76, ML: +/- 3.5, DV: -8.5 from dura). Following behavioral testing, cannulated animals were sacrificed and cannula placement was assessed on cryostat-sectioned tissue. Animals with both cannula correctly placed either i.c.v. or within the amygdala were included for analysis.

Startle Apparatus: Animals were trained and tested in 8 X 15 X 15 cm Plexiglas and wire-mesh cages, with floors consisting of four 6.0-mm-diameter stainless-steel bars spaced 18 mm apart. Each cage was suspended between compression springs within a steel frame and located within a custom-designed 90 X 70 X 70 cm ventilated sound-attenuating chamber. Background noise (60-dB wide-band) was provided by a General Radio Type 1390-B noise generator (Concord, MA) and delivered through high-frequency speakers (Radio Shack Supertweeter; Tandy, Fort Worth, TX) located 5 cm from the front of each cage. Sound level measurements (sound pressure level) were made with a Bruel & Kjaer (Marlborough, MA) model 2235 sound-level meter (A scale; random input) with the microphone (Type 4176) located 7 cm from the center of the speaker (approximating the distance of the rat's ear from the speaker). Startle responses were evoked by 50-msec, 95-dB white-noise bursts generated by a Macintosh G3

computer soundfile (0-22 kHz), amplified by a Radio Shack amplifier (100 W; model MPA-200; Tandy), and delivered through the same speakers used to provide background noise. An accelerometer (model U321AO2; PCB Piezotronics, Depew, NY) affixed to the bottom of each cage produced a voltage output proportional to the velocity of cage movement. This output was amplified (model 483B21; PCB Piezotronics) and digitized on a scale of 0-2500 U by an InstruNET device (model 100B; GW Instruments, Somerville, MA) interfaced to a Macintosh G3 computer. Startle amplitude was defined as the maximal peak-to-peak voltage that occurred during the first 200 msec after onset of the startle-eliciting stimulus. The CS was a 3.7-sec light (82 lux) produced by an 8 W fluorescent bulb (100 μsec rise time) located 10 cm behind each cage. Luminosity was measured using a VWR light meter (Atlanta, GA). The US was a 0.5-sec shock, delivered to the floorbars and produced by a shock generator (SGS-004; Lehigh Valley, Beltsville, MD). Shock intensities (measured as in Cassella et al., 1986) were 0.4 mA. The presentation and sequencing of all stimuli were under the control of the Macintosh G3 computer using custom-designed software (The Experimenter; Glassbeads Inc., Newton, CT).

Drugs: NPY (Bachem Biosciences, King of Prussia, Pennsylvania) and (R)-N-[[4-aminocarbonylaminomethyl)-phenyl]methyl]-N^2-(diphenylacetyl)-argininamide trifluoroacetate (BIBO 3304, given as a generous gift from Dr. Marcus Schindler, Boehringer-Ingelheim, Biberach, Germany) were suspended in artificial cerebrospinal fluid with 1% bovine serum albumin. All infusions were given through microinjection cannulae (28-gauge) connected with PE-20 tubing

to a 10 μL Hamilton syringe. I.c.v. infusions were administered at a flow rate of 1 μL/min with a total injection volume of 5 μL. Intra-amygdala infusions were administered at a flow rate of 0.25 μL/min with a total injection volume of 0.5 μL/side. Microinjection cannulae were left in place for 2-5 min (intra-BLA and i.c.v., respectively) to allow for diffusion away from cannulae to prevent backflow.

Baseline Startle Testing: Animals were placed in the startle chambers for 20 min on each of 2 days prior to training to habituate them to the test procedures and chambers and to minimize the effects of contextual conditioning. Baseline startle testing consisted of a 5 minute habituation period followed by 30 startle stimuli (50-msec, 95-dB white-noise burst).

Fear Conditioning: On 2 consecutive days following baseline testing, rats were returned to the same chambers and presented with 10 pairings of a light (3.7 sec) co-terminating with a 0.4-mA, 0.5-sec shock (4 min variable inter-trial interval).

Matching: Twenty-four hours following the last fear-conditioning session, animals were returned to the same chambers and presented with startle stimuli (50-msec, 95-dB white-noise bursts) in the presence or absence of the light conditioned stimulus [5 light-noise compounds (LN) and 5 noise-alone trials (NA)]. Increased startle in the presence of the light-CS was taken as a measure of conditioned fear, and the magnitude of the fear response was calculated as the percentage by which startle increased when the light-CS was presented in

compound with the startle stimulus versus when it was omitted [% fear-potentiated startle, ((LN-NA)/NA)*100]. Using these measurements, animals were divided into groups displaying equivalent levels of fear-potentiated startle prior to expression testing.

Behavioral Procedures, Experiment 1, The effect of central NPY on baseline startle: Animals (n = 16) were tested for effects of NPY on baseline startle using a within-subjects repeated measures design in which each animal received an i.c.v. infusion of 0, 1, 3, or 10 μg NPY on each of four days in a counterbalanced fashion. Drug was infused 60 min prior to baseline startle testing and statistics among the four drug groups were analyzed using One-Way Repeated Measures ANOVA.

Behavioral Procedures, Experiment 2, The effect of central NPY on the expression of fear-potentiated startle: To evaluate levels of expression of fear-potentiated startle, animals were returned to the chamber and presented with a 46 min test similar to that described for matching, but consisting of a 5 min acclimation period followed by 30 LN and 30 NA trials. Animals (n=11) were infused i.c.v. with 10 μg NPY or vehicle 60 min prior to testing. Interval between infusion and testing and drug dose were based on previous studies (Heilig et al., 1989; Broqua et al., 1995). A within-subjects crossover design was employed in which half the rats were infused with NPY on Day 1 and vehicle on Day 2 and the other half administered drugs in the opposite

pattern with 48 hrs between Day 1 and Day 2. The data were analyzed using a paired t-test.

Behavioral Procedures, Experiment 3, The effect of amygdala administration of NPY on the expression of fear-potentiated startle: To evaluate levels of expression of fear-potentiated startle, animals were returned to the chamber and presented with a 46 min test consisting of a 5 min acclimation period followed by 30 LN and 30 NA trials. We used a within-subjects crossover design, and animals were given 1 day of re-training before their second expression test to reduce the probability that extinction during the Day 1 test session would influence the Day 2 test data. The magnitude of the fear response was calculated as for matching above. Animals were infused bilaterally immediately prior to the expression test with vehicle or 10 pmol NPY/side into either the basolateral (n = 13) or medial amygdala (n = 10). This dose has been used previously and has consistently shown behavioral effects in anxiety paradigms (Sajdyk et al., 1999). Values for % fear-potentiated startle were grouped into blocks of 5 trials each and statistics were performed using a Two-Way Repeated Measures ANOVA with block (1-5) and treatment (vehicle, NPY) as factors.

Behavioral Procedures, Experiment 4, The effect of amygdala administration of an NPY Y1 receptor antagonist on the expression of fear-potentiated startle: For Experiment 4, animals were infused bilaterally with vehicle (n = 20) or 200 pmol/side BIBO 3304 into either the

basolateral (n = 15) or medial (n = 7) amygdala immediately prior to an expression test consisting of a 5 min acclimation period followed by 30 LN and 30 NA trials. Comparisons between groups were performed using Two-Way Repeated Measures ANOVA. The intra-amygdala dose of BIBO 3304 was chosen from previous studies in an anxiety paradigm (Sajdyk et al., 1999; Wieronska et al., 2004).

Results:

Experiment 1, Central NPY activation inhibits baseline startle:

Before preceding with an evaluation of the effects of NPY in our learning paradigms, we generated a dose-response curve (Figure 1) for the effect of i.c.v. administration of 0,1,3, and 10 μg of NPY on baseline startle. One-Way ANOVA Repeated Measures ANOVA identified that NPY administration had a dose-dependent effect on decreasing baseline startle, with an overall effect between treatments ($p < 0.05$, $F_{(3,61)} = 4.09$). Student-Newman-Keuls post-hoc analysis indicated a significantly lower levels of baseline startle between 3 and 10 μg NPY and vehicle ($p < 0.05$) and a trend-level decrease in baseline startle between 1 μg NPY and vehicle ($p = 0.08$).

Experiment 2, Central NPY activation inhibits the expression of fear-potentiated startle:

Administration of NPY i.c.v. inhibited the expression of fear-potentiated startle (Figure 2B), evidenced by a reduction in % fear-potentiated startle (paired t-test, $p < 0.05$). Figure 2A demonstrates that we observed not only a reduction

in fear-potentiated startle, but an overall reduction in startle amplitude as well, with noise-alone and light-noise values both significantly reduced compared to the vehicle condition, as well as a significant reduction in the difference score (LN-NA) between conditions (paired t-test, $p < 0.01$).

Experiment 3, Intra-basolateral amygdala activation of NPY inhibits the expression of fear-potentiated startle:

NPY infusion into the basolateral, and not the medial, nucleus of the amygdala inhibited the expression of fear-potentiated startle (Figure 3A). Two-Way Repeated Measures ANOVA for the basolateral amygdala group identified a significant overall effect of time ($p < 0.001$, $F_{(5,155)} = 8.704$) and a time by treatment interaction ($p < 0.05$, $F_{(5,155)} = 2.897$). Student-Newman-Keuls post hoc analysis revealed a significant difference between NPY and vehicle for block 1 ($p < 0.05$). No effect was found for the medial amygdala group (Figure 3B). NPY had no effect on baseline startle responding in either region (Figure 3C), indicating that the observed effect on expression of fear-potentiated startle is not merely an effect on the startle reflex itself and that the effects of i.c.v. NPY on baseline startle was probably not due to effects on these amygdala nuclei.

Experiment 4, Intra-amygdala antagonism of NPY Y1 receptors has no effect on the expression of fear-potentiated startle:

Two-Way Repeated Measures ANOVA indicated that infusion of the NPY Y_1 receptor antagonist BIBO3304 into the basolateral or medial amygdala immediately prior to testing has no effect of on the expression of fear-potentiated

startle (Figure 4A). There was no observed effect on baseline startle in either region, as measured by NA values during the expression test (Figure 4B).

Discussion:

These data demonstrate that administration of exogenous NPY inhibits the expression of fear-potentiated startle. The blockade of fear-potentiated startle was observed after i.c.v. and intra-BLA, but not intra-MeA, infusion of NPY. Moreover, we observed a reduction in baseline startle following i.c.v. administration of NPY, as demonstrated by decreased startle values during the NA trials, and found that this reduction was dose-dependent. However, this was not seen in the amygdala, at least at the dose that was used, indicating that the effects of NPY on fear potentiated startle were not an artifact of a change in baseline startle.

The reduction in baseline startle with i.c.v. NPY is in contrast to a previous report that NPY did not alter baseline startle levels (Broqua et al., 1995). However, we observed this effect in several different experiments and saw this result reliably in both untrained (Figure 1) and trained (Figure 2A) animals. It is possible that this reduction in baseline startle reflects a reduction in anxiety-related behavior in our paradigm. There is some correlation between changes in baseline startle amplitude and presumed anxiety states in rats. For example, isolation stress increases baseline startle in rats and this was shown to correlate with neuropeptide receptor levels in a presumed social anxiety circuit (Nair et al., 2005). Furthermore, results from human studies demonstrate that

administration of a benzodiazepine anxiolytic drug blocked increases in startle due to contextual fear but had no effect on responding to the cue (Grillon et al., 2006), supporting the notion that increases in baseline startle are anxiety-related in nature. A large body of literature has implicated the bed nucleus of the stria terminalis in anxiety-related behaviors (Davis, 1998; Walker et al., 2003). Future studies will evaluate whether NPY administration into the bed nucleus of the stria terminalis would affect baseline startle.

As mentioned earlier, the basolateral amygdala has been implicated as a key site in the modulation of fear and anxiety behaviors, including the expression of fear-potentiated startle, and the highest concentration of NPY is located in this region (Davis et al., 1997; Sajdyk et al., 2006). Therefore, we predicted that administration of NPY in the basolateral amygdala would reproduce the deficit observed with i.c.v. administration. Here, we used the medial amygdala as a control for regional specificity because it also expresses NPY receptors and peptide (Chronwall et al., 1985; Parker and Herzog, 1999; Kopp et al., 2002). NPY in the medial amygdala has been associated with GABA receptor modulation and neuroactive steroids (Oberto et al., 2000; Ferrara et al., 2001; Oberto et al., 2002).

Our finding that NPY infusion into the basolateral amygdala, but not the medial amygdala, inhibits the expression of fear-potentiated startle is consistent with previous findings and is consistent with the previously defined role for the basolateral amygdala as an important site for NPY signaling in emotional

systems. Electrophysiological data have shown that NPY agonists inhibit glutamate release in the hippocampus (Colmers et al., 1987; Qian et al., 1997). Furthermore, NPY is able to inhibit excitatory transmission in the amygdala (Molosh and Rainnie, 2007, unpublished observations). Moreover, pharmacological studies have shown that the expression fear-potentiated startle is dependent on AMPA glutamate receptors in the basolateral amygdala (Kim et al., 1993; Walker and Davis, 1997; Walker et al., 2005). Therefore, it is likely that NPY is acting to decrease glutamatergic transmission in the basolateral amygdala, thereby suppressing excitatory output from the amygdala and leading to the observed inhibition of fear-potentiated startle.

While we attempted to determine the receptor subtype involved in the reduction of fear-potentiated startle, our data remain inconclusive with regards to which NPY receptor mediates the response. We were not particularly surprised by the lack of effect of the Y_1 receptor antagonist BIBO 3304 on the expression of fear-potentiated startle. In fact, other studies have similarly found no effect of BIBO 3304 alone on baseline behavioral measures. For example, when administered alone at doses of 100 and 200 pmol/side, BIBO 3304 had no significant effect on baseline levels of social interaction (Sajdyk et al., 1999). Since years of research have implicated glutametergic systems as the major player in the expression of conditioned fear, basal tone of NPY in the basolateral amygdala may not be high enough during the expression of fear to observe effects of an antagonist alone. This does not diminish the significance of our findings regarding the effects of NPY on the expression of fear-potentiated startle; it

suggests that while enhanced NPY signaling can inhibit the expression of conditioned fear, endogenous NPY is not *necessary* for normal expression of fear to occur.

Alternatively, it is possible that another NPY receptor subtype is involved in the inhibition of the expression of conditioned fear. Broqua *et al.* reported that while i.c.v. infusion of the Y_1 agonist [Leu^{31},Pro^{34}]-NPY reduced fear-potentiated startle, a Y_2 agonist, NPY_{13-36}, had no effect (Broqua et al., 1995). However, since [Leu^{31},Pro^{34}]-NPY is a ligand at the Y_5 receptors as well as the Y_1 receptor, these results do not exclude the possibility that Y_5 receptors are involved in our effect. Y_5 receptors in the basolateral amygdala have been implicated in anxiety-related behaviors (Sajdyk et al., 2002a) and could underlie the NPY effects observed in these studies. However, findings in Chapter 3 that evaluate BIBO 3304 and extinction of fear-potentiated startle support the former rather than the latter conclusion and suggest that while activation of the Y_1 receptor is not necessary for the reduction of fear-potentiated startle, it is the receptor subtype important for regulation of learning in the basolateral amygdala.

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Figure 1: *Dose-response curve showing that activation of central NPY receptors inhibits baseline startle.* Each animal received an infusion of vehicle, 1, 3, or 10 μg NPY on each of four days in a counterbalanced fashion 60 min prior to baseline startle testing. NPY had a dose-dependent effect on decreasing baseline startle. (overall effect between treatments ($F_{(3,61)} = 4.09$, $p < 0.05$); values shown are the average of all trials; error bars indicate +/- SEM; * denotes $p < 0.05$ compared to vehicle)

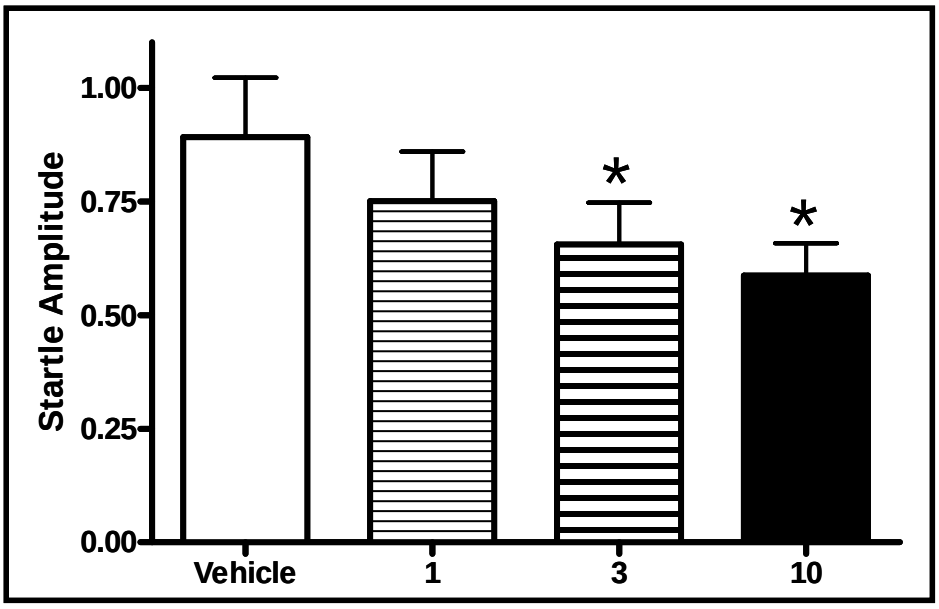

Startle Amplitude
1.00
0.75
0.50
0.25
0.00
*
*
Vehicle
1
3
10

Figure 2: *Central NPY activation inhibits the expression of fear-potentiated startle.* Animals (n=11) were infused i.c.v. with 10 μg NPY or vehicle 60 min prior to testing. **(A)** Following NPY infusion, animals exhibited an overall reduction in startle amplitude with NA and LN values significantly lower than in the vehicle condition. **(B)** NPY administration led to a significant reduction in % fear-potentiated startle during the expression test. (error bars indicate +/- SEM, * denotes $p < 0.05$, ** denotes $p < 0.01$)

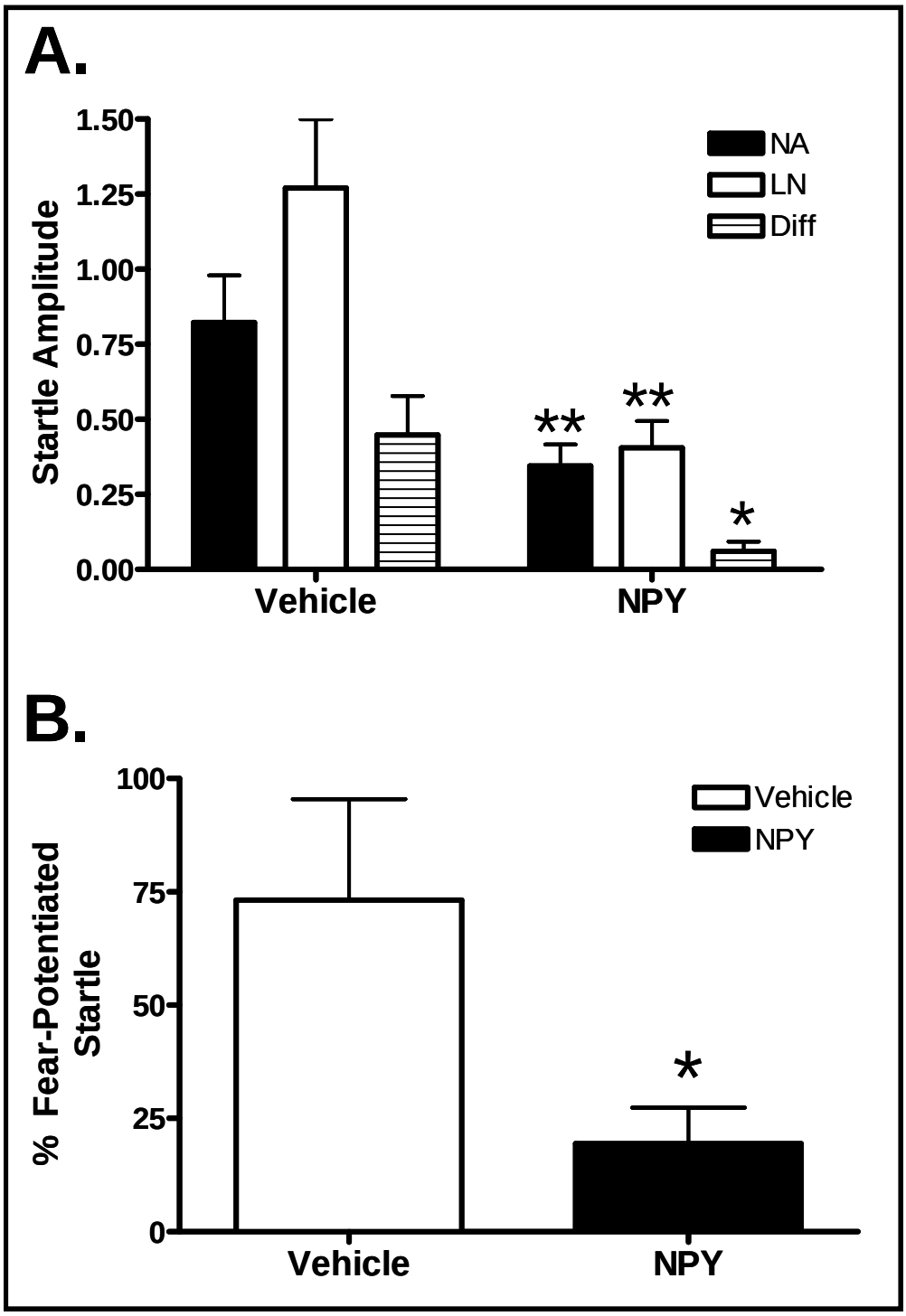

A.
Startle Amplitude
1.50
1.25
1.00
0.75
0.50
0.25
0.00
NA
LN
Diff
**
**
*
Vehicle
NPY
B.
% Fear-Potentiated Startle
100
75
50
25
0
Vehicle
NPY
*
Vehicle
NPY

Figure 3: *Intra-basolateral amygdala activation of NPY inhibits the expression of fear-potentiated startle.* Animals were implanted with bilateral cannulae in the basolateral or medial amygdala 7-10 days prior to behavioral training. Immediately prior to the expression test, animals were infused bilaterally with vehicle or 10 pmol NPY/side. **(A)** Following NPY infusion, animals exhibited a significant decrease in the expression of fear-potentiated startle. There was a significant overall effect of time ($p < 0.001$, $F_{(5,155)} = 8.704$) and a time by treatment interaction ($p < 0.05$, $F_{(5,155)} = 2.897$). (values shown are in blocks of 5 trials; error bars indicate +/- SEM; pairwise comparison identified a difference between groups in block 1, * denotes $p < 0.05$) **(B)** There was no difference between vehicle and NPY conditions following infusion into the medial amygdala. **(C)** NPY had no effect on baseline startle in either region, indicating that the effect shown in **(A)** is not merely an effect on the startle reflex.

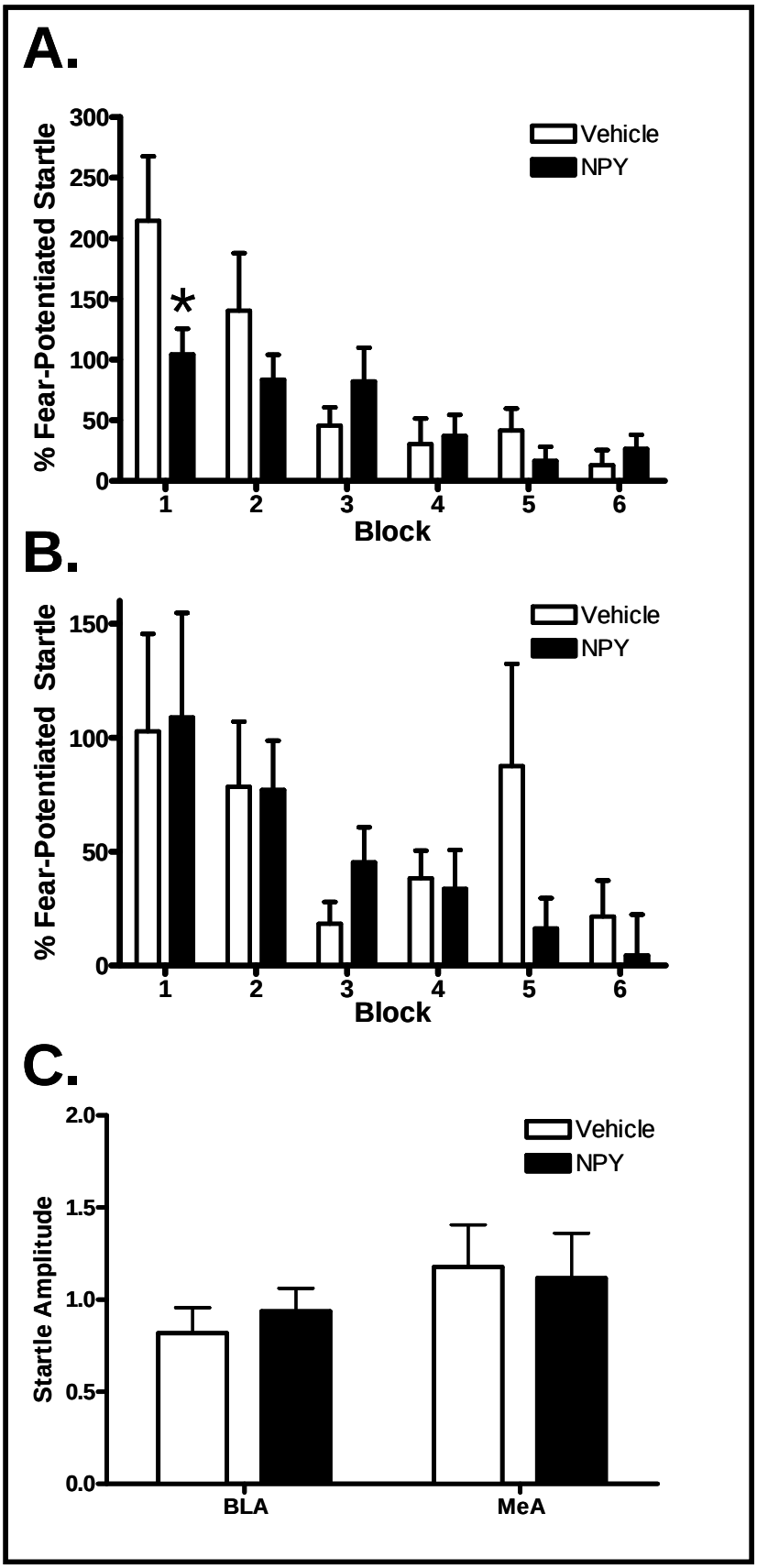
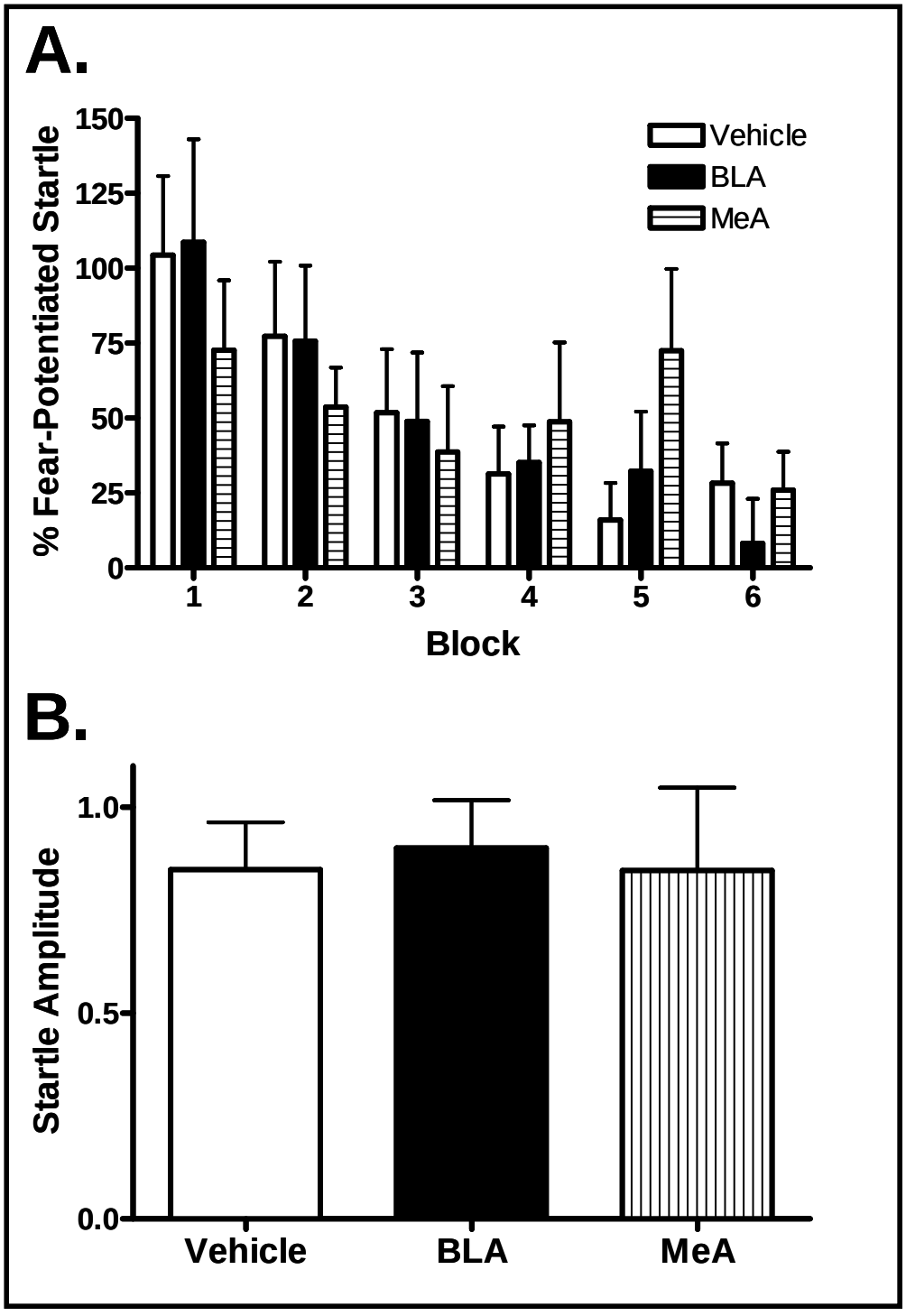

A.
% Fear-Potentiated Startle
300
250
200
150
100
50
0
*
Vehicle
NPY
1
2
3
4
5
6
Block
B.
% Fear-Potentiated Startle
150
100
50
0
Vehicle
NPY
1
2
3
4
5
6
Block
C.
Startle Amplitude
2.0
1.5
1.0
0.5
0.0
Vehicle
NPY
BLA
MeA

Figure 4: *Intra-amygdala antagonism of NPY Y1 receptors has no effect on the expression of fear-potentiated startle:* Animals were implanted with bilateral cannulae in the basolateral or medial amygdala 7-10 days prior to behavioral training. Immediately prior to the expression test, animals were infused bilaterally with vehicle (n = 20) or 200 pmol/side BIBO 3304 into either the basolateral (n = 15) or medial (n = 7) amygdala. **(A)** Intra-basolateral and intra-medial amygdala administration of BIBO 3304 had no effect on the expression of fear-potentiated startle. **(B)** Infusion of BIBO 3304 had no effect on baseline startle in either region.


A.
% Fear-Potentiated Startle
150
125
100
75
50
25
0
1
2
3
4
5
6
Block
Vehicle
BLA
MeA
B.
Startle Amplitude
1.0
0.5
0.0
Vehicle
BLA
MeA

Chapter 3

The Role of NPY in the Extinction of Fear-Potentiated Startle

Characterization of the effects of central administration of NPY and intra-amygdala administration of an NPY Y_1 receptor antagonist on the extinction of fear-potentiated startle

Introduction:

Traditionally, the major focus of psychiatry research has been aimed at understanding 'what goes wrong' in individuals with psychiatric disease. More recently, the field has begun to search in earnest for 'what goes right' in individuals who do not develop psychopathology even when exposed to trauma and other risk factors for illness. More precisely, the zeitgeist of research in psychiatry has expanded to include identification of resilience factors in addition to vulnerability factors for psychiatric morbidity. This concept of psychobiological resilience is not new, but increased interest is helping to generate new treatment strategies for a variety of diseases, including post-traumatic stress disorder (PTSD).

An elegant series of studies comparing Special Forces soldiers with regular army soldiers supports the notion that enhanced levels of NPY are associated with resilience against developing stress-related pathology. Here, the authors used a powerful strategy to ask 'What makes an individual resilient?' by comparing neuropeptide levels during military survival training, an acute uncontrollable stressor, between two army populations (and not compared with healthy non-traumatized controls). Plasma NPY levels increased following exposure to interrogation stress and then dipped below baseline values during prolonged stress in both groups, with a significantly higher increase and a more rapid return to baseline in Special Forces soldiers than non-Special Forces

soldiers, whose NPY values remained decreased 24 hours after trauma exposure (Morgan et al., 2000).

In addition to blood samples, survival instructors assessed an 'interrogation behavior score.' Special forces soldiers were rated with higher mental alertness and are identified by the army as more "stress hardy" than most other soldiers. While these data are only correlational, the higher and more prolonged NPY levels in this behaviorally identified resilient population might indicate that differences in NPY levels can lead to differences in response to stress. Recent work in Rachel Yehuda's laboratory has corroborated this association and further suggests that it is a state phenomena in which high levels of NPY are found following trauma in individuals who do not go on to develop PTSD (Yehuda et al., 2006). As mentioned earlier, NPY fiber staining in the hippocampus and basolateral amygdala was increased when rats were exposed to a fearful context (Teppen, 2003) and we have similarly found an increase in NPY mRNA in the amygdala after fear conditioning (Gutman, Ressler, and Davis, unpublished observations). The association between NPY and resiliency and the fact that NPY seems to be activated by conditioned fear led us to ask the mechanistic question, '*How could increased levels of NPY be protective?*'

Results from Chapter 1 demonstrate that central and intra-BLA infusion of NPY can suppress the expression of learned fear. This has helped to extend the known effects of NPY in the basolateral amygdala to fear systems as well as anxiety-like behaviors. This was an important first step in evaluating the role of

NPY in fear-potentiated startle. We then wanted to address whether NPY might also modulate emotional learning beyond this effect on expression of fear and thereby underly some of the clinical observations that increased NPY is associated with protection from stress-related psychopathology.

A few studies have suggested that NPY may affect learning processes at several different stages. Post-training NPY injection in rats into the amygdala and caudal hippocampus impaired memory retention for footshock avoidance in a T-maze, whereas injection into the rostral hippocampus and septum improved retention (Flood et al., 1989). Furthermore, intra-third cerebroventricular injection of NPY improved consolidation and retrieval in a step-down passive avoidance test (Nakajima et al., 1994). In an NPY Y_2 receptor knockout mouse, deficits were observed in the probe trial of the Morris Water Maze task and in an object recognition test (Redrobe et al., 2004). These results are among the only studies that have evaluated the involvement of NPY in explicit learning, whereas the majority of relevant literature speaks to broader questions about how NPY affects anxiety-related behaviors. The relevant clinical literature coupled with observed effects of NPY on learning led us to the hypothesis that NPY might enhance the extinction of conditioned fear.

Extinction of Conditioned Fear

If the CS is presented repeatedly in the absence of a US, extinction of conditioned fear will occur and lead to a decrement in the CR. In other words, if the light in our example is shown repeatedly in the absence of shock, the light no

longer predicts shock and fear is no longer elicited by the light. For extinction, we distinguish among 1) **extinction training**, the procedure used to produce a decrement in the CR, 2) **extinction retention**, the effect of this procedure on the CR as measured during an expression test, and 3) **extinction**, the theoretical process underlying both of the preceding phenomena (Davis et al., 2003).

Extinction is believed to be context-dependent and mediated by new learning of an inhibitory memory and not by forgetting of the original association. This is supported by several phenomena that can restore the original CR after extinction: 1) **spontaneous recovery**, in which the expression of extinction dissipates over time, 2) **renewal**, in which the CR returns when the animal is tested in a different context than extinction training, and 3) **reinstatement**, in which administration of an unsignaled US (i.e. shock alone trial) leads to return of the CR (Davis et al., 2003).

As with the expression of conditioned fear, extinction of conditioned fear is also dependent on NMDA glutamate receptors in the amygdala. Intra-amygdala infusion of AP5, an NMDA antagonist, prior to extinction training dose-dependently blocked extinction of fear-potentiated startle (Falls et al., 1992). This was replicated with conditioned freezing wherein intra-BLA infusion of AP5 interfered with extinction of conditioned fear to tone, light, and context stimuli (Lee and Kim, 1998). Additional evidence for the role of NMDA in extinction comes with the finding that systemic or amygdala administration of D-cycloserine, a partial agonist at the glycine site on the NMDA receptor, facilitates

the extinction of fear-potentiated startle (Walker et al., 2002) and conditioned freezing (Ledgerwood et al., 2003). While other brain regions, most notably the prefrontal cortex, have been implicated as important sites for extinction learning (Quirk et al., 2006), we focus here on the role of the amygdala in extinction.

Current Approaches to Treatment for Affective Disorders

The finding that D-cycloserine facilitated extinction in rats was extended to human extinction learning by combining exposure therapy for acrophobia with the administration of D-Cycloserine. When D-Cycloserine was administered prior to a virtual reality exposure therapy, subjects experienced a significantly greater reduction in acrophobia symptoms (Ressler et al., 2004). This finding has been further validated by replication in individuals with social anxiety disorder (Hofmann et al., 2006) and obsessive compulsive disorder (Kushner et al., in press). These results are a perfect example of the type of translational research that benefits from the multi-species applicability of fear learning paradigms.

Developing adjuncts for cognitive behavioral therapy can decrease the amount of psychotherapy needed, thereby decreasing overall monetary and time costs for patients and physicians alike. Perhaps the most exciting thing about this type of intervention is that it offers not merely an opportunity for chronic treatment, but a possible cure. This is virtually unheard of with respect to therapeutic intervention for affective disorders.

Accordingly, Chapter 3 addressed the question of whether administration of NPY modulates the extinction of fear-potentiated startle and attempted to determine where and via which receptor these effects take place.

Materials and Methods:

Animals, Surgery, Apparatus, and Drugs were identical to those used in Chapter 2. However, because we had found previously that NPY significantly decreased baseline startle, which can cause interpretive problems when evaluating extinction data, with the exception of determining the effects of NPY on within-session extinction all other tests were performed 48 hours after NPY infusion at a time we knew would be long enough for the drug to no longer effect baseline startle.

Baseline Startle Testing: Animals were placed in the startle chambers for 20 min on each of 2 days prior to training to habituate them to the test procedures and chambers and to minimize the effects of contextual conditioning. Baseline startle testing consisted of a 5 minute habituation period followed by 30 startle stimuli (50-msec, 95-dB white-noise burst).

Fear Conditioning: On 2 consecutive days following baseline testing, rats were returned to the same chambers and presented with 10 pairings of a light (3.7 sec) co-terminating with a 0.4-mA, 0.5-sec shock (3.6-min inter-trial interval).

Pre-Extinction Test: Twenty-four hours following the last fear-conditioning session, animals were returned to the same chambers and presented with startle stimuli (50-msec, 95-dB white-noise bursts) in the presence or absence of the light conditioned stimulus [randomized presentations of light-noise compounds (LN) and noise-alone trials (NA)]. Increased startle in the presence of the light-CS was taken as a measure of conditioned fear, and the magnitude of the fear response was calculated as the percentage by which startle increased when the light-CS was presented in compound with the startle stimulus versus when it was omitted [% fear-potentiated startle, ((LN-NA)/NA)*100]. Using these measurements, animals were divided into groups displaying equivalent levels of fear-potentiated startle prior to extinction training.

Behavioral Procedures, Experiment 1, Effect of i.c.v. NPY on within-session extinction: Animals were returned to the chamber and presented with a 46 min test consisting of a 5 min acclimation period followed by 30 LN and 30 NA trials to evaluate levels of within-session extinction. Animals (n=11) were infused i.c.v. with 10 μg NPY or vehicle 60 min prior to testing. Interval between infusion and testing and drug dose were based on previous studies (Heilig et al., 1989; Broqua et al., 1995). A within-subjects crossover design was employed and drug was allowed to wash out for 48 hours before testing with the other condition. Data were analyzed using a Two-Way Repeated Measures ANOVA with block (5 trials/block, blocks 1-6) and treatment (vehicle, NPY) as factors.

Behavioral Procedures, Experiment 2, Effect of i.c.v. NPY on extinction retention: Five days following the last fear conditioning session, animals were administered 10 μg NPY (n = 10) or vehicle (n = 10) i.c.v. 30 min prior to the presentation of 30 light-CS stimuli (light-alone, LA) in the absence of footshock (3.7-sec light, 30-sec intertrial interval). Relative level of extinction retention was evaluated 48 hours later with a retention test (15 LN and 15 NA). Fear-potentiated startle was calculated as for the pre-extinction test and data were analyzed using an unpaired t-test to compare % fear-potentiated startle and Two-Way Repeated Measures ANOVA to compare NA values in the baseline startle, pre-extinction, and post-extinction tests.

Behavioral Procedures, Experiment 3, Effect of i.c.v. NPY on extinction retention with context shift: Because Experiment 2 showed that NPY facilitated extinction of context conditioning, which made it difficult to evaluate its effects on cued conditioning, animals in this study were fear conditioned in one context (A), as previously described, but then given extinction training in another context (B). Context B consisted of the following elements: 1) sandpaper inserts placed over the floorbars, 2) 4 chains hanging down from the top of the cage, 3) Velcro inserts placed over the side walls, 4) cotton soaked in 100% ethanol below the cage inside of the chamber. Five days following the last fear conditioning session, animals were placed into Context B and administered 10 μg NPY (n = 7) or vehicle (n = 7) i.c.v. 30 min prior to the presentation of 30 LA. Relative level of extinction retention was evaluated 48 hours later in Context B with a retention test (15 LN and 15 NA). Due to often large amounts of within-

session extinction during extinction retention tests, we have found that data from the first block of the extinction retention test best exemplifies levels of between-session extinction. Fear-potentiated startle was calculated as for the pre-extinction test and data were analyzed using unpaired t-test to compare block 1 for the vehicle and NPY groups and paired t-test to compare pre-extinction and post-extinction values for each group.

Behavioral Procedures, Experiment 4, Effect of BIBO 3304 in the amygdala on extinction retention: Five days following the last fear conditioning session, animals were infused bilaterally with vehicle (n = 20) or 200 pmol/side BIBO 3304 into either the basolateral (n = 15) medial (n = 7) amygdala immediately prior to extinction training consisting of 30 LN and 30 NA stimuli in the absence of footshock (30-sec intertrial interval). Animals were then tested 48 hours later with a test (15 LN, 15 NA) to evaluate levels of extinction retention. The intra-amygdala dose of BIBO 3304 used was chosen from previous studies in an anxiety paradigm (Sajdyk et al., 1999; Wieronska et al., 2004). In contrast to previous experiments, in this extinction training session startle stimuli were given 3.2 sec after each light (i.e. a LN trial) to evaluate the effects of drug on the expression and extinction of fear-potentiated startle in the same group of animals (results from the expression data are found in Chapter 2). A prior parametric study comparing the difference between extinction training with light-alone trials and light-noise trials indicated that both paradigms yield similar levels of extinction. It should be noted that we did not observe a difference in noise-alone values between groups when infusing into the amygdala

in previous studies employing infusion of BIBO 3304 into the amygdala, and therefore used our standard context for this experiment. Data were analyzed using One-Way ANOVA to compare % fear-potentiated startle in block 1 for the three groups.

Results:

Experiment 1, Central NPY activation enhances within-session extinction of fear-potentiated startle:

Figure 1 demonstrates a significant enhancement of within-session extinction in the NPY condition as compared to vehicle. Two-Way Repeated Measures ANOVA indicates significant main effects for both treatment ($F_{(1,131)}$ = 7.235, $p < 0.05$) and block ($F_{(5,131)}$ = 5.444, $p < 0.001$), although the interaction was not significant. However, Student-Newman-Keuls post-hoc analysis indicated that fear-potentiated startle in the NPY group is significantly lower than in the vehicle condition at blocks 4 ($p < 0.01$) and 6 ($p < 0.01$).

Experiment 2, Central NPY activation enhances extinction to the contextual component of fear-potentiated startle:

We did not observe an overall effect of NPY on % fear-potentiated startle in our extinction retention test (Figure 2B). However, we did observe an overall decrease in startle amplitude in the NPY group (Two-Way Repeated Measures ANOVA, significant main effect of treatment ($F_{(1,35)}$ = 4.902, $p < 0.05$)) and a significant reduction during noise-alone trials (Figure 2C, $p < 0.05$). The baseline change between the vehicle and NPY groups made a comparison

between % fear difficult to verify, and compelled us to examine this decrease in noise-alone trials further.

In comparing noise-alone startle responses before training (Baseline), after training (Pre-Ext), and after extinction (Post-Ext), Two-Way Repeated Measures ANOVA identified a significant main effect of test session ($F_{(2,59)}$ = 6.545, $p < 0.01$) and Student-Newman-Keuls post hoc analysis revealed an increase in startle response for both groups from Baseline to Pre-Ext ($p < 0.05$), corresponding to contextual fear conditioning (Figure 3B). In the vehicle group, we observed no difference between our Pre-Ext and Post-Ext values; whereas, in the NPY group, there was a reduction in startle response from Pre-Ext to Post-Ext ($p < 0.05$) and no difference between the Baseline and Post-Ext startle amplitude. These data suggest an enhancement of extinction to the contextual component of fear conditioning in our NPY group.

Experiment 3, Central NPY activation enhances the extinction of fear-potentiated startle in an ABB paradigm:

Animals who received NPY prior to extinction training showed significantly lower fear-potentiated startle in the post-extinction test than vehicle controls ($p < 0.05$). Furthermore, there was significant extinction in the NPY group ($p < 0.001$) but not in the vehicle group, as measured by a decrease in % fear-potentiated startle from pre-extinction to post-extinction. Interestingly, there was no longer a significant difference in baseline startle during the extinction retention test in our groups (Figure 4C), indicating that the shift in context was effective at alleviating the confound of contextual conditioning.

Experiment 4, Intra-basolateral amygdala antagonism of NPY Y1 receptors blocks the extinction of fear-potentiated startle:

In comparing levels of % fear-potentiated startle among groups receiving vehicle, BIBO 3304 into the basolateral amygdala, and BIBO 3304 into the medial amygdala prior to extinction training, One-Way ANOVA identified a significant main effect of treatment (Figure 5, $F_{(2,40)} = 3.281$, $p < 0.05$). Student-Newman-Keuls post hoc analysis indicated that animals who received BIBO3304 into the basolateral amygdala showed significantly greater fear-potentiated startle during the extinction retention test as compared to vehicle ($p < 0.05$). No effect was observed following BIBO3304 infusion into the medial amygdala. Results from Chapter 2 indicated that BIBO 3304 had no effect on baseline startle in either region.

Discussion:

Literature from human studies showing a role for NPY in resilience is largely correlative. We suggest that an important aspect of resilience may be the maintenance of intact extinction learning. If application of exogenous NPY could enhance the rate or magnitude of extinction learning, or if blockade of NPY could block the rate or magnitude of extinction learning, then this would be a plausible mechanisms underlying NPY's role in resiliency. We found that i.c.v. administration of NPY enhanced the rate of within-session extinction and extinction retention for both contextual and cued aspects of conditioned fear. Furthermore, we demonstrated that antagonism of NPY Y_1 receptors in the

basolateral amygdala results in decreased extinction retention, suggesting that endogenous NPY is important under normal conditions for the acquisition of extinction.

Enhanced retention of extinction measured by lower levels of fear-potentiated startle could conceivably be explained by a lingering effect of NPY at test. However, we have observed in several other experiments, including the baseline startle experiment described in Chapter 2, that any effects of NPY on baseline startle are gone by 48 hours after infusion, if not much sooner. Most extinction studies are carried out using freezing as the behavioral output measure. For extinction studies assayed with freezing, high levels of contextual fear in the fear conditioned context necessitates the use of an ABB extinction protocol in which the context for the acquisition and expression of extinction (Context B) must differ from that used in the initial fear conditioning (Context A). In contrast, for fear-potentiated startle, we generally do not see much context conditioning, possibly because of pre-exposure to the apparatus and a very salient light. Thus, we typically use the same context for fear conditioning and extinction training and testing (AAA protocol). However, the results in Experiment 2 indicated considerable context conditioning and suggested that NPY was facilitating its extinction, thereby masking measurement of its possible effect on cue extinction.

While the enhancement of extinction to contextual fear was interesting in and of itself, we wanted to determine whether or not central administration of

NPY could also enhance extinction of cued fear. For this reason, we repeated the previous experiment but altered our testing context for extinction training and post-extinction testing. We no longer saw a difference in noise-alone startle amplitude between our NPY and vehicle groups in the Post-Ext test. We interpreted this to indicate that our modified paradigm had effectively removed the confounding effects of contextual conditioning, and were therefore able to compare % fear in our groups since we no longer had differential shifts in our baseline startle values. Figure 3 shows that the NPY group had significantly lower % fear-potentiated startle in the extinction retention test and we therefore conclude that exogenous administration of NPY can enhance both within-session and between-session extinction of fear-potentiated startle.

Significantly higher levels of % fear-potentiated startle during the extinction retention test were found following infusion of the NPY Y_1 receptor antagonist BIBO 3304 into the basolateral amygdala. This demonstrates that blockade of NPY interferes with normal extinction and therefore suggests that under normal conditions endogenous NPY Y_1 receptors are activated during the extinction of conditioned fear. BIBO 3304 is a highly selective antagonist for the Y_1 receptor with subnanomolar affinity for both the human and rat receptors (IC_{50} values 0.38 ± 0.06 nM and 0.72 ± 0.42 nM, respectively) and shows minimal activity in more than 75 different receptor binding and enzyme systems, including 1,000-10,000 fold lower affinity for the NPY Y_2, Y_4, and Y_5 receptors (IC_{50} values > 1000 nM) (Wieland et al., 1998). Antagonist studies provide a more direct approach for evaluating how endogenous systems function. These

results provide evidence that endogenous NPY is important for the extinction of fear-potentiated startle. Remarkably, results from Chapter 2 showed that BIBO 3304 had no effect on baseline startle or expression of fear-potentiated startle when infused into the basolateral amygdala. This supports the notion that the antagonist was modulating learning processes in the basolateral amygdala and our observed effects on extinction retention are not due to any lingering effects of the drug itself.

As mentioned earlier, extinction of conditioned fear is dependent on NMDA glutamate receptors in the amygdala. GABAergic interneurons mediate inhibition of glutamatergic excitatory transmission in the basolateral amygdala (Rainnie et al., 1991). Studies evaluating the electrophysiological effects of NPY in the arcuate nucleus indicate that NPY can inhibit GABAergic transmission (Acuna-Goycolea et al., 2005). It is thereby possible that NPY could be inhibiting a subpopulation of interneurons in the basolateral amygdala, and thereby disinhibiting the glutamatergic neurons responsible for extinction.

We have previously shown that infusion of NPY both centrally and in the basolateral amygdala inhibits the expression of fear-potentiated startle. However, while modulation of fear-potentiated startle is often relevant to anxiety, these results more specifically identify changes in processes that underly fear learning. While a few studies had begun to establish a role for NPY in learning and memory, there is a paucity of recent literature on the subject and we feel that these studies fill this gap and begin to examine how NPY's previously established

roles in both learning and anxiety paradigms may come together to help explain how NPY acts in humans to enhance resiliency against stress-related pathology.

Manipulation of extinction processes has already shown efficacy in human psychotherapy studies. Our laboratory demonstrated that administration of the partial NMDA agonist D-Cycloserine prior to extinction training can facilitate the acquisition of extinction learning in rats (Walker et al., 2002). This finding was extended to human extinction learning by combining exposure therapy for acrophobia with the administration of D-Cycloserine. When D-Cycloserine was administered prior to a virtual reality exposure therapy, subjects experienced a significantly greater reduction in acrophobia symptoms (Ressler et al., 2004). This finding has been further validated by replication in individuals with social anxiety disorder (Hofmann et al., 2006).

Based on the studies described here, we believe that enhancement of NPY Y_1 receptor activation could be a potential adjunct for extinction-based psychotherapy, such as exposure therapy. Currently, the lack of available nonpeptide systemic NPY Y_1 receptor agonists prohibits the direct testing of this approach clinically. However, an alternative strategy would take advantage of the presynaptic autoreceptor function of NPY Y_2 receptors, which have been found to inhibit transmitter release (Michel et al., 1998). Administration of antagonists to the NPY Y_2 autoreceptor could lead to an endogenous enhancement of NPY Y_1 receptor activation, and thereby produce the desired enhancement of extinction. Further studies are necessary to evaluate the merit of this approach.

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Figure 1: *Central NPY activation enhances within-session extinction of fear-potentiated startle.* **(A)** Implantation of i.c.v. cannulae was performed 7-10 days prior to behavioral training. Animals (n=11) were infused i.c.v. with 10 μg NPY or vehicle 60 min prior to testing. **(B)** Fear-potentiated startle is lower in the NPY condition, especially during the second half of the test session, indicating increased within-session extinction. (values shown are blocks of 5 trials; significant main effect for both treatment ($F_{(1,131)} = 7.235$, $p < 0.05$) and block ($F_{(5,131)} = 5.444$, $p < 0.001$); post-hoc analysis identified significant difference between vehicle and NPY at blocks 4 and 6; error bars indicate +/- SEM,** denotes $p < 0.01$)

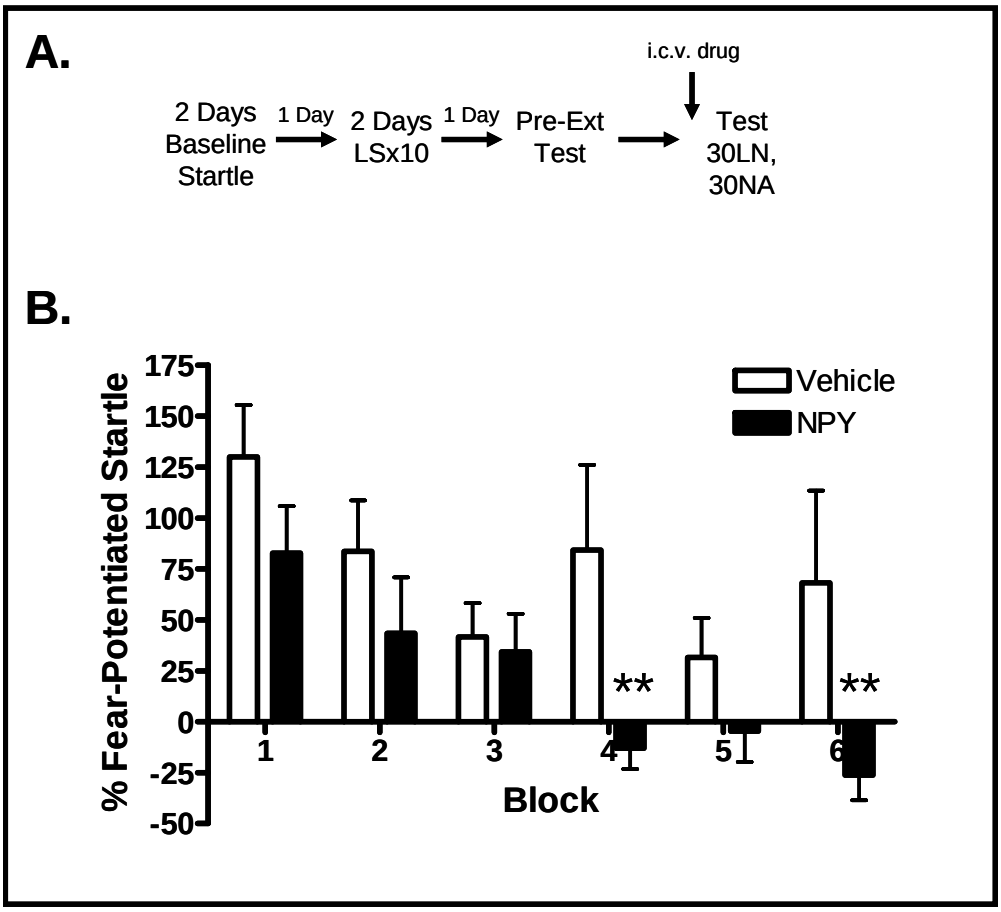

A.
i.c.v. drug
2 Days Baseline Startle
1 Day
2 Days LSx10
1 Day
Pre-Ext Test
Test 30LN, 30NA
B.
% Fear-Potentiated Startle
175
150
125
100
75
50
25
0
-25
-50
Vehicle
NPY
**
**
1
2
3
4
5
6
Block

Figure 2: *Central administration of NPY prior to extinction training decreased overall startle amplitude but not % fear-potentiated startle.* **(A)** Animals were implanted with i.c.v. cannulae 7-10 days prior to behavioral training. 30 min prior to extinction training, vehicle or 10 μg NPY was infused. Animals were tested 48 hours later with no drug onboard (Post-Ext). **(B)** There was no significant difference in % fear-potentiated startle between the vehicle and NPY groups. (values shown represent average of first five trials of test) **(C)** We observed an overall decrease in startle amplitude and a significant decrease in NA startle values in the NPY group during the Post-Ext test. (significant main effect of treatment ($F_{(1,35)} = 4.902$, $p < 0.05$); error bars indicate +/- SEM; * denotes $p < 0.05$)

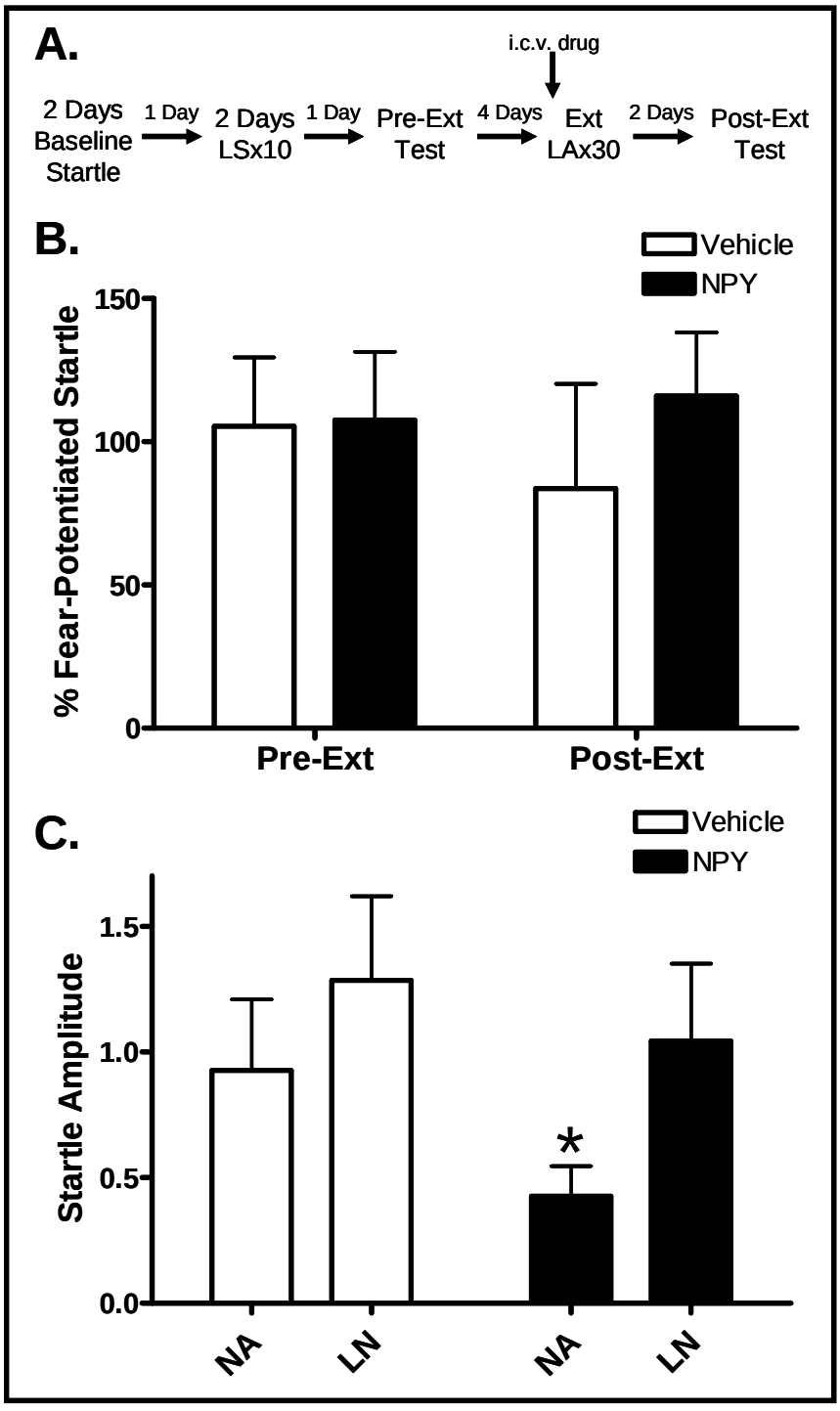

A.
i.c.v. drug
2 Days Baseline Startle
1 Day
2 Days LSx10
1 Day
Pre-Ext Test
4 Days
Ext LAx30
2 Days
Post-Ext Test
B.
Vehicle
NPY
% Fear-Potentiated Startle
150
100
50
0
Pre-Ext
Post-Ext
C.
Vehicle
NPY
Startle Amplitude
1.5
1.0
0.5
0.0
*
NA
LN
NA
LN

Figure 3: *Central administration of NPY enhances the extinction to the contextual component of fear-potentiated startle.* **(A)** A comparison of noise-alone startle was made among values from three days of the behavioral procedure shown here: 1) Baseline (startle amplitude measured prior to fear conditioning, 2) Pre-Ext Test (startle amplitude measured after fear conditioning, prior to extinction training), 3) Post-Ext Test (startle amplitude measured after extinction training). **(B)** In the vehicle group, startle response increases from Baseline to Pre-Ext and then remains constant, corresponding to the development and maintenance of contextual fear. However, in the NPY group startle response increases from Baseline to Pre-Ext and then decreases to levels comparable to Baseline during the Post-Ext test. This pattern corresponds to extinction of contextual fear. (significant main effect of test session ($F_{(2,59)} = 6.545$, $p < 0.01$); error bars indicate +/- SEM; * denotes $p < 0.05$)

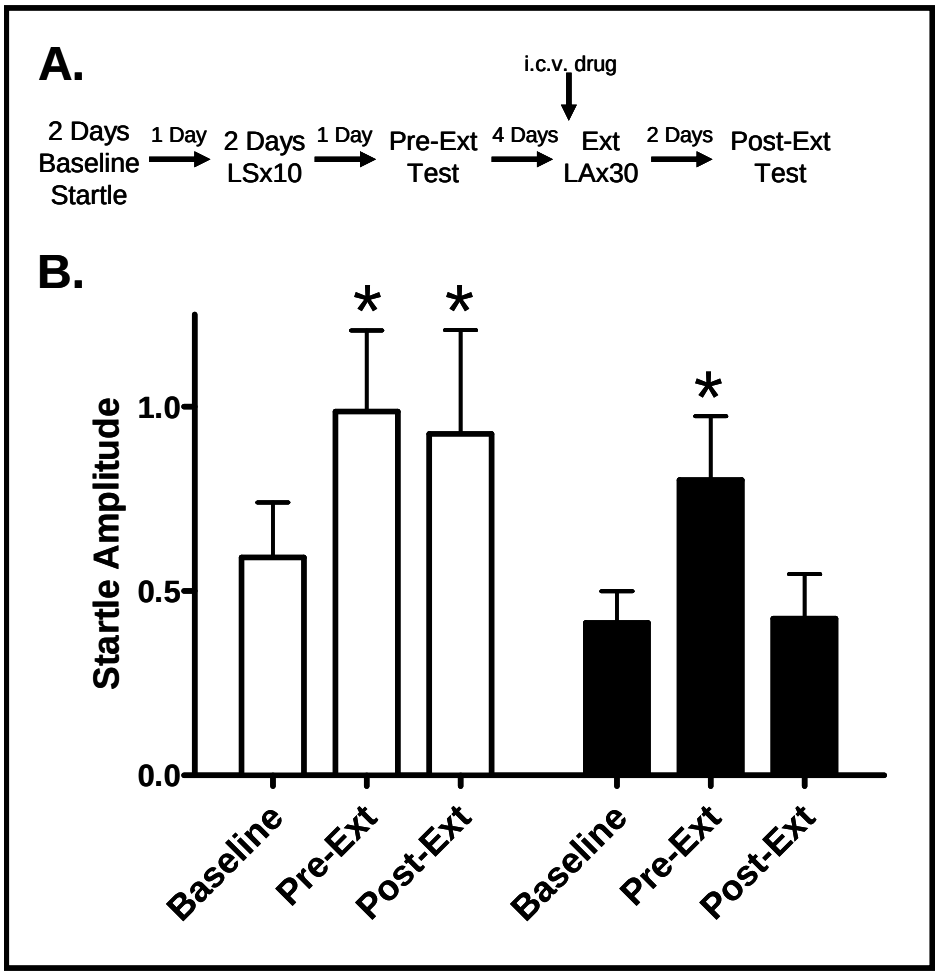

A.
i.c.v. drug
2 Days Baseline Startle
1 Day
2 Days LSx10
1 Day
Pre-Ext Test
4 Days
Ext LAx30
2 Days
Post-Ext Test
B.
Startle Amplitude
1.0
0.5
0.0
*
*
*
Baseline
Pre-Ext
Post-Ext
Baseline
Pre-Ext
Post-Ext

Figure 4: *Central administration of NPY enhances the extinction of fear-potentiated startle.* **(A)** Animals were implanted with i.c.v. cannulae 7-10 days prior to behavioral testing. Animals were baseline tested, trained, and matched with a pre-extinction test (Pre-Ext) in our standard context. The context was altered for extinction training and testing to decrease the possible confounding effects of contextual conditioning. Prior to extinction training (30 min), animals were infused with either vehicle or 10 μg NPY. Animals were tested for extinction retention (Post-Ext) 48 hours later off drug. **(B)** Animals infused with NPY had significantly lower % fear-potentiated startle during the Post-Ext test than vehicle controls. Additionally, the NPY group shows a significant reduction in % fear-potentiated startle from Pre-Ext to Post-Ext, while there is no significant change in the vehicle group. (error bars indicate +/- SEM; ** denotes $p < 0.05$ compared to vehicle Post-Ext, $p < 0.001$ compared to NPY Pre-Ext) **(C)** There was no difference in overall startle amplitude or baseline startle between groups.

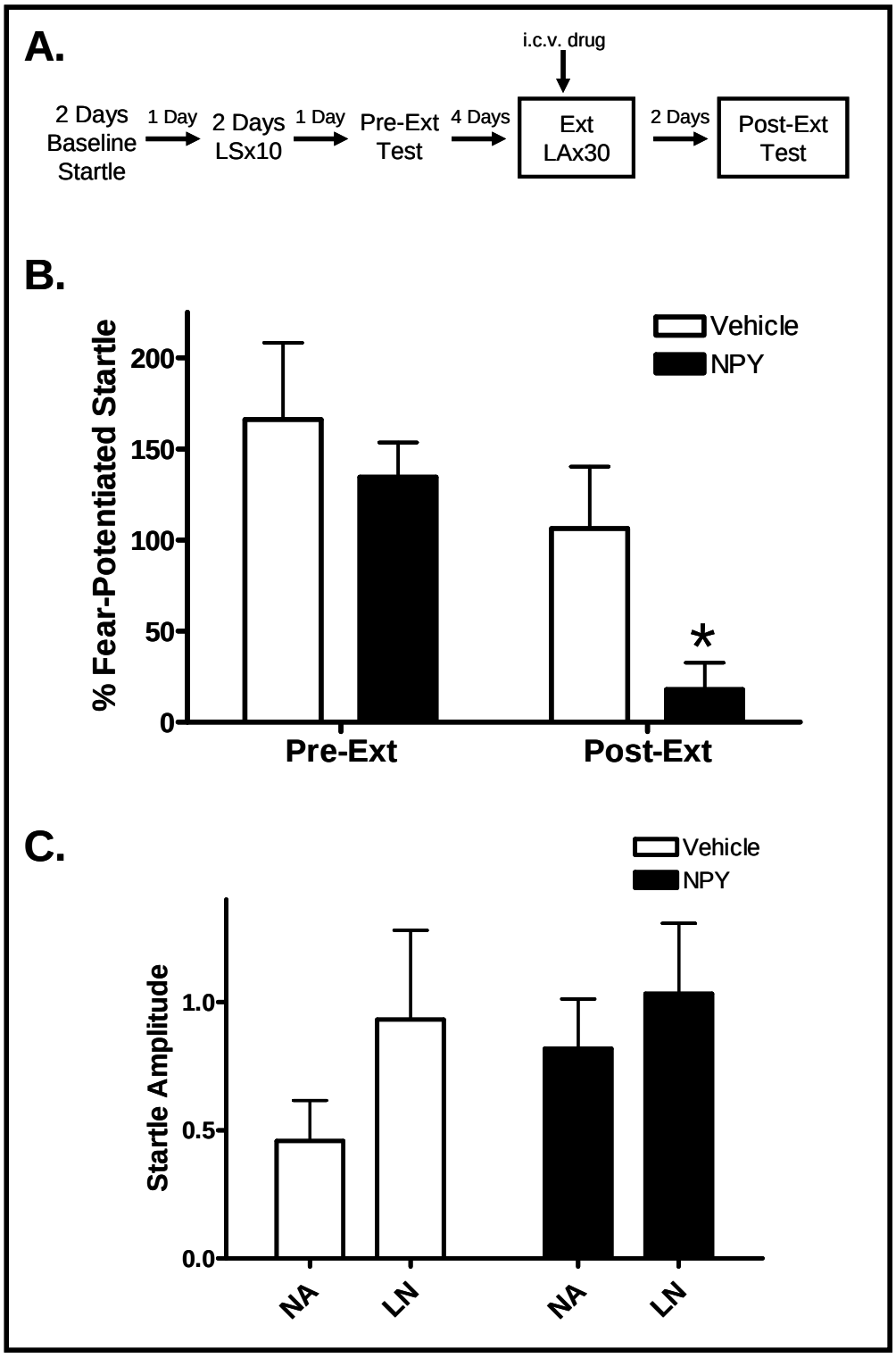
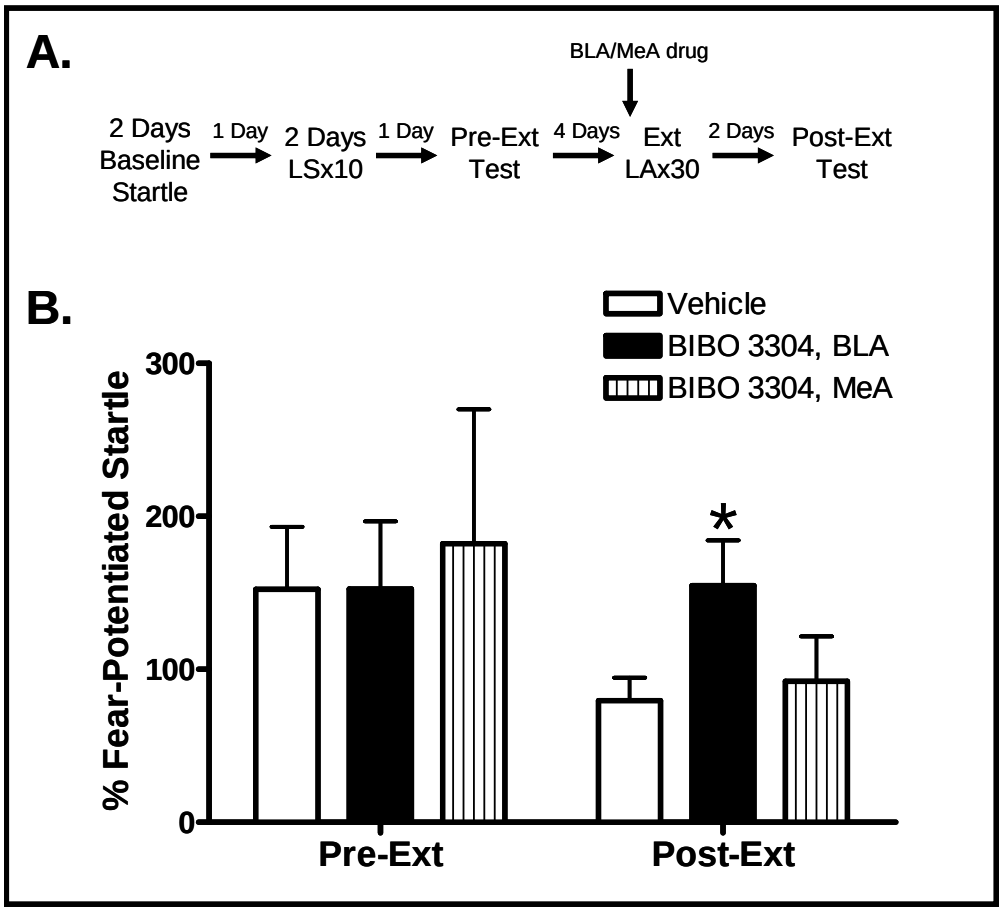

A.
i.c.v. drug
2 Days Baseline Startle
1 Day
2 Days LSx10
1 Day
Pre-Ext Test
4 Days
Ext LAx30
2 Days
Post-Ext Test
B.
% Fear-Potentiated Startle
200
150
100
50
0
Vehicle
NPY
*
Pre-Ext
Post-Ext
C.
Startle Amplitude
1.0
0.5
0.0
Vehicle
NPY
NA
LN
NA
LN

Figure 5: *Infusion of the NPY Y_1 receptor antagonist BIBO 3304 in the basolateral amygdala blocks the extinction of fear-potentiated startle.* **(A)** Animals were implanted with bilateral cannulae in the basolateral or medial amygdala 7-10 days prior to behavioral training. Immediately prior to extinction training, animals were infused with either vehicle (n=20) or 200 pmol/side BIBO 3304 into either the basolateral (n=15) or medial (n=7) amygdala. Animals were tested off drug 48 hours later to assess extinction retention (Post-Ext). **(B)** Animals who received BIBO 3304 into the basolateral amygdala had significantly greater fear-potentiated startle during Post-Ext than vehicle controls. No difference was found for animals who received intra-medial amygdala BIBO 3304. (significant main effect of treatment ($F_{(2,40)} = 3.281$, $p < 0.05$); error bars indicate +/- SEM; * denotes $p < 0.05$ compared to vehicle)


A.
BLA/MeA drug
2 Days Baseline Startle
1 Day
2 Days LSx10
1 Day
Pre-Ext Test
4 Days
Ext LAx30
2 Days
Post-Ext Test
B.
Vehicle
BIBO 3304, BLA
BIBO 3304, MeA
% Fear-Potentiated Startle
300
200
100
0
*
Pre-Ext
Post-Ext

Chapter 4

The Role of Cholecystokinin in the Extinction of Fear-Potentiated Startle

Analysis of the effects of central administration of a CCK_2 receptor agonist and systemic and intra-amygdala administration of a CCK_2 receptor antagonist on the extinction of fear-potentiated startle and the interaction between the cholecystokinin and endocannabinoid systems

Introduction:

The Cholecystokinin System

While not as consistent as the correlation between NPY and anxiolysis, a large body of literature has implicated cholecystokinin (CCK) as an anxiogenic compound. Indeed, a series of pharmacological studies evaluating the behavioral effects of CCK agonists and antagonists have demonstrated that activation of CCK is associated with anxiety-like behavior in a variety of animal models. As with NPY, CCK is similarly abundant and widely distributed in the mammalian brain. CCK was originally identified as a 33 amino acid gut peptide (Ivy and Janecek, 1959), and was later found to be one of the most abundant neuropeptides in the brain (Vanderhaeghen et al., 1975). Since its initial discovery, CCK has been found to exist in several endogenous forms that are cleaved from a 115 amino acid precursor (Deschenes et al., 1984), the most prevalent brain form being the sulphated octapeptide (CCK-8S) (Rehfeld and Hansen, 1986).

Early immunocytochemistry and radioimmunochemistry showed that CCK is particularly abundant in the cortex, hippocampus, amygdala, and hypothalamus (Larsson and Rehfeld, 1979). CCK neurons in the rat basolateral amygdala can be subdivided into two populations of nonpyramidal interneurons based on morphological characteristics; these have been designated type L (large somata, thick dendrites) and type S neurons (small somata, thin dendrites). Double label immunofluorescence indicates that 30-40% of type L interneurons exhibit calbindin immunoreactivity, whereas type S interneurons colocalize with

calretinin and vasoactive intestinal polypeptide (Mascagni and McDonald, 2003). Of particular significance to our studies is the finding that cannabinoid type 1 receptors (CB1) are highly enriched on CCK-positive GABAergic interneurons in the adult mouse forebrain (Marsicano and Lutz, 1999).

CCK Receptors

Two CCK receptor subtypes have been identified, originally identified as CCK-A (alimentary) and CCK-B (brain), but re-designated as CCK_1 and CCK_2 receptors, respectively (Noble et al., 1999). CCK_2 receptors were first identified in the brain (Innis and Snyder, 1980), but further anatomical studies have revealed that both receptors are widely distributed in the central nervous system. CCK_1 and CCK_2 receptors belong to the G-protein coupled receptor superfamily. CCK_1 receptor activation has been shown to stimulate phospholipase C, phospholipase A_2/arachidonic acid pathways, and adenylyl cyclase. These varied effects suggest that the CCK_1 receptor is capable of coupling to both G_q and G_s. While the signal transduction pathways associated with CCK_2 receptor activation is less understood, evidence suggests that they also couple to a pertussis toxin-insensitive G protein (G_q). Furthermore, both receptors have also been shown to couple to mitogen-activated protein kinase pathways (MAPK) and subsequent expression of transcription factors (i.e. c-myc, c-jun, c-fos) (Noble et al., 1999).

Receptor mRNA for both CCK_1 and CCK_2 were expressed in cerebral cortex, olfactory regions, hippocampus, septum, and interpeduncular nucleus. Several hypothalamic nuclei expressed only CCK_1 mRNA, including the arcuate

nucleus and paraventricular nucleus. Expression of CCK_2 mRNA was observed in the striatum and nucleus accumbens. Importantly, most amygdala subnuclei expressed only CCK_2 mRNA, including the basolateral and cortical nuclei as well as the bed nucleus of the stria terminalis (Honda et al., 1993; Jagerschmidt et al., 1994). Autoradiographical data from receptor binding studies is relatively consistent with the *in* situ hybridization studies with substantial receptor binding in cortex, olfactory regions, basal ganglia, hippocampus, and amygdala (Gaudreau et al., 1983; Zarbin et al., 1983). Most immunohistochemical studies are similarly consistent with the mRNA data; however, some studies with different antiserum have suggested a wider distribution of CCK_1 receptor-like immunoreactivity (Mercer and Beart, 1997).

Behavioral Effects of CCK

Following an early study suggesting that CCK is anxiogenic in rats (Csonka et al., 1988), a series of animal studies demonstrated that CCK peptide administration is anxiogenic and CCK receptor antagonists are anxiolytic (Harro et al., 1990c; Harro et al., 1990a; Harro et al., 1990b; Hughes et al., 1990). The introduction of subtype selective receptor antagonists led to the conclusion that anxiogenic effects of CCK were the result of CCK_2 receptor activation (Hughes et al., 1990; Harro and Vasar, 1991; Singh et al., 1991). It is important to note that while the behavioral and clinical data indicating that CCK_2 agonist-induced anxiety can be reduced with CCK_2 antagonists, results showing the effects of CCK_2 antagonists alone remain controversial. While some studies indicate that CCK_2 antagonists can be anxiolytic, other studies show no effect (Harro, 2006).

CCK has also been shown to have effects on acoustic startle. Central infusion of pentagastrin, a CCK_2 receptor agonist, dose-dependently increased baseline startle in rats (Frankland et al., 1996). This effect was localized to the amygdala, where infusions of pentagastrin reproduced the potentiation originally observed with central infusions and could not be attributed to an overall effect on locomotor activity. Informatively, intra-amygdala infusion of a CCK_2 receptor antagonist blocked the effects of i.c.v. pentagastrin, suggesting that amygdala CCK receptors are an important site of action for central CCK effects (Frankland et al., 1997).

Intravenous administration of cholecystokinin-4 peptide (CCK-4), a CCK_2 receptor agonist, produced panic-like attacks in healthy volunteers and this effect was blocked by pretreatment with the benzodiazepine lorazepam, suggesting an anxiety related mechanism (de Montigny, 1989). Pentagastrin has similarly been found to have anxiogenic properties when administered to humans. In a structured social interaction task, intravenous pentagastrin dose-dependently increased blood pressure, pulse, cortisol, ACTH, and physical symptoms of panic (McCann et al., 1994). Pentagastrin has been found to precipitate panic-type symptoms, including increased anxiety, heart rate, and blood pressure, in not only social phobics and panic disorder patients, but also healthy controls, although to a lesser extent (Abelson and Nesse, 1994; McCann et al., 1997).

Clinical evidence has further corroborated a role for CCK in anxiogenesis with increased CCK receptor binding in frontal and cingulate cortex of suicide victims (Harro 1992) and decreased CCK in the CSF of panic disorder patients (Lydiard 1992). Recently, a CCK_2 receptor gene polymorphism has been associated with panic disorder (Hosing 2004) and CCK-related genes have been implicated as contributing to a higher risk for anxiety disorders (Maron et al 2005).

CCK and the Engodenous Cannabinoid System

As mentioned above, anatomical evidence has suggested an interaction between CCK and the endogenous cannabinoid system, namely the presence of CB1 receptors on the pre-synaptic terminals of GABAergic interneurons coexpressing CCK (Katona et al., 1999; Marsicano and Lutz, 1999; McDonald and Mascagni, 2001). Activation of CB1 receptors leads to decreased excitability of the pre-synaptic terminal by enhancing inwardly-rectifying potassium currents, decreasing calcium influx, and inhibiting adenylyl cyclase activity (Pertwee, 1997; Schlicker and Kathmann, 2001). From this anatomical and molecular evidence, it follows that CB1 receptor activation would lead to decreased GABA and/or CCK release from CCK+/CB1+ interneurons, and several electrophysiological studies support this notion (Katona et al., 1999; Beinfeld and Connolly, 2001; Burdyga et al., 2004; Fride, 2005).

Among the most clinically relevant findings regarding the endocannabinoid system is that CB1 receptor knockout mice or mice or rats

administered CB1 receptor antagonists show deficits in the extinction of conditioned fear (Marsicano et al., 2002; Chhatwal et al., 2005). These behavioral findings suggest that CB1 receptor activation is necessary for normal extinction learning. In light of the electrophysiological data described above, activity-dependent reductions in neurotransmitter release from CCK+/CB1+ neurons (as well as other CB1+ neurons) may play an important role in the neurobiology of extinction learning. For the field of translational psychiatry research, this suggests a possible therapeutic intervention for illnesses wherein altered extinction learning is believed to be the underlying pathophysiology, such as post-traumatic stress disorder, specific phobias, and other anxiety disorders.

Rationale

CCK activation is clearly associated with anxiogenesis and we hypothesized that CCK administration may inhibit the extinction of conditioned fear. Due to the aforementioned link between cannabinoid receptor activation and extinction learning and the anatomical and physiological association of these two systems, we further hypothesized that CB1 antagonist-induced deficits in extinction learning may be mediated by an inability of endocannabinoids to reduce CCK receptor activation during extinction. Therefore, blockade of the CCK system should reverse the effects of CB1 antagonist on extinction. First, we find that central CCK activation blocks the extinction of fear-potentiated startle. Then, we demonstrate that systemic and intra-basolateral amygdala administration of a CCK_2 antagonist can reverse the effects of systemic cannabinoid receptor antagonists on extinction without having effects on

baseline startle or nociception. This series of experiments was done in collaboration with Jasmeer Chhatwal in Kerry Ressler's laboratory.

Materials and Methods:

Animals, Surgery, and Apparatus were identical to those used in Chapters 2 and 3. For Experiment 1, which employed intracerebroventricular (i.c.v.) drug administration, 22-gauge stainless-steel guide cannulae cut 6 mm below the pedestal were implanted under ketamine/xylazine anesthesia, and secured using dental cement and 1/8" cap screws (coordinates: AP:0, ML:-1.6, DV:-5.0; nosebar:+5.0 from bregma). Similar procedures were used in Experiments 3 and 4 to implant bilateral cannulae aimed at the basolateral complex of the amygdala (22-gauge cannulae, AP:-3.1, ML:+/-5.4, DV:-8.4 from bregma; nosebar:-3.6) and striatum (22-gauge cannulae, AP: -1.0, ML: +/- 4.0, DV: 5.0 from bregma; nosebar: -3.6). Following behavioral testing, cannulated animals were sacrificed and cannula placement was assessed on cryostat-sectioned tissue. Animals with both cannulae correctly placed either i.c.v., in the amygdala, or in the striatum were included for analysis.

Drugs: All infusions were given through microinjection cannulae (28-gauge) connected with PE-20 tubing to a 10 µL Hamilton syringe. SR141716A (NIMH Drug Supply Program, Bethesda, MD) and CR2945 (Sigma-Aldrich, St. Louis, MO) were dissolved in 100% DMSO. 25 mg pentagastrin (Sigma-Aldrich) was first dissolved in 2.5 mLs 100% DMSO, and then serially diluted to generate 100 nM and 500 nM working solutions. I.c.v. infusions were performed using a flow

rate of 1.0 µl/minute with a total infused volume of 5 µl. For local infusion of CR2945, a 1 mg/mL solution of CR2945 in 100% DMSO was diluted to generate a working solution of 2 µg/uL CR2945 in 5% DMSO/95% sterile PBS. Intra-BLA infusions were performed using a flow rate of 0.1µL/minute with a total infused volume of 0.5 µL per side (1µg of drug/side).

Baseline Startle Testing: Animals were placed in the startle chambers for 20 min on each of 2 days prior to training to habituate them to the test procedures and chambers and to minimize the effects of contextual conditioning. Baseline startle testing consisted of a 5 minute habituation period followed by 30 startle stimuli (50-msec, 95-dB white-noise burst).

Fear Conditioning: On 2 consecutive days following baseline testing, rats were returned to the same chambers and presented with 10 pairings of a light (3.7 sec) co-terminating with a 0.4-mA, 0.5-sec shock (3.6-min inter-trial interval).

Pre-Extinction Test: Twenty-four hours following the last fear-conditioning session, animals were returned to the same chambers and presented with startle stimuli (50-msec, 95-dB white-noise bursts) in the presence or absence of the light conditioned stimulus [randomized presentations of light-noise compounds (LN) and noise-alone trials (NA)]. Increased startle in the presence of the light-CS was taken as a measure of conditioned fear, and the magnitude of the fear response was calculated as the percentage by which startle increased when the light-CS was presented in compound with the startle stimulus versus when it was

omitted [% fear-potentiated startle, ((LN-NA)/NA)*100]. Using these measurements, animals were divided into groups displaying equivalent levels of fear-potentiated startle prior to extinction training.

Extinction Training and Testing: Five days following the last fear conditioning session, animals were administered drug or vehicle prior to the presentation of 90 light-CS stimuli (light-alone, LA) in the absence of footshock (3.7-sec light, 30-sec intertrial interval). Relative level of extinction retention was evaluated 48 hours later with a retention test (15 LN and 20 NA). For experiment 3, this paradigm was repeated three times in order to more extensively evaluate effects of the combined manipulation of CCK and cannabinoid systems on extinction (see Figures 3A and 4A). Fear-potentiated startle was calculated as for the pre-extinction test and data were analyzed using Two-Way Repeated Measures ANOVA and Student-Newman-Keuls post-hoc test for pairwise analysis.

Behavioral Procedures, Experiment 1, Effect of the CCK_2 agonist pentagastrin on the extinction of fear-potentiated startle: Animals were implanted with i.c.v. cannula, fear conditioned, and matched into four groups based on their pre-extinction levels of fear-potentiated startle. Animals were administered 500 nM pentagastrin (n = 17) or vehicle (n = 18) i.c.v. 30 min prior to extinction training and tested for extinction retention 48 hours later. In order to generate an abbreviated dose-response curve for the effect of

pentagastrin on extinction, another group of animals (n = 8) was administered 100 nM pentagastrin 30 min prior to extinction training.

Behavioral Procedures, Experiment 2, Effect of co-administration of a CCK_2 antagonist and a CB1 antagonist on the extinction of fear-potentiated startle: Animals were fear conditioned and matched into four groups based on their pre-extinction levels of fear-potentiated startle. 30 minutes prior to extinction training, animals were intraperitoneally administered vehicle (n = 7, 100% DMSO), SR141716A (n = 7, 5 mg/kg), CR2945 (n = 6, 3 mg/kg), or a combination of SR141716A and CR2945 (n = 7, 5 and 3 mg/kg, respectively).

Behavioral Procedures, Experiment 3, Effect of coadministration of an intra-basolateral amygdala CCK_2 antagonist and a systemic CB1 antagonist on the extinction of fear-potentiated startle: Rats were implanted with bilateral cannulae aimed at the basolateral amygdala and subsequently fear conditioned and matched into two groups showing equivalent levels of fear-potentiated startle based on the pre-extinction test. Thirty minutes prior to extinction training (90 light without shocks), all animals were given i.p. injections of SR141716A (5 mg/kg), along with bilateral infusions of either vehicle (5% DMSO in PBS, n = 8) or CR2945 (1ug/0.5µl/side, n = 8) into the basolateral amygdala. Because we observed relatively little extinction at the first post-extinction test, two additional blocks of extinction training (with similar drug treatment) and testing were given (Figure 3A). For analysis, we focused on the

amount of fear-potentiated startle demonstrated on the first five trials of each post-extinction testing session and used this to assess extinction retention, as a great deal of within-session extinction was observed on days 2 and 3 of testing.

Behavioral Procedures, Experiment 4, Anatomical specificity of administration of a CCK_2 antagonist and a systemic CB1 antagonist on the extinction of fear-potentiated startle: In order to provide a confirmation of our results in Experiment 3 and to assess the anatomical specificity of our finding, a second group of animals was implanted with bilateral cannulae aimed at either the basolateral amygdala or striatum, fear conditioned, and matched into three groups. These groups were administered 1) i.p. vehicle and intra-basolateral amygdala vehicle (vehicle/vehicle, n = 8); 2) i.p. SR141716A and intra-basolateral amygdala CR2945 (SR/CRbla, n = 7); or 3) i.p. SR141716A and intra-striatal CR2945 (SR/CRstr, n = 7) 30 min prior to extinction training.

Results:

Experiment 1, CCK_2 activation impairs the extinction of fear-potentiated startle:

Figure 1 demonstrates that animals administered pentagastrin had significantly greater fear-potentiated startle in an extinction retention test (post-extinction) than vehicle controls. Two-Way Repeated Measures ANOVA identified a significant main effect of test session ($p < 0.05$, $F_{(1,69)} = 5.908$) and Student-Newman-Keuls post-hoc analysis revealed a significant difference of treatment in the post-extinction test ($p < 0.05$). In the vehicle group, fear-

potentiated startle was significantly reduced post-extinction as compared to pre-extinction ($p < 0.01$). No difference was found between pre- and post-extinction tests for the pentagastrin group.

Animals receiving 100nM pentagastrin manifested levels of FPS intermediate between vehicle- and 500nM pentagastrin-treated animals, suggesting that the impairment of extinction retention with pentagastrin may be dose-dependent (linear contrast ANOVA $F_{(1,50)}=5.074$; post-hoc 500nM vs. vehicle $p<.05$).

Experiment 2, The CCK_2 antagonist CR2945 reverses the impairment of extinction caused by treatment with the CB1 antagonist SR141716A:

Administration of SR141716A inhibited extinction learning, as animals receiving SR141716A showed higher levels of fear-potentiated startle when tested both 48 and 96 hours post-extinction (Figures 2B and C, respectively). Furthermore, rats treated with a combination of SR141716A and CR2945 showed significantly less fear than those receiving SR141716A alone (Figure 2B $F_{(3,24)}=3.876$, $p<.05$; Figure 2C $F_{(3,24)}=3.060$, $p<.05$), and statistically similar levels of fear-potentiated startle to vehicle-treated controls. Rats that received CR2945 alone prior to extinction training did not show enhanced extinction retention as compared to vehicle-treated animals.

Experiment 3, Intra-amygdala infusions of the CCK_2 antagonist CR2945 partially reverse the blockade of extinction caused by treatment with the CB1 antagonist SR141716A:

Animals who received intra-basolateral amygdala infusions of CR2945 showed significant extinction retention on the second and third post-extinction tests as compared to their test 1 and pre-extinction test values (significant main effect of testing day, repeated measures ANOVA $F_{(3,18)}=4.344$, $p<.05$; post-hoc tests comparing days 2 and 3 to pre-extinction and test 1, $p<.05$; Figure 3B). In contrast, vehicle-infused controls failed to show significant extinction on any of the three testing days (repeated measures ANOVA $F_{(3,18)}=0.383$, $p=.766$), consistent with earlier data showing that SR141716A attenuated extinction (Chhatwal et al., 2005).

Experiment 4, Intra-striatal infusions of CR2945 do not reverse the SR141716A-induced blockade of extinction:

Animals in the vehicle/vehicle group showed significant extinction retention on all three test days (Figure 4B; significant main effect of testing day, overall repeated measures ANOVA $F_{(3,87)}=5.902$, $p<.01$; post-hoc tests comparing day 1 to pre-extinction $p < 0.05$, days 2 and 3 to pre-extinction, $p < 0.001$). We also found that, as in the reversal experiment above, the intra-amygdala CR2945 group extinguished significantly faster than the intra-striatum group, which did not show significant extinction during the testing (Figure 4B; post-hoc tests comparing intra-amygdala group days 2 and 3 to pre-extinction, $p<.05$).

Discussion:

We found that CCK_2 receptor activation via i.c.v. administration of pentagastrin inhibited the extinction of fear-potentiated startle. Additionally, both systemic and intra-basolateral administration of a CCK_2 antagonist reversed the blockade of extinction observed following i.p. injection of the CB1 receptor antagonist SR141716A. This effect was found to be specific to the basolateral amygdala, as striatal infusions of the CCK_2 antagonist could not reverse the effects of SR141716A.

Our data demonstrating that pentagastrin impairs extinction is consistent with previous studies described above showing that CCK_2 receptor activation is acutely anxiogenic. Perhaps most relevant is the finding that the CCK_2 antagonist L-365,260 dose-dependently decreases the expression of fear-potentiated startle (Josselyn et al., 1995). This suggests that activation of the CCK system may enhance fear expression, and that the observed deficits in extinction retention in our pentagastrin treated animals might represent inadequate reductions in fear during extinction training. Future studies can further assess this hypothesis by evaluating within-session extinction following manipulation of the CCK system. Interestingly, this is similar to effects observed in CB1 receptor knockout mice and in animals administered CB1 receptor antagonists (Marsicano et al., 2002; Chhatwal et al., 2005).

While the effect was significant and we found it in two independent groups of animals, the amelioration of the CB1 receptor antagonist effect was less

pronounced when CR2945 was given locally into the amygdala than in prior experiments where CR2945 was given systemically. This suggests that while the amygdala is a critically important site of interaction between the CCK and CB1 systems, sites other than the amygdala may be important mediators of the CCK/CB1 interaction as well.

Interestingly, rats that received the CCK_2 antagonist alone prior to extinction training did not show enhanced extinction retention as compared to vehicle-treated animals, as might be predicted by the prior finding that the CCK_2 agonist pentagastrin inhibited extinction retention. There are several possible explanations for this observation. First, there is the procedural possibility that the level of extinction that we normally see in these studies is already maximal so that we cannot detect further facilitation of extinction. To further address this question, we would need to evaluate CR2945 using an extinction paradigm with fewer light alone presentations, such as was used in Chapter 3 to evaluate the effects of NPY and we have performed previously in examining agents that facilitate extinction (Walker et al., 2002).

Conceptually, the finding that the CCK_2 receptor antagonist is only effective in combination with the CB1 receptor antagonist fits into our working circuit model of how these systems interact in the basolateral amygdala. CB1 receptors are located on interneurons that release both GABA and CCK. GABA is critical for extinction (Harris and Westbrook, 1998) whereas CCK interferes with extinction (current manuscript). So, when CB1 receptors are blocked, GABA

release would be reduced (bad for extinction) but CCK release would also be blocked (good for extinction). Giving the CCK antagonist alone would prevent CCK from interfering with extinction (good for extinction), but endogenous release of cannabinoids would prevent GABA release (bad for extinction), therefore the net result would be no change in extinction, as was found. However, when the CCK antagonist is given in combination with the CB1 antagonist, GABA would no longer be blocked (good for extinction) and the deleterious effects of CCK on extinction would be blocked by the CCK antagonist (good for extinction), leading to exactly what we observed in these experiments: greater extinction with the combination of drugs than either drug alone. Rather than being a problem for the theory, we believe the behavior reflects the complicated multiple interactions taking place.

Furthermore, results from both human and animal literature suggest that while CCK antagonists reverse the anxiogenic effects of CCK activation, they do not consistently have effects when administered alone (Harro, 2006). This is evident in clinical trials of CCK_2 antagonists wherein no effect was found following administration of several CCK_2 antagonist in patients with generalized anxiety disorder or panic disorder (Adams et al., 1995; Kramer et al., 1995; van Megen et al., 1997; Pande et al., 1999). Several results from the animal literature also support the notion that CCK_2 antagonists alone are not necessarily anxiolytic when administered alone (Dawson et al., 1995; Johnson and Rodgers, 1996). These results suggest that we would not expect the CCK antagonist to facilitate extinction alone, and are consistent with our hypothesis that perturbation of the

endogenous system with the CB1 antagonist is necessary to reveal the underlying behavioral effects of CCK in the extinction of conditioned fear. Further studies aimed at examining pre- and post-synaptic effects of CB1 receptor and CCK_2 receptor manipulation on extinction learning hope to further dissect these interacting mechanisms.

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Figure 1: *Pentagastrin, a CCK_2 receptor agonist, impairs the extinction of fear-potentiated startle.* **(A)** Animals were implanted with i.c.v. cannulae 7-10 days prior to behavioral training. Thirty minutes prior to extinction training, animals received 5 μL infusions of 0, 100, or 500 nM pentagastrin. Animals were tested 48 hours following extinction off drug. **(B)** Animals treated with pentagastrin showed significantly greater fear-potentiated startle during the post-extinction test (Post-Ext) as compared to vehicle controls. Animals in the vehicle group showed significant extinction of fear-potentiated startle. **(C)** Dose-response curve showing that animals receiving 100 nM pentagastrin have intermediate levels of fear-potentiated startle during the Post-Ext test. (values shown are the average of all test trials; error bars indicate +/- SEM; * denotes $p < 0.05$, ** denotes $p < 0.01$)

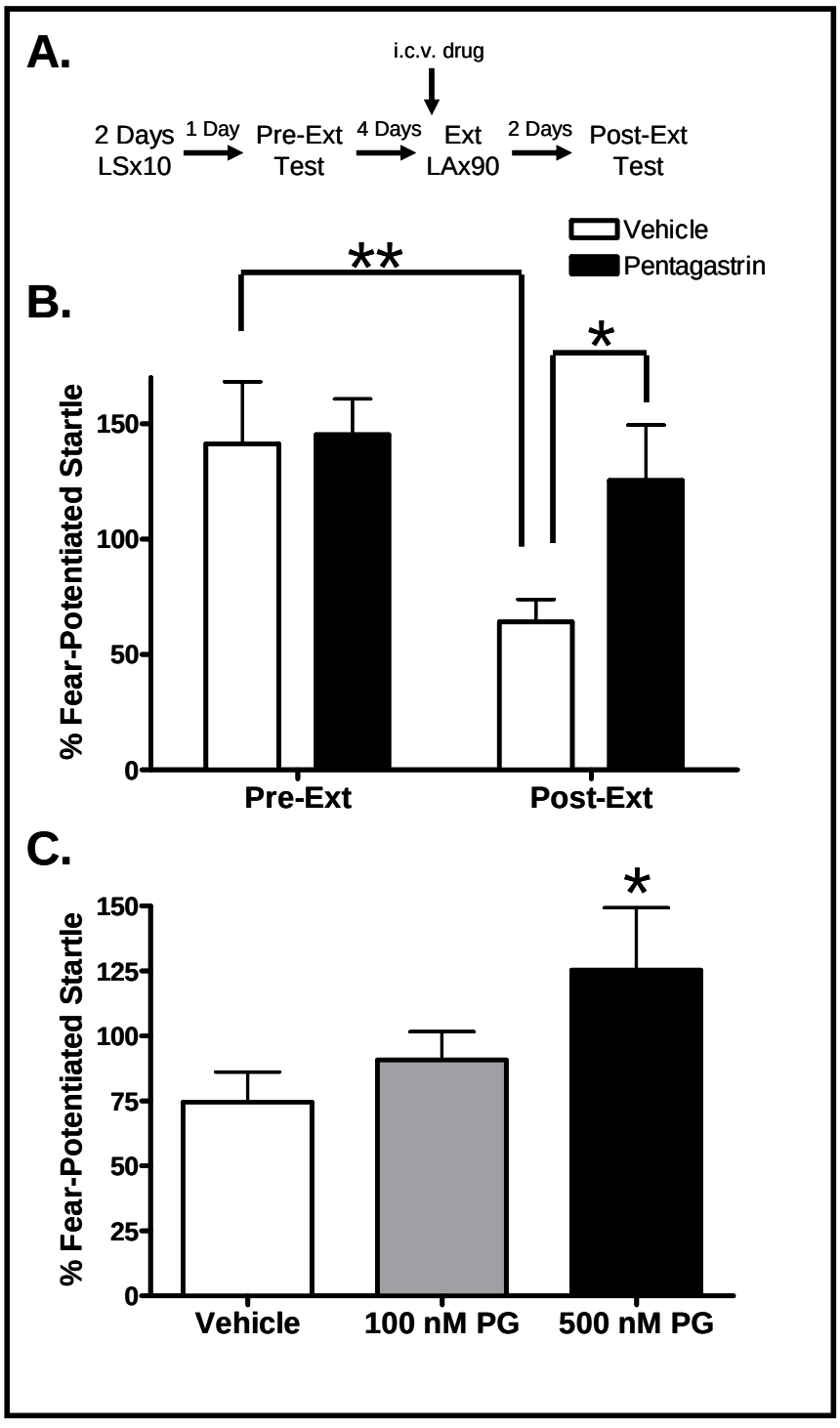
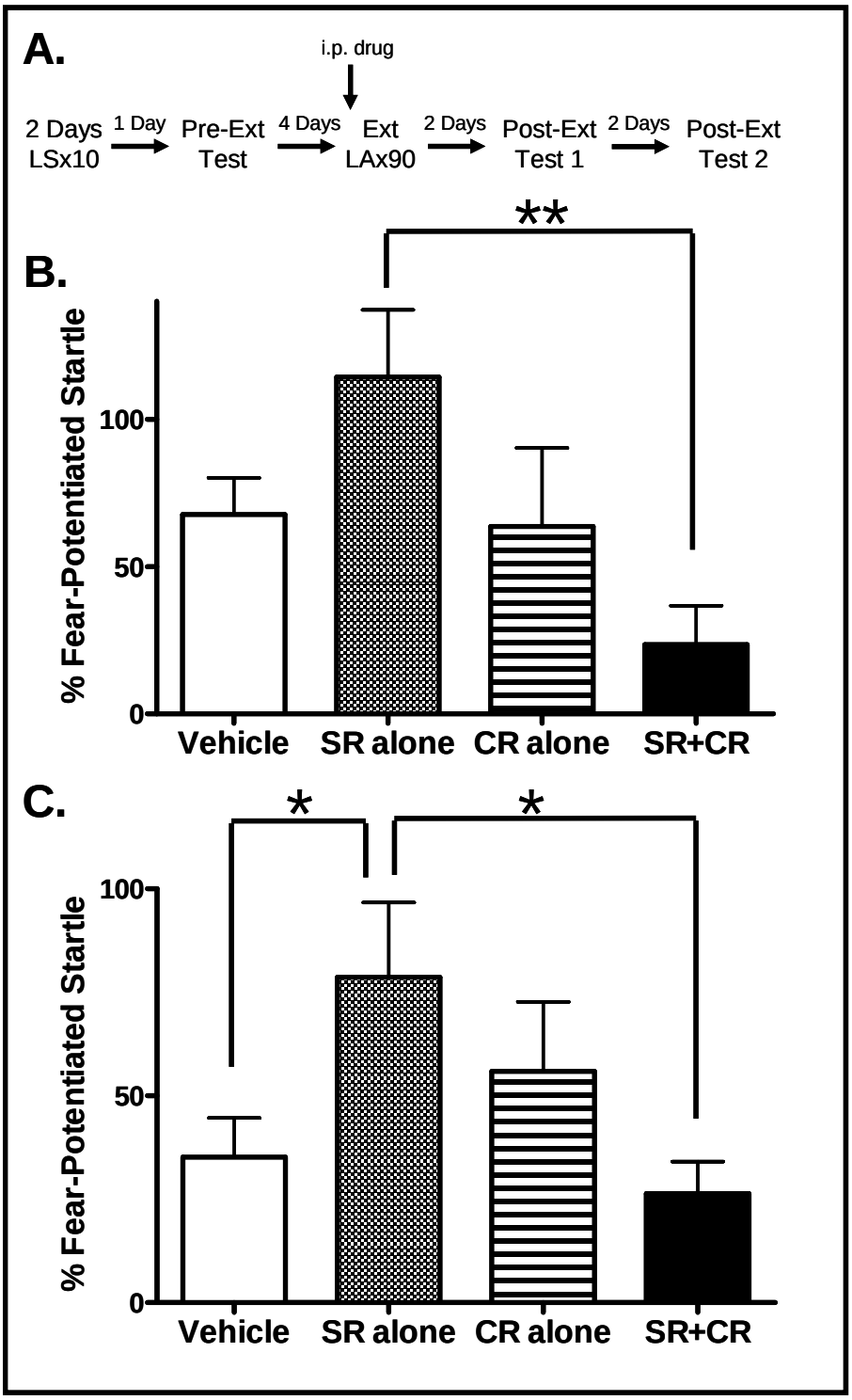

A.
i.c.v. drug
2 Days LSx10
1 Day
Pre-Ext Test
4 Days
Ext LAx90
2 Days
Post-Ext Test
B.
Vehicle
Pentagastrin
**
*
% Fear-Potentiated Startle
150
100
50
0
Pre-Ext
Post-Ext
C.
*
% Fear-Potentiated Startle
150
125
100
75
50
25
0
Vehicle
100 nM PG
500 nM PG

Figure 2: *Systemic administration of a CCK_2 receptor antagonist reverses the blockade of extinction normally seen with CB1 receptor antagonist treatment.* **(A)** Animals were administered vehicle, the CB1 antagonist SR141716A (SR, 5 mg/kg), the CCK_2 receptor antagonist CR2945 (CR, 3 mg/kg), or a combination of SR and CR i.p. 30 min prior to extinction training. Vehicle treated animals and animals co-administered SR and CR showed significantly less fear-potentiated startle in extinction retention tests at 48 hours (**B**, SR+CR group) and 96 hours (**C**, vehicle and SR+CR group) as compared to those receiving SR alone. (n = 7/group; values shown are average of all trials in each test, error bars indicate +/- SEM; * denotes $p < 0.05$, ** denotes $p < 0.01$)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i.p. drug
2 Days LSx10
1 Day
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4 Days
Ext LAx90
2 Days
Post-Ext Test 1
2 Days
Post-Ext Test 2
B.
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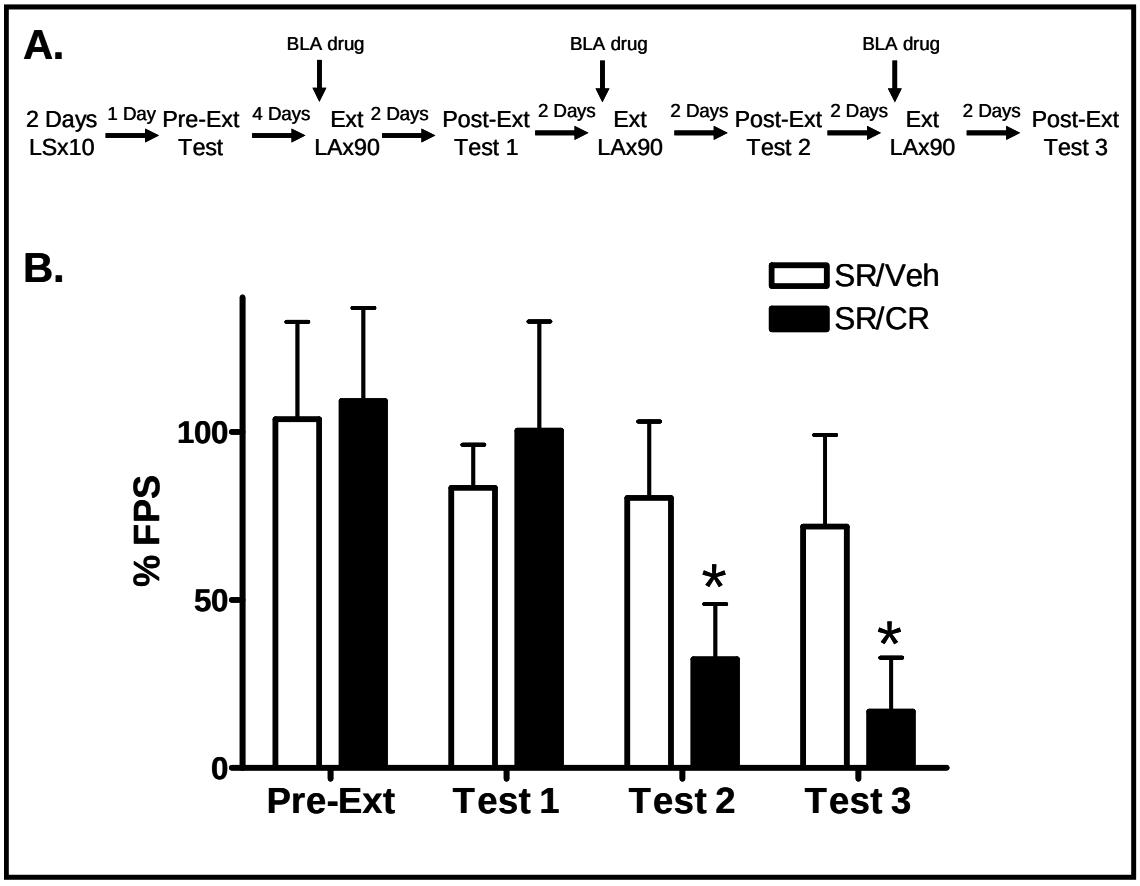
% Fear-Potentiated Startle
100
50
0
Vehicle
SR alone
CR alone
SR+CR
C.
*
*
% Fear-Potentiated Startle
100
50
0
Vehicle
SR alone
CR alone
SR+CR

Figure 3: *Intra-amygdala infusion of a CCK_2 receptor antagonist partially reverses the blockade of extinction seen with CB1 receptor antagonist treatment.* **(A)** Animals were implanted with bilateral cannulae aimed at the basolateral amygdala (BLA) 7-10 days prior to behavioral training. 30 min prior to extinction training, all animals were injected i.p. with 5 mg/kg SR141716A. In addition, either vehicle or 1 μg CR2945 (CR) was bilaterally infused into the BLA (volume=0.5μL over 5 min). Animals were tested for extinction retention (Post-Ext) 48 hours after each extinction training session off drug. **(B)** Animals receiving intra-BLA CR showed significant extinction retention on Post-Ext Tests 2 and 3. Animals receiving intra-BLA vehicle did not show significant extinction retention on any of the three Post-Ext tests. (n = 8/group), values shown are averages of the first 5 trials in each test; error bars indicate +/- SEM; * denotes $p < 0.05$ comparing Pre-Ext to Post-Ext tests)


A.
BLA drug
BLA drug
BLA drug
2 Days
LSx10
1 Day
Pre-Ext
Test
4 Days
Ext
LAx90
2 Days
Post-Ext
Test 1
2 Days
Ext
LAx90
2 Days
Post-Ext
Test 2
2 Days
Ext
LAx90
2 Days
Post-Ext
Test 3
B.
SR/Veh
SR/CR
% FPS
100
50
0
*
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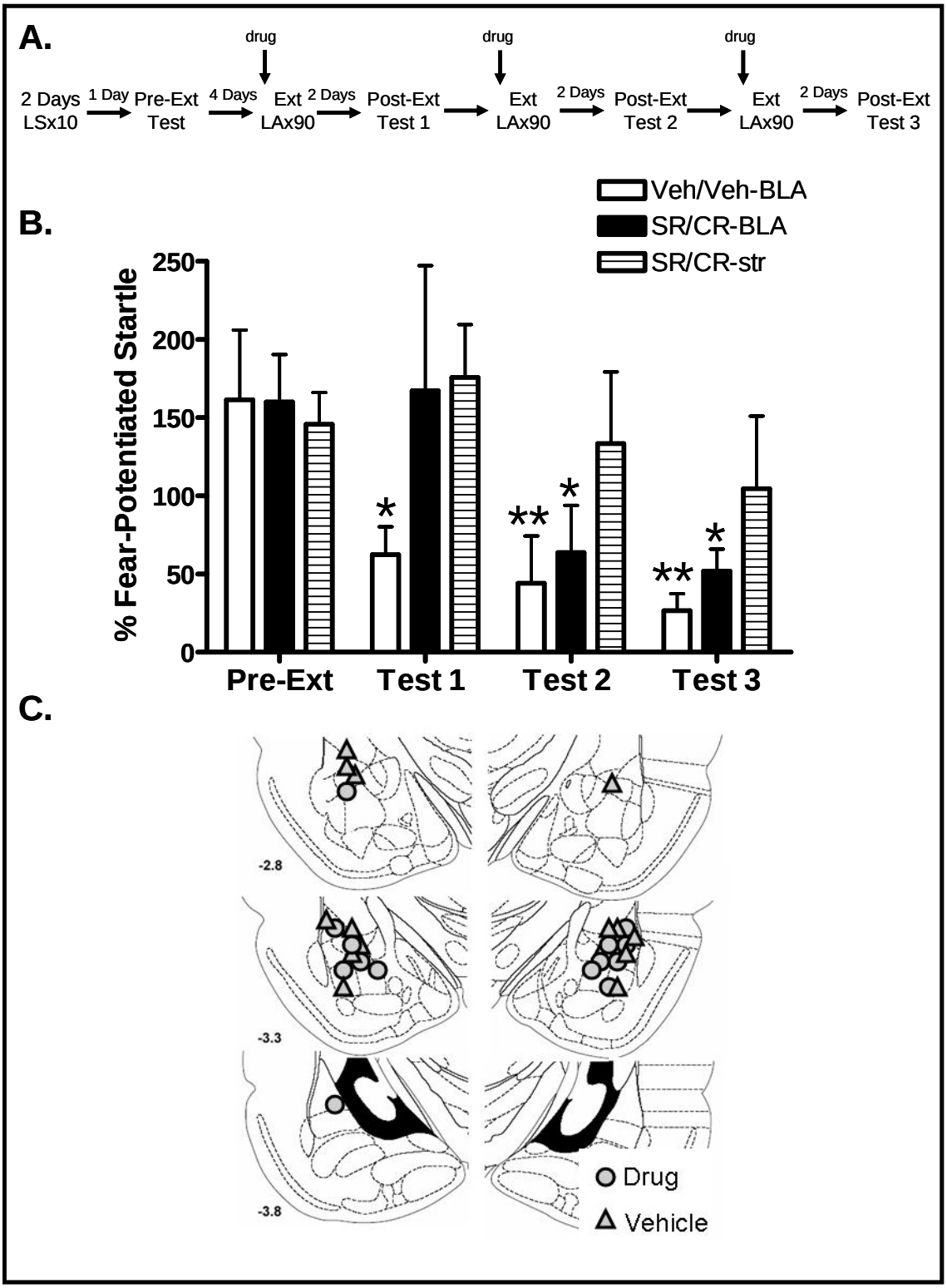
Pre-Ext
Test 1
Test 2
Test 3

Figure 4: *Reversal of systemic cannabinoid antagonist blockade of extinction by local infusion of a CCK_2 receptor antagonist in the basolateral amygdala but not the striatum.* **(A)** Animals were implanted with bilateral cannulae aimed at the basolateral amygdala (BLA) or striatum (str) 7-10 days prior to behavioral training. Prior to each extinction training, animals were administered either 1) i.p. vehicle, intra-BLA vehicle (Veh/Veh-BLA, n=8); 2) i.p. SR141716A, intra-BLA CR2945 (SR/CR-BLA, n=7); 3) i.p. SR141716A, intra-STR CR2945 (SR/CR-str, n=7) and tested 48 later off drug (Post-Ext). **(B)** Animals in the SR/CR-BLA group demonstrate extinction levels similar to vehicle treated animals on the second and third Post-Ext test. (values shown are averages of all trials in each test; error bars indicate +/- SEM; * denotes $p < 0.05$, ** denotes $p < 0.001$ difference from Pre-Ext) **(C)** Cannulae locations with the BLA of rats included within this experiment (striatal cannulae not shown).


A.
drug
drug
drug
2 Days LSx10
1 Day
Pre-Ext Test
4 Days
Ext LAx90
2 Days
Post-Ext Test 1
Ext LAx90
2 Days
Post-Ext Test 2
Ext LAx90
2 Days
Post-Ext Test 3
B.
Veh/Veh-BLA
SR/CR-BLA
SR/CR-str
% Fear-Potentiated Startle
250
200
150
100
50
0
*
**
*
**
*
Pre-Ext
Test 1
Test 2
Test 3
C.
-2.8
-3.3
-3.8
Drug
Vehicle

Chapter 5

CONCLUSION

Summary of Findings, Discussion of Gut Peptides and Anxiety, Future Directions, and Clinical Implications

Summary of Findings

We found that central administration of NPY inhibits both baseline acoustic startle and the expression of fear-potentiated startle. Infusion of NPY into the basolateral, but not the medial, nucleus of the amygdala reproduced the impairment of the expression of fear-potentiated startle. This is consistent with previous findings that NPY is anxiolytic in several behavioral paradigms. Based on literature showing that fear-potentiated startle is dependent on glutamate transmission in the basolateral amygdala (Kim et al., 1993; Walker and Davis, 1997; Walker et al., 2005) and electrophysiological studies showing that NPY can inhibit glutamate release (Colmers et al., 1987; Qian et al., 1997), it is likely that the suppression of fear-potentiated startle results from NPY acting to decrease glutamate transmission in the basolateral amygdala. While we were unable to determine the receptor subtype involved in this effect, we are confident based on the relevant literature and results from Chapter 3 that the NPY Y_1 and Y_5 receptors are the most likely candidates for the observed inhibition of fear expression.

Our characterization of the effects of NPY on fear expression revealed that central administration of NPY also enhances within-session extinction of fear-potentiated startle. This finding, coupled with the growing body of literature correlating NPY with resilience in humans, led us to the hypothesis that NPY may enhance the extinction of conditioned fear. We were then able to demonstrate that when NPY is administered i.c.v. prior to extinction training, extinction retention for both the contextual and cued components of conditioned fear is

enhanced when measured off drug 48 hours later. Additionally, we found that intra-basolateral amygdala administration of the NPY Y_1 receptor antagonist BIBO 3304 prior to extinction training led to a profound deficit in extinction retention at 48 hours, thereby identifying both the brain locus and receptor subtype likely underlying the central effects. We believe that the role of NPY in the extinction of conditioned fear may, at least in part, explain the mechanism underlying the association between NPY and psychobiological resilience in humans.

Central infusion of pentagastrin, a CCK_2 receptor agonist, prior to extinction training yields impaired extinction retention when measured 48 and 96 hours later. Anatomical studies have shown overlap between the CCK and endocannabinoid systems (Marsicano and Lutz, 1999; McDonald and Mascagni, 2001), and genetic and pharmacological studies indicate that CB1 receptors are involved in extinction (Marsicano et al., 2002; Chhatwal et al., 2005). Based on this, we performed a series of experiments assessing interactions between the endocannabinoid and CCK systems. These studies indicate that both systemic and intra-basolateral amygdala administration of the CCK_2 antagonist CR2945 prior to extinction training reverses the blockade of extinction that we find following i.p. injection of the CB1 receptor antagonist SR141716A.

Gut Peptides and Anxiety

The series of studies described here begins to evaluate how two neuropeptides, NPY and CCK, affect the extinction of fear-potentiated startle.

NPY was originally isolated from porcine brain due to structural similarities with peptide YY (PYY), a peptide isolated from extracts of porcine intestine. In fact, the authors thought that the isolated peptide amide was PYY until they determined that this peptide had a novel amino acid sequence (Tatemoto, 1982; Tatemoto et al., 1982). NPY was later found in the intestine as well (Tatemoto et al., 1985) and has since been identified as one of the most potent orexigenic agents known . Similarly, CCK was originally identified as a gut peptide (Ivy and Janecek, 1959) and was later characterized as on of the most abundant neuropeptides in the brain (Vanderhaeghen et al., 1975). CCK was the first gut peptide shown to inhibit feeding when administered peripherally (Gibbs et al., 1973).

This relationship, wherein the anxiolytic NPY induces feeding and the anxiogenic CCK inhibits feeding, is not unique to these two peptides (e.g. corticotropin releasing factor is anxiogenic and inhibits feeding). From an evolutionary biology perspective, it is interesting to consider why these gut peptides are irrevocably intertwined with anxiety systems. Since feeding is among the most essential and basic behaviors, ensuring that hunger is accompanied by decreased fear to forage or hunt would be beneficial. Reciprocally, hunger might interfere with the appropriate response to a high stress situation. Therefore, a system in which the same compound could signal both decreased fear/anxiety and increased hunger, or increased fear/anxiety and decreased hunger would be ideally suited to the task. This setup resonates in the developmental biology of these systems, as both the enteric and central nervous

systems develop from the same embryonic neural crest cells (Gershon, 1997). This may help explain why these peptides are important in both the gut and the brain.

More evidence that these systems function together comes from occasions when they malfunction together in a modern environment. Indeed, evidence from both psychiatric and gastrointestinal research supports this association. For example, 50-90% of individuals seeking treatment for irritable bowel syndrome have combordid psychiatric disorders (Lydiard, 2001). Changes in eating patterns, both increases and decreases in hunger, are part of the diagnostic criteria for depression. Early life trauma, long known to be associated with the development of psychiatric disorders (Heim and Nemeroff, 2001; Sanchez et al., 2001; Gutman and Nemeroff, 2002), has also been found to increase vulnerability of gut mucosa to stress and impair defense against bacteria (Soderholm et al., 2002; Gareau et al., 2006); both of these phenomena have been attributed to mechanisms involving the peptide corticotropin releasing factor. While a complete review of the literature is outside the scope of this dissertation, these and many other examples strongly support a profound connection between gastrointestinal and brain disorders.

Future Directions

We would like to characterize the effect of NPY administration into the bed nucleus of the stria terminalis (BNST) to answer questions raised in Chapters 2 and 3. First, we would like to determine if infusion of NPY into the BNST would

reproduce the reduction in baseline startle described in Experiment 1 of Chapter 2. Changes in baseline startle may represent changes in anxiety states (Nair et al., 2005; Grillon et al., 2006) and a growing body of literature has implicated the BNST in anxiety-related behaviors (Davis, 1998; Walker et al., 2003), making the BNST a likely locus for our effect. In addition, in order to further explore the enhancement of extinction to the contextual component of fear-potentiated startle described in Experiment 2 of Chapter 3, we would like to evaluate the effect of intra-BNST NPY on extinction of contextual fear.

While we have focused in Chapter 4 on teasing apart the complicated interactions between two systems, CCK and endocannabinoids, we are interested in further evaluating the relationships among these and several other neuropeptide systems. A recent study showed that cannabinoid activation can augment the release of NPY in the rat hypothalamus (Gamber et al., 2005). As described earlier, cannabinoids decrease release from CCK+/CB1+ interneurons (Katona et al., 1999; Beinfeld and Connolly, 2001; Burdyga et al., 2004; Fride, 2005). We find this interesting in light of our results that NPY enhances and CCK impairs the extinction of fear-potentiated startle. It is possible that cannabinoids act upstream of both CCK and NPY, decreasing CCK release and increasing NPY release to facilitate extinction learning. In a pilot study, we found that i.c.v. infusion of NPY could partially reverse the blockade of extinction caused by i.p. injection of SR141716A. These results are promising and warrant further investigation.

A particularly interesting direction involves the interaction between NPY and the melanocortin α-MSH, a pro-opiomelanocortin (POMC) derivative. Anatomically, both NPY and α-MSH have dense peptide and mRNA in the arcuate nucleus of the hypothalamus (Bai et al., 1985; Broberger et al., 1997) and are expressed in the amygdala (Kask et al., 2002; Kishi et al., 2003). Many arcuate POMC neurons express NPY Y_1 receptor mRNA and protein (Broberger et al., 1997), and there is an association between NPY fibers and POMC neurons (Csiffary et al., 1990; Garcia de Yebenes et al., 1995). Furthermore, NPY-positive cell bodies in the arcuate nucleus co-express agouti-related peptide (AGRP), an endogenous antagonist at the melanocortin 3 (MC3) and melanocortin 4 (MC4) receptors (Broberger et al., 1998). Behaviorally, i.c.v. infusion of α-MSH is anxiogenic and anorexigenic (Fan et al., 1997; Hansen and Morris, 2002; Rao et al., 2003), in contrast to the anxiolytic and orexigenic NPY.

A recent study showed that pretreatment with α-MSH attenuates the anxiolytic-like effect of intra-amygdala NPY and an NPY Y_1 receptor agonist in the elevated plus-maze. Additionally, combined intra-amygdala administration of the MC4 receptor antagonist HS014 with normally subeffective doses of NPY or an NPY Y_1 receptor agonist produces an anxiolytic-like effect (Kokare et al., 2005). In a small pilot study, we found that α-MSH may enhance the expression of fear-potentiated startle. We believe that α-MSH may also block the extinction of fear-potentiated startle and would like to characterize this in a series of studies similar to Chapter 3. Moreover, the functionally antagonistic relationship between these two peptides could help to explain why there were occasions when

our NPY effect was weaker in some animal cohorts than others; a group of rats with greater melanocortin signaling, as might occur with stress-induced increases in POMC, would likely respond less to activation of NPY receptors. It is therefore possible that co-administration of an MC4 receptor antagonist with NPY may further facilitate the extinction of fear-potentiated startle seen in Chapter 3.

Clinical Implications

In general, current treatment strategies for anxiety disorders act as 'hammers' on neural circuitry by targeting GABAergic or glutamatergic systems. For example, benzodiazepines, which work at a modulatory site on the GABA receptor, are often prescribed for anxiety. D-cycloserine, which is being used as an adjunct for psychotherapy, works at a modulatory site on the NMDA glutamate receptor. While these treatment strategies are effective for many individuals, a more subtle manipulation of the pathological neural circuitry may enhance treatment response rates while decreasing the risk of side effects. Selective serotonin reuptake inhibitors (SSRIs), which target the modulatory monoamine neurotransmitter serotonin, modify neural systems in a more discrete manner. Still, while these drugs work well for some individuals, they often have a slow onset of action and are only effective in a subset of patients.

Unlike glutamate or GABA, which are ubiquitous throughout the brain with regards to both anatomical distribution and general function (i.e. glutamate is excitatory and GABA is inhibitory), neuropeptides play a modulatory role in the brain and are found in more restricted regions. This makes them an

interesting drug target for human disorders in that manipulation of neuropeptides is likely to confer enhanced specificity of function. A better understanding of which neuropeptides are involved in extinction and what role they play in extinction learning is likely to either directly or indirectly lead to new treatments. The data presented here suggests that enhancement of the NPY system and blockade of the CCK system could be an effective treatment strategy for post-traumatic stress disorders and other anxiety disorders.

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