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Mallory Elva Bowers Date

Interaction between the cholecystokinin and endogenous cannabinoid systems in cued fear expression and extinction retention.

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Interaction between the cholecystokinin and endogenous cannabinoid systems in cued fear expression and extinction retention.

By

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An abstract of a dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Neuroscience
2014

Abstract

Interaction between the cholecystokinin and endogenous cannabinoid systems in cued fear expression and extinction retention.

By Mallory Elva Bowers

Posttraumatic stress disorder (PTSD) is thought to develop, in part, from improper inhibition of fear. Accordingly, one of the most effective treatment strategies for PTSD is exposure-based psychotherapy. Pavlovian fear conditioning and extinction using rodent models is a valid analog of trauma consolidation and exposure therapy. Pavlovian fear conditioning involves repeated co-presentation of a neutral stimulus, often an auditory tone, with an aversive, unconditioned stimulus (US) so that the test subject learns that the neutral, now conditioned, stimulus (CS) predicts an incoming US. As the subject learns that the CS is predictive of the US the subject will exhibit fear behavior in response to the CS. Conversely, extinction involves repeated presentations of the CS so that the test subject learns that the CS no longer signals an incoming US and inhibits fear behaviors. Ideally, neuroscience would inform adjunct therapies that target the neurotransmitter systems involved in extinction processes. Separate studies have implicated the cholecystokinin (CCK) and endocannabinoid systems in fear; however, there is a high degree of anatomical colocalization between the cannabinoid 1 receptor (Cnr1) and CCK in the basolateral amygdala (BLA), which is critical for Pavlovian fear conditioning and extinction and emotion regulation. Although most research has focused on GABA and GABAergic plasticity as the mechanism by which Cnr1 mediates fear inhibition, we hypothesize that an interaction between Cnr1 and CCKBR is critical for fear extinction processes. This dissertation reports on a behavioral interaction between the CCK and endocannabinoid systems in cued fear expression and extinction retention that is likely mediated by functional CCKBR/Cnr1 cross-talk in the amygdala. First, the behavioral effect of Cnr1 antagonist administration was measured in C57BL/6J and *CCKBR* transgenic mice. Additionally, BLA Cnr1 and CCKBR immunoreactivity was examined. Second, the behavioral effect of CCKBR antagonist administration in Cnr1 transgenic mice was measured. In the same set of experiments, functional and genetic interactions between Cnr1 and CCKBR were assessed. Finally, sex differences in anxiety-like behavior of *Cnr1* transgenic mice were assessed. These results provide much needed, novel evidence that Cnr1 contributes to cued fear expression via an interaction with the CCK system. Dysfunctional Cnr1-CCKBR interactions might contribute to the etiology of, or result from, fear-related psychiatric disease.

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"A child does not grow up only in a single home." - Ugandan proverb

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Chapter 1:

A brief overview and framework

1.1 An overall framework and perspective on the dissertation

The following dissertation presents evidence of anatomical, genetic, and functional interactions between the cholecystokinin and endogenous cannabinoid system that may be critical for a behavioral interaction observed between the cannabinoid 1 receptor (Cnr1) and the cholecystokinin B receptor (CCKBR) during cued fear expression and extinction retention. The endogenous cannabinoid system is consistently implicated in learning processes that underlie Pavlovian fear conditioning and extinction, a valid analog of PTSD and other fear-related disorders. In this vein, the goal of this dissertation is two-fold: to uncover the mechanism by which Cnr1 modulates fear learning and to move towards a better understanding of normal and pathological extinction learning, particularly within the amygdala – a region critical for Pavlovian fear conditioning and extinction, as well as emotion regulation. Determining the nature of an interaction between Cnr1 and CCKBR during extinction could aid in the discovery of pharmacotherapies for the enhancement of exposure therapy - particularly within the CCK system.

In the body of the dissertation, we first report the effect of Cnr1 antagonist administration on C57BL/6J and *CCKBR* transgenic mouse fear behavior (chapter 3). We find Cnr1 antagonist administration increases fear behavior during cued fear expression in C57BL/6J and *CCKBR*-wild-type subjects, but not *CCKBR*-knockout subjects, suggesting that activation of Cnr1 is upstream of CCKBR during cued fear expression. In the same chapter, we present anatomical data that show Cnr1-positive fibers form perisomatic baskets in the basolateral amygdala (BLA), a region critical for Pavlovian fear conditioning and extinction, as mentioned, and the putative site of a Cnr1-CCKBR interaction during cued fear expression. In chapter 4, the behavioral effect of CCKBR antagonist administration in *Cnr1* transgenic mice is presented. We find CCKBR antagonist administration enhances cued fear expression and extinction retention in

Cnr1 knockout subjects, but not wild-type littermates, supporting evidence in chapter 3 that CCKBR is downstream of Cnr1 in cued fear expression and extinction retention. *Ex vivo* amygdala slice experiments suggest that activation of Cnr1 inhibits release of CCK, providing a mechanism by which Cnr1 inhibits CCKBR during cued fear expression. Finally, we present evidence of sex differences in anxiety-like behavior of *Cnr1* transgenic mice (chapter 5). Here, we report that female *Cnr1* knockout subjects are buffered against increased anxiety-like behavior seen in male *Cnr1* knockout subjects (which is in line with prior literature and observed in our own experiments). Pharmacological and ovariectomy experiments suggest that female gonadal hormones may be protective early in development against anxiety-like behavior observed in adult *Cnr1* knockout males.

The results of the original experiments reported in this dissertation are contextualized with a comparison to prior literature on the cholecystokinin and endogenous cannabinoid systems, as well as previous evidence of an interaction between the two neuromodulator systems, particularly as they relate to fear- and anxiety-like behavior. Further, our data is situated among general overviews of the literature regarding neuropeptide regulation of fear and anxiety (chapter 2) and translationally informed treatments for posttraumatic stress disorder (PTSD, chapter 6). These reviews present a broader picture of the field of learning and memory, and, in particular, the role of the amygdala and specific neuromodulators in aversive learning as they relate to PTSD. These reviews are included here to provide context and a rationale for the research conducted under the current dissertation. Further, the literature summarized in chapter 2 and chapter 6 (particularly chapter 6), point to the future directions of the field of amygdala-dependent fear learning, highlighting the potential impact of the research at hand. Already, pharmacotherapies targeting the cannabinoid and CCK systems are being tested in healthy human subjects and individuals with PTSD. Although more work is needed to clarify the functional relationship between CCK and Cnr1, the results of this study suggest that dysfunction

in a putative Cnr1-CCKBR interaction might be critical to understand the etiology, and ultimately treatment, of fear-related disorders.

Chapter 2:

Neuropeptide regulation of fear and anxiety: implications of cholecystokinin, endogenous opioids, and neuropeptide Y

2.1 Context, Author's Contribution, and Acknowledgement of Reproduction

The following chapter reviews neuropeptide modulation of fear circuitry that likely underlies anxiety and fear-related disorders such as specific and social phobia, panic disorder, and posttraumatic stress disorder. The work presented here was conceptualized, organized, researched, and written by the dissertation author under the guidance of Dr. Ressler. The chapter is reproduced with from sections with minor edits from Bowers, M.E., Choi, D.C., and Ressler, K.J.. Neuropeptide regulation of fear and anxiety: implications of cholecystokinin, endogenous opiods, and neuropeptide Y. *Physiology & Behavior* (2012)

2.2 Introduction

Anxiety and fear-related disorders are thought to involve dysregulation of the fear system. There are several aspects of the pathology of these disorders that can be modeled in the laboratory. Pre-existing sensitivity involving genetic background and environment can be analyzed using human genome-wide association studies in the human population, knockout and transgenic mice, and environmental manipulations in animal models. Fear acquisition is often modeled with a Pavlovian associative fear learning paradigm to assess freezing behavior in response to a conditioned context or cue. Fear learning can also be assayed using fear-potentiated startle, passive avoidance, and active avoidance. Because the above assays are robust, easily reproducible, and amenable to manipulation, there has been an exponential increase in data contributing to the understanding of fear acquisition. Therefore, for the purpose of this review, we will examine studies employing these assays.

Perhaps the most worthwhile aspect of fear-related disorders to model, in terms of clinical relevance, is the extinction of aversive memories. Resilient individuals likely extinguish fear

memories normally, even if they are not conscious of this process. In contrast, those who are vulnerable to fear-related disorders often are unable to normally extinguish aversive memories and continue to have high levels of disruptive, even pathological fear (Jovanovic and Ressler 2010). To overcome anxiety and fear-related pathology, those with fear-related disorders require the aid of professionals in order to extinguish their fear memories – this is known as exposure therapy. Exposure therapy is modeled in the laboratory via an extinction learning paradigm, in which the aversive stimulus is presented repeatedly until inhibition of the fear response is achieved. Because of its face validity, extinction provides an excellent opportunity for bench to bedside translational research. Additionally, enhancing extinction learning or interfering with the consolidation of fear memories may also provide novel therapeutic approaches. Overall, a broader perspective on all aspects of fear will provide a better understanding of anxiety and fear-related disorders.

Although sensory cortex, periaqueductal gray, lateral septum, striatum, inferior colliculus, and bed nucleus of the stria terminalis (BNST) have all been implicated in fear, most research has focused on the amygdala, hippocampus, and medial prefrontal cortex (**Figure 2.2-1**). Human imaging studies, as well as pharmacological, lesion, and single unit recordings in animal models have pegged the amygdala as the central fear nucleus. Pathways that convey information about the conditioned (neutral) stimulus and unconditioned (aversive) stimulus are thought to converge at the lateral (LA) and basolateral amygdala (BLA) in associative / Pavlovian learning paradigms. The BLA then sends information to the central amygdala, which controls the expression of fear responses by projecting to brainstem areas. In this model, multiple pairings of the conditioned stimulus and the unconditioned stimulus induce plasticity, resulting in conditioned stimulus-elicited responses at the level of the LA and BLA. Data suggests that extinction is a not an erasure of fear memories, but rather new learning that suppresses fear memories via an inhibitory memory trace. This new learning process may proceed through

multiple mechanisms (Quirk, Pare, et al. 2010). For review of extinction processes, (see review (Myers and Davis 2007)).

While the BLA is critical in mediating cued fear conditioning, studies implicate the hippocampus in contextual fear conditioning (Goosens 2011). It is hypothesized that the hippocampus processes information related to the environment and relays this information to the BLA to be associated with an aversive stimulus. More recent studies have shown medial prefrontal cortex (mPFC) can influence fear learning (see review (Quirk, Garcia, et al. 2006)). The laboratory of Gregory Quirk has shown differential roles for prelimbic and infralimbic subregions of mPFC, where infralimbic activity reduces the expression of conditioned fear while prelimbic activity increases the expression of conditioned fear. The opposing influences of these subregions are thought to occur via activation of different circuits. While the prelimbic subregion sends excitatory input to BLA, the infralimbic projects to a largely GABAergic nucleus adjacent to BLA known as the intercalated mass (ITC) (Vertes 2004). The ITC then sends inhibitory input to the central amygdala, inhibiting output that will control expression of fear.

While the two major neurotransmitter systems in the brain, GABA and glutamate, figure prominently in the fear system, perhaps the study of neuromodulators will yield the most successful therapeutics for the treatment of fear-related disorders. Most neuropeptides modulate the biochemistry of the cell via activation of G-protein coupled receptors. G-protein coupled receptors interact with three main subtypes of G proteins - Gs, Gq, and Gi, and less often Go. G proteins Gs and Gq are generally thought to enhance excitation, as they activate adenylyl cylase, protein kinases, and cause release of intracellular calcium stores. The G proteins Gi and Go, which often couple to the same receptor, are thought to be mainly inhibitory - they activate inwardly rectifying potassium channels and cause inhibition of adenylyl cyclase. These properties of G-protein coupled receptors make them appealing targets for drug development –

they offer finer grade control of neuronal excitation and behavior. In this review, we will discuss behavioral investigations relating to the influence of neuropeptides on fear learning. We will review several of the relevant neuropeptides which have been less examined in recent years, focusing on the opioids, cholecystokinin, and neuropeptide Y. We will not review plasticity-related peptides such as brain derived neurotrophic factor (BDNF), nor corticotrophin releasing factor (CRF), as there are large literatures related to these peptide systems in fear and anxiety models, and have merited reviews of their own.

2.3 Opioids

The endogenous opioid peptides that act throughout the brain and periphery include endorphin, enkephalin, dynorphin, and endomorphin. There are three principal classes of opioid receptors – μ, κ, and δ, although up to 17 have been reported. The opioid receptors belong to the super family of G-protein coupled receptors and generally couple to heterotrimeric Gi/Go proteins, although coupling to Gs has also been reported. Activation of the opioid receptors inhibits adenylyl cyclase and voltage-gated calcium channels while stimulating inwardly rectifying potassium channels and phospholipase C_β (Hughes and Kosterlitz 1983, Waldhoer, Bartlett, et al. 2004). Although the opioid system is most recognized for its role in antinociception, many studies now attribute a memory-based function to the opioids as well. Here we review a large body of evidence implicating endogenous opioids, in particular the μ opioid receptor, in fear learning and extinction (Summarized in **Table 2.3-1**).

Research in the Fanselow laboratory initially demonstrated that pre-treatment with naloxone, an opiate antagonist, increased post-shock freezing levels in rats (Fanselow and Bolles 1979). This effect was dose and shock intensity-dependent. Notably, naloxone pre-treatment did not enhance freezing to one or zero footshocks, an increase was only observed after multiple

footshocks. This suggested that there is release of endorphins to an initial footshock which act as natural analgesics to reduce the aversiveness of subsequent footshocks. A follow up study attempted to determine the locus of naloxone's effects on freezing behavior. Citing an unpublished study and observing that post-shock freezing is due to Pavlovian conditioning of fear to contextual stimuli, the authors proposed that naloxone may increase freezing by enhancing fear conditioning (Erhman 1979). To test this, naloxone was administered intraperitoneally (IP) every day before testing, where each animal was placed in one context (A) for four minutes and then subsequently placed in a different context (B) for four minutes. During the first two days termed "adaptation" subjects were simply observed without administration of footshock. The following 12 days, subjects were shocked in one of the two chambers. This was followed by 8 days of extinction. Naloxone enhanced freezing in the chamber associated with footshock during the extinction phase of the experiment, but not during conditioning, when compared to freezing in the neutral chamber. In a second experiment, the authors used a reduced shock intensity and a greater context shift between chambers to examine whether the effects found in the prior experiment were due directly to context or ceiling effects. The authors found that naloxone also enhanced freezing in the conditioned context during acquisition, indicating that naloxone exerts its effects during conditioning as well as extinction (Fanselow 1981). Together these results were consistent with the hypothesis that endogenous opioids are released at the time of an expected fearful or painful stimulus, possibly as an endogenous protective mechanism to a learned fear response.

These initial studies were unable to distinguish between central and peripheral opioid effects on freezing, as the authors used systemic injections of drugs that readily cross the blood brain barrier (BBB). Fanselow et al used an opioid receptor antagonist, QNTX, which is not able to cross the BBB, to specifically characterize opioid effects on freezing in the periphery. Fanselow and colleagues found that intracerebroventricular (ICV), and not systemic, infusion of QNTX

enhanced freezing, confirming a central effect of endogenous opioids on fear responses. To dissect which of the three opioid receptors are involved in fear acquisition, the authors administered selective antagonists during fear acquisition in a follow up study. In the first experiment, animals received ICV infusions of vehicle, a μ opioid antagonist, a δ opioid antagonist, or a κ opioid antagonist before conditioning. During conditioning, animals received three successive footshocks in the chamber after a three minute acclimation period. The following day, animals were returned to the chamber and freezing behavior was observed. Treatment with a μ opioid antagonist almost doubled freezing levels compared to vehicle administered animals, mimicking effects observed with pre-treatment of naloxone in other studies. In contrast, freezing was attenuated with administration of a κ receptor antagonist, whereas the δ opioid receptor antagonists exerted no effect on freezing levels. These data suggested that the μ opioid receptor is the primary target of endogenous opioids in reducing fear responses.

To further examine specificity, Fanselow and colleagues assessed the contribution of the μ_1 receptor subtype to conditioned freezing by administering a μ_1 receptor antagonist, naloxonazine, prior to training. Pre-treatment with naloxonazine caused enhancement of freezing compared to saline controls (Fanselow, Kim, et al. 1991). They further analyzed μ opioid receptor involvement in fear conditioning using μ opioid receptor (MOR) knockout mice. These mice show enhanced baseline sensitivity to painful stimuli in some tests, such as the tail flick assay and paw pressure test. Notably, no effect of genotype was found with contextual freezing following 5 footshocks when measured 24 hours after fear conditioning. To more sensitively measure differences in learning, the authors administered only a single footshock per day for five days. Freezing behavior pre and post-shock was analyzed each day. There was a slight freezing deficit observed in KOs, with the biggest difference occurring on day 4 and 5. This is surprising, given the pharmacological data showing enhancement of freezing with

administration of a μ opioid receptor antagonist. The authors observed no effect of genotype on footshock reactivity (Sanders, Kieffer, et al. 2005). These findings could be due to compensatory changes which may occur in the endogenous opioid system in a developmental knockout of the MOR.

While the initial fear acquisition opioid studies focused on naloxone interactions with unconditioned stimulus intensity, many studies pointed to opioid modulation of learning without the involvement of footshock. McNally and Westbrook set out to investigate the role of opioids in extinction learning based on preliminary reports that proved to be conflicting (McNally and Westbrook 2003). In experiment 1, the authors wanted to characterize the effects of opioid receptor antagonism on the extinction of Pavlovian fear conditioning in rats. Instead of contextual fear conditioning, the authors used cued fear conditioning, pairing auditory tone with a brief footshock. Naloxone or vehicle was administered systemically before extinction learning 24 hours after fear conditioning. Naloxone impaired extinction learning suggesting that actions at opioid receptors are critical for the extinction of Pavlovian fear conditioning. Experiment 2 was designed to address the question of peripheral versus central opioid involvement in extinction learning. Rats were fear conditioned and then 24 hours later, prior to extinction learning, they were administered vehicle, naloxone, or naloxone methiodide – a derivative of naloxone that cannot cross the blood brain barrier. Only naloxone was able to inhibit a decrease in the fear response, suggesting that central endogenous opioids are required for extinction modulation.

To make sure that opioid peptides were not involved in some sort of impairment of memory processes, the authors examined the effects of post-extinction injections of naloxone on subsequent cued freezing. Rats were fear conditioned and extinction trained as described, however drugs were administered after extinction learning. Rats were placed in one of four

groups receiving either vehicle or naloxone immediately after extinction, naloxone 30 minutes after extinction, or naloxone 120 minutes after extinction. All groups showed an equivalent level of freezing 24 hours later to the conditioned stimulus, suggesting that it is extinction learning and not consolidation of extinction that is critical for opioid involvement, and that administration of naloxone is not involved in memory impairment. In the 4th experiment, the authors demonstrated that opioid receptors regulate the development but not the expression of Pavlovian fear conditioning. Naloxone or vehicle was administered before extinction learning. Naloxone blocked extinction learning as expected. Each group was then administered naloxone or vehicle 24 hours later and tested for expression of fear, yielding four groups – vehicle/vehicle, vehicle/naloxone, naloxone/vehicle, and naloxone/naloxone. Impairment of extinction was observed independently of the presence of naloxone versus vehicle on test, suggesting there is no state-dependent effect on learning. Additionally, injection of naloxone on test did not reverse any extinction. These results reflect similar findings in the Fanselow study suggesting that opioids modulate the learning process. Based on their results, McNally and Westbrook proposed that the endogenous opioids contribute to error correction. To lend support for this hypothesis, McNally and colleagues looked at the effects of naloxone on blocking and overexpectation of fear (McNally and Westbrook 2003). Blocking involves two stages. In the first stage, subjects undergo cued fear conditioning to a CS. In the second stage, the same subjects are presented with the CS plus a different, additional CS, as well as the US. Prior conditioning to the original CS will "block" conditioning from accruing to the new CS despite 100% reinforcement. Overexpectation also involves two stages. In the first stage, subjects are conditioned separately to two different CS. In stage two, half of the subjects receive compound presentations of both CS with the US, while the other half of subjects receive additional training to just one CS. Compound training reduces the amount of fear provoked by either CS alone on a subsequent test. McNally et al found that naloxone prevented both blocking and overexpectation (McNally, Pigg, et al. 2004). From these data, they suggested that the

endogenous opioids may be acting as the error signal that promotes learning during fear conditioning and extinction.

The error correction process occurs when there is a discrepancy between the predicted and actual unconditioned stimulus. When the US is not fully predicted, e.g. during fear conditioning, excitatory learning occurs. This is dependent on repeated pairings of a conditioned stimulus with an unconditioned stimulus. When the US is overpredicted, e.g. during extinction learning, the model proposes that inhibitory learning is occurring. No learning occurs when the US is accurately predicted as when the US has been paired with the CS multiple times (Rescorla 1972). The McNally model predicts that endogenous opioid release represents expected shock input. At the beginning of fear conditioning, the US is not fully predicted and there is no release of opioids. There is a large discrepancy between actual and expected shock and excitatory learning occurs. As CS-US pairings increase, opioids are increasingly released during the CS until the discrepancy between the actual and predicted shock is zero and no further learning occurs. During extinction, there is a large release of endogenous opioids upon presentation of the CS, without reinforcement with shock. Now the discrepancy between expected and actual shock drives inhibitory learning.

Data on the effects of naltrexone in an overshadowing paradigm support the endogenous opioid error signal hypothesis. Overshadowing is similar to blocking in that both suggest fear learning is dependent on the degree to which the US is surprising, i.e. there is a discrepancy between the actual and predicted CS which drives learning. In overshadowing, compound presentation of a light CS and a tone CS with a US reduces the degree to which the light CS can be fear conditioned (Mackintosh 1979). Subjects trained with a tone-light compound froze less to light presentation than subjects just trained to light. The more salient CS (tone) and the US build an association rapidly and bring the discrepancy between the predicted and actual shock to zero,

preventing further learning of an association between the less salient CS and US. Administration of naltrexone attenuated action of endogenous opioids and rescued responding to the light in compound trained animals, thereby preventing overshadowing (Zelikowsky and Fanselow 2010).

Given the great amount of opioid receptors within the PAG and multiple lines of evidence suggesting PAG influence on freezing, McNally and colleagues used microinjections of an opioid receptor antagonist to determine PAG opioid contribution to extinction learning (Carrive 1993, Fanselow, Kim, et al. 1991). Rats received two tone shock pairings . The following five days, subjects received infusions of vehicle or naloxone into ventrolateral PAG (vlPAG) before extinction learning. Naloxone infusions significantly blocked extinction. Rats were then returned to the test chamber and presented with the CS for ten minutes on the sixth day; no differences were observed between freezing while drug-free. The authors also found no differences in freezing levels on a crossover extinction reinstatement test, indicating that naloxone did not alter expression of an already extinguished conditioned response. The authors further analyzed the effects of naloxone on expression of extinction, by administering two days of extinction training plus drug infusion into the vlPAG. There were significant differences between freezing levels in vehicle versus naloxone groups during the drug-free third day of testing. As the dorsal PAG (dPAG) has also been implicated in freezing, the authors examined the effect of microinjection of naloxone into dPAG on extinction learning. The authors did not observe any blockade of extinction; in fact, they saw an enhancement of extinction on the first day of training. There were no differences in freezing levels between groups on a third drug-free test day, indicating infusion of naloxone in dPAG did not impair development of freezing. Finally, the authors demonstrate dose-dependent impairment of extinction with naloxone infusions into the vlPAG. To dissect which opioid receptor mediates opioid-induced blockade of extinction, McNally and colleagues infused antagonists specific to μ, κ, or δ opioid receptors into

the vlPAG. Fear extinction was retarded by infusion of the μ opioid receptor antagonist CTAP into vlPAG prior to extinction training. Given the evidence that activation of opioid receptors can inhibit adenylyl cyclase and decrease intracellular cAMP, the authors next studied the effects of increasing cAMP within vlPAG on extinction behavior. Extinction learning was impaired in a dose-dependent manner by infusion of the membrane permeable cAMP analog 8-Br-cAMP into the vlPAG; however there were no significant differences in extinction behavior with infusion of a PKA activator or an inhibitor of MAPKK/MEK kinase activity c ompared to vehicle (McNally, Lee, et al. 2005). In a separate study, McNally found enhancement of extinction learning with administration of RB101(s), an inhibitor of enkephalin-degrading enzymes (McNally 2005).

Several human studies mirror results observed by McNally and colleagues. In a 1988 study, Kelly Egan and John Carr found that simple phobics who received intravenous injection of naloxone prior to systematic desensitization treatment did not show a reduction in symptomatology (measured by the SCL-90 Global Severity Index), nor a reduction in the number of feared items endorsed as eliciting much or very much fear (Fear Survey Schedule) (Egan, Carr, et al. 1988). Studies by Peter de Jong and Thomas Merluzzi also demonstrate blockade of extinction in spider phobics with administration of naltrexone (Arntz, Merckelbach, et al. 1993).

In an effort to identify more subtypes of the classical opioid receptors, the Opioid Receptor Like 1 (ORL1) was discovered, alternatively known as the nociceptin or orphanin FQ receptor (Meunier, Mollereau, et al. 1995), which we will refer to as the NOP receptor. Although NOP shares a high degree of structural homology with the δ, μ, and κ opioid receptors, it bears no pharmacological homology with the classic opioid receptors. As the BLA expresses a high density of NOP receptors and drugs that act on NOP alter levels of norepinephrine within the

BLA, Roozendaal and colleagues decided to look at the activation of NOP and its effects on step-through latency in the inhibitory avoidance retention test (Roozendaal, Lengvilas, et al. 2007). Immediate post-training infusion of the heptadecapeptide orphanin FQ/nociceptin (OFQ/N) into the BLA induced a dose-dependent impairment of retention. This impairment of retention was replicated when an optimal dose of OFQ/N was infused 3 hours post-training, but not 6 hours – suggesting that OFQ/N modulates consolidation of learning. Post-training infusions of the NOP receptor antagonist into the BLA enhanced retention latencies and co-administration with a beta-adrenergic receptor antagonist, atenolol, blocked this memory enhancement. Atenolol administered alone had no influence on retention latencies. This supports an earlier finding by Manabe and colleagues who showed that deletion of the NOP receptor increased step-through latencies (Manabe, Noda, et al. 1998). The Roozendaal study also supports data from the Grottick group showing increased latency on step-through retention using OFQ/N peptide knockout mice (Higgins, Kew, et al. 2002). These mice also exhibited enhanced fear conditioning, however the authors did not address whether this was contextual versus cued fear conditioning (Higgins, Kew, et al. 2002). To get at effects of OFQ/N on fear conditioning, Fornari and colleagues administered OFQ/N peptide ICV before context and cued fear conditioning. Rats showed impaired context and cued fear conditioning with high doses of OFQ/N, but only an impairment of context conditioning with lower doses. The authors suggest the impairment of cued conditioning at higher doses could be due to non-specific effects. Interestingly, they found no effects on conditioning with post-training infusions of the peptide (Fornari, Soares, et al. 2008).

While studies have demonstrated the importance of amygdala NOP in fear learning, recent evidence has also proven κ opioid receptors (KOR) to be critical at the same locus. Systemic treatment with KOR antagonists attenuated fear-potentiated startle without affecting baseline startle (Knoll, Meloni, et al. 2007). A follow up study by the same group found that this

inhibition of fear-potentiated startle is specific to basolateral and central amygdala, as determined by site-specific infusions of KOR antagonists. The same group also found increased KOR mRNA in the BLA after fear conditioning and decreased mRNA after extinction training (Knoll, Muschamp, et al. 2011).

Altogether, the large body of evidence examining the role of the opioids in fear and anxiety points to a highly critical role played by the endogenous opioid systems in a potential error signal. The model predicts that endogenous opioid release represents expected shock input and the discrepancy between actual shock input and predicted shock input drives learning. This effect has been localized to the ventrolateral PAG. As the opioid system is so divergent, including multiple isoforms of the receptor with various natural ligands at several different levels of the brain, it will be very interesting to narrow in on how the opioid system orchestrates specific functions within the fear response and fear modulation cascade.

2.4 Cholecystokinin (CCK)

Cholecystokinin (CCK) was originally isolated in the gastrointestinal system, but is found extensively throughout the nervous system, with particularly high concentrations distributed throughout the limbic system (Vanderhaeghen, Signeau, et al. 1975). CCK is synthesized as a 115 amino acid preprohormone and is converted into multiple isoforms. The predominant form of CCK in the CNS is a sulfated octapeptide, CCK-8S, however, CCK-8 nonsulphated, CCK-5, and CCK-4 isoforms exist in lesser concentrations within the brain (Derrien, McCort-Tranchepain, et al. 1994, Rehfeld 1985). There are two CCK receptors – CCK-A and CCK-B. Their designations refer to their primary localization, “A” for alimentary and “B” for brain, although CCK-B is found in the stomach and vagus nerve and CCK-A receptor distribution in the brain is wider than originally thought (Hill, Campbell, et al. 1987, Mercer and Beart 2004). Both receptors belong to

the super family of G-protein coupled receptors, and couple to Gq. CCK-A has a high affinity for sulphated CCK-8 (CCK-8S), where CCK-B is equally selective for CCK-8S, non-sulphated CCK-8 (CCK-8N) , CCK-4, and CCK-5 (Fink, Rex, et al. 1998, Lotti and Chang 1989, Schafer, Harhammer, et al. 1994).

Initial behavioral studies showed impairment of acquisition of active avoidance with IP administration of sulphated and non-sulphated CCK-8. Both versions of the peptide were also able to enhance extinction of active avoidance (Fekete, Lengyel, et al. 1984). In a separate study, the authors found no effect of IP injection with CCK-8S or CCK-8N on step-through passive avoidance during the first learning trial. However, when CCK was administered immediately after the first learning trial, latencies significantly increased, suggesting a role for CCK in memory consolidation. The authors were able to replicate these effects with CCK ICV infusion (Kadar, Fekete, et al. 1981). However, according to a review by the Belcheva group, the Fekete studies and other early reports may be slightly contradictory in their proposed roles for CCK due to their use of high doses (Hadjiivanova, Belcheva, et al. 2003). Nevertheless, data has continuously supported the idea that CCK plays a crucial role in anxiety and fear (Summarized in **Table 2.4-1**). CCK-8S and CCK-8N have been shown to increase anxiety-like behavior in elevated plus maze, the marble burying test, light-dark test, and open field test. Pharmacological experiments seem to implicate the CCK-B receptor in mediating these effects (for review, see (Wang, Wong, et al. 2005)).

A report by Claude de Montigny sparked a flurry of interest in CCK when it was found that intravenous (IV) injection of CCK-4 caused panic attacks in healthy subjects. Based on reports of benzodiazepine antagonism of CCK behavioral effects, de Montigny hypothesized that administration of CCK should induce anxiety in human subjects. The author selected the CCK-4 isoform based on chemical properties allowing blood brain barrier passage and maximal

activation of central receptors with minimal peripheral activation. De Montigny also includes an anecdote from a personal communication with JF Rehfeld, who reported “a very unpleasant anxiety” immediately after self-administration. This panicogenic effect found by de Montigny was blocked with pre-treatment of lorazepam, but not meprobamate, or naloxone (de Montigny 1989). This study was followed up by Bradwejn and colleagues, who found that IV CCK-4 induced panic attacks in all subjects previously diagnosed with panic disorder. Panic disorder is a type of anxiety disorder characterized by repeated attacks of intense fear that something bad will occur when not expected. In a second controlled study, Bradwejn found that patients with panic disorder were more sensitive to the panicogenic effect of CCK-4 compared to healthy controls. Although this was not a complete dose-response study with administration of two doses, the results suggest a dose-response effect for duration and time onset until symptoms. The authors suggest that the threshold for panic attack may be lower in those with panic disorder (Bradwejn, Koszycki, et al. 1991). Importantly, the authors found that pre-treatment with a CCK-B receptor antagonist, L-365,260, blocked CCK-4 induced panic attacks in a separate study (Bradwejn, Koszycki, et al. 1994). Jim Abelson and Randolph Neese found a similar sensitivity in patients with panic disorder compared to healthy controls with IV administration of pentagastrin, a synthetic peptide identical to CCK-4 (Abelson and Nesse 1990). Positron emission tomography studies conducted on patients experiencing CCK-4 induced panic attacks show regional cerebral blood flow (rCBF) changes in anterior cingulate gyrus, the claustrum-insular-amygdala region, and cerebellar vermis (Benkelfat, Bradwejn, et al. 1995, Javanmard, Shlik, et al. 1999). Kennedy and Bradwejn found evidence supporting an association between panic disorder and CCK-B, suggesting that a single nucleotide polymorphism in the coding region may confer susceptibility to the disorder (Kennedy, Bradwejn, et al. 1999). Recently, the Estivill group found several human microRNAs that are associated with panic disorder. Micro-RNAs are endogenous small non-coding RNAs that bind to target mRNAs,fine tuning gene expression via translational repression, degradation, and

deadenylation (Bartel 2004). Luciferase assays showed miR-488 and and miR-148 reduced luciferase activity of CCK-B (Muinos-Gimeno, Espinosa-Parrilla, et al. 2011).

Given the increasing amount of data attributing fear and anxiety type properties to CCK, Markus Fendt used the acoustic startle response model to further characterize CCK mechanism of action (Fendt, Koch, et al. 1995). The acoustic startle response pathway is elegantly simple, with inputs from the auditory nerve sending information to the pontine reticular formation (PnC) which project to spinal cord and muscle (Davis, Gendelman, et al. 1982). The PnC receives inputs from the amygdala, central gray, and laterodorsal tegmental area. The authors found that infusion of CCK-8 (the authors do not specify whether they used the sulfated or non-sulfated form of the octapeptide) into PnC potentiated the acoustic startle response. They also found that CCK increased tone evoked activity in PnC neurons by about 30%. In the discussion, the authors suggest that CCK-containing projection neurons from the central amygdala or the midbrain central gray are capable of releasing CCK into the PnC, mediating excitatory effects.

In parallel with the above work, Sheena Josselyn and colleagues found that systemic L-365,260, a CCK-B antagonist, attenuated fear-potentiated startle, but did not alter baseline startle (Josselyn, Frankland, et al. 1995). A follow up study by the same group showed that ICV administration of pentagastrin enhanced acoustic startle, without affecting locomotion (Frankland, Josselyn, et al. 1996). They found a similar behavioral effect with intra-amygdala infusions of pentagastrin, not attributable to changes in locomotion. This potentiation was mildly attenuated with systemic pre-treatment with L-365,260. Infusion of a different CCK-B antagonist into the amygdala blocked potentiation of startle caused by systemic injection of pentagastrin (Frankland, Josselyn, et al. 1997). These findings suggest that the potentiation of startle is mediated by CCK-B in the amygdala, however it does not rule out the contribution of CCK-B in other regions, such as PnC, as suggested by Fendt.

Our laboratory has also shown involvement of the CCK system in extinction learning, suggesting that the effect of CCK may be dependent on endocannabinoid activation. Pentagastrin administered ICV dose-dependently impaired extinction of fear-potentiated startle (Chhatwal, Gutman, et al. 2009). Previous studies have firmly established a specific role in extinction learning for the endocannabinoids. Antagonism of the cannabinoid 1 receptor (Cnr1) blocks extinction of aversive memories across several different paradigms, with a groundbreaking study by the Marsicano study demonstrating that global knockout of Cb1 receptor blocks fear extinction (Marsicano, Wotjak, et al. 2002). Interestingly, the Cnr1-expressing neurons within the amygdala are highly overlapping with CCK-expressing neurons (Mascagni and McDonald 2003). Hippocampal data suggested that Cnr1 activation prevents presynaptic release of CCK. On the heels of this data, Chhatwal and colleagues demonstrated that blockade of fear extinction with a systemic Cnr1 antagonist was reversed with intra-amygdala infusion of a CCK-B antagonist (Chhatwal, Gutman, et al. 2009). These results suggest that the effects of endogenous cannabinoid activation in mediating extinction of fear may be through the prevention of presynaptic CCK release, which may normally serve to maintain fear responses and impair extinction.

Given the role of CCK-B in fear and acoustic startle responses, the Vaccarino group hypothesized that perhaps individual behavioral differences were associated with individual differences in the CCK system. The authors measured fear-potentiated startle responses, acoustic startle responses, and percent time spent in the open arm of an elevated plus maze. Animals were split into high and low responding groups based on mean startle response and on anxiety-like responses in the elevated plus maze. Using autoradiography, the authors found less binding of a CCK-B specific radiolabeled ligand in the BLA and CeA of high fear-potentiated startle responders. They also found less binding in the BLA, but not CeA, in high anxiety-like

responders. They saw no differences in binding between low and high acoustic startle responders. Given the large body of evidence suggesting that increased CCK peptide contributes to high anxiety/fear states, the authors suggest that decreased binding of CCK-B in high responders may be due to receptor down-regulation in response to increased activity (Wunderlich, Raymond, et al. 2002).

Other groups, however, have produced data that conflicts with the results of Vaccarino. Harro and colleagues separated rats into "anxious" and "non-anxious" groups according to time spent in the open arms of an elevated plus maze. They observed decreased numbers of CCK receptors in hippocampus of anxious rats compared to non-anxious rats and increased number of CCK receptors in frontal cortex of anxious rats compared to non-anxious rats (Harro, Kiivet, et al. 1990). When rats are socially isolated, the authors noted a decrease in their exploratory behavior, as well as an increase in CCK receptor binding in the frontal cortex, but not hippocampus (Vasar, Peuranen, et al. 1993). Another group found increased CCK receptor binding in hippocampus in a group of "anxious" rats, as assigned by their behavior in the elevated plus maze assay (Koks, Vasar, et al. 1997). These early studies do not differentiate between CCK-A and CCK-B receptor binding, and none of the binding studies so far have included correlational analyses. Additionally, baseline levels of stress may differ between studies, accounting for differences in binding levels. Nevertheless, these studies are interesting as they contribute to the prediction that dysregulation of the CCK system may play a substantial role in the pathology of fear-related and anxiety disorders.

Around this time, the Koyama group tested the effects of three non-peptide CCK receptor antagonists on rat fear behavior assayed by conditioned fear stress. Rats were individually subjected to five minutes of inescapable footshock – 2.5 mA of scrambled shock presented for 30 seconds on an interval schedule. Twenty-four hours after footshock the animals were

returned to the original chamber and observed for five minutes. Aside from administering a particularly intense and lengthy footshock, conditioned fear stress is nearly identical to contextual fear conditioning. LY288513, a CCK-B antagonist, blocked acquisition of conditioned freezing when administered systemically 30 minutes prior to the footshock conditioning procedure. LY288513 also blocked expression of conditioned fear when administered 30 minutes prior to re-exposure to the conditioned context. LY288513 did not seem to alter consolidation, as administration 5 minutes after conditioning did not affect expression of freezing the following day. A CCK-A antagonist, lorglumide, had no effect on the acquisition of fear, however, it blocked expression of fear at the highest dose administered (Izumi, Inoue, et al. 1996). Another group found a similar effect of rats with PD135158, a different CCK-B antagonist, in the conditioned fear stress paradigm. PD135158 blocked acquisition and expression of conditioned fear but not fear consolidation (Tsutsumi, Akiyoshi, et al. 1999). In a follow-up study, this same group found differences in the conditioned fear stress paradigm following continuous administration of ICV saline, CCK-B antisense, and CCK-B sense oligonucleotides. CCK-B antisense significantly suppressed the expression of conditioned fear, without affecting motor behavior. Autoradiography showed decreased binding in rats infused with CCK-B antisense (Tsutsumi, Akiyoshi, et al. 2001).

Several knockout mouse models have been used to explore the role of CCK-B in fear and anxiety. Raud and colleagues found that CCK-B receptor knockout mice have an anxiolytic phenotype as assayed by dark-light box exploration paradigm and elevated plus maze. There were no significant differences between genotypes in expression of context and or cued fear conditioning, however neither acquisition nor extinction behavior were analyzed (Raud, Innos, et al. 2005). The Tang group overexpressed CCK-B in the mouse forebrain using a tTA/tetO-inducible transgenic approach. The authors propose that CCKergic tone is dependent on receptor number and that enhanced CCKergic tone plays a role in anxiogenesis. The authors

used doxycycline to inhibit transgene expression. Mutant mice (increased CCK-B density) spent less time and made fewer entries into the center of an open field chamber, but exhibited no motor deficits. Doxycycline treatment, which should 'turn-off' the inducible CCKB overexpression, reversed this phenotype. CCK-B overexpression also resulted in increased expression of freezing in the conditioned fear stress paradigm. This result supports prior findings that systemic treatment with CCK-B antagonists blocks expression of conditioned fear stress. Because of previous reports suggesting an antagonistic relationship between GABA and CCK, the authors repeated the open-field test and conditioned fear stress test with administration of diazepam. They found that treatment with diazepam in mutant (CCK-B overexpressing) mice reversed anxiety-like behavior measured by the open-field test. Diazepam also reversed the increase in expression of conditioned freezing observed in mutant mice (Chen, Nakajima, et al. 2006). A follow up study by the Tang group examined the role of CCK-B in mild versus intense contextual fear conditioning. CCK-B overexpression mutants showed impaired expression of contextual freezing with one trial of footshock compared to wild-types. There was an enhanced fear response observed in these same mice with 36 trials of footshock as compared to wild-type. In order to study whether the increased fear response following 36 trials of footshock was relevant to an anxiety-like phenotype, three groups of mutant mice were subjected to no footshock, one trial of footshock, or 36 trials of footshock and were examined by the open-field test. Together with naïve wild-type mice, they found an interaction between the transgene and extensive, but not mild, stress in the anxiogenesis observed. An elevated plus maze test revealed similar results. This study suggests that increased expression of CCK-B disables the turning point from enhancement to impairment of fear memory in response to stress. By testing six groups of wild-type mice to 1, 3, 6, 12, 24, or 36 footshocks in context and cued fear conditioning, they observed a typical inverted "U" shaped freezing curve, where there is an initial enhancement of freezing as the number of trials increases. An impairment of freezing began at 12 trials and decreased further with 24 and 36 footshocks. This "U" curve was

not observed in mutant mice with CCK-B overexpression, who exhibited a linear increase in freezing behavior (Chen, Tang, et al. 2010).

A large amount of research has been driven by cholecystokinin's dramatic panic-inducing effects on humans. Numerous studies have demonstrated CCK to be anxiolytic, utilizing specific pharmacological agents to suggest that this anxiety phenotype is mediated via CCK-B. Additional studies have found that CCK-B agonists potentiate acoustic startle response and block extinction of conditioned fear. Further analysis has shown that these effects may be specific to the amygdala and dependent on cannabinoid receptors. Given new data suggesting more extensive CNS localization of CCK-A, it will be interesting to explore CCK-A's role in anxiety and fear (Mercer and Beart 2004).

2.5 Neuropeptide Y

Neuropeptide Y (NPY) is a 36 amino acid peptide initially discovered as part of the pancreatic polypeptide family (Tatemoto, Carlquist, et al. 1982). Immunocytochemistry and radioimmunoassay show NPY to be the most highly concentrated and widely expressed peptide in the mammalian brain (Allen, Adrian, et al. 1983), exceeding those of cholecystokinin (CCK) and somatostatin. In particular, NPY is notably dense in the cortical, limbic and hypothalamic regions, in particular, basal ganglia, hippocampus, hypothalamus, amygdala, nucleus accumbens, cortex, PAG, and lower brain stem (Adrian, Allen, et al. 1983, Allen, Adrian, et al. 1983, Chronwall, DiMaggio, et al. 1985).

With the highest levels of NPY mRNA being found in the hypothalamic arcuate nucleus (Morris 1989), extensive studies have shown NPY to be critical in stimulating food intake and regulating energy stores (see review (Beck 2006) (Kuo, Kitlinska, et al. 2007). Additionally, NPY is also

found to target the paraventricular nucleus (PVN), where it stimulates synthesis of corticotropin-releasing factor (CRF) (Haas and George 1989) and induces (hypothalamic-pituitary-adrenal) HPA axis stress responses. (Hanson and Dallman 1995, King, Widdowson, et al. 1999, Pomonis, Levine, et al. 1997, White, Dean, et al. 1994). Additionally, literature indicates the role of NPY in circadian rhythms (Yannielli and Harrington 2001), epilepsy (Baraban 2004), addiction (Thiele, Sparta, et al. 2004), reproduction (Kalra and Kalra 2004), immune regulation (Groneberg, Folkerts, et al. 2004), neuroprotection (Silva, Xapelli, et al. 2005) and anxiety and fear (Heilig 2004) (Summarized in **Table 2.5-1**).

There are six known receptors for NPY designated Y_1 through Y_6 (Wahlestedt and Reis 1993), and their effects are mediated by G-protein-coupled downstream signaling (Michel, Lewejohann, et al. 1995). Among these subunit variants, the Y_1, Y_2, Y_4, and Y_5 are functional subtypes located in the human brain (Holmes et al. 2003), and are activated by the three peptides in the neuropeptide Y hormone family: NPY, pancreatic polypeptide, and peptide YY (Lindner, Stichel, et al. 2008). NPY receptors are expressed differentially in many areas of the brain (Dumont, Fournier, et al. 1993) and in particular, with mRNA expression of Y_1, Y_2, Y_4, and Y_5 observed in the amygdala, including the basolateral amygdala.

The expression of NPY-immunoreactive cells have been identified in the amygdala of rat (Chronwall, DiMaggio, et al. 1985) and humans (Caberlotto, Fuxe, et al. 2000, Walter, Mai, et al. 1990). mRNA expression from four functional Y-receptor subtypes (NPY Y1, Y2, Y4, and Y5) has also been observed in the amygdala, including the basolateral amygdala. In contrast, the central amygdala only expresses NPY Y1 and Y5 receptor mRNA (Parker and Herzog 1999, Rostkowski, Teppen, et al. 2009, Wolak, DeJoseph, et al. 2003). Overall, this positions NPY as a prime candidate for the regulation of emotional and learning and memory of fear. The literature indicates NPY to have a major role in regulating anxiety. Intracerebro-ventricular

(ICV) or intra-amygdala infusion of NPY leads to an anxiolytic behavioral profile in several animal models (Britton, Southerland, et al. 1997, Broqua, Wettstein, et al. 1995, Flood, Baker, et al. 1989, Heilig, McLeod, et al. 1992, Heilig 1995, Kokare, Dandekar, et al. 2005, Sajdyk, Vandergriff, et al. 1999). The anxiolytic behavioral effects of NPY seems to be mediated primarily through the Y1 receptor (Heilig, Soderpalm, et al. 1989, Kask, Kivastik, et al. 1999, Sajdyk, Vandergriff, et al. 1999, Wahlestedt and Reis 1993, Wieland, Willim, et al. 1995). Overexpression of NPY in the amygdala attenuated behavioral responses to stress and reduced anxiety-like behavior on the elevated plus maze, while the Y1 antagonist BIBP 3226 also enhanced anxiety (Primeaux, Wilson, et al. 2005). Additionally, Y2 and Y5 receptors have also been implicated (Sajdyk, Schober, et al. 2002, Sajdyk, Schober, et al. 2002). Further, Sajdyk et al. found that injections of NPY into the BLA blocked the anxiogenic effects of a chemical or physical stressor, an effect that persisted for 8 weeks after a series of NPY infusions into the BLA (Sajdyk, Johnson, et al. 2008). Also, ten days of repeated daily stressors caused behavioral habituation and an upregulation of amygdala NPY expression (Thorsell, Carlsson, et al. 1999) – thus NPY may act as a buffer promoting a behavioral adaptation to stress. It was found that acute restraint stress reduced anxiogenic responses on the elevated plus maze for WT but not transgenic rats overexpressing NPY (Thorsell, Michalkiewicz, et al. 2000). Furthermore, another study examined expression of NPY during recovery from a chronic variable stress (CVS) model of repetitive trauma in rats. ELISA for NPY peptide was reduced in the amygdala 7 days after CVS, while a significant increase in prefrontal NPY was observed at the same recovery time-point (McGuire, Larke, et al. 2011).

Neuropeptide Y is implicated in affecting learning and memory through different processes. Following footshock avoidance training in rats, post-training injections of NPY into the amygdala and hippocampus impaired memory retention for footshock avoidance in a T-maze, whereas injection into the rostral hippocampus and septum improved retention (Flood, Baker,

et al. 1989). Furthermore, third ventricular injections of NPY improved consolidation and retrieval in a step-down passive avoidance test (Nakajima, Inui, et al. 1994). In NPY Y2 receptor knockout mice, deficits were observed in the probe trial of the Morris Water Maze task and in an object recognition test (Redrobe, Dumont, et al. 2004).

NPY is ideally expressed and localized to modulate fear learning circuitry, as NPY colocalizes with GABA in local circuit neurons of the BLA (McDonald and Pearson 1989) and likely exerts inhibitory control on BLA projection neurons. Additionally, the NPY Y1 receptor is robustly expressed in the BLA (Rostkowski, Teppen, et al. 2009). Throughout the BLA, Y1r-immunoreactivity was predominately found on soma with negligible fiber staining. High levels of co-expression of Y1r (99.9%) in CaMKII-immunoreactive cells were seen, suggesting thatthese receptors colocalize on pyramidal cells. Further, it suggests that NPY may influence BLA output by directly regulating the activity of these projection neurons. Additionally, Y1r-immunoreactivity was also colocalized with the interneuronal marker, parvalbumin. Parvalbumin interneurons participate in feedforward inhibition of BLA pyramidal cells, representing the largest number of Y1r expressing interneurons in the BLA (but only 4% of the total neuronal population). Therefore NPY could modulate the activity of the BLA via actions on both projection cells and interneuron cell populations.

One report found that ICV injections of NPY did not affect startle amplitude, however it dose-dependently inhibited fear-potentiated startle. Central administration of Y1 agonist increased time in the open arms of the EPM and inhibited FPS, while no such effects were seen with a Y2 agonist (Broqua, Wettstein, et al. 1995). These data indicates NPY to be anxiolytic, but possibly playing important role in blunting fear responsiveness as well.

Additional mouse studies have investigated central administration of NPY, Y1, Y2 and Y5 receptor agonists and a Y1 receptor antagonist on heart rate after fear conditioning (Tovote, Meyer, et al. 2004). With ICV injections 15 min before cued memory recall test, NPY induced bradycardia and blunted the stress-induced tachycardic response. Additionally, Y1 receptor antagonist BIBO 3304 blocked the NPY- and Y1-receptor agonist-induced suppression of conditioned tachycardia without affecting basal HR. The tachycardia elicited by both conditioned and unconditioned stressor was effectively attenuated by the Y1 receptor agonist. These results suggest NPY mediates central inhibition of sympathetic response, through a specific contribution of Y1, but not Y2 and Y5 receptors, to modulate emotional responses. In another experiment, ICV NPY (0.5, 1.0 nmol) produced clear anxiolytic-like effects in the elevated plus-maze and light. NPY (0.5 nmol) also increased locomotor activity in the open field test. In the fear conditioning paradigm, NPY administered prior to training reduced freezing to context (0.5, 1.0 nmol) and auditory cue (1.0 nmol) (Karlsson, Holmes, et al. 2005) 24 and 48 hours later.

Work from our group found that ICV administration of NPY inhibits baseline acoustic startle and expression of fear potentiated startle (FPS) (Gutman, Yang, et al. 2008). Intra-BLA infusions of NPY also inhibited FPS but did not attenuate acoustic startle, while there was no effect of NPY infused into the medial amygdala on fear responses. In contrast, expression of fear was not affected by infusions of a Y1 antagonist (BIBO 3304) into the BLA. Central NPY activation was found to enhance extinction of FPS, and extinction of contextual fear - consistent with the fear expression data. Moreover, infusion of a NPY Y1 antagonist BIBO 3304 into BLA blocks extinction of FPS following conditioned fear in rats (Gutman, Yang, et al. 2008).

Another report utilized conditioned fear in the passive avoidance test, and found that following fear conditioning in rats, there was increased NPY-like immunoreactivity in the amygdala,

hypothalamus, nucleus accumbens, while there was decreased NPY-like immunoreactivity in the frontal cortex (Krysiak, Obuchowicz, et al. 2000). Moreover, diazepam and buspirone dose-dependently inhibited passive avoidance and attenuated the fear induced changes in NPY immunoreactivity. Buspirone attenuated the fear-induced changes in NPY-expression in all regions studied. In the amygdala, the effect of diazepam was dose-dependent. The effect of diazepam on both behavior and NPY-LI was antagonized by flumazenil. Apart from supporting the role of the NPY system in fear and anxiety, the results of this study suggest that NPY is involved in the anxiolytic effects of diazepam and buspirone and that the effect of diazepam is mediated by benzodiazepine receptors.

Using a model of fear incubation, (where mass fear conditioning - 100 tone-shock pairings over 10 days) it was found that both incubated and non-incubated fear responses were attenuated by central administration of NPY (Pickens, Adams-Deutsch, et al. 2009). In contrast, D-Phe CRF(12-41), MTIP, BIBO3304, or BIIE0246 had no effect on conditioned fear at the different time points. Another report found that intra-amygdala injections of NPY decreased the expression of conditioned fear measured by conditioned freezing and fear-potentiated startle (Fendt, Burki, et al. 2009). Additionally, these NPY effects were not replicated by intra-amygdala injections of the Y1R agonists Y-28 or Y-36, and co-infusion of the Y1R antagonist BIBO 3304 did not block the NPY effects. Moreover, Y1R-deficient mice were also fear conditioned and no significant differences between wild type and mutant littermates in fear expression (freezing) were found. Finally, when NPY was injected into the amygdala of Y1R-deficient mice, the local infusion of NPY had no effect on reducing fear.

Most recently, Verma and colleagues performed fear conditioning and extinction on NPY knockout mice as well as Y receptor knockout mice (Y_1, Y_2, Y_4 and Y_1/Y_2 double KO) using a discriminative delay fear-conditioning paradigm. NPYKO mice acquired higher freezing levels

and showed increased expression and impaired extinction of conditioned fear (Verma 2011). Y_1-KO mice show faster conditioning and delayed extinction, whereas Y_2-KO mice are similar to wildtype mice. In contrast, Y_1/Y_2 double KO mice exhibited enhanced fear acquisition and impaired between-session extinction, indicating an important role of Y_2 receptors in these processes. Interestingly, Y_4-KO mice showed normal fear conditioning but impaired extinction. Similarly, adeno-associated viral (AAV) vector-mediated over-expression of NPY in the BLA of NPY-KO mice normalized the increased fear acquisition of NPY-KO mice. In addition, extinction was significantly improved after AAV-induced over-expression of NPY in the BLA of NPY-KO mice (Verma 2011).

Overall the literature consistently demonstrates that NPY within the BLA has an inhibitory role in fear acquisition and facilitates extinction of conditioned fear. Y1R does not appear to be involved in the mediation of the observed intra-amygdala NPY effects suggesting that these effects are mediated via other NPY receptors. However, Y1R may be more important for fear extinction circuitry in the BLA. These effects seem to be mediated predominantly in the BLA. However, the knockout studies suggest the Y_1 receptor may modulate the acquisition of fear (in regions other than the amygdala), whereas extinction may involve $Y_1$and Y_4 receptors. Future studies may further dissect in which regions of the brain NPY is likely regulating fear learning and extinction, as well as the specific NPY receptors involved.

NPY is also thought be an important factor in resilience or development of psychiatric disease states. Abnormally low levels of plasma and cerebrospinal fluid levels of NPY have been found in patients with depression and anxiety disorders (Heilig, Zachrisson, et al. 2004, Rasmusson, Hauger, et al. 2000). Further data indicates that genetic variations of NPY predispose certain individuals to have low NPY levels, which can increase responsiveness to aversive stimuli in the mPFC and anterior cingulate resulting in greater risk to depression and other affective disorders

(Mickey, Zhou, et al. 2011). These findings further for the idea that NPY may be critical to the control of normal emotional responses.

An interesting comparison study investigated resiliency during military survival training (uncontrollable stress / trauma) in terms of neuropeptide regulation (Morgan, Wang, et al. 2000). They compared Special Forces soldiers versus non-Special Forces soldiers, with the hypothesis that enhanced levels of NPY will be associated with resilience against developing stress and trauma related pathology such as PTSD. Interestingly Special Forces had greater increases in plasma NPY levels following interrogation stress, while NPY levels also returned to baseline much more rapidly. In contrast, the non-Special Forces soldiers also had lower levels of NPY compared to Special Forces 24 hours after the trauma exposure. Although this is only correlational data, the higher and more prolonged NPY levels identified in the resilient Special Forces implicate NPY in an important role in controlling stress and fear responsiveness.

PTSD patients are known to have augmented sympathetic responses. Administration of yohimbine, a noradrenergic α(2)-antagonist, has been found to enhance sympathetic responses and PTSD symptoms. Another study found that PTSD patients had lower baseline plasma NPY levels and a blunted increase in NPY following yohimbine administration, compared to healthy controls (Rasmusson, Hauger, et al. 2000). Additionally, the baseline NPY levels were also negatively correlated with combat exposure scale scores and PTSD symptoms. Overall, the findings are consistent with prior data and suggest that combat stress-induced decreases in plasma NPY may mediate, in part, the noradrenergic system hyper-reactivity observed in combat-related PTSD. The persistence of this decrease in plasma NPY may contribute to symptoms of hyperarousal and the expression of exaggerated alarm reactions, anxiety reactions, or both in combat veterans with PTSD.

Consistent with these data, the Yehuda laboratory also found that high levels of NPY are found following trauma in individuals who do not go on to develop PTSD (Yehuda, Brand, et al. 2006). These data are consistent with the previously mentioned increases in NPY expression following fear training in animal models and further support the idea that NPY may be important for resiliency and is protective against the development of fear and trauma related pathology. Consistent evidence in the literature suggests that NPY likely promotes resilience because it blunts fear expression and/or enhances extinction of conditioned fear (Gutman, Yang, et al. 2008).

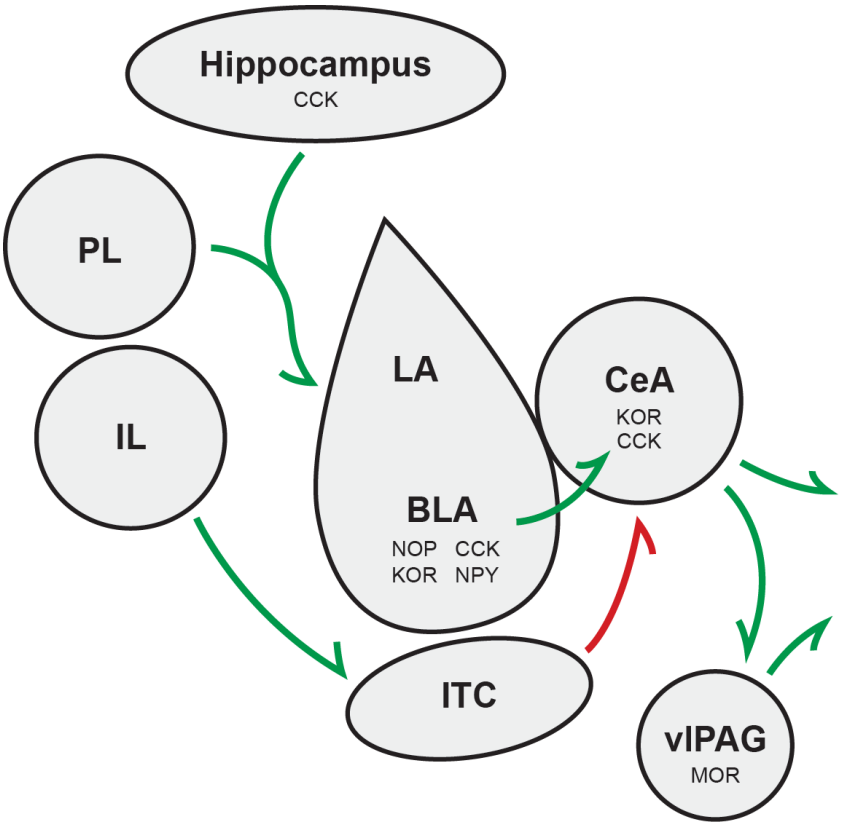
2.6 Discussion

In summary, CCK, opioids and NPY systems each have potent effects on modulating fear and anxiety circuitry in combination with effects on stress responsiveness. While NPY is anxiolytic, and within the BLA has an inhibitory role in fear acquisition and facilitates extinction of conditioned fear, the CCK system is anxiogenic and is critical in the amygdala to drive fear expression or blunt extinction. The opioid system seems to be pivotal for fear acquisition and extinction, driving learning by contributing to error correction. This does not rule out interactions between systems, but suggests unique subpopulations of neurons within the amygdala that may be more specific to on and off of fear expression and extinction. Long term changes in expression are implicated in potential differences in resilience or susceptibility to PTSD, panic attacks or other anxiety disorders. As some of the most abundantly expressed neuropeptides in the brain (CCK and NPY) this makes for attractive drug targets for future pharmacological approaches.

As mentioned, extinction of fear, modeled in the laboratory, is quite similar procedurally to real world inhibition of aversive memories via exposure therapy. Both involve repeated

presentations of the fear-inducing stimulus until the fear behavior is inhibited. As exposure therapy is currently the most effective and prescribed treatment for those with fear-related disorders, learning more about extinction from a basic science perspective is of great interest. For example, D-cycloserine (DCS) as an adjunct to exposure therapy has had promising success in augmenting the treatment of phobias and social anxiety(Norberg, Krystal, et al. 2008). DCS, a partial agonist of the NMDA receptor, was initially found to facilitate extinction learning of conditioned fear in the laboratory (Walker, Ressler, et al. 2002), and then translated to extinction studies in humans(Ressler, Rothbaum, et al. 2004). In this way, studies of conditioned fear and the neuropeptides in the laboratory may be the first step in translating these indications from the bench to the clinic. The neuropeptides are particularly appealing with respect to their modulatory properties – drugs targeting the various neuropeptide systems might be expected to shift extinction learning curves without the danger of neuronal over-excitation. CCK, the opioids, and NPY have each been shown to exhibit some system dysregulation in fear-related disorders, specifically PTSD, specific phobias, and panic disorder. Given the demonstrated role these neuropeptides play in fear-related disorders and the ease of bench to bedside translation, it is expected that future therapeutic strategies will likely exploit these systems.

Figure. 2.2-1 Schematic Diagram of Mammalian Fear Circuitry



Prelimbic (PL) and infralimbic (IL) regions of the medial prefrontal cortex, hippocampus, and amygdala (shown are lateral amygdala (LA), basolateral amygdala (BLA), and central amygdala (CeA) subnuclei) are all regions critical to processing fear; green arrows signify excitatory connections, red arrows represent inhibitory connections from the intercalated cell mass (ITC); some of the neuropeptides discussed here and their respective receptors have been demonstrated to act locally within specific nuclei to effect fear and anxiety behavioral output

Table. 2.3-1 The effect of opioid manipulation on fear/anxiety models

Authors (Year)	Manipulation/Drug Type	Drug Name	Route of Admin	Species	Behavioral Paradigm	Observation
Fanselow (1981)	Broad opioid receptor antagonist	naloxone	IP	Rat (female; Long-Evans)	contextual fear conditioning	Enhanced freezing
Fanselow et al (1988)	Broad opiod receptor antagonist; not BBB permeable	QNTX (naltrexone methobromide)	IP, ICV	Rat (female; Long-Evans)	Contextual fear conditioning	Enhanced freezing with IP, not ICV infusion
Fanselow et al (1991)	μ,δ, and κ opiod receptor antagonists	CTOP and naloxonazine (μ), 16-methyl cyprenorphine and naltrindole (δ), nor-binaltorphimine (κ)	ICV	Rat (female; Long-Evans)	Contextual fear conditioning	Enhanced freezing with μ receptor antagonists
Sanders et al (2005)	μ receptor gene deletion			Mouse (male; C57)	Contextual fear conditioning	Slight freezing deficit
McNally and Westbrook 2003	Broad opioid receptor antagonist	naloxone	SC	Rat (male; Wistar)	Extinction of cued fear	Impaired extinction learning
Zelikowsky and Fanselow (2010)	Broad opioid receptor antagonist	naltrexone	IP	Rat (male; Long-Evans)	overshadowing	Prevention of overshadowing
McNally et al (2004)	Broad opioid receptor antagonist	naloxone	SC	Rat (male; Wistar)	Blocking, overexpectation	Prevention of blocking and overshadowing
McNally et al (2004)	Broad opioid receptor antagonist	naloxone	vlPAG and dPAG infusion	Rat (male; Wistar)	Extinction of cued fear	Blockage of extinction (vlPAG)
McNally et al (2005)	μ,δ, and κ opiod receptor antagonists	CTAP (μ), naltrindole (δ), nor-BNI (κ)	vlPAG infusion	Rat (male; Wistar)	Extinction of cued fear	Blockade of extinction by μ receptor antagonist
Roozendaal et al (2007)	NOP agonist	OFQ/N	BLA infusion	Rat (male; Sprague-Dawley)	Inhibitory avoidance retention	Impairment of retention
Knoll et al (2007)	κ receptor antagonists	Nor-BNI, JDTic	IP	Rat (male; Sprague-Dawley)	EPM, FPS	Decreased anxiety, decreased conditioned fear
Knoll et al (2011)	κ receptor antagonist	JDTic	BLA or CeA infusion	Rat (male; Sprague-Dawley)	EPM, FPS	Decreased anxiety and conditioned fear with BLA and CeA infusion

Table. 2.4-1 Modulation of the cholecystokinin system in fear/anxiety models

Authors (Year)	Manipulation/Drug Type	Drug Name	Route of Admin	Species	Behavioral Paradigm	Observation
Fekete et al (1984)	CCK receptor agonist	CCK-8S, CCK-8N	IP, ICV	Rat (male; Sprague-Dawley)	Active avoidance	Impairment of acquisition; enhancement of extinction
Fekete et al (1981)	CCK receptor agonist	CCK-8S, CCK-8N	IP, ICV	Rat (male; CFY)	Passive avoidance	Enhancement of retention
Fendt et al (1995)	CCK receptor agonist	CCK-8	PnC infusion	Rat (male; Wistar)	ASR	Enhanced ASR
Josselyn et al (1995)	CCK-B antagonist	L-365,260	IP	Rat (male; Wistar)	FPS	Attenuated FPS
Frankland et al (1996)	CCK-B agonist	Pentagastrin	ICV	Rat (Wistar)	ASR	Potentiation of ASR
Frankland et al (1997)	CCK-B agonist and CCK-B antagonist	Pentagastrin and PD135158	ICV (pentagastrin) and intra-BLA (PD-135158)	Rat (Wistar)	ASR	Blockade of potentiation caused by pentagastrin
Chhatwal et al (2009)	CCK-B agonist	Pentagastrin	ICV	Rat (male; Sprague-Dawley)	Extinction of FPS	Blockade of extinction
Chhatwal et al (2009)	Cnr1 antagonist and CCK-B antagonist	SR151716a (Cnr1 antagonist) and CR2945 (CCK-B antagonist)	IP	Rat (male; Sprague-Dawley)	Extinction of FPS	CR2945 reverses blockade of extinction by SR141716a
Izumi et al (1996)	CCK-B antagonist	LY288513	SC	Rat (male; Sprague-Dawley)	Conditioned fear stress	Blockade of acquisition and expression
Tsutsumi et al (1999)	CCK-B antagonist	PD135158		Rat (male; Wistar)	Conditioned fear stress	Blockade of acquisition and expression
Raud et al (2005)	CCK-B gene deletion			Mouse (female; C57)	Dark-light box exploration; EPM	Anxiolytic phenotype
Chen et al (2006)	Forebrain CCK-B overexpression			Mouse	OFT; conditioned fear stress	Anxiogenic phenotype; enhanced freezing

Table. 2.5-1 The effect of NPY manipulation on fear/anxiety models

Authors (Year)	Manipulation/Drug Type	Drug Name	Route of Admin	Species	Behavioral Paradigm	Observation
Flood et al (1989)	NPY receptor agonist	NPY	Local infusion	Mouse (male; CD-1)	Footshock avoidance T-maze	Impairment of retention with amygdalar and hippocampal infusion
Nakajima et al (1994)	NPY receptor agonist	NPY	ICV	Mouse (male; ddY)	Step-down passive avoidance	Enhanced consolidation and retrieval
Broqua et al (1995)	Y1 receptor agonist	[Leu^{31}, Pro^{34}]-NPY	ICV	Rat (male; Sprague-Dawley and Long-Evans)	FPS	Inhibition of FPS
Karlsson et al (2005)	NPY receptor agonist	NPY	ICV	Mouse (male; C57Bl/6)	Cued and contextual fear conditioning	Inhibition of cued and context freezing on test
Gutman et al (2008)	NPY receptor agonist	NPY	BLA infusion	Rat (male; Sprague-Dawley)	FPS and ASR	Inhibition of FPS; no effect on ASR
Gutman et al (2008)	NPY receptor agonist	NPY	ICV	Rat (male; Sprague-Dawley)	Extinction of FPS	Enhancement of extinction of FPS
Gutman et al (2008)	Y1 receptor antagonist	BIBO 3304	BLA infusion	Rat (male; Sprague-Dawley)	Extinction of FPS	Blockade of extinction of FPS
Pickens et al (2009)	NPY receptor agonist	NPY	ICV	Rat (male; Long-Evans)	Fear incubation	Reduced expression of incubated fear
Fendt et al (2009)	NPY receptor agonist	NPY	Amygdala infusion	Mouse (DBA/1J)	FPS and expression of fear conditioning	Reduced freezing and FPS on expression test

Chapter 3:

Interaction between the cholecystokinin and endogenous cannabinoid system in cued fear expression

3.1 Context, Author's Contribution, and Acknowledgement of Reproduction

The following chapter presents evidence of a behavioral and anatomical interaction between the cannabinoid 1 receptor (Cnr1) and the cholecystokinin B receptor (CCKBR) that is critical for fear extinction processes. The context of the study was an effort to better understand a potential interaction between Cnr1 and CCKBR, as Cnr1 is thought to mediate fear inhibition primarily via GABA and GABAergic plasticity. The results of this paper were compared to prior literature and a study from the Ressler laboratory dissecting a Cnr1-CCKBR interaction mediating extinction of fear-potentiated startle in rats. The dissertation author contributed to the paper by designing and running experiments, analyzing the data, and was a main contributor to the writing of the paper. The chapter is reproduced with minor edits from Bowers, M.E. and Ressler, K.J. Interaction between the cholecystokinin and endogenous cannabinoid system in cued fear expression. *Neuropsychopharmacology* (2014)

3.2 Introduction

Evidence suggests that posttraumatic stress disorder (PTSD) and other fear-related disorders might manifest from dysfunction in the inhibition, or extinction, of fear (Myers and Davis 2007). One of the most effective treatment strategies for fear-related disorders is exposure therapy, in which the feared object, context, or memory is repeatedly presented or recalled until fear is inhibited. While exposure therapy is an often prescribed and efficacious treatment, its mechanisms are still poorly understood. Extinction of conditioned fear in animal models can be used as an analog of exposure therapy to try to dissect the mechanisms of fear learning. In this way, translational approaches can be used to augment currently prescribed therapies.

The exposure therapy/extinction literature demonstrates a critical role played by the amygdala in fear learning (LeDoux 2000, Muller, Corodimas, et al. 1997, Quirk, Repa, et al. 1995, Quirk, Likhtik, et al. 2003). The amygdala processes emotionally relevant stimuli via the interactions of neurotransmitters (Bowers, Choi, et al. 2012) and it is highly enriched in a number of neuromodulators, in particular the endogenous cannabinoids and cholecystokinin (CCK) (Herkenham, Lynn, et al. 1990, Larsson and Rehfeld 1979). Studies of the cannabinoid system suggest that the cannabinoid 1 receptor (Cnr1) and the endogenous cannabinoids are critical for emotion, pain, feeding, addiction, anxiety, and memory (Mechoulam and Parker 2013, Richard, Guesdon, et al. 2009). Global knockout or systemic antagonism of Cnr1 increases freezing behavior during a fear expression test and causes a persistent blockade of within-session extinction of cued fear (Marsicano, Wotjak, et al. 2002, Reich, Mohammadi, et al. 2008). Data from the same study demonstrate an increase in the synthesis of the two major endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), in the basolateral amygdala (BLA) during extinction training (Marsicano, Wotjak, et al. 2002).

Intriguingly, Cnr1 shows a high degree of colocalization with CCK in the BLA at the mRNA and protein level (Chhatwal, Gutman, et al. 2009, McDonald and Mascagni 2001). CCK is one of the most highly expressed central nervous system (CNS) neuropeptides, particularly within limbic structures (Mascagni and McDonald 2003, Vanderhaeghen, Signeau, et al. 1975). There are two CCK receptor isoforms – CCKAR and CCKBR (IUPHAR – CCK1/2) (Hill, Campbell, et al. 1987, Mercer and Beart 2004). A number of studies demonstrate a role for CCK in fear and anxiety, primarily through activation of G_q-coupled CCKBR (Areda, Raud, et al. 2006, Bradwejn, Koszycki, et al. 1991, de Montigny 1989, Frankland, Josselyn, et al. 1996, Frankland, Josselyn, et al. 1997, Joseph, Tang, et al. 2013, Josselyn, Frankland, et al. 1995, Rasmussen, Helton, et al. 1993). Interestingly, CCK and the endocannabinoids seem to engender opposite fear responses. CCK elicits panic attacks in humans, and elevates anxiety-like behavior and the expression of

cued fear in rodents (Bradwejn, Koszycki, et al. 1990, Bradwejn, Koszycki, et al. 1991, Chhatwal, Gutman, et al. 2009, de Montigny 1989). In contrast, increasing endocannabinoid tone enhances extinction of cued fear and fear-potentiated startle, and can be anxiolytic (Chhatwal, Davis, et al. 2005, Gunduz-Cinar, MacPherson, et al. 2013). Notably, some data suggest that Cnr1 activation leads to an inhibition of CCK release in the hippocampus (Beinfeld and Connolly 2001).

Although most research has focused on GABA and GABAergic plasticity as the mechanism by which Cnr1 mediates fear inhibition (Azad, Eder, et al. 2003, Azad, Monory, et al. 2004, Kamprath, Romo-Parra, et al. 2011, Katona, Rancz, et al. 2001, Lin, Yang, et al. 2011, Marsicano, Wotjak, et al. 2002, Uriguen, Garcia-Gutierrez, et al. 2011), we hypothesize that the anatomical colocalization of CCK and Cnr1 indicates a potential functional relationship that may be critical for extinction learning. We propose that activation of pre-synaptic Cnr1 during extinction causes a decrease in probability of release (Pertwee 1997, Schlicker and Kathmann 2001) at GABAergic CCK terminals, inhibiting CCK transmission. Thus, by preventing initiation of CCK-activated fear circuitry via CCKBR, Cnr1 promotes inhibition of freezing during cued fear extinction. Here, we test the hypothesis that CCKBR knockout mice will exhibit enhanced extinction of cued fear. Furthermore, we propose that normal blockade of extinction in wild-type mice by a Cnr1 antagonist (Marsicano, Wotjak, et al. 2002) will not be observed in CCKBR knockout littermates, as we hypothesize that Cnr1 is upstream of CCKBR.

3.3 Methods

3.3.1 Animals

Adult male C57BL/6J and 129-*Cckbr*^{tm1Kpn}/J (Jackson Laboratories) 8 to 12 weeks old were group housed in a temperature-controlled (24 ºC) animal colony, with *ad libitum* access to food and water, on a 12 hour light-dark cycle. Experimental animals were genotyped by PCR using primers olMR6447 (reverse: 5' CTTAGCCTGGACAGAGAA GC), olMR6916 (knockout forward: 5' CTTGGGTGGAGAGGCTAT TC), and olMR7283 (wild-type forward: 5' CCAAGCTGCTGGCTAAGAAG). Homozygous CCKBR knockout and wild-type littermates from in house heterozygous breeding pairs were used for experiments. All behavioral procedures were performed during the light cycle. Separate cohorts of transgenic mice were tested on elevated plus maze, open field test, shock reactivity and associative fear learning and extinction paradigms.

3.3.2 Behavior

3.3.2.1 Elevated Plus Maze

Mice were handled once per day for two days prior to testing. Subjects were placed in the elevated plus maze apparatus to explore for 5 minutes in dim lighting. Behavior was hand-scored for time on open arms, time on closed arms, time in center, and number of entries into the open and closed arms.

3.3.2.2 Open Field Test

Mice were handled once per day for two days prior to testing. The open field consisted of an open box (27.9 cm x 27.9 cm) made of PLEXIGLAS. Subjects were placed in the apparatus to explore for 10 minutes, and then returned to their home cage. All testing was conducted under

standard room lighting. Activity data was analyzed using the Open Field Activity Software (Med Associates Inc., St. Albans, VT) for locomotor activity and anxiety-like behavior.

3.3.2.3 Shock Reactivity

Shock reactivity was assayed by averaging immediate shock reactivity to five 0.5 mA shocks separated by a 5 minute inter-trial interval (Med Associates).

3.3.2.4 Associative Fear Conditioning and Extinction

All mice were handled once per day for two days and then pre-exposed once to the test chambers (Med Associates Inc., St Albans, VT) the day prior to training. Fear conditioning and extinction experiments were performed in different contexts, where light, odor, and tactile cues were shifted. FreezeFrame and FreezeView software (Coulbourn Instruments, #ACT-100, Allentown, PA) were used to examine percent time spent freezing during tone presentations as a measure of fear behavior.

For the Cnr1 antagonist (SR141716A) and fatty acid amide (FAAH) inhibitor (URB597) experiments, C57BL/6J mice received two days of 10 paired conditioned stimulus (CS) tones (30 s, 6 kHz, 75-80 dB) which co-terminated with the unconditioned stimulus (US) shock (500 ms, 1.0 mA). Three days after fear conditioning, subjects were presented with a brief fear expression test of 3 CS trials ("grouping"). Average freezing in response to the 3 CS trial test was used to organize subjects into separate groups. The following day, subjects were administered vehicle or drug and exposed to 10 CS trials (30 s tone, 30 s ITI) to assess cued fear expression. Twenty-four hours later, subjects were tested to 15 CS trials (30 s tone, 30 s ITI), off drug, to assess extinction retention. In the SR141716A experiment, one and two subjects were removed from

analysis on extinction retention and fear expression/extinction training days, respectively, as subjects were obscured from the camera and thus not able to be accurately scored.

For experiments with CCKBR transgenic mice, subjects received one day of 5 CS-US pairings. Three days after fear conditioning, subjects were presented with a brief fear expression test of 3 CS trials ("grouping"). Average freezing in response to the 3 CS trial test was used to organize subjects into separate groups. The following day, subjects were administered 30 CS trials (30 s tone, 30 s ITI). Two experiments were conducted with CCKBR transgenic mice. In the first experiment, subjects were not administered drug prior to the cued fear expression test. In the second experiment, subjects were administered vehicle or 3 mg/kg SR141716A 20 minutes prior to the 30 CS fear expression test. In both experiments, freezing to the first 10 CS of the 30 CS trials was averaged and compared between groups to dissociate cued fear expression and within-session extinction. Within-session extinction was analyzed by parsing freezing behavior into three 10 CS bins. Average freezing to CS 11-20 and CS 21-30 were normalized to average freezing during CS 1-10. The extinction retention test occurred similarly in test chambers 24 hours later, off drug, where mice were exposed to 15 trials of the 30 second CS tone (30 s ITI). Extinction retention was defined as average freezing to CS 1-10 to dissociate retention from within-session extinction.

3.3.3 Drugs

The Cnr1 antagonist, SR141716A (Cayman Chemical 9000484), was dissolved in a vehicle of 2.5% DMSO/0.1% Tween-80 in saline to yield a final drug concentration of 3 mg/kg. The FAAH inhibitor, URB597 (Sigma U4133), was dissolved in DMSO and then diluted to 10% to yield final drug concentrations of 0.1, 0.3, and 1 mg/kg. SR141716A and URB597 were systemically administered intraperitoneally (IP) 20 and 30 minutes prior to extinction training, respectively.

3.3.4 Immunohistochemistry

Immunofluorescence experiments were performed on 4% paraformaldehyde-fixed mouse brain sections derived from three adult mice. Animals were anesthetized with sodium pentobarbital and then transcardially perfused with ice-cold 0.05 M phosphate buffer saline (PBS, pH 7.4) followed by 20 mL of 4% paraformaldehyde in PBS. Brains were removed and post-fixed in 4% paraformaldehyde for 2 hours before being cryoprotected in 30% sucrose in PBS for 48 hours at 4°C. Coronal brain sections (45 µm) were cut on a Leica CM 3050S cryostat and stored at 20°C in a cryoprotective medium consisting of 25% glycerol and 30% ethylene glycol in 0.05 M phosphate buffer until needed. Representative sections were rinsed 3 times for 10 min in PBS, permeabilized with 0.5% Triton-X 100 in PBS, and incubated for 48 hours at 4°C with primary antibody in 0.5%Triton-X/PBS solution (all antibodies catalogued in Table 1). Sections were then rinsed three times for 10 min in PBS and incubated at room temperature for 2 hours with either Alexa-Fluor 488 or Alexa-Fluor 568 (1:500, Molecular Probes, Invitrogen, Carlsbad, CA, USA) against the primary antibody's host. Sections were then rinsed two times for 10 min in PBS and one time for 10 minutes in phosphate buffer (PB). Sections were then mounted on glass slides and cover slipped using Mowiol mounting media. Confocal laser scanning microscopy was used to obtain high-resolution photomicrographs using an Orca R2 cooled CCD camera (Hammamatsu, Bridgewater, NJ, USA) mounted on a Leica DM5500B microscope (Leica Microsystems, Bannockburn, IL, USA).

3.3.5 Statistics

Statistics were performed using IBM SPSS Statistics 21 software. Two-tailed, one- or two-way repeated-measures ANOVA, followed by Least Significant Difference (LSD) *post-hoc*

comparisons, or Student's *t*-test (two-tailed) for independent samples were used where appropriate, unless otherwise noted. The results are presented as mean + SEM, with $\alpha \leq 0.05$.

3.4 Results

3.4.1 The cannabinoid system is critical for cued fear expression

To address our overarching hypothesis, we first performed auditory fear conditioning experiments in wild-type mice to assess the role of Cnr1 in extinction learning. Two separate experiments were conducted in which Cnr1 receptor function was blocked or anandamide tone was enhanced via pharmacological intervention. In the first experiment, 3 mg/kg SR141716A, a Cnr1 antagonist, was administered prior to fear expression/extinction training. At a dose of 3 mg/kg, SR141716A has been shown to be effective at blocking extinction in C57BL/6J mice (Marsicano, Wotjak, et al. 2002). In our model, SR141716A treatment significantly increased freezing behavior compared to vehicle group during cued fear expression/extinction training (repeated-measures ANOVA $F_{1,19}=6.06$, $p<0.05$, **Figure 3.4-1A**). Vehicle and SR141716A-treated groups did not differ significantly in freezing behavior the following day on an extinction retention test (**Figure 3.4-1A'**).

Next, we administered the FAAH inhibitor, URB597, prior to cued fear expression/extinction training. FAAH, fatty-acid amide hydrolase, is an enzyme that catalyzes degradation of anandamide, one of the two major endocannabinoids (Cravatt, Giang, et al. 1996). We injected URB597 at a dose of 0.1, 0.3, or 1 mg/kg prior to cued fear expression/extinction training. We found that at a dose of 1 mg/kg, URB597-treated subjects exhibited a significant decrease in freezing behavior during cued fear expression/extinction training (repeated-measures ANOVA $F_{3,26}=3.2$, $p<0.05$, *post hoc* (LSD): vehicle vs. 1 mg/kg, $p<0.05$, **Figure 3.4-2A**). Vehicle- and

URB597-treated groups did not significantly differ in freezing behavior 24 hours later on an extinction retention test (**Figure 3.4-2A'**).

3.4.2 Global CCKB receptor knockout has no effect on baseline measures of weight, shock reactivity, or anxiety-like behavior

Next, we examined the role of CCKBR in anxiety-like behavior and baseline measures of weight, shock reactivity, and locomotion. To do this, we tested mice with a targeted mutation of the CCKB receptor, in which insertion of a neomycin selection cassette deleted sequence-encoding transmembrane domains V through VII. No receptor function was detected in a competition binding assay of brains in mutant mice (Langhans, Rindi, et al. 1997). Body weights were not different between genotypes (**Figure 3.4-3A**), and CCKBR knockouts did not exhibit significantly different shock reactivity relative to wild-type littermates (**Figure 3.4-3B**). Wild-type and CCKBR knockout littermates did not significantly differ on distance traveled during the open field test (**Figure 3.4-4**). Additionally, CCKBR knockout mice did not exhibit an anxiety-like phenotype when tested on an open field (**Figure 3.4-3C**) or elevated plus maze (**Figure 3.4-3D**, **Figure 3.4-5**).

3.4.3 CCKBR knockout mice exhibit normal cued fear acquisition, expression, and extinction

Next, we performed auditory fear conditioning and extinction tests to determine whether knockout of CCKBR has an effect on cued fear learning and memory. Wild-type and CCKBR knockout littermates did not exhibit significant differences in freezing behavior during cued fear acquisition, expression, or extinction retention (**Figure 3.4-6**). Additionally, there was no effect of genotype on rate of within-session extinction during extinction training (**Figure 3.4-**

6). Thus, by our measures, constitutive, global knockout of CCKBR does not affect cued fear acquisition, fear expression, extinction retention, or within-session extinction.

3.4.4 Knockout of CCKBR blunts Cnr1 antagonist-mediated increases in freezing during cued fear expression and extinction retention

To address our central hypothesis of a potential Cnr1-CCKBR interaction, we next administered CCKBR knockout and wild-type littermates with SR141716A prior to cued fear expression/extinction training. We previously demonstrated that acute administration of SR141716A increased freezing behavior during cued fear expression/extinction training in C57BL/6J mice. Here, we examined this effect in CCKBR knockout mice. Here, we examined this effect in CCKBR knockout mice, dissociating cued fear expression and extinction retention from within-session extinction to explicitly examine the effect of SR141716A on each phase of learning. Again, we observed no significant effect of genotype on freezing behavior during cued fear acquisition (**Figure 3.4-7A**). As expected, SR141716A-administered wild-type subjects froze significantly more during the cued fear expression test compared to vehicle-administered wild-type subjects (**Figure 3.4-7B**). In contrast, SR141716A-treated CCKBR knockout mice exhibited virtually identical levels of freezing behavior compared to vehicle-administered CCKBR knockout mice ((**Figure 3.4-7C**) across cued fear expression and extinction retention test days (repeated-measures ANOVA, genotype x drug interaction, $F_{1,55}=2.87$, $p<0.05$, one-tailed). One-tailed statistical analysis was performed as we predicted *a priori* a directional effect of a Cnr1-CCKBR interaction. We did not observe a significant interaction between genotype x drug x test day. *Post hoc* tests reveal a significant difference between wild-type subjects administered vehicle versus wild-type littermates administered SR141716A ($F_{1,31}=4.82$, $p<0.05$) across cued fear expression and extinction retention test days. Statistical analysis revealed a significant effect of CS bin on freezing ($F_{1.56,85.63}=31.49$, $p<0.05$) during training, suggesting that

subjects exhibited within-session extinction; however, we found no significant main effect of genotype or drug, nor an interaction effect, on within-session extinction (**Figure 3.4-7D**). These results suggest that CCKBR is downstream of Cnr1 activation during cued fear expression and extinction retention.

3.4.5 Cnr1-positive fibers form perisomatic baskets around CCKBR-positive cell bodies in the BLA

Next, we performed immunohistochemistry experiments to determine the cellular localization of Cnr1 and CCKBR in the BLA. The amygdala processes emotionally relevant stimuli via the interactions of neurotransmitters (Bowers, Choi, et al. 2012) and is enriched in Cnr1, CCK, and CCKBR (Herkenham, Lynn, et al. 1990, Larsson and Rehfeld 1979, Lein, Hawrylycz, et al. 2007). Prior studies have demonstrated a high degree of colocalization between Cnr1 and CCK mRNA and protein in the BLA (Chhatwal, Gutman, et al. 2009, McDonald and Mascagni 2001). We first performed single-labeling experiments to separately determine immunoreactivity of Cnr1 and CCKBR in the amygdala (for information on development and testing of these antibodies see (Morisset, Julien, et al. 2003, Rooman, Lardon, et al. 2001, Tsou, Brown, et al. 1998)). Cnr1 was detected in the lateral (LA) and basolateral (BLA) amygdala, but only minimally within the central amygdala (CeA) (**Figure 3.4-8A**). CCKBR was detected in all three amygdala subnuclei (LA, BLA, and CeA) (**Figure 3.4-8B**). At higher magnifications, Cnr1 immunoreactivity appeared primarily on fibers in the BLA (**Figure 3.4-8c**). In contrast, CCKBR localized specifically to cell bodies in the amygdala (**Figure 3.4-8D**). To better understand how Cnr1 and CCKBR might functionally interact, we performed serial, double-labeling immunohistochemistry (as antibodies against Cnr1 and CCKBR share the same host). Incubations were performed in the following order: rabbit anti-CCKBR, goat anti-rabbit Alexa-Fluor 488, rabbit anti-Cnr1, goat anti-rabbit Alexa-Fluor 568. High magnification

photomicrographs of the BLA showed Cnr1-positive fibers form perisomatic baskets around CCKBR-positive cell bodies (**Figure 3.4-8AE-F**). We detected ectopic Cnr1 immunoreactivity on cell bodies. This was likely the result of goat anti-rabbit Alexa-Fluor 568 secondary reacting with unbound rabbit anti-CCKBR antibody, rather than true detection of Cnr1 on cell bodies. The results of these immunohistochemistry experiments support our behavioral findings, in that CCKBR appears to be downstream of Cnr1 modulation through perisomatic input of Cnr1-containing synapses onto CCKBR cell bodies.

3.4.6 CCKBR colocalizes with markers for excitatory and inhibitory neurons in the BLA

To generate a clearer picture of how inhibition of CCK might influence BLA neurotransmission, we performed double labeling experiments with antibodies against CCKBR and markers of excitatory and inhibitory neurons. We found that CCKBR colocalizes with calbindin and calretinin (**Figure 3.4-9A-A", C-C").** Calbindin and calretinin are calcium-binding proteins expressed in the two major non-overlapping populations of interneurons in the BLA. Further, CCKBR colocalizes with parvalbumin (**Figure 3.4-9B-B").** Parvalbumin is a calcium-binding protein that is co-expressed in a proportion of calbindin interneurons. Additionally, CCKBR colocalizes with calcium/calmodulin-dependent protein kinase II alpha (CaMKIIα), which is expressed almost exclusively in excitatory cells in the BLA (**Figure 3.4-9D-D"**).

3.5 Discussion

The present study demonstrates: (1) systemic SR141716A, a Cnr1 antagonist, increases cued fear expression in C57BL/6J mice; (2) systemic URB597, an FAAH inhibitor – which increases activation of Cnr1, decreases cued fear expression in C57BL/6J mice; (3) global CCKBR knockout has no effect on weight, shock reactivity, fear- or anxiety-like behavior; (4) SR141716A

increases freezing behavior during cued fear expression and extinction retention tests in wild-type littermates, as expected, but has no effect on freezing behavior in CCKBR knockouts; (5) Cnr1-positive fibers form perisomatic baskets around CCKBR-positive cell bodies in the BLA; and (6) CCKBR colocalizes with markers of both excitatory and inhibitory neurons in the BLA.

As has been demonstrated previously, we find that Cnr1 is critical for inhibition of cued fear (Marsicano, Wotjak, et al. 2002). In this study, we show that manipulation of the cannabinoid system alters cued fear expression. These results are consistent with a prior study testing the effect of the Cnr1 antagonist AM251 on delay fear conditioning (Reich, Mohammadi, et al. 2008). Other studies, however, demonstrate that knockout or antagonism of the Cnr1 receptor has no effect of fear expression, but persistently blocks within-session extinction (Marsicano, Wotjak, et al. 2002).

Additionally, we find that enhancement of anandamide tone via administration of URB597, an FAAH inhibitor, decreases cued fear expression. This is consistent with reports demonstrating that URB597 promotes extinction of conditioned aversion (Manwell, Satvat, et al. 2009). Our laboratory has previously shown that AM404, an inhibitor of anandamide uptake, attenuates fear-potentiated startle (Chhatwal, Davis, et al. 2005). Furthermore, AM3506, a different FAAH inhibitor, decreases freezing behavior during a retrieval test when administered prior to extinction (Gunduz-Cinar, MacPherson, et al. 2013). Human studies demonstrate that carriers of a low-expressing FAAH variant (385A allele; rs 324420) exhibit decreased amygdala reactivity and faster habituation of amygdala reactivity in response to threat. Additionally, these carriers have lower scores on the personality trait of stress-reactivity (Gunduz-Cinar, MacPherson, et al. 2013, Hariri, Gorka, et al. 2009). Importantly, neither SR141716A nor URB597 - at the doses tested - have effects on locomotion (Compton, Aceto, et al. 1996, Tzavara, Davis, et al. 2003)(**Figure 3.4-10**). Altogether, the results of these experiments are in line with

the literature, suggesting that the cannabinoid system plays a critical role in the expression of cued fear.

Separately, we observe no effect of global CCKBR knockout on baseline measures of weight, shock reactivity, locomotion, or anxiety-like behavior in mice. Previous studies show that CCK can increase anxiety, eliciting panic attacks in humans (Bradwejn, Koszycki, et al. 1990, Bradwejn, Koszycki, et al. 1991, de Montigny 1989). However, evidence for the role of CCKBR in rodent anxiety-like behavior is mixed. While some studies show an anxiolytic effect of CCKBR antagonists (Matto, Harro, et al. 1997, Revel, Mennuni, et al. 1998, Tsutsumi, Akiyoshi, et al. 1999), others report null results (Griebel, Perrault, et al. 1997, Johnson and Rodgers 1996). Still, others report that CCKBR antagonists only have anxiolytic effects when co-administered with CCK (Hernandez-Gomez, Aguilar-Roblero, et al. 2002). The mixed conclusions of the literature suggest that CCKBR likely plays a role in anxiety-like behavior, but that the CCK system might be particularly sensitive to environmental conditions, prior stress, and/or specific testing parameters.

We find no significant effect of global CCKBR knockout on a number of measures of cued fear. Although we proposed that CCKBR knockout mice would show enhanced extinction, our results are consistent with our central hypothesis. If we predict that CCKBR activation during cued fear expression/extinction is minimal, due to Cnr1-mediated inhibition of CCK, global CCKBR knockout should not exert an effect on cued fear expression/extinction. Like the anxiety literature, evidence for the role of CCKBR in cued freezing and fear-potentiated startle is somewhat mixed. Work from our laboratory demonstrates that administration of a CCKBR antagonist prior to extinction training has no effect on fear-potentiated startle in rats 48 hours later (Chhatwal, Gutman, et al. 2009). Raud et al. find no differences in cued fear between wild-type and CCKBR knockout littermate females, although extensive testing of extinction was not

conducted (Raud, Innos, et al. 2005). However, pentagastrin, a CCKBR agonist, enhances acoustic startle and blocks extinction of fear-potentiated startle in rats (Chhatwal, Gutman, et al. 2009, Frankland, Josselyn, et al. 1996). Additionally, Josselyn et al. find that administration of a CCKBR antagonist prior to startle testing (after cued fear acquisition) attenuates fear-potentiated startle (Josselyn, Frankland, et al. 1995). The behavioral differences observed across studies might be attributed to species-specific organization of the CCK system (Dietl and Palacios 1989, Kuwahara, Kudoh, et al. 1993, Sekiguchi and Moroji 1986). Additionally, a caveat of our study is the use of a constitutive knockout line with a 129S strain background. Compared to C57BL/6 mice, the 129S strain shows delayed extinction of conditioned fear (Camp, Norcross, et al. 2009, Hefner, Whittle, et al. 2008). Further, future studies should test transgenic strains with precise spatial and temporal control of CCKBR expression to avoid potential genetic compensation issues.

Importantly, we find that Cnr1 antagonist treatment, which increases freezing during a cued fear expression test in wild-type subjects, has no effect on freezing behavior in CCKBR knockout littermates. These results suggest that CCKBR is downstream of Cnr1 activation during cued fear expression. We propose that administration of a Cnr1 antagonist prevents Cnr1-mediated suppression of CCK release and subsequent activation of CCKBR, increasing freezing during cued fear expression. Knockout of CCKBR reverses this Cnr1 antagonist-mediated increase in freezing behavior. Prior data from our laboratory demonstrates that Cnr1 and CCKBR, when using pharmacological probes, interact to mediate extinction of fear-potentiated startle in rats (Chhatwal, Gutman, et al. 2009). Interestingly, the endocannabinoids and cholecystokinin are thought to interact to modulate appetite, via central and/or peripheral mechanisms (Alen, Ramirez-Lopez, et al. 2013, Orio, Crespo, et al. 2011). Slice physiology studies propose an alternative Cnr1-CCK interaction in the hippocampus. According to the “Cnr1 receptor hypothesis”, CCK activation of CCKBR initiates endocannabinoid synthesis, activating pre-

synaptic Cnr1 on CCK-containing interneurons to inhibit GABA transmission. Separately, CCK strongly depolarizes parvalbumin interneurons via CCKBR, increasing firing frequency of inhibitory currents (Foldy, Lee, et al. 2007, Karson, Whittington, et al. 2008, Lee and Soltesz 2011, Lee, Foldy, et al. 2011). Although our results suggest that CCKBR is downstream of Cnr1, cell-type specific behavioral studies will better clarify the differences in our respective models of a Cnr1-CCKBR interaction

Consistently, our results suggest that Cnr1 and a Cnr1-CCKBR interaction is critical to cued fear expression, rather than within-session extinction, as we originally predicted. As mentioned, this effect is in contrast to prior data suggesting that Cnr1 primarily contributes to within-session extinction (Marsicano, Wotjak, et al. 2002, Plendl and Wotjak 2010). However, these studies used individually-housed subjects conditioned with one tone-shock pairing. Individual housing has been shown to have significant effects on fear- and anxiety-like behavior (Voikar, Polus, et al. 2005). In contrast, we fear conditioned group-housed subjects with at least five tone-shocks. From a clinical perspective, this suggests that pharmacological interventions aimed at the CCK and/or cannabinoid systems may differentially affect within- or between-session extinction based on the level of prior trauma and the type of exposure protocol. Likewise, the effect of SR141716A on C57BL/6J versus wild-type CCKBR freezing behavior on extinction retention might be explained by training and strain differences. Future studies should address whether Cnr1 and CCKBR interact to mediate within-session extinction.

Our behavioral results are supported by immunofluorescence evidence, showing that Cnr1-positive fibers form perisomatic baskets around CCKBR-positive cell bodies in the BLA. Although our behavioral experiments were performed systemically, we chose to perform immunofluorescence on the BLA. Ample evidence implicates this area in cue-dependent fear learning (Davis 1992, Fanselow and LeDoux 1999, LeDoux 2000, Maren and Fanselow 1996).

Recent evidence, however, suggests that activity in the medial prefrontal cortex (mPFC) is critical for cued fear learning, in addition to the amygdala (Quirk, Repa, et al. 1995, Quirk, Likhtik, et al. 2003). As anatomical evidence demonstrates that CCK, CCKBR and Cnr1 localize to prefrontal cortex (Herkenham, Lynn, et al. 1990, Larsson and Rehfeld 1979, Zarbin, Innis, et al. 1983) and behavioral studies implicate prefrontal Cnr1 in fear (Ganon-Elazar and Akirav 2013, Kuhnert, Meyer, et al. 2013, Laviolette and Grace 2006, Lin, Mao, et al. 2008, Lin, Mao, et al. 2009), similar immunofluorescence experiments should be performed in mPFC. Additionally, site-specific behavioral experiments should be conducted in follow-up studies to determine whether the observed Cnr1-CCKBR interaction occurs in BLA.

To determine how inhibition of CCK might contribute to cued fear expression, we examined CCKBR immunoreactivity in the amygdala. We initially hypothesized that CCKBR would localize primarily to excitatory projection neurons, as prior evidence suggests that CCK is an axiogenic/panicogenic peptide (de Montigny 1989). We find that CCKBR colocalizes with markers of excitatory projection neurons, as well as local inhibitory neurons in the BLA. This suggests that CCK, as well as putative Cnr1-mediated inhibition of CCK, may exert a complex, computational effect on amygdala-dependent fear circuits. To more quantitatively asses how widely CCKBR is distributed throughout inhibitory and excitatory networks, stereology experiments will need to be performed. Our immunohistochemistry results are consistent with the physiology literature, which shows that CCK excites interneurons and enhances inhibitory transmission in rat BLA projection neurons (Chung and Moore 2007, Chung and Moore 2009). CCK directly initiates an inward, depolarizing current in projection neurons (Meis, Munsch, et al. 2007). CCK-activated currents, via CCKBR, seem to be mediated by TRP channels (Chung and Moore 2009, Meis, Munsch, et al. 2007). CCK, via activation of interneurons - in particular, parvalbumin interneurons, could modulate BLA oscillations. BLA parvalbumin interneurons can innervate approximately 150 projections neurons, a property which is thought to be critical

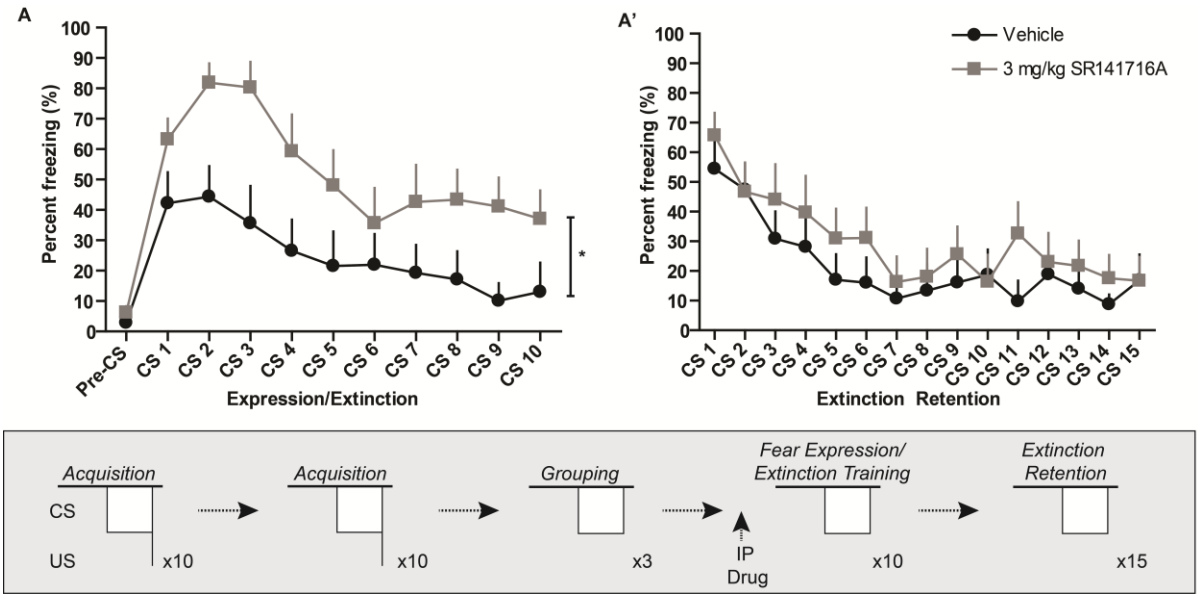
for coordinating projection neuron activity and facilitating oscillations (Muller, Mascagni, et al. 2005, Ryan, Ehrlich, et al. 2012, Woodruff and Sah 2007). These parvalbumin interneurons form perisomatic baskets around projections neuron cell bodies, strongly inhibiting excitatory output (McDonald, Mascagni, et al. 2005). Interestingly, CCK can elicit rhythmic, compound IPSPs in rat BLA projection neurons (Chung and Moore 2009) and activation of Cnr1 has been shown to inhibit hippocampal network oscillations (Hajos, Katona, et al. 2000, Robbe, Montgomery, et al. 2006). In this way, Cnr1-mediated inhibition of CCK could disrupt or dampen synchronous output to the CeA from BLA projection neurons, decreasing activation of the HPA axis, PAG, and other regions critical for mediating fear behavior output.

Taken together, these findings suggest that Cnr1 affects cued fear expression, in part, by decreasing activation of CCKBR, potentially via inhibition of the anxiogenic neuropeptide CCK (**Figure 3.4-11**). Human studies demonstrate that individuals with PTSD have greater CNS Cnr1 availability compared to controls. Elevated Cnr1 availability is thought to be driven by increased receptor upregulation caused by low anandamide levels (Neumeister, Normandin, et al. 2013). Our data suggest that decreased anandamide levels in individuals with PTSD could drive excess/aberrant CCK signaling. In fact, a number of studies support a link between the CCK system and panic, which may share a similar neurobiological mechanism with posttraumatic flashbacks (Bradwejn, Koszycki, et al. 1990, Bradwejn, Koszycki, et al. 1991, de Montigny 1989, Kellner, Wiedemann, et al. 2000, Mellman and Davis 1985).

How, exactly, CCK promotes fear and anxiety is still unclear, but some studies suggest that CCK may act, in part, through the corticotropin-releasing factor (CRF) system (Biro, Sarnyai, et al. 1993, Kellner, Yassouridis, et al. 1997, Shlik, Aluoja, et al. 1997). Our results suggest that BLA CCK likely activates a complex network of excitatory and inhibitory circuitry, which is modulated via Cnr1 regulation. Although more work is needed to clarify the functional

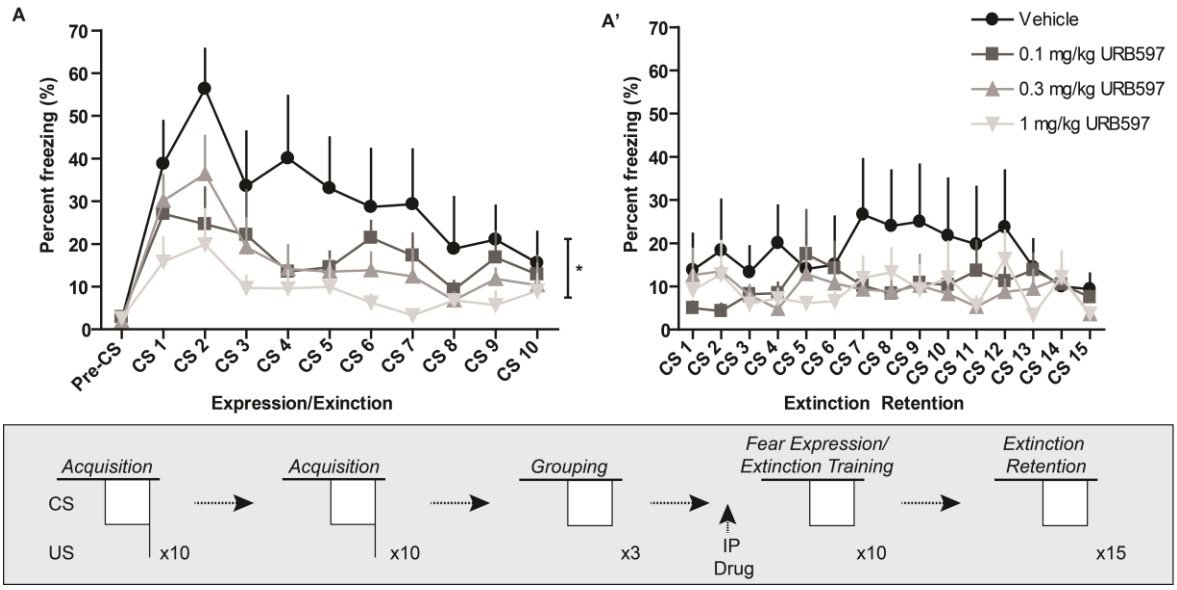
relationship between CCK and Cnr1, the results of this study suggest that dysfunction in a putative Cnr1-CCKBR interaction might be critical to understand the etiology, and ultimately treatment, of fear-related disorders. Indeed, a synthetic cannabinoid has recently been shown to reduce treatment-resistant nightmares in a majority of PTSD patients (Fraser 2009). These studies show promise that the use of CCKBR antagonists alone, or in combination with cannabinoid-targeted treatments, may prove to be ameliorative with exposure-based psychotherapy.

Figure. 3.4-1 Manipulation of the cannabinoid system acutely alters cued fear expression



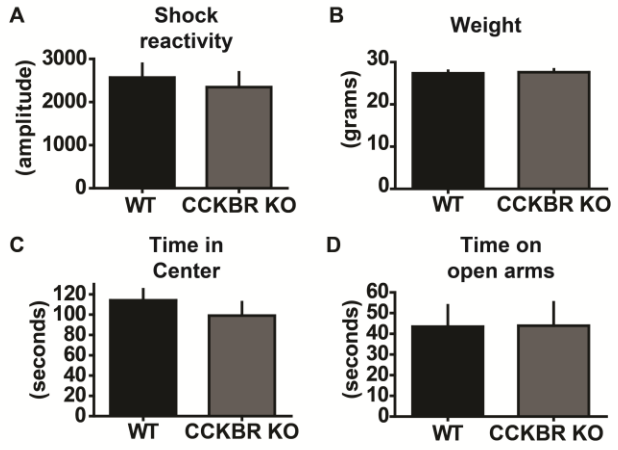
(**A**) Intraperitoneal (IP) administration of the cannabinoid 1 receptor (Cnr1) antagonist, SR141716A, increases freezing during cued fear expression/extinction training. (**A'**) 24 hours after drug administration, vehicle- and SR141716A-administered groups do not exhibit significantly different freezing behavior during an extinction retention test. Average freezing in response to a 3 CS "grouping" test was used to organize subjects into separate groups (grouping data not shown). Asterisk denotes $p<0.05$, main effect of drug.

Figure. 3.4-2 Manipulation of the cannabinoid system acutely alters cued fear expression



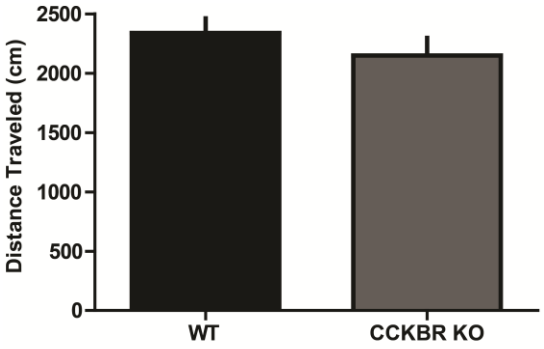
(**A**) IP administration of a fatty acid amide hydrolase (FAAH inhibitor), URB597, decreases freezing during cued fear expression/extinction training at a dose of 1 mg/kg. (**A'**) 24 hours after drug administration, vehicle- and URB597-administered groups do not exhibit significantly different freezing behavior during an extinction retention test. Average freezing in response to a 3 CS "grouping" test was used to organize subjects into separate groups (grouping data not shown). Asterisk denotes $p<0.05$, vehicle versus 1 mg/kg URB597.

Figure. 3.4-3 Baseline measures in cholecystokinin B receptor (CCKBR) knockout mice



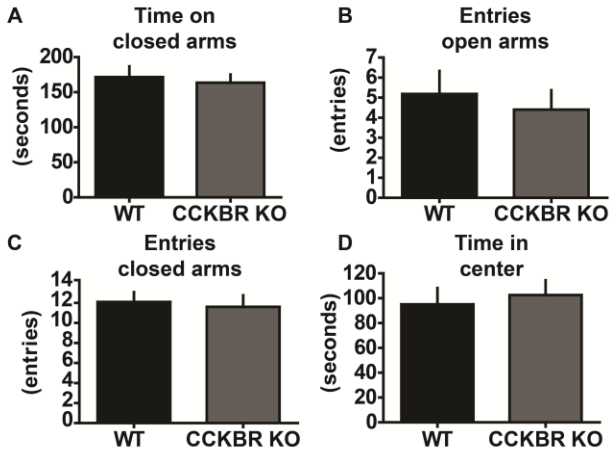
(**A**) CCKBR knockout mice exhibit similar levels of shock reactivity compared to wild-type littermates (**B**) CCKBR knockout mice do not weigh significantly different from wild-type littermates at 9 weeks of age. (**C**) There is no significant effect of genotype on time spent in the center of an open field chamber. (**D**) Additionally, there is no effect of genotype on amount of time on the open arms of an elevated plus maze.

Figure. 3.4-4 CCKBR knockout mice exhibit normal locomotion



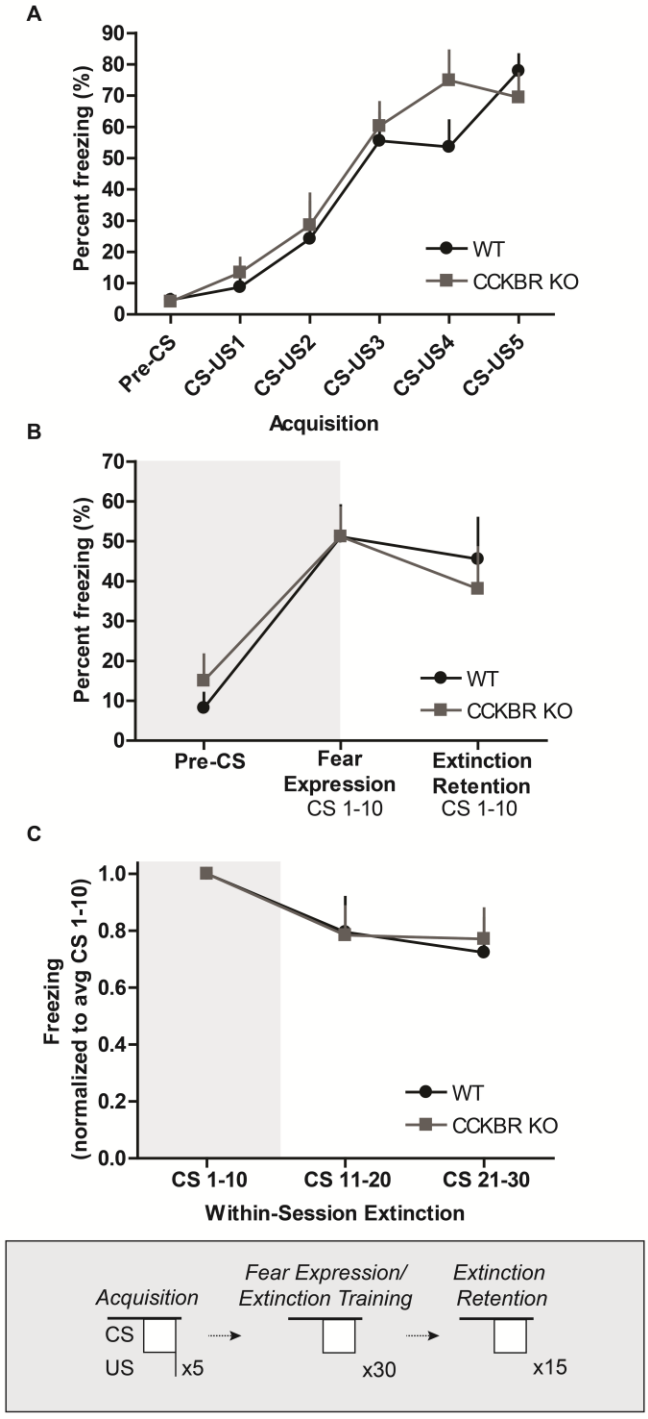
CCKBR knockout mice exhibit normal locomotion. $t(53)=0.99$, NS

Figure. 3.4-5 Wild-type and CCKBR knockout littermates do not differ significantly on elevated plus maze measures of time spent on closed arms, entries on open arms, entries on closed arms, and time in center



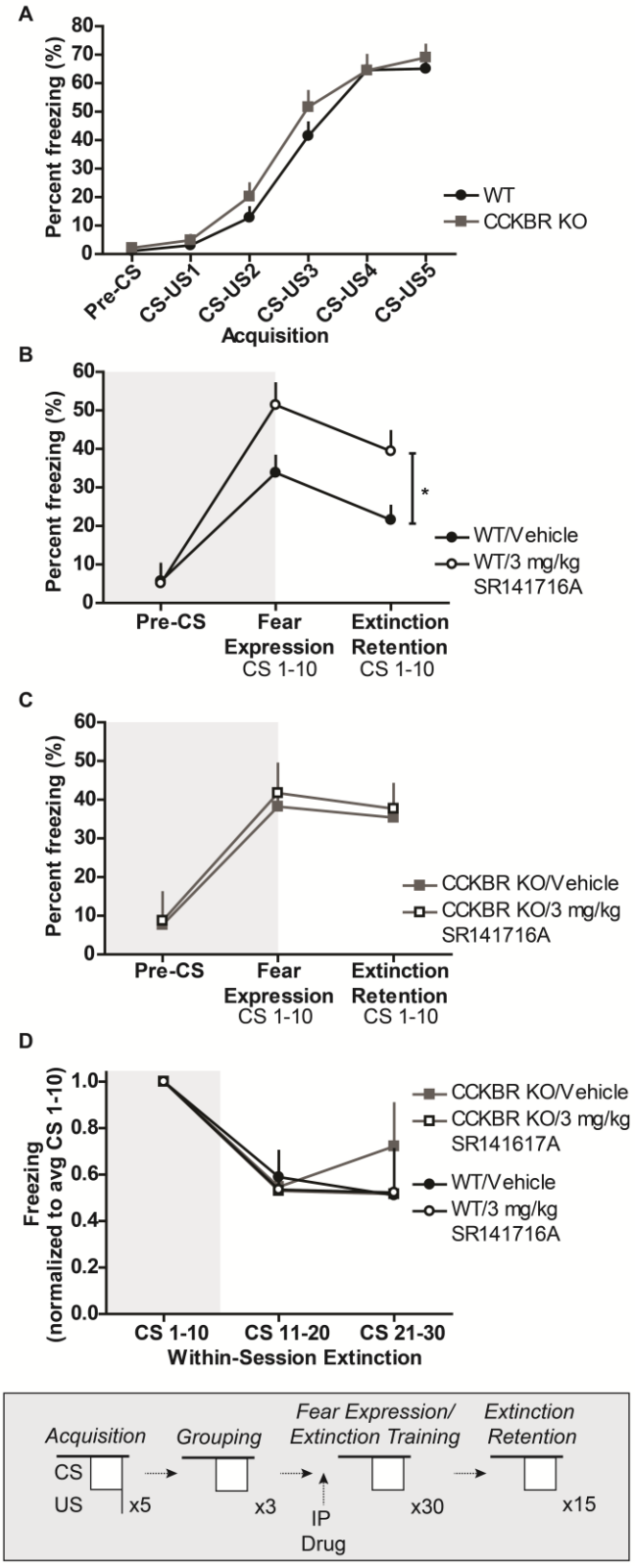
(**A**) Time on closed arms: $t(19)=0.41$, NS. (**B**) Entries open arms: $t(19)=0.51$, NS. (**C**) Entries closed arms: $t(19)=0.33$, NS. (**D**) Time in center $t(19)=0.41$, NS.

Figure. 3.4-6 CCKBR knockout mice exhibit normal cued fear learning



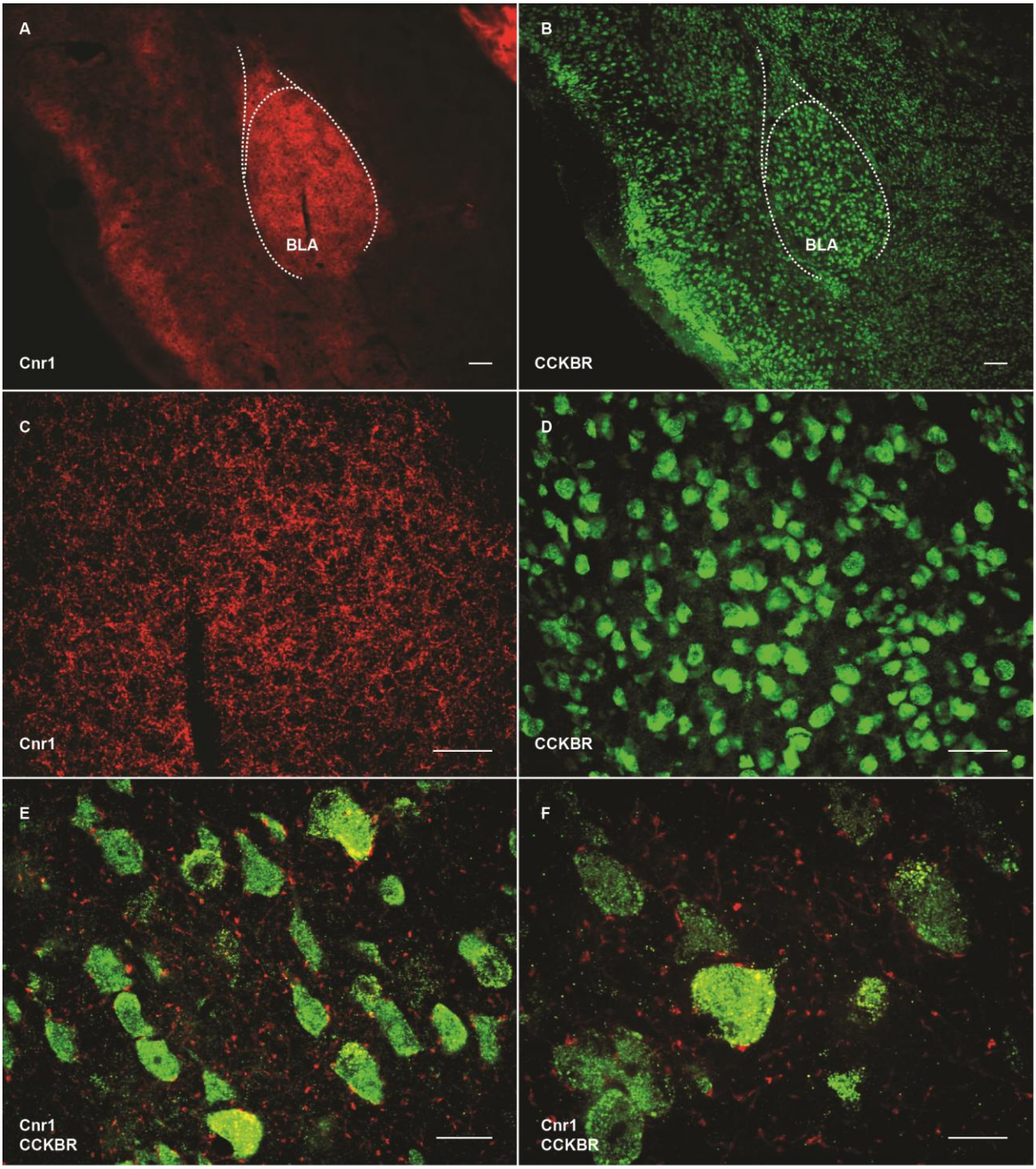
(**A**) CCKBR knockout and wild-type littermates do not exhibit significantly different freezing behavior during cued fear acquisition, (**B**) fear expression, or extinction retention. (**C**) There is no effect of genotype on within-session extinction.

Figure. 3.4-7 Knockout of CCKBR blunts Cnr1 antagonist-mediated increases in freezing across cued fear expression and extinction retention test days



(**A**) CCKBR knockout and wild-type littermates do not exhibit significantly different freezing behavior during cued fear acquisition. (**B**) IP administration of 3 mg/kg SR141716A increases freezing behavior across cued fear expression and extinction retention test days in wild-type mice. (**C**) Vehicle- and SR141716A-treated CCKBR knockout mice do not exhibit significantly different freezing behavior across cued fear expression and extinction retention test days. (**D**) All groups show similar rates of within-session extinction. Average freezing in response to a 3 CS "grouping" test was used to organize subjects into separate groups (grouping data not shown). Asterisk denotes $p<0.05$.

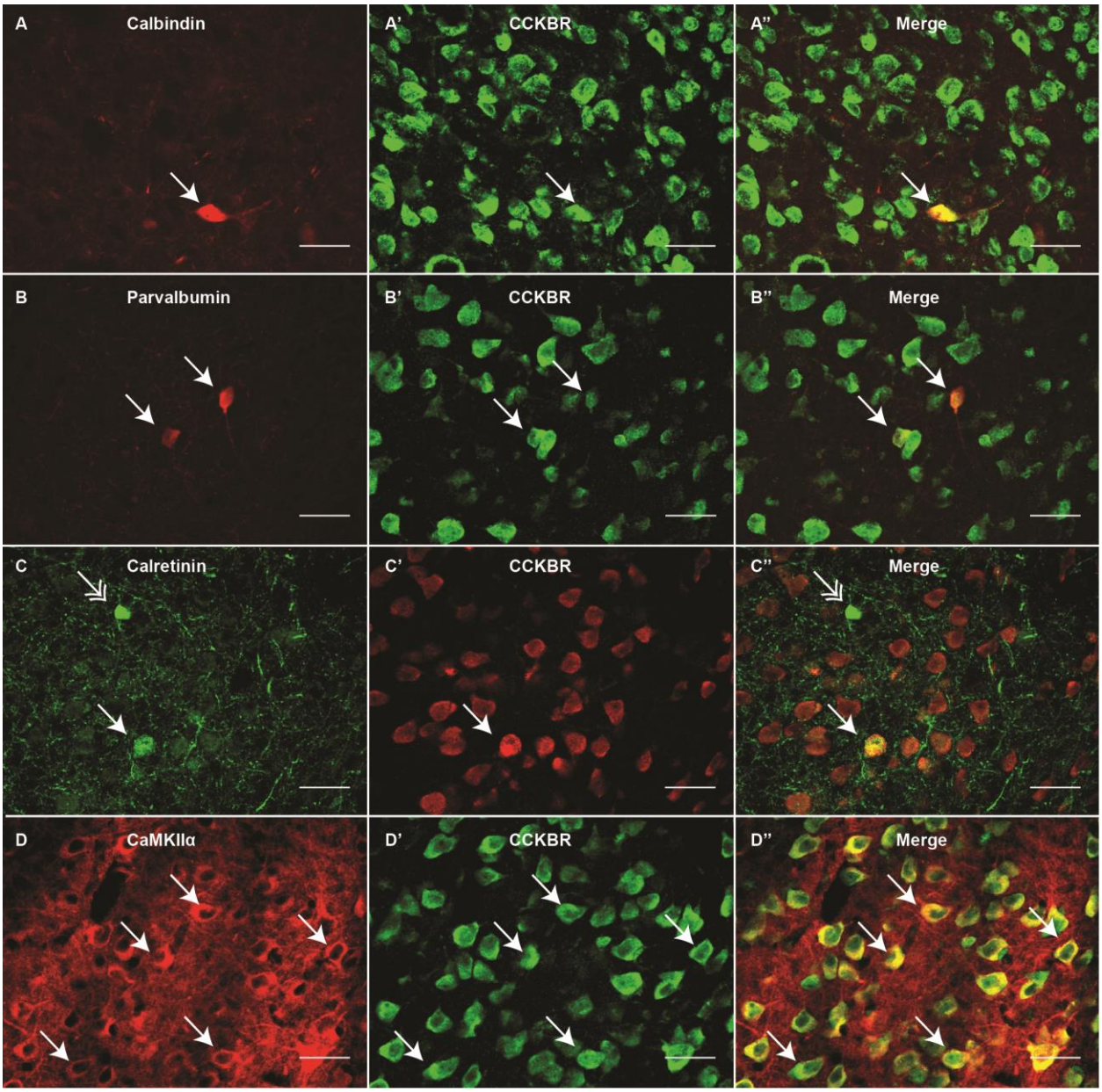
Figure. 3.4-8 Cnr1-positive fibers form perisomatic baskets around CCKBR-positive cell bodies in the BLA



(**A**) Photomicrograph showing immunoreactivity of Cnr1 in the amygdala. Cnr1 localizes to lateral (LA), basolateral (BLA), but not central (CeA) amygdala (4x magnification, 100 μm scale

bar). (**B**) Photomicrograph showing immunoreactivity of CCKBR in the amygdala. CCKBR localizes to LA, BLA, and CeA (4x magnification, 100 μm scale bar). (**C**) Cnr1 localizes to fibers in the BLA (20x magnification, 50 μm scale bar). (**D**) CCKBR localizes to cell bodies in the BLA (20x magnification, 50 μm scale bar). (**E-F**) Photomicrograph showing immunoreactivity of Cnr1 (red) and CCKBR (green) in the BLA (63x and 100x magnification with 15 and 10 μm scale bars, respectively).

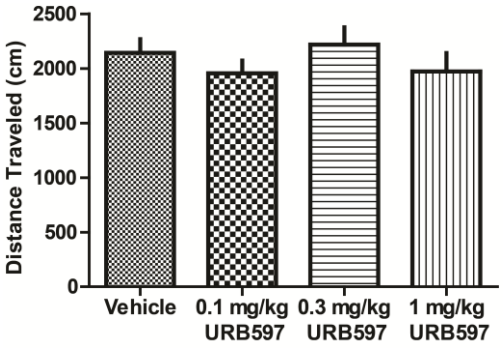
Figure. 3.4-9 CCKBR colocalizes with markers of excitatory and inhibitory neurons in the BLA



(**A-A”**) Photomicrographs showing colocalization of calbindin (**A**, red) and CCKBR (**A’**, green) in the BLA (**A”**, merged). (**B-B”**) Photomicrographs showing colocalization of parvalbumin (**B**, red) and CCKBR (**B’**, green) in the BLA (**B”**, merged). (**C-C”**) Photomicrographs showing colocalization of calretinin (**C**, green) and CCKBR (**C’**, red) in the BLA (**C”**, merged). (**D-D”**) Photomicrograph showing colocalization of calcium/calmodulin-dependent protein kinase II

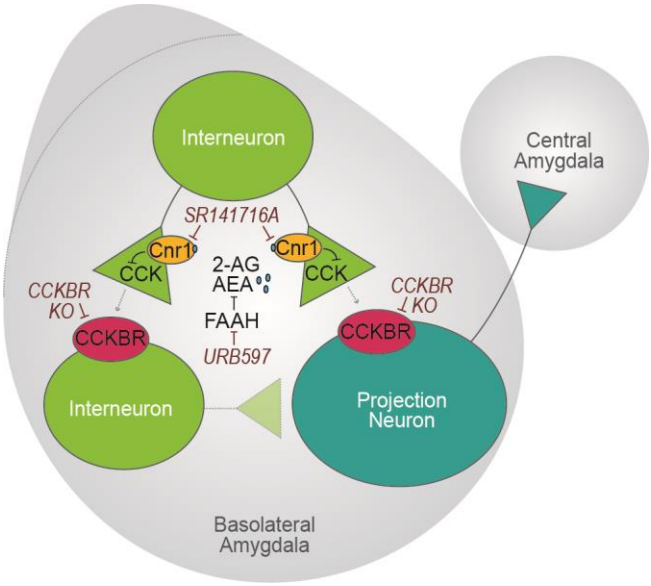
alpha (CaMKIIα) (**D**, red) with CCKBR (**D'**, green) in the BLA (**D"**, merged). (40x magnification, 30 μm scale bar).

Figure. 3.4-10 At a dose of 0.1, 0.3, and 1 mg/kg, URB597 does not affect locomotion



At a dose of 0.1, 0.3, and 1 mg/kg, URB597 does not affect locomotion. $F_{3,29}$=0.83, NS.

Figure3.4-11 Schematic of putative Cnr1-CCKBR interaction during cued fear expression: future directions



We hypothesize that activation of pre-synaptic Cnr1 during cued fear expression decreases probability of release via regulation of ion channels. CCK transmission is inhibited, preventing activation of CCKBR on excitatory and inhibitory neurons in the BLA. As a result, depolarizing TRP (transient receptor potential) channel currents on projection neurons are not activated (Meis, Munsch, et al. 2007). Similarly, CCKBR-induced excitation of inhibitory neurons is unable to coordinate projection neuron firing. We speculate that Cnr1-CCKBR-mediated weakening of projection neuron excitation and potential disruption of BLA oscillations could contribute to decreased signaling to the CeA, thus dampening fear behavior during cued fear expression. In contrast, when Cnr1 activation is inhibited, CCKBR activation leads to enhanced projection neuron firing concomitant with potentially increased interneuronal coordination of firing patterns. Although our experiments suggest that Cnr1 and CCKBR interact to mediate cued fear expression, future experiments will need to address the hypotheses put forth in this schematic.

Table. 3.4-12 Antibodies used in immunohistochemistry experiments

Antibody	Host	Company	Dilution	Catalog Number	Antigen
Parvalbumin	Mouse monoclonal	Swant	1:1000	PV 235	^{45}Ca-binding spot of parvalbumin
CaMKIIα	Mouse monoclonal	Cell Signaling Solutions	1:1000	NB12	~50 kDa phosphorylated and unphosphorylated α subunit
Calbindin	Mouse monoclonal	Sigma	1:1000	C9848	Purified bovine kidney calbindin-D-28K
Calretinin	Goat polyclonal	Millipore	1:2500	AB1550	Rat calretinin
Cnr1	Rabbit polyclonal	Dr. Ken Mackie	1:1000		Last 15 amino acid residues of rat Cnr1
CCKBR	Rabbit polyclonal	CURE/Gastroenteric Biology Center, Antibody/RIA Core/Dr. Bradley Alger	1:1000		Against amino acids 418-429 (C-terminus)

Chapter 4:

Genetic and functional interaction between the endogenous cannabinoid and cholecystokinin systems may underlie expression of cued fear

4.1 Context, Author's Contribution, and Acknowledgement of Reproduction

The following chapter presents evidence of a behavioral, functional, and genetic interaction between the cannabinoid 1 receptor (Cnr1) and the cholecystokinin B receptor (CCKBR). The context of the study was an effort to further elaborate on evidence for an interaction between Cnr1 and CCKBR. The results of this paper were compared to prior literature and our recent paper describing a Cnr1-CCKBR anatomical and behavioral interaction during cued fear expression and extinction retention (Bowers and Ressler 2014). The dissertation author contributed to the paper by designing and running experiments, analyzing the data, and was a main contributor to the writing of the paper. The chapter is reproduced with minor edits from Bowers, M.E., Ehrlich, D.E., Maddox, S.A., Rainnie, D.G., and Ressler, K.J. Genetic and functional interaction between the endogenous cannabinoid and cholecystokinin systems may underlie expression of cued fear. *In preparation*.

4.2 Introduction

Posttraumatic stress disorder (PTSD) is a psychiatric disease that manifests after traumatic experience. PTSD is characterized by symptoms that are organized into three main categories: hyperarousal, re-experiencing, and avoidance (American Psychiatric Association. and American Psychiatric Association. DSM-5 Task Force. 2013). Approximately 94% of trauma survivors will experience acute PTSD-like symptoms, however, for most individuals, these symptoms will abate over time (Kessler, Sonnega, et al. 1995, Yehuda 2002). Because of this, researchers and clinicians hypothesize that PTSD is fundamentally a disorder in the learned inhibition, or extinction, of aversive memory (Yehuda 2004). Consequently, exposure-based psychotherapy - where the feared object, context, or memory, is repeatedly presented or recalled in a safe

environment until fear is inhibited - is one of the most effective treatment strategies for PTSD (Difede, Olden, et al. 2014).

Pavlovian fear conditioning and extinction in rodent models is a valid analog of trauma consolidation and exposure therapy. Procedurally, cued fear extinction and exposure therapy are nearly identical. Furthermore, drugs that enhance extinction learning and retention in animal subjects facilitate fear inhibition in humans when used in combination with exposure therapy. However, selective serotonin reuptake inhibitors (SSRIs) are the only approved pharmacotherapy for PTSD presently, and evidence for therapeutic benefit of SSRI use in combination with exposure therapy is mixed (Burghardt, Sigurdsson, et al. 2013, Deschaux, Spennato, et al. 2011, Lebron-Milad, Tsareva, et al. 2013). Accordingly, Pavlovian fear extinction in rodent subjects is an ideal model to test translationally promising treatment strategies, as exposure therapy is time-consuming and anxiogenic for patients (Kessler 2000).

Consistently, the endogenous cannabinoid system has been implicated in stress, fear, and anxiety across species (Gunduz-Cinar, MacPherson, et al. 2013, Marsicano, Wotjak, et al. 2002, Neumeister, Normandin, et al. 2013). Cnr1 is thought to mediate emotion via modulation of short- and long-term plasticity of the GABA- and glutamatergic systems in limbic regions critical for anxiety, fear, and emotion regulation - the medial prefrontal cortex (mPFC), bed nucleus of the stria terminalis (BNST), and various subnuclei of the amygdala (Lisboa, Reis, et al. 2010, Marsicano, Wotjak, et al. 2002, Puente, Elezgarai, et al. 2010, Ramikie, Nyilas, et al. 2014, Ruehle, Remmers, et al. 2013). In rodents, manipulation of the two major endocannabinoids – anandamine (AEA) and 2-arachidonoylglycerol (2-AG), as well as the cannabinoid 1 receptor (Cnr1), modulates cue and context fear acquisition, expression, and extinction (Bowers and Ressler 2014, Marsicano, Wotjak, et al. 2002, Reich, Mohammadi, et al. 2008). In particular, within-session extinction of cued fear is thought to be controlled via Cnr1 activation in the

amygdala, as increased synthesis of 2-AG and AEA is observed exclusively in the amygdala after a short extinction test(Marsicano, Wotjak, et al. 2002). Interestingly, accumulating evidence suggests that Cnr1 regulates fear via interaction with another neuromodulator – cholecystokinin (CCK) (Bowers and Ressler 2014, Chhatwal, Gutman, et al. 2009). CCK, through activation of the CCK B receptor (CCKBR), is generally categorized as an anxiogenic neuropeptide, as it elicits panic attacks in humans, and increases anxiety-like behavior and cued fear in rodents (Bowers, Choi, et al. 2012, de Montigny 1989).

Here, we present further evidence of a behavioral interaction between the cholecystokinin and endogenous cannabinoid systems in cued fear expression. Administration of a CCKBR antagonist enhances extinction retention in *Cnr1* knockout subjects, but not in wild-type littermates, indicating that global deletion of *Cnr1* enhances CCKBR activation during extinction retention. Although we do not observe blockade of within-session extinction in *Cnr1* knockout subjects as has been previously reported (Marsicano, Wotjak, et al. 2002), and is exhibited by C57BL/6J mice administered a Cnr1 antagonist, this behavioral discrepancy might be explained by differential gene expression of CCKBR in *Cnr1* knockout subjects. Finally, we provide novel evidence of CCK inhibition via Cnr1 activation in the amygdala. Cnr1 inhibition of CCK release supports the hypothesis that activation of Cnr1 during cued fear expression/extinction blocks CCK transmitter release to promote inhibition of fear.

4.3 Methods

4.3.1 Animals

Adult male C57BL/6J and Cnr1 transgenic mice were group housed in a temperature-controlled (24° C) animal colony, with *ad libitum* access to food and water, on a 12 hour light-dark cycle.

Cnr1 transgenic mice were a generous gift from Dr. Carl Lupica at NIDA (Zimmer, Zimmer, et al. 1999). Experimental subjects were genotyped by PCR using primers CB1F (wild-type forward: 5' GTA CCA TCA CCA CAG ACC TCC T), CB1wt (wild-type reverse: 5' GGA TTC AGA ATC ATG AAG CAC TC) and CNK03 (mutant reverse: 5' AAG AAC GAG ATC AGC AGC CTC T). Homozygous Cnr1 knockout and wild-type littermates from in house heterozygous breeding pairs were used for experiments. All behavioral procedures were performed during the light cycle. Separate cohorts of transgenic mice were tested on elevated plus maze, open field test, and associative fear learning and extinction paradigms.

4.3.2 Behavior

4.3.2.1 Elevated plus maze

Subjects were handled once per day for the two days prior to testing. Subjects were placed in the elevated plus maze apparatus to explore for 5 minutes in dim lighting. Behavior was hand-scored for time on open arms, time on closed arms, time in center, and number of entries into the open and closed arms.

4.3.2.2 Open field test

Subjects were handled once per day for two days prior to testing. The open field consisted of an open box (27.9 cm x 27.9 cm) made of PLEXIGLAS. Subjects were placed in the apparatus to explore for 10 minutes, and then returned to their home cage. All testing was conducted under standard room lighting. Activity data was analyzed using the Open Field Activity Software (Med Associates Inc., St. Albans, VT) for locomotor activity (distance traveled in cm over 10 minutes)

and anxiety-like behavior (time spent in center of chamber in sec, where center is defined as 6 cm from the perimeter of chamber walls).

4.3.2.3 Associative fear conditioning and extinction

All mice were handled once per day for two days and then pre-exposed once to the test chambers (Med Associates Inc., St Albans, VT) the day prior to training. Fear conditioning and extinction experiments were performed in different contexts, where light, odor, and tactile cues were shifted. FreezeFrame and FreezeView software (Coulbourn Instruments, #ACT-100, Allentown, PA) were used to examine percent time spent freezing during tone presentations as a measure of fear behavior.

For the experiments shown in **Figure 4.4-2**, **4.4-3**, and **4.4A-B**, the behavioral protocol of Mariscano et al. was replicated (Marsicano, Wotjak, et al. 2002). In **Figure 4.4-2** and **Figure 4.4-3**, *Cnr1* wild-type and knockout littermates received one paired conditioned stimulus (CS) tone (180 sec, 9 kHz, 75-80 dB) which co-terminated with an unconditioned stimulus (US) shock (1 sec, 0.7 mA). Three days after fear conditioning, subjects were exposed to one CS trials (180 sec tone) to assess cued fear expression/extinction. One, two, three, and four days after fear expression/extinction training, subjects were again tested to one CS trial (180 sec tone) to assess extinction retention. For the Cnr1 antagonist experiment depicted in **Figure 4.4-4A-B**, subjects received one day of one paired conditioned stimulus (CS) tone (180 sec, 9 kHz, 75-80 dB) which co-terminated with the unconditioned stimulus (US) shock (1 s, 0.7 mA). Three days after fear conditioning, C57BL/6J mice were injected with vehicle or 3 mg/kg SR141716A, a potent Cnr1 antagonist, 20 minutes prior to cued fear expression/extinction training (one CS trial, 180 s). Subjects were assessed for extinction retention (one CS trial, 180 sec) 24 hours after cued fear expression/extinction training. For experiments shown in **Figure 4.4-4C-D**, the

same behavioral protocol was replicated as in the prior experiments, however the intensity and length of US was reduced to 0.4 mA, 0.5 sec. CS tone (180 sec) was averaged according to 30 second bins for analysis.

4.3.3 Ex vivo CCK release in amygdala slice

Adult C57BL/6J males were decapitated after anesthetization with isoflurane. Brains were sliced in cutting solution (contains (in mM):130 NaCl, 3.5 KCl, 1.1 KH_2PO_4, 6 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 2 kynurenic acid, 30 $NaHCO_3$) bubbling with 95% O_2/5% CO_2 into 300 μm-thick coronal sections using a vibratome. The amygdala was isolated in separate dish using a $2mm^3$ punch instrument. N was equal to approximately 3 bilateral amygdala punches (approximately 6 punches total). Punches were incubated in bubbling cutting solution at 37° C for 40 minutes. For the first experiment, punches were transferred from cutting solution to artificial cerebrospinal fluid (ACSF) with constant bubbling for 20 minutes. Basal ACSF contained 130 mM NaCl, 3.5 mM KCl, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.1 mM KH_2PO_4, 30 mM $NaHCO_3$, and 10 mM glucose pH of 7.4. After 20 minutes, punches were transferred to a 0.5 mL bath of ACSF. After two minutes, the ACSF bath was collected in a microcentrifuge tube and punches were transferred to a separate 0.5 mL bath of high potassium (KCl) ACSF. High KCl ACSF contained 40 mM potassium; equimolar amounts of NaCl was replaced by KCl to maintain iso-osmotic conditions. After two minutes, punches were discarded and the high KCl ACSF bath was collected in a microcentrifuge tube. In the second experiment, punches were transferred from a 40 minute incubation in cutting solution to artificial cerebrospinal fluid (ACSF) with vehicle or 1 μM WIN 55-212,2 with constant bubbling for 20 minutes. Punches were then transferred to a 0.5 mL bath of ACSF with vehicle or 1 μM WIN 55-212,2. After two minutes, punches were discarded and the ACSF bath (with vehicle or 1 μM WIN 55-212,2) was collected in a microcentrifuge tube. All samples were kept at -80° C until immunoassay analysis.

4.3.4 CCK octapeptide (non-sulfated) enzyme immunoassay

The mouse CCK octapeptide (non-sulfated) enzyme immunoassay kit was purchased from Phoenix Pharmaceuticals (EK-069-04). Procedure was followed as indicated by the manufacturer.

4.3.5 Drugs

The Cnr1 antagonist, SR141716A (Cayman Chemical 9000484), was dissolved in a vehicle of 2.5% DMSO/0.1% Tween-80 in saline to yield a final drug concentration of 3 mg/kg. SR141716A was systemically administered intraperitoneally (IP) 20 minutes prior to fear expression/extinction training. The CCKBR antagonist, L-365,260 (L4795 Sigma), was dissolved in a vehicle of 2.5% DMSO/0.1% Tween-80 in saline to yield a final drug concentration of 0.3, 1, and 3 mg/kg. L-365,260 was systemically administered intraperitoneally (IP) 30 minutes prior to fear expression/extinction training. The Cnr1 agonist, WIN 55,212-2 (W102 Sigma), was dissolved in DMSO and added to ACSF for *ex vivo* experiments, yielding a final concentration of 1 μM WIN 55,212-2 at 0.2% DMSO.

4.3.6 RNA extraction, cDNA synthesis, and quantitative PCR (qPCR)

Brains were extracted and frozen on dry ice until processing. $1mm^3$ bilateral punches of the amygdala were taken from each brain using a sliding-freezing microtome and punches were stored at -80°C until processed for RNA extraction. RNA was extracted from bilateral amygdala punches using QIAGEN's RNeasy extraction kit (74104). RLT buffer was added to each sample before being briefly homogenized using a probe homogenizer. RNA extraction then followed the

standard protocol from the kit. Following extractions RNA was quantified using a Nanodrop spectrophotometer and the amount of RNA used for reverse transcription was standardized across all samples. RNA was reverse transcribed to cDNA using QIAGEN's RT² First Strand kit (330421) and processed using the kit provided protocol. cDNA was then used for quantitative polymerase chain reaction (qPCR) analysis to determine gene transcription using Taqman primer assays. All primers were purchased from Life Technologies: CCKBR (Mm00432329_m1) and GAPDH (Mm99999915_g1) to serve as a house keeping control gene. qPCR conditions were as follows: 50 ºC for 2 min, 95 ºC for 10 min, and 40 cycles of 95 ºC for 15 sec and 60 ºC for 1 min. Ct values were normalized using the established 2^ delta delta Ct method and normalized to GAPDH Cts. Wild-type C57BL/6J values were set to 1.0 and all values were expressed as a fold change relative to the wild-type control group.

4.3.7 Statistics

Statistics were performed using IBM SPSS Statistics 21 software. Student's *t*-test (two-tailed) for independent samples or one- or two-way repeated-measures ANOVA were used where appropriate. The results are presented as mean + SEM, with $p \leq 0.05$ as the statistical measure of significance.

4.4 Results

4.4.1 Cnr1 knockout increases anxiety-like behavior

Wild-type and Cnr1 knockout littermates were assessed for anxiety-like behavior and locomotion on an open field and elevated plus maze. We detected significant differences between wild-type and Cnr1 knockouts on open arm time ($t(36)=2.78$, $p \leq 0.05$), open arm

entries ($t(36)=5.34$, $p \leq 0.05$), and closed arm entries ($t(36)=2.24$, $p \leq 0.05$) on the elevated plus maze, indicating that Cnr1 knockout subjects exhibit an increase in anxiety-like behavior (**Figure 4.4-1A-C**). We did not observe a significant difference in closed arm entries (elevated plus maze, **Figure 4.4-1D**) or a significant difference in time spent in center (open field test, **Figure 4.4-1E**). There was a significant difference in distance traveled between wild-type and Cnr1 knockout littermates, indicating that Cnr1 knockouts exhibit differences in activity levels ($t(22)=2.92$, $p \leq 0.05$, **Figure 4.4-1F**).

4.4.2 Administration of the CCKBR antagonist, L-365,260, decreases freezing across multiple days of cued fear extinction in Cnr1 knockout mice, not in wild-type littermates

To address our central hypothesis – a Cnr1/CCKBR interaction is critical for cued fear behavior – we administered the CCKBR antagonist, L-365,260, at a dose of 0.3, 1, and 3 mg/kg prior to a fear expression/extinction test. At a dose of 1 mg/kg, we observed a significant drug x genotype interaction on extinction retention day 1 ($F_{1,31}=4.75$, $p \leq 0.05$), extinction retention day 3 ($_{F1,31}=5.60$, $p \leq 0.05$, not shown), and across all five extinction days ($F_{1,31}=5.27$, $p \leq 0.05$). We observed a drug x genotype interaction trend during cued fear expression/extinction training ($F_{1,31}=4.04$, $p=0.053$, **Figure 4.4-2D**) and extinction retention day 2 ($F_{1,31}=3.69$, $p=0.06$, not shown). *Post hoc* analysis revealed a significant difference between *Cnr1* knockout subjects administered vehicle versus 1 mg/kg L-365,260 on extinction retention day 1 ($F_{1,13}=4.90$, $p \leq 0.05$, **Figure4.4-2E**), extinction retention day 3 ($F_{1,13}=5.92$, $p \leq 0.05$), and across days ($F_{1,13}=5.86$, $p \leq 0.05$, **Figure 4.4-2F**). We did not observe a difference in freezing behavior between wild-type littermates administered vehicle versus 1 mg/kg L-365,260 during cued fear expression, extinction retention days 1-4, or across days (**Figure 4.4-2A-C**). Statistical analysis revealed a main effect of genotype during cued fear expression/extinction training, on extinction retention days 1 and 2, and across days, however, *post hoc* testing suggested that this was an

artifact of a genotype x drug interaction, as we did not detect a significant difference in freezing behavior between wild-type and *Cnr1* knockout littermates. However, we detected a genotype x day interaction ($F_{4,64}$=3.41, p≤0.05). In fact, wild-type ($F_{4,36}$=7.82, p≤0.001), but not *Cnr1* knockout subjects ($F_{4,28}$=0.09, p=0.98), exhibit a significant difference in freezing behavior across days, indicating that wild-type subjects - but not *Cnr1* knockout mice - extinguish normally after multiple days of training. We did not detect a main effect of drug or genotype, or an interaction, in our statistical analysis of 0.3 and 3 mg/kg doses of L-365,260 (**Figure 4.4-3**).

4.4.3 Administration of a Cnr1 antagonist, SR141716A, blocks within-session extinction of cued freezing; expression of CCBR mRNA may underlie differences in within-session extinction between C57BL/6J versus Cnr1 transgenic mice

Similar to the effect observed in Marsicano *et al.*, vehicle and SR141716A-treated subjects did not differ in their initial freezing response to the CS. However, across multiple CS bins, we detected a significant drug x 30 s CS bin interaction during extinction training ($F_{5,120}$=2.68, p≤0.05, **Figure 4.4-4A**), indicating a significant difference between drug groups on within-session extinction. Similarly, we observed a drug x 30 s CS bin interaction trend the following day when analyzing extinction retention ($F_{5,120}$=2.63, p=0.058, **Figure 4.4-4B**). To try to dissect differences in within-session extinction behavior between wild-type and Cnr1 knockout subjects, we fear conditioned wild-type and *Cnr1* knockout littermates with a milder unconditioned stimulus (0.4 mA, 0.5 sec). We hypothesized that differences in within-session extinction behavior between wild-type and Cnr1 knockout subjects were masked by the intensity of fear conditioning in the prior experiments. Using a milder fear conditioning protocol, however, we did not detect a significant main effect of genotype, or a genotype x 30 s CS bin interaction during cued fear expression/extinction or during an extinction retention test (**Figure 4.4-4C-D**). We hypothesized that *Cnr1* knockout littermates might not exhibit a

blockade in within-session extinction, as previously reported and observed in Cnr1 antagonist administered subjects, due to genetic compensation of the CCK system. Specifically, we predicted that *Cnr1* knockout subjects would express less CCKBR compared to wild-types. Although, according to our model, *Cnr1* knockout subjects release more CCK during cued fear expression (due to removal of Cnr1-mediate inhibition of CCK release), we hypothesized that decreased CCKBR expression might explain a milder deficit in extinction behavior exhibited by *Cnr1* knockout subjects. To test this hypothesis, we performed quantitative PCR (qPCR) for CCKBR on bi-lateral amygdala punches of age-matched C57BL/6J mice, *Cnr1*-wild-type, and *Cnr1*-knockout subjects (*Cnr1*-wild-type and *Cnr1*-knockout subjects were littermates). Interestingly, one-way ANOVA revealed a significant difference between groups ($F_{2,27}$=4.75, p≤0.05), where *Cnr1* knockout subjects exhibit higher levels of CCKBR mRNA in the amygdala compared to C57BL/6J mice and *Cnr1*-wild-type littermates (p≤0.05, **Figure 4.4-4E**). This suggests that there is dynamic genetic regulation of the CCK system with knockout of *Cnr1*, and that differential expression of CCK family genes might account for differences in extinction behavior between *Cnr1* genotypes.

4.4.4 Cnr1 activation inhibits release of CCK from amygdala punch

Our behavioral results suggest that there is endogenous activation of CCKBR in *Cnr1* knockout subjects during fear expression/extinction, but not in wild-type littermates, as CCKBR antagonist administration decreases freezing behavior in *Cnr1* knockout subjects, but has no behavioral effect in wild-type littermates. These results might be explained by an increase in CCK release in *Cnr1* knockout subjects during cued fear expression/extinction, supporting our hypothesis that activation of Cnr1 inhibits CCK transmission during cued fear expression/extinction. To test this hypothesis, we developed an *ex vivo* assay based on the work of Beinfeld and Connolly (Beinfeld and Connolly 2001). Here, we tested CCK release from

amygdala punches in response to high potassium (KCl) ACSF and in response to the Cnr1 agonist, WIN 55,212-2. We focused on the amygdala, given the overwhelming evidence implicating the amygdala in cued fear acquisition and extinction. Furthermore, anatomical and physiological data suggest that there is an increase in the synthesis of the endocannabinoids specifically in the amygdala (not mPFC) with cued fear extinction and that Cnr1-positive fibers form perisomatic baskets around CCKBR positive cells bodies in the BLA. In our first experiment, we bath applied high KCl ACSF to depolarize neuronal membrane potential in order to induce neurotransmitter release. Importantly, we found a significant increase in CCK release from the amygdala with bath application of high KCl ACSF (paired-samples *t* test, $t(13)=-2.95$, $p \leq 0.05$, **Figure 4.4-5A**), indicating that 40 mM potassium is sufficient to induce neurotransmitter release, in particular, CCK release. Furthermore, we observed a significant decrease in CCK release with administration of 1 μM WIN 55,212-2, a Cnr1 agonist (independent samples *t* test, $t(11)=2.42$, $p \leq 0.05$, **Figure 4.4-5B**). This suggests that activation of Cnr1 inhibits release of CCK in the amygdala.

4.5 Discussion

Here, we propose that activation of Cnr1 during cued fear expression inhibits release of the anxiogenic neuropeptide CCK to promote fear inhibition. We find: 1.) *Cnr1* knockout subjects exhibit increased anxiety-like behavior compared to wild-type littermates; 2.) administration of a CCKBR antagonist decreases freezing during cued fear expression and extinction retention in *Cnr1* knockout subjects, but not in wild-type littermates; 3.) *Cnr1* knockout subjects exhibit increased freezing across multiple days of extinction training compared to wild-type littermates, however *Cnr1* knockout subjects do not exhibit blockade of within-session extinction as has been previously reported and is observed in Cnr1 antagonist-treated mice; 4.) *Cnr1* knockout subjects exhibit increased CCKBR mRNA expression in the amygdala compared to Cnr1 wild-

type littermates and C57BL/6J subjects; finally, 5.) activation of Cnr1 in the amygdala inhibits release of CCK.

Cnr1 knockouts exhibit increased anxiety-like behavior, spending less time on the open arms, making fewer open arm entries, and spending more time on the closed arms of an elevated plus maze. The cannabinoid system is consistently implicated in anxiety, where *Cnr1* knockout or antagonist treatment increases rodent anxiety-like behavior on a number of different paradigms (Ruehle, Rey, et al. 2012). Cnr1 is thought to regulate anxiety-like behavior via modulation of excitatory and inhibitory input onto BNST neurons with downstream projections to the hypothalamus (Puente, Elezgarai, et al. 2010, Ruehle, Remmers, et al. 2013). The results of the present study confirm the findings of an anxiety-like behavioral phenotype in *Cnr1* knockout subjects reported in the literature.

Importantly, we report a drug x genotype behavioral interaction between Cnr1 and CCKBR, where administration of the CCKBR antagonist significantly decreases freezing behavior in *Cnr1* knockouts, but not wild-type littermates, across multiple days of extinction. Although we are only able to detect a significant interaction on extinction retention day 1, day 3, and across days, we observe a trend towards interaction between drug treatment and genotype during cued fear expression/extinction training day 1 ($p=0.053$), suggesting that Cnr1 and CCKBR likely interact to mediate cued fear expression. This supports our prior finding that Cnr1 antagonist treatment increases freezing across cued fear expression and extinction retention days in wild-type subjects, but not *CCKBR* knockout littermates (Bowers and Ressler 2014). Results from amygdala immunohistochemistry experiments match what is predicted by the behavioral results of the present study – CCK and Cnr1 colocalize at the mRNA and protein level, Cnr1-positive fibers form perisomatic baskets are CCKBR-positive cell bodies, and diacylglyerol lipase (DGLα, which synthesizes 2-AG, one of the major endocannabinoids) clusters selectively appose

interneuron terminals expressing Cnr1 and CCK (Bowers and Ressler 2014, Chhatwal, Gutman, et al. 2009, McDonald and Mascagni 2001, Yoshida, Uchigashima, et al. 2011). Based on the anatomical data and the large body of literature implicating the amygdala in Pavlovian fear conditioning and extinction, we propose that a Cnr1-CCKBR interaction mediating cued fear expression occurs in the amygdala. The locus of this interaction will further be informed by future site-specific behavioral experiments. Altogether, these results suggest that Cnr1 is upstream of CCKBR, where activation of Cnr1 inhibits release of CCK during cued fear expression.

We do not observe blockade of within-session extinction in *Cnr1* knockout subjects, as has been previously reported (Marsicano, Wotjak, et al. 2002). Although we replicate their fear conditioning and extinction paradigm, Mariscano et al. present findings from individually-housed subjects. Solitary housing can have significant effects on fear- and anxiety-like behavior (Voikar, Polus, et al. 2005). As the cannabinoid system is particularly sensitive to stress (Hill, Hillard, et al. 2011, Roozendaal, McEwen, et al. 2009), differences in extinction behavior across studies could be attributed to respective housing conditions. However, we observe a blockade in within-session extinction in C57BL/6J mice with administration of a Cnr1 antagonist, SR141716A, suggesting that a potential effect of individual housing on the cannabinoid system is negligible. We initially hypothesized that the observed behavioral discrepancy between *Cnr1* wild-type and knockout littermates might be explained by differential gene expression of CCKBR in *Cnr1* knockout subjects. Specifically, we predicted that *Cnr1* knockout subjects would express less CCKBR compared to wild-types. Although, according to our model, *Cnr1* knockout subjects release more CCK during cued fear expression (due to removal of Cnr1-mediate inhibition of CCK release), we hypothesized that decreased CCKBR expression might explain a milder deficit in extinction behavior exhibited by *Cnr1* knockout subjects. To the contrary, we find an increase in amygdala CCKBR amygdala expression in *Cnr1* knockout subjects compared

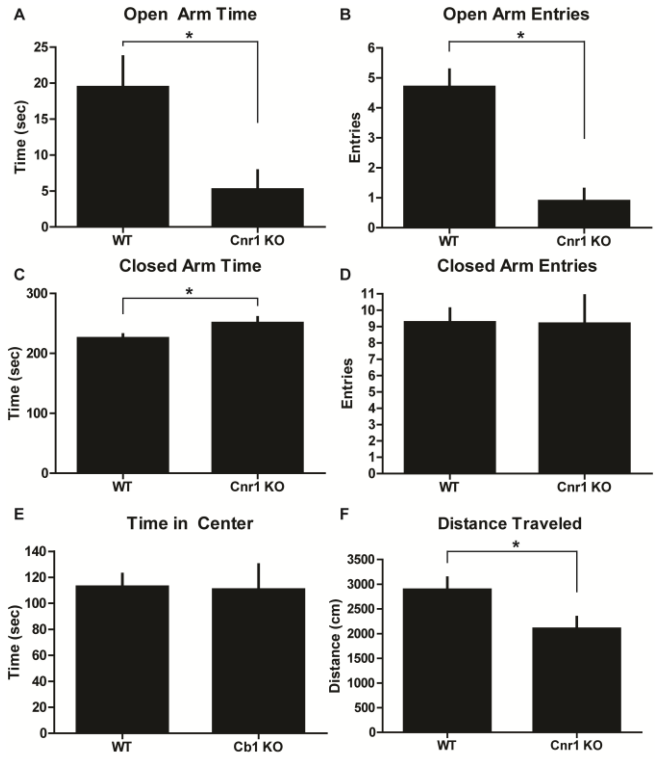
to wild-type littermates. Increased CCKBR expression might reflect an increase in CCKBR autoreceptors for the purpose of controlling excess release of CCK in *Cnr1* knockout subjects. Although the literature on CCK autoreceptors is scant, some hypothesize that CCK modulates dopaminergic transmission via autoreceptors (Markstein and Hokfelt 1984). To assemble a more holistic understanding of how genetic compensation of the CCK system might affect extinction behavior in *Cnr1* knockout subjects, additional CCK system genes must be tested – specifically, quantified mRNA and protein must be assessed.

Finally, we provide novel evidence of CCK inhibition via Cnr1 activation in the amygdala. We first elicited an increase in CCK release with bath application of high potassium ACSF to demonstrate that changes in CCK release from amygdala punch are detected by immunoassay. Importantly, we also observe a decrease in CCK release with extended application of a Cnr1 agonist WIN 55,212-2, at a concentration of 1 μM. WIN 55,212-2 was bath applied to punches for 20 minutes prior to sample collection to allow for activation and onset of second messenger systems downstream of Cnr1. The results of this experiment support the conclusions of a similar study conducted in hippocampal slice (Beinfeld and Connolly 2001). *Ex vivo* amygdala Cnr1 inhibition of CCK release supports the hypothesis that activation of Cnr1 during cued fear expression blocks CCK transmitter release to promote inhibition of fear.

As Cnr1 and other cannabinoid family genes are implicated in PTSD, human via genetic association and neuroimaging data, future studies should identify potential interactions between the cannabinoid system, the cholecystokinin system, and PTSD (and other anxiety and fear-related disorders). For instance, PET data reveals that individuals with PTSD have increased brain Cnr1 availability, possibly due to lower peripheral levels of anadamide (Neumeister, Normandin, et al. 2013). One hypothesis is that lower circulating levels of anandamide removes Cnr1-mediated inhibition of CCK, perhaps contributing to PTSD symptomatology. Critically, a

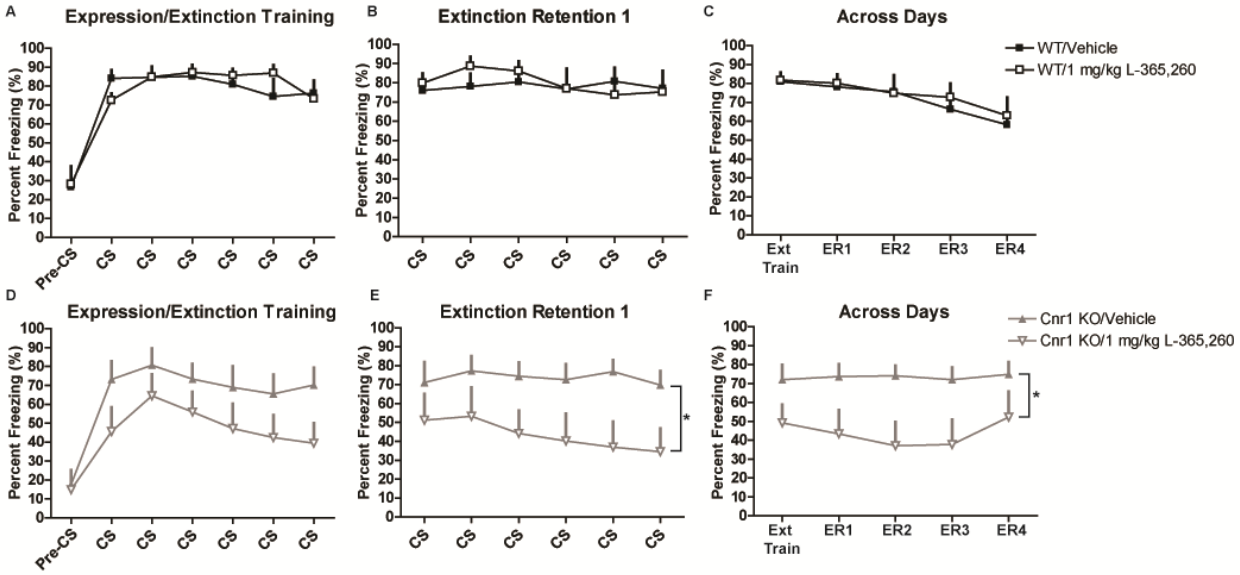
number of studies support a link between the CCK system and panic, which may share a similar neurobiological mechanism with posttraumatic flashbacks (Bradwejn, Koszycki, et al. 1990, Bradwejn, Koszycki, et al. 1991, de Montigny 1989, Kellner, Wiedemann, et al. 2000, Mellman and Davis 1985) .

Figure. 4.4-1 Cnr1 knockout mice exhibit increased anxiety-like behavior



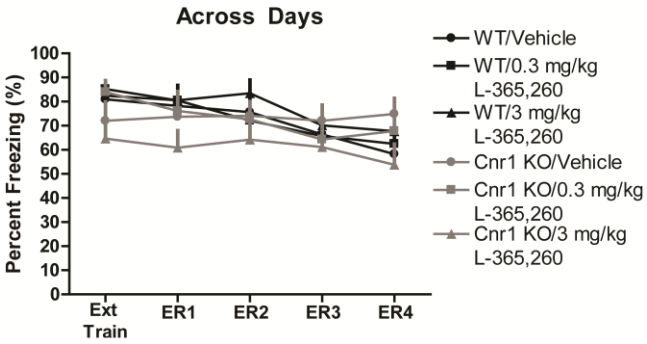
(**A-D**) *Cnr1* knockouts exhibit increased anxiety-like behavior as determine by a five minute elevated plus maze test, (**E**) *Cnr1* knockout subjects and wild-type littermates spend comparable amount of time in the center of an open field, (**F**) *Cnr1* knockouts exhibit significant motor differences compared to wild-type littermates.

Figure. 4.4-2 At a dose of 1 mg/kg, the CCKBR antagonist L-365,260 decreases freezing across multiple days of cued fear extinction in Cnr1 knockout mice, not in wild-type littermates



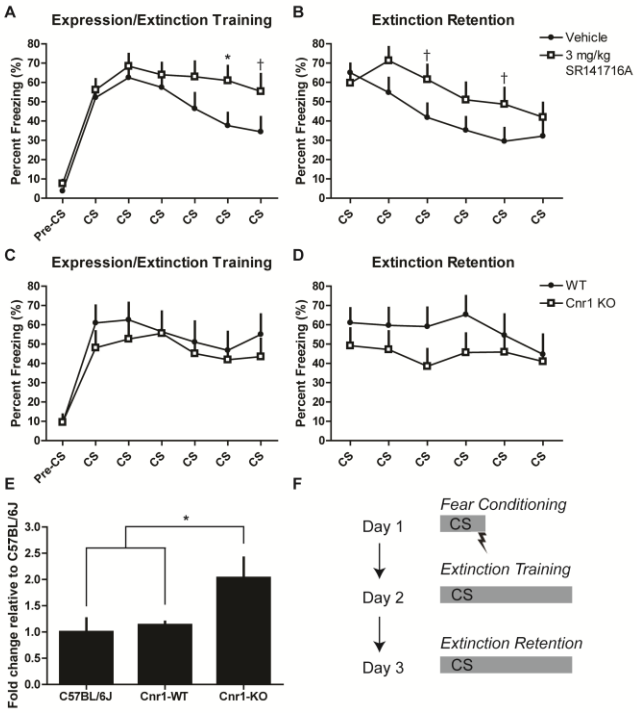
(**A-C**) At a dose of 1 mg/kg, L-365,260, a CCKBR antagonist has no effect on freezing during cued fear expression (**A**), extinction retention (**B**), or across multiple days of extinction (**C**) in wild-type subjects, (**D-F**) At the same dose, L-365,260 decreases freezing during an extinction retention test (**E**) and across days.

Figure. 4.4-3 At a dose of 0.3 and 3 mg/kg, the CCKBR antagonist L-365, 260 does not affect freezing across multiple days of extinction in Cnr1 wild-type and knockout littermates



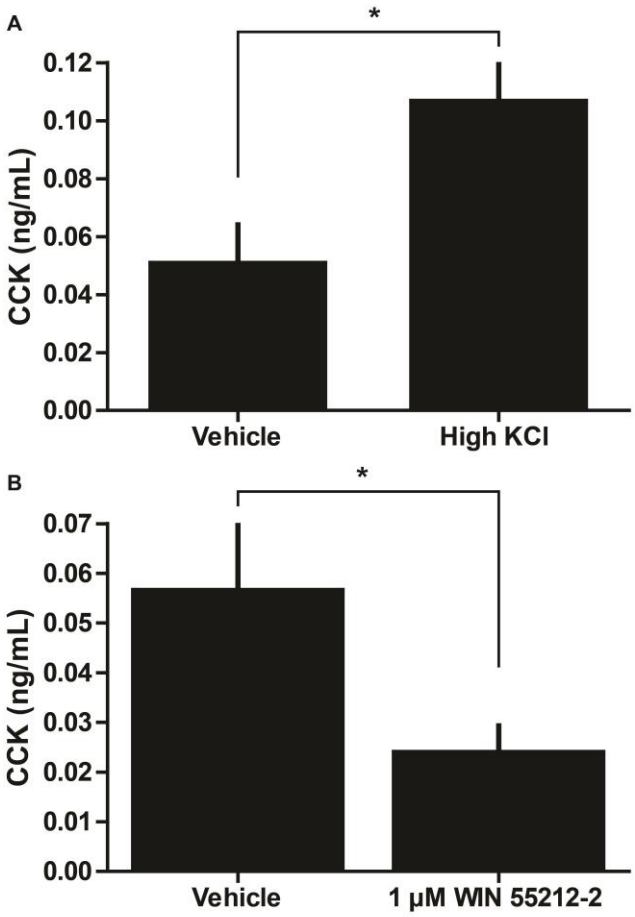
At a dose of 0.3 and 3 mg/kg, the CCKBR antagonist L-365,260 does not affect freezing across multiple days of extinction in wild-type or *Cnr1* knockout littermates.

Figure. 4.4-4 Administration of a Cnr1 antagonist, SR141716A, blocks within-session extinction of cued freezing; expression of CCBR mRNA may underlie differences in within-session extinction between C57BL/6J versus Cnr1 transgenic mice



(**A-B**) At 3 mg/kg, the Cnr1 antagonist blocks within-session of extinction, (**C-D**) Conversely, global knockout of Cnr1 does not affect within-session extinction, (**E**) CCKBR mRNA expression is increased in the amygdala of Cnr1 knockout subjects, (**F**) Fear conditioning protocol for subjects tested in panels **A-D**.

Figure. 4.4-5 Cnr1 activation inhibits release of CCK from amygdala punch



(**A**) Bath application of high potassium ACSF increases CCK release from amygdala punch,

(**B**) Incubation with 1 uM WIN 55,212-2, a Cnr1 agonist, decreases release of CCK.

Chapter 5:

Anxiety-like behavior in cannabinoid 1 receptor (*Cnr1*) knockout mice is sex-dependent

5.1 Context, Author's Contribution, and Acknowledgement of Reproduction

The following chapter presents evidence of a genotype x sex interaction in anxiety-like behavior, where female *Cnr1* knockout mice do not exhibit increased anxiety-like behavior compared to female wild-type littermates, as is seen in male *Cnr1* transgenic subjects. The context of the study was an effort to better understand the effect of sex on anxiety-like behavior, as women are at increased risk for certain anxiety disorders. The dissertation author contributed to the paper by designing and running experiments, analyzing the data, and was a main contributor to the writing of the paper. The chapter is reproduced with minor edits from Bowers, M.E. and Ressler, K.J. Anxiety-like behavior in cannabinoid receptor (*Cnr1*) knockout mice is sex-dependent. *Submitted*

5.2 Introduction

Human epidemiological data consistently suggest that women are at increased risk for major depression and anxiety disorders (Gavranidou and Rosner 2003). As behavioral neuroscience studies have historically modeled psychiatric disease in male subjects, it is increasingly imperative to include a sex-dependent component of analysis. Comprehensive models will allow researchers to determine how gonadal hormones interact with central transmitter systems and neural circuits, particularly those that underlie emotion.

Evidence indicates that the endogenous cannabinoid system, which is critical for emotion, pain, anxiety, and memory, bidirectionally interacts with gonadal hormones (Craft 2005, Gorzalka and Dang 2012, Mechoulam and Parker 2013). The endocannabinoid system modulates release of androgens and estrogens via the central regulation of luteinizing hormone (LH) and gonadotropin releasing hormone (GnRH). In turn, gonadal hormones influence central

endocannabinoid signaling and cannabinoid 1 receptor (Cnr1) expression in adult rodents (Gorzalka and Dang 2012, Rodriguez de Fonseca, Cebeira, et al. 1994). Furthermore, gonadal hormones can exert early organizational effects on the endocannabinoid system, as sex differences in Cnr1 expression and distribution have also been reported in rat pups (Llorente, Llorente-Berzal, et al. 2008, Suarez, Llorente, et al. 2009).

There is much evidence for the role of endocannabinoids in modulating emotion and inhibiting anxiety-related behavior, however there is a dearth of evidence on sex differences underlying this role. Given prior evidence supporting an interaction between gonadal hormones and the endocannabinoid system, we hypothesize that global knockout of *Cnr1* may have a differential sex-dependent effect on anxiety-like behavior. In male subjects, global knockout or antagonism of *Cnr1* increases anxiety-like behavior on a number of different paradigms (Chhatwal and Ressler 2007, Marsicano, Wotjak, et al. 2002, Ruehle, Rey, et al. 2012). Here, we demonstrate differential anxiety-like behaviors of male and female *Cnr1* transgenic mice on an elevated-plus maze test. To our knowledge, no one has yet reported on an anxiety-like behavioral effect of endogenous cannabinoid system manipulation in female subjects.

5.3 Methods

5.3.1 Animals

Adult male and female C57BL/6J and *Cnr1* transgenic mice were group housed in a temperature-controlled (24° C) animal colony, with *ad libitum* access to food and water, on a 12 hour light-dark cycle. *Cnr1* transgenic mice were a generous gift from Dr. Carl Lupica at NIDA (Zimmer, Zimmer, et al. 1999). Experimental subjects were genotyped by PCR using primers "CB1F" (wild-type forward: 5' GTA CCA TCA CCA CAG ACC TCC T), "CB1wt" (wild-type reverse:

5’ GGA TTC AGA ATC ATG AAG CAC TC) and “CNK03” (mutant reverse: 5’ AAG AAC GAG ATC AGC AGC CTC T). Homozygous *Cnr1* knockout and wild-type littermates from in house heterozygous breeding pairs were used for experiments. All behavioral procedures were performed during the light cycle.

5.3.2 Surgery

Ovariectomy or sham surgeries were performed on 8-week old female *Cnr1* transgenic mice. Anesthesia was induced and maintained using isoflurane. For the ovariectomies, ovaries were gently pulled out and removed via cauterization and then cutting of the fallopian tubes. Sham surgeries were conducted identical to ovariectomy with the exception of ovary removal. Behavioral testing commenced two weeks after surgery.

5.3.3 Elevated plus maze

Subjects were handled once per day for the two days prior to testing. Subjects were placed in the elevated plus maze apparatus to explore for 5 minutes in dim lighting. Behavior was hand-scored for time on open arms, time on closed arms, time in center, and number of entries into the open and closed arms. Total number of entries (closed arm entries plus open arm entries) was analyzed as a proxy for motor behavior.

5.3.4 Drugs

The Cnr1 antagonist, SR141716A (Cayman Chemical 9000484), was dissolved in a vehicle of 2.5% DMSO/0.1% Tween-80 in saline to yield a final drug concentration of 3 mg/kg. SR141716A

was systemically administered intraperitoneally (IP) 20 minutes prior to elevated plus maze behavior.

5.3.5 Statistics

Two-way ANOVA was performed using IBM SPSS Statistics 21 software. The results are presented as mean + SEM. Exact p-values are reported. *Post hoc* Least Significant Difference (LSD) tests were used where appropriate.

5.4 Results

5.4.1 Genetic validation of Cnr1 knockout; Wild-type and Cnr1 knockout male and female littermates exhibit normal locomotor behavior

Wild-type and *Cnr1* knockout male and female littermates were weaned at 3 weeks of age and assessed on an elevated plus maze at 8-12 weeks old (**Figure 5.4-1A**). Wild-type and *Cnr1* knockout male and female genotypes were validated using PCR (wild-type amplicon ~300 bp, *Cnr1* knockout amplicon ~150 bp, **Figure 5.4-1B**). We did not detect a main effect of genotype or sex, or a genotype x sex interaction on total number of entries (closed arm entries + open arm entries) during a five minute elevated plus maze test, indicating that wild-type and *Cnr1* knockout subjects of both sexes exhibit comparable motor behavior (**Figure 5.4-1C**).

5.4.2 Female Cnr1 knockout subjects do not exhibit increased anxiety-like behavior compared to male Cnr1 knockout littermates

Wild-type and *Cnr1* knockout male and female littermates were tested on an elevated plus maze and assessed for 1) time spent on open arms, 2) time spent on closed arms, 3) open arm entries, and 4) closed arm entries (same cohort as in **Figure 5.4-1**). We detected a significant main effect of genotype on open arm entries ($F_{1,47}$=5.10, p=0.029, **Figure 5.4-2B**). We also found a significant genotype x sex interaction in our analysis of open arm entries ($F_{1,47}$=4.53, p=0.039). *Post hoc* LSD tests revealed a significant difference between male wild-type versus male *Cnr1* knockouts (p=0.002), male *Cnr1* knockouts versus female wild-type subjects (p=0.025), and male *Cnr1* knockouts versus female *Cnr1* knockouts (p=0.049). We did not detect a significant difference between female wild-type versus female *Cnr1* littermates. Furthermore, there were no significant differences observed when comparing open arm entries between male wild-type subjects versus female wild-type subjects and male wild-type subjects versus female *Cnr1* knockout subjects. We did not detect a main effect of sex or genotype, or a genotype x sex interaction on closed arm time (**Figure 5.4-2C**), closed arm entries (**Figure 5.4-2D**), or open arm time (**Figure 5.4-2E**). These data suggest that female *Cnr1* knockout subjects do not exhibit an increase in anxiety-like behavior compared to male *Cnr1* knockout littermates, suggesting that female gonadal hormones may mediate a protective effect against anxiety-like behavior in *Cnr1* knockout mice.

5.4.3 Ovariectomy does not increase anxiety-like behavior in Cnr1 knockout females

To test the hypothesis that female gonadal hormones (estrogen and progesterone) mediate a protective effect against anxiety-like behavior in the presence of *Cnr1* deletion, we next performed ovariectomy and sham surgeries on 8-week old wild-type and *Cnr1* knockout females. Two weeks after surgery, subjects were assessed for anxiety-like behavior on a five minute elevated plus maze, as in the prior experiment (**Figure 5.4-3A**). We did not detect a significant main effect of surgery or sex, nor did we observe a surgery x sex interaction on open

arm entries (**Figure 5.4-3B**), closed arm time (**Figure 5.4-3C**), closed arm entries (**Figure 5.4-3D**), or open arm time (**Figure 5.4-3E**). These data replicate our previous finding - global knockout of *Cnr1* does not increase anxiety-like behavior in female mice as in male mice. Interestingly, ovariectomy does not increase anxiety-like behavior in female *Cnr1* knockout subjects, suggesting that female gonadal hormones might buffer against *Cnr1* knockout-mediated increases in anxiety-like behavior by differentially organizing neural circuitry early in development, but with no effect in adulthood.

5.4.4 A Cnr1 antagonist, SR141716A, increases anxiety-like behavior in male and female C57BL/6J mice

Next, we administered male and female adult C57BL/6J mice the Cnr1 antagonist SR141716A at a dose of 3 mg/kg to further examine whether the previously observed interaction between sex and *Cnr1* knockout is developmentally-mediated (**Figure 5.4-4A**). We observed a significant main effect of drug on open arm entries ($F_{1,43}$=11.49, p=0.002, **Figure 5.4-4B**), closed arm time ($F_{1,43}$=16.49, p=0.0001, **Figure 5.4-4C**), closed arm entries ($F_{1,43}$=5.62, p=0.022, **Figure 5.4-4D**), and open arm time ($F_{1,43}$=5.14, p=0.029, **Figure 5.4-4E**). We did not detect a significant drug x sex interaction on any measure of anxiety-like behavior. For open arm entries, *post hoc* LSD tests revealed a significant difference between males administered vehicle versus males administered SR141716A (p=0.004), males administered SR141716A versus females administered vehicle (p=0.022), and males administered vehicle versus females administered SR141716A (p=0.02). We observed a trend when comparing females administered vehicle versus females administered SR141716A (p=0.081). *Post hoc* LSD tests for closed arm time, closed arm entries, and open arm time are as follows: 1.) closed arm time (male vehicle vs. male SR141716A: p=0.006, male vehicle vs. female SR141716A: p=0.021, female vehicle vs. male SR141716A:

p=0.002, female vehicle vs. female SR141716A: p=0.007), 2.) closed arm entries (male vehicle vs. female SR141716A: p=0.015), 3.) open arm time (male vehicle vs. male SR141716A: p=0.04).

These data suggest that in the background of normative sexual development, Cnr1 may be similarly sensitive to inhibition and anxiogenesis in adulthood, but that deletion of *Cnr1* during development leads to differential sex-dependent angiogenesis. Overall, these results support the findings from our prior experiments, suggesting that sex, potentially via female gonadal hormones, is developmentally protective against *Cnr1* knockout-mediated increases in anxiety-like behavior.

5.5 Discussion

These data demonstrate an interaction between sex and the endocannabinoid system in anxiety-like behavior, where: 1) female *Cnr1* knockout subjects do not exhibit increased anxiety-like behavior compared to male *Cnr1* knockout littermates; 2) ovariectomy in adult females is not sufficient to produce increased anxiety-like behavior in *Cnr1* knockout females; however, 3) the Cnr1 antagonist, SR141716A, increases anxiety-like behavior across sexes in wild-type mice, with no significant difference between males and females administered drug.

These data suggest that there is an interaction between female gonadal hormones and the endogenous cannabinoid system that is limited to development. This interaction is protective against anxiety-like behavior in female adults with developmental deletion; but, it does not lead to differential effects of Cnr1 pharmacological antagonism in normally developing adults. Specifically, our data indicate that activity of female gonadal hormones during early development may detect and compensate for aberrant Cnr1 signaling that contributes to increased anxiety-like behavior in adult males. Future experiments, outside the scope of this

study, should investigate the potentially protective effect of female gonadal hormones during early development in order to determine the exact nature of a Cnr1/female gonadal hormone interaction in anxiety-like behavior.

Our results are supported by prior literature, where global knockout of *Cnr1* or administration of a Cnr1 antagonist increases anxiety-like behavior in male rodents (Ruehle, Rey, et al. 2012). Cnr1 is thought to regulate anxiety-like behavior via modulation of excitatory and inhibitory input onto BNST neurons with downstream projections to the hypothalamus (Puente, Elezgarai, et al. 2010). A recent study, however, suggests that replacement of Cnr1 on dorsal telencephalic glutamatergic neurons is sufficient to rescue increased anxiety-like behavior observed in global *Cnr1* knockout mice (Ruehle, Remmers, et al. 2013).

Although a number of studies reveal baseline sex differences in rodent anxiety-like behavior, to our knowledge, this is the first time a sex difference in *Cnr1*-mediated anxiety-like behavior has been reported (Imhof, Coelho, et al. 1993, Johnston and File 1991, Lucion, Charchat, et al. 1996). A potential site of action for this difference is the bed nucleus of the stria terminalis (BNST). Sexual dimorphism of the BNST, which is implicated in long-lasting unconditioned fear/anxiety, is thought to mediate sex differences in rodent anxiety-like behavior (Walker and Davis 1997, Walker, Toufexis, et al. 2003). Volume of the encapsulated region is approximately 97% larger in male rodents compared to females (Hines, Allen, et al. 1992). Sexual dimorphism of the BNST is also observed in humans (Allen and Gorski 1990, Chung, De Vries, et al. 2002). As in rodents, volume of the BNST is larger in men compared to women.

Furthermore, the cholecystokinin (CCK) system, particularly within the BNST, is differentially organized in male and female rodents. Interestingly, data suggests that endocannabinoids inhibit activation of the CCK system, potentially via Cnr1 inhibition of the

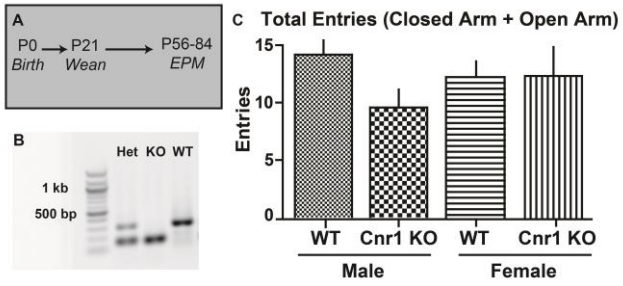
panicogenic/anxiogenic neuropeptide CCK, to mediate cued fear expression and extinction retention, as well as extinction of fear-potentiated startle (Bowers and Ressler 2014, Chhatwal, Gutman, et al. 2009). Female rats have fewer CCK immunoreactive cells in the BNST compared to males, and this difference is not reversed by ovariectomy (Micevych, Akesson, et al. 1988). One possibility is that Cnr1-mediated differences in anxiety-like behavior between males and females stems from early development and sexual regulation of the CCK system.

Data from investigations of stress and anxiety-like behavior and sex in rodent models, however, do not often reflect observed sex/gender differences in psychiatric disorders (Cohen and Yehuda 2011). Where estrogen appears to be protective against stress and anxiety in rodent models, overwhelming epidemiological data suggests that women are more vulnerable to major depression and anxiety disorders (Cohen and Yehuda 2011, Gavranidou and Rosner 2003). Yet, studies of the cannabinoid system may offer a more translatable model for study of sex/gender and stress and anxiety. Human genetic association studies implicate *Cnr1* in posttraumatic stress disorder (PTSD) and attention deficit hyperactivity disorder (ADHD) (Lu, Ogdie, et al. 2008). Furthermore, Cnr1 availability in the amygdala mediates threat processing in trauma survivors and carriers of a low-expressing FAAH allele (rs324420) exhibit faster habituation of amygdala activity to threat and have lower scores on the personality-trait of stress-reactivity (Gunduz-Cinar, MacPherson, et al. 2013, Pietrzak, Huang, et al. 2014). Interestingly, males with a specific SNP haplotype (rs806377) are at greater risk for ADHD compared to females with the same haplotype (Lu, Ogdie, et al. 2008). *In vivo* PET demonstrates differential binding of a Cnr1 ligand between men and women according to age and brain region (Van Laere, Goffin, et al. 2008).

Future studies, particularly pharmacologic and genetic association studies, should address potential interactions between the cannabinoid system and sex/gender. Ideally, neuroscience

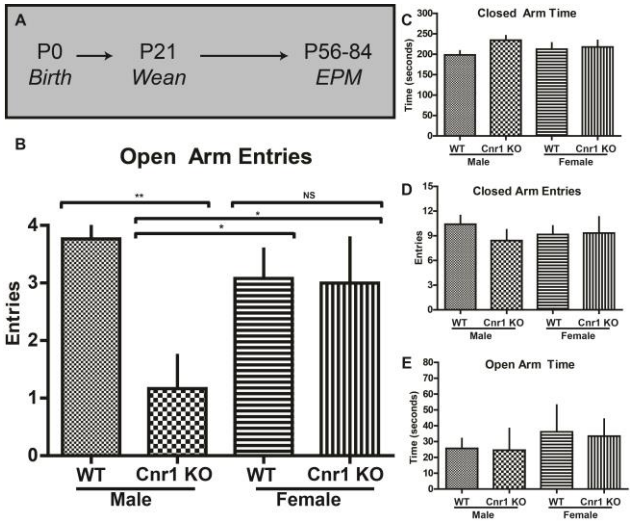
and psychology will inform the discrepancy between rodent models and human epidemiological data, as identifying risk according to sex/gender becomes more critical.

Figure. 5.4-1 Schematic of behavioral protocol, genotype validation, and measure of motor behavior



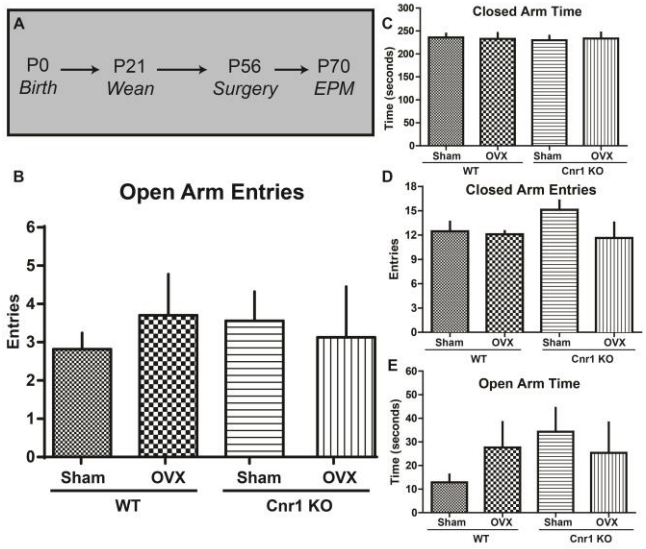
(**A**) Wild-type and *Cnr1* knockout male and female littermates were weaned at 3 weeks of age and assessed on an elevated plus maze at 8-12 weeks old. (**B**) Genotypes were validated using PCR (wild-type amplicon ~300 bp, *Cnr1* knockout amplicon ~150 bp). (**C**) We did not detect a main effect of genotype or sex, or a genotype x sex interaction on total number of entries (closed arm entries + open arm entries) during a five minute elevated plus maze test, indicating that wild-type and *Cnr1* knockout subjects of both sexes exhibit comparable motor behavior.

Figure. 5.4-2 Unlike males, female *Cnr1* knockout subjects do not exhibit increased anxiety-like behavior compared to *Cnr1* knockout littermates



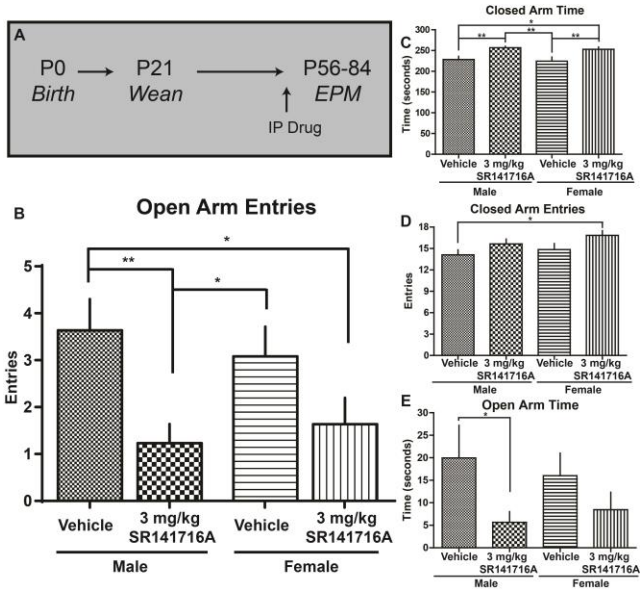
(**A**) Wild-type and *Cnr1* knockout male and female littermates were weaned at 3 weeks of age and assessed on an elevated plus maze at 8-12 weeks old. (**B**) We detected a significant main effect of genotype ($F_{1,47}$=5.10, p=0.029) and a significant genotype x sex interaction ($F_{1,47}$=4.53, p=0.039) in our analysis of open arm entries on a five minute elevated plus maze task. We did not detect a main effect of sex or genotype, or a genotype x sex interaction on (**C**) closed arm time, (**D**) closed arm entries, or (**E**) open arm time. LSD *post hoc* tests, * indicates p<0.05, ** indicates p<0.01.

Figure. 5.4-3 Ovariectomy does not increase anxiety-like behavior in *Cnr1* knockout females



(**A**) Wild-type and *Cnr1* knockout male and female littermates were weaned at 3-weeks old, underwent surgery at 8-weeks old, and tested on an elevated plus maze at 10-weeks old. We did not detect a significant main effect of surgery or sex, nor did we observe a surgery x sex interaction on number of (**B**) open arm entries, (**C**) closed arm time, (**D**) closed arm entries, or (**E**) open arm time made on a five minute elevated plus maze.

Figure. 5.4-4 A Cnr1 antagonist, SR141716A, increases anxiety-like behavior in adult male and female C57BL/6J mice



(**A**) Wild-type male and female littermates were weaned at 3-weeks of age and assessed on an elevated plus maze at 8-12-weeks old. (**B**) We observed a significant main effect of drug on open arm entries ($F_{1,43}$=11.49, p=0.002) when 3 mg/kg SR141716A is administered intraperitoneally 20 minutes prior to a five minute elevated plus maze test. Furthermore, we observed a significant main effect of drug on (**C**) closed arm time ($F_{1,43}$=16.49, p=0.0001), (**D**) closed arm entries ($F_{1,43}$=5.62, p=0.022), and (**E**) open arm time ($F_{1,43}$=5.14, p=0.029). We did not detect a significant drug x sex interaction on any measure of anxiety-like behavior. LSD *post hoc* tests, * indicates p<0.05, ** indicates p<0.01.

Chapter 6:

Translationally informed treatments for PTSD

6.1 Context, Author's Contribution, and Acknowledgement of Reproduction

The following chapter reviews established and emerging treatment strategies for PTSD that are supported by pre-clinical and clinical data. The work presented here was conceptualized, organized, researched, and written by the dissertation author under the guidance of Dr. Ressler. The chapter is reproduced with from sections with minor edits from Bowers, M.E. and Ressler, K.J.. (In preparation). "Translationally informed treatments for PTSD." *Biological Psychiatry*

6.2 Introduction

Posttraumatic stress disorder (PTSD) is a psychiatric disorder that manifests after exposure to a traumatic event and is characterized by avoidance/numbing, flashbacks, and hyperarousal. An event is considered traumatic if it threatens an individual's life or physical integrity and involves a subjective response of fear, helplessness, or horror (American Psychiatric Association. and American Psychiatric Association. DSM-5 Task Force. 2013). While most individuals recover from acute trauma, 8-9% of the United States population will develop PTSD over their lifetime (Breslau, Davis, et al. 1991, Kessler, Sonnega, et al. 1995).

Data suggests that approximately 94% of individuals who experience trauma develop acute PTSD-like symptoms (Kessler, Sonnega, et al. 1995, Yehuda 2004). For most individuals, these symptoms will abate over time, indicating that hyper-consolidation of trauma and/or deficient extinction might underlie development of PTSD. Animal studies of fear acquisition and extinction provide valid models of PTSD that allows investigation of pathological learning and dissection of underlying neurobiology. Through these studies, along with human neuroimaging data, researchers have identified the amygdala (interacting critically with the hippocampus and medial prefrontal cortex) as the primary locus of fear learning and extinction (Davis 1992,

LaBar, Gatenby, et al. 1998, LeDoux 2007, Milad and Quirk 2002). Further, manipulations of various transmitter systems during different phases of aversive learning point to a number of potential pharmacotherapies and specific treatment windows. Based on pre-clinical data, pilot and large-scale clinical studies have now been conducted on a number of treatments with a variety of administration protocols, e.g. chronically, administered in the immediate aftermath of trauma, in conjunction with exposure therapy, and during reconsolidation. Additionally, researchers are also exploring efficacy of device-based treatments for PTSD and PTSD-like symptoms in humans and rodents, given the success of deep-brain stimulation (DBS) for the treatment of depression (Mayberg, Lozano, et al. 2005).

In this review, we explore established and emerging treatment strategies for PTSD that are supported by pre-clinical and clinical data. Although the number of approved treatments is small, with SSRIs as the only class of drug approved for treatment of PTSD, exciting new evidence points to a number of promising pharmacotherapies and device-based treatments with a variety of treatment protocols.

6.3 Pharmacotherapies

6.3.1 D-cycloserine (DCS)

In combination with cognitive behavioral therapy, D-cycloserine (DCS), a partial agonist of the NMDA receptor, may offer a promising treatment strategy for PTSD. DCS initially showed therapeutic indication in rodent models of aversive learning. Systemic or intra-amygdala administration of DCS facilitates extinction of fear-potentiated startle and cued freezing in rats (FPS) (Ledgerwood, Richardson, et al. 2005, Mao, Hsiao, et al. 2006, Walker, Ressler, et al. 2002, Weber, Hart, et al. 2007). DCS also blocks increases in freezing caused by reinstatement,

but has no effect on renewal processes (Ledgerwood, Richardson, et al. 2004, Woods and Bouton 2006). DCS is thought to act on consolidation of learning, as post-training administration has been shown to facilitate extinction (Ledgerwood, Richardson, et al. 2005).

While DCS has shown efficacy in the facilitation of extinction learning, some suggest that fear-behavior of prior-stressed subjects during extinction learning is a more valid model of PTSD and exposure therapy. Fear extinction is impaired in stressed rats or mice (Andero, Daviu, et al. 2012, Izquierdo, Wellman, et al. 2006, Miracle, Brace, et al. 2006). DCS has been shown to reverse deficits in fear extinction caused by single prolonged stress (SPS – 2 hour restraint stress, 20 minute forced swim, and ether anesthesia) (Yamada, Wada, et al. 2011, Yamamoto, Morinobu, et al. 2008). DCS has also shown efficacy when administered after extinction training in 129S1/SvImJ (S1), a genetic mouse model that exhibits impaired fear extinction (Whittle, Schmuckermair, et al. 2013).

In healthy human volunteers, DCS facilitates consolidation of cued fear acquisition and extinction (Kalisch, Holt, et al. 2009, Kuriyama, Honma, et al. 2011). Other studies, however, do not observe a reduction in conditioned fear with administration of DCS (Guastella, Lovibond, et al. 2007, Klumpers, Denys, et al. 2012, Kuriyama, Honma, et al. 2013).

DCS has shown promise for the treatment of social anxiety, obsessive compulsive disorder (OCD), panic disorder, acrophobia, and nicotine dependence (Hofmann, Meuret, et al. 2006, Kushner, Kim, et al. 2007, Otto, Tolin, et al. 2010, Ressler, Rothbaum, et al. 2004, Santa Ana, Rounsaville, et al. 2009, Storch, Murphy, et al. 2010). Data on the efficacy of DCS in treating PTSD is mixed. DCS seems to be particularly effective when administered with virtual reality exposure (VRE) (Difede, Cukor, et al. 2014, Rothbaum, Price, et al. 2014). Furthermore, DCS reduced cortisol and startle reactivity compared to placebo in one study (Rothbaum, Price, et al.

2014). Other studies do not report increased symptom remission with DCS compared to placebo (when in administered in combination with cognitive behavioral therapy) (Litz, Salters-Pedneault, et al. 2012, Scheeringa and Weems 2014). Despite inconsistencies in the literature, meta-analyses suggest that DCS enhances fear extinction/exposure therapy in both animal subjects and humans (Norberg, Krystal, et al. 2008, Rodrigues, Figueira, et al. 2014). Conflict in the data might be explained by more thorough analyses. For instance, in one study that did not observe an overall effect of DCS on PTSD, DCS yielded greater reductions in PTSD-symptoms in subjects with more severe pre-treatment PTSD (de Kleine, Hendriks, et al. 2012). Furthermore, participants with high conscientiousness and low extraversion exhibit better outcomes with DCS and exposure therapy, compared to placebo (de Kleine, Hendriks, et al. 2014). DCS also appears to selectively enhance exposure therapy when administered with successful sessions (Smits, Rosenfield, et al. 2013). This effect is reflected in rodent models, where subjects who exhibit successful within-session extinction show better long-term extinction with DCS (Bolkan and Lattal 2014, Weber, Hart, et al. 2007).

6.3.2 Cannabinoids

Overwhelming evidence from rodent models suggest that the endocannabinoids are critically involved in stress, fear, and anxiety (Chhatwal and Ressler 2007, Ruehle, Rey, et al. 2012). Knockout or antagonism of Cnr1 increases anxiety-like behavior across rodent models (Chhatwal and Ressler 2007, Marsicano, Wotjak, et al. 2002, Moise, Eisenstein, et al. 2008). Increased synthesis of the endocannabinoids and subsequent activation of Cnr1 in the amygdala is thought to mediate fear extinction in mice and rats, potentially via inhibition of the anxiogenic neuropeptide cholecystokinin (CCK) and/or modulation of the GABAergic system (Bowers and Ressler 2014, Chhatwal, Gutman, et al. 2009, Marsicano, Wotjak, et al. 2002). Cnr1 has also been implicated in acquisition, retrieval, and extinction of both cue and context fear, as well as

reconsolidation of cued fear memory (Bowers and Ressler 2014, Kuhnert, Meyer, et al. 2013, Ratano, Everitt, et al. 2014, Reich, Mohammadi, et al. 2008). Cnr1 involvement in fear is thought to be mediated primarily by amygdala and mPFC Cnr1 (Chhatwal, Gutman, et al. 2009, Kuhnert, Meyer, et al. 2013, Ratano, Everitt, et al. 2014). The endocannabinoid system has also been heavily implicated in stress and stress-sensitization of fear behavior, where Cnr1 is thought to modulate glutamatergic and GABAergic signaling primarily in the bed nucleus of the stria terminalis, the basolateral amygdala, and the central amygdala (Campos, Ferreira, et al. 2012, Ganon-Elazar and Akirav 2012, Korem and Akirav 2014, Laricchiuta, Centonze, et al. 2013, Puente, Elezgarai, et al. 2010, Ramikie, Nyilas, et al. 2014, Reich, Iskander, et al. 2013). Recently, administration of a Cnr1 agonist acutely after shock has been shown to prevent PTSD-like symptoms in rats, suggesting that cannabinoid drugs might be administered acutely after trauma to prevent development of PTSD (Korem and Akirav 2014).

Evidence implicating Cnr1 involvement in stress, fear, and anxiety in rodent models has stimulated investigation of Cnr1 involvement in PTSD and fear processes in humans. Studies suggest delta-9-tetrahydrocannabinoil (Δ9-THC) facilitates extinction of conditioned fear in healthy human volunteers (Klumpers, Denys, et al. 2012, Rabinak, Angstadt, et al. 2013). PTSD diagnosis is significantly associated with greater marijuana use, indicating that Δ9-THC may be used as a form of self-medication to compensate for potential cannabinoid system dysregulation (Calhoun, Sampson, et al. 2000). In fact, several genetic association studies reveal specific Cnr1 and FAAH allelic risk factors for threat processing, stress-coping, and PTSD (Gunduz-Cinar, MacPherson, et al. 2013, Lu, Ogdie, et al. 2008, Pardini, Krueger, et al. 2012). Furthermore, PET studies suggest that individuals with PTSD have increased brain Cnr1 availability, possibly due to lower peripheral levels of anadamide (Neumeister, Normandin, et al. 2013). Although the data is preliminary, several studies show that the synthetic cannabinoid receptor agonist,

nabilone, improved insomnia, subjective chronic pain, nightmares, and symptoms related to PTSD (Cameron, Watson, et al. 2014, Fraser 2009).

6.3.3 Glucocorticoids

Evidence for enhanced memory of emotionally salient experiences is well-established. Stressful events induce the release of the adrenal hormone cortisol (human)/corticosterone (rodent), which is a critical component of emotional memory consolidation. Glucocorticoid enhancement of memory consolidation is mediated via glucocorticoid receptor activation in the amygdala. Conversely, glucocorticoids impair memory retrieval processes (de Quervain, Roozendaal, et al. 1998, Roozendaal, Hahn, et al. 2004). Consequently, dysregulation of the HPA-axis can lead to behavioral changes reflective of PTSD psychopathology, which is thought to involve hyper-consolidation of traumatic memory and/or enhanced traumatic memory retrieval. PTSD-like behavioral changes in stressed rodents are more prevalent in strains with a blunted corticosterone stress response (Cohen, Zohar, et al. 2006). Acute pharmacological intervention with corticosterone after stress prevents PTSD-like behavioral effects and underlying dysregulated BLA connectivity in rodents (Cohen, Matar, et al. 2008, Daskalakis, Cohen, et al. 2014, Rao, Anilkumar, et al. 2012).

In humans, a number of studies reveal an association between PTSD and low basal cortisol, as well as a lower cortisol awakening response (Wessa, Rohleder, et al. 2006, Witteveen, Huizink, et al. 2010, Yehuda, Southwick, et al. 1990). Pre-existing high glucocorticoid receptor number and low cortisol in the aftermath of trauma is thought to precipitate development of PTSD (Delahanty, Raimonde, et al. 2000, van Zuiden, Geuze, et al. 2011). Notably, individuals with PTSD have higher CSF concentrations of corticotropin-releasing factor (CRF) (Baker, West, et al. 1999, Bremner, Licinio, et al. 1997). Enhanced suppression of cortisol by dexamethasone

supports evidence of HPA-axis dysregulation in PTSD (de Kloet, Vermetten, et al. 2007, Yehuda, Halligan, et al. 2002, Yehuda, Golier, et al. 2004, Yehuda, Halligan, et al. 2004).

Given the immense amount of data that suggests PTSD is associated with HPA-axis dysregulation, a number of researchers have begun to test the effect of glucocorticoids on PTSD symptomatology using a variety of administration paradigms. Administration of 4 mg/kg hydrocortisone in combination with traumatic memory reactivation ameliorates PTSD symptoms compared to placebo (Suris, North, et al. 2010). One month of daily oral administration of hydrocortisone (10 mg/day) reduces re-experiencing and avoidance symptoms (Aerni, Traber, et al. 2004). As in rodent models, administration of hydrocortisone within six hours of a traumatic event has been shown to reduce the risk of PTSD development (Schelling, Kilger, et al. 2004, Schelling, Roozendaal, et al. 2006, Zohar, Yahalom, et al. 2011).

6.3.4 Opioids/Morphine

PTSD is frequently associated with substance abuse. Along with marijuana and alcohol, opiates are one of the most commonly abused substances among individuals with PTSD, indicating that aberrant endogenous opioid signaling may underlie PTSD (Mills, Teesson, et al. 2006).

In rodents, administration of opioid antagonists increase conditioned fear by enhancing fear acquisition or blocking fear extinction (Fanselow, Calcagnetti, et al. 1988, Hernandez and Powell 1980, McNally and Westbrook 2003). Conversely, morphine administration blocks conditioned fear acquisition in normal and stressed models (Good and Westbrook 1995, Szczytkowski-Thomson, Lebonville, et al. 2013). Opioid signaling in ventrolateral periaqueductal gray matter (vlPAG) regulates conditioned fear extinction, potentially via activation of mPFC and the BLA (McNally, Pigg, et al. 2004, Parsons, Gafford, et al. 2010).

Research addressing specificity of opioid regulation of conditioned fear has implicated the mu and kappa opioid and nociception (NOP)/orphanin FQ receptors. Antagonism of the mu opioid receptor facilitates contextual fear conditioning (Fanselow, Kim, et al. 1991) and blocks extinction of cued fear (McNally, Lee, et al. 2005). Similarly, antagonism of the kappa opiod receptor blocks conditioned fear on the fear potentiated startle paradigm (Knoll, Meloni, et al. 2007, Knoll, Muschamp, et al. 2011) In PTSD-like rodent models, differential levels of CSF nociception (NOP)/orphanin FQ and nociception (NOP)/orphanin FQ receptor mRNA are observed (Andero, Brothers, et al. 2013, Zhang, Gandhi, et al. 2012). Furthermore, nociception (NOP)/orphanin FQ receptor agonist administration blocks contextual and cue fear consolidation in normal and PTSD-like rodent models (Andero, Brothers, et al. 2013, Fornari, Soares, et al. 2008, Goeldner, Reiss, et al. 2009).

In humans, tag single nucleotide polymorphism analysis (SNP) reveals a significant interaction between the *OPRL1* (opiod receptor-like 1 gene) SNP rs6010719 and childhood trauma that is associated with PTSD, where GG/GC carriers with high levels of childhood trauma exhibit greater number of PTSD symptoms compared to CC carriers with comparable levels of trauma (Andero, Brothers, et al. 2013). Further, GG/GC carriers are unable to discriminate between aversive and safety cues on a fear-potentiated startle test. The authors suggest that carrier-specific differences between amygdala-insula functional connectivity might underlie observed behavioral responses and psychopathology. Other studies have also found allelic risk factors within the opioid system that associate with PTSD. Participants were genotyped for the rs1799971 (A118G) polymorphism within the *OPRM1* gene (opioid receptor μ-1). In this study, G alleles were associated with less severe PTSD symptoms (Nugent, Lally, et al. 2012).

Increased opioid-mediated analgesia after trauma across species (Fanselow and Bolles 1979, Kraus, Geuze, et al. 2009) suggests that individuals with PTSD might have aberrant opioid signaling compared to healthy controls. Although some report increased CSF β-endorphin levels in veterans with PTSD (Baker, West, et al. 1997), others find lower serum levels of β-endorphin associated with PTSD (Hoffman, Burges Watson, et al. 1989). Conflict in the data might be explained by a lack of significant correlation between CSF and plasma β-endorphin levels (Baker, West, et al. 1997).

Increasingly, evidence suggests that morphine may be effective at secondary prevention of PTSD. Children administered morphine after acute burns have decreased PTSD symptoms months to years after treatment, and this effect appears to be dependent on dosage of morphine (Saxe, Stoddard, et al. 2001, Sheridan, Stoddard, et al. 2014, Stoddard, Sorrentino, et al. 2009). Studies of traumatized adults administered morphine mirror results found in these pediatric data sets. The use of morphine in early trauma care of combat veterans is associated with lower risk of PTSD (Holbrook, Galarneau, et al. 2010). Prospective studies find that patients who meet criteria for PTSD at 3 months post-trauma received significantly less morphine acutely after injury (Bryant, Creamer, et al. 2009). Data from healthy volunteers, where opioid agonists inhibit and antagonists promote fear acquisition, support the conclusions of studies conducted with PTSD patients (Eippert, Bingel, et al. 2008, Ipser, Terburg, et al. 2013). Although data is limited and further study is needed, consistent evidence suggests that morphine and other opiates might be effective secondary prevention treatments against PTSD.

6.3.5 SSRIs/Antidepressants

Prescription of SSRIs for the treatment of PTSD stems from the observed efficacy of SSRIs for depression and the high prevalence of PTSD-depression co-morbidity (Campbell, Felker, et al.

2007). The initial discovery of antidepressants was made during clinical trials on anti-tuberculosis agents (Selikoff and Robitzek 1952). Through pre-clinical testing, researchers refined the first generation of antidepressants and developed the current class of SSRIs. Only through pre-clinical studies, conducted after the discovery of antidepressant efficacy in humans, have researchers begun to determine the mechanism of action of SSRIs - inverting the bench to bedside translational model. The biogenic amine hypothesis of depression suggests that disturbances in serotonin, dopamine, and norepinephrine underlie the pathology of depression (Harvey 1997). SSRIs are thought to ameliorate symptoms of depression via inhibition of pre-synaptic reuptake of extracellular serotonin in the CNS (Harvey 1997). Evidence from rodent and human studies implicates brain serotonin systems in the neurobiology of PTSD, additionally (Murrough, Huang, et al. 2011, Wellman, Izquierdo, et al. 2007, Xie, Kranzler, et al. 2009).

Although selective serotonin reuptake inhibitors (SSRIs) are the only class of drugs approved for the treatment of PTSD, efficacy is controversial (Difede, Olden, et al. 2014, Steckler and Risbrough 2012). While a 2008 report from the Insitute of Medicine (IOM) concludes that SSRIs, among other all other classes of drugs, do not demonstrate efficacy in the treatment of PTSD, a recent meta-analysis supports the efficacy of long-term treatment of PTSD with SSRIs (Ipser and Stein 2012). Furthermore, professional organizations offer conflicting first-line treatment recommendations – some suggest that SSRIs are as effective as psychotherapy as a first-line treatment, while others recommend SSRIs as a second-line treatment, after cognitive behavioral therapy (Difede, Olden, et al. 2014). Notably, chronic SSRI treatment in rats impairs cued fear extinction, indicating that SSRIs should not be combined with exposure therapy for the treatment of PTSD (Burghardt, Sigurdsson, et al. 2013). Given the questionable efficacy of SSRIs for the treatment of PTSD, evidence for an effective combinatorial approach (SSRIs and exposure therapy) is scant (Hetrick, Purcell, et al. 2010).

6.3.6 Norepinephrine/Propranolol

Researchers and clinicians hypothesize that hyper-consolidation of trauma and/or poor extinction might contribute to development of PTSD. Given the vast amount of data implicating the noradrenergic system in memory consolidation, some suggest that noradrenergic dysfunction might underlie pathology of PTSD, in particular, deficits in fear acquisition and extinction, as well as hyperarousal (McGaugh 2004, O'Donnell, Hegadoren, et al. 2004, Rodrigues, LeDoux, et al. 2009, Roozendaal, McEwen, et al. 2009).

In rodents, stress-induced release of norepinephrine (NE) into the amygdala, specifically the BLA, is critical for emotional memory consolidation (Galvez, Mesches, et al. 1996). Although numerous studies implicate NE, via β-adrenergic receptors, in consolidation of aversive memory (inhibitory avoidance learning, in particular), the role of NE in associative fear learning is less clear (McGaugh 2004, Roozendaal, Schelling, et al. 2008). Some studies find evidence of noradrenergic activity in consolidation of associative fear learning and extinction (Berlau and McGaugh 2006, LaLumiere, Buen, et al. 2003, Mueller, Porter, et al. 2008, Roozendaal, Hui, et al. 2006). Others, however, report that treatment with NE or propranolol (β-adrenergic receptor antagonist) has no effect on consolidation of auditory fear learning. Furthermore, propranolol administration significantly impairs auditory fear acquisition (whereas, treatment with an α1-adrenergic receptor antagonist facilitates fear acquisition) (Bush, Caparosa, et al. 2010, Debiec and Ledoux 2004, Lazzaro, Hou, et al. 2010).

Interestingly, noradrenergic signaling is critical for reconsolidation of fear learning across multiple paradigms (Debiec and Ledoux 2004, Muravieva and Alberini 2010, Przybyslawski, Roullet, et al. 1999). Reconsolidation involves transiently rendering memories labile through reactivation (Nader, Schafe, et al. 2000). Through this reactivation, memories again undergo a

stabilization process that is sensitive to protein-synthesis inhibitors (as in the original consolidation phase). Propanolol administered systemically or intra-amygdala blocks reconsolidation of cue and context fear conditioning (Debiec and Ledoux 2004, Muravieva and Alberini 2010). Intra-LA infusion of Isoproterenol, a β-adrenergic receptor agonist, enhances reconsolidation, blocking extinction of cued fear (Debiec, Bush, et al. 2011).

Studies in healthy human subjects support a role for norepinephrine in memory consolidation, additionally. Propranolol attenuates responses to aversively conditioned stimuli and memory for emotionally arousing stories when administered during the consolidation window (Grillon, Cordova, et al. 2004, Orr, Milad, et al. 2006, Reist, Duffy, et al. 2001). Memory retrieval, however, is not impaired by propranolol (Tollenaar, Elzinga, et al. 2009, Tollenaar, Elzinga, et al. 2009). As in rodents, decreases in recall might be due, in part, to diminished amygdala response to emotionally relevant stimuli, as propranolol inhibits amygdala reactivity to facial expressions (Hurlemann, Walter, et al. 2010).

As noradrenergic activity is implicated in memory consolidation processes, drugs like propranolol are being tested for their efficacy in blocking primary consolidation or reconsolidation of traumatic memory in PTSD. Studies show that propranolol administration in the immediate aftermath of trauma might be effective at secondary prevention of PTSD, as rates and symptoms of PTSD were lower over a period of weeks to months post-trauma in individuals who received propranolol (Pitman, Sanders, et al. 2002, Taylor and Cahill 2002, Vaiva, Ducrocq, et al. 2003). However, a recent double-blind pilot study in children finds weak evidence for a decrease in PTSD symptoms in boys acutely administered propranolol, and an increase in symptoms in similarly-treated girls (Nugent, Christopher, et al. 2010). Increasingly, research has focused on the effect of propranolol on weakening emotional associations during reconsolidation of traumatic memory. Propranolol administered with trauma reactivation

decreased physiological responses, such as heart beat and skin conductance, during subsequent mental imagery of the event (Brunet, Orr, et al. 2008)(Brunet et al. 2008). In separate studies, propranolol significantly improved PTSD symptoms compared to placebo after administration with six brief trauma reactivation sessions (Brunet, Poundja, et al. 2011). Several other studies report improvement of PTSD symptoms with propranolol treatment, however, dosage and administration is either unknown or not reported (Famularo, Kinscherff, et al. 1988, McGhee, Maani, et al. 2009).

6.4 Device-based treatments

Increasingly, researchers are investigating device-based treatments to alter pathological brain activity and connectivity in psychiatric disease. A number of different stimulation tools - including deep-brain stimulation (DBS), vagus nerve stimulation (VNS), transcranial direct current stimulation (tDCS), and transcranial magnetic stimulation (TMS) - are under investigation, and each are at various stages of development and testing at the pre-clinical and clinical level (Marin, Camprodon, et al. 2014).

Relative to other device-based treatments, DBS has been studied the most extensively with a comparatively large amount of evidence accumulated supporting efficacy in the treatment of psychiatric disorders. DBS has demonstrated efficacy in the treatment of Parkinson's, its original indication, and is now being investigated for the treatment of and depression, obsessive compulsive disorder (OCD), and PTSD (Koek, Langevin, et al. 2014, Marin, Camprodon, et al. 2014, Mayberg, Lozano, et al. 2005, Mian, Campos, et al. 2010, Yu and Neimat 2008). At the pre-clinical level, several studies find enhanced cued fear extinction with DBS of the ventral striatum that may be mediated by enhanced BDNF expression (Do-Monte, Rodriguez-Romaguera, et al. 2013, Rodriguez-Romaguera, Do Monte, et al. 2012, Whittle, Schmuckermair,

et al. 2013). Others find decreased PTSD-like symptoms and cued fear expression in rats with DBS of the amygdala (Langevin, De Salles, et al. 2010, Stidd, Vogelsang, et al. 2013, Sui, Huang, et al. 2014). Based on these studies, participants are now being recruited to evaluate the efficacy of BLA DBS for the treatment of PTSD (Koek, Langevin, et al. 2014). Because the mechanism of action is still relatively unclear (i.e. whether DBS activates or inhibits targeted brain regions), future rodent studies will be critical for the interpretation, and thus refinement, of DBS and DBS treatment protocols.

Transcranial magnetic stimulation has also shown promise for the treatment of psychiatric disorders, where non-invasive electrical current is delivered via magnetic coil placed on the scalp (Rossi, Hallett, et al. 2009). TMS of mPFC ameliorates PTSD symptoms when administered repetitively over two weeks, in combination with a brief trauma re-exposure with script driven imagery, and in combination with exposure therapy (Boggio, Rocha, et al. 2010, Isserles, Shalev, et al. 2013, Osuch, Benson, et al. 2009). These studies, along with evidence that transcranial direct current stimulation (tDCS) of dorsolateral prefrontal cortex modulates consolidation of cued fear, underline the importance of mPFC in fear learning, which has been extensively studied in rodents (Asthana, Nueckel, et al. 2013, Milad and Quirk 2002, Vidal-Gonzalez, Vidal-Gonzalez, et al. 2006).

6.5 Discussion

Here, we examine evidence for efficacy of specific treatment strategies informed by rodent pre-clinical studies. Fear conditioning and extinction experiments in animal subjects allow researchers to cleanly model aversive learning processes that underlie development of PTSD, as well as inhibition of fear via exposure therapy. While the number of approved treatments for

PTSD is still limited, evidence across species indicates new pharmacotherapies and device-based treatments that are at various stages of development.

In surveying the literature, it is clear that rodent models inform clinical studies, however the linear bench to bedside translational paradigm has shifted. Pharmacotherapies that are being tested in humans stemming from studies of rodent neurotransmitter systems, for instance, are being further developed in their original models to safely refine treatment windows, dosages, etc. In the case of SSRIs or DBS, rodent models are being tested based on indications from human studies. While the traditional translational model has uncovered a number of therapies – D-cycloserine, hydrocortisone, and others discussed in this review – it is clear from a number of other therapies that a new, circular translational model is emerging. In this vein, researchers have begun investigating the efficacy of MDMA (3,4-methylenedioxy-methamphetamine), ACE (angiotensin-converting enzyme) inhibitors, PACAP (pituitary adenylate cyclase-activating polypeptide), etc (Marvar, Goodman, et al. 2014, Mithoefer, Wagner, et al. 2011, Ressler, Mercer, et al. 2011).

Although the number of approved treatments for PTSD is minimal, translational models of PTSD and PTSD treatment are among the best in psychiatric research. Through these models, researchers and clinicians have begun to establish specific therapies for PTSD and uncover exciting new evidence that point to a number of promising pharmacotherapies and device-based treatments.

Chapter 7:

Discussion

7.1 Summary of results

Based on prior evidence in the literature, this dissertation proposes that Cnr1 and CCKBR interact to mediate cued fear extinction, where activation of Cnr1 inhibits release of the anxiogenic peptide CCK to decrease fear and promote extinction. Altogether, the results of the dissertation support the hypothesis that Cnr1 and CCKBR interact during cued fear expression and extinction retention. Genetic, anatomical, and functional corroborate the observed behavioral interaction and provide a mechanism by which Cnr1 and CCKBR interface. Specifically, we first report the effect of Cnr1 antagonist administration on C57BL/6J and *CCKBR* transgenic mouse fear behavior. We find that Cnr1 antagonist administration increases fear behavior during cued fear expression in C57BL/6J and *CCKBR*-wild-type subjects, but not *CCKBR*-knockout subjects, suggesting that activation of Cnr1 is upstream of CCKBR during cued fear expression. In the same chapter, we present anatomical data that show Cnr1-positive fibers form perisomatic baskets in the basolateral amygdala (BLA), a region critical for Pavlovian fear conditioning and extinction and the putative site of a Cnr1-CCKBR interaction during cued fear expression. Furthermore, the behavioral effect of CCKBR antagonist administration in *Cnr1* transgenic mice is presented. We find CCKBR antagonist administration enhances cued fear expression and extinction retention in *Cnr1* knockout subjects, but not wild-type littermates, supporting our prior behavioral findings that CCKBR is downstream of Cnr1 in cued fear expression and extinction retention. *Ex vivo* amygdala slice experiments suggest that activation of Cnr1 inhibits release of CCK, providing a mechanism by which Cnr1 inhibits CCKBR during cued fear expression. Finally, we present evidence of sex differences in anxiety-like behavior of *Cnr1* transgenic mice. Here, we report that female *Cnr1* knockout subjects are buffered against increased anxiety-like behavior seen in male *Cnr1* knockout subjects (which is in line with prior literature and observed in our own experiments). Pharmacological and ovariectomy experiments

suggest that female gonadal hormones may be protective early in development against anxiety-like behavior observed in adult *Cnr1* knockout males.

7.2 Integration of findings

Prior data from our laboratory demonstrates that Cnr1 and CCKBR, when using pharmacological probes, interact to mediate extinction of fear-potentiated startle in rats (Chhatwal, Gutman, et al. 2009). This dissertation extends those findings in mice using extinction of conditioned fear. However, Chhatwal et al. observe an interaction between Cnr1 and CCKBR during within-session extinction, whereas our findings suggest that a Cnr1-CCKBR interaction occurs primarily during cued fear expression. This within-session effect supports the conclusion of Marsicano et al., which reports comparable levels of freezing during cued fear expression, but persistent blockade of within-session extinction in *Cnr1* knockout and Cnr1 antagonist administered subjects (Marsicano, Wotjak, et al. 2002). Nevertheless, an interaction between Cnr1 and CCKBR persists through extinction retention tests in the studies conducted under the purview of this dissertation and in the experiments of Chhatwal et al.

Interestingly, hippocampal slice physiology studies suggest an alternative Cnr1-CCK interaction than our hypothesized behavioral model. According to the "Cnr1 receptor hypothesis", CCK activation of CCKBR initiates endocannabinoid synthesis, activating pre-synaptic Cnr1 on CCK-containing interneurons to inhibit GABA transmission. Separately, CCK strongly depolarizes parvalbumin interneurons via CCKBR, increasing firing frequency of inhibitory currents (Foldy, Lee, et al. 2007, Karson, Whittington, et al. 2008, Lee and Soltesz 2011, Lee, Foldy, et al. 2011). Although our results suggest that CCKBR is downstream of Cnr1, cell-type and region-specific behavioral studies will better clarify the differences in our respective models of a Cnr1-CCKBR interaction during aversive learning. As studies demonstrate, the endocannabinoids modulate

fear using a variety of unique mechanisms across different brain regions, suggesting that the cannabinoid and cholecystokinin system may interact in variety of ways apart from the mechanism used during cued fear expression (Ruehle, Rey, et al. 2012).

7.3 Implications and future directions

Although this dissertation provides evidence of an interaction between Cnr1 and CCKBR during cued fear expression, future experiments addressing the nature of this interaction are needed to corroborate the present evidence and further the implications of this research. Site-specific experiments, including lentivirus and local drug infusion, are needed to precisely determine the locus of a Cnr1-CCKBR interaction. Furthermore, differences in findings across studies should be addressed by streamlining experimental protocols. As mentioned, the findings of this dissertation are in contrast with prior data suggesting that Cnr1 primarily contributes to within-session extinction (Marsicano, Wotjak, et al. 2002, Plendl and Wotjak 2010). However, these prior studies used individually-housed subjects. Individual housing has been shown to have significant effects on fear- and anxiety-like behavior (Voikar, Polus, et al. 2005). As the cannabinoid system is sensitive to stress, differences in housing and behavioral paradigms must be accounted for during experimental design and interpretation of future studies.

Additionally, we initially proposed designing microdialysis experiments to detect amygdala CCK release in awake, behaving mice during cued fear extinction, technological limitations prevented these studies from being conducted. Should these technological barriers be minimized or eliminated in the future, microdialysis would allow researchers to directly test whether Cnr1 activation inhibits CCK release during cued fear expression.

As Cnr1 and other cannabinoid family genes are implicated in PTSD, future studies could identify potential interactions between the cannabinoid system, the cholecystokinin system, and PTSD (and other anxiety and fear-related disorders). For instance, PET data reveals that individuals with PTSD have increased brain Cnr1 availability, possibly due to lower peripheral levels of anadamide. One hypothesis is that lower circulating levels of anandamide removes Cnr1-mediated inhibition of CCK, perhaps contributing to PTSD symptomatology. Interestingly, a number of studies support a link between the CCK system and panic, which may share a similar neurobiological mechanism with posttraumatic flashbacks (Bradwejn, Koszycki, et al. 1990, Bradwejn, Koszycki, et al. 1991, de Montigny 1989, Kellner, Wiedemann, et al. 2000, Mellman and Davis 1985) .

So far, translational studies addressing the promise of pharmacotherapies targeting the cannabinoid and cholecystokinin systems are few. Human studies suggest delta-9-tetrahydrocannabinoil (Δ9-THC), a partial agonist of Cnr1, facilitates extinction of conditioned fear in healthy volunteers (Klumpers, Denys, et al. 2012, Rabinak, Angstadt, et al. 2013). Furthermore, several genetic association studies reveal specific Cnr1 and FAAH allelic risk factors for threat processing, stress-coping, and PTSD (Gunduz-Cinar, MacPherson, et al. 2013, Lu, Ogdie, et al. 2008, Pardini, Krueger, et al. 2012). As mentioned, human neuroimaging data suggest that individuals with PTSD have increased brain Cnr1 availability, possibly due to lower peripheral levels of anadamide (Neumeister, Normandin, et al. 2013). Although the data is preliminary, several studies show that the synthetic cannabinoid receptor agonist, nabilone, improves insomnia, subjective chronic pain, nightmares, and symptoms related to PTSD (Cameron, Watson, et al. 2014, Fraser 2009). Fewer studies exist that address the efficacy of CCK targeted pharmacotherapies, and none exist that address efficacy of treating PTSD. As intravenously administered CCK induces panic attacks in healthy human volunteers and individuals with panic disorder, researchers have investigated the ability of CCKBR antagonist

to mitigate panic attacks (Adams, Pyke, et al. 1995, Kramer, Cutler, et al. 1995, Pande, Greiner, et al. 1999). So far, these studies have yielded negative results.

Looking forward, the studies of this dissertation contribute to the growing body of literature suggesting that the cannabinoid and cholecystokinin critically interact within limbic regions to mediate emotion. It is our hope that these studies propel new and ongoing research in humans to develop therapies for the treatment of PTSD and other fear-related and anxiety disorders.

References

Abelson, JL and Nesse, RM (1990) Cholecystokinin-4 and panic. Arch Gen Psychiatry 47: 395.

Adams, JB, Pyke, RE, Costa, J, Cutler, NR, Schweizer, E, Wilcox, CS, Wisselink, PG, Greiner, M, Pierce, MW and Pande, AC (1995) A double-blind, placebo-controlled study of a CCK-B receptor antagonist, CI-988, in patients with generalized anxiety disorder. J Clin Psychopharmacol 15: 428-34.

Adrian, TE, Allen, JM, Bloom, SR, Ghatei, MA, Rossor, MN, Roberts, GW, Crow, TJ, Tatemoto, K and Polak, JM (1983) Neuropeptide Y distribution in human brain. Nature 306: 584-6.

Aerni, A, Traber, R, Hock, C, Roozendaal, B, Schelling, G, Papassotiropoulos, A, Nitsch, RM, Schnyder, U and de Quervain, DJ (2004) Low-dose cortisol for symptoms of posttraumatic stress disorder. Am J Psychiatry 161: 1488-90.

Alen, F, Ramirez-Lopez, MT, Gomez de Heras, R, Rodriguez de Fonseca, F and Orio, L (2013) Cannabinoid receptors and cholecystokinin in feeding inhibition. Vitam Horm 92: 165-96.

Allen, LS and Gorski, RA (1990) Sex difference in the bed nucleus of the stria terminalis of the human brain. J Comp Neurol 302: 697-706.

Allen, YS, Adrian, TE, Allen, JM, Tatemoto, K, Crow, TJ, Bloom, SR and Polak, JM (1983) Neuropeptide Y distribution in the rat brain. Science 221: 877-9.

Andero, R, Daviu, N, Escorihuela, RM, Nadal, R and Armario, A (2012) 7,8-dihydroxyflavone, a TrkB receptor agonist, blocks long-term spatial memory impairment caused by immobilization stress in rats. Hippocampus 22: 399-408.

Andero, R, Brothers, SP, Jovanovic, T, Chen, YT, Salah-Uddin, H, Cameron, M, Bannister, TD, Almli, L, Stevens, JS, Bradley, B, Binder, EB, Wahlestedt, C and Ressler, KJ (2013) Amygdala-dependent fear is regulated by Oprl1 in mice and humans with PTSD. Sci Transl Med 5: 188ra73.

Areda, T, Raud, S, Philips, MA, Innos, J, Matsui, T, Koks, S, Vasar, E, Karis, A and Asser, T (2006) Cat odour exposure decreases exploratory activity and alters neuropeptide gene expression in CCK(2) receptor deficient mice, but not in their wild-type littermates. Behav Brain Res 169: 212-9.

Arntz, A, Merckelbach, H and de Jong, P (1993) Opioid antagonist affects behavioral effects of exposure in vivo. J Consult Clin Psychol 61: 865-70.

Asthana, M, Nueckel, K, Muhlberger, A, Neueder, D, Polak, T, Domschke, K, Deckert, J and Herrmann, MJ (2013) Effects of transcranial direct current stimulation on consolidation of fear memory. Front Psychiatry 4: 107.

Azad, SC, Eder, M, Marsicano, G, Lutz, B, Zieglgansberger, W and Rammes, G (2003) Activation of the cannabinoid receptor type 1 decreases glutamatergic and GABAergic synaptic transmission in the lateral amygdala of the mouse. Learn Mem 10: 116-28.

Azad, SC, Monory, K, Marsicano, G, Cravatt, BF, Lutz, B, Zieglgansberger, W and Rammes, G (2004) Circuitry for associative plasticity in the amygdala involves endocannabinoid signaling. J Neurosci 24: 9953-61.

Baker, DG, West, SA, Orth, DN, Hill, KK, Nicholson, WE, Ekhator, NN, Bruce, AB, Wortman, MD, Keck, PE, Jr. and Geracioti, TD, Jr. (1997) Cerebrospinal fluid and plasma beta-endorphin in combat veterans with post-traumatic stress disorder. Psychoneuroendocrinology 22: 517-29.

Baker, DG, West, SA, Nicholson, WE, Ekhator, NN, Kasckow, JW, Hill, KK, Bruce, AB, Orth, DN and Geracioti, TD, Jr. (1999) Serial CSF corticotropin-releasing hormone levels and adrenocortical activity in combat veterans with posttraumatic stress disorder. Am J Psychiatry 156: 585-8.

Baraban, SC (2004) Neuropeptide Y and epilepsy: recent progress, prospects and controversies. Neuropeptides 38: 261-5.

Bartel, DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281-97.

Beck, B (2006) Neuropeptide Y in normal eating and in genetic and dietary-induced obesity. Philos Trans R Soc Lond B Biol Sci 361: 1159-85.

Beinfeld, MC and Connolly, K (2001) Activation of CB1 cannabinoid receptors in rat hippocampal slices inhibits potassium-evoked cholecystokinin release, a possible mechanism contributing to the spatial memory defects produced by cannabinoids. Neurosci Lett 301: 69-71.

Benkelfat, C, Bradwejn, J, Meyer, E, Ellenbogen, M, Milot, S, Gjedde, A and Evans, A (1995) Functional neuroanatomy of CCK4-induced anxiety in normal healthy volunteers. Am J Psychiatry 152: 1180-4.

Berlau, DJ and McGaugh, JL (2006) Enhancement of extinction memory consolidation: the role of the noradrenergic and GABAergic systems within the basolateral amygdala. Neurobiol Learn Mem 86: 123-32.

Biro, E, Sarnyai, Z, Penke, B, Szabo, G and Telegdy, G (1993) Role of endogenous corticotropin-releasing factor in mediation of neuroendocrine and behavioral responses to cholecystokinin octapeptide sulfate ester in rats. Neuroendocrinology 57: 340-5.

Boggio, PS, Rocha, M, Oliveira, MO, Fecteau, S, Cohen, RB, Campanha, C, Ferreira-Santos, E, Meleiro, A, Corchs, F, Zaghi, S, Pascual-Leone, A and Fregni, F (2010) Noninvasive brain stimulation with high-frequency and low-intensity repetitive transcranial magnetic stimulation treatment for posttraumatic stress disorder. J Clin Psychiatry 71: 992-9.

Bolkan, SS and Lattal, KM (2014) Opposing effects of D-cycloserine on fear despite a common extinction duration: interactions between brain regions and behavior. Neurobiol Learn Mem 113: 25-34.

Bowers, ME, Choi, DC and Ressler, KJ (2012) Neuropeptide regulation of fear and anxiety: Implications of cholecystokinin, endogenous opioids, and neuropeptide Y. Physiol Behav 107: 699-710.

Bowers, ME and Ressler, KJ (2014) Interaction between the Cholecystokinin and Endogenous Cannabinoid Systems in Cued Fear Expression and Extinction Retention. Neuropsychopharmacology

Bradwejn, J, Koszycki, D and Meterissian, G (1990) Cholecystokinin-tetrapeptide induces panic attacks in patients with panic disorder. Can J Psychiatry 35: 83-5.

Bradwejn, J, Koszycki, D and Shriqui, C (1991) Enhanced sensitivity to cholecystokinin tetrapeptide in panic disorder. Clinical and behavioral findings. Arch Gen Psychiatry 48: 603-10.

Bradwejn, J, Koszycki, D, Couetoux du Tertre, A, van Megen, H, den Boer, J and Westenberg, H (1994) The panicogenic effects of cholecystokinin-tetrapeptide are antagonized by L-365,260, a central cholecystokinin receptor antagonist, in patients with panic disorder. Arch Gen Psychiatry 51: 486-93.

Bremner, JD, Licinio, J, Darnell, A, Krystal, JH, Owens, MJ, Southwick, SM, Nemeroff, CB and Charney, DS (1997) Elevated CSF corticotropin-releasing factor concentrations in posttraumatic stress disorder. Am J Psychiatry 154: 624-9.

Breslau, N, Davis, GC, Andreski, P and Peterson, E (1991) Traumatic events and posttraumatic stress disorder in an urban population of young adults. Arch Gen Psychiatry 48: 216-22.

Britton, KT, Southerland, S, Van Uden, E, Kirby, D, Rivier, J and Koob, G (1997) Anxiolytic activity of NPY receptor agonists in the conflict test. Psychopharmacology (Berl) 132: 6-13.

Broqua, P, Wettstein, JG, Rocher, MN, Gauthier-Martin, B and Junien, JL (1995) Behavioral effects of neuropeptide Y receptor agonists in the elevated plus-maze and fear-potentiated startle procedures. Behav Pharmacol 6: 215-222.

Brunet, A, Orr, SP, Tremblay, J, Robertson, K, Nader, K and Pitman, RK (2008) Effect of post-retrieval propranolol on psychophysiologic responding during subsequent script-driven traumatic imagery in post-traumatic stress disorder. J Psychiatr Res 42: 503-6.

Brunet, A, Poundja, J, Tremblay, J, Bui, E, Thomas, E, Orr, SP, Azzoug, A, Birmes, P and Pitman, RK (2011) Trauma reactivation under the influence of propranolol decreases posttraumatic stress symptoms and disorder: 3 open-label trials. J Clin Psychopharmacol 31: 547-50.

Bryant, RA, Creamer, M, O'Donnell, M, Silove, D and McFarlane, AC (2009) A study of the protective function of acute morphine administration on subsequent posttraumatic stress disorder. Biol Psychiatry 65: 438-40.

Burghardt, NS, Sigurdsson, T, Gorman, JM, McEwen, BS and LeDoux, JE (2013) Chronic antidepressant treatment impairs the acquisition of fear extinction. Biol Psychiatry 73: 1078-86.

Bush, DE, Caparosa, EM, Gekker, A and Ledoux, J (2010) Beta-adrenergic receptors in the lateral nucleus of the amygdala contribute to the acquisition but not the consolidation of auditory fear conditioning. Front Behav Neurosci 4: 154.

Caberlotto, L, Fuxe, K and Hurd, YL (2000) Characterization of NPY mRNA-expressing cells in the human brain: co-localization with Y2 but not Y1 mRNA in the cerebral cortex, hippocampus, amygdala, and striatum. J Chem Neuroanat 20: 327-37.

Calhoun, PS, Sampson, WS, Bosworth, HB, Feldman, ME, Kirby, AC, Hertzberg, MA, Wampler, TP, Tate-Williams, F, Moore, SD and Beckham, JC (2000) Drug use and validity of substance use self-reports in veterans seeking help for posttraumatic stress disorder. J Consult Clin Psychol 68: 923-7.

Cameron, C, Watson, D and Robinson, J (2014) Use of a synthetic cannabinoid in a correctional population for posttraumatic stress disorder-related insomnia and nightmares, chronic

pain, harm reduction, and other indications: a retrospective evaluation. J Clin Psychopharmacol 34: 559-64.

Camp, M, Norcross, M, Whittle, N, Feyder, M, D'Hanis, W, Yilmazer-Hanke, D, Singewald, N and Holmes, A (2009) Impaired Pavlovian fear extinction is a common phenotype across genetic lineages of the 129 inbred mouse strain. Genes Brain Behav 8: 744-52.

Campbell, DG, Felker, BL, Liu, CF, Yano, EM, Kirchner, JE, Chan, D, Rubenstein, LV and Chaney, EF (2007) Prevalence of depression-PTSD comorbidity: implications for clinical practice guidelines and primary care-based interventions. J Gen Intern Med 22: 711-8.

Campos, AC, Ferreira, FR and Guimaraes, FS (2012) Cannabidiol blocks long-lasting behavioral consequences of predator threat stress: possible involvement of 5HT1A receptors. J Psychiatr Res 46: 1501-10.

Carrive, P (1993) The periaqueductal gray and defensive behavior: functional representation and neuronal organization. Behav Brain Res 58: 27-47.

Chen, Q, Nakajima, A, Meacham, C and Tang, YP (2006) Elevated cholecystokininergic tone constitutes an important molecular/neuronal mechanism for the expression of anxiety in the mouse. Proc Natl Acad Sci U S A 103: 3881-6.

Chen, Q, Tang, M, Mamiya, T, Im, HI, Xiong, X, Joseph, A and Tang, YP (2010) Bi-directional effect of cholecystokinin receptor-2 overexpression on stress-triggered fear memory and anxiety in the mouse. PLoS One 5: e15999.

Chhatwal, JP, Davis, M, Maguschak, KA and Ressler, KJ (2005) Enhancing cannabinoid neurotransmission augments the extinction of conditioned fear. Neuropsychopharmacology 30: 516-24.

Chhatwal, JP and Ressler, KJ (2007) Modulation of fear and anxiety by the endogenous cannabinoid system. CNS Spectr 12: 211-20.

Chhatwal, JP, Gutman, AR, Maguschak, KA, Bowser, ME, Yang, Y, Davis, M and Ressler, KJ (2009) Functional interactions between endocannabinoid and CCK neurotransmitter systems may be critical for extinction learning. Neuropsychopharmacology 34: 509-21.

Chronwall, BM, DiMaggio, DA, Massari, VJ, Pickel, VM, Ruggiero, DA and O'Donohue, TL (1985) The anatomy of neuropeptide-Y-containing neurons in rat brain. Neuroscience 15: 1159-81.

Chung, L and Moore, SD (2007) Cholecystokinin enhances GABAergic inhibitory transmission in basolateral amygdala. Neuropeptides 41: 453-63.

Chung, L and Moore, SD (2009) Cholecystokinin excites interneurons in rat basolateral amygdala. J Neurophysiol 102: 272-84.

Chung, L and Moore, SD (2009) Neuropeptides modulate compound postsynaptic potentials in basolateral amygdala. Neuroscience 164: 1389-97.

Chung, WC, De Vries, GJ and Swaab, DF (2002) Sexual differentiation of the bed nucleus of the stria terminalis in humans may extend into adulthood. J Neurosci 22: 1027-33.

Cohen, H, Zohar, J, Gidron, Y, Matar, MA, Belkind, D, Loewenthal, U, Kozlovsky, N and Kaplan, Z (2006) Blunted HPA axis response to stress influences susceptibility to posttraumatic stress response in rats. Biol Psychiatry 59: 1208-18.

Cohen, H, Matar, MA, Buskila, D, Kaplan, Z and Zohar, J (2008) Early post-stressor intervention with high-dose corticosterone attenuates posttraumatic stress response in an animal model of posttraumatic stress disorder. Biol Psychiatry 64: 708-17.

Cohen, H and Yehuda, R (2011) Gender differences in animal models of posttraumatic stress disorder. Dis Markers 30: 141-50.

Compton, DR, Aceto, MD, Lowe, J and Martin, BR (1996) In vivo characterization of a specific cannabinoid receptor antagonist (SR141716A): inhibition of delta 9-tetrahydrocannabinol-induced responses and apparent agonist activity. J Pharmacol Exp Ther 277: 586-94.

Craft, RM (2005) Sex differences in behavioral effects of cannabinoids. Life Sci 77: 2471-8.

Cravatt, BF, Giang, DK, Mayfield, SP, Boger, DL, Lerner, RA and Gilula, NB (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature 384: 83-7.

Daskalakis, NP, Cohen, H, Cai, G, Buxbaum, JD and Yehuda, R (2014) Expression profiling associates blood and brain glucocorticoid receptor signaling with trauma-related individual differences in both sexes. Proc Natl Acad Sci U S A

Davis, M, Gendelman, DS, Tischler, MD and Gendelman, PM (1982) A primary acoustic startle circuit: lesion and stimulation studies. J Neurosci 2: 791-805.

Davis, M (1992) The role of the amygdala in fear and anxiety. Annu Rev Neurosci 15: 353-75.

de Kleine, RA, Hendriks, GJ, Kusters, WJ, Broekman, TG and van Minnen, A (2012) A randomized placebo-controlled trial of D-cycloserine to enhance exposure therapy for posttraumatic stress disorder. Biol Psychiatry 71: 962-8.

de Kleine, RA, Hendriks, GJ, Smits, JA, Broekman, TG and van Minnen, A (2014) Prescriptive variables for d-cycloserine augmentation of exposure therapy for posttraumatic stress disorder. J Psychiatr Res 48: 40-6.

de Kloet, CS, Vermetten, E, Heijnen, CJ, Geuze, E, Lentjes, EG and Westenberg, HG (2007) Enhanced cortisol suppression in response to dexamethasone administration in traumatized veterans with and without posttraumatic stress disorder. Psychoneuroendocrinology 32: 215-26.

de Montigny, C (1989) Cholecystokinin tetrapeptide induces panic-like attacks in healthy volunteers. Preliminary findings. Arch Gen Psychiatry 46: 511-7.

de Quervain, DJ, Roozendaal, B and McGaugh, JL (1998) Stress and glucocorticoids impair retrieval of long-term spatial memory. Nature 394: 787-90.

Debiec, J and Ledoux, JE (2004) Disruption of reconsolidation but not consolidation of auditory fear conditioning by noradrenergic blockade in the amygdala. Neuroscience 129: 267-72.

Debiec, J, Bush, DE and LeDoux, JE (2011) Noradrenergic enhancement of reconsolidation in the amygdala impairs extinction of conditioned fear in rats--a possible mechanism for the persistence of traumatic memories in PTSD. Depress Anxiety 28: 186-93.

Delahanty, DL, Raimonde, AJ and Spoonster, E (2000) Initial posttraumatic urinary cortisol levels predict subsequent PTSD symptoms in motor vehicle accident victims. Biol Psychiatry 48: 940-7.

Derrien, M, McCort-Tranchepain, I, Ducos, B, Roques, BP and Durieux, C (1994) Heterogeneity of CCK-B receptors involved in animal models of anxiety. Pharmacol Biochem Behav 49: 133-41.

Deschaux, O, Spennato, G, Moreau, JL and Garcia, R (2011) Chronic treatment with fluoxetine prevents the return of extinguished auditory-cued conditioned fear. Psychopharmacology (Berl) 215: 231-7.

Dietl, MM and Palacios, JM (1989) The distribution of cholecystokinin receptors in the vertebrate brain: species differences studied by receptor autoradiography. J Chem Neuroanat 2: 149-61.

Difede, J, Cukor, J, Wyka, K, Olden, M, Hoffman, H, Lee, FS and Altemus, M (2014) D-cycloserine augmentation of exposure therapy for post-traumatic stress disorder: a pilot randomized clinical trial. Neuropsychopharmacology 39: 1052-8.

Difede, J, Olden, M and Cukor, J (2014) Evidence-based treatment of post-traumatic stress disorder. Annu Rev Med 65: 319-32.

Do-Monte, FH, Rodriguez-Romaguera, J, Rosas-Vidal, LE and Quirk, GJ (2013) Deep brain stimulation of the ventral striatum increases BDNF in the fear extinction circuit. Front Behav Neurosci 7: 102.

Dumont, Y, Fournier, A, St-Pierre, S and Quirion, R (1993) Comparative characterization and autoradiographic distribution of neuropeptide Y receptor subtypes in the rat brain. J Neurosci 13: 73-86.

Egan, KJ, Carr, JE, Hunt, DD and Adamson, R (1988) Endogenous opiate system and systematic desensitization. J Consult Clin Psychol 56: 287-91.

Eippert, F, Bingel, U, Schoell, E, Yacubian, J and Buchel, C (2008) Blockade of endogenous opioid neurotransmission enhances acquisition of conditioned fear in humans. J Neurosci 28: 5465-72.

Erhman, RN, Josephson, J., Schull, J., Sparich, C. (1979) An assessment of the behavioral effects of the endorphin system with instrumental and classical conditioning paradigms Paper presented at the meeting of the Eastern Psychological Association New York

Famularo, R, Kinscherff, R and Fenton, T (1988) Propranolol treatment for childhood posttraumatic stress disorder, acute type. A pilot study. Am J Dis Child 142: 1244-7.

Fanselow, MS and Bolles, RC (1979) Naloxone and shock-elicited freezing in the rat. J Comp Physiol Psychol 93: 736-44.

Fanselow, MS (1981) Naloxone and Pavlovian fear conditioning. Learning and Motivation 12: 398-419.

Fanselow, MS, Calcagnetti, DJ and Helmstetter, FJ (1988) Peripheral versus intracerebroventricular administration of quaternary naltrexone and the enhancement of Pavlovian conditioning. Brain Res 444: 147-52.

Fanselow, MS, Kim, JJ, Young, SL, Calcagnetti, DJ, DeCola, JP, Helmstetter, FJ and Landeira-Fernandez, J (1991) Differential effects of selective opioid peptide antagonists on the acquisition of pavlovian fear conditioning. Peptides 12: 1033-7.

Fanselow, MS and LeDoux, JE (1999) Why we think plasticity underlying Pavlovian fear conditioning occurs in the basolateral amygdala. Neuron 23: 229-32.

Fekete, M, Lengyel, A, Hegedus, B, Penke, B, Zarandy, M, Toth, G and Telegdy, G (1984) Further analysis of the effects of cholecystokinin octapeptides on avoidance behaviour in rats. Eur J Pharmacol 98: 79-91.

Fendt, M, Koch, M, Kungel, M and Schnitzler, HU (1995) Cholecystokinin enhances the acoustic startle response in rats. Neuroreport 6: 2081-4.

Fendt, M, Burki, H, Imobersteg, S, Lingenhohl, K, McAllister, KH, Orain, D, Uzunov, DP and Chaperon, F (2009) Fear-reducing effects of intra-amygdala neuropeptide Y infusion in animal models of conditioned fear: an NPY Y1 receptor independent effect. Psychopharmacology (Berl) 206: 291-301.

Fink, H, Rex, A, Voits, M and Voigt, JP (1998) Major biological actions of CCK--a critical evaluation of research findings. Exp Brain Res 123: 77-83.

Flood, JF, Baker, ML, Hernandez, EN and Morley, JE (1989) Modulation of memory processing by neuropeptide Y varies with brain injection site. Brain Res 503: 73-82.

Foldy, C, Lee, SY, Szabadics, J, Neu, A and Soltesz, I (2007) Cell type-specific gating of perisomatic inhibition by cholecystokinin. Nat Neurosci 10: 1128-30.

Fornari, RV, Soares, JC, Ferreira, TL, Moreira, KM and Oliveira, MG (2008) Effects of nociceptin/orphanin FQ in the acquisition of contextual and tone fear conditioning in rats. Behav Neurosci 122: 98-106.

Frankland, PW, Josselyn, SA, Bradwejn, J, Vaccarino, FJ and Yeomans, JS (1996) Intracerebroventricular infusion of the CCKB receptor agonist pentagastrin potentiates acoustic startle. Brain Res 733: 129-32.

Frankland, PW, Josselyn, SA, Bradwejn, J, Vaccarino, FJ and Yeomans, JS (1997) Activation of amygdala cholecystokininB receptors potentiates the acoustic startle response in the rat. J Neurosci 17: 1838-47.

Fraser, GA (2009) The use of a synthetic cannabinoid in the management of treatment-resistant nightmares in posttraumatic stress disorder (PTSD). CNS Neurosci Ther 15: 84-8.

Galvez, R, Mesches, MH and McGaugh, JL (1996) Norepinephrine release in the amygdala in response to footshock stimulation. Neurobiol Learn Mem 66: 253-7.

Ganon-Elazar, E and Akirav, I (2012) Cannabinoids prevent the development of behavioral and endocrine alterations in a rat model of intense stress. Neuropsychopharmacology 37: 456-66.

Ganon-Elazar, E and Akirav, I (2013) Cannabinoids and traumatic stress modulation of contextual fear extinction and GR expression in the amygdala-hippocampal-prefrontal circuit. Psychoneuroendocrinology 38: 1675-87.

Gavranidou, M and Rosner, R (2003) The weaker sex? Gender and post-traumatic stress disorder. Depress Anxiety 17: 130-9.

Goeldner, C, Reiss, D, Wichmann, J, Kieffer, BL and Ouagazzal, AM (2009) Activation of nociceptin opioid peptide (NOP) receptor impairs contextual fear learning in mice through glutamatergic mechanisms. Neurobiol Learn Mem 91: 393-401.

Good, AJ and Westbrook, RF (1995) Effects of a microinjection of morphine into the amygdala on the acquisition and expression of conditioned fear and hypoalgesia in rats. Behav Neurosci 109: 631-41.

Goosens, KA (2011) Hippocampal regulation of aversive memories. Curr Opin Neurobiol 21: 460-6.

Gorzalka, BB and Dang, SS (2012) Minireview: Endocannabinoids and gonadal hormones: bidirectional interactions in physiology and behavior. Endocrinology 153: 1016-24.

Griebel, G, Perrault, G and Sanger, DJ (1997) CCK receptor antagonists in animal models of anxiety: comparison between exploration tests, conflict procedures and a model based on defensive behaviours. Behav Pharmacol 8: 549-60.

Grillon, C, Cordova, J, Morgan, CA, Charney, DS and Davis, M (2004) Effects of the beta-blocker propranolol on cued and contextual fear conditioning in humans. Psychopharmacology (Berl) 175: 342-52.

Groneberg, DA, Folkerts, G, Peiser, C, Chung, KF and Fischer, A (2004) Neuropeptide Y (NPY). Pulm Pharmacol Ther 17: 173-80.

Guastella, AJ, Lovibond, PF, Dadds, MR, Mitchell, P and Richardson, R (2007) A randomized controlled trial of the effect of D-cycloserine on extinction and fear conditioning in humans. Behav Res Ther 45: 663-72.

Gunduz-Cinar, O, MacPherson, KP, Cinar, R, Gamble-George, J, Sugden, K, Williams, B, Godlewski, G, Ramikie, TS, Gorka, AX, Alapafuja, SO, Nikas, SP, Makriyannis, A, Poulton, R, Patel, S, Hariri, AR, Caspi, A, Moffitt, TE, Kunos, G and Holmes, A (2013) Convergent translational evidence of a role for anandamide in amygdala-mediated fear extinction, threat processing and stress-reactivity. Mol Psychiatry 18: 813-23.

Gutman, AR, Yang, Y, Ressler, KJ and Davis, M (2008) The role of neuropeptide Y in the expression and extinction of fear-potentiated startle. J Neurosci 28: 12682-90.

Haas, DA and George, SR (1989) Neuropeptide Y-induced effects on hypothalamic corticotropin-releasing factor content and release are dependent on noradrenergic/adrenergic neurotransmission. Brain Res 498: 333-8.

Hadjiivanova, C, Belcheva, S and Belcheva, I (2003) Cholecystokinin and learning and memory processes. Acta Physiol Pharmacol Bulg 27: 83-8.

Hajos, N, Katona, I, Naiem, SS, MacKie, K, Ledent, C, Mody, I and Freund, TF (2000) Cannabinoids inhibit hippocampal GABAergic transmission and network oscillations. Eur J Neurosci 12: 3239-49.

Hanson, ES and Dallman, MF (1995) Neuropeptide Y (NPY) may integrate responses of hypothalamic feeding systems and the hypothalamo-pituitary-adrenal axis. J Neuroendocrinol 7: 273-9.

Hariri, AR, Gorka, A, Hyde, LW, Kimak, M, Halder, I, Ducci, F, Ferrell, RE, Goldman, D and Manuck, SB (2009) Divergent effects of genetic variation in endocannabinoid signaling on human threat- and reward-related brain function. Biol Psychiatry 66: 9-16.

Harro, J, Kiivet, RA, Lang, A and Vasar, E (1990) Rats with anxious or non-anxious type of exploratory behaviour differ in their brain CCK-8 and benzodiazepine receptor characteristics. Behav Brain Res 39: 63-71.

Harvey, BH (1997) The neurobiology and pharmacology of depression. A comparative overview of serotonin selective antidepressants. S Afr Med J 87: 540-50, 552.

Hefner, K, Whittle, N, Juhasz, J, Norcross, M, Karlsson, RM, Saksida, LM, Bussey, TJ, Singewald, N and Holmes, A (2008) Impaired fear extinction learning and cortico-amygdala circuit abnormalities in a common genetic mouse strain. J Neurosci 28: 8074-85.

Heilig, M, Soderpalm, B, Engel, JA and Widerlov, E (1989) Centrally administered neuropeptide Y (NPY) produces anxiolytic-like effects in animal anxiety models. Psychopharmacology (Berl) 98: 524-9.

Heilig, M, McLeod, S, Koob, GK and Britton, KT (1992) Anxiolytic-like effect of neuropeptide Y (NPY), but not other peptides in an operant conflict test. Regul Pept 41: 61-9.

Heilig, M (1995) Antisense inhibition of neuropeptide Y (NPY)-Y1 receptor expression blocks the anxiolytic-like action of NPY in amygdala and paradoxically increases feeding. Regul Pept 59: 201-5.

Heilig, M (2004) The NPY system in stress, anxiety and depression. Neuropeptides 38: 213-24.

Heilig, M, Zachrisson, O, Thorsell, A, Ehnvall, A, Mottagui-Tabar, S, Sjogren, M, Asberg, M, Ekman, R, Wahlestedt, C and Agren, H (2004) Decreased cerebrospinal fluid neuropeptide Y (NPY) in patients with treatment refractory unipolar major depression: preliminary evidence for association with preproNPY gene polymorphism. J Psychiatr Res 38: 113-21.

Herkenham, M, Lynn, AB, Little, MD, Johnson, MR, Melvin, LS, de Costa, BR and Rice, KC (1990) Cannabinoid receptor localization in brain. Proc Natl Acad Sci U S A 87: 1932-6.

Hernandez-Gomez, AM, Aguilar-Roblero, R and Perez de la Mora, M (2002) Role of cholecystokinin-A and cholecystokinin-B receptors in anxiety. Amino Acids 23: 283-90.

Hernandez, LL and Powell, DA (1980) Effects of anloxone on Pavlovian conditioning of eyeblink and heart rate responses in rabbits. Life Sci 27: 863-9.

Hetrick, SE, Purcell, R, Garner, B and Parslow, R (2010) Combined pharmacotherapy and psychological therapies for post traumatic stress disorder (PTSD). Cochrane Database Syst Rev CD007316.

Higgins, GA, Kew, JN, Richards, JG, Takeshima, H, Jenck, F, Adam, G, Wichmann, J, Kemp, JA and Grottick, AJ (2002) A combined pharmacological and genetic approach to investigate the role of orphanin FQ in learning and memory. Eur J Neurosci 15: 911-22.

Hill, DR, Campbell, NJ, Shaw, TM and Woodruff, GN (1987) Autoradiographic localization and biochemical characterization of peripheral type CCK receptors in rat CNS using highly selective nonpeptide CCK antagonists. J Neurosci 7: 2967-76.

Hill, MN, Hillard, CJ and McEwen, BS (2011) Alterations in corticolimbic dendritic morphology and emotional behavior in cannabinoid CB1 receptor-deficient mice parallel the effects of chronic stress. Cereb Cortex 21: 2056-64.

Hines, M, Allen, LS and Gorski, RA (1992) Sex differences in subregions of the medial nucleus of the amygdala and the bed nucleus of the stria terminalis of the rat. Brain Res 579: 321-6.

Hoffman, L, Burges Watson, P, Wilson, G and Montgomery, J (1989) Low plasma beta-endorphin in post-traumatic stress disorder. Aust N Z J Psychiatry 23: 269-73.

Hofmann, SG, Meuret, AE, Smits, JA, Simon, NM, Pollack, MH, Eisenmenger, K, Shiekh, M and Otto, MW (2006) Augmentation of exposure therapy with D-cycloserine for social anxiety disorder. Arch Gen Psychiatry 63: 298-304.

Holbrook, TL, Galarneau, MR, Dye, JL, Quinn, K and Dougherty, AL (2010) Morphine use after combat injury in Iraq and post-traumatic stress disorder. N Engl J Med 362: 110-7.

Hughes, J and Kosterlitz, HW (1983) Opioid Peptides: introduction. Br Med Bull 39: 1-3.

Hurlemann, R, Walter, H, Rehme, AK, Kukolja, J, Santoro, SC, Schmidt, C, Schnell, K, Musshoff, F, Keysers, C, Maier, W, Kendrick, KM and Onur, OA (2010) Human amygdala reactivity is diminished by the beta-noradrenergic antagonist propranolol. Psychol Med 40: 1839-48.

Imhof, JT, Coelho, ZM, Schmitt, ML, Morato, GS and Carobrez, AP (1993) Influence of gender and age on performance of rats in the elevated plus maze apparatus. Behav Brain Res 56: 177-80.

Ipser, JC and Stein, DJ (2012) Evidence-based pharmacotherapy of post-traumatic stress disorder (PTSD). Int J Neuropsychopharmacol 15: 825-40.

Ipser, JC, Terburg, D, Syal, S, Phillips, N, Solms, M, Panksepp, J, Malcolm-Smith, S, Thomas, K, Stein, DJ and van Honk, J (2013) Reduced fear-recognition sensitivity following acute buprenorphine administration in healthy volunteers. Psychoneuroendocrinology 38: 166-70.

Isserles, M, Shalev, AY, Roth, Y, Peri, T, Kutz, I, Zlotnick, E and Zangen, A (2013) Effectiveness of deep transcranial magnetic stimulation combined with a brief exposure procedure in post-traumatic stress disorder--a pilot study. Brain Stimul 6: 377-83.

Izquierdo, A, Wellman, CL and Holmes, A (2006) Brief uncontrollable stress causes dendritic retraction in infralimbic cortex and resistance to fear extinction in mice. J Neurosci 26: 5733-8.

Izumi, T, Inoue, T, Tsuchiya, K, Hashimoto, S, Ohmori, T and Koyama, T (1996) Effect of the selective CCKB receptor antagonist LY288513 on conditioned fear stress in rats. Eur J Pharmacol 300: 25-31.

Javanmard, M, Shlik, J, Kennedy, SH, Vaccarino, FJ, Houle, S and Bradwejn, J (1999) Neuroanatomic correlates of CCK-4-induced panic attacks in healthy humans: a comparison of two time points. Biol Psychiatry 45: 872-82.

Johnson, NJ and Rodgers, RJ (1996) Ethological analysis of cholecystokinin (CCKA and CCKB) receptor ligands in the elevated plus-maze test of anxiety in mice. Psychopharmacology (Berl) 124: 355-64.

Johnston, AL and File, SE (1991) Sex differences in animal tests of anxiety. Physiol Behav 49: 245-50.

Joseph, A, Tang, M, Mamiya, T, Chen, Q, Yang, LL, Jiao, J, Yu, N and Tang, YP (2013) Temporal association of elevated cholecystokininergic tone and adolescent trauma is critical for posttraumatic stress disorder-like behavior in adult mice. Proc Natl Acad Sci U S A 110: 6589-94.

Josselyn, SA, Frankland, PW, Petrisano, S, Bush, DE, Yeomans, JS and Vaccarino, FJ (1995) The CCKB antagonist, L-365,260, attenuates fear-potentiated startle. Peptides 16: 1313-5.

Jovanovic, T and Ressler, KJ (2010) How the neurocircuitry and genetics of fear inhibition may inform our understanding of PTSD. Am J Psychiatry 167: 648-62.

Kadar, T, Fekete, M and Telegdy, G (1981) Modulation of passive avoidance behaviour of rats by intracerebroventricular administration of cholecystokinin octapeptide sulfate ester and nonsulfated cholecystokinin octapeptide. Acta Physiol Acad Sci Hung 58: 269-74.

Kalisch, R, Holt, B, Petrovic, P, De Martino, B, Kloppel, S, Buchel, C and Dolan, RJ (2009) The NMDA agonist D-cycloserine facilitates fear memory consolidation in humans. Cereb Cortex 19: 187-96.

Kalra, SP and Kalra, PS (2004) NPY--an endearing journey in search of a neurochemical on/off switch for appetite, sex and reproduction. Peptides 25: 465-71.

Kamprath, K, Romo-Parra, H, Haring, M, Gaburro, S, Doengi, M, Lutz, B and Pape, HC (2011) Short-term adaptation of conditioned fear responses through endocannabinoid signaling in the central amygdala. Neuropsychopharmacology 36: 652-63.

Karlsson, RM, Holmes, A, Heilig, M and Crawley, JN (2005) Anxiolytic-like actions of centrally-administered neuropeptide Y, but not galanin, in C57BL/6J mice. Pharmacol Biochem Behav 80: 427-36.

Karson, MA, Whittington, KC and Alger, BE (2008) Cholecystokinin inhibits endocannabinoid-sensitive hippocampal IPSPs and stimulates others. Neuropharmacology 54: 117-28.

Kask, A, Kivastik, T, Rago, L and Harro, J (1999) Neuropeptide Y Y1 receptor antagonist BIBP3226 produces conditioned place aversion in rats. Prog Neuropsychopharmacol Biol Psychiatry 23: 705-11.

Katona, I, Rancz, EA, Acsady, L, Ledent, C, Mackie, K, Hajos, N and Freund, TF (2001) Distribution of CB1 cannabinoid receptors in the amygdala and their role in the control of GABAergic transmission. J Neurosci 21: 9506-18.

Kellner, M, Yassouridis, A, Jahn, H and Wiedemann, K (1997) Influence of clonidine on psychopathological, endocrine and respiratory effects of cholecystokinin tetrapeptide in patients with panic disorder. Psychopharmacology (Berl) 133: 55-61.

Kellner, M, Wiedemann, K, Yassouridis, A, Levengood, R, Guo, LS, Holsboer, F and Yehuda, R (2000) Behavioral and endocrine response to cholecystokinin tetrapeptide in patients with posttraumatic stress disorder. Biol Psychiatry 47: 107-11.

Kennedy, JL, Bradwejn, J, Koszycki, D, King, N, Crowe, R, Vincent, J and Fourie, O (1999) Investigation of cholecystokinin system genes in panic disorder. Mol Psychiatry 4: 284-5.

Kessler, RC, Sonnega, A, Bromet, E, Hughes, M and Nelson, CB (1995) Posttraumatic stress disorder in the National Comorbidity Survey. Arch Gen Psychiatry 52: 1048-60.

Kessler, RC (2000) Posttraumatic stress disorder: the burden to the individual and to society. J Clin Psychiatry 61 Suppl 5: 4-12; discussion 13-4.

King, PJ, Widdowson, PS, Doods, HN and Williams, G (1999) Regulation of neuropeptide Y release by neuropeptide Y receptor ligands and calcium channel antagonists in hypothalamic slices. J Neurochem 73: 641-6.

Klumpers, F, Denys, D, Kenemans, JL, Grillon, C, van der Aart, J and Baas, JM (2012) Testing the effects of Delta9-THC and D-cycloserine on extinction of conditioned fear in humans. J Psychopharmacol 26: 471-8.

Knoll, AT, Meloni, EG, Thomas, JB, Carroll, FI and Carlezon, WA, Jr. (2007) Anxiolytic-like effects of kappa-opioid receptor antagonists in models of unlearned and learned fear in rats. J Pharmacol Exp Ther 323: 838-45.

Knoll, AT, Muschamp, JW, Sillivan, SE, Ferguson, D, Dietz, DM, Meloni, EG, Carroll, FI, Nestler, EJ, Konradi, C and Carlezon, WA, Jr. (2011) Kappa opioid receptor signaling in the basolateral amygdala regulates conditioned fear and anxiety in rats. Biol Psychiatry 70: 425-33.

Koek, RJ, Langevin, JP, Krahl, SE, Kosoyan, HJ, Schwartz, HN, Chen, JW, Melrose, R, Mandelkern, MJ and Sultzer, D (2014) Deep brain stimulation of the basolateral amygdala for treatment-refractory combat post-traumatic stress disorder (PTSD): study protocol for a pilot randomized controlled trial with blinded, staggered onset of stimulation. Trials 15: 356.

Kokare, DM, Dandekar, MP, Chopde, CT and Subhedar, N (2005) Interaction between neuropeptide Y and alpha-melanocyte stimulating hormone in amygdala regulates anxiety in rats. Brain Res 1043: 107-14.

Koks, S, Vasar, E, Soosaar, A, Lang, A, Volke, V, Voikar, V, Bourin, M and Mannisto, PT (1997) Relation of exploratory behavior of rats in elevated plus-maze to brain receptor binding properties and serum growth hormone levels. Eur Neuropsychopharmacol 7: 289-94.

Korem, N and Akirav, I (2014) Cannabinoids Prevent the Effects of a Footshock Followed by Situational Reminders on Emotional Processing. Neuropsychopharmacology

Kramer, MS, Cutler, NR, Ballenger, JC, Patterson, WM, Mendels, J, Chenault, A, Shrivastava, R, Matzura-Wolfe, D, Lines, C and Reines, S (1995) A placebo-controlled trial of L-365,260, a CCKB antagonist, in panic disorder. Biol Psychiatry 37: 462-6.

Kraus, A, Geuze, E, Schmahl, C, Greffrath, W, Treede, RD, Bohus, M and Vermetten, E (2009) Differentiation of pain ratings in combat-related posttraumatic stress disorder. Pain 143: 179-85.

Krysiak, R, Obuchowicz, E and Herman, ZS (2000) Conditioned fear-induced changes in neuropeptide Y-like immunoreactivity in rats: the effect of diazepam and buspirone. Neuropeptides 34: 148-57.

Kuhnert, S, Meyer, C and Koch, M (2013) Involvement of cannabinoid receptors in the amygdala and prefrontal cortex of rats in fear learning, consolidation, retrieval and extinction. Behav Brain Res 250: 274-84.

Kuo, LE, Kitlinska, JB, Tilan, JU, Li, L, Baker, SB, Johnson, MD, Lee, EW, Burnett, MS, Fricke, ST, Kvetnansky, R, Herzog, H and Zukowska, Z (2007) Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome. Nat Med 13: 803-11.

Kuriyama, K, Honma, M, Soshi, T, Fujii, T and Kim, Y (2011) Effect of D-cycloserine and valproic acid on the extinction of reinstated fear-conditioned responses and habituation of fear conditioning in healthy humans: a randomized controlled trial. Psychopharmacology (Berl) 218: 589-97.

Kuriyama, K, Honma, M, Yoshiike, T and Kim, Y (2013) Valproic acid but not D-cycloserine facilitates sleep-dependent offline learning of extinction and habituation of conditioned fear in humans. Neuropharmacology 64: 424-31.

Kushner, MG, Kim, SW, Donahue, C, Thuras, P, Adson, D, Kotlyar, M, McCabe, J, Peterson, J and Foa, EB (2007) D-cycloserine augmented exposure therapy for obsessive-compulsive disorder. Biol Psychiatry 62: 835-8.

Kuwahara, T, Kudoh, T, Nakano, A, Yoshizaki, H, Takamiya, M, Nagase, H and Arisawa, M (1993) Species specificity of pharmacological characteristics of CCK-B receptors. Neurosci Lett 158: 1-4.

LaBar, KS, Gatenby, JC, Gore, JC, LeDoux, JE and Phelps, EA (1998) Human amygdala activation during conditioned fear acquisition and extinction: a mixed-trial fMRI study. Neuron 20: 937-45.

LaLumiere, RT, Buen, TV and McGaugh, JL (2003) Post-training intra-basolateral amygdala infusions of norepinephrine enhance consolidation of memory for contextual fear conditioning. J Neurosci 23: 6754-8.

Langevin, JP, De Salles, AA, Kosoyan, HP and Krahl, SE (2010) Deep brain stimulation of the amygdala alleviates post-traumatic stress disorder symptoms in a rat model. J Psychiatr Res 44: 1241-5.

Langhans, N, Rindi, G, Chiu, M, Rehfeld, JF, Ardman, B, Beinborn, M and Kopin, AS (1997) Abnormal gastric histology and decreased acid production in cholecystokinin-B/gastrin receptor-deficient mice. Gastroenterology 112: 280-6.

Laricchiuta, D, Centonze, D and Petrosini, L (2013) Effects of endocannabinoid and endovanilloid systems on aversive memory extinction. Behav Brain Res 256: 101-7.

Larsson, LI and Rehfeld, JF (1979) Localization and molecular heterogeneity of cholecystokinin in the central and peripheral nervous system. Brain Res 165: 201-18.

Laviolette, SR and Grace, AA (2006) Cannabinoids Potentiate Emotional Learning Plasticity in Neurons of the Medial Prefrontal Cortex through Basolateral Amygdala Inputs. J Neurosci 26: 6458-68.

Lazzaro, SC, Hou, M, Cunha, C, LeDoux, JE and Cain, CK (2010) Antagonism of lateral amygdala alpha1-adrenergic receptors facilitates fear conditioning and long-term potentiation. Learn Mem 17: 489-93.

Lebron-Milad, K, Tsareva, A, Ahmed, N and Milad, MR (2013) Sex differences and estrous cycle in female rats interact with the effects of fluoxetine treatment on fear extinction. Behav Brain Res 253: 217-22.

Ledgerwood, L, Richardson, R and Cranney, J (2004) D-cycloserine and the facilitation of extinction of conditioned fear: consequences for reinstatement. Behav Neurosci 118: 505-13.

Ledgerwood, L, Richardson, R and Cranney, J (2005) D-cycloserine facilitates extinction of learned fear: effects on reacquisition and generalized extinction. Biol Psychiatry 57: 841-7.

LeDoux, J (2007) The amygdala. Curr Biol 17: R868-74.

LeDoux, JE (2000) Emotion circuits in the brain. Annu Rev Neurosci 23: 155-84.

Lee, SH and Soltesz, I (2011) Requirement for CB1 but not GABAB receptors in the cholecystokinin mediated inhibition of GABA release from cholecystokinin expressing basket cells. J Physiol 589: 891-902.

Lee, SY, Foldy, C, Szabadics, J and Soltesz, I (2011) Cell-type-specific CCK2 receptor signaling underlies the cholecystokinin-mediated selective excitation of hippocampal parvalbumin-positive fast-spiking basket cells. J Neurosci 31: 10993-1002.

Lein, ES, et al. (2007) Genome-wide atlas of gene expression in the adult mouse brain. Nature 445: 168-76.

Lin, HC, Mao, SC, Chen, PS and Gean, PW (2008) Chronic cannabinoid administration in vivo compromises extinction of fear memory. Learn Mem 15: 876-84.

Lin, HC, Mao, SC, Su, CL and Gean, PW (2009) The role of prefrontal cortex CB1 receptors in the modulation of fear memory. Cereb Cortex 19: 165-75.

Lin, QS, Yang, Q, Liu, DD, Sun, Z, Dang, H, Liang, J, Wang, YX, Chen, J and Li, ST (2011) Hippocampal endocannabinoids play an important role in induction of long-term potentiation and regulation of contextual fear memory formation. Brain Res Bull 86: 139-45.

Lindner, D, Stichel, J and Beck-Sickinger, AG (2008) Molecular recognition of the NPY hormone family by their receptors. Nutrition 24: 907-17.

Lisboa, SF, Reis, DG, da Silva, AL, Correa, FM, Guimaraes, FS and Resstel, LB (2010) Cannabinoid CB1 receptors in the medial prefrontal cortex modulate the expression of contextual fear conditioning. Int J Neuropsychopharmacol 13: 1163-73.

Litz, BT, Salters-Pedneault, K, Steenkamp, MM, Hermos, JA, Bryant, RA, Otto, MW and Hofmann, SG (2012) A randomized placebo-controlled trial of D-cycloserine and exposure therapy for posttraumatic stress disorder. J Psychiatr Res 46: 1184-90.

Llorente, R, Llorente-Berzal, A, Petrosino, S, Marco, EM, Guaza, C, Prada, C, Lopez-Gallardo, M, Di Marzo, V and Viveros, MP (2008) Gender-dependent cellular and biochemical effects of maternal deprivation on the hippocampus of neonatal rats: a possible role for the endocannabinoid system. Dev Neurobiol 68: 1334-47.

Lotti, VJ and Chang, RS (1989) A new potent and selective non-peptide gastrin antagonist and brain cholecystokinin receptor (CCK-B) ligand: L-365,260. Eur J Pharmacol 162: 273-80.

Lu, AT, Ogdie, MN, Jarvelin, MR, Moilanen, IK, Loo, SK, McCracken, JT, McGough, JJ, Yang, MH, Peltonen, L, Nelson, SF, Cantor, RM and Smalley, SL (2008) Association of the cannabinoid receptor gene (CNR1) with ADHD and post-traumatic stress disorder. Am J Med Genet B Neuropsychiatr Genet 147B: 1488-94.

Lucion, AB, Charchat, H, Pereira, GA and Rasia-Filho, AA (1996) Influence of early postnatal gonadal hormones on anxiety in adult male rats. Physiol Behav 60: 1419-23.

Mackintosh, NJ, Reese, B (1979) One-Trial Overshadowing. Quarterly Journal of Experimental Psychology 31: 519-526.

Manabe, T, Noda, Y, Mamiya, T, Katagiri, H, Houtani, T, Nishi, M, Noda, T, Takahashi, T, Sugimoto, T, Nabeshima, T and Takeshima, H (1998) Facilitation of long-term potentiation and memory in mice lacking nociceptin receptors. Nature 394: 577-81.

Manwell, LA, Satvat, E, Lang, ST, Allen, CP, Leri, F and Parker, LA (2009) FAAH inhibitor, URB-597, promotes extinction and CB(1) antagonist, SR141716, inhibits extinction of

conditioned aversion produced by naloxone-precipitated morphine withdrawal, but not extinction of conditioned preference produced by morphine in rats. Pharmacol Biochem Behav 94: 154-62.

Mao, SC, Hsiao, YH and Gean, PW (2006) Extinction training in conjunction with a partial agonist of the glycine site on the NMDA receptor erases memory trace. J Neurosci 26: 8892-9.

Maren, S and Fanselow, MS (1996) The amygdala and fear conditioning: has the nut been cracked? Neuron 16: 237-40.

Marin, MF, Camprodon, JA, Dougherty, DD and Milad, MR (2014) Device-based brain stimulation to augment fear extinction: implications for PTSD treatment and beyond. Depress Anxiety 31: 269-78.

Markstein, R and Hokfelt, T (1984) Effect of cholecystokinin-octapeptide on dopamine release from slices of cat caudate nucleus. J Neurosci 4: 570-5.

Marsicano, G, Wotjak, CT, Azad, SC, Bisogno, T, Rammes, G, Cascio, MG, Hermann, H, Tang, J, Hofmann, C, Zieglgansberger, W, Di Marzo, V and Lutz, B (2002) The endogenous cannabinoid system controls extinction of aversive memories. Nature 418: 530-4.

Marvar, PJ, Goodman, J, Fuchs, S, Choi, DC, Banerjee, S and Ressler, KJ (2014) Angiotensin type 1 receptor inhibition enhances the extinction of fear memory. Biol Psychiatry 75: 864-72.

Mascagni, F and McDonald, AJ (2003) Immunohistochemical characterization of cholecystokinin containing neurons in the rat basolateral amygdala. Brain Res 976: 171-84.

Matto, V, Harro, J and Allikmets, L (1997) The effects of cholecystokinin A and B receptor antagonists on exploratory behaviour in the elevated zero-maze in rat. Neuropharmacology 36: 389-96.

Mayberg, HS, Lozano, AM, Voon, V, McNeely, HE, Seminowicz, D, Hamani, C, Schwalb, JM and Kennedy, SH (2005) Deep brain stimulation for treatment-resistant depression. Neuron 45: 651-60.

McDonald, AJ and Pearson, JC (1989) Coexistence of GABA and peptide immunoreactivity in non-pyramidal neurons of the basolateral amygdala. Neurosci Lett 100: 53-8.

McDonald, AJ and Mascagni, F (2001) Localization of the CB1 type cannabinoid receptor in the rat basolateral amygdala: high concentrations in a subpopulation of cholecystokinin-containing interneurons. Neuroscience 107: 641-52.

McDonald, AJ, Mascagni, F, Mania, I and Rainnie, DG (2005) Evidence for a perisomatic innervation of parvalbumin-containing interneurons by individual pyramidal cells in the basolateral amygdala. Brain Res 1035: 32-40.

McGaugh, JL (2004) The amygdala modulates the consolidation of memories of emotionally arousing experiences. Annu Rev Neurosci 27: 1-28.

McGhee, LL, Maani, CV, Garza, TH, Desocio, PA, Gaylord, KM and Black, IH (2009) The effect of propranolol on posttraumatic stress disorder in burned service members. J Burn Care Res 30: 92-7.

McGuire, JL, Larke, LE, Sallee, FR, Herman, JP and Sah, R (2011) Differential Regulation of Neuropeptide Y in the Amygdala and Prefrontal Cortex during Recovery from Chronic Variable Stress. Front Behav Neurosci 5: 54.

McNally, GP and Westbrook, RF (2003) Opioid receptors regulate the extinction of Pavlovian fear conditioning. Behav Neurosci 117: 1292-301.

McNally, GP, Pigg, M and Weidemann, G (2004) Blocking, unblocking, and overexpectation of fear: a role for opioid receptors in the regulation of Pavlovian association formation. Behav Neurosci 118: 111-20.

McNally, GP, Pigg, M and Weidemann, G (2004) Opioid receptors in the midbrain periaqueductal gray regulate extinction of pavlovian fear conditioning. J Neurosci 24: 6912-9.

McNally, GP (2005) Facilitation of fear extinction by midbrain periaqueductal gray infusions of RB101(S), an inhibitor of enkephalin-degrading enzymes. Behav Neurosci 119: 1672-7.

McNally, GP, Lee, BW, Chiem, JY and Choi, EA (2005) The midbrain periaqueductal gray and fear extinction: opioid receptor subtype and roles of cyclic AMP, protein kinase A, and mitogen-activated protein kinase. Behav Neurosci 119: 1023-33.

Mechoulam, R and Parker, LA (2013) The endocannabinoid system and the brain. Annu Rev Psychol 64: 21-47.

Meis, S, Munsch, T, Sosulina, L and Pape, HC (2007) Postsynaptic mechanisms underlying responsiveness of amygdaloid neurons to cholecystokinin are mediated by a transient receptor potential-like current. Mol Cell Neurosci 35: 356-67.

Mellman, TA and Davis, GC (1985) Combat-related flashbacks in posttraumatic stress disorder: phenomenology and similarity to panic attacks. J Clin Psychiatry 46: 379-82.

Mercer, LD and Beart, PM (2004) Immunolocalization of CCK1R in rat brain using a new anti-peptide antibody. Neurosci Lett 359: 109-13.

Meunier, JC, Mollereau, C, Toll, L, Suaudeau, C, Moisand, C, Alvinerie, P, Butour, JL, Guillemot, JC, Ferrara, P, Monsarrat, B and et al. (1995) Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature 377: 532-5.

Mian, MK, Campos, M, Sheth, SA and Eskandar, EN (2010) Deep brain stimulation for obsessive-compulsive disorder: past, present, and future. Neurosurg Focus 29: E10.

Micevych, P, Akesson, T and Elde, R (1988) Distribution of cholecystokinin-immunoreactive cell bodies in the male and female rat: II. Bed nucleus of the stria terminalis and amygdala. J Comp Neurol 269: 381-91.

Michel, MC, Lewejohann, K, Farke, W, Bischoff, A, Feth, F and Rascher, W (1995) Regulation of NPY/NPY Y1 receptor/G protein system in rat brain cortex. Am J Physiol 268: R192-200.

Mickey, BJ, Zhou, Z, Heitzeg, MM, Heinz, E, Hodgkinson, CA, Hsu, DT, Langenecker, SA, Love, TM, Pecina, M, Shafir, T, Stohler, CS, Goldman, D and Zubieta, JK (2011) Emotion processing, major depression, and functional genetic variation of neuropeptide Y. Arch Gen Psychiatry 68: 158-66.

Milad, MR and Quirk, GJ (2002) Neurons in medial prefrontal cortex signal memory for fear extinction. Nature 420: 70-4.

Mills, KL, Teesson, M, Ross, J and Peters, L (2006) Trauma, PTSD, and substance use disorders: findings from the Australian National Survey of Mental Health and Well-Being. Am J Psychiatry 163: 652-8.

Miracle, AD, Brace, MF, Huyck, KD, Singler, SA and Wellman, CL (2006) Chronic stress impairs recall of extinction of conditioned fear. Neurobiol Learn Mem 85: 213-8.

Mithoefer, MC, Wagner, MT, Mithoefer, AT, Jerome, L and Doblin, R (2011) The safety and efficacy of {+/-}3,4-methylenedioxymethamphetamine-assisted psychotherapy in subjects with chronic, treatment-resistant posttraumatic stress disorder: the first randomized controlled pilot study. J Psychopharmacol 25: 439-52.

Moise, AM, Eisenstein, SA, Astarita, G, Piomelli, D and Hohmann, AG (2008) An endocannabinoid signaling system modulates anxiety-like behavior in male Syrian hamsters. Psychopharmacology (Berl) 200: 333-46.

Morgan, CA, 3rd, Wang, S, Southwick, SM, Rasmusson, A, Hazlett, G, Hauger, RL and Charney, DS (2000) Plasma neuropeptide-Y concentrations in humans exposed to military survival training. Biol Psychiatry 47: 902-9.

Morisset, J, Julien, S and Laine, J (2003) Localization of cholecystokinin receptor subtypes in the endocine pancreas. J Histochem Cytochem 51: 1501-13.

Morris, BJ (1989) Neuronal localisation of neuropeptide Y gene expression in rat brain. J Comp Neurol 290: 358-68.

Mueller, D, Porter, JT and Quirk, GJ (2008) Noradrenergic signaling in infralimbic cortex increases cell excitability and strengthens memory for fear extinction. J Neurosci 28: 369-75.

Muinos-Gimeno, M, Espinosa-Parrilla, Y, Guidi, M, Kagerbauer, B, Sipila, T, Maron, E, Pettai, K, Kananen, L, Navines, R, Martin-Santos, R, Gratacos, M, Metspalu, A, Hovatta, I and Estivill, X (2011) Human microRNAs miR-22, miR-138-2, miR-148a, and miR-488 are associated with panic disorder and regulate several anxiety candidate genes and related pathways. Biol Psychiatry 69: 526-33.

Muller, J, Corodimas, KP, Fridel, Z and LeDoux, JE (1997) Functional inactivation of the lateral and basal nuclei of the amygdala by muscimol infusion prevents fear conditioning to an explicit conditioned stimulus and to contextual stimuli. Behav Neurosci 111: 683-91.

Muller, JF, Mascagni, F and McDonald, AJ (2005) Coupled networks of parvalbumin-immunoreactive interneurons in the rat basolateral amygdala. J Neurosci 25: 7366-76.

Muravieva, EV and Alberini, CM (2010) Limited efficacy of propranolol on the reconsolidation of fear memories. Learn Mem 17: 306-13.

Murrough, JW, Huang, Y, Hu, J, Henry, S, Williams, W, Gallezot, JD, Bailey, CR, Krystal, JH, Carson, RE and Neumeister, A (2011) Reduced amygdala serotonin transporter binding in posttraumatic stress disorder. Biol Psychiatry 70: 1033-8.

Myers, KM and Davis, M (2007) Mechanisms of fear extinction. Mol Psychiatry 12: 120-50.

Nader, K, Schafe, GE and Le Doux, JE (2000) Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. Nature 406: 722-6.

Nakajima, M, Inui, A, Teranishi, A, Miura, M, Hirosue, Y, Okita, M, Himori, N, Baba, S and Kasuga, M (1994) Effects of pancreatic polypeptide family peptides on feeding and learning behavior in mice. J Pharmacol Exp Ther 268: 1010-4.

Neumeister, A, Normandin, MD, Pietrzak, RH, Piomelli, D, Zheng, MQ, Gujarro-Anton, A, Potenza, MN, Bailey, CR, Lin, SF, Najafzadeh, S, Ropchan, J, Henry, S, Corsi-Travali, S, Carson, RE and Huang, Y (2013) Elevated brain cannabinoid CB1 receptor availability in post-traumatic stress disorder: a positron emission tomography study. Mol Psychiatry 18: 1034-40.

Norberg, MM, Krystal, JH and Tolin, DF (2008) A meta-analysis of D-cycloserine and the facilitation of fear extinction and exposure therapy. Biol Psychiatry 63: 1118-26.

Nugent, NR, Christopher, NC, Crow, JP, Browne, L, Ostrowski, S and Delahanty, DL (2010) The efficacy of early propranolol administration at reducing PTSD symptoms in pediatric injury patients: a pilot study. J Trauma Stress 23: 282-7.

Nugent, NR, Lally, MA, Brown, L, Knopik, VS and McGeary, JE (2012) OPRM1 and diagnosis-related posttraumatic stress disorder in binge-drinking patients living with HIV. AIDS Behav 16: 2171-80.

O'Donnell, T, Hegadoren, KM and Coupland, NC (2004) Noradrenergic mechanisms in the pathophysiology of post-traumatic stress disorder. Neuropsychobiology 50: 273-83.

Orio, L, Crespo, I, Lopez-Moreno, JA, Reyes-Cabello, C, Rodriguez de Fonseca, F and Gomez de Heras, R (2011) Additive effects of cannabinoid CB1 receptors blockade and cholecystokinin on feeding inhibition. Pharmacol Biochem Behav 98: 220-6.

Orr, SP, Milad, MR, Metzger, LJ, Lasko, NB, Gilbertson, MW and Pitman, RK (2006) Effects of beta blockade, PTSD diagnosis, and explicit threat on the extinction and retention of an aversively conditioned response. Biol Psychol 73: 262-71.

Osuch, EA, Benson, BE, Luckenbaugh, DA, Geraci, M, Post, RM and McCann, U (2009) Repetitive TMS combined with exposure therapy for PTSD: a preliminary study. J Anxiety Disord 23: 54-9.

Otto, MW, Tolin, DF, Simon, NM, Pearlson, GD, Basden, S, Meunier, SA, Hofmann, SG, Eisenmenger, K, Krystal, JH and Pollack, MH (2010) Efficacy of d-cycloserine for

enhancing response to cognitive-behavior therapy for panic disorder. Biol Psychiatry 67: 365-70.

Pande, AC, Greiner, M, Adams, JB, Lydiard, RB and Pierce, MW (1999) Placebo-controlled trial of the CCK-B antagonist, CI-988, in panic disorder. Biol Psychiatry 46: 860-2.

Pardini, M, Krueger, F, Koenigs, M, Raymont, V, Hodgkinson, C, Zoubak, S, Goldman, D and Grafman, J (2012) Fatty-acid amide hydrolase polymorphisms and post-traumatic stress disorder after penetrating brain injury. Transl Psychiatry 2: e75.

Parker, RM and Herzog, H (1999) Regional distribution of Y-receptor subtype mRNAs in rat brain. Eur J Neurosci 11: 1431-48.

Parsons, RG, Gafford, GM and Helmstetter, FJ (2010) Regulation of extinction-related plasticity by opioid receptors in the ventrolateral periaqueductal gray matter. Front Behav Neurosci 4:

Pertwee, RG (1997) Pharmacology of cannabinoid CB1 and CB2 receptors. Pharmacol Ther 74: 129-80.

Pickens, CL, Adams-Deutsch, T, Nair, SG, Navarre, BM, Heilig, M and Shaham, Y (2009) Effect of pharmacological manipulations of neuropeptide Y and corticotropin-releasing factor neurotransmission on incubation of conditioned fear. Neuroscience 164: 1398-406.

Pietrzak, RH, Huang, Y, Corsi-Travali, S, Zheng, MQ, Lin, SF, Henry, S, Potenza, MN, Piomelli, D, Carson, RE and Neumeister, A (2014) Cannabinoid Type 1 Receptor Availability in the Amygdala Mediates Threat Processing in Trauma Survivors. Neuropsychopharmacology

Pitman, RK, Sanders, KM, Zusman, RM, Healy, AR, Cheema, F, Lasko, NB, Cahill, L and Orr, SP (2002) Pilot study of secondary prevention of posttraumatic stress disorder with propranolol. Biol Psychiatry 51: 189-92.

Plendl, W and Wotjak, CT (2010) Dissociation of within- and between-session extinction of conditioned fear. J Neurosci 30: 4990-8.

Pomonis, JD, Levine, AS and Billington, CJ (1997) Interaction of the hypothalamic paraventricular nucleus and central nucleus of the amygdala in naloxone blockade of neuropeptide Y-induced feeding revealed by c-fos expression. J Neurosci 17: 5175-82.

Primeaux, SD, Wilson, SP, Cusick, MC, York, DA and Wilson, MA (2005) Effects of altered amygdalar neuropeptide Y expression on anxiety-related behaviors. Neuropsychopharmacology 30: 1589-97.

Przybyslawski, J, Roullet, P and Sara, SJ (1999) Attenuation of emotional and nonemotional memories after their reactivation: role of beta adrenergic receptors. J Neurosci 19: 6623-8.

Puente, N, Elezgarai, I, Lafourcade, M, Reguero, L, Marsicano, G, Georges, F, Manzoni, OJ and Grandes, P (2010) Localization and function of the cannabinoid CB1 receptor in the anterolateral bed nucleus of the stria terminalis. PLoS One 5: e8869.

Quirk, GJ, Repa, C and LeDoux, JE (1995) Fear conditioning enhances short-latency auditory responses of lateral amygdala neurons: parallel recordings in the freely behaving rat. Neuron 15: 1029-39.

Quirk, GJ, Likhtik, E, Pelletier, JG and Pare, D (2003) Stimulation of medial prefrontal cortex decreases the responsiveness of central amygdala output neurons. J Neurosci 23: 8800-7.

Quirk, GJ, Garcia, R and Gonzalez-Lima, F (2006) Prefrontal mechanisms in extinction of conditioned fear. Biol Psychiatry 60: 337-43.

Quirk, GJ, Pare, D, Richardson, R, Herry, C, Monfils, MH, Schiller, D and Vicentic, A (2010) Erasing fear memories with extinction training. J Neurosci 30: 14993-7.

Rabinak, CA, Angstadt, M, Sripada, CS, Abelson, JL, Liberzon, I, Milad, MR and Phan, KL (2013) Cannabinoid facilitation of fear extinction memory recall in humans. Neuropharmacology 64: 396-402.

Ramikie, TS, Nyilas, R, Bluett, RJ, Gamble-George, JC, Hartley, ND, Mackie, K, Watanabe, M, Katona, I and Patel, S (2014) Multiple mechanistically distinct modes of endocannabinoid mobilization at central amygdala glutamatergic synapses. Neuron 81: 1111-25.

Rao, RP, Anilkumar, S, McEwen, BS and Chattarji, S (2012) Glucocorticoids protect against the delayed behavioral and cellular effects of acute stress on the amygdala. Biol Psychiatry 72: 466-75.

Rasmussen, K, Helton, DR, Berger, JE and Scearce, E (1993) The CCK-B antagonist LY288513 blocks effects of diazepam withdrawal on auditory startle. Neuroreport 5: 154-6.

Rasmusson, AM, Hauger, RL, Morgan, CA, Bremner, JD, Charney, DS and Southwick, SM (2000) Low baseline and yohimbine-stimulated plasma neuropeptide Y (NPY) levels in combat-related PTSD. Biol Psychiatry 47: 526-39.

Ratano, P, Everitt, BJ and Milton, AL (2014) The CB1 Receptor Antagonist AM251 Impairs Reconsolidation of Pavlovian Fear Memory in the Rat Basolateral Amygdala. Neuropsychopharmacology 39: 2529-37.

Raud, S, Innos, J, Abramov, U, Reimets, A, Koks, S, Soosaar, A, Matsui, T and Vasar, E (2005) Targeted invalidation of CCK2 receptor gene induces anxiolytic-like action in light-dark exploration, but not in fear conditioning test. Psychopharmacology (Berl) 181: 347-57.

Redrobe, JP, Dumont, Y, Herzog, H and Quirion, R (2004) Characterization of neuropeptide Y, Y(2) receptor knockout mice in two animal models of learning and memory processing. J Mol Neurosci 22: 159-66.

Rehfeld, JF (1985) Neuronal cholecystokinin: one or multiple transmitters? J Neurochem 44: 1-10.

Reich, CG, Mohammadi, MH and Alger, BE (2008) Endocannabinoid modulation of fear responses: learning and state-dependent performance effects. J Psychopharmacol 22: 769-77.

Reich, CG, Iskander, AN and Weiss, MS (2013) Cannabinoid modulation of chronic mild stress-induced selective enhancement of trace fear conditioning in adolescent rats. J Psychopharmacol 27: 947-55.

Reist, C, Duffy, JG, Fujimoto, K and Cahill, L (2001) beta-Adrenergic blockade and emotional memory in PTSD. Int J Neuropsychopharmacol 4: 377-83.

Rescorla, RA, Wagner, A.R. (1972) A Theory of Pavlovian Conditioning: Variations in the Effectiveness of Reinforcement and Nonreinforcement. New York: Appleton-Century-Crofts

Ressler, KJ, Rothbaum, BO, Tannenbaum, L, Anderson, P, Graap, K, Zimand, E, Hodges, L and Davis, M (2004) Cognitive enhancers as adjuncts to psychotherapy: use of D-cycloserine in phobic individuals to facilitate extinction of fear. Arch Gen Psychiatry 61: 1136-44.

Ressler, KJ, Mercer, KB, Bradley, B, Jovanovic, T, Mahan, A, Kerley, K, Norrholm, SD, Kilaru, V, Smith, AK, Myers, AJ, Ramirez, M, Engel, A, Hammack, SE, Toufexis, D, Braas, KM, Binder, EB and May, V (2011) Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. Nature 470: 492-7.

Revel, L, Mennuni, L, Garofalo, P and Makovec, F (1998) CR 2945: a novel CCKB receptor antagonist with anxiolytic-like activity. Behav Pharmacol 9: 183-94.

Richard, D, Guesdon, B and Timofeeva, E (2009) The brain endocannabinoid system in the regulation of energy balance. Best Pract Res Clin Endocrinol Metab 23: 17-32.

Robbe, D, Montgomery, SM, Thome, A, Rueda-Orozco, PE, McNaughton, BL and Buzsaki, G (2006) Cannabinoids reveal importance of spike timing coordination in hippocampal function. Nat Neurosci 9: 1526-33.

Rodrigues, H, Figueira, I, Lopes, A, Goncalves, R, Mendlowicz, MV, Coutinho, ES and Ventura, P (2014) Does D-cycloserine enhance exposure therapy for anxiety disorders in humans? A meta-analysis. PLoS One 9: e93519.

Rodrigues, SM, LeDoux, JE and Sapolsky, RM (2009) The influence of stress hormones on fear circuitry. Annu Rev Neurosci 32: 289-313.

Rodriguez-Romaguera, J, Do Monte, FH and Quirk, GJ (2012) Deep brain stimulation of the ventral striatum enhances extinction of conditioned fear. Proc Natl Acad Sci U S A 109: 8764-9.

Rodriguez de Fonseca, F, Cebeira, M, Ramos, JA, Martin, M and Fernandez-Ruiz, JJ (1994) Cannabinoid receptors in rat brain areas: sexual differences, fluctuations during estrous cycle and changes after gonadectomy and sex steroid replacement. Life Sci 54: 159-70.

Rooman, I, Lardon, J, Flamez, D, Schuit, F and Bouwens, L (2001) Mitogenic effect of gastrin and expression of gastrin receptors in duct-like cells of rat pancreas. Gastroenterology 121: 940-9.

Roozendaal, B, Hahn, EL, Nathan, SV, de Quervain, DJ and McGaugh, JL (2004) Glucocorticoid effects on memory retrieval require concurrent noradrenergic activity in the hippocampus and basolateral amygdala. J Neurosci 24: 8161-9.

Roozendaal, B, Hui, GK, Hui, IR, Berlau, DJ, McGaugh, JL and Weinberger, NM (2006) Basolateral amygdala noradrenergic activity mediates corticosterone-induced enhancement of auditory fear conditioning. Neurobiol Learn Mem 86: 249-55.

Roozendaal, B, Lengvilas, R, McGaugh, JL, Civelli, O and Reinscheid, RK (2007) Orphanin FQ/nociceptin interacts with the basolateral amygdala noradrenergic system in memory consolidation. Learn Mem 14: 29-35.

Roozendaal, B, Schelling, G and McGaugh, JL (2008) Corticotropin-releasing factor in the basolateral amygdala enhances memory consolidation via an interaction with the beta-adrenoceptor-cAMP pathway: dependence on glucocorticoid receptor activation. J Neurosci 28: 6642-51.

Roozendaal, B, McEwen, BS and Chattarji, S (2009) Stress, memory and the amygdala. Nat Rev Neurosci 10: 423-33.

Rossi, S, Hallett, M, Rossini, PM and Pascual-Leone, A (2009) Safety, ethical considerations, and application guidelines for the use of transcranial magnetic stimulation in clinical practice and research. Clin Neurophysiol 120: 2008-39.

Rostkowski, AB, Teppen, TL, Peterson, DA and Urban, JH (2009) Cell-specific expression of neuropeptide Y Y1 receptor immunoreactivity in the rat basolateral amygdala. J Comp Neurol 517: 166-76.

Rothbaum, BO, Price, M, Jovanovic, T, Norrholm, SD, Gerardi, M, Dunlop, B, Davis, M, Bradley, B, Duncan, EJ, Rizzo, A and Ressler, KJ (2014) A randomized, double-blind evaluation of D-cycloserine or alprazolam combined with virtual reality exposure therapy for posttraumatic stress disorder in Iraq and Afghanistan War veterans. Am J Psychiatry 171: 640-8.

Ruehle, S, Rey, AA, Remmers, F and Lutz, B (2012) The endocannabinoid system in anxiety, fear memory and habituation. J Psychopharmacol 26: 23-39.

Ruehle, S, Remmers, F, Romo-Parra, H, Massa, F, Wickert, M, Wortge, S, Haring, M, Kaiser, N, Marsicano, G, Pape, HC and Lutz, B (2013) Cannabinoid CB1 receptor in dorsal telencephalic glutamatergic neurons: distinctive sufficiency for hippocampus-dependent and amygdala-dependent synaptic and behavioral functions. J Neurosci 33: 10264-77.

Ryan, SJ, Ehrlich, DE, Jasnow, AM, Daftary, S, Madsen, TE and Rainnie, DG (2012) Spike-timing precision and neuronal synchrony are enhanced by an interaction between synaptic inhibition and membrane oscillations in the amygdala. PLoS One 7: e35320.

Sajdyk, TJ, Vandergriff, MG and Gehlert, DR (1999) Amygdalar neuropeptide Y Y1 receptors mediate the anxiolytic-like actions of neuropeptide Y in the social interaction test. Eur J Pharmacol 368: 143-7.

Sajdyk, TJ, Schober, DA and Gehlert, DR (2002) Neuropeptide Y receptor subtypes in the basolateral nucleus of the amygdala modulate anxiogenic responses in rats. Neuropharmacology 43: 1165-72.

Sajdyk, TJ, Schober, DA, Smiley, DL and Gehlert, DR (2002) Neuropeptide Y-Y2 receptors mediate anxiety in the amygdala. Pharmacol Biochem Behav 71: 419-23.

Sajdyk, TJ, Johnson, PL, Leitermann, RJ, Fitz, SD, Dietrich, A, Morin, M, Gehlert, DR, Urban, JH and Shekhar, A (2008) Neuropeptide Y in the amygdala induces long-term resilience to stress-induced reductions in social responses but not hypothalamic-adrenal-pituitary axis activity or hyperthermia. J Neurosci 28: 893-903.

Sanders, MJ, Kieffer, BL and Fanselow, MS (2005) Deletion of the mu opioid receptor results in impaired acquisition of Pavlovian context fear. Neurobiol Learn Mem 84: 33-41.

Santa Ana, EJ, Rounsaville, BJ, Frankforter, TL, Nich, C, Babuscio, T, Poling, J, Gonsai, K, Hill, KP and Carroll, KM (2009) D-Cycloserine attenuates reactivity to smoking cues in nicotine dependent smokers: a pilot investigation. Drug Alcohol Depend 104: 220-7.

Saxe, G, Stoddard, F, Courtney, D, Cunningham, K, Chawla, N, Sheridan, R, King, D and King, L (2001) Relationship between acute morphine and the course of PTSD in children with burns. J Am Acad Child Adolesc Psychiatry 40: 915-21.

Schafer, U, Harhammer, R, Boomgaarden, M, Sohr, R, Ott, T, Henklein, P and Repke, H (1994) Binding of cholecystokinin-8 (CCK-8) peptide derivatives to CCKA and CCKB receptors. J Neurochem 62: 1426-31.

Scheeringa, MS and Weems, CF (2014) Randomized placebo-controlled D-cycloserine with cognitive behavior therapy for pediatric posttraumatic stress. J Child Adolesc Psychopharmacol 24: 69-77.

Schelling, G, Kilger, E, Roozendaal, B, de Quervain, DJ, Briegel, J, Dagge, A, Rothenhausler, HB, Krauseneck, T, Nollert, G and Kapfhammer, HP (2004) Stress doses of hydrocortisone, traumatic memories, and symptoms of posttraumatic stress disorder in patients after cardiac surgery: a randomized study. Biol Psychiatry 55: 627-33.

Schelling, G, Roozendaal, B, Krauseneck, T, Schmoelz, M, D, DEQ and Briegel, J (2006) Efficacy of hydrocortisone in preventing posttraumatic stress disorder following critical illness and major surgery. Ann N Y Acad Sci 1071: 46-53.

Schlicker, E and Kathmann, M (2001) Modulation of transmitter release via presynaptic cannabinoid receptors. Trends Pharmacol Sci 22: 565-72.

Sekiguchi, R and Moroji, T (1986) A comparative study on characterization and distribution of cholecystokinin binding sites among the rat, mouse and guinea pig brain. Brain Res 399: 271-81.

Selikoff, IJ and Robitzek, EH (1952) Tuberculosis chemotherapy with hydrazine derivatives of isonicotinic acid. Dis Chest 21: 385-438.

Sheridan, RL, Stoddard, FJ, Kazis, LE, Lee, A, Li, NC, Kagan, RJ, Palmieri, TL, Meyer, WJ, 3rd, Nicolai, M, Stubbs, TK, Chan, G, Hinson, MI, Herndon, DN and Tompkins, RG (2014) Long-term posttraumatic stress symptoms vary inversely with early opiate dosing in children recovering from serious burns: effects durable at 4 years. J Trauma Acute Care Surg 76: 828-32.

Shlik, J, Aluoja, A, Vasar, V, Vasar, E, Podar, T and Bradwejn, J (1997) Effects of citalopram treatment on behavioural, cardiovascular and neuroendocrine response to cholecystokinin tetrapeptide challenge in patients with panic disorder. J Psychiatry Neurosci 22: 332-40.

Silva, AP, Xapelli, S, Grouzmann, E and Cavadas, C (2005) The putative neuroprotective role of neuropeptide Y in the central nervous system. Curr Drug Targets CNS Neurol Disord 4: 331-47.

Smits, JA, Rosenfield, D, Otto, MW, Powers, MB, Hofmann, SG, Telch, MJ, Pollack, MH and Tart, CD (2013) D-cycloserine enhancement of fear extinction is specific to successful exposure sessions: evidence from the treatment of height phobia. Biol Psychiatry 73: 1054-8.

Steckler, T and Risbrough, V (2012) Pharmacological treatment of PTSD - established and new approaches. Neuropharmacology 62: 617-27.

Stidd, DA, Vogelsang, K, Krahl, SE, Langevin, JP and Fellous, JM (2013) Amygdala deep brain stimulation is superior to paroxetine treatment in a rat model of posttraumatic stress disorder. Brain Stimul 6: 837-44.

Stoddard, FJ, Jr., Sorrentino, EA, Ceranoglu, TA, Saxe, G, Murphy, JM, Drake, JE, Ronfeldt, H, White, GW, Kagan, J, Snidman, N, Sheridan, RL and Tompkins, RG (2009) Preliminary evidence for the effects of morphine on posttraumatic stress disorder symptoms in one- to four-year-olds with burns. J Burn Care Res 30: 836-43.

Storch, EA, Murphy, TK, Goodman, WK, Geffken, GR, Lewin, AB, Henin, A, Micco, JA, Sprich, S, Wilhelm, S, Bengtson, M and Geller, DA (2010) A preliminary study of D-cycloserine augmentation of cognitive-behavioral therapy in pediatric obsessive-compulsive disorder. Biol Psychiatry 68: 1073-6.

Suarez, J, Llorente, R, Romero-Zerbo, SY, Mateos, B, Bermudez-Silva, FJ, de Fonseca, FR and Viveros, MP (2009) Early maternal deprivation induces gender-dependent changes on the expression of hippocampal CB(1) and CB(2) cannabinoid receptors of neonatal rats. Hippocampus 19: 623-32.

Sui, L, Huang, S, Peng, B, Ren, J, Tian, F and Wang, Y (2014) Deep brain stimulation of the amygdala alleviates fear conditioning-induced alterations in synaptic plasticity in the cortical-amygdala pathway and fear memory. J Neural Transm 121: 773-82.

Suris, A, North, C, Adinoff, B, Powell, CM and Greene, R (2010) Effects of exogenous glucocorticoid on combat-related PTSD symptoms. Ann Clin Psychiatry 22: 274-9.

Szczytkowski-Thomson, JL, Lebonville, CL and Lysle, DT (2013) Morphine prevents the development of stress-enhanced fear learning. Pharmacol Biochem Behav 103: 672-7.

Tatemoto, K, Carlquist, M and Mutt, V (1982) Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. Nature 296: 659-60.

Taylor, F and Cahill, L (2002) Propranolol for reemergent posttraumatic stress disorder following an event of retraumatization: a case study. J Trauma Stress 15: 433-7.

Thiele, TE, Sparta, DR, Hayes, DM and Fee, JR (2004) A role for neuropeptide Y in neurobiological responses to ethanol and drugs of abuse. Neuropeptides 38: 235-43.

Thorsell, A, Carlsson, K, Ekman, R and Heilig, M (1999) Behavioral and endocrine adaptation, and up-regulation of NPY expression in rat amygdala following repeated restraint stress. Neuroreport 10: 3003-7.

Thorsell, A, Michalkiewicz, M, Dumont, Y, Quirion, R, Caberlotto, L, Rimondini, R, Mathe, AA and Heilig, M (2000) Behavioral insensitivity to restraint stress, absent fear suppression of behavior and impaired spatial learning in transgenic rats with hippocampal neuropeptide Y overexpression. Proc Natl Acad Sci U S A 97: 12852-7.

Tollenaar, MS, Elzinga, BM, Spinhoven, P and Everaerd, W (2009) Psychophysiological responding to emotional memories in healthy young men after cortisol and propranolol administration. Psychopharmacology (Berl) 203: 793-803.

Tollenaar, MS, Elzinga, BM, Spinhoven, P and Everaerd, W (2009) Immediate and prolonged effects of cortisol, but not propranolol, on memory retrieval in healthy young men. Neurobiol Learn Mem 91: 23-31.

Tovote, P, Meyer, M, Beck-Sickinger, AG, von Horsten, S, Ove Ogren, S, Spiess, J and Stiedl, O (2004) Central NPY receptor-mediated alteration of heart rate dynamics in mice during expression of fear conditioned to an auditory cue. Regul Pept 120: 205-14.

Tsou, K, Brown, S, Sanudo-Pena, MC, Mackie, K and Walker, JM (1998) Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. Neuroscience 83: 393-411.

Tsutsumi, T, Akiyoshi, J, Isogawa, K, Kohno, Y, Hikichi, T and Nagayama, H (1999) Suppression of conditioned fear by administration of CCKB receptor antagonist PD135158. Neuropeptides 33: 483-6.

Tsutsumi, T, Akiyoshi, J, Hikichi, T, Kiyota, A, Kohno, Y, Katsuragi, S, Yamamoto, Y, Isogawa, K and Nagayama, H (2001) Suppression of conditioned fear by administration of CCKB receptor antisense oligodeoxynucleotide into the lateral ventricle. Pharmacopsychiatry 34: 232-7.

Tzavara, ET, Davis, RJ, Perry, KW, Li, X, Salhoff, C, Bymaster, FP, Witkin, JM and Nomikos, GG (2003) The CB1 receptor antagonist SR141716A selectively increases monoaminergic neurotransmission in the medial prefrontal cortex: implications for therapeutic actions. Br J Pharmacol 138: 544-53.

Uriguen, L, Garcia-Gutierrez, MS and Manzanares, J (2011) Decreased GABAA and GABAB receptor functional activity in cannabinoid CB1 receptor knockout mice. J Psychopharmacol 25: 105-10.

Vaiva, G, Ducrocq, F, Jezequel, K, Averland, B, Lestavel, P, Brunet, A and Marmar, CR (2003) Immediate treatment with propranolol decreases posttraumatic stress disorder two months after trauma. Biol Psychiatry 54: 947-9.

Van Laere, K, Goffin, K, Casteels, C, Dupont, P, Mortelmans, L, de Hoon, J and Bormans, G (2008) Gender-dependent increases with healthy aging of the human cerebral cannabinoid-type 1 receptor binding using [(18)F]MK-9470 PET. Neuroimage 39: 1533-41.

van Zuiden, M, Geuze, E, Willemen, HL, Vermetten, E, Maas, M, Heijnen, CJ and Kavelaars, A (2011) Pre-existing high glucocorticoid receptor number predicting development of posttraumatic stress symptoms after military deployment. Am J Psychiatry 168: 89-96.

Vanderhaeghen, JJ, Signeau, JC and Gepts, W (1975) New peptide in the vertebrate CNS reacting with antigastrin antibodies. Nature 257: 604-5.

Vasar, E, Peuranen, E, Harro, J, Lang, A, Oreland, L and Mannisto, PT (1993) Social isolation of rats increases the density of cholecystokinin receptors in the frontal cortex and abolishes

the anti-exploratory effect of caerulein. Naunyn Schmiedebergs Arch Pharmacol 348: 96-101.

Verma, D, Tasan, R.O., Mietzsch, M., Weger, S., Heilbronn, R., Herzog, H., Sperk, G. (2011) Reduced fear conditioning after viral vector mediated neuropeptide Y administration into the basolateral amygdala. BMC Pharmacology 11:

Vertes, RP (2004) Differential projections of the infralimbic and prelimbic cortex in the rat. Synapse 51: 32-58.

Vidal-Gonzalez, I, Vidal-Gonzalez, B, Rauch, SL and Quirk, GJ (2006) Microstimulation reveals opposing influences of prelimbic and infralimbic cortex on the expression of conditioned fear. Learn Mem 13: 728-33.

Voikar, V, Polus, A, Vasar, E and Rauvala, H (2005) Long-term individual housing in C57BL/6J and DBA/2 mice: assessment of behavioral consequences. Genes Brain Behav 4: 240-52.

Wahlestedt, C and Reis, DJ (1993) Neuropeptide Y-related peptides and their receptors--are the receptors potential therapeutic drug targets? Annu Rev Pharmacol Toxicol 33: 309-52.

Waldhoer, M, Bartlett, SE and Whistler, JL (2004) Opioid receptors. Annu Rev Biochem 73: 953-90.

Walker, DL and Davis, M (1997) Double dissociation between the involvement of the bed nucleus of the stria terminalis and the central nucleus of the amygdala in startle increases produced by conditioned versus unconditioned fear. J Neurosci 17: 9375-83.

Walker, DL, Ressler, KJ, Lu, KT and Davis, M (2002) Facilitation of conditioned fear extinction by systemic administration or intra-amygdala infusions of D-cycloserine as assessed with fear-potentiated startle in rats. J Neurosci 22: 2343-51.

Walker, DL, Toufexis, DJ and Davis, M (2003) Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. Eur J Pharmacol 463: 199-216.

Walter, A, Mai, JK and Jimenez-Hartel, W (1990) Mapping of neuropeptide Y-like immunoreactivity in the human forebrain. Brain Res Bull 24: 297-311.

Wang, H, Wong, PT, Spiess, J and Zhu, YZ (2005) Cholecystokinin-2 (CCK2) receptor-mediated anxiety-like behaviors in rats. Neurosci Biobehav Rev 29: 1361-73.

Weber, M, Hart, J and Richardson, R (2007) Effects of D-cycloserine on extinction of learned fear to an olfactory cue. Neurobiol Learn Mem 87: 476-82.

Wellman, CL, Izquierdo, A, Garrett, JE, Martin, KP, Carroll, J, Millstein, R, Lesch, KP, Murphy, DL and Holmes, A (2007) Impaired stress-coping and fear extinction and abnormal corticolimbic morphology in serotonin transporter knock-out mice. J Neurosci 27: 684-91.

Wessa, M, Rohleder, N, Kirschbaum, C and Flor, H (2006) Altered cortisol awakening response in posttraumatic stress disorder. Psychoneuroendocrinology 31: 209-15.

White, BD, Dean, RG, Edwards, GL and Martin, RJ (1994) Type II corticosteroid receptor stimulation increases NPY gene expression in basomedial hypothalamus of rats. Am J Physiol 266: R1523-9.

Whittle, N, Schmuckermair, C, Gunduz Cinar, O, Hauschild, M, Ferraguti, F, Holmes, A and Singewald, N (2013) Deep brain stimulation, histone deacetylase inhibitors and glutamatergic drugs rescue resistance to fear extinction in a genetic mouse model. Neuropharmacology 64: 414-23.

Wieland, HA, Willim, KD, Entzeroth, M, Wienen, W, Rudolf, K, Eberlein, W, Engel, W and Doods, HN (1995) Subtype selectivity and antagonistic profile of the nonpeptide Y1 receptor antagonist BIBP 3226. J Pharmacol Exp Ther 275: 143-9.

Witteveen, AB, Huizink, AC, Slottje, P, Bramsen, I, Smid, T and van der Ploeg, HM (2010) Associations of cortisol with posttraumatic stress symptoms and negative life events: a study of police officers and firefighters. Psychoneuroendocrinology 35: 1113-8.

Wolak, ML, DeJoseph, MR, Cator, AD, Mokashi, AS, Brownfield, MS and Urban, JH (2003) Comparative distribution of neuropeptide Y Y1 and Y5 receptors in the rat brain by using immunohistochemistry. J Comp Neurol 464: 285-311.

Woodruff, AR and Sah, P (2007) Inhibition and synchronization of basal amygdala principal neuron spiking by parvalbumin-positive interneurons. J Neurophysiol 98: 2956-61.

Woods, AM and Bouton, ME (2006) D-cycloserine facilitates extinction but does not eliminate renewal of the conditioned emotional response. Behav Neurosci 120: 1159-62.

Wunderlich, GR, Raymond, R, DeSousa, NJ, Nobrega, JN and Vaccarino, FJ (2002) Decreased CCK(B) receptor binding in rat amygdala in animals demonstrating greater anxiety-like behavior. Psychopharmacology (Berl) 164: 193-9.

Xie, P, Kranzler, HR, Poling, J, Stein, MB, Anton, RF, Brady, K, Weiss, RD, Farrer, L and Gelernter, J (2009) Interactive effect of stressful life events and the serotonin transporter 5-HTTLPR genotype on posttraumatic stress disorder diagnosis in 2 independent populations. Arch Gen Psychiatry 66: 1201-9.

Yamada, D, Wada, K and Sekiguchi, M (2011) Facilitating actions of an AMPA receptor potentiator upon extinction of contextually conditioned fear response in stressed mice. Neurosci Lett 488: 242-6.

Yamamoto, S, Morinobu, S, Fuchikami, M, Kurata, A, Kozuru, T and Yamawaki, S (2008) Effects of single prolonged stress and D-cycloserine on contextual fear extinction and hippocampal NMDA receptor expression in a rat model of PTSD. Neuropsychopharmacology 33: 2108-16.

Yannielli, PC and Harrington, ME (2001) Neuropeptide Y in the mammalian circadian system: effects on light-induced circadian responses. Peptides 22: 547-56.

Yehuda, R, Southwick, SM, Nussbaum, G, Wahby, V, Giller, EL, Jr. and Mason, JW (1990) Low urinary cortisol excretion in patients with posttraumatic stress disorder. J Nerv Ment Dis 178: 366-9.

Yehuda, R (2002) Post-traumatic stress disorder. N Engl J Med 346: 108-14.

Yehuda, R, Halligan, SL, Grossman, R, Golier, JA and Wong, C (2002) The cortisol and glucocorticoid receptor response to low dose dexamethasone administration in aging

combat veterans and holocaust survivors with and without posttraumatic stress disorder. Biol Psychiatry 52: 393-403.

Yehuda, R (2004) Risk and resilience in posttraumatic stress disorder. J Clin Psychiatry 65 Suppl 1: 29-36.

Yehuda, R, Golier, JA, Halligan, SL, Meaney, M and Bierer, LM (2004) The ACTH response to dexamethasone in PTSD. Am J Psychiatry 161: 1397-403.

Yehuda, R, Halligan, SL, Golier, JA, Grossman, R and Bierer, LM (2004) Effects of trauma exposure on the cortisol response to dexamethasone administration in PTSD and major depressive disorder. Psychoneuroendocrinology 29: 389-404.

Yehuda, R, Brand, S and Yang, RK (2006) Plasma neuropeptide Y concentrations in combat exposed veterans: relationship to trauma exposure, recovery from PTSD, and coping. Biol Psychiatry 59: 660-3.

Yoshida, T, Uchigashima, M, Yamasaki, M, Katona, I, Yamazaki, M, Sakimura, K, Kano, M, Yoshioka, M and Watanabe, M (2011) Unique inhibitory synapse with particularly rich endocannabinoid signaling machinery on pyramidal neurons in basal amygdaloid nucleus. Proc Natl Acad Sci U S A 108: 3059-64.

Yu, H and Neimat, JS (2008) The treatment of movement disorders by deep brain stimulation. Neurotherapeutics 5: 26-36.

Zarbin, MA, Innis, RB, Wamsley, JK, Snyder, SH and Kuhar, MJ (1983) Autoradiographic localization of cholecystokinin receptors in rodent brain. J Neurosci 3: 877-906.

Zelikowsky, M and Fanselow, MS (2010) Opioid regulation of Pavlovian overshadowing in fear conditioning. Behav Neurosci 124: 510-9.

Zhang, Y, Gandhi, PR and Standifer, KM (2012) Increased nociceptive sensitivity and nociceptin/orphanin FQ levels in a rat model of PTSD. Mol Pain 8: 76.

Zimmer, A, Zimmer, AM, Hohmann, AG, Herkenham, M and Bonner, TI (1999) Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. Proc Natl Acad Sci U S A 96: 5780-5.

Zohar, J, Yahalom, H, Kozlovsky, N, Cwikel-Hamzany, S, Matar, MA, Kaplan, Z, Yehuda, R and Cohen, H (2011) High dose hydrocortisone immediately after trauma may alter the trajectory of PTSD: interplay between clinical and animal studies. Eur Neuropsychopharmacol 21: 796-809.