Neurobiologically-motivated treatments for post-traumatic stress disorder in an animal model

By

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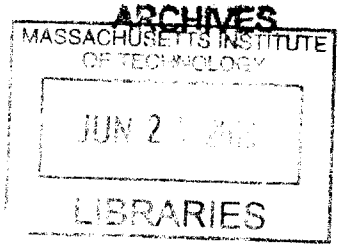

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Abstract

This thesis demonstrates that chronic immobilization stress administered to rats enhances fear learning and increases plasma acylated ghrelin. This effect is independent of the hypothalamus-pituitary-adrenal (HPA) axis since it was unaffected by prior bilateral adrenalectomy. Chronic exposure to a ghrelin receptor agonist, without stress, enhanced associational fear learning without altering HPA hormone levels. This effect was replicated by repeated direct infusions of a ghrelin receptor agonist in the basolateral amygdala (BLA), a brain region involved in emotional memory and altered by stress, suggesting a direct action of ghrelin at ghrelin receptors in the BLA. Administration of a ghrelin receptor inverse-agonist concurrent with stress exposure prevented stress-induced enhancement of fear learning. Other forms of chronic stress increase plasma acylated ghrelin as well, suggesting ghrelin is a novel mediator of long-term stress and a causal agent for stress-increased fear. Furthermore, this thesis identifies patterns in stress-induced feeding changes and body weight alterations that may offer an etiological explanation of the recruitment of the ghrelin pathway during periods of chronic stress. The final scientific chapter of this thesis describes a non-pharmacological intervention to enhance extinction of learned fear. In this work, optimization of extinction training yields resistance to spontaneous recovery of fear and demonstrates a weakening of potentiated synapses in the amygdala after optimal, but not suboptimal, behavioral extinction training.

Thesis Supervisor: Ki Goosens
Title: Assistant Professor of Neuroscience

Foreword

*"**Men ought to know that from the brain, and from the brain only, arise our pleasures, joys, laughter and jests, as well as our sorrow, pains, griefs and tears**. Through it, in particular, we think, see, hear and distinguish the ugly from the beautiful, the bad from the good, the pleasant from the unpleasant... It is the same thing that makes us mad or delirious, inspires us with dread and fear, whether by night or by day, brings sleeplessness, inopportune mistakes, aimless anxieties, absent-mindedness, and acts that are contrary to habit. These things that we suffer all come from the brain, when it is not healthy ... **But when the brain is still, a man can think properly."***

– **Hippocrates,** circa 500 BCE

Every investigation which is guided by principles of Nature fixes it ultimate aim entirely on gratifying the stomach.

- **Athenaeus,** circa 200 CE

Dedication

This thesis is dedicated to the memory of my father, **Colonel Michael D. Meyer**, who inspired my curiosity and love of the natural world, instilled my work ethic and moral compass, and provided the love and enduring confidence that encouraged me to be the scientist I am today.

To my husband, **Reuben L. Goodman**, my best friend, my partner in life, who supports me every day in every way. Your love and encouragement allow me to believe that I can solve the intractable and take on the world.

And finally to the family of **Sean Collier**, the MIT policeman killed in the line of duty not far from where I was working to complete this document. And all survivors of acts of violation and violence. Traumatic events can threaten to break us, can imperil the very core of our being. My hope, that science can find ways to help keep our minds whole in the face of these forces, nourishes the passion with which I am able to do the work that led to the discoveries within these pages.

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And to my teachers and mentors:

*"It is the supreme art of the teacher to awaken **joy** in creative expression and knowledge."*

-Albert Einstein

My high school science teacher: **Mr. John Leach** for his support and inspiration.

My first psychology professor, **Dr. W Jake Jacobs,** who inspired me to aim my curiosity at the brain, served as my mentor from the day he helped me to declare my Psychology major to the day I submitted my graduate applications and to this day. He is a beloved teacher, mentor, and friend.

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[1] *MIT CBCL provided coffee, bubble water, sandwiches, printing services, editing, and friendship.*

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And to my friends,

"The glory of friendship is not the outstretched hand, not the kindly smile, nor the joy of companionship; it is the spiritual inspiration that comes to one when you discover that someone else believes in you and is willing to trust you with a friendship."

— Ralph Waldo Emerson

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Chapter 1

Introduction

The prevalence and cost of mental illness

Psychiatric illnesses represent a heavy burden in terms of human and economic costs. Mental illnesses comprise any behavioral or psychological syndromes associated with distressful or painful symptoms, disability and impairment in important areas of functioning, or significantly increased risk of death, pain, disability or loss of freedom (1). Recently, it was estimated that as many as 32% of Americans will be diagnosed with a psychiatric disorder in their lifetime (2-4). The burden of disease can be calculated in many ways but is most often computed as a combination of mortality and disability. Mortality is shortened lifespan compared to average expectancy for the population and disability is interference with normal functioning. Although mental disorders are responsible for only 1% of all deaths, they produce 47% of all disability in economically developed countries and 28% of all disability world-wide (5-7). Mental disorders are the second highest source of disease burden in developed countries and, therefore, one of the world's greatest health challenges.

In addition to its impact on quality of life, there are also economic consequences of disease. While the total economic cost of mental illness is not easy to calculate, assessments using a combination of costs have been made (8). Easily calculated are the health care costs of mental illness. The Agency for Healthcare Research and Quality reports a cost of $57.5 billion in 2006 for mental health care in the United States, equivalent to the cost of cancer care (9). However, unlike cancer, much of the economic burden of mental illness is not the cost of care, but a range of indirect costs: loss of income due to unemployment, expenses for social

supports, and chronic disability (10). This expense is not unique to the United States. The World Health Organization (WHO) has reported that mental illnesses are the leading causes of loss of disability-adjusted life years worldwide, accounting for 37% of all healthy years lost due to non-communicable diseases (11). Another WHO report estimated the global cost of mental illness at nearly $2.5 trillion in 2010; two-thirds of these costs are considered indirect costs. This number is projected to increase to over $6 trillion by 2030 (11). If we wish to slow this trend, it is imperative that research be done to close knowledge gaps in the prevention and treatment of mental illness. Medical science will benefit from a deeper understanding of how these conditions evolve over time, both in identifying putative treatments and optimizing rehabilitation outcomes.

Diathesis-Stress model of mental illness

Research aimed at understanding psychiatric illness has uncovered several genetic underpinnings that lead to disease vulnerability. However, these do not account for 100% of disease development except in a few rare genetic or chromosomal disorders. For a variety of psychiatric diseases, there are significant environmental components leading to the development or exacerbation of the illness such as viral infection, high fat, high sugar diet (12), drug use (13), and low socio-economic status (14). One environmental component that has been found to exacerbate a wide range of psychiatric illnesses is stress, especially when prolonged or intense in nature. Stress appears to play a role in the pathophysiology of most psychiatric illnesses (15-18). Stress is known to exacerbate or even cause the onset

of schizophrenia, unipolar and bipolar depression disorders, anxiety disorders, post-traumatic stress disorder and even addiction. The Diathesis-Stress model of psychopathology suggests that an abnormal biological substrate is acted on by stress to produce or exacerbate symptoms. In other words, genetically or biologically vulnerable individuals may develop symptoms or signs of illness after bouts of chronic stress (19-22). Understanding how the activation of the stress response and exposure to stress hormones increases disease vulnerability will advance the development of much needed preventatives or treatments for a wide range of mental illnesses.

While psychiatric illnesses are complex in their etiology, the cause of post-traumatic stress disorder (PTSD) is considered to be known. By definition, to be diagnosed with PTSD a patient must experience an event that involves threat to the physical integrity of oneself or others that induces a response of intense fear, helplessness, or horror (1, 23). Importantly, not everyone who experiences trauma develops PTSD. This implies that the etiology of the disorder involves more than just the experience of the trauma itself. PTSD requires a Diathesis-Stress model of vulnerability in which aspects of event exposure interact with individual characteristics and response patterns. One component of this vulnerability is previous periods of stress or exposure to trauma. Overall, PTSD is one of the psychiatric illnesses with the greatest stress component (19-22, 24).

PTSD is an anxiety disorder that develops after a traumatic event. It involves three major symptom categories: re-experiencing, avoidance, and arousal (1). For

patients diagnosed with PTSD, the traumatic event is often re-experienced in nightmares, intrusive thoughts, or by great physiological reactivity upon exposure to cues reminiscent of the event. Patients begin to avoid activities, places, or people associated with the event and may even avoid activities or people that were once very important to them. Finally, patients with PTSD exhibit hyperarousal. This may manifest as insomnia, irritability, or hypervigilance. It is associated with a high resting heart rate and exaggerated norepinephrine response to startling stimuli (25). PTSD is an extremely disruptive disorder that interferes with normal functioning.

History, prevalence, and persistence of post-traumatic stress disorder

In the United States, post-traumatic psychopathology has been recognized under various names. It has historically been associated with soldiers at war. During the Civil War it was known as Soldier's Heart, in World War I as Shell Shock, World War II as Combat Fatigue, and after the Vietnam War as Delayed Stress (26). But accounts of the impact of war, violence and natural disasters on human mental health have been with us for millennia. In the Iliad, written by Homer circa 700 BCE, Achilles experiences traumatic battles and suffers pervasive reactions similar to those documented in cases of combat stress after modern wars. In ancient Egypt, physicians reported hysterical reactions to disasters in the first medical textbooks published circa 1990 BCE (26). Despite its long history, post-traumatic stress disorder (PTSD) was not officially added to the Diagnostic and

Statistical Manual of Mental Disorders (DSM) until the third edition was published in 1980 and was not expanded to include non-war trauma until the fourth edition.

A recent study found that nearly 8% of people living in the United States will experience PTSD at some point in their lives (27). The 2008 RAND study investigating the prevalence of mental illness in returning soldiers found that one in five develop PTSD, depression, or both (28). This number is increasing as soldiers are required to deploy multiple times and are suffering multiple traumas. It is known that previous exposure to trauma leads to greater risk of PTSD from subsequent trauma (29, 30). In the civilian population, studies show that more than 60% of people will experience a significant traumatic event during their lives and up to 20% of people in the United States may experience a trauma in any given year (20). By definition, trauma is necessary for the development of PTSD, but it is not sufficient. Though researchers state that anyone can develop PTSD given a sufficiently intense trauma, after severe disasters it is found that only 9-35% of those exposed will go on to develop PTSD (30-35).

Not only is PTSD a prevalent illness to which the entire population is, to some degree, vulnerable, it is also a persistent illness. The disorder is not only unique in the nature of the onset of the symptoms (i.e. the traumatic event), but also the longevity of the associated impairment (21, 36). By definition, patients with PTSD must exhibit symptoms for at least three months (1). However, patients often suffer much longer. Forty years after the end of World War II, retrospective diagnoses found that many prisoners of war (POWs) had suffered PTSD after the

war. Of the POWs who had PTSD, only 30% had fully recovered by this 40 year follow-up. As many as 60% still suffered mild to moderate PTSD and 10% failed to recover or were deteriorating at the time of the study (21, 36). In the general population, recovery is better but not complete (27, 37). While half of patients diagnosed with PTSD will recover within three years, 40% still suffer symptoms of PTSD six years post-trauma. At ten years post-trauma, 40% are still suffering (Fig. 1). In other words, at six years patients are either cured of their PTSD or are likely to continue to suffer from PTSD for the rest of their lives.

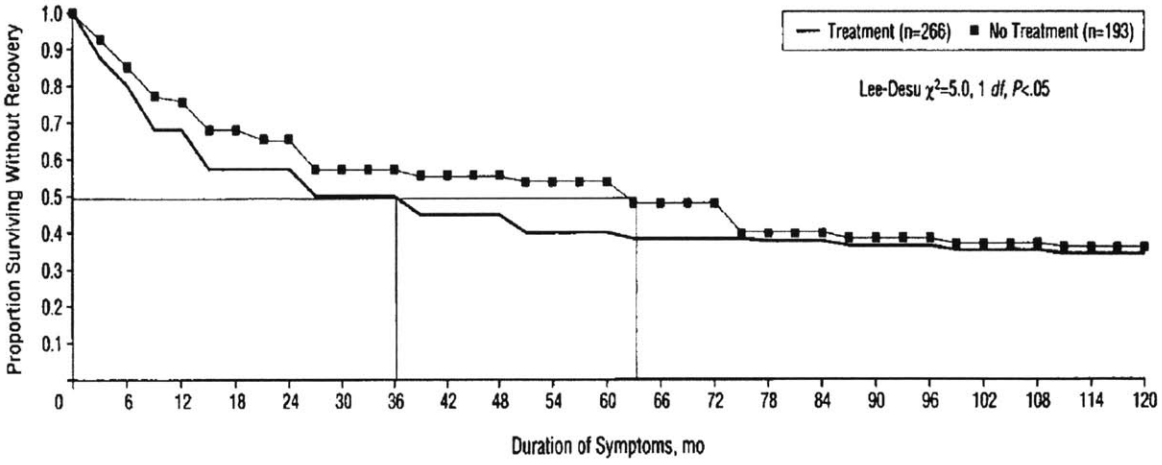


Figure 1. Survival curves based on duration of symptoms for respondents who did and did not receive treatment for posttraumatic stress disorder. *Adapted from Kessler, Sonnega, Bromet, Hughes, and Nelson, 1995.*

Treatments for PTSD: an unmet need

Much research has been dedicated to the development of treatments for PTSD. Due to the large stress component in PTSD etiology and symptomology, most

researchers have targeted the hormones of the hypothalamic-pituitary-adrenal axis (HPA axis), the purported driver of the stress response. The HPA cascade is activated by the brain during threat. Neurons from the paraventricular nucleus (PVN) of the hypothalamus release corticotropin-releasing hormone (CRH) directly in the pituitary which, in turn, releases adrenocorticotropic hormone (ACTH) into the bloodstream. The ACTH activates the release of catecholamines and glucocorticoids from the adrenal glands. These hormones have actions across the body and brain.

Elevated levels of CRH have been found in the CSF and blood of patients with PTSD (38-45). CRH is anxiogenic when given exogenously, making it a logical target for PTSD interventions. However, CRH was found to decrease in PTSD patients undergoing exposure therapy and recent clinical trials with CRH antagonists were prematurely ended due to toxicity (46-54).

One drug targeting the catecholamine system demonstrated potential in preliminary studies: propranolol. Propranolol is a ß-adrenergic receptor antagonist that crosses the blood-brain barrier. Given immediately after trauma, propranolol reduced physiological indicators of PTSD. However, these results have not been consistently replicated and, in the long-term, propranolol failed to alleviate PTSD symptoms (25). Additionally, the side effects of propranolol include decreased heart rate and blood pressure, limiting its use in patients suffering from physical trauma or loss of blood.

The glucocorticoid system was also a logical target. Patients with PTSD have been found to have higher number of glucocorticoid receptors in lymphocytes and some exhibit increased phasic cortisol responses to CRH or ACTH administration (48, 49, 53). However, PTSD is not characterized by increased tonic cortisol levels and some subsets of PTSD patients exhibit abnormally low cortisol levels (50). Therapeutic results targeting the glucocorticoid system have been mixed with interventions exacerbating the illness in some patients (25).

Antidepressants, including selective serotonin reuptake inhibitors (SSRIs), are often prescribed to treat PTSD. Other anti-anxiety and sleep medications have also been used. However, many of these pharmacological treatments aim to alleviate symptoms, such as general anxiety and nightmares, but seldom cure the pathophysiology underlying PTSD (25, 55-57).

In the absence of pharmaceutical interventions to treat PTSD, the current best treatment is cognitive behavioral therapy (CBT). In CBT, symptom reduction is attained by encouraging patients to remember the traumatic event and process it in a safe environment. Over time, the memories of the event become less frightening. As seen in Fig. 1, this form of treatment (solid line) can result in a quicker recovery process for those who *will* cease to have symptoms, but 40% of treated patients will still exhibit the disease state for years afterward. This proportion is equivalent for those who go untreated, indicating that a substantial proportion of patients receive no benefit from CBT (37).

Recent work suggested that adjuvant therapy, a combination of both psychological therapy (CBT) and pharmacotherapy, may enhance treatment response in those who have not responded to either intervention in isolation (58). However, a meta-analysis examining this hypothesis revealed no significant differences between combination and single intervention groups (58). Despite multiple avenues of research into the treatment of PTSD, there is currently an unmet need in the prevention and treatment of PTSD.

Biological studies of PTSD: Clinical findings

Although PTSD is an illness that primarily exists as a pathology of psychology, biological research is necessary to understand factors that alter vulnerability to develop the disorder, the neurological changes that occur after trauma, and the molecular pathways involved. Understanding the neurobiological mechanisms of PTSD will lead to advancements in each of these areas and ultimately to treatment or even prevention of the disorder itself.

Much work examining the neurobiological sequelae of PTSD has been done in the human patient population. Symptoms of PTSD include anxiety and avoidance of anxiety-provoking stimuli, hyperarousal such as elevated heart rate, and overpowering, intrusive reliving of the fearful event. These symptoms suggest a disorder of the limbic system, the brain system responsible for both memory and emotional processing. Indeed, much research has shown dysfunction in both the amygdala and hippocampus of patients who have developed or go on to develop PTSD (59). Recent work has also found alterations in the prefrontal cortex (PFC)

(42). People with PTSD show alterations in behaviors dependent on these brain regions, changes in functional activation, and even structural alterations.

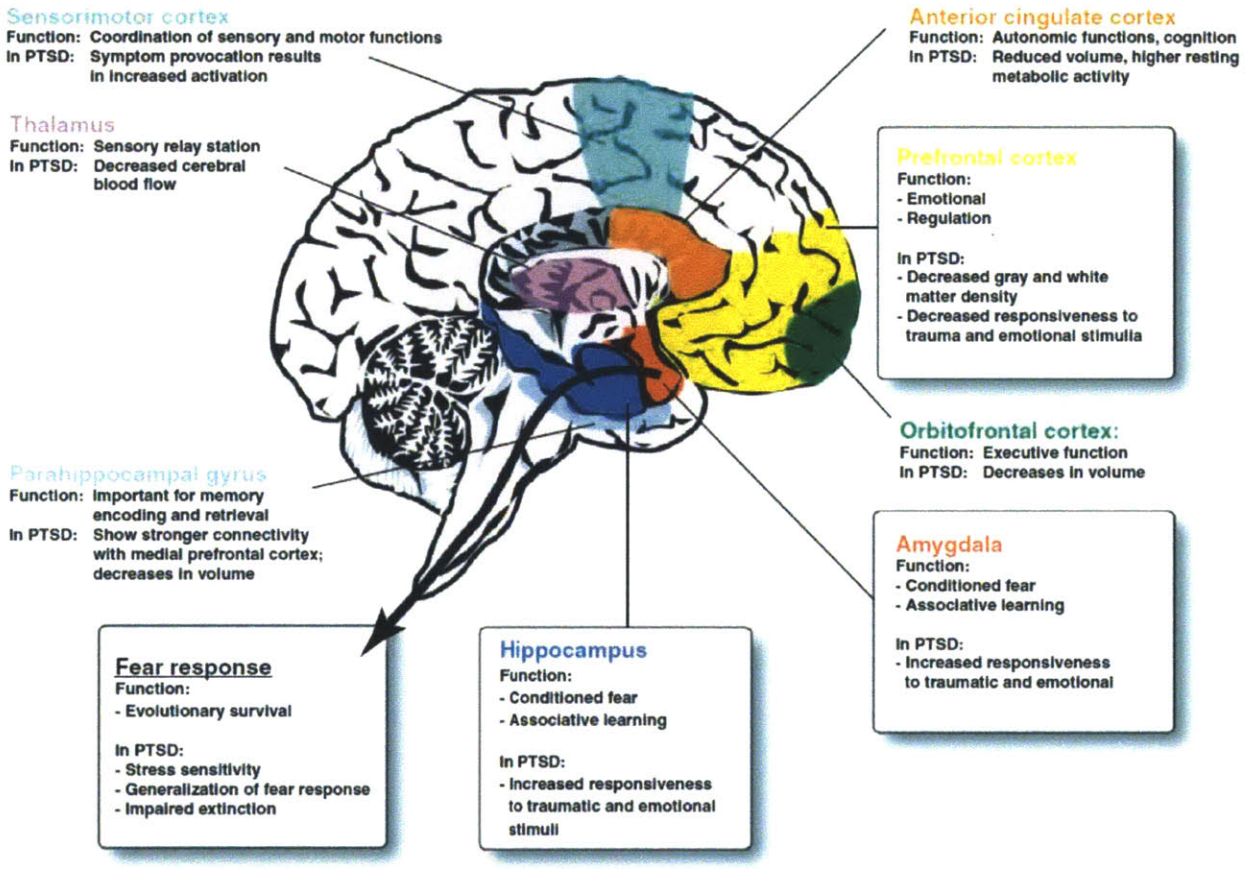


Figure 2. A schematic of the human brain illustrating how the limbic system is involved in PTSD. *Adapted from Mahan and Ressler, 2012*

The hippocampus is a major site of memory acquisition in the brain. Patients with PTSD are known to have problems with declarative memory function such as memorizing numbers or word lists, in addition to intrusive memories colloquially referred to as "flashbacks" (60-64). Furthermore, patients with PTSD have significantly smaller hippocampi than people without PTSD (64). While PTSD, by

definition, is caused by a psychologically traumatic event, it is not necessary that any biological abnormality found to accompany PTSD be trauma-induced. Rather, the abnormality may pre-date the traumatic event, rendering the individual vulnerable to the development of the disorder (25). In the case of hippocampal size, a study of monozygotic twins discordant for combat exposure (e.g. one twin was exposed to combat while the other was not) found evidence that smaller hippocampal volume was a pre-existing factor that made patients with PTSD more vulnerable to the development of the disorder.

The PFC is a brain region involved in working memory and cognitive flexibility. Behaviorally, patients with PTSD show inhibited function of the PFC such as limited working memory and impaired fear extinction (65). Neuroimaging experiments have also found volume reductions in the PFC of patients with PTSD. Unlike the hippocampus, the volume loss in the PFC appears to be acquired rather than a risk factor (66, 67). This suggests shrinking of the PFC is a symptom of the pathology of PTSD rather than a cause.

The amygdala regulates fear and fear associations and receives input from the thalamus, hippocampus, PFC and other cortical regions. Behaviorally, patients with PTSD show facilitated fear conditioning and increased memory for emotionally salient stimuli. Structural or volumetric changes in the amygdala of PTSD patients have been inconclusive but, functionally, they exhibit higher amygdala activation in the presence of fear-inducing stimuli (68, 69) as well as greater recruitment during

the acquisition of conditioned fear (70). Together, these results guide researchers and provide evidence of limbic dysfunction in the etiology of PTSD.

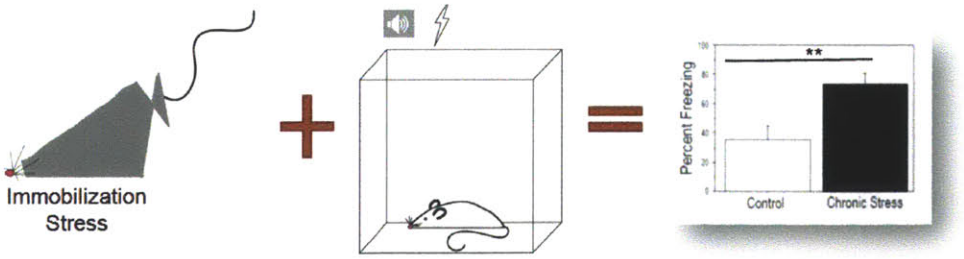
Furthermore, the pattern of biological findings suggests that the fear response is sensitized in PTSD. The perception of threat in the environment, real or imagined, activates the HPA axis. It has been suggested that heightened reactivity in PTSD may be due to the failure of the stress response system to shut down. When functioning normally, the hippocampus and PFC inhibit the HPA axis by targeting inhibitory neurons in the PVN of the hypothalamus at the head of the cascade (71-74). The amygdala inhibits these inhibitory neurons, thereby disinhibiting the HPA axis (74). With reduced activation of the PFC and hippocampus and the increased amygdala activity, these neurological changes could result in heightened activation of the stress response and the HPA axis. Furthermore, hormones of the HPA cascade have been shown to damage the hippocampus while activating the amygdala. This dysregulation may provide a positive feedback loop thereby exacerbating the hyperactivity of the stress response in PTSD.

Biological studies of PTSD: Laboratory findings

The ultimate goals of biological research are to elucidate the mechanisms involved in the development of PTSD, establish biomarkers, and generate novel preventative and therapeutic interventions to alleviate the suffering that PTSD and other disorders involving heightened emotional reactivity impose (25). In order to delve deeper into the neurobiology of PTSD, animal models are needed. Fortunately, the neural circuitry of fear and the hormones involved in the stress response are

preserved across vertebrates (75). Therefore, researchers can study the neurobiology of fear and stress, with relevance to potential treatments for trauma-related anxiety disorders, using animal models.

Animal models of PTSD must characterize the disorder at the behavioral level while also illuminating the neuronal and physiological mechanisms of the illness. The fact that exposure to severe stress leads to the development of PTSD in humans provides the basic rationale for all rodent models of the disorder. Stress-exaggerated fear is one such model. Stress-exaggerated fear conditioning (SEFC) entails exposing an animal to a chronic or prolonged stressor followed by administration of a traumatizing event, such as auditory Pavlovian fear conditioning. The stressed animals become vulnerable to the development of exaggerated fear associations after the chronic stress (*see summary below).*

	Stress-induced vulnerability	Trauma	Result
Human	War conditions Poverty Starvation	IED Rape Natural Disaster ...	Increased vulnerability to develop PTSD
Rodent	Immobilization	Pavlovian Fear Conditioning	Stress-enhanced fear conditioning
			
SEFC models PTSD symptoms	Intrusive Memories	Increased Fear	Hyper-arousal

There are parallels between SEFC and PTSD symptoms. After a traumatic event, animals show an enhanced reaction to a similar, less aversive event (76). Patients with PTSD also experience excessive reactions to things that are similar to the trauma that they experienced (77). Similarly, after the exposure to stress, animals demonstrate facilitation of fear learning similar to the finding that humans with PTSD are more likely to develop additional phobias (78). Lastly, like PTSD, SEFC can be long lasting (76).

In addition to replicating behavioral changes, this model also produces similar neuronal and functional changes in the brain areas most associated with PTSD. Many experiments have shown that chronic stress paradigms like those used in SEFC decrease hippocampal volume and animal work demonstrates that the loss of hippocampal volume is due to simplification of hippocampal neurons (79-85). As in PTSD, the opposite effect is found in the amygdala. For example, while hippocampal pyramidal neurons from chronically stressed animals show dendritic atrophy, basolateral amygdala pyramidal and stellate cells show increases in dendritic branching and spine density (Figure 3, (86)). Further modeling the changes seen in patient populations, animals administered chronic stress exhibit increased amygdala activity (81, 87). SEFC is a robust animal model and has allowed for better understanding and investigation of the neurobiology of trauma-induced anxiety disorders such as PTSD.

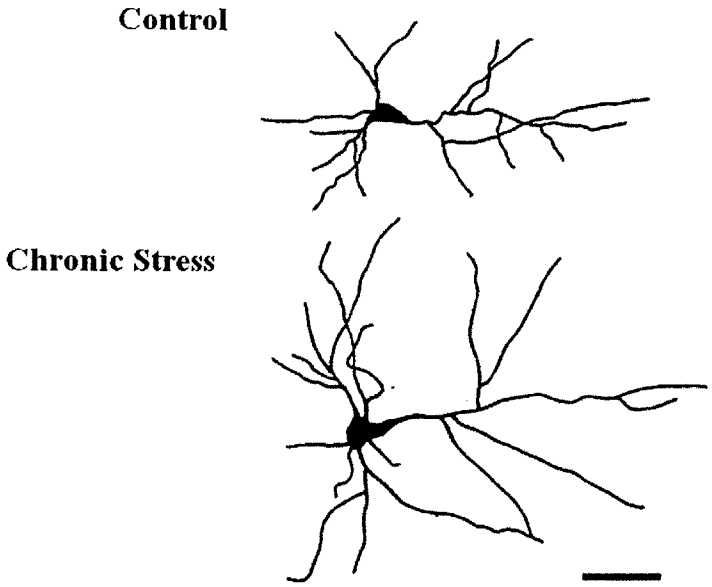


Figure 3. Chronic stress increases dendritic arborization in BLA pyramidal neurons. *Adapted from Vyas et al, 2002.*

Brief overview

The objective of the work in this thesis is to examine the effects of chronic stress on an area of the brain important for emotional memory, the amygdala. Stress responses, while protective during a threatening encounter, become detrimental if repeatedly experienced. Chronic stress increases a person's susceptibility to the development of fear and anxiety disorders, such as PTSD. The most pertinent paradigm used in humans and animals to study the psychological and physiological alterations associated with fear and anxiety disorders is Pavlovian fear conditioning, in which a neutral conditional stimulus becomes predictive of danger after pairing with an aversive unconditional stimulus. Fear

learning is modulated by stress and this modulation likely plays a role in PTSD. Stress-induced alterations in learning are thought to be mediated by structural and functional changes in the amygdala and other areas of fear circuitry (61, 86-89). Behavioral data demonstrate that amygdala-dependent aspects of fear conditioning are enhanced during chronic stress and serve as an animal model of PTSD and other trauma-induced anxiety disorders.

While consequences of chronic stress on fear are fairly well-documented, the molecular pathways that contribute to the stress-induced modulation of fear learning are unknown. It has been found that ghrelin, an orexigenic hormone produced by the stomach, is modulated by chronic stress (90-92). Over a course of chronic stress, levels of ghrelin rise (91, 93). Ghrelin secretion remains elevated for at least four weeks after cessation of the stress (90). Ghrelin is known to cross the blood-brain barrier, and the ghrelin receptor is found in the amygdala (94-96). Exogenous ghrelin is also known to promote synapse formation (97). Taken together, this prior work suggests that ghrelin may contribute to stress-induced sequelae in fear. A series of experiments performed within the scope of this thesis explore the hypothesis that chronic stress alterations of fear are dependent on the ghrelin pathway (Chapter 2) and discover that stress-induced increases in ghrelin are sufficient and necessary for stress-enhanced fear conditioning. Additionally, experiments in Chapter 2 provide evidence that ghrelin is mediating these effects independently of the HPA axis and directly in the amygdala.

In spite of the widely appreciated magnitude of stress-induced mental illness, little is known about how chronic stress actually leads to these disorders and how to ameliorate stress-related alterations in brain function. The research here provides insight into the relationship between the established effects of stress in fear behavior and the cognitive dysfunction that follows chronic stress (89). This thesis explores the recruitment of several hormonal pathways as stress duration accumulates and becomes chronic or prolonged in nature (Chapter 3). Chapter 3 identifies ghrelin and leptin as hormones uniquely altered by chronic stress whereas glucocorticoids and growth hormone are predominantly altered by short-term stress. The work herein also suggests risk factors and biomarkers that would indicate people who are most vulnerable to stress-induced exacerbation of a psychiatric illness (Chapters 2 and 3). Further, it provides evidence of pharmacological treatments to prevent increased emotional reactivity after chronic stress (Chapter 2). In the final data chapter, this thesis identifies a behavioral therapy paradigm that could increase the efficacy of CBT for fear and anxiety disorders (Chapter 4).

These investigations provide new scientific insight into the molecular mechanisms involved in chronic stress and fear learning. This work is also clinically significant because understanding the molecular mechanisms of stress-induced changes in the brain will facilitate development of new pharmacological treatments for mental illnesses involving neuronal network dysfunction, emotional dysregulation, and those with significant stress components. As an outcome of

these studies, I suggest treatments that prevent the development of pathological anxiety disorders. The savings in human cost will be dramatic if it can be taken to fruition (see Appendix B) and is expected to have a significant positive impact on the mental health of soldiers returning from war, victims of child abuse, or casualties of natural or man-made disasters. This, in turn, will produce dramatic reduction in mental health costs, and will have major benefits for the millions of people suffering from stress-related mental illness worldwide while also advancing our scientific understanding of the neurobiology of psychiatric illnesses.

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Chapter 2

Persistently elevated ghrelin drives stress-induced vulnerability to enhanced fear

Abstract

Hormones in the hypothalamus-pituitary-adrenal (HPA) axis mediate many of the bodily responses to stressors, yet there is not a clear relationship between the levels of these hormones and stress-associated mental illnesses such as post-traumatic stress disorder (PTSD). Therefore, other hormones are likely to be involved in this effect of stress. Here we use a rodent model of PTSD to show that stress-related increases in ghrelin are necessary and sufficient for stress-associated vulnerability to exacerbated fear learning. Furthermore, these effects can be dissociated from HPA activity. Virus-mediated overexpression of growth hormone, a downstream effector of the ghrelin receptor, in the amygdala also increases fear. These results suggest that ghrelin mediates a novel branch of the stress response and highlight a previously unrecognized role for ghrelin in maladaptive changes following prolonged stress.

Introduction

When a cue or event threatens the well-being of an organism, stress responses are engaged to promote coping and adaptation (3). Despite the utility of these responses, repeated or prolonged activation of the stress response causes detrimental effects, including increased susceptibility to mental illnesses such as depression, anxiety, or post-traumatic stress disorder (PTSD) (4-10). It is thought that the stress response is principally coordinated by hormones of the hypothalamic-pituitary-adrenal (HPA) axis, however strong correlations between stress-induced alterations in these hormones and mental illness are lacking, and not all effects of chronic stress can be simulated with exogenous administration of HPA hormones (11, 12). Furthermore, while excessive HPA activity has been linked to heightened fear and anxiety in rodents, there has been little success in the clinical application of these findings (13). Both high and low levels of HPA activity have been observed in humans with stress-sensitive mental disorders and, in some cases, patients respond positively to treatment with exogenous glucocorticoids, one of the adrenal stress hormones (14). Thus, there is a crucial need for novel biomarkers and therapeutic targets.

Recently, it has been found that ghrelin, a gastric peptide, is modulated by exposure to stress (15, 16). Ghrelin is activated by post-translational acylation before being transported from endocrine cells of the stomach into the blood stream. It can then cross the blood-brain barrier (*13*) where it binds to the Growth Hormone Secretagogue Receptor 1a (GHSR1a, or ghrelin receptor). Interestingly, the ghrelin receptor is found in the basolateral complex of the amygdala (BLA) (17), a brain

region that regulates negative emotional states such as fear. Single infusions of exogenous ghrelin into the amygdala can alter behavior in tasks such as the elevated plus maze and inhibitory avoidance (18), but a relationship between emotional learning and endogenous ghrelin has not been explored. Furthermore, growth hormone (GH), which is released by cells in response to ghrelin receptor activation and altered in the brain by stress (19), is present in BLA neurons (20). Ghrelin's stress-sensitivity, together with the localization of its receptor and major signaling molecule (GH) in BLA, make it an attractive candidate mechanism by which emotional memories may be altered following periods of stress.

Patients with PTSD exhibit heightened fear learning (21) and enhanced amygdala activity (22, 23). Animal studies report similar changes in fear learning and amygdala function following stress exposure (24-26). To explore the mechanisms underlying the relationship between stress and increased propensity for affective disorders, we use a rodent model of PTSD and uncover an essential and novel role for ghrelin in stress-induced susceptibility to exacerbated fear.

Results

Stress-related changes in fear and ghrelin are independent of adrenal stress hormones

We used an animal model of PTSD in which rats were repeatedly exposed to immobilization stress (4h/d for 14d) and subsequently administered auditory fear conditioning. Many studies suggest that the development of affective illness following stress, including disorders involving fear (11, 12, 27), is due to repeated activation of the HPA axis, which results in elevated adrenal stress hormone release (8, 28). To determine whether stress-induced increases in fear learning require adrenal stress hormones, such as corticosterone or adrenaline, we examined the impact of adrenalectomy on stress-related enhancement of fear conditioning. Following adrenalectomy (ADX) or sham surgery (SHAM), animals were exposed to immobilization stress (STR) or daily handling (no stress, or NS). One subset of animals underwent auditory fear conditioning 24h after the final stress or handling session. Fear to the tone was assessed 48h post-conditioning. Though a slight enhancement of fear acquisition was seen in stressed rats, this did not reach statistical significance (Fig. 1a, stress: $F(1, 22)=3.98$, $p<0.10$, ns). However, stress produced a robust enhancement of long-term fear memory (Fig. 1b, stress: $F(1,22)=12.17$, $p<0.01$). Surprisingly, this was observed in the complete absence of adrenal stress hormones (Fig. 1b, Surgery X Stress interaction, $F(1,22)=1.3$, p=ns; corticosterone verified as undetectable in all ADX animals, Fig. 1c.).

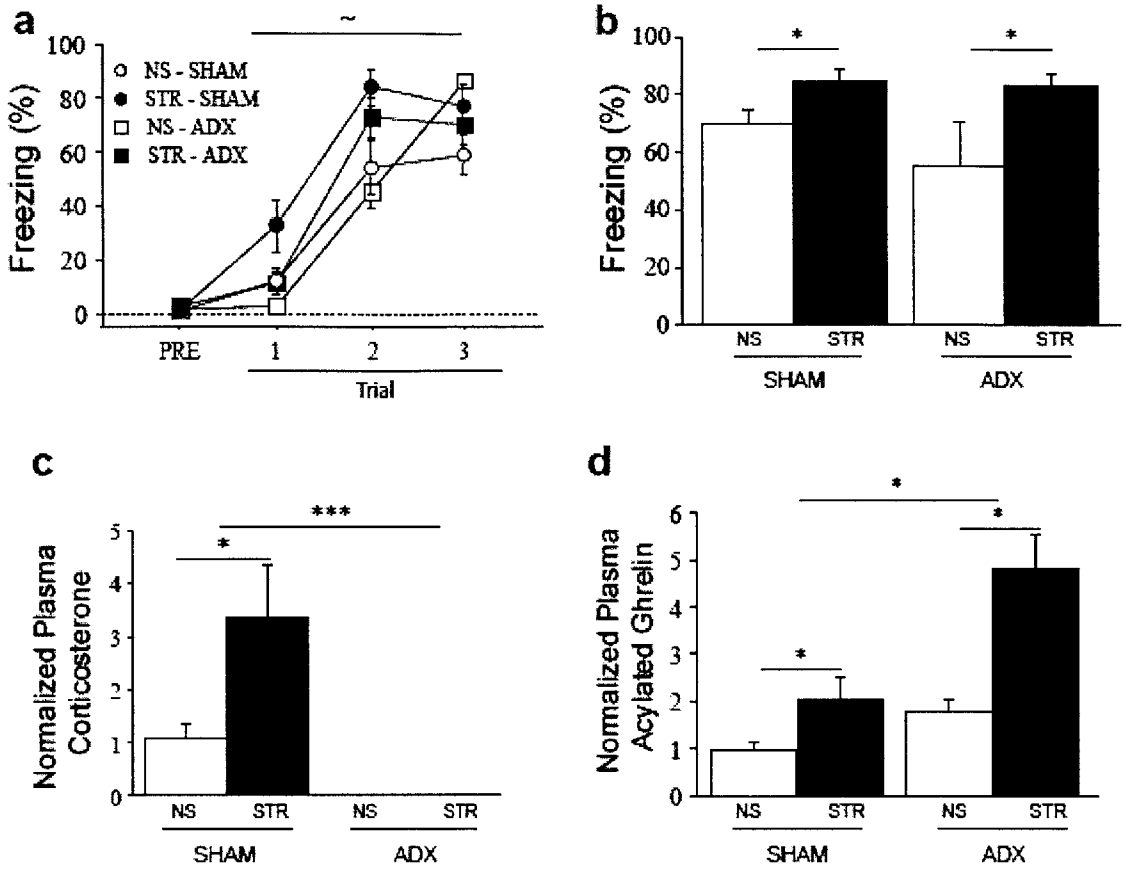
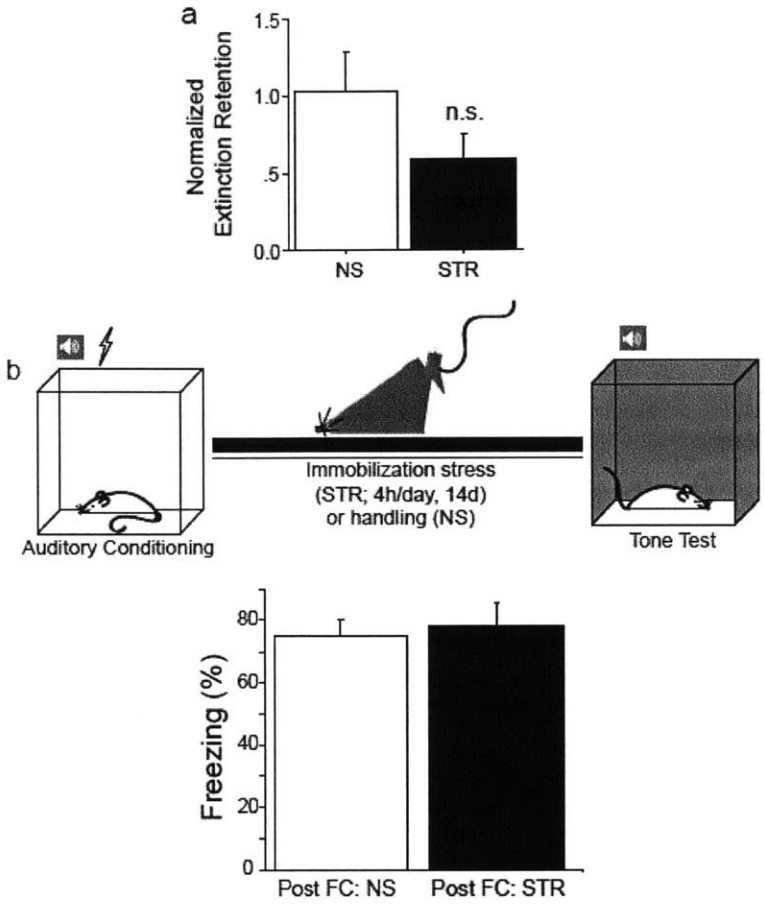
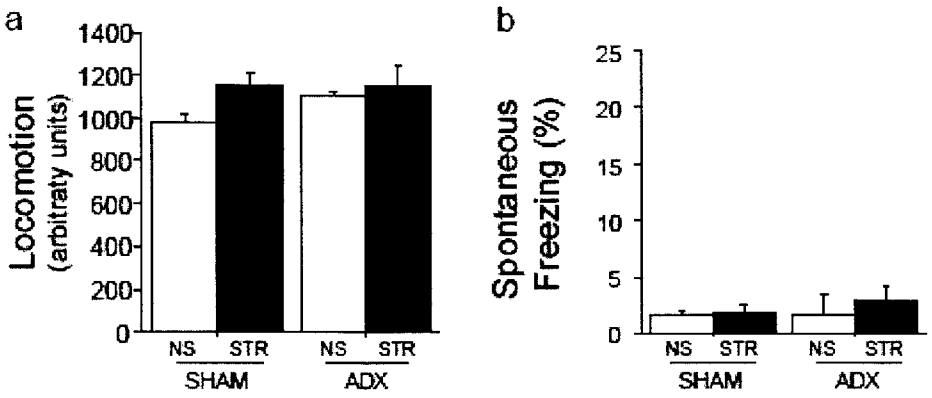


Figure 1. Stress-related changes in fear and ghrelin are independent of adrenal stress hormones. Animals received adrenalectomy (ADX) or sham surgery (SHAM). After at least a week of recovery, animals received either 14 days (4h/d) immobilization stress (STR) or gentle handling (NS). (a) Some animals received auditory Pavlovian fear conditioning 24h after the last stress or handling session. (b) Fear to the tone was assessed 48h later in a novel context. In a separate group of animals, trunk blood was collected 24h after the lass stress session. Plasma level corticosterone (c) and acylated ghrelin (d) were determined with ELISA. All data are mean ±s.e.m. * $p<0.05$, *** $p<0.001$, ~ $p<0.10$ in planned comparisons.

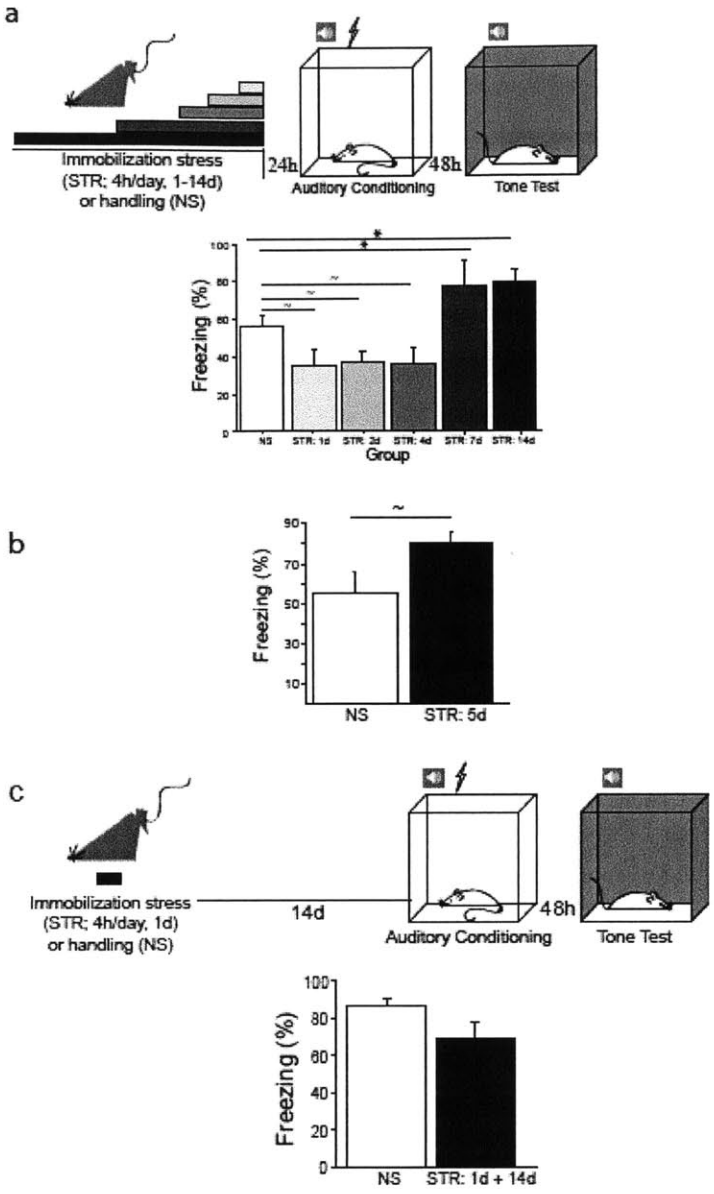
The enhancement of fear was specific to fear acquisition and/or consolidation rather than fear expression, locomotion, extinction, or spontaneous immobility (see Supplementary Text and Supp. Fig. 1-3). These results indicate that stress-enhanced fear learning is not mediated by glucocorticoids or adrenaline, consistent with the limited clinical benefit of pharmacological manipulations targeting adrenal hormone signaling in PTSD patients (14, 29). Importantly, these data also suggest that other stress hormones drive this behavioral change.



Supplementary Figure 1. Chronic stress does not alter extinction or expression of previously acquired fear memories. Rats in the SHAM group from Figure 1 received either daily handling (NS) or immobilization stress (STR). Animals received Pavlovian fear conditioning 24h after the last stress or handling session. Fear memory was assessed by placing the animals in a novel context 48h after the conditioning session and measuring conditional freezing following tone presentation. (a) An extinction test was performed 48h after the fear memory test during which animals were returned to the extinction context and presented with additional tones. Extinction retention measures the memory strength for the extinction learning acquired during the first extinction session. It is calculated as the difference in initial freezing levels between the first and the second extinction sessions. This value is normalized to the NS-SHAM group. (b) In a second group, intact animals received Pavlovian fear conditioning 24h before beginning daily handling (Post FC: NS) or immobilization stress (Post FC: STR) for 14d. Fear memory was assessed by placing the animals in a novel context and measuring conditional freezing following tone presentation 24h after the last stress or handling session. All data are mean ±s.e.m.



Supplementary Figure 2. Adrenalectomy and immobilization stress do not alter locomotion or spontaneous freezing levels. Rats from Figure 1 received either daily handling (NS) or immobilization stress (STR). Locomotion (a) and spontaneous freezing (b) in a novel environment were assessed prior to fear conditioning. All data are mean ±s.e.m.



Supplementary Figure 3. Stress-induced enhancement of fear arises after 5 consecutive days of stressor exposure. (a) Rats received either daily handling (30s/d, 14d, NS) or immobilization stress (4h/d, STR) for 1, 2, 4,7 or 14 days. Animals received auditory Pavlovian fear conditioning 24h after the last stress or handling session. Fear to the tone was assessed 48h later. (b) In a separate group of animals, rats received either daily handling (30s/d, 5d, NS) or immobilization stress (4h/d, STR) for 5 days. Animals received auditory Pavlovian fear conditioning 24h after the last stress or handling session. Fear to the tone was assessed 48h later. (c) In a third group of animals, rats received a single handling (NS) or immobilization stress (STR) session. They were then returned to the vivarium for 14d before receiving auditory Pavlovian fear conditioning. Fear to the tone was assessed 48h later. All data are mean ±s.e.m. * $p<0.05$, ~ $p<0.10$ in planned comparisons.

Circulating acylated ghrelin is elevated following chronic stress (15), raising the possibility that it may function as a stress hormone but its relationship with HPA hormones is unclear. To clarify this, we examined the impact of adrenalectomy on stress-induced increases in acylated ghrelin. Animals were administered surgical and stress treatments as per the previous experiment but sacrificed 24h after the final stress or handling session for the collection of blood samples. This was performed during a narrow window surrounding the circadian trough of ghrelin release to minimize hunger-induced variability in ghrelin levels. As expected, corticosterone was significantly elevated by immobilization stress in the SHAM group but undetectable in the ADX group (Fig. 1c, Stress X Surgery interaction: $F(1, 17)=8.37$, $p<0.05$). In contrast, acylated ghrelin was elevated by stress regardless of the presence or absence of the adrenal glands (Fig. 1d, stress: $F(1, 17)=13.19$, $p<0.01$, and Stress X Surgery interaction: $F(1, 17)=2.99$, p=ns). Interestingly, stress-related increases in acylated ghrelin were amplified by adrenalectomy (Fig. 1d, surgery: $F(1,17)=9.97$, $p<.01$), showing that adrenal hormones inhibit rather than facilitate ghrelin release (30). Ghrelin is not only elevated by psychological stressors such as immobilization stress, but also by other stressors involving environmental factors (water stress, Supp. Fig. 4, stress: $F(1, 14)=33.46$, $p<.0001$) and social status [social defeat, (15)]. Together, these data reveal that ghrelin is not simply a downstream effector of adrenal hormone recruitment during chronic stress, and may instead represent an independent hormonal pathway of the stress response, broadly recruited by different stressors.

Additionally, the elevation of ghrelin by stress in the absence of adrenal hormones raises the intriguing possibility that the ghrelin pathway mediates stress-related enhancement of fear.

Repeated activation of the ghrelin receptor is sufficient for enhanced fear in the absence of stress and independent of the HPA

To determine whether increased activation of the ghrelin receptor is sufficient for enhancement of fear memory, we conducted experiments using pharmacological agonism of GHSR-1a in non-stressed animals. Stress-induced changes in acylated ghrelin were observed at the nadir of the diurnal ghrelin cycle, suggesting that stress-related increases in ghrelin persist throughout the day. Because the half-life of acylated ghrelin is short [~30m (31)], we used MK-0677, a highly selective GHSR-1a agonist with a half-life of at least 5-6h (32), instead of exogenous acylated ghrelin in order to more closely model the prolonged stress-induced increases in GHSR activation by endogenous ghrelin. We systemically administered MK-0677 (MK: 5d) or saline (VEH: 5d) once a day for five consecutive days in non-stressed rats to determine whether repeated ghrelin receptor agonism in the absence of stress is sufficient to increase fear learning and whether HPA hormones may play a role in this effect. Five days of treatment were used because this reflects the minimum number of sessions our immobilization stress must be repeated to see stress-related enhancement of fear (see Supplementary Text and Supp. Fig. 3). One subset of animals was administered auditory fear conditioning 24h after the last injection. This drug regimen significantly enhanced long-term fear

memory (Fig. 2b, injection: $F(1,31)=4.21$, $p<0.05$), but did not alter acquisition during conditioning (Fig. 2a, injection: $F(1, 31)=1.54$, p=ns).

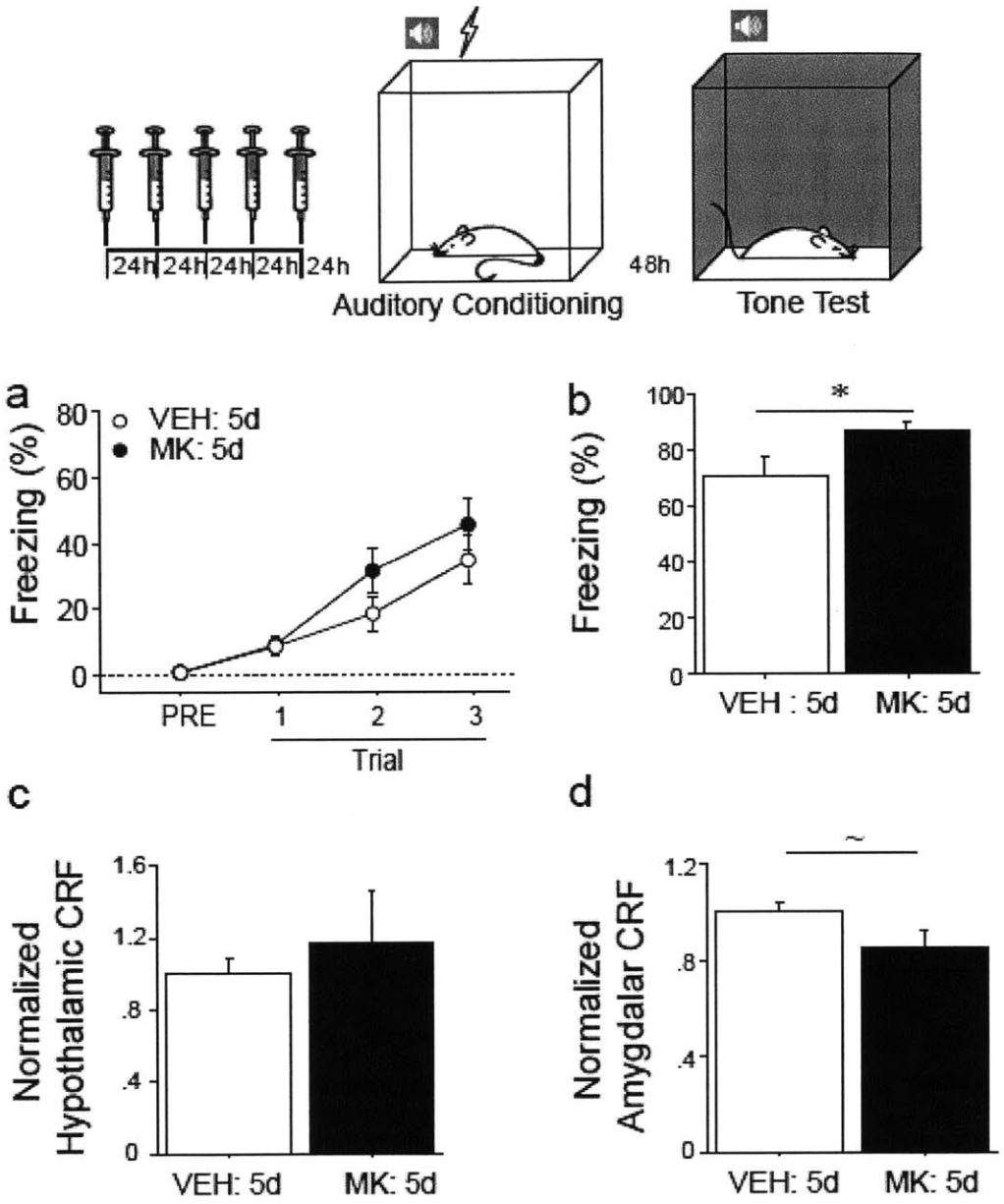
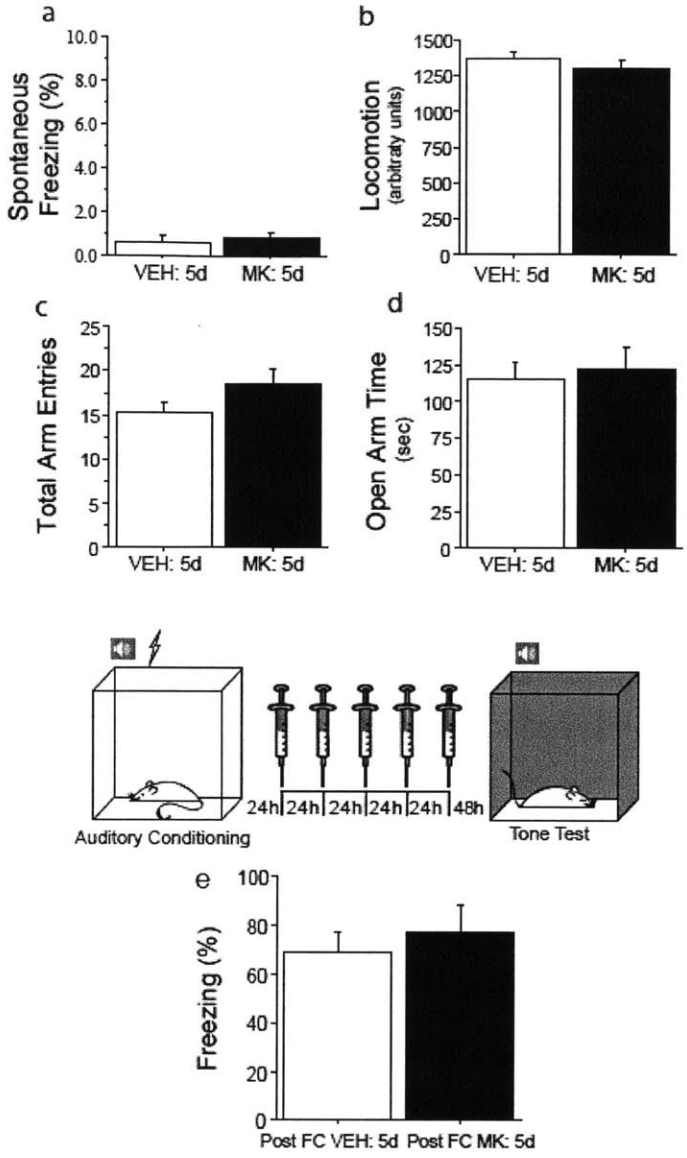


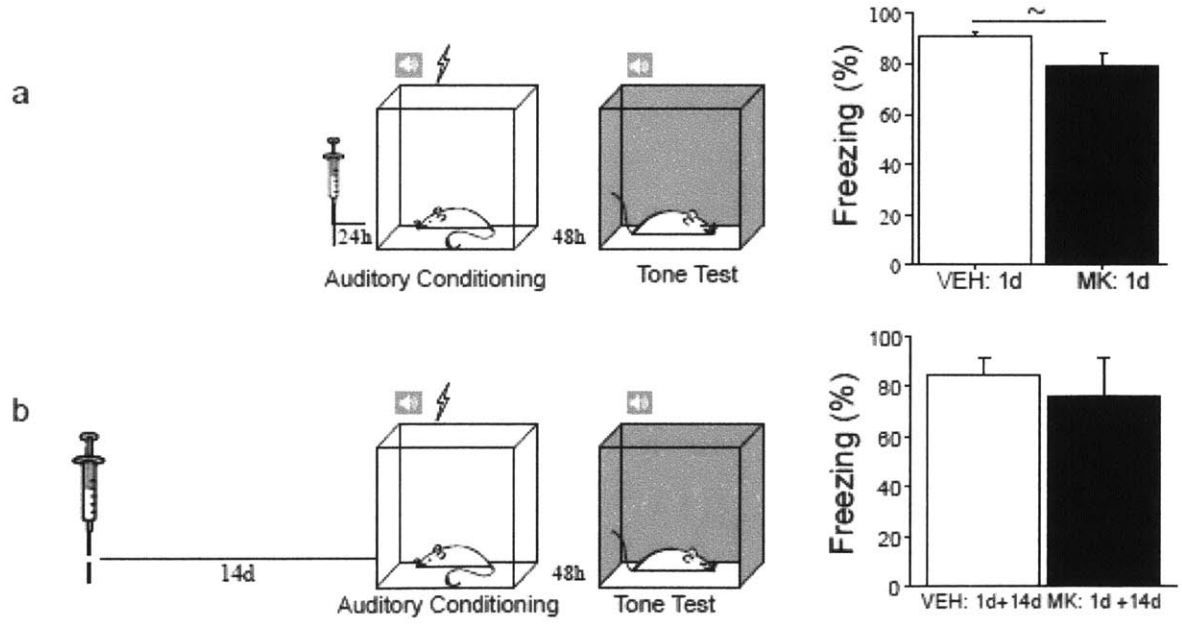
Figure 2. Long-term pharmacological stimulation of ghrelin receptor activity enhances fear memory without altering other stress hormones. Rats received daily systemic injections of MK-0677 (MK: 5d), a GHSR-1a agonist, or saline (VEH: 5d) for five days at the endogenous ghrelin signaling nadir. (a) One group underwent auditory fear conditioning 24h following the final injection. Fear acquisition was assessed by monitoring freezing levels. (b) Conditional freezing to the tone was assessed in a novel context 48h following fear conditioning. A separate group was sacrificed 24h following the final injection and microdissections of hypothalamus and amygdala performed. Brain CRF levels were measured using ELISA (c, hypothalamus, d, basolateral complex of the amygdala). * $p<0.05$, ** $p<0.01$, ~ $p<0.10$ in planned comparisons.

This enhancement was similar to the effect of chronic immobilization stress and was not attributable to spontaneous freezing (Supp. Fig. 5a, injection: $F(1, 31)=.25$, p=ns) or a drug-induced decrease in locomotor activity (Supp. Fig. 5b and c, injection: $F(1,31)=0.95$, $F(1, 15)=2.44$, p=ns all). Additionally, it was specific to associative aversive processing, as innate anxiety was not altered (Supp. Fig. 5d, treatment; $F(1,15)=.15$, p=ns). Furthermore, just as we observed following chronic immobilization stress (see Supplementary Text), fear expression was not altered following chronic ghrelin receptor agonism: previously acquired auditory fear memory was not affected by chronic ghrelin receptor agonism (Supp. Fig. 5e, injection: $F(1, 10)=.30$, p=ns).



Supplementary Figure 5. Chronic ghrelin receptor agonism does not alter locomotion, innate anxiety, or expression of previously acquired fear memories. Rats received a daily systemic injection of MK-0677 (MK: 5d), an agonist of GHSR-1a, or vehicle (VEH: 5d) for five days at the endogenous ghrelin signaling nadir. Spontaneous freezing (a) and locomotion (b) were assessed in a novel context 24h after the last injection. A separate group was assessed on the elevated plus maze in a single 8 minute session 24h after the last injection. Total arm entries (c) and open arm time (d) were measured to assess exploratory behavior and innate anxiety, respectively. (e) Animals received Pavlovian fear conditioning 24h *before* beginning daily handling injections of MK-0677 (Post FC MK:5d) or vehicle (Post FC VEH: 5d) for 5d. Fear memory was assessed by placing the animals in a novel context and measuring conditional freezing following tone presentation 24h after the last injection. All data are mean ±s.e.m.

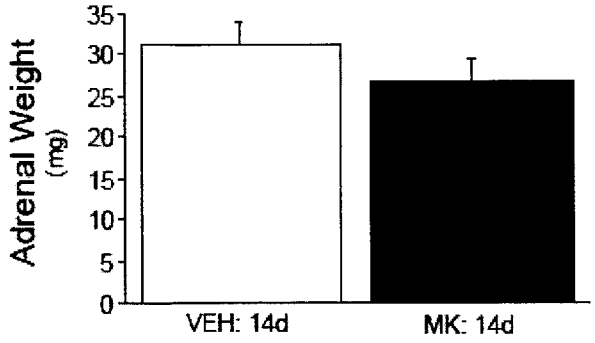
Additionally, the enhancement of fear memory by repeated ghrelin receptor agonism cannot be attributed to effects of the most recent drug treatment (Supp. Fig. 6a, injection; $F(1, 19)=3.70$, $p<0.10$) or delayed effects arising from the first drug treatment (Supp. Fig. 6b; injection: $F(1, 6)=.22$, p=ns). Interestingly, there is a trend towards impairment of fear learning after a single dose of the ghrelin receptor agonist (Supp. Fig. 6a, injection; $F(1, 19)=3.7$, $p<0.10$). This effect is similar to the effect of a single immobilization session (See Supp. Fig. 3a and Supplementary Text). These data suggest that long-term activation of the ghrelin receptor is sufficient to enhance fear memory, with strong parallels to the temporal dynamics of stress exposure.



Supplementary Figure 6. Single injections of ghrelin receptor agonist are not sufficient to enhance fear. (a) Rats received a single injection of saline (vehicle, VEH: 1d) or MK-0677 (MK: 1d) and received Pavlovian Auditory Fear Conditioning 24h later. Fear to the tone was assessed 48h after fear conditioning. (b) Rats received a single injection of saline (vehicle, VEH: 1d+14d) or MK-0677 (MK: 1d+14d) and were then returned to the vivarium for 14d before receiving auditory Pavlovian fear conditioning. Fear to the tone was assessed 48h later. All data are mean ±s.e.m. ~ $p<0.10$ in planned comparisons.

While stress-related increases in ghrelin are not triggered by the HPA axis, ghrelin could interact with the HPA axis in other ways to enhance fear. For example, the hypothalamic stress hormone corticotrophin-releasing factor (CRF) is secreted by neurons of the paraventricular nucleus, an area dense with GHSR-1a (33), and ghrelin increases CRF mRNA in this area (34). Moreover, hypothalamic CRF neurons project to the amygdala and amygdalar CRF can modulate fear memory (35, 36). Thus, systemic ghrelin receptor agonism could mediate effects on fear learning by increasing CRF release in the amygdala. Additionally, ghrelin receptors have been identified in the adrenal cortex (37). Therefore systemic ghrelin receptor agonism could mediate effects on fear learning by increasing release of adrenal hormones. To determine whether the effects of ghrelin on fear learning are mediated through the HPA axis, we examined CRF peptide levels in both the hypothalamus and the amygdala of animals treated as above. There was no change in hypothalamic CRF (Fig. 2c, injection: $F(1, 10)=.32$, p=ns) and a trend for repeated ghrelin receptor activation to decrease amygdalar CRF levels (Fig. 2d, injection: $F(1, 10)=3.55$, $p<0.10$). In a third group of animals, we examined adrenal weights following a more prolonged period of ghrelin receptor agonism. Increased adrenal weight is seen following prolonged recruitment of adrenocorticotrophin (ACTH) from the pituitary and repeated glucocorticoid and adrenaline production and release from the adrenal glands. Animals received systemic administration of MK-0677 (MK: 14d) or saline (VEH: 14d) once a day for 14 days. Repeated systemic ghrelin receptor agonism did not alter this measure (Supp. Fig. 7, injection:

F(1,14)=1.24, p=ns). This suggests that repeated ghrelin receptor agonism at the doses used here does not stimulate the HPA axis.



Supplementary Figure 7. Prolonged ghrelin receptor agonism does not alter adrenal weights. Rats received a daily injections of saline (vehicle, VEH: 14d) or MK-0677 (MK: 14d) for 14 days. Adrenal glands were dissected perimortem 24h after the last injection. All data are mean ±s.e.m.

Fear memory requires plasticity in numerous brain regions but the basolateral complex of the amygdala (BLA) is particularly important for both formation and storage of learned fear. Recently, it has also been identified as the region of the amygdala with the highest density of ghrelin receptors (17). To determine whether repeated ghrelin receptor activation in the BLA is sufficient to enhance fear memory, we infused either MK-0677 (MK-Inf: 5d) or artificial cerebrospinal fluid (vehicle, VEH-Inf: 5d) directly into the BLA daily for five days prior to auditory fear conditioning. Freezing during fear conditioning was not altered by the treatment (Fig. 3a, infusion: $F(1,8)=.36$, p=ns) but long-term fear memory was significantly enhanced (Fig. 3b, infusion: $F(1,8)=13.75$, $p<0.01$).

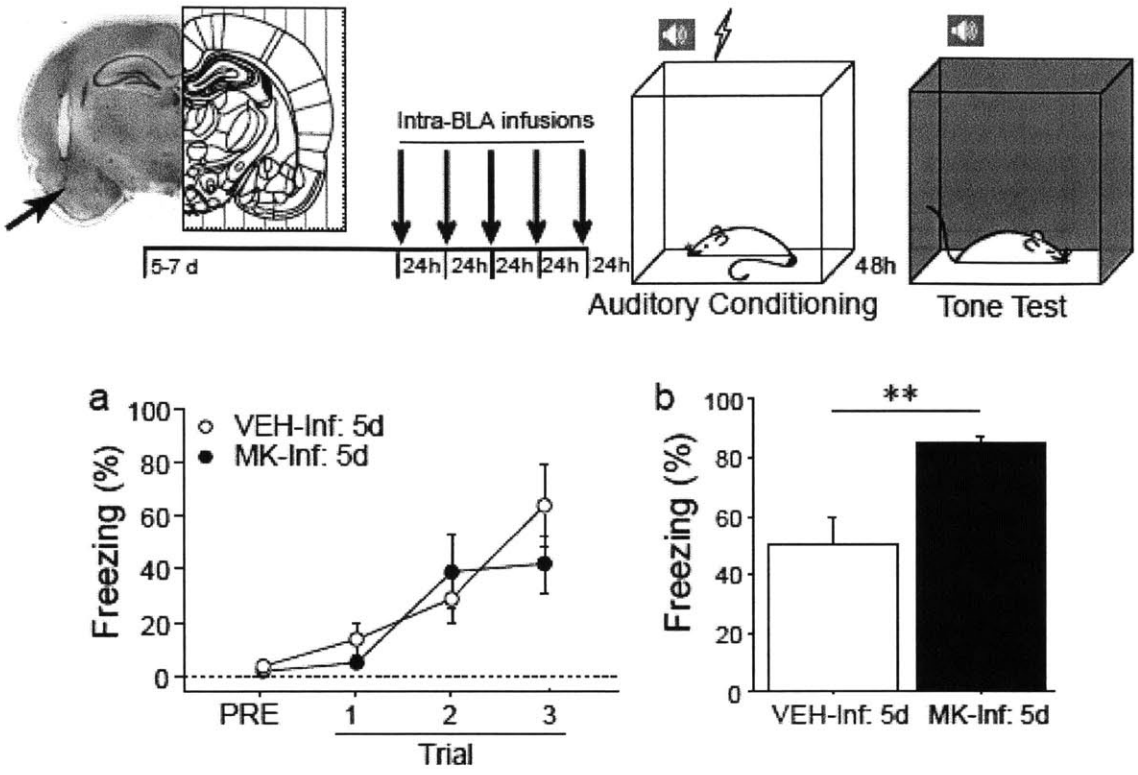
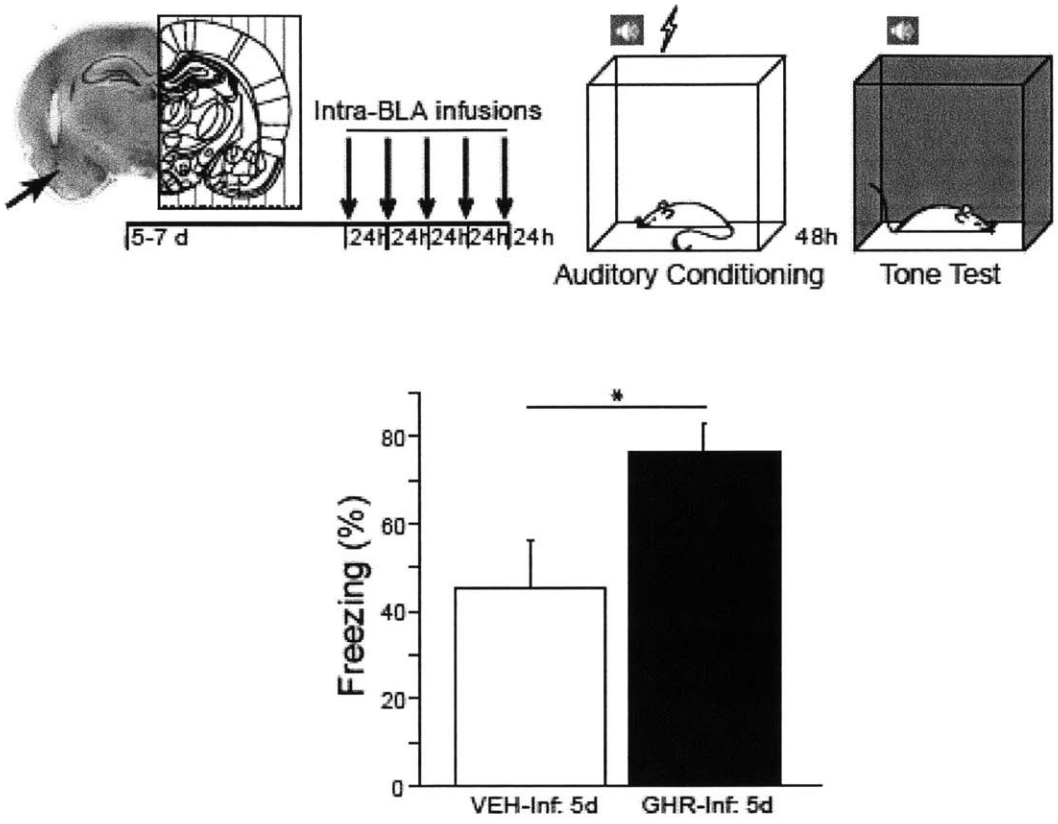


Figure 3. Long-term pharmacological stimulation of ghrelin receptor activity in the amygdala enhances fear memory. Rats were implanted with bilateral cannulae aimed at the basolateral amygdala (BLA). The arrow indicates the tip of the injector within a representative coronal brain section. Following recovery, intra-BLA infusions of either MK-0677 (MK-Inf: 5d) or aCSF (VEH-Inf: 5d) were administered daily for five consecutive days and, 24h following the final infusion, (a) auditory fear conditioning was administered. (b) Fear memory was assessed in a novel context 48h following fear conditioning. Brain illustration adapted from (1). All data are mean ±s.e.m. * $p<0.05$, ** $p<0.01$, ~ $p<0.10$ in planned comparisons.

A similar potentiation of fear memory was observed when acylated ghrelin (GHR) was infused into the BLA daily for five days (Supp. Fig. 8; infusion: $F(1,8)=6.07$, $p<0.05$). Collectively, these data show that repeated activation of the ghrelin receptor directly in BLA is sufficient for heightened fear memory. This finding indicates that stress-induced increases in circulating ghrelin may enhance fear through actions in the BLA. Additionally, because direct intra-BLA

manipulations are unlikely to increase either CRF or ACTH (34), this provides further support for the claim that ghrelin alters fear by direct actions in the amygdala, rather than through interactions with the HPA axis.



Supplementary Figure 8. Repeated intra-amygdala ghrelin infusions enhance fear memory. Rats were implanted with bilateral cannulae aimed at the basolateral amygdala (BLA). The arrow indicates the tip of the injector within the coronal brain section. Following recovery, intra-BLA infusions of either acylated ghrelin (GHR-Inf: 5d) or artificial cerebrospinal fluid (vehicle, VEH-Inf: 5d) were administered daily for five consecutive days and, 24h following the final infusion, auditory fear conditioning was administered. Fear memory was assessed during tone presentation in a novel context. Brain illustration adapted from (1) . All data are mean ±s.e.m. * $p<0.05$ in planned comparisons.

The ghrelin pathway is necessary for stress-induced vulnerability to fear during chronic stress

To determine whether ghrelin signaling is necessary for stress-related enhancement of fear memory, we blocked ghrelin receptor signaling during repeated stress sessions. Rats were administered immobilization stress (STR) or daily handling (NS) and given either a systemic injection of D-[Lys3]-GHRP-6 (DLys3), a highly specific inverse agonist of GHSR-1a that crosses the blood-brain barrier, or saline (VEH) at the start of each session (32). Twenty-four hours following the final stress or handling session, we administered auditory fear conditioning and assessed fear to the tone in a subsequent session. Long-term fear memory was enhanced by stress in saline-treated control animals, but DLys3 completely reversed stress-enhanced fear (Fig. 4b; Injection X Stress interaction: $F(1,27)=6.36$, $p<0.05$ and post-hoc comparisons). In contrast, ghrelin receptor antagonism had no effect on fear memory in non-stressed controls (Fig. 4b; post-hoc comparison, NS-SAL vs. NS-Dlys3). Stress enhanced fear acquisition in the saline treated group (Fig. 4a, planned comparisons, treatment, STR-VEH vs NS-VEH; $F(1, 13)=5.03$, $p<.05$) but this effect was not seen when the ghrelin receptor was antagonized during immobilization stress (Fig. 4a, Trial 3, Stress X Injection interaction: $F(1,27)=3.94$, $p<0.10$). Moreover, DLys3 treatment did not blunt stress-induced HPA activation as measured by corticosterone secretion in stressed animals (Fig. 4c; injection: $F(1, 5)=.10$,p=ns). These data show that ghrelin-mediated signaling is necessary for stress-related enhancement of fear and suggest that other peripheral or central

stress hormones are not sufficient to mediate this effect in the absence of heightened ghrelin signaling.

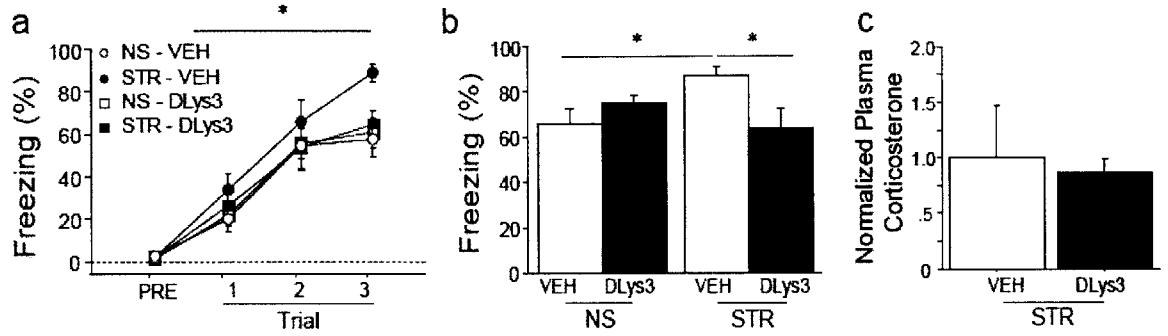


Figure 4. Ghrelin receptor antagonism during chronic stress abolishes stress-related enhancement of fear memory without affecting corticosterone release. Rats received either daily handling (NS) or immobilization stress (STR). Each day, a systemic injection of either D-lys3-GHRP-6 (Dlys3), an antagonist of GHSR-1a, or saline (VEH) was administered within 30m of handling or stress initiation. (a) Animals received auditory fear conditioning 24h after the last stress or handling session. (b) Fear memory was assessed 48h after the conditioning session by placing the animals in a novel context and measuring conditional freezing during tone presentation. (c) In a subset of animals in the STR group, tail bleeds were performed during the final 30m of the final stress session and plasma corticosterone levels were measured using ELISA. All data are mean ±s.e.m. * $p<0.05$, ~ $p<0.10$ in planned comparisons.

Growth hormone, a major effector of the ghrelin receptor, enhances fear memory in the amygdala

One of the best-characterized consequences of ghrelin receptor activation is release of GH (38). While the pituitary expresses the highest levels of GH, it is also expressed in other brain regions, including the BLA (20). In one region, GH levels have been shown to increase following acute stress (19). However, it is not known

how prolonged stress alters GH in the BLA. To test this, we examined the impact of repeated immobilization stress (STR) or daily handling (NS) on GH levels in the BLA. We found that GH was readily detected in BLA homogenate and significantly upregulated 24h after chronic stress (Fig. 5a, group: $F(1,16)=6.44$, $p<.05$), the time point at which we observe increases in circulating ghrelin and fear conditioning. This suggests that ghrelin receptor-mediated signaling in the BLA may be amplified following stress.

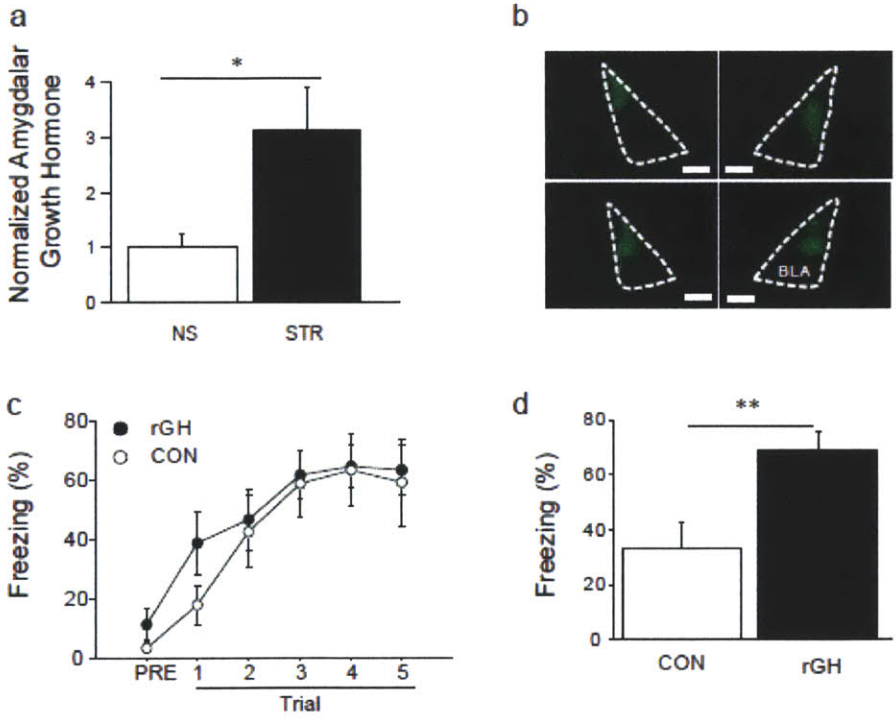


Figure 5. Amygdalar growth hormone is increased by chronic stress and is sufficient to enhance fear memory. (a) Rats received either daily handling (NS) or immobilization stress (STR) for 14d. Animals were sacrificed 24h after the last stress or handling session and the BLA was dissected. Growth hormone levels were measured using ELISA. (b) An HSV-based viral vector expressing either GFP (CON) or recombinant growth hormone (rGH) was infused in the BLA and expression was assessed 3 days after surgery (c). In a second group of rats, auditory Pavlovian fear conditioning was performed 3 days after surgery. (d) Fear memory was assessed by placing the animals in a novel context and measuring conditional freezing following tone presentation 48h after the conditioning session. Scale bar is 500 microns. All data are mean ±s.e.m. * $p<0.05$, ** $p<0.01$, ~ $p<0.10$ in planned comparisons.

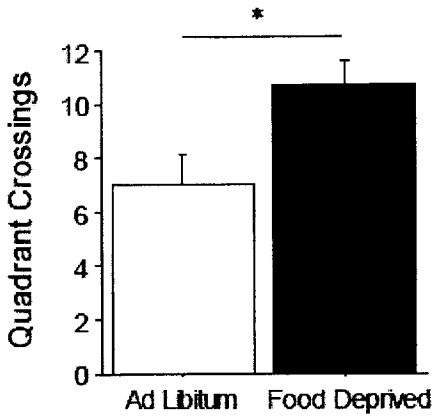
GH can induce synaptic plasticity (39) and is increased in response to learning (40), but it is unclear how it affects amygdala function. Herpes simplex virus (HSV)-based viral vectors were used to express recombinant rat GH (rGH) and a green fluorescent protein (GFP) reporter or GFP only. Naive rats received intra-BLA infusions of either the rGH virus (rGH) or the GFP-only control virus (CON). After three days, when HSV-mediated transgene expression is at its maximum (41), auditory fear conditioning was administered. Fear to the tone was assessed in a subsequent session. Overexpression of rGH did not alter fear acquisition (Fig. 5d; Infusion X Trial interaction: $F(4, 52)=.57$, p=ns) but did enhance fear memory (Fig. 5e, infusion: $F(1, 13)=9.97$, $p<.01$). These data show that high levels of GH in the BLA are sufficient to enhance fear learning and suggest that ghrelin receptor-mediated alterations in fear memory could be due to increased GH action in BLA.

Discussion

In conclusion, we first demonstrate that ghrelin acts in parallel to the HPA axis: adrenalectomy does not affect the ability of stress to enhance fear learning or increase circulating acylated ghrelin. This finding indicates that the observed effects of stress are not simply downstream from HPA hormones. We also show that increased ghrelin receptor activity is sufficient and necessary for stress-enhanced fear and is dissociable from HPA activity. Repeated activation of ghrelin receptors in non-stressed animals significantly enhances fear learning without elevating HPA stress hormones, while systemic blockade of the ghrelin receptor during chronic stress prevents stress-related enhancement of fear, even in the presence of elevated

adrenal stress hormones. Finally, we show that GH, the downstream effector of ghrelin receptor activation, is increased in the BLA by stress and can enhance fear learning.

Our study is the first to explicitly examine the effects of protracted exposure to elevated ghrelin, as observed following chronic stress. We show that there are profound differences in the behavioral consequences of ghrelin exposure following different exposure durations, similar to the cumulative nature of stress. We also provide the first evidence to link prolonged exposure to elevated ghrelin with a specific, detrimental consequence of stress: enhanced fear memory. In contrast, prior studies have argued that ghrelin promotes adaptive changes during stress, including antidepressant effects (15) and reduction in anxiety (42). However, these studies either focused exclusively on acute ghrelin manipulations or used short- and long-term ghrelin manipulations interchangeably. Additionally, the alterations in ghrelin levels were achieved through artificial states: heightened ghrelin levels were attained by extreme food deprivation or a single bolus injection of the short-lived peptide. These treatments potentiate locomotor activity (see Supplementary Text and Supp. Fig. 9) which may contribute to the behavioral effects previously reported. Here we demonstrate changes in endogenous ghrelin following stress and then use an appropriately low dose, long acting agonist to replicate the naturally occurring ghrelin state. Moreover, it is important to note that the changes in fear reported here occurred following small, but persistent, changes in ghrelin signaling, and all were in the absence of any locomotor effects.



Supplementary Figure 9. Food restriction to 90% body weight increases exploratory locomotion in a novel context. Rats were food deprived for 15 days at 4g chow per 100g rat. When rats reached 90% of their initial body weight, they were placed in a novel context with a 3x3 grid floor. The number of border crossings was recorded. All data are mean ±s.e.m. * $p<0.05$ in planned comparisons.

Our data show that stress-related changes in amygdala-based aversive processing are not dependent on HPA activity and that ghrelin plays an important role in stress-related affective dysfunction by actions independent of the HPA axis. This does not discount the role of the HPA axis in coordinating other aspects of the stress response. It is clear that the HPA hormonal cascade can account for numerous stress effects [for review see (43)]. However, this work may need to be re-examined through the lens of putative parallel stress pathways such as ghrelin. Future work will be needed to explore the possible synergistic effects of co-activation of the HPA axis and the ghrelin system during chronic stress.

No current treatments exist for preventing stress-related affective disorders, suggesting that our most intriguing and important finding is that blockade of ghrelin signaling during stress is sufficient to prevent stress-related vulnerability to

excessive fear. This raises the possibility that such a strategy might reduce or prevent the development of stress-sensitive affective disorders like PTSD during prolonged or extreme stress load. While there are some non-HPA molecules which might be targeted in the treatment of PTSD [such as brain-derived neurotrophic factor, tissue plasminogen activator, or FKBP5; for review, see (44)], the dysregulation of these molecules in PTSD models is brain region-specific. To effectively treat PTSD, pharmaceuticals for these molecules would need to cross the blood-brain barrier and act in a brain region-specific manner to minimize off-target side effects. Furthermore, there are no pharmaceuticals that can readily affect these molecules in humans. In contrast, because ghrelin is a peripheral hormone, it can be targeted using therapeutics that act in the periphery. Also, many anti-ghrelin treatments already have been tested for human use due to its putative role in the development of obesity (45). Thus, the discovery that ghrelin plays a role in stress-related affective dysregulation reveals an especially attractive target for treating stress-sensitive affective disorders.

Supplementary Discussion

The enhancement in long-term fear memory after stress cannot be explained by stress-related changes in extinction nor memory retrieval: no difference in extinction retention was observed in a second extinction test performed 48h after the initial extinction session (Supp. Fig. 1a; stress: $F(1, 12)=2.15$, p=ns), and stress administered after fear conditioning did not alter the expression of previously acquired fear memories (Supp. Fig. 1b; stress: $F(1, 17)=.107$, p=ns). The high levels of conditional freezing seen in rats in the STR or ADX groups also cannot be explained by non-specific decreases in locomotor activity (Supp. Fig. 2a; stress: $F(1, 22)=1.52$, p=ns and surgery: $F(1,22)=.56$, p=ns) or increases in spontaneous freezing (Supp. Fig. 2b; stress: $F(1, 22)=.37$, p=ns and surgery: $F(1,22)=.23$, p=ns). Stress-related enhancement of fear was also not due to the most recent stress session: a single session of immobilization stress was not sufficient to increase subsequent fear learning (Supp. Fig. 3a). Stress-related enhancement of fear also did not stem from delayed effects of the first stress exposure as has been shown in other aspects of stress (11, 46): a single exposure to immobilization stress did not affect fear conditioning administered 14d later (Supp. Fig. 3b, stress: $F(1, 6)=2.90$, p=ns). Rather, stress-related increases in fear memory appeared after cumulative stress exposure of approximately five or more days for this particular stressor (Supp. Fig. 3a and b, group: $F(5, 46)=5.01$, $p<.01$ and $F(1, 13)=4.62$, $p<0.10$), respectively).

The antidepressant effect of ghrelin requires extremely high levels of ghrelin, as found in food-restricted rodents after 10-15% weight loss (15). We find that this level of food deprivation leads to increased exploratory motor activity (Supp. Fig. 9;

$F(1, 13)=7.51$, $p<0.05$). A recent study reports similar motor effects following acute ghrelin manipulations (47). These motor effects can be a significant confound.

We suggest that, as a stress hormone, ghrelin may be similar to glucocorticoids: under "normal" conditions, there is an optimal level of the hormone (48) and too little (49, 50) or too much hormonal signaling (18) can lead to dysfunction in neuronal circuits. According to such a model, repeated activation of these two hormone pathways contributes to stress-induced wear and tear on the body, or allostatic load. In this regard, heightened ghrelin signaling may have both advantageous and undesirable consequences, but these must be carefully considered with respect to the length and level of elevated ghrelin exposure.

Further Questions:

Do immobilization stress or chronic ghrelin receptor agonism increase freezing in general?

Much of the data within this chapter depends on the reliability of behavioral measures to reflect an associative learning event, such as the tone-evoked fear behavior we measure here: freezing. If immobilization stress or chronic ghrelin receptor agonism increased freezing or immobility behaviors arbitrarily, then this confound would be insurmountable and would make it impossible to make any conclusions based on these metrics. However, control data shown here demonstrate that there is no change in spontaneous freezing levels nor is there a change in activity within a novel environment after immobilization (Supplementary Figure 2). Furthermore, I have demonstrated that animals administered chronic ghrelin do not demonstrate changes in either locomotion or spontaneous freezing (Supplementary Figure 5). Other data (not shown) demonstrate no differences in exploratory locomotion duration or distance on the elevated plus maze for either treatment (chronic immobilization or chronic ghrelin receptor agonism,). Additionally, Supplementary Figure 9 demonstrates that animals with chronically elevated endogenous ghrelin due to severe food restriction manifest a *hyperactivity phenotype.*

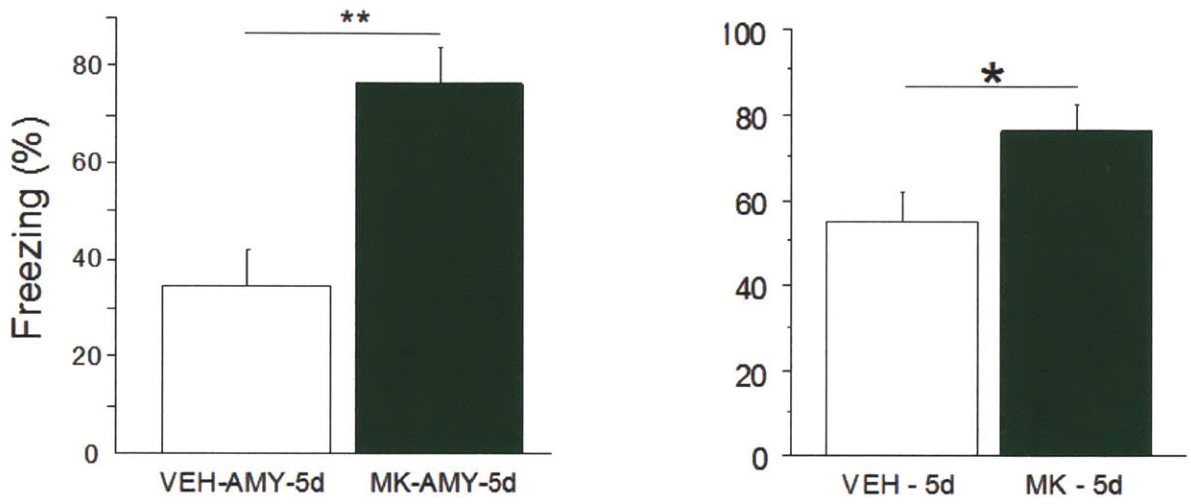
Moreover, other laboratories using immobilization stress have demonstrated enhanced fear behaviors of more active nature such as increases in defensive burying. Other measures that demonstrate chronic immobilization alters affective

behaviors include changes in exploration of the open field, increases in neophobia as measured by object exploration, expression of an anhedonia phenotype in the sucrose consumption test, and changes in social interaction that did not manifest as locomotion changes. Finally, chronic ghrelin administration or elevation of endogenous ghrelin by food deprivation has demonstrated an increase in locomotion and mobility rather than a decrease as mentioned above and as demonstrated by exogenous treatments peripherally or centrally. **Rather than displaying a phenotype that would inappropriately inflate our measure, animals undergoing these treatments display a phenotype that would challenge the very nature of our measure: increased freezing.**

What is the endogenous source of ghrelin? Are you sure that it is from the periphery?

The major source of ghrelin is the stomach, specifically the A/X-like endocrine cells in the mucosa of the glandular fundus. Radio-labeled ghrelin studies have demonstrated that peripheral ghrelin is transported *across the blood-brain barrier* by a saturable transporter. Ghrelin-O-Acyl-Transferase (GOAT) is required to activate the ghrelin peptide and the acylated form of ghrelin is the only form able to activate the primary ghrelin receptor (GHSR1a). While a few cells within the hypothalamus and pituitary express GOAT mRNA, the cells are non-projecting, suggesting an autocrine mechanism (Gahete et al, 2010). The predominance of GOAT containing cells in the body lie in the stomach. Another reason that I am

confident that the peripheral ghrelin signaling is the major source of the effect is that I observed a similar sized effect on fear learning whether the ghrelin receptor agonist was applied systemically or peripherally. Furthermore, exogenous ghrelin administered centrally demonstrated an equal effect to both stress and the agonist.



In conclusion, I am confident that the stress-induced increase in endogenous ghrelin mediates fear enhancing effects at the ghrelin receptors of the amygdala, at least in part, and that the major source of the circulating ghrelin is the stomach.

Would the popular roux-en-y gastric bypass surgery alter PTSD rates?

The roux-en-y gastric bypass surgery entails removing a portion of the patient's stomach and then closing the resection in order to reduce the volume of the stomach and help promote weight-loss. In the process, a portion of the glandular fundus is traditionally removed. It has been found recently that this portion

contains the ghrelin producing cells and that the patients no longer produce the large majority of their circulating ghrelin. Interestingly, roux-en-y patients do much better than gastric sleeve patients in terms of weight-loss and this is hypothesized to be due to the huge reductions in ghrelin production. **Because these patients do not produce ghrelin and because this chapter has shown that ghrelin plays an integral role in stress-induced disorders, it would be interesting to measure the rate of stress disorders in roux-en-y patients; however, this has not yet been examined.** What has been done are single nucleotide polymorphism (SNP) studies which have demonstrated three major SNPs on the preproghrelin gene in the human population. One SNP, rs4684677, a missense mutation located in exon 4 that is responsible for an amino acid exchange from Leu to Gln, has been positively correlated with Panic Disorder. This suggests that ghrelin changes play a role in anxiety disorders in humans as well as animals.

What is the composition of the rat chow? How did you control food access?

Rats were fed, *ad libitum*, a high protein (25%): carbohydrate (52%) diet (Prolab RMH 3000, standard rat chow, Purina, LabDiet, Richmond, Indiana, USA) 20h/day. This diet is especially designed for growth of lab rats. It contains all the nutrients required by the animal and uses high quality animal protein to provide optimal balance of amino acids. This diet has a metabolizable energy content of 3.20 kcal/g. Rats will eat up to 30 grams per day. Cage feeders contained a minimum of 200g of chow each day of the experiment. Food access was restricted 4h/day for both the non-stressed and stressed rats during the stress session.

Does immobilization stress administration alter the circadian rhythm?

Work from the lab and within the literature demonstrate that immobilization stress does not alter the circadian rhythm on a variety of measures. Here animals were administered immobilization stress 4h/day for 10 days. Home cage locomotion was measured 18h/day outside the stress session. Stress treatment did not alter the rhythm of this measure. Marti et al (1993) demonstrated that the chronic immobilization stress, while altering the levels of each, did not alter the peaks or nadirs of corticosterone, growth hormone, or food consumption. Timing of sampling within this thesis coincides with the endogenous nadir of ghrelin.

Materials and Methods

Subjects. All experiments used adult male Long Evans rats (250–350 g, Taconic, Germantown, NY), housed individually (68-72°F; 12-h light-dark cycle, 7AM lights on). Food and water (or 0.9% saline for adrenalectomy experiments) was provided *ad libitum*. Stressed and unstressed animals were housed in separate cubicles. All procedures were in accordance with the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the MIT Institutional Animal Care and Use Committee and the Animal Care and Use Review Office of the USAMRMC.

Adrenalectomy. Rats that were adrenalectomized were allowed to recover 1 week before being subjected to experimentation. Briefly, bilateral adrenalectomy was carried out through lateral incisions under 3.5% isoflurane anesthesia. Sham adrenalectomy was performed by making the incision through the skin and muscle and locating the adrenal glands. Muscle incisions were closed with chromic gut sutures and skin incisions were closed with wound clips. Some adrenalectomized rats were purchased from Taconic (Germantown, NY). Gland amputation was verified by dissection after removal and further verified by plasma corticosterone analysis.

Cannulae implants. Rats were anesthetized with a cocktail of 10mg/kg acepromazine, 100mg/kg xylazine, and 100mg/kg ketamine (1ml/kg; i.p.). Rats were mounted into a dual arm stereotaxic frame (Kopf Instruments; Tujunga, CA). The

rats were then bilaterally implanted with 23-gauge stainless steel guide cannulae aimed 1mm above the lateral amygdala: A/P -2.0, M/L +/-5.3, D/V -5.4, relative to brain surface and bregma (1). The cannulae were secured by the placement of three jeweler screws in the skull and dental acrylic. Dummy cannulae extending 1mm past the tip of the guide cannulae were placed into the guide cannulae after surgery and changed every other day. Rats received 0.03mg/kg of Buprenex (1ml/kg; s.c.) as post-operative pain management every 12h for at least 24h and up to 3 days. All rats recovered for a minimum of 5 days before experimentation commenced.

Virus Preparation. Virus was packaged with the 5*dl*1.2 helper virus and 2-2 cells using standard methods (51). Virus was purified on a sucrose gradient, pelleted, and resuspended in 10% sucrose in D-PBS. Titers were ~1X10^{8}IU/ml.

Virus Infusions. Pulled glass pipettes were backfilled with silicone oil and the appropriate virus solution. The pipettes were mounted in stereotaxic barrel holder and the pipette plunger was placed against a custom-made apparatus designed to control the plunger via a syringe pump (Harvard Apparatus, Hollison, MA). Rats were anesthetized and mounted in a stereotaxic frame as described for cannulae implants. Small holes were drilled for intra-cranial placement of a pulled glass pipette aimed within the lateral amygdala: A/P -2.0, M/L +/-5.3, D/V -6.4, relative to brain surface and bregma (1). Virus was infused at 0.1ul/m for 20m (2ul total volume per side). The glass pipette remained in the brain for 10m before being

withdrawn. Incisions were closed with wound clips and Buprenex was administered as for cannulae implants.

Drug preparation. For systemic drug delivery, rats were injected with 1ml/kg (i.p.) of the appropriate solution. All drugs were solubilized in 0.9% saline (vehicle) such that injection volumes remained constant for each experiment. MK-0677 (Merck; Whitehouse, NJ) is a highly specific GHSR1a agonist that readily crosses the blood-brain barrier and has a half-life of over six hours (52, 53). A dose of 0.5 mg/ml, diluted in vehicle, was selected because it is well-tolerated and results in significant and prolonged increases in growth hormone release (53). D-Lys^3-GHRP-6 (Tocris Biosciences; Minneapolis, MN) was diluted to 2.74ug/ml in vehicle. D-Lys3-GHRP-6 is a selective and potent inhibitor of GHSR 1a (54, 55) with an IC_{50} of 0.9 μM (56) (Tocris Bioscience literature). It also crosses the blood brain barrier (57). The only other known receptor class with affinity for D-Lys3-GHRP-6 is the melanocortin receptors but the K_i = 26-120 μM so the dilute dose used here would not be expected to affect these receptors. D-Lys^3-GHRP-6 was injected within 30m of the start of immobilization stress or following handling.

For experiments using intra-BLA drug delivery, drugs were solubilized in physiological artificial cerebrospinal fluid (vehicle; pH=7.35). MK-0677 was solubilized to 0.5ug/μl. For bioactive ghrelin, a dose of 5 nmol/μl, diluted in vehicle was selected as it was previously shown to have behavioral effects following a single infusion into the amygdala(18).

Drug Infusion. For intra-cranial infusions, rats were placed in 5-gallon buckets containing bedding. The dummy cannulae were removed and injectors (30G stainless steel cannulae; extending 1mm beyond the cannulae end) were inserted. The injectors were attached to Hamilton syringes (10ul; Hamilton Co., Reno, NV) via polyethylene tubing, and the syringes were mounted in a Harvard syringe pump (Harvard Apparatus; Holliston, MA). Infusions were given at a rate of 0.1ul/m for 5m for a total volume of 0.5ul/side, with 1m for diffusion, before the injectors were removed and new dummy cannulae were inserted.

Immobilization Stress. Immobilization stress was administered 4h per day for 1-14 consecutive days, depending on the experiment. Animals were placed in Decapicone plastic bags (Braintree Scientific; Braintree, MA), which were secured at the tail. Stress occurred in a lab room used for no other procedures. All stress sessions were performed between 10AM and 4PM. Non-stressed control rats were handled daily for 30s. For further parallel control parameters, food was removed from the unstressed rats daily for the same 4h period in which stressed animals were immobilized and, therefore, without food.

Water Stress. Water stress was administered 1h per day for 14 consecutive days. Animals were placed in cages with room temperature water 1.5 to 2 inches deep. All sessions were performed between 12pm and 2pm. Unstressed control rats were handled daily for 30s. For further parallel control parameters, food was removed from the unstressed rats daily for the same 1h period as above.

Pavlovian Fear Conditioning. Fear conditioning experiments were conducted in a modified chamber (MED Associates; St. Albans, VT) housed in a sound-attenuated cubicle. The animals were placed in individual chambers and infrared video of each session was recorded. Each experiment used auditory fear conditioning wherein rats received 3-5 tone (2kHz, 85dB, 10-16s)-footshock (1-2s, 0.4-0.7mA) pairings in a unique context (metal shock grid floors, chamber fan on, 0.3% PineSol odor, house and room lights on). Animals were allowed 2-3m to habituate to the chamber before tone-footshock pairings were given at intervals of 1-4m. Fear memory was tested 24-72h later by placing the animals in a novel context (white Plexiglas plastic floors, curved Plexiglas wall inserts, fans off, 1% acetic acid odor, and house and room lights off). One to 5m after placement in the novel context, fear to the tone was assessed either by presenting a continuous tone (2 kHz, 85dB, 8 minutes) or several discrete tones (15 10-16s tones with 1-4m ISI). Freezing was measured using commercial software (VideoFreeze, MedAssociates, St. Albans, VT).

Elevated Plus Maze. Rats were tested for anxiety using an elevated plus maze (Hamilton Kinder; Poway, CA). The maze had two open arms (51cm x 12cm each) and was located in a moderately lit room. Open arms consisted of black Plexiglas floors and no walls. The closed arms had black Plexiglas walls 40cm high. Animals were placed on an open arm 88cm above the ground facing away from the center of the maze. Automated software (Motor Monitor 4.14) recorded the second by second movements of the animals while the experimenter made observations in an

obscured corner. Each session lasted 8m and both the observer and the software recorded the latency to exit the first arm and the number of entries into the open and closed arms. Additionally, the software recorded time spent in the each region of the maze. The maze was cleaned with 70% ethanol after each animal's session.

Trunk blood collection. Perimortem blood was collected from the trunk after decapitation in a tube which contained 1:100 v/v 0.5 M EDTA and 1:100 v/v HALT (Pierce; Rockford, IL). Immediately after collection, plasma was extracted by centrifugation (2,100g at 4°C for 10-15m). The plasma layer was then collected and half the volume treated with 10% v/v 1M HCl in order to stabilize the acylated form of ghrelin. Samples were stored at -20°C or -80°C.

Histology. Following completion of the experiment, animals were anesthetized with an overdose of isoflurane and intracardially perfused with physiological saline followed by 4% formalin fixative in saline. Brains were harvested and placed in 4% formalin for 24-72h. The brains were then transferred to a 30% sucrose/4% formalin solution for a minimum of 3 days. For brains infused with virus, solutions containing paraformaldehyde were used in lieu of formalin. Coronal sections (40μm) were made and mounted on gelatinized slides. Tissue that did not contain virus was stained with 0.1% cresyl violet. Slides were then assessed for cannulae position or GFP florescence. Animals with incorrect placements were excluded from all analyses.

Hormone Assays. Corticosterone, acylated ghrelin, growth hormone and CRF levels were determined using commercial ELISA kits. For corticosterone, non-acidified plasma was diluted 1:25 in assay buffer 15 (Enzo Life Sciences; Farmingdale, NY). For acylated ghrelin, the acidified sample was used for the active ghrelin ELISA (Millipore; Billerica, MA) and processed according to the manufacturer's protocol. Samples were excluded from analysis if they displayed signs of hemolysis or lipemia. For growth hormone, brain tissue was homogenized 1:6 in lysis buffer and was assayed as per manufacturer's protocol (Millipore; Billerica, MA). For CRF, brain tissue collected by micro-dissection was homogenized 1:30 w/v in lysis buffer and assayed per manufacturer's protocol (Kamiya Biomedical Company, Seattle, WA).

Statistics. For each fear memory session, conditional freezing was assessed as a percentage of time spent freezing, a probability estimate that is amenable to analysis with parametric statistics. These probability estimates of freezing, along with other measures, were analyzed using ANOVA. Post hoc comparisons in the form of Fisher's PLSD tests were performed after a significant omnibus F-ratio ($p<0.05$). Statistical trends are noted in the text when omnibus F-ratio did not reach $p<0.05$ but were $p<0.10$. All data where $p>0.10$ are identified as not significant (ns).

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Chapter 3

Cumulative stress exposure dynamically regulates the physiological and hormonal signature of stress

Abstract

Stress is the body's response to perceived challenge or threat. While several studies have started to parse the differential effects following acute and chronic stress, little is known about the shift from an acute to chronic stress state. In the present study, we evaluated the timing of this shift on behavioral, physiological, and hormonal measures in adult rats using repeatedly administered immobilization stress. We demonstrate divergence in responses to early stress sessions (acute stress) and later stress sessions (chronic stress). Additionally, shifts in acute and chronic stress food consumption and body weight responses are maintained in the absence of the peripheral glucocorticoid and catecholamine systems suggesting other hormones to be involved. Surprisingly, the timing of the shift on each measure is concentrated before a narrow time window. The acute stress response was characterized by a shift in metabolism, an increase in stress-induced corticosterone, and a near-complete suppression of growth hormone. These measures all peaked early in stress exposure, and showed habituation over days, though the rate of habituation was different for each measure. The chronic stress response was characterized by a gradual increase in ghrelin and decrease in leptin which persisted throughout the entirety of stress exposure. Collectively, these data show that physiological and hormonal stress responses are temporally coordinated and depend on cumulative exposure to stress in an animal model of psychological stress.

Introduction

When a cue or event threatens the well-being of an organism, physiological and behavioral responses are engaged to promote coping and adaptation (3, 58). These stress responses are induced by physical, immunological, environmental, emotional, and even psychological challenge (59). The stress system allows an organism to adapt to the demands of the stressor by undergoing allostasis. The concept of allostasis was first introduced to describe how the cardiovascular system adjusts to changing body states in order to maintain blood pressure and means "maintaining stability through change" (60). More generally, allostasis is the process of achieving functional stability through physiological or behavioral change. It has been expanded to apply to the processes that occur during stress. In this context, it describes the physiological responses to the demands of the stressor, such as the secretion of cortisol or catecholamines from the adrenal glands. Despite the utility of the stress responses in the short-term, repeated or prolonged activation of the stress response can lead to maladaptive changes (61, 62). To include this, the concept of allostasis was further adapted into the theory of "allostatic load". Allostatic load refers to the "wear and tear" that the body experiences due to prolonged or repeated cycles of allostasis with frequent recruitment of the neural and endocrine stress systems (63). Allostatic load is proposed as the measure of the cumulative biological burden exacted on the body through attempts to adapt and has been correlated with increased risk of several stress-related disorders, such as metabolic syndrome and psychiatric illness (4, 62, 64-73).

Despite the well-documented problems correlated with increasing allostatic load, little is known about the differences in systemic stress responses following short- or long-term exposure to stress due to the static nature of the report of these measurements (9, 74). In the stress literature, a few differences have been described. For example, within the cardiovascular system, acute stress increases blood pressure, aiding muscles by providing oxygen and energy needed for optimal function. However, chronic recruitment of this adaptive mechanism leads to changes that cause susceptibility to disease states such as atherosclerosis or cardiovascular disease (75, 76). The digestive system also reveals the dichotomy of acute and chronic stress. Acute stress stops digestion, moving those resources to more urgently needed systems. Chronic activation of this system leads to increased net acid load buildup and subsequent increased acid delivery which, in turn, leads to ulcers of the fundic mucosa of the stomach and proximal duodenum (77-79). Interestingly, in animal models of ulcer-inducing, psychological stress, a similar pattern is seen though gastric motility will return to normal levels after about 5 bouts of repeated restraint stress (80).

The brain also responds differently to acute and chronic stress. For example, we have recently shown that immobilization stress repeated for 4 or fewer days impairs amygdala-dependent fear learning. However, if the stress repeats for 5 to 14 sessions, fear learning is increased (Meyer et al. 2013). Many other brain regions, such as the prefrontal cortex and the hippocampus, show similar dichotomies between acute and chronic stress but traditionally in the opposite

direction: acute stress increasing efficiency and chronic stress impairing function (28, 43, 81). Finally, weight loss or growth suppression is a consequence of most forms of chronic or repeated stress (82, 83). Even with this evidence for detrimental effects of increasing stress-induced allostatic load, few studies have looked for the point at which the accumulation becomes detrimental.

In this paper, we aim to identify how and when stress responses diverge from early to late stress exposures. We analyze behavioral, physiological, and hormonal data across time as animals undergo a repeated, chronic stress protocol. We show that responses to early stress sessions exhibit an acute stress phenotype, but these responses evolve over time to a new phase the chronic stress state. We then correlate the behavioral changes with the hormonal changes to examine which known stress hormones show similar time courses or dynamic patterns. Surprisingly, the shift in responses on each of our measures occurs in a narrow window. We term this shift in responses the "pivot point". Finally, we demonstrate that these physiological dynamics are maintained in the absence of the glucocorticoid and catecholamine systems, suggesting other systems are coordinating this signature chronic stress response.

Results

It has been suggested that allostatic load increases with cumulative exposure to stress (74, 84). However, few studies have systematically compared the development of acute versus chronic stress effects as stressor exposure repeats. Here we acquired within-animal measures of behavioral, physiological, and hormonal responses throughout repeated stress sessions. Rats underwent immobilization stress for four hours a day for 14 days (STR groups) or received 30s of gentle handling daily for 14 days (no stress, or NS, groups). Daily measurements of body weight, food consumption, and feeding efficiency were collected throughout stress exposure.

Stress caused an initial weight loss that reversed during later stress sessions

Across the stress exposure, weight changes were not constant. Instead, weight loss reached a plateau and actually reversed. These changes in directionality of ponderal growth were statistically significant, but not obvious when animal weight was observed at single points in time rather than across the treatment period. Stressed animals showed the weight loss phenotype typical of animals undergoing either acute or chronic stress (Fig. 1a and 1b, treatment group: $F(1,35)=49.70$, $F(1,35)= 51.31$, grams and percent body weight, respectively, $P < 0.001$) (85, 86). When the direction and magnitude of the daily weight change was plotted (Fig. 1c) a remarkable pattern emerged: the significant failure to thrive did not endure across repeated stress sessions (Fig. 1c, treatment group x time interaction: $F(12, 420)=4.58$, $P< 0.0001$). Interestingly, stress-induced weight loss

reached a plateau after the fourth immobilization session and no further significant weight loss was seen (Fig. 1c, Wilcoxon signed-rank test, stress group only: *Day 4: Z= -0.06, Day 5: Z= -0.471, Day 6: Z= -0.065, Day 7: Z= -1.475, P= n.s.*, no change). The weight loss then reversed after the eighth immobilization session when the stressed animals began to grow (*Z=-2.451, P* < 0.05). By the twelfth session, stressed animals put on weight at the same rate as non-stressed animals (Fig. 1c, stress group only: *Z*=-3.85, P < 0.0001).

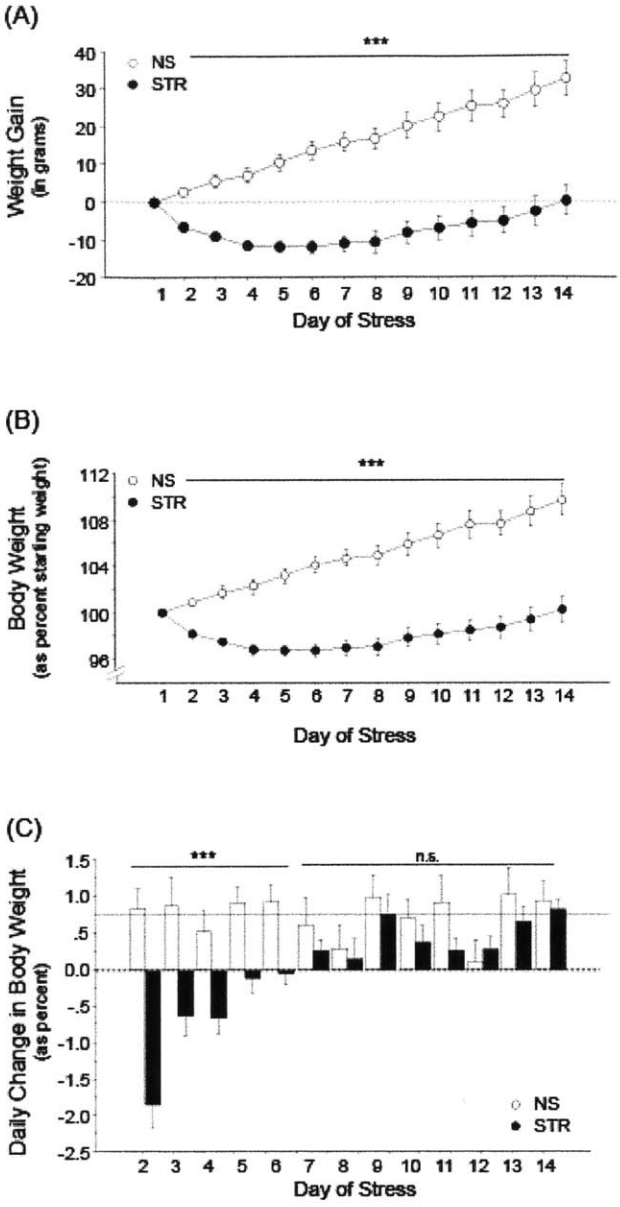
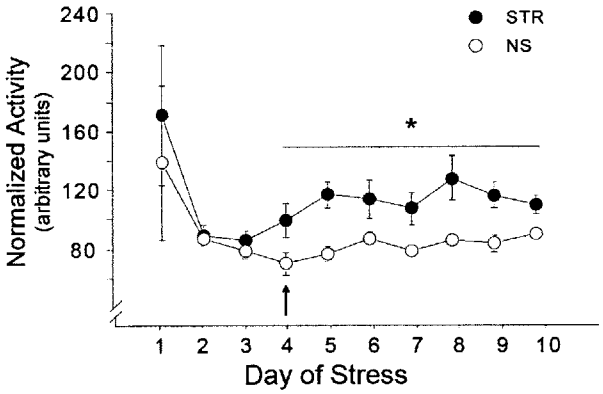


Figure 1. Effect of chronic administration of immobilization stress on body weight of adult male Long Evans rats. Daily administration of immobilization stress (4h/d for 14 days) (●, filled circles) caused a significant failure to thrive compared to non-stressed control animals (○, open circles). (A) Daily weight gain or loss in grams. (B) Daily changes in body weight expressed as a percentage of the starting weight. (C) Direction and magnitude of daily change in body weight. Weight loss continued as the stress sessions continued until stress day 4, when a plateau in weight loss was seen (Wilcoxon signed-rank test). The plateau ended and weight gain began at stress day 8 (Wilcoxon signed-rank test). Non-stressed animals grew at a rate of 0.74% of their starting weight daily, indicated by the solid line. Values represent mean±s.e.m., *** $P \leq 0.0001$ compared to non-stressed controls.

Weight changes are seen when metabolic demand and caloric intake are not in equilibrium. Therefore, stress-induced weight loss could be due to either increased motor output or decreased food consumption. To monitor activity outside the stress session, animals were implanted with telemetric implants. There were no differences between the stress and un-stressed animals at first but stress significantly *increased* motor activity after four stress sessions (Supp. Fig. 1, treatment group: $F(1, 9)=10.193$, $P < 0.05$) and beyond (planned comparisons, $P < 0.05$). Therefore, the initial weight-loss was not due to increased activity and subsequent recovery of growth was not due to a compensatory decrease in gross motor activity. Rather, the changes were in the opposite directions expected from the growth curve. These data indicate that the stress-induced alterations in growth cannot be explained by congruent changes in activity.



Supplementary Figure 1. Effect of chronic immobilization stress on gross motor activity in the home-cage. Post-stress gross motor activity was measured daily in the home cage for 6h following handling or immobilization stress (NS, n=4; STR, n=5). This coincided with the first 6h of the dark cycle. The arrow indicates the first day on which differences in activity were observed between the STR and NS groups. Values represent mean±s.e.m., * $P \leq 0.05$.

Stress caused an initial anorexia and negative feeding efficiency that reversed during later stress sessions

Changes in appetite are observed in stress-sensitive disorders such as major depression (2) and are important for animal models of such disorders (87). Additionally, because alterations in motor output cannot account for weight changes, we hypothesized the changes related to stress-induced alterations in food consumption. We measured food consumed by the animals over each 24h period during which immobilization stress was administered. We observed that stressed animals showed the hypophagia or anorexia typical of animals undergoing either acute or chronic stress (88-91). Typically, this is reported as the average amount of food consumed throughout the experiment and is seen as a significant decrease when compared to non-stressed controls (92-95). Chronic immobilization stress clearly led to a significant decrease in food consumption (Fig. 2a, treatment group: $F(1, 35)=39.50$, $P < 0.0001$). Since a uniform reduction in food consumption could not account for the dynamics of the changes seen in body weight, we explored how food consumption changed over cumulative stress exposure.

Cumulative food consumption is a metric that does account for time (92-94, 96). Again, we found a significant decrease in food consumption that persisted throughout later stress exposures (Fig.2b, treatment group: $F(1,35)=4.88$, $P < 0.05$; treatment group X time interaction: $F(13, 455)=3.21$, $P < 0.0001$). However, this metric also failed to capture the dynamics in ponderal growth. Thus we determined the overall effect could be biased by substantial differences at a single, early time point when the largest differences occur.

To further increase sensitivity and accuracy to changes in food consumption, we normalized the food consumption to the daily body weights. The weight of each group of animals was the same before the administration of stress (345±30 versus 355±25, NS versus STR, respectively, P = *n.s.*). However, weight significantly diverged after the first stress session (see Fig. 1). To normalize food consumption to rat size, grams of food consumed over the previous 24h period was divided by the morning's body weight. With this measure, we finally captured the dynamic pattern in food consumption as the stress sessions repeated. Stress produced an immediate anorexia (Fig. 2c; treatment group: $F(1,35)=16.43$, $P < 0.001$; treatment group X time interaction: $F(35,455)=9.02$, $P < 0.0001$). This severe reduction in food consumption coincided with the severe drop in body weight seen in Figure 1. The hypophagia was most severe after the first stress session (planned comparisons, maximum mean difference 2.47, $P< 0.0001$) and lessened after subsequent sessions. After six stress sessions, stress-induced hypophagia reversed: food consumption increased in stressed animals to the level of the non-stressed controls (planned comparisons, $P < 0.05$). Strikingly, this was the same time-point at which stress-induced weight loss began to reverse.

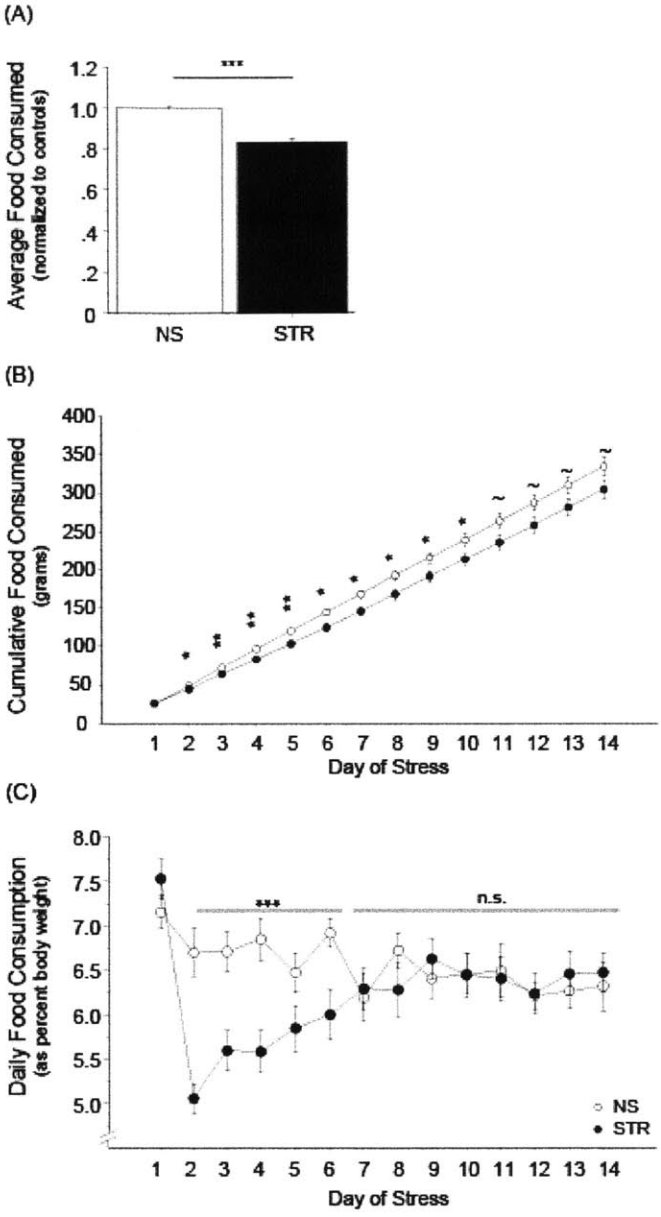


Figure 2. Effect of chronic administration of immobilization stress on food consumption behavior in adult male Long Evans rats. Daily administration of immobilization stress (4h/d for 14 days) (•, filled bars or circles) caused a significant stress-induced hypophagia compared to non-stressed control animals (○, open bars or circles). (A) Average daily food consumption during 14d of daily stress sessions normalized to control levels. (B) Cumulative food consumption throughout experiment expressed in grams. (C) Daily food consumption normalized to body weight. This is food consumed expressed as a percentage of body weight (24h food intake (g)/body weight (g)). Stress-induced hypophagia continued until stress day 6, when the stressed animals consumed chow at a similar rate as the non-stressed controls. Values represent mean±s.e.m., ~ $P \leq 0.10$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.0001$.

The data above indicate that metabolic changes occurred over the course of stress exposure. To investigate the nature of these changes further, we investigated "feeding efficiency" over time. Feeding efficiency is the ratio of weight gained to food consumed (94, 96). This benchmark is informative because it takes into account the metabolic fitness or state of the animal on a triadic axis, accounting for calories consumed, calories expended, and weight change in a single value. Here, a similar pattern was discovered: stressed rats were much less efficient than non-stressed controls from sessions 1 through 6 (Fig. 3a, treatment group: $F(1,35)=24.47$, $P < 0.0001$, treatment group X time interaction: $F(12, 420)=3.56$, $P < 0.0001$). After the sixth stress session, they regained efficiency and returned to control levels (planned comparisons, $P=$ *n.s.*).

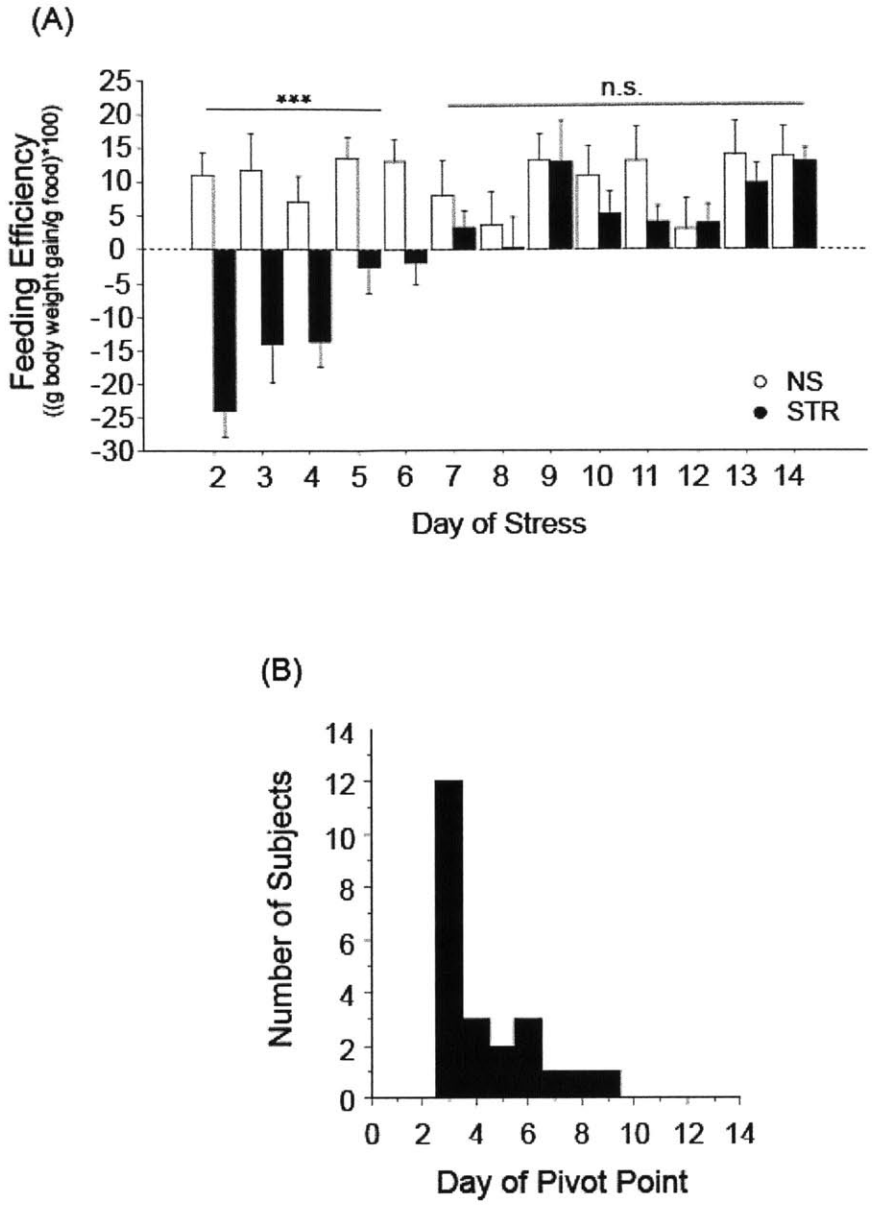
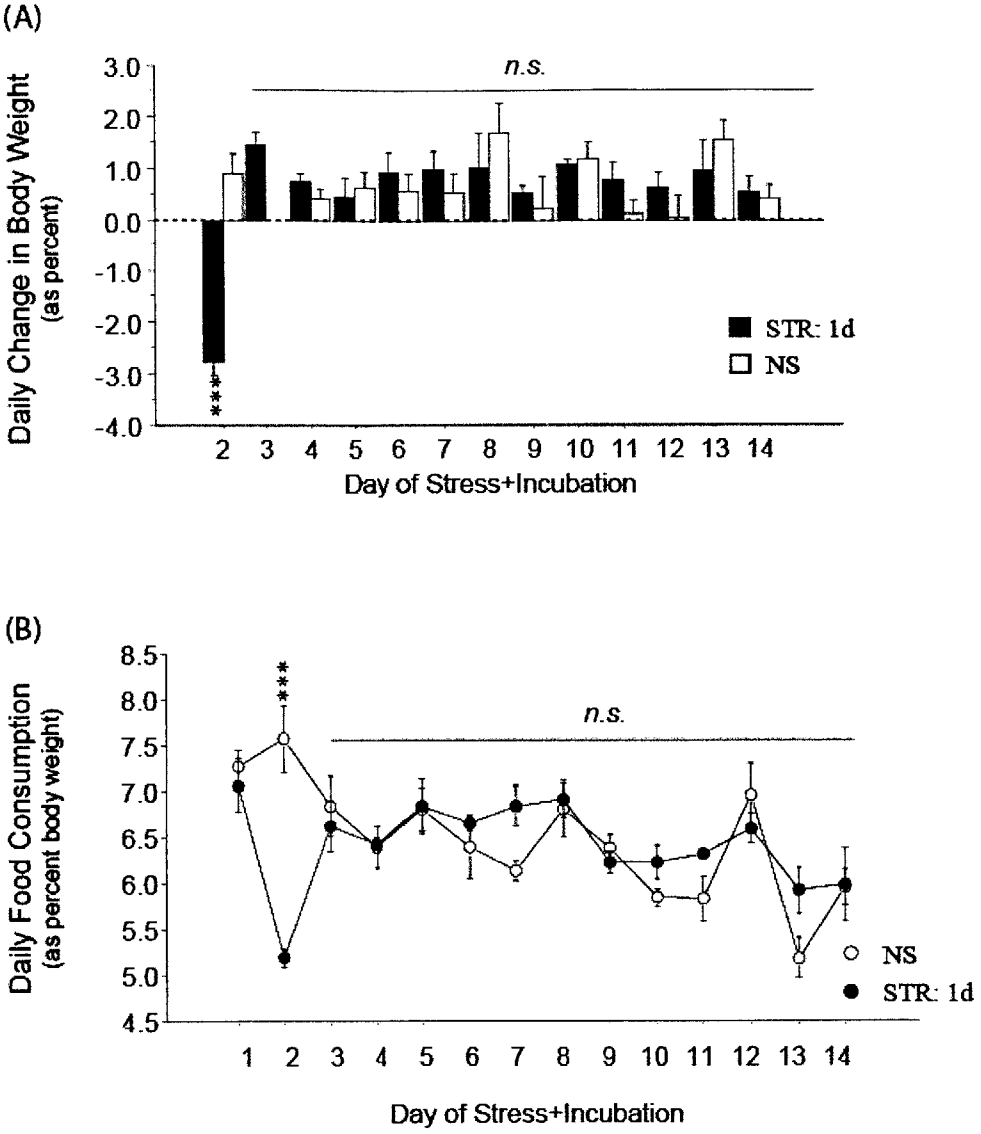


Figure 3. Effect of chronic administration of immobilization stress on feeding efficiency in adult male Long Evans rats. Daily administration of immobilization stress (4h/d for 14 days) (●, filled bars) caused dynamic changes in feeding efficiency compared to non-stressed control animals (○, open bars). (A) Daily feeding efficiency ((grams of weight gain/grams of food consumed)*100). (B) Histogram of population hypophagia reversal point or "pivot point". This is the day stress induced hypophagia reverses and animals began to consume more. The median reversal day is stress day 3. The population average is stress day 4. By day 9, all the animals have engaged in this reversal. Values represent mean±s.e.m., *** $P \leq 0.0001$.

Researchers have found that a single stress session can have delayed effects (46). To determine whether the first stress session could account for the above effects or if the effects were dependent on cumulative exposure to stress, we performed an experiment wherein animals underwent a single 4h stress session and were then monitored for 14d following the session. Changes in body weight (Supp. Fig. 2a) and food consumption (Supp. Fig. 2b) did not recapitulate the patterns seen during repeated stress sessions (Figs. 1 and 2). After a single day of stress-induced weight-loss, stressed animals returned to normal growth rates (Supp. Fig. 2a, treatment group: $F(1,13)=0.97$, $P = n.s.$). Similarly, the single stress session resulted in severe hypophagia for 1d but a return to normal feeding levels the next day (Supp. Fig. 2b, treatment group: $F(1,13)=0.09$, $P = n.s.$). These data demonstrate that the patterns of stress induced changes in caloric intake and ponderal growth are not due to the effects of the first stress session and suggest that cumulative exposure to several sessions of stress is responsible for the changes.



Supplementary Figure 2. Effect of administration of a single immobilization stress session on body weight and food consumption in adult male Long Evans rats. A single immobilization stress session (4h) was administered on day 1. Daily measures of body weight and food consumption were monitored for 14d after the single stress session. (A) Daily changes in body weight (magnitude and direction). (B) Daily food consumption normalized to daily body weight (expressed as a percentage of body weight). Values represent mean±s.e.m., *** $P \leq 0.0001$.

These data show that short- and long-term stress exposures have profoundly distinct effects on physiological response to repeated stressors. For our specific stressor, the first through sixth days of stress exposure defined a window after which differential effects of short- versus long-term stress were first observed in the population. To determine how this window was shaped by individual animals, we examined food consumption in greater detail. For each stressed animal, we computed the day on which food consumption first increased above the stress-induced minimum (Fig. 3b). This analysis revealed that a reversal day occurred within a variable but narrow time window. We identified this day as the "pivot point." Analysis of the histogram of individual animals' "pivot point" revealed that the median reversal day was stress day 3, the population average was stress day 4, and by day 9, all the animals had engaged in this reversal. This demonstrates that in the population the shift from short-term stress to long-term stress states occurs within a narrow window.

Dynamic hormonal changes over repeated immobilization stress

Many hormones regulate or are regulated by stress. However, data for this area of endocrinology are fragmentary and few studies take into account differing stressor durations and cumulative exposures. Hormones are often only assayed at a single time point and often at the end of the stressor exposure. Here, we provide an integrated hormonal analysis across accumulating stress exposure. We examined five circulating hormones previously reported to be regulated by stress (97):

corticosterone (the glucocorticoid stress hormone in rodents), acylated ghrelin(15), growth hormone (98-101), leptin (90, 102, 103), and neuropeptide Y (104-106).

To explore the molecular mechanisms behind the differences in short- and long-term stress exposure further, peripheral hormone signatures were sampled throughout the stress sessions of this 14d protocol. Animals underwent daily immobilization sessions as above. Each day, a subset of animals underwent tail-bleeding during the last 15 minutes of the 4h stress session. Though daily bleeding of each individual subject would be optimal for such analyses, every third day was the maximum permitted to minimize pain activation and avoid blood-loss induced changes in the tested hormones (107-109). The horizontal line on the graph represents baseline levels taken before stress sessions began (Fig. 4a). From each blood sample, corticosterone, acylated ghrelin, growth hormone, leptin, and Neuropeptide Y were measured. The fold change from baseline is indicated (Fig. 4a) and the statistical significance of the changes are illustrated by the heat map below (Fig. 4b).

Corticosterone was regulated by stress but differentially regulated over time. Corticosterone was significantly elevated from the first stress session (○, open circles, Fig. 4a) and reached its peak during the second stress session. Corticosterone measures began to dampen during the third session, and this downward trajectory persisted until it reached baseline levels. Changes were no long statistically significant by the tenth session (Fig. 4b, first row). This corroborates the importance of corticosterone during the early stress responses.

The mirror image of corticosterone changes was seen in the dynamics of growth hormone levels. Stress significantly blunted peripheral growth hormone (■, filled squares, Fig. 4a). As with corticosterone, growth hormone reached its peak effect (in this case, its minimum levels) during the second stress session. Growth hormone levels then monotonically increased throughout the rest of the stress sessions, approaching baseline levels. Changes were no longer statistically significant by the 10th session (Fig. 4b, second row).

Unlike corticosterone and growth hormone, acylated ghrelin levels showed a gradual increase from the first stress session (●, filled circles, Fig. 4a) and continued to monotonically increase throughout the duration and repetition of the stress. Ghrelin peaked in later stress sessions, reaching its highest levels during the ninth session and remaining elevated for the remainder of the stress exposure (Fig. 4b, third row).

Leptin was blunted in the immobilization stress paradigm (□, open squares, Fig. 4a). Like ghrelin, the changes were gradual and initially slow. Leptin changes did not reach significance until during the third stress session (Fig. 4b, fourth row). Leptin continued to decrease, reaching its minimum during the sixth stress session and remaining at this level throughout the rest of the sessions. Interestingly, the prolonged, sustained decrease in leptin mirrored the sustained increase in circulating ghrelin.

Neuropeptide Y demonstrated unique dynamics within our subset of hormones. NPY was upregulated (⊠, hatched squares, Fig. 4a) during the first

stress session and was maintained through the third; however, this elevation did not reach statistical significance (Fig. 4b, fifth row). During the fourth session, NPY levels dropped below baseline. Levels continued to decrease, reaching statistically significant levels from the sixth through ninth stress sessions. Following this, NPY levels returned to normal in a subset of animals, while remaining below baseline for other animals, contributing to variability in the long-term effects of stress on NPY.

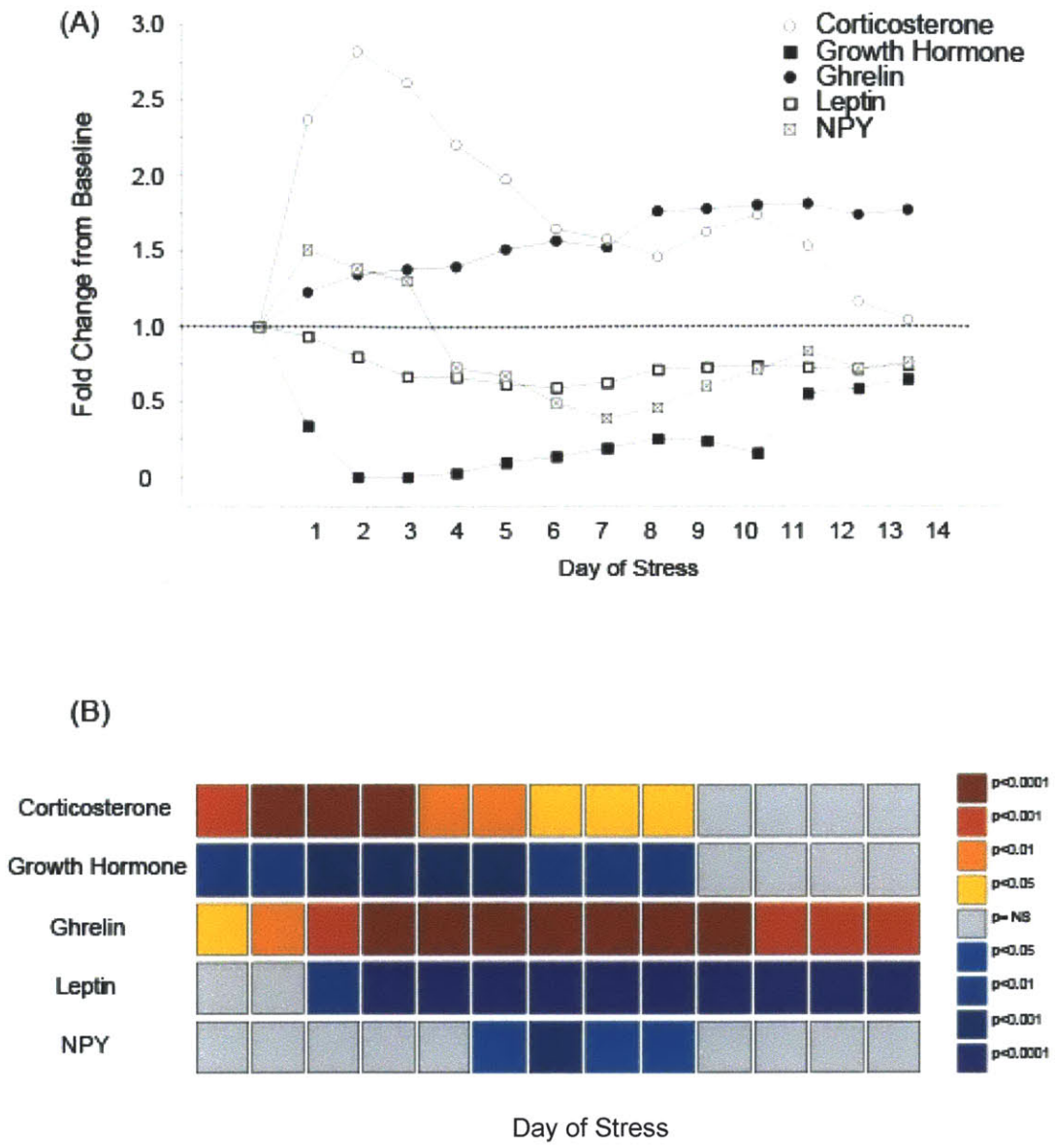


Figure 4. Hormonal dynamics throughout chronic administration of immobilization stress. Animals were administered daily immobilization stress (4h/d for 14 days). Tail blood samples were collected during the last 15 minutes of each stress session and several hormones were measured. (A) All hormones were normalized to pre-stress baseline levels. Each line represents the fold change from baseline levels of the respective hormone. Corticosterone (○), growth hormone (■), ghrelin (●), leptin (□), and NPY (⊠). (B) Statistical significance of (A).Warm colors indicate statistically significant increases, cool colors represent statistically significant decreases, and grey indicates that the changes seen are not significant.

Food consumption and body weight were measured daily for all animals in which hormones were examined. Thus, we explored relationships within and across the hormones as well as these two metabolic measures (Table 1). Corticosterone was strongly, negatively correlated with food consumption and body weight (Table 1A). NPY was strongly negatively correlated with food consumption, as predicted above. Each of the hormones examined was associated with the changes in ponderal growth, though these correlations did not reach significance after Bonferroni correction (Table 1A, column 3, Δ Body Weight, $P < 0.05$). This suggests that each of the hormones selected plays a major role in changes in body weight. Next we examined the relationships between the hormonal changes plotted in Fig. 4a (Table 1B). Growth hormone and corticosterone had the strongest correlation. As described above, stress-induced changes in these hormones are tightly anti-correlated. Ghrelin and leptin are the next most strongly correlated. Again, their dynamics are mirror images and the relationship is a statistically significant, negative correlation. These findings demonstrate that the dynamics of stress hormones are complex, change across time, and some pathways are coordinated in these changes.

A

	Food Consumption	Body Weight	Δ Body Weight
Ghrelin	0.22	0.03	0.29 †
CORT	***-0.41*** **	***-0.35*** *	-0.33 †
Leptin	-0.20 †	0.29 †	-0.28 †
NPY	***-0.36*** *	-0.06	-0.26 †
Growth Hormone	0.28†	0.28 †	0.25 †

B

	Ghrelin	Corticosterone	Leptin	NPY	GH
Ghrelin		-0.11	***-0.36*** *	0	0.27 †
CORT	-0.11		-0.09	0.23	***-0.41*** **
Leptin	***-0.36*** *	-0.09		0.16	-0.01
NPY	0	0.23	0.16		-0.03
Growth Hormone	0.27 †	***-0.41*** **	-0.01	-0.03	

Table 1. Summary of regression analysis. Linear regression analysis was performed with each hormone as the independent variable and (A) physiological measures or (B) other hormones as the dependent variables. Correlation coefficients are reported. Sign indicates direction of modulation and value indicates strength of the relationship. Bonferroni correction is applied to statistics. † $P < 0.05$, * $P \leq 0.003$, ** $P \leq 0.001$.

Adrenalectomy did not alter the dynamics of the physiological responses to repeated stress

Many studies suggest that the development of allostatic load is due to repeated activation of the HPA axis, which results in elevated adrenal stress hormone release, including glucocorticoids (8, 9, 81, 97, 110). Weight-loss is one measure of allostatic load and the above regression analysis strongly suggests the glucocorticoids are the coordinator of stress-induced weight loss. However, recently we have shown that the adrenal hormones are not necessary for certain consequences of chronic stress (Meyer et al., 2013). Furthermore, the clinical application of these findings has had little success (14, 111). To determine whether stress-induced dynamics in body weight, food consumption, and hypophagia reversal *require* adrenal stress hormones, such as corticosterone and adrenaline, we examined the impact of adrenalectomy on these measures.

Following adrenalectomy (ADX) to remove the source of endogenous glucocorticoids and adrenaline or sham surgery (SHAM), animals received immobilization stress (STR) or daily handling (NS). We found that the stress-induced direction and magnitude of daily change in body weight was not altered by adrenalectomy (Fig. 5a, stress only, surgery X time interaction: $F(13, 325)=0.28$, $P=$ *n.s.*). Dynamics in daily food consumption were not altered by adrenalectomy either (Fig. 5b, surgery: $F(1,48)=0.05$, $P=$ *n.s.*). Furthermore, neither surgery significantly altered the distribution of "pivot points" in stress-induced hypophagia (Fig. 5c, $\chi^2(13, N=12)=18.071$ and $\chi^2(13, N=15)=6.5$, for sham surgery and adrenalectomy,

respectively, *P= n.s.)*. The average pivot point for both surgical groups was stress day 5, and all the animals had engaged in this reversal by day 9. These data suggest that the adrenal hormones are not required for the dynamic changes that occur throughout cumulative stress exposure.

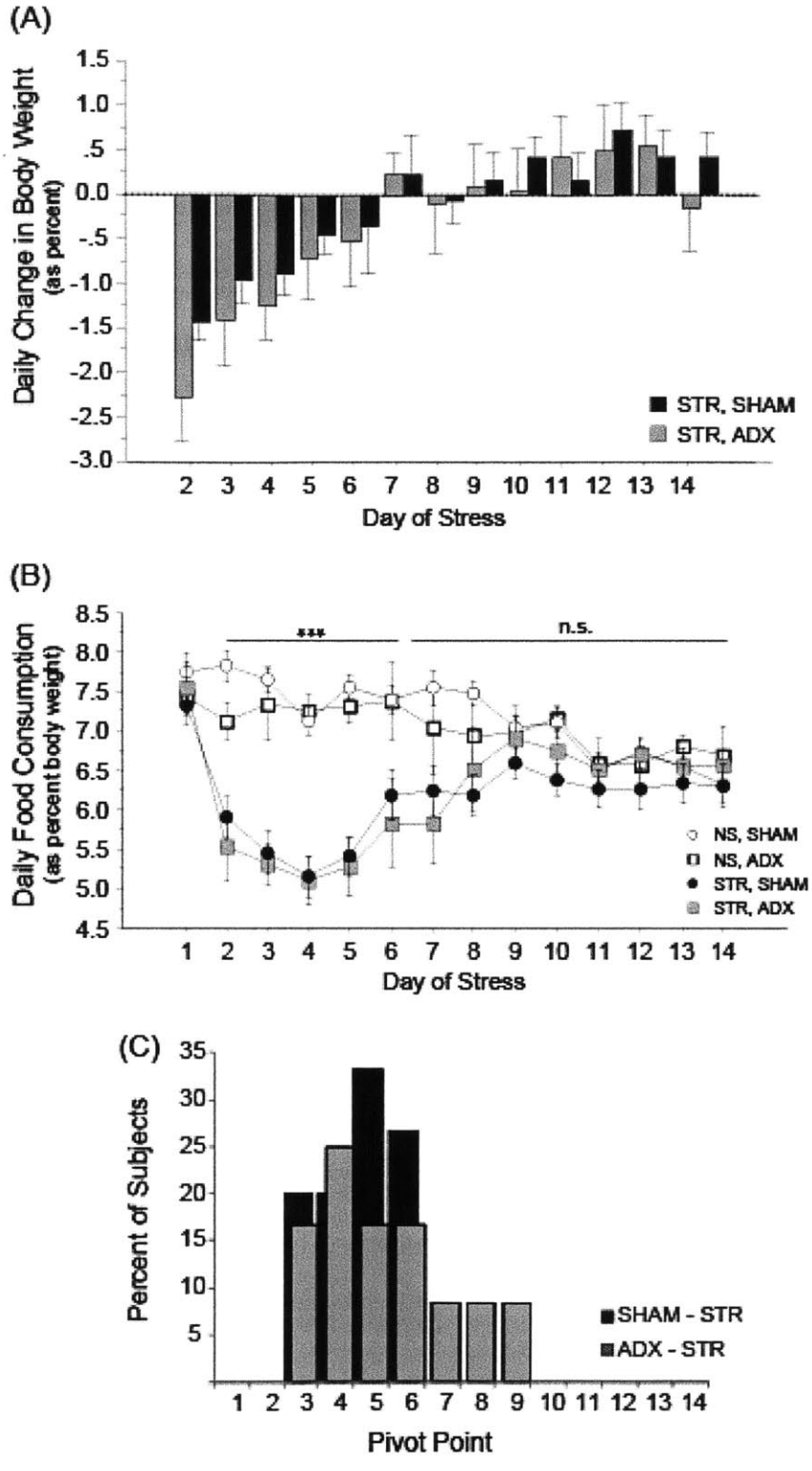

(A)
Daily Change in Body Weight (as percent)
1.5
1.0
.5
0.0
-.5
-1.0
-1.5
-2.0
-2.5
-3.0
2 3 4 5 6 7 8 9 10 11 12 13 14
Day of Stress
STR, SHAM
STR, ADX
(B)
Daily Food Consumption (as percent body weight)
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
***
n.s.
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Day of Stress
NS, SHAM
NS, ADX
STR, SHAM
STR, ADX
(C)
Percent of Subjects
35
30
25
20
15
10
5
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Pivot Point
SHAM - STR
ADX - STR

Figure 5. Effect of adrenalectomy on chronic immobilization stress-induced changes in body weight, food consumption, and the pivot point. Animals underwent adrenalectomy (ADX) or sham surgery (SHAM). After at least one week of recovery, animals were administered daily immobilization stress (4h/d for 14 days). (A) Daily stress-induced changes (magnitude and direction) in body weight were not altered by adrenalectomy. (B) Daily food consumption normalized to daily body weight (expressed as a percentage of body weight). Adrenalectomy did not alter this measure. (C) Histogram of individual animals' "pivot point" in stress-induced hypophagia: the day at which animals' stress induced hypophagia reverses and they begin to consume more. The median reversal day for SHAM animals (black bars) is stress day 5 and for ADX (grey bars) is stress day 4. The population average for both SHAM and ADX animals is stress day 5. By day 9, all the animals have engaged in this reversal. Values represent mean±s.e.m., *** $P \leq 0.0001$.

Discussion

Many studies have examined the consequences of a single stress exposure or several repeated stress exposures at static time points, taking snapshots of the effects of different stress exposures post-experience, but few have examined the evolution and onset of the changes through cumulative stress exposures. Here we observe that acute and chronic exposure to a stressor show divergent physiological and hormonal responses within the same animal and that the time course to the onset of this dichotomy is surprisingly aligned along these measures. Additionally, it is clear that the bodily responses to stress are not uniform or stable but change across time.

Here we recapitulate the findings of the existing acute and chronic stress literatures, but we extend those findings by identifying the time course of the onset of the differences between the two stages. Strikingly, this time-point is similar across all of our measures. We term this shift the "pivot point" and explore the

contributions of many hormonal pathways. For the first time, we demonstrate that the physiological dynamics and the timing of the "pivot point" are independent of the adrenal hormones. The data suggest that while the glucocorticoids and catecholamines are integral to the functioning stress system, they are not needed for the timing of the physiological changes observed here, which significantly extends the understanding of the coordinated stress response.

The concepts underlying allostasis and allostatic load suggest that measuring multiple stress mediators and consequences will allow early detection of disease vulnerability, which presumes the measures are cumulative and have a binary healthy-unhealthy score. They do not account for gradual shifts or alignment of those shifts. Additionally, the score from each measure is taken independently (74). However, our data show an interesting synchrony across growth, feeding, and hormones, suggesting an orchestration in the shift from acute to chronic phenotypes. A parsimonious explanation is that the changes seen in feeding and growth are the results of a hormonal "tug of war" and that stress recruits different systems to elicit different effects at different time points. Initially, during the early stress sessions, one set of hormones is strongest. However, these signals begin to weaken and, over repeated sessions, a second set of hormones may be coordinating the responses to further stress exposures. As shown in Figure 4, corticosterone and growth hormone are most altered during the short-term stress. After the sixth session of stress, corticosterone and growth hormone begin to return to normal. At this time, ghrelin increases and leptin deficiencies become more significant. These

measures then persist through subsequent stress sessions.

In the "tug of war" hypothesis, the initial increase of corticosterone and synchronous blunting of growth hormone dominate the bodily responses in the short-term, decreasing food consumption and interfering with growth. However, as stress endures, ghrelin increases and concomitant leptin blunting counteract the hypophagic signals to stimulate feeding and growth. Eventually, as seen in Fig. 4b, the initial signals (corticosterone and growth hormone) return to baseline and the delayed-onset signals (ghrelin and leptin) begin to dominate. The results of this competition may be reflected best in Fig. 2c: initial extreme anorexia gradually returns to baseline in spite of continued stress sessions, and this timing (acute <5d, chronic after 6d) closely follows the changes in the hormone pathways discussed here. In this model, each hormone may serve a different function at each stage of stress and the balance across multiple systems gives rise to the differing phenotypes.

Perhaps the most interesting data presented here are those collected from stressed animals that underwent adrenalectomy. These findings reveal that the glucocorticoids and catecholamines are not coordinating the feeding behavior or ponderal growth. Neither the direction, magnitude, nor timing of our measures was altered. The preponderance of stress endocrinology suggests that the HPA axis drives the effects of stress and that the changes after repeated stress exposure are the results of adaptation to the repeated hits by the adrenal hormones (112). However, recently we have shown that the adrenal hormones are not necessary for

certain consequences of chronic stress and that stress-induced changes in ghrelin are sufficient, necessary, and independent of the HPA axis (Meyer et al., 2013). The data here show that timing of stress-induced changes in feeding and growth are not reliant on the HPA axis and further support the idea that alternative pathways are involved in the symphony of stress. In lieu of the HPA axis, the measures observed here may depend on the balance between other hormonal systems, may be completely dominated by a heretofore unspecified hormonal pathway, or are dependent on coordination from the autonomic nervous system. Further work to identify the source of the dynamics is necessary.

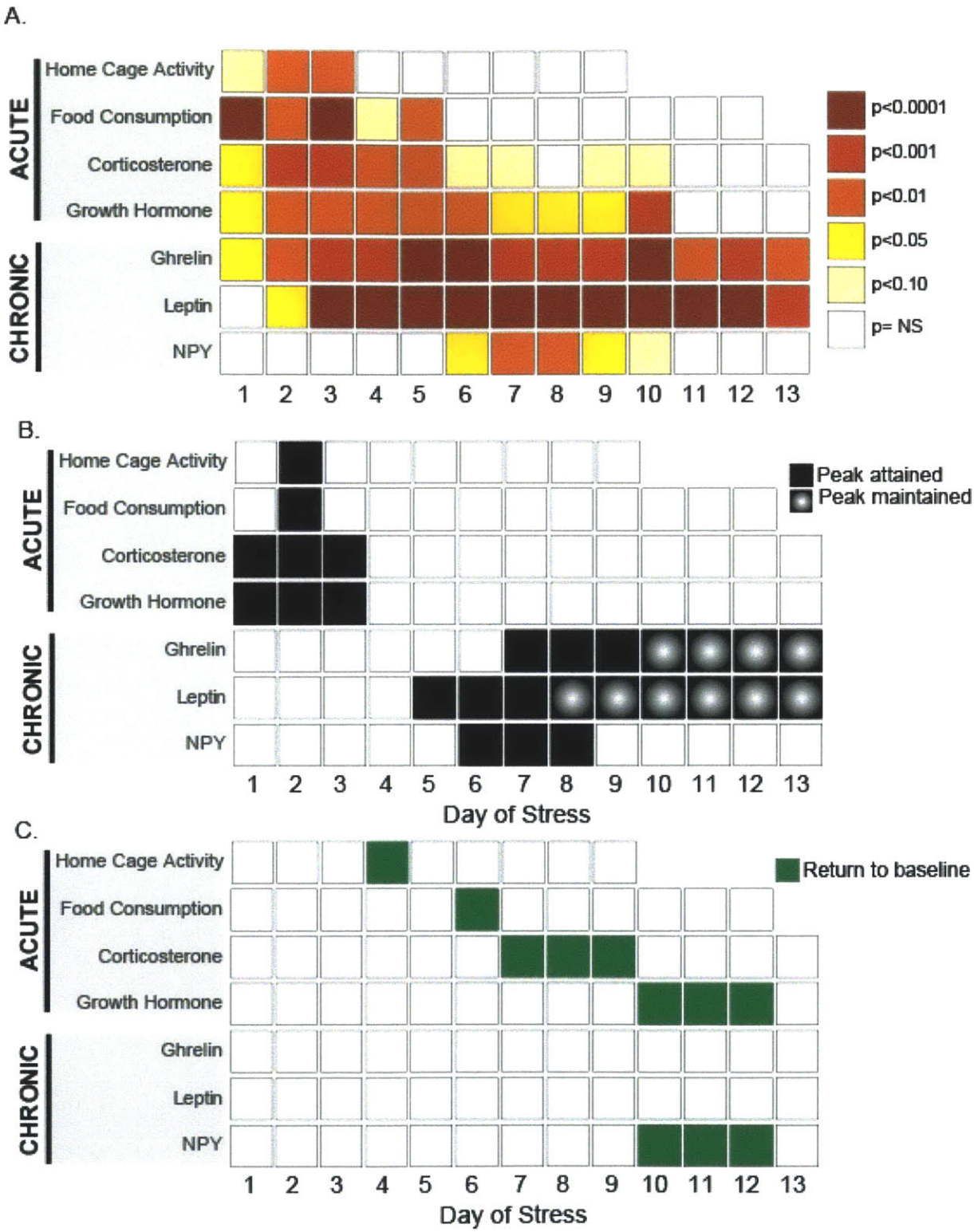
Additional studies will translate these findings and relate them to the extensive study of allostatic "load" in human medical literature. In the United States, there is an increasing incidence of obesity as well as mood disorders and, because each of these disorders has a stress component, physicians use markers of allostatic load to predict vulnerability to development of disease states (4, 9, 69-71, 74, 113-116). However, in practice, attention is largely placed on values reaching clinically significant levels, and many of the hormones discussed here are neglected. By integrating additional biomarkers, better prediction of disease states can be achieved.

Supplementary Discussion

Supporting the idea that different allostatic mechanisms may be engaged over the course of stress exposure, studies have reported different stress-induced changes on a broad range of measures following a single exposure versus multiple exposures to a stressor. For example, in the periphery, chronic, but not acute, stress leads to glucocorticoid resistance in regulators of the immune system (Rohleder, 2012). Chronic, but not acute, stress also leads to increased activation of the hypothalamus-pituitary-adrenal (HPA) stress axis to novel stressors, but not familiar, predictable stressors (Mizoguchi et al., 2001; Nyuyki et al., 2012). Within the central nervous system, dendritic arborization within stress-sensitive brain regions is altered by chronic, but not acute, stress (Mitra et al., 2005). Finally, chronic and acute stress can lead to different changes in emotional behaviors, including depressive behaviors (Suvrathan et al., 2010) and associative fear learning (Meyer et al, 2013).

The acute and chronic stress responses are temporally distinct

To examine the timing of each stress response in greater detail, we examined the day on which the peak change on each measure above was observed and when each certain measures returned to baseline (Supplementary Figure 3). Responses can be clearly distinguished as part of the "acute" stress response (showing immediate change on the first day of stress and a habituation of this response), or "chronic" stress response (showing a later stress-induced change, and not



Supplementary Figure 3. Dichotomy of acute and chronic stress signatures. Daily administration of immobilization stress (4h/d for 14 days). (A) Statistical significance of stress-induced changes. (B) Timing of peak stress-induced changes. (C) Timing of return to baseline levels. Note, ghrelin and leptin do not return to baseline. For significance, see legend.

habituating with repeated stress exposure). Notably, all aspects of the acute stress response measured here (depression in home cage activity, hypophagia, increased corticosterone, and decreased growth hormone) peaked on the second day of stress and habituated as stress was repeated. In contrast, changes that comprised the chronic stress response showed a slower onset and two of the three measures did not show habituation over time.

The acute stress response was characterized by a shift in metabolism, an increase in stress-induced corticosterone, and a near-complete suppression of growth hormone. These measures all peaked early in stress exposure, and showed habituation over days, though the rate of habituation was different for each measure. The chronic stress response was characterized by a gradual increase in ghrelin and decrease in leptin which persisted throughout the entirety of stress exposure.

Our findings reveal a clear dichotomy in the acute and chronic stress response. The acute stress response was characterized by changes on most measures, observable after a single stress session, but peaking at the second stress exposure. These changes include elevated corticosterone (De Kloet et al., 1998; Herman et al,, 2003), near complete suppression of circulating growth hormone (Kant et al., 1983, Savendahl, 2012), decreased home cage activity, and decreased body weight, food consumption, and caloric efficiency (Tamashirto et al., 2007, Flak et al., 2011). It was perhaps surprising that all of these acute stress responses showed habituation over the course of stress exposure, including those measures

that were unaffected by HPA "end point" activity (Fig. 4). As future studies examine day-to-day stress-induced changes in other measures, it will be interesting to determine whether habituation (Grissom and Bhatnagar, 2009) is a general property of the acute stress response.

The chronic stress response was distinguished by gradual increases in ghrelin, and decreases in leptin, a hormone profile observed in humans who are stressed by sleep deprivation (Knutson and Van Cauter, 2008). In rodents, stress-induced changes in ghrelin and leptin persist well beyond the last day of stress exposure (Kumar et al., 2013). Collectively, these findings suggest that levels of leptin and ghrelin may be useful peripheral biomarkers of a chronic stress state

It is important to note that the exact time course of stress-induced changes in all possible measures is expected to vary with the duration of each exposure. For example, brief, but repeated, stressors may lead to a slower process of response habituation than what was reported here (Ma and Lightman, 1998; Garcia et al., 2000). In addition, the stress hormones that are recruited as part of the stress response may also vary with the nature of a stressor (for example, physical versus psychological stressors; Kuo et al., 2007; Kavushansky et al., 2009). Thus, the composition and timing of the acute and chronic stress responses reported here should not be expected to apply to all stressors, but should be expected to differ between these two distinct phases of the stress response, regardless of the stressor used.

The observation that adrenalectomy has no impact on stress-induced changes in body weight or food consumption may appear surprising, given the strong relationship observed between daily changes in corticosterone and daily changes in these measures (Table 1). However, an alternative possibility is that HPA activity and hypophagia are induced by a shared mechanism. Indeed, other studies have suggested that stress activates brain-based satiety signals to drive hypophagia (Calvez et al 2011, Maniscalco et al 2013) and that neurons in the paraventricular nucleus (PVN) of the hypothalamus, the brain region important for initiating HPA stress responses, are important for triggering satiety (Takayanagi et al 2008; Aldaheff et al 2012). Thus, activation of the PVN during stress could explain the correlation between corticosterone and hypophagia, despite the lack of a causative relationship.

Further Questions:

Is this timing, a shift in the stress response between 4 to 7 days, seen by others?

Interestingly, this work is not the only group of studies to find significant changes in hormones or other physiological responses after 4 to 7 days of repeated stress. Ochi et al (2008) found ghrelin was uniquely recruited only after 5 days of simple restraint stress. Ghrelin was not recruited after 1 or 3 days of stress. Interestingly, they also found after 5 days of restraint that stress-induced decreases in gastric motility were reversed. Furthermore, after 5 stress session, prolonged stressor-induced release of noradrenaline had habituated and returned to baseline levels. Another recent paper found that hypermetabolic syndrome and immune responses were altered after 4.5 days of twice daily alternating acoustic stress and mild restraint stress (Depke et al). While these studies demonstrate similar timing of a chronic stress phenotype, more work needs to be done to observe whether this timing is a universal among stressors and how the timing would scale to human life span.

Do other types of stress recruit ghrelin as well?

Ghrelin as a stress hormone is relatively novel. However, in the last few years exploration of the topic has demonstrated that several types of stress recruit ghrelin across a variety of animals (see table below).

Stressor	Species	Ghrelin	Citation
Water Stress	Rat	↑ Acyl-ghrelin	Meyer et al 2013
Cold Stress	Rat	↑ Acyl-ghrelin	Stengel et al, 2010
Cold Stress	Human	↑ Total ghrelin	Tomasik et al, 2010
Restraint Stress	Rat	↑ Acyl-ghrelin	Ochi et al, 2008
Social Defeat	Mouse	↑ Acyl-ghrelin	Lutter et al, 2008
Chronic Unpredictable stress	Mouse	↑ Acyl-ghrelin	Patterson et al, 2010
Immobilization Stress	Rat	↑ Acyl-ghrelin	Meyer et al 2013
Trier Social Stress Test	Human	↑ Total ghrelin	Rouach et al, 2007

However, endotoxin infection, surgical trauma, and exercise have been found to decrease ghrelin levels (Stengel et al, 2011).

Methods and Materials

Animals

Subjects were adult male Long Evans rats (250–350 g, Taconic, Germantown, NY). Animals were individually housed (68-72°F; 12-h light-dark cycle, 7AM lights on). Food and water (or 0.9% saline for adrenalectomy experiments) was provided *ad libitum* in the home cage. Stressed and non-stressed animals were housed in separate cubicles. All experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (CAC) of the Massachusetts Institute of Technology and the Animal Care and Use Review Office of the USAMRMC. All surgery was performed under isoflurane anesthesia. Buprenorphine (.03mg/ml/kg b.w., s.c.) was administered for post-operative pain. Concerted efforts were made to minimize unnecessary suffering.

Immobilization stress

Immobilization stress was administered 4h per day. Stress sessions occurred for 14 consecutive days. Animals were placed in plastic bags (Decapicone, Braintree Scientific; Braintree, MA) and were secured at the tail. All stress sessions were performed between 10AM and 4PM in a lab room used for no other procedures. Non-stressed control rats were handled daily for 30s. Food was removed from the

unstressed rats daily for the same 4h period in which stressed animals were immobilized and, therefore, without food.

Blood collection and plasma fractionation

For hormone analysis, tail blood was collected from the lateral tail vein. One sample was collected from each rat prior to stress or handling for baseline assessment. Daily samples were collected during the last 15 minutes of each four hour stress session, a time point coinciding with the approximate endogenous circadian ghrelin nadir (31). Samples were collected in a tube which contained 1:10 v/v 0.5 M EDTA and 1:100 v/v HALT (Pierce; Rockford, IL). Immediately after collection, plasma was extracted by centrifugation (2,100g at 4°C for 10-15min). The plasma layer was then collected and half the volume treated with 10% v/v 1M HCl in order to stabilize the acylated form of ghrelin. Samples were stored at -20°C or -80°C. Each sample yielded at least 200 μL of whole blood. Because of CAC restrictions on the total volume of blood collected from individual animals within a given time frame, each animal could only be sampled every 72 hours across the stress sessions. Thus, different groups of animals were sampled for each time point, with each group repeatedly sampled every third day of stress exposure.

Adrenalectomy

Rats that were adrenalectomized were allowed to recover one week before being subjected to experimentation. Bilateral adrenalectomy was carried out through lateral incisions under 3.5% isoflurane anesthesia. Sham adrenalectomy

was performed by making the incision through the skin and muscle and locating the adrenal glands. Muscle incisions were closed with chromic gut sutures and skin incisions were closed with wound clips. Some adrenalectomized rats were purchased from Taconic (Germantown, NY). Gland amputation was verified by dissection after removal and further verified by plasma corticosterone analysis.

E-mitter implantation

Gross motor activity was measured using biotelemetric E-Mitters (Minimitter Co., Bend, OR). G2 E-mitters were surgically implanted into the abdominal cavity through a midline abdominal incision and attached to the ventral right side of the peritoneal membrane with silk sutures. Incisions were closed with chromic gut sutures. The procedure was performed under ketamine-xylazine-acepromazine anesthesia. Rats were allowed to recover for 5d before additional manipulation.

Activity monitoring

Long-term recordings of motion were performed using the VitalView data acquisition system and software. Data were sampled once every 5min for 18h/day for all rats (no monitoring occurred for any rats during the 4h daily immobilization stress period). Locomotion was measured in arbitrary units (as measured by VitalView) and data were binned in 1h increments. Data are reported for the 6h period at the beginning of the dark cycle of each day (no data was collected one hour prior to, four hours during, and one hour following each stress session).

Food

Rats were fed, *ad libitum*, a high protein (25%): carbohydrate (52%) diet (Prolab RMH 3000, standard rat chow, Purina, LabDiet, Richmond, Indiana, USA). This diet is especially designed for growth of lab rats. It contains all the nutrients required by the animal and uses high quality animal protein to provide optimal balance of amino acids. This diet has a metabolizable energy content of 3.20 kcal/g. Rats will eat up to 30 grams per day. Cage feeders contained a minimum of 200g of chow each day of the experiment.

Hormone analysis

Corticosterone, acylated ghrelin, growth hormone, NPY, and leptin levels were determined using commercial ELISA kits. For corticosterone, non-acidified plasma was used. Plasma was diluted 1:25 in assay buffer 15 for the corticosterone assay (Enzo Life Sciences; Farmingdale, NY). For acylated ghrelin, NPY, growth hormone, and leptin, acidified samples were used (Millipore; Billerica, MA).. Samples were excluded from analysis if they displayed signs of hemolysis or lipemia. All samples were assayed according to the manufacturer's protocols, corrected for dilution, and normalized as described in the text.

Data analysis

Repeated measurement analysis of variance (ANOVA) was used to determine differences among groups (significance level, $P \leq 0.05$), with stress and surgical treatments serving as independent variables. Post hoc comparisons in the form of

Fisher's PLSD tests were performed after a significant omnibus *F*-ratio ($P < 0.05$). For paired, non-parametrical measures, the Wilcoxon Signed-Rank Test was used (significance indicated by $\alpha<0.05$). Relationships and correlations between each measure were assessed using simple linear regression analysis corrected for Familywise Error Rates with the conservative Bonferroni correction with $\alpha \leq 0.003$ necessary significance. A Chi-square goodness-of-fit test was used to determine similarity of population distributions. Statistical trends are noted in the text when the omnibus F-ratio did not reach $P < 0.05$ but had $P < 0.10$. All data where $P > 0.10$ are identified as not significant (*n.s.*).

Data are presented as mean ± s.e.m.

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Chapter 4

Fear memory destabilization in the amygdala through optimized exposure training

Abstract

Strategies enhancing extinction learning and its retrieval over time are necessary for enhancing clinical efficacy of exposure therapy. Fear conditioning and extinction learning serve as laboratory models of phobia and exposure therapy, respectively. It is not clear how different exposure paradigms regulate the neurobiological underpinnings of extinction learning. In this study, we used a variety of exposure paradigms to examine the effects of conditional stimulus (CS) characteristics during extinction training on the acquisition and retention of extinction. Repeated, discrete trials of short duration CS presentations promoted better between-session extinction than longer tone presentations. Prolonged context exposure did not alter the strength of the CS exposure paradigm on extinction retention. Numerous discrete CS exposures produced a significant change in AMPA receptor phosphorylation states in the lateral amygdala. Finally, maximal number of discrete CS exposures prevented spontaneous recovery of fear, even when reduced cumulative CS exposure or unpredictable CS exposures were used. The results demonstrate that a lasting weakening of the fear memory can be achieved by presenting many discrete CS exposures during extinction training.

Introduction

The annual prevalence of phobias and other fear-based anxiety disorders is as much as 17% in the United States (117). The first line of treatment is exposure therapy (118, 119). Exposure therapy is a form of cognitive behavioral therapy which involves exposing the patient to the object of fear in a safe environment under the guidance of a trained therapist to overcome their anxiety (120). Despite widespread use in patients, the timing, presentation, and duration of fear inducing stimuli vary from clinic to clinic and optimization of these parameters are not well understood (121). The neurophysiology of differences between effective and ineffective exposure therapy are a mystery but studies of extinction learning in animal models are leading to a better understanding. Establishing the optimal parameters for these stimuli has the potential to improve the effectiveness of exposure therapy immediately. Understanding the neurophysiological changes of effective treatment might lead to pharmacological interventions in the longer term.

Pavlovian fear conditioning and subsequent extinction training are laboratory models used to study the neural basis of cognitive behavioral therapy of psychiatric disorders involving fear (120, 122-126). During fear conditioning, a neutral stimulus, such as a tone, is tightly paired with a noxious stimulus, such as an electric foot-shock. The tone is known as the conditional stimulus (CS) and the shock is known as the unconditional stimulus (US). The animal quickly learns that the presence of the CS predicts the onset of the US and responds to the CS with fear reactions (conditional responses, CR, such as freezing).

Fear extinction training is used to eliminate the CR and requires the animal

to learn that the CS is no longer predictive of the US. Extinction is achieved by exposing the animal to the CS in the absence of the US (127-129). Decreases in the CR in the presence of the CS are used to measure the effectiveness of extinction learning (127-129). It is known that over time, the CR can return even after the animal has undergone significant extinction training. This phenomenon is known as spontaneous recovery (130) and is seen as evidence that the original fear engram persists following extinction training (120, 123, 131-133). Declining levels of fear expressed during an extinction training session is used as a measure of effective extinction learning (121). However, recent work indicates that decreases in the CR during training do not necessarily represent enduring CR inhibition (i.e. extinction retention) (121, 134). Strategies to enhance extinction learning are necessary for enhancing exposure therapy and will depend on understanding the neurobiological mechanisms of persistent extinction.

Information about the CS and US converge in the lateral amygdala, the brain structure where fear memories form (135, 136). Extinction depends on learning of the new CS- no US contingency. While previous work indicates that extinction depends on new learning to inhibit the fear memory, recent work also provides evidence that extinction training (or CS exposure) leads to weakening of the original fear memory. This suggests that during extinction training the associational memory in the amygdala is altered in addition to the formation of a new fear-inhibiting association in other brain areas (128, 137-140). Associational memory is dependent on synaptic plasticity. It is known that increases in ionotropic glutamate

receptors (AMPA receptors) at synapses result in the long-term potentiation (LTP) or strengthening of that synapse. On the other hand, removal of synaptic AMPA receptors leads to long-term depression (LTD) (141). Change in AMPA receptor phosphorylation states precede the insertion or removal of synaptic AMPA receptors (141). Decreases in the phosphorylation of the serine 845 GluR1 subunit have been shown to be important for the reconsolidation based erasure of fear memory (140). We suggest that the same molecular change may be found when effective CS exposures are used during extinction training to produce persistent extinction of the CR.

To determine which properties of CS exposure presentations yield transient versus persistent learning of the new CS-no US contingency, we measured the effects of five different CS exposure paradigms on the behavioral correlates of extinction learning: namely, reduction of fear expression both within- and between-training sessions. Because the familiarity of the training context during extinction training can yield differing rates of extinction retention (132), we varied the duration of extinction context exposure within CS exposure groups to observe context modulation of extinction retention. Next, we varied the CS exposure paradigms to test the hypothesis that, like reconsolidation, CS exposure that leads to maximal extinction learning also leads to plasticity in the amygdala. Finally, we demonstrated that CS exposures that maximize between-session extinction also result in persistent retention of extinction learning, preventing spontaneous recovery of fear.

Materials and methods

Animals

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (CAC) of the Massachusetts Institute of Technology and the Animal Care and Use Review Office of the USAMRMC. Subjects were adult male Long Evans rats (250–350 g, Taconic, Germantown, NY). Animals were individually housed (68-72°F; 12-h light-dark cycle, 7AM lights on). Food and water was provided *ad libitum* in the vivarium.

Behavioral apparatus

All sessions were conducted in four identical rodent observation chambers constructed of aluminum and Plexiglas (30 × 24 × 21 cm; MED Associates), situated in sound-attenuating chambers and located in an isolated room. The floor of each chamber consisted of a stainless steel grid floor. The grid floor was connected to a shock source and a solid-state grid scrambler (MED Associates) which delivered the foot-shock unconditioned stimulus (US). Mounted on one wall of the chamber was a speaker to provide a distinct auditory conditioned stimulus (CS). The chamber also contained a stimulus light, a house light, and a fan, which provided background noise (65 dB). Infrared cameras mounted to the door of the sound-attenuating chambers were used to record behavior, which was scored offline.

Two unique contexts were created by manipulating auditory, visual, and olfactory cues: Context A (light context) comprised a 0.03% diluted PineSol solution placed in trays at the bottom of the chambers, the house and stimulation lights on, and fans on in the chambers; Context B (dark context) comprised a 1% acetic acid solution in chambers, no visible lights (IR only), and fans off. We also used two different transportation boxes for each context: Context A had clear transport boxes and Context B had dark transport boxes.

Behavior

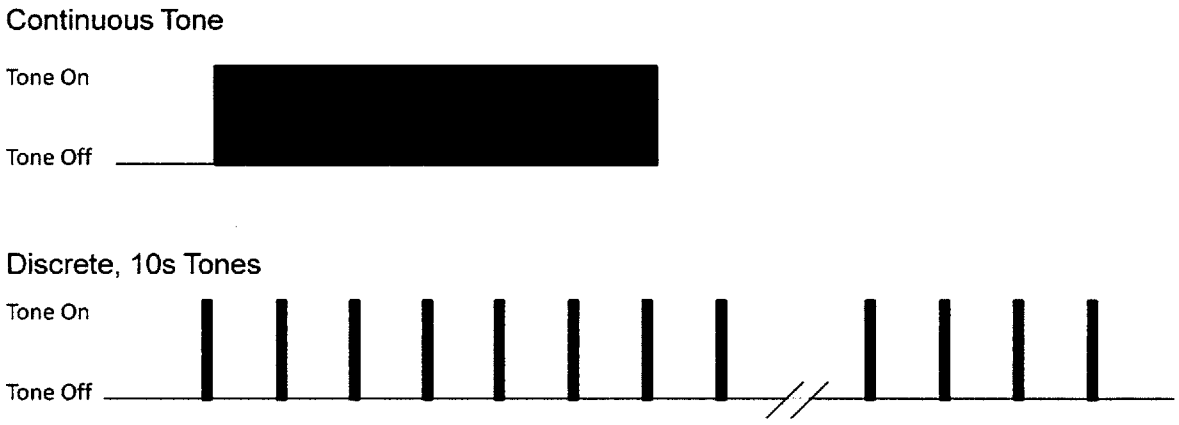
To bias the fear towards the tone rather than the context and increase the strength of this initial CS-US association, fear conditioning and context exposure occurred in Context A, whereas CS exposure (extinction training) occurred in Context B. Animals were randomly assigned to each tone group such that freezing behavior during fear conditioning and context exposure did not differ.

Experiment 1: Continuous and discrete CS presentations on within-session and between-session extinction

On Day 1, 29 naïve rats (Continuous Tone= 13; 10s Discrete Tones= 16) were transported from their home cages in squads of four and placed in the conditioning context (Context A). After 3 minutes of context habituation, rats received five tone (10s, 80dB, 2 kHz)-foot-shock pairings (0.7 mA, 1 seconds). The inter-trial interval was 70 seconds and the rats remained in the chambers for 59 seconds after the last tone-foot-shock pairing. One day after conditioning (Day 2), all rats were placed

back into Context A for 10 minutes without any presentations of either the US or the CS in order to extinguish fear responding to the context. On Days 3 and 4, all rats were placed into Context B to extinguish fear of the tone. The "Continuous Tone" group was presented with a single 5 minute tone (2 kHz, 80dB) that began 120s after being placed in the chambers. The 10s Discrete Tones group was presented with 30 10s Tones (2 kHz, 80dB, 60s inter-stimulus interval (ISI)).

Experiment 1. Schematic of CS exposure paradigms



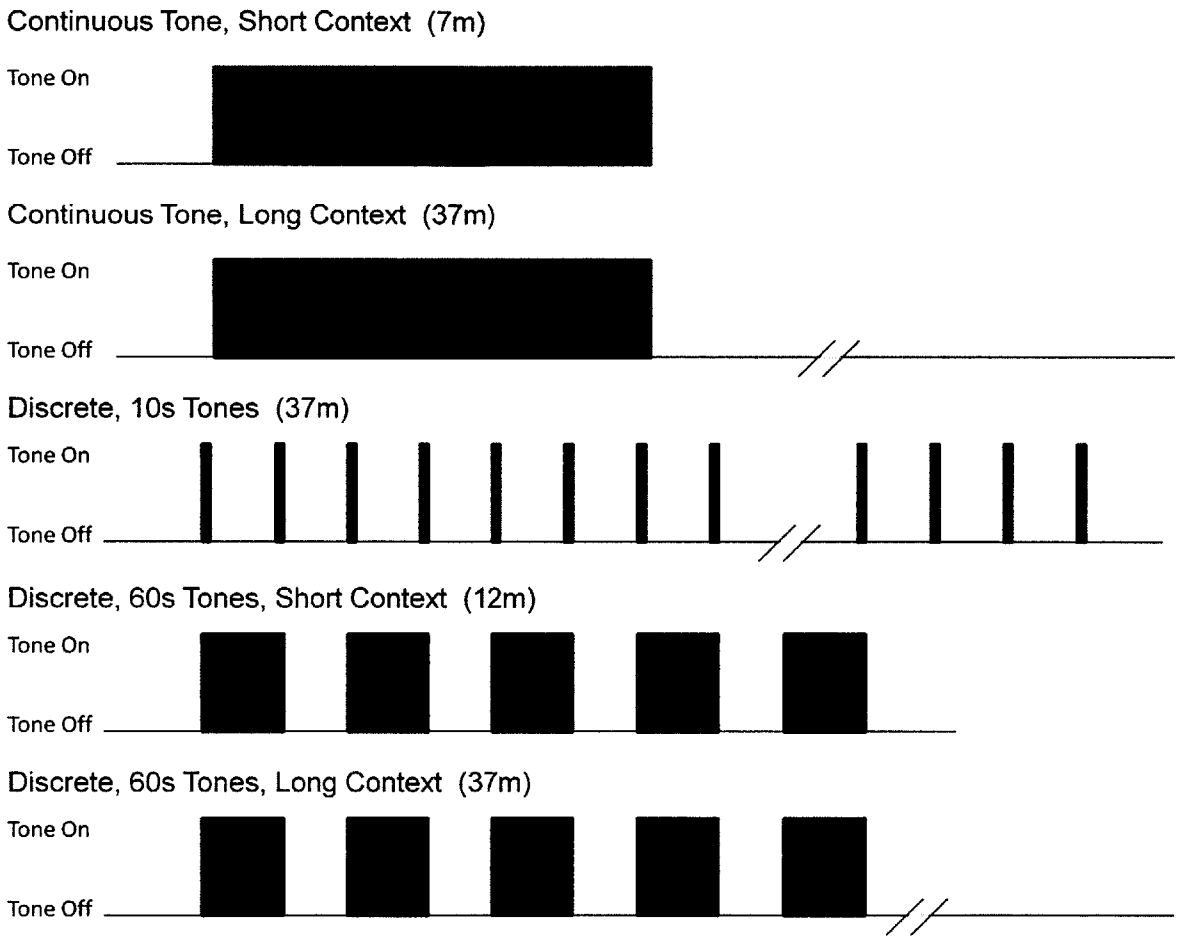
Experiment 2: Tone presentation and context exposure on within-session and between-session extinction

On Day 1, 64 naïve rats (Continuous -Short= 12; Continuous-Long=13, 10s Tones = 12, 60s Tones-Short= 14, 60s Tones-Long = 13) were transported from their home cages in squads of four and placed in the conditioning context (Context A). After 3 minutes of context habituation, rats received five tone (10s, 80dB, 2 kHz)-foot-shock pairings (0.7 mA, 1 seconds). The inter-trial interval was 70 seconds and the rats remained in the chambers for 59 seconds after the last tone-foot-shock pairing. One day after conditioning (Day 2), all rats were placed back into Context A for 10 minutes without any presentations of either the US or the CS in order to extinguish fear responding to the context. On Days 3, 4, and 5, all rats were placed into Context B to extinguish fear of the tone. The "Continuous Tone" groups were presented with a single 5 minute tone (2 kHz, 80dB) that began 120s after being placed in the chambers. The 10s Discrete Tones group was presented with 30x10s Tones (2 kHz, 80dB, 60s ISI) that began 120s after being placed in the chamber. The 60s Discrete Tones groups were presented with 5x60s Tones (2 kHz, 80dB, 60s ISI) that began 120s after being placed in the chamber. For both discrete tone groups, the ISI was 60 seconds. Because the 10s tone group received more exposure to the extinction context (37 minutes total), two additional control groups were added to take into account context exposure for the continuous and 60s tone groups. Continuous-Short animals were removed immediately after cessation of the tone (7m). Continuous-Long animals were removed 30m after the end of the tone (37m).

Discrete 60s – Short animals were removed 60s after the end of the last tone (12m).

Discrete 60s – Long animals were removed 25m after the last tone (37m).

Experiment 2. Schematic of CS exposure paradigms



Experiment 3: Continuous and discrete CS presentations on AMPA-R phosphorylation states

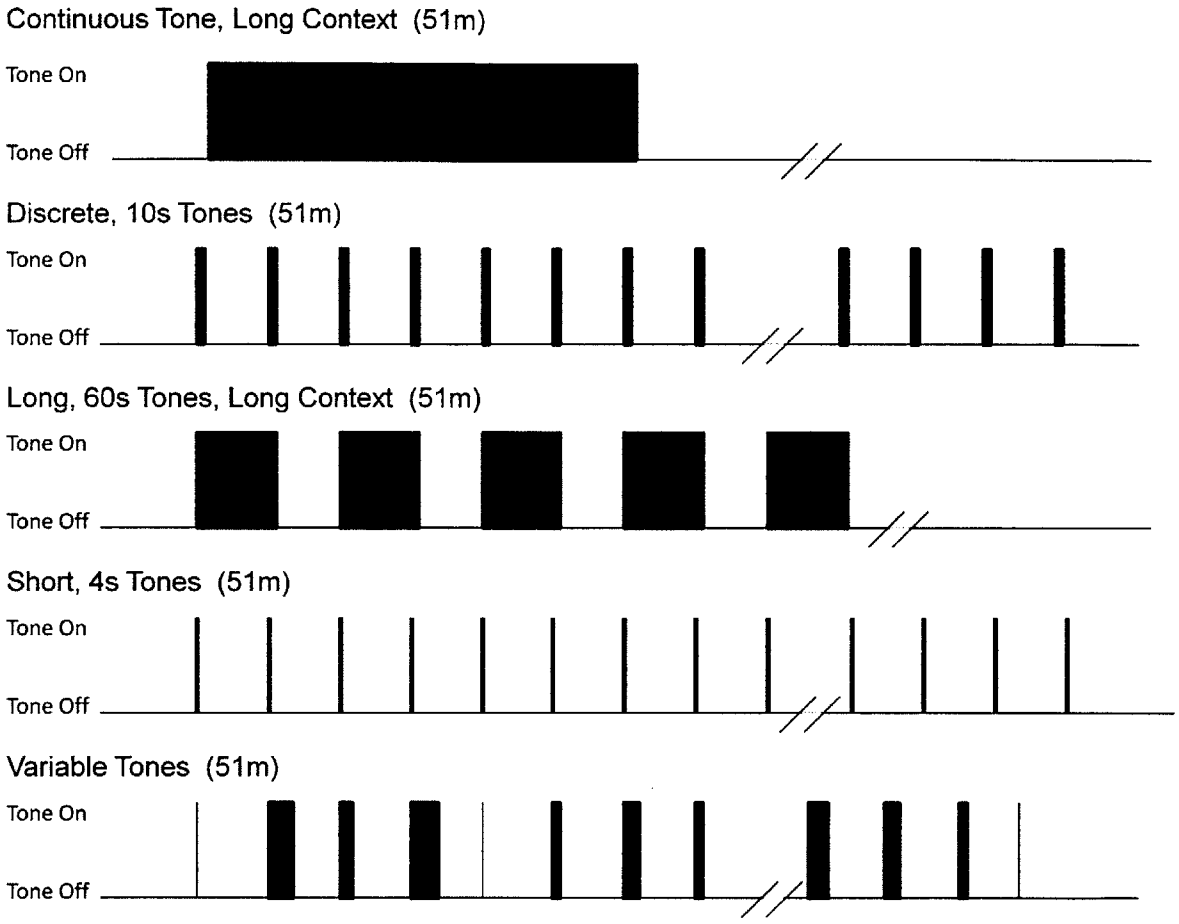
On Day 1, 15 naïve rats (Context Exposure = 5; Continuous Tone= 5; 10s Discrete Tones= 5) were transported from their home cages in squads of four and placed in the conditioning context (Context A). After 3 minutes of context habituation, rats received five tone (10s, 80dB, 2 kHz)-foot-shock pairings (0.45 mA, 1 sec). The inter-trial interval was 70 seconds and the rats remained in the chambers for 59 seconds after the last tone-foot-shock pairing. One day after conditioning (Day 2), all rats were placed back into Context A for 10 minutes without any presentations of either the US or the CS in order to extinguish fear responding to the context. On Day 3, all rats were placed into Context B for 51 minutes. The Context Exposure group did not receive any CS exposure. The 10s Discrete Tones group was presented with 42x10s tones (2 kHz, 80dB, 60s ISI). The Continuous Tone group was presented with a single 7 minute tone (2 kHz, 80dB) and left in the context for the remainder of the time. Animals were sacrificed within 2 minutes of finishing this extinction session.

Experiment 4: Equal CS Number, Variable CS Duration, Variable CS Exposure

On Day 1, 43 naïve rats (Continuous = 3; 60s Tones=12, 10s Tones = 11, 4s Tones = 9, Variable duration Tones = 8) were transported from their home cages in squads of four and placed in the conditioning context (Context A). After 3 minutes of context habituation, rats received five tone (10s, 80dB, 2 kHz)-foot-shock pairings (0.45 mA, 1 second). The inter-trial interval was 70 seconds and the rats remained

in the chambers for 59 seconds after the last tone-foot-shock pairing. One day after conditioning (Day 2), all rats were placed back into Context A for 10 minutes without any presentations of either the US or the CS in order to extinguish fear responding to the context. On Days 3, 4, and 5, all rats were placed into Context B to extinguish fear of the tone. The Continuous Tone group was presented with a single 7 minute tone (2 kHz, 80dB) that began 120s after being placed in the chambers. The 10s Discrete Tones group was presented with 42x10s Tones (2 kHz, 80dB, 60s ISI) that began 120s after being placed in the chamber. The 60s Discrete Tones group was presented with 7x60s Tones (2 kHz, 80dB, 60s ITI) that began 120s after being placed in the chamber. The 4s Tones group was presented with 42x4s tones (2 kHz, 80dB, 60s ITI) that began 120s after being placed in the chamber (Cumulative CS exposure = 168s). The Variable Tones group was presented with 42 variable duration tones (1-19s, 2 kHz, 80dB, 60s ITI) that began 120s after being placed in the chamber (cumulative CS exposure = 420s, mean tone duration = 10s). For all discrete tone groups the ISI was 60 seconds. Context exposure duration was equal for each tone exposure group (51m).

Experiment 4. Schematic of CS exposure paradigms



Western Blot

The lateral amygdala was microdissected at time of sacrifice. It was flash frozen and stored at -80° C. Protein samples were processed and reconstituted in homogenization buffer (320 mM sucrose, 2 mM DTT, 2 mM EDTA, 4 mM HEPES, ph 7.4, 1:100 HALT with phosphatase inhibitors). 16.6 micrograms of protein were run on an in-house gradient 0.21%:40% bisacrylamide:acrylamide gel and blotted onto PVDF membranes in a methanol-based tris-glycine buffer overnight. Before each probe, membranes were blocked in 5% milk - 0.1% Tween 20 – Tris Buffered Saline. Each primary antibody was suspended in blocking solution and incubated overnight at 4° C. Primary antibodies included Rabbit anti-GluR1 Phospho-Ser845 (1:500, from Novus Biologicals, NB300-171) and Rabbit anti-Total GluR1 (1:500, from Millipore, AB1504). Membranes were incubated in secondary antibody (Anti-Rabbit-HRP, 1:35,000) diluted in blocking solution at room temperature for 1 hour. After rinsing, membranes were developed with enhanced chemiluminescence fluid (Thermo Scientific Pierce, #32209) for 5 minutes and then exposed to Kodak film for 1 to 10 seconds. Membranes were stripped with stripping buffer (10% SDS, ß-mercaptoethanol-Tris buffer) at 55°C for 30 minutes before being blocked and reprobed with the next antibody. Blots were analyzed using ImageJ (NIH, Bethesda, Md) and PhotoShop (Adobe, San Jose, Ca). Phospho-Ser845 GluR1 was normalized to total GluR1 and both experimental groups were expressed as fold change from context exposure controls. Two Western Blots were run in parallel and both were included in quantification.

Data analysis

All statistics were performed using StatView (Apple, Cupertino, Ca). Repeated measurement analysis of variance (ANOVA) was used to determine differences among groups (significance level, $P \leq 0.05$), with extinction paradigm (tone type) serving as the independent variable. Post hoc comparisons in the form of Fisher's PLSD tests were performed after a significant omnibus F-ratio ($P < 0.05$). Statistical trends are noted in the text when the omnibus F-ratio did not reach $P < 0.05$ but were $P < 0.10$. All data where $P > 0.10$ were identified as not significant (*n.s.*). Data are presented as mean ± s.e.m.

Results

Experiment 1: Extinction paradigms that result in excellent within-session extinction do not necessarily provide good between-session extinction

In this experiment, we compared two classic tone presentation paradigms for extinction of the CR: a single continuous tone of 300s duration or 30 discrete tones lasting 10s each (for detailed schematics see Methods section). To understand which CS exposure paradigm led to the highest amount of extinction learning, we compared freezing behavior from these two CS exposure protocols.

The quality of extinction can be assessed either within-session or between-session. We define between-session extinction as the optimal form of CR inhibition because goals in the clinic are to yield persistent inhibition of fear.

Within single extinction sessions, there was no significant difference between the two common CS exposure paradigms. One day after context exposure, rats were placed in a novel context (Context B) and exposed to either a continuous 5m tone or thirty discrete 10s tones. Cumulative CS exposure was 300s for both groups. Analysis of CS-evoked fear behavior during this first tone extinction session revealed a significant main effect of time (within-session CR inhibition)(Fig. 1A, $F(29, 783)= 5.59$, $P < 0.0001$) but no main effect of CS exposure (tone presentation) group ($F(1, 27)= 0.51$, P = n.s.). When the freezing in response to the first 60s of CS exposure was compared to the last 60s of CS exposure, a significant reduction in fear behavior was seen for both CS exposure groups (Fig. 1B, $F(1, 27)= 21.18$, $P < 0.0001$). These results indicate that animals in both tone groups had equivalent within-session extinction.

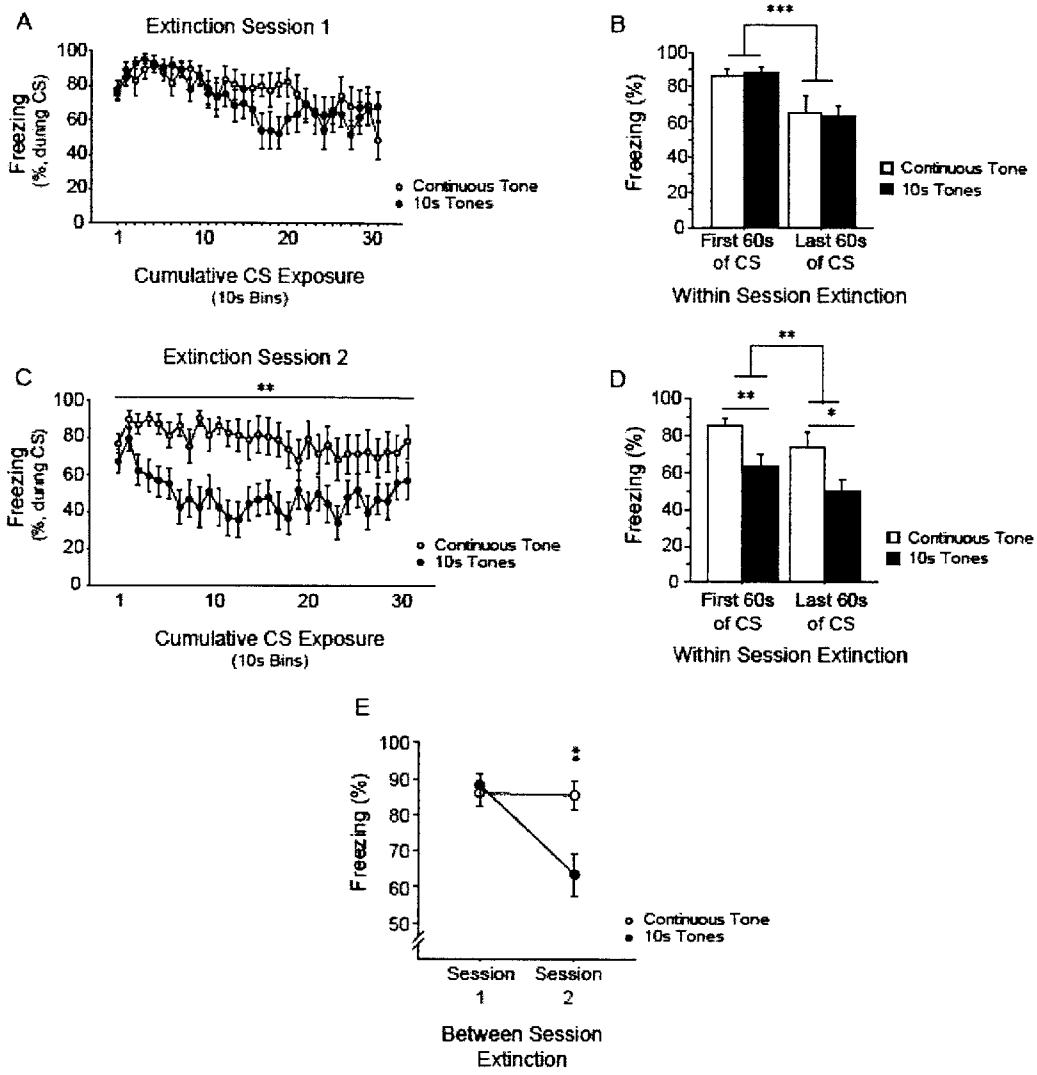


Figure 1. Extinction paradigms that result in excellent within-session extinction do not necessarily provide good between-session extinction. After fear conditioning and context extinction, fear to CS (tone) was assessed by measuring freezing levels when exposed to 300s of the CS presented in a novel environment. Animals were presented with either a single 5 minute tone (○, open circles) or thirty, discrete 10s tones (●, filled circles). (A) Freezing levels throughout CS exposure during the first extinction session. (B) Average freezing levels during first 60s of CS exposure and last 60s of CS exposure demonstrate that both groups have significant within-session extinction. (C) Freezing levels throughout CS exposure during the second extinction session. (D) Average freezing levels during first 60s of CS exposure and last 60s of CS exposure demonstrate that while again both groups have significant within-session extinction, the group exposed to a single continuous tone freezes more to the CS than the animals exposed to discrete tones. (E) Average freezing levels during first 60s of CS exposure of each extinction session demonstrate efficient between-session extinction in the 10s Discrete Tones group but not within the Continuous Tone group. Values represent mean±s.e.m., * $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$.

Many studies have shown that while generally fear declines within an exposure trial, there is little evidence to indicate that such declines are indicative of learning or long-lasting improvement (121). The next day, rats were returned to Context B for a second tone extinction session to assess both between-session extinction retention and further within-session extinction. Analysis of CS-evoked freezing behavior revealed a significant effect of time (Fig. 1c, $F(29, 783)= 2.24$, $P < 0.001$) and CS exposure group ($F(1, 27)= 10.18$, $P < 0.01$) but no interaction effect ($F(29, 783)= 1.30$, P = n.s.). When the freezing in response to the first 60s of CS exposure was compared with the last 60s of CS exposure, a significant reduction in fear behavior was seen for both CS exposure groups (Fig. 1D, $F(1, 27)= 9.52$, $P < 0.01$), however, the discrete 10s tone group had significantly less freezing during either epoch ($F(1, 27)= 7.25$, $P < 0.05$).

There was a significant effect of CS exposure subtype on between-session extinction. An ANOVA of the first 60s of CS exposure in both extinction sessions (Fig. 1E) revealed a main effect of treatment on between-session extinction ($F(1, 27)= 8.51$, $P < 0.01$). These results indicated that while within-session extinction of CS-evoked freezing occurred in both groups, the 10s Discrete Tones group retained previous extinction memory. This was reflected in higher freezing in the Continuous Tone group at the start of the second extinction session when compared to the discrete 10s tone group. These results demonstrated that robust within-session extinction does not portend persistent retention of extinction and also demonstrate

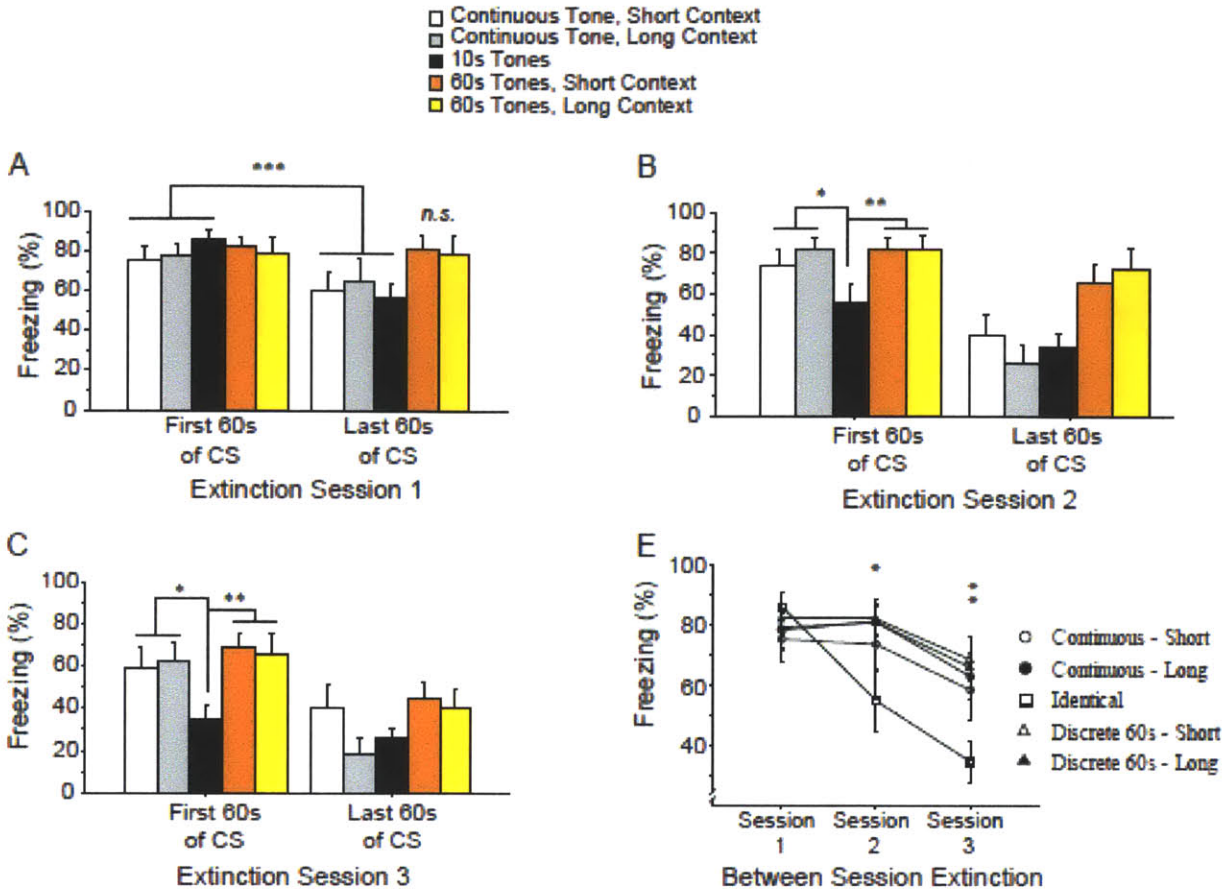
that the continuous tone was inferior to discrete tones in eliciting between-session extinction.

Furthermore, we demonstrated that extinction learning can best be seen by the decreased conditioned fear response at the beginning of later extinction sessions. Therefore, to more directly measure the effect of repeated extinction training on between-session extinction retention, we focused on the initial fear recall and extinction recall in subsequent extinction sessions for the remainder of the experiments presented in this paper. To measure the effect of repeated extinction training on between-session extinction retention, we defined an Extinction Retention Index to show cumulative between-session extinction across multiple sessions. The Extinction Retention Index is equal to the fear recall expressed as percent freezing at the beginning of the first extinction session minus the fear recall expressed as percent freezing from the beginning of the subsequent extinction session. A positive value indicates a suppression of the CR occurred during the subsequent session and that there was significant between-session extinction, whereas a null or negative value indicates no suppression of CR during the subsequent extinction session and, therefore, no between-session extinction occurred.

Experiment 2: Context exposure does not alter resistance to between-session extinction of inferior extinction paradigms

In this experiment, we evaluated the duration of extinction context exposure and the effectiveness of multiple discrete tones for within-session and between-session extinction learning. Many groups have shown that context modulates expression of fear (131) and so controls for context exposure duration were included because Context B exposure was longest in the 10s Discrete Tones group. Also, because the 10s Discrete Tones CS paradigm in the previous experiment yielded the better between-session extinction, a third tone group was added in which animals were exposed to discrete 60s tones. As in Experiment 1, fear conditioning and context exposure occurred in Context A and animals were pseudo-randomly assigned to each tone group such that freezing behavior during fear conditioning and context exposure did not differ. One day after context exposure, rats were placed in a novel context (Context B) and exposed to a continuous 5m tone, thirty discrete 10s tones, or five discrete 60s tones. Cumulative CS exposure was 300s for all groups. Two context equivalent groups were added for the Continuous and Discrete 60s Tones groups (Continuous, Long Context and 60s Tones, Long Context groups). Fear recall was equivalent for all groups (Fig. 2A, left bars, ($F(4, 59)= 0.44$, P = n.s.). There was a significant effect of time (Fig. 2A, $F(1, 59)= 13.49$, $P< 0.001$) but no effect of CS exposure group ($F(4, 59)= 0.69$, P = n.s.). Post-hoc analysis of within-session extinction in each extinction group revealed a significant reduction in CRs for the continuous and discrete 10s tone groups but not for the discrete 60s tone groups (planned comparisons). There was no effect of context duration on the

levels of within-session extinction between the two Continuous Tone groups or Discrete 60s Tones groups (P = n.s.). However, context effects would not be revealed at the beginning of the extinction training since the context equilibration is attached to the end of the tone presentations. Additionally, because the Discrete 60s Tones groups did not demonstrate significant within-session extinction, cumulative CS exposure duration was not responsible for the inhibition of the CR within the extinction session because all groups were exposed to 300s of CS.


Continuous Tone, Short Context
Continuous Tone, Long Context
10s Tones
60s Tones, Short Context
60s Tones, Long Context
A
Freezing (%)
***
n.s.
First 60s of CS
Last 60s of CS
Extinction Session 1
B
Freezing (%)
*
**
First 60s of CS
Last 60s of CS
Extinction Session 2
C
Freezing (%)
*
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First 60s of CS
Last 60s of CS
Extinction Session 3
E
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Continuous - Short
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Discrete 60s - Short
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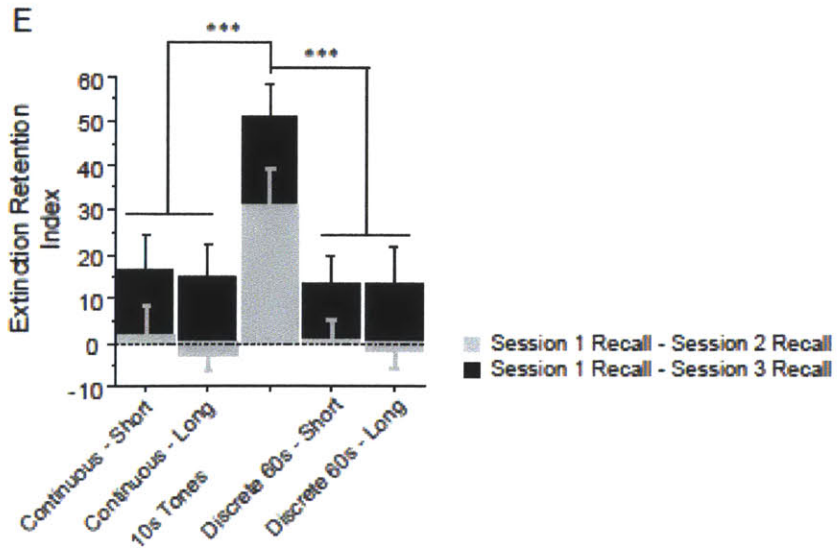
Session 1
Session 2
Session 3
Between Session Extinction


E
Extinction Retention Index
***
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Session 1 Recall - Session 2 Recall
Session 1 Recall - Session 3 Recall
Continuous - Short
Continuous - Long
10s Tones
Discrete 60s - Short
Discrete 60s - Long

Figure 2. Context exposure does not alter resistance to between-session extinction of suboptimal extinction paradigms and optimized extinction results in cumulative between-session extinction. After fear conditioning and context extinction, fear to CS (tone) was assessed in a novel environment. A variety of CS presentations were used for a cumulative 300s of CS exposure in each group: a single 5m tone, five 60s tones, or thirty 10s tones. Two context durations were used for the Continuous Tone and Discrete, 60s Tones groups: short (7m total for continuous, 12m total for 60s tones) or long (37m for all). (A) Average freezing levels during first 60s of CS exposure and last 60s of CS exposure demonstrate within-session extinction during the first extinction session. Continuous Tone groups and 10s Discrete Tones group have significant within-session extinction but Discrete 60s Tones groups do not. (B) Average freezing levels during first 60s of CS exposure and last 60s of CS exposure demonstrate within-session extinction during the second extinction session. Initial freezing levels demonstrate extinction retention from the first extinction session. The 10s Discrete Tones group freezes significantly less than the other groups during the first 60s of CS exposure. All tone groups have significant within-session extinction. (C) Average freezing levels during first 60s of CS exposure and last 60s of CS exposure demonstrates within-session extinction during the third extinction session. Initial freezing levels demonstrate extinction retention from the first and second extinction sessions. The 10s Discrete Tones group freezes significantly less than the other groups during the first 60s of CS exposure. All tone groups have significant within-session extinction. (D) Average freezing levels during first 60s of CS exposure of each extinction session demonstrates efficient between-session extinction in the 10s Discrete Tones group and not the Continuous Tone or Discrete 60s Tones groups. (E) Extinction retention index demonstrates the reduction in freezing behavior representative of between-session extinction. Gray bars represent between-session extinction from the first to second extinction session. Black bars represent cumulative between-session extinction for all extinction sessions. Values represent mean±s.e.m., * $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$.

The next day, rats were returned to Context B for a second tone extinction session to assess between-session extinction retention. Here analysis of CS-evoked freezing behavior in the first 60s and last 60s of CS exposure during the second tone extinction session revealed a significant effect of time (Fig. 2B, $F(1, 59)= 59.96$, $P < 0.0001$), CS exposure group ($F(4, 59)= 3.38$, $P < 0.05$), and CS exposure group x time interaction ($F(4, 59)= 5.43$, $P < 0.001$.). Unlike the first extinction session, post-hoc analysis of within-session extinction in each extinction group revealed a significant reduction in fear behavior for all tone groups (planned comparisons). There was no

effect of context duration on the levels of within-session extinction in the continuous and discrete 60s tone groups (P = n.s.). These results indicate that within-session CS extinction learning can occur repeatedly. Furthermore, because the behavior of the long context exposure groups did not differ from their short context counterparts, duration in the extinction context did not modulate extinction acquisition.

A third extinction session occurred 24h later to assess cumulative between-session extinction retention and further assess the repeatability of within-session extinction. Analysis of CS-evoked freezing behavior in the first 60s and last 60s of CS exposure revealed a significant main effect of time (Fig. 2C, $F(1, 59)= 32.39$, $P < 0.0001$), a near significant main effect of CS exposure group ($F(4, 59)= 2.39$, $P = 0.06$), and no CS exposure group x time interaction ($F(4, 59)= 1.95$, P =n.s..). Post-hoc analysis of within-session extinction in each extinction group revealed a significant reduction in fear behavior for all tone groups (planned comparisons). Again, there was no effect of context duration on the levels of within-session extinction in the continuous and discrete 60s tone groups (P = n.s.). These results further indicate that within-session CS extinction learning can occur repeatedly and is not dependent on extinction context exposure duration.

Analysis of between-session extinction across all three extinction sessions (Fig. 2D) revealed a main effect of CS exposure group x time interaction ($F(8, 118)= 3.76$, $P < 0.001$). Analysis of extinction retention using an extinction retention index revealed that discrete 10s tones yielded the best between-session extinction (Fig.

2E, $F(4, 59)= 4.68$, $P < 0.01$). Due to the failure of the discrete 60s tones to construe significant extinction retention, these results indicate that the discrete nature of the 10s Discrete Tones group alone is not responsible for the robust extinction retention compared to the Continuous Tone group. Interestingly, despite equivalent CS exposure, very little within-session extinction occurred with the Discrete 60s Tones groups. Again, we showed that despite within-session extinction behavior in the Continuous Tone group groups, extinction retention is not robust. Finally, perhaps due to tone-biased fear association, context exposure did not modulate extinction retention.

Experiment 3: Optimized extinction lead to AMPA receptor dephosphorylation in amygdala

Previously, other labs have demonstrated that training which yields between-session fear extinction results in changes in AMPA receptor phosphorylation states. Specifically, disruptive extinction shows a down-regulation in Protein Kinase A dependent phosphorylation of serine 845 of the GluR1 subunit of the AMPA receptor which is known to regulate the stability of synaptic receptors (140). Here, we evaluated the physiological changes of the amygdala after the two CS exposure paradigms which yielded equivalent within-session extinction but differential between-session extinction: continuous tone exposure versus discrete 10s tones. As in the previous experiments, animals were pseudo-randomly assigned to each tone group such that freezing behavior during fear conditioning and context exposure did not differ. The next day animals underwent a single extinction session. Both

amygdalae were collected within at the end of the extinction session and synaptosomal fractions were prepared and analyzed for total GluR1 and pS845 GluR1 (Fig. 3A). A significant effect of CS exposure was found (Fig. 3B, $F(2, 12)= 7.56$, $P < 0.01$) and revealed that pS845-GluR1 is significantly down-regulated following discrete 10s tones ($p<0.01$) but not changed after a continuous tone relative to context exposure only controls (P = n.s.). There was no difference in total GluR1 for either group (P = n.s.). These results show that the discrete 10s tones resulted in dephosphorylation of synaptic pS845-GluR1 whereas the continuous tone did not, suggesting dephosphorylation is not a consequence of within-session fear inhibition and that the retention of extinction may be dependent on this change in GluR1 phosphorylation.

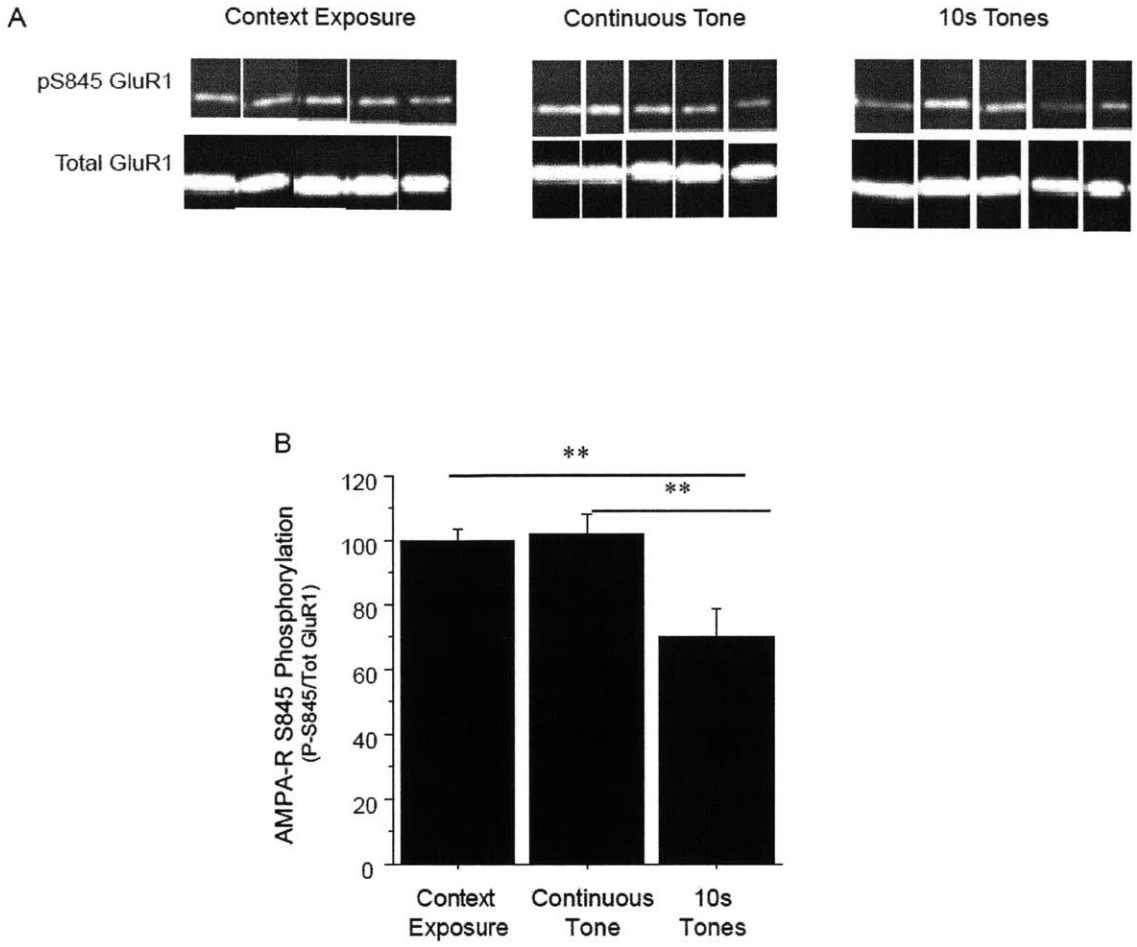

Figure 3. AMPA receptor phosphorylation state differences between CS exposures that result in equivalent within-session extinction but different between-session extinction. After fear conditioning and context extinction, animals were placed in a novel context and either allowed to explore for 51 minutes with no tone or administered extinction training with either a 7 minute continuous tone or 42 discrete 10s tones. Animals remained in the extinction context for a total of 51 minutes in all groups. Animal were sacrificed within 2 minutes of the end of the session and the lateral amygdala dissected. (A) Synaptosomal preparations were run on western blot for AMPA receptor phosphorylation state analysis. (B) Compared to context exposure control group, 10s Discrete Tones resulted in significant dephosphorylation of S845. Values represent mean±s.e.m., ** $P \leq 0.01$.

Experiment 4: CS exposure paradigms that result in the best between-session extinction also prevent spontaneous recovery of fear

We found that discrete 10s tone CS exposure resulted in the best extinction retention and dephosphorylation of pS845-GluR1. This CS exposure paradigm differed from the continuous tone and the discrete 60s tones in two ways: (1) the 10s tones "matched" the CS presentation used during fear conditioning and (2) there were more individual, discrete CS exposures. However, in all the tested paradigms, animals received equivalent cumulative CS exposure and predictable within-session presentation.

Here we explored the effect of the number of CS exposures, the necessity of matching across fear conditioning and extinction trials, the predictability within extinction training, and the cumulative CS exposure on extinction retention and spontaneous recovery. Animals were pseudo-randomly assigned to each tone group such that freezing behavior during fear conditioning and context exposure did not differ. The next day the animals were placed in the novel context (Context B) for the first extinction session. Rats underwent one of five CS exposure paradigms: a single seven minute continuous tone (Continuous), forty-two discrete 10s tones (10s Tones), seven discrete 60s tones (Long, 60s), forty-two discrete 4s tones (Short, 4s), or forty-two discrete variable duration (1s – 20s, mean: 10s, Variable) tones.

The Short, 4s Tones group tested the necessity of cumulative CS exposure and matching of the CS presentation between extinction training and fear conditioning: this group received only a fraction (40%) of cumulative CS exposure compared to the other four groups and CS presentation did not match the CS used

for fear conditioning. However, this group did have the maximum forty-two trials. If maximal trial number is sufficient for optimal extinction retention, this group should perform as well as the 10s Discrete Tones or the Variable Tones groups and significantly better than the Continuous or Long, 60s tone groups. However, if maximal CS exposure is necessary, the Short, 4s group should fail to exhibit between-session extinction.

The Variable Tones group tested the necessity of within-session predictability and matching to fear conditioning. This group underwent extinction training where the duration of each CS presentation was unpredictable and did not match the CS used for fear conditioning. However, this group had the maximum CS exposure of 420s as well as the maximum number of trials (42). Again, if maximal trial number is sufficient for optimal extinction retention, this group should perform as well as the 10s Discrete Tones or the Short, 4s Tones groups. However, if predictability across fear conditioning and extinction training is necessary, both the Variable Tones and Short, 4s Tones groups should exhibit compromised extinction learning. Furthermore, if predictability within the extinction training is necessary for extinction retention, then the Variable Tone group should exhibit compromised extinction retention compared to the other groups which have trial-by-trial predictability.

Interestingly, we found that all paradigms with the maximal number of CS exposure number (10s tones, Short-4s tones, and Variable tones, 42 trials each) yielded significant between-session extinction retention. During the first extinction

session, all groups exhibited equivalent levels of CS-evoked fear behavior (Fig. 4A. ($F(4, 38)= 0.13$, P = n.s.). The next day, rats were returned to Context B for a second tone extinction session. Here analysis of CS-evoked freezing behavior in the first 60s revealed a significant main effect of CS exposure group (Fig. 4B, $F(1, 38)= 3.61$, $P < 0.05$) and revealed a significant reduction in fear behavior for each tone group that received 42 trials compared to the Continuous Tone group (Fig. 4B, planned comparisons, $P <0.05$). Rats were returned to Context B for a third tone extinction session 24h later. CS-evoked freezing behavior revealed a significant effect of CS exposure group (Fig. 4C, $F(1, 38)= 7.72$, $P < 0.0001$) due to a reduction in fear behavior for each tone group that received 42 trials compared to both the Continuous Tone group and the Long-60s Tones group (Fig. 4C, planned comparisons, $P <0.05$). Analysis of extinction retention revealed that each group that received 42 CS exposures yielded significant between-session extinction compared to the Continuous Tone or Long-60s Tones group (Fig. 4D, planned comparisons, $P < 0.05$). Long-60s tones group also demonstrated significant extinction retention compared to the Continuous Tone group (Fig. 4D, planned comparisons, $P < 0.05$).

Most interestingly, when tested four weeks later, animals in the groups that received 42 CS exposures still expressed reduced fear behavior compared to the Continuous or Long-60s Tone groups (Fig. 4E, planned comparisons, $P < 0.05$), demonstrating persistent extinction retention and resistance to spontaneous recovery of fear. These results suggest that it is CS exposure number and not

cumulative CS exposure, trial-by-trial predictability, or fear conditioning-extinction training matching that confers between-session extinction.

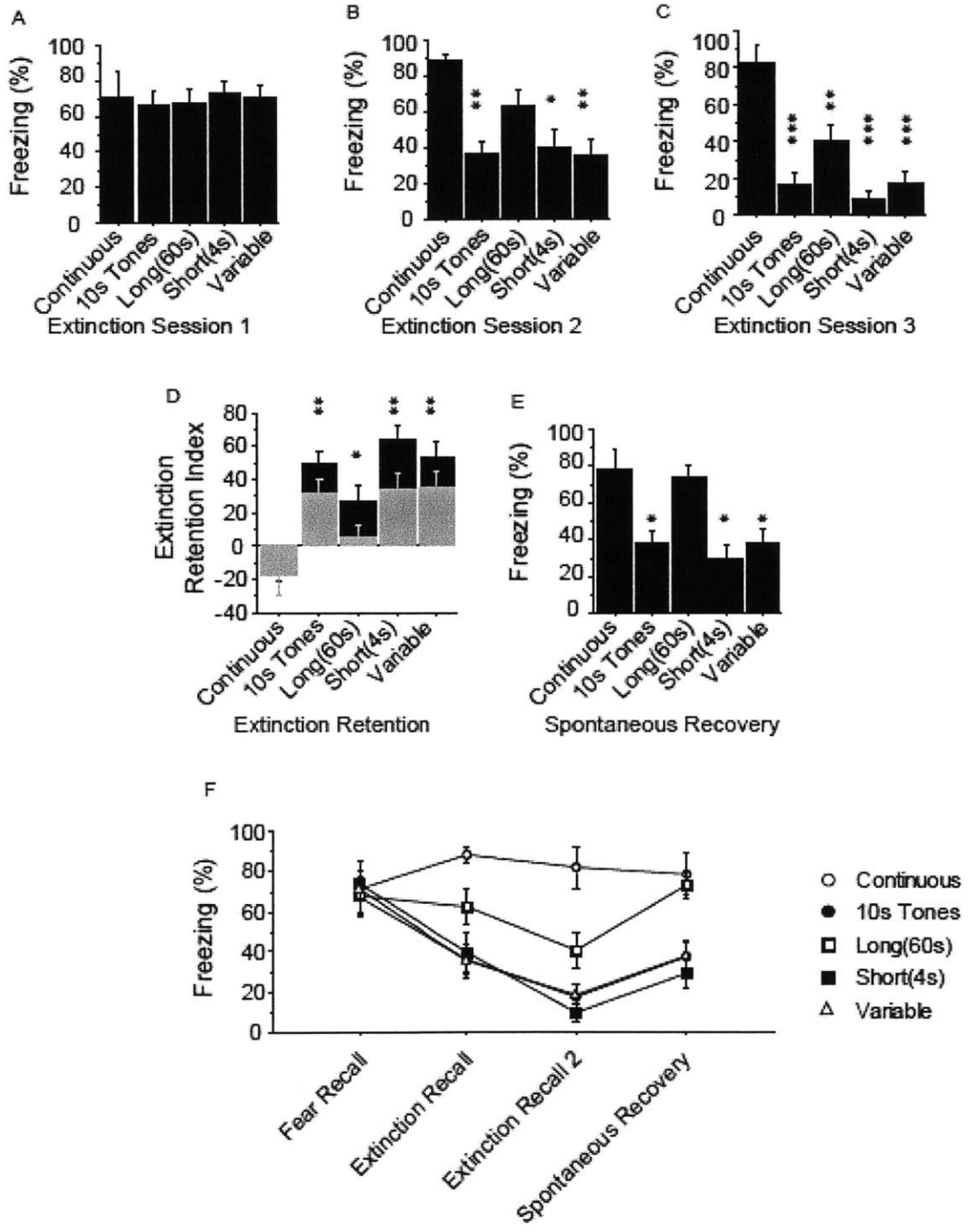

Figure 4. CS exposure paradigms that result in the best between-session extinction and decreased pS845 also prevent spontaneous recovery of fear. After fear conditioning and context extinction, fear to CS (tone) was extinguished. A variety of CS presentations were used for a total of 420s CS exposure: a continuous 7 minutes tone, seven 60s tones, forty-two 10s tones, forty-two 4s tones, or variable duration tones (range: 1s to 20s, mean: 10s). All inter-tone intervals were 60 seconds and all context exposure was equivalent (51 minutes). (A) Average freezing levels during first 60s of CS exposure during the first extinction session. The freezing levels demonstrate fear recall in the presence of the CS. (B) Average freezing levels during first 60s of CS exposure during the second extinction session. The freezing levels demonstrate extinction retention from the first extinction session. (C) Average freezing during first 60s of CS exposure during the third extinction session. Initial freezing levels demonstrate cumulative extinction retention from the first and second extinction sessions. (D) The Extinction Retention Index calculates the reduction in freezing behavior indicative of between-session extinction. Gray bars represent between-session extinction from the first and second extinction session. Black bars represent cumulative between-session extinction for all extinction sessions. (E) Average freezing levels during first 60s of CS exposure during spontaneous recovery test. The freezing levels demonstrate persistent extinction retention. (F) Summary of average freezing levels during first 60s of CS exposure of each extinction session and spontaneous recovery. Values represent mean±s.e.m., * $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$.

Discussion

We demonstrate that different presentations of a CS during extinction training can affect the strength of extinction of learned fear behaviors, as measured by between-session reduction of fear behavior. We show differences in between-session reduction of fear behavior across exposure paradigms, despite equivalent effects within a single extinction session (Fig. 1). The dissociation of within- versus between-session extinction has been raised as a concern in efficiency of exposure therapy and laboratory models (121). Previous work has dissociated within-session extinction from between-session extinction showing that progressive inhibition of fear behavior during an extinction training session is neither sufficient nor necessary for long-term extinction retention (134, 142). Our data corroborate those findings. The striking decrease in expression of the conditioned fear response during the first extinction session in response to two traditional CS exposure types (Fig. 1, continuous and discrete, 10s) suggest the decreased freezing within a single extinction session is dependent on cumulative duration of CS exposure and that the reaction to the Continuous Tone is not extinction learning but instead may be short-term habituation (143). Habituation is a distinct process from extinction learning and is a response to the present environment rather than a long-term associative process.

We extend previous work by demonstrating that a CS exposure paradigm that leads to significant between-session extinction retention also leads to dephosphorylation of pS845GluR1 and prevents spontaneous recovery of fear. This result suggests a persistent weakening of the original fear memory in optimized

extinction training. Finally, we establish that extinction retention and spontaneous recovery of fear depended upon the number of trials within a CS exposure paradigm, rather than predictability, matching to the CS used during fear conditioning, or even in total CS exposure duration.

Laboratory studies of fear disorders must be optimized to ensure that the models being tested are appropriate for the psychological mechanisms being studied. These optimizations should also aim to promote translational efficacy. Scientists use a variety of auditory fear conditioning and tone extinction paradigms across the field and even within individual laboratories. Indeed, procedural variations in CS exposure paradigms between experimenters can make replication difficult, both between and within a lab. The data presented here may help explain this phenomenon: some CS exposure paradigms may yield permanent inhibition of the fear response while other exposure paradigms do not (Figure 4). A researcher using a continuous tone paradigm may observe spontaneous recovery of fear, whereas a scientist using discrete tones may not. However, those using a continuous tone observe spontaneous recovery of fear at the cost of studying long-term between-session extinction, which may be more relevant for exposure therapy rather than an epiphenomenon of habituation.

Studies aiming to optimize extinction behavior have focused on inter-trial intervals and have found conflicting results: some note that closely spacing the CS exposure leads to better extinction while others demonstrate enhanced extinction with longer inter-stimulus intervals (ISI) (121, 144-146). One study has found that

differences in CS presentation can affect the strength of extinction retention (134). However, that work did not control for context exposure and used variable ISI that lead to profound differences in extinction context exposure. Here we maintain an ISI of 60s for each CS exposure paradigm (except Continuous Tone group which had no ISIs) and explored the characteristics of the CS presentation as the primary mediator of extinction efficacy. Surprisingly, we found that discrete, 4s tones yield similar results to discrete, 10s tones or variable tones despite substantially less CS exposure. Furthermore, the continuous tone and the discrete, 60s tones do not yield enduring between-session extinction despite equivalent CS exposure. Therefore, total number of CS exposures, or trial number, is more important than cumulative CS exposure.

A potential explanation for the above finding stems from the diversity of cellular responses within the amygdala. The examination of distinct amygdalar cell subtypes offers an explanation why extinction of fear responses may depend on the number of exposure trials. Neurons in the lateral amygdala do not show sustained firing during the presentation of a CS. Rather, subsets of neurons, referred to as "tone-on" and "tone-off" cells, are activated by either the start or end of the CS, respectively. Clearly, the CS exposure paradigms explored here would recruit these populations differently depending on trial number. More specifically, increasing or decreasing the number of CS exposures would result in these neurons being activated more or less, respectively. In this light, it is not surprising that the

increase in the number of discrete CS exposures was the most important characteristic of the CS exposure paradigm for between-session extinction retention.

If laboratory work studying extinction processes can be translated to the clinic, then these results suggest that exposure therapy would be more effective when more frequent exposures to stimuli are used. Interestingly, exposures during extinction training may be significantly shorter than the fear conditioning presentations while still achieving effective fear extinction. Clinically, shorter CS presentations during exposure therapy would have the added benefit of not requiring a patient to endure long bouts of fear engagement. Because exposure therapy often yields secondary conditioning and fear of the therapy itself, shorter yet more numerous exposures might result in less fear of exposure therapy and, therefore, higher compliance (147). The numerous, short exposures paradigm, while optimizing the learning procedures, may also have the effect of increasing the probability of finishing the recommended treatment course.

Another area of the brain implicated in extinction learning is the prefrontal cortex (PFC). Neurons in the PFC, unlike those in the amygdala, are active for the duration of the CS. The PFC projects to GABA-ergic cells within the amygdaloid complex which then synapse on cells in the lateral nuclei, inhibiting the fear expressing activity of the lateral amygdala. Much work has shown that these projections are necessary for extinction (128, 148). Although the work within this paper does not preclude the involvement of the PFC, it does demonstrate a significant amygdalar effect. It is likely that both the PFC and amygdala are

involved in effective extinction retention. We propose that extinction requires both weakening of the fear memory within the amygdala, and inhibition from the PFC. In other words, we hypothesize that the depotentiation of engrams within the lateral amygdala results in reduced CS-evoked activity in the amygdala that permits the depotentiated engrams to be further inhibited by projections from PFC and result in the reduced expression of fear behavior. Thus, the inhibitory association acquired as a result of the extinction may be gated by the strength of the amygdalar engram, and extinction depends on a strong CS-no US association as well as a weak CS-US memory. Within this framework, future work should assess the balance of the PFC inhibitory function and the amygdala engram strength on persistent inhibition of the CR.

Previous work has demonstrated that fear memories become labile after CS exposure (138-140, 149-152). Our results corroborate these findings and suggest that effective CS exposure paradigms may change memory strength of the original engram in the amygdala. We show that dephosphorylation of the AMPA receptor and long lasting weakening of the fear association in the case of the spontaneous recovery were possible with the proper CS presentation. AMPA receptor phosphorylation states and subsequent subunit compositional changes can lead to a synaptic depotentiation through regulated removal of AMPAR from post synaptic membranes (140, 150, 153-155). In the present study, optimization of extinction training led to profound changes in GluR1 phosphorylation states. This suggests that attributes of the discrete, 10s tone training paradigm permitted depotentiation

of the fear memory through alterations in GluR1 phosphorylation. Future studies should examine how this discrete, 10s tone paradigm can lead to such profound molecular changes. Comparing the effects of the 4s tones, 10s tones, and variable tones paradigms on pS845GluR1 state would test whether factors other than number of trials may affect the degree of depotentiation.

While many anxiolytics are available that treat the symptoms of fear based psychiatric illness, few pharmacological treatments permanently reverse these disorders. The most promising treatments available are cognitive behavioral therapies such as exposure therapy (118, 156-158). Clinicians have aimed to improve exposure therapy by prescribing a combination of pharmacological and behavioral interventions. However, a meta-analysis of adjuvant anxiolytic or anti-depressant pharmacology and therapy compared to therapy alone showed no added benefit of many of these compounds (159). Researchers anticipate that understanding the neural circuit underlying fear extinction will translate into the clinical realm in the form of better treatments and compounds. The goal is to treat anxiety disorders by augmenting the learning process that occurs during exposure-based procedures with cognitive enhancers. However, many of these adjuvants have not been effective in the clinic. Research has shown that GABA receptor agonist based treatments, such as benzodiazepines, interfere with the consolidation of the exposure therapy learning and have a high incidence of abuse. Yohimbine, an adrenergic agonist, has undesirable side-effects such as hyperarousal and increased blood pressure, in some cases exacerbating the illness (160). Furthermore, a number

of animal studies have failed to replicate the yohimbine augmentation effect, and some have even shown impairment in extinction learning (161). The most promising laboratory research investigated the use of a well tolerated NMDA receptor partial agonist, D-Cycloserine, which increases the level of extinction learning when administered prior to the CS exposure (162). However, the effects of D-Cycloserine on extinction are not well understood and results with human studies are mixed (163, 164). Based on the work in this paper and others, we suggest that treatments that lead to destabilization of the fear memory via molecular changes in the amygdala, rather than enhancement of the extinction learning of novel associations dependent on the PFC, may provide a promising avenue of investigation.

Summary

The first-line treatment for fear-related anxiety disorders is cognitive behavioral therapy. The current study used a Pavlovian fear conditioning model of associative fear and an extinction model of exposure therapy to explore differences across treatment paradigms for extinction training. We also ascertained neurobiological changes that were associated with a successful treatment paradigm. Our examination of different CS exposures revealed that numerous short exposures hold an advantage versus a single prolonged exposure to achieve optimal retention of extinction. This result may be rapidly evaluated for translation to human exposure therapy for fear related anxiety disorders. Furthermore, while adjuvant pharmacological treatments have been used in the clinic in the past, these have focused on preventing fear expression or strengthening the inhibitory extinction

memory. The current study suggests that pharmacological destabilization of the original fear engram within amygdalar circuits may be another promising avenue of adjuvant therapy.

Supplementary Discussion

Here, we measured the effects of five different CS extinction paradigms on the reduction of fear expression both within and between training sessions to determine whether different paradigms would yield extinction memories of differing strength. We found that the number of discrete CS presentations during extinction was the primary determinant of between-session extinction, with more trials leading to stronger extinction retention. Measures of within-session extinction, cumulative CS exposure during extinction, and total time spent in the extinction context were not predictive of between-session extinction *per se,* as robust within-session extinction, and lengthy cumulative CS or extinction context exposures were not sufficient to drive between-session extinction in the absence of large numbers of discrete CS presentations. Extinction paradigms that produced robust between-session extinction were preceded by dephosphorylation of AMPA receptors in the lateral amygdala, indicating destabilization of the original fear conditioning memory. Finally, robust between-session extinction was also followed by resistance to spontaneous recovery of the original fear memory. Collectively, this work demonstrates that extinction paradigms, even those that are quantitatively similar across many measures, vary widely in their ability to produce enduring fear extinction memories, and that paradigms yielding robust between-session extinction were associated both with molecular destabilization of the original fear memory and persistence of the extinction memory over weeks.

The idea that fear extinction can weaken the original, excitatory fear memory is not without controversy. Some studies have suggested that extinction engages

depotentiation-like mechanisms to actually reverse synaptic plasticity in the amygdala following fear conditioning (Kim et al., 2007; Hong et al., 2009; Hong et al., 2011). However, the mere existence of spontaneous recovery of fear with the passage of time following extinction training suggests that extinction only temporarily inhibits the expression of fear, leaving the original fear memory intact. This latter mechanistic model of extinction is compatible with a substantial literature showing that the medial prefrontal cortex (mPFC) is important for inhibiting fear expression following extinction training (Milad and Quirk, 2002; Vidal-Gonzalez et al., 2006; Maroun et al., 2012; Shehadi and Maroun, 2013). We suggest that both mechanisms are likely to be engaged by fear extinction, but the extent to which each mechanism is employed is influenced by the exact parameters used in the extinction procedure, with the number of distinct CS presentations being critical for determining whether the original fear memory trace is destabilized during extinction. Our results support the idea that extinction paradigms that lead to destabilization of the original memory trace are especially resistant to spontaneous recovery of the original fear memory (Klavir, Genud-Gabai, and Paz, 2012; Schiller et al., 2010).

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Chapter 5

Conclusions

Ghrelin-based therapeutics for mood disorders

The preponderance of stress research has focused on interactions with the hormones in the hypothalamus-pituitary-adrenal (HPA) axis. While these mediate many of the bodily responses to stressors, direct links to specific consequences of chronic stress are lacking. In Chapter 2, I explored the involvement of ghrelin and the hormones of the HPA axis on one consequence of stress: stress-exaggerated fear conditioning (SEFC). I found that the ghrelin pathway was ***necessary and sufficient*** for SEFC. Furthermore, I show that ghrelin-dependent increases in fear learning are ***independent of the HPA axis***, a finding that is novel to the field and challenges the dominant view that changes in the HPA axis mediate stress-related changes in fear (81, 110, 113, 165-207). Finally, I found that ghrelin-dependent increases in fear learning can be replicated with ***direct*** agonism in the amygdala. With these results, I suggest that anti-ghrelin treatments could prevent stress vulnerability to trauma-induced anxiety disorders such as PTSD.

The ghrelin pathway is an excellent pharmaceutical target because there are many junctures from gene to active peptide to receptor that are easily targeted by pharmaceuticals administered in the periphery (Conclusions Figure 1). Ghrelin is a peptide made, activated, and released from the A/X-like (P/D1 cells in human) endocrine cells of the mucosa of the glandular fundus of the stomach. Ghrelin production and release is stimulated by nutritional deficiency and suppressed by nutritional abundance. The preproghrelin gene encodes a 117 amino acid peptide. Once translated the peptide is translocated through the endoplasmic reticulum membrane followed by proteolytic cleavage of the N-terminal 23 amino acid signal

sequence to produce proghrelin. Proghrelin is then cleaved by **Prohormone Convertatases** (PC2 and Furin) to yield the 28 amino acid ghrelin protein. In this form, the ghrelin peptide is inactive and unable to be transported into the circulation. Post-translational addition of an octanoyl group must be added to the third serine residue by active enzymatic function of **ghrelin-O acyl-transferase**.

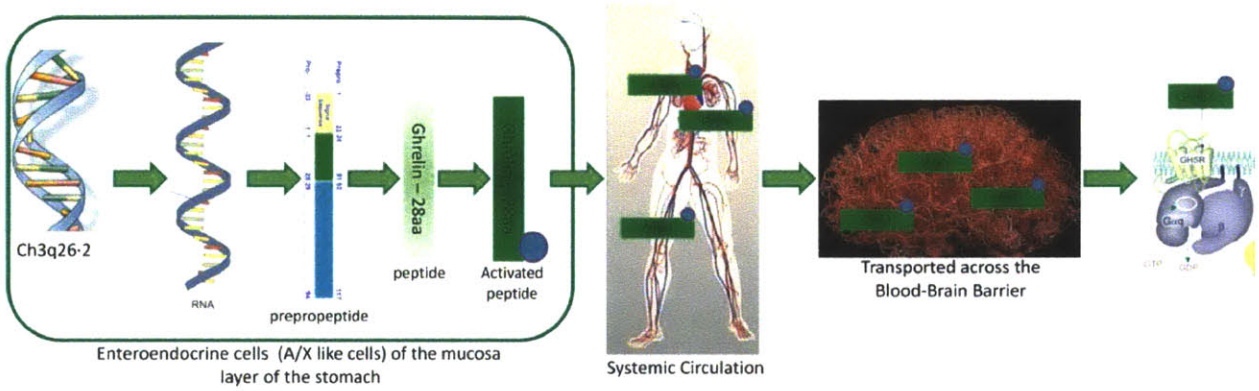


Figure 1. Ghrelin pathway from gene to receptor. *The ghrelin pathway provides many targetable junctures for pharmaceutical interventions.*

Once it is in its active, acylated form, acyl-ghrelin can now be **transported into circulation** where it binds to the Growth Hormone Secretagogue Receptor (GHSR1a, recently renamed the ghrelin receptor).

The **ghrelin receptor** is distributed across the brain and body. It is a g-protein coupled receptor (GPCR) that activates the G_q α-subunits though activation of the ghrelin receptor has also been found to engage the G_s α-subunit. It has been found that acyl-ghrelin is able to cross the blood brain barrier using a heretofore unidentified **saturable transporter**. Interestingly, the half-life of the active form of

the peptide is only 10-15 minutes in circulation. It is actively degraded by **butyrylcholinesterase** in circulation. The production, activation, and receptor activity of ghrelin is well-characterized and provides a plethora of targetable sites for pharmaceutical interventions.

In Chapter 2, I demonstrated that global blockade of the ghrelin receptor prevented stress-induced increases in fear learning. In the future, experiments should be performed to test whether blocking the ghrelin pathway at another junction is effective at preventing stress-exaggerated fear. Some of the most promising treatments available in the literature are ghrelin vaccines. Three are in development from laboratories in California, Texas, and Portugal (208-213). The vaccines create an immune response against ghrelin. When administered in animals, the immune system releases antibodies that attach to ghrelin and prevent it from being transported out of the bloodstream. This renders the peptide unable to cross the blood-brain barrier. Ghrelin vaccines were originally developed to prevent obesity but may be repurposed to prevent increases in anxiety and emotional reactivity after stress, thereby preventing stress exacerbation of mental illness. The vaccine is just one of many pharmacological agents that can be used to target the ghrelin system.

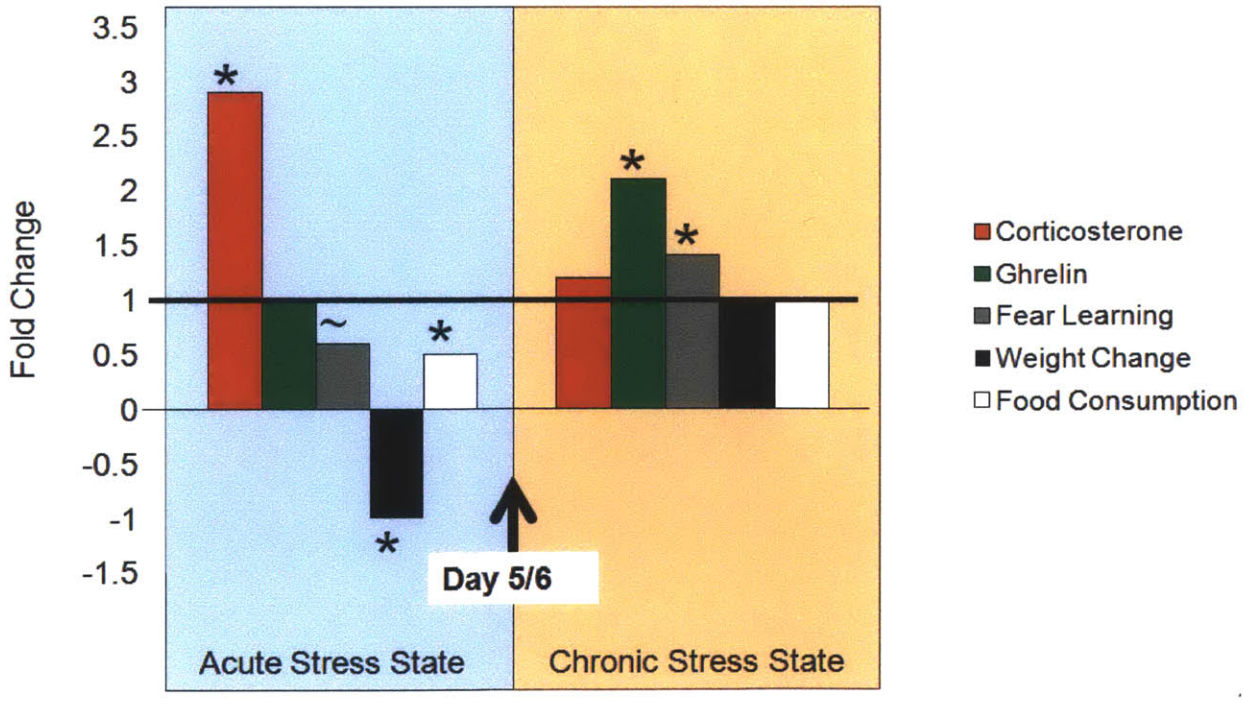
Updating allostatic load formulations

While studies have started to parse differential effects following acute and chronic stress, little is known about the timing of the shift from an acute to chronic stress state. In Chapter 3, I examined changes along several physiological measures

throughout cumulative stress exposure. Surprisingly, I found that the state changes were tightly aligned along the many measures. Unpredictably, I found that timing of the shift was stable even in the absence of the adrenal hormones. Collectively, these data show that physiological and hormonal stress responses are temporally coordinated and depend on cumulative exposure to stress.

Allostatic load is proposed to arise from the inappropriate recruitment of processes of allostasis. A healthy response is initiated by a stressor, sustained for an appropriate interval, and then turned off. Allostatic load is thought to arise in one of four proposed dysregulation conditions (*adapted from McEwen, 1998, (62)*). The first subtype of dysregulation of an allostatic system is when the physiological response is repeatedly recruited, thereby, repeatedly hitting the subject. Second, the system can fail to adapt appropriately, repeatedly responding with magnified response. Third, the system could fail to shut off in the presence of repeated stressor, exposing the subject to prolonged and unremitting response. Finally, dysregulation from repeated stress can lead to inadequate response in one system, thereby causing the other systems to compensate with hyperactive responses. The concepts behind allostasis and allostatic load do not require an alignment of the shift from adaptive to dysregulated across different physiological measures. However, our data suggest an uncanny synchrony across changes in growth, feeding, and hormones which I deemed the "pivot point". A parsimonious explanation is that the changes seen in feeding and growth are the results of a hormonal "tug of war" or shift in "hormonal balance." Initially, during the earlier

stress sessions, one set of hormones is strongest and exert more force on the behavior and physiology of the animal. However, the changes in these systems begin to diminish and, over repeated sessions, a second set of hormones comes to coordinate the response. When reviewing the results in Chapter 3 (summarized in Conclusions Figure 2), corticosterone and growth hormone are most altered during short-term stress. After the sixth session of stress, corticosterone recruitment begins to decline and growth hormone begins to return to normal. At the same time, ghrelin increases and leptin deficiency become more significant and dominate the response to subsequent stress sessions. It is this shift in dominance that I suggest represents a state-change: the new hormone balance represents a chronic stress state. Correspondingly, this is the time point at which feeding and growth return demonstrating the orexigenic dominance of these hormones.



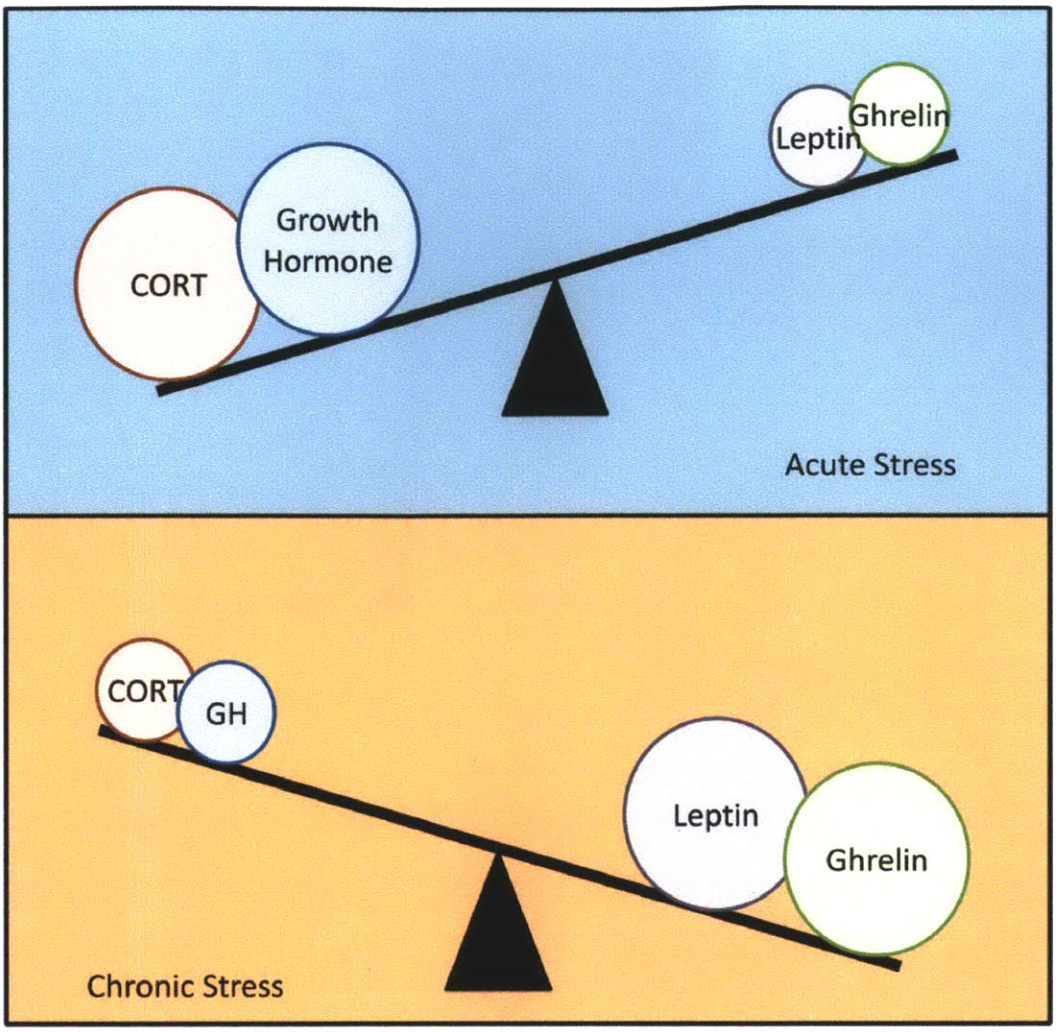


Figure 2. The shift from acute to chronic stress state in hormonal signatures. *Summary of Chapter 3.*

The traditional method of observing stress responses in static snapshots was excellent at establishing the different phenotypes seen after acute or chronic stress exposure. Here I demonstrate a clear benefit to observing measures across time rather than at single, post-treatment time points. These data demonstrate that physiological responses gradually change over cumulative stress exposure, a finding that required daily sampling to discover. Unfortunately, studies of this type require

increased number of subjects and are time intensive for the researcher. However, the extra demands on data collection are necessary for such findings and would allow future experiments to complement the potential of this initial data set.

The work in this thesis suggests important changes in the use and calculation of allostatic load scores. Currently, allostatic load comprises of a score as calculated using ten measures. The allostatic load score is used to predict a single outcome: death. However, high allostatic load measures are not only predictive of mortality but have been shown to be highly correlated with several disease states such as mild cognitive impairment associated with aging, metabolic syndrome, and cardiovascular disease. These disease outcomes may be lost in the current death-dependent formulations and may require alterations to the calculation of allostatic load. The first change to the equation of allostatic load I would suggest is the creation of several ***disease specific equations***. As mentioned above, the score is taken at one time-point In Chapter 3 I established that measures may change over time and if taken at a single point in time will not provide evidence of an increasing or decreasing trajectory. I suggest using patients' ***scores across time*** to calculate allostatic load. The measures used could be run during annual doctor visits and the score of allostatic load calculated across the lifetime of the patient.

All measures included in the calculation of allostatic load are given binary scores: a score of '1' if the patient's results are in the highest quintile on that measure or '0' if they are not. The dependency on a binary scoring system blunts the nuances that may be seen by examining hormones in 'balance'. In other words, I

suggest ***relative scoring***: formulating the equation for calculating allostatic load such that the scores depend on their levels relative to the other hormones. Finally, the 10 measures of allostatic load currently used today are highly HPA dependent. As the work in Chapters 2 and 3 suggests, stress and vulnerability to develop stress exacerbated illnesses may be dependent on other hormonal pathways. This does not preclude the heavy involvement of the HPA measures currently used, but rather suggests that allostatic scores ***include a greater range of important pathways.***

$$f(x,t) = \alpha_t(corticosterone) + \beta_t(ghrelin) + \gamma_t(growth\ \ hormone) + \delta_t(leptin) + \varepsilon_t(NPY) + \ldots$$

Figure 3. Proposed changes to the calculation of allostatic load in the clinic.

1. ***f*(x)** - independent functions: the function can be any effect of stress, body weight, cardiovascular disease development, or vulnerability to the development of psychiatric disorders.
2. ***t*** - time: the measures should be observed over time to follow changes and evolution of the state of allostatic load.
3. ***α, β, etc*** - the weighting coefficients: Each function would depend on differential weighting of the hormones involved. This may include a weight of zero in cases where the disease is independent of the pathway. They can also be used to alter the dependence of the balance amongst the measures.
4. ***Ghrelin, etc*** - the measures used: More pathways should be included in the calculation of allostatic load as new findings reveal HPA independence of many of the disease symptoms.

Stress responses are complicated due to their multifaceted nature. However, if given enough observations, doctors could one day be able to predict the response to the subsequent stress given the current state of the hormones, such as in equation above. In order to bring such predictors to fruition, many observations and

measures are needed. Here we identify several hormonal signatures as they evolve across stress. Further study is needed to complete the potential of this initial data set and translate the findings to patients in the clinic.

Improving Exposure Therapy

Fear and fear based psychiatric disorders are often treated with cognitive behavioral therapy or, more specifically, exposure therapy. During exposure therapy, patients confront the object or places associated with fear or anxiety. Strategies enhancing extinction learning and its retrieval over time are necessary for enhancing clinical efficacy of exposure therapy. Fear conditioning and extinction learning serve as laboratory models of phobia and exposure therapy, respectively. In Chapter 4, I use these models of fear and exposure therapy to identify stimulus exposure paradigms that yield better and longer lasting reduction of fear. The results show that using the maximal number of discrete stimulus exposures prevented spontaneous recovery of fear, even when reduced cumulative stimulus exposure was used. Furthermore, it demonstrates that one molecular difference between stimuli presentations that yield transient fear inhibition and presentations that yield persistent fear inhibition is a significant decrease of pS845 GluR1. The results demonstrate that a lasting weakening of the fear memory can be achieved by presenting many discrete stimulus exposures during extinction training.

This work may be rapidly evaluated for translation to human exposure therapy for fear related anxiety disorders. It suggests that during exposure therapy, rather than presenting the stimulus for a long time, patients should be presented

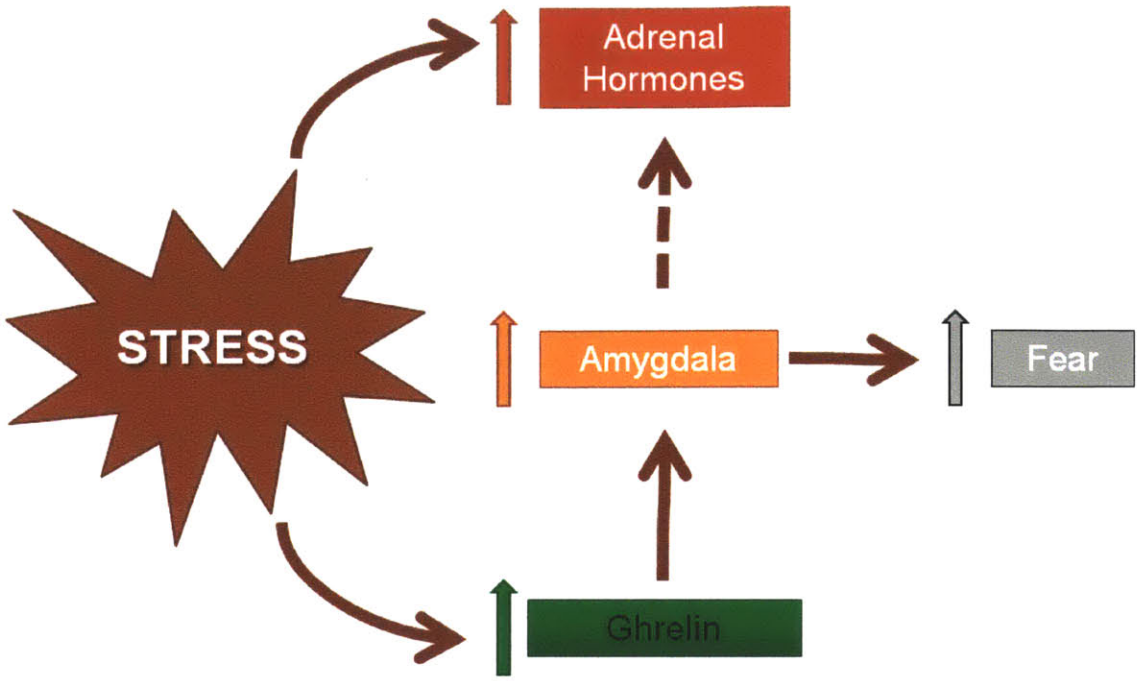
the stimulus several times in a single session. These exposures can be short in duration, as demonstrated by experiment four of Chapter 4. If the exposures are shorter, they may provoke less fear which, in turn, could promote great compliance for the course of exposure therapy.

Work from this chapter also suggests that multiple mechanisms are occurring in parallel during extinction of fear. The prefrontal cortex has been shown to be necessary to inhibit expression of fear behaviors. Here I suggest that weakening of the excitatory circuits in the amygdala is also part of the extinction process. Fear memory destabilization in the amygdala may be permissive of the inhibition from the cortex. Future work will have to examine this possibility. Furthermore, while adjuvant pharmacological treatments have been used in the clinic in the past, these have focused on preventing fear expression or strengthening the inhibitory extinction memory. The current study suggests that pharmacological destabilization of the original fear engram within amygdalar circuits may be another promising avenue of combination pharmacological and psychological therapy.

Final thoughts

The finding that the emotional dysregulation after stress is independent of the HPA axis is surprising within the current paradigm. Though the experiments that were performed to establish the independence of these consequences of stress from these pathways were simple, they went against the current school of thought and so had not been done until now. It is widely held that the hormones of the HPA axis and especially the adrenal hormones are the coordinators and exacerbators of the stress response and, therefore, would play a role in each of the consequences. The work with the animals who had undergone adrenalectomy demonstrates otherwise.

But this is not to say that decades of findings are incorrect. Indeed, patients with psychiatric illnesses display dysregulation of their HPA hormones. However, these appear to be symptoms of the disorders and, while tightly correlated, not causal. As I mentioned in the first chapter of this thesis, amygdalar hyperactivity could cause this HPA dysregulation. The amygdala activates the HPA axis. As the HPA activity increases, so do the basal levels of adrenal hormones. The adrenal hormones act as negative feedback, inhibiting the HPA. Therefore, the dysregulation of the HPA axis could be the body's coping mechanism as it aims to down-regulate the cascade. The recruitment of ghrelin after periods of chronic stress could be creating the overactive amygdala and, therefore, creating the dysregulation of the HPA axis. Further work is needed to explore this hypothesis.



Finally, emotional dysregulation is a component of a wide number of mental illnesses exacerbated by stress. *By understanding how stress alters an area of the brain responsible for emotional memory, the* ***amygdala****, we may be able to glean how stress increases susceptibility to the development of these psychiatric disorders.*

Further Questions:

How long will it take the animal to recover from the chronic stress treatment?

Recovery after stress is a very interesting question that has been explored by myself, members of the Goosens lab, and other labs. As I mentioned in the introduction, the amygdala, hippocampus, and prefrontal cortex are each altered by stress. In the amygdala, neurons undergo hypertrophy extending more dendrites with more branches and more synapses. The prefrontal cortex and hippocampus, conversely, demonstrate atrophy, loss of dendrite complexity, and decreased number of spines. Animal experiments have demonstrated that the prefrontal cortex will regain pre-stress levels of dendritic branching within 10 days and neurons of the hippocampus will return to non-stressed levels of complexity within 14 days but, in the case of the hippocampus, this recovery will not fully resemble the non-stressed state with shorter primary dendrites and altered synapse distribution. The amygdala, however, does not appear to recover. Researchers have looked as far as two months after the cessation of stress. In the rat, this is a significant portion of its lifespan. The hypertrophy of the amygdalar neurons is maintained. **This demonstrates that, while the hippocampus and prefrontal cortex will return upon cessation of stress, the amygdala is maintained in this hypertrophic state.** This suggests that the mechanisms responsible for these sequelae of chronic stress differ as well.

Furthermore, amygdala dependent fear conditioning has been shown to have stress-enhanced effects as long as 60 days after the end of the stressor. I have

examined persistent elevation of ghrelin levels two months after the cessation of stress and found that the levels are still significantly elevated and, in fact, are at levels equivalent with those seen immediately after stress. I propose that ghrelin may mediate the maintenance of the amygdalar hypertrophy and promote this long-term increase in stress-enhanced fear conditioning.

How is circulating ghrelin increased?

Ghrelin is a peptidyl hormone that is processed through the endoplasmic reticulum and acted on by several enzymes in order to cleave and acylate the peptide for secretion and activity. Several factors regulate ghrelin's translation and release. It has been found that the increases in endogenous medium chain fatty acids (MCFAs), such as levels that occur when free glucose has been depleted and adipose tissue becomes the main source of energy, provide ghrelin's activating enzyme, GOAT, with the substrate to activate ghrelin. Therefore, metabolic state regulates ghrelin activation. The autonomic nervous system, a major system recruited during stressor exposure, has also been found to play a role in ghrelin secretion and release. Stimulation of gut sympathetic nerves and exogenous administration of catecholamines have also been shown to increase ghrelin secretion. Furthermore, vagotomy has been shown to reduce circulating ghrelin levels while vagal stimulation increases ghrelin in the circulation. Finally, due to the fast deactivation of acylated-ghrelin to its inactive des-acyl form (the half-life is under 15 minutes), I hypothesize that the increases in ghrelin after chronic stress are due to increases in production and release rather than decreases in catabolism.

Summary

The objective of this thesis was to examine the effects of chronic stress on an area of the brain important for emotional memory, the amygdala. As an outcome of the investigations within, we now better understand the molecular mechanisms involved chronic stress that underlie alterations in fear learning. This thesis explores the recruitment of several hormonal pathways as stress duration becomes chronic or prolonged in nature (Chapter 3). The work herein also suggests risk factors and biomarkers that would indicate when people are most vulnerable to stress-induced exacerbation of a psychiatric illness (Chapters 2 and 3). Further, it provides evidence of pharmacological preventions of increased emotional reactivity after chronic stress (Chapter 2) as well as a behavioral therapy paradigm that could alleviate suffering of those with fear and anxiety disorders (Chapter 4). This thesis represents novel findings in the field of neuroscience and comprises significant contributions to our understanding of nature.

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Declaration of Data Contributions

Chapter 2:

- Retsina M. Meyer conceived experiments, designed protocols, performed experiments, and completed histology and data analyses for Figs 1, 2, 3, 4, SF2, SF3, SF5, SF6, SF7 - all panels, as well as SF1 panel a and SF4 panel c.
- Goosens Lab members contributed data for Fig. 5, SF1 panel b, and SF8.

Chapter 3:

- Retsina M. Meyer conceived experiments, designed protocols, performed surgeries, collected and tested samples, and performed data analysis and statistics, for Figs 1, 2, 3, 4, 5, and SF2.
- Goosens Lab members contributed data for Supplementary Figure 1.

Chapter 4:

- Retsina M. Meyer conceived experiments, designed protocols, collected and tested samples, and performed data analysis and statistics, for all figures.

Appendix A:

- Retsina M. Meyer conceived experiments, designed protocols, collected and tested samples, and performed data analysis and statistics, for all figures.

Appendix A

Prolonged effects of chronic stress

Specific consequences of a traumatic event, such as nightmares, avoidance, and hyperarousal, are exhibited within large sections of the population after trauma and represent a normal response to distress. However, the majority of the population will return to normal within days or weeks. The persistence of these symptoms represents an aspect of the PTSD pathology. Here I demonstrate that in the laboratory model of psychological stress, effects can also be long lasting.

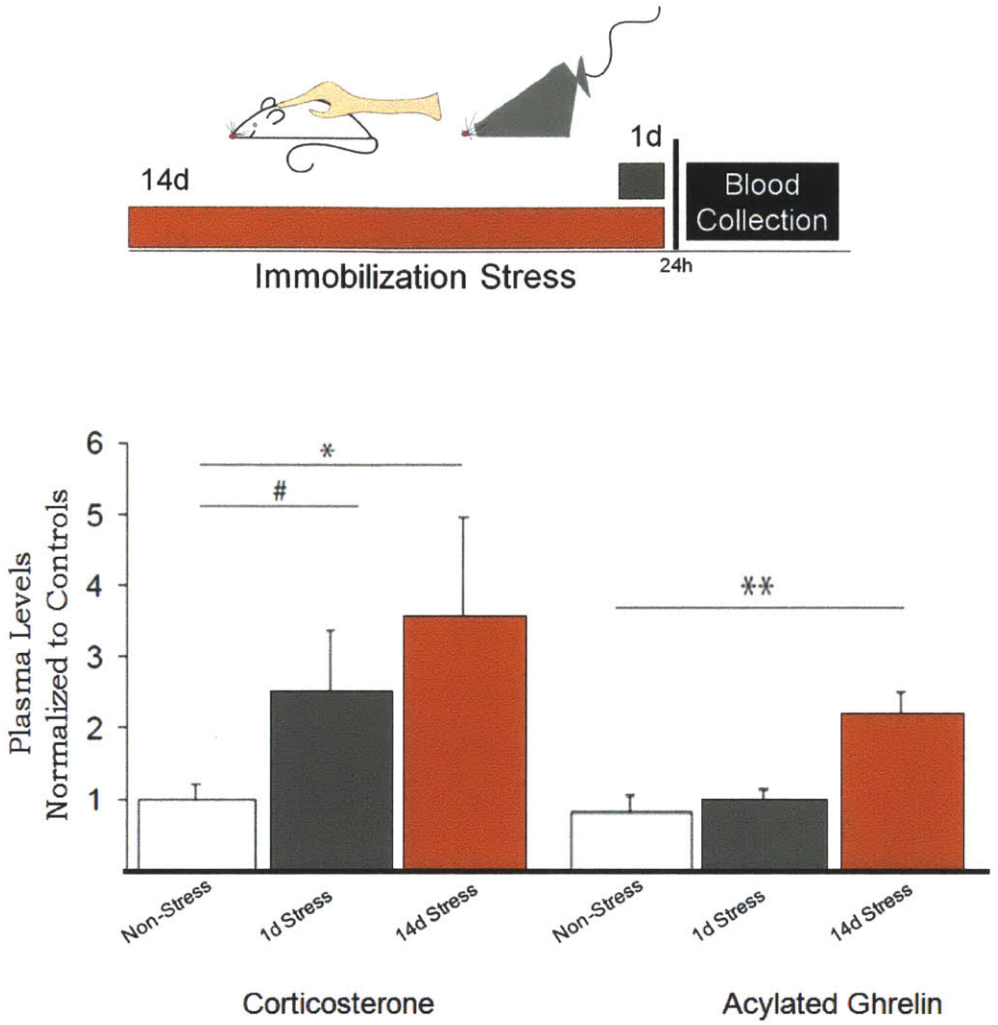

Figure 1. Corticosterone is elevated after acute and chronic stress whereas acylated-ghrelin is uniquely elevated after chronic stress.
Animals underwent 1d or 14d of immobilization stress (Stress) or daily handling (Non-stress). The day after the last stress session, animals were sacrificed and trunk blood fractionated. Corticosterone was elevated after both 1d or 14d of immobilization stress compared to non-stressed controls. Ghrelin was elevated only in the 14d immobilization treatment group. All data are mean ±s.e.m. # P <0.10 * P <0.05 ** P <0.01

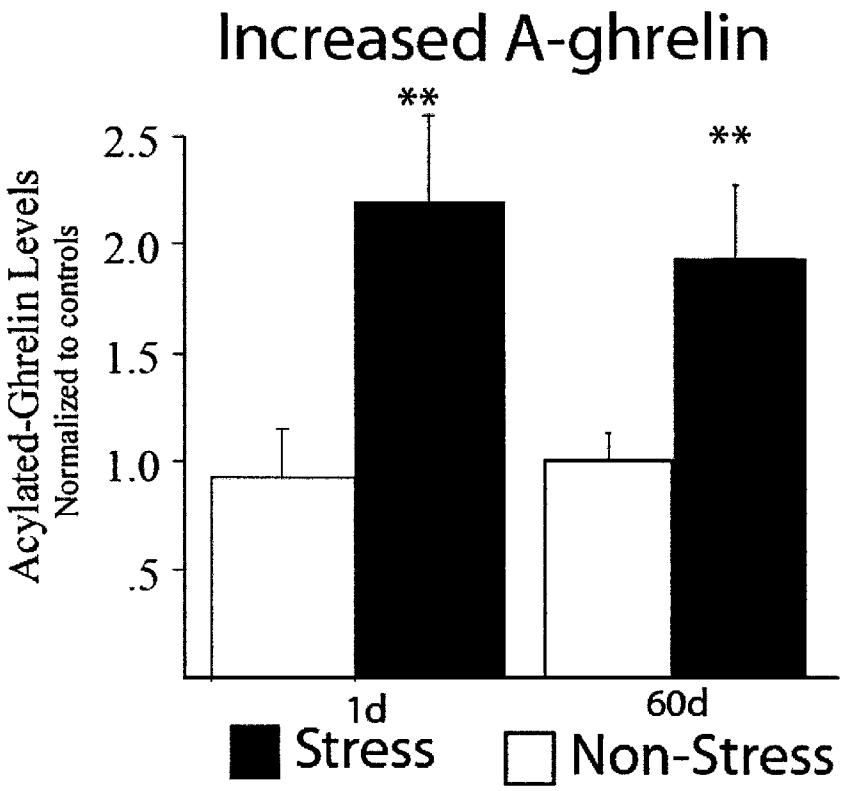

Figure 2. Acylated-Ghrelin levels 1 day and 60 days after stress cessation.
Animals underwent 14d of chronic immobilization stress (Stress) or daily handling (Non-stress). Blood samples were taken 1d or 60d after cessation of stress. These results demonstrate that ghrelin changes are long lasting and may contribute to stress-induced vulnerability to exaggerated fear long after stress exposure has stopped. All data are mean ±s.e.m. ** $P < 0.01$

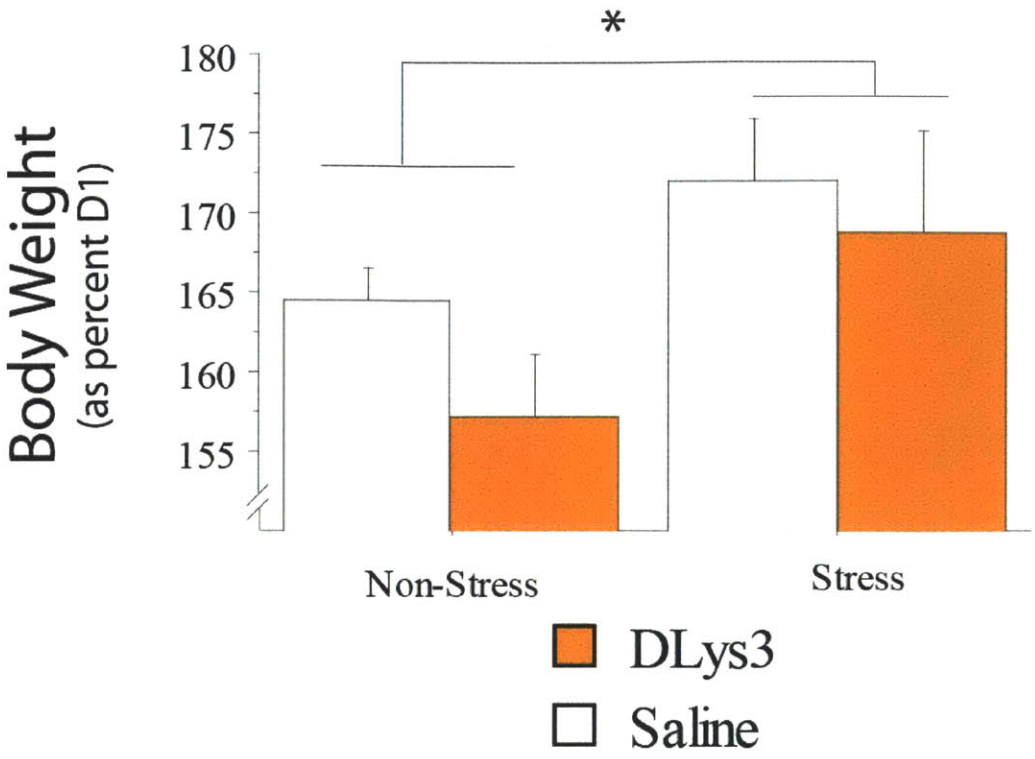

Figure 3. Body weight 130 days after stress cessation.
Animals underwent 14d of chronic immobilization stress (Stress) or daily handling (Non-stress). Animals were administered either saline or the ghrelin receptor inverse agonist DLys3-GHRP6 daily throughout stress. After stress ended, drugs were no longer administered. Body weight was measured 130 days after the end of stress. Stress animals weighed significantly more than Non-Stress animals. These results demonstrate that stress alters weight gain long after stress exposure has ended. Additionally, animals treated with DLys3 during stress did not differ from Stress animals treated with saline, indicating the drug did not alter stress-induced changes in weight gain. All data are mean ±s.e.m. * $P < 0.05$

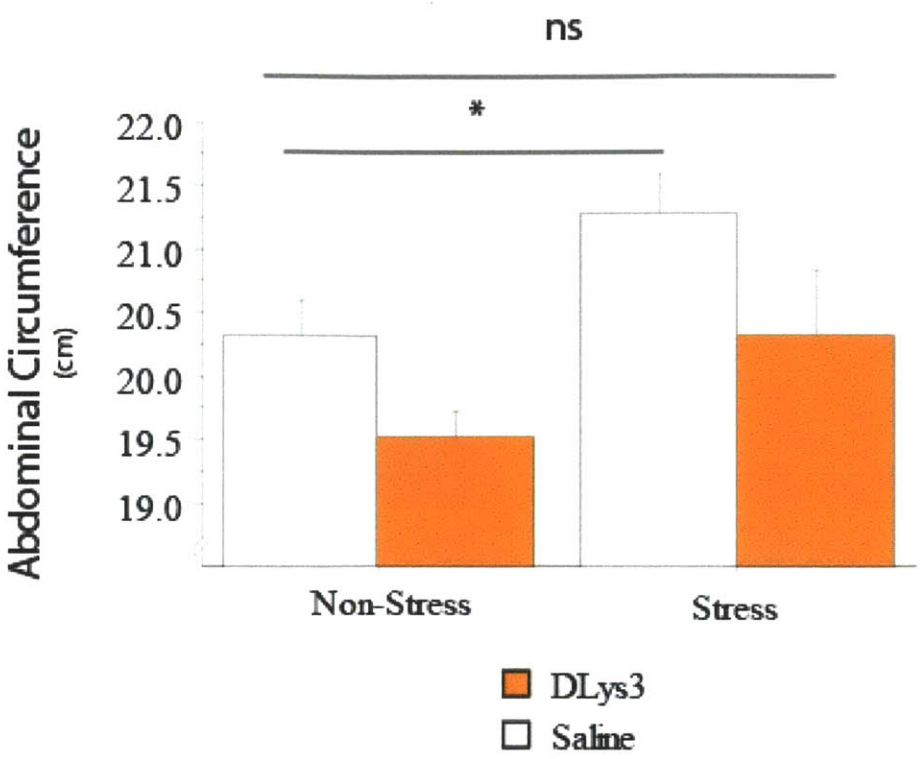

Figure 4. Abdominal circumference 130 days after stress cessation.
Animals underwent 14d of chronic immobilization stress (Stress) or daily handling (Non-Stress). Animals were administered either saline or the ghrelin receptor inverse agonist DLys3-GHRP6 daily throughout stress. After stress ended, drugs were no longer administered. Prone abdominal circumference was measured 130 days after the end of stress. These results demonstrate that stress alters distribution of body weight long after stress exposure has ended. Additionally, animals treated with DLys3 during stress did not differ from Non-Stress control animals, indicating the drug prevented this change in body morphology. All data are mean ±s.e.m. * $P < 0.05$

Appendix B

Use of antagonists of ghrelin or ghrelin receptor to prevent or treat stress-sensitive psychiatric illness

The following section contains a summary of the U.S. and World patent filed based on the work in Chapter 2 and 3 in this thesis filed in February 2013 (provisional patent U.S.S.N. 61/595,845, filed February 7, 2012, entitled "USE OF ANTAGONISTS OF GHRELIN OR GHRELIN RECEPTOR TO PREVENT OR TREAT STRESSSENSITIVE PSYCHIATRIC ILLNESS")

ABSTRACT

The invention relates to methods of protecting against chronic stress in a subject and treating stress sensitive disorders in a subject by antagonizing ghrelin or ghrelin receptor.

FIELD OF THE INVENTION

The invention relates to antagonism of ghrelin or ghrelin receptor to protect against, treat or prevent disorders associated with chronic stress.

SUMMARY OF INVENTION

Ghrelin is a peptide hormone produced primarily by gastrointestinal cells. Receptors for ghrelin are highly expressed in regions of the hypothalamus that control feeding. Accordingly, ghrelin has been extensively studied for its ability to induce feeding behavior. However, ghrelin receptors are also expressed in other brain regions not traditionally associated with feeding behavior, such as the hippocampus. Ghrelin signaling is linked to obesity, diabetes and cardiovascular function. It has also been reported that increasing the levels of ghrelin leads to anti-depressant effects and that mice carrying a null mutation in the ghrelin receptor have increased depressive symptoms, suggesting that active ghrelin signaling has anti-depressant activity (*13*).

Here, it is surprisingly shown that ghrelin, in fact, enhances, rather than ameliorates, the deleterious effects of chronic stress. Ghrelin was found to be both necessary and sufficient for stress-induced vulnerability to excessive fear. Accordingly, in contrast to previous reports, antagonizing, rather than activating ghrelin signaling, is desirable for combatting stress-related symptoms.

Aspects of the invention relate to methods of protecting against chronic stress in a subject

in need thereof, including administering to the subject a therapeutically effective amount of an agent that inhibits the level or activity of ghrelin or ghrelin receptor. In some embodiments, the agent is administered before, during and/or after exposure of the subject to chronic stress.

In some embodiments, the agent targets the ghrelin receptor. In certain embodiments, the agent is a GHSr1a antagonist or a GHSr1a inverse antagonist. In other embodiments, the agent targets ghrelin. In certain embodiments, the agent is an anti-ghrelin vaccine. In other embodiments, the agent targets ghrelin O-acyltransferase (GOAT). In certain embodiments, the agent is an anti-GOAT vaccine. In other embodiments, the agent is a compound that reduces or inhibits the synthesis or release of ghrelin by the stomach. In other embodiments, the agent is a compound that reduces or prevents ghrelin from crossing the blood-brain barrier. In other embodiments the treatment involves reducing levels in circulation using agents that inactivate (deacylate) ghrelin or by agents that increase plasma esterases responsible for endogenous ghrelin deacylation, such as APT1 and other putative esterases. Thus, esterases such as APT1 are agents targeting ghrelin or the ghrelin receptor according to the invention.

In some embodiments, the chronic stress is associated with military service or a natural disaster. In some embodiments, the chronic stress is associated with low socioeconomic status. In some embodiments, the chronic stress is associated with acutely traumatic experiences.

Further aspects of the invention relate to methods of treating a stress-sensitive disorder in a subject exposed to chronic stress, including administering to the subject a therapeutically effective amount of an agent that inhibits the level or activity of ghrelin or ghrelin receptor. In some embodiments, the stress-sensitive disorder is post-traumatic stress disorder (PTSD), depressive disorder, major depressive disorders, bipolar disorder, acute stress disorder,

generalized anxiety disorder, obsessive-compulsive disorder, schizophrenia , panic disorders or trichotillomania. In some embodiments, the agent is administered before, during and/or after exposure of the subject to chronic stress. In some embodiments, the stress-sensitive disorder is not schizophrenia.

In some embodiments, the agent targets the ghrelin receptor. In certain embodiments, the agent is a GHSr1a antagonist or a GHSr1a inverse antagonist. In other embodiments, the agent targets ghrelin. In certain embodiments, the agent is an anti-ghrelin vaccine. In other embodiments, the agent targets ghrelin O-acyltransferase (GOAT). In certain embodiments, the agent is an anti-GOAT vaccine. In other embodiments, the agent is a compound that reduces or inhibits the synthesis or release of ghrelin by the stomach. In other embodiments, the agent is a compound that reduces or prevents ghrelin from crossing the blood-brain barrier. In some embodiments, the chronic stress is associated with military service or a natural disaster. In some embodiments the subject is not overweight or obese.

Further aspects of the invention relate to methods for determining whether a subject exposed to chronic stress has an increased risk of developing a stress-sensitive disorder, including conducting an assay to measure the ghrelin levels in the subject after the subject has been exposed to chronic stress, wherein elevation of ghrelin levels in the subject after the subject has been exposed to chronic stress relative to normal ghrelin levels indicates that the subject has an increased risk of developing a stress-sensitive disorder.

In some embodiments, normal ghrelin levels correspond to ghrelin levels in the subject prior to exposure to chronic stress. In some embodiments, ghrelin levels are measured at multiple time points after the subject has been exposed to chronic stress to detect long-term elevation of ghrelin levels. In some embodiments, the assay is performed on a blood sample

from the subject. In some embodiments, if the subject has elevated ghrelin levels after exposure to chronic stress, then the subject is administered a therapeutically effective amount of an agent that inhibits the level or activity of ghrelin or ghrelin receptor.

In some embodiments, the stress-sensitive disorder is post-traumatic stress disorder (PTSD), depressive disorder, major depressive disorders, bipolar disorder, acute stress disorder, generalized anxiety disorder, obsessive-compulsive disorder, panic disorders, schizophrenia or trichotillomania.

In some embodiments, the agent targets the ghrelin receptor. In certain embodiments, the agent is a GHSr1a antagonist or a GHSr1a inverse antagonist. In other embodiments, the agent targets ghrelin. In certain embodiments, the agent is an anti-ghrelin vaccine. In other embodiments, the agent targets ghrelin O-acyltransferase (GOAT). In certain embodiments, the agent is an anti-GOAT vaccine. In other embodiments, the agent is a compound that reduces or inhibits the synthesis or release of ghrelin by the stomach. In other embodiments, the agent is a compound that reduces or prevents ghrelin from crossing the blood-brain barrier. In some embodiments, the chronic stress is associated with military service or a natural disaster.

These and other aspects of the invention, as well as various embodiments thereof, will become more apparent in reference to the drawings and detailed description of the invention.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention.

What is claimed is:

CLAIMS

1. A method of protecting against chronic stress in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of an agent that inhibits the level or activity of ghrelin or ghrelin receptor.
2. The method of claim 1 wherein the agent is administered before, during and/or after exposure of the subject to chronic stress.
3. The method of claim 1 or 2 wherein the agent targets the ghrelin receptor.
4. The method of claim 3 wherein the agent is a GHSr1a antagonist or a GHSr1a inverse antagonist.
5. The method of claim 1 or 2 wherein the agent targets ghrelin.
6. The method of claim 5 wherein the agent is an anti-ghrelin vaccine.
7. The method of claim 1 or 2 wherein the agent targets ghrelin O-acyltransferase (GOAT).
8. The method of claim 7, wherein the agent is an anti-GOAT vaccine.
9. The method of claim 1 or 2 wherein the agent is a compound that reduces or inhibits the synthesis or release of ghrelin by the stomach.
10. The method of claim 1 or 2 wherein the agent is a compound that reduces or prevents ghrelin from crossing the blood-brain barrier.
11. The method of any one of claims 1-9 wherein the chronic stress is associated with military service or a natural disaster.
12. A method of treating a stress-sensitive disorder in a subject exposed to chronic stress comprising administering to the subject a therapeutically effective amount of an agent that inhibits the level or activity of ghrelin or ghrelin receptor.

13. The method of claim 12 wherein the stress-sensitive disorder is post-traumatic stress disorder (PTSD), depressive disorder, major depressive disorders, bipolar disorder, acute stress disorder, generalized anxiety disorder, obsessive-compulsive disorder, panic disorders, schizophrenia, or trichotillomania.
14. The method of claim 12 or 13 wherein the agent is administered before, during and/or after exposure of the subject to chronic stress.
15. The method of any one of claims 12-14 wherein the agent targets the ghrelin receptor.
16. The method of claim 15 wherein the agent is a GHSr1a antagonist or a GHSr1a inverse antagonist.
17. The method of any one of claims 12-14 wherein the agent targets ghrelin.
18. The method of claim 17 wherein the agent is an anti-ghrelin vaccine.
19. The method of any one of claims 12-14 wherein the agent targets ghrelin Oacyltransferase (GOAT).
20. The method of claim 19, wherein the agent is an anti-GOAT vaccine.
21. The method of any one of claims 12-14 wherein the agent is a compound that reduces or inhibits the synthesis or release of ghrelin by the stomach.
22. The method of any one of claims 12-14 wherein the agent is a compound that reduces or prevents ghrelin from crossing the blood-brain barrier.
23. The method of any one of claims 12-21 wherein the chronic stress is associated with military service or a natural disaster.
24. A method for determining whether a subject exposed to chronic stress has an increased risk of developing a stress-sensitive disorder, comprising conducting an assay to measure the ghrelin levels in the subject after the subject has been exposed to chronic stress, wherein

elevation of ghrelin levels in the subject after the subject has been exposed to chronic stress relative to normal ghrelin levels indicates that the subject has an increased risk of developing a stress-sensitive disorder.

25. The method of claim 24 wherein normal ghrelin levels correspond to ghrelin levels in the subject prior to exposure to chronic stress.

26. The method of claim 24 or 25 wherein ghrelin levels are measured at multiple time points after the subject has been exposed to chronic stress to detect long-term elevation of ghrelin levels.

27. The method of any one of claims 24-26 wherein the assay is performed on a blood sample from the subject.

28. The method of any one of claims 24-27 wherein if the subject has elevated ghrelin levels after exposure to chronic stress, then the subject is administered a therapeutically effective amount of an agent that inhibits the level or activity of ghrelin or ghrelin receptor.

29. The method of any one of claims 24-28 wherein the stress-sensitive disorder is post-traumatic stress disorder (PTSD), depressive disorder, major depressive disorders, bipolar disorder, acute stress disorder, generalized anxiety disorder, obsessive-compulsive disorder, panic disorders, schizophrenia, or trichotillomania.

30. The method of claim 28 or 29 wherein the agent targets the ghrelin receptor.

31. The method of claim 30 wherein the agent is a GHSr1a antagonist or a GHSr1a inverse antagonist.

32. The method of claim 28 or 29 wherein the agent targets ghrelin.

33. The method of claim 29 wherein the agent is an anti-ghrelin vaccine.

34. The method of claim 28 or 29 wherein the agent targets ghrelin O-acyltransferase (GOAT).

35. The method of claim 34, wherein the agent is an anti-GOAT vaccine.

36. The method of claim 28 or 29 wherein the agent is a compound that reduces or inhibits the synthesis or release of ghrelin by the stomach.

37. The method of claim 28 or 29 wherein the agent is a compound that reduces or prevents ghrelin from crossing the blood-brain barrier.

38. The method of any one of claims 24-36 wherein the chronic stress is associated with military service or a natural disaster.