Inhibition of Diacylglycerol Lipase Impairs Fear Extinction in Mice

By

Victoria S. Cavener

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

September 30, 2018

Nashville, Tennessee

Approved:

Bruce Carter, Ph.D.

Danny Winder, Ph.D.

Ron Emeson, Ph.D.

Sachin Patel M.D., Ph.D.

Dedicated in loving memory of: Thomas Lewis Cavener and Richard Scott Bryant.
I kept my promise to you both. You believed that I would, long before it was something I could imagine for myself.

ACKNOWLEDGMENTS

Many, *many* years ago, I was a high school freshman hanging around Vanderbilt campus waiting for my father to finish up in his lab so that we could grab quick dinner at the SATCO and catch a Vandy basketball game. I had recently attended a Holocaust symposium where I listened to several survivors tell their terrible stories. I remember seeing a sign for the graduate school and pledging that someday, I would come back here, earn a Ph.D. and spend the rest of my life trying to figure out why humans behave that way, how it was possible, and work to fix it….and here we are.

The path was winding and haphazard. I joined the military to see the world in all of its complexity and beauty. From those experiences I started to really understand people and philosophies, how our similarities align with culture to shape our societies. I learned how poverty and limited resources change the expression of priorities and how those priorities forge bonds to bridge the gaps in resources. I learned from my military brothers and sisters the extraordinary power of total commitment and perseverance. To all of you I offer my first mention of thanks. When this was the most difficult, I marched on in your name.

I owe an enormous debt of gratitude towards Vanderbilt, the Vanderbilt Interdisciplinary Graduate program, and the Neuroscience Graduate program. Vanderbilt is an extraordinary school. The leaders, scientists, professors and staff that assisted me throughout, exceed all of my expectations. Thank you for making this such a rewarding challenge, and seeing me through to the end.

Thank you to my committee for endless patience. It is difficult to remember sometimes that a grad student's committee is there to help, but you did, even when I forgot or was reluctant to ask. Thank you to the professional staff and management at Murine Neurobehavioral Core

(Dr. John Allison especially) for allowing the flexibility and care that made this project possible. Thank you also to the BRET office for your constant work to enrich my education.

Thank you to Dr. Roger Colbran for painstakingly holding my feet to the fire until my lofty dreams reshaped into functional skills. I did not make this easy. It is hard to turn a dreamer into a scientist, but you did. I will always be grateful. Thank you to Dr. Sachin Patel. You gave me the chance, in the nick of time, that made all of the difference. You also have inspired me and given me words of hope for the veterans I meet and know, struggling with PTSD. I can tell them that the smartest person I have ever known in real life, is working on it. I tell them to hold on. You might be surprised in how much that means to us.

Thank you to my labmates and friends, Dr. Brian Shonesy, Dr. Xiaohan Wang, Christian Marks, Tyler Perfitt, Dr Franklin Echevarria, Dr. Chris Reissaus, Dr. Rita Baldi, Dr. Gaurav Bedse, Dr. Mandy Morgan, Dr. Becca Bluett, Nolan Hartley, Oakleigh Folks, Nathan Winters, David Marcus, Megan Altemus, Toni Patrick, and Andrew Gauldin. I appreciate all of you immensely, and cannot wait to see what you guys do in your own careers.

A special thanks to the two greatest friend a person could hope for, Dr. Barb McGrath and Jason Adams. Both of you fielded the worst of my frustration and tantrums, without you there is no way I would have made it through. 'Blood on the tracks' indeed, Barb, and you were spot-on -PH ended up being pretty important….

Finally, I want to thank my parents and sisters. I am extremely lucky. I have two of the most extraordinary sisters imaginable. Beth and Mia Cavener, you two are a constant inspiration. Thank you to my mother Nancy Jacobsohn, who showed me that if you cannot find the things you need and want, make them yourself. You taught me all about the power of creativity and strength. Last but certainly not least, thank you to my father. You helped me love and trust

science from the very beginning. You were the one, who recognized that I *could* be a scientist and you kept faith in me when I could not find it myself. You are the one, who told me I *could do this*, and you were right.

TABLE OF CONTENTS

LIST OF FIGURES

.

LIST OF ABBREVIATIONS

2-AG.....2-arachidonoylglycerol
AA .....arachidonic acid
ABHD6, ABHD12 ..... alpha/beta hydrolase domain containing 6, 12
AEA.....arachidonoylethanolamine, anandamide
ANOVA..... analysis of variance
ANS ..... autonomic nervous system
BLA .....basolateral amygdala
Ca2+ ..... calcium cation
CB1R, CB2.....cannabinoid receptor subtype 1, 2
CNS ..... central nervous system
CRH.....corticotropin releasing hormone
DAGL.....diacylglycerol lipase
DSM5 .....diagnostic and statistical manual 5
eCB..... endogenous cannabinoid
EPSC.....excitatory postsynaptic current
FS.....foot-shock
HPA ..... hypothalamic-pituitary-adrenal
MAGL .....monoacylglycerol lipase
MDD.....major depressive disorder
mGluR .....metabotropic glutamate receptor
mPFC..... medial prefrontal cortex
NMDAR ..... n-methyl-d-aspartate receptor
PIP2 .....phosphatidylinositol 4,5-bisphosphate
PTSD ..... posttraumatic stress disorder
SAG .....steroyl-arachidonoylglycerol
SSRI..... selective serotonin reuptake inhibitor
THC.....Δ9-tetrahydrocannabinol

CHAPTER I

INTRODUCTION

Lifetime Prevalence, Presentation and Prognosis of Post Traumatic Stress Disorder

Post Traumatic Stress Disorder (PTSD) is a mental disorder with a life time prevalence of 6.8%-8.0% of the population (Kessler, Berglund et al. 2005, Alegria, Jackson et al. 2016). Categorized until recently as a subclass of anxiety disorder, PTSD is now listed in the Diagnostic Statistical Manual V (DSMV) as a stress related, post trauma phenomenon in which 69.7% of those diagnosed experience severe to moderate impairment of daily life (Kessler, Chiu et al. 2005, Alegria, Jackson et al. 2016). During any given year it is estimated that approximately 3.6-8% of adults (approximately 13.3- 24.4 million) currently meet the diagnostic criteria stated in the DSMV for the PTSD (Kilpatrick, Resnick et al. 2013, Alegria, Jackson et al. 2016). Women are diagnosed with PTSD at a higher rate than men at a rate of 10.4% and 5% respectively (Arnatt 2015, Alegria, Jackson et al. 2016). War time military and combat Veterans are a subpopulation that is particularly vulnerable to this illness as a result of high levels of sustained unpredictable stress and the increased probability for exposure to life threatening trauma. The estimated lifetime prevalence for PTSD among Veterans was 26.9% for women and 30.9% for men (Kang, Natelson et al. 2003). The lower frequency of PTSD diagnosed in women from this group, is attributed to women historically being barred from combat roles(Kilpatrick, Resnick et al. 2013)

PTSD is diagnosed using 5 symptom categories dependent on duration of symptoms and the exclusion other diagnostic factors. Symptom categories are listed in the DSMV as follows: (Association 2013).

A. Stressor(s)

Exposure to actual or threatened death, serious injury, or sexual violence by:

- Directly experiencing
- Witnessing as occurred to others
- Learning that the traumatic event(s) occurred to a close family member or close friend
- Experiencing repeated or extreme exposure to aversive details of the traumatic event(s)

B. Intrusion Symptoms

One (or more) of the following intrusion symptoms associated with the traumatic event(s):

- Recurrent, involuntary, and intrusive distressing memories
- Recurrent distressing dreams
- Dissociative reactions (e.g., flashbacks)
- Distress associated with traumatic event cues
- Physiological reactions to internal or external cues

C. Avoidance & Negative Symptoms

Persistent avoidance of stimuli associated with the traumatic event(s) or negative alterations in cognitions and mood:

- Avoidance of, or efforts to avoid, distressing memories, thoughts, or feelings
- Avoidance of, or efforts to avoid, external reminders
- Negative symptomology

D. Cognitions & Mood

Negative alterations in cognitions and mood that began or worsened after the traumatic event

- Inability to recall key features of the traumatic event
- Persistent negative beliefs about oneself or the world
- Persistent distorted blame of self or others for causing the traumatic event or for resulting consequences
- Persistent negative trauma-related emotions
- Markedly diminished interest in (pre-traumatic) significant activities
- Feeling alienated from others (e.g. detachment or estrangement)
- Constricted affect: persistent inability to experience positive emotions

E. Arousal & Reactivity

Marked alterations in arousal and reactivity associated with the traumatic event(s)

- Irritable or aggressive behavior
- Self-destructive or reckless behavior
- Hypervigilance
- Exaggerated startle response
- Problems in concentration
- Sleep disturbance

F. Duration of the disturbance (Criteria B, C, D, and E) is more than 1 month.

G. The disturbance causes clinically significant distress or impairment in social, occupational, or other important areas of functioning

H. The disturbance is not attributable to the physiological effects of a substance (e.g., medication, alcohol) or another medical condition.

Multiple studies have shown patients diagnosed with PTSD have markedly diminished quality of life, high rates of comorbid mental and physical health issues, as well as poorer treatment outcomes compared to the normal population (Kang, Natelson et al. 2003, Kessler, Chiu et al. 2005, Senneseth, Alsaker et al. 2012). Moderate to severe PTSD symptoms can result in diminished social functioning/networks, difficulty maintaining employment, and deteriorating health combined with an increased risk of substance abuse, addiction and suicide. (Bremner, Southwick et al. 1996, Volpicelli, Balaraman et al. 1999, Kessler, Chiu et al. 2005, Jakupcak, Vannoy et al. 2010, Berenz and Coffey 2012, Meier, Lambert-Harris et al. 2014, Arnatt 2015, Patel, Elmaadawi et al. 2017). Researchers found an increased risk of suicide among Operations Iraqi Freedom and Enduring Freedom veterans of 25% among those who had been exposed to significant trauma (Kang and Bullman 2008). In addition, alarming statics report the tendency for patients to self-medicate with alcohol, marijuana, heroin, and benzodiazepines, in order to assuage

hyper arousal and anxiety-like symptoms (Patel, Elmaadawi et al. 2017). The risk for addiction gives physicians fewer treatment options for patients suffering from anxiety, injury and chronic pain, which in turn, exacerbates the patient's PTSD symptoms (Brady, Killeen et al. 2000, Fareed, Eilender et al. 2013).

Patients suffering from PTSD have high rates of comorbid anxiety, depressive, compulsive and addictive disorders. Many of the symptoms of PTSD overlap with other anxiety, compulsive, and depressive pathologies and thus give physicians options for pharmacological treatments without addictive properties. Unfortunately medications such as Selective Serotonin Reuptake Inhibitors (SSRIs), Selective Norepinephrine Reuptake Inhibitors (SNRIs) have proven to be only moderately effective in an unpredictable subset of patients, despite the significant overlap of symptoms. (Jonas, Cusack et al. 2013, Flory and Yehuda 2015). To illustrate this point, comorbidity rates of a Major Depressive Episode (MDE) or Major Depressive Disorder (MDD) and PTSD are estimated to be as high as 48.5% and 42% (Kessler, Sonnega et al. 1995, Rojas, Bujarski et al. 2014).

There are no known specific causes for why approximately 20% of those who experience a traumatic event eventually develop PTSD (Iribarren, Prolo et al. 2005). Intense study of the neural networks involved in stress regulation, adaptation to stress, and return to homeostasis after a traumatic event have revealed that PTSD is a syndrome driven by a multilayered dysregulation of both bottom up and top down emotional processing that results in an extreme pervasive reactivity to stress in the environment. One way to conceptualize symptom clusters in patients with PTSD is to consider the changes in the human brain affected by PTSD seem to mimic the cognitive and emotional patterns apparent when comparing top predators to vulnerable prey animals in the wild. These observations have led some clinicians to reconsider the postulation that PTSD is

neuronal dysfunction, and instead is a hyper adaptation that selects for hypervigilance focused on survival, over the very human drive for enrichments based on quality of life (Diamond and Zoladz 2015, St-Cyr and McGowan 2018). Regardless of whether PTSD is a result of an evolutionary adaptation more profoundly expressed in some people over others, or is in fact a product of a pervasive degenerative disease processes in the brain, PTSD symptoms are not suited for modern human existence and cause the sufferer considerable difficulty in maintaining a reasonable standard of living. In addition these adaptations or disease processes do in fact contribute to considerable risk for other health problems and shortened life expectancy. Genetic risk factors, low socio economic status, early childhood trauma and high levels of neurosis are notable common risk factors in multiple symptom clusters resulting in diagnosis of anxiety, depressive, and compulsive disorders as well as PTSD (Hoffman and Mathew 2008, Rojas, Bujarski et al. 2014, Flory and Yehuda 2015). Due to the overlap in symptoms and risk factors, is not surprising that people diagnosed with PTSD are more often than not, also diagnosed with another co-occurring mental disorder. This has lead practitioners to consider using treatments effective for other mental disorders as first line treatments for PTSD. Providers have started characterizing patients with PTSD as belonging in sub categories based on comorbid disorders and symptoms (i.e. PTSD-depressive type, dissociative type etc.) (Brown, Stout et al. 1998, Randall D. Marshall, Katherine L. Beebe et al. 2001, Lanius, Vermetten et al. 2010, Flory and Yehuda 2015). Besides the additional complexity that comorbid disorders add to the overall treatment prognosis and the probability of accurate diagnosis for patients with PTSD, treatments indicated for the additional diagnosis have less efficacy for a substantial percentage of PTSD patients. For instance, of the patients with MDD treated with SSRIs, approximately 40% reach full remittance of symptoms (Rush, Trivedi et al. 2006, Hoffman and Mathew 2008, Flory and Yehuda 2015) SSRI efficacy in

treating the same symptoms in patients also diagnosed with PTSD is as low as 20-30% (Brady, Pearlstein et al. 2000, Davidson, Rothbaum et al. 2001, Flory and Yehuda 2015). Combination therapies designed to target separate symptom clusters are often time consuming, expensive and greatly increase the probability of patient non-compliance due to side effects.

Exposure Therapy is a behavioral intervention for PTSD that has been moderately effective at reducing fear related anxiety, dissociative and depressive symptoms of PTSD by employing techniques to stimulate post-trauma fear extinction learning. PTSD symptoms, including traumatic reexperiencing the event in a dissociative state (flashbacks), hyperarousal, and negative changes in beliefs and feelings, are some of the most disruptive aspects of PTSD affecting a person's productivity and quality of life. Exposure therapy relies on exposing an individual to aspects of the original trauma without the consequences (pain, injury, physical discomfort, separation from resources, etc.) or context of the original stressor. Studies have shown significant improvements in these symptom clusters over other psychological treatments in approximately 50% of patients, however the effect sizes of clinically significant change were small (η^2 =0.16/0.24) (Taylor, Thordarson et al. 2003). For reviews of the efficacies and treatment outcomes involving pharmacological and psychological treatment options for PTSD see (Hoffman and Mathew 2008, Steckler and Risbrough 2012, Jonas, Cusack et al. 2013).

Along with the significant suffering PTSD patients endure, there are important societal and economic burdens associated with this illness. The overall cost born by society for all anxiety type disorders is 42.3 billion dollars annually. This figure includes: indirect work place costs, psychiatric and non-psychiatric medical care, prescription drugs and mortality costs (Kessler, Berglund et al. 2005, Alegria, Jackson et al. 2016). Patients with PTSD have one of the highest rates of medical service utilization within this category, and it is estimated that 50% of all

outpatient therapy clients are suffering from PTSD (Kessler, Sonnega et al. 1995, Kessler, Chiu et al. 2005). Costs estimated by the Veterans Administration to treat PTSD patients for 2 years, post military deployment, ranges between $5904- $25,757 per patient (Terra C. Holdeman 2009). Given the long term health consequences and enduring symptomology of PTSD, the overall cost of treating thousands of veterans is enormous. in addition, PTSD is the fastest growing disability benefit payout category within the United States government's 49 billion dollar annual veteran's disability budget.

There is abundant preclinical and clinical data to indicate that research concerning abnormalities in fear learning and subsequent fear extinction is essential to understanding and treating PTSD. Pharmacological treatments and psychological interventions like Exposure Therapy is limited and the complete reversal of PTSD symptoms is uncommon. Understanding the molecular and circuit level mechanisms that prevent successful fear extinction and normalization of the stress response after a traumatic event, may lead to a targeted pharmaceutical solution that garners better treatment outcomes with fewer side effects. However, given the lack of comprehensive treatments available, the clear multilayered neural complexity of the disease, and significant societal burden associated with PTSD, research to discover how PTSD develops in hopes to discover an intervention to halt the progression of the disorder in early stages after trauma, is an important step towards lowering the net cost and suffering associated with this disorder. The focus of the following research is to investigate the complex molecular pathways associated with retention of fear learning following acute stress in rodents as a model for the development of PTSD.

The Hypothalamic-Pituitary-Adrenal Axis and the Stress Response in PTSD

The normal stress response is an adaptive, protective reaction to perceived danger or heightened stimulation from the organism's physical or emotional environment. The activation of the autonomic nervous system and hypothalamic-pituitary-adrenal axis (HPA axis) occurs in response to potential risk. It redirects the organism's physiological resources from homeostasis to physically prepare the body to illicit a reaction ideally suited towards survival of the organism (fight or flight response) (McEwen 2007). In response to stress, the brain secretes several neuropeptides through a complex neural network that converge on the hypothalamic paraventricular nucleus and results in activation of the HPA axis (Fig. 1.1).

Activation of the HPA axis initiates a cascade of hormone and hormone releasing factors to be released. This cascade regulates specific autonomic sequences effecting important survival mechanisms including blood glucose levels, energy metabolism, blood pressure, cognition and arousal, and the immune system (McEwen 2007). In addition, adrenal corticosteroid hormones bind to two types of nuclear receptors [glucocorticoid receptors (GR) and mineralocorticoid receptors (MR)] that act as transcriptional regulators that target a variety of genes involved in cognition and emotion regulation. The HPA axis through release of glucocorticoids, functions as a "master switch" providing jurisdiction over neuronal and network responses that underlie behavioral adaptation (de Kloet, Joels et al. 2005). For a more in depth overview of specific regulation of these pathways see (de Kloet, Joels et al. 2005, Smith and Vale 2006).

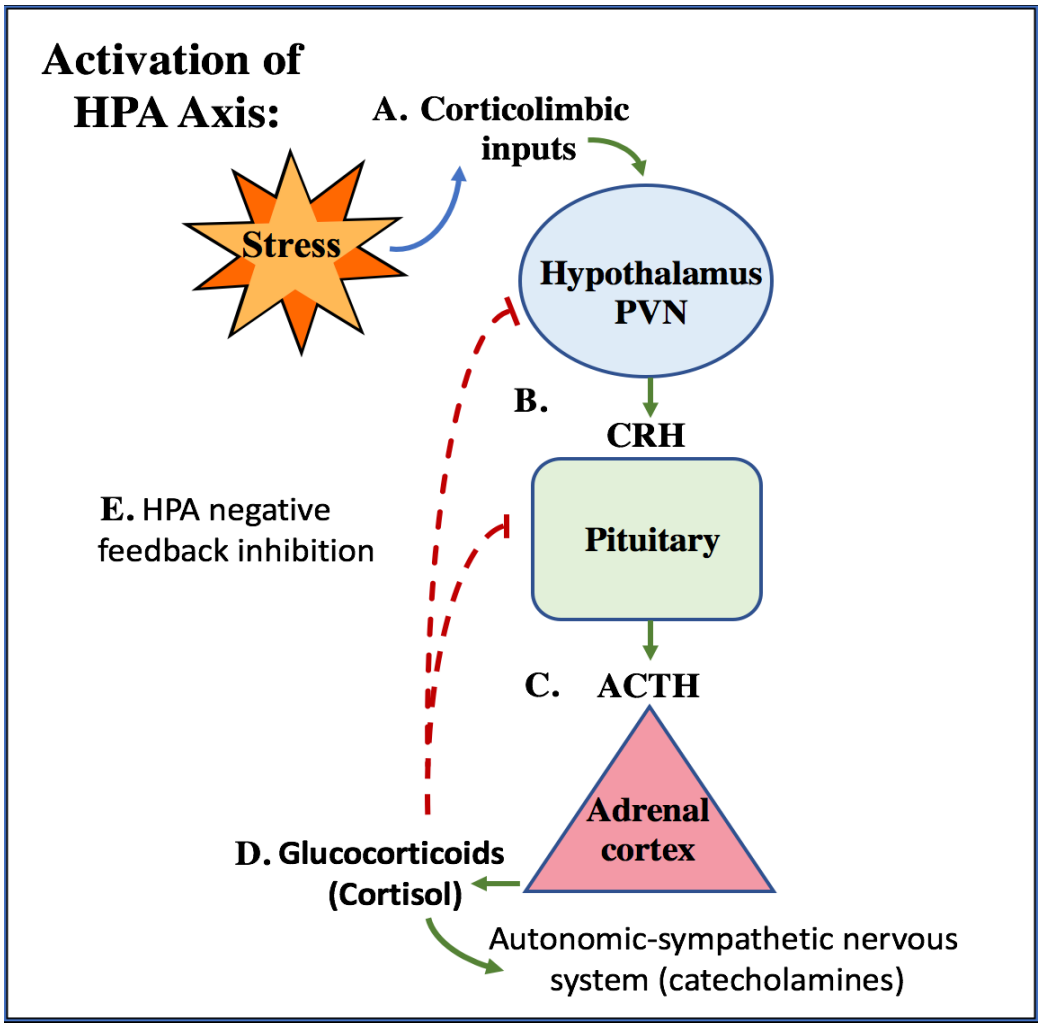


Figure 1.1. Activation of the HPA axis A) Sensory information regarding stressor is processed through the corticolimbic system and relays information to the hypothalamic paraventricular nucleolus (PVN). B) CRH travels through the hypophyseal portal system to the anterior pituitary to stimulate secretion of adrenocorticotropic hormone (ACTH). C) ACTH is carried through the blood stream to adrenal cortex and triggers the synthesis and release of Glucocorticoids (cortisol). D) Cortisol circulates through the body and activates the autonomic sympathetic nervous system responsible for production and release of Catecholamines (Epinephrine (Norepinephrine, and Aldosterone) that control the flight or fight response. E) Increases of cortisol inhibit further release of CRH and ACTH from the hypothalamus and pituitary in order to allow the organism to return to homeostasis once the threat is resolved.

While activation of the stress response is adaptive and protective in the short term, over stimulation and prolonged exposure to stress inducing conditions has deleterious effects on an organism's physical and emotional well-being. Allostatic Overload is a term used to describe the physiological consequences of sustained hyper activation of the HPA axis (McEwen and Stellar 1993). Over time and with repeated challenges to normal physiological adaptations required for optimal and efficient survival of the organism, the processes involved in allostasis "achieving stability through change" are overwhelmed. This hyperactivity results in a failure to return to and maintain homeostasis through disruptions in HPA negative feedback functioning that contributes to dysregulation of important mechanisms required for proper immune function, energy expenditure, cognition and appropriate scaling during threat assessment. (McEwen 1998, McEwen and Seeman 1999, Smith and Vale 2006, McEwen 2007). Over exposure to stress can lead to allostatic modifications in one system that results in hyper activation of another (McEwen 1998). For example, chronic elevation of cortisol is a risk factor for metabolic diseases such as diabetes and obesity, cardiovascular disease, anxiety disorders, addiction, cancer and can result in a significantly shorter life span (Schnall, Landsbergis et al. 1994, Whitworth, Williamson et al. 2005, Sinha 2008, Hackett, Kivimaki et al. 2016, Daimon, Kamba et al. 2017, Papale, Seltzer et al. 2018).

Cortisol levels are used as a barometer to measure HPA axis activity at basal levels throughout the day, and in response to stress. Temporal levels of cortisol are regulated by the suprachiasmatic nucleus and hypothalamus in response to circadian rhythm signaling that regulates cortisol levels to support arousal during the sleep wake cycle (Weitzman, Czeisler et al. 1981). Other factors causing fluctuations in cortisol are from external cues that can influence this pattern as a residual consequence of HPA hyper activity in response to stress, or lower activity due to relaxing circumstances that can carry over into the following day. It is well established that the

circadian cortisol pattern in people with psychological disturbances is altered, causing changes in a variety of activities in daily living (Stetler, Dickerson et al. 2004).

Specific dysregulation of the HPA axis in patients with PTSD has been difficult to define. Surprisingly, patients suffering from PTSD have been reported to have decreased basal levels of cortisol, enhanced HPA feedback function and progressive sensitization of the HPA axis overall (Yehuda and Seckl 2011). However, there has been some conflicting data regarding cortisol levels in patients with PTSD and this issue has not been completely resolved (Yehuda, Southwick et al. 1990, Yehuda 2002, Meewisse, Reitsma et al. 2007, Yehuda and Seckl 2011, Morris, Compas et al. 2012, Tajima-Pozo, Montes-Montero et al. 2013). It does appear that cortisol levels can be expressed differently depending on comorbid factors, gender, and the type of trauma experienced. The most well studied comorbid diagnosis concerning this issue is MDD (Kendall-Tackett 2000, Meewisse, Reitsma et al. 2007, Morris, Compas et al. 2012).

Patients with a diagnosis of MDD show increased levels of basal cortisol, impaired HPA feedback function and progressive desensitization of the HPA axis (Kendall-Tackett 2000). Given opposing cortisol levels were reported for MDD and PTSD, it stands to reason that patients with comorbid disorders will present with yet another pattern of HPA dysfunction. A meta-analysis of HPA feedback function involving 47 independent studies compared levels of cortisol in patient diagnosed with PTSD, PTSD and MDD, trauma exposed participants (TE) (that did not meet criteria for PTSD or MDD), and normal controls. Patients diagnosed with PTSD had lower basal cortisol levels in the am, pm, and "daily output cortisol levels" than normal controls. TE participants revealed lower levels of pm cortisol but no significant changes in other measures compared to the normal controls. In the final comparison of cortisol output PTSD+MDD

participants had lower am cortisol levels, higher pm, and lower daily output cortisol levels than normal controls (Morris, Compas et al. 2012).

Morris et. al next analyzed studies measuring the HPA feedback function that utilized the dexamethasone suppression test (DST). The DST measures the production of cortisol following administration of a synthetic glucocorticoid that inhibits cortisol production in the HPA axis. Measuring "post DST" cortisol levels after exposure to a controlled stressor indicates the efficiency of HPA negative feedback inhibition (Tajima-Pozo, Montes-Montero et al. 2013). Lower levels of cortisol measurements after stress exposure indicates a stronger HPA negative feedback inhibition. Enhanced negative feedback inhibition on the hypothalamus and anterior pituitary within the HPA axis will result in decreased levels of CRH and ACTH secretion and diminished cortisol output (Yehuda and Seckl 2011, Yoshida, Uchigashima et al. 2011). While it may seem counter intuitive that patients with PTSD have lower levels of the major hormone related to stress, chronic decreases in levels of cortisol results in changes in glucocorticoid receptor (GR) surface expression and sensitivity to fluctuations in cortisol (de Kloet, Joels et al. 2005, Gola, Engler et al. 2014). Evidence suggests that patients with PTSD have a lower threshold required for activation of the HPA axis and may explain why symptoms of PTSD manifest as a sensitivity to sudden noises, exaggerated startle response, lower tolerance for noxious stimuli.

Meta-analysis of the data revealed that PTSD, PTSD+MDD, and TE participants exhibited enhanced HPA negative feedback inhibition compared to normal controls. This analysis supports the hypothesis that exposure to trauma causes hyperactivation of the HPA axis and negative feedback inhibition. The authors of this study caution that it is still uncertain as to whether having enhanced negative HPA feedback inhibition is a risk factor for developing PTSD or if PTSD causes a change in the regulation of this process given there was no significant difference in "post DST"

cortisol levels between TE and PTSD participants (Morris, Compas et al. 2012). A cohesive explanation for how trauma results in the pervasive symptoms found in PTSD requires an extended examination of the cortico-limbic system upstream of the HPA axis. Understanding cortico-limbic signaling that results in triggering or suppressing stress responses may lead to novel approaches for reducing or preventing dysfunction precipitated by stress exposure.

Cortico-limbic Regulation of Stress, Fear Learning, and Fear Extinction

The diagnostic features specific to PTSD are hypothesized to be a failure of higher order brain regions in the cortico-limbic system to dampen exaggerated symptoms of arousal and distress mitigated through the brain's main threat detection nucleus known as the amygdala. Brain regions that have been found be functionally disrupted in persons suffering from PTSD have in common that they are all involved in some aspect of visuospatial processing, fear learning, fear retention and memory. The following section gives a brief overview of the key brain structures within this system and differences in function related to PTSD pathology (Fig. 1.2).

Specific characteristics of distinct stressors determine the degree and extent of HPA axis stress responses. Stressor type and intensity illicit different responses from specific brain regions that converge onto the PVN via precisely regulated pathways that vary based not only on the type of stress, but also on the individual's experience, gender, and age. Limbic structures synthesize sensory information from sensory cortices and combined with cue information regarding the potential threat, assign emotional valence to the stimuli for an optimized response. The limbic system is comprised of multiple sub-nuclei and cortices with multiple layers of inhibitory and excitatory efferent and afferent connections between them. The limbic system is often referred to as the "feeling and reacting" center of the brain. Psychological stress elicited during Pavlovian fear

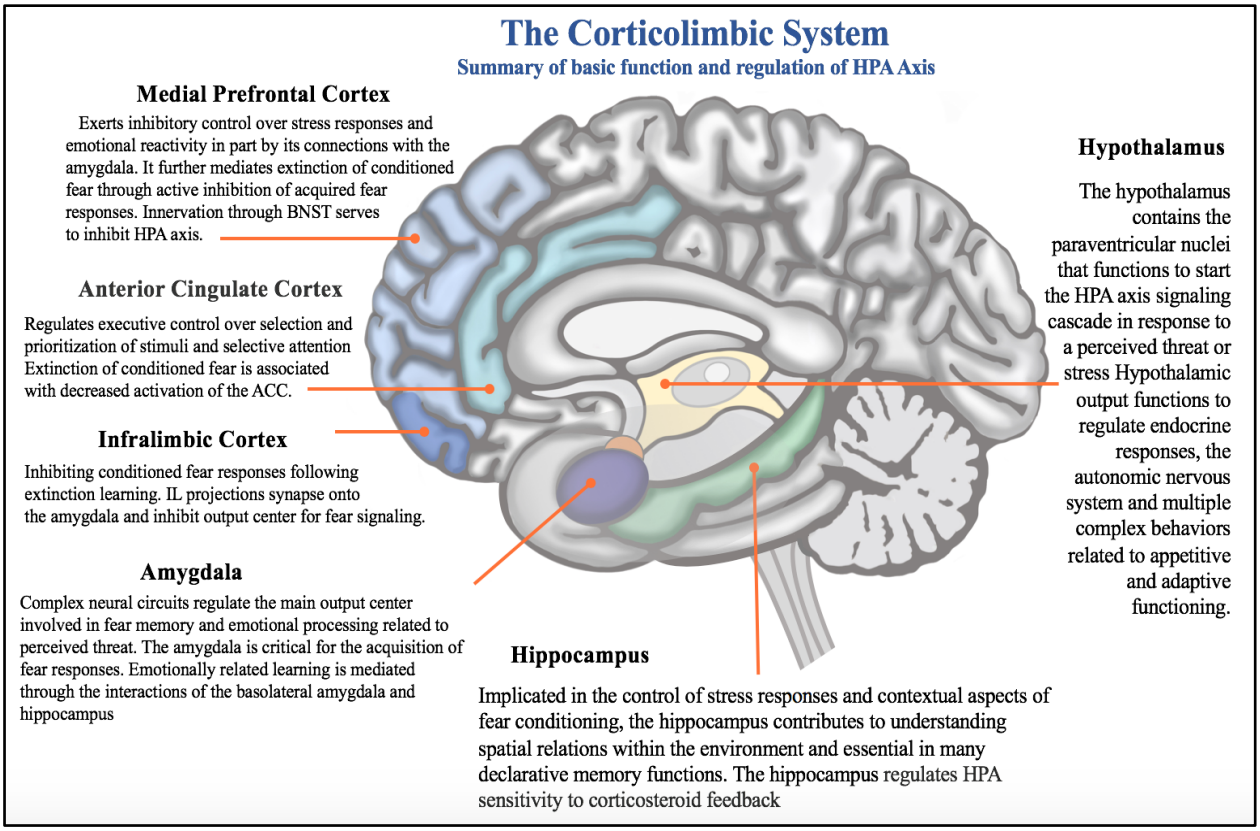


Figure 1.2. Summary of key brain regions in the cortico-limbic regions involved in regulating HPA axis. Many of the brain regions involved in fear conditioning and fear extinction that are known to directly or indirectly regulate the HPA axis. The structures included in this figure are also regions that have been correlated with significant disruptions related to PTSD symptoms and pathophysiology.

conditioning and fear extinction will be the primary focus of this review of the literature concerning corticolimbic regulations of the stress response.

Fear conditioning and fear extinction learning is a model for processes involved in normal adaption after exposure to acute stress. Multiple studies in humans and rodent show similarities in brain activity during tasks involved in fear learning and fear extinction. Given these similarities and the inability to study human molecular pathways in depth, the fear conditioning and fear

extinction paradigm is a useful model for exploration of the molecular pathways involving regulation of fear learning and normalization of fear behavior after trauma that prevents development of maladaptive generalized anxiety to other contexts. Fear conditioning and fear extinction paradigms are models used to mimic fear learning and extinction in the environment. Fear learning as well as extinction of that fear is important for survival. Animals must learn to both recognize and respond to threats, as well adapt that learning to specific contexts to facilitate the mouse's ability to return to foraging for food and activities of daily life also required for survival. PTSD is thought to be a failure of the fear extinction learning paradigm mediated by a complex neural and molecular system of layered fear processing and learning. Figure 1.3 shows a schematic drawing of a stereotypical fear conditioning and fear extinction paradigm used in research and is the model utilized in the study described in Chapter II. Figure 1.3 shows a schematic drawing of a stereotypical fear conditioning and fear extinction paradigm and is the model utilized in the study described in Chapter II.

Fear extinction is dependent on inhibition of fear responses learned during fear conditioning. Both human and rodents will respond to a conditioned stimuli at pre-conditioned levels if fear extinction is sufficiently learned. However, even after a conditioned response has been neutralized with fear extinction training, over time if the organism is returned to the original context experienced during conditioning, or is subsequently experiences another trauma, the conditioned fear response will reappear. (Pavlov 1927, Rescorla and Heth 1975, Bouton and Bolles 1979). This behavioral observation led to the hypothesis that during fear extinction a conditioned fear memory is not over written or "forgotten" but rather a new learning results in a memory capable of inhibiting the conditioned response.

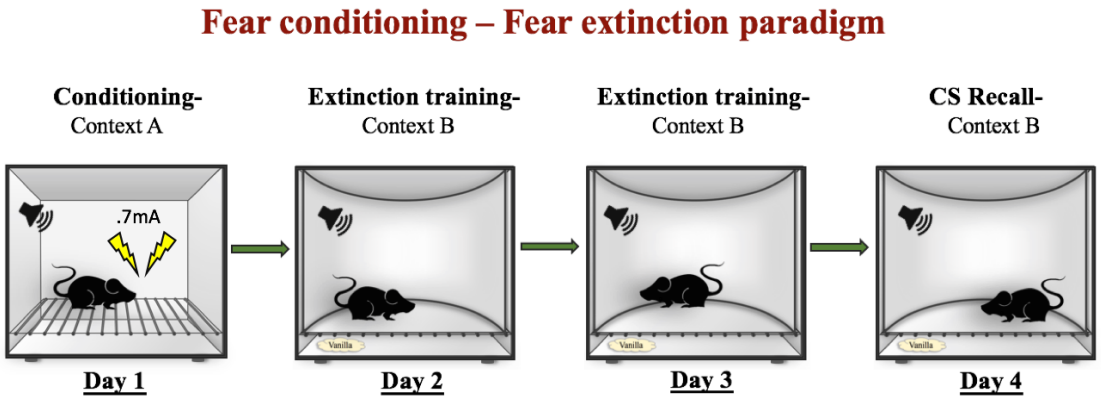


Figure 1.3. Fear conditioning paradigm. Fear conditioning requires a learning to pair a neutral stimuli conditioned stimuli (CS) with an adverse event unconditioned stimuli (US). In the paradigm depicted above mice are placed in a chamber with a metal grid capable of delivering a shock (US) at the end of a 30 second auditory cue (CS). After a series of several tone/shock pairings the mouse will start to anticipate a shock when it hears the tone and freeze as an autonomic response to acute stress and fear. 24 hours after this training session the mouse will return to the chamber, however the walls and flooring will be fitted with inserts to change the visual and tactical cues from the preceding training days. Vanilla scent will be added under the floor insert to remove any learned olfactory association the mouse may have learned during the training day. Consecutive 30 second tones will play without the shock 20 times for 2 consecutive days. As the mouse learns that in this chamber context the tone will not accompany a shock the freezing behavior will subside. On the final day in this paradigm mice will remain in the chamber for longer baseline measurement (no tones) to establish residual generalized fear and a final test of freezing behavior post fear extinction learning.

A substantial amount of research has been dedicated to describing and searching for the underlying mechanisms involved in fear learning and fear extinction memories with hopes that revealing normal fear responses will shed light on maladaptive adaptations, by comparison. For reviews on this topic see, (Myers and Davis 2007, Quirk and Mueller 2008, Herry, Ferraguti et al.

2010, Pape and Pare 2010, Radulovic and Tronson 2010). The hypothalamus, hippocampus, prefrontal cortex, and amygdala have emerged as the principal brain regions involved in fear conditioning, fear learning and the stress response. Examples of their roles in these processes are summarized in the remainder of this section.

The hypothalamus is the primary output center for the limbic system. This structure is made up of multiple sub nuclei that regulate temperature, hunger, sexual desire, and monitor internal signals related to homeostasis such as blood glucose, sodium levels, and osmolarity. It receives inputs from most of the body, as well as receives information directly from retina and olfactory bulb. Hypothalamic output functions to regulate endocrine responses, the autonomic nervous system and multiple complex behaviors related to appetitive and adaptive functioning. As discussed earlier, the hypothalamus contains the paraventricular nucleus, which functions to initiate the HPA axis signaling cascade in response to a perceived threat or stress. Multiple studies have revealed significant sensitivity to cortisol fluctuations within the hypothalamus. The paraventricular nucleus has one of the highest levels of GR expression in the brain. GR expression is also high in the hippocampus and other limbic neurons projecting to the GABA (γ-aminobutyric acid) inhibitory network surrounding the PVN (peri-PVN region) which functions primarily to inhibit the HPA axis and regulate HPA inhibitory tone (Herman 1993). GR distributions in both structures are significantly changed in PTSD pathology to favor HPA axis hypersensitivity (de Kloet, Joels et al. 2005). Tonic disruptions of endocannabinoid signaling, which further regulates HPA axis sensitivity, is also implicated in PTSD and will be discussed in further detail later in this section (Cota 2008).

The hippocampus and medial prefrontal cortex (mPFC) primarily serve to exert an inhibitory action on the HPA axis. This top-down control over the HPA axis is weakened as a

result of PTSD. The hippocampus synapses onto the hypothalamus through the fornix and is an important inhibitory mediator of HPA signaling. In the context of fear conditioning the hippocampus has been shown to contribute more to contextual fear memory than that elicited from an auditory cue. When the hippocampus was lesioned 24 hours after fear conditioning, rats exhibited fear retention to an auditory cue but context specific fear retention was abolished (Maren, Aharonov et al. 1997). However, when specific regions of the ventral hippocampus were lesioned in rodents, it resulted in a marked decrease in freezing behavior in response to both a contextual and auditory conditioned stimulus and contextual stimuli (Maren 1999). There is still some debate on the specific role the hippocampus may play in generation and retention of fear learning and retention (Kim and Jung 2006).

During fear extinction the hippocampus regulates important aspects of context-dependent fear expression and fear extinction. Data acquired through fMRI imaging of the human brain reveals that hippocampal activation to the conditioned stimulus occurs in the extinction context but not in the conditioning context, implying that hippocampal dysfunction may be a contributing factor to generalized fear responses independent of the original context of an acquired fear (Kalisch, Korenfeld et al. 2006). In addition hippocampal activity was correlated with activity in the mPFC. Hippocampal and mPFC interactions may be important for regulation of how the context in which the stressor is experienced effects fear extinction learning (Kalisch, Korenfeld et al. 2006).

Studies involving patients with PTSD have revealed a significant reduction in hippocampal volume. This reduction has been hypothesized to be a result of neurotoxic effects of repeated exposure to stress and glucocorticoids (Sapolsky, Krey et al. 1985, Gilbertson, Shenton et al. 2002, Shin, Rauch et al. 2006, Conrad 2008). Some doubt that this volume decrease is a result of PTSD

and suggest it may be a contributing factor in developing the disorder, based on twin studies showing that the twin not exposed to trauma also had a smaller hippocampal volume (Gilbertson, Shenton et al. 2002, Pitman, Sanders et al. 2002, Shin, Rauch et al. 2006). Hippocampal deficits may decrease the ability to adequately distinguish between safe and unsafe environments and increase the likelihood of HPA axis hyperactivity and impaired fear extinction (Sherin and Nemeroff 2011). It is also possible that PTSD symptoms related to memory and hippocampal function develop as a result of the disorder and not have a specific role in contributing to the development of the disease. Various nuclei located in the prefrontal cortex and analogous structures in rodent models have been implicated in the regulation of the HPA axis and contribute to both diminished function in PTSD and fear learning/extinction paradigms.

Important inhibitory control over the stress response and emotional regulation is mediated by through the mPFC both directly and through indirect connections with the amygdala and hippocampus. The mPFC also regulates important aspects of the extinction of conditioned fear by inhibiting learned fear responses (Shin, Rauch et al. 2006). In addition experiments with rodents show that lesions in the mPFC render the animal incapable of fear extinction following a fear conditioning paradigm (Morgan, Romanski et al. 1993). Neuroimaging data reveal that patients with PTSD have decreased activation of mPFC in response to a variety of stress inducing stimuli (Bremner, Narayan et al. 1999, Lanius, Williamson et al. 2003, Bremner, Vermetten et al. 2004, Britton, Phan et al. 2005). Studies involving patients with PTSD have revealed decreased function and volumes within specific regions in the frontal cortex most notably within the Anterior Cingulate Cortex (ACC). Reductions in ACC volume are inversely proportional to the severity of PTSD symptoms reported in patients (Woodward, Kaloupek et al. 2006). The ACC has been shown to regulate executive control over selection and prioritization of stimuli and selective

attention. In PTSD pathology, ACC dysfunction results a failure to filter stressful stimuli and adequately drive appropriate distinction between the stimulus and conditioned fear associations resulting from the original trauma (Bench, Frith et al. 1993). In contrast to the studies related to hippocampal volume, the decreased volume of frontal cortex seems to be as a result of PTSD and not a predisposing risk factor for developing the disorder (Kasai, Yamasue et al. 2008).

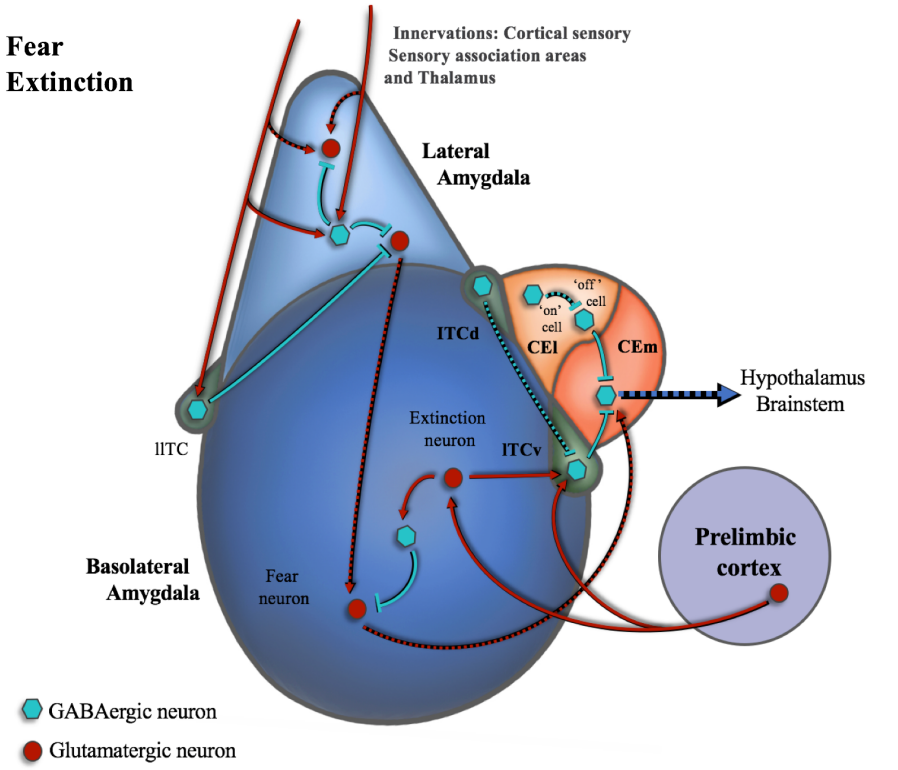
The infralimbic cortex (IL) plays an important role in inhibiting conditioned fear responses following extinction learning. In an auditory cue-fear conditioning paradigm, lesioning the IL in rats resulted in animals that exhibited normal within session fear extinction, however 24 hours later, the rats had spontaneous recovery of 87% of freezing behavior on cue compared to 27% of non-lesioned controls. Rats with lesions of the mPFC that did not include the IL showed no impairment of fear extinction learning or recall of that learning on the following day. The authors interpret these findings that IL signaling is not required for fear extinction learning but is required for recall of extinction after a 24 hour delay (Quirk, Russo et al. 2000). Projections from the IL synapse directly onto the GABAergic cells in the lateral subdivision of the central nucleus (CeL) and intercalated (ITC) cell masses of the amygdala and are thought exert inhibition of CeL output (Cassell and Wright 1986). Micro-stimulation of the IL paired with conditioned tones in rats reduced freezing behavior in response to the tone, and markers of neuronal excitability were detected in the IL during extinction recall (Cassell and Wright 1986, Quirk, Russo et al. 2000, Herry and Garcia 2002). One interesting study found that "brief uncontrollable stress" resulted in dendritic retraction in IL neurons, which the authors hypothesized facilitated resistance to fear extinction during acute trauma (Izquierdo, Wellman et al. 2006). This data supports the hypothesis that the IL serves to inhibit amygdala activity to facilitate behavioral expression of fear extinction learning.

Anatomical studies and electrophysiology data show that regulation of the HPA axis by the hippocampus and prefrontal cortex projections are indirect and converge on secondary structures such as the bed nucleus of the stria terminalis (BNST) that in turn regulate CRH release from the PVN. Additionally there is complex circuitry between these structures implicated in PTSD pathology. For a review of these circuits see (Herman, Ostrander et al. 2005, Choi, Furay et al. 2007, Jin and Maren 2015).

In contrast to the hippocampus and mPFC's indirect inhibitory drive on the HPA axis, the amygdala supports activation via direct and indirect connections to the hypothalamus (Feldman, Conforti et al. 1995). The amygdala is the structure in the brain responsible for emotional processing, and is critical for associative fear learning and the acquisition of fear responses. The amygdala is the most strongly associated structure with the pathophysiology of PTSD and hyperactivity within the HPA axis. Acute stimulation of the amygdala in rodents results in increased synthesis and release of glucocorticoids (Matheson, Branch et al. 1971, Gray, Carney et al. 1989, Feldman, Conforti et al. 1995, Van de Kar and Blair 1999). Activation of the HPA axis in response to noxious fumes and restraint stress was diminished after bilateral amygdala lesions (Feldman, Conforti et al. 1995). In human studies, amygdala hyperactivity in response to negative stimuli has been consistently measured by fMRI in patients presenting with PTSD (Yehuda and Seckl 2011). Patients with amygdala lesions fail to respond to threat predictive cues and although the interpretation must be guarded, deep brain stimulation which inhibited the basolateral amygdala (BLA) resulted in dramatic relief of severe PTSD symptoms in one patient (LaBar, Gatenby et al. 1998, Funayama, Grillon et al. 2001, Whalen, Kagan et al. 2005, Langevin, Koek et al. 2016).

Given the extensive study of the rodent amygdala and associated fear neurobiology, circuitry involving amygdala's 13 sub nuclei are relatively well understood. Complex intra-amygdala circuits regulate fear conditioning via glutamatergic inputs from the sensory cortices and the thalamus (Fig 1.4). Not surprisingly, most of the studies involving regulation of fear conditioning and fear extinction learning involving amygdala circuitry have been done with rodent models. Human studies using fMRI have at least confirmed that there are increases in activity in the amygdala during a simple fear conditioning task and that amygdala activity is reduced in parallel to fear extinction learning. The rodent amygdala nuclei can be categorized into roughly into the 3 major sections with specific roles in acquisition of fear and fear expression during fear conditioning, and fear extinction. The basolateral complex (BLA) is the input center of the amygdala and is subdivided further into the basal nucleus (BA) and the lateral nuclei (LA). Intercalated cells (ITC) are three distinct masses of inhibitory cells including: dorsal (ITCd), ventral (ITCV), and lateral (ITCl). The central nucleus (CeA) is major output center of the amygdala and is divided into the centromedial (CEm) and centrolateral (CEl) nuclei (Maren 2001).

A.


Fear
Extinction
Innervations: Cortical sensory
Sensory association areas
and Thalamus
Lateral
Amygdala
'off' cell
'on' cell
ITCd
CEl
CEm
Hypothalamus
Brainstem
Extinction
neuron
llTC
ITCv
Prelimbic
cortex
Basolateral
Amygdala
Fear
neuron
GABAergic neuron
Glutamatergic neuron

B.

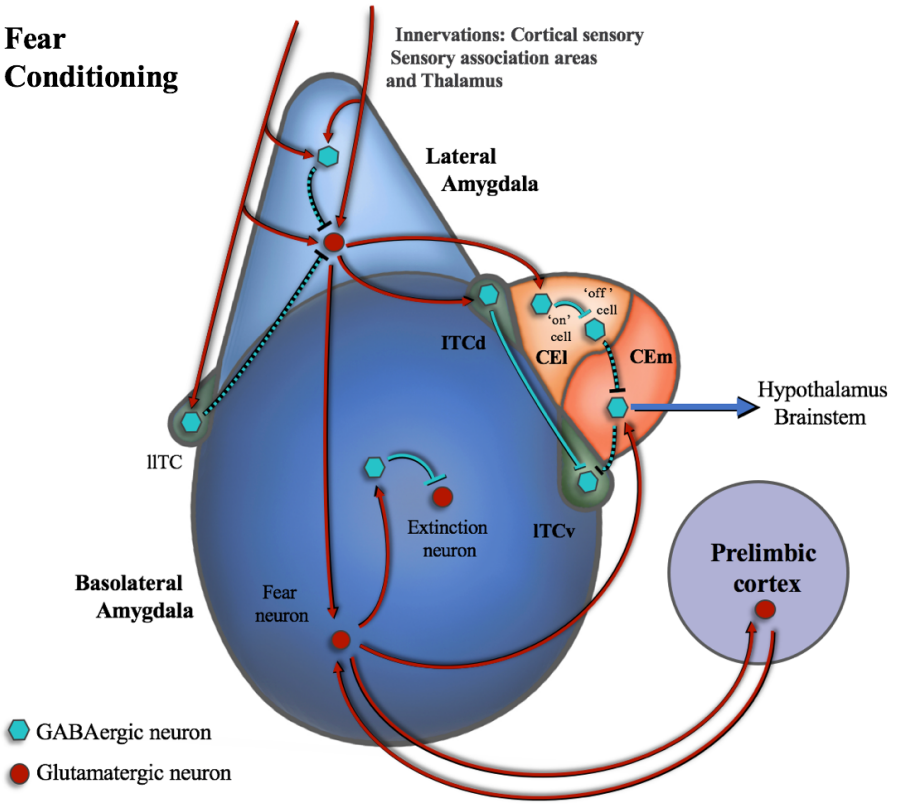

Fear
Conditioning
Innervations: Cortical sensory
Sensory association areas
and Thalamus
Lateral
Amygdala
'off' cell
'on' cell
ITCd
CEl
CEm
Hypothalamus
Brainstem
llTC
Extinction
neuron
ITCv
Prelimbic
cortex
Basolateral
Amygdala
Fear
neuron
GABAergic neuron
Glutamatergic neuron

Figure 1.4. Representative amygdala circuitry involved in fear conditioning (a) and (b) extinction. Solid lines indicate neural connections strengthened during the acquisition of fear or extinction learning. Dotted lines indicate pathways that are less active or partially inhibited within these paradigms. Red lines indicate glutamatergic projections, Blue lines indicate GABAergic projections. A) During fear conditioning excitatory inputs from cortical sensory, sensory association areas, and the thalamus disinhibit LA glutamatergic neurons projecting into the BA. Excitatory synapses within the BA excite inhibitory neurons involved in inhibition of extinction specific populations of neurons and also and directly stimulate CEm MSNs with net result of increased output from the CeA. B) During fear extinction excitatory inputs from cortical sensory, sensory association areas, and the thalamus stimulate inhibitory neurons and dampen signals to fear neurons in the BA. Projections from the infralimbic areas excite extinction specific microcircuits within the BA as well as ITCv MSNs that serve to increase the inhibitory drive on CeA pathways. The net result of these circuits is decreased output from the CeA to the hypothalamus and brainstem (flight or fight response). This figure is based on and modified after (Lee, Kim et al. 2013).

The BLA is the main "sensory interface" of the amygdala. Lesions in the BLA produce deficits in the animal's Pavlovian fear response and expression (LeDoux, Cicchetti et al. 1990, Campeau and Davis 1995, Cousens and Otto 1998). Specifically the BA seems to be important for fear conditioning as the LA is associated with the behavioral response to learned fear (Amorapanth, LeDoux et al. 2000).

The CeA functions as the direct and indirect output nuclei for fear response systems. Direct stimulation of the CeA will cause the organism to respond in the same innate conditioned fear

behavior independent of conditioning or conditioned stimuli (Iwata, Chida et al. 1987). Lesions of the CeA cause disruptions in fear acquisition and fear conditioning (Hitchcock and Davis 1986, Iwata, Chida et al. 1987, Kim and Davis 1993, Young and Leaton 1996). Studies that lesioned brain regions receiving inputs from the CeA show deficits in fear responses particular to that region's function which indicates the CeA is the "final common pathway for the generation of learned fear responses" (LeDoux, Cicchetti et al. 1990, De Oca, DeCola et al. 1998, Amorapanth, LeDoux et al. 2000, Maren 2001). The CeA is not simply an outward relay station, it also plays a role in fear conditioning and fear extinction through CeA microcircuits. Within the CeA, the CEm plays a role in fear expression, and the CEl is important for fear learning. Furthermore discrete "CEl_{on}" and "CEl_{off}" neurons are capable of a biphasic inhibition of CEm projections to the brain stem in response to a conditioned stimulus. CEl_{off} neurons disinhibit CEm output neurons and facilitate behavioral expression of fear conditioning. Fear conditioning increases the proportion of CEl_{off} cells and fear extinction increases the proportion of CEl_{on} cells that regulate CEm output (Duvarci, Popa et al. 2011).

The ITC are distinct clusters of GABAergic neurons interspersed between the BLA and CeA and are thought to mediate signals between these two amygdala sub regions (Nitecka and Ben-Ari 1987, McDonald and Augustine 1993). The BLA synapses onto the ITCs which then project to the CeA (Royer, Martina et al. 1999). Interestingly, systematic microsimulation of these clusters revealed a spatiotemporal inhibition pattern that indicates that ITCs function as a complex feedforward inhibition within the amygdala to regulate the flow of information between the receiving and output nuclei. This distinct amygdalar neural network results in computational capabilities within the amygdala, wherein CeA output is not just dependent on the type and intensity of sensory input to the BLA, but can be further distinguished based on input timing and

spatial properties from cell populations within the BLA (Royer, Martina et al. 1999). Other brain regions besides the amygdala are known to synapse directly onto the ITC. The infralimbic area of the mPFC has glutamatergic synapses on ITCs and are thought to increase inhibitory drive on the CeA during fear extinction (Likhtik, Popa et al. 2008).

The cortico-limbic circuitry involved in regulating activation of the HPA axis, and fear conditioning/fear extinction is clearly a tightly regulated network reliant on highly plastic synaptic connections regulated by multiple layers of neurotransmitter release, receptor expression, and tightly controlled protein-protein interactions within distinct synapse specific microdomains. The endocannabinoid system has immerged in the past decade as extremely important for the regulation of homeostasis, protection from neurotoxic effects of stress, and short term plasticity required for adaptation to a stress laden environment. The remaining section will cover the molecular regulation and expression of eCB signaling with a particular emphasis on what is known regarding eCBs during acute stress, fear learning and extinction.

The Endogenous Cannabinoid System

The endogenous cannabinoid (eCB) system is a retrograde signaling pathway that uniquely allows for the post-synaptic neuron to regulate neuro transmitter release by the pre-synaptic neuron. Generally this mechanism is employed in response to high levels of activity and is protective against excitotoxicity as well as being important for transient regulation of neuronal circuits involved in multiple physiological circumstances. The study of eCBs was initially motivated by the search for the underlying mechanism responsible for the effects of the Cannabis sativa plant and its primary psychoactive component, Δ9 -tetrahydrocannabinol (THC) (Gaoni and Mechoulam 1971). Cannabinoid Receptor 1 (CB1R) and Cannabinoid Receptor 2 (CB2R) are the

two cannabinoid receptors that were discovered and have only been understood to be important neuromodulators within the past two decades. CB1R is mainly expressed in the central nervous system (CNS) with the CB2 subtype preferentially expressed in the periphery(Matsuda, Lolait et al. 1990, Munro, Thomas et al. 1993, Glass and Felder 1997). The two eCB endogenous ligands arachidonoylethanolamine (anandamide; AEA) and 2-arachidonoylglycerol (2-AG) are expressed ubiquitously throughout the brain (Devane, Hanus et al. 1992, Sugiura, Kondo et al. 1995). There are other possible lipid binding partners for CB1R and CB2R, however none have been shown to be physiologically relevant in the mammalian brain thus far (Huang, Lo et al. 2001).

The CB1R is a transmembrane G-protein coupled receptor (GPCR) which predominantly couples to Gαi/o expressed in presynaptic axon terminals. CB1R is evolutionarily conserved across species and is approximately 97% homologous between rodents and humans (Chakrabarti, Onaivi et al. 1995). A brief over view of the eCB pathway including important enzymes regulating the most abundant eCB (2-AG) synthesis and hydrolysis can be found in figure 1.5 (Pertwee 1997, Kano, Ohno-Shosaku et al. 2009).

There is still a frustrating lack of evidence regarding how eCBs traffic from the post synaptic to the presynaptic neuron. There is an ongoing debate concerning whether there is active transportation of eCBs via a pump/carrier molecule or passive diffusion. The CB1R ligand binding pockets are located within the transmembrane helices which is consistent with the indication that eCBs do in fact diffuse through the membrane at some point in their journey to the presynaptic target (Song and Bonner 1996, Beltramo, Stella et al. 1997). However, eCB signaling has such tight control over synaptic function in perisynaptic microdomains that it seems unlikely to be left to passive diffusion alone.

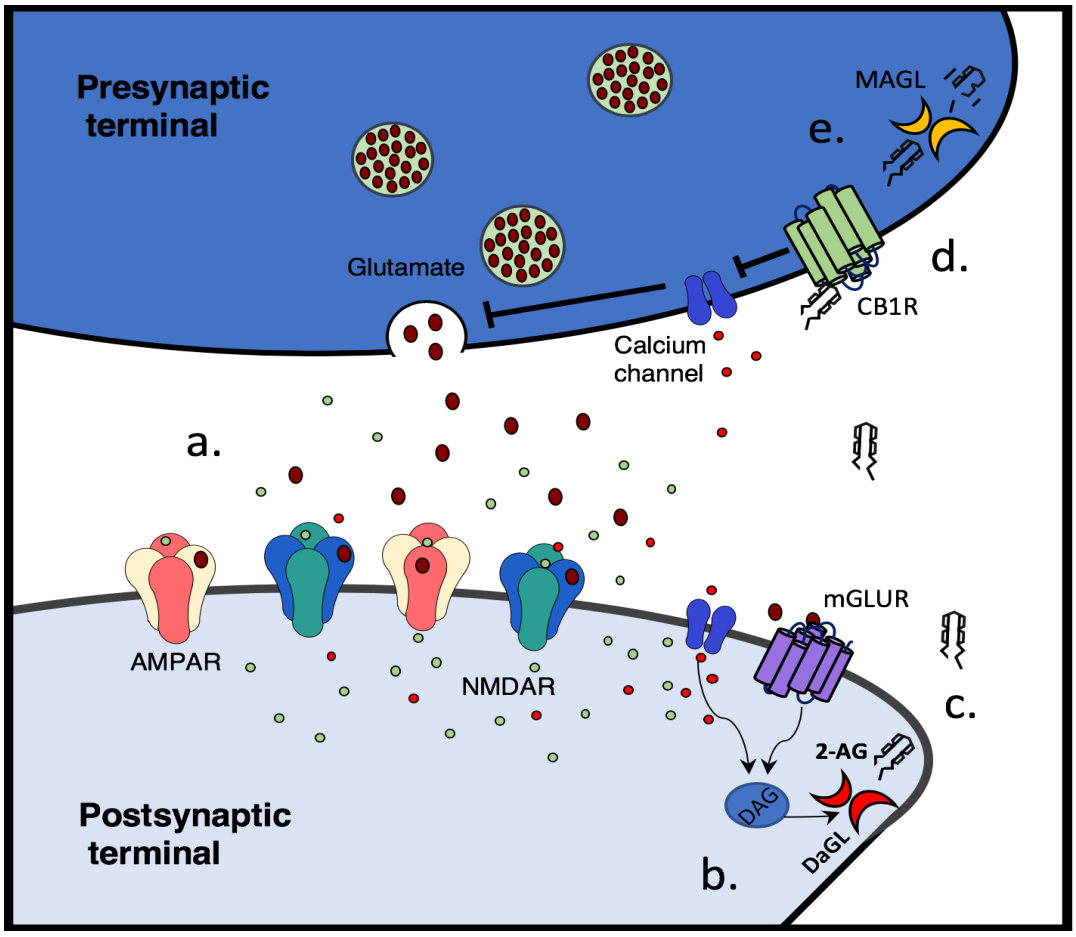


Figure 1.5 Endocannabinoid retrograde signaling dampens presynaptic release of neurotransmitter via a postsynaptic molecular cascade resulting in decreased calcium signaling in presynaptic neuron. (a) Endocannabinoids are synthesized in the postsynaptic cell in response to increased concentrations of calcium levels after presynaptic release of neurotransmitter and binds postsynaptic Ca^{2+} permeable ion channels and/or activate G-protein coupled protein receptors (GPCRs) (a). Neuronal 2-AG is synthesized on demand in the postsynaptic membrane via cleavage of DAG by diacylglycerol lipase α (DAGLα) (b), then is trafficked to the presynaptic cell via mechanism that is still not well understood (c). 2-AG then binds and activates the CB1R (d). Activation of the CB1R (inhibitory GPCR) results in a decrease of calcium influx into the presynaptic cell and subsequently reduces the amount of neurotransmitter release from the presynaptic neuron. Finally, 2-AG is degraded by MAGL in the presynaptic cell. (e) This pathway in effect enables the postsynaptic cell to dampen presynaptic signaling inducing a short term depression of the neuronal circuit.

By in large, CB1Rs are localized to the perisynaptic membrane on the presynaptic terminal in many highly plastic limbic brain regions relevant for fear conditioning, emotional processing and fear extinction learning (Kawamura, Fukaya et al. 2006, Yoshida, Uchigashima et al. 2011, Ramikie, Nyilas et al. 2014). Indeed, eCB signaling is highly involved in the regulation of important neural circuitry that regulates downstream activation of the HPA axis and autonomic nervous system(Wamsteeker, Kuzmiski et al. 2010, Gray, Vecchiarelli et al. 2015) (Morena, Patel et al. 2016). AEA is the less abundant of the two eCBs and acts as only a partial agonist at the CB1R. There are several pathways for AEA synthesis and it is still unclear which pathway is preferential in specific circumstances (Astarita and Piomelli 2009) (Simon and Cravatt 2008, Ueda, Suzukamo et al. 2013). Fatty acid amide hydrolase (FAAH) is the primary enzyme that terminates AEA signaling by hydrolysis (Cravatt, Giang et al. 1996).

2-AG is the most abundant eCB in the CNS. 2-AG has been found to be important for return to homeostasis after acute stress. It was originally thought to be simply an intermediate in a mechanism to convert diacylglycerols into free arachidonic acid (AA) (Prescott and Majerus 1983). 2-AG is synthesized 'on demand' in response to postsynaptic depolarization and Ca2+ influx, activation of Gq/11 coupled GPCRs, and in greater amounts in response to synchronized Ca2+ influx and Gq/11 8 activity (Kreitzer and Regehr 2001),(Kano, Ohno-Shosaku et al. 2009, Ohno-Shosaku and Kano 2014, Ramikie, Nyilas et al. 2014) (Hashimotodani, Ohno-Shosaku et al. 2013). Multiple pathways can result in 2-AG signaling, however it is generally accepted that 2-AG is synthesized by DAGLα in response to GPCR activity is the predominate pathway utilized in the CNS. DAGLα activity is required for 2-AG facilitated retrograde synaptic inhibition, its primary function in the CNS (Stella, Schweitzer et al. 1997, Bisogno, Howell et al. 2003) The precursor for 2-AG is the DAG, 1-stearoyl-2-arachidonoyl-snglycerol (SAG), which is produced

by the phospholipase Cβ (PLCβ)-mediated cleavage of PIP2, a component of the plasma membrane that is ubiquitously dispersed across cell types (Aaltonen, Riera Ribas et al. 2014). SAG is thought to be the precursor molecule for most of the 2-AG synthesized in the CNS as it is readily available and abundant in the CNS lipid membrane profile. 2-AG synthesis is stimulated by the activation of Gq/11 coupled metabotropic receptors, (metabotropic glutamate receptors mGluR 1 and 5) and muscarinic acetylcholine receptors. The PLCβ-DAGLα pathway likely regulates 'on demand' synthesis of 2-AG due to there being a calcium requirement for PLCβ activity and both calcium influx and PLCβ activity results in increased eCB-mediated synaptic signaling (Ohno-Shosaku, Maejima et al. 2001, Kim and Chouikha 2002, Ohno-Shosaku, Matsui et al. 2003, Fukudome, Ohno-Shosaku et al. 2004, Ramikie, Nyilas et al. 2014).

Two diacylglycerol lipase isoforms (DAGL α and β) have been cloned and the characterization of the functional and regulation of these enzymes is ongoing. The α isoform has a longer C-terminal tail with multiple potential regulatory and scaffolding protein binding sites and is more abundantly expressed in the CNS. (Bisogno et al. 2003, Jung et al. 2007, Oudin et al. 2011). DAGLα is mostly found in mature neurons localized adjacent to CB1R expressing axon terminals. DAGLβ is expressed more broadly in peripheral tissues and visceral organs such as the liver (Bisogno, Howell et al. 2003, Katona, Urban et al. 2006, Yoshida, Uchigashima et al. 2011) (Uchigashima, Narushima et al. 2007).

DAGLα-/- mice have several stress and anxiety relevant phenotypes (Shonesy, Bluett et al. 2014, Shonesy, Bluett et al. 2014, Jenniches, Ternes et al. 2016, Patel, Shonesy et al. 2016) (Fig.1.6). DAGLα-/- produce up to 80% less 2-AG in the CNS and exhibit grossly diminished 2-AG signaling in the CNS. DAGLβ-/- mice show a 50% reduction in CNS 2-AG content do not have 2-AG mediated synaptic signaling deficiencies (Gao, Vasilyev et al. 2010) (Tanimura,

DAGLα -/- mice	Shonesy et al., 2014	Jenniches et al., 2016
Anxiety		
Light/dark (dark preference)	↑	↑
NIH assay (latency to drink)	↑	–
Zero maze (DT in open arms	↑	↑
Fear extinction	–	↓
Depression		
Tail suspension (immobility)	**NC**	–
Forced swim (immobility)	–	
Sucrose preference	♀↓ **NC** ♂	**NC**

Figure 1.6. Comparison of anxiety and depression-like behaviors in two separate Dagl-/- mice. (-) not done, (NC) no change (Shonesy, Bluett et al. 2014, Jenniches, Ternes et al. 2016). Figure adapted from (Shonesy, Bluett et al. 2014).

Yamazaki et al. 2010). Monoacylglycerol lipase (MAGL) hydrolyzes 87% of 2-AG to form arachidonic acid (Dinh, Carpenter et al. 2002, Blankman, Simon et al. 2007, Hoover, Blankman et al. 2008, Hermanson, Hartley et al. 2013). Two additional serine hydrolases, ABHD6 and ABHD12, account for a further 13% of 2- AG hydrolysis in rat brain membrane preparations (Blankman, Simon et al. 2007). MAGL inhibition results in antidepressant and anxiolytic effects in mice, however as discussed in Chapter II, inhibition of MAGL 2 hours prior to fear extinction

after auditory cue fear conditioning, resulted in a gross impairment of fear extinction (Fiskerstrand, H'Mida-Ben Brahim et al. 2010, Kinsey, O'Neal et al. 2011, Kinsey, Wise et al. 2013, Tchantchou and Zhang 2013, Nomura, Morrison et al. 2011, Hartley, Gunduz-Cinar et al. 2016). Knockdown of MAGL in cultured cells enhances the accumulation of 2-AG following application of ionomycin, which stimulates the production of 2-AG by elevating intracellular Ca^{2+} (Dinh, Carpenter et al. 2002). DAGLα is expressed in the postsynaptic neuron and MAGL is co-expressed with CB1R in tightly regulated presynaptic microdomains to facilitate precise control over signaling and rapid hydrolysis (Gulyas, Cravatt et al. 2004). Whether this regulation is driven mainly to clear 2-AG from the synaptic terminal or in response to a demand for AA synthesis, remains unclear. It is important to note that 2-AG was originally thought to have little function except as a an intermediate in the production of arachidonic acid (AA) (Prescott and Majerus 1983). In this past decade 2-AG has been found to be an important regulator of the stress response. However it should not be overlooked that when 2-AG is hydrolyzed by MAGL to produce AA, AA also has important signaling capabilities in the presynaptic bouton. AA has been shown to mediate receptor expression, lipid trafficking, and calcium channel regulation (Dh, Sladek et al. 1995, Horimoto, Nabekura et al. 1997, Fink, Lesage et al. 1998, Meves 2008, Yang, Zhang et al. 2008, Pertwee 2015). Presynaptic AA activity related to outcomes of the following study are discussed in more detail in Chapter III. It is likely that both 2-AG and AA signaling are important synaptic modulators. Both AEA and 2-AG signaling serve to modulate stress effects with downstream consequences for HPA axis activity and the capacity to return to homeostasis after exposure to stress. In the following Chapter we have attempted to clarify how 2-AG synthesis and signaling mitigates fear extinction in mice using a auditory cue fear conditioning- fear extinction paradigm by inhibiting DAGL at key time points throughout the study.

Our overarching hypothesis is that DAGL regulates fear extinction learning in male and female mice following fear conditioning and is not a consequence of adaptations resulting from a genetic deficiency. Overall, our convergent pharmacological and genetic data demonstrate an important role for DAGL in the regulation of fear extinction and further suggest deficient 2-AG-mediated eCB signaling may be an important susceptibility endophenotype that promotes the risk for the development of trauma-related psychiatric disorders.

CHAPTER II

INHIBITION OF DIACYLGLYCEROL LIPASE IMPAIRS FEAR EXTINCTION IN MICE

Victoria S. Cavener1,2, Andrew Gaulden1, Dante Pennipede1, Puja Jagasia1, Md. Jashim Uddin3, Lawrence J. Marnett3, Sachin Patel1,2,*
1Department of Psychiatry and Behavioral Sciences, Vanderbilt University Medical Center, Nashville, TN, USA
2Vanderbilt Brain Institute, Vanderbilt University, Nashville, TN, USA
3Departments of Biochemistry, Chemistry, and Pharmacology, A.B. Hancock Jr. Memorial Laboratory for Cancer Research, Vanderbilt Institute of Chemical Biology, Vanderbilt University School of Medicine, Nashville, TN, USA

Abstract

Elucidating the underlying molecular mechanisms regulating fear and extinction learning may offer insights that can lead to novel treatments for debilitating anxiety and trauma-related disorders including posttraumatic stress disorder (PTSD). The endocannabinoid (eCB) system is a retrograde inhibitory signaling pathway involved in regulating central responses to stress. The eCB 2-arachidonoylglycerol (2-AG) has recently been proposed to serve as a homeostatic signal mitigating adverse effects of stress exposure, however, less well understood is 2-AG's role in fear learning and fear extinction. In this study, we have sought to explore 2-AG's role in fear conditioning and fear extinction by disrupting 2-AG synthesis utilizing the DAGL inhibitor (DO34) and DAGLα knock-out mice (DAGLα-/-). We found that DAGLα-/- mice, and male and female C57B6/J mice treated with DO34, exhibited impairment in extinction learning in an auditory cue fear-conditioning paradigm. DO34 did not increase unconditioned freezing. Interestingly, inhibition of fatty-acid amide hydrolase (FAAH) was not able to restore normal fear extinction in DO34-treated mice suggesting increased Anandamide (AEA) cannot compensate for

deficient 2-AG signaling in the regulation of fear extinction. Moreover, augmentation of CB1R signaling with tetrahydrocannabinol (THC) also failed to restore normal fear extinction in DO34-treated mice. Overall, these data support the hypothesis that DAGLα plays an important role in fear extinction learning. Although genetic and pharmacological disruption of DAGL activity causes widespread lipidomic remodeling, these data combined with previous studies putatively suggest that deficient 2-AG signaling could be a susceptibility endophenotype for the development of trauma-related psychiatric disorders.

Introduction

Over the past 25 years, studies have shown that the endocannabinoid (eCB) system is a key regulator of an organism's response to stress and plays an important role in facilitating recovery after exposure to stress (Lutz, Marsicano et al. 2015, Patel, Hill et al. 2017, Hill, Campolongo et al. 2018). Dysregulation of fear learning, fear extinction learning, and the abnormal retention of a heightened fear response have been implicated in many anxiety-related mental illnesses including posttraumatic stress disorder (PTSD) (Parsons and Ressler 2013, Maren and Holmes 2016, Deslauriers, Toth et al. 2017) . Understanding the molecular mechanisms involved in fear learning, fear extinction, and the development of generalized anxiety with persistent hyper responsiveness to stressful situations, could lead to important insights into the pathophysiological mechanisms underlying fear adaptations and potentially novel treatment approaches for stress-related mental disorders. In this study, we investigate the role of eCBs in fear learning and fear extinction via pharmacological and genetic modulation of 2-arachidonoylglycerol (2-AG) synthesis in male and female mice.

The eCB system is a retrograde inhibitory signaling pathway composed of the presynaptic cannabinoid CB1 receptor (CB1R) and its endogenous ligands AEA (arachidonoylethanolamine) and 2-AG (Herkenham, Lynn et al. 1990, Matsuda, Lolait et al. 1990, Devane, Hanus et al. 1992, Piomelli 2003, Ohno-Shosaku and Kano 2014). 2-AG is the most abundant eCB in the brain and is acutely increased by stress exposure (Kondo, Kondo et al. 1998, Patel, Roelke et al. 2005, Patel, Kingsley et al. 2009, Dubreucq, Matias et al. 2012, Bedse, Hartley et al. 2017). It is hypothesized that this stress-induced increase in 2-AG signaling serves to counteract some of the adverse behavioral consequences of stress exposure (Hohmann, Suplita et al. 2005, Evanson, Tasker et al. 2010, Hill, McLaughlin et al. 2011, Wang, Hill et al. 2012, Bluett, Gamble-George et al. 2014, Bedse, Hartley et al. 2017, Bluett, Baldi et al. 2017). Conversely, AEA is decreased in response to stress, and plays a role in activating the stress response within the HPA-axis (Dubreucq, Matias et al. 2012, McLaughlin, Hill et al. 2012, Wang, Hill et al. 2012, Gray, Vecchiarelli et al. 2015, Gray, Wilson et al. 2016). Studies have shown that augmenting 2-AG reduces stress-induced anxiety-like and depressive-like behaviors and can promote resilience to the adverse effects of acute and repeated stress (Kinsey, O'Neal et al. 2011, Sciolino, Zhou et al. 2011, Sumislawski, Ramikie et al. 2011, Roberts, Stuhr et al. 2014, Zhang, Wang et al. 2015, Bedse, Hartley et al. 2017, Bluett, Baldi et al. 2017, Heinz, Genewsky et al. 2017). 2-AG augmentation can also increase active fear responses to threats (Heinz, Genewsky et al. 2017). Surprisingly, 2-AG augmentation promotes the expression of conditioned freezing and impairs conditioned fear extinction learning (Llorente-Berzal, Terzian et al. 2015, Hartley, Gunduz-Cinar et al. 2016). This surprising contradiction highlights the complexity of eCB signaling in the brain and motivated us to further investigate the role of 2-AG signaling in the regulation of fear learning and extinction.

DAGLα is a key enzyme responsible for 2-AG synthesis in the postsynaptic neuron in response to increased synaptic activity (Bisogno, Howell et al. 2003, Tanimura, Yamazaki et al. 2010, Shonesy, Bluett et al. 2014). Repeated homotypic stress results in increased 2-AG production via DAG hydrolysis by DAGLα (Patel, Kingsley et al. 2009). DAGLα-/- mouse models show reduced levels of 2-AG (and in some cases AEA (Tanimura, Yamazaki et al. 2010, Jenniches, Ternes et al. 2016)), increases in anxiety associated behaviors and increased susceptibility to adverse behavioral consequences of stress exposures (Shonesy, Bluett et al. 2014, Jenniches, Ternes et al. 2016, Bluett, Baldi et al. 2017). These studies are consistent with work demonstrating reducing 2-AG levels via MAGL overexpression also increases anxiety-like behaviors (Guggenhuber, Romo-Parra et al. 2015). In addition, acute treatment with the DAGL inhibitor DO34 increases innate anxiety levels and promotes susceptibility to the adverse behavioral consequences of stress in mice (Bedse, Hartley et al. 2017, Bluett, Baldi et al. 2017). Interestingly, DAGLα-/- also exhibit impairment in extinction of conditioned fear responses (Jenniches, Ternes et al. 2016). Here we aim to replicate and extend these findings to gain insight into how DAGL regulates fear extinction learning using a combination of pharmacological and genetic approaches in male and female mice. Overall, our convergent pharmacological and genetic data demonstrate an important role for DAGL in the regulation of fear extinction and further suggest deficient 2-AG-mediated eCB signaling may be an important susceptibility endophenotype subserving risk for the development of trauma-related psychiatric disorders.

Results

DAGLα-/- mice have impaired fear extinction

Given that it has been previously shown that global DAGLα-/- mice exhibit impaired fear extinction (Jenniches, Ternes et al. 2016), we first aimed to replicate these findings in our line of global DAGLα-/- mice in a fear conditioning and extinction protocol we and others have previously utilized extensively (Hartley, Gunduz-Cinar et al. 2016) (Fig. 1a). There was no significant difference in freezing to tone during fear-conditioning on day 1 between DAGLα-/- and WT littermate controls (Fig. 1b-c). However, freezing behavior in response to tone presentation during extinction was increased in both male and female DAGLα-/- mice compared to WT littermate controls (Fig. 1b-c, Female day 2 and 3: p= 0.0154/0.0124, F(1,7) = 6.422/11.18, eta2= 0.23/0.32, males day 2 and 3: (p= 0.0390/0.0261, F(1,7) = 10.13/7.901, $\eta 2$ = 0.26/0.30). Freezing levels were also significantly higher during extinction recall in DAGLα-/- male and female mice compared to WT mice during both baseline and tone presentation (females day 4:p= 0.0389, F(1,7) = 6.431, $\eta 2$ = 0.28, males day 4: p= 0.0045, F(1,7) = 16.87, eta2= 0.59).

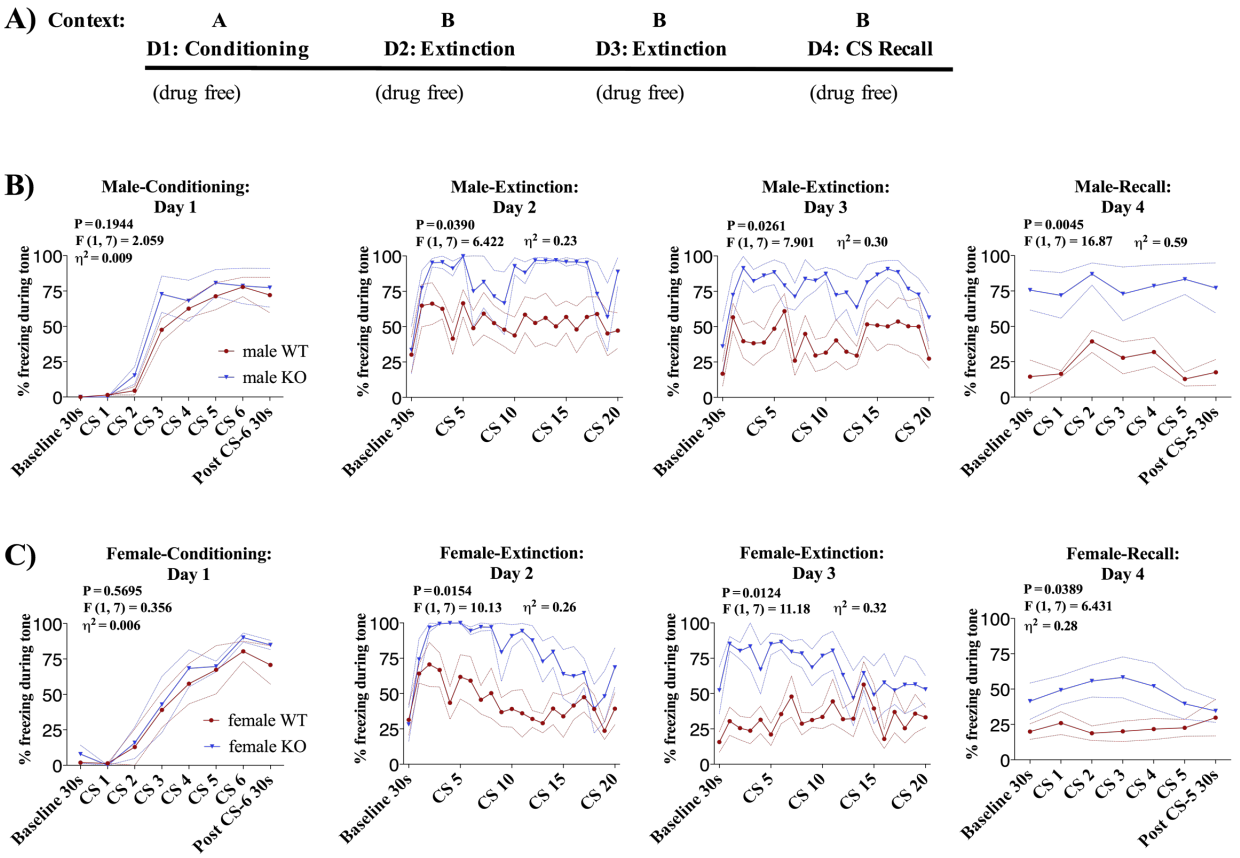


Figure 2.1. Male and female DAGLα-/- mice have impaired fear extinction. (A) Schematic diagram of the experimental paradigm. (B) (Far left panel) % freezing by male DAGLα-/- and WT mice during acquisition of cue-conditioned fear. (Middle panels) % freezing during auditory cue during extinction training days 2 and 3. (Far right panel) % freezing during extinction recall (n=5 WT, n=4 KO mice) (C) (Far left panel) % freezing by female DAGLα-/- and WT mice during acquisition of cue-conditioned fear. (Middle panels) % freezing during auditory cue during extinction training days 2 and 3. (Far right panel) % freezing during extinction recall (n=5 WT, n=4 KO mice). F, P and η2 values for genotype effect shown in each panel. All values are presented as mean ± SEM.

Pharmacological DAGL inhibition does not affect acquisition of conditioned-fear

In order to confirm the effects observed in DAGLα-/- mice were mediated via impaired enzymatic activity during adulthood and to gain insight into the temporal regulation of fear learning and extinction by DAGL, we utilized a pharmacological inhibitor of DAGL, DO34 (Ogasawara, Deng et al. 2016). We first tested whether acquisition of conditioned fear was regulated by DAGL activity via administration of DO34 or vehicle 2 hours prior to fear conditioning on day one in Context A (Fig. 2a). We observed a very small but significant decrease in freezing behavior in DO34-treated male mice compared to vehicle-treated controls during conditioning (Fig. 2b, day 1: $p= 0.050$, $F(1,38) = 8.875$, $\eta 2 = 0.025$) suggesting a slight delay in the acquisition of conditioned freezing behavior. In contrast, there was no significant difference in subsequent freezing behavior during tone presentation during extinction training on days 2-3 nor during extinction recall on day 4 in the drug-free states.

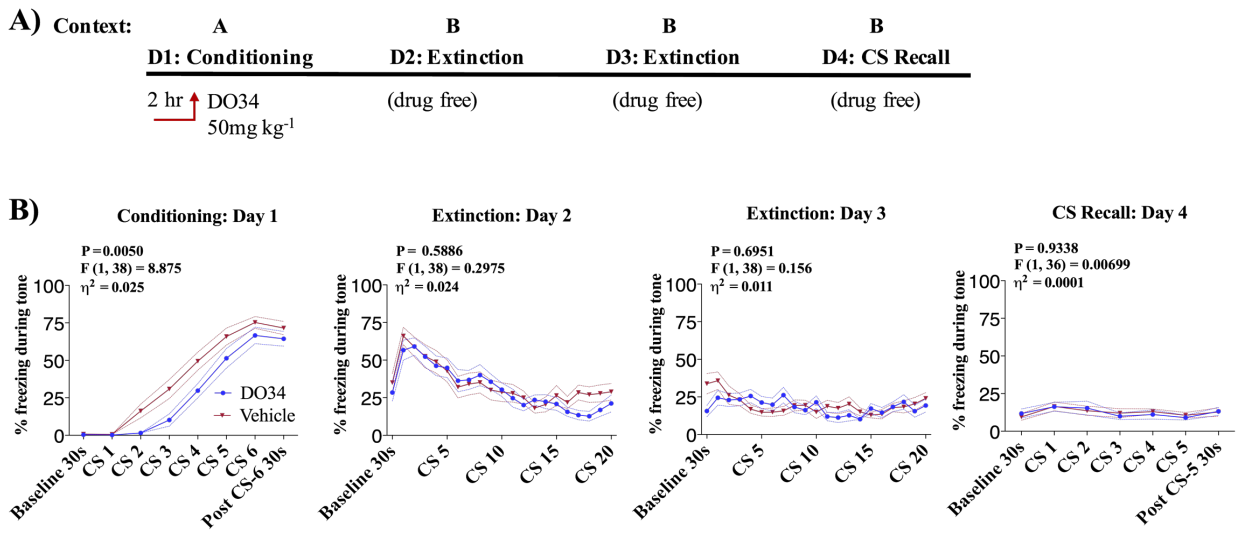


Figure 2.2. Pharmacological DAGL inhibition does not affect acquisition of conditioned-fear. (A) Schematic diagram of the experimental paradigm. (B) (Far left panel) % freezing by C57BL/6 J male mice during acquisition of cue-conditioned fear when DO34 (50mg kg-1) was injected IP 2 hours prior to trial. (Middle panels) % freezing during auditory cue during extinction training days 2 and 3. (Far right panel) % freezing during extinction recall (n=20 male mice per condition). F, P and η2 values for main effect of drug treatment show in each panel. All values are presented as mean ± SEM.

Pharmacological DAGL inhibition impairs fear extinction

Data derived from global DAGLα-/- mice suggest a potential role for 2-AG signaling in the regulation of fear extinction; however, limitations of global knock-out models make conclusive interpretations in this regard difficult (Jenniches, Ternes et al. 2016). To circumvent these limitations, we next determined whether acute depletion of 2-AG using the pharmacological

DAGL inhibitor DO34 would also inhibit fear extinction learning in male and female mice (Fig. 3a). We utilized a dose of 50 mg/kg which we have shown causes a near-complete elimination of measurable 2-AG throughout the brain (Bedse, Hartley et al. 2017, Bluett, Baldi et al. 2017). Prior to conditioning, mice were assigned to vehicle or DO34 treatment groups. There were no differences in freezing response to tone-sock pairings between groups (Fig. 3b), confirming similar conditioning efficiency in both treatment groups. On day two, DO34 or vehicle was injected 2 hours prior to extinction training. DO34-treated male and female mice showed a significant increase in percent freezing time during tone presentation on both extinction day 2 and extinction day 3 (Fig. 3b-c Fig. 3b-c, day 2 and 3: female p= 0.0023/0.0006 $F(1,38)= 10.69/14.13$, $\eta2 = 0.097/0.142$, male p= 0.0015/0.0023, $F(1,33) = 11.96/10.93$, eta2= 0.11/0.11) suggesting a slight delay). During the extinction recall test on day 4, performed under drug-free conditions, previously DO34-treated mice showed a sensitized freezing response during baseline and subsequent tone presentation relative to vehicle-treated mice Fig. 3b-c, day 4: female p=0.0005, $F(1,36)= 14.5$, $\eta2= 0.195$, male p= 0.0015, $F(1,33) = 11.96$, eta2= 0.11). Similar effects were observed in female mice (Fig. 3c). The effect of DO34 treatment during extinction training was similar to that observed in mice that did not undergo extinction training compared to those that did (Fig. S1 day 4: p=0.0075, $F(1,28)= 8.136$, $\eta2 = 0.166$), indicating DAGL inhibition during extinction training reduces the effectiveness of fear extinction training, mirroring effects obtained in mice which had not undergone extinction training at all.

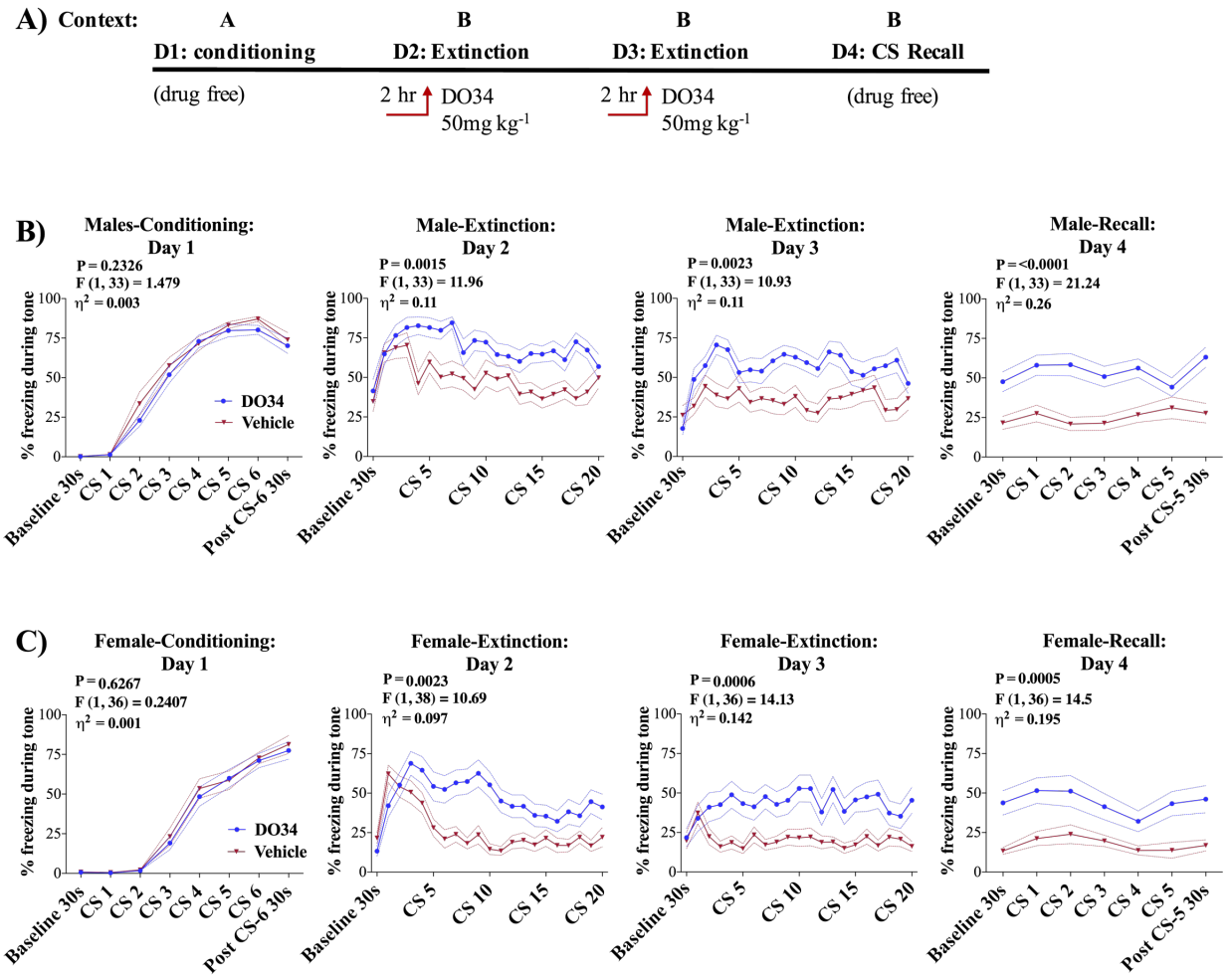


Figure 2.3. Pharmacological DAGL inhibition impairs fear extinction (A) Schematic diagram of the experimental paradigm. (B) (Far left panel) % freezing by C57BL/6 J male mice during acquisition of cue-conditioned fear. (Middle panels) % freezing during auditory cue by mice during extinction training days 2 and 3 when D034 (50mg kg-1) was injected IP 2 hours prior to trial. (Far right panel) % freezing during extinction recall (n=17 DO34-treated male mice, n=18 vehicle-treated male mice). (C) (Far left panel) % freezing by C57BL/6 J female mice during acquisition of cue-conditioned fear when DO34 (50mg kg-1) was injected IP 2 hours prior to trial. (Middle panels) % freezing during auditory cue during extinction training days 2 and 3. (Far right panel) % freezing during extinction recall (n=18 DO34-treated female mice n=20 vehicle-treated female mice). F, P and η2 values for main effects of drug treatment shown in each panel. All values are presented as mean ± SEM.

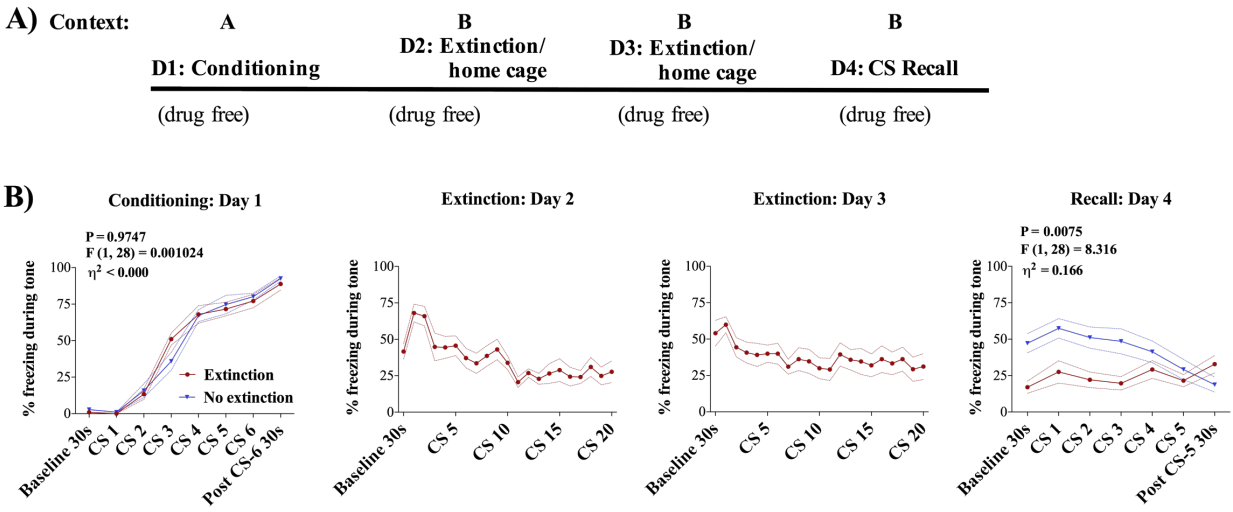


Figure 2.4. (S1) DO34 treatment prior to extinction training mimics the effect of no fear extinction training. (A) Schematic diagram of the experimental paradigm. (B) (Far left panel) % freezing by C57BL/6 J male mice during conditioning assay (Middle panels) % freezing during auditory cue during extinction training days 2 and 3. "No extinction" group remained in home cage during extinction trials day 2-3. (Far right panel) % freezing during extinction recall (n=15 male mice per condition). F, P and η2 values for main effect of condition (extinction vs. no extinction) shown in relevant panels. All values are presented as mean ± SEM.

Pharmacological DAGL inhibition does not affect unconditioned freezing

Given that DAGLα inhibition can increase unconditioned anxiety (Shonesy, Bluett et al. 2014, Jenniches, Ternes et al. 2016), we wanted to rule out the possibility that DO34 increased freezing behavior independent of a fear-conditioning. To explicitly test this, mice were tested in a sham conditioning paradigm where, on day one in Context A, mice were exposed to tone without successive shocks (i.e. CS only) (Fig. 4a). On days 2-3 mice were injected with DO34 2 hours prior to sham fear extinction training. Freezing behavior was not different between DO34-treated

and vehicle-treated mice on days 2-3 of sham extinction, or on day 4 of sham extinction recall (Fig. 4b). These data indicate that DO34 does not increase freezing behavior independent of conditioning.

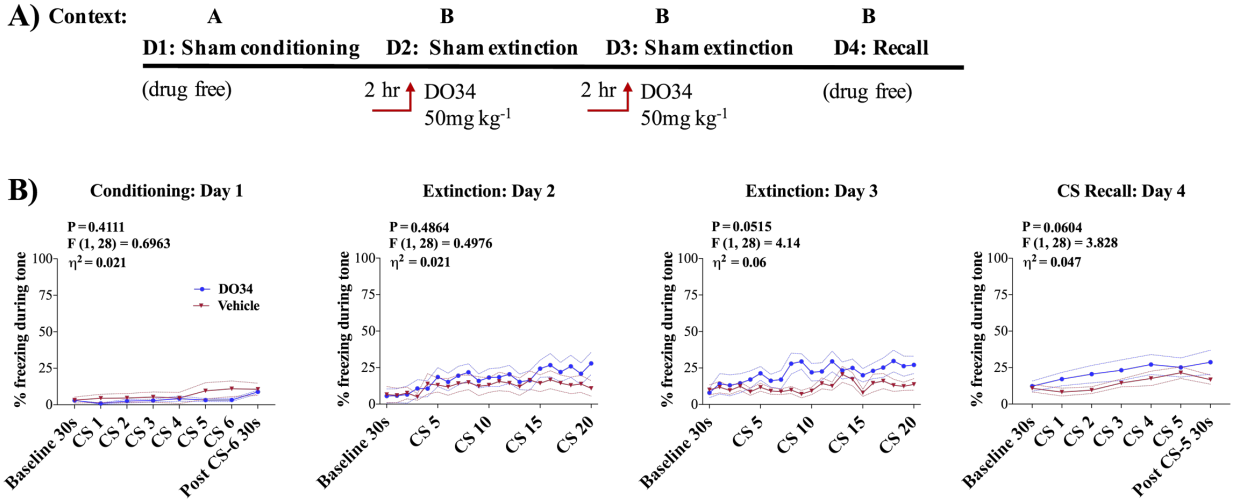


Figure 2.5. Pharmacological DAGL inhibition does not affect unconditioned freezing. (A) Schematic diagram of the experimental paradigm. (B) (Far left panel) % freezing by C57BL/6 J male mice during sham-conditioning assay-no shock was administered after tone. (Middle panels) % freezing during auditory cue during extinction training days 2 and 3. (Far right panel) % freezing during extinction recall (n=15 male mice per condition). F and P values for main effect of drug treatment shown in each panel. All values are presented as mean ± SEM.

AEA augmentation does not reverse impaired extinction after DAGL inhibition

In order to determine whether augmentation of AEA signaling could compensate for deficient 2-AG synthesis and promote successful fear extinction, we blocked AEA degradation using the FAAH inhibitor PF-3845 concomitantly with DO34 treatment during extinction training

(Fig. 5a). We found no significant difference between the groups' freezing behaviors when mice were given DO34 + FAAH Inhibitor or DO34 alone, prior to fear extinction training on days 2 or 3 (Fig. 5b).

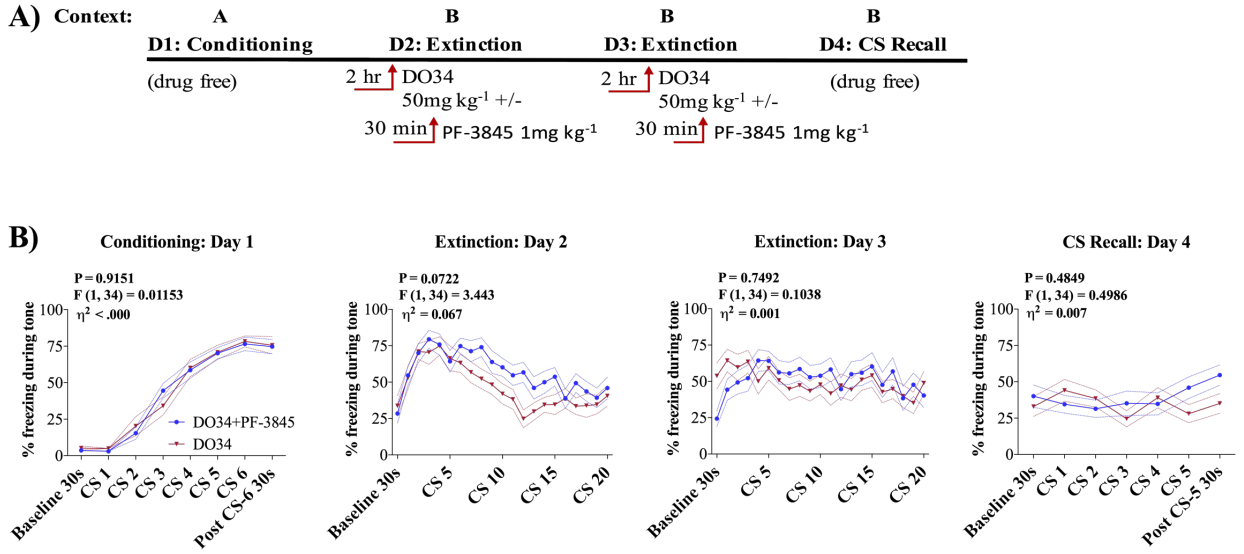


Figure 2.6. AEA augmentation does not reverse impaired extinction after DAGL inhibition (A) Schematic diagram of the experimental paradigm. (B) (Far left panel) % freezing by C57BL/6 J male mice during acquisition of cue-conditioned fear. (Middle panels) % freezing during auditory cue by mice during extinction training days 2 and 3 when DO34 (50mg kg-1) was injected alone or in addition to PF-3845 (FAHH inhibitor 1 mgkg-1) IP 2 hours prior to trial. (Far right panel) % freezing during extinction recall (n=18 male mice per condition). F, P and η2 values for main effect of drug shown in each panel. All values are presented as mean ± SEM.

CB1R partial agonist THC does not reverse impaired extinction after DAGL inhibition

In order to determine whether activation of CB1R receptors could compensate for deficient 2-AG synthesis and promote successful fear extinction, we injected THC (0.3-0.6 mg/kg) 30 minutes before extinction training on days 2 and 3. We found no significant difference between

the groups' freezing behaviors when mice were given DO34 + THC or DO34 alone, prior to fear extinction training on days 2 or 3 (Fig. S2).

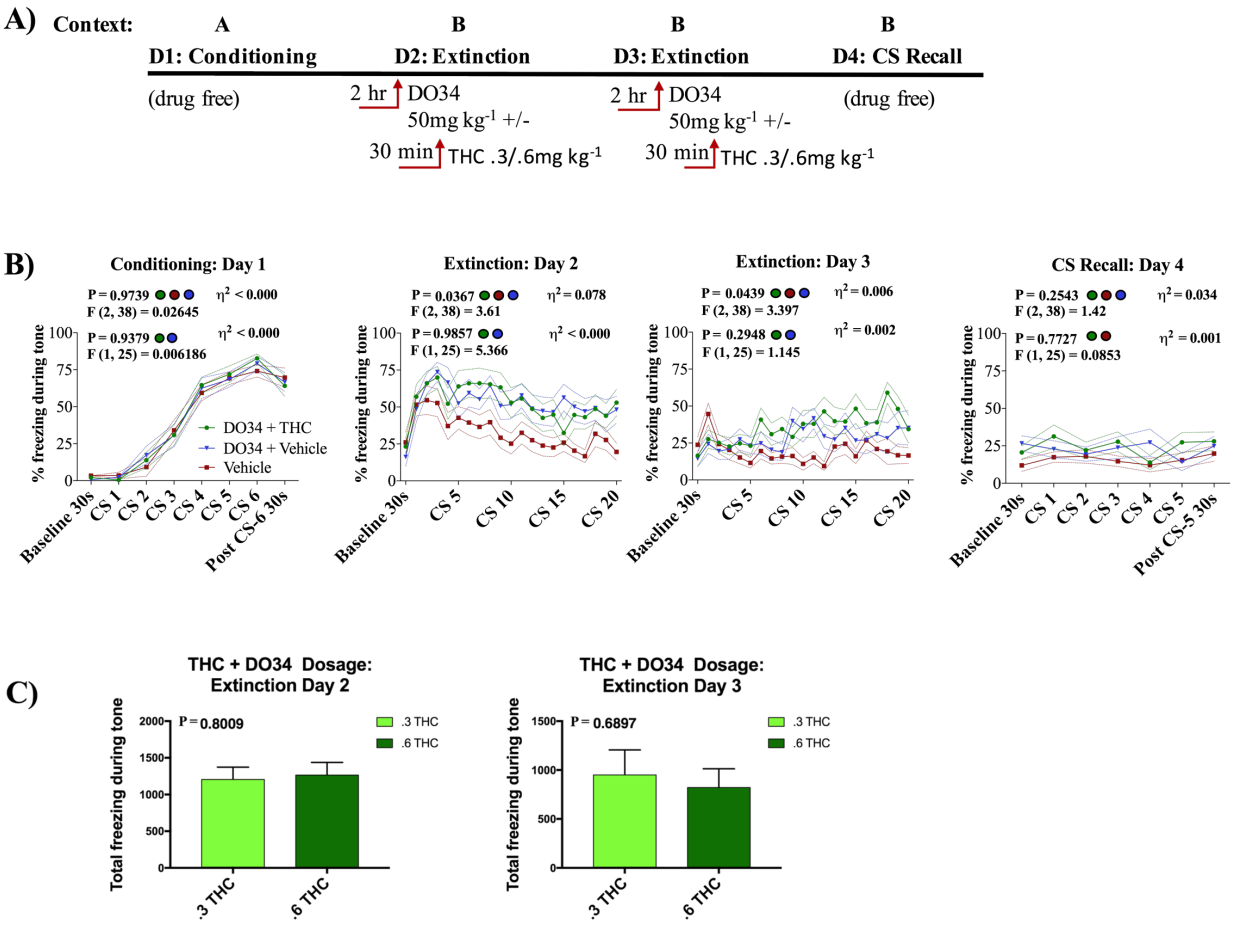


Figure 2.7. (S2). THC does not reverse impaired extinction after DAGL inhibition (A) Schematic diagram of the experimental paradigm. (B) (Far left panel) % freezing by C57BL/6 J male mice during acquisition of cue-conditioned fear. (Middle panels) % freezing during auditory cue by mice during extinction training days 2 and 3 when DO34 (50mg kg-1) was injected IP 2 hours prior to trial, alone or with an additional injection of THC 30 minutes prior to extinction training (THC 0.6 mgkg-1) (Far right panel) % freezing during auditory cue during CS recall (n=14 DO34+THC-treated male mice, n=13 DO34+vehicle=treated male mice, n=14 vehicle+vehicle-treated male mice). F and P and η2

values obtained by repeated measures two-way ANOVA representing effect of DAGL+THC treatment, DO34+vehicle treatment, and vehicle only treated animals, prior to fear extinction training. A separate ANOVA was conducted to compare DO34+THC and DO34+vehicle-treated animals, and is shown. Colored dots corresponding to treatment legend delineates which treatments are represented in each analysis. All values are given as mean ± SEM. (C) In a separate experiment there was no significant difference in total freezing behavior between two doses of THC (THC 0.3 and 0.6 mgkg-1) on extinction days 2 and 3. Significance values obtained by student t-test of total freezing behavior during the entire trial.

Discussion

eCB signaling is a well-established regulator of fear extinction, however, the role of 2-AG in the regulation of these processes has only recently been studied due to previously limited pharmacological and genetic tools to interrogate 2-AG signaling *in vivo* (Llorente-Berzal, Terzian et al. 2015, Hartley, Gunduz-Cinar et al. 2016, Jenniches, Ternes et al. 2016). Generation of pharmacological tools for 2-AG augmentation and depletion (Ogasawara, Deng et al. 2016), and development of global and conditional DAGL$\alpha^{-/-}$ mice (Shonesy, Bluett et al. 2014, Bluett, Baldi et al. 2017), has significantly enhanced our ability to examine the role of 2-AG within multiple stress-related biological processes. In this context, we aimed to utilize convergent pharmacological and genetic modulation of DAGL to elucidate the role of 2-AG signaling in fear-learning and extinction. The main findings of the present study are that 1) global life-long DAGLα deletion results in impaired extinction of conditioned fear, 2) acute pharmacological DAGL inhibition impairs extinction of conditioned fear behavior, and 3) Neither FAAH inhibition nor THC were able to correct these deficits in fear extinction caused by acute DAGL inhibition. These data provide further support for the notion that 2-AG deficiency states could predispose to the development of stress-related psychopathology and that pharmacological approaches aimed at

counteracting this deficiency could represent novel approaches to the treatment of an array of anxiety and trauma-related disorders (Bedse, Hartley et al. 2017, Bluett, Baldi et al. 2017, Hill, Campolongo et al. 2018).

With regard to the effects of 2-AG modulation on acquisition of conditioned fear behaviors, our pharmacological data suggest 2-AG signaling may be important for aversive learning as DO34 administered prior to conditioning slowed the acquisition of conditioned freezing behavior. This finding is consistent with data showing accelerated acquisition of conditioned freezing in a trace fear conditioning paradigm after 2-AG augmentation with the monoacylglycerol lipase (MAGL) inhibitor JZL184 (Xu, Antion et al. 2014). These data suggest 2-AG signaling may be important for optimal cognitive function consistent with the proposed nootropic effects of CB1 receptor stimulation in ageing (Bilkei-Gorzo, Albayram et al. 2017). However, cognitive impairment of CB1 stimulation have also been demonstrated (Kruk-Slomka, Dzik et al. 2017), suggesting a potentially complex and/or divergent roles of eCB signaling relative to exogenous cannabinoid agonist administration.

Our findings that both genetic and pharmacological inhibition of DAGL impair fear extinction are consistent with a recent study showing impaired long-term fear extinction in DAGL$\alpha^{-/-}$ mice (Jenniches, Ternes et al. 2016). Our studies extend these findings by demonstrating impaired fear extinction after acute pharmacological DAGL inhibition, suggesting findings in DAGL$\alpha^{-/-}$ mice are not a consequence of developmental abnormalities or compensatory adaptations resulting from life-long DAGLα deletion. We further show that the effects of DAGL inhibition are not confounded by increases in unconditioned freezing. Overall, these data are consistent with long-standing findings that blockade or genetic deletion of CB1 receptors robustly impairs fear extinction (see (Lutz 2007, Ruehle, Rey et al. 2012, Rabinak and Phan 2014, Hill,

Campolongo et al. 2018) for review), supporting the notion that 2-AG depletion secondary to DAGL inhibition may be the cause of the behavioral effects observed here. However, DAGL inhibition causes widespread changes in lipidomic networks and thus lack of ability to conclusively ascribe 2-AG deficiency as causally related to the observed phenotypes remains a limitation of the work. Lastly, extinction impairing effects of DO34 were only observed when relatively high shock intensities were used for the US (0.7 mA), but not lower (0.4 mA) intensities (data not shown). This caused significant fear generalization as evidence by unconditioned freezing during the baseline period in all mice on days 2, 3 and 4. Whether the effects of DAGL inhibition are explicitly dependent on US intensity remains to be determined.

That acute pharmacological DAGL inhibition is associated with impaired extinction of conditioned fear is also globally consistent with the increased unconditioned anxiety and increased stress-susceptibility observed after DGAL inhibition and in DAGL$\alpha^{-/-}$ mice (Shonesy, Bluett et al. 2014, Jenniches, Ternes et al. 2016, Bedse, Hartley et al. 2017, Bluett, Baldi et al. 2017). These data are also consistent with the anxiolytic effects of 2-AG augmentation in a variety of unconditioned anxiety models and the ability or 2-AG augmentation to reduce, and promote resilience to, the adverse effects of stress exposure (Busquets-Garcia, Puighermanal et al. 2011, Kinsey, O'Neal et al. 2011, Sciolino, Zhou et al. 2011, Sumislawski, Ramikie et al. 2011, Zhang, Wang et al. 2015, Morena, Leitl et al. 2016, Bluett, Baldi et al. 2017). Taken together, these recent and compelling findings suggest 2-AG is an important signaling molecule involved in reducing unconditioned anxiety and the adverse effects of stress, and facilitating appropriate extinction of aversive memories. They also suggest 2-AG deficiency could represent a susceptibility endophenotype predisposing to anxiety and trauma-related psychiatric disorders (see (Hill, Campolongo et al. 2018)). A corollary to these conclusions is that 2-AG augmentation may

represent a novel approach for the treatment of anxiety and stress-related neuropsychiatric disorders, however, there are some contradictory findings in this regard. Specifically, as noted above (Fig. 2), 2-AG augmentation increased the acquisition of conditioned fear responses, increased the expression of conditioned fear (Llorente-Berzal, Terzian et al. 2015), and impairs fear extinction (Hartley, Gunduz-Cinar et al. 2016). Thus, there appears to be an emerging distinction between the effects of 2-AG augmentation on conditioned versus unconditioned fear behaviors, with only the latter being consistently reduced in response to pharmacological 2-AG augmentation.

A critical question that arises from these findings is how both augmenting and depletion of 2-AG results in similar fear extinction deficits. The fear promoting effects of 2-AG augmentation are absent in mice with GABA neuron-specific CB1 deletion, supporting the importance of 2-AG acting on GABAergic neurons to impair extinction and promote conditioned fear (Llorente-Berzal, Terzian et al. 2015). The retrograde inhibition of GABA release may regulate important circuits within the basolateral amygdala thereby enhancing neuronal output to the central amygdala controlling freezing behavior. It is also important to point out that CB1 deletion from forebrain glutamatergic neurons itself impairs fear extinction (Llorente-Berzal, Terzian et al. 2015). Based on these data we propose the extinction-impairing and anxiogenic effects of 2-AG deficiency are due to reduced activity at CB1 on limbic glutamatergic terminals, which may be tonically suppressed by 2-AG signaling. In contrast, the fear promoting and extinction impairing effects of MAGL inhibition are mediated via 2-AG accumulation, synaptic spillover, and subsequent activation of CB1 on GABAergic neurons controlling freezing behavior. We hypothesize that these synapses may physiologically be under less tonic inhibition by 2-AG than glutamatergic CB1, thus 2-AG depletion does not significantly change CB1 activity on GABAergic cells and does not

produce anxiolytic effects *per se*. A critical assumption in this model is the differential tonic inhibition of glutamate and GABA release mediated via 2-AG-CB1 signaling, an effect which remains to be tested experimentally. Additionally, that 2-AG augmentation reduces unconditioned anxiety suggests 2-AG may be acting on distinct neural circuits to affect excitation/inhibition balance to ultimately differentially affect conditioned versus unconditioned behaviors.

With regard to the therapeutic potential of eCB signaling, pharmacological AEA augmentation via inhibition of FAAH has also been demonstrated to have anxiolytic and anti-stress effects (Piomelli, Tarzia et al. 2006, Gunduz-Cinar, Hill et al. 2013, Hill, Campolongo et al. 2018), and to facilitate extinction of conditioned fear in some models (Gunduz-Cinar, MacPherson et al. 2013, Llorente-Berzal, Terzian et al. 2015). Furthermore, we have recently demonstrated that FAAH inhibition can prevent the unconditioned anxiety associated with acute 2-AG depletion (Bedse, Hartley et al. 2017). However, FAAH inhibition at the same dose used in Bedse et al. was unable to enhance fear extinction in mice treated with DO34, suggesting AEA cannot compensate for all aspects of DAGL inhibition. Furthermore, the addition of the CB1 partial agonist, THC was unable to overcome 2-AG-deficiency-induced impairments in fear extinction at dose that has mitigated anxiety like behaviors in non-fear conditioning paradigms (Bedse, Hartley et al. 2017). In Bedse et al. when animals were treated with 0.3mg/kg dose of THC + 50mg/kg of DO34 they showed decreases in anxiety like behaviors versus animals treated with DO34 alone. In this study, we tested both 0.3 and 0.6mg/kg doses and found no significance between doses in total freezing behavior or the ability to reverse fear extinction impairments caused by DAGL inhibition. Whether direct full CB1 agonists or CB1 positive allosteric modulators, or alternative doses of THC, could overcome DAGL inhibition-induced impairments in fear extinction is a critical open question in the field of cannabinoid therapeutics development.

In summary, by utilizing genetic and pharmacological approaches, we have demonstrated a that DAGL activity plays an important role in fear extinction learning in both male and female mice. We have also shown that augmentation of AEA levels during fear extinction cannot reverse fear extinction deficits caused by disrupting the molecular mechanisms regulating 2-AG synthesis. Our data combined with data in previous studies highlights the intriguing paradoxical findings that both depletion and augmentation of 2-AG levels impairs fear extinction. There are some limitations to the present work that should be considered in the context of the above interpretations. For example, DAGL inhibition decreases levels of several monoacylglycerols in addition to 2-AG and reduces arachidonic acid levels (Shonesy, Bluett et al. 2014, Ogasawara, Deng et al. 2016), both of which could affect physiology and behavior independent of 2-AG-mediated eCB signaling. In addition, both genetic and pharmacological approaches utilized here are systemic, therefore the specific brain regions and circuits responsible for the behavioral effects of DAGL inhibition are not currently known. Despite these limitations, the present data provide a solid framework from which to test critical hypotheses regarding the potential therapeutic benefits of eCB modulating compounds on stress-related behavioral outcomes.

Methods And Materials

Animals

9-12-week-old male and female C57BL/6 J were used as subjects (Jackson Laboratory, Bar Harbor, ME, USA). Male and female DAGL$\alpha^{-/-}$ mice on a C57BL/6J background were bred in house as described previously and used in one experiment (Shonesy, Bluett et al. 2014, Bluett, Baldi et al. 2017). All mice were habituated for one week at Vanderbilt Murine Neurobehavior Core prior to behavior testing. Three to five mice were housed per cage in a temperature and

humidity controlled housing facility under a 12-h light/dark cycle, with access to food and water ad libitum. Behavior experiments were performed during dark cycle under red light. All studies were approved by the Vanderbilt University Animal Care and Use Committees, and were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Drug Treatment

Mice were given an intraperitoneal (IP) injection of DO34 (50mg/kg) synthesized as previously described (Ogasawara, Deng et al. 2016) dissolved in an 18:1:1 solution of saline, ethanol and kolliphor EL (Sigma Aldrich, St. Louis, MO, USA), or vehicle alone (18:1:1 solution of saline, ethanol and kolliphor EL) 2 hours prior to behavioral testing at a volume of 10 ml/kg. In one experiment, mice were treated with a combination of DO34 (50mg/kg) and PF-3845 (FAAH Inhibitor) at 1 mg/kg (A gift from Pfizer Central Research), via IP injection 2 hours prior to behavioral testing, or just DO34 (50mg/kg) alone. In two experiments, mice were treated with a combination of IP injection of DO34 (50mg/kg) 2 hours prior to extinction training and Tetrahydrocannabinol (THC; CB1R partial agonist) at 0.3 mg/kg (in one experiment) or 0.6mg/kg (Cayman Chemical Company-18:1:1 solution of saline, ethanol and kolliphor EL), via IP injection 30 minutes prior to behavioral testing, or just DO34 (50mg/kg) alone. Doses utilized in this study were similar to those previously described in (Bedse, Hartley et al. 2017).

Fear-Condition and Extinction Paradigm

Freezing behavior was measured using video analysis software (Video Freeze-Med Associates) during all of the fear conditioning and extinction trials exactly as described previously

(Hartley, Gunduz-Cinar et al. 2016). The mice were placed in an auxiliary room directly adjacent to the testing room and allowed to acclimate for 30 minutes prior to each trial under red light, with consistent temperature and humidity conditions between the auxiliary and trial room. At the end of each protocol, the mice were placed in their respective home cages in the auxiliary room and later returned to the housing facility.

On testing day 1, the mice were placed in a square Plexiglas chamber (dimensions: 30.5 x 24.1 x 21.0 cm) housed in a sound proofing box developed by Med. Associates. Context A consists of a bare metal grid floor, no insert along the walls, and no added scent. Baseline measurement were taken for 90 seconds. After the baseline, six 30 second tones - conditioned stimulus (CS), were played through a chamber wall mounted speaker, each tone was followed by followed by a 2 second .70mA shock (unconditioned stimulus-US). There was a 30 second delay between each tone. All mice who failed to freeze at least 50% by final CS on conditioning day were removed from analysis except in figure 2 where effects of DO34 on conditioning *per se* were examined.

On testing days 2 and 3, mice were placed in the conditioning chamber in Context B for extinction training. Context B has a smooth white plastic floor insert to cover the metal grid, as well as a curved plastic insert along the side and back walls made of the same white plastic material as the floor. The wall insert has perforations that align with the wall mounted speaker in order to ensure the sound quality of the (CS). Additionally, a paper towel soaked in vanilla extract was placed under the floor insert and grid as a novel olfactory queue, specific to Context B. A baseline measure of freezing behavior was recorded for 30s followed by a series of 20 30s tones with a 30s delay between each tone. No shock was administered during the extinction training.

On day 4, the mice were put back in Context B for CS recall. No drug was administered prior to CS recall. Baseline measure was taken for 2.5 min followed by five 30s tones with a 30s

delay between each tone. A final measure of extinction recall was taken after the final tone for 2.5 minutes.

Statistical analysis

The freezing data for each group was analyzed using a repeated measures two-way ANOVA factoring trial block (time) and drug treatment/or genotype. Total freezing time was entered as reported by the video analysis software. All statistical analyses were conducted using Prism Graphpad 7. $P<0.05$ was considered significant throughout. F and P values for significant effects of drug treatment or genotype can be found within figure panels. Effect size was calculated using formula for η^2 to reflect the proportion of total variability in the freezing behavior that is accounted for by variation in genotypes or drug treatment during each trial (Tabachnick and Fidell 2007).

CHAPTER III

Discussion And Future Directions

Discussion

The data presented in Chapter II demonstrate a clear need for further research concerning the role of DAGL in fear extinction learning. Given that exposure therapy is modeled from fear extinction research and is the clinical standard of care for patients suffering from PTSD, better understanding of the molecular underpinnings that enhance or diminish the effects of the treatment may lead to medications that enhance the process or intercede to stop normal post traumatic stress responses from becoming maladaptive. In this study we have confirmed that acute DAGL activity is an important factor in inhibitory learning that is required to suppress a conditioned response rather than a consequence of developmental adaptions due to deletion of the gene. The results of this study also gave rise to several questions about how 2-AG signaling might spatially and temporally regulate fear extinction in mice. In addition this study also calls into question if fear extinction learning can be further regulated down stream of CB1R activity. Our main conclusions and hypothesis regarding these questions are discussed in the following section.

Freezing responses were clearly CS dependent and not simple anxiety as result of the DO34 treatment. In another experiment, data not shown, there were no significant differences when mice were conditioned to context rather than tone, and mice were injected with DO34 or vehicle 2 hours prior to context specific extinction training. This result is in contrast to the significant differences seen in fear extinction/freezing behavior exhibited by mice that were fear conditioned to context and treated with JZL184 prior to extinction training (Hartley, Gunduz-Cinar et al. 2016). Although in Hartley et al. the protocol used to context condition mice was longer (multiple days) and was done with the ICR strain as opposed to C57 mice, these results could indicate that the extinction

deficits seen with 2-AG augmentation versus 2-AG depletion are mediated by different neuronal circuits and/or molecular pathways. To further support the hypothesis that 2-AG augmentation affects circuits differently than 2-AG depletion, when mice were treated with JZl184 plus the CB1R antagonist Rimonabant, fear extinction deficits were reversed in both auditory cue (identical protocol/ICR mice) and context specific fear conditioning paradigms.

In contrast to other studies showing anxiolytic effects of CB1R stimulation, two partial CB1R agonists did not reverse fear extinction learning deficits in this study (Piomelli, Tarzia et al. 2006, Gunduz-Cinar, Hill et al. 2013, Hill, Campolongo et al. 2018). Using a CB1R antagonist, Hartley et al. reversed fear extinction deficits mediated by increased 2-AG after both context and auditory cue fear conditioning (Hartley, Gunduz-Cinar et al. 2016). Taken together with the other contrasting data, our hypothesis to explain this phenomenon is necessarily theoretical and warrants further investigation. Given that MAGL is expressed in presynaptic invaginating GABAergic synapses in the BLA, and less in glutamatergic presynaptic terminals, we hypothesize that increases in 2-AG by MAGL inhibition would cause significant spillover and inhibition of GABAergic signaling. Fear extinction is dependent on specific inhibition of glutamatergic fear promoting neurons within the BLA, and GABA release during fear extinction would be vital for this suppression, (Fig. 3.1a). DAGL is thought have a short half-life, and 2-AG is known to be synthesized on demand (Di Marzo, Melck et al. 1998, Ogasawara, Deng et al. 2016). Unpublished data from this lab has shown that 24 hours after fear conditioning 2-AG levels are down from basal pre-fear conditioned levels in the brain. At first glance this data may seem counter intuitive given the fear extinction learning deficits revealed by our data when 2-AG synthesis is blocked. However, these results may indicate that 2-AG and DAGL activity are increased as a consequence of the fear extinction paradigm and necessary for fear extinction

to occur. Excitatory signals from the LA to fear neurons in the BLA may need CB1R retrograde signaling to dampen activity in order to augment GABAergic inhibition of that circuit. 2-AG signaling may have only short term effects on particular synapses that express higher levels of MAGL in the BLA, making rapid degradation of 2-AG more likely (Fig. 3.1b). Again, by inhibiting MAGL, GABA inhibition would be diminished and the net effect of increased fear signaling would be the same It is highly likely that given the complexity of amygdalar neural circuitry, that 2-AG synthesis, and DAGL is tightly regulated in both a spatial and temporal manner. 2-AG augmentation my inhibit specific synapses not normally regulated by 2-AG at a particular time points, and result in similar behavioral deficits exhibited when 2-AG is depleted. Augmentation of CB1R during fear extinction without the capability to specificity target specific circuits, may have had the same effect as 2-AG augmentation and potentiated critical fear circuits. It is also possible that due to the fact that 2-AG is a full agonist of the CB1R, partial agonists are not capable of simulating the normal 2-AG signaling capacity. An experiment using a full CB1R agonist may offer insight to this question.

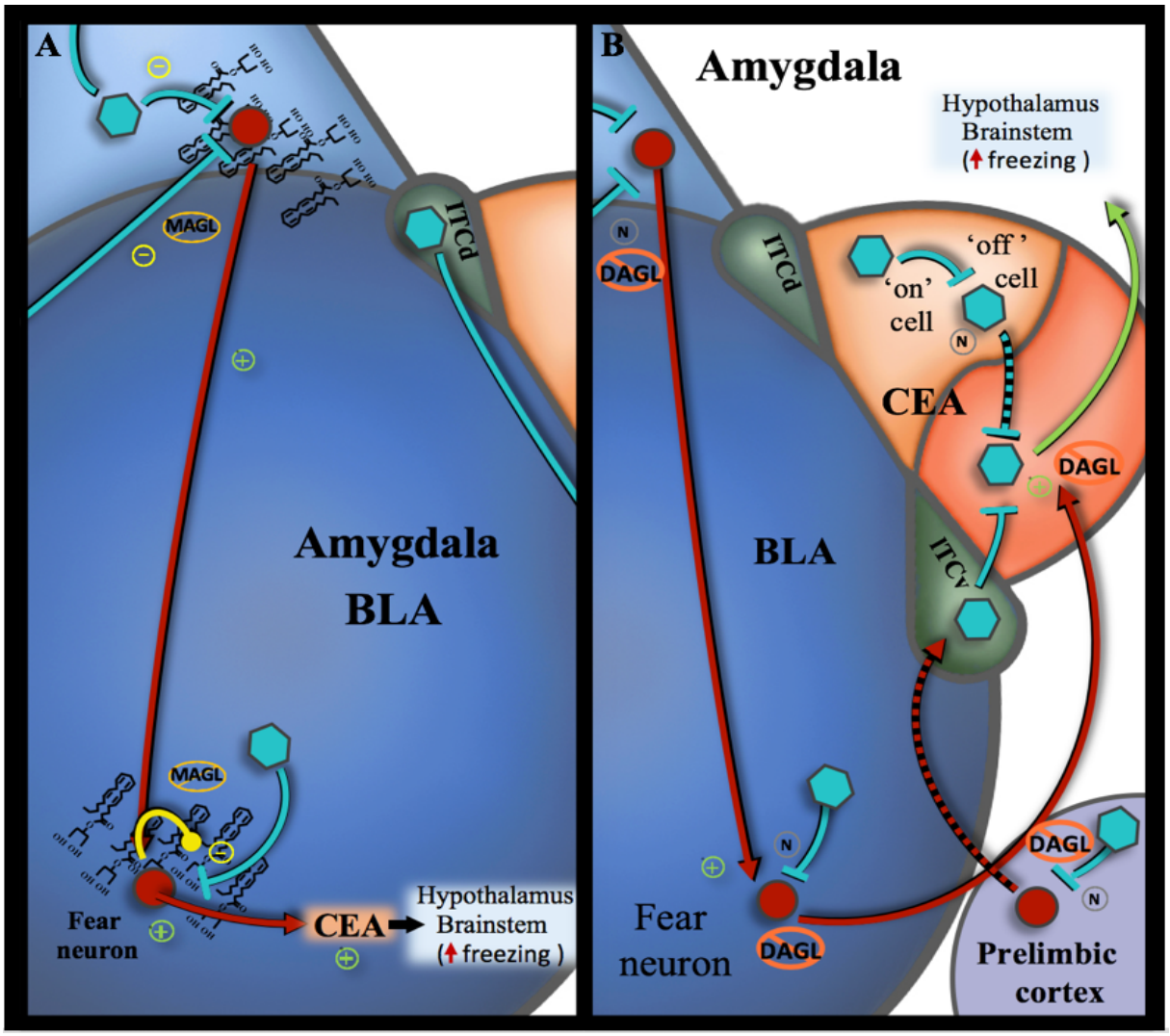


Figure 3.1. Theoretical models of fear neuron circuit regulation as a result of 2-AG augmentation [(a) MAGL inhibitor-JZL184] or depletion [(b)DAGL inhibitor-DO34]. Red lines indicate glutamatergic neurons and projections, blue lines indicate GABAergic neurons and projections. Green circles with plus signs indicate a synapse which is potentiated, yellow circles with a minus symbol indicates a synapse which is inhibited, and gray circles marked by the letter 'N' indicate synapses with a net neutral effect of 2-AG depletion in figure (b). Dotted lines indicate a projection with a weakened signaling output as a result of 2-AG depletion (b).

Injecting mice with JZL184 and inhibition the degradation of 2-AG by MAGL did not significantly affect fear conditioning. Injecting DO34 and inhibition the synthesis of 2-AG via DAGL, had a small but significant effect on fear conditioning. Mice given DO34 prior to conditioning had no significant difference in the percent of freezing behavior during the sixth conditioning CS/US stimulus. Neither JZL184 nor DO34 treatment administered before auditory-cue fear conditioning had any effect on subsequent 'drug free' fear extinction on days 2-3 compared to vehicle controls. Mice in both experiments however, did exhibit freezing behavior to tone at the beginning of the extinction trials (Hartley, Gunduz-Cinar et al. 2016). These results indicate fear learning is not overtly dependent on 2-AG/CB1R signaling, and neither increases or decreases of 2-AG is important for fear learning and memory consolidation processes.

One caveat to the data presented in Chapter II, continues to be somewhat puzzling. The lack of fear extinction deficit on days 2 and 3 when DO34 was administered prior to fear conditioning indicates that DO34 does not impair DAGL activity for more than 24 hours. A biochemical analysis measuring how long various DAGL inhibitors were able to decrease DAGL activity in brain tissue collected from mice injected with each molecule, showed that inhibiting DAGL activity with D034 resulted in an approximately 4 fold difference in DAGL activity 24 hours after administration of the drug (Ogasawara, Deng et al. 2016). If DAGL inhibition by DO34 knocks down DAGL expression/activity for 24 hours, we would have expected to there to be significant impairments on day 2 and 3 during fear extinction. The authors of this paper also contend that DAGL is a short half-life protein and that the duration of DAGL inhibition is a result of the drug remaining in the tissue for an extended period of time. One explanation for why we did not see extinction deficits on day 2-3 when mice were injected with DO34 prior to fear

conditioning is that DAGL may be rapidly trafficked to the synapse on demand in response to increases in synaptic activity. This increase may overcome the inhibitor after 24 hours and restore activity in response to acute stress. It is tempting to hypothesize that the inhibition of DAGL activity that causes deficits in fear extinction learning exhibited on day 4, can be compared to mice that had no day 2-3 extinction training. Given the lack of evidence showing restored DAGL activity mitigated by on demand synthesis, our data showing that fear extinction deficits and residual increased freezing behavior on CS recall day 4 (drug free) must be viewed with caution. It is possible that the impairment of fear extinction after DAGL inhibition is not mediated by 2-AG deficiency, but rather other mechanisms secondary to the widespread lipidomic changes induced by DAGL inhibition.

Another explanation for our results showing that depletion of DAGL yields similar behavioral deficits to the augmentation of 2-AG is downstream of CB1R activity. It cannot be ruled out that interruption of AA synthesis due to inhibition of either DAGL or MAGL regulates mechanisms involving fear extinction. Cyclooxygenase (COX) is an enzyme that facilitates the oxygenation of AA which in turn, produces prostaglandins (Marnett, Rowlinson et al. 1999). COX inhibitors, which would promote accumulation of AA in the presynaptic terminal secondary to 2-AG signaling, have resulted in anxiolytic effects in mice during some behavioral tasks, with and without prior stress exposure, and disrupted contextual memory after fear conditioning (Hein, Stutzman et al. 2007, Hermanson, Hartley et al. 2013, Gamble-George, Baldi et al. 2016). These findings force consideration that the downstream blockade of AA acid synthesis by either post synaptic DAGL and pre-synaptic MAGL inhibition, both ultimately result in a modest decrease of AA in the pre synaptic microdomain surrounding MAGL, within the axon terminal.

CB1 receptors inhibit N-, P-, and Q-type calcium channels, activate inwardly rectifying potassium channels and block others in heterologous cells (Felder, Joyce et al. 1995, Pan, Ikeda et al. 1996, Glass, Dragunow et al. 1997, Pertwee 2015, Pertwee 2015). In contrast, AA has been shown to activate specific types of presynaptic potassium channels, increase synaptic vesicle fusion, and increase calcium channel currents, hypothetically counterbalancing or modulating the effect of CB1R activation (Dh, Sladek et al. 1995, Horimoto, Nabekura et al. 1997, Fink, Lesage et al. 1998, Huang, Woodruff et al. 2006, Meves 2008, Pertwee 2015). Inhibiting DAGL activity and blocking AA synthesis would not theoretically cause a change in net presynaptic activity given the CB1R is not activated in this paradigm. However, when MAGL is inhibited and CB1R activity is induced, the depletion of AA synthesis could result in sustained inhibition of the affected synaptic currents. BLA MAGL expression was found in presynaptic GABAergic terminals that also expressed CB1R and post synaptic DAGL which seemed to be absent in glutamatergic synapses (Yoshida, Uchigashima et al. 2011). As stated in Chapter II, we suspect 2-AG accumulation and synaptic spillover enhances subsequent activation of CB1 on GABAergic neurons controlling freezing behavior. In theory, AA acid depletion resulting from MAGL inhibition, may block counteracting AA activity and result in CB1R hyperactivity in BLA GABAergic synapses. This could account for the increased freezing behavior during fear extinction training observed by Hartley et al. 2016 when MAGL was inhibited by JZL184 (Hartley, Gunduz-Cinar et al. 2016).

We doubt the effects of AA are a major contributing factor in fear extinction learning deficits observed with administration of DAGL or MAGL inhibitors as 2-AG induces a CB1R-dependent suppression of COX-2 activity and expression (Yang, Zhang et al. 2008). It is also possible that AA activity is important for aspects of fear extinction independent of calcium and

potassium channels and is not important for dampening CB1R signaling. An experiment testing fear extinction using a COX inhibitor with or without a CB1R antagonist could give us insight to the role of AA in fear extinction and whether or not CB1R activity mediates this response.

Future Directions

The open question concerning how DAGL activity and subsequent production of 2-AG specifically regulates fear extinction can be further explored using several different approaches. Understanding the spatial and temporal regulation of 2-AG following acute stress is needed in order to narrow down specific circuits for intervention. Based on prior work looking at 2AG level changes in response to stress, combined with finding that acute disruption of DAGL activity by DO34 injections prior to fear conditioning and extinction training leads to probable learning deficits and short term anxiety-related behavior, we hypothesize that there will be measurable differences in DAGL activity within specific brain regions and at specific time points during the fear conditioning and fear extinction paradigm. In order to test DAGL activity, a synthetic DAGL substrate (MRJ-20) fitted with a FRET pair on the N and C terminals of the substrate can be utilized to measure DAGL activity in homogenized brain tissue before and after each experimental trial in a FRET plate assay. Florescence will increase in proportion to substrate cleavage by DAGL. Micro- dissected brain regions including the amygdala, BNST, hippocampus, and mPFC at specific key time points during training and extinction should give an indication of where DAGL activity is increased or decreased in response to the conditioning and extinction paradigm. Given that DAGL is expressed at various levels across the brain structures we have targeted, we will also need to run western blots in order to measure the expression of DAGL within these brain regions samples to gather a rough estimate of protein expression so that it can be correlated with DAGL

activity. To measure 2-AG abundance, this lab has collected micro-dissected brain regions to be processed and analyzed via mass spectrometry at similar time points. From this data we will learn which brain regions have significant changes in DAGL activity in response to fear conditioning and in multiple stages of fear extinction. We will learn if DAGL activity and expression is increased during fear extinction learning, and if DAGL activity correlates with changes in freezing behavior. We can also test DAGL activity post DO34 treatments by intervals to understand how long DAGL activity is suppressed through DO34 inhibition.

Once we determine the specific brain regions where 2-AG signaling is important for fear learning and extinction, we can confirm these results with titrated micro infusions of both DAGL inhibitors and CB1R agonist/antagonists to learn how CB1R activity regulates particular regions important for fear learning and extinction training. Using this technique we could add on GABAR agonists/antagonists to confirm that fear extinction learning is dependent on inhibition of specific regional signals with in the brain. This data would offer insight not only to eCB signaling during fear learning and extinction but also the unique signaling patterns of potentiation and inhibition after fear learning that promote fear extinction in mice. Physicians have started using Deep Brain Stimulation in some patients with severe PTSD to relieve his or her symptoms, and this data may offer guidance into how fear signaling patterns are disrupted in patients with PTSD. Experimentally, this data will narrow down the possible circuits to target for further study in order to better understand how fear learning and fear extinction are regulated. Electrophysiology techniques can then be utilized to study specific circuits and synaptic behaviors related to stress, fear learning/extinction regulation by eCB retrograde signaling.

Conclusion

In order to understand enhanced fear retention and how post traumatic stress becomes maladaptive, we must have a deeper understanding of the normal regulation involved in fear expression and extinction. Once the brain region, circuit level and molecular regulation of fear learning and extinction is well understood, we can start elucidate maladaptive patterns and study factors that promote resilience to trauma at a molecular level.

Insight into the normal neural fear conditioning and fear extinction processes will identify potential targets for novel treatments for this debilitating anxiety/stress disorders, such as PTSD, as well as suggest interventions to prevent others from developing the disorder after trauma. Clearly the mammalian brain has evolved to enable an intricate fear learning network to facilitate fine discrimination between stimuli in a complex environment. The benefits of learning how these processes are regulated have implications for the prevention of suffering on an enormous scale. Understanding the molecular underpinning of fear learning and extinction could lead to treatment and prevention breakthroughs that may alleviate economic and societal burdens due to PTSD, boost economic recovery and potential for those recovering from war-torn and developing countries, prevent long term underachievement by those who experience trauma, and offer relief to those who have sacrificed mental stability and health in the service of our country. Current estimates show that up to 70.4-89.7% of people will experience some form of trauma in their lifetime with an average of 3.4 traumas per person (Kilpatrick, Resnick et al. 2013, Kessler, Aguilar-Gaxiola et al. 2017). Of those approximately 10% will develop some degree of PTSD (Breslau 2009). Research involving fear learning and fear extinction as a model for understanding complex fear adaptation has broad applications and is crucially important.

REFERENCES

Aaltonen, N., C. Riera Ribas, M. Lehtonen, J. R. Savinainen and J. T. Laitinen (2014). "Brain regional cannabinoid CB1 receptor signalling and alternative enzymatic pathways for 2-arachidonoylglycerol generation in brain sections of diacylglycerol lipase deficient mice." European Journal of Pharmaceutical Sciences **51**: 87-95.

Alegria, M., J. S. Jackson, R. C. Kessler and D. Takeuchi (2016). Collaborative Psychiatric Epidemiology Surveys (CPES), 2001-2003 [United States], Inter-university Consortium for Political and Social Research [distributor].

Amorapanth, P., J. E. LeDoux and K. Nader (2000). "Different lateral amygdala outputs mediate reactions and actions elicited by a fear-arousing stimulus." Nature Neuroscience **3**: 74.

Arnatt, M. (2015). Post-traumatic Stress Disorder (PTSD): A Diagnostic Overview and Treatment Options.

Association, A. P. (2013). Diagnostic and statistical manual of mental disorders. Arlington, VA, Author.

Astarita, G. and D. Piomelli (2009). "Lipidomic analysis of endocannabinoid metabolism in biological samples." J Chromatogr B Analyt Technol Biomed Life Sci **877**(26): 2755-2767.

Bedse, G., N. D. Hartley, E. Neale, A. D. Gaulden, T. A. Patrick, P. J. Kingsley, M. J. Uddin, N. Plath, L. J. Marnett and S. Patel (2017). "Functional Redundancy Between Canonical Endocannabinoid Signaling Systems in the Modulation of Anxiety." Biol Psychiatry **82**(7): 488-499.

Beltramo, M., N. Stella, A. Calignano, S. Y. Lin, A. Makriyannis and D. Piomelli (1997). "Functional role of high-affinity anandamide transport, as revealed by selective inhibition." Science **277**(5329): 1094-1097.

Bench, C. J., C. D. Frith, P. M. Grasby, K. J. Friston, E. Paulesu, R. S. Frackowiak and R. J. Dolan (1993). "Investigations of the functional anatomy of attention using the Stroop test." Neuropsychologia **31**(9): 907-922.

Berenz, E. C. and S. F. Coffey (2012). "Treatment of Co-occurring Posttraumatic Stress Disorder and Substance Use Disorders." Current psychiatry reports **14**(5): 469-477.

Bilkei-Gorzo, A., O. Albayram, A. Draffehn, K. Michel, A. Piyanova, H. Oppenheimer, M. Dvir-Ginzberg, I. Racz, T. Ulas, S. Imbeault, I. Bab, J. L. Schultze and A. Zimmer (2017). "A chronic low dose of Delta(9)-tetrahydrocannabinol (THC) restores cognitive function in old mice." Nat Med **23**(6): 782-787.

Bisogno, T., F. Howell, G. Williams, A. Minassi, M. G. Cascio, A. Ligresti, I. Matias, A. Schiano-Moriello, P. Paul, E. J. Williams, U. Gangadharan, C. Hobbs, V. Di Marzo and P. Doherty (2003). "Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain." J Cell Biol **163**(3): 463-468.

Blankman, J. L., G. M. Simon and B. F. Cravatt (2007). "A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol." Chem Biol **14**(12): 1347-1356.

Bluett, R. J., R. Baldi, A. Haymer, A. D. Gaulden, N. D. Hartley, W. P. Parrish, J. Baechle, D. J. Marcus, R. Mardam-Bey, B. C. Shonesy, M. J. Uddin, L. J. Marnett, K. Mackie, R. J. Colbran, D. G. Winder and S. Patel (2017). "Endocannabinoid signalling modulates susceptibility to traumatic stress exposure." Nat Commun **8**: 14782.

Bluett, R. J., J. C. Gamble-George, D. J. Hermanson, N. D. Hartley, L. J. Marnett and S. Patel (2014). "Central anandamide deficiency predicts stress-induced anxiety: behavioral reversal through endocannabinoid augmentation." Transl Psychiatry **4**: e408.

Bouton, M. E. and R. C. Bolles (1979). "Role of conditioned contextual stimuli in reinstatement of extinguished fear." J Exp Psychol Anim Behav Process **5**(4): 368-378.

Brady, K., T. Pearlstein, G. M. Asnis, D. Baker, B. Rothbaum, C. R. Sikes and G. M. Farfel (2000). "Efficacy and safety of sertraline treatment of posttraumatic stress disorder: a randomized controlled trial." Jama **283**(14): 1837-1844.

Brady, K. T., T. K. Killeen, T. Brewerton and S. Lucerini (2000). "Comorbidity of psychiatric disorders and posttraumatic stress disorder." J Clin Psychiatry **61 Suppl 7**: 22-32.

Bremner, J. D., M. Narayan, L. H. Staib, S. M. Southwick, T. McGlashan and D. S. Charney (1999). "Neural correlates of memories of childhood sexual abuse in women with and without posttraumatic stress disorder." Am J Psychiatry **156**(11): 1787-1795.

Bremner, J. D., S. M. Southwick, A. Darnell and D. S. Charney (1996). "Chronic PTSD in Vietnam combat veterans: course of illness and substance abuse." Am J Psychiatry **153**(3): 369-375.

Bremner, J. D., E. Vermetten, M. Vythilingam, N. Afzal, C. Schmahl, B. Elzinga and D. S. Charney (2004). "Neural correlates of the classic color and emotional stroop in women with abuse-related posttraumatic stress disorder." Biol Psychiatry **55**(6): 612-620.

Breslau, N. (2009). "The Epidemiology of Trauma, PTSD, and Other Posttrauma Disorders." Trauma, Violence, & Abuse **10**(3): 198-210.

Britton, J. C., K. L. Phan, S. F. Taylor, L. M. Fig and I. Liberzon (2005). "Corticolimbic blood flow in posttraumatic stress disorder during script-driven imagery." Biol Psychiatry **57**(8): 832-840.

Brown, P. J., R. L. Stout and J. Gannon-Rowley (1998). "15Substance Use Disorder-PTSD Comorbidity: Patients' Perceptions of Symptom Interplay and Treatment Issues." Journal of Substance Abuse Treatment **15**(5): 445-448.

Busquets-Garcia, A., E. Puighermanal, A. Pastor, R. de la Torre, R. Maldonado and A. Ozaita (2011). "Differential role of anandamide and 2-arachidonoylglycerol in memory and anxiety-like responses." Biol Psychiatry **70**(5): 479-486.

Campeau, S. and M. Davis (1995). "Involvement of the central nucleus and basolateral complex of the amygdala in fear conditioning measured with fear-potentiated startle in rats trained concurrently with auditory and visual conditioned stimuli." J Neurosci **15**(3 Pt 2): 2301-2311.

Cassell, M. D. and D. J. Wright (1986). "Topography of projections from the medial prefrontal cortex to the amygdala in the rat." Brain Res Bull **17**(3): 321-333.

Chakrabarti, A., E. S. Onaivi and G. Chaudhuri (1995). "Cloning and sequencing of a cDNA encoding the mouse brain-type cannabinoid receptor protein." DNA Seq **5**(6): 385-388.

Choi, D. C., A. R. Furay, N. K. Evanson, M. M. Ostrander, Y. M. Ulrich-Lai and J. P. Herman (2007). "Bed nucleus of the stria terminalis subregions differentially regulate hypothalamic-pituitary-adrenal axis activity: implications for the integration of limbic inputs." J Neurosci **27**(8): 2025-2034.

Conrad, C. D. (2008). "Chronic Stress-Induced Hippocampal Vulnerability: The Glucocorticoid Vulnerability Hypothesis." Reviews in the neurosciences **19**(6): 395-411.

Cota, D. (2008). "The Role of the Endocannabinoid System in the Regulation of Hypothalamic-Pituitary-Adrenal Axis Activity." Journal of Neuroendocrinology **20**(s1): 35-38.

Cousens, G. and T. Otto (1998). "Both pre- and posttraining excitotoxic lesions of the basolateral amygdala abolish the expression of olfactory and contextual fear conditioning." Behav Neurosci **112**(5): 1092-1103.

Cravatt, B. F., D. K. Giang, S. P. Mayfield, D. L. Boger, R. A. Lerner and N. B. Gilula (1996). "Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides." Nature **384**(6604): 83-87.

Daimon, M., A. Kamba, H. Murakami, S. Mizushiri, S. Osonoi, K. Matsuki, E. Sato, J. Tanabe, S. Takayasu, Y. Matsuhashi, M. Yanagimachi, K. Terui, K. Kageyama, I. Tokuda, S. Kurauchi and S. Nakaji (2017). "Dominance of the hypothalamus-pituitary-adrenal axis over the renin-angiotensin-aldosterone system is a risk factor for decreased insulin secretion." Scientific Reports **7**: 11360.

Davidson, J. R., B. O. Rothbaum, B. A. van der Kolk, C. R. Sikes and G. M. Farfel (2001). "Multicenter, double-blind comparison of sertraline and placebo in the treatment of posttraumatic stress disorder." Arch Gen Psychiatry **58**(5): 485-492.

de Kloet, E. R., M. Joels and F. Holsboer (2005). "Stress and the brain: from adaptation to disease." Nat Rev Neurosci **6**(6): 463-475.

De Oca, B. M., J. P. DeCola, S. Maren and M. S. Fanselow (1998). "Distinct regions of the periaqueductal gray are involved in the acquisition and expression of defensive responses." J Neurosci **18**(9): 3426-3432.

Deslauriers, J., M. Toth, A. Der-Avakian and V. B. Risbrough (2017). "Current Status of Animal Models of Posttraumatic Stress Disorder: Behavioral and Biological Phenotypes, and Future Challenges in Improving Translation." Biol Psychiatry.

Devane, W. A., L. Hanus, A. Breuer, R. G. Pertwee, L. A. Stevenson, G. Griffin, D. Gibson, A. Mandelbaum, A. Etinger and R. Mechoulam (1992). "Isolation and structure of a brain constituent that binds to the cannabinoid receptor." Science **258**(5090): 1946-1949.

Dh, K., C. Sladek, C. Aguado-Velasco and J. Mathiasen (1995). Arachidonic acid activation of a new family of K+ channels in cultured rat neuronal cells.

Di Marzo, V., D. Melck, T. Bisogno and L. De Petrocellis (1998). "Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action." Trends Neurosci **21**(12): 521-528.

Diamond, D. and P. Zoladz (2015). Dysfunctional or hyperfunctional? The amygdala in posttraumatic stress disorder is the bull in the evolutionary China shop.

Dinh, T. P., D. Carpenter, F. M. Leslie, T. F. Freund, I. Katona, S. L. Sensi, S. Kathuria and D. Piomelli (2002). "Brain monoglyceride lipase participating in endocannabinoid inactivation." Proc Natl Acad Sci U S A **99**(16): 10819-10824.

Dubreucq, S., I. Matias, P. Cardinal, M. Haring, B. Lutz, G. Marsicano and F. Chaouloff (2012). "Genetic dissection of the role of cannabinoid type-1 receptors in the emotional consequences of repeated social stress in mice." Neuropsychopharmacology **37**(8): 1885-1900.

Duvarci, S., D. Popa and D. Pare (2011). "Central amygdala activity during fear conditioning." J Neurosci **31**(1): 289-294.

Evanson, N. K., J. G. Tasker, M. N. Hill, C. J. Hillard and J. P. Herman (2010). "Fast feedback inhibition of the HPA axis by glucocorticoids is mediated by endocannabinoid signaling." Endocrinology **151**(10): 4811-4819.

Fareed, A., P. Eilender, M. Haber, J. Bremner, N. Whitfield and K. Drexler (2013). "Comorbid posttraumatic stress disorder and opiate addiction: a literature review." J Addict Dis **32**(2): 168-179.

Felder, C. C., K. E. Joyce, E. M. Briley, J. Mansouri, K. Mackie, O. Blond, Y. Lai, A. L. Ma and R. L. Mitchell (1995). "Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors." Mol Pharmacol **48**(3): 443-450.

Feldman, S., N. Conforti and J. Weidenfeld (1995). "Limbic pathways and hypothalamic neurotransmitters mediating adrenocortical responses to neural stimuli." Neurosci Biobehav Rev **19**(2): 235-240.

Fink, M., F. Lesage, F. Duprat, C. Heurteaux, R. Reyes, M. Fosset and M. Lazdunski (1998). "A neuronal two P domain K+ channel stimulated by arachidonic acid and polyunsaturated fatty acids." Embo j **17**(12): 3297-3308.

Fiskerstrand, T., D. H'Mida-Ben Brahim, S. Johansson, A. M'Zahem, B. I. Haukanes, N. Drouot, J. Zimmermann, A. J. Cole, C. Vedeler, C. Bredrup, M. Assoum, M. Tazir, T. Klockgether, A. Hamri, V. M. Steen, H. Boman, L. A. Bindoff, M. Koenig and P. M. Knappskog (2010). "Mutations in ABHD12 Cause the Neurodegenerative Disease PHARC: An Inborn Error of Endocannabinoid Metabolism." The American Journal of Human Genetics **87**(3): 410-417.

Flory, J. D. and R. Yehuda (2015). "Comorbidity between post-traumatic stress disorder and major depressive disorder: alternative explanations and treatment considerations." Dialogues in Clinical Neuroscience **17**(2): 141-150.

Fukudome, Y., T. Ohno-Shosaku, M. Matsui, Y. Omori, M. Fukaya, H. Tsubokawa, M. M Taketo, M. Watanabe, T. Manabe and M. Kano (2004). Two distinct classes of muscarinic action on

hippocampal inhibitory synapses: M2-mediated direct suppression and M1/M3-mediated indirect suppression through endocannabinoid signalling.

Funayama, E. S., C. Grillon, M. Davis and E. A. Phelps (2001). "A double dissociation in the affective modulation of startle in humans: effects of unilateral temporal lobectomy." J Cogn Neurosci **13**(6): 721-729.

Gamble-George, J. C., R. Baldi, L. Halladay, A. Kocharian, N. Hartley, C. G. Silva, H. Roberts, A. Haymer, L. J. Marnett, A. Holmes and S. Patel (2016). "Cyclooxygenase-2 inhibition reduces stress-induced affective pathology." Elife **5**.

Gao, Y., D. V. Vasilyev, M. B. Goncalves, F. V. Howell, C. Hobbs, M. Reisenberg, R. Shen, M. Y. Zhang, B. W. Strassle, P. Lu, L. Mark, M. J. Piesla, K. Deng, E. V. Kouranova, R. H. Ring, G. T. Whiteside, B. Bates, F. S. Walsh, G. Williams, M. N. Pangalos, T. A. Samad and P. Doherty (2010). "Loss of retrograde endocannabinoid signaling and reduced adult neurogenesis in diacylglycerol lipase knock-out mice." J Neurosci **30**(6): 2017-2024.

Gaoni, Y. and R. Mechoulam (1971). "The isolation and structure of delta-1-tetrahydrocannabinol and other neutral cannabinoids from hashish." J Am Chem Soc **93**(1): 217-224.

Gilbertson, M. W., M. E. Shenton, A. Ciszewski, K. Kasai, N. B. Lasko, S. P. Orr and R. K. Pitman (2002). "Smaller hippocampal volume predicts pathologic vulnerability to psychological trauma." Nature neuroscience **5**(11): 1242-1247.

Glass, M., M. Dragunow and R. L. Faull (1997). "Cannabinoid receptors in the human brain: a detailed anatomical and quantitative autoradiographic study in the fetal, neonatal and adult human brain." Neuroscience **77**(2): 299-318.

Glass, M. and C. C. Felder (1997). "Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor." J Neurosci **17**(14): 5327-5333.

Gola, H., A. Engler, J. Morath, H. Adenauer, T. Elbert, I.-T. Kolassa and H. Engler (2014). "Reduced Peripheral Expression of the Glucocorticoid Receptor α Isoform in Individuals with Posttraumatic Stress Disorder: A Cumulative Effect of Trauma Burden." PLOS ONE **9**(1): e86333.

Gray, J. M., H. A. Vecchiarelli, M. Morena, T. T. Lee, D. J. Hermanson, A. B. Kim, R. J. McLaughlin, K. I. Hassan, C. Kuhne, C. T. Wotjak, J. M. Deussing, S. Patel and M. N. Hill (2015). "Corticotropin-releasing hormone drives anandamide hydrolysis in the amygdala to promote anxiety." J Neurosci **35**(9): 3879-3892.

Gray, J. M., C. D. Wilson, T. T. Lee, Q. J. Pittman, J. M. Deussing, C. J. Hillard, B. S. McEwen, J. Schulkin, I. N. Karatsoreos, S. Patel and M. N. Hill (2016). "Sustained glucocorticoid exposure recruits cortico-limbic CRH signaling to modulate endocannabinoid function." Psychoneuroendocrinology **66**: 151-158.

Gray, T. S., M. E. Carney and D. J. Magnuson (1989). "Direct projections from the central amygdaloid nucleus to the hypothalamic paraventricular nucleus: possible role in stress-induced adrenocorticotropin release." Neuroendocrinology **50**(4): 433-446.

Guggenhuber, S., H. Romo-Parra, L. Bindila, J. Leschik, E. Lomazzo, F. Remmers, T. Zimmermann, R. Lerner, M. Klugmann, H. C. Pape and B. Lutz (2015). "Impaired 2-AG Signaling in Hippocampal Glutamatergic Neurons: Aggravation of Anxiety-Like Behavior and Unaltered Seizure Susceptibility." Int J Neuropsychopharmacol **19**(2).

Gulyas, A. I., B. F. Cravatt, M. H. Bracey, T. P. Dinh, D. Piomelli, F. Boscia and T. F. Freund (2004). "Segregation of two endocannabinoid-hydrolyzing enzymes into pre- and postsynaptic compartments in the rat hippocampus, cerebellum and amygdala." Eur J Neurosci **20**(2): 441-458.

Gunduz-Cinar, O., M. N. Hill, B. S. McEwen and A. Holmes (2013). "Amygdala FAAH and anandamide: mediating protection and recovery from stress." Trends Pharmacol Sci **34**(11): 637-644.

Gunduz-Cinar, O., K. P. MacPherson, R. Cinar, J. Gamble-George, K. Sugden, B. Williams, G. Godlewski, T. S. Ramikie, A. X. Gorka, S. O. Alapafuja, S. P. Nikas, A. Makriyannis, R. Poulton, S. Patel, A. R. Hariri, A. Caspi, T. E. Moffitt, G. Kunos and A. Holmes (2013). "Convergent translational evidence of a role for anandamide in amygdala-mediated fear extinction, threat processing and stress-reactivity." Mol Psychiatry **18**(7): 813-823.

Hackett, R. A., M. Kivimaki, M. Kumari and A. Steptoe (2016). "Diurnal Cortisol Patterns, Future Diabetes, and Impaired Glucose Metabolism in the Whitehall II Cohort Study." J Clin Endocrinol Metab **101**(2): 619-625.

Hartley, N. D., O. Gunduz-Cinar, L. Halladay, O. Bukalo, A. Holmes and S. Patel (2016). "2-arachidonoylglycerol signaling impairs short-term fear extinction." Translational Psychiatry **6**(3): e749.

Hashimotodani, Y., T. Ohno-Shosaku, A. Tanimura, Y. Kita, Y. Sano, T. Shimizu, V. Di Marzo and M. Kano (2013). "Acute inhibition of diacylglycerol lipase blocks endocannabinoid-mediated retrograde signalling: evidence for on-demand biosynthesis of 2-arachidonoylglycerol." J Physiol **591**(19): 4765-4776.

Hein, A. M., D. L. Stutzman, S. T. Bland, R. M. Barrientos, L. R. Watkins, J. W. Rudy and S. F. Maier (2007). "Prostaglandins are necessary and sufficient to induce contextual fear learning impairments after interleukin-1 beta injections into the dorsal hippocampus." Neuroscience **150**(4): 754-763.

Heinz, D. E., A. Genewsky and C. T. Wotjak (2017). "Enhanced anandamide signaling reduces flight behavior elicited by an approaching robo-beetle." Neuropharmacology **126**: 233-241.

Herkenham, M., A. B. Lynn, M. D. Little, M. R. Johnson, L. S. Melvin, B. R. de Costa and K. C. Rice (1990). "Cannabinoid receptor localization in brain." Proc Natl Acad Sci U S A **87**(5): 1932-1936.

Herman, J. P. (1993). "Regulation of adrenocorticosteroid receptor mRNA expression in the central nervous system." Cell Mol Neurobiol **13**(4): 349-372.

Herman, J. P., M. M. Ostrander, N. K. Mueller and H. Figueiredo (2005). "Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis." Prog Neuropsychopharmacol Biol Psychiatry **29**(8): 1201-1213.

Hermanson, D. J., N. D. Hartley, J. Gamble-George, N. Brown, B. C. Shonesy, P. J. Kingsley, R. J. Colbran, J. Reese, L. J. Marnett and S. Patel (2013). "Substrate-selective COX-2 inhibition decreases anxiety via endocannabinoid activation." Nat Neurosci **16**(9): 1291-1298.

Hermanson, D. J., N. D. Hartley, J. Gamble-George, N. Brown, B. C. Shonesy, P. J. Kingsley, R. J. Colbran, J. Reese, L. J. Marnett and S. Patel (2013). "Substrate-selective COX-2 inhibition decreases anxiety via endocannabinoid activation." Nature Neuroscience **16**: 1291.

Herry, C., F. Ferraguti, N. Singewald, J. J. Letzkus, I. Ehrlich and A. Luthi (2010). "Neuronal circuits of fear extinction." Eur J Neurosci **31**(4): 599-612.

Herry, C. and R. Garcia (2002). "Prefrontal cortex long-term potentiation, but not long-term depression, is associated with the maintenance of extinction of learned fear in mice." J Neurosci **22**(2): 577-583.

Hill, M. N., P. Campolongo, R. Yehuda and S. Patel (2018). "Integrating Endocannabinoid Signaling and Cannabinoids into the Biology and Treatment of Posttraumatic Stress Disorder." Neuropsychopharmacology **43**(1): 80-102.

Hill, M. N., R. J. McLaughlin, B. Pan, M. L. Fitzgerald, C. J. Roberts, T. T. Lee, I. N. Karatsoreos, K. Mackie, V. Viau, V. M. Pickel, B. S. McEwen, Q. S. Liu, B. B. Gorzalka and C. J. Hillard (2011). "Recruitment of prefrontal cortical endocannabinoid signaling by glucocorticoids contributes to termination of the stress response." J Neurosci **31**(29): 10506-10515.

Hitchcock, J. and M. Davis (1986). "Lesions of the amygdala, but not of the cerebellum or red nucleus, block conditioned fear as measured with the potentiated startle paradigm." Behav Neurosci **100**(1): 11-22.

Hoffman, E. J. and S. J. Mathew (2008). "Anxiety disorders: a comprehensive review of pharmacotherapies." Mt Sinai J Med **75**(3): 248-262.

Hohmann, A. G., R. L. Suplita, N. M. Bolton, M. H. Neely, D. Fegley, R. Mangieri, J. F. Krey, J. M. Walker, P. V. Holmes, J. D. Crystal, A. Duranti, A. Tontini, M. Mor, G. Tarzia and D. Piomelli (2005). "An endocannabinoid mechanism for stress-induced analgesia." Nature **435**(7045): 1108-1112.

Hoover, H. S., J. L. Blankman, S. Niessen and B. F. Cravatt (2008). "Selectivity of inhibitors of endocannabinoid biosynthesis evaluated by activity-based protein profiling." Bioorg Med Chem Lett **18**(22): 5838-5841.

Horimoto, N., J. Nabekura and T. Ogawa (1997). "Arachidonic acid activation of potassium channels in rat visual cortex neurons." Neuroscience **77**(3): 661-671.

Huang, C. C., S. W. Lo and K. S. Hsu (2001). "Presynaptic mechanisms underlying cannabinoid inhibition of excitatory synaptic transmission in rat striatal neurons." J Physiol **532**(Pt 3): 731-748.

Huang, F.-D., E. Woodruff, R. Mohrmann and K. Broadie (2006). "Rolling Blackout Is Required for Synaptic Vesicle Exocytosis." The Journal of Neuroscience **26**(9): 2369.

Iribarren, J., P. Prolo, N. Neagos and F. Chiappelli (2005). "Post-Traumatic Stress Disorder: Evidence-Based Research for the Third Millennium." Evidence-based Complementary and Alternative Medicine **2**(4): 503-512.

Iwata, J., K. Chida and J. E. LeDoux (1987). "Cardiovascular responses elicited by stimulation of neurons in the central amygdaloid nucleus in awake but not anesthetized rats resemble conditioned emotional responses." Brain Research **418**(1): 183-188.

Izquierdo, A., C. L. Wellman and A. Holmes (2006). "Brief uncontrollable stress causes dendritic retraction in infralimbic cortex and resistance to fear extinction in mice." J Neurosci **26**(21): 5733-5738.

Jakupcak, M., S. Vannoy, Z. Imel, J. W. Cook, A. Fontana, R. Rosenheck and M. McFall (2010). "DOES PTSD MODERATE THE RELATIONSHIP BETWEEN SOCIAL SUPPORT AND SUICIDE RISK IN IRAQ AND AFGHANISTAN WAR VETERANS SEEKING MENTAL HEALTH TREATMENT?" Depression and anxiety **27**(11): 1001-1005.

Jenniches, I., S. Ternes, O. Albayram, D. M. Otte, K. Bach, L. Bindila, K. Michel, B. Lutz, A. Bilkei-Gorzo and A. Zimmer (2016). "Anxiety, Stress, and Fear Response in Mice With Reduced Endocannabinoid Levels." Biol Psychiatry **79**(10): 858-868.

Jin, J. and S. Maren (2015). "Prefrontal-Hippocampal Interactions in Memory and Emotion." Frontiers in Systems Neuroscience **9**: 170.

Jonas, D. E., K. Cusack, C. A. Forneris, T. M. Wilkins, J. Sonis, J. C. Middleton, C. Feltner, D. Meredith, J. Cavanaugh, K. A. Brownley, K. R. Olmsted, A. Greenblatt, A. Weil and B. N. Gaynes

(2013). Psychological and Pharmacological Treatments for Adults With Posttraumatic Stress Disorder (PTSD), Agency for Healthcare Research and Quality (US), Rockville (MD).

Kalisch, R., E. Korenfeld, K. E. Stephan, N. Weiskopf, B. Seymour and R. J. Dolan (2006). "Context-dependent human extinction memory is mediated by a ventromedial prefrontal and hippocampal network." J Neurosci **26**(37): 9503-9511.

Kang, H. K. and T. A. Bullman (2008). "Risk of suicide among US veterans after returning from the Iraq or Afghanistan war zones." Jama **300**(6): 652-653.

Kang, H. K., B. H. Natelson, C. M. Mahan, K. Y. Lee and F. M. Murphy (2003). "Post-traumatic stress disorder and chronic fatigue syndrome-like illness among Gulf War veterans: a population-based survey of 30,000 veterans." Am J Epidemiol **157**(2): 141-148.

Kano, M., T. Ohno-Shosaku, Y. Hashimotodani, M. Uchigashima and M. Watanabe (2009). "Endocannabinoid-mediated control of synaptic transmission." Physiol Rev **89**(1): 309-380.

Kasai, K., H. Yamasue, M. W. Gilbertson, M. E. Shenton, S. L. Rauch and R. K. Pitman (2008). "Evidence for acquired pregenual anterior cingulate gray matter loss from a twin study of combat-related posttraumatic stress disorder." Biol Psychiatry **63**(6): 550-556.

Katona, I., G. M. Urban, M. Wallace, C. Ledent, K. M. Jung, D. Piomelli, K. Mackie and T. F. Freund (2006). "Molecular composition of the endocannabinoid system at glutamatergic synapses." J Neurosci **26**(21): 5628-5637.

Kawamura, Y., M. Fukaya, T. Maejima, T. Yoshida, E. Miura, M. Watanabe, T. Ohno-Shosaku and M. Kano (2006). "The CB1 cannabinoid receptor is the major cannabinoid receptor at excitatory presynaptic sites in the hippocampus and cerebellum." J Neurosci **26**(11): 2991-3001.

Kendall-Tackett, K. A. (2000). "Physiological correlates of childhood abuse: chronic hyperarousal in PTSD, depression, and irritable bowel syndrome." Child Abuse Negl **24**(6): 799-810.

Kessler, R. C., S. Aguilar-Gaxiola, J. Alonso, C. Benjet, E. J. Bromet, G. Cardoso, L. Degenhardt, G. de Girolamo, R. V. Dinolova, F. Ferry, S. Florescu, O. Gureje, J. M. Haro, Y. Huang, E. G. Karam, N. Kawakami, S. Lee, J.-P. Lepine, D. Levinson, F. Navarro-Mateu, B.-E. Pennell, M.

Piazza, J. Posada-Villa, K. M. Scott, D. J. Stein, M. Ten Have, Y. Torres, M. C. Viana, M. V. Petukhova, N. A. Sampson, A. M. Zaslavsky and K. C. Koenen (2017). "Trauma and PTSD in the WHO World Mental Health Surveys." European Journal of Psychotraumatology **8**(sup5): 1353383.

Kessler, R. C., P. Berglund, O. Demler, R. Jin, K. R. Merikangas and E. E. Walters (2005). "Lifetime prevalence and age-of-onset distributions of dsm-iv disorders in the national comorbidity survey replication." Archives of General Psychiatry **62**(6): 593-602.

Kessler, R. C., W. T. Chiu, O. Demler, K. R. Merikangas and E. E. Walters (2005). "Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication." Arch Gen Psychiatry **62**(6): 617-627.

Kessler, R. C., A. Sonnega, E. Bromet, M. Hughes and C. B. Nelson (1995). "Posttraumatic stress disorder in the National Comorbidity Survey." Arch Gen Psychiatry **52**(12): 1048-1060.

Kilpatrick, D. G., H. S. Resnick, M. E. Milanak, M. W. Miller, K. M. Keyes and M. J. Friedman (2013). "National Estimates of Exposure to Traumatic Events and PTSD Prevalence Using DSM-IV and DSM-5 Criteria." Journal of traumatic stress **26**(5): 537-547.

Kim, C. and M. Chouikha (2002). Attenuation Characteristics of High RateHome-Networking PLC Signals.

Kim, J. J. and M. W. Jung (2006). "Neural circuits and mechanisms involved in Pavlovian fear conditioning: A critical review." Neuroscience & Biobehavioral Reviews **30**(2): 188-202.

Kim, M. and M. Davis (1993). "Lack of a temporal gradient of retrograde amnesia in rats with amygdala lesions assessed with the fear-potentiated startle paradigm." Behav Neurosci **107**(6): 1088-1092.

Kinsey, S. G., S. T. O'Neal, J. Z. Long, B. F. Cravatt and A. H. Lichtman (2011). "Inhibition of endocannabinoid catabolic enzymes elicits anxiolytic-like effects in the marble burying assay." Pharmacol Biochem Behav **98**(1): 21-27.

Kinsey, S. G., L. E. Wise, D. Ramesh, R. Abdullah, D. E. Selley, B. F. Cravatt and A. H. Lichtman (2013). "Repeated low-dose administration of the monoacylglycerol lipase inhibitor JZL184 retains cannabinoid receptor type 1-mediated antinociceptive and gastroprotective effects." J Pharmacol Exp Ther **345**(3): 492-501.

Kondo, S., H. Kondo, S. Nakane, T. Kodaka, A. Tokumura, K. Waku and T. Sugiura (1998). "2-Arachidonoylglycerol, an endogenous cannabinoid receptor agonist: identification as one of the major species of monoacylglycerols in various rat tissues, and evidence for its generation through CA2+-dependent and -independent mechanisms." FEBS Lett **429**(2): 152-156.

Kreitzer, A. C. and W. G. Regehr (2001). "Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells." Neuron **29**(3): 717-727.

Kruk-Slomka, M., A. Dzik, B. Budzynska and G. Biala (2017). "Endocannabinoid System: the Direct and Indirect Involvement in the Memory and Learning Processes-a Short Review." Mol Neurobiol **54**(10): 8332-8347.

LaBar, K. S., J. C. Gatenby, J. C. Gore, J. E. LeDoux and E. A. Phelps (1998). "Human amygdala activation during conditioned fear acquisition and extinction: a mixed-trial fMRI study." Neuron **20**(5): 937-945.

Langevin, J. P., R. J. Koek, H. N. Schwartz, J. W. Y. Chen, D. L. Sultzer, M. A. Mandelkern, A. D. Kulick and S. E. Krahl (2016). "Deep Brain Stimulation of the Basolateral Amygdala for Treatment-Refractory Posttraumatic Stress Disorder." Biol Psychiatry **79**(10): e82-e84.

Lanius, R. A., E. Vermetten, R. J. Loewenstein, B. Brand, C. Schmahl, J. D. Bremner and D. Spiegel (2010). "Emotion Modulation in PTSD: Clinical and Neurobiological Evidence for a Dissociative Subtype." The American journal of psychiatry **167**(6): 640-647.

Lanius, R. A., P. C. Williamson, J. Hopper, M. Densmore, K. Boksman, M. A. Gupta, R. W. Neufeld, J. S. Gati and R. S. Menon (2003). "Recall of emotional states in posttraumatic stress disorder: an fMRI investigation." Biol Psychiatry **53**(3): 204-210.

LeDoux, J. E., P. Cicchetti, A. Xagoraris and L. M. Romanski (1990). "The lateral amygdaloid nucleus: sensory interface of the amygdala in fear conditioning." J Neurosci **10**(4): 1062-1069.

Lee, S., S.-J. Kim, O.-B. Kwon, J. H. Lee and J.-H. Kim (2013). "Inhibitory networks of the amygdala for emotional memory." Frontiers in Neural Circuits **7**: 129.

Likhtik, E., D. Popa, J. Apergis-Schoute, G. A. Fidacaro and D. Paré (2008). "Amygdala intercalated neurons are required for expression of fear extinction." Nature **454**: 642.

Llorente-Berzal, A., A. L. Terzian, V. di Marzo, V. Micale, M. P. Viveros and C. T. Wotjak (2015). "2-AG promotes the expression of conditioned fear via cannabinoid receptor type 1 on GABAergic neurons." Psychopharmacology (Berl) **232**(15): 2811-2825.

Lutz, B. (2007). "The endocannabinoid system and extinction learning." Mol Neurobiol **36**(1): 92-101.

Lutz, B., G. Marsicano, R. Maldonado and C. J. Hillard (2015). "The endocannabinoid system in guarding against fear, anxiety and stress." Nat Rev Neurosci **16**(12): 705-718.

Maren, S. (1999). "Neurotoxic or electrolytic lesions of the ventral subiculum produce deficits in the acquisition and expression of Pavlovian fear conditioning in rats." Behav Neurosci **113**(2): 283-290.

Maren, S. (2001). "Neurobiology of Pavlovian fear conditioning." Annu Rev Neurosci **24**: 897-931.

Maren, S., G. Aharonov and M. S. Fanselow (1997). "Neurotoxic lesions of the dorsal hippocampus and Pavlovian fear conditioning in rats." Behavioural Brain Research **88**(2): 261-274.

Maren, S. and A. Holmes (2016). "Stress and Fear Extinction." Neuropsychopharmacology **41**(1): 58-79.

Marnett, L. J., S. W. Rowlinson, D. C. Goodwin, A. S. Kalgutkar and C. A. Lanzo (1999). "Arachidonic Acid Oxygenation by COX-1 and COX-2: MECHANISMS OF CATALYSIS AND INHIBITION." Journal of Biological Chemistry **274**(33): 22903-22906.

Matheson, G. K., B. J. Branch and A. N. Taylor (1971). "Effects of amygdaloid stimulation on pituitary-adrenal activity in conscious cats." Brain Res **32**(1): 151-167.

Matsuda, L. A., S. J. Lolait, M. J. Brownstein, A. C. Young and T. I. Bonner (1990). "Structure of a cannabinoid receptor and functional expression of the cloned cDNA." Nature **346**(6284): 561-564.

McDonald, A. J. and J. R. Augustine (1993). "Localization of GABA-like immunoreactivity in the monkey amygdala." Neuroscience **52**(2): 281-294.

McEwen, B. S. (1998). "Protective and damaging effects of stress mediators." N Engl J Med **338**(3): 171-179.

McEwen, B. S. (2007). "Physiology and neurobiology of stress and adaptation: central role of the brain." Physiol Rev **87**(3): 873-904.

McEwen, B. S. and T. Seeman (1999). "Protective and damaging effects of mediators of stress. Elaborating and testing the concepts of allostasis and allostatic load." Ann N Y Acad Sci **896**: 30-47.

McEwen, B. S. and E. Stellar (1993). "Stress and the individual. Mechanisms leading to disease." Arch Intern Med **153**(18): 2093-2101.

McLaughlin, R. J., M. N. Hill, F. R. Bambico, K. L. Stuhr, G. Gobbi, C. J. Hillard and B. B. Gorzalka (2012). "Prefrontal Cortical Anandamide Signaling Coordinates Coping Responses to Stress Through a Serotonergic Pathway." European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology **22**(9): 664-671.

Meewisse, M. L., J. B. Reitsma, G. J. de Vries, B. P. Gersons and M. Olff (2007). "Cortisol and post-traumatic stress disorder in adults: systematic review and meta-analysis." Br J Psychiatry **191**: 387-392.

Meier, A., C. Lambert-Harris, M. P. McGovern, H. Xie, M. An and B. McLeman (2014). "Co-occurring prescription opioid use problems and posttraumatic stress disorder symptom severity." Am J Drug Alcohol Abuse **40**(4): 304-311.

Meves, H. (2008). "Arachidonic acid and ion channels: an update." British Journal of Pharmacology **155**(1): 4-16.

Morena, M., K. D. Leitl, H. A. Vecchiarelli, J. M. Gray, P. Campolongo and M. N. Hill (2016). "Emotional arousal state influences the ability of amygdalar endocannabinoid signaling to modulate anxiety." Neuropharmacology **111**: 59-69.

Morena, M., S. Patel, J. S. Bains and M. N. Hill (2016). "Neurobiological Interactions Between Stress and the Endocannabinoid System." Neuropsychopharmacology **41**(1): 80-102.

Morgan, M. A., L. M. Romanski and J. E. LeDoux (1993). "Extinction of emotional learning: contribution of medial prefrontal cortex." Neurosci Lett **163**(1): 109-113.

Morris, M. C., B. E. Compas and J. Garber (2012). "Relations among Posttraumatic Stress Disorder, Comorbid Major Depression, and HPA Function: A Systematic Review and Meta-Analysis." Clinical Psychology Review **32**(4): 301-315.

Munro, S., K. L. Thomas and M. Abu-Shaar (1993). "Molecular characterization of a peripheral receptor for cannabinoids." Nature **365**(6441): 61-65.

Myers, K. M. and M. Davis (2007). "Mechanisms of fear extinction." Mol Psychiatry **12**(2): 120-150.

Nitecka, L. and Y. Ben-Ari (1987). "Distribution of GABA-like immunoreactivity in the rat amygdaloid complex." Journal of Comparative Neurology **266**(1): 45-55.

Nomura, D. K., B. E. Morrison, J. L. Blankman, J. Z. Long, S. G. Kinsey, M. C. Marcondes, A. M. Ward, Y. K. Hahn, A. H. Lichtman, B. Conti and B. F. Cravatt (2011). "Endocannabinoid hydrolysis generates brain prostaglandins that promote neuroinflammation." Science **334**(6057): 809-813.

Ogasawara, D., H. Deng, A. Viader, M. P. Baggelaar, A. Breman, H. den Dulk, A. M. van den Nieuwendijk, M. Soethoudt, T. van der Wel, J. Zhou, H. S. Overkleeft, M. Sanchez-Alavez, S. Mori, W. Nguyen, B. Conti, X. Liu, Y. Chen, Q. S. Liu, B. F. Cravatt and M. van der Stelt (2016).

"Rapid and profound rewiring of brain lipid signaling networks by acute diacylglycerol lipase inhibition." Proc Natl Acad Sci U S A **113**(1): 26-33.

Ohno-Shosaku, T. and M. Kano (2014). "Endocannabinoid-mediated retrograde modulation of synaptic transmission." Curr Opin Neurobiol **29**: 1-8.

Ohno-Shosaku, T., T. Maejima and M. Kano (2001). "Endogenous cannabinoids mediate retrograde signals from depolarized postsynaptic neurons to presynaptic terminals." Neuron **29**(3): 729-738.

Ohno-Shosaku, T., M. Matsui, Y. Fukudome, J. Shosaku, H. Tsubokawa, M. M. Taketo, T. Manabe and M. Kano (2003). "Postsynaptic M1 and M3 receptors are responsible for the muscarinic enhancement of retrograde endocannabinoid signalling in the hippocampus." European Journal of Neuroscience **18**(1): 109-116.

Pan, X., S. R. Ikeda and D. L. Lewis (1996). "Rat brain cannabinoid receptor modulates N-type Ca2+ channels in a neuronal expression system." Mol Pharmacol **49**(4): 707-714.

Papale, L. A., L. J. Seltzer, A. Madrid, S. D. Pollak and R. S. Alisch (2018). "Differentially Methylated Genes in Saliva are linked to Childhood Stress." Sci Rep **8**(1): 10785.

Pape, H.-C. and D. Pare (2010). "Plastic Synaptic Networks of the Amygdala for the Acquisition, Expression, and Extinction of Conditioned Fear." Physiological reviews **90**(2): 419-463.

Parsons, R. G. and K. J. Ressler (2013). "Implications of memory modulation for post-traumatic stress and fear disorders." Nat Neurosci **16**(2): 146-153.

Patel, R. S., A. Elmaadawi, S. Nasr and J. Haskin (2017). "Comorbid Post-Traumatic Stress Disorder and Opioid Dependence." Cureus **9**(9): e1647.

Patel, S., M. N. Hill, J. F. Cheer, C. T. Wotjak and A. Holmes (2017). "The endocannabinoid system as a target for novel anxiolytic drugs." Neurosci Biobehav Rev **76**(Pt A): 56-66.

Patel, S., P. J. Kingsley, K. Mackie, L. J. Marnett and D. G. Winder (2009). "Repeated Homotypic Stress Elevates 2-Arachidonoylglycerol Levels and Enhances Short-Term Endocannabinoid

Signaling at Inhibitory Synapses in Basolateral Amygdala." Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology **34**(13): 2699-2709.

Patel, S., C. T. Roelke, D. J. Rademacher and C. J. Hillard (2005). "Inhibition of restraint stress-induced neural and behavioural activation by endogenous cannabinoid signalling." Eur J Neurosci **21**(4): 1057-1069.

Patel, S., B. C. Shonesy, R. J. Bluett, D. G. Winder and R. J. Colbran (2016). "The Anxiolytic Actions of 2-Arachidonoylglycerol: Converging Evidence From Two Recent Genetic Endocannabinoid Deficiency Models." Biological Psychiatry **79**(10): e78-e79.

Pavlov, I. P. (1927). Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex. Oxford, England, Oxford Univ. Press.

Pertwee, R. G. (1997). "Pharmacology of cannabinoid CB1 and CB2 receptors." Pharmacology & Therapeutics **74**(2): 129-180.

Pertwee, R. G. (2015). "cannabinoids."

Pertwee, R. G. (2015). "Endocannabinoids and Their Pharmacological Actions." Handb Exp Pharmacol **231**: 1-37.

Piomelli, D. (2003). "The molecular logic of endocannabinoid signalling." Nat Rev Neurosci **4**(11): 873-884.

Piomelli, D., G. Tarzia, A. Duranti, A. Tontini, M. Mor, T. R. Compton, O. Dasse, E. P. Monaghan, J. A. Parrott and D. Putman (2006). "Pharmacological profile of the selective FAAH inhibitor KDS-4103 (URB597)." CNS Drug Rev **12**(1): 21-38.

Pitman, R. K., K. M. Sanders, R. M. Zusman, A. R. Healy, F. Cheema, N. B. Lasko, L. Cahill and S. P. Orr (2002). "Pilot study of secondary prevention of posttraumatic stress disorder with propranolol." Biol Psychiatry **51**(2): 189-192.

Prescott, S. M. and P. W. Majerus (1983). "Characterization of 1,2-diacylglycerol hydrolysis in human platelets. Demonstration of an arachidonoyl-monoacylglycerol intermediate." J Biol Chem **258**(2): 764-769.

Quirk, G. J. and D. Mueller (2008). "Neural mechanisms of extinction learning and retrieval." Neuropsychopharmacology **33**(1): 56-72.

Quirk, G. J., G. K. Russo, J. L. Barron and K. Lebron (2000). "The role of ventromedial prefrontal cortex in the recovery of extinguished fear." J Neurosci **20**(16): 6225-6231.

Rabinak, C. A. and K. L. Phan (2014). "Cannabinoid modulation of fear extinction brain circuits: a novel target to advance anxiety treatment." Curr Pharm Des **20**(13): 2212-2217.

Radulovic, J. and N. C. Tronson (2010). "Molecular specificity of multiple hippocampal processes governing fear extinction." Rev Neurosci **21**(1): 1-17.

Ramikie, T. S., R. Nyilas, R. J. Bluett, J. C. Gamble-George, N. D. Hartley, K. Mackie, M. Watanabe, I. Katona and S. Patel (2014). "Multiple mechanistically distinct modes of endocannabinoid mobilization at central amygdala glutamatergic synapses." Neuron **81**(5): 1111-1125.

Randall D. Marshall, Katherine L. Beebe, Mary Oldham and Rocco Zaninelli (2001). "Efficacy and Safety of Paroxetine Treatment for Chronic PTSD: A Fixed-Dose, Placebo-Controlled Study." American Journal of Psychiatry **158**(12): 1982-1988.

Rescorla, R. A. and C. D. Heth (1975). "Reinstatement of fear to an extinguished conditioned stimulus." J Exp Psychol Anim Behav Process **1**(1): 88-96.

Roberts, C. J., K. L. Stuhr, M. J. Hutz, H. Raff and C. J. Hillard (2014). "Endocannabinoid signaling in hypothalamic-pituitary-adrenocortical axis recovery following stress: effects of indirect agonists and comparison of male and female mice." Pharmacol Biochem Behav **117**: 17-24.

Rojas, S. M., S. Bujarski, K. A. Babson, C. E. Dutton and M. T. Feldner (2014). "Understanding PTSD comorbidity and suicidal behavior: associations among histories of alcohol dependence, major depressive disorder, and suicidal ideation and attempts." J Anxiety Disord **28**(3): 318-325.

Royer, S., M. Martina and D. Paré (1999). "An Inhibitory Interface Gates Impulse Traffic between the Input and Output Stations of the Amygdala." The Journal of Neuroscience **19**(23): 10575.

Ruehle, S., A. A. Rey, F. Remmers and B. Lutz (2012). "The endocannabinoid system in anxiety, fear memory and habituation." J Psychopharmacol **26**(1): 23-39.

Rush, A. J., M. H. Trivedi, S. R. Wisniewski, A. A. Nierenberg, J. W. Stewart, D. Warden, G. Niederehe, M. E. Thase, P. W. Lavori, B. D. Lebowitz, P. J. McGrath, J. F. Rosenbaum, H. A. Sackeim, D. J. Kupfer, J. Luther and M. Fava (2006). "Acute and longer-term outcomes in depressed outpatients requiring one or several treatment steps: a STAR*D report." Am J Psychiatry **163**(11): 1905-1917.

Sapolsky, R. M., L. C. Krey and B. S. McEwen (1985). "Prolonged glucocorticoid exposure reduces hippocampal neuron number: implications for aging." J Neurosci **5**(5): 1222-1227.

Schnall, P. L., P. A. Landsbergis and D. Baker (1994). "Job strain and cardiovascular disease." Annu Rev Public Health **15**: 381-411.

Sciolino, N. R., W. Zhou and A. G. Hohmann (2011). "Enhancement of endocannabinoid signaling with JZL184, an inhibitor of the 2-arachidonoylglycerol hydrolyzing enzyme monoacylglycerol lipase, produces anxiolytic effects under conditions of high environmental aversiveness in rats." Pharmacol Res **64**(3): 226-234.

Senneseth, M., K. Alsaker and G. K. Natvig (2012). "Health-related quality of life and post-traumatic stress disorder symptoms in accident and emergency attenders suffering from psychosocial crises: a longitudinal study." Journal of Advanced Nursing **68**(2): 402-413.

Sherin, J. E. and C. B. Nemeroff (2011). "Post-traumatic stress disorder: the neurobiological impact of psychological trauma." Dialogues in Clinical Neuroscience **13**(3): 263-278.

Shin, L. M., S. L. Rauch and R. K. Pitman (2006). "Amygdala, medial prefrontal cortex, and hippocampal function in PTSD." Ann N Y Acad Sci **1071**: 67-79.

Shonesy, B. C., R. J. Bluett, T. S. Ramikie, R. Baldi, D. J. Hermanson, P. J. Kingsley, L. J. Marnett, D. G. Winder, R. J. Colbran and S. Patel (2014). "Genetic disruption of 2-arachidonoylglycerol synthesis reveals a key role for endocannabinoid signaling in anxiety modulation." Cell Rep **9**(5): 1644-1653.

Shonesy, B. C., R. J. Bluett, T. S. Ramikie, R. Baldi, D. J. Hermanson, P. J. Kingsley, L. J. Marnett, D. G. Winder, R. J. Colbran and S. Patel (2014). "Genetic disruption of 2-arachidonoylglycerol synthesis reveals a key role for endocannabinoid signaling in anxiety modulation." Cell Rep **9**(5): 1644-1653.

Simon, G. M. and B. F. Cravatt (2008). "Anandamide biosynthesis catalyzed by the phosphodiesterase GDE1 and detection of glycerophospho-N-acyl ethanolamine precursors in mouse brain." J Biol Chem **283**(14): 9341-9349.

Sinha, R. (2008). "Chronic Stress, Drug Use, and Vulnerability to Addiction." Annals of the New York Academy of Sciences **1141**: 105-130.

Smith, S. M. and W. W. Vale (2006). "The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress." Dialogues in Clinical Neuroscience **8**(4): 383-395.

Song, Z. H. and T. I. Bonner (1996). "A lysine residue of the cannabinoid receptor is critical for receptor recognition by several agonists but not WIN55212-2." Molecular Pharmacology **49**(5): 891.

St-Cyr, S. and P. O. McGowan (2018). "Adaptation or pathology? The role of prenatal stressor type and intensity in the developmental programing of adult phenotype." Neurotoxicology and Teratology **66**: 113-124.

Steckler, T. and V. Risbrough (2012). "Pharmacological Treatment of PTSD – Established and New Approaches." Neuropharmacology **62**(2): 617-627.

Stella, N., P. Schweitzer and D. Piomelli (1997). "A second endogenous cannabinoid that modulates long-term potentiation." Nature **388**(6644): 773-778.

Stetler, C., S. S. Dickerson and G. E. Miller (2004). "Uncoupling of social zeitgebers and diurnal cortisol secretion in clinical depression." Psychoneuroendocrinology **29**(10): 1250-1259.

Sugiura, T., S. Kondo, A. Sukagawa, S. Nakane, A. Shinoda, K. Itoh, A. Yamashita and K. Waku (1995). "2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain." Biochem Biophys Res Commun **215**(1): 89-97.

Sumislawski, J. J., T. S. Ramikie and S. Patel (2011). "Reversible gating of endocannabinoid plasticity in the amygdala by chronic stress: a potential role for monoacylglycerol lipase inhibition in the prevention of stress-induced behavioral adaptation." Neuropsychopharmacology **36**(13): 2750-2761.

Tabachnick, B. G. and L. S. Fidell (2007). Using multivariate statistics, 5th ed. Boston, MA, Allyn & Bacon/Pearson Education.

Tajima-Pozo, K., A. Montes-Montero, I. Guemes, S. Gonzalez-Vives, M. Diaz-Marsa and J. L. Carrasco (2013). "[Contributions of cortisol suppression tests to understanding of psychiatric disorders: a narrative review of literature]." Endocrinol Nutr **60**(7): 396-403.

Tanimura, A., M. Yamazaki, Y. Hashimotodani, M. Uchigashima, S. Kawata, M. Abe, Y. Kita, K. Hashimoto, T. Shimizu, M. Watanabe, K. Sakimura and M. Kano (2010). "The endocannabinoid 2-arachidonoylglycerol produced by diacylglycerol lipase alpha mediates retrograde suppression of synaptic transmission." Neuron **65**(3): 320-327.

Taylor, S., D. S. Thordarson, L. Maxfield, I. C. Fedoroff, K. Lovell and J. Ogrodniczuk (2003). "Comparative efficacy, speed, and adverse effects of three PTSD treatments: exposure therapy, EMDR, and relaxation training." J Consult Clin Psychol **71**(2): 330-338.

Tchantchou, F. and Y. Zhang (2013). "Selective inhibition of alpha/beta-hydrolase domain 6 attenuates neurodegeneration, alleviates blood brain barrier breakdown, and improves functional recovery in a mouse model of traumatic brain injury." J Neurotrauma **30**(7): 565-579.

Terra C. Holdeman , M. D., M.S. , (2009). "Invisible Wounds of War: Psychological and Cognitive Injuries, Their Consequences, and Services to Assist Recovery." Psychiatric Services **60**(2): 273-273.

Uchigashima, M., M. Narushima, M. Fukaya, I. Katona, M. Kano and M. Watanabe (2007). "Subcellular arrangement of molecules for 2-arachidonoyl-glycerol-mediated retrograde signaling and its physiological contribution to synaptic modulation in the striatum." J Neurosci **27**(14): 3663-3676.

Ueda, T., Y. Suzukamo, M. Sato and S. Izumi (2013). "Effects of music therapy on behavioral and psychological symptoms of dementia: a systematic review and meta-analysis." Ageing Res Rev **12**(2): 628-641.

Van de Kar, L. D. and M. L. Blair (1999). "Forebrain pathways mediating stress-induced hormone secretion." Front Neuroendocrinol **20**(1): 1-48.

Volpicelli, J., G. Balaraman, J. Hahn, H. Wallace and D. Bux (1999). "The role of uncontrollable trauma in the development of PTSD and alcohol addiction." Alcohol Res Health **23**(4): 256-262.

Wamsteeker, J. I., J. B. Kuzmiski and J. S. Bains (2010). "Repeated stress impairs endocannabinoid signaling in the paraventricular nucleus of the hypothalamus." J Neurosci **30**(33): 11188-11196.

Wang, M., M. N. Hill, L. Zhang, B. B. Gorzalka, C. J. Hillard and B. E. Alger (2012). "Acute restraint stress enhances hippocampal endocannabinoid function via glucocorticoid receptor activation." J Psychopharmacol **26**(1): 56-70.

Weitzman, E. D., C. A. Czeisler, J. C. Zimmerman and M. C. Moore-Ede (1981). "Biological rhythms in man: relationship of sleep-wake, cortisol, growth hormone, and temperature during temporal isolation." Adv Biochem Psychopharmacol **28**: 475-499.

Whalen, P., J. Kagan, R. Cook, F. Davis, H. Kim, S. Polis, D. McLaren, L. H Somerville, A. A McLean, J. Maxwell and T. Johnstone (2005). Human Amygdala Responsivity to Masked Fearful Eye Whites.

Whitworth, J. A., P. M. Williamson, G. Mangos and J. J. Kelly (2005). "Cardiovascular Consequences of Cortisol Excess." Vascular Health and Risk Management **1**(4): 291-299.

Woodward, S. H., D. G. Kaloupek, C. C. Streeter, C. Martinez, M. Schaer and S. Eliez (2006). "Decreased anterior cingulate volume in combat-related PTSD." Biol Psychiatry **59**(7): 582-587.

Xu, J., M. D. Antion, T. Nomura, S. Kraniotis, Y. Zhu and A. Contractor (2014). "Hippocampal metaplasticity is required for the formation of temporal associative memories." J Neurosci **34**(50): 16762-16773.

Yang, H., J. Zhang, K. Andreasson and C. Chen (2008). "COX-2 oxidative metabolism of endocannabinoids augments hippocampal synaptic plasticity." Molecular and cellular neurosciences **37**(4): 682-695.

Yehuda, R. (2002). "Post-traumatic stress disorder." N Engl J Med **346**(2): 108-114.

Yehuda, R. and J. Seckl (2011). "Minireview: Stress-Related Psychiatric Disorders with Low Cortisol Levels: A Metabolic Hypothesis." Endocrinology **152**(12): 4496-4503.

Yehuda, R., S. M. Southwick, G. Nussbaum, V. Wahby, E. L. Giller, Jr. and J. W. Mason (1990). "Low urinary cortisol excretion in patients with posttraumatic stress disorder." J Nerv Ment Dis **178**(6): 366-369.

Yoshida, T., M. Uchigashima, M. Yamasaki, I. Katona, M. Yamazaki, K. Sakimura, M. Kano, M. Yoshioka and M. Watanabe (2011). "Unique inhibitory synapse with particularly rich endocannabinoid signaling machinery on pyramidal neurons in basal amygdaloid nucleus." Proc Natl Acad Sci U S A **108**(7): 3059-3064.

Young, B. J. and R. N. Leaton (1996). "Amygdala central nucleus lesions attenuate acoustic startle stimulus-evoked heart rate changes in rats." Behav Neurosci **110**(2): 228-237.

Zhang, Z., W. Wang, P. Zhong, S. J. Liu, J. Z. Long, L. Zhao, H. Q. Gao, B. F. Cravatt and Q. S. Liu (2015). "Blockade of 2-arachidonoylglycerol hydrolysis produces antidepressant-like effects and enhances adult hippocampal neurogenesis and synaptic plasticity." Hippocampus **25**(1): 16-26.

ACKNOWLEDGEMENTS

These studies were supported by NIH Grant MH107435 (S.P.). Behavioral studies were conducted at the Vanderbilt Neurobehavioral Core Facility. The work is the responsibility of the authors and does not represent the official views of the NIH.