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Endocannabinoid modulation of predator stress-induced long-term anxiety in rats

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Endocannabinoid modulation of predator stress-induced long-term anxiety in rats

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Science

by

James Lim

Dissertation Committee:
Professor Daniele Piomelli, Chair
Professor Christine Gall
Professor James McGaugh

2016
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ABSTRACT OF THE DISSERTATION

Endocannabinoid Modulation of Predator Stress-Induced Long-Term Anxiety in Rats.

By

James Lim

Doctor of Philosophy in Biomedical Science
University of California, Irvine, 2016
Professor Daniele Piomelli, Chair

Individuals who experience life-threatening psychological trauma are at risk of developing a series of chronic neuropsychiatric pathologies that include generalized anxiety, depression and drug addiction. The endocannabinoid system has been implicated in the modulation of these responses by regulating the activity of the amygdala and the hypothalamic-pituitary-adrenal axis. However, the relevance of this signaling complex to the long-term consequences of traumatic events is unclear. Here, we use an animal model of predatory stress-induced anxiety-like behavior to investigate the role of the endocannabinoid system in the development of persistent anxiety states. Our main finding is that rats exposed to the fox pheromone 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), a life-threatening stimulus for rodents, display a marked and selective increase in the mobilization of the endocannabinoid, 2-arachidonoyl-sn-glycerol (2-AG), in the amygdala. This effect lasts for at least 14 days after the stress has occurred. Additionally, systemic or local pharmacological inhibition of monoacylglycerol lipase (MGL) – a lipid hydrolase that degrades 2-AG in presynaptic nerve terminals – elevates 2-AG levels and suppresses the anxiety-like behavior elicited by exposure to TMT. In addition, peripherally restricted pharmacological inhibition of fatty acid amid hydrolase (FAAH) – the enzyme that degrades anandamide – also suppresses the anxiety-like
behavior elicited by exposure to TMT. The results suggest that predator threat triggers long-term changes in 2-AG-mediated endocannabinoid signaling in the amygdala, and there is a peripheral endocannabinoid component that influences long-term anxiety states. Overall our findings indicate that pharmacological interventions targeting MGL or FAAH might provide a therapeutic strategy for the treatment of chronic brain disorders initiated by trauma.
CHAPTER 1

Stress, Fear, Anxiety, and Post Traumatic Stress Disorder

Introduction

In the human clinical literature, anxiety is defined as a long-term trait characterized by non-adaptive hypervigilance and overestimation of the potential for threat in uncertain situations (Sylvers et al, 2011). In comparison, the animal literature often defines anxiety as a temporary behavioral state induced by diffuse threatening and stressful stimuli (Sylvers et al, 2011), such as bright lights and open spaces (Crawley, 1985; Pellow et al, 1985). Despite the discrepancy between the definitions, similar brain structures are involved in both human and rodent models of anxiety (Boehme et al, 2014; Duvarci et al, 2009; Moreira et al, 2007a; Yassa et al, 2012). In addition, drugs that are anxiolytic in humans reduces avoidance towards bright lights and open spaces in rodents (Bhattacharya and Satyan, 1997; Pellow and File, 1986; Rodgers and Dalvi, 1997; Schmitt and Hiemke, 1998; Walker and Davis, 1997). These striking similarities indicates that the study of rodent anxiety paradigms can provide profound insights into human anxiety disorders. Anxiety disorders are often associated with significant negative impact on the quality life of an individual, which includes large financial and emotional burdens (Lecrubier, 2007).

In general, the stress response is a biological cascade of events that occurs in response to a real or perceived threat. It requires the coordinated activation of a collection of physiological systems that act to promote the survival of the organism. Typically, the response to physiological and/or psychological stressors requires the
concerted activation of both an autonomic response and a neuroendocrine response. However, when the stress response system is altered due to prolonged activation, the anxiety response can become maladaptive. This dysfunction can often occur after an organism is confronted with a life-threatening stimulus.

Individuals exposed to a single emotionally traumatic event can have a lifelong impact on their psychological health, including triggering anxiety-related disorders like post-traumatic stress disorder (PTSD). It is estimated that as many as 80% of all people are exposed to traumatic events during their lifetimes (Breslau et al, 1998; Frans et al, 2005; Kessler et al, 1995; Norris et al, 2008). Approximately 8% of the trauma victims will then go on to develop PTSD, a condition characterized with hyperactivity to mild stressors, especially when it closely resembles the original traumatic event. PTSD patients often develop comorbidity of mental disorder such as depression, general anxiety and high rates of drug and alcohol abuse (Brady et al, 2000; Brady et al, 2005).

Exposure cognitive therapy is the most frequently used treatment for anxiety disorders, and unfortunately it has been relatively ineffective in treating PTSD (Hembree et al, 2003). In recent years psychopharmacological therapies has been gaining attention in the treatment for anxiety disorder and PTSD. Our understanding of the neurobiology of anxiety provides a theoretical framework for the use of pharmacotherapy in the secondary prevention of chronic anxiety after life-threatening events (Davidson, 2004, 2006; Pitman and Delahanty, 2005). Evidence suggests that pharmacological agents such as β-adrenergic antagonists, opiates, D-cycloserine and anxiolytics are at least partially effective in managing the symptoms of PTSD (Ducrocq and Vaiva, 2005; Zatzick and Roy-Byrne, 2003; Zatzick and Roy-Byrne, 2006; Zhang
and Davidson, 2007). However, few studies have thoroughly examined the mechanisms of action and efficacy of psychotherapeutic drugs to prevent persistent anxiety states that develop in the aftermath of major trauma (Adshead, 2007; Davidson, 2004, 2006; Giuffrida et al., 2004; Pitman et al., 2005). PTSD is implicated with significant occupational, social, and quality-of-life impairments, as well as increased risk for developing a variety of chronic neuropsychiatric conditions. Given the high prevalence of PTSD and its negative impact on society and individual, it is essential to understand the mechanisms by which stress induces changes in neurological function, as well as the molecular mechanisms that maintain long-term expression of maladaptive behavior.

The primary goal of this chapter is to give an overview of anxiety and the neurobiological process that are involved in mounting a stress response to understand the mechanism of maladaptive anxiety states brought on by a traumatic event. We will briefly outline the different aspect of fear learning and maintenance of anxiety disorders. Then we will describe PTSD and how it is an aberrant and extreme form of fear learning. Lastly the current chapter will explore different pharmacological intervention that are available today for treating maladaptive long-term anxiety and PTSD.

**Overview on Animals Models of Anxiety**

In humans, anxiety is assessed by self-report. Since rodents cannot self-report their anxiety levels, we can instead measure their innate avoidance of bright lights and open spaces. Rodents avoid bright and open areas presumably because they are more vulnerable to predators. Avoidance towards bright open space is commonly studied in the elevated plus maze (EPM), a behavioral test consisting of a plus-shaped maze on an elevated platform. It contains two brightly lit open arms and two dark closed arms.
enclosed by walls. This test capitalizes on the conflict between the innate curiosity of rodents to explore and their fearfulness of open spaces (Pellow et al, 1985). Another frequently used paradigm is the open field test, which consists of a brightly lit square arena with enclosed walls. As expected, rodents spend more time exploring the walled periphery of the open field test and avoid its brightly lit center. This avoidance behavior has pharmacological correlation to anxiety in humans, as drugs that are anxiolytic in humans such as benzodiazepines, decrease the bright open space aversion in rodents (File and Pellow, 1985; Schmitt et al, 1998)

Rodents also show innate aversion to bright lights has been extensively studied using the startle paradigm, in which rodents exhibit a startle reflex when presented with an unpredictable burst of noise. Interestingly, the amplitude of the startle response is augmented when the behavior test is coupled with an innately aversive stimulus, such as a predator (Blundell et al, 2005) or bright illumination (Walker et al, 1997). This paradigm has been validated pharmacologically, as the amplitude of the light-potentiated startle is decreased by the anxiolytic drug buspirone (Walker et al, 1997).

While there are indeed other behavioral paradigms that measure other types of anxiety, such as social anxiety (Pobbe et al, 2011), the current thesis will focus on EPM paradigm measuring aversion towards bright light and open spaces, as it is one of the most commonly used and well accepted anxiety paradigms.

**Overview of the Amygdala Circuitry and Anxiety.**

Substantial number of studies implicates the amygdala in anxiety both in humans and rodents. For example, greater amygdala volume is correlated with increased anxiety in humans (Machado-de-Sousa et al, 2014; Qin et al, 2014), and functional
magnetic resonance imaging study show patients with social anxiety disorder have higher blood-oxygen-level dependent signals within the amygdala during anticipatory anxiety compared to healthy subjects (Boehme et al, 2014). Moreover, immediate early gene expression studies in rodents show increased amygdala activation following exposure to anxiogenic contexts (Butler et al, 2012; Silveira et al, 1993), and pharmacological inhibition of the amygdala has an anxiolytic-like effect in the EPM (Moreira et al, 2007a). Therefore, both human and rodent studies suggests that the amygdala is a critical structure in the anxiety circuitry.

The basolateral amygdala (BLA) and the central nucleus of the amygdala (CeA) are the most studied sub-region of interest within the amygdala. An extensive body of research has established that stressful sensory information across multiple modalities enters the amygdala through the BLA and the centrolateral amygdala (CeAL), both of which project to the centromedial amygdala (CeAM) (LeDoux, 2000). The CeAM, in turn, projects to rostral and caudal brain regions such as the hypothalamus, involved in the expression of stress, fear, and anxiety (Figure 1.1) (LeDoux, 2000). Functional studies have shown that direct activation of CeAL strongly inhibits CeAM output neurons through GABA (gamma-aminobutyric)-ergic projections and reduces fear and anxiety responses, while direct activation of the BLA increases fear and anxiety responses through glutamatergic projections to the CeAM (Etkin et al, 2009; Li et al, 2013). Additionally, results from the fear conditioning literature show the BLA integrates highly processed information about the environment and encodes behaviorally relevant cues (LeDoux, 2000). The CeA also plays crucial role in mediating behaviors induced by threatening stimuli. The inactivation of the CeAM significantly reduces freezing
during tone presentation in an auditory fear conditioning paradigm (Ciocchi et al, 2010). This effect is thought to be mediated by projections of the central medial nucleus to hypothalamic and brain stem targets which modulate various anxiety states and stress responses (Price and Amaral, 1981).

Optogentic studies have shown that activation of the entire BLA significantly increases anxiety-like behavior in mice, while selective activation of the projection from the BLA to the central lateral nucleus has an anxiolytic-like effect (Tye et al, 2011). These results are supported by anatomy, as the CeAL inhibits the CeAM (Tye et al, 2011), which is the main output structure of the amygdala (LeDoux, 2000; Price et al, 1981). These data demonstrate that the amygdala is made up of complex set of neurons with different functions depending on their post-synaptic targets (Tye et al, 2011). In addition to the BLA and CeA, The amygdala has other sub-regions that mediate defensive behaviors. For example, the medial amygdala is necessary to react to olfactory cues from a predator as it projects out to the hypothalamus (Li et al, 2004), whereas the basomedial amygdala mediates avoidance of potentially threatening visual and auditory cues (Gross and Canteras, 2012). In summary, previous studies demonstrate the amygdala is essential for generating anxiety. However, some sub-regions have been more extensively studied than others. Furthermore, with the advent of optogenetics and other novel genetic tools, the study of functional differentiation of sub-regions in the amygdala has only just begun.

**Post-Traumatic Stress Disorder and Prolonged Anxiety**

When a fear response is surpasses the severity of a threat, it can interfere with other adaptive behaviors, compromising mental function and fitness of an individual
Inappropriate fear responses in humans can lead to the development of anxiety disorders (Rosen and Schulkin, 1998), including PTSD. People who are exposed to a traumatic life event are at risk of developing PTSD and currently it affects about 7% of the United States population (Kessler et al., 2005a). Symptoms include constant re-experiencing of the incident, avoiding stimuli associated with the traumatic event, and increased arousal, exhibited by exaggerated startle responses (American Psychiatric Association. and American Psychiatric Association. DSM-5 Task Force., 2013). In threatening situations, these symptoms are adaptive for coping with the potential danger (Bonne et al., 2004; Charney, 2004; Christopher, 2004). For example, avoiding stimuli associated with the traumatic event decreases the probability of encountering the same threat or dangerous situations similar to the event. Additionally, hypervigilance may help increase awareness and perception of surroundings and more readily detect potential threats.

However, patients with PTSD have significant impairment in their daily lives because these responses become over exaggerated and dysfunctional by occurring in non-threatening situations that do not require intense reactions. As a result, avoiding trauma-related cues can lead to a social isolation, re-experiencing the event can lead to sudden panic attacks and sleep disturbances, and hypervigilance can lead to paranoia and exhaustion (Brewin et al., 2000; Gmitrowicz and Kucharska, 1994; Kessler et al., 2005b; Speckens et al., 2006; Stam, 2007a, b). One key feature of PTSD is a highly defensive reaction to a mild stressor, a response more suitable for the original traumatic event. (Bremner et al., 1995; Dykman et al., 1997; Friedman, 1994). Additionally, PTSD is often accompanied by other psychiatric symptoms such as phobias, depression, and
anxiety (Brady et al, 2000; Brady et al, 2005). Other reports have also shown that PTSD leads to a predilection towards drug and alcohol addiction (Dutton et al, 2014; Stander et al, 2014; van Dam et al, 2013). These studies demonstrate that PTSD is a serious mental illness and there is a critical need for developing novel and effective treatments for this disorder.

**The General Stress Response**

Exposure to stress appears to increase the sensitivity of a constellation of physiological systems designed to increase the rate of survival of the organism. The common stress response to both physiological and psychological stressors can be simplified as the combined activation of an autonomic response and a neuroendocrine response. The autonomic response involves stimulation of sympathetic motor and hormonal outputs via descending neural circuits originating in hypothalamic pre-autonomic control centers (Pecoraro et al, 2006). The neuroendocrine stress response is mediated by activation of the hypothalamic-pituitary-adrenal (HPA) axis, which results in an increase in circulating epinephrine and glucocorticoids (Pecoraro et al, 2006).

It is likely that both the peripheral and central nervous systems work in concert to enhance fear responses through the autonomic response and the neuroendocrine system (Brunet et al, 2001; Carrasco and Van de Kar, 2003; Hurlemann et al, 2010; Johnson et al, 1992; O'Donnell et al, 2004; Pitman, 1989). Generally, the activation of these systems causes the body to initiate a set of responses that facilitate coping with the stress and restore homeostasis once the threat is eliminated. These responses, well known as the flight or fight response, include allocating energy and other resources to sustain the brain, heart, and muscles, preparing the immune system, enhancing
cognitive functioning, and inhibiting behaviors and bodily processes that are unnecessary for survival (Christopher, 2004; Johnson et al., 1992; Sapolsky, 2000).

The activation of the HPA-axis to mediate stress response has been heavily studied. Stressful stimuli activate neural inputs to the paraventricular nucleus (PVN) of the hypothalamus, inducing the release of corticotropin-releasing hormone (CRH), which is then transported to the anterior pituitary gland and stimulates the release of adrenocorticotropic hormone (ACTH). Adrenocorticotropic hormone then travels through the bloodstream and initiates glucocorticoid release from the adrenal cortex (Figure 1.2) (Antoni, 1986; Owens and Nemeroff, 1991; Pecoraro et al., 2006; Vale et al., 1981). Glucocorticoids (cortisol in humans and corticosterone in rodents; CORT) is involved in increasing energy mobilization and aid to restore homeostasis through a negative feedback mechanisms (involving multiple brain regions and systems) after a threat has passed (Munck et al., 1984; Sapolsky et al., 2000). Glucocorticoids are lipophilic, thus readily crosses the blood brain barrier and are actively transported into the brain. In the brain, glucocorticoids bind to the membrane nuclear receptors or diffuse across neuronal cell membranes and bind to cytosolic nuclear receptors (De Kloet et al., 1998; McEwen et al., 1970; Tasker and Herman, 2011). Glucocorticoid have a high affinity for either mineralocorticoid or glucocorticoid receptors (GR), which can modulate the release of neurotransmitters and/or translocate into the nucleus and alter gene expression (Tasker et al., 2011). These glucocorticoid-mediated mechanisms help exert negative feedback control over the HPA-axis and modulate stress response (Figure 1.2) (McEwen et al., 1970; Perusini et al., 2015; Reichardt and Schutz, 1998; Tasker et al., 2011). In chapter 2, we will discuss in detail about our current understanding of the
endocannabinoid-mediated glucocorticoid negative feedback regulation of the HPA-axis.

In rodent studies, mimicking HPA-axis activation with administration of CORT have consistent results in stress and fear conditioning paradigms. Rats that were given CORT injections for 5 days before contextual fear conditioning or rats given just a single injection of CORT after contextual fear conditioning both showed increase in conditioned freezing during a context test compared to the vehicle treated rats (Cordero et al., 2003; Perusini et al., 2015; Thompson et al., 2004). These studies suggest a role for stress hormones in mediating stress induced enhancement of behavioral response.

It has been hypothesized that changes in HPA-axis responsiveness is a contributing factor to the PTSD patients who exhibit severe defensive responses in a benign environment (Perusini et al., 2015). In other words, PTSD symptoms may develop through a sensitization process involving the HPA-axis that causes low-intensity stressors to be perceived as life-threatening (Rasmusson and Charney, 1997; Yehuda, 1997). It is thought that the initial traumatic event evoke a stress response; upon receiving constant reminders of the trauma shortly after the event, the stress circuit goes through a continuous cycle of reactivation even in absence of the original stress stimulus. Repeated activation alters the HPA-axis circuitry and the individual becomes more susceptible to triggering a stress response. This sensitization results in a lowered activation threshold for subsequent stress stimuli, facilitating an anxiety response towards an innocuous stimuli (Hageman et al., 2001; Rosen et al., 1998). In addition, other studies have provided compelling evidence that the presence of a prolonged adrenergic activation during a life-threatening event contributes to over
consolidation of memory for the trauma and thereby supports the development of the intrusive symptoms found in PTSD (Hurlemann et al., 2010; O’Donnell et al., 2004; Pitman, 1989). Potentiated activity in fear circuitry due to repeated activation of the stress response may contribute to dysfunction of the central and peripheral component of the HPA-axis negative feedback mechanism leading to a long-term anxiety disorders commonly found in PTSD.

**Fear Conditioning and Fear Extinction**

The neural systems mediating associative fear learning are heavily studied, which provides valuable information to model fear responses and help explain some PTSD related anxiety symptoms (Fendt and Fanselow, 1999). Experimentally, fear conditioning occurs when a conditioned stimulus (CS) such as a tone or light, is paired with an aversive, unconditioned stimulus (US; e.g. electric shock to the forearm in humans, mild foot shock in rodents), resulting in a CS–US association where the CS alone evokes a conditioned fear response (e.g. increased skin conductance in humans, freezing in rodents) (Maren, 2001; Singewald et al., 2015). Following a CS–US association, aversive memories are consolidated though a cascade of molecular and cellular events that modifies synaptic efficacy, as well as a prolonged changes in the connectivity between different brain regions (Maren, 2001; McGaugh, 2000). Once consolidated, fear memories can be attenuated by various pharmacological and psychological interventions, where some producing temporary reduction of fear behaviors and others causing more long-lasting relief (Lee et al., 2006; Nader et al., 2000).
More naturally, fear memories can also be extinguished. Extinction is a learning process driven by re-association of the original CS-US paring to CS-nothing (McNally and Westbrook, 2006; Pearce and Bouton, 2001). Fear extinction was originally demonstrated by Pavlov (Pavlov, 1951), which involves repeated exposure to anxiety-provoking cues to establish a new memory that counters the original fear memory. The process is highly relevant to fear, anxiety, and trauma-related disorders which are associated with negative emotional reactions triggered by specific situations, objects, or internal and external cues that are closely related to the original trauma but benign. Moreover, extinction in animals is procedurally similar to forms of cognitive behavior therapy (CBT) that rely on exposing patients to anxiety-provoking cues without any danger, in order to overcome their anxiety (Milad and Quirk, 2012), since human anxiety disorders are associated with an inability to extinguish learned fear and to respond properly to safety signals (Jovanovic et al, 2012; Michael et al, 2007; Milad et al, 2009; Wessa and Flor, 2007). Therefore, fear extinction has considerable translational utility. It is important to note that extinction memories are prone to re-emergence (Tsai and Graff, 2014). The re-emergence of extinguished fear occurs under following circumstances: renewal, when the CS is presented in a different context to that in which extinction training occurred; reinstatement, when the original US or another stressor is given unexpectedly; and spontaneous recovery, when a significant period of time has elapsed following successful extinction training (Herry et al, 2010; Myers and Davis, 2007). Spontaneous recovery, reinstatement and renewal are all observable in clinical settings, and can be readily exploited in the laboratory to identify drugs and other interventions that can prevent fear re-emergence in animals and relapse in humans.
recovery (Effting and Kindt, 2007; Rowe and Craske, 1998a, b; Schiller et al, 2008; Vervliet et al, 2013).

**Neural System Involved in Associational Fear Learning**

Extensive neuroanatomical studies show that several key brain areas including the amygdala, hippocampus, medial prefrontal cortex (mPFC), periaqueductal gray (PAG), bed nucleus of the stria terminalis (BNST) and other brain regions have been implicated in fear conditioning and extinction (Duvarci and Pare, 2014; Ehrlich et al, 2009; Herry et al, 2010; Knapska et al, 2012). During conditioned fear learning, excitatory sensory input relating to both the CS and US converge on the BLA complex, where a CS-US association is encoded through long-term potentiation at the BLA synapses (Kim and Jung, 2006; Rogan et al, 1997). This synaptic plasticity is dependent upon the glutamate activation of the N-methyl-D aspartates receptors (NMDAR) and is modulated through the GABAergic neurons in rodents (Ehrlich et al, 2009; Fanselow and Kim, 1994; Makkar et al, 2010; Miserendino et al, 1990). The BLA is necessary for encoding the memory of the US as either pleasant or aversive, as well as for storing it long-term (Fanselow and Gale, 2003; Gale et al, 2004). Consistent with rodent studies, human functional magnetic resonance imaging studies show enhanced amygdala activity in PTSD patients during encoding and exposure to aversive stimuli (Brohawn et al, 2010; Rauch et al, 2000; Shin et al, 2006).

The BLA projects to CeA both directly and indirectly, via a link through the intercalated cell masses that lie between these two regions (Pare et al, 2004; Pitkanen et al, 1997). As mentioned above, BLA neurons project to the CeAL, which sends GABAergic projections to the CeAM (Haubensak et al, 2010; LeDoux, 2000). The fear
response (freezing in rodents) is controlled by projections from the CeAM to the periaqueductal grey (PAG) (Perusini et al, 2015). Besides the CeA, the BLA projects to the bed nucleus of the stria terminalis (BNST), which in turn projects to the PAG for fear response (Waddell et al, 2006; Walker et al, 2003). Extinction training has been shown to cause a rapid reduction of CS-evoked responses of BLA neurons possibly through depotentiation of thalamic inputs (Duvarci et al, 2014). It is hypothesized that the BLA contains extinction-encoding neurons which drive GABAergic cells in the medial intercalated cell masses and neurons in the CeAL to inhibit the CeAM in order to reduce the expression of fear (Duvarci et al, 2014).

Other important regions in the fear learning and memory network involve cortical regions such as infralimbic (IL), prelimbic (PL), and the PFC (Milad and Quirk, 2002; Santini et al, 2004). Previous studies have shown that the PL cortex projects to the BLA to enhance fear response, while the IL cortex directly projects to the extinction encoding neurons in the BLA and indirectly projects to the CeAM by projecting to the intercalated cells to promote fear extinction (Perusini et al, 2015; Quirk et al, 2003). The dichotomous behavioral outputs of the PL and the IL cortexes has been shown to be modulated by the descending projections of the mPFC (Quirk et al, 2003). Additionally, inputs from the ventral hippocampus onto the BLA, either directly or indirectly through the PL cortex, mediate contextual control of fear and reinstatement of conditioned fear after extinction (Orsini et al, 2011).

**Strategies for Treating Pathological Fear and Anxiety**

As mentioned above, traumatic events are reasonably common, with around 6.4% of the population developing PTSD (Breslau et al, 1998; Frans et al, 2005; Kessler...
et al, 1995; Norris et al, 2008). Around half of those who develop PTSD will recover within a year without professional help but, unfortunately, up to 15% of people with the disorder will show little or no improvement even after receiving treatment (Bradley et al, 2005). Given the relative frequency of no-response to current method of treatments, and the pervasive and debilitating effect of the disorder, preventing PTSD would be of great benefit in reducing the burden to both to society and individuals.

Available pharmacological and psychotherapeutic treatments which aim to reduce fear and anxiety have shown to be somewhat effective in alleviating the severity of the symptoms, where only about 40% of anxiety patients show partial long-term benefit, and a majority of them fail to achieve complete remission (Baldwin et al, 2014; Bandelow et al, 2007; Bandelow et al, 2012; Hofmann and Smits, 2008; Stein et al, 2009) clearly emphasizing the need for a better option. Current pharmacological approaches either have acute anxiolytic effects (e.g. benzodiazepines, some antipsychotics) or require chronic treatment (e.g. selective serotonin reuptake inhibitors, other antidepressants) to attenuate symptoms of pathological fear and anxiety (Singewald et al, 2015). Commonly adapted psychotherapeutic interventions include using CBT techniques such as exposure therapy to help patients overcome the maladaptive anxiety and avoidance behaviors towards fear-triggering cues associated with the pathology (Singewald et al, 2015). Previous studies indicate that CBT does have some efficacy over anxiety disorders, including PTSD (Choy et al, 2007; Singewald et al, 2015). However, majority of the patients have difficulty bearing the demanding and exhausting process of therapy and many who do manage to cope with it respond only partially and often relapse with time (Choy et al, 2007).
In order to address the unresponsiveness to CBT, attempts were made to combine psychotherapy with pharmacological treatments. Indeed, pharmacological agents in combination with exposure therapy shows some synergistic effective in managing anxiety and symptoms of PTSD (Ducrocq et al, 2005; Zatzick et al, 2003; Zatzick et al, 2006; Zhang et al, 2007). There has been an increase in search to discover and identify pharmacological agents that serve as more effective adjuncts to CBT. Here we list out some of options for alleviating anxiety and PTSD symptoms that are currently available today.

**Alcohol:** Of all the available drugs with the potential to treat anxiety brought on by trauma, alcohol probably has the longest history. For centuries soldiers and military combatants have consumed alcohol before battle to mitigate the stress of the experience (Maes et al, 2001; McFarlane, 1998). Recent meta-analysis studies have found that individuals who were intoxicated during a fire or a motor vehicle accident have a decreases likelihood of developing PTSD (Maes et al, 2001; Mellman et al, 1998). For anyone who have experience the effect of alcohol, it is easy to imagine that the protective effect of alcohol is most likely due to interference with the encoding and storage of memories (Maes et al, 2001; Mellman et al, 1998). Additionally, it has be proposed that alcohol allows the drinker to perceive and focus on only the most immediate cues, thus distracting the individual from the source of the stress (Sayette, 1999). Furthermore, alcohol likely impairs perception of relevant threat information, thus the event is not seen as severe or life-threatenning (Clum et al, 2002; Sayette, 1999). Alcohol may be desirable as a primary preventative medication for PTSD for several reasons. It is relatively cheap, readily available, and particularly useful in the military
where combat and traumatic event is somewhat predictable. Despite these advantages, however, alcohol is unlikely to be widely utilized for preventing PTSD. This is because alcohol protects against stress only if consumed before a trauma (Clum et al., 2002; Sayette, 1999). In other words, alcohol cannot be used as a secondary prevention for PTSD. The use of alcohol to prevent PTSD can only be capitalized by those individuals who are fairly certain that a traumatic experience is imminent, such as members of the emergency services or military. However, regular consumption of alcohol as a preventative strategy in case a stressful event is unwise for these professions, since many of these individuals require motor precision and unimpaired judgement (Simon and Gorman, 2004).

**Glucocorticoid:** As described above, stress activates the HPA-axis and initiates the production of glucocorticoid which serves to shut down the stress response through a negative feedback mechanism (McEwen et al., 1970; Perusini et al., 2015; Reichardt et al., 1998; Tasker et al., 2011). In individuals who develop PTSD, there is some evidence that the negative feedback regulation of the HPA-axis fails and post-traumatic glucocorticoid levels are lower than in subjects who recover (Ehring et al., 2008; McFarlane et al., 1997; Meewisse et al., 2007). With persistently low glucocorticoid levels relating to an increase risk for PTSD, we can hypothesize that artificially increasing glucocorticoid levels would provide some protection against the development of disorder. Indeed, experiments with survivors of septic shock and cardiac surgery shows that patients administered hydrocortisone in the intensive care unit were less likely to develop PTSD than those who received a placebo (Schelling et al., 2001; Schelling et al.,
Morphine: When faced with a highly stressful event, the brain increases its production of norepinephrine which enhances consolidation of aversive memories (Bryant et al, 2009; McGaugh et al, 2002a; McGaugh and Roozendaal, 2002b; Pitman et al, 2002). This mechanism is thought to have evolved to ensure we properly distinguish between potentially dangerous and less threatening situations (Pitman et al, 2005). The extreme hyperarousal associated with trauma leads to repeated consolidation of the trauma memory, which results in pathological anxiety and PTSD (Henry et al, 2007; McCleery and Harvey, 2004). The protective effect of morphine comes from its inhibition of norepinephrine release (Bryant et al, 2009; Simon et al, 2004). Generally, it was observed that survivors from motor vehicle accidents who were given high doses of morphine in the first 48 hours were free of PTSD while patients that were given lower doses went on to develop PTSD (Bryant et al, 2009). Acute morphine administration has also been associated with a lower likelihood of subsequent PTSD in US military personnel serving in Iraq (Holbrook et al, 2010). Although in theory acute administration of morphine as a secondary prevention for PTSD looks promising, further empirical investigation is needed.

β-adrenergic receptor antagonist: Developed in the 1950s, propranolol is a β-adrenergic receptor antagonist that was first used in the treatment of hyper tension and heart problems (McCleery et al, 2004). Propranolol acts to reduce sympathetic arousal via blockade of noradrenergic signaling and is hypothesized to reduce the likelihood of developing PTSD when administered within hours of trauma exposure (Debiec et al,
Although propranolol gained enormous attention in the literature, the evidence for its effectiveness as an agent for secondary prevention of PTSD remains controversial.

Initial randomized trial found that in trauma-exposed individuals, propranolol recipients (within 8 hours post-trauma) showed no significant difference from placebo recipients in the risk of developing PTSD (Pitman \textit{et al}, 2002). However, propranolol recipients were exhibited reduced physiological response to trauma related cues 3 months post-trauma (Pitman \textit{et al}, 2002), suggesting that propranolol may be specifically efficacious at reducing the conditioned fear-related symptoms of PTSD. In contrast, results from a non-randomized trial showed that trauma patients who refused propranolol were significantly more likely to suffer PTSD than those who took the drug within the first 24 hours post-trauma. (Vaiva \textit{et al}, 2003). A subsequent randomized trial found no differences between patients receiving propranolol versus placebo at either 4 or 12 weeks post-trauma (Stein \textit{et al}, 2007). Contradictory findings are likely be the result of differences in timing of drug administration and doses given post-trauma. Despite these ambiguous findings, there is some support for the efficacy of early propranolol administration as a secondary preventative measure for PTSD symptoms.

\textbf{D-Cycloserine:} D-cycloserine (DCS) is a partial NMDAR agonist that has been approved by the United States Federal Drug Administration for the treatment of tuberculosis. As discussed above, fear learning is dependent upon neural activity in the amygdala and involves the action of glutamate through NMDARs, it is believed that acute DCS administration stimulates the NMDA glutamate synapses involved in
emotional learning and strengthens extinction learning that takes place in the context of exposure-based treatments (Ledgerwood et al, 2004; Walker and Davis, 2000).

In randomized controlled trials, subjects had enhanced therapeutic outcomes for PTSD for participants receiving DCS-augmented exposure therapy relative to placebo (de Kleine et al, 2012; Difede et al, 2014). An in depth analysis found that subjects with a more severe PTSD symptom experienced greater effects from DCS-augmented exposure therapy (de Kleine et al, 2012). However, similar to β-adrenergic receptor antagonist, the efficacy of DCS in the secondary prevention for PTSD is mixed. Litz et al (2012) identified that participants receiving placebo-augmented therapy had greater positive therapeutic outcomes compared with DCS on post-traumatic symptoms (Litz et al, 2012). Additionally, another group found no significant difference between DCS or placebo-augmented virtual reality exposure therapy on the risk of developing long-term anxiety and PTSD (Rothbaum et al, 2014). Once again the contradictory findings are likely be the result of differences in drug administration timing, doses given post-trauma, and the type of trauma.

In summary, while there are several promising options, there is currently no compelling evidence that any of the drugs above can effectively prevent PTSD. Further studies are essential to find pharmacological agents to help prevent long-term anxiety and PTSD.

Conclusions

In summary, fear, anxiety and trauma-related disorders are associated with excessive fear reactions triggered by specific objects, situations or internal and external
cues in the absence of any actual danger, and often include an inability to extinguish learned fear and to show adequate safety learning signals (Jovanovic et al., 2012; Michael et al., 2007; Milad et al., 2009; Rasmusson et al., 1997; Wessa et al., 2007; Yehuda, 1997). There have been copious amount of studies that focus on the neural structures, pathways, systems, and mechanisms of fear conditioning paradigm. The systems and circuits discussed in this chapter is highly integrated. As we can postulate, this integrated system for fear and anxiety is essential for survival. The stress response system is able to rapidly discriminate and encode relevant associative relationships over a multitude of environmental stimuli that could indicate a major threat. Furthermore, these systems have redundancies with alternate and compensatory pathways for persistent operation. However, repeated or over activation the stress systems can cause individuals to become more susceptible to triggering a stress response, facilitating an anxiety response towards an innocuous stimuli (Hageman et al., 2001; Rosen et al., 1998). Despite our profound understanding in fear learning and the HPA-axis response to stress, systematic studies on fear and anxiety responses brought on by a life-threatening trauma is still lacking.

Additionally, maladaptive long-term anxiety and PTSD is associated with severe occupational, social, and quality-of-life impairments and there is a dire need for developing novel and effective treatments for this disorder. As mentioned above there are several promising options, but all the drugs covered in this section show partial efficacy in treating anxiety and PTSD. In addition for the need to determine appropriate dosing and duration of administration, as well as determining the ideal timing of...
administration to prevent post-traumatic distress, it is essential to explore novel pharmacological targets for secondary preventative intervention for PTSD.
**Figure 1.1: A simplified circuit of the amygdala.** The basolateral amygdala (BLA) consists of the lateral amygdala (LA) and basal amygdala (BA) and projects to many different brain regions involved in learning and memory, including the prefrontal cortex, the hippocampus, the caudate nucleus and the nucleus accumbens. The BA also projects to the central amygdala (CeA) and the medial amygdala (MeA). The CeA projects to autonomic, behavioural and hormonal regulatory centers in the hypothalamus, the midbrain, the pons and the bed nucleus of the stria terminals (BNST). The MeA sends efferents to the BNST and hypothalamus.
**Figure 1.2: The hypothalamic-pituitary-adrenal axis.** Stressful stimuli activate neural inputs to the paraventricular nucleus (PVN) of the hypothalamus, inducing the release of corticotropin-releasing hormone (CRH), which is then transported to the anterior pituitary gland and stimulates the release of adrenocorticotropic hormone (ACTH). Adrenocorticotropic hormone then travels through the bloodstream and initiates glucocorticoid and epinephrine release from the adrenal cortex.
CHAPTER 2

The Endocannabinoid System and Anxiety.

Introduction

Marijuana has been used for recreational and therapeutic purposes for thousands of years. Marijuana, a derivative of the plant Cannabis sativa, inhalation causes variety of psychotropic effects including relaxation and euphoria, grandiosity, altered perception of time, hallucinations, diminished coordination, memory impairment, or memory enhancement (Iversen, 2000; Kano, 2014; Lujan et al, 1996; Piomelli, 2003, 2014). Marijuana can also increase appetite, reduce nausea, and act as a chronic analgesic, which led to the use of cannabinoids for therapeutic purposes (Iversen, 2000; Kano, 2014; Piomelli, 2014). The endocannabinoid (endocannabinoid) system nomenclature derives from the discovery that endocannabinoids and plant-derived cannabinoids, Δ⁹-tetrahydrocannabinol (Δ⁹-THC), bind to the same molecular receptor target (Mechoulam and Parker, 2013; Piomelli, 2014). The endocannabinoid system is a neuromodulatory lipid system, which consists of the cannabinoid receptor type 1 and type 2 (CB₁ and CB₂ receptors, respectively) and two major endogenous ligands, N-arachidonyl ethanolamine (anandamide) and 2-arachidonoyl glycerol (2-AG) (Lujan et al, 1996; Piomelli, 2003). The CB receptors are seven-transmembrane receptors coupled to Gi/o protein and display distinct expression patterns in animal's body. CB₁ receptors are richly expressed in the brain, while CB₂ receptors are mainly expressed in the immune system (Kano et al, 2009).
Perhaps the most significant aspect of the endocannabinoids is that they mediate retrograde signaling at synapses. It has be shown extensively that endocannabinoids are released from postsynaptic neurons upon postsynaptic depolarization and/or receptor activation, and act on presynaptic CB₁ receptors to induce transient or long-term suppression of neurotransmitter release at variety of synapses throughout the central and peripheral nervous systems (PNS) (Castillo et al, 2012; Kano et al, 2009; Katona and Freund, 2012; Ohno-Shosaku et al, 2012). Consistently, immunohistochemical studies demonstrate that the receptors and enzymes associated with endocannabinoid signaling are arranged around synapses in functionally relevant manner (Katona et al, 2006; Uchigashima et al, 2007; Uchigashima et al, 2011; Yoshida et al, 2006; Yoshida et al, 2011). These endocannabinoid signaling components are expressed in various brain regions including the hippocampus, cerebral cortex, amygdala, dorsal and ventral striatum, hypothalamus, cerebellum, and spinal cord (Herkenham et al, 1991; Kano et al, 2009; Piomelli, 2003). Behavioral studies with pharmacological and genetic manipulation of retrograde endocannabinoid signaling have demonstrated that it is involved in various aspects of neural functions, including learning and memory, mood and anxiety, drug addiction, feeding behavior, motor learning and analgesia (Kano et al, 2009; Mechoulam et al, 2013; Piomelli, 2003). For the purpose of this thesis, this chapter will primarily focus on reviewing the endocannabinoid system, and its role in relation to fear, stress, and anxiety.

**Endocannabinoid Signaling Components**

Within the central nervous system (CNS), CB₁ receptors are expressed on glutamatergic, GABAergic, serotonergic, noradrenergic, and dopaminergic axon
terminals (Azad et al, 2008; Haring et al, 2007; Hermann et al, 2002; Kano et al, 2009; Morozov et al, 2009; Oropeza et al, 2007; Piomelli, 2003) However, because most of the brain is composed of excitatory and inhibitory neurons, and the high levels of CB₁ receptor expression on these terminals, the majority of endocannabinoid signaling occur at GABAergic and glutamatergic synapses (Katona et al, 2012). CB₂ receptors are typically located in immune cells (e.g. microglia in the brain) and, when activated, can modulate immune cell migration and cytokine release both in the CNS and the PNS (Pertwee, 2005). CB₁ receptors are preferentially localized to the presynaptic axon terminals and expression level can vary greatly depending on brain regions and synapse types. Activation of CB₁ receptor triggers multiple signal transduction pathways mainly through the Gi/o proteins to inhibit adenylyl cyclase activity and decrease cyclic AMP (cAMP) levels (Kano et al, 2009; Piomelli, 2003). This in turn reduces protein kindase A (PKA) activity that leads to the activation of potassium channels and the inhibition voltage-gated calcium channels at the axon terminal (Howlett et al, 2002; Kano et al, 2009). The activation of the CB₁ receptors results in a robust suppression of neurotransmitter release into the synaptic cleft (Figure 2.1) (Kano et al, 2009; Piomelli, 2003).

Anandamide and 2-AG are synthesized in an activity dependent manner from phospholipid precursors in the postsynaptic membrane by calcium (Ca²⁺)-dependent and Ca²⁺-independent mechanisms (Kano et al, 2009) and exerts its influence in a retrograde manner onto presynaptic terminals, thus suppressing presynaptic neurotransmitter release via activation of CB₁ receptors (Ohno-Shosaku and Kano, 2014). The process of how lipid signaling molecules such as the endocannabinoids are
released and transported across the synapse is still a mystery, but the collective electrophysiological and biochemical evidence to date overwhelmingly supports a model of postsynaptic synthesis and a presynaptic site of action.

**Anandamide:** Anandamide is a partial agonist for CB\textsubscript{1} receptor, CB\textsubscript{2} receptor, and a strong agonist for the transient receptor potential vanilloid type 1 receptor (Starowicz et al, 2007). A complete biochemical pathways for the synthesis of anandamide have still unknown. Studies up to date suggest that the main pathway of anandamide biosynthesis begins with the transfer of an arachidonate group from the \textit{sn}-1 position of phosphatidylcholine to the amino group of phosphatidylethanolamine by \textit{N}-acyltransferase, yielding \textit{N}-arachidonoyl-phosphatidylethanolamine (NAPE). NAPE is hydrolyzed by \textit{N}-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD), producing anandamide (Cadas \textit{et al}, 1997; Jin \textit{et al}, 2007). Anandamide deactivation is primarily mediated by the serine hydrolase, fatty acid amide hydrolase (FAAH) localized at the postsynaptic spine (Figure 2.1) (Kano \textit{et al}, 2009; Piomelli, 2003; Vandevoorde and Lambert, 2007).

**2-AG:** 2-AG is a strong agonist of CB\textsubscript{1} receptor and CB\textsubscript{2} receptor, and its content in the brain is significantly higher than that of anandamide (Sugiura \textit{et al}, 2006). Biochemical studies have found several pathways for 2-AG biosynthesis. In the central nervous system (CNS), 2-AG is produced in a receptor-dependent manner through enzyme-mediated cleavage of a phospholipid precursor in cell membranes (Pertwee, 2008). 2-AG biosynthesis starts with the activation of phospholipase C-\(\beta\), which converts phosphatidylinositol-4,5-bisphosphate (PIP\textsubscript{2}) into 1,2-diacylglycerol (DAG). DAG is hydrolyzed by diacylglycerol lipase-\(\alpha\) (DGL-\(\alpha\)) to produce 2-AG. Newly formed 2-AG is
released from postsynaptic spines, where DGL-α is localized, and stimulates CB1 receptors on presynaptic axon terminals (Katona and Freund, 2008). The biological effects of 2-AG are predominantly terminated through enzyme-mediated hydrolysis, which is catalyzed by monoacylglycerol lipase (MGL) at the presynaptic terminal (Dinh et al, 2002; Jung et al, 2012; Katona et al, 2008; Marrs et al, 2010). Additionally, a small proportion of 2-AG is also metabolized by the α-β-hydrolase domain-6 hydrolase (ABHD) class of enzymes, specifically ABHD6 and ABHD12 (Blankman et al, 2007; Marrs et al, 2010) (Besides hydrolysis, oxidation by cyclooxygenase 2 (COX-2) is another pathway for 2-AG degradation (Kano, 2014). Since morphological data suggest that Cox-2 is expressed at postsynaptic sites this enzyme may play complementary roles with MGL in 2-AG degradation (Figure 2.1) (Kaufmann et al, 1996).

As mentioned above, the activation of the endocannabinoid system at the synapse leads to short or long-term suppression of neurotransmitter release (Castillo et al, 2012; Kano et al, 2009; Katona et al, 2012; Ohno-Shosaku et al, 2012). Although both anandamide and 2-AG act to modulate presynaptic neurotransmitter release via activation of CB receptors, it is believed that these two molecules may operate differentially in mediating short-term and long-term synaptic plasticity processes throughout the brain (Ahn et al, 2008; Katona et al, 2012). It is hypothesized that anandamide represents the “tonic” signaling agent of the endocannabinoid system that acts to regulate basal synaptic transmission, whereas 2-AG represents the “phasic” signaling agent that is mobilized during sustained neuronal depolarization (Ahn et al, 2008; Morozov et al, 2009).
Overview of Endocannabinoid and Stress

Substantial evidence implicates the endocannabinoid system is involved in the regulation of various stress-coping responses (Bortolato et al., 2006; Gaetani et al., 2003; Griebel et al., 2005; Lutz, 2009; Moreira et al., 2007b; Patel and Hillard, 2006, 2008; Rodgers et al., 2003; Steiner et al., 2008). First, cannabis consumption in humans typically results in a reduction of perceived stress, an increase in relaxation, and a dampening of feelings of anxiety (Green et al., 2003). Given that the physiological actions of cannabis are mediated by activation of the CB₁ receptor, this suggested that endocannabinoid system could also dampen or buffer against the effects of stress. Consistently, animal experiments suggest that anandamide produced in the amygdala modulates the response to stressful events (Gaetani et al., 2003). For example, exposure to an auditory fear-conditioning paradigm increases anandamide levels in the basolateral amygdala (BLA) of mice (Marsicano et al., 2002) and pharmacological inhibition of FAAH activity enhances stress-coping responses and exerts anxiolytic-like and anti-depressant-like effects in rodents through a CB₁-dependent mechanism (Hill et al., 2006; Kathuria et al., 2003). Additional studies of pharmacological or genetic disruption of the endocannabinoid signaling consistently generates a neurobehavioral phenotype that includes activation of the HPA axis, increased anxiety, suppressed feeding behavior, hypervigilance and arousal, reduced responsiveness to rewarding stimuli, enhanced grooming behavior, and impaired cognitive flexibility (Bellocchio et al., 2013; Friemel et al., 2014; Gaetani et al., 2003; Haller et al., 2004; Marsicano et al., 2002; Patel et al., 2004; Sanchis-Segura et al., 2004; Santucci et al., 1996; Shonesy et al., 2014; Tallett et al., 2007; Varvel and Lichtman, 2002). These research indicate that the
endocannabinoid system is involved in inhibiting stress, fear, and anxiety responses. Anatomically, within the stress response circuit, enzyme involved in the synthesis and the degradation of endocannabinoids and CB$_1$ receptors are prominently expressed in the amygdala, hippocampus, medial prefrontal cortex (mPFC), ventral striatum, and the hypothalamus, where they modulate overall activity (Herkenham et al., 1991; McPartland et al., 2007; Ramikie et al., 2014; Ramikie and Patel, 2012). Additionally, many studies report that stress induces changes in the mobilization of endocannabinoids in various brain structures involved in stress.

**Brain Anandamide Levels Following Acute Stress:** Studies have shown that exposure to acute stress generally causes a rapid reduction in brain anandamide content in response to an array of psychological stressors. In particular, exposure of both rats and mice to restraint stress or social defeat stress causes a reduction in the anandamide levels within the amygdala and the hippocampus immediately after the stressor (Dubreucq et al., 2012; Gray et al., 2015; Hill et al., 2009; Rademacher et al., 2008). This reduction in anandamide content in the amygdala and the hippocampus is suggested to be mediated by an acute stress-induced increase in FAAH activity which leads the rise in anandamide hydrolysis (Gray et al., 2015; Hill et al., 2009; Navarria et al., 2014). In the mPFC, the exposure to swim stress causes a robust reduction of anandamide content (McLaughlin et al., 2012), but neither acute restraint nor acute social defeat has any effect on anandamide content in the mPFC (Dubreucq et al., 2012; Gray et al., 2015; Rademacher et al., 2008).

Contrary to restraint stress and social defeat stress, studies have found that exposure to footshock elevates anandamide levels in the mPFC, amygdala, hippocampus, and
periaqueductal gray immediately after the stimulus (Hohmann et al, 2005; Morena et al, 2014). It is likely that the different nature of the stressful stimulus, or the different behavioral paradigm activate specific neuronal pathways that mobilize anandamide to modulate diverse aspects of the stress response (Hohmann et al, 2005; Morena et al, 2014). Interestingly, a recent study has demonstrated a brain-wide reduction in anadamide content 24 h following exposure to footshock (Bluett et al, 2014). This suggests that, there is also a temporal component to the endocannabinoid response as footshock initially elevate anandamide content which is followed by a delayed reduction in anandamide levels (Bluett et al, 2014).

**Brain 2-AG levels Following Acute Stress:** Unlike the effects of stress on anandamide, the majority of studies suggest that stress induces an increase in brain 2-AG signaling. Specifically, about 30-60 minutes after an acute restraint stress, moderate increases in 2-AG content is observed within the mPFC, hippocampus, and hypothalamus (Evanson et al, 2010; Hill et al, 2011; Wang et al, 2012), while footshock increases 2-AG content in the periaqueductal gray (Hohmann et al, 2005). Additionally, rats exposed to an acute predator-stress induces a prolonged increase in 2-AG content in the amygdala that lasts for at least 2 weeks (Lim et al, 2015). Contrary to the rapid changes in anandamide following stress, the increases in 2-AG show a prominent delay. Relevant to this point, a recent study has shown that acute activation of muscarinic acetylcholine receptors in the amygdala causes a short-lived form of anandamide-mediated synaptic depression, while prolonged activation (>1 h) of the same receptor causes a tonic 2-AG-mediated depression (Ramikie et al, 2014). These data indicate significant differences in the temporal dynamics of anandamide and 2-AG
mobilization following stress exposure, which may also indicate that the two are involved in different phases of stress response.

**Endocannabinoid Regulation of the Hypothalamic-Pituitary-Adrenal Axis.**

Studies have documented the existence of a link between the endocannabinoid system and the hypothalamic-pituitary-adrenal (HPA) axis. Stressful stimuli activate neural inputs to the paraventricular nucleus (PVN) of the hypothalamus, inducing the release of corticotropin-releasing hormone (CRH), which is then transported to the anterior pituitary gland and stimulates the release of adrenocorticotropic hormone (ACTH). Circulating ACTH initiates the release of CORT (CORT; glucocorticoids in humans) and epinephrine from the adrenal gland (Pecoraro et al, 2006).

Interestingly, administration of CB₁ receptor antagonist to a non-stressed rodent has shown to increase circulating levels of ACTH and CORT in a dose dependent manner (Atkinson et al, 2010; Gonzalez et al, 2004; Hill et al, 2010a; Manzanares et al, 1999; Newsom et al, 2012) Consistent with these findings, CB₁ KO mice have generally been found to exhibit increased basal HPA axis activity with the increase in CRH mRNA within the PVN, and increasing circulating levels of ACTH and CORT (Barna et al, 2004; Cota et al, 2003; Cota et al, 2007). Series of more detailed studies have uncovered that disruption of CB₁ receptors in the BLA increases HPA-axis activity elevating the plasma concentrations of CORT (Hill and Tasker, 2012). Histological data show that CB₁ receptors in the BLA are located on both glutamatergic and GABAergic terminals (Domenici et al, 2006; Katona et al, 2001; Marsicano and Lutz, 1999). However, electrophysiological work in the BLA has demonstrated that CB₁ receptor-mediated glutamate suppression overwhelms the suppression of GABA, as the net effect of CB₁
receptor activation in the BLA is a reduction of excitability of the principal neurons (Azad et al., 2003). These data suggests that, under basal conditions, there is an endocannabinoid tone that suppresses the excitatory inputs into the BLA and thus constrains the excitability of the BLA under non-stressed conditions. Disruption of this endocannabinoid tone increases BLA activity leading to the increased outflow to the PVN and results in an increase in HPA activity (Hill et al., 2012). While the BLA does not project directly to the PVN, it does regulate PVN activity through a series of indirect pathways via the central amygdala, medial amygdala, the bed nucleus of the stria terminalis, and neighboring hypothalamic nuclei (Herman et al., 2005; Ulrich-Lai and Herman, 2009).

In addition to the anandamide-mediated basal control of the HPA axis activity through the BLA, evidence has also mounted that acute stress induces a rapid loss of this anandamide signal in the BLA is leading to an acute activation of the HPA axis. Specifically, exposure to restraint stress results in a reduction in the tissue content of anandamide in the amygdala (Hill et al., 2009; Patel et al., 2005; Rademacher et al., 2008). The extent of the stress induced decline in anandamide content within the amygdala has a strong negative correlation with the magnitude of HPA axis activation (Hill et al., 2012). In other words, larger reductions in amygdala anandamide levels in response to restraint stress are associated with greater increases in plasma CORT (Blankman et al., 2007; Hill et al., 2009). Further studies have also revealed that systemic or intra-BLA reduction of anandamide-hydrolysis via inhibition of FAAH, dampens stress-induced activation of the HPA axis (Bedse et al., 2014; Patel et al., 2004; Sanchis-Segura et al., 2004). Moreover, the ability of systemic or intra-BLA administration of a
FAAH inhibitor to decrease the neuronal activation within the PVN is completely reversible by local CB₁ receptor antagonism within the BLA (Bedse et al., 2014; Hill et al., 2009). Taken together, these data indicate that elevations of anandamide signaling within the BLA are capable of attenuating stress-induced activation of the HPA axis, likely through a various indirect projections from the BLA to the PVN.

Within the PVN, CB₁ receptors are localized to glutamatergic axon terminals projecting to the CRH neurosecretory neurons, and CB₁ receptor activation inhibits glutamate release onto these neurons (Di et al., 2003; Wamsteeker et al., 2010). Therefore theoretically, endocannabinoid signaling in the PVN should also regulate the HPA axis activity. However, CB₁ receptor antagonism in PVN slices does not increase excitatory synaptic inputs to CRH neurons and local infusion of a CB₁ receptor antagonist in the PVN in vivo does not change basal HPA axis output (Di et al., 2003; Evanson et al., 2010). Interestingly under stressful conditions, local administration of a CB₁ receptor antagonist into the PVN impairs glucocorticoid mediated rapid feedback inhibition of the HPA (Evanson et al., 2010). These data suggest that while under non-stress conditions endocannabinoid within the PVN does not contribute to the regulation of basal HPA axis activity, but under stressful conditions endocannabinoid activity in the PVN can have a significant impact on regulation of HPA axis output.

Albeit restraint stress-induced reduction in BLA anandamide content leads to the activation of the HPA axis, this mechanism should be examined in other fear and stress paradigms. Additionally, further investigations are needed to examine the role of 2-AG in modulating the HPA axis activity, as such studies are extremely lacking. Moreover, since 2-AG is thought to be the phasic signaling agent, longer time-course studies
(greater than 3 hours) are essential to examine the full extent of how the endocannabinoid system controls the neuroendocrine response following stress. Nevertheless, the current body of work shows that under steady-state conditions, there is an endocannabinoid tone within the BLA that suppresses the incoming excitatory neurotransmission. Disruption of this endocannabinoid tone either through the blockade of CB₁ receptor signaling or a reduction in endocannabinoid content via exposure to stress increases the excitability of principal neurons in the BLA that activates the HPA axis and increasing glucocorticoid circulation.

**Endocannabinoid and Stress Induced Anxiety**

For centuries, medicinal preparations containing cannabis were widely used in many societies for its anxiolytic properties, and today, anxiety is one of the most common conditions in which physicians prescribe medical marijuana (Reinarman et al, 2011). Accordingly, about a decade of research has shown that endocannabinoids play a prominent role in reducing behavioral signs of anxiety specifically under stressful, aversive, or environmentally challenging conditions. Our lab was first to demonstrate that inhibition of anandamide hydrolysis by FAAH results in a reduction of anxiety-like behavior (Kathuria et al, 2003). The anxiolytic-like effect of enhanced anandamide signaling seems to be highly specific to the stressful nature of the environmental context. In other words, the inhibition of FAAH, through both pharmacological and genetic means, is most effective at reducing anxiety-like behaviors under challenging environmental conditions or after extreme stressor exposure (Bluett et al, 2014; Kathuria et al, 2003). Additional studies show that pharmacological or genetic disruption of CB₁ receptor signaling can moderately increase anxiety-like
behavior under basal conditions (Haller et al., 2002; Haller et al., 2004; Martin et al., 2002), as well as dramatically enhances anxiety induced by stress (Haller et al., 2004; Martin et al., 2002). Further studies demonstrate that central anandamide levels are negatively correlated with anxiety-like behaviors, and elevating anandamide signaling can effectively limit anxiety induced by both acute and chronic stress (Bluett et al., 2014; Campos et al., 2010; Lomazzo et al., 2015; Rossi et al., 2010). These findings suggest that the changes in endocannabinoids signaling in response to stress may contribute to fluctuations in anxiety-like behavior.

There is evidence suggesting that endocannabinoid manipulations in various brain regions and circuits involved in stress response can modulate anxiety. For example, CRH released from the PVN can trigger FAAH activity in the BLA, that results in the generation of anxiety (Gray et al., 2015), and local overexpression of FAAH within the mPFC has also been found to be sufficient to induce anxiety (Rubino et al., 2008). Finally, an additional report has found that administration of the anadamide reuptake inhibitor, AM404, into the ventral hippocampus can reverse stress-induced anxiety (Campos et al., 2010). Together, it is likely that changes in anadamide signaling within a discrete brain region may be capable of modulating anxiety.

In addition to anandamide, a role for 2-AG signaling in the regulation of anxiety has been examined using genetic and pharmacological approaches. Similar to the effects seen with FAAH inhibition, treatment with a MGL inhibitor (to inhibit 2-AG hydrolysis), seems reduce anxiety particularly under high stress testing conditions (Aliczki et al., 2012; Aliczki et al., 2013; Busquets-Garcia et al., 2011; Kinsey et al., 2011; Lim et al., 2015; Sciolino et al., 2011). As mentioned above, acute stress can increase 2-
AG mobilization in various brain regions, one interpretation of these data would be that the mobilization of 2-AG has a protective role against stress-induced anxiety and that enhancement of this signal through the inhibition of MGL has a potent anxiolytic effect. Consistent with this, genetic 2-AG deficiency results in increased anxiety that can be reversed by acute pharmacological normalization of 2-AG levels (Shonesy et al, 2014). These studies clearly support the notion that 2-AG also has an important role in modulating anxiety and stress response. Collectively, these data indicate that elevating both anandamide and 2-AG signaling alleviates stress-induced anxiety. Future work will require combined genetic and neuroanatomical approaches to clearly define the circuits by which endocannabinoid signaling regulates stress-induced anxiety.

**The Role of Endocannabinoids in Stress Learning and Memory**

Anecdotally, it has long been recognized that $\Delta^9$-THC intake can cause memory impairment in humans. In laboratory animals, effects of exogenously applied cannabinoid agonists on learning and memory have been intensively investigated using various behavioral paradigms (Davies et al, 2002; Lichtman et al, 2002). These studies have revealed that in cannabinoid-treated animals, certain aspects of memory are impaired, while other aspects are largely intact or even improved. In general, cannabinoids affect the acquisition and learning of new memory, while memory retrieval is resistant to the effects cannabinoids.

The Morris water maze is one of the most widely used hippocampus-dependent spatial learning task. In this task, the rodents are required to navigate in a water pool to locate and learn the position of a hidden platform using its visual cues (Vorhees and Williams, 2006). Generally the hidden platform remains in a fixed location between trials.
but when testing for working memory, the location of the platform is changed before each session (Vorhees et al, 2006). Systemic administration of cannabinoid agonists impairs the learning in both the fixed hidden platform task and in the working memory version, with the latter being more sensitive (Ferrari et al, 1999; Varvel et al, 2001). Effects of CB1 blockade on spatial memory have been examined by using genetic and pharmacological tools. While the administration of CB1 antagonist rimonabant had no effect in the performance in the fixed hidden platform task in CB1-knockout mice and in the wildtype mice, CB1-knockout mice showed memory impairment when the platform was moved to a new location after the mice had acquired the task (Varvel et al, 2005; Varvel et al, 2002). Generally, when the platform is moved in the Morris water maze task, the wild-type mice gradually ceased returning to the previous platform location and learns the new location (Vorhees et al, 2006). In contrast, the CB1-knockout mice continued to return to the previous location and exhibited a significant deficit in learning the new location, suggesting the impairment of extinction process (Varvel et al, 2005).

Collectively, these data suggest that the endocannabinoid system is involved in the acquisition and the extinction of spatial memory but not recall.

The Role of Endocannabinoids in Aversive Memory

In life people go through many situations that potentially impose a physical or psychological threat. The subjective experience of these threats is frequently called stress. The strength of our memories often reflect the degree of stress and emotional arousal perceived from these threats (McGaugh, 2015). Stress, norepinephrine, and glucocorticoids typically facilitate learning and enhance memory consolidation (Akirav et al, 2004; Joels and Baram, 2009; McGaugh, 2004a; Salehi et al, 2010). Particularly, the
BLA appears to have a crucial role in mediating the stress hormone dependent enhancement of memory consolidation (McGaugh, 2004a; Roozendaal and McGaugh, 1997). In addition, it seems that the endocannabinoid signaling in the amygdala is important for the facilitation of consolidation that occurs in highly arousing situations. For example in inhibitory avoidance learning, post training intra-BLA Infusions of the CB$_1$ agonist, WIN55,212-2, induces enhancement of inhibitory avoidance retention, while post training intra-BLA infusions of the CB$_1$ antagonist, AM251, induces memory impairment (Campolongo et al, 2009). Additionally, infusion of the AM251 into the BLA blocks the memory-enhancing effect of WIN55,212-2 (Campolongo et al, 2009). Consistent to this result, another group reported that blockade of CB$_1$ receptor activity in the BLA prevents acquisition fear memory with a supra-threshold footshock intensity in a fear conditioning paradigm while, intra-BLA administration of AM404 or WIN55,212-2 strongly potentiated fear memory acquisition under sub-threshold footshock intensity condition (Tan et al, 2011). Supporting these findings, others have reported that infusion of AM251 into the amygdala disrupts the consolidation of long-term memory, possibly by blocking long-term potentiation (Bucherelli et al, 2006; de Oliveira Alvares et al, 2006). Taken together, these data indicate that endocannabinoid signaling in the amygdala is importantly in regulating the memory consolidation that occurs after aversive situations.

Interestingly, rats trained in an inhibitory avoidance task with a higher footshock intensity present better memory retention than rats trained with a lower footshock intensity, and this effect is paralleled by an increase in anandamide levels within the amygdala, hippocampus, and mPFC (Morena et al, 2014b). In a separate study, intra-
hippocampal infusion of AM251 impaired the consolidation conditioning training with a high intensity shock, but it had no effect on procedures with lower intensity shock (de Oliveira Alvares et al, 2010). Although more evidence is needed, these results suggest that the level of stress elicited by the behavioral task results in differential engagement of endocannabinoids to modulate fear memory consolidation. In other words, similar to the relationship between endocannabinoids and anxiety, the endocannabinoid influence on memory seem to depend on the degree of stress and aversion experienced by the organism.

Fear conditioning is widely used to study aversive memory in laboratory animals. In fear conditioning, a conditioned stimulus (CS), is paired with an aversive, unconditioned stimulus (US) (Maren, 2001; Singewald et al, 2015). After conditioning, the animal shows fear response such as freezing when re-exposed to the CS, indicating acquisition of aversive memory (Maren, 2001). The fear response is extinguished gradually when the conditioned stimulus is applied repeatedly without the US (Maren, 2001). Systemic administration of the cannabinoid agonist WIN55,212-2 impaired the acquisition of contextual, but not auditory fear conditioning in rats (Pamplona and Takahashi, 2006). While auditory fear conditioning requires the basolateral amygdala, contextual fear conditioning is known to depend on the hippocampus (Anagnostaras et al, 2001). Therefore, WIN55,212-2 selectively affects acquisition of the hippocampus-dependent aversive memory. Additionally, administration of WIN55,212-2 right before extinction trials facilitated the extinction of fear conditioning (Sutherland et al, 1998). Consistent with this finding, studies with pharmacological or genetic disruption of CB1 have repeatedly demonstrated impaired extinction of aversive memory.
In auditory fear conditioning paradigm, tone presentation during extinction trials results in elevated levels of endocannabinoids in the BLA (Gunduz-Cinar et al., 2013; Marsicano et al., 2002), and genetic deletion of CB₁ receptors, as well as systemic or intra-BLA blockade of endocannabinoid signaling, robustly inhibits fear extinction, with normal acquisition of the fear memory (Akirav et al., 2004; Chhatwal et al., 2005; Ganon-Elazar and Akirav, 2009; Marsicano et al., 2002; Suzuki et al., 2004). Additional studies show that intraperitoneal or intracerebroventricular injection of AM404 enhances fear extinction through a CB₁-dependent mechanism (Bitencourt et al., 2008; Chhatwal et al., 2005; Pamplona et al., 2008). A more recent study also found that intra-BLA infusions of the FAAH inhibitor, AM3506, enhances memory extinction through a CB₁-dependent manner (Gunduz-Cinar et al., 2013). Similarly, the CB₁ antagonist, rimonabant, impaired the extinction in wild-type mice when injected subcutaneously just before the first extinction trial, whereas rimonabant had no effect on the acquisition as well as extinction when applied before auditory fear conditioning (Suzuki et al., 2004). These result indicates that CB₁ receptors are required at the moment of aversive-memory extinction. The extinction of contextual fear conditioning in mice was also suppressed by systemic administration of rimonabant (Suzuki et al., 2004). Moreover, in fear conditioning with light, systemic administration of rimonabant just before extinction trials impaired the extinction (Chhatwal et al., 2005).

The BLA is known to be involved in extinction of conditioned fear (McGaugh, 2004a). Therefore, it has been hypothesized that the endocannabinoid signaling in the BLA is essential in the extinction of aversive memories (Kano et al., 2009). Several lines of evidence support this hypothesis. First, the CB₁ receptor is highly expressed in the
BLA (Chhatwal et al., 2005; Katona et al., 2001; Ramikie et al., 2014). Second, a CS during extinction trials elevates levels of endocannabinoids in the BLA (Marsicano et al., 2002). Third, endocannabinoid-mediated synaptic plasticity can be induced by neural activity in the basolateral amygdala (Marsicano et al., 2002). However, it is still unclear how the endocannabinoids and the CB$_1$ receptors mediate the extinction. From a clinical stand point, these studies suggest that drugs activating the endocannabinoid system may be useful for the treatments of psychiatric disorders associated with retrieval of fear memories, including panic disorders, phobias, and posttraumatic stress disorder (PTSD).

**Conclusions**

Collectively, this chapter briefly reviews the rapidly growing field of research investigating the neurobiological interactions between stress and the endocannabinoid system. To summarize, exposure to stress appears to generally result in a changes in the brain content of the two major endocannabinoid ligands anandamide and 2-AG, with stress-induced reduction in anandamide and 2-AG being increased from stress. The endocannabinoids have a district temporal nature such that the reduction of anandamide appears to occur relatively quickly in response to stress, while the increase in 2-AG is delayed. This significant differences in the temporal dynamics between brain anandamide and 2-AG content changes following stress exposure suggest that the two are involved in different phases of stress response. In the acute response to stress, this decline in anandamide signaling seems to contribute to the manifestation of an anxiety state, the activation of the HPA axis, and the impairment in fear extinction. As such, inhibition of FAAH is able to reverse many of the effects of acute stress. With respect to
acute stress, the delayed increase in 2-AG seem to buffer and constrain the effects of stress on the brain and facilitate termination of the stress response. Ongoing research is seeking to determine the mechanism of this 2-AG increase it is thought that a reduction in MGL expression at the presynaptic membrane appears to be one potential factor resulting in enhanced 2-AG signaling capacity after stress (Sumislawski et al, 2011). Overall, both anandamide and 2-AG providing a stress inhibitory and anxiolytic effect. However, anandamide signaling seems to be relevant for the initiation and expression of the effects of stress, while 2-AG is relevant for moderating and terminating the stress response.

For future studies, it will be important to identify the mechanisms by which these changes in endocannabinoid signaling occur, the circuit-specificity of these effects, and their impact on synaptic transmission. The endocannabinoid system plays an important role in the modulation of fear, stress, and anxiety. Dysfunctions in this system could lead to maladaptive stress response leading to an acute or chronic psychiatric disorders. Future work, both basic and clinical, should further investigate whether impairments in the endocannabinoid system is a potential threat to developing long-term neuropsychiatric pathologies, such as panic disorders, general anxiety disorders, phobias, depression and PTSD. Indeed based on these studies, there has been a significant interest in therapeutics development around endocannabinoid augmenting agents for stress-related neuropsychiatric disorders.

In conclusion, the endocannabinoid system is a vital component of the neural regulation of the stress response. This is consistent with the fact that humans have been consuming cannabis for centuries, largely because of its stress-reducing qualities.
(Reinarman et al., 2011). As it is becoming increasingly appreciated that endocannabinoid signaling likely a mediator of homeostasis, both at the synaptic level and at the systems level of neural activity, it will be important to understand whether perturbation of this system could lead to changes in stress sensitivity and anxiety. Further research using pharmacology, genetics, and other neuroscientific techniques will hopefully provide us with a more profound insight into the importance of endocannabinoid signaling in stress regulation to help us deal with the rapidly growing burden of stress and anxiety in our society.
Figure 2.1: The structure of endocannabinoid ligands.
Figure 2.2: The endocannabinoid system. Postsynaptic depolarization causes increased intracellular Ca\(^{2+}\) levels through activation of AMPA, NMDA receptors and/or Gq-coupled receptors (e.g., mGluR1/5) and voltage-gated Ca\(^{2+}\) channels. Elevation of intracellular Ca\(^{2+}\) increases endocannabinoid biosynthesis, though there is evidence for Ca\(^{2+}\)-independent pathway of endocannabinoid synthesis as well. This cartoon illustrates the two primary biosynthetic pathways for anandamide (AEA) and 2-arachidonoyl glycerol (2-AG). AEA is synthesized from phospholipid precursors such as...
phosphatidylethanolamine (PE) by a Ca\textsuperscript{2+}-dependent transacylase, N-acyltransferase (NAT), yielding N-arachidonoyl phosphatidylethanolamine (NAPE). NAPE is hydrolyzed by a NAPE-phospholipase D (NAPE-PLD) to output AEA. For the synthesis of 2-AG, Ca\textsuperscript{2+} influx and/or the activation of Gq-coupled receptors stimulate phospholipase C (PLC), which hydrolyses phosphatidylinositol (PI) into diacylglycerol (DAG). DAG is converted to 2-AG by diacylglycerol lipase-α (DGL-α). Through an unknown mechanism, AEA and 2-AG then migrate in retrograde from postsynaptic neurons to presynaptic cannabinoid type 1 (CB\textsubscript{1}) receptors. Once activated, CB\textsubscript{1} receptors couple through Gi/o proteins to inhibit adenylyl cyclase and suppress neurotransmitter release. Endocannabinoid signaling is then terminated by degrading enzymes. AEA is mainly hydrolyzed to arachidonic acid (AA) and ethanolamine (EA) by fatty acid amide hydrolase (FAAH), located postsynaptically. 2-AG is hydrolyzed presynaptically to AA and glycerol (Glyc) by monoacylglycerol lipase (MGL), which accounts for ~85% of 2-AG hydrolysis, and postsynaptically by alpha-beta-hydrolase 6/12 (ABHD6/12), which accounts for the remainder of 2-AG hydrolysis. AEA and 2-AG are also oxidized by cyclo-oxygenase 2 (COX-2) to form prostaglandin-ethanolamides (PG-EAs) and prostaglandin-glycerols (PG-Gs).
CHAPTER 3

2,5-Dihydro-2,4,5-Trimethylthiazoline as a Fear Stimulus.

Introduction

In threatening situations, anxiety is a normal and adaptive reaction. It allows the assessment of the potential risk of the situation and executes the appropriate autonomic and defensive behavioral responses. However when anxiety is persistent and excessive or triggered in inappropriate environments, it becomes a significant mental health issue. Understanding how environmental stimuli influences and motivate behavior is one of the fundamental questions in behavioral neuroscience and an enormous amount of studies were conducted regarding to stress, anxiety, and fear. Rodent models are the most well studied and intensively used for investigating defensive behaviors produced by dangerous, threatening, and fearful stimuli (Davis et al, 2010; LeDoux, 2012). This has primarily been studied using associative fear conditioning, and this paradigm has been extremely informative for understanding how threat is processed across different levels in the brain.

Although the ability to adapt, anticipate, and learn during situations of threat and danger is critical for survival, another way to increase survival in the face of danger is to have hardwired systems that does not rely on associational learning. In other words, a system that triggers a defensive behavior to threatening stimuli without any prior learning. Many animals, including humans, appear to have species specific innate sensitivity to various stimuli. Rodents have a natural propensity to fear predator and their odors and have therefore been used to study innate threat or fear in the laboratory
(Blanchard et al, 2013; Brennan and Keverne, 2015; Dielenberg and McGregor, 2001; Masini et al, 2006; Rosen, 2004). The inborn fear to predators is evident by the fact that the stimuli produce robust defensive behaviors upon the first exposure in laboratory rodent strains that have never encountered these predators for generations.

Although using live predators are more natural and provide a robust multimodal threat stimuli, the use of predator odors have allowed for the precise examination of defensive behaviors produced by stimulating a single sensory modality. Predator odors reliably indicate danger for rodent and innately initiates a number of different defensive behaviors (Apfelbach et al, 2005; Dielenberg et al, 2001; Kavaliers and Choleris, 2001; Masini et al, 2006; Takahashi et al, 2005), and an increasing numbers of studies are being carried out with these odors in neuroscience laboratories. Specifically, a single molecule kairomones isolated from fox feces, 2,5-dihydro-2,4,5-trimethylthiazoline (TMT; Figure 3.1) has been extensively studied and generated the most progress toward understanding the neuroanatomical circuits necessary for producing predator odor-induced defensive behaviors (Brennan et al, 2015; Fendt and Endres, 2008; Fendt et al, 2005; Rosen, 2004; Takahashi, 2014).

Because TMT is a single molecule which can be synthesized, the use of TMT has some advantages. For example, odor concentration can be exactly controlled. In addition, TMT as a synthesized molecule has constant properties whereas natural predator odors like cat odor are inconstant in their composition depending on individual animals and other variables (Berton et al, 1998). The current chapter will mainly focus on fear-inducing qualities of TMT, the neural systems and circuitry for innate fear, and defensive behaviors elicited by TMT. Overall, based on the assumption that anxiety
disorders are the result of inappropriate or exaggerated activation of normal anxiety responses (Nutt, 2005), studying predator odor-induced anxiety may help us better understand human anxiety disorders such as post-traumatic stress disorder (PTSD).

**TMT as an Unconditioned Fear Stimulus in Rodents**

TMT is a compound that was originally isolated from fox feces in the 1980s (Vernet-Maury et al, 1984). Of a number of compounds isolated from fox feces, TMT exhibited the robust effect on avoidance, and therefore was marketed as a natural rodent repellent (Vernet-Maury et al, 1984). TMT is a highly volatile, water insoluble molecule. The TMT odor is thought to be an unconditioned hostile stimulus because naive laboratory bred rats and mice display fear-like and anxiety-like responses on their first exposure to TMT (Holman et al, 2014; Lim et al, 2015; Wallace and Rosen, 2001). Examining freezing as a fear response, TMT elicits level of freezing similar to footshock-induced conditioned freezing (Wallace et al, 2001). Additionally, rats exposed to higher concentration of TMT exhibit higher level of freezing, demonstrating a dose-response relationship between TMT and the amount of freezing or unconditioned fear (Endres et al, 2005; Wallace et al, 2001). Moreover, repeated exposure to TMT lacks habituation and sensitization in freezing behavior in rats (Blanchard et al, 2003; McGregor et al, 2002; Wallace et al, 2001).

**Fear Inducing Property of TMT is Specie Specific**

Although a number of laboratories reported robust fear behavior during exposure to TMT (Fendt et al, 2005), some laboratories did not observe fear behavior to TMT (McGregor et al, 2002; Morrow et al, 2000). Such observations are not unique to TMT
and are also observed with other predator odors. While many laboratories show robust fear response in rodents after cat exposure, some laboratories reported lack of fear behavior in response to cat odor (Day et al., 2004). This controversy can be explained by a high inter-individual variability in the fear response to cat odor (Hogg and File, 1994), or other factors like sex and strain of the experimental animal, source of the odor, diet of the odor donor as well as the test environment (Berton et al., 1998; Fendt, 2006; Masini et al., 2005; McGregor et al., 2002; Perrot-Sinal et al., 2004; Staples and McGregor, 2006). Each of these factors is has shown to affect type and the intensity of the defensive behavior induced by cat odor. So far, very similar observations were made with TMT. For example, some rat strains are less sensitive to TMT than others (Rosen et al., 2006; Staples et al., 2006). TMT induced a significant increase in freezing in Sprague-Dawley and Long–Evans rats but not in Wistar rats (Fendt et al., 2008). The reasons for this different sensitivity for TMT is currently unknown. As with other fear-inducing stimuli, the test environment, the test protocol, and the way that the odor is presented strongly modulate fear behavior during TMT exposure. For example, TMT was effective in a large, illuminated open field but not in a small and dimly lit open field (Morrow et al., 2002). In parallel, conditioning to TMT was only observed with a specific testing environment (Fendt et al., 2008; Rosen et al., 2015).

**Efficacy of TMT in Inducing Fear**

TMT is the most widely studied single molecule predator odor because it produces robust levels of fear induced freezing behavior under the right conditions (Hagenaars et al., 2014; Roelofs et al., 2010). However, for several years, scientists have argued whether TMT is rather a noxious than a fear-inducing stimulus. In general, when
animals are exposed to a live predator, the animals show long-lasting generalized anxiety and express more defensive behavior for hours, even days, after exposure in animal models of anxiety such as the elevated plus maze (EPM), the light/dark box, or the acoustic startle response (Adamec et al, 2006; Korte and De Boer, 2003). Therefore, the question was whether such generalized anxiety can also be observed after exposure to TMT (Dielenberg et al, 2001). Many studies have emerged to address whether TMT is indeed a fear-inducing stimulus.

When the fear-inducing properties of TMT is compared with butyric acid, an aversive and acrid odor that induces avoidance behavior, only TMT is able to induce freezing behavior (Fendt et al, 2008). As freezing is a strong indicator for fear in rodents, this result suggested that unconditioned freezing to TMT odor is due to fear inducing properties instead of its unpleasant and pungent qualities that elicit avoidance (Ayers et al, 2013; Fendt et al, 2008). Importantly, it should also be noted that even at extreme doses of butyric acid, the odor is not able to induce freezing in rats (Wallace et al, 2001). Furthermore in our laboratory, we challenged a group of rats to either TMT or butyric acid and demonstrated that only TMT exposure rats exhibited a statistically detectable increase in anxiety-like behavior on the EPM (Holman et al, 2014; Lim et al, 2015). Taken together, these studies clearly indicate that the fear behavior which is induced by TMT exposure is not simply due to its aversive, noxious or repugnant properties

**Neuroanatomy of Predator Odor Induced Fear**

Studies investigating the neuroanatomy of predator odors have also made significant progress over the years. As one would expect, the initial perception of TMT
odor starts at the olfactory bulb. It has been identified that a neurons in the glomeruli with the olfactory receptor genes called dorsal domain class II receptors (DII) are necessary for innate avoidance of TMT (Kobayakawa et al, 2007). This landmark study demonstrated that mutant mice lacking neurons with DII receptors showed a lack of fear (freezing) and anxiety-like responses to TMT (Kobayakawa et al, 2007). A separate study found that transection of the axons of the Grueneberg ganglion cells, group of neurons in the nasal epithelium that are sensitive to temperature and pheromones, blocks TMT-induced freezing (Brechbuhl et al, 2013). In addition to the Grueneberg ganglions, olfactory sensory neurons (OSN) in zone II of the nasal epithelium are also sensitive to TMT (Rosen et al, 2015). Interestingly, both the TMT-responsive Grueneberg ganglion cells and OSN in zone II projects from the nasal epithelium and converges onto the DII domain cells in glomeruli (Brechbuhl et al, 2013; Kobayakawa et al, 2007; Matsumoto et al, 2010). Together these studies suggest that TMT’s fear inducing qualities are transmitted to the brain via the olfactory system to drive freezing and anxiety-like responses (Fendt et al, 2008; Galliot et al, 2012; McGregor et al, 2002).

Further studies traced the TMT-responsive DII neurons from the glomeruli synapse onto mitral and tufted cells that project to the principal neurons in the amygdala (Miyamichi et al, 2011; Sosulski et al, 2011). These studies speculated that the trans-synaptic projection from the olfactory circuits to the amygdala are responsible for the generation of innate behavior to olfactory stimuli. Functional studies using optogenetics demonstrated that TMT-induced innate avoidance and freezing are reduced by inhibition of mitral cells projections from the DII domain of the olfactory bulb to the amygdala (Root et al, 2014). Together, these studies identify an olfactory-amygdalar
circuit for innate fear behavior to TMT. This circuit initiates fear behavior from Grueneberg ganglion cells or OSN in zone II in the nasal epithelium, which synapse onto the amygdala projecting mitral cells in the DII domain of the glomeruli.

Conclusions

The current chapter discusses whether TMT, a component of fox odor, really represents a predator odor and induces fear in rodents. First, the fear inducing property of TMT is very specific to species of rodents and the testing environment. In addition, we presented several studies demonstrating that the fear behavior which is induced by TMT exposure is not simply caused by aversive, noxious or repugnant properties of TMT. Anatomical studies with TMT uncovered the functional neural pathways responsible for perception of predator odor threat and the generation of defensive behavior. Finally, our lab demonstrated that TMT exposed rats display marked anxiety-like behavior on the EPM test which lasts for at least 14 days after the stress has occurred (Lim et al., 2015). Further examination, of the innate properties of TMT-induced behavior and the fear learning supported by TMT and other predator odors will be important for understanding innate and acquired fears (Rosen et al., 2008). These studies may give us valuable insights into analogues human conditions such as general anxiety disorder, phobias, and PTSD.
Figure 3.1: The structure of 2,5-dihydro-2,4,5-trimethylthiazoline (TMT).
CHAPTER 4

Endocannabinoid Modulation of Predator Stress-Induced Long-Term Anxiety in Rats.

Introduction

Traumatic life events heighten the risk of developing neuropsychiatric pathologies such as post-traumatic stress disorder (PTSD), substance abuse and depression (Adshead, 2007). In addition to being confronted with the intrusive re-experiencing of trauma-related memories, subjects with PTSD exhibit persistent anxiety states that greatly impair their quality of life (Brewin et al, 2000; Gmitrowicz et al, 1994; Kessler et al, 2005b; Speckens et al, 2006). Our understanding of the neurobiology of anxiety provides a theoretical framework for the use of pharmacotherapy in the secondary prevention of chronic anxiety after life-threatening events (Davidson, 2004, 2006; Pitman et al, 2005). Evidence suggests that pharmacological agents such as adrenergic antagonists, opiates, D-cycloserine and anxiolytics are at least partially effective in managing the symptoms of PTSD (Ducrocq et al, 2005; Zatzick et al, 2003; Zatzick et al, 2006; Zhang et al, 2007). However, few studies have thoroughly examined the mechanisms of action and efficacy of psychotherapeutic drugs to prevent persistent anxiety states that develop in the aftermath of major trauma (Adshead, 2007; Davidson, 2004, 2006; Giuffrida et al, 2004; Pitman et al, 2005).

The endocannabinoid system is a signaling complex that consists of G-protein-coupled receptors, cannabinoid receptor 1 (CB₁) and cannabinoid receptor 2 (CB₂), endogenous lipid-based ligands (mainly anandamide and 2-arachidonoyl-sn-glycerol, 2-

Multiple studies have documented the existence of a link between the endocannabinoid system and the hypothalamic-pituitary-adrenal (HPA) axis. Stressful stimuli activate neural inputs to the paraventricular nucleus (PVN) of the hypothalamus, inducing the release of corticotropin-releasing hormone (CRH), which is then transported to the anterior pituitary gland and stimulates the release of adrenocorticotropic hormone (ACTH). Circulating ACTH initiates the release of cortisol (corticosterone in rodents) from the adrenal cortex (Pecoraro et al, 2006). Interestingly, disruption of CB1 receptors in the BLA increases HPA-axis activity in non-stressed rats,
elevating the plasma concentrations of corticosterone (Hill et al, 2010b). Accordingly, local administration of an FAAH inhibitor in the BLA suppresses stress-induced activation of the HPA-axis in a CB₁-dependent manner (Hill et al, 2009). In addition, a human positron emission tomography study on PTSD patients revealed a significant up-regulation of CB₁ receptors within the amygdala-hippocampal-corticostriatal neural circuit, associated with abnormally low levels of circulating anandamide (Neumeister et al, 2013). These data suggest that traumatic events can activate endocannabinoid signaling in the brain, which may act in turn as an intrinsic modulator of the response to such events. Indeed, FAAH inhibitors are currently under investigation as potential treatment for anxiety disorders and secondary prevention for PTSD (Finn, 2010). In addition to anandamide, changes in 2-AG have also been implicated in the regulation of stress-induced responses (Evanson et al, 2010; Hill et al, 2011; Sciolino et al, 2011; Sutt et al, 2008). However, a systematic investigation of the long-term impact of trauma-induced stress on 2-AG-mediated signaling is still lacking.

In the current study, we show that an acute life-threatening stress in rats, i.e. exposure to the red fox pheromone 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), selectively heightens 2-AG-mediated signaling in the amygdala, but not in the prefrontal cortex, hippocampus, striatum, hypothalamus and cerebellum. We further show that systemic or intra-amygdalar inhibition of monoacylglycerol lipase (MGL) activity suppresses anxiety-like behavior triggered by exposure to TMT, an effect prevented by CB₁ receptor blockade. The results suggest that pharmacological strategies aimed at enhancing 2-AG signaling at CB₁ receptors may offer a novel therapeutic approach to
the treatment of pathological sequelae of psychological trauma, such as PTSD and substance abuse.

Materials and Methods

Animals

We purchased male Sprague-Dawley rats (8-9 weeks, 225-250 g) from Charles River (Wilmington, MA) and housed them in groups of 3 per cage. We also used an in-house bred colony of C57BL6J mice (25-30 g). The animals were maintained on a 12 h light/dark cycle (6:30 AM to 6:30 PM) and received food (2020X, Harlan, Madison, WI) and water ad libitum. Animals undergoing surgery were individually housed and acclimated to laboratory conditions for one week prior to surgery. All procedures met the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Chemicals

We purchased TMT from Contech Enterprises (Delta, B.C., Canada) and butyric acid from Aldrich (St. Louis, MO). JZL184 was either purchased from Cayman Chemical (Ann Arbor, MI) or provided by the National Institute on Drug Abuse (NIDA). Rimonabant was supplied by NIDA and NF1819 was synthesized in the laboratory of G. Campiani.
Drug administration

We dissolved rimonabant in a vehicle of propylene glycol/Tween-80/sterile saline (0.9%) (1/1/18, vol/vol) and JZL184 in polyethylene glycol-400/Tween-80 (4/1). NF1819 was dissolved in 100% DMSO for intraperitoneal (i.p.) injections and dimethyl sulfoxide (DMSO)/sterile saline (1/39 v/v) for intracerebral infusions. We administered drugs and their vehicle by i.p. injection in a volume of 1 mL/kg.

Surgery

We anesthetized the rats with ketamine (100 mg/kg, i.p., Phoenix Manufacturing Inc., Phoenix, AZ) and xylazine (10 mg/kg; Western Medical Supply, Arcadia, CA), given buprenorphine (0.05 mg/kg, i.p., Reckitt Benckiser, Slough, Berkshire, UK) to control pain and placed them in a stereotaxic frame (Kopf Instruments, Tujunga, CA) with the nose bar maintained at -3.3 mm relative to the interaural line. In some experiments, we implanted two stainless-steel guide cannulae (7.5 mm; 23 gauge) bilaterally with the tips aimed at the BLA (2.8 mm posterior and 5.0 mm lateral to bregma and 6.2 mm from the skull surface). In other experiments, we placed a single-guide cannula (7.5 mm; 23 gauge) with the tip aimed at the third ventricle (2.3 mm posterior to bregma and 6.5 mm from the skull surface). The cannulae were affixed to the skull with three anchoring screws and dental cement. Dummy cannulae (7.5 mm) were used to maintain patency. The coordinates were histologically verified by taking the flash frozen brain and mounting 20 μm sections on slides and performing a cresyl violet stain (Sigma-Aldrich, St. Louis, MO). Once the animal had recovered from anesthesia, a second dose of buprenorphine (0.02 mg/kg, i.p.) was administered. After surgery, the rats were individually housed and allowed to recover for 8 days before
training and handling. The topical antibiotic, neosporin, was applied daily to prevent infections.

**Intracerebral infusion**

We dissolved NF1819 in a vehicle of dimethyl sulfoxide (DMSO)/sterile saline (1/39 v/v). We infused individual doses of the drug into the BLA (5 ng, 0.2 µL) or the third ventricle (75 ng, 3 µL) using a 10-µL Hamilton syringe and a 30-gauge injector (Plastics One, Chicago, IL) connected to the cannula via polyethylene tubing. The infusion needle extended 2 mm beyond the end of the guide cannula. An automated pump PHD 2000 (Harvard, Holliston, MA) drove the syringe at the rate of 0.1 µL/min. The infusion needles were left in place for an additional 30 s to allow the solution to diffuse. Immediately following the procedure, the animals were returned to their home cages.

**Brain tissue preparation**

We excised brain regions of interest by taking micropunches from frozen brains mounted on a Microm HM525 cryostat (Thermo Fisher Scientific, Waltham, MA). The following brain regions were selected (coordinates, in mm from bregma)(Paxinos and Watson, 1998): hypothalamus, single 2 mm×4 mm punch (from −0.8 to −4.8); amygdala, bilateral 2 mm×2 mm punch (from −1.6 mm to −3.6); dorsal hippocampus, bilateral 2 mm×2 mm punch (from −1.6 mm to −3.6); ventral hippocampus, bilateral 1.5 mm×2 mm punch (from −4.16 mm to −6.16); cerebellum, single 2 mm×2 mm punch (from −10.3 mm to −11.3 mm). The punches were transferred to 8-mL glass vials on dry ice and kept frozen at −80 °C until time of processing.
**Lipid analyses**

We homogenized brain tissue samples (7-15 mg) in methanol (1 mL) containing deuterium-labeled 2-AG (0.5 nmol) and anandamide (10 pmol) as internal standards (Cayman, Ann Arbor, MI). We extracted lipids with chloroform (2 mL) and water (1 mL). The organic phases were dried under N₂, reconstituted in chloroform (2 mL) and fractionated by open-bed silica gel column chromatography as described (Fu et al., 2007). The eluted fractions containing 2-AG and anandamide were dried under N₂, and residues were suspended in chloroform/methanol (1/3, vol/vol; 60 μL). Analyses were conducted using a liquid chromatography (LC) apparatus consisting of an Agilent 1100 system and 1946D mass spectrometer (MS) detector equipped with electrospray ionization interface (Agilent Technologies, Santa Clara, CA, USA). Anandamide and 2-AG were separated on a ZORBAX Eclipse XDB-C18 column (2.1 x100 mm, 1.8 μm, Agilent Technologies) using an acetonitrile gradient. Solvent A consisted of water containing 0.1% formic acid, and Solvent B consisted of acetonitrile containing 0.1 % formic acid. We used the following gradient profile: 0-15 min, 65% B; 15-16 min, 65-100% B linear gradient; 16-26 min, 100% B; 26-28 min, 100-65% B linear gradient; 28-30 min, 65% B. The column temperature was kept at 15°C and the flow rate at 0.3 mL/min. ESI was in the positive ionization mode, capillary voltage was set at 3 kV, and the fragmentor voltage was set at 70 V. Nitrogen gas was used as a drying gas at a flow rate of 13 liters/min and a temperature of 350°C. The nebulizer pressure was set at 40 psi. Absolute amounts of 2-AG and anandamide were quantified using a calibration curve.
**MGL activity assay**

We homogenized frozen brain samples in 10 volumes of ice-cold Tris-HCl (50 mM, pH 7.5) containing sucrose (0.32 M), centrifuged the homogenates at 1,000×g for 10 min, and incubated the supernatants (0.1 mg of protein) at 37°C for 30 min in Tris-HCl (0.5 mL) containing heptadecenoylglycerol (10 μM; NuCheck Prep, Elysian, MN) as substrate. Reactions were stopped by adding 1.5 mL of chloroform-methanol (2/1) containing heptadecanoic acid (NuCheck Prep) as internal standard. After centrifugation at 1,000×g at 4°C for 10 min, we collected the organic layers, dried them under N₂, suspended the residues in chloroform/methanol (1/3, 60 μL) and analyzed them by LC/MS (see above).

**Reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative PCR**

We extracted RNA from brain punches using a TRIzol (Invitrogen)/ RNeasy (QIAGEN) hybrid protocol. First-strand complementary DNAs were synthesized from 1 μg of the total RNA using SuperScript II RNase H reverse transcriptase (Invitrogen) and oligo-dT12–18 primers, for 50 min at 42°C. Quantitative PCR was conducted using Mx3000P system (Stratagene) by a TaqMan method. All mRNA levels were normalized using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as an internal standard. The primer/probe set were as follows: for rat c-fos forward, 5’-TTCCGGCATCATCTAGGCC-3’; reverse, 5’-ACAGGTCCACATCTGGCACA-3’; TaqMan probe, 5’-AGTGGCTCGAGACTGCCCCG-3’; for rat CB₁ receptor forward, 5’-CAAGGCACGCAACAACACAG-3’; reverse, 5’-TCTTAACG GTGCTCTTGATGCA-3’; TaqMan probe, 5’-TGCACAGGGGCCGC GGAGAG-3’. for rat diacylglycerol lipase-α (DGL-α) forward, 5’-CCAGGCCTTTTGCGGCG-3’; reverse, 5’-
GCCTACCACAATCAGGCCAT-3’; TaqMan probe, 5’-
ACCTGGGCGGTGAACCAAACA-3’; for rat MGL forward, 5’-
CCCAGTGACACCCAAG-3’; reverse, 5’-TAACGACAGTGGTCCC-3’; TaqMan
probe, 5’-CCCTCATCTCTGTTGCCCCAGGC-3’.
We used TaqMan gene expression
assays for rat GAPDH (Rn01775763_g1) (Life Technologies, Carlsbad, CA).

**Odor Exposure**

For 5 consecutive days, we handled each rat for 2 min and placed it for 10 min in
a plastic exposure box (45 x 25 x 20 cm) containing a square of gauze (5 x 5 cm)
doused with saline (35 μL). The box was housed in a fume hood. On the day of the
experiment, we randomly assigned the animals to the saline or TMT group. The rats
were placed for 10 min in the exposure box containing a gauze doused either with
saline, TMT (4.1 M, 35 μL), or butyric acid (10.3 M, 35 μL). After the procedure, the
animals were immediately returned to their home cages.

**Elevated plus maze**

We conducted the behavioral tests between 8:00 AM and 2:00 PM. The elevated
plus maze (EPM) apparatus included two open (50×10 cm) and two closed (50×10×40
cm) arms extending from a central platform (10 ×10 cm) elevated 60 cm above the floor.
We placed each rat in the central platform of the maze, facing an open arm opposite to
the experimenter, and videotaped test sessions of 5-min duration for each trial.
Observers blinded to treatment measured the amount of time spent in the open arm, the
number of open-arm entries, and the anxiety index. The latter was calculated as 1-
(average of percent time in open arm and percent open arm entry). Between tests the
apparatus was cleaned with a 20% ethanol solution and was allowed to dry thoroughly. The open arms of the maze were illuminated at 150-170 lux, and the closed arms at 30-40 lux.

**Experimental design**

To determine the time-course of the effects of TMT exposure, rats were habituated to the setting, exposed to saline or TMT, and then subjected to the EPM test 24 h, 7 days, or 14 days following odor exposure. The rats were sacrificed 3 h after the test and their brains were snap-frozen. Regions of interest were collected from frozen brains and analyzed as described above. A separate group of rats was exposed to butyric acid and tested on the EPM 24 hours later. Each animal was subjected to the test only once. In separate experiments, the rats were habituated to the experimental setting, exposed to saline, TMT or butyric acid, and then sacrificed at various time points (30 m, 3 h, 1 h, 24 h, 7 days, 14 days) following odor exposure. The brains were removed and snap-frozen. To test the effect of systemic MGL inhibition on TMT-induced long-term fear, the rats were habituated to the experimental setting and in addition to the handling procedure, received daily injections of vehicle (4:1 PEG-400:Tween-80, 1 mL/kg, i.p) for 5 days. Eighteen h after odor exposure, vehicle or JZL184 (16 mg/kg, i.p) was injected. Six h or 6 days after JZL184 injections, the animals were subjected to the EPM test. In a separate group of rats, we administered the CB₁ antagonist/inverse agonist, rimonabant (1 mg/kg, i.p.), 30 min before JZL184 and performed an EPM test 6 days later. The rats were sacrificed 3 h after the test and their brains were snap-frozen for analyses. Lastly, we investigated the effect of MGL inhibition within the BLA or the hypothalamus. The rats were habituated to the experimental setting as outlined above.
Eighteen h after odor exposure, vehicle or NF1819 was infused into the BLA or the third ventricle and the EPM test was performed 6 days later. The animals were sacrificed 3 h after the test and their brains were snap-frozen for analyses.

**Statistical analyses**

All results are presented as mean±s.e.m. Data were analyzed by two-way analysis of variance (ANOVA). Post-hoc comparisons, when appropriate, were performed by Tukey’s multiple comparisons test. In all cases, differences with a P<0.05 were considered significant. For all data an extreme Studentized deviate method with α=0.05 was performed to identify significant outliers and removed from statistical analysis. TMT-resistant vs TMT-sensitive animal groups were parsed out by calculating the k-means for each cluster and determining the center point.

**Results**

**TMT causes long-term anxiety-like behavior in rats**

We challenged male Sprague-Dawley rats with a single 10-min exposure to the fox pheromone TMT (Fendt et al., 2005) and measured anxiety-like behavior in the EPM at various time points after odor exposure (24 h, 7 days and 14 days). Compared to saline-exposed animals, rats challenged with TMT exhibited a statistically detectable increase in anxiety-like behavior, which lasted for the entire duration of the experiment (14 days; Figure 4.1a-c). Exposure to TMT reduced the amount of time spent in the open arms of the maze (TMT effect, F_{1,42}=16.93, p=0.0002; Figure 4.1a) and the number of open arm entries (TMT effect, F_{1,42}=22.67, p<0.0001; Figure 4.1b), and increased the anxiety index (TMT effect, F_{1,42}=21.17 p<0.0001; Figure 4.1c). To verify
the behavioral selectivity of the effect of TMT, we challenged a separate group of rats for 10 min with butyric acid, whose odor is pungent but non-threatening to rats. In contrast to TMT, exposure to butyric acid failed to induce EPM anxiety-like responses 24 h after odor presentation (anxiety index: one-way ANOVA, $F_{2,20}=13.68$, $P=0.0002$; Table 4.1).

**Exposure to TMT mobilizes 2-AG in the amygdala**

To test whether exposure to TMT affects endocannabinoid signaling in the brain, we challenged rats with the odor and sacrificed them 30 min, 3 h, 24 h, 7 days, and 14 days later. Of the seven brain regions surveyed (Table 4.2), two showed statistically detectable changes in 2-AG content, compared to saline-exposed controls: the amygdala, where 2-AG levels increased 24 h after TMT exposure and remained significantly elevated for the following 13 days (TMT effect $F_{1,50}=20.26$ $p<0.001$; time after odor $F_{3,50}=4.62$ $p=0.003$; Figure 4.1d); and the hypothalamus, where a transient increase in 2-AG (3 h after TMT) was followed by a short-lasting decrease (24 h after TMT) (interaction $F_{1,50}=2.63$, $p=0.045$; Figure 4.1e). In addition to the 2-AG changes, mRNA analyses indicate an increase in c-Fos mRNA levels in the amygdala 24 h after TMT exposure, which returned to baseline levels 6 days later (time after odor, $F_{1,23}=4.82$ $p=0.038$; interaction, $F_{1,23}=7.33$ $p=0.013$; Figure 4.1f). We did not observe significant changes in the transcription of genes encoding for CB1 (Figure 4.1g), MGL, and diacylglycerol lipase-α (DGL-α), a key enzyme of 2-AG production (Figure 4.2a-f). No change in 2-AG mobilization was detected in the other brain regions included in the survey (Table 4.3). Additionally, anandamide levels in those regions were not affected by TMT under the present experimental conditions (Table 4.4). Confirming the
selectivity of the response to TMT, exposure to butyric acid had no effect on either 2-AG or anandamide levels (Table 4.2).

Consistent with published data (Fendt et al, 2005; Holman et al, 2014), we found that approximately 25% of the rats exposed to TMT did not develop long-term anxiety-like behavior. This led us to hypothesize that TMT exposure might differentially regulate 2-AG levels in resistant versus sensitive rats. We applied the k-means cluster analysis to the “ratio of time spent in the open arm” data from TMT-exposed rats and determined the center of the clusters (TMT-sensitive vs. TMT-resistant) to be 14.3%. Using this percentage as a threshold, we separated TMT-resistant versus TMT-sensitive rats. In a separate experiment with 12 saline-exposed and 12 TMT-exposed animals, we parsed out 8 TMT-resistant and 4 TMT-sensitive rats. As expected, the TMT-sensitive rats showed higher levels of anxiety-like behavior relative to TMT-resistant and saline-treated rats (one-way ANOVA, $F_{2,21}=10.59$, $p=0.0007$; Figure 4.1h), which was associated with elevated 2-AG levels in the amygdala (Figure 4.1i). Compared to TMT-sensitive rats, TMT-resistant rats had 2-AG levels that were similar to those measured in saline-exposed control rats (one-way ANOVA, $F_{2,21}=9.29$, $p=0.0013$; Figure 4.1i).

**MGL inhibition prevents TMT-induced long-term anxiety-like behavior**

To examine the functional role of 2-AG mobilization in the response to TMT exposure, we interrupted 2-AG degradation *in vivo* using the MGL inhibitor, JZL184 (Pan et al, 2009). A single injection of JZL184 (16 mg/kg, i.p.) decreased MGL activity (JZL184 effect, $F_{1,20}=23.58$ $p<0.0001$; Figure 4.2a) and increased 2-AG levels (JZL184 effect, $F_{1,20}=11.46$, $p=0.0029$; Figure 4.2b) in the brain, irrespective of whether the rats were exposed to saline or TMT. As previously reported for other models of anxiety
(Sciolino et al, 2011), treatment with the MGL inhibitor resulted in a reduction in anxiety-like behavior. Administration of JZL184 (16 mg/kg, i.p.) 18 h after TMT and 6 h before the tests normalized anxiety-like responses in TMT-exposed, but not saline-exposed rats (TMT effect, \( F_{1,30}=14.72 \) \( p=0.0006 \); JZL184 effect, \( F_{1,30}=9.89 \) \( p=0.0037 \); interaction, \( F_{1,30}=10.18 \) \( p=0.0033 \); Figure 4.2c).

Next, we tested whether an early intervention with the MGL inhibitor might influence the subsequent development of long-lasting TMT-induced anxiety-like responses. We administered JZL184 (i.p.) 18 h after TMT exposure followed by behavioral tests 6 days later. We found that JZL184 reduced anxiety-like responses in TMT-exposed, but not saline-exposed rats (TMT effect, \( F_{1,42}=11.66 \) \( p=0.0014 \); JZL184 effect, \( F_{1,42}=5.61 \) \( p=0.023 \); interaction, \( F_{1,42}=6.138 \) \( p=0.017 \); Figure 4.2d). As expected, MGL activity (JZL184 effect, \( F_{1,20}=0.34 \) \( p=0.57 \); Figure 4.2e) and amygdalar 2-AG content (JZL184 effect, \( F_{1,20}=0.28 \) \( p=0.6 \); Figure 4.2f) were at basal levels 6 days after administration of the drug. The results suggest that temporary enhancement of 2-AG-mediated signaling is sufficient to prevent long-term behavioral changes evoked by predator stress.

**The anxiolytic-like effect of JZL184 is CB\(_1\) receptor-dependent**

Next we examined whether the long-term effect of JZL184 might be due to 2-AG-mediated activation of CB\(_1\) receptors. First, we tested whether the CB\(_1\) inverse agonist, rimonabant, has a direct effect on TMT-induced anxiety-like behavior. We administered the drug (1 mg/kg, i.p.) 17.5 h after TMT exposure and tested the behavior after 6 days. The treatment did not affect the time spent in the open arm (TMT effect \( F_{1,18}=9.24 \), \( p=0.007 \); rimonabant effect \( F_{1,18}=0.022 \), \( p=0.88 \); Figure 4.3a), the number of open arm
entries (TMT effect $F_{1.18}=4.88$, $p=0.04$, rimonabant effect $F_{1.18}=0.077$, $p=0.78$; Figure 4.3b), or the anxiety index (TMT effect, $F_{1.18}=7.87$, $p=0.012$, rimonabant effect, $F_{1.18}=0.054$, $p=0.82$; Figure 4.3c) of rats exposed either to saline or TMT. In another group of rats, we administered rimonabant (1 mg/kg, i.p.) 17.5 h after TMT exposure (all rats were exposed to TMT), followed by JZL184 injection after 30 min and behavioral testing after 6 days. Rimonabant suppressed the anxiolytic-like effects of JZL184 (ratio time open arm: interaction, $F_{1.43}=4.85$, $p=0.033$; ratio entry open arm: interaction, $F_{1.43}=6.13$, $p=0.017$; anxiety index: interaction, $F_{1.43}=6.85$ $p=0.012$; Figure 4.3d-f), providing evidence that that such effect requires CB1 receptor activation.

**MGL inhibition in the BLA blocks TMT-induced anxiety-like behavior**

To further explore the mechanism underlying the anxiolytic-like effect of MGL inhibition, we examined whether the amygdala or the hypothalamus, in each of which 2-AG levels change in response to TMT, is responsible for the suppression of TMT-induced anxiety-like behavior. Because JZL184 is poorly soluble, we used a newly disclosed compound, NF1819, which is a potent, selective and water-soluble MGL inhibitor. The synthesis and general properties of this agent will be reported elsewhere.

To test the efficacy of NF1819, we first administered the drug systemically to adult male mice (5 mg/kg, i.p.). We found that NF1819 markedly reduces MGL activity ($p<0.0001$) and increases 2-AG content ($p<0.0001$) in brain tissue within one h of administration (Figure 4.4a, b). We then infused NF1819 either into the BLA or the third ventricle of rats (Figure 4.4c, f). Compared to vehicle, MGL activity was decreased and 2-AG levels were increased in BLA micropunches (MGL activity: $p=0.045$; 2-AG levels: $p=0.0007$; Figure 4.4d, e; micropunch A), but not in micropunches taken from
immediately adjacent brain regions (Figure 4.4d, e; micropunches B and C). Similarly, infusion of NF1819 into the third ventricle resulted in significant changes in both MGL activity and 2-AG accumulation (MGL activity: $p=0.027$; 2-AG levels: $p=0.0008$; Figure 4.4g, h).

Having confirmed that NF1819 effectively inhibits MGL activity and accrues 2-AG availability after intracerebral administration, we targeted the BLA or the hypothalamus for behavioral testing. We first exposed all the rats to TMT and then infused NF1819 (5 ng, 0.2 µL) into the BLA 18 h after TMT exposure and 6 days before the behavioral tests. NF1819 reduced anxiety-like responses in rats, compared to vehicle ($p=0.0045$ Figure 4.5a). In contrast, infusion of NF1819 into the third ventricle (75 ng, 3 µL) 18 h after TMT exposure and 6 days before the behavioral tests had no significant changes in EPM behavior ($p=0.82$; Figure 4.5d-f). The results suggest that the anxiolytic-like effects of MGL blockade are mediated through the BLA.

**Discussion**

In this study, we report that the long-term anxiety-like state caused in rats by exposure to the fox pheromone TMT is paralleled by a sustained elevation in amygdalar levels of the endocannabinoid 2-AG. We further show that pharmacological inhibition of the 2-AG-hydrolyzing enzyme, MGL, increases 2-AG accumulation and produces marked anxiolytic-like effects, which are abrogated by the CB$_1$ cannabinoid receptor antagonist, rimonabant. The amygdala appears to be critical to this response, because local infusion of an MGL inhibitor into this structure recapitulates the actions of systemic MGL blockade. Lastly, inhibiting MGL within 24 h of exposure to TMT completely prevents the development of long-term anxiety-like behavior. The results suggest that 2-
AG mobilization in the amygdala acts as an intrinsic feedback mechanism that protects rats against the chronic consequences of TMT-evoked stress.

An extensive body of research has established that stressful sensory information across multiple modalities enters the amygdala through the BLA and the centrolateral amygdala (CeAL), both of which project to the centromedial amygdala (CeAM) (LeDoux, 2000). The CeAM, in turn, projects to rostral and caudal brain regions such as the hypothalamus, involved in the expression of stress, fear, and anxiety (LeDoux, 2000). Functional studies have shown that direct activation of CeAL strongly inhibits CeAM output neurons through GABA (gamma-aminobutyric)-ergic projections and reduces fear and anxiety responses, while direct activation of the BLA increases fear and anxiety responses through glutamatergic projections to the CeAM (Etkin et al., 2009; Li et al., 2013). Because CB₁ receptors expression in the amygdala is restricted to the BLA (Ramikie et al., 2012), it is tempting to hypothesize that the persistent increase in amygdalar 2-AG mobilization after TMT exposure provides a negative feedback signal that inhibits the BLA and attenuates CeAM activation. Additionally, although infusion of NF1918 into the third ventricle caused no behavioral effect, the changes of 2-AG levels in this region after TMT exposure are suggestive of a link between hypothalamus and amygdala in the modulation of fear and anxiety. Identifying the precise neural mechanism underlying 2-AG mobilization is an important question that needs to be addressed by future studies.

Previous reports indicate that anandamide plays a protective role in the response to environmental stressors (Hill et al., 2013; Hill et al., 2011; Kathuria et al., 2003; Patel et al., 2006) and that stressful stimuli enhance anandamide mobilization in brain regions
such as BLA and prefrontal cortex (Bortolato *et al*, 2006; Gaetani *et al*, 2003; Kathuria *et al*, 2003; Lutz, 2009; Marsicano *et al*, 2002; Patel *et al*, 2006, 2008; Steiner *et al*, 2008). In the auditory fear-conditioning paradigm, administration of FAAH inhibitors into the BLA enhances fear extinction and impairs fear memory retrieval (Gunduz-Cinar *et al*, 2013; Marsicano and Lutz, 2006; Marsicano *et al*, 2002). How do anandamide and 2-AG cooperate to modulate the response to stress? While the available data do not allow us to answer this question, it is interesting to note that previous studies have reported a temporal disconnect between the mobilization of anandamide and that of 2-AG. For example, foot shock induces a rapid and short-lived increase in 2-AG content in the dorsal midbrain of rats, which is accompanied by a slower, more sustained increase in anandamide levels (Hohmann *et al*, 2005). The lack of changes in anandamide levels observed in the present study, compared to those previously documented (Hill *et al*, 2009; Pecoraro *et al*, 2006), might be explained by differences in the time course of mobilization of the two endocannabinoid transmitters in the amygdala. Relevant to this point, a recent study has shown that acute activation of muscarinic acetylcholine receptors in the amygdala causes a short-lived form of anandamide-mediated synaptic depression, while prolonged activation (>1 h) of the same receptor causes a tonic 2-AG-mediated depression (Ramikie *et al*, 2014). Based on those data, it is tempting to speculate that short-term changes in anandamide mobilization might play a role in acute stress-coping responses, whereas 2-AG might be involved in the sustained response to a stressor.

The anxiolytic-like effects elicited by MGL blockade, and their sensitivity to rimonabant, suggest that endogenously produced 2-AG is involved in the regulation of
TMT-induced long-term anxiety through the activation of CB₁ receptors in the BLA. These findings complement a growing body of evidence suggesting a role for 2-AG in the modulation of emotional responses to stress (Evanson et al, 2010; Hill et al, 2011; Sciolino et al, 2011; Sutt et al, 2008). For example, studies have shown that cat odor exposure causes rapid changes in the expression of enzymes involved in the biosynthesis and degradation of 2-AG in various rat brain regions, including amygdala and periaqueductal grey area, immediately after odor exposure (Sutt et al, 2008). These studies did not examine the long-term consequences of odor exposure, which were the object of the present investigation. Additionally, it has been reported that systemic administration of JZL184 reduces anxiety-like behaviors in rats in a CB₁-dependent manner, but only when the animals are exposed to high-stress conditions (i.e., strong environmental light) (Sciolino et al, 2011). In agreement with this evidence, the current work suggests that 2-AG acts as an endogenous modulator of anxiety-like behavior, and that this action might be particularly relevant during conditions of high stress.

Exposure to TMT evokes a prolonged anxiety-like state that is paralleled by an increase in 2-AG levels within the amygdala. A single intra-BLA administration of NF1819 18 h after TMT-exposure evoked anxiolytic-like effects 7 days after the exposure. This suggests that enhancing the mobilization of endogenous 2-AG protects rats against an inappropriately severe response to a life-threatening stressor. The persistent increase in 2-AG content after TMT exposure may be a consequence of temporary increase in neuronal activity in specific sets of synapses feeding into the BLA from higher brain regions involved in the stress response. Consistent with this view, we observed a significant increase in amygdalar c-Fos mRNA expression 24 h, but not
seven days after TMT-exposure. One possibility is that MGL in the BLA modulates neurotransmission between specific synapses engaged in 2-AG signaling, thus destabilizing essential connections and promoting long-term anxiety. Conversely, it is also possible that synapses that are not engaged in 2-AG mediated signaling might be responsible for triggering the long-term anxiety-like state in rats, and the 2-AG engaged synapses prevent over activation of this circuit. Therefore, elevated 2-AG content in unengaged synapses may destabilize and silence all communications from the BLA. This is plausible since other studies show that increase of endocannabinoid activity can drive CB1-dependent synaptic depression in the amygdala (Gunduz-Cinar et al, 2013; Ramikie et al, 2014). Further investigations are necessary to determine the molecular mechanism(s) of 2-AG mobilization and its relation to long-term anxiety responses.

Not all people respond to a traumatic event in the same way (Adshead, 2007; Davidson, 2004, 2006; Giuffrida et al, 2004; Pitman et al, 2005). Studying individual differences that influence post trauma adaptation may generate clues about how anxiety states are triggered. Consistent with published data, about 25% of TMT-exposed rats in our study failed to develop long-term anxiety-like behavior in the EPM (Fendt et al, 2005; Holman et al, 2014). Additionally, TMT-resistant rats did not exhibit elevated levels of 2-AG in the amygdala compared to TMT-sensitive animals. This suggests that the persistent increase in 2-AG content in TMT-sensitive animals may serve as a natural coping mechanism to an overwhelming stressor. For TMT-resistant animals, the TMT exposure alone may not have been adequate enough to trigger a significant fear to activate a 2-AG-mediated coping response. Further investigation of the difference in endocannabinoid regulation between TMT-resistant and TMT-sensitive rats should
provide valuable insights into the mechanisms underlying the induction of long-term anxiety states.

In conclusion, our study suggests that exposure to predator odor initiates changes in amygdalar 2-AG signaling, which might play a modulatory/protective role in the response to a traumatic event. The results further suggest that pharmacological agents that enhance 2-AG signaling may attenuate anxiety-like responses in stressed animals and anxiety symptoms in human trauma victims.
Figure 4.1: Rats exposed to TMT show significantly increased anxiety-like behavior and changes in 2-AG mobilization. (a-c) Rats were subjected to EPM 24 h, 7 days or 14 days after exposure to TMT. (a) Ratio of time in open arm, (b) ratio of open
arm entry, and (c) anxiety index indicate that exposure to TMT generates long-term anxiety in rats. The anxiety index was calculated as 1-(average of percent time in open arm and percent open arm entry) (n = 8 rats per group). (d) 2-AG content remained elevated in the amygdala for at least 14 days (n = 6 rats per group). (e) Fluctuations in 2-AG level were observed in the hypothalamus for the first 24 h (n = 6 rats per group). In addition to 2-AG content, (f) we observed an increase in c-fos mRNA expression in the amygdala at 24 h but (g) no significant change in CB₁ receptor gene expression. The mRNA data are shown in relative quantity (copies) ratio to GAPDH as normalizer (n = 6-8 rats per group). (h, i) We parsed out TMT-resistant and TMT-sensitive rats. (h) Anxiety index of TMT-resistant and TMT-sensitive rats indicates that TMT-sensitive rats show heightened level of anxiety-like behavior. (i). TMT-resistant did not exhibit increased levels of 2-AG 7 days after the TMT exposure. N = 4-12 rats per group. Results are expressed as mean±SEM; *P<0.05, **P<0.01, ***P<0.001.
Figure 4.2: Expression of 2-AG-related genes in the amygdala and hypothalamus of TMT-exposed rats. (a-b) No significant changes in mRNA expression of (a) MGL and (b) DGL-α in the amygdala after TMT exposure (n = 7-8 rats per group). Effects of TMT exposure on (c) c-Fos (d) CB₁, (e) MGL, and (f) DGL-α mRNA expression in the
hypothalamus. The mRNA data are shown in relative quantity (copies) ratio to GAPDH as normalizer (n = 6 rats/group). Results are expressed as mean±SEM; *P<0.05.
Figure 4.3: A single administration of MGL inhibitor, JZL184, exerts anxiolytic-like effects in TMT-exposed rats. (a-c) Acute, single i.p. injection of JZL184 18 h after TMT exposure followed by behavioral test after 6 h after exerts an anxiolytic-like effect. (a) JZL184 (i.p.) administration inhibits brain MGL activity and (b) increase 2-AG content after 6 h (n = 6 rats per group.) (c) Anxiety index data (n = 8-9 rats per group). (d) Administration of JZL184 (i.p.) 18 h after TMT exposure, followed by behavioral tests 7 d post TMT exposure reduces anxiety-like responses in rats (n = 11-12). (e-f) JZL184 (i.p.) administration 6 days before measurement causes no change in brain (e) MGL activity or (f) 2-AG content (n= 6 rats per group). Results are expressed as mean±SEM; *P<0.05, **P<0.01.
Figure 4.4: The anxiolytic-like effects of JZL184 are CB₁ dependent. (a-c) Intraperitoneal injection of rimonabant 17.5 h after TMT exposure causes no significant behavioral changes. (a) Ratio of time spent in open arm, (b) ratio of open arm entry and (c) anxiety index indicate that rimonabant (1 mg/kg) administration 18 h after TMT exposure has no effect on EPM behavior 7 days later (n= 5-6 rats per group). (d-f) Intraperitoneal injection of rimonabant 30 min prior to a single JZL184 injection 18 h after TMT exposure blocks the anxiolytic-like effect of JZL184 that lasts for at least 7 days. (d) Ratio of time spent in open arm, (e) ratio of open arm entry and (f) anxiety index indicate that the anxiolytic-like effects of JZL184 are CB₁-dependent (n= 11-12 rats per group). Results are expressed as mean ±SEM; *P<0.05, **P<0.01.
Figure 4.5: Effects of NF1819 on MGL activity and 2-AG mobilization. (a) Intraperitoneal injection of NF1819 (5 mg/kg) lowers rat MGL activity and (b) increases 2-AG content in mouse brain. (c-e) Infusion of NF1819 into the BLA inhibits local MGL activity and increases 2-AG level. (c) Diagram of the needle tract and location of brain punches taken to measure MGL activity and 2-AG levels in the amygdala. (d) NF1819 infusion into the BLA inhibits MGL activity and (e) increases 2-AG levels at the site of injection (A) but not in nearby tissue (B-D) (n = 4 rats per group). (f-h) Infusion of NF1819 into the third ventricle inhibits MGL activity and increases 2-AG level in the
hypothalamus. (f) Diagram of the needle tract and location of brain punches taken to measure MGL activity and 2-AG levels in the hypothalamus. (g) NF1819 infusion into the third ventricle inhibits MGL-activity and (h) increases 2-AG levels in the hypothalamus (n = 4 rats per group). Results are expressed as mean ±SEM; *P<0.05, **P<0.01, ***P<0.001.
Figure 4.6: The anxiolytic-like effects of MGL inhibition on TMT-induced anxiety-like behavior are mediated by the BLA. (a-c) Infusion of NF1819 into the BLA 18 h after TMT exposure has an anxiolytic-like effect that lasts for at least 7 days. (a) Ratio of time spent in open arm, (b) ratio of open arm entry and (c) anxiety index suggest that the anxiolytic-like effects of the MGL inhibitor occur in the BLA. (d-f) Infusion of NF1819 into the third ventricle has no overt behavioral effect in TMT-exposed rats. (d) Ratio of time spent in open arm, (e) ratio of open arm entry, and (f) Anxiety index (n = 5 per group). Results are expressed as mean ±SEM; n=6-10 rats/group; *P<0.05, **P<0.01.
Figure 4.7: Acute administration of MGL inhibitor, JZL184, prevents TMT-induced open arm avoidance. (a, b) Acute, single i.p. injection of JZL184 18 h after TMT exposure followed by the behavioral test after 6 h significantly increases (a) ratio time open arm and (b) ratio open arm entry in TMT exposed rats. N = 8-9 rats per group. (c, d) Administration of JZL184 (i.p.) 18 h after TMT exposure, followed by behavioral tests 7 d post TMT exposure increases (c) ratio time open arm and (d) ratio open arm entry in TMT exposed rats (n = 11-12). Results are expressed as mean±SEM; *P<0.05, **P<0.01.
Table 4.1: Elevated plus maze measurement 24 h after butyric acid exposure.

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Butyric Acid</th>
<th>TMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio Time in Open Arm (%)</td>
<td>42.7±3.6</td>
<td>48.7±3.9</td>
<td>15.3±5.9**</td>
</tr>
<tr>
<td>Ratio of Open Arm Entry (%)</td>
<td>41.9±1.8</td>
<td>44.7±3.5</td>
<td>17.4±6.7**</td>
</tr>
<tr>
<td>Anxiety Index</td>
<td>0.6±0.04</td>
<td>0.5±0.04</td>
<td>0.8±0.06**</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SEM.
Significant association: **P<0.01.
N = 7-8 rats per group.
Table 4.2: Endocannabinoid levels in brain regions of rats 24 hours after butyric acid exposure.

<table>
<thead>
<tr>
<th></th>
<th>2-AG (nmol/g)</th>
<th>AEA (pmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Butyric Acid</td>
</tr>
<tr>
<td>Amygdala</td>
<td>10.2±1.1</td>
<td>12.3±1.4</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>26.5±4.0</td>
<td>29.1±2.6</td>
</tr>
<tr>
<td>Ventral Striatum</td>
<td>14.4±1.3</td>
<td>15.0±0.5</td>
</tr>
<tr>
<td>Dorsal Hippocampus</td>
<td>18.7±1.2</td>
<td>19.1±1.6</td>
</tr>
<tr>
<td>Ventral Hippocampus</td>
<td>13.7±0.6</td>
<td>14.0±0.6</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>13.8±1.6</td>
<td>16.8±1.0</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SEM. 
N = 6 rats per group.
Table 4.3: 2-AG levels in different brain regions of TMT-exposed rats (nmol/g)

<table>
<thead>
<tr>
<th>Region</th>
<th>3 hours</th>
<th>24 hours</th>
<th>7 days</th>
<th>14 days</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>TMT</td>
<td>Saline</td>
<td>TMT</td>
</tr>
<tr>
<td>Prefrontal Cortex</td>
<td>22.5±1.2</td>
<td>24.7±1.3</td>
<td>21.3±1.8</td>
<td>21.8±1.7</td>
</tr>
<tr>
<td>Amygdala</td>
<td>17.1±0.9</td>
<td>19.5±2.3</td>
<td>20.6±1.8</td>
<td>30.2±1.2**</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>59.3±4.2</td>
<td>73.7±4.2*</td>
<td>63.1±4.7</td>
<td>46.4±2.8*</td>
</tr>
<tr>
<td>Ventral Striatum</td>
<td>25.1±1.8</td>
<td>22.9±1.1</td>
<td>15.0±1.0</td>
<td>13.9±1.1</td>
</tr>
<tr>
<td>Dorsal Hippocampus</td>
<td>24.8±2.0</td>
<td>27.3±2.2</td>
<td>16.2±3.4</td>
<td>22.7±1.8</td>
</tr>
<tr>
<td>Ventral Hippocampus</td>
<td>29.6±0.8</td>
<td>29.9±2.7</td>
<td>20.7±3.1</td>
<td>18.1±1.6</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>25.7±3.3</td>
<td>24.5±2.4</td>
<td>17.1±2.6</td>
<td>18.1±3.0</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SEM.
Significant association: *P<0.05, **P<0.01.
N = 6 rats per group.
<table>
<thead>
<tr>
<th>Brain Region</th>
<th>3 hours Saline</th>
<th>3 hours TMT</th>
<th>24 hours Saline</th>
<th>24 hours TMT</th>
<th>7 days Saline</th>
<th>7 days TMT</th>
<th>14 days Saline</th>
<th>14 days TMT</th>
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</thead>
<tbody>
<tr>
<td>Prefrontal Cortex</td>
<td>11.6±0.7</td>
<td>13.3±1.2</td>
<td>12.8±3.1</td>
<td>9.1±2.0</td>
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<td>9.6±1.5</td>
<td>10.0±2.5</td>
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<tr>
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<td>3.8±0.28</td>
<td>4.3±1.1</td>
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<td>5.4±0.7</td>
<td>5.8±0.6</td>
<td>3.6±0.9</td>
<td>5.0±1.2</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>3.8±0.9</td>
<td>3.1±0.2</td>
<td>5.3±1.1</td>
<td>4.9±0.9</td>
<td>5.7±1.4</td>
<td>5.2±1.0</td>
<td>3.4±0.2</td>
<td>4.5±0.7</td>
</tr>
<tr>
<td>Ventral Striatum</td>
<td>11.8±2.4</td>
<td>11.0±1.6</td>
<td>16.4±1.4</td>
<td>15.3±1.3</td>
<td>12.4±1.7</td>
<td>13.3±1.0</td>
<td>16.4±1.4</td>
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<tr>
<td>Dorsal Hippocampus</td>
<td>17.8±3.6</td>
<td>14.7±2.1</td>
<td>19.5±2.7</td>
<td>21.5±2.5</td>
<td>24.5±1.8</td>
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<td>26.8±3.3</td>
<td>27.5±3.3</td>
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<tr>
<td>Ventral Hippocampus</td>
<td>12.4±2.0</td>
<td>9.0±1.9</td>
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<td>21.8±1.4</td>
<td>24.4±1.6</td>
<td>12.5±1.0</td>
<td>11.9±3.4</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>20.5±2.2</td>
<td>22.6±4.3</td>
<td>26.5±1.2</td>
<td>26.0±2.9</td>
<td>28.1±3.3</td>
<td>27.7±1.6</td>
<td>26.2±2.6</td>
<td>27.0±1.2</td>
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</table>

Results are expressed as mean±SEM.
N = 6 rats per group.
CHAPTER 5

Inhibition of Peripheral Fatty Acid Amide Hydrolase Suppresses Predator Stress-Induced Long-Term Anxiety in Rats

Introduction

Acute stress is considered as a normal response to a major traumatic event and an evolutionarily adaptive function in humans. While majority of people naturally recover from these acute stress, a variety of persistent emotional distress is observed within substantial proportion of trauma victims. Posttraumatic stress disorder (PTSD) is one of the most common disorders that may occur after exposure to life-threatening events. PTSD has a lifetime prevalence of approximately 8% in the United States (Breslau et al., 1998; Kessler et al., 2005c; Kessler et al., 1995), and it is characterized as the re-experiencing of the extreme traumatic event accompanied by symptoms of increased arousal and avoidance of stimuli associated with the trauma (American Psychiatric Association. et al, 2013). Comorbidity is common among patients with PTSD, with anxiety and substance abuse as two of the most common co-diagnoses following trauma. These symptoms of PTSD commonly leads to poor quality of life, greater level of physical disability, and have a significantly negative impact on educational and occupational success (Byers et al, 2014; Kessler, 2000).

A subgroup of trauma victims experiences a panic-like state that can include hyperventilation, trembling, sweating, and tachycardia, which suggest an immediate adrenergic activation at the time of trauma exposure (Brunet et al, 2001). Studies have provided compelling evidence that the presence of a prolonged peripheral adrenergic
activation during a life-threatening event contributes to over consolidation of memory for the trauma and thereby supports the development of the intrusive symptoms found in PTSD (Hurlemann et al., 2010; O'Donnell et al., 2004; Pitman, 1989). A growing area of research interest is focused on the secondary prevention of PTSD after a psychological trauma. Large body of evidence indicate that administration of pharmacological agents, such as the lipophilic β-adrenergic receptor antagonist, propranolol, shortly following the trauma has shown to be useful for mitigating PTSD symptoms or perhaps even preventing the development of PTSD (Argolo et al., 2015; Grillon et al., 2004; Hoge et al., 2012). However, few studies have thoroughly examined the possible interaction between the central and the peripheral adrenergic systems in generating a persistent anxiety states that develop in the aftermath of major trauma. Although lipophilic β-adrenergic receptor antagonist (systemic antagonist), such as propanolol, has been studied extensively as a secondary preventative measure for PTSD, the investigation on hydrophilic β-adrenergic receptor antagonist (peripherally restricted antagonist) is lacking.

In chapter 4, we reported that long-term anxiety-like state caused in rats by exposure to the fox pheromone TMT is paralleled by a sustained elevation in amygdalar levels of the endocannabinoid, 2-arachidonoylglycerol (2-AG) (Lim et al., 2015). We further showed that pharmacological inhibition of the 2-AG-hydrolyzing enzyme, monoacylglycerol lipase (MGL), increases 2-AG accumulation and produces marked anxiolytic-like effects, which are abrogated by the cannabinoid receptor type 1 (CB1) antagonist, rimonabant (Lim et al., 2015). These results indicates a strong involvement of the endocannabinoid system in the initiation, maintenance, and modulation of long-
term anxiety brought on by a traumatic event. A recent study reported that administration of a rimonabant has a significant anxiogenic-like effect and this effect is abrogated by a pretreatment of peripherally restricted β-adrenergic receptor antagonist, sotalol (Bellocchio et al, 2013). This finding suggests a link between the endocannabinoid system and the peripheral adrenergic system, and the disruption of the peripheral adrenergic system alone may be sufficient to inhibit a stress response.

In the current study, we use an acute life-threatening stress in rats, i.e. exposure to the red fox pheromone 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), to induce a persistent anxiety state. We show that systemic inhibition of fatty acid amid hydrolase (FAAH) activity suppresses anxiety-like behavior triggered by TMT in a dose dependent manner, and the effect prevented by CB1 receptor blockade. Significantly, we demonstrate that an acute administration of the peripherally restricted FAAH inhibitor is also capable of preventing the development of TMT-induced long-term anxiety states. Interestingly, the peripheral FAAH inhibitor does not act as an anxiolytic under acute stress conditions, while the systemic FAAH inhibitor exerts a potent anxiolytic-like effect. The results suggest that the peripheral stress response system may play a significant role in the consolidation of prolonged anxiety states. This study indicates that pharmacological strategies aimed at enhancing peripheral FAAH may offer a novel secondary preventative measure for the treatment of pathological consequence of psychological trauma, such as PTSD.
**Materials and Methods**

**Animals**

We purchased male Sprague-Dawley rats (8-9 weeks, 225-250 g) from Charles River (Wilmington, MA) and housed them in groups of 3 per cage. The animals were maintained on a 12 h light/dark cycle (6:30 AM to 6:30 PM) and received food (2020X, Harlan, Madison, WI) and water ad libitum. All procedures met the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

**Chemicals**

We purchased TMT from Contech Enterprises (Delta, B.C., Canada) and butyric acid from Aldrich (St. Louis, MO). URB597 and URB937 were synthesized at the Istituto Italiano di Tecnologia. Rimonabant was provided by the National Institute on Drug Abuse (NIDA).

**Drug administration**

We dissolved rimonabant in a vehicle of propylene glycol/Tween-80/sterile saline (0.9%) (1/1/18, vol/vol) and URB597 and URB937 in polyethylene glycol-400/Tween-80/saline (1/1/8). We administered drugs and their vehicle by i.p. injection in a volume of 1 mL/kg.

**Odor Exposure**

For 5 consecutive days, we handled each rat for 2 min and placed it for 10 min in a plastic exposure box (45 x 25 x 20 cm) containing a square of gauze (5 x 5 cm)
doused with saline (35 µL). The box was housed in a fume hood. On the day of the experiment, we randomly assigned the animals to the saline or TMT group. The rats were placed for 10 min in the exposure box containing a gauze doused either with saline, TMT (4.1 µM, 35 µL). After the procedure, the animals were immediately returned to their home cages.

**Elevated plus maze**

We conducted the behavioral tests between 8:00 AM and 2:00 PM. The elevated plus maze (EPM) apparatus included two open (50×10 cm) and two closed (50×10×40 cm) arms extending from a central platform (10×10 cm) elevated 60 cm above the floor. We placed each rat in the central platform of the maze, facing an open arm opposite to the experimenter, and videotaped test sessions of 5-min duration for each trial. Observers blinded to treatment measured the amount of time spent in the open arm, the number of open-arm entries, and the anxiety index. The latter was calculated as 1-(average of percent time in open arm and percent open arm entry). Between tests the apparatus was cleaned with a 20% ethanol solution and was allowed to dry thoroughly. The open arms of the maze were illuminated at 150-170 lux, and the closed arms at 30-40 lux. For the high intensity light experiments, the open arms of the maze were illuminated at 800-850 lux, and the closed arms at 30-40 lux.

**Experimental design**

To test the effect of systemic FAAH inhibition on TMT-induced long-term fear, the rats were habituated to the experimental setting and in addition to the handling procedure, received daily injections of vehicle (1:1:8 PEG-400:Tween-80:saline, 1
mL/kg, i.p) for 5 days. Eighteen h after odor exposure, vehicle or URB597 (0, 0.03, 0.1, 0.3 mg/kg, i.p) was injected. Six days after URB597 injections, the animals were subjected to the EPM test. In a separate group of rats, we administered the CB₁ antagonist/inverse agonist, rimonabant (1 mg/kg, i.p.), 30 min before URB597 (0.3 mg/kg) and performed an EPM test 6 days later. The rats were sacrificed 3 h after the test and their brains were snap-frozen for analyses.

Lastly, we investigated the effect of peripherally restricted FAAH inhibition in comparison with global FAAH inhibition. The rats were habituated to the experimental setting as outlined above. Eighteen h after odor exposure, vehicle or URB937 (0, 0.3, 3 mg/kg) was injected i.p. and the EPM test was performed 6 days later. The animals were sacrificed 3 h after the test and their brains were snap-frozen for analyses. To test whether URB937 act as an anxiolytic, new cohort of rats were injected with either vehicle, URB937 (3 mg/kg), or URB597 (0.3 mg/kg) and was placed on a high intensity light EPM test for behavior. In a separate experiment, we administered, URB937 (3 mg/kg), or URB597 (0.3 mg/kg) 7 days post TMT exposure and were subjected to an EPM test 1 hour later.

Statistical analyses

All results are presented as mean±s.e.m. Data were analyzed by two-way analysis of variance (ANOVA). Post-hoc comparisons, when appropriate, were performed by Tukey’s multiple comparisons test. In all cases, differences with a P<0.05 were considered significant. For all data an extreme Studentized deviate method with α=0.05 was performed to identify significant outliers and removed from statistical
analysis. TMT-resistant vs TMT-sensitive animal groups were parsed out by calculating the k-means for each cluster and determining the center point.

**Results**

**Systemic FAAH inhibitor, URB597, prevents TMT-induced long-term anxiety-like behavior in a CB₁ receptor-dependent manner.**

As previously reported from rodent models of anxiety, anandamide produced in the amygdala modulates the response to stressful events (Gaetani et al, 2003). Similar to the studies conducted in chapter 4, we tested whether an early intervention with the FAAH inhibitor might influence the subsequent development of long-lasting TMT-induced anxiety-like responses. To examine this, we interrupted anandamide degradation *in vivo* using the global FAAH inhibitor, URB597. Administration of URB597 (0.3 mg/kg, i.p.) 18 h after TMT exposure followed by behavioral tests 6 days later resulted in a significant increase in ratio time spent in open arm (TMT effect, $F_{1,20} = 6.17 \ p = 0.022$; URB597 effect, $F_{1,20} = 8.38 \ p = 0.009$; interaction, $F_{1,20} = 4.35 \ p = 0.049$; Figure 5.1a), increase in ratio open arm entry (TMT effect, $F_{1,20} = 22.07 \ p = 0.0001$; URB597 effect, $F_{1,20} = 20.34 \ p = 0.0002$; interaction, $F_{1,20} = 11.59 \ p = 0.0028$; Figure 5.1b), and reduction in anxiety index score (TMT effect, $F_{1,20} = 12.94 \ p = 0.0018$; URB597 effect, $F_{1,20} = 12.94 \ p = 0.0032$; interaction, $F_{1,20} = 7.82 \ p = 0.011$; Figure 5.1c) in TMT-exposed, but not saline-exposed rats.

To further investigate the effects of URB597, we conducted a dose response experiment. We administered URB597 with various doses (0, 0.03, 0.1, 0.3 mg/kg) 18 h after TMT exposure and 6 days before the behavioral tests. As expected, increase in
dose of URB597 resulted the rise of anxiolytic-like response in rats (ratio time open arm: $F_{3,44}=7.77$, $p=0.0003$; ratio entry open arm: $F_{3,44}=8.97$, $p<0.0001$; anxiety index: $F_{3,44}=8.48$, $p=0.0001$; Figure 5.1d-f).

Next we examined whether the long-term effect of URB597 might be due to anadamide-mediated activation of CB$_1$ receptors. We administered rimonabant (1 mg/kg, i.p.) 17.5 h after TMT exposure (all rats were exposed to TMT), followed by URB597 injection after 30 min and behavioral testing after 6 days. Rimonabant suppressed the anxiolytic-like effects of URB597 (ratio time open arm: interaction, $F_{1,32}=9.668$, $p=0.0039$; ratio entry open arm: interaction, $F_{1,32}=12.44$, $p=0.0013$; anxiety index: interaction, $F_{1,32}=11.5$, $p=0.0019$; Figure 5.2a-c). The results suggest that temporary enhancement of anadamide-mediated activation of CB$_1$ receptors signaling is sufficient to prevent long-term behavioral changes evoked by predator stress.

**Peripherally restricted FAAH inhibitor, URB937, prevents TMT-induced long-term anxiety-like behavior.**

We investigated whether an early intervention with a peripherally restricted FAAH inhibitor, URB937, would have the same influence as URB597 in preventing the development of long-lasting TMT-induced anxiety-like responses like URB597. To examine this, we interrupted peripheral anadamide degradation *in vivo* using the peripherally restricted FAAH inhibitor, URB937. Administration of URB937 (3 mg/kg, i.p.) 18 h after TMT exposure followed by behavioral tests 6 days later resulted in a significant increase in ratio time spent in open arm (TMT effect, $F_{1,31}=11.39$ $p=0.002$; URB937 effect, $F_{1,31}=5.00$ $p=0.033$; interaction, $F_{1,31}=8.81$ $p=0.0057$; Figure 5.3a), increase in ratio open arm entry (TMT effect, $F_{1,31}=17.24$ $p=0.0002$; URB937 effect,
F_{1,31}=4.17 \ p=0.0498; \ interaction, \ F_{1,31}=7.7 \ p=0.0093; \ Figure\ 5.3b), \ and \ reduction \ in \ anxiety \ index \ score \ (TMT \ effect, \ F_{1,31}=9.99 \ p=0.0035; \ URB937 \ effect, \ F_{1,31}=4.43 \ p=0.044; \ interaction, \ F_{1,31}=5.141 \ p=0.031; \ Figure \ 5.3c) \ in \ TMT\text{-}exposed, \ but \ not \ saline\text{-}exposed \ rats.

Similar to the experiments investigating the effect of URB597, we conducted a dose response experiment with URB937. We administered URB937 with various doses (0, 0.3, 3 mg/kg) 18 h after TMT exposure and 6 days before the behavioral tests. Unfortunately, we saw maximal anxiolytic-like response in rats at 0.3 mg/kg rats (ratio time open arm: F_{2,20}=14.75, \ p=0.0001; ratio entry open arm: F_{2,20}=8.51, \ p=0.0021; anxiety index: F_{2,20}=12.79, \ p=0.0003; Figure 5.3d-f). Future studies looking at doses between 0-0.3 mg/kg will be necessary.

**URB597 has an acute anxiolytic-like effect while URB937 does not.**

To further explore the mechanism underlying the anxiolytic-like effect of the peripherally restricted FAAH inhibition, we examined whether the URB937 would have an anxiolytic-like effect under an acute stress paradigm. One hour after injecting the rats with either URB597 (0.3 mg/kg) or URB937 (3 mg/kg), we placed rats on the EPM with high intensity light (800-850 lux in open arms, 30-40 lux in closed arms). Rats that were given URB597 showed a significantly reduced anxiety-like behavior, while URB937 failed to induce an anxiolytic-like response (ratio time open arm: F_{2,33}=27.19, \ p<0.0001; ratio entry open arm: F_{2,33}=14.23, \ p<0.0001; anxiety index: F_{2,33}=21.98, \ p<0.0001; Figure 5.4a-c).
Next we examined whether URB937 has an anxiolytic-like effect if administered at 7 days post TMT exposure. We administered either URB937 or URB597 (1 mg/kg, i.p.) 7 days after TMT exposure and 1 hour before the behavior test. While URB597 showed significant anxiolytic-like effect, URB937 did not affect the ratio time spent in the open arm (F_{2,33}=14.22, p<0.0001; Figure 5.4d), the ratio of open arm entries (F_{2,33}=7.4, p=0.0022; Figure 5.4e), or the anxiety index (F_{2,33}=11.08, p=0.0002; Figure 5.4f) of rats exposed to TMT. These results indicate that the global FAAH inhibitors acts as an anxiolytic while peripherally restricted FAAH inhibitors does not.

Discussion

In this study, we report that systemic inhibition of the anandamide-hydrolyzing enzyme, FAAH, when administered within 24 h of exposure to TMT prevents the development of the long-term anxiety-like behavior in a dose dependent manner, which is abrogated by the CB$_1$ antagonist, rimonabant. Importantly, we demonstrate that administration of peripherally restricted FAAH inhibitor, URB937, 18 hours post TMT-exposure also prevents the onset of long-term anxiety-like behavior. Interestingly, although systemic FAAH inhibitor, URB597, has a potent anxiolytic-like effect when administered 7 days post TMT-exposure or in high lightning conditions, URB937 fail to exert an anxiolytic-like influence under these conditions. The results suggest that there may be a time window when an interaction between the central and peripheral stress systems in necessary to potentiate an anxiety responses to make it long lasting in TMT-evoked stress. Overall, these results suggest that disruption of the peripheral endocannabinoid system is sufficient to prevent the development of the long-term anxiety-like behavior triggered by TMT.
Extensive evidence shows an interplay between the central and the peripheral systems during aversive memory consolidation. Centrally, it is well established that the BLA is an important locus for modulating acute stress driven memory consolidation process (McGaugh, 2000). Infusions of norepinephrine into the BLA in rodents immediately after training on emotionally arousing learning tasks enhance the consolidation of memory of the task, whereas blocking action of training-induced norepinephrine with β-adrenoceptor antagonists impairs memory consolidation (Hatfield and McGaugh, 1999; Liang et al, 1990). Interestingly, the effects of peripherally secreted stress hormones, such as epinephrine and glucocorticoids also enhance memory consolidation and is dependent on the integrity of the amygdala (McGaugh, 2000). Several important studies demonstrated that systemic epinephrine administered immediately after inhibitory avoidance training increases norepinephrine levels in the amygdala (Williams et al, 1998), whereas others showed that infusions of a β-adrenoceptor antagonist into the amygdala block the memory-enhancing effects of peripherally administered epinephrine (Liang et al, 1986; McGaugh, 2004b). As peripheral epinephrine enters the brain poorly due to its hydrophilic composition, a pathway running from the periphery to the CNS is most likely involved in mediating peripheral epinephrine’s effects on amygdala modulation of memory consolidation (Clayton and Williams, 2000; McGaugh et al, 1996). It is now well established that epinephrine can activate peripheral β-adrenoceptors located on vagal afferents that terminate in the nucleus of the solitary tract (NST) (McGaugh, 2015; Roozendaal et al, 2009). In turn, norepinephrine releasing cell groups in this brain region send direct projections to the amygdala (McGaugh, 2015; Roozendaal et al, 2009). Additionally, the
NST regulates the norepinephrine activity of the forebrain through indirect projections to the locus coeruleus (McGaugh, 2015; Roozendaal et al, 2009). Moreover, studies have found that inactivation of the NTS with microinfusions of lidocaine impaired memory consolidation and blocked peripheral epinephrine effects on memory (McIntyre et al, 2012; Williams and McGaugh, 1992, 1993).

In our study, a single i.p. injection of peripherally restricted FAAH inhibitor, URB937, 18 h after TMT-exposure evoked anxiolytic-like effects 7 days after the exposure. Because the anxiogenic-like effect of rimonabant can be blocked by a pretreatment of peripherally restricted β-adrenergic receptor antagonist, sotalol (Bellocchio et al, 2013), it is tempting to hypothesize that the vagal afferents are involved in the anxiolytic-like effect of URB937. Blockade of the peripheral CB₁ receptor could increase the activity of the peripheral β-adrenoceptors expressing vagal afferents, thus increasing the sympathetic feedback to the brain and heightening the stress response. Conversely, increase of peripheral anandamide via URB937 would increase CB₁ activation which leads to the suppression of the β-adrenergic receptor expressing vagal afferents, attenuating the stress response. However, we must still test whether the effect of URB937 is indeed a CB₁ receptor mediated response by conducting and experiment with a peripherally restricted CB₁ receptor antagonist, AM6545. Additionally, it would be important for future studies to investigate a possible link between the amygdalar norepinephrine levels and the peripheral FAAH inhibition, and how these aspects may be associated with TMT-induced long-term anxiety state. Taken together, the current findings suggest that TMT-exposure may initiate a positive feedback response between the central and peripheral stress systems to consolidate the life-
threatening stimulus. This hypothesis requires further investigation and it may provide us with a valuable insight into the mechanism of how a long-term anxiety states are developed. Overall, the exposure to TMT-initiates a stress response leading to the development of the long-term anxiety-like behavior that requires the engagement of the peripheral stress systems.

In chapter 4, we demonstrated that the long-term anxiety-like state caused in rats by exposure to the fox pheromone TMT is paralleled by a sustained elevation in amygdalar levels of the endocannabinoid 2-AG. We further showed that pharmacological inhibition of the 2-AG-hydrolyzing enzyme, MGL, increases 2-AG accumulation and produces marked anxiolytic-like effects. Here we find that systemic FAAH inhibitor, URB597 elicits a similar effect which is likely to be driven by anandamide action on the CB₁ receptor. It is possible that simply activating the amygdalar CB₁ receptor may be enough to suppress the TMT-induced long term anxiety. However, the effect of FAAH inhibition might be mediated through brain regions other than amygdala. Detailed investigation of anandamide and FAAH activity in other brain regions will be necessary.

The finding that acute administration of peripherally restricted FAAH inhibitor prevents the onset of TMT-induced long-term anxiety is very surprising. To investigate further, we first tested whether there is a difference between global and peripheral FAAH inhibition on acute anxiety. When tested under very bright lightening conditions, URB597 had a very potent anxiolytic-like effect while URB937 had no effect. Additionally, when both drugs were administered 7 days after TMT exposure, only URB597 exerted an anxiolytic-like effect. These data strongly indicate that URB937
does not act as an anxiolytic, and yet it is able to prevent the TMT-induced long-term anxiety-like behavior in rats when administered with 24 hours of odor exposure. Collectively, the data suggest that while the peripheral systems may not be involved in acute stress response, but it is essential for the development of persistent stress response. We could postulate that there must be an interplay between the central and the peripheral stress system which is necessary to establish a long-term anxiety-like responses in rodents after TMT exposure, and disruption of the endocannabinoid signaling on either the central or the peripheral side will prevent the onset of persistent anxiety states. Further investigation of how the peripherally restricted FAAH inhibition prevents the development of long-term anxiety will be paramount to understanding the mechanism of chronic human stress conditions such as PTSD.

In conclusion, our study suggests that increasing the mobilization of the peripheral endocannabinoid system can suppress long-term anxiety brought on by a life threatening stimulus. Additionally, the peripheral systems may act to stabilize the consolidation of long-term anxiety but have minor influence on the acute stress response. The results further suggest that pharmacological agents that peripheral endocannabinoid signaling may attenuate anxiety-like responses in stressed animals and anxiety symptoms in human trauma victims without the psychotropic side effects.
Figure 5.1: A single administration of FAAH inhibitor, URB597, exerts dose dependent anxiolytic-like effects in TMT-exposed rats. (a-c) Acute, single i.p. injection of URB597 18 h after TMT exposure, followed by behavioral tests 7 d post TMT exposure indicate reduces anxiety-like responses in rats. (a) Ratio of time spent in open arm, (b) ratio of open arm entry, and (f) anxiety index suggest an acute FAAH inhibition can suppress TMT induced long-term anxiety (n = 6 rats per group). (d-f) Administration of URB597 at 0, 0.03, 0.1, or 0.3 mg/kg 18 h after TMT exposure, followed by behavioral tests 7 d post TMT exposure show dose dependent anxiolytic-like responses in rats. (d) Ratio of time spent in open arm, (e) ratio of open arm entry, and (f) anxiety index (n = 12 rats per group). Results are expressed as mean±SEM; *P<0.05, **P<0.01.
Figure 5.2: The anxiolytic-like effects of URB597 are CB₁ dependent. (a-c) Intraperitoneal injection of rimonabant 30 min prior to a single URB597 injection 18 h after TMT exposure blocks the anxiolytic-like effect of URB597 that lasts for at least 7 days. (d) Ratio of time spent in open arm, (e) ratio of open arm entry and (f) anxiety index indicate that the anxiolytic-like effects of URB597 are CB₁-dependent (n= 9 rats per group). Results are expressed as mean ±SEM; *P<0.05, **P<0.01.
Figure 5.3: Administration of peripherally restricted FAAH inhibitor, URB937, exerts anxiolytic-like effects in TMT-exposed rats. (a-c) Acute, single i.p. injection of URB937 18 h after TMT exposure, followed by behavioral tests 7 d post TMT exposure indicate reduces anxiety-like responses in rats. (a) Ratio of time spent in open arm, (b) ratio of open arm entry, and (f) anxiety index suggest an acute peripheral FAAH inhibition can suppress TMT induced long-term anxiety (n = 9 rats per group). (d-f) Administration of URB937 at 0, 0.3, or 3 mg/kg 18 h after TMT exposure, followed by behavioral tests 7 d post TMT exposure show maximum anxiety-like responses at 0.3 mg/kg in rats. (d) Ratio of time spent in open arm, (e) ratio of open arm entry, and (f) anxiety index (n = 7-8 rats per group). Results are expressed as mean±SEM; *P<0.05, **P<0.01.
Figure 5.4: URB597 has an anxiolytic-like effect, while URB937 does not. (a-c) Acute, single i.p. injection of either URB597 or URB937 1 h prior to EPM test with high intensity light show reduction in anxiety-like responses in rats with URB597 administration but not with URB937. (a) Ratio of time spent in open arm, (b) ratio of open arm entry, and (f) anxiety index suggest that only systemic FAAH inhibition can act as an anxiolytic under high lighting conditions (n = 12 rats per group). (d-f) Acute, single i.p. injection of either URB597 or URB937 7 d after TMT exposure and 1 h before behavioral tests indicate reduction in anxiety-like responses in rats with URB597 administration but not with URB937. Again (d) ratio of time spent in open arm, (e) ratio of open arm entry, and (f) anxiety index suggest that only systemic FAAH inhibition can act as an anxiolytic 7 days after TMT exposure (n = 12 rats per group). Results are expressed as mean±SEM; *P<0.05, **P<0.01, ***P<0.001.
CHAPTER 6

General Discussion

Introduction

Stress response is essential for the survival of an organism. In addition to preparing an individual for the acute consequences of threatening situations and the return to homeostasis, an important function of stress is to initiate long-term adaptive responses (McEwen, 1998). Enhanced memory for emotionally arousing or stressful events is a highly adaptive trait that helps us remember important information about dangerous situations. In general, people have exceptional memory after experiencing a major earthquake or have witnessed an accident (Bohannon, 1988; Neisser et al, 1996). Similarly, rodents remember the location of a hidden escape platform in a Morris water maze or the context of an apparatus where they received a footshock (Roozendaal et al, 2009). There is extensive evidence that the amygdala, is critically involved in regulating stress effects on memory (McGaugh, 2004a; McGaugh et al, 2002b; Packard, 2009). Stress hormones and stress activated neurotransmitters enhance the consolidation of memory for emotionally arousing experiences through the amygdala (McGaugh, 2004a). However, although stress and emotional arousal enhances the consolidation of new memories, they can also impair our remembrance through amygdala interactions with other brain regions (Roozendaal et al, 2009).

It is now evident that intensely emotional events or chronic exposure to stressful experiences can create traumatic memories and even result in the development of mood and anxiety disorders, including post-traumatic stress disorder (PTSD) (Adshead,
Animal models indicate that acute and chronic stress induces a persistent functional and morphological alterations in the amygdala and other associated brain regions, such as the hippocampus and the prefrontal cortex, that leads to the increases in anxiety-like behavior (Roozendaal et al, 2009). In our study, we demonstrated that the endocannabinoid mobilization in the amygdala may play a significant role in the modulation of TMT-induced long-term anxiety-like behavior in rats. Here I will discuss some of my thoughts and unresolved issues on the endocannabinoid modulation of TMT-induced long-term anxiety and how it might relate to the human condition, PTSD.

**General Discussion**

To summarize, in the current thesis we report that the long-term anxiety-like state caused in rats by exposure to the fox pheromone TMT is paralleled by a sustained elevation in amygdalar levels of the endocannabinoid 2-arachidonoylglycerol (2-AG). We further show that systemic pharmacological inhibition of the 2-AG-hydrolyzing enzyme, monoacylglycerol lipase (MGL), or inhibition of the anandamide hydrolyzing enzyme, fatty acid amid hydrolase (FAAH), 18 h after exposure to TMT completely prevents the development of long-term anxiety-like behavior, which are abrogated by the CB₁ cannabinoid receptor antagonist, rimonabant. The amygdala appears to be crucial to this response, because local infusion of an MGL inhibitor into this structure recapitulates the actions of systemic MGL and FAAH blockade. We also demonstrate that administration of peripherally restricted FAAH inhibitor, URB937, 18 h post TMT-exposure prevents the development of the long-term anxiety-like behavior. Lastly, although URB597 and JZL184 has an acute anxiolytic effect, URB937 administration
does not induce an immediate anxiolytic-like influence. The results suggest that both the central and peripheral endocannabinoid system has a significant role in the development of long-term anxiety induced by severe trauma. Centrally, 2-AG mobilization in the amygdala acts as an intrinsic feedback mechanism that protects rats against the chronic consequences of TMT-evoked stress. Peripherally, CB₁ activation may dampen the stress response by excreting its influence on the brain through the ascending vagus nerve.

It has been hypothesized that robust emotional arousal induced by stress or anxiety leads to the facilitation and predilection towards the use of habit memory (Packard, 2009; Packard and Goodman, 2012). In a recent study, Leong and Packard (2014) show that TMT exposure biases rats towards response learning in a dual solution plus-maze task (Leong and Packard, 2014). In this study, the researchers trained the rats to swim from a consistent start arm (e.g. south) to a hidden escape platform located in a consistent goal arm (e.g. west) (Leong et al, 2014). Then the trained rats are given a probe trial starting from the opposite start box (Leong et al, 2014; Packard, 2009). Rats were tested for whether they use the knowledge of the spatial location of the platform employing hippocampus-dependent place learning or use a specific body turn to choose the opposite goal arm employing dorsal striatal-dependent response learning (Packard et al, 2012; Packard and McGaugh, 1996). Relative to control animals, rats exposed to TMT prior to maze training predominantly displayed response learning on the probe trial (Leong et al, 2014). In support of this finding, work from other groups has shown that rats exposed to a cat (predator stress) exhibit a suppression of hippocampal synaptic plasticity (Mesches et al, 1999; Packard,
2009), and an impairment of spatial memory (Diamond et al, 1999; Park et al, 2008; Sandi et al, 2005; Woodson et al, 2003). Moreover in one of these studies, while the predator stress exposure induced impairment in hippocampus dependent special learning, animals begins to employ response learning by resorting to body turn to find the hidden platform in a radial water maze (Park et al, 2008). It has been suggested that the basolateral amygdala (BLA) might be involved in the switch between the two memory systems (McGaugh, 2004a; Packard, 2009). Furthermore, many studies report that predator odors are incompatible with context fear conditioning (Fendt et al, 2008; Rosen et al, 2015; Staples, 2010), suggesting that life-threatening stress impairs of context dependent learning.

In addition, extreme stress conditions can allow fear to be generalize to cues that did not originally predict danger. For example, Kaouane et al, (2012) demonstrated that contextually fear conditioned animals with high threat stimulus (high intensity footshock) along with CORT (CORT) injections begin freezing to tones that were never presented previously (Kaouane et al, 2012). Additionally, animals that are exposed to high threat and CORT injections are no longer able to properly associate the shock to the context, while animals exposed to low threat conditions were able to correctly freeze to the context they were given the shock (Kaouane et al, 2012). The same group also demonstrated that there is a threshold CORT level where contextual fear conditioned animals fail to respond to the proper context and start freezing to novel tones (Kaouane et al, 2012). However, the group did not observe generalization of fear when the CORT injection was coupled with a lower intensity shock (Kaouane et al, 2012). This suggests
that high level of CORT in combination with an intense stressor may push the stress response system to a generalize fear and impair hippocampus function.

The research detailed in this thesis along with the data we collected indicates a critical and complex role of the endocannabinoid system in the regulation of stress and anxiety. Looking at the complete picture, we see that the endocannabinoid system is involved in the hypothalamic-pituitary-adrenal (HPA) axis regulation, aversive memory consolidation, and fear extinction. There is also evidence that the severity of stress can determine the engagement of different memory systems to respond to danger predicting stimuli. Under the exposure of moderate stress stimulus, anandamide content within the BLA rapidly decreases, which disinhibits the BLA and results in an activation of the HPA axis and glucocorticoid hormone secretion into the circulation (Hill et al, 2012; Morena et al, 2015). As glucocorticoid hormone concentrations rise and penetrate the brain, they bind to cytosolic or membrane glucocorticoid receptors in the paraventricular nucleus (PVN) of the hypothalamus and the amygdala, to indirectly induce endocannabinoid synthesis (Hill et al, 2012; Morena et al, 2015). Within the PVN, this increase in endocannabinoid signaling suppresses excitatory synaptic input to the CRH neurons and mediate the feedback inhibition of the HPA axis. Within the amygdala, the increase in endocannabinoid signaling functions to decrease the excitatory inputs to the BLA and reduces HPA axis activity (Hill et al, 2012; Morena et al, 2015). Therefore bringing the stress system back to homeostasis. Additionally, mild stress induced increase of glucocorticoid, norepinephrine, and the endocannabinoids in the amygdala, which facilitates the consolidation of aversive-memory (Campolongo et al, 2009; McGaugh, 2004a, 2015).
On the contrary exposure to a life-threatening stimulus induces a different type of neuronal response that may lead to a maladaptive long-term anxiety state. Similar to mild stress, exposure to extreme stress would likely result in immediate decrease in anandamide content within the BLA, leading to the increase of circulating glucocorticoids. As reported in several groups, rodents exposed to a severe stressor such as TMT exhibit an extreme level (9-11 fold increase) of plasma CORT levels (Morrow et al, 2002; Morrow et al, 2000; Tsutsumi et al, 2002). Consequently, the high level of CORT in the hippocampus can lead to a PTSD-like memory impairments causing rodents generalize fear cues that did not originally predict danger (Kaouane et al, 2012). Furthermore, extreme elevation of CORT coupled with a highly aversive stimulus have shown to cause animal to inappropriately generalizing fear to the proper context (Kaouane et al, 2012; Leong et al, 2014; Packard et al, 2012; Park et al, 2008), suggesting an impairment of a hippocampus driven behavior. Together, the research suggests that life-threatening stressor can increase CORT to unusual levels that decreases animal’s ability to restrict fear to the appropriate context. Thus possibly causing the TMT-exposed rats in our experiments to have persistent anxiety-like behavior on the EPM. This is similar to PTSD, since it is associated with negative emotional reactions triggered by specific situations, objects, or internal and external cues that are related to the original trauma but benign.

In our experiments, the exposure to TMT evokes a prolonged anxiety-like state that is paralleled by an increase in 2-AG levels within the amygdala. Our data indicates that, a single intra-BLA administration of NF1819, an MGL inhibitor, 18 h after TMT-exposure induced anxiolytic-like effects 7 days after the exposure. This suggests that
enhancing the mobilization of endogenous 2-AG in the BLA protects rats against an inappropriately severe response to a life-threatening stressor. It is also possible that the additional increase in 2-AG content in the BLA allows the animal to switch from dorsal striatal-dependent response memory to hippocampus-dependent place memory, restoring the ability to place the fear to the appropriate context. Therefore preventing the development of long-term anxiety-like behavior on the EPM. Furthermore, because previous research indicates that both the CORT and the endocannabinoid signaling is required for aversive memory consolidation (Campolongo et al, 2009), there may be a mechanism in which the endocannabinoid system closely interacts with the circulating glucocorticoids to modulate the different memory systems after a traumatic event. The balance of these two system in the BLA during or after trauma exposure might be critical for the development of long-term anxiety.

Peripherally, stress causes the release of CORT and epinephrine from the adrenal gland (Munck et al, 1984; Sapolsky et al, 2000). While the CORT readily enters the brain and exerts its modulatory effect on different brain regions involved in stressful memory, peripheral epinephrine cannot (Tasker et al, 2011). The epinephrine released from the adrenal gland activate peripheral β-adrenoceptors located on vagal afferents that terminate in the nucleus of the solitary tract (NST) (McGaugh, 2004a; Packard, 2009). In turn, noradrenergic cell groups in this brain region send direct projections to the amygdala and release norepinephrine (McGaugh, 2004a, 2015; Packard, 2009). The norepinephrine activity in the BLA is essential in mediating the modulatory effects of other hormones and neurotransmitters on aversive memory consolidation (Figure 6.1) (McGaugh, 2004a, 2015; Packard, 2009). In our study, we demonstrate that blockade of
peripheral FAAH inhibitor can completely suppress the TMT induced the long-term anxiety-like behavior. This is possibly due to the dampening of the peripheral vagus innervation through the activation of the vagal CB$_1$ receptors. Therefore preventing the release of norepinephrine in the BLA which is essential for consolidating aversive memory. However, several studies report that peripherally restricted beta adrenergic receptor antagonist, atenolol, is significantly less effective in attenuating PTSD symptoms when compared to a global beta blocker, propranolol (Bhuvaneswar et al, 2014; Bobo et al, 2007; Padala et al, 2007). This suggest that peripheral endocannabinoid signaling may have a much more complex mechanism in modulating long-term anxiety brought on by severe stress. Further studies should determine whether TMT induces a change in peripheral endocannabinoid signaling, and whether the peripheral FAAH inhibitor indeed causes the reduction of norepinephrine in the BLA via the vagal afferents. Moreover, the amygdalar norepinephrine may moderate CORT and endocannabinoid activity in the BLA to further modulate aversive memory consolidation.

Taking everything together, I hypothesize that within the BLA, there is a delicate balance among glucocorticoids, endocannabinoids, and norepinephrine that modulates the consolidation of aversive memory and long-term anxiety. During an exposure to a highly stressful stimuli, high level of glucocorticoids in the brain drives animals to resort to dorsal striatal-dependent response memory, thus decreasing animal’s ability to restrict fear to the appropriate context. However, if the delayed increase in 2-AG level reaches a threshold, the system reverts back towards hippocampus-dependent place memory, restoring the animal's ability to place fear into the appropriate context. In
addition, studies have provided compelling evidence that the presence of a prolonged peripheral adrenergic activation during a life-threatening event contributes to over consolidation of memory for the trauma and thereby supports the development of the intrusive symptoms found in PTSD (Hurlemann et al., 2010; O'Donnell et al., 2004; Pitman, 1989). With the possible link between the peripheral endocannabinoid system and the β-adrenergic system, the peripheral endocannabinoid dependent suppression of norepinephrine release in the BLA may have a significant impact on aversive memory consolidation and long-term anxiety development. (Figure 6.2). However, there is very little evidence of this idea and should be further investigated.

**Unresolved Issues and Future Directions**

There are many unresolved issues that should be addressed in future experiments. Here I briefly list out few major issues that I feel should be addressed to better understand the predator stress induced long-term anxiety.

**Unresolved Issue 1: Finding the molecular mechanism that causes the persistent increase in 2-AG after TMT exposure.** Although we have screened for several enzymes involved in the synthesis and the degradation of 2-AG, the mechanism still a mystery. Future experiments should look for whether there are changes in the number of synapses after TMT exposure or a possible epigenetic mechanism that could explain this persistent change.

**Unresolved Issue 2: Time window during which MGL or FAAH inhibition suppresses TMT-induced anxiety.** In our study, we demonstrated that a single administration of either FAAH or MGL inhibitor 18 hours after TMT exposure causes
reduction anxiety-like behavior 7 days after. It will be important to identify the precise time window after TMT exposure during which FAAH or MGL inhibition is most effective at suppressing TMT-induced anxiety-like responses. The results of this investigation will show if there is indeed a consolidation window where the long-term anxiety is established and how long after the trauma the intervention will be effective. Once this window is identifies, further studies can tease out the neurochemical changes that regulate this window.

**Unresolved Issue 3: How does the early administration of peripherally restricted FAAH inhibition prevent TMT-induced long-term anxiety?** It will be essential to investigate how the peripheral FAAH inhibitor is involved in the development of long-term anxiety after trauma. This will not only advance our knowledge on the role of the peripheral system in modulating the adaptive response to stress, but it will help identify novel pharmacological targets to treat trauma victims without the psychotropic side effects most drugs available today.

**Unresolved Issue 4: Potential interaction between amygdalar glucocorticoids, endocannabinoid, and norepinephrine in modulating aversive memory consolidation and development of long-term anxiety.** I hypothesize that an imbalance of glucocorticoids, endocannabinoids, and norepinephrine in the BLA is responsible for the over generalization of fear and stress cues. Further studies should investigate the possibility that high level of glucocorticoid and norepinephrine may push the animals to resort to response learning, while high levels of endocannabinoids may restore place learning under extreme stress conditions.
Conclusions

In conclusion, the amygdala has unique features of structural and functional plasticity that modulates the memory of emotionally arousing, aversive, and fearful experiences. However, abnormal changes to the amygdala functions can have pathological consequences of chronic and severe stress. Furthermore, any functional changes in the amygdala seems to propagate to other interconnected brain regions and the peripheral systems. Overall, the central and the peripheral systems needs work in concert to maintain homeostasis, and disruptions in either side may result to a maladaptive outcome. Our study suggests that exposure to predator odor initiates changes in amygdalar 2-AG signaling, which might play a modulatory/protective role in the response to a traumatic event. Additionally, manipulations in the peripheral endocannabinoid system can suppress long-term anxiety brought on by a life threatening stimulus. These results further suggest that pharmacological agents that enhance brain 2-AG signaling or selectively enhance peripheral endocannabinoid signaling may attenuate anxiety-like responses in stressed animals and anxiety symptoms in human trauma victims.
Figure 6.1: Emotional arousal-induced modulation of memory consolidation.
Emotionally arousing and aversive experiences induces the release epinephrine and glucocorticoids from the adrenal gland and induce the release of norepinephrine (NE) in the basolateral amygdala (BLA). Peripheral epinephrine, which does not cross the blood–brain barrier, induces the release of NE in the BLA by activating vagal afferents to the nucleus of the solitary tract (NST). Adrenergic neurons in the NST project both directly and indirectly to the BLA. Glucocorticoids freely enter the brain and can directly bind to glucocorticoid receptors in the BLA. The Glucocorticoid can induce the release
of endocannabinoids (eCB) modulating the activity within the BLA. Such stress-induced BLA activity modulates memory consolidation via influencing neuroplasticity of downstream brain targets that are involved in stress response. In addition, glucocorticoids can directly activate other brain regions to enhance or impair memory consolidation. Overall the BLA may be involved in recruiting the hippocampus for spatial or contextual information or the caudate nucleus (dorsal striatum) for procedural information.
Figure 6.2: High stress and glucocorticoid may recruit multiple memory systems to modulate long-term anxiety. During exposure to highly stressful stimuli, high level of glucocorticoids drives animals to resort to dorsal striatal-dependent response memory, thus decreasing animal's ability to restrict fear to the appropriate context. However, if the delayed increase in 2-AG level reaches a threshold, the system reverts back towards hippocampus-dependent place memory system, restoring the animal's ability to place the fear to the appropriate context. When an individual have moderate glucocorticoid increase during a stress response, they do not develop long-term anxiety and properly contextualize fear. The difference in the initial glucocorticoid levels and the delayed increase in endocannabinoid levels in the amygdala may account for the sub-group of the population that experiences trauma, but fail to develop PTSD.
REFERENCES


