

**Effects of LTD-blocking Tat-GluR2 peptide on contextual fear memory  
impairments induced by cannabinoids**

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## **ABSTRACT**

The mechanisms underlying cannabinoid impairment of fear memory is not clear. This study investigated the effects of the synthetic cannabinoid HU210 and the endocannabinoid hydrolysis inhibitor JZL 195 on fear memory following contextual fear conditioning (CFC; an animal model of fear). The long-term depression (LTD)-blocking peptide Tat-GluR2 was utilized to investigate whether the expression of cannabinoid-induced LTD (CB-LTD) is required for the cannabinoid impairment of acquisition and consolidation of contextual fear memory. HU210 reduced freezing throughout the test phase of the acquisition protocol, which was not affected by pre-administration of Tat-GluR2. High and moderate doses of HU210 reduced freezing during the first and last half, respectively, of the test phase of the consolidation protocol, which was prevented by pre-treatment with Tat-GluR2. HU210 did not affect freezing during the test phase of the retrieval protocol. Thus, these results suggest that HU210 impairs acquisition and consolidation, but not retrieval of contextual fear memory, and that *in vivo* CB-LTD expression is required for HU210 impairment of the consolidation, but not acquisition, of contextual fear memory. We also observed that HU210 and JZL 195 do not facilitate the acquisition of contextual fear memory extinction.

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## LIST OF ABBREVIATIONS

<b>2-AG</b>	2-arachidonylglycerol
<b>ACh</b>	acetylcholine
<b>AEA</b>	arachidonylethanolamide
<b>AMPAR</b>	$\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor
<b>ANS</b>	autonomic nervous system
<b>B</b>	basal nucleus of the amygdala
<b>BII</b>	blood-injection-injury
<b>BLA</b>	basolateral amygdala complex
<b>Ca<sup>2+</sup></b>	calcium
<b>CB-LTD</b>	cannabinoid-induced long-term depression
<b>CBF</b>	cerebral blood flow
<b>CBT</b>	cognitive-behavioural therapy
<b>CB<sub>1</sub>R</b>	cannabinoid type 1 receptor
<b>CB<sub>1</sub>R-KO</b>	cannabinoid type 1 receptor knockout
<b>CE</b>	central nucleus of the amygdala
<b>CFC</b>	contextual fear conditioning
<b>CG</b>	central gray
<b>CR</b>	conditioned response
<b>CS</b>	conditioned stimulus
<b>DAGL</b>	diacylglycerol lipase
<b>EMDR</b>	eye movement desensitization and reprocessing
<b>EMT</b>	endocannabinoid membrane transporter
<b>FAAH</b>	fatty acid amide hydrolase

<b>fMRI</b>	functional magnetic resonance
<b>GABA</b>	gamma-aminobutyric acid
<b>GABA<sub>A</sub>R</b>	gamma-aminobutyric acid type A receptor
<b>Glu</b>	glutamate
<b>GPCR</b>	G-protein coupled receptor
<b>HFS</b>	high frequency stimulation
<b>ICM</b>	intercalated masses
<b>IL</b>	infralimbic subregion of the mPFC
<b>ITI</b>	inter-trial interval
<b>LA</b>	lateral nucleus of the amygdala
<b>LH</b>	lateral hypothalamus
<b>LTD</b>	long-term depression
<b>LTP</b>	long-term potentiation
<b>MGL</b>	monoacylglycerol lipase
<b>mPFC</b>	medial prefrontal cortex
<b>MWM</b>	Morris water maze
<b>NMDAR</b>	N-methyl-D-aspartic acid receptor
<b>NS</b>	neutral stimulus
<b>PE</b>	phosphatidylethanolamine
<b>PET</b>	positron emission tomography
<b>PI</b>	phosphatidylinositol
<b>PL</b>	prelimbic subregion of the mPFC
<b>PLD</b>	phospholipase D
<b>PTSD</b>	posttraumatic stress disorder

<b>PVN</b>	paraventricular nucleus of the hypothalamus
<b>THC</b>	delta-9-tetrahydrocannabinol
<b>US</b>	unconditioned stimulus
<b>VAB</b>	ventral angular bundle
<b>WT</b>	wild-type

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# **INTRODUCTION**

## **1. Fear and Anxiety**

### **1.1. Overview**

Fear and anxiety are functionally adaptive emotional responses to danger or threats that elicit relevant defensive reactions that can reduce risk or harm (Rosen and Schulkin, 1998). More specifically, fear is an instantaneous alarm response to immediate danger or an urgent situation. Fear is beneficial because it activates the fight or flight response, a biological reaction to distressing stimuli that accumulates the body's resources to defend against or escape the threat. Anxiety is a negative mood state in which a person displays tension and uneasiness about the future. As with fear, a modest amount of anxiety is favourable and can facilitate a person's performance (Barlow et al., 2006). Typical characteristics of fear and anxiety in humans and animals include: increased startle, heart rate changes, frequent defecation and urination, hypervigilance, and persistent avoidance. Additionally, animals exhibit fighting, biting, freezing, and escape behaviours, while humans can experience feelings of numbness and/or irritability and have problems sleeping (Rosen and Schulkin, 1998).

The amygdala is commonly stated as the main brain structure involved in the production of both normal and pathological fear responses (Davis, 1992; LeDoux, 2000). However, it is not the only key structure involved (Chhatwal and Kessler, 2007; LeDoux, 2007; Maren, 2008). For instance, Bechara et al. (1995) found that humans with bilateral damage to the amygdala did not demonstrate conditional fear responses to stimuli paired with loud noise, but had unharmed declarative memory for the visual or auditory stimuli

associated with the loud noise. Furthermore, Vazdarjanova and McGaugh (1998) determined that rats that received bilateral excitotoxic lesions of the basolateral amygdala complex (BLA) did not freeze in response to contextual cues associated with shock, but did avoid the compartment in which they were given the footshocks. Thus, as was alluded to by the studies mentioned, the hippocampus and the medial prefrontal cortex (mPFC) are also involved in the formulation of an aversive emotional experience (Chhatwal and Kessler, 2007; LeDoux, 2007; Maren, 2008). The hippocampus allows for the ability to discriminate between threat levels posed by stimuli in various situations, adding contextual regulation (LeDoux, 2007; Maren, 2008). For example, the hippocampus will give an individual the ability to recognize that one should be more afraid of a bear on one's campsite than a bear at the circus. The mPFC, on the other hand, is responsible for adjusting the degree of fear that the amygdala conveys (LeDoux, 2007; Maren, 2008).

## **1.2. Psychopathophysiology**

Cue-specific (i.e., anxiety and fear is triggered by a specific object or situation; Cryan and Holmes, 2005) fear-related psychiatric disorders associated with exaggerated fear responses and persistent aversive memories include specific phobia and posttraumatic stress disorder (PTSD), respectively (Rosen and Schulkin, 1998; Cryan and Holmes, 2005; Moreira and Wotjak, 2010).

### **1.2.1. Specific Phobia**

Specific phobia is the most common type of anxiety disorder (Paquette et al., 2003; Stein and Matsunaga, 2006, Straube et al., 2006a), with a lifetime prevalence of 6-

12% (Kessler et al., 2010). A specific phobia is described as an illogical fear of a specific situation or object that noticeably interferes with the individual's daily life (Barlow et al., 2006), and induces an immediate, amplified and enduring fear reaction (American Psychiatric Association, 1994). Specific phobia comprises four major subtypes, including animal type, natural environment type (e.g., water or heights), blood-injection-injury (BII) type, and situational type (e.g., public transportation or enclosed spaces). There is also a fifth category entitled "other" that consists of phobias that do not fall under any of the four major subtypes (e.g., events that could lead to choking; Veltman et al., 2004; Barlow et al., 2006). Importantly, approximately 80% of BII phobics undergo a biphasic response characterized by an initial increase, followed by a subsequent decrease, in heart rate and blood pressure, while all other specific phobias generate increased arousal and peripheral activation (Caseras et al., 2010). Animal, natural environment, and BII subtypes tend to emerge in children with average ages of onset being seven (Öst, 1987; Antony et al., 1997), seven (Barlow et al., 2006), or nine (Öst, 1989b; Antony et al., 1997), respectively. However, situational phobia transpires between the ages of twenty to twenty-five, and tends to run in families like BII phobia (Curtis et al., 1990; Barlow et al., 2006).

According to Rachman (1977), there are at least three ways to acquire specific phobias, including via a direct experience, a vicarious experience (i.e., viewing someone endure a fearful incident), or via information transmission (i.e., repeatedly being informed about possible danger). Additionally, experiencing a panic attack in a specific situation can also generate a specific phobia. For example, a study conducted by Munjack (1984) found that 40% of those telephone interviewed indicated that their

specific phobia of driving was a consequence of them experiencing a panic attack while driving on the freeway.

The functional neuroanatomy that underlies specific phobias has yet to be clearly determined. Brain imaging studies that have attempted to determine the neural correlates underlying specific phobias, particularly animal phobias, have produced inconsistent results (Veltman et al., 2004; Straube et al., 2006b). For instance, a positron emission tomography (PET) study conducted by Mountz et al. (1989) found that after accounting for anxiety-induced hyperventilation, significant differences in absolute global and regional cerebral blood flow (CBF) between animal type phobics and control participants disappeared during a rest-fear-rest-fear-rest repeated measures task. In contrast, a PET investigation by Wik et al. (1993) revealed increased regional CBF in the secondary visual cortex, but decreased regional CBF in the hippocampus and prefrontal, orbitofrontal, temporopolar, and posterior cingulate cortices, upon phobic stimuli presentation. Furthermore, an event-related functional magnetic resonance (fMRI) imaging study found that spider phobics had increased activation in the amygdala, compared to controls, in response to visually presented phobia-related stimuli (Dilger et al., 2003), whereas other studies have failed to show amygdala activation (Mountz et al., 1989; Wik et al., 1993; Paquette et al., 2003). Discrepancies in results may be attributed to differences in methodology, and thus more standardized studies need to be conducted. Importantly, an fMRI study by Caseras et al. (2010) revealed the importance of comparing different specific phobia subtypes and determined that phobia-related visual stimuli lead to increased activation mainly in the thalamus and occipito-temporo-parietal cortex of BII phobics, while increased activation was seen in the dorsal anterior cingulate

and anterior insula of animal phobics. Thus, additional standardized investigations that compare the different subtypes of specific phobia are necessary to determine the underlying brain circuits of this anxiety disorder.

Although there are countless specific phobias that an individual could develop via numerous means, there is a consensus about what treatment presently has proven to be most successful in treating specific phobias. Cognitive-behavioural therapy (CBT) is an effective treatment for specific phobias. It consists of exposure-based exercises involving multiple presentations of phobogenic stimuli, gradually increasing intensity with each subsequent presentation (e.g., a spider phobic might initially be asked to look at pictures of spiders, then look at live spiders, and eventually hold a live spider in his/her hand, during multiple succeeding therapy sessions), as well as instructions about how to modify the misbeliefs associated with the stimuli (Öst, 1989a; Paquette et al., 2003; Veltman et al., 2004; Barlow et al., 2006). For instance, Öst (1996) performed a study in which participants with spider phobia received a single three hour session of CBT, in either a group of three or four participants, or a group of seven or eight participants. Ultimately, Öst (1996) determined that one session of group exposure therapy was just as effective in treating spider phobias, regardless of group size, and was almost as successful as individual treatment. This gold standard treatment of exposure therapy utilizes extinction procedures that are based on the idea that repeated exposure to the fear-eliciting stimuli will lead to a reduction of fear responses (Veltman et al., 2004; Milad et al., 2006).

### 1.2.2. PTSD

Of all anxiety disorders, PTSD is the third most common, with a lifetime prevalence of 1-2% in Western Europe, 6-9% in North America, and over 10% in countries where there is long-lasting incidents of sectarian violence (Kessler et al., 2010). PTSD, which cannot be diagnosed until at least one month after the ordeal, is characterized by persistent feelings of fear, helplessness, or terror in response to a traumatic experience (e.g., war, rape, natural disaster), or an unexpected loss of a loved one. The victim re-experiences the ordeal via memories and nightmares, evades cues associated with it, and acquires increased arousal and vigilance, and a numbing of emotional responsiveness (King et al., 1996; Barlow et al., 2006). Furthermore, PTSD can be diagnosed as being acute or chronic, lasting between one and three months, or more than three months, respectively, after the traumatic occurrence. Individuals who display only a few, if any, symptoms instantly after an ordeal, but form clinical PTSD, possibly even years afterwards, are said to have delayed-onset PTSD (Barlow et al., 2006).

PTSD is the only anxiety disorder that has a known etiology, which entails the sufferer to personally endure a traumatic experience (Barlow et al., 2006). However, not everyone who experiences distress necessarily acquires PTSD. A couple of studies determined that a close connection with the traumatic event appears to be essential in the development of PTSD (King et al., 1996; Keane and Barlow, 2002). Resnick et al. (1993) utilized telephone interviews to carry out an investigation employing American adult women, and determined that the percentage of those who experienced direct assault

(i.e., sexual or physical) and developed PTSD increased as a function of the seriousness of the distressing experience. Moreover, twin studies suggest that the probability of developing PTSD also depends on an individual's own biological and psychological (i.e., personality characteristics that influence whether you enjoy risky or safe environments) vulnerabilities. For instance, True et al. (1993) surveyed 4042 Vietnam era veteran monozygotic (55%) and dizygotic (45%) twin pairs, and found that a monozygotic twin was more likely to acquire PTSD compared to a dizygotic twin, given the same quantity of combat exposure and one twin with PTSD. Furthermore, Stein et al. (2002) utilized over 400 identical and fraternal twin pairs, and determined that PTSD symptoms resulting from noncombat trauma are also heritable, and that experiencing particular types of trauma (i.e., assaultive but not nonassaultive) was also associated with genetics. Lastly, social and cultural factors have also been recognized to play a significant part in the generation of PTSD, in that an individual is much less likely to develop PTSD if they possess a strong, supportive network of people (Barlow et al., 2006).

A neurocircuitry model of PTSD has indicated three brain regions that are hypothesized to underlie the pathophysiology of PTSD: the amygdala (i.e., important in the evaluation of threat-related stimuli and fear conditioning) is thought to be hyperresponsive in PTSD, the mPFC (including the anterior cingulate cortex, subcallosal cortex, and medial frontal gyrus; i.e., important to the process and retention of extinction of fear conditioning) is believed to be hypo-responsive, and in conjunction with the hippocampus (i.e., important to explicit memory processing and context encoding during fear conditioning) is hypothesized to fail to inhibit the amygdala in PTSD (Shin et al., 2006). Although some neuroimaging studies have shown that there are no significant

differences in amygdala volumes between adult PTSD patients, who were traumatized by war (Bremner et al., 2005) or were victims of childhood sexual abuse (Bremner et al., 1997), and controls, the severity of PTSD symptoms have been found to be positively correlated with amygdala activation (Protopopescu et al., 2005). Importantly, a PET study by Bremner et al. (2005) employed female PTSD patients, who were sexually abused as children, and found that they had increased amygdala activation and decreased anterior cingulate function, during fear acquisition and extinction, respectively. These findings provide support for the hypothesis that PTSD victims have hyperresponsive amygdalae because of the lack of inhibitory modulation from the mPFC. Also, many studies have illustrated that areas within the mPFC of PTSD patients have reduced volumes (Rauch et al., 2003; Yamasue et al., 2003; Woodward et al., 2006). Both neuroimaging and neuropsychological studies have implicated the hippocampus as being a key brain structure involved in PTSD (Bremner, 2006). For example, Bremner et al. (1995) found that patients with war-related PTSD, compared to controls, had significantly smaller right hippocampal volumes, which were correlated with short-term verbal memory deficits. Additionally, Gurvits et al. (1996) determined that Vietnam veterans with PTSD had significantly smaller left and right hippocampi compared to controls. PTSD patients, who were adult survivors of childhood sexual abuse, were also revealed to have left hippocampal volumes that were significantly smaller compared to controls (Bremner et al., 1997).

Similar to treatment for specific phobias, most clinicians concur that exposure therapy is necessary to produce successful coping skills to manage and/or conquer PTSD (Foa and Meadows, 1997; Keane and Barlow, 2002). However, unlike with most specific

phobias that concern objects, the distressing ordeals that cause PTSD are hard to replicate, and thus imaginal, as opposed to *in vivo*, exposure is utilized (Foa and Meadows, 1997; Barlow et al., 2006). Other psychosocial treatments for PTSD include Donald Meichenbaum's CBT, in which the patient learns to modify his/her meaning associated with the distressing ordeal and acquires adaptive coping skills and a survivorship awareness (Meichenbaum, 1994), eye-movement desensitization and reprocessing (EMDR), in which the patient is asked to keep his/her eyes on the therapist's moving finger while thinking about the traumatic experience (Shapiro, 1995; Foa and Meadows, 1997; Shapiro, 1999), resulting in prompt re-encoding of the disturbing situation (Shapiro, 1999), and relaxation therapy. A study that compared these three psychosocial PTSD treatments was conducted by Taylor et al. (2003) and determined that exposure therapy was better than EMDR and relaxation therapy because it yielded greater reductions in avoidance and cognitive re-experiencing, generated more rapid reductions in avoidance, and resulted in more PTSD-free patients after treatment. In spite of this, exposure therapy is unsuccessful in treating some patients with PTSD (Foa, 2000; van Minnen et al., 2002), alluding to the fact that there is a deficit in extinction learning (Milad et al., 2006).

In regards to the use of pharmacotherapy, although existing anxiolytics are effective, undesirable side effects, such as impaired arousal, amnesic effects, tolerance and dependence, sanction the need for novel treatments for fear-related anxiety disorders, such as PTSD (Chhatwal and Ressler, 2007). The endocannabinoid system seems to be an optimal place to start looking for new drug targets for the development of novel

anxiolytics with fewer side effects, considering the fact that ample research has revealed its involvement in fear and anxiety, and memory modulation.

## **2. Animal Models of Fear and Anxiety**

Animal research concerning fear and anxiety has been conducted to gain insight into the neural mechanisms that underlie fear and anxiety in humans (Cryan and Holmes, 2005). Unlike human research, animal research allows for the use of invasive techniques (e.g., cannula implantation) and molecular technologies (e.g., gene targeting) to help deduce these neural mechanisms (Cryan and Holmes, 2005).

### **2.1. Unconditioned Fear and Anxiety**

The fundamental idea behind animal models of unconditioned fear and anxiety is the existence of a struggle between two contrasting innate incentives, one being the desire to examine a new environment, probably to attain food, shelter, or a mate, or to escape, and the other being the need to evade potentially unsafe places (Rodgers and Dalvi, 1997; Moreira and Wotjak, 2010). The elevated plus maze (EPM), the light-dark avoidance test, and the open field test are the main behavioural tasks utilized under these circumstances (Fraser et al., 2010). The commonality amongst these paradigms are that they all evaluate avoidance of aversive compartments, be it elevated open arms in the EPM, brightly lit compartments in the light-dark avoidance apparatus, or the centre of an open space of an open field apparatus. These exploratory-based approach-avoidance tasks have face validity because several anxiety disorders are characterized by a persistent avoidance of a feared item or circumstance (Cryan and Holmes, 2005). However, more important is the fact that these tests have predictive validity in that

clinically efficacious anxiolytics, although mainly of the benzodiazepine class including diazepam, have been shown to reduce avoidance behaviours (Rodgers, 1997).

It is generally acknowledged that approach-avoidance conflict tests, such as the EPM, the light-dark avoidance test, and the open field test, do not measure identical anxiety-related behaviours. However, how these inter-task differences correlate with different types of human anxiety is still unknown (Rodgers, 1997; Cryan and Holmes, 2005). Another limitation of these behavioural tests is that, since they are based on the interaction among approach and avoidance behaviours, they do not adequately distinguish whether the behaviours result from diminished anxiety-related avoidance, or from enhanced novelty-seeking or impulsivity-related approach (Cryan and Holmes, 2005). Furthermore, behavioural performance on these tests is greatly dependent on intact sensory and motor function, which poses a problem when utilizing certain drugs, if they have sedative effects for instance (Cryan and Holmes, 2005). Thus, although exploratory-based approach-avoidance tests have a high degree of ecological validity (Rodgers, 1997), their limitations highlight the need for alternate methods to aid in the investigation of anxiety- and fear-related behaviour in animals.

## **2.2. Conditioned Fear**

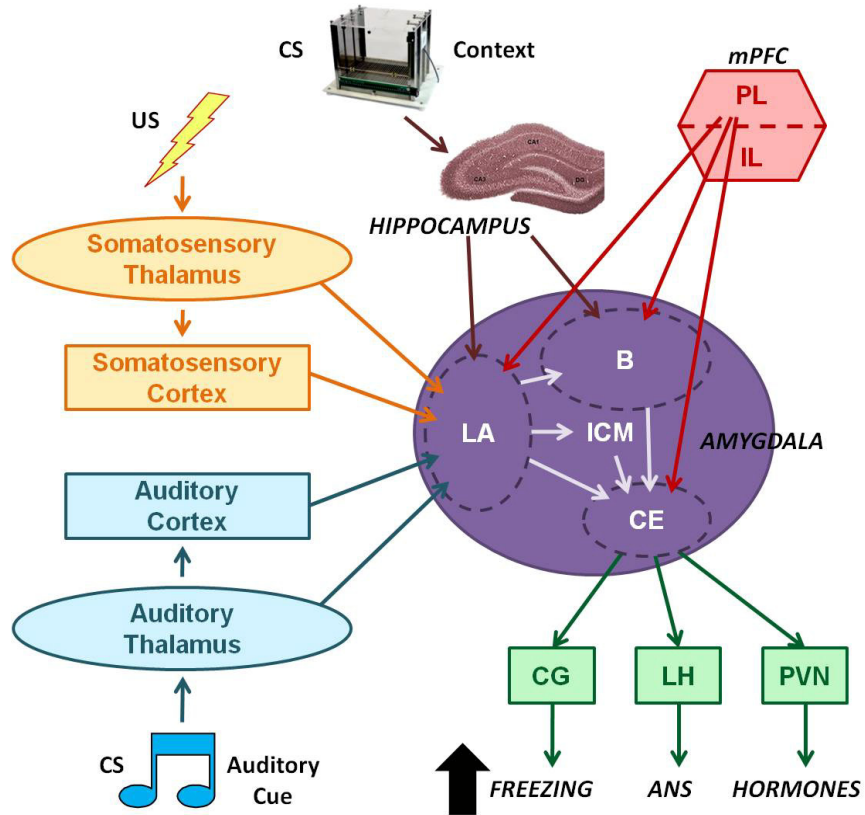
Animal models of conditioned fear are based on Pavlovian conditioning, with which animals associate a previously neutral stimulus (NS; i.e., cued fear conditioning) or environment (i.e., contextual fear conditioning; CFC) with an innately aversive unconditioned stimulus (US; e.g., footshock). Subsequent encounters with the stimulus or environment (i.e., now called a conditioned stimulus; CS) then evoke typified fear

responses (i.e., conditioned response; CR), including freezing and potentiated startle responses, in the absence of the US (LeDoux, 2000; LeDoux, 2007; Maren, 2008). Investigations about fear have been successful mainly because fear conditioning is an effective neurobiological tool that comprises measurable physiological and behavioural responses that are consistently elicited by a known, experimenter-regulated stimulus, upon learning. This seemingly unnatural technique emulates everyday life occurrences, in which the US produces injury and the CS inadvertently becomes associated with the US, consequently evoking a fear response upon a future encounter (Johansen et al, 2011). For instance, a rat that was injured by a snake, but managed to get away, might develop a fear response when hearing the sound of the rustling leaves on the trees in the forest as was heard during the snake attack.

The utilization of freezing behaviour as a measure of learned fear was established in 1969 (Blanchard and Blanchard, 1969), and has become the indicator of learned fear in the most commonly used fear conditioning paradigms (i.e., cued and contextual fear conditioning) employed in investigations of the neural mechanisms underlying emotional learning and memory (Maren, 2008). Cued fear conditioning can be broken down into two types, one being delay conditioning and the other being trace conditioning. In delay conditioning, the US co-terminates with, or is presented directly after the CS (i.e., cue). Conversely, in trace conditioning, the US is presented after a variable time interval has passed after CS (i.e., cue) termination, and thus requires supplementary brain regions, such as the hippocampus, in order to construct a temporal association between the CS and US. Since the animal learns to associate the US with, not only the cue, but also the context, in cued fear conditioning, researchers can perform a pre-exposure trial before

conditioning, in which the animal is exposed to the conditioning chamber in absence of the US, in an attempt to separate context from cue conditioning. However, this tactic is not entirely sufficient (Curzon et al., 2009). CFC, on the other hand, is the most simple of the fear conditioning paradigms, in which subjects' freezing behaviour is measured in response to associative learning of an aversive US with a particular context (i.e., CS; Curzon et al., 2009). CFC animal models have demonstrated that different signalling components have been found to play a role in distinct stages of memory processing: acquisition (i.e., association of the US with the CS), consolidation (i.e., stabilization of the learned association into long-term memory), and retrieval (i.e., utilization of the memory; Garelick and Storm, 2005). Several studies have conveyed that CFC findings delineate dorsal, as opposed to ventral, hippocampal-dependent memory (Anagnostaras et al., 2001; Curzon et al., 2009), specifically in the acquisition and consolidation of contextual representations (Anagnostaras et al., 2001). For instance, Young et al. (1994) found that rats that were pre-exposed to the conditioning context twenty-eight days before they were given electrolytic lesions to the dorsal hippocampus were protected from anterograde amnesia (i.e., the inability to associate the conditioning context with a US), suggesting that the dorsal hippocampus must create a contextual representation during the pre-exposure that is eventually stored in a different brain region. Importantly, although lesions of the dorsal hippocampus tend to spare cued fear conditioning, neurotoxic lesions of the ventral hippocampus have been shown to produce cued fear conditioning acquisition and expression deficits (Maren and Holt, 2004). Accordingly, cued and contextual fear conditioning are mediated by similar, but not identical, neural systems.

Numerous investigations have revealed that the same neural circuits, involving the amygdala, hippocampus, and the mPFC, implicated in human anxiety, also underlie conditioned fear behaviour (Cryan and Holmes, 2005). The fundamental neural circuit underlying cued and contextual fear conditioning is as follows (Figure 1). Sensory modality specific thalamic and cortical CS (i.e., cue) and US (e.g., footshock) inputs converge on the lateral nucleus of the amygdala (LA; LeDoux, 2007), thus providing the CS, in absence of the US, with the ability to elicit freezing (Sotres-Bayon et al., 2006). For example, Romanski et al. (1993) recorded single-unit activity in the LA of anaesthetized rats while presenting acoustic (i.e., clicks) and somatosensory (i.e., footshock) stimuli, and determined that neurons in the dorsal part of the LA reacted to both auditory and somatosensory stimuli, suggesting that the LA is the site of convergence of CS and US inputs in auditory fear conditioning. More specifically, the convergence of CS and US inputs to single LA projection neurons results in enhancement (i.e., long-term potentiation; LTP) of excitatory postsynaptic potentials (EPSP) induced by the CS. In other words, this synaptic plasticity increases the output of the LA projection neurons in response to the CS (Sah et al., 2003). CS-induced information is then conveyed from the LA to the central nucleus of the amygdala (CE) directly and via other regions of the amygdala, including the intercalated masses (ICM), which gate the output, and the basal nucleus of the amygdala (B), which processes hippocampal contextual information (Sotres-Bayon et al., 2006; LeDoux, 2007; LeDoux, 2012). Noteworthy is a study by Maren and Fanselow (1995) that found that long and numerous trains of high frequency stimulation (HFS) of the ventral angular bundle (VAB; i.e., axonal projections from the hippocampus to the BLA) induced N-Methyl-D-aspartic acid



**Figure 1. Neural circuit of cued and contextual fear conditioning.** During cued fear conditioning, sensory modality specific thalamic and cortical conditioned cue (i.e., conditioned stimulus; CS) and unconditioned stimulus (US; e.g., footshock) inputs converge on the lateral nucleus of the amygdala (LA). Contextual fear conditioning differs slightly, however, in that contextual information (i.e., CS) is processed in the hippocampus. The hippocampus then conveys configural representations of the context to the LA and basal nucleus of the amygdala (B), where they are associated with the aversive US. Convergence of CS and US inputs on the LA leads to increases in LA projection neuronal output in response to the CS. CS-induced information is then conveyed from the LA to the central nucleus of the amygdala (CE) directly and via other regions of the amygdala, including the intercalated masses (ICM), which gate the output, and the B, which processes hippocampal contextual information. Additionally, glutamatergic excitatory projections from the prelimbic (PL) subregion of the medial prefrontal cortex (mPFC) synapse onto the LA, B, and CE, augmenting CE output. Finally, CE projections terminate in brainstem and hypothalamic areas, including the central gray (CG), the lateral hypothalamus (LH), and the paraventricular nucleus of the hypothalamus (PVN), which mediate the physiological responses, including freezing behaviour, autonomic nervous system (ANS) responses, and hormonal responses, respectively.

receptor (NMDAR) -dependent LTP in the BLA, and that electrolytic lesions of the BLA and hippocampal regions that project to the BLA diminished fear conditioning to a contextually conditioned stimulus, implying that plasticity at hippocampal-BLA synapses is important in CFC. More specifically, the hippocampus is essential for processing contextual information and conveying configural representations of the context to the amygdala, where they are correlated with the aversive US (Rudy et al., 2004).

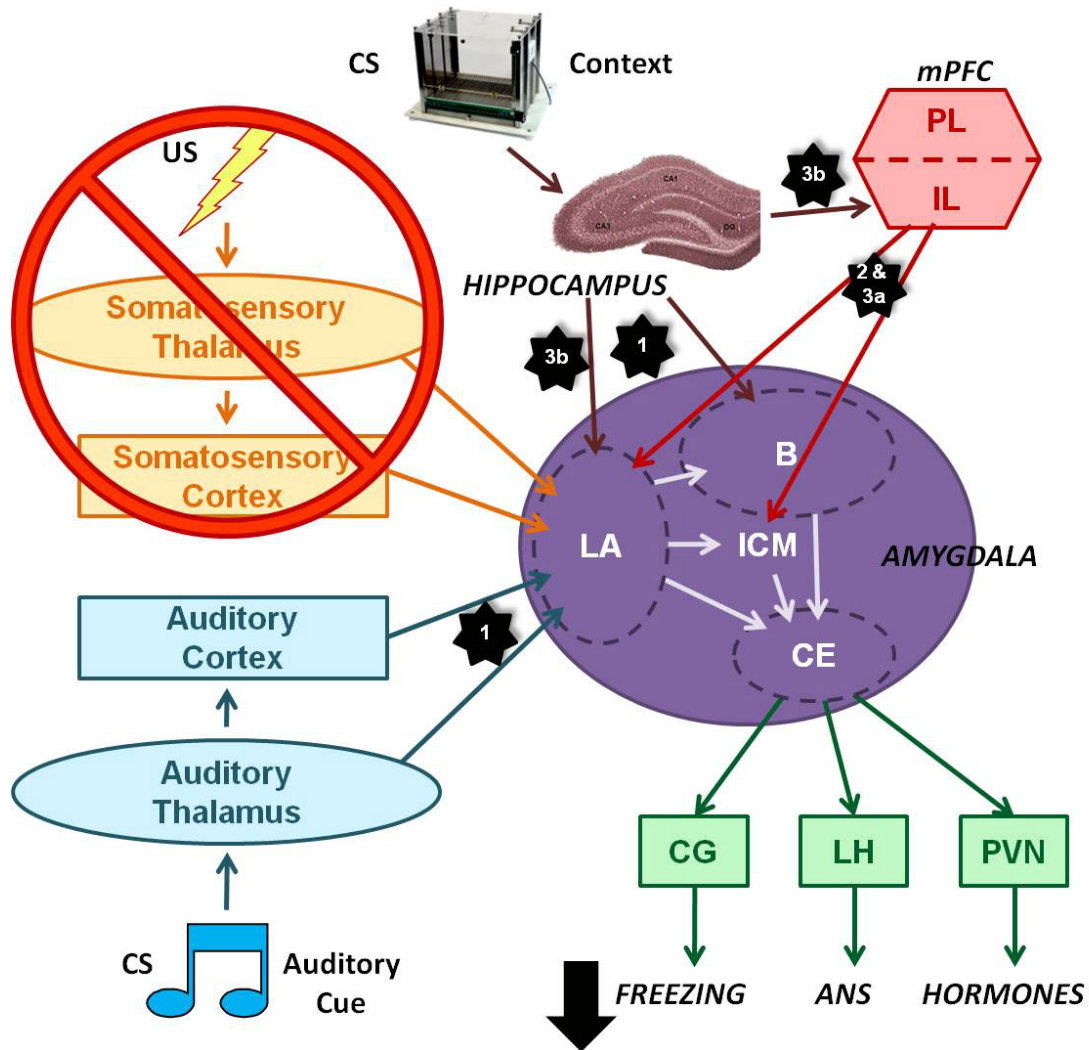
Furthermore, glutamatergic excitatory projections from the prelimbic subregion of the mPFC (PL) synapse onto the BLA and CE, augmenting CE output (Kaplan et al., 2011).

Lastly, CE projections terminate in brainstem and hypothalamic areas, including the central gray (CG), the lateral hypothalamus (LH), and the paraventricular nucleus of the hypothalamus (PVN), which mediate the physiological responses, including freezing behaviour, autonomic nervous system (ANS) responses, and hormonal responses, respectively (Sah et al., 2003; LeDoux, 2007; LeDoux, 2012). This is supported by CE lesion studies (LeDoux et al., 1988) and suggests that the CE is the last step in the final common pathway for the generation of conditioned fear responses.

Given that fear-related anxiety disorders, including specific phobias and PTSD, are characterized by a resistance to extinguish learned fear responses to anxiety-inducing stimuli or situations, and considering the gold standard for treatment of these disorders is exposure therapy, which utilizes extinction procedures, the investigation about how conditioned fear is extinguished is perhaps even more important than that of conditioned fear itself. In order to extinguish a conditioned fear, the CS is repeatedly presented in the absence of the US, which leads to the attenuation of the CS-elicited CR (Sotres-Bayon et al., 2006). For example, a rat that initially learned that being put in the conditioning

chamber (i.e., CS), as opposed to its home cage, predicted that it would subsequently receive a variable amount of footshocks (i.e., US), and consequently freeze (i.e., CR), would eventually learn that it no longer needs to freeze (i.e., new CR) when it is put into the conditioning chamber (i.e., CS) on another occasion and does not receive any footshocks (i.e., US). Importantly, extinction is generally viewed, not as a loss of the CR (i.e., freezing), but as new learning based on the new association of the CS (i.e., the context) with the lack of the US (i.e., footshock), which inhibits the original CS-aversive US association (Cammarota et al., 2007; Kaplan et al., 2011). The idea that this original CS-US association is impeded, and not erased, is supported by behavioural phenomena such as renewal (i.e., extinguished CR restoration when the CS is presented in the conditioning context, but not in the context in which it was extinguished), reinstatement (i.e., extinguished CR restoration after presentation of the aversive US only in the context where extinction training took place), and spontaneous recovery (extinguished CR restoration after the CS is presented after a period of time has passed since extinction training; Wilson et al., 1995; Myers and Davis, 2007).

Research studies exploring the underlying neural circuitry of cued and contextual fear extinction have implicated the amygdala, medial prefrontal cortex and hippocampus, once again, as key players. Three supposed fear extinction stages are as follows (Figure 2). In the first stage, CS-no US (i.e., cue or context alone) presentations initiate a decrease in the firing rate of amygdala neurons, via new inhibitory learning (Sotres-Bayon et al., 2006). For instance, Repa et al. (2001) determined that CS (i.e., cue)-no US presentations decreased freezing and CS-elicited spike firing in the majority of LA neurons in rats. During the second stage, the inhibitory memory is created between the



**Figure 2. Neural circuit of cued and contextual fear extinction.** There are three supposed fear extinction stages. (1) Conditioned stimulus (i.e., cue or context)-no unconditioned stimulus (CS-no US) presentations initiate a decrease in the firing rate of amygdala neurons via new inhibitory learning. (2) The inhibitory fear memory is created between the infralimbic (IL) subregion of the medial prefrontal cortex (mPFC) and the lateral nucleus of the amygdala (LA) and/or the intercalated masses (ICM). (3a) Consolidation of the fear extinction memory promotes activation of IL, which inhibits amygdala output via activation of inhibitory LA interneurons and/or activation of ICM inhibitory projections to the central nucleus of the amygdala (CE), resulting in the inhibition of fear responses on succeeding experiences with the fear stimuli. (3b) Concurrently, contextual modulation of fear extinction involves hippocampal modulation of LA and/or IL neural activity.

infralimbic subregion of the mPFC (IL) and the LA and/or the ICM (Sotres-Bayon et al., 2006). The third stage occurs when the animal needs to retrieve the consolidated extinction memory. During this time, the IL inhibits amygdala output via activation of inhibitory LA interneurons (Rosenkranz et al., 2003) and/or activation of ICM inhibitory projections to the CE (Kaplan et al., 2011), consequently leading to decreased freezing. For example, Milad and Quirk (2002) determined via single unit recordings that IL neurons only fire in response to the CS (i.e., tone) when the rats recall extinction after extinction training has occurred, and that rats that demonstrated the most amplified CS-evoked IL neuronal responses, also froze the least. Furthermore, it was illustrated that low levels of freezing in rats that did not undergo extinction training could be evoked by the pairing of conditioned tones with brief IL electrical stimulation, thus emulating extinction memory. Together these results imply that consolidation of extinction memory promotes activation of the IL, resulting in the inhibition of fear responses on succeeding experiences with the fear stimuli. Given that fear extinction is context-dependent and is expressed predominantly only in the context in which extinction training occurred (Maren and Quirk, 2004), it is important to report that concurrently during stage three, the hippocampus is responsible for contextual modulation of extinction (Bouton et al., 2006; Sotres-Bayon et al., 2006; Myers and Davis, 2007). For instance, Corcoran et al. (2005) inactivated the dorsal hippocampi of rats via the infusion of muscimol, a gamma-aminobutyric acid type A receptor (GABA<sub>A</sub>R) agonist, either before extinction training or test. It was revealed that dorsal hippocampal inactivation before extinction training did not disrupt extinction learning, but reduced the rate at which extinction was acquired, since the muscimol-infused rats showed a gradual decline in freezing throughout

extinction training, but freezing was still more compared to the saline-infused controls by the end of extinction training. When tested later in a drug-free state, either in the extinction context or a different context, muscimol-infused rats were found to freeze similarly in both contexts, implying that the dorsal hippocampus is involved in establishing the association between the CS (i.e., cue) and the context that causes extinction to be context-dependent. Furthermore, dorsal hippocampal inactivation before the extinction test was also shown to impede context-specific fear towards an already extinguished CS. Collectively, these findings demonstrate that the dorsal hippocampus is important for the acquisition, contextual encoding, and context-specific retrieval of fear extinction (Corcoran et al., 2005; Maren, 2008). It is thought that the contextual modulation of extinction involves hippocampal modulation of LA and/or IL neural activity (Sotres-Bayon et al., 2006). For example, in a study by Hobin et al. (2003), rats were fear conditioned to two different auditory stimuli (i.e., CSs), which were subsequently extinguished independently in two distinct contexts, and then rats were tested with each CS in each context while LA neuronal activity was recorded. LA neurons were found to respond more to the CS when it was presented in the context in which it was not extinguished, compared to their response to the same CS in the extinction context. This renewal of CS-evoked LA neuronal activity in the no-extinction context correlated to the rats' renewed fear responses to the CSs in their no-extinction context. Importantly, a follow-up study revealed that muscimol infusion into the dorsal hippocampus impeded the context-dependent LA neuronal firing in rats (Bouton et al., 2006). Additionally, it has been proposed that the hippocampus might inhibit IL activation when an extinguished CS is presented in a context other than the extinction

context (Maren, 2005), resulting in disinhibition of amygdala output, and a subsequent increase in freezing.

Since fear extinction is the basis for exposure therapy, which is the treatment of choice for anxiety disorders, such as specific phobia and PTSD, acquiring a more in depth understanding about the mechanism behind how conditioned fear is extinguished will continue to be beneficial. More precisely, further research about how and what compounds facilitate or impede acquisition of fear extinction will prove to be valuable when deciding which drugs will accelerate or hinder, respectively, exposure therapy. Since recurrence of fear is often context-dependent, the development of drugs that interfere with dorsal hippocampal-dependent context encoding, but not extinction, also has potential to increase the efficacy of exposure therapy.

### **3. Cannabinoids and LTD**

For over 5000 years, the plant *Cannabis sativa* has been utilized for medical and recreational reasons (Murray et al., 2007; Moreira and Wotjak, 2010). However, it was not until the 1960s, that the purification and chemical structure of the major psychoactive ingredient of cannabis, known as delta-9-tetrahydrocannabinol (THC), was determined (Gaoni and Mechoulam, 1964; Neumeyer and Shagoury, 1971; Mechoulam and Hanus, 2000). The discovery of phytocannabinoids (e.g., THC) led to the production of synthetic cannabinoids, such as HU210 and WIN 55212-2, which are capable of imitating THC effects, but with higher potency (Moreira and Wotjak, 2010). The psychoactive effects of THC and related compounds are primarily mediated by cannabinoid type 1 receptors (CB<sub>1</sub>Rs; Mackie, 2005).

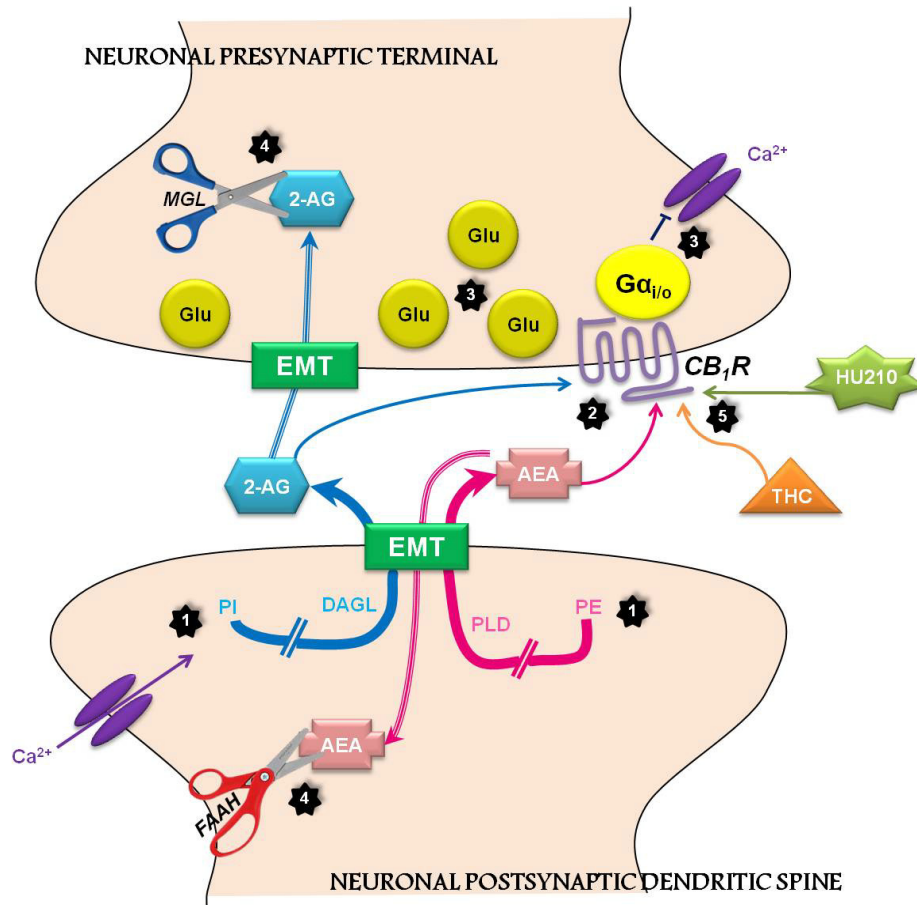
Rodent brain research utilizing radioactively labelled synthetic cannabinoids brought about the discovery of the first cannabinoid receptor, the CB<sub>1</sub>R, in 1988 (Devane et al., 1988), which was subsequently cloned in 1990 (Matsuda et al., 1990). CB<sub>1</sub>Rs are G-protein coupled receptors (GPCRs), and have a characteristic extracellular N-terminal, intracellular C-terminal, and seven transmembrane domains (Matsuda et al., 1990; Galiègue et al., 1995). Moreover, they are the predominant cannabinoid receptor subtype found within the brain (Demuth and Molleman, 2006; Ashton and Moore, 2011). Interestingly, the localization of CB<sub>1</sub>R expression levels correlates well with the impaired function of the subserved brain region after cannabis use, including the hippocampus, amygdala, and prefrontal cortex (Mackie, 2005), which are essential in learning and memory (Kemp and Manahan-Vaughan, 2007), emotion, and cognition, respectively (Abood and Martin, 1992; Demuth and Molleman, 2006). More specifically, CB<sub>1</sub>Rs are highly enriched in all subfields of the hippocampus (Herkenham et al., 1991; Jansen et al., 1992; Tsou et al., 1998), highly expressed in the BLA, but only meagrely in the ICM and CE (Mackie, 2005), and abundantly expressed in the IL (Tsou et al., 1998). Furthermore, CB<sub>1</sub>Rs are located on the presynaptic terminals (Saito et al., 2010) of, both, gamma-aminobutyric acid (GABA)ergic and glutamatergic neurons (Katona et al., 1999; Katona et al., 2006), accentuating their role in modulating neurotransmission at certain synapses (Freund et al., 2003; Mackie, 2005).

Naturally, the discovery of CB<sub>1</sub>Rs initiated a pursuit to find their endogenous agonists (Murray et al., 2007). The first endocannabinoid was detected in 1992, and called arachidonylethanolamide (AEA, anandamide; Devane et al., 1992). In 1995, a second endocannabinoid, 2-arachidonylglycerol (2-AG), that is more abundant in the

brain than AEA (Hashimotodani et al., 2007), was subsequently discovered (Mechoulam et al., 1995). In contrast to conventional neurotransmitters, such as amino acids (e.g., glutamate [Glu], GABA), monoamines (e.g., dopamine) and acetylcholine (ACh), endocannabinoids are not stored in synaptic vesicles, but are synthesized on-demand in postsynaptic neurons from membrane phospholipids, and act as retrograde signals (Saito et al., 2010). More specifically, AEA and 2-AG are synthesized from phosphatidylethanolamine (PE) and phosphatidylinositol (PI), respectively, in the dendritic spines of the postsynaptic neuron after calcium influx and subsequent activation of phospholipase D (PLD) and diacylglycerol lipase (DAGL), respectively (Freund et al., 2003; Hashimotodani et al., 2007; Heifets and Castillo, 2009; Saito et al., 2010). AEA and 2-AG are then released from the postsynaptic neuron into the synaptic cleft via an uncharacterized bidirectional endocannabinoid membrane transporter (EMT; Hermann et al., 2006; Murray et al., 2007; Heifets and Castillo, 2009), and bind to CB<sub>1</sub>Rs on neuronal presynaptic terminals (Murray et al., 2007; Heifets and Castillo, 2009; Saito et al., 2010). Presynaptic neuronal CB<sub>1</sub>R activation, via intricate intracellular signalling processes, eventually leads to decreased presynaptic calcium influx, and ultimately results in reduced neurotransmitter release (Murray et al., 2007; Heifets and Castillo, 2009; Saito et al., 2010). Finally, AEA and 2-AG are removed from the synaptic cleft via up-take, and are primarily hydrolyzed by fatty acid amide hydrolase (FAAH) in the postsynaptic (Egertová et al., 2003) and monoacylglycerol lipase (MGL) in the presynaptic neuron, respectively (Gulyas et al., 2004; Saito et al., 2010). Noteworthy is the fact that like endocannabinoids, exogenous cannabinoids, such as THC and HU210, also activate

neuronal presynaptic CB<sub>1</sub>Rs, resulting in the inhibition of presynaptic calcium influx, and consequently decreased neurotransmitter release (Murray et al., 2007; Figure 3).

Brain areas such as the hippocampus that express high levels of CB<sub>1</sub>Rs are also rich in NMDARs, which are fundamental to memory formation (Demuth and Molleman, 2006). NMDARs and  $\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPA) have been implicated in the processes of LTP (i.e., a short high-frequency burst of synaptic activity, followed by an instant and sustained increase in synaptic efficacy) and long-term depression (LTD; i.e., triggered by more prolonged low-frequency synaptic activity; Bliss and Schoepfer, 2004; Ge et al., 2010), which are the cellular models of learning and memory (Abush and Akirav, 2010). NMDARs are ionotropic glutamate receptors comprised of two NR1 and two NR2 subunits (i.e., NR2A-NR2D; Cull-Candy et al., 2001), while AMPARs are ionotropic glutamate receptors that are composed of four GluR subunits (i.e., GluR1-4; Hollmann and Heinemann, 1994). An *in vitro* study conducted by Liu et al. (2004), which utilized hippocampal slice preparations, found that selectively blocking NR2A-containing or NR2B-containing NMDARs, prevents LTP or LTD induction, respectively, findings that were replicated *in vivo* (Fox et al., 2006). Additionally, studies have verified the contribution of postsynaptic membrane insertion and internalization of AMPARs in the manifestation of LTP (Rumpel et al., 2005) and LTD (Ahmadian et al., 2004), respectively. For instance, Rumpel et al. (2005) demonstrated that auditory (i.e., cued) fear conditioning triggers the insertion of AMPARs into the postsynaptic membranes of a portion of neurons in the rat LA, and that inhibition of this AMPAR insertion results in diminished fear memory.



**Figure 3. Schematic representation of cannabinoid action at neuronal synapses in the brain.** (1) Endocannabinoids, such as arachidonylethanolamide (AEA) and 2-arachidonylglycerol (2-AG), are synthesized on-demand from the membrane phospholipids, phosphatidylethanolamine (PE) and phosphatidylinositol (PI), respectively, in the dendritic spines of postsynaptic neurons following calcium ( $\text{Ca}^{2+}$ ) influx and subsequent activation of phospholipase D (PLD) and diacylglycerol lipase (DAGL), respectively. (2) AEA and 2-AG are then released from the postsynaptic neuron into the synaptic cleft via an uncharacterized bidirectional endocannabinoid membrane transporter (EMT), and bind to cannabinoid type 1 receptors ( $\text{CB}_1\text{Rs}$ ) on neuronal presynaptic terminals. (3) Presynaptic neuronal  $\text{CB}_1\text{R}$  activation, via intricate intracellular signalling processes, eventually leads to decreased presynaptic calcium influx, and ultimately results in reduced neurotransmitter release. (4) Finally, AEA and 2-AG are removed from the synaptic cleft via up-take, and are primarily hydrolyzed by fatty acid amide hydrolase (FAAH) in the postsynaptic and monoacylglycerol lipase (MGL) in the presynaptic neuron, respectively. (5) Additionally, exogenous cannabinoids, such as delta-9-tetrahydrocannabinol (THC) and HU210, also activate neuronal presynaptic  $\text{CB}_1\text{Rs}$ , resulting in the inhibition of presynaptic calcium influx, and consequently decreased neurotransmitter release. Glu, glutamate.

Numerous studies investigating LTD expression, characterized by a decrease in regulated cell surface expression of AMPARs (Brebner et al., 2005) that is either NMDAR-dependent or independent (Man et al., 2000; Dalton et al., 2008; Collingridge et al., 2010), in several different brain regions, including the amygdala, hippocampus, and mPFC, have utilized a peptide known as Tat-GluR2 (Collingridge et al., 2010). Tat-GluR2 is a membrane-permeable GluR2-derived peptide that blocks the GluR2-dependent, regulated, but not constitutive (i.e., the steady-state level of the cell surface expression of AMPARs, which mediates basal synaptic transmission), AMPAR endocytosis (Brebner et al., 2005; Dalton et al., 2008) *in vitro* and *in vivo* (Kim et al., 2007). Importantly, the Tat-GluR2 peptide is thought to prevent LTD expression, delineated by AMPAR endocytosis, by disrupting protein-protein interactions between the carboxyl tail of the GluR2 subunit and the endocytic machinery via competitive inhibition of the intracellular endocytic machinery (Ahmadian et al., 2004; Fox et al., 2007). For instance, Kim et al. (2007) illustrated that microinfusion of Tat-GluR2, but not its control peptide, into the LA disrupted extinction of auditory fear memory in rats, suggesting that LTD expression in the LA is required for cued fear extinction. Additionally, Wong et al. (2007) demonstrated that systemic injections of Tat-GluR2 blocked the stress-induced impairment of spatial memory retrieval in a hippocampal-dependent Morris water maze (MWM) task, which was consistent with findings that showed that LTD underlies stress-induced memory impairment. This implies that the stress-induced impairment of spatial memory retrieval requires LTD expression. Furthermore, a study by Van den Oever et al. (2008), which employed a rat self-administration model, found that intra-ventral, but not dorsal, mPFC Tat-GluR2

injections hindered cue-induced reinstatement of heroin-seeking behaviour, without influencing seeking behaviour of a natural reinforcer (i.e., sucrose), suggesting that vmPFC LTD expression is required for cued-induced reinstatement of heroin-seeking behaviour. Together, these studies exhibit that the Tat-GluR2 peptide can be utilized to decipher how LTD expression underlies various types of memory impairments and behaviour, which are dependent on different brain regions.

Logically, cannabinoids have also been shown to induce *in vitro* LTD in various brain regions, including the amygdala, hippocampus, and the prefrontal cortex (Gerdeman and Lovinger, 2003). Importantly, a recent study conducted in our laboratory (Han et al., 2012) has provided the first evidence that in rats a single *in vivo* exposure to HU210 or THC, CB<sub>1</sub>R full (Maćkowiak et al., 2009) and partial (Shen and Thayer, 1999) agonists, respectively, induce the expression of LTD at the hippocampal CA3-CA1 synapses. This cannabinoid-elicited hippocampal LTD (CB-LTD) occurs via the following signalling pathway: cannabinoids activate astrocyte CB<sub>1</sub>Rs leading to an increase in extracellular levels of glutamate, which activates postsynaptic NMDA receptors to induce postsynaptic AMPA receptor endocytosis, resulting in LTD expression (Han et al., 2012). Thus, a probable mechanism has been elucidated to explain cannabinoid-induced effects on the cellular and molecular aspects of memory. Han et al. (2012) also demonstrated that the above mechanism accounts for the cannabinoid-induced impairment of hippocampal-dependent spatial working memory, as was investigated using a delayed-matching-to place version of the Morris water maze test, a T-maze using a delayed non-match to sample protocol, and the Tat-GluR2 LTD

expression-blocking peptide. However, whether this mechanism relates to other types of memory has yet to be determined.

#### **4. Cannabinoids and Their Role in Conditioned Fear**

Globally, cannabis is the most extensively used illegal drug (Beseler and Hasin, 2010). Cannabinoids, the active constituents of cannabis (Misner and Sullivan, 1999), induce behavioural consequences, the most frequently reported being learning and memory impairments (Lichtman et al., 1995; Pamplona and Takahashi, 2006). Typically, the thought of cannabinoid-induced learning and memory impairments has a negative connotation. However, in the case of fear-related anxiety disorders that are characterized by excessive fear responses and persistent aversive memories, including specific phobias and PTSD (Moreira and Wotjak, 2010), cannabinoid-induced memory impairments can be considered beneficial. Thus, research concerning cannabinoid-induced impairments of conditioned fear memory will prove to be valuable in providing insights into how manipulation of the endocannabinoid system might be useful for the treatment of fear-related anxiety disorders.

##### **4.1. Endocannabinoids**

###### **4.1.1. Inhibition of Endocannabinoid Action**

One way of investigating how endocannabinoids influence conditioned fear and its extinction is by impeding the effects of endocannabinoids, which can be done by utilizing CB<sub>1</sub>R antagonists or by employing mutant mice, which lack the expression of CB<sub>1</sub>Rs (i.e., CB<sub>1</sub>R-KO mice; Moreira and Wotjak, 2010; Saito et al., 2010). An alternative strategy would be to use endocannabinoid synthesis inhibitors. However, this

approach has not been used in fear conditioning paradigms, and is less commonly exploited in general (Saito et al., 2010).

Cued fear conditioning studies that have used CB<sub>1</sub>R-KO mice and/or CB<sub>1</sub>R antagonists have reported mixed findings. For instance, Marsicano et al. (2002) demonstrated that CB<sub>1</sub>R-KO mice were able to acquire and consolidate the tone-footshock association, but showed impaired acquisition of extinction, and that these results were replicated using wild-type (WT) mice that received SR141716 (i.e., rimonabant, CB<sub>1</sub>R antagonist) before fear conditioning or extinction training. Additionally, elevated levels of AEA & 2-AG in the BLA were found in mice that received tone-alone presentations during extinction training, compared to non-extinguished mice or mice that received unpaired tone and shock presentations during conditioning. Together, these results imply that extinction acquisition, but not acquisition or consolidation of cued fear conditioning, is dependent on CB<sub>1</sub>R activation. In contrast to these SR141716 findings, two other investigations reported conflicting findings concerning another CB<sub>1</sub>R antagonist, AM251, where AM251 hindered (Sink et al., 2010), or enhanced (Reich et al., 2008) the acquisition of cued fear conditioning.

Contextual, like cued, fear conditioning studies employing different CB<sub>1</sub>R antagonists have also showed conflicting results. For example, investigations concerning CFC have revealed that inhibition of CB<sub>1</sub>R via AM251 facilitates acquisition (Sink et al., 2010) and impairs consolidation (Bucherelli et al., 2006) of contextual fear memory. However, a study by Suzuki et al. (2004) illustrated that preconditioning administration of another CB<sub>1</sub>R antagonist, SR141716, had no effect on acquisition of CFC. Moreover,

pretreatment with SR141716 before extinction training was found to impede acquisition of extinction of the contextual fear memory (Suzuki et al., 2004; Pamplona et al., 2006; Pamplona et al., 2008). Similarly, de Oliveira Alvares et al. (2008) found that intra-CA1 injections of AM251 impaired acquisition of contextual fear memory extinction.

Taken together, both cued and contextual fear conditioning studies utilizing CB<sub>1</sub>R-KOs or CB<sub>1</sub>R antagonists, have conflicting findings regarding acquisition and consolidation of fear memory. Interestingly, different CB<sub>1</sub>R antagonists seem to consistently block acquisition of extinction of, both, cued and contextual fear memory.

#### **4.1.2. Enhancement of Endocannabinoid Action**

Since endocannabinoids are synthesized and released from the postsynaptic cell on-demand, temporal and spatial specificity of CB<sub>1</sub>R signalling in conditioned fear studies could be improved by employing compounds that disrupt endocannabinoid uptake and hydrolysis (i.e., FAAH and MGL inhibitors; Moreira and Wotjak, 2010; Saito et al., 2010). However, unlike inhibition of only FAAH, MGL inhibition produces tetrad effects (i.e., the four main consequences of systemic cannabinoid treatment: hypolocomotion, catalepsy, hypothermia, and analgesia; Long et al., 2009). Another way to investigate the facilitation of endocannabinoid action in conditioned fear paradigms is to directly inject endocannabinoids into the test subjects (de Oliveira Alvares et al., 2008).

Minimal fear conditioning studies that use freezing behaviour as an index of fear have utilized endocannabinoids, or inhibitors of their uptake or degradation to explore how increased endocannabinoid activity impacts the stages of conditioned fear memory

processing or fear memory extinction. In spite of this, one study revealed that intra-mPFC injections of AEA or AM404, an AEA transport inhibitor, attenuated retrieval of contextual fear memory, an effect that was prevented by local pretreatment of AM251 (Lisboa et al., 2010). Additionally, various investigations have consistently shown that facilitation of acquisition of contextual fear memory extinction can be induced by bilateral intra-CA1 injections of AEA (de Oliveira Alvares et al., 2008), and systemic (Pamplona et al., 2008) and intracerebroventricular (Bitencourt et al., 2008) injections of AM404.

#### **4.2. Exogenous Cannabinoids**

Intriguingly, research about cannabis' effect on conditioned fear commenced not long after THC was purified and characterized (González et al., 1972). Enquiries about how exogenous cannabinoids impact conditioned fear and its extinction have utilized phytocannabinoids, such as THC (i.e., a partial CB<sub>1</sub>R agonist; Shen and Thayer, 1999), and synthetic cannabinoids, such as WIN 55212-2 (i.e., a full CB<sub>1</sub>R agonist; Clarke et al., 2008) and HU210 (i.e., a full CB<sub>1</sub>R agonist; Maćkowiak et al., 2009).

Various studies have shown that exogenous cannabinoid-induced CB<sub>1</sub>R activation can lead to impairments of different memory processing stages in contextual fear conditioning. An investigation conducted by Pamplona and Takahashi (2006) found that WIN 55212-2 impairs acquisition, but not retrieval, of hippocampal-dependent contextual fear memory via the activation of CB<sub>1</sub>Rs. Moreover, Maćkowiak et al. (2009) and Puighermanal et al. (2009) demonstrated that HU210 and THC, respectively, disturb the consolidation of contextual fear memory through activation of CB<sub>1</sub>Rs. Furthermore,

exogenous cannabinoids have been shown to facilitate the extinction of contextual fear memory. More specifically, WIN 55212-2 (Pamplona et al., 2006; Pamplona et al., 2008) has been found to enhance the acquisition of contextual fear memory extinction in rats.

In summary, the enhancement or inhibition of endocannabinoid action, as well as exogenous cannabinoids have produced contrasting insights about how cannabinoids influence different memory processing stages of conditioned fear. Conflicting results could be due to differences in methodology and techniques utilized, and possibly the fact that multiple brain regions are involved. However, the above studies have consistently demonstrated that CB<sub>1</sub>R activation is essential for the facilitation of the acquisition of both cued and contextual fear memory extinction. This discovery is important for people suffering from pathological cue-specific, fear-related anxiety disorders, such as PTSD, because PTSD is thought to be caused by impaired extinction of normal responses to an ordeal, which are persistently elicited by trauma-related cues for a long time after the initial distressing occurrence (Rothbaum and Davis, 2003; Varvel et al., 2007). Thus, manipulations of the endocannabinoid system might prove to be beneficial in the development of treatments for people with fear-related anxiety disorders.

## **5. Aims**

Although current treatments for fear-related anxiety disorders, such as specific phobias and PTSD, are generally effective, exposure therapy is an ineffective treatment for some patients, and existing anxiolytics produce adverse side effects (Degroot, 2008). Thus, the development of novel treatments is needed. In view of the fact that CFC

possesses more ecological validity compared to cued fear conditioning (i.e., human fear is usually elicited by multiple, as opposed to one, environmental cues), and that several studies have established that exogenous cannabinoids impair various stages of contextual fear memory processing via CB<sub>1</sub>R activation (Pamplona and Takahashi, 2006; Maćkowiak et al., 2009; Puighermanal et al., 2009), in an animal model of fear, the first goal of this study was to confirm the effects of exogenous cannabinoids on the acquisition, consolidation, and retrieval of hippocampal-dependent contextual fear memory. In accordance with previous research, HU210 was hypothesized to impair acquisition (Pamplona and Takahashi, 2006) and consolidation (Maćkowiak et al., 2009; Puighermanal et al., 2009), but not retrieval (Pamplona and Takahashi, 2006), of hippocampal-dependent contextual fear memory via the activation of CB<sub>1</sub>Rs.

Since exogenous cannabinoids have been shown to induce *in vitro* LTD in brain regions implicated in fear-related anxiety disorders and animal models of conditioned fear, including the amygdala, hippocampus, and the prefrontal cortex (Gerdeman and Lovinger, 2003), Tat-GluR2 has inhibited LTD expression in these same brain areas (Collingridge et al., 2010), and a single *in vivo* exposure of exogenous cannabinoids elicited hippocampal LTD expression at the CA3-CA1 synapses, which resulted in an impairment of spatial working memory (Han et al., 2012), this study explored whether *in vivo* CB-LTD expression also causatively contributed to contextual fear memory impairments. Thus, the second objective of this investigation was to examine the effects of the LTD expression-blocking peptide, Tat-GluR2, on the cannabinoid-induced impairment of acquisition and consolidation of hippocampal-dependent contextual fear memory. Systemic injections of Tat-GluR2, but not its control peptide Tat-GluR2S, was

hypothesized to abolish the HU210-induced impairment of acquisition and consolidation of hippocampal-dependent contextual fear memory.

Given that exogenous cannabinoids and the endogenous cannabinoid system (i.e., CB<sub>1</sub>Rs, endocannabinoids and their metabolizing enzymes; Moreira and Wotjak, 2010) have been implicated in the facilitation of the acquisition of contextual fear memory extinction (Suzuki et al., 2004; Pamplona et al., 2006; Bitencourt et al., 2008; de Oliveira Alvares et al., 2008; Pamplona et al., 2008), this investigation examined whether this finding was applicable to other exogenous cannabinoids (i.e., HU210) and JZL 195, a potent inhibitor of FAAH and MGL. In other words, the third objective of this study was to confirm the effects of exogenous cannabinoids and the enhancement of endocannabinoid action on the acquisition of hippocampal-dependent contextual fear memory extinction. In accordance with previous research (Suzuki et al., 2004; Pamplona et al., 2006; Bitencourt et al., 2008; de Oliveira Alvares et al., 2008; Pamplona et al., 2008), HU210 and JZL 195 were hypothesized to enhance the acquisition of hippocampal-dependent contextual fear memory extinction.

Lastly, if objective three was fulfilled, this study also aimed to explore if this cannabinoid-induced facilitation of extinction acquisition required *in vivo* LTD expression for several reasons: cannabinoids induce LTD in brain regions implicated in fear-related anxiety disorders and animal models of conditioned fear and extinction, including the amygdala, hippocampus, and the prefrontal cortex (Gerdeman and Lovinger, 2003), numerous studies that employed the Tat-GluR2 peptide have illustrated LTD expression in these same brain areas (Collingridge et al., 2010), and Dalton et al.

(2008) determined that the disruption of AMPAR endocytosis impaired acquisition of cued fear memory extinction. Therefore, the conditional fourth objective of this investigation was to examine the effects of the LTD expression-blocking peptide, Tat-GluR2, on the cannabinoid-induced enhancement of the acquisition of hippocampal-dependent contextual fear memory extinction. Systemic injections of Tat-GluR2, but not its control peptide Tat-GluR2S, was hypothesized to abolish the HU210- and JZL 195-induced enhancement of acquisition of hippocampal-dependent contextual fear memory extinction.

## **MATERIALS AND METHODS**

### **1. Animals**

Adult male Long-Evans rats (Charles River, Saint-Constant, QC, Canada) weighing 225-275 g at the time of arrival were used. Rats were housed 2 per cage at standard experimental conditions (12 h light/dark cycle, with lights on at 7:00 am; temperature  $21\pm 1^{\circ}\text{C}$ , 40-50% relative humidity) with access to food and water *ad libitum*. All procedures were performed in accordance with the guidelines established by the Canadian Council on Animal Care as approved by the Animal Care Committee of the University of Ottawa Institute of Mental Health Research.

### **2. Drugs**

HU210 (Tocris Bioscience, Ellisville, USA) and JZL195 (Cayman Chemical Company, Ann Arbor, USA) were dissolved in DMSO:TWEEN 80: 0.9% NaCl (1:1:8).

### **3. Peptides**

Tat-GluR2 (YG-20, YGRKKRRQRRRYKEGYNVYGG, GL Biochem, Ltd., Shanghai, China) and its control scrambled peptide Tat-GluR2S (YE-20, YGRKKRRQRRRVYKYGGYNE, GL Biochem, Ltd., Shanghai, China) were dissolved in distilled water. Previous studies have established that fluorescently tagged Tat-GluR2 (1.5  $\mu\text{mol/kg}$ , i.v.) reached peak concentrations in the brain after 90 min with a single exponential decay and a 4 h half-life. Undetectable brain levels of Tat-GluR2 were reached 24 h after injection (Brebner et al., 2005; Dalton et al., 2008).

## **4. Contextual Fear Conditioning**

### **4.1. Apparatus**

The dimensions of the conditioning chamber (Coulbourn Instruments, L.L.C., Allentown, USA) was 31x25x30 cm, and consisted of back and front walls made of clear Plexiglas, two side walls made of stainless steel, and a floor made up of 16 stainless steel rods (4 mm diameter, 1.4 cm apart). The floor was connected to a constant current shock generator (Coulbourn Instruments model H13-16).

### **4.2. CFC Procedures**

All rats were handled for 2-3 days before undergoing CFC. All tests were conducted between 10:00 am and 2:30 pm, and were video-recorded with a Canon 8 MP digital camera (Canon Inc., Japan).

#### **4.2.1. Contextual fear memory acquisition**

Four groups of rats were conditioned: 1) Vehicle, 2) HU210, 3) Tat-GluR2S + HU210 and 4) Tat-GluR2 + HU210. In order to examine the acquisition of contextual fear memory, Tat-GluR2S + HU210 and Tat-GluR2 + HU210 groups were injected with Tat-GluR2S or Tat-GluR2 (1.5  $\mu\text{mol/kg}$ , i.p), respectively, followed by HU210 (50  $\mu\text{g/kg}$ , i.p.) 1.5 h later and immediately before conditioning. Vehicle and HU210 groups were injected with vehicle (DMSO:TWEEN 80:0.9% NaCl = 1:1:8, 1 ml/kg, i.p.) or HU210 (50  $\mu\text{g/kg}$ , i.p.), respectively, immediately before conditioning. Rats were then placed in the conditioning chamber for a habituation period of 200 s, followed by 5 footshocks (1 s, 1 mA) with an inter-trial interval (ITI) of 120 s. During the test phase, which occurred 24 h after conditioning, rats were put back into the conditioning chamber

for 20 min (Figure 4A). As for all of the CFC that was conducted, the Observational Data Logging (ODLog) software program (Macropod Software) was used to measure freezing behaviour, defined as the absence of movement excluding involuntary respiratory movements (Blanchard and Blanchard, 1969). Freezing behaviour was analyzed for the entire duration of the 20 min test phase, which was also divided into four 5 min blocks, and compared between all 4 groups.

#### **4.2.2. Contextual fear memory consolidation**

Four groups of rats were conditioned: 1) Vehicle, 2) HU210, 3) Tat-GluR2S + HU210 and 4) Tat-GluR2 + HU210. During the conditioning phase, rats were placed in the conditioning chamber for a habituation period of 120 s, followed by 6 footshocks (1 s, 1 mA) on a random schedule with an average ITI of 60 s. Immediately after conditioning, Tat-GluR2S + HU210 and Tat-GluR2 + HU210 groups were injected with Tat-GluR2S or Tat-GluR2 (1.5  $\mu\text{mol/kg}$ , i.p.), respectively, and the Vehicle and HU210 groups were injected with vehicle (1 ml/kg, i.p.) or HU210 (50  $\mu\text{g/kg}$ , i.p., Figure 5; 100  $\mu\text{g/kg}$ , i.p., Figure 6), respectively. One and a half hours later, the Tat-GluR2S + HU210 and Tat-GluR2 + HU210 groups were injected with HU210 (50  $\mu\text{g/kg}$ , i.p., Figure 5; 100  $\mu\text{g/kg}$ , i.p., Figure 6). Twenty-four hours after conditioning, the test phase commenced, in which the rats were placed back in the conditioning chamber for 20 min. Freezing behaviour was analyzed for the entire duration of the 20 min test phase, which was also divided into four 5 min blocks, and compared between all 4 groups.

### **4.2.3. Contextual fear memory retrieval**

Two groups of rats were conditioned: 1) Vehicle and 2) HU210. During the conditioning phase, rats were placed in the conditioning chamber for a habituation period of 120 s, followed by 6 footshocks (1 s, 1 mA) on a random schedule with an average ITI of 60 s. Twenty-four hours after conditioning, and immediately before the test phase, the Vehicle and HU210 groups were injected with vehicle (1 ml/kg, i.p.) or HU210 (50 µg/kg, i.p.), respectively (Figure 7A). The rats were then placed back into the conditioning chamber for a 20 min test phase. Freezing behaviour was analyzed for the entire duration of the 20 min test phase, which was also divided into four 5 min blocks, and compared between groups.

## **4.3. CFC Extinction Acquisition Procedures**

### **4.3.1. Procedure 1**

During the conditioning phase, rats were placed in the conditioning chamber for a habituation period of 120 s, followed by 6 footshocks (1 s, 0.8 mA) on a random schedule with an average ITI of 60 s. Twenty-two and a half hours, 23.5 h, and 24 h after conditioning, the rats were injected with Tat-GluR2S or Tat-GluR2 (1.5 µmol/kg, i.p., Figure 11), JZL 195 (10 mg/kg, i.p.) or its vehicle (DMSO:TWEEN 80:0.9% NaCl = 1:1:8, 1 ml/kg, i.p., Figure 9), or HU210 (50 µg/kg, i.p.) or its vehicle (DMSO:TWEEN 80:0.9% NaCl = 1:1:8, 1 ml/kg, i.p., Figure 8), respectively. Twenty-four hours after conditioning, the rats were then placed back into the conditioning chamber for a 40 min extinction training session. Freezing behaviour was analyzed for the entire 40 min

extinction training phase, which was also broken down into eight 5 min blocks, and compared between groups. This procedure was adapted from Dalton et al., (2008).

#### **4.3.2. Procedure 2**

During the conditioning phase, rats were placed in the conditioning chamber for a habituation period of 180 s, followed by 1 footshock (1 s, 1.5 mA), and remained in the chamber for an additional 1 min. Twenty-four hours after conditioning, the rats were injected with vehicle (DMSO:TWEEN 80:0.9% NaCl = 1:1:8, 1 ml/kg, i.p.), or HU210 (10 µg/kg or 50 µg/kg, i.p.), and immediately placed back into the conditioning chamber for a 30 min extinction training session (Figure 10A). Freezing behaviour was analyzed for the entire 30 min extinction training phase, which was also separated into ten 3 min blocks, and compared between all 3 groups.

### **5. Statistical Analysis**

Student SPSS 16.0 for Windows was used for statistical analysis. Results were reported as mean  $\pm$  SE. Statistical comparisons were carried out using one-way ANOVAs for overall test phase analyses and mixed repeated measures ANOVAs for block analyses, followed by LSD *post hoc* test. Statistical significance was set at  $p < 0.05$ .

## RESULTS

### 1. Cannabinoid-induced effects on the acquisition of hippocampal-dependent contextual fear memory

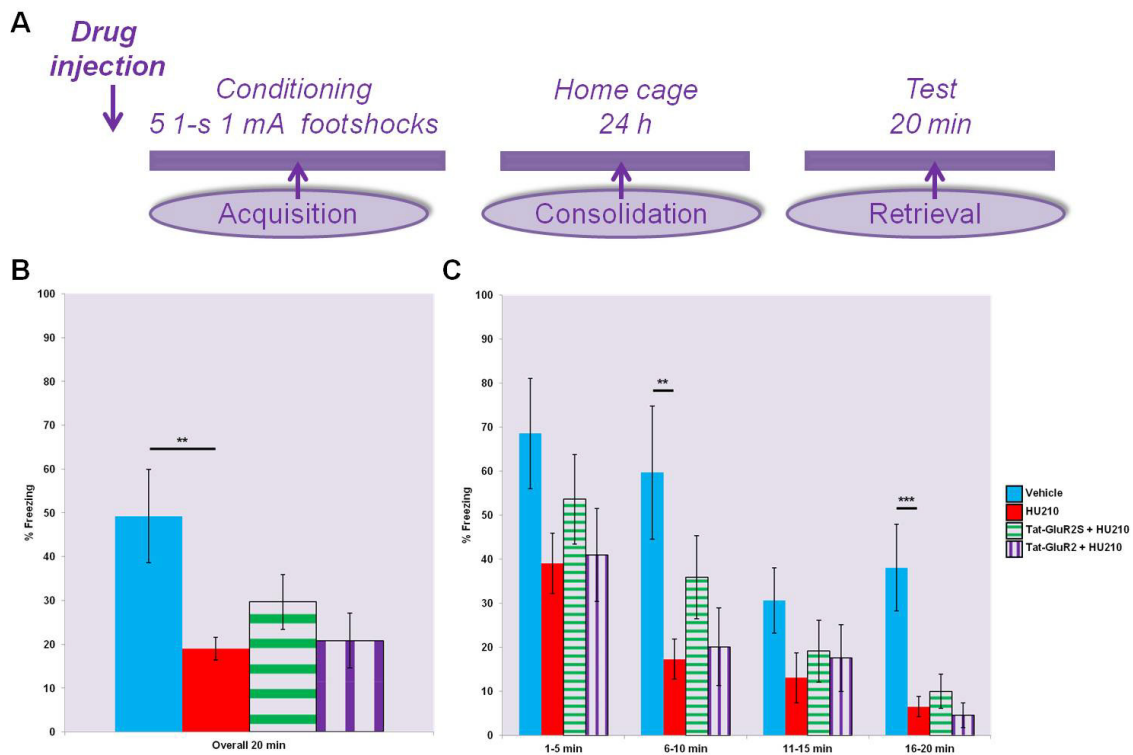
Systemic injections of HU210 (50 µg/kg) impaired acquisition of hippocampal-dependent contextual fear memory, as exemplified by the significantly greater amount of freezing by the Vehicle group compared to the HU210 group during the overall 20 min ( $F_{3,28} = 3.87$ ,  $p = 0.005$ , LSD *post hoc* test, Figure 4B) and during the second and last 5 min blocks ( $F_{3,28} = 3.64$ ,  $p = 0.006$  and  $F_{3,28} = 7.81$ ,  $p < 0.001$ , respectively, LSD *post hoc* test, Figure 4C) of the test phase.

### 2. Effects of Tat-GluR2 peptide on the cannabinoid-induced impairment of acquisition of hippocampal-dependent contextual fear memory

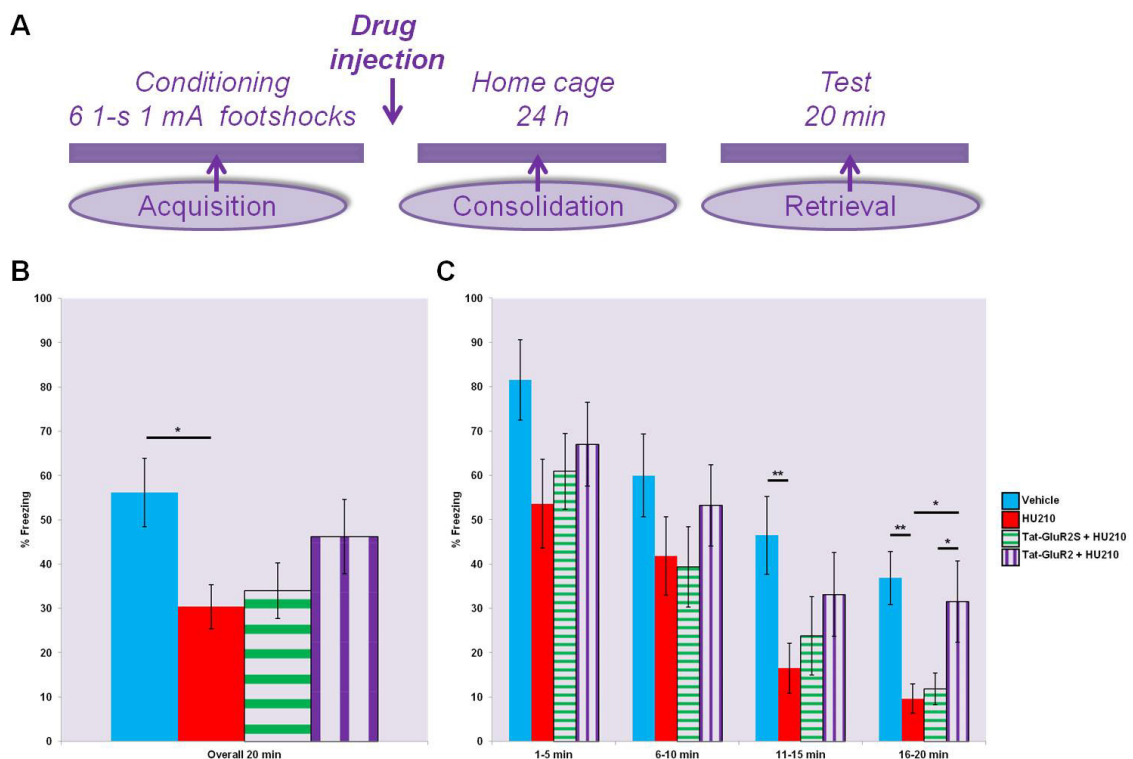
Systemic injections of Tat-GluR2 did not abolish the HU210 (50 µg/kg)-induced impairment of acquisition of hippocampal-dependent contextual fear memory, as illustrated by the lack of significant differences in the amount of freezing between the Tat-GluR2S + HU210 group, the Tat-GluR2 + HU210 group, and the HU210 group during the entire 20 min test phase ( $p > 0.05$ , LSD *post hoc* test, Figure 4).

### 3. Cannabinoid-induced effects on the consolidation of hippocampal-dependent contextual fear memory

Systemic injections of HU210 (50 µg/kg) significantly impaired hippocampal-dependent contextual fear memory consolidation during the overall 20 min ( $F_{3,44} = 2.86$ ,  $p = 0.012$ , LSD *post hoc* test, Figure 5B) and during the last two 5 min blocks ( $F_{3,44} = 2.42$ ,  $p = 0.014$  and  $F_{3,44} = 5.26$ ,  $p = 0.002$ , respectively, LSD *post hoc* test, Figure 5C)



**Figure 4. Tat-GluR2 peptide did not diminish the HU210-induced impairment of hippocampal-dependent contextual fear memory acquisition.** (A) Representative diagram of experimental protocol. (B-C) Mean  $\pm$  SE percentage of time spent freezing during the entire 20 min, 1-5 min, 6-10 min, 11-15 min, and 16-20 min of the test phase, which occurred 24 h after CFC (5 1 s, 1 mA footshocks). Tat-GluR2S + HU210 (green horizontal stripes,  $n=8$ ) and Tat-GluR2 + HU210 (purple vertical stripes,  $n=8$ ) rats were injected with Tat-GluR2S or Tat-GluR2 (1.5  $\mu\text{mol/kg}$ , i.p.), respectively, followed by HU210 (50  $\mu\text{g/kg}$ , i.p.) 1.5 h later and immediately before CFC. Vehicle (blue,  $n=8$ ) and HU210 (red,  $n=8$ ) rats were injected with vehicle (1 ml/kg, i.p.) or HU210 (50  $\mu\text{g/kg}$ , i.p.), respectively, immediately before CFC.  $***p < 0.001$ ,  $**p < 0.01$  (LSD *post hoc* test).



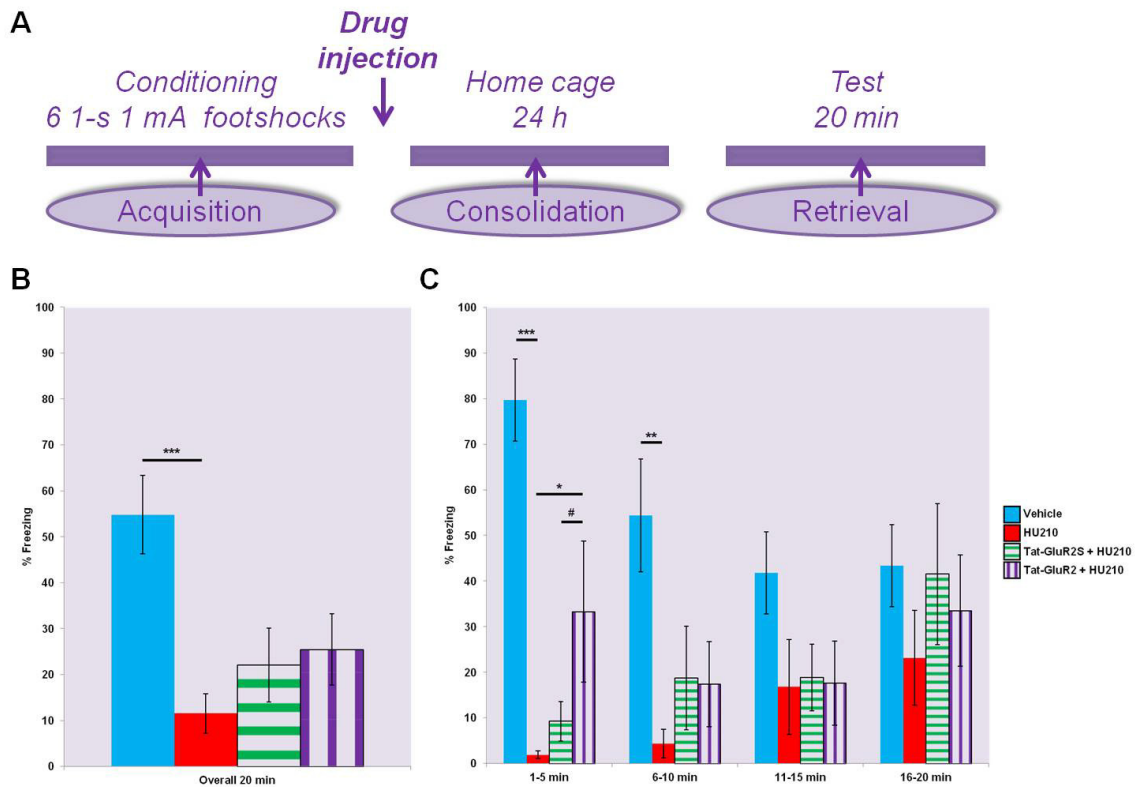
**Figure 5. Effects of Tat-GluR2 on HU210 (50  $\mu\text{g}/\text{kg}$ )-induced impairment of contextual fear memory consolidation.** (A) Representative diagram of experimental protocol. (B-C) Mean  $\pm$  SE percentage of time spent freezing during the entire 20 min, 1-5 min, 6-10 min, 11-15 min, and 16-20 min of the test phase, which occurred 24 h after CFC (6 1 s, 1 mA footshocks). Tat-GluR2S + HU210 (green horizontal stripes,  $n=12$ ) and Tat-GluR2 + HU210 (purple vertical stripes,  $n=12$ ) rats were injected with Tat-GluR2S or Tat-GluR2 (1.5  $\mu\text{mol}/\text{kg}$ , i.p.), respectively, immediately after CFC, followed by HU210 (50  $\mu\text{g}/\text{kg}$ , i.p.) 1.5 h later. Vehicle (blue,  $n=12$ ) and HU210 (red,  $n=12$ ) rats were injected with vehicle (1 ml/kg, i.p.) or HU210 (50  $\mu\text{g}/\text{kg}$ , i.p.), respectively, immediately after CFC. **\*\*** $p < 0.01$ , **\*** $p < 0.05$  (LSD *post hoc* test).

of the test phase, as demonstrated by the significantly reduced amount of freezing by the HU210 group compared to the Vehicle group throughout those time periods. Since these results differed slightly from previous research (Maćkowiak et al., 2009; Puighermanal et al., 2009), the experiment was repeated using the same consolidation protocol described above, except utilizing HU210 at a dose of 100 µg/kg, in order to investigate whether increasing the dose of HU210 would reveal a significant difference in freezing between the HU210 and Vehicle groups at an earlier block of the test phase.

When the experiment was conducted again using HU210 at a concentration of 100 µg/kg, as opposed to 50 µg/kg, the HU210 group was found to freeze significantly less than the Vehicle group during the overall 20 min ( $F_{3,28} = 6.39, p < 0.001$ , LSD *post hoc* test, Figure 6B) and during the first two 5 min blocks ( $F_{3,28} = 14.50, p < 0.001$  and  $F_{3,28} = 4.84, p = 0.001$ , respectively, LSD *post hoc* test, Figure 6C) of the test phase. Thus, while both high (i.e., 100 µg/kg) and moderate (i.e., 50 µg/kg) doses of HU210 impair the consolidation of contextual fear memory, high and moderate doses impair the first and last half of the test phase, respectively.

#### **4. Effects of Tat-GluR2 peptide on the cannabinoid-induced impairment of hippocampal-dependent contextual fear memory consolidation**

When 50 µg/kg of HU210 was injected, the Tat-GluR2 + HU210 group was found to freeze significantly more compared to the Tat-GluR2S + HU210 group ( $F_{3,44} = 5.26, p = 0.025$ , LSD *post hoc* test, Figure 5) and the HU210 group ( $F_{3,44} = 5.26, p = 0.013$ , LSD *post hoc* test, Figure 5) only during the last 5 min block of the 20 min test phase. When 100 µg/kg of HU210 was injected, however, the Tat-GluR2 + HU210



**Figure 6. Effects of Tat-GluR2 on HU210 (100  $\mu\text{g}/\text{kg}$ )-induced impairment of contextual fear memory consolidation.** (A) Representative diagram of experimental protocol. (B-C) Mean  $\pm$  SE percentage of time spent freezing during the entire 20 min, 1-5 min, 6-10 min, 11-15 min, and 16-20 min of the test phase, which occurred 24 h after CFC (6 1 s, 1 mA footshocks). Tat-GluR2S + HU210 (green horizontal stripes,  $n=8$ ) and Tat-GluR2 + HU210 (purple vertical stripes,  $n=8$ ) rats were injected with Tat-GluR2S or Tat-GluR2 (1.5  $\mu\text{mol}/\text{kg}$ , i.p.), respectively, immediately after CFC, followed by HU210 (100  $\mu\text{g}/\text{kg}$ , i.p.) 1.5 h later. Vehicle (blue,  $n=8$ ) and HU210 (red,  $n=8$ ) rats were injected with vehicle (1 ml/kg, i.p.) or HU210 (100  $\mu\text{g}/\text{kg}$ , i.p.), respectively, immediately after CFC. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ , # $p < 0.10$  (LSD *post hoc* test).

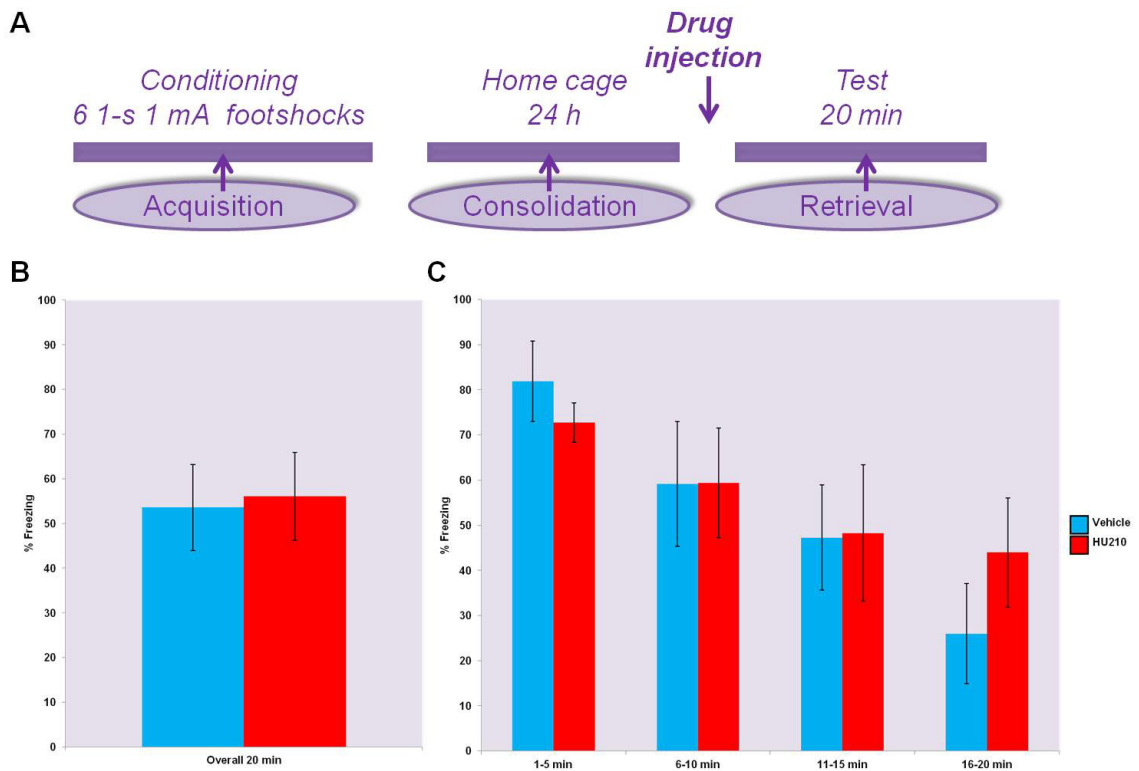
group was found to freeze significantly more compared to the HU210 group ( $F_{3,28} = 14.50, p = 0.023$ , LSD *post hoc* test, Figure 6), but not the Tat-GluR2S + HU210 group ( $F_{3,28} = 14.50, p = 0.076$ , LSD *post hoc* test, Figure 6) only during the first 5 min block of the 20 min test phase. Thus, Tat-GluR2 inhibited the first and last blocks of HU210-impaired contextual fear memory consolidation when high and moderate doses of HU210 were administered, respectively. Additionally, no significant difference in the amount of freezing was found between the HU210 (50  $\mu\text{g}/\text{kg}$  or 100  $\mu\text{g}/\text{kg}$ ) groups and the Tat-GluR2S + HU210 group during the entire 20 min test phases ( $p > 0.05$ , LSD *post hoc* test, Figure 5& 6), indicating that the Tat-GluR2S peptide acted as a proper control in these experiments.

## **5. Cannabinoid-induced effects on the retrieval of hippocampal-dependent contextual fear memory**

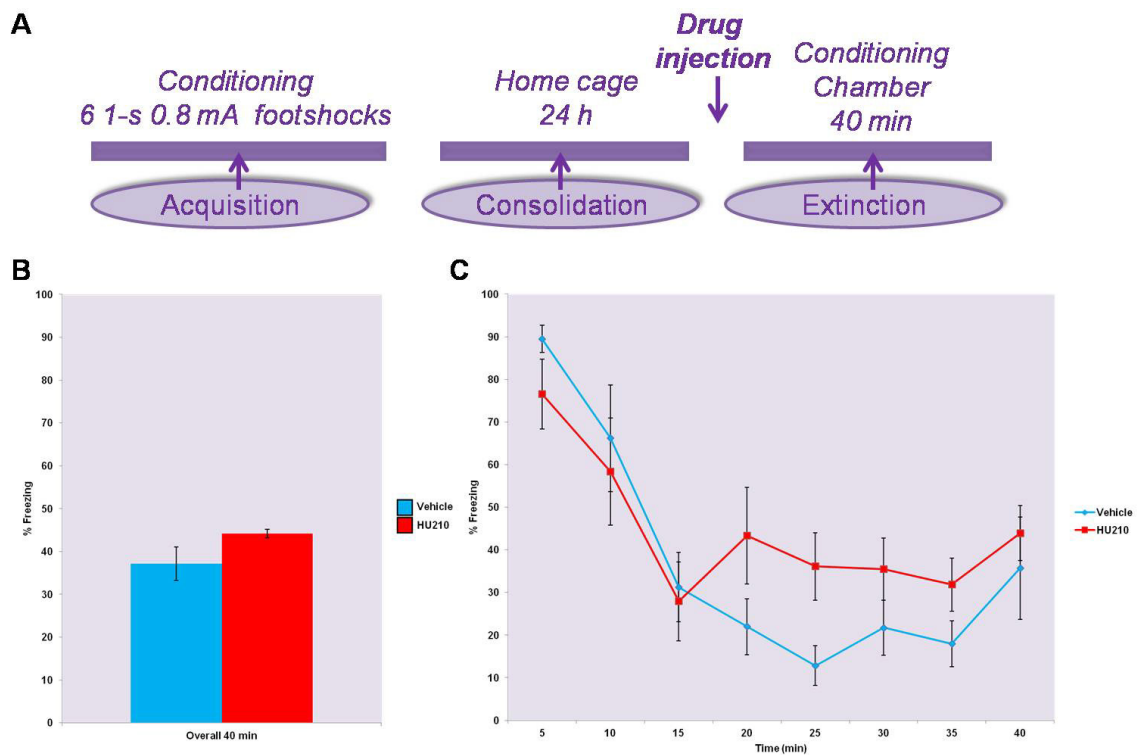
Systemic injections of HU210 (50  $\mu\text{g}/\text{kg}$ ) did not impair retrieval of hippocampal-dependent contextual fear memory, as demonstrated by the lack of significant difference in the amount of freezing between the Vehicle group and the HU210 group during the entire 20 min test phase ( $p > 0.05$ , Figure 7).

## **6. Cannabinoid-induced effects on the acquisition of hippocampal-dependent contextual fear memory extinction**

Systemic injections of HU210 (50  $\mu\text{g}/\text{kg}$ ) and JZL 195 (10  $\text{mg}/\text{kg}$ ) did not significantly enhance the acquisition of hippocampal-dependent contextual fear memory extinction, as illustrated by the lack of significant difference in the amount of freezing between the HU210 group and its Vehicle group ( $p > 0.05$ , Figure 8), and between the



**Figure 7. HU210 did not impair retrieval of hippocampal-dependent contextual fear memory.** (A) Representative diagram of experimental protocol. (B-C) Mean  $\pm$  SE percentage of time spent freezing during the entire 20 min, 1-5 min, 6-10 min, 11-15 min, and 16-20 min of the test phase, which occurred 24 h after CFC (6 1 s, 1 mA footshocks). Vehicle (blue, n=8) and HU210 (red, n=8) rats were injected with vehicle (1 ml/kg, i.p.) or HU210 (50  $\mu$ g/kg, i.p.), respectively, immediately before the test phase.  $p > 0.05$  compared to the Vehicle group.



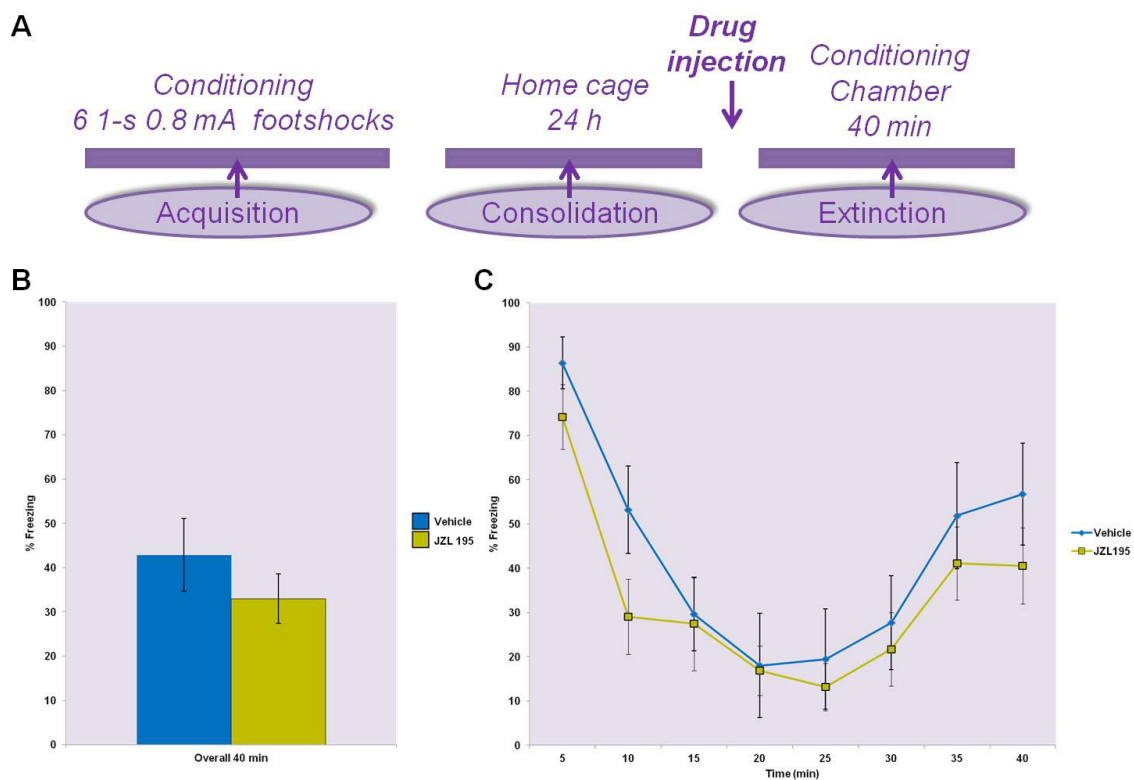
**Figure 8. Effects of HU210 on the acquisition of contextual fear memory extinction.** (A) Representative diagram of experimental protocol. (B-C) Mean  $\pm$  SE percentage of time spent freezing during the entire 40 min extinction training phase, and divided into 5 min bins, which occurred 24 h after CFC (6 1 s, 0.8 mA footshocks). HU210 (red, n=8) and Vehicle (blue, n=8) rats were injected with HU210 (50  $\mu$ g/kg, i.p.) or vehicle (1 ml/kg, i.p.), respectively, immediately before extinction training.  $p > 0.05$  compared to the Vehicle group. *Methods adapted from Dalton et al. (2008).*

JZL 195 group and its Vehicle group ( $p > 0.05$ , Figure 9) during the 40 min extinction training phases.

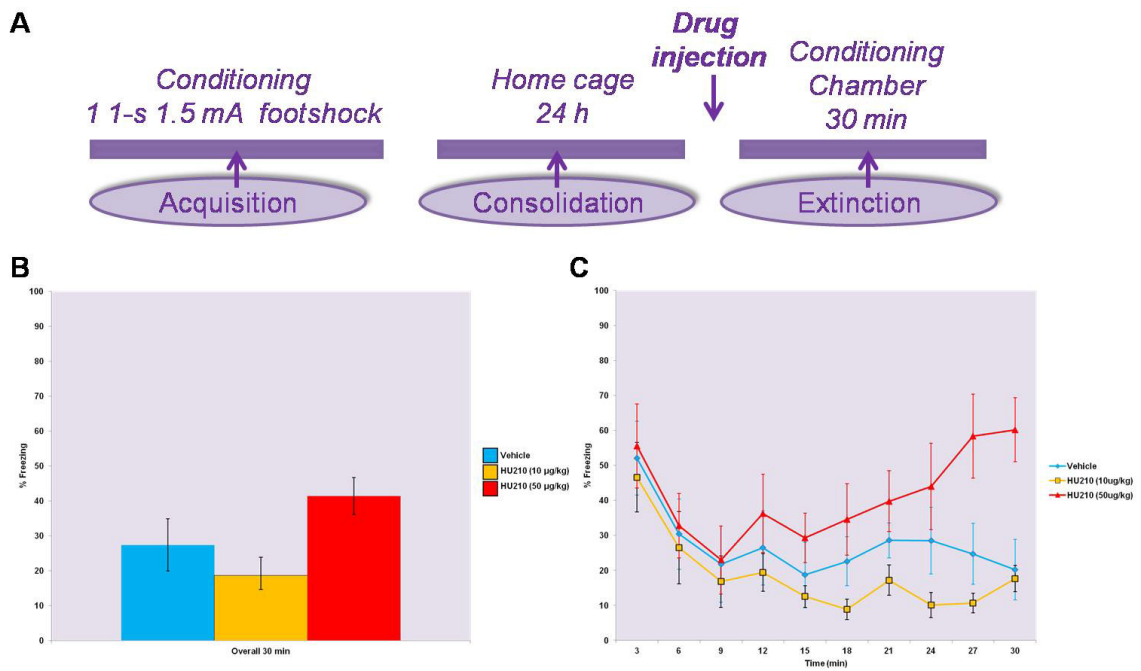
Since the above results conflicted with previous literature (Pamplona et al., 2006; Bitencourt et al., 2008; Pamplona et al., 2008), it was hypothesized that perhaps another CFC extinction protocol would generate findings that were in accordance with published results. Furthermore, since Pamplona et al. (2006) found that different concentrations of WIN 55 212-2, a full CB<sub>1</sub>R agonist, caused rats to have variable freezing times compared to control rats during extinction training (i.e., 2.5 mg/kg, 1.25 mg/kg, and 0.25 mg/kg doses of WIN 55 212-2, respectively, generated increased, similar, and decreased freezing times compared to controls), an HU210 (10 µg/kg) group was added to the experiment. Similar to the results obtained via the first CFC extinction protocol, systemic injections of both low (10 µg/kg) and moderate (50 µg/kg) doses of HU210 did not significantly enhance the acquisition of hippocampal-dependent contextual fear memory extinction, as illustrated by the lack of significant difference in the amount of freezing between the Vehicle group and the HU210 (10 µg/kg or 50 µg/kg) groups during the 30 min extinction training phase ( $p > 0.05$ , LSD *post hoc* test, Figure 10). Thus, even under various protocols, various doses of HU210 do not facilitate the acquisition of hippocampal-dependent contextual fear memory extinction.

## **7. Effects of Tat-GluR2 peptide on the acquisition of hippocampal-dependent contextual fear memory extinction**

Although this study did not find that cannabinoids facilitate the acquisition of contextual fear memory extinction, Tat-GluR2 was employed to investigate whether

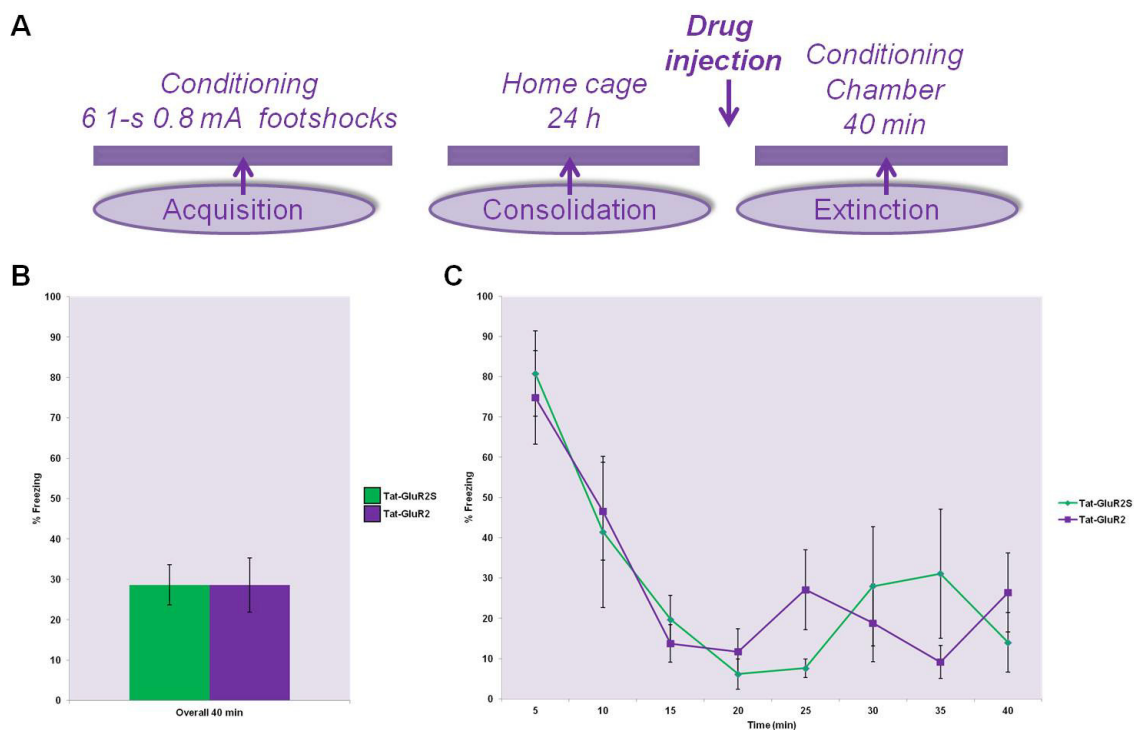


**Figure 9. JZL 195 did not significantly enhance the acquisition of the contextual fear memory extinction.** (A) Representative diagram of experimental protocol. (B-C) Mean  $\pm$  SE percentage of time spent freezing during the entire 40 min extinction training phase, and divided into 5 min bins, which occurred 24 h after CFC (6 1 s, 0.8 mA footshocks). JZL195 (yellow, n=8) and Vehicle (blue, n=8) rats were injected with JZL195 (10 mg/kg, i.p.) or vehicle (1 ml/kg, i.p.), respectively, 30 min before extinction training.  $p > 0.05$  compared to the Vehicle group. *Methods adapted from Dalton et al. (2008).*



**Figure 10. HU210 did not significantly enhance the acquisition of contextual fear memory extinction.** (A) Representative diagram of experimental protocol. (B-C) Mean  $\pm$  SE percentage of time spent freezing during the entire 30 min extinction training phase, and divided into 3 min bins, which occurred 24 h after CFC (1 1 s, 1.5 mA footshock). HU210 (orange or red,  $n=8$  per group) and Vehicle (blue,  $n=8$ ) rats were injected with HU210 (10 ug/kg or 50 ug/kg, i.p.) or vehicle (1 ml/kg, i.p.), respectively, immediately before extinction training.  $p > 0.05$  compared to the Vehicle group (LSD *post hoc* test).

*in vivo* LTD expression was required for the acquisition of contextual fear memory extinction. Systemic injections of Tat-GluR2 did not significantly impact the acquisition of hippocampal-dependent contextual fear memory extinction, as exemplified by the lack of significant difference in the amount of freezing between the Tat-GluR2S and the Tat-GluR2 groups during the entire 40 min extinction training phase ( $p > 0.05$ , Figure 11).



**Figure 11. Tat-GluR2 peptide did not significantly impact the acquisition of contextual fear memory extinction compared to its control peptide Tat-GluR2S.** (A) Representative diagram of experimental protocol. (B-C) Mean  $\pm$  SE percentage of time spent freezing during the entire 40 min extinction training phase, and divided into 5 min bins, which occurred 24 h after CFC (6 1 s, 0.8 mA footshocks). Tat-GluR2S (green,  $n=5$ ) and Tat-GluR2 (purple,  $n=6$ ) rats were injected with Tat-GluR2S or Tat-GluR2 (1.5  $\mu\text{mol/kg}$ , i.p.), respectively, 1.5h before extinction training.  $p > 0.05$  compared to the Tat-GluR2S group. *Methods adapted from Dalton et al. (2008).*

## DISCUSSION

There is a need for the development of new treatments for fear-related anxiety disorders, such as specific phobias and PTSD, since modern treatments including exposure therapy, which is analogous to extinction training in animals, and anxiolytics are sometimes ineffective and produce adverse side effects, respectively (Degroot, 2008). Numerous studies employing CFC, an animal model of fear, have revealed that exogenous cannabinoids impair various stages of contextual fear memory processing via the activation of CB<sub>1</sub>Rs, although no study has investigated how the same exogenous cannabinoid has impacted all three stages. Thus, the first aim of this study was to confirm the effects of the same exogenous cannabinoid on the acquisition, consolidation, and retrieval of hippocampal-dependent contextual fear memory. In accordance with previous literature (Pamplona and Takahashi, 2006), systemic injections of HU210 were found to impair acquisition, but not retrieval of hippocampal-dependent contextual fear memory. Results concerning the effects of HU210 on consolidation of hippocampal-dependent contextual fear memory were more complex. Systemic injections of HU210 (50 µg/kg) was found to significantly impair the last half of blocks of the 20 min test phase. When the experiment was conducted again using a higher dose of HU210 (100 µg/kg), systemic injections of HU210 (100 µg/kg) was found to significantly impair the first half of blocks of the 20 min test phase. This result is in accordance with previous research in that HU210 (100 µg/kg) was found to impair hippocampal-dependent contextual fear memory consolidation during the first 5-10 min of the test phase (Maćkowiak et al., 2009; Puighermanal et al., 2009). Therefore, both high (i.e., 100 µg/kg) and moderate (i.e., 50 µg/kg) doses of HU210 impaired contextual fear

memory consolidation, with high and moderate doses impairing the first and last half of the 20 min test phase, respectively. This first line of evidence suggests the phase-specific transient impairment of contextual fear memory following administration of different doses of exogenous cannabinoids.

Although CFC is believed to be a dorsal hippocampal-dependent task (Anagnostaras et al., 2001; Curzon et al., 2009), other brain regions, including the amygdala and mPFC are also involved (Cryan and Holmes, 2005; Figure 1). Thus, investigations employing bilateral injections of exogenous cannabinoids into specific subregions of the hippocampus, amygdala and mPFC should be conducted in order to deduce which of these subregions and their interactions underlie this cannabinoid-induced impairment of acquisition and consolidation of contextual fear memory. CB<sub>1</sub>Rs exist in all subfields of the hippocampus (Herkenham et al., 1991; Jansen et al., 1992; Tsou et al., 1998) and in the BLA (Mackie, 2005). All projections in the neural circuit underlying CFC except those from the CE are glutamatergic (Sah et al., 2003). Therefore, it is plausible to hypothesize that exogenous cannabinoids activate CB<sub>1</sub>Rs in both the hippocampus and BLA, decreasing neurotransmitter release from excitatory neurons, reducing CE output and then resulting in a decreased fear response (i.e., freezing). Future experiments should also be conducted to examine whether endogenous cannabinoids, directly or indirectly via manipulations of the endocannabinoid system (e.g., CB<sub>1</sub>R antagonists, CB<sub>1</sub>R-KOs, or endocannabinoid synthesis or hydrolysis inhibitors) impact the processing stages of contextual fear memory in a similar manner as exogenous cannabinoids.

Exogenous cannabinoids have been found to induce *in vitro* LTD-like synaptic depression in the hippocampus, amygdala, and mPFC (Gerdeman and Lovinger, 2003). These brain regions are implicated in fear-related anxiety disorders (Davis, 1992; LeDoux, 2000; Chhatwal and Kessler, 2007; LeDoux, 2007; Maren, 2008) and animal models of conditioned fear (Cryan and Holmes, 2005), and are also areas where studies utilizing Tat-GluR2 have shown that LTD expression occurs (Collingridge et al., 2010). These findings, together with the evidence that a single *in vivo* exposure of exogenous cannabinoids (i.e., HU210 or THC) impairs spatial working memory through CB-LTD expression at hippocampal CA3-CA1 synapses (Han et al., 2012), directed this study to examine whether cannabinoid impairment of acquisition and consolidation of contextual fear memory could be abolished by the LTD expression-blocking peptide, Tat-GluR2. Systemic injections of Tat-GluR2 did not significantly affect HU210 impairment of contextual fear memory acquisition. This result suggests that HU210 impairment of acquisition of contextual fear memory does not require *in vivo* CB-LTD expression. However, when high (i.e., 100 µg/kg) and moderate (i.e., 50 µg/kg) doses of HU210 were injected 1.5 h after systemic injections of Tat-GluR2, Tat-GluR2 significantly inhibited HU210-impaired consolidation of contextual fear memory. These data suggest that inhibition of CB-LTD expression abolishes HU210-induced impairment of contextual fear memory consolidation. Therefore, high and moderate doses of HU210 impair consolidation of contextual fear memory, which likely requires *in vivo* CB-LTD expression. When a high (i.e., 100 µg/kg) dose of HU210 was injected, the Tat-GluR2 + HU210 group, compared to the Tat-GluR2S + HU210 group, froze almost significantly more during the first block of the test phase ( $F_{3,28} = 14.50, p = 0.076$ , LSD *post hoc* test,

Figure 6C). This finding indicates that blockade of CB-LTD expression with Tat-GluR2 may partially block the impairment of contextual fear memory consolidation elicited by a high dose of HU210. Alternatively, a higher dose of Tat-GluR2 may be needed to block the effects induced by a higher, as opposed to moderate, dose of HU210. In order to test this hypothesis, future CFC experiments utilizing a higher dose of Tat-GluR2 and HU210 (100  $\mu\text{g}/\text{kg}$ ) need to be conducted. Furthermore, if this hypothesis is confirmed, investigations employing intra-hippocampal and intra-amygdala injections of Tat-GluR2 would help decipher where the CB-LTD expression that underlies the contextual fear memory consolidation impairment occurs.

Numerous studies have revealed that the acquisition of contextual fear memory extinction can be facilitated or inhibited either via exogenous cannabinoids (Pamplona et al., 2006; Pamplona et al., 2008) or via the enhancement (Bitencourt et al., 2008; de Oliveira Alvares et al., 2008; Pamplona et al., 2008) or inhibition (Suzuki et al., 2004; Pamplona et al., 2006; de Oliveira Alvares et al., 2008; Pamplona et al., 2008) of endocannabinoid action. However, in contrast to previous literature (Pamplona et al., 2006; Pamplona et al., 2008) we observed that systemic injections of HU210 (50  $\mu\text{g}/\text{kg}$ ) before extinction training under various protocols did not significantly affect the acquisition of contextual fear memory extinction during the extinction training phases (Figures 8 & 10). Since increasing doses of WIN 55212-2, another CB<sub>1</sub>R full agonist, positively correlate with increased amounts of freezing throughout extinction training (Pamplona et al., 2006), we further utilized a lower dose of HU210 (10  $\mu\text{g}/\text{kg}$ ). Interestingly, this lower dose of HU210 did not significantly affect freezing times (Figure 10). Together, these results imply that various doses of HU210 affect the

acquisition of contextual fear memory extinction similarly, with moderate (i.e., 50 µg/kg) and low (i.e., 10 µg/kg) doses eliciting amounts of freezing not significantly different from controls, during the 30 min extinction training phase. Furthermore, systemic injections of a potent inhibitor of both FAAH and MGL, JZL 195(10 mg/kg), were also found to not facilitate the acquisition of contextual fear memory extinction (Figure 9). Together, these results are in contrast to previous literature that have illustrated that exogenous cannabinoids (Pamplona et al., 2006; Pamplona et al., 2008) and the enhancement of endocannabinoid action (Bitencourt et al., 2008; de Oliveira Alvares et al., 2008; Pamplona et al., 2008) facilitate the acquisition of contextual fear memory extinction. In spite of this, our findings are plausible because as mentioned previously, our results (Figure 4) and previous literature (Pamplona and Takahashi, 2006) have already shown that exogenous cannabinoids impair hippocampal-dependent contextual fear memory acquisition. Since extinction is generally viewed, not as a loss of the CR (i.e., freezing), but as new learning based on the new association of the CS (i.e., the context) with the lack of the US (i.e., footshock; Cammarota et al., 2007), it is feasible that cannabinoids injected directly before extinction training impair and/or do not facilitate the acquisition of extinction.

Alternatively, discrepancies in the results concerning the effects of cannabinoids on acquisition of contextual fear memory extinction could be due to the fact that, like CFC, the hippocampus, mPFC, and amygdala have been implicated in its extinction (Sotres-Bayon et al., 2006). It has been hypothesized that during contextual fear extinction, the hippocampus modulates LA and/or IL neural activity (Sotres-Bayon et al., 2006), and the IL inhibits amygdala output via the activation of inhibitory LA

interneurons (Rosenkranz et al., 2003) and/or the activation of ICM inhibitory projections to the CE (Kaplan et al., 2011), resulting in the inhibition of fear responses (e.g., freezing) on succeeding experiences with the fear stimuli (Figure 2). Since CB<sub>1</sub>Rs have been found to be highly expressed in all subfields of the hippocampus (Herkenham et al., 1991; Jansen et al., 1992; Tsou et al., 1998), in the IL (Tsou et al., 1998), and in the BLA (Mackie, 2005), it is plausible that activation of CB<sub>1</sub>Rs in one or all of these brain regions could affect acquisition of contextual fear memory extinction. Since the hippocampus and IL are thought to inhibit amygdala output in contextual fear memory extinction (Sotres-Bayon et al., 2006), CB<sub>1</sub>R activation in both of these brain regions would disinhibit CE output, eventually resulting in an increased fear response compared to controls, reflecting an impairment of the acquisition of contextual fear memory extinction. However, since activation of CB<sub>1</sub>Rs typically leads to reduced neurotransmitter release (Murray et al., 2007; Heifets and Castillo, 2009; Saito et al., 2010), CB<sub>1</sub>R activation in the BLA would logically lead to a decreased CE output, which would result in a decreased fear response, possibly reflecting a facilitation of acquisition of contextual fear memory extinction, as was found by previous studies (Pamplona et al., 2006; Bitencourt et al., 2008; de Oliveira Alvares et al., 2008; Pamplona et al., 2008). Thus, investigations employing bilateral injections of exogenous cannabinoids and/or drugs that enhance endocannabinoid action into specific subregions of the hippocampus, mPFC, and amygdala should be conducted in order to clarify which of these subregions and their interactions underlie which cannabinoid-induced effects on the acquisition of contextual fear memory extinction.

Although this study did not find that exogenous and endogenous (i.e., indirectly via an endocannabinoid hydrolysis inhibitor) cannabinoids facilitate the acquisition of contextual fear memory extinction, the LTD expression-blocking Tat-GluR2 peptide was utilized to see if the acquisition of contextual fear memory extinction, as opposed to cannabinoid-induced, required *in vivo* LTD expression. In contrast to previous research investigating cued fear extinction (Dalton et al., 2008), systemic injections of Tat-GluR2 did not significantly impact the acquisition of contextual fear memory extinction any differently than its control peptide, Tat-GluR2S. This finding suggests that, unlike cued fear extinction, contextual fear extinction does not require *in vivo* LTD expression. Future investigations, utilizing the Tat-GluR2 peptide, need to be conducted to determine whether the effects of cannabinoids on acquisition of contextual fear memory extinction require CB-LTD expression.

In summary, this study confirmed that exogenous cannabinoids (e.g., HU210) impair acquisition and consolidation, but not retrieval of contextual fear memory. Interestingly, various doses of HU210 were found to impair contextual fear memory consolidation differently. Furthermore, while the HU210-induced impairment of acquisition does not require *in vivo* CB-LTD expression, the HU210-induced impairment of contextual fear memory consolidation may require *in vivo* CB-LTD expression. In contrast to previous literature, this investigation also found that even under various CFC extinction protocols, systemic injections of exogenous (i.e., HU210) and endogenous cannabinoids (i.e., indirectly via an endocannabinoid hydrolysis inhibitor) do not facilitate the acquisition of extinction of contextual fear memory. Interactions between various subregions of the hippocampus, amygdala, and mPFC, all of which highly

express CB<sub>1</sub>Rs (Herkenham et al., 1991; Jansen et al., 1992; Tsou et al., 1998; Mackie, 2005) and have demonstrated LTD expression (Collingridge et al., 2010), have been determined to be involved in CFC (Cryan and Holmes, 2005; Figure 1) and its extinction (Sotres-Bayon et al., 2006; Figure 2). Thus, future investigations should inject exogenous cannabinoids, drugs that enhance endocannabinoid action, and Tat-GluR2, directly into these subregions to deduce which of these subregions and their interactions underlie the cannabinoid-induced impairment of contextual fear memory acquisition and consolidation and which underlie the cannabinoid-induced effects on the acquisition of contextual fear memory extinction. Accordingly, this animal research will provide insights into how exogenous cannabinoids and manipulations of the endocannabinoid system can augment or even replace the current treatments of cue-specific fear-related anxiety disorders (e.g., specific phobias and PTSD), including exposure therapy (i.e., analogous to extinction training in animals) and anxiolytics, which are sometimes ineffective and produce adverse side effects, respectively (Degroot, 2008).

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