

Exploring novel treatment approaches for post-traumatic stress disorder

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Abstract

Post-traumatic stress disorder is a disorder characterized by an inability to extinguish traumatic memories and heightened reactivity to emotional stimuli. Due to the heightened resistance of traumatic memories to extinction, treatment for PTSD has been challenging and is limited to behavioral therapies targeted at reducing responsivity to threatening stimuli. Currently there are no standard pharmacological interventions that are specific to PTSD; rather, drugs used appear to target symptoms of some of the co-morbid conditions, such as anxiety (e.g. benzodiazepines) or depression (antidepressants) - which may also affect fear-memory. In this thesis, we explore the effects of natural health products (NHPs) including naturally occurring peptides and some medical botanicals on fear memory in order to explore the efficacy of natural products as potential pharmacological targets for fear-based disorders.

Fear-conditioning has been used effectively in both rodents and humans to study fear-learning. Fear-conditioning is a learning paradigm during which an unconditioned aversive stimulus (such as foot shock) is paired with a neutral stimulus (such as light or tone), such that the neutral stimulus becomes associated with aversion. Fear-learning has several well-characterized stages, including acquisition, consolidation, reconsolidation, expression, and extinction that can be manipulated in order to study the pharmacological action(s) on the attenuation of learned-fear. Blockade of reconsolidation, the state during which formed memories are briefly rendered susceptible to change following recall, may provide a window of opportunity to pharmacologically diminish learned fear. In Chapter 1 of the thesis, we discuss fear-conditioning as a pre-clinical model of PTSD to explore the effects of novel pharmacological treatments on the reconsolidation process in rodents. We ultimately hope to

provide a framework for translational work in humans for attenuating conditioned responses to trauma-related stimuli among humans with PTSD.

In Chapter 2, we present evidence that systemic administration of gastrin-releasing peptide attenuates the reconsolidation of conditioned fear in rodents. Similarly, in chapter 3, we explore the effects of Δ^9 -Tetrahydrocannabinol (THC) and Cannabidiol (CBD) on the reconsolidation of learned-fear, and provide evidence that cannabinoid molecules may similarly prove effective at blocking the reconsolidation of conditioned fear memories. In chapter 4, we present evidence demonstrating that extracts of medical botanical *Souroubea sympetala* and its components may similarly block reconsolidation of conditioned fear-memory, and also exert more general anxiolytic-like activity in the elevated plus maze paradigm. Finally, in chapter 5 a general discussion considers the relative therapeutic potential for future human clinical trials of each of the three tested groups of compounds.

Co-Authorship

In all cases, A. Murkar contributed to the study design, data collection, data analysis, and preparation of manuscripts. P. Kent contributed to the study design in chapters 2 and 3. J. James and C. Cayer contributed to data collection in chapters 2-4. Z. Merali contributed to the conceptualization of the study design and support to execution of all studies.

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List of Abbreviations

ACC: Anterior cingulate cortex

BA: Betulinic acid

BE: Betulin

BLA: Basolateral amygdala

CB1: Cannabinoid receptor type 1

CBD: Cannabidiol

CeA: Central amygdala

CeL: Lateral nucleus of the central amygdala

CeM: Medial nucleus of the central amygdala

CER: Conditioned emotional response

GABA: Gamma-amino butyric acid

GRP: Gastrin releasing peptide

GRPR (BB2): Gastrin releasing peptide receptor

LA: Lateral amygdala

mPFC: Medial-prefrontal cortex

SNS: Sympathetic nervous system

THC: Δ^9 -Tetrahydrocannabinol

Summary

Post-traumatic stress disorder (PTSD) is characterized by enhanced fear response and physiological alterations to both the stress system, consisting largely of the neurohormonal networks of the hypothalamic-pituitary-adrenal (HPA) axis, and the central fear circuitry consisting of the limbic system and prefrontal cortex. PTSD is also characterized by the “over-consolidation,” or excessive strengthening, of traumatic memories – thus, memories of trauma are abnormally resistant to extinction and therefore difficult to treat.

In addition, due to the lack of standardized pharmacologic interventions for PTSD, many individuals seek complementary and alternative medicine (CAM) as replacements of supplements for traditional medical treatment. These therapeutic practices are often unguided, with limited involvement of health care practitioners – yet there is evidence to suggest that natural health products (NHPs) may possess significant memory-altering and anxiolytic qualities. NHPs identified by native healers, for example, are often used to treat disorders which – according to local descriptions – bear resemblance to many mental disorders categorized by western medicine. Thus, the overall objective of the studies presented in this thesis was to determine the fear-memory altering and/or anxiolytic properties of naturally occurring peptides, crude extracts of medicinal botanicals, and isolated phytochemicals in rodent models which may in the future translate to studies with human participants. The findings presented here provide strong evidence that NHPs can modulate fear-memory, and may therefore have therapeutic use for the treatment of human fear-based disorders which are resistant to extinction (such as PTSD and phobias). With these studies, we hope to have laid the groundwork for future research in

clinical trials with humans for fear-based disorders. This summary highlights the main findings presented in this thesis.

1. Gastrin-releasing peptide (GRP) is the homologue of the amphibian peptide bombesin, a peptide originally isolated from the fire-bellied toad (*Bombina bombina*), and its receptor may play a role in fear-learning. If bombesin-like peptides are involved in the mediation of fear memory (as has been suggested), it is possible that gastrin-releasing peptide (GRP) could affect the reconsolidation of learned fear. BLA inhibitory interneurons express GRP receptors, and central injections of GRPR agonists mediate fear-learning in rats via this mechanism. In addition, although GRP is not believed to cross the blood-brain-barrier, peripheral administration of GRP produces effects on feeding and grooming which are mediated centrally (and which are blocked by central administration of GRPR antagonists). Evidence suggests GRPR agonists transmit signals centrally via multiple pathways from the periphery, including the brain-gut axis and vagus nerve. Thus, it was clear that although peripherally administered GRP does not enter the brain directly, it modulates central processes via a peripheral binding site.

We predicted that if peripheral binding of GRP signals central changes for fear-learning (similarly to how it does for feeding and grooming behaviors), peripherally administered GRP might be effective at blocking the reconsolidation of learned-fear. Our findings suggested that administration of GRP immediately after retrieval (recall) of contextual fear-conditioning attenuated the freezing response (a measure of fear expression) in rats 24h and 5d later. The effect was blocked by co-administration of a GABA_A BZD-antagonist Flumazenil, suggesting that the effects of GRP on learned fear may be mediated via a GABAergic mechanism. The effects of GRP persisted 5d later (day 7), suggesting that the effects were long-lasting and that

memories targeted by reconsolidation blockade were not susceptible to spontaneous recovery. In addition, GRP failed to attenuate the learned fear response when administered 30- or 60-min post-retrieval. This suggests that the effectiveness of GRP was quite sensitive to the time-course of the reconsolidation window, which is consistent with an initial peripheral binding site responsible for transmitting signals centrally. These findings suggest that (1) GRP may hold therapeutic potential for the treatment of fear-based disorders, but also that (2) that therapeutic potential may be limited by the sensitivity to the time-course of the reconsolidation window.

2. The second set of experiments focused on phytocannabinoids as potential mediators of reconsolidation of learned fear. Cannabis is frequently used to self-medicate (or, more recently, by prescription of medical marijuana) for PTSD and other conditions. In addition, sufferers of PTSD exhibit alterations of the central endocannabinoid system. Δ^9 -Tetrahydrocannabinol (THC), the primary active component of *Cannabis spp.*, has been shown to exert effects on fear expression in rodents. Cannabidiol (CBD) also exerts effects on fear-learning and expression, although the mechanisms of action of CBD are extremely complex and poorly understood. Various synthetic cannabinoids and CB1 antagonists have also been shown to modulate fear-learning.

Very little research has explored isolated THC and CBD (rather than whole plant material or synthetic cannabinoids) within the context of fear-memory reconsolidation. Here, we tested the effects of both isolated THC and CBD in varying concentrations as well as in combination with whole plant background material (all remaining plant components). Our findings suggested that CBD, but not THC, successfully attenuated the reconsolidation of learned fear. CBD alone administered immediately following recall of conditioned fear yielded significant reductions in

subsequent fear expression (freezing). THC yielded similar effects only when combined with plant background material. However, plant background material alone also successfully attenuated the learned fear response – suggesting the effects may be due to either the presence of synergist molecules affecting THC, or the action of other active principles in the background material on their own. These findings support the therapeutic potential of CBD alone (which is non-psychoactive), but also highlight that other molecules in the plant (e.g. triterpenoids) which are known to possess anxiolytic qualities (e.g. β -caryophyllene) might also hold therapeutic potential either on their own or as THC/CBD synergists.

3. Our final set of experiments focused on the therapeutic potential of *S. sympetala* (a neotropical vine found in South America). *S. sympetala* is used by local indigenous healers to treat a disorder which, according to local descriptions, bears a marked resemblance to PTSD. Evidence also suggests that betulinic acid (BA), one of the active principle components of the plants, is an intermediate agonist for the GABA_A BZD-binding site. Importantly, *S. sympetala* extract has been found to not produce withdrawal/dependency symptoms typically associated with Benzodiazepine use when administered in rodents. Thus, we sought to explore whether *S. sympetala* extracts would similarly be able to attenuate the reconsolidation of learned fear when administered immediately post-recall. In addition, since previous work has shown that the effects of BA are synergized by amyryns contained within the plant, we also explored whether BA could be synergized by the addition of betulin (BE). Betulin is a much more abundant molecule closely related to BA which can be more easily sourced from the bark of *Platanus spp.* (sycamore) which is indigenous to North America. In addition, since BA has been shown to exert anxiolytic-

like action in rodents, we also explored the effects of isolated phytochemicals BA and BE on fear-expression in the elevated plus maze (EPM) paradigm.

Our findings suggested that *S. sympetala* leaf extract significantly attenuated the reconsolidation of learned fear in a dose-dependent manner, and that a combination of BA + BE similarly attenuated the learned fear response. Leaf extract and BA + BE similarly exerted an anxiolytic-like effect in the EPM paradigm. Our findings suggest that *S. sympetala* leaf extract and isolated phytochemicals BA and BE may have therapeutic benefit for anxiety and fear-based disorders.

In conclusion, these investigations have provided strong evidence for the therapeutic benefits of three separate natural health products, and supports our hypothesis that both phytochemicals and naturally occurring peptides may be strong modulators of the reconsolidation of learned fear. In addition, the memory-altering properties of NHPs may be of use to the development of novel pharmacotherapies and CAM treatments for use as safe, effective, clinician-guided treatments for fear-based disorders such as PTSD.

1.0 General Introduction

Post-traumatic stress disorder (PTSD) is a psychological condition defined by hyper-reactivity to emotional stimuli and an inability to extinguish memories of trauma whose onset is triggered by exposure to actual or threatened death, serious injury, or sexual violation. PTSD affects up to 37.4% of individuals following exposure to a traumatic event [1], and although it is a fairly pervasive disorder, limited treatments exist (with little or no pharmacological intervention). Psychological interventions such as eye-movement desensitization and reprocessing (EMDR) and trauma-focused cognitive behavioural therapy (CBT) are somewhat effective, and have comparable response rates [2,3]. Exposure therapy can also reduce re-experiencing of the traumatic memory and avoidance behaviours significantly [3] – yet for numbing and hyperarousal, two core features of PTSD, EMDR and exposure therapy are ineffective for >50% of respondents at sustaining reduced symptomatology [3]. In addition, approximately half of those suffering from PTSD who do respond to treatment will remit within three years (although rates vary greatly and are lower for physical trauma than exposure to natural disasters; [4]).

1.1 Onset and symptoms clusters of PTSD

PTSD is characterized by a host of symptom clusters (summarized in Table 1) in addition to being persistent (duration of symptoms lasting greater than 30 days), causing clinically significant distress or impairment, and not being due to the physiological effects of a substance or other medical condition. PTSD also arises following exposure to a trauma (see Table 1, A1-A4) either through first-hand experience or, in the most recent version of the DSM (DSM-5), vicariously through first-hand observation of a traumatic event (or learning of a traumatic event) having occurred to a loved one or primary caregiver [5]. Although previously categorized as an

anxiety disorder (in DSM-IV-TR), PTSD is now categorized as a trauma- and stressor-related condition in DSM-5 [5]. Additionally, although PTSD is categorized as a single disorder (excluding childhood PTSD, which is categorized separately as a condition for children under 6 years of age), the phenotypic expression of PTSD can vary widely [6]. As a result of the varied expressions of the condition and the nature of diagnostic criteria for each symptom clusters, it is possible for two individuals to share the same diagnosis while expressing only a moderate degree of symptom overlap.

PTSD symptoms fall into four main clusters (see Table 1 B-E): intrusion symptoms (i.e. re-experiencing the trauma via intrusive thoughts or cognitions either during waking-day thought or while asleep in the form of recurrent distressing dreams), avoidance symptoms (i.e. avoiding triggers – people, places, or things – that remind the individual of the traumatic experience), negative alterations in cognition or mood (i.e. negative affect or inability to experience positive emotions), and arousal symptoms (i.e. hyperarousal/enhanced startle response, alterations in sleep-wake patterns, etc.). In addition, diagnosis of PTSD is also specified as being with or without dissociative symptoms (i.e. feelings of de-personalization or de-realization).

Table 1	
<i>Trauma criteria and major symptom clusters for post-traumatic stress disorder according to the DSM-5 [5].</i>	
A.	Exposure to actual or threatened death, serious injury, or sexual violence in one (or more) of the following ways: 1. Directly experiencing the traumatic event(s) 2. Witnessing, in person, the event(s) as it occurred to others 3. Learning that the traumatic event(s) occurred to a close family member or close friend. In cases of actual or threatened death of a family member or friend, the event(s) must have been violent or accidental 4. Experiencing repeated or extreme exposure to aversive details of the traumatic event(s)

- B. Presence of one (or more) of the following intrusion symptoms associated with the traumatic event(s), beginning after the traumatic event(s) occurred:
1. Recurrent, involuntary, and intrusive distressing memories of the traumatic event(s). In children older than 6 years, repetitive play may occur in which themes or aspects of the traumatic event(s) are expressed
 2. Recurrent distressing dreams in which the content and/or affect of the dream are related to the traumatic event(s). In children, there may be frightening dreams without recognizable content.
 3. Dissociative reactions (i.e. flashbacks) in which the individual feels or acts as if the traumatic event(s) were recurring. (Such reactions may occur on a continuum, with the most extreme expression being a complete loss of awareness of present surroundings.) In children, trauma-specific re-enactment may occur in play
 4. Intense or prolonged psychological distress at exposure to internal or external cues that symbolize or resemble an aspect of the traumatic event(s)
 5. Marked physiological reactions to internal or external cues that symbolize or resemble an aspect of the traumatic event(s)
- C. Persistent avoidance of stimuli associated with the traumatic event(s), beginning after the traumatic event(s) occurred, as evidenced by one or both of the following:
1. Avoidance of or efforts to avoid distressing memories, thoughts, or feelings about or closely associated with the traumatic event(s)
 2. Avoidance of or efforts to avoid external reminders (people, places, conversations, activities, objects, situations) that arouse distressing memories, thoughts, or feelings about or closely associated with the traumatic event(s)
- D. Negative alterations in cognitions and mood associated with the traumatic event(s), beginning or worsening after the traumatic event(s) occurred, as evidenced by two (or more) of the following:
1. Inability to remember an important aspect of the traumatic event(s) (typically due to dissociative amnesia, and not to other factors such as head injury, alcohol, or drugs)
 2. Persistent and exaggerated negative beliefs or expectations about oneself, others, or the world
 3. Persistent, distorted cognitions about the cause or consequences of the traumatic event(s) that lead the individual to blame himself/herself or others
 4. Persistent negative emotional state
 5. Markedly diminished interest or participation in significant activities.
 6. Feelings of detachment or estrangement from others
 7. Persistent inability to experience positive emotions (e.g., inability to experience happiness, satisfaction, or loving feelings)

- E. Marked alterations in arousal and reactivity associated with the traumatic event(s), beginning or worsening after the traumatic event(s) occurred, as evidenced by two (or more) of the following:
1. Irritable behavior and angry outbursts (with little or no provocation), typically expressed as verbal or physical aggression toward people or objects
 2. Reckless or self-destructive behavior
 3. Hypervigilance
 4. Exaggerated startle response.
 5. Problems with concentration
 6. Sleep disturbance

Although the symptoms of PTSD can begin to manifest themselves soon after exposure to trauma, not all individuals exposed to a traumatic experience develop the condition. Evidence suggests that a variety of risk factors are associated with vulnerability to PTSD following trauma-exposure, including (but not limited to) epidemiological and socioeconomic factors [7], sex [8,9], and the type of trauma [10] (i.e. sexual violence versus physical trauma). Evidence from functional imaging studies further suggests that PTSD subtypes (including dissociative and hyperarousal symptom clusters) may be functionally distinct [11,12]. Herein, the focus is not on modelling PTSD *per se*. Rather, the primary focus of this work is on modelling phenotypic expression of diagnostic criteria A and E (i.e. using learned fear responses as a model for trauma exposure and subsequent hyperarousal and fear-associated symptoms) as core features of the disorder rather than PTSD as a whole. In subsequent sections, therapeutic compounds are introduced, and the theoretical framework of fear-memory is discussed in more detail as a model of trauma and the hyperarousal/fear-based symptom cluster.

1.2 Natural products as treatment

Among sufferers of PTSD, traumatic memories may become over-consolidated (reinforced to such an extent that they become resilient to extinction; [13]). Individuals with PTSD tend to

express intensified memory for the trauma [14]. In addition, PTSD is characterized by a number of functional and structural brain changes. For example, it has been suggested that amygdala hyper-responsivity to emotional stimuli is characteristic of the disorder. Although limited treatments exist for PTSD, pharmacologic alteration of established fear-memories may be one route for developing treatments that target anxious-arousal symptoms of the disorder, and extensive research suggests that some natural products may be of use for attenuating learned fear responses and/or anxiety-like symptoms. Natural products also play a pivotal role in drug discovery, and it is likely that the same will remain true for discovery of treatments for fear-based disorders. As a result, it is probable that natural products will exert significant therapeutic effects relevant for fear-based disorders such as PTSD. There is some evidence that both naturally occurring peptides and plant extracts do indeed possess anxiolytic and/or fear-memory altering qualities, and some have shown promise in pre-clinical rodent models of learned fear and PTSD [15–22].

Ethnomedicine (or ethnobotanic medicine) is broadly defined as the use of plants as medicine by humans [23–25], and traditional medicine is a broader term that characterizes all medical practices that fall outside of the scope of modern western-medicine. However, traditional medicines derived from natural sources are not only limited to plant-derived products. A wide variety of natural products from both plants and animals have been the source of numerous medications (both historically and in recent research). Within the past 20 years, for example, the FDA has approved medications originally derived from cone snails, tropical sea squirts (invertebrate ocean-dwelling filter feeders often found in shallow water on rocks or coral), cyanobacteria, and other organisms including bacteria living in extreme environments (such as at extreme depths in the oceans and in very alkaline environments) [26–31].

Before the advent of high-throughput screening (HTS), a drug discovery technique that utilizes robotics to rapidly identify potential therapeutic compounds (and thereby greatly reduces the difficulty of testing a very large numbers of molecules that might possess therapeutic effects), over 80% of drugs had origins that could be traced to natural products [32]. This remained to be the case until the late 1990s - although after the industry transitioned away from reliance on natural products and looked to other methods of drug discovery, there was a sharp reduction in the number of new drugs approved per year. That number then remained fairly consistent or decreased further from 1989 until at least 2007 [31]. There are other concerns which threaten the use of natural products in drug discovery as well. Identification of new drugs from natural products relies very heavily on the availability of those natural products. As a result, extinction of species that are endemic to a single region is a large concern due to deforestation and climate change; 21-30% of medicinal plant species are believed to be threatened with extinction [33]. Combined with other concerns that affect availability of natural products (such as long delays in legally accessing botanicals from some forests), the result is a net decrease in the pool of natural resources that may yield medicinal botanicals that harbor potential for the development of compounds with therapeutic benefit.

Despite this the rate at which successful drugs are developed based on screening of natural products, their isolated phytochemicals, and analogues has been extremely fruitful (with a relative success rate of 0.3%, versus less than 0.001% for other methods) [34]. Compared to current methods, an ethnomedical approach has been much more successful [35]. Of the thirteen drugs derived from plants identified and approved by the FDA in the United States from 2005-2007, five of them were the first members of entirely new classes of drugs [36,37], and in 2015 the Nobel prize in medicine was awarded for the discovery of a medication derived from a

natural health product. That medication, artemisinin (or ‘Quinhaosu’ in Chinese), led to significant reductions in mortality rates for patients afflicted by malaria [38] and was the product of a significant push to develop NHPs that could be beneficial for the treatment of disease by modern medical practitioners, but which were also derived from practices used in traditional Chinese medicine [39].

There is also historical precedent for the importance of naturally occurring molecules from plants & animals in drug discovery. Many drug classes still in use today, for example, were first derived based on insights from native traditions whose origins date back to before the Spanish first visited Latin America in the late 1400s [40]. The first pharmacologically active pure compound was derived from cut seeds of the opium poppy *Papaver Somniferum* by Friedrich Sertüner for postoperative pain relief over 200 years ago [41]. That isolated alkaloid, morphine, is still in use for pain management today.

As a result, it is clear that products derived from natural sources often yield significant and long-lasting advances in medicine. Despite this, overall there has been a relative decrease in the number of plant- and animal-derived medications being tested in translational studies and later-phase clinical trials in humans. This decrease is also partially attributable to the perceived difficulty of natural product chemistry (since it is sometimes more labour intensive to identify and test active principle components from plant extracts which are complex mixtures containing many different groups of molecules that may interact) and difficulty of obtaining access to products from remote regions of the world [37], since many plant-derived medicines are identified by anthropologists (rather than biochemists) based on the traditions of remote - and sometimes difficult to access – native healers. It is clear, however, that despite these difficulties natural products from plants and animals may have a large role to play in the discovery of new

medications for the treatment of human disorders, and they may hold promise for the discovery of entirely new classes of pharmaceuticals that might prove advantageous for the treatment of human conditions that have otherwise proved resistant to treatment with currently available interventions (e.g. major depressive disorders, post-traumatic stress disorder, etc.).

In this thesis, the primary aim is to explore the effects of some of these natural products on reconsolidation (the process by which formed memories are rendered susceptible to change) as a more general approach to targeting learned-fear, which may have practical applications in treating fear-based disorders such as PTSD and phobias which may be in part based in learned fear associations. In the following sections we explore the role of fear-learning in the pathophysiology of PTSD and the use of Pavlovian conditioning as a model for fear-based disorders. In subsequent sections, three natural products are discussed as possible novel interventions that may have therapeutic benefit for the treatment of fear-based disorders.

1.3 Rodent Models of fear-learning and PTSD

Human PTSD consists of a host of neurochemical and biological alterations to the hormonal stress system, SNS activity, and the central neurological fear circuitry (which are discussed in more detail in later sections). There are several different approaches to studying fear-learning in rodents, each of which may involve these systems to varying degrees.

Although not necessarily identical to human PTSD, animal models recruit a number of the same brain regions thought to play a key role in anxious-arousal symptoms of PTSD (e.g. amygdala, hippocampus, and mPFC). It is difficult (if not impossible) to entirely isolate the contributions of the stress and fear systems of the brain in behavioural models. However, there are a number of stress- and fear-based models which attempt to mimic PTSD or a specific subset of PTSD symptoms. Single prolonged stress (SPS), for example, is considered to be a stress-

based model intended to mimic exposure to actual or threatened death (one of the triggers of PTSD highlighted in the DSM-5) wherein rodents are exposed to several severe, consecutive stressors over a long period of time (two hour physical restraint, twenty minutes of forced swim, and anaesthetisation with diethyl ether; [42]). Indeed, evidence suggests that SPS may produce many of the physical and neurological signs of PTSD in rodents including enhanced startle response and HPA-axis abnormalities [43–45]. However, although SPS in some cases yields PTSD-like responses in rodents, results are mixed and the paradigm may not reflect human PTSD. In addition, SPS is comprised of a mix of both stressors and fear-memory cues (contextual, physical, olfactory, etc.), making it difficult to tease apart the networks which mediate its effects.

Classical conditioning (the learning process by which an aversive stimulus is paired with a neutral stimulus until the unconditioned response is evoked by the neutral stimulus alone), in contrast, is shorter than SPS and more acute in nature – and has similarly been used extensively in PTSD research. Although fear-conditioning is not in itself intended to mimic PTSD, it may be used to address mechanisms of fear-learning whilst also having the advantage of targeting responses to more specific triggers. Exposure to a traumatic stressor and hyperarousal are two core features of PTSD (See Table 1 A and Table 1 E), and classical conditioning captures the learned-fear aspect of the condition as well as hypersensitivity features of the condition. SPS, in contrast, is a model intended to reproduce PTSD as a whole, and has been demonstrated to require the combination of all three aversive triggers [46]. In the case of conditioned emotional response (CER), an aversive foot-shock (unconditioned stimulus; US) is paired with either a tone or the conditioning chamber (conditioned stimulus; CS). Following this procedure, presentation of the CS alone will elicit a fearful response (freezing). Thus, CER has the advantage of

addressing some of the core features PTSD while also having greater specificity in terms of which symptom clusters are being addressed and which triggers are associated with acquired fear responses. Additionally, since it is not a model of PTSD *per se*, results are more generalizable to other fear-based disorders (e.g. phobias). Fear conditioning allows the observer to examine, via various methods of intervention, effects of pharmacological manipulation on all stages of fear-learning (acquisition, consolidation, expression, reconsolidation, and extinction), and has been used successfully to model fear-learning under a variety of conditions [15–17,47]. Here, we pay particular focus to reconsolidation blockade as a model for pharmacologic intervention which may have the benefit of targeting memories which are resistant to extinction.

1.4 Consolidation, reconsolidation, & memory updating

Consolidation refers to the process by which recently acquired memories are strengthened for long-term storage; according to the ‘lingering consolidation’ hypothesis, memories become stronger and more resilient over time [48] (see figure 1).

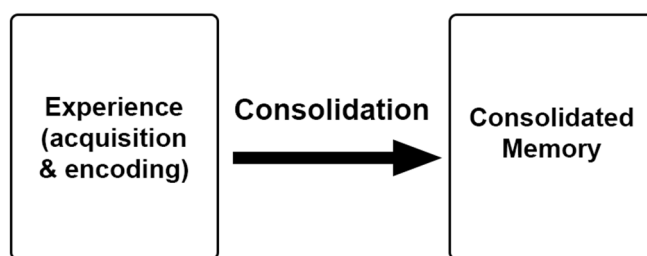


Figure 1. Newly acquired memories are encoded and then consolidated for long-term storage.

Limbic regions such as the amygdala and hippocampus play a key role in the consolidation of newly acquired memories into a more stable, long-term form. Infusion of β -

adrenergic agonists into the basolateral amygdala (BLA) post-learning, for example, enhances memory for learned fear responses while antagonism and BLA lesioning have the opposite effect [49,50]. Similarly, consolidation of long-term spatial memory is blocked by mRNA synthesis inhibition at the CA1 region of the hippocampus [51].

Reconsolidation, by contrast, refers to the process by which recalled memories are rendered labile, producing a state of limited duration during which memories are vulnerable to change or interference (the reconsolidation window). Reconsolidation was initially identified in animal models of electroconvulsive shock therapy which produced significant memory loss [52–54]. Evidence has also shown that reconsolidation at the BLA is blocked by administration of protein synthesis inhibitor anisomycin [55], and it has been suggested that memories must be reconsolidated following recall in order to be maintained for long-term storage. Interestingly, a meta-analysis demonstrated that recently acquired memories are usually less susceptible to change than older memories [56] – although this is not universally accepted [57]. While the lingering consolidation hypothesis would suggest that memories become stronger and more resilient to change over time [58], these findings instead suggest that this is not always the case. This is particularly relevant for development of treatments for PTSD, since trauma-memories are often remote. Thus, reconsolidation may offer a viable target for development of novel treatment regimens.

It has been suggested that consolidation and reconsolidation are distinct processes mediated by different patterns of regional activation and neurochemical transmission. For example, consolidation requires hippocampal BDNF signaling while reconsolidation does not [59]. Crucially, this highlights that consolidation and reconsolidation are controlled by distinct molecular mechanisms within the same region. Similarly, consolidation of inhibitory avoidance

memory requires hippocampal expression of some transcription factors that are not required for reconsolidation [60]. Although the processes bear some similarity, recall also does not identically recapitulate patterns of activation seen during consolidation [61]. As well, expression of c-fos at the intermediate medial hyperstriatum ventrale (IMHV) is enhanced during consolidation but not during reconsolidation [61]. Taken together, these findings collectively suggest that reconsolidation is a unique memory process which is distinct from consolidation.

Reconsolidation also appears to be distinct from extinction learning (the process whereby new learning inhibits older memories). CB1 antagonism and blockade of L-type voltage gated calcium channels block extinction but not reconsolidation in one study [57]. In addition, reconsolidation of learned fear is blocked by protein synthesis inhibition at the amygdala and hippocampus, while blockade of extinction is achieved by protein synthesis inhibition at mPFC [62].

Yet a means of reliably manipulating consolidation but not reconsolidation (or vice versa) has not yet been identified for emotional memory – and while the two appear distinct in some respects, the molecular mechanisms of consolidation and reconsolidation also share some overlap. Pharmacological blockade of hippocampal protein synthesis, for example, blocks both consolidation and reconsolidation of contextual fear conditioning – and the same is true of the amygdala for auditory fear conditioning [55,63]. Propranolol, which has been tested in human trials of reconsolidation blockade [64], similarly blocks both consolidation and reconsolidation of inhibitory avoidance in rodents [65].

Our current understanding of the mechanisms of reconsolidation is incomplete, and many questions remain to be addressed. For example, does reconsolidation produce amnesia for learned fear responses as some authors suggest [55], or are memories updated during

reconsolidation in order to facilitate incorporation of new information? One approach suggests that following recall, long-term memories are updated only when there is new information pertinent to the long-term memory [66]. According to this approach, retrieval alone is insufficient to trigger memory reconsolidation. Instead, retrieval and reconsolidation are dissociable processes in which reconsolidation is only sometimes triggered (when updating of long-term memory is required). Some research suggests that prediction error (the degree to which predicted outcomes, based on older memories, differ from new experiences) may mediate memory updating. According to this perspective, reconsolidation is only triggered when present experience varies significantly from expectation (i.e. initiation of reconsolidation depends on the need to update memories with new, pertinent information) [67–69]. This would suggest reconsolidation is a facet of adaptive learning and memory updating, and not a process triggered each time memories are recalled in order for them to be maintained for long-term storage.

In support of the memory updating hypothesis, negative emotional arousal following retrieval (thought to trigger the need for memory updating) enhances later recall [70]. As well, recall of an older procedural sequence memory followed immediately by new sequence learning negatively impacts performance on the original task [71]. This suggests new learning immediately after recall, without pharmacological intervention, is sufficient to induce attenuation of recall for older memories. The same is true of declarative task learning [72] and episodic learning [73], lending support to the notion that reconsolidation updates and incorporates new information to old learning (rather than solely strengthening or re-stabilizing memories for maintenance and long-term storage). Using a sequential finger-tapping task, Gabitov et al. also demonstrated that genuine impairments in consolidated motor skill memories can be induced by

new learning which interferes with the original task when introduced immediately (but not 8h) following training [74].

It may also be the case that reconsolidation is a more complex process capable of both destabilizing recalled memories and incorporating new information. Some authors suggest that reconsolidation has dissociable phases during which recalled memories are destabilized (or labilized) and then re-stabilized – thus, reconsolidation may include *both* facets of memory destabilization and memory updating [67]. Protein degradation is required for the destabilization of recalled memories but not for their re-stabilization [75], highlighting that memory destabilization and re-stabilization are also dissociable processes with different underlying mechanisms. As a result, it may be the case that some interventions modulate one process but not the other. As in the case of Nader et al. for example [55], it is possible that protein synthesis inhibition interferes with memory re-stabilization but not destabilization (since destabilization has been shown to be resistant to protein synthesis inhibition). Conversely, non-pharmacological interventions or those enhancing or altering the way in which memories are re-stabilized [76] may induce memory alterations (updating) during re-stabilization without blocking re-stabilization (i.e. reconsolidation blockade) or enhancing memory destabilization (instead producing changes in subsequent fear responses by incorporating new learning with older memories). Interestingly, although they appear similar, this process (in which older memories might be changed so as to reduce subsequent fear expression) is thought to be distinct from extinction learning (in which new learning inhibits the expression of an older learned response) [62]. A more modern understanding of memory processes thus includes both facets of acquisition and consolidation of recently acquired memories for long-term storage as well as the destabilization and re-stabilization of those stored memories (see figure 2).

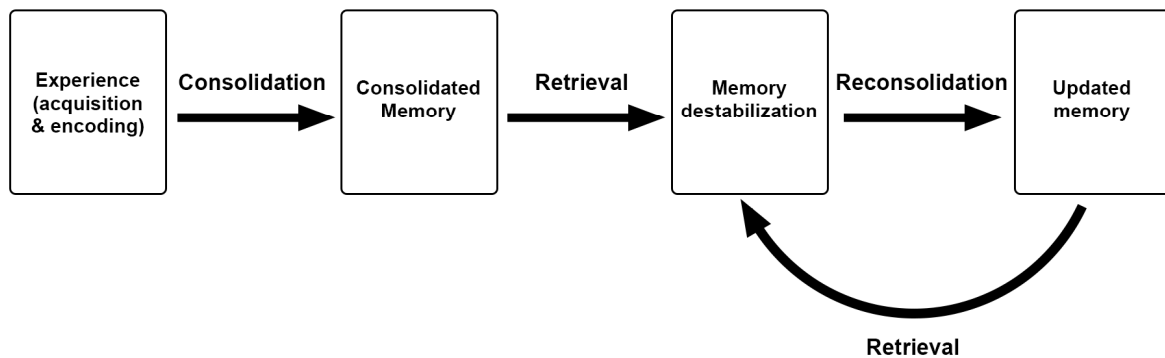


Figure 2. Consolidated long-term memories may be destabilized and re-stabilized during reconsolidation in order to incorporate new, relevant information.

It has been suggested that memory updating during reconsolidation may be the mechanism responsible for the efficacy of a variety of non-pharmacological therapeutic treatment regimens [77] - although this hypothesis has yet to be sufficiently tested. In sum, it can be stated that reconsolidation is a complex process distinct from both consolidation and extinction, and is characterized by dissociable stages of destabilization and re-stabilization. Reconsolidation may also constitute memory updating (incorporation of new information with old learning). In addition, recall alone may be insufficient to induce reconsolidation. Instead, reconsolidation may require a sufficient degree of prediction error signaling the need to update

older memories to incorporate newer, unexpected information with previously formed associations.

1.5 Reconsolidation Blockade

In terms of therapeutic approaches, the principle benefit of targeting reconsolidation lies in the fact that memories targeted in this fashion seem less vulnerable to spontaneous reinstatement [55,77,78]. This is an important concern for PTSD, since memories of trauma are often prone to reinstatement [79] and are notoriously difficult to extinguish [80–82]. In addition, reconsolidation may be able to update or destabilize older memories which are resistant to extinction. This is an important consideration for PTSD since the time between the initial trauma and when an individual first seeks treatment could be quite long (in some cases years or decades). During the reconsolidation window, pharmacological disruption can potentially alter or inhibit the updating or re-storage of previously formed fear-memories – and thus diminish responsiveness to conditioned aversive stimuli (see figure 3).

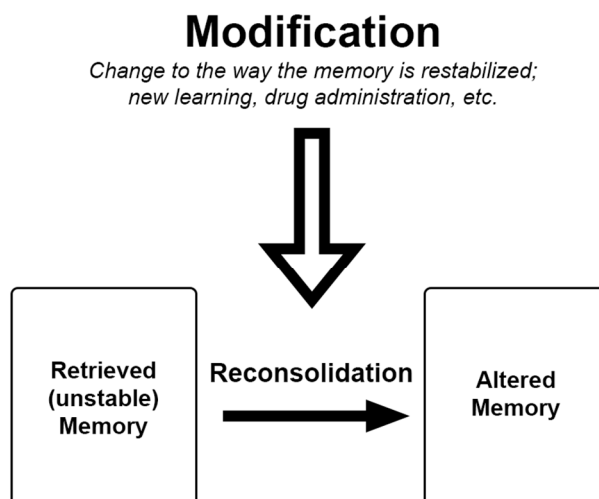


Figure 3. Modifying the reconsolidation process (i.e. via introducing new learning, drugs, etc.) after recall may affect memory re-stabilization, thus altering the resulting re-stabilized memory trace.

It has been demonstrated that subsequent expression of learned fear responses can be attenuated pharmacologically by targeting reconsolidation with drugs administered immediately after retrieval of a learned fear association, while no effect is produced if drugs are administered in same time frame but in the absence of fear-memory re-exposure (i.e. recall/retrieval) [78]. This model of interventions has been used in animal research to identify potential therapeutic targets for fear-based disorders such as PTSD [13,83,84]. For example, the β -adrenergic antagonist propranolol (which is typically used to treat high blood pressure and/or heart arrhythmias) has been shown to disrupt fear memory in rats when administered immediately following recall of the fearful memory trace [83]. Similar findings have also been observed in animal models with xenon, and NMDA receptor antagonist [78], and cannabidiol, a phytocannabinoid [85]. However, with regards to human studies, results have been mixed. Propranolol administration following fear-memory retrieval reduced physiologic responses to

traumatic imagery in sufferers of PTSD in one study [86]. However, subsequent follow-up studies failed to replicate this [64]. As a result, new therapeutic targets are needed for future translational work to determine whether reconsolidation blockade can be successfully implemented in studies with humans. Natural health products which have been shown to exert effects on other aspects of learned fear (i.e. consolidation, extinction, and expression) may offer one such avenue for diminishing learned fear.

1.6 Pathophysiology of PTSD: Role of the HPA-axis and sympathetic nervous system

PTSD is a complex disorder whose onset likely necessitates the involvement of several systems – and at the minimum, PTSD appears to consist of alterations to the stress system, sympathetic nervous system (SNS), and central nervous system fear-networks. Although this thesis focuses principally on fear-learning (rather than stress as a model of PTSD), it is important to consider that neurohormonal stress responses, SNS, and fear-circuitry of the prefrontal cortex and limbic system all play a role in the condition (and are all interconnected to some degree). Thus, although the experiments in the following chapters do not attempt to directly address these systems, it is important to discuss them briefly. In response to stress, organisms react with a variety of adaptive responses involving the central nervous and neuroendocrine systems [87] involving both limbic systems and the hypothalamic-pituitary-adrenal (HPA) axis [88–90] and some evidence suggests PTSD may be characterized by permanent or long-lasting alterations to these systems [42,91–97].

In terms of the stress response, stressor exposure is thought to trigger the activation of ascending peripheral nervous system (PNS) neurons which signal information about important homeostatic changes (e.g. distress, pain, and blood loss) [98] to spinal cord and brainstem sites. These brainstem neurons project to the periventricular nucleus (PVN) of the hypothalamus [99],

and in response PVN initiates HPA-axis activity and the begins release of neurohormones [100–102]. The PVN contains both sympathetic- and parasympathetic-projecting neurons, suggesting it plays a role both in the activation and inactivation of HPA-axis activity as well as the NE dependent activity of the SNS [103,104].

Yet PVN also receives inhibitory GABAergic inputs indirectly from several limbic regions [105–109], indicating a complex reciprocal role of fear and stress systems in both the activation and inactivation of HPA and SNS activity. Activation of the medial nucleus of the amygdala (CeM; a key amygdala region implicated in fear expression) is triggered by psychological stress [110–114], and CeM lesioning blunts HPA-axis response to psychological stress [115] – thus, the initiation of the stress response would seem to be (at least in part) dependent upon the fear-associated networks of the amygdala. However, CeM sparsely projects to PVN [116] – thus, it is likely that CeM only modulates PVN activity via an indirect mechanism.

Sufferers of PTSD exhibit a number of stereotypic HPA-axis alterations. For example, lower levels of plasma cortisol despite elevated corticotropin-releasing factor (CRF, a polypeptide hormone secreted by the hypothalamus as a part of stress-induced HPA-axis activity) are common among individuals with PTSD [117–120]. CRF is released by PVN, which binds to sites at the anterior pituitary gland and promotes the release of adrenocorticotrophic hormone (ACTH); ACTH then acts at the adrenal cortex to promote the release of glucocorticoid hormones (cortisol in humans or corticosterone in the rat) [99]. Aside from their role in stress, release hormones (e.g. CRF) and glucocorticoid hormones (cortisol/corticosterone) may also play a role in consolidation of traumatic memories. In rodents, for example, a CRF₁ antagonist blocked consolidation of stressor effects on startle magnitude in one study [121], suggesting a role for CRF in fear-learning.

Nocturnal administration of hydrocortisone also reduced subsequent amygdala reactivity to negative stimuli in one study in humans [122]. Hypocortism in PTSD might thus also play a role in preserving reactivity to emotional stimuli, which is partly sleep-dependent and influenced by both waking-day and nocturnal glucocorticoid secretion [123].

The HPA-axis receives negative feedback from secretion of glucocorticoids by the adrenal gland. Mice lacking cortical glucocorticoid receptors have elevated basal glucocorticoid levels, suggesting a failure of negative feedback inhibition of upstream HPA-axis structures (e.g. PVN) [124,125], while GR overexpression has the opposite effect on both ACTH and glucocorticoid availability [126,127]. Thus, HPA-axis activation produces a negative feedback loop in which the products of its activation will in turn exert an inhibitory influence at PVN (see figure 1).

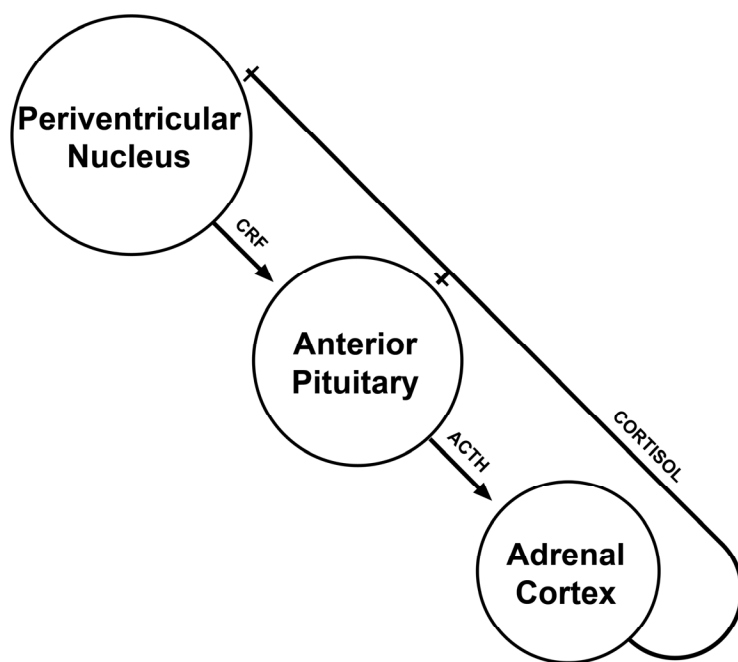


Figure 4. Negative feedback loop of stressor-induced neurohormone secretion by the hypothalamus, pituitary, and adrenal gland.

In addition to alterations to the activation of the HPA axis, PTSD is also characterized by changes to SNS activity – the division of the nervous system primarily responsible for activation of the body’s fight-or-flight response. The release of norepinephrine (NE) stimulates adrenergic receptors, and initiates SNS-associated effects in peripheral tissues involved in mobilization of bodily resources to cope with threat and danger (e.g. increased blood flow, reduced digestive activity, hypertension, etc.). NE is one of the most widely distributed neurotransmitters in the human body. In the CNS, NE is primarily released by the locus coeruleus (LC) [128], which projects to most other regions of the neocortex (including fear-associated regions like the basolateral amygdala). In the peripheral nervous system (PNS), NE is used in neurotransmission by SNS ganglia localized to the spinal cord, chest, and abdomen, and is also released into the bloodstream by the adrenal cortex following SNS activation [128] from which it gains wider access to peripheral tissues.

Chronic stress and PTSD are characterized by an elevation of SNS activity (SNS reactivity, disrupted sleep, and tonic activity of the SNS) [129], and individuals with PTSD have been shown in some cases to exhibit enhanced levels of norepinephrine in cerebro-spinal fluid (CSF) [130]. Some authors suggest that chronic activation of stress-related mechanisms, including the release of CRF, can lead to a reduction in the normal activity of other systems (including PTSD-related hypocortisolism and NE/SNS alterations). While acute release of glucocorticoid hormones may exert a protective effect (i.e. increased resiliency to the effects of trauma) [131], chronic stress (i.e. the chronic release of CRF) may instead result in long-term downstream changes to various systems – including enhanced sensitivity of glucocorticoid receptors, enhanced NE/SNS activity, and reduced secretion of cortisol [130]. These alterations in PTSD have been characterized by some authors as a condition of “allostatic load” [132] – that is, that the biological cost of allostasis

(achieving homeostasis through biological change or adaptation of bodily mechanisms including the stress response) [132,133] has resulted in a maladaptive long-term change to the regulation of both the HPA axis and noradrenergic systems of the SNS. This is further supported by evidence which suggests that, in addition to enhanced CRF and reduced cortisol secretion, PTSD is also characterized by behavioural indications of enhanced SNS activation (including increased heart rate, skin conductance, and blood pressure) [134–136]. Finally, levels of urinary catecholamines have been similarly demonstrated to be enhanced in sufferers of PTSD in a variety of studies [137–139], as have plasma NE levels [140,141].

1.7 Pathophysiology of PTSD: Fear Circuitry

In addition to altered HPA-axis and SNS/NE activity, PTSD is also characterized by alterations in fear-associated prefrontal and limbic system signaling. Among those with PTSD there is a high prevalence of heightened amygdala responsivity to emotional stimuli which has been observed upon presentation of a wide variety of fearful stimuli in humans [142–146], suggesting that hyper-responsivity of the amygdala is characteristic of PTSD pathophysiology. Imaging studies also suggest that this activity also correlates with symptom severity [147], lending support to the notion that amygdala hyper-responsivity may be a defining feature of PTSD. Fear-learning and fear expression are believed to be controlled by distinct amygdala microcircuits, with the central nucleus (CeA) playing a more significant role in fear expression [148]. Conversely, it has been suggested that LA acts as the point of convergence for relevant signals from the periphery while BLA plays a role in fear-learning. GABAergic transmission in the BLA mediates fear-learning [149], and GABAergic signaling affects fear expression within the central nucleus and activity-dependent plasticity in the lateral nucleus [148,150–152].

In addition to amygdala hyper-responsivity, there is also evidence to suggest decreased activation of the mPFC in individuals with PTSD (as well as some differences in morphology of the frontal cortex in general). Structural MRI studies have noted a decreased volume of the anterior cingulate cortex (ACC), and ACC volume correlates with symptom severity [153–155]. Right amygdala activation in response to emotional-provoking facial images also negatively correlates with ventral PFC activity in humans [156]. Additionally, the ability of participants to regulate their own emotional states in one study was associated with increased dorsal- and ventral-lateral PFC activity [12], further suggesting a role for PFC in inhibitory control of amygdala responsivity. Expectation also plays a large role in regional activation in humans; expectation of aversive stimuli, for example, produces increased activation of the amygdala and cingulate cortex [157–159], while expectation of reward activates the amygdala and nucleus accumbens [160,161]. Anticipation of emotion-related events has been shown to recruit mPFC under a variety of rewarding or aversive conditions in humans [158,161–163], further suggesting a role for mPFC in subsequent emotion-related regional brain activation (and might suggest altered anticipatory responses toward emotional events among sufferers of PTSD).

Almost all of what we know about the role of mPFC and amygdala microcircuits in fear learning comes from rodent studies. With consideration for human PTSD, it is important to consider findings in terms of human brain anatomy. In the rest of this section, we examine some of the differences in rodent versus human/primate physiology with regards to fear learning. Unfortunately, there is very little evidence of specific fear-related circuitry from human *in vivo* recording studies (most of which, due to being studies in patients with epilepsy, have low numbers of participants and high variability in electrode placement). Evidence of human functional connectivity of the amygdala and prefrontal cortex is therefore largely limited to

imaging and electrophysiology studies, and many of the networks discussed are very poorly explored in humans – although some evidence from non-human primates exists, which can help to give a clearer picture of how human pathophysiology of PTSD might look versus rodents.

In rodents, mPFC pre-stimulation drastically reduces the firing of brainstem-projecting CeA neurons, suggesting mPFC likely acts as a primary top-down inhibitor of CeM-mediated fear expression [164]. Interestingly, in humans greater levels of prefrontal theta activity are associated with resiliency to PTSD [165]; thus, the role of mPFC in mediating amygdala reactivity may extend to humans to some degree. In rodents, extinction training reduces the efficacy of mPFC excitatory projections to the basolateral amygdala (BLA), while inhibitory projections are left unaffected [166].

In addition, in rodents the inhibitory control of CeA by mPFC is indirect; mPFC only sparsely projects to central medial nucleus of the amygdala. Instead, mPFC projections likely inhibit CeM by exciting GABAergic neurons (intercalated cells of the BLA) which receive inputs from mPFC and project to CeM [167–169]. Together, these findings collectively suggest a possible role for dampened mPFC activity in PTSD in disinhibition of amygdala reactivity. Reduced efficacy of brainstem-projecting CeM neurons in PTSD may therefore lead to excessive fear expression and reduced ability to extinguish traumatic memories.

In rodents, the infralimbic cortex (analogous to human vmPFC) and prelimbic cortex (analogous to human dACC) are associated with fear learning and inhibitory control of central amygdala output. In non-human primates, the densest concentration of amygdala-projecting neurons are similarly localized to the posterior orbitofrontal cortex (OFC) [170]. This suggests a similar mechanism of top-down inhibitory control may exist in humans. However, amygdala-prefrontal connections display a high degree of complexity in primates. In addition, in contrast to

the more straight-forward inhibitory/excitatory networks so far uncovered in rats, all prefrontal regions appear to have some degree of interconnectedness with amygdala structures in rhesus monkeys and macaques [170]. Although unconfirmed, it is rather likely that this complexity extends to human physiology due to the greater degree of similarity between the brains of non-human primates and humans (versus the similarities between the rodent brain and the human brain).

Like in rats, the primate vmPFC also contains excitatory projections to intercalated cells of the amygdala which exhibit a top-down inhibitory influence over CeA [171]. vmPFC is therefore thought to fulfill the role in humans that IL performs in rodents. However, while IL inhibits CeM via an indirect mechanism in rodents (and IL does not project directly to CeM [148]), vmPFC also contains excitatory projections directly to CeA (and other amygdala structures) in primates [172,173]. vmPFC therefore fulfills a more complex role in primates allowing it to both inhibit and activate CeA. In addition, vmPFC also projects directly to the hypothalamus in macaques and plays a role in mediating HPA-axis activity [174]. In humans, the amygdala, mPFC, and hippocampus are reciprocally connected [175–177] and the hippocampus and amygdala are both highly activated during recall of fearful stimuli [178].

Since vmPFC acts as a homologue for the rodent IL (in terms of exerting a top-down inhibitory influence over brainstem-projecting CeA neurons), one might suspect there would be a reduction in vmPFC inhibitory control over CeA among sufferers of PTSD. Some studies have supported this; for example, one study showed reduced orbitofrontal cortex (OFC) volume among sufferers of PTSD [179], for example. Another study showed increased regional cerebral blood flow (rCBF) among PTSD sufferers during a script-guided task of traumatic imagery recall [180]. However, importantly – and, unlike in rodents - vmPFC in primates contains both

inhibitory projections to ITC *and* excitatory projections to CeA. The importance of regional activation and/or regional cerebral blood flow is therefore very difficult to interpret - since either might be associated with inhibition *or* excitation of CeA.

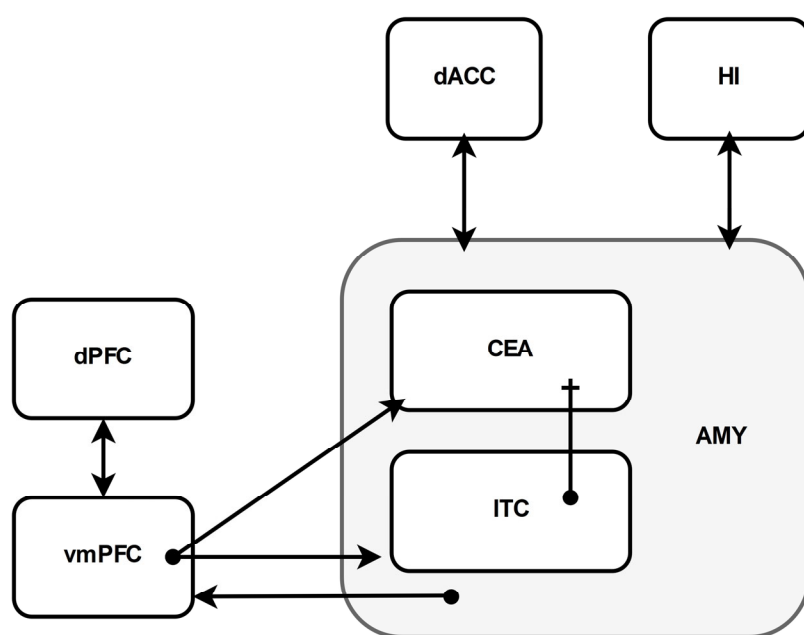


Figure 5. Networks mediating fear-learning in non-human primates, which may extend to human physiology.

With these insights in mind, we can consider how memory-circuits implicated in fear-learning and in PTSD might be affected by NHPs whose constituent molecules act as ligands for cellular networks that might affect neuron signaling at these sites. In the following sections, we first very briefly explore the role of sleep, since sleep disturbances are a core feature of PTSD

and sleep may also play a role in fear-memory. In subsequent sections, the NHPs that were primarily explored in this paper are introduced.

1.8 The Role of Sleep

In addition to alterations in neurohormone secretion and limbic structure activity, sleep disturbances are also a core feature of PTSD – and sleep-dependent activity of mPFC and limbic regions is known to play a role in fear-learning. Typically, sleep disturbances in PTSD are characterized by REM fragmentation and nightmares [181], and some authors suggest increased amygdala reactivity may directly contribute to REM sleep disturbance in PTSD [182]. The combination of increased amygdala output and REM fragmentation is also a good candidate mechanism for explaining PTSD nightmares [123]. Since the amygdala is also interconnected with regions associated with REM onset and arousal centers of the brain, there may also be a reciprocal relationship whereby amygdala hyper-reactivity drives both maladaptive processing of traumatic memories during REM in the initial stages of consolidation post-trauma and frequent awakenings from REM sleep [123].

Sleep increases memory for negative emotional stimuli and decreases memory for neutral stimuli [183], suggesting that sleep prioritizes storage of emotional memories (possibly at the expense of memory for neutral stimuli). Sleep enhances memory for emotional objects while reducing memory for neutral scenes and objects [184]. Specifically, REM sleep also appears to be more involved in emotional learning than NREM (stages I, II, and SWS), although NREM may play a role in PTSD-related sleep disturbances [182,185]. REM deprivation also enhances reactivity to emotional stimuli the following morning, while NREM interruption has no such effect [186]. During REM sleep there is also coupled theta band activity among mPFC and limbic regions, and synchronized amygdalohippocampal and medial-prefrontal activity during

REM sleep correlates with individual differences in consolidation efficacy (suggesting theta oscillations among limbic system structures and mPFC may be involved in sleep-dependent consolidation in rodents) [187–190].

There is also some evidence to suggest REM-dependent changes in emotional reactivity are mediated by glucocorticoid hormones. Nocturnal administration of enhanced memory for emotional stimuli in one study, while also reducing amygdala reactivity during testing the next day [122]. Similar effects are observed during wakefulness, since elevated cortisol levels during encoding also enhances long-term recall for emotional stimuli in humans during the day [191]. Nocturnal cortisol secretion is also typically highest during the REM-dense second half of the night rather than the earlier SWS dense period that is typical during the first half of the night [192]. As a result, nocturnal secretion of cortisol is a strong candidate mechanism for mediating sleep-dependent fear learning and altered glucocorticoid signaling could play a role in PTSD-related sleep disturbances. Indeed, some authors have already highlighted the potential role of nocturnal stress-hormone secretion in fear learning [193].

It is clear that both waking and sleep-dependent networks may play a role in fear-memory. In terms of therapeutic benefit, during wakefulness (and possibly also during sleep) the application of NHPs believed to affect memory processes may attenuate subsequent expression of older fear associations. In this thesis, the primary aim was to determine whether administration of NHPs could successfully modulate the reconsolidation of a learned fear association. In the following sections, we will discuss the three NHPs primarily explored in our experiments: (1) Gastrin-releasing peptide, (2) *Cannabis spp.* extracts and isolated phytocannabinoids, and (3) *Souroubea Sympetala* extracts and isolated triterpenoids.

1.9 Gastrin-releasing peptide

Current mainline treatments for PTSD are limited. Benzodiazepines are often used to address comorbid anxiety disorders or PTSD itself. However, while benzodiazepines act as potent anxiolytics (and act to reduce amygdala responsivity), prolonged use causes strong biological dependence [194–196]. As a result, there is great need for interventions that act through other mechanisms. Naturally occurring peptides may be one avenue for the development of novel treatment strategies. Naturally occurring peptides are implicated in a wide array of human medical conditions, including diabetes and Alzheimer’s disease [197,198]. Gastrin-releasing peptide (GRP; a 27 amino-acid peptide) may show promise as a novel therapeutic target, and evidence suggests it may play a role in memory-related processes [199].

GRP is a mammalian peptide, one of two homologues of the amphibian peptide Bombesin (BB; the other homologue being neuromedin B, or NMB). GRP binds to the bombesin (or BB2 receptor/GRPR), which is widely distributed throughout the hypothalamus, brain stem, and limbic system including the amygdala and hippocampus [200–204]. Bombesin (a 14-amino acid peptide) was originally isolated from the skin of *Bombina bombina* (the European fire-bellied toad) in the early 1970s; [205,206], and fulfills a role as a satiety peptide both centrally and peripherally. Mice lacking bombesin-like peptide receptors BB1, GRPR, and BRS-3 for example exhibit obesity and abnormal social behaviors [207]. In mammals, peripheral GRP stimulates the release of gastrin from G-cells of the stomach, and thus plays an important role in mediating the subsequent secretion of gastric acid in the stomach [208]. GRP also plays an important role in mediating control of food intake [208], alongside other peptides such as Ghrelin [209–212].

Interestingly, evidence also suggests that GRP plays a role in fear memory [213]. Within the amygdala, the GRPR is highly expressed in the basolateral complex (which comprises both the lateral nucleus [LA] and the BLA), a key region that has been implicated in various aspects of fear-memory [148]. In addition, the GRPR is more specifically expressed on inhibitory GABAergic interneurons, and infusion of GRP into the LA causes GABA release and the inhibition of principal amygdala neurons [213]. Reduced GABAergic inhibition within the LA is associated with increased activity-dependent plasticity [151,152], and there is evidence suggesting that GABAergic transmission within the amygdala mediates fear learning [149]. Increased fear expression is also associated with decreased GABA_A receptor expression in the amygdala, highlighting the role of GABAergic signaling within the amygdala in fear-learning and expression [150].

Research suggests that GRP and RC-3095 (a GRP receptor partial agonist) modulate the expression of conditioned fear when given intra-cerebro-ventricularly [16,47]. BLA GRPR activity also plays a role in consolidation of recently acquired fear-memories; post-training injection of RC-3095 blocked consolidation of inhibitory avoidance memory in rats one study [214] and impaired aversive (but not recognition) memory in another [215].

Although some research has explored the effects of bombesin-like peptide receptors on memory consolidation and expression, research has yet to address their effects on the reconsolidation of learned fear. As such, GRPR is a valid target for exploration of pharmacological manipulation of learned-fear in rodent models using the reconsolidation blockade paradigm. In Chapter 2, we explore the effects of peripherally administered gastrin-releasing peptide on reconsolidation of conditioned fear in rats.

1.10 The complex nature of cannabinoids

Plant chemicals (PCs) are among the most widely recognized source of NHPs, largely due to the relative availability and ease of extraction of plant-based chemicals versus those sourced from animals (e.g. GRP). Among these, *Cannabis spp.* is one plant that is already common for recreational use and alternative medicine which is believed to have anxiety- and memory-altering properties. *Cannabis* extracts and whole plant material have historically been frequently used for self-medication of fear-based disorders such as post-traumatic stress disorder. In more recent years, it has also been prescribed by health care practitioners. The role of cannabinoids in reconsolidation of fear memory has also been only minimally explored. In Canada, medical costs of marijuana for medical purposes (MMP) among veterans with combat-related PTSD have been paid for by Veterans Affairs Canada (VAC) since 2008 [216]. Like GRP, *Cannabis spp.* extracts and whole plant material contain molecules which may have memory- and/or anxiety-altering properties and may thus be of use for the development of novel pharmacotherapies for fear-based disorders.

Of the compounds discussed in this paper, cannabinoids may be simultaneously both the best explored and the most poorly understood. This is partly due to the notoriously inconsistent results of central injection studies using cannabinoid receptor agonists and antagonists, but also due to the complex mechanisms of the compounds themselves (since many cannabinoids such as Cannabidiol are not believed to exert their therapeutic effects via a single mechanism, but rather by exerting a variety of complex effects on various aspects of human biology simultaneously). In addition, phytocannabinoids are much more poorly explored than synthetic cannabinoids despite that plant-derived cannabinoids are much more relevant to human use of *Cannabis spp.* As a federally restricted Schedule I prohibited substance in the United States (the most stringent

classification of prohibited substances), *Cannabis spp.* extracts and whole plant material are highly restricted in most states – thus, although prevalent elsewhere in the world, research from the United States is largely limited to synthetic cannabinoids and antagonists rather than plant material. This has resulted in a gap in research on naturally occurring cannabinoids.

In this thesis, our experiments focus primarily on the behavioural effects of orally administered plant-derived compounds (phytochemicals) which are known to have much more consistent behavioural effects than those typically observed following central injection studies or in studies utilizing synthetic CB1 agonists and antagonists (many of which are - as we discuss in the following sections - notoriously inconsistent and non-selective in nature). This approach is most relevant for human consumption of medical cannabis, cannabis extracts, and/or isolated phytocannabinoids. However, in order to fully understand the role of cannabinoids in fear-learning, it is important to discuss the many ways in which cannabinoids exert a complex influence on fear-learning and behavior.

Δ^9 -Tetrahydrocannabinol (THC), the primary psychoactive component of *Cannabis (sativa, indica, and ruderalis)*, has been shown to play a role in the mediation of fear memory processes. Cannabidiol (or CBD), a second component of the plants, may also affect fear-learning. In rodents, peripheral and central injection of THC affects fear expression [217]. Activation of CB1 receptors in the dorsolateral periaqueductal grey is also associated with reduced expression of contextual learned fear [218]. Thus, it is clear that activation of the cannabinoid receptor CB1 may play a role in both fear-learning and expression.

THC acts as an agonist for both the CB1 and CB2 receptors [219,220], although it binds to CB2 with a lower affinity than CB1. In contrast, the medicinal effects of CBD are generally not attributed to action as a receptor agonist or antagonist. Instead, CBD is thought to exert its effects

indirectly, primarily by inhibiting endogenous CB1 and CB2 ligands. As a result, in many respects CBD produces opposite effects to those produced by THC when compounds are administered both centrally and peripherally. Some studies have found evidence that this also translates to behavioural effects in humans. One functional magnetic resonance imaging (fMRI) study, for example, showed that CBD and THC had opposite effects on regional activation in numerous areas including the hippocampus and amygdala in humans [221] (key regions of fear-related circuitry), highlighting that CBD and THC may have opposite effects on fear-learning. The same study demonstrated that 5 mg CBD intravenously (IV) attenuated THC-induced psychotic symptoms.

CB1 receptors are thought to be some of the most widely expressed receptors in the brain, while CB2 is more predominant in peripheral tissue (although CB2 is also expressed in the brain to a lesser degree than CB1; the role of CB2 in central processes relating to memory and fear-learning is much less well explored than CB1) [222]. CB1 is highly expressed in the hippocampus, amygdala, and medial prefrontal cortex (mPFC) [223], regions implicated in fear-learning and fear-expression. Evidence from positron emission tomography (PET) imaging further suggests that among individuals with PTSD there is a widespread upregulation of CB1 receptor density [224], and individuals with PTSD have altered availability of several endogenous cannabinoids including 2-arachydonylglycerol (2-AG) and anandamide [224–226]. Thus, much like how sufferers of PTSD often exhibit altered neurohormonal responses to learned fear and altered activity of brain sites relevant to fear learning and expression, there is also evidence that neurochemical alterations of the endocannabinoid system may be a core feature of human PTSD. Therefore, it is clear that the endocannabinoid system may be an important avenue for explaining and possibly treating PTSD (and possibly other related disorders).

Some research has already indicated positive effects of oral cannabinoids. Since sufferers of PTSD also exhibit decreased availability of endogenous cannabinoids 2-AG and anandamide, treatment with a chronic low dose of THC may be beneficial – and has been shown to exert some beneficial effects in early trials [227]. Anandamide has been shown to play a role in stress-reactivity in rodents [228]. Chronic treatment studies suggest that decreased availability of anandamide is a core feature of the disorder, and overexpression of CB1 may be a compensatory mechanism - thus supplementation with exogenous cannabinoids as a corrective measure may return CB1 to basal levels. Although positive behavioural effects have been observed [227], PET studies have yet to confirm whether those effects correlate with changes in regional CB1 expression.

With regards to fear-related circuits mediated by CB1, evidence from region-specific studies is much less clear – although there have been several recent insights lending some clarity to the field. In the following sections, the roles of cannabinoid receptor binding and region-specific effects of CB1 activation are explored in greater detail.

1.10.1 Role of CB1 in fear expression & extinction

Fear expression in rodents is mediated by brainstem-projecting neurons of the central-medial nucleus of the amygdala (CeM) [229,230]. CB1 receptors are expressed in the hippocampus, basolateral and lateral amygdala, and medial prefrontal cortex (mPFC) [223] – key regions implicated in fear learning - but are absent in the central amygdala (CeA) including CeM [231]. Although CB1 is absent in CeM, oral administration of THC tends to be anxiogenic in both humans and rodents – suggesting an indirect CB1-mediated mechanism responsible for activation of brainstem-projecting CeM neurons (or inactivation of CeM inhibiting pathways). Activation of the prelimbic mPFC (PL) is required for expression of learned (but not innate) fear

in rodents [232] while infralimbic mPFC (IL) is implicated in consolidation and retrieval of extinction learning [233–235]. CB1 agonism reduces firing of GABAergic BLA interneurons, disinhibiting BLA efferent projections to regions such as PLC [236]. BLA projections induce long-term potentiation at PLC, and this effect is reversed by blockade of CB1 receptor signaling (suggesting that CB1 mediated activity of BLA is important for Amygdala-PLC connectivity) [236]. IL also plays a role in top-down inhibition of CeM during fear expression, and intra-IL administration of anandamide (which agonizes CB1 receptors) reduces fear expression; the anxiolytic effects of intra-IL anandamide are also reversed by pre-treatment with CB1 antagonist AM251 [237]. AM251 is also anxiogenic when administered peripherally on its own [238]. These findings suggest CB1 activation at IL might produce anxiolytic-like effects by facilitating inhibition of brainstem-projection CeM neurons by inhibitory IL projections. The same study showed that CB1 receptor mRNA increased at IL 48 hours after fear conditioning. Similar effects were achieved by central administration of anandamide into the ventrolateral periaqueductal gray (vlPAG), and effects were similarly blocked by CB1 antagonist AM251 [218].

Extinction, in contrast to expression, is the process by which new learning inhibits the expression of older memory [233,234,239] – thus extinction memory must be encoded, consolidated, and retrieved. Acquisition of extinction induces basolateral amygdala (BLA) plasticity [240], and IL activation is also necessary for subsequent consolidation and retrieval of emotional extinction learning [233,241]. vmPFC, believed to act as a homologue to the rat IL [242–244], also becomes activated during extinction recall in humans [245]. Amygdala activity is also implicated in extinction of conditioned fear in humans and rats [245–249] in both cued

and contextual paradigms, while contextual conditioning also recruits the hippocampus [245,250].

Intra-IL CBD (which antagonizes endogenous CB1 agonists) blocked extinction-learning in several studies [84,251] while CB1 agonism enhanced it [150]. IL administration of a CB1 agonist also blocks expression [251]. As a result, it would seem that CB1 activation at IL may exert an inhibitory influence over amygdala regions involved in fear expression while having the opposite effect on extinction. Lemos et al. [252] showed that intra-IL CBD produced enhanced fear-expression, while intra-IL CB1 agonists reduced fear-expression. This lends further support to the notion that fear-associated infralimbic circuits show differential response to CB1 agonism for expression versus extinction. It is important to note, however, that central studies with cannabinoids are notoriously inconsistent. Some studies have shown opposite effects with intra-IL administration of a CB1 agonist, and blockade of the effect with CBD [253–255].

Overall, however, it seems that CB1 agonism at IL generally inhibits CeM activity and results in reduced fear expression. This may also allow for new learning without central amygdala involvement (thus de-potentiating the amygdala response of fear-memories; i.e. extinction). Consequently, CB1 agonism at IL would also inhibit expression through this mechanism. In support of this, some evidence has shown that co-activation of CB1 receptors with mGluR5 at mPFC reduces firing of central amygdala neurons in rodents [256]. IL has also been shown to inhibit central amygdala output in other (non-cannabinoid) research. IL contains projections to intercalated cells of the amygdala (ITC), which in turn inhibit brainstem-projecting neurons of the CeM [148]. Thus, there is prefrontal control of CeM output via an indirect pathway (since mPFC sparsely projects to CeM itself) and infralimbic CB1 receptors may in part mediate this effect in rodents.

While this seems a likely mechanism for cannabinoid mediation of prefrontal control over the amygdala, it is important to note that a direct link between CB1 activation at IL and the activation of inhibitory GABAergic intercalated cells (ITC) has not yet been confirmed. In addition, it is also important to consider that some of these insights come from studies with CBD – yet the actions of CBD are very complex in nature. CBD affects both CB1 and CB2 receptor ligands (i.e. CBD is non-selective in nature and therefore cannot be used on its own to infer selective blockade of CB1 ligands), and some evidence suggests CBD may also act as a partial agonist at 5HT_{1A} receptors [257]. As a result, it is very difficult to interpret findings from studies with CBD at the cellular and molecular level.

1.10.2 Role of CB1 in consolidation of learned fear

Consolidation following initial acquisition of emotional learning involves many of the same regions implicated in consolidation of extinction learning. These include the basolateral amygdala (BLA), hippocampus, and mPFC [240,258]. Like for extinction learning, CB1 also plays a role in memory consolidation. However, the findings of studies on central CB1 activation and inactivation on consolidation of learned fear are much less consistent. When injected into the basolateral amygdala (BLA), for example, CB1 agonist WIN 55,212-2 (WIN) enhanced 48-hour retention of inhibitory avoidance in one study [259] but reduced consolidation of fear-potentiated startle in another [260]. CB1 antagonism at BLA also blocks reconsolidation of Pavlovian fear conditioning [261].

Contextual fear conditioning induces an increase in polysialylated neural cell adhesion molecule (PSA-NCAM) immunoreactivity (believed to mediate memory consolidation in the hippocampus) at the dentate gyrus (DG) [262], and this was blocked by CB1 agonist HU-210. The same study showed that the effects of HU-210 on both PSA-NCAM and fear expression are

also reversed by AM-251. This suggests CB1 activation at DG may act to disrupt consolidation of contextual fear memory. Other groups have also corroborated this; Yim et al. [263] showed that post-training activation of CB1 receptors by CB1 agonist WIN blocked consolidation when administered both systemically and centrally (into the dorsal hippocampus). However, in other studies intra-hippocampal infusions of AM251 produced an amnesic effect on consolidation of inhibitory avoidance in one study while anandamide had the opposite effect [264], and these results are consistent with prior work by the same group [265].

Seemingly contradictory findings might be in part explained by differences in selectivity of agonists and antagonists. AM251 for example binds not just a CB1 antagonist, but also displays some affinity for μ -opioid receptors [266]. CB1/ μ -opioid synergism might thus offer an explanation for why some findings with AM251 contradict work with anandamide (which does not bind to μ -opioid receptors). Some evidence suggests that both AM251 and Rimonabant may act at μ -opioid receptors; CB1 antagonist AM281 is perhaps one of the only antagonists that appears to be truly selective to CB1, but (possibly due to the ease of access and availability of other compounds like Rimonabant, AM251, and WIN) it has been used very infrequently [266,267].

Overall, the effects of central administration of compounds acting at CB1 on consolidation appear less consistent than those looking solely at expression - and this may be due in part to the non-selective nature of synthetic CB1 agonists and antagonists. The use of more selective CB1 agonists and antagonists such as AM281 may provide a cleaner picture of role of CB1 in fear-learning.

1.10.3 Differential effects cannabinoids modulation at infralimbic versus prelimbic cortex

In addition to playing complex and different roles in the consolidation, expression, and extinction of fear-memory, region-specific cannabinoid receptors may also have distinct (and sometimes opposing) effects on fear-learning. Research suggests opposite action of CBD at IL versus PL, for example. Intra-IL CBD enhances fear-expression while intra-PL CBD has the opposite effect [252]. CB1 agonism at BLA also induces BLA-PLC connectivity and prelimbic LTP, while intra-PL CBD also inhibits c-fos expression at the hippocampus (CA1, CA2, CA3, CA4, and dentate gyrus) as well as in the BLA [268]; this may in part be because BLA is reciprocally connected with the ventral hippocampus in rodents [269,270]. PL projects to the BLA [243,271] and evidence suggests PL inactivation attenuates expression of learned (but not innate) fear [232]; this further suggests a role for PL activation in promoting expression of conditioned fear. Overall, with some exceptions, research tends to support that CB1 activation at IL is anxiolytic, while CB1 activation at PL has the opposite effect [218,237].

As discussed in a prior section, inhibitory control of CeA by IL is indirect, since IL sparsely projects to CeM [272]. Instead, IL projections inhibit CeM via excitation of GABAergic neurons (intercalated cell masses, ITC) [273] that project to CeM [168,169,273–275], and the anxiolytic action of CB1 agonists administered directly at IL may act through this network. *In vivo* stimulation of the IL-amygdala pathway in rodents also enhances extinction, while optogenetic silencing impairs it [276], and IL inputs to BLA potentiate ITC activity, thus inhibiting CeM indirectly via ITC efferents [271,277]. However, while IL contains neurons that project to CeM [272] and inhibit its output [164], one study demonstrated that co-activation of infralimbic CB1 receptors with mGluR5 inhibits central amygdala output [256]. Thus, the role of CB1 at IL may in part depend on co-activation of other receptors.

Many questions remain to be addressed. Importantly, although anxiolytic action of intra-IL CB1 agonism and the role of IL on dampening amygdala activity have been explored, a direct link between CB1 agonism and IL efferents to ITC has not yet been confirmed. Since mPFC has been shown to be largely involved in top-down inhibitory control over the amygdala [148,164,167,274] and this network plays a critical role in fear-learning and expression [166,235,241,258], it will be important for future studies to determine whether CB1-mediated prefrontal control of CeM is via ITC or another (as of yet unidentified) inhibitory network.

The experiments discussed thus far by-and-large suggest that cannabinoid signaling is complex, and the effects of CB1 activation may depend on host of factors including receptor co-activation. In addition, many endogenous and exogenous cannabinoids are non-selective in nature and exert their effects through complex co-activation of various receptor types (including CB2). In the following section, the role of CB2 receptors is discussed.

1.10.4 The role of CB2 receptors

CB2 has been much less well studied in the context of fear-learning than CB1. Similarly to CB1 (although to a lesser degree) CB2 is expressed in brain, including in the hippocampus and amygdala [278]. Evidence also suggests that in addition to effecting CB1 ligands, CBD also antagonizes endogenous CB2 ligands. Thus, central experiments with CBD are not specific to CB1 and are difficult to interpret.

There is some evidence to suggest that CB2 may also be involved in fear-learning and anxiety-like behavior. Acute treatment with a CB2 receptor antagonist increased anxiety-like effects in rodents in one study, and the effect was blocked by pre-treatment with CB2 receptor agonist JWH133 [279]. Further, the same study found that chronic treatment with a CB2 antagonist produces alterations in GABA_A receptor gene and protein expression in the cortex.

Reduced GABAergic inhibition within the lateral amygdala (LA) is associated with increased activity-dependent plasticity [151,152] and increased fear expression following acquisition is also associated with decreased expression of the GABA_A receptor within the amygdala [280]. GABA agonist Midazolam administered post-reactivation also reduces conditioned freezing on subsequent trials [281,282]. Since GABA_A receptors are implicated in fear and anxiety processes, it is possible that the effects of CB2 on fear-learning and expression are mediated via an indirect mechanism involving GABA_A receptors. β -Caryophyllene (BCP), a plant-derived sesquiterpene, also binds to the CB2 receptor [283,284] and may exert anxiolytic effects [285,286]. Interestingly, while the effects of *Cannabis spp.* have been traditionally ascribed to their THC and CBD content, most (if not all) strains of *Cannabis* contain BCP in varying quantities. Therefore, the effects of cannabis extracts on learned fear may not be solely attributable to their cannabinoid molecule content.

1.10.5 Summary - Cannabinoids & Learned fear

In sum, it can be stated that the actions of cannabinoids are extremely complex – and although research has uncovered some of the fear-associated networks affected by cannabinoid molecules, a clear consensus has not yet been reached in all cases. In addition, cannabinoid molecules (both endogenous and exogenous) are very often non-selective in nature, and exert a wide variety of behavioural effects through receptor co-activation. Some cannabinoids also often exhibit bi-phasic effects at different doses.

While the central effects of THC and CBD application are not well understood, in this thesis we approach cannabinoids principally from a behavioural perspective using combinations of isolated phytochemicals THC and CBD as well as whole plant material (which, as a behavioural paradigm, is much more relevant to human use of MMP). In Chapter 3, we explore

the effects of orally administered isolated phytocannabinoids THC and CBD, as well as whole plant background material, on the reconsolidation of learned fear.

1.11 *S. Sympetala* leaf extract and betulinic acid modulate learned fear

Souroubea sympetala is a neo-tropical vine indigenous to South America, which may also possess anxiolytic properties. Similar to GRP, much less is known about the action of this NHP than is known about cannabinoids. Indeed, there are only a handful of studies examining the effects of *S. sympetala* and its constituent phytochemicals on fear-learning and anxiety. *S. Sympetala* falls under the umbrella of ethnopharmacology: the pharmacologic study of medicinal botanicals used by native/indigenous healers as part of their traditional medical practices. Many modern medications have been derived from ethnomedicine. Acetylsalicylic acid (Aspirin®, or ASA), for example, was originally derived from bark infusions of *Salix spp.* (*Salicaceae*, or willow trees) containing salicin (which is metabolized to salicylic acid) [40]. Similarly, the origin of local anaesthetics (including many preparations that are still commonly used today such as benzocaine, novocaine/procaine, and lidocaine) can be traced to the use of *Erythroxylaceae spp.* (Coca) leaves by natives of the Andean region as part of very old traditional medical practices from the region's indigenous healers that date back to long before the discovery of North America by the Spanish in the late 1400s [40]. As a result, it is clear that ethnomedicine is a powerful avenue for drug discovery that may lead to the development of new and effective treatments.

Traditional plant-based medicine is also often sought as complementary and alternative medicine (CAM): approaches to the treatment of illness that falls outside of the scope of standard medical care. Many individuals seek CAM in addition to or as an alternative to their standard medical treatment. Evidence suggests as many as 56.7% of individuals with anxiety disorders

use alternative medicine as a supplement to traditional treatments, while only 20% of those sought the guidance of a health practitioner (i.e. a large percentage self-medicate) [287]. This is especially relevant for PTSD, since current treatment approaches issued by general practitioners are limited. In addition, pharmacologic treatments are largely limited to benzodiazepines, which can have serious long-term complications and cause biological dependence. As a result, it is important to seek new alternatives to traditional treatments. NHPs derived from indigenous medicine, often falling under the umbrella of CAM, may offer a novel approach to safe and effective treatment of human fear-based disorders.

Much less research has explored the effects of *S. sympetala* extracts than cannabinoids – however, there is evidence (both from traditional use by native indigenous healers and some pre-clinical animal studies) to suggest it may possess anxiolytic qualities. Within the Q’eqchi’ community (Belize), tea brewed from fresh or dried crushed *S. sympetala* leaves is referred to as *Sin Susto* [288]. The term *susto* refers to an illness described by the Q’eqchi healers as the “fright sickness,” while *sin* translates as “without.” *Sin susto* therefore means “without fear”, and the plant is consumed as a natural remedy for fear [289]. More specifically, local descriptions of *susto* bear marked resemblance to the western-medicine notion of PTSD: *susto* is a long-term condition that begins at onset with exposure to a frightening event involving a startle, and causes depression, weakness, vertigo, irritability, and difficulty sleeping [290–293]. Furthermore, evidence suggests that *susto* likely contains an underlying neurological and/or psychological component linking it to anxiety and/or fear-learning [294].

Evidence from studies with rodents suggests *S. sympetala* leaf extract may be effective as an anxiolytic, and that betulinic acid (BA; one of the components of the plant) may be the active component responsible for mediating this effect [20]. *S. sympetala* and BA exert some of their

effects on fear-learning and expression by acting as an intermediate acting agonist for the Benzodiazepine (BZD) binding site of the GABA_A receptor [289]. Anxiolytic botanicals frequently possess agonistic qualities for the GABAergic system [21]. Valerian Root for example, which contains an active principle component that is structurally similar to BA, also possesses anxiolytic activity which is primarily mediated by GABA_A BZD-receptor agonism [295].

Research has demonstrated that reconsolidation of learned-fear can be inhibited by GABAergic neurotransmission in animal models when administered following recall [296]. As such, *S. sympetala* leaf extract and its active components may be of use to the development of novel treatment approaches for fear-based disorders. In chapter 4, we explore the effects of orally administered *S. sympetala* leaf extract and isolated triterpenoids betulinic acid and betulin on the reconsolidation of learned fear.

1.12 Thesis overview, objectives, & hypotheses

It is clear that there is a gap in safe, effective treatment regimens for anxiety and fear-based disorders and - more specifically - for post-traumatic stress disorder. Here, by exploring the effects of three separate compounds in models of reconsolidation blockade we aimed to lay the groundwork for future safety and behavioural translational studies with particular focus on using natural products as a means to pharmacologically alter the reconsolidation process. Particular attention is paid to clinical relevance for the treatment of PTSD and other fear-based disorders, although throughout the following chapters we have examined effects primarily through the lens of Pavlovian fear-conditioning (which, although not a model of PTSD *per se*, addresses some of the core anxious-arousal symptoms of PTSD and other fear-based disorders). This work aimed primarily to address the basic-research level questions of whether there is

sufficient evidence from behavioural trials with animals to justify future human clinical trials and safety studies with gastrin-releasing peptide, exogenous cannabinoids THC and CBD, and *S. sympetala* leaf extract (and its constituents as well as some closely related extracts).

In Chapter 2, the efficacy of gastrin-releasing peptide is explored. We examined the effects of GRP on reconsolidation of learned fear, and attempted to block those effects with a GABA_A benzodiazepine receptor antagonist (Flumazenil). Furthermore, we explored the significance of treatment timelines, and demonstrated that reconsolidation blockade with GRP is quite sensitive to the time-course of drug administration. In chapter 3, we similarly explored the effects of CBD and THC on reconsolidation blockade, and also explored the effects of plant background material to determine whether whole plant material also plays a role in the effects of marijuana on fear-memory reconsolidation. In chapter 4, we aimed to assess the effects of *S. sympetala* leaf extract and BA on fear-memory reconsolidation using the same fear-conditioning paradigm as chapters 2 and 3. In addition, we also sought to determine whether the effects of BA could be synergized by the addition of betulin (BE), since the effects of BA on anxiety-like behavior are known to be synergized by the closely related triterpenoid group amyrins.

In chapter 2, it was hypothesized that (Hypothesis 1) GRP would significantly attenuate the reconsolidation of conditioned fear-memory, leading to decreased freezing response. It was also hypothesized that (Hypothesis 2) this effect would be reversed by co-administration of GABA_A BZD receptor antagonist Flumazenil. In Chapter 3, it was hypothesized that (Hypothesis 3) CBD and THC would significantly attenuate reconsolidation of conditioned fear. In addition, it was also hypothesized that (Hypothesis 4) the combination of CBD and THC would block reconsolidation. Conditions examining the effects of remaining plant background material were exploratory in nature. In Chapter 4, we hypothesized that (Hypothesis 5) *S. sympetala* leaf

extract and BA would block the reconsolidation of conditioned fear. In addition, we hypothesized that (Hypothesis 6) this effect would be synergized by BE, producing a greater effect on reconsolidation blockade than administration of BA alone. We also hypothesized that (Hypothesis 7) BA and BE would also exert an anxiolytic-like effect on fear-expression in the elevated plus maze.

Chapter 2: Gastrin-Releasing Peptide attenuates reconsolidation of conditioned fear memory

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2.1 Abstract

Background: Gastrin Releasing Peptide (GRP) may play a role in fear learning. The GRP Receptor is expressed in the basolateral amygdala and hippocampus, and central administration of GRP modulates fear learning. The effects of GRP on reconsolidation, however, have been minimally explored. Reconsolidation, the process by which formed memories are rendered labile following recall, provides a window of opportunity for pharmacological manipulation. Although evidence suggests the window of opportunity to alter reactivated consolidation memory can be as long as 6 h, shorter intervals have not been extensively investigated.

Method: Male Sprague-Dawley rats received six 1.0 mA continuous footshocks. 24 h later, rats were re-exposed to the context (shock chamber). Immediately following memory retrieval rats received i.p. injection of GRP (10 nmol/kg), Flumazenil (1 mg/kg), GRP + Flumazenil (10 nmol/kg GRP with 1 mg/kg Flumazenil), or Vehicle. Other groups received GRP or Vehicle at 0, 10, 30, or 60 min post-reactivation. 24 h and 5 days later rats were assessed for fear expression upon re-exposure to the fearful stimulus.

Results: GRP significantly attenuated the reconsolidation of learned fear when administered immediately (but not 10 min or longer) following recall. As peripheral treatments are aimed at disrupting fear memories are, in part, governed by the time-course of the reconsolidation window, this may explain why reconsolidation blockade has had varied success in translational research. The effect of immediate administration persisted for 5 days. Co-administration of benzodiazepine-receptor antagonist Flumazenil blocked this effect, suggesting the effect is mediated via a GABAergic mechanism.

2.2 Introduction

While it was once believed that consolidated memories were relatively permanent, this notion has been largely refuted by evidence showing that fear memories can be returned to an unstable or labile state upon retrieval and must then be reprocessed (or reconsolidated) to be maintained [55]. During this period of reconsolidation, a pharmacological disruption can potentially alter the re-storage (or enhance re-learning) and thus diminish its expression. This model of fear-memory interventions has been used in pre-clinical animal research to identify potential therapeutic targets for fear-based disorders [13,297,298], and interventions which can effectively diminish subsequent expression of learned-fear may be relevant for the treatment of numerous disorders (phobias, PTSD, etc.).

It has been suggested that traumatic memories become over-consolidated in PTSD, resulting in their excessive endurance and persistence [13]; thus, treatments which diminish the expression of learned fear may be of use for reducing well-established trauma memories. Interestingly, a recent meta-analysis indicated that older memories may be more susceptible to change via reconsolidation than newer memories, thus opening valuable avenues of treatment for fear-based disorders rooted in older experience (although this is not universally accepted) [56]. Few studies have targeted reconsolidation in translational research with humans, although many potential targets have been identified in research with rodents. The administration of a β -adrenergic antagonist propranolol, for example, immediately after memory retrieval (during the reconsolidation window) disrupts fear memory in rats [297]. Similar results have been observed in animal models with xenon (which blocks NMDA receptors; [78]) and the phytocannabinoid cannabidiol [85].

Translational studies on reconsolidation blockade in humans are few in number; however, there is some evidence to suggest it may have practical applications in human subjects. Propranolol administration following fear-memory retrieval, for example, was reported to reduce physiologic responses to traumatic imagery in sufferers of PTSD [86]. In addition, one study showed that extinction training during reconsolidation reduced fear responses in humans up to one year later [299], suggesting that updating of older fear memories with non-fearful experiences may successfully reduce fear expression in humans long-term. However, follow up studies with propranolol failed to replicate earlier findings [64], highlighting that new pharmacologic targets are required which may have therapeutic benefit that translates well to humans.

Gastrin-releasing peptide (GRP), a 27 amino-acid neuropeptide which appears to be involved in fear memory [213], may offer one such target. GRP binds to the bombesin or BB2 receptor (also termed GRPR) which is widely distributed throughout the hypothalamus, brainstem and limbic system including regions such as the amygdala and hippocampus [200–204]. More specifically, within the amygdala the GRPR is highly expressed in the basolateral complex (which comprises both the Lateral and Basolateral nuclei) - a key region implicated in fear learning [148,213]. Further, the GRPR is expressed on inhibitory GABAergic interneurons and infusion of GRP into the lateral nucleus of the amygdala (LA) causes GABA release and the inhibition of principal amygdala neurons. Reduced GABAergic inhibition within the LA is associated with increased activity-dependent plasticity [151,152], and increased fear expression following acquisition is also associated with decreased expression of the GABA_A receptor within the amygdala [280].

Evidence suggests that both GRP and the GRPR antagonist RC-3095 modulate the expression of conditioned fear when administered centrally or directly into the BLA [16,47]. In addition, when injected into the dorsal hippocampus low doses of GRPR antagonist RC-3095 blocked consolidation of memory for an inhibitory avoidance task [214]. Surprisingly, high doses of GRPR antagonist RC-3095 also enhanced fear memory consolidation when infused into the dorsal hippocampus, an effect blocked by pretreatment with GABA_A receptor agonist muscimol [300] suggesting involvement of a GABAergic mechanism in the hippocampus as well. The role of this peptidergic system in fear memory reconsolidation has only been minimally explored, with one study showing that post-reactivation intra-hippocampal infusion of RC-3905 blocks fear memory reconsolidation [301]. As a result of GRPR's known role in fear-learning, we predicted that GRP agonists may have an attenuating effect on reconsolidation of learned fear (thus providing new therapeutic targets for future translation studies with humans). With this in mind, the present study sought to examine the effects of intraperitoneal (i.p.) administration of GRP on the reconsolidation of contextual fear memory. We also sought to determine whether GRP acts to modulate fear memory reconsolidation via a GABAergic mechanism using co-administration of GRP with the selective benzodiazepine antagonist Flumazenil to block the effects of GRP.

In addition, research also suggests that immediate central infusion of anisomycin (a protein synthesis inhibitor) disrupts reconsolidation in rodents [55], while delayed infusion (6h post-retrieval) has no such effect. However, although evidence suggests that the time-window for blockade is limited [78], the exact time course during which blockade/disruption can be achieved at intervals shorter than 6 h has remained relatively unexplored. As a result, an

exploratory analysis was also conducted to plot the effectiveness of GRP administered at different time points during the reconsolidation window.

2.3 Materials & Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratories International, Inc.; 200-250g on arrival) were individually housed and maintained on a 12-h light/dark cycle (lights on at 07:00-h). Temperature was maintained at 23° C, and relative humidity at 37%. Throughout the duration of the study, animals had free access to food and water. All experiments were conducted in accordance with the guidelines established by the Canadian Council on Animal Care and approved by the University of Ottawa Animal Care Committee.

Drugs and Injections

Flumazenil (1mg/kg), Porcine GRP (Tocris Bioscience; 10nmol/kg for all GRP conditions), and co-administration solution of GRP and Flumazenil (10nmol/kg GRP with 1mg/kg Flumazenil) were each dissolved in saline solution with 12.5% cyclodextrin. Each solution was injected intraperitoneally (i.p.) in a 1ml/kg volume.

Flumazenil is a partial agonist [302,303] which selectively antagonizes the BZD binding site of the GABA_A-receptor. Flumazenil disperses rapidly in the body and brain tissue following administration, maintaining antagonistic activity for 2-3 hours following administration [304]. GRP is a 27 amino-acid neuropeptide, homologue of the 14 amino-acid peptide Bombesin, originally discovered in amphibians (*Bombina bombina*) [205,206] and BB2 agonist. In contrast to Flumazenil, as a large neuropeptide with a short biological half-life (~2.8 min) [305], it is unlikely that peripherally administered GRP readily crosses the blood brain barrier.

Contextual fear conditioning

The conditioning chambers (Coulbourn Instruments) measured 31cm x 25cm x 30cm. The front and back walls were made of clear acrylic, and the two side walls and top made of stainless steel. The floor was composed of 16 stainless steel bars (4mm diameter spaced 1.4cm apart) connected to Coulbourn precision regulated animal shockers, which delivered scrambled footshock (1.0mA). Animals ($n = 7-10/\text{group}$) were randomly distributed into treatment groups. Sample sizes were consistent with previous experiments employing similar methods [47]. Subjects that did not learn the conditioned response (failed to achieve a minimum baseline freezing level of 40% during memory reactivation assessed on Day 2) were removed from the analyses.

Experimental Procedure

Experiment 1: Effects of GRP and co-administration of GRP and Flumazenil on reconsolidation blockade

Animals were exposed to 6 consecutive 1-s footshocks over the course of 11 min. Contextual conditioning was used (pairing of footshock with the conditioning chamber). 24 h later animals were re-exposed to the context in which they received the footshock (conditioning chamber) for a duration of 5 min, and freezing (total time spent in complete immobility) was measured (Day 2; reactivation). Cage placement and assignment to drug treatment groups was randomized and counterbalanced.

Immediately following the 5 min reactivation session, animals were administered drugs in one of the experimental conditions (GRP, Flumazenil, co-administration of both Flumazenil and GRP, or vehicle). 24 h later (Day 3; testing), animals were re-exposed to the conditioning chamber and freezing was measured over the course of 15 min. Freezing on Day 3 was scored in

5-min time blocks of 0-5, 5-10, and 10-15 min. The timeline of procedures used for fear conditioning is illustrated in Fig 1.

Experiment 2: Effects of GRP administered at different time intervals during post-reactivation on memory disruption

These same procedures were also used to test for the effects of GRP when administered at different time intervals following memory reactivation. Animals in this second experiment were administered drugs in one of three experimental conditions post reactivation on Day 2 (GRP administered immediately following reactivation, GRP administered 10 min following reactivation, or vehicle administration 10 min following recall). Animals were re-exposed to the conditioning chamber on Day 3 for 15 min, and freezing was measured in 5-min blocks of 0-5, 5-10, and 10-15 min. The timeline of procedures used for this experiment is illustrated in Fig 2.

A follow up experiment was also conducted to confirm that no effects were present when GRP was administered at longer time intervals post reactivation. In this case, animals were administered either vehicle or GRP at 30- or 60-min post reactivation (Day 2) and then tested on Day 3 where freezing behavior was assessed (data not shown).

Experiment 3: Effects of GRP administered in the absence of memory reactivation

A control experiment was also conducted whereby the same procedure was used, but reactivation of the fearful memory trace on Day 2 was absent. Animals in this experiment were administered drugs in one of two conditions (GRP or vehicle) administered on Day 2 in the home cage (no reactivation). Animals were then exposed to the conditioning chamber on Day 3, and freezing was measured over the course of 15 min in 5-min blocks of 0-5, 5-10, and 10-15 min. The timeline of procedures for this non-reactivation control is illustrated in Fig 4.

Experiment 4: Long-term effects of GRP on reconsolidation blockade

Finally, the same training procedures were also utilized to test for long-term effects of GRP on fear memory reconsolidation blockade. Immediately after the 5 min reactivation session on Day 2, animals were administered either GRP or vehicle. 5 days later (Day 7), animals were re-exposed to the conditioning chamber and freezing was measured over the course of 15 min in blocks of 0-5, 5-10, and 10-15 min. The timeline of procedures for the long-term experiment are illustrated in Fig 5.

Statistical Analyses

All statistical analyses were conducted using IBM Statistics Package for the Social Sciences® (SPSS) 20. Data were analyzed through mixed measures analysis of variance (ANOVA) in which drug treatment was the between groups variable and time was the repeated measure. Follow-up comparisons of significant main effects or simple effects of significant interactions were conducted using Bonferroni corrected t-tests.

2.4 Results

Experiment 1: Effects of GRP and co-administration of GRP and Flumazenil on reconsolidation blockade

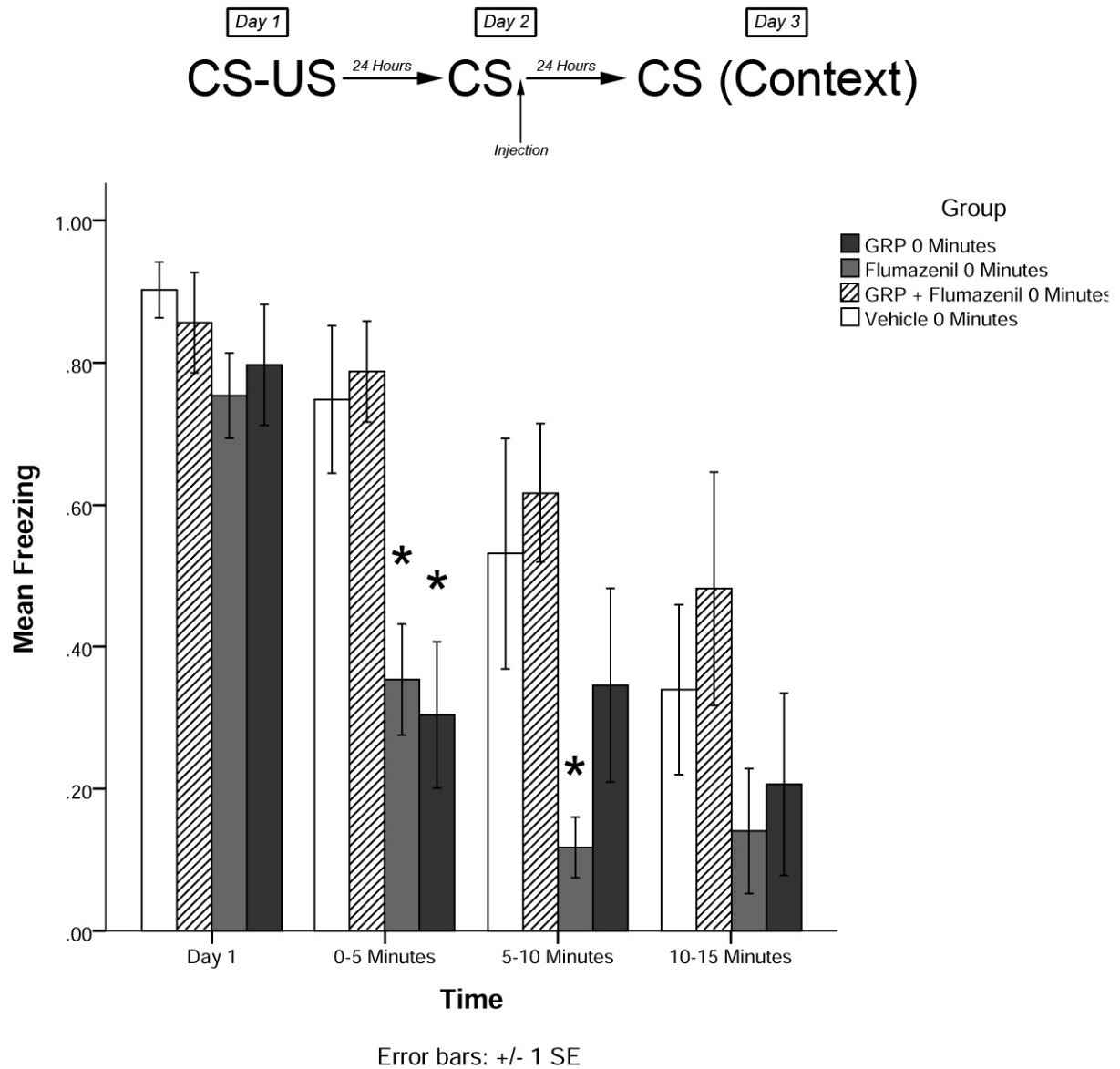


Figure 1. Peripheral GRP significantly attenuates the reconsolidation of conditioned fear memory; GABA_A receptor antagonist Flumazenil blocks this effect. $*p < 0.05$.

Figure 1 shows the effects of peripheral GRP administration or co-administration of GRP and Flumazenil administered immediately post reactivation on freezing behavior as measured during testing on Day 3. Three animals were excluded from the analyses. The mixed measures ANOVA revealed a significant main effect of treatment group on freezing behavior, $F(3,21) = 4.124, p < 0.05$. The follow-up analyses indicated that animals who received peripheral GRP immediately following memory reactivation on Day 2 displayed significantly less Freezing than vehicle treated animals during the first 5 min of testing on Day 3, $p < 0.05$ (See Fig 1), suggesting that GRP attenuates fear-memory reconsolidation. Additionally, Flumazenil alone also yielded a significant reduction in Freezing at both the 0-5 min and 5-10 min time blocks during testing on Day 3 ($p < 0.05$), while the co-administration of GRP and Flumazenil yielded no effect on freezing behavior at any time point versus controls. Co-administration of flumazenil and GRP also differed significantly from Flumazenil alone condition at both 0-5 min ($p < 0.05$) and 5-10 min ($p < 0.05$) time blocks.

Experiment 2: Effects of GRP administered at different time intervals post-reactivation on memory disruption

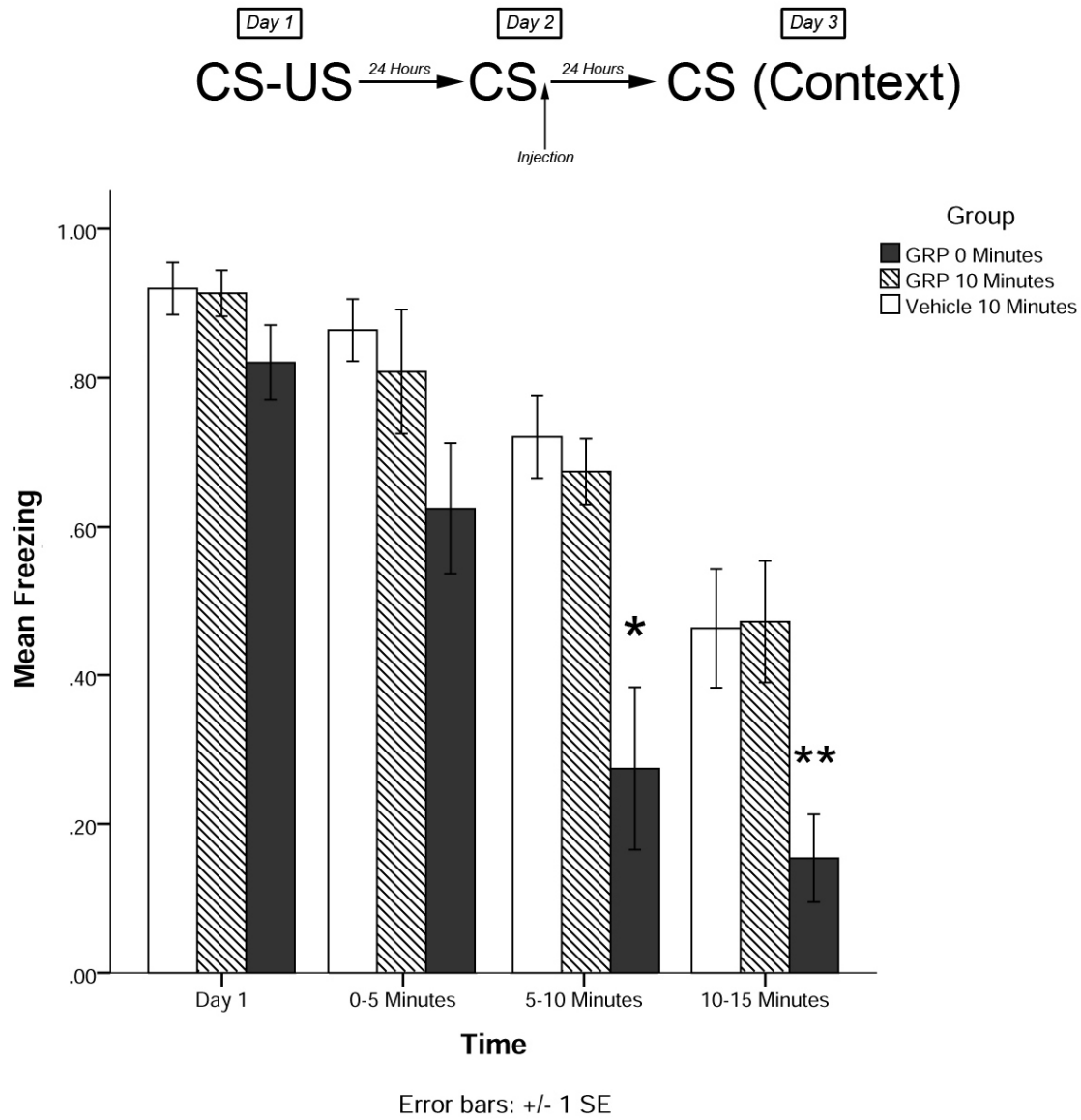


Figure 2. Peripheral GRP blocks reconsolidation of conditioned fear when administered immediately, but not 10 min following fear memory reactivation. $*p < 0.05$, $**p < 0.01$.

To examine the time window of effectiveness of GRP at disrupting memory reconsolidation, we administered GRP at different time intervals post reactivation on Day 2 and then measured freezing behavior during testing on Day 3 (see Fig 2). No animals were excluded from the analyses. ANOVA revealed a significant main effect of Treatment on levels of freezing, $F(2,24) = 8.671, p < 0.01$. Post-hoc analyses further revealed that animals who received peripheral GRP immediately following memory reactivation on Day 2 displayed significantly less freezing than vehicle treated animals on Day 3 at 5-10 min ($p < 0.01$) and 10-15 min ($p < 0.01$) time-blocks. GRP administration at 10 min following fear-memory reactivation, however, did not yield a significant reduction in freezing at any time point versus controls ($p > 0.05$). Results therefore suggest that the time-window for the effectiveness of peripheral GRP for blockade of fear-memory reconsolidation is quite short.

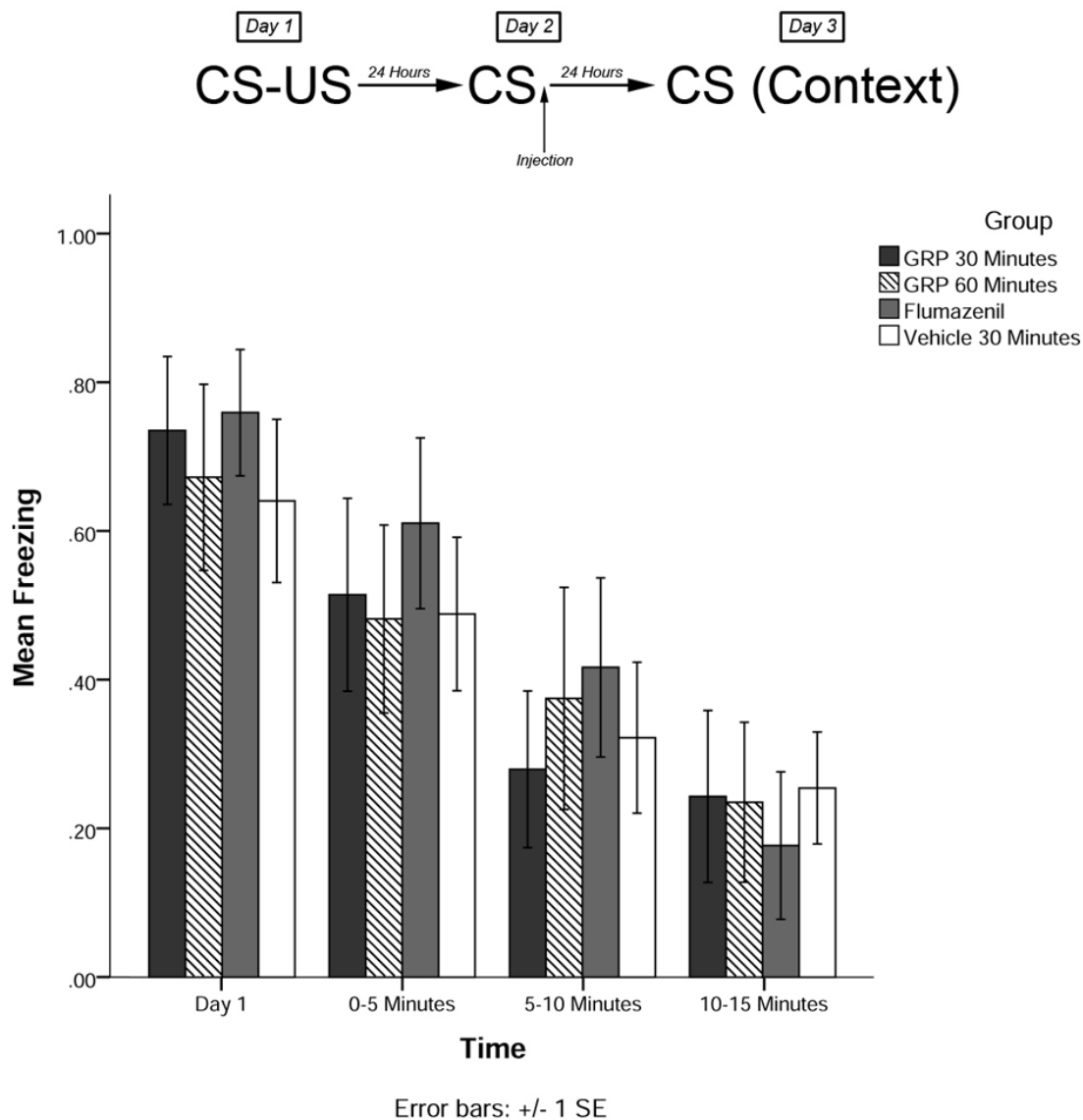


Figure 3. Peripheral GRP fails to block reconsolidation of conditioned fear when administered 30 or 60 min post-recall. Flumazenil administered 30 min post-recall similarly fails to block the effect.

In addition, additional analyses further indicated that there were no significant effects when GRP was administered at longer intervals of 30 and 60 min following fear-memory recall

or when Flumazenil was administered at 30 minutes post-recall, $F(3,32) = 0.91, p > 0.05$ (see Fig 3; no animals were excluded from the analyses).

Experiment 3: Effects of GRP administered in the absence of memory reactivation

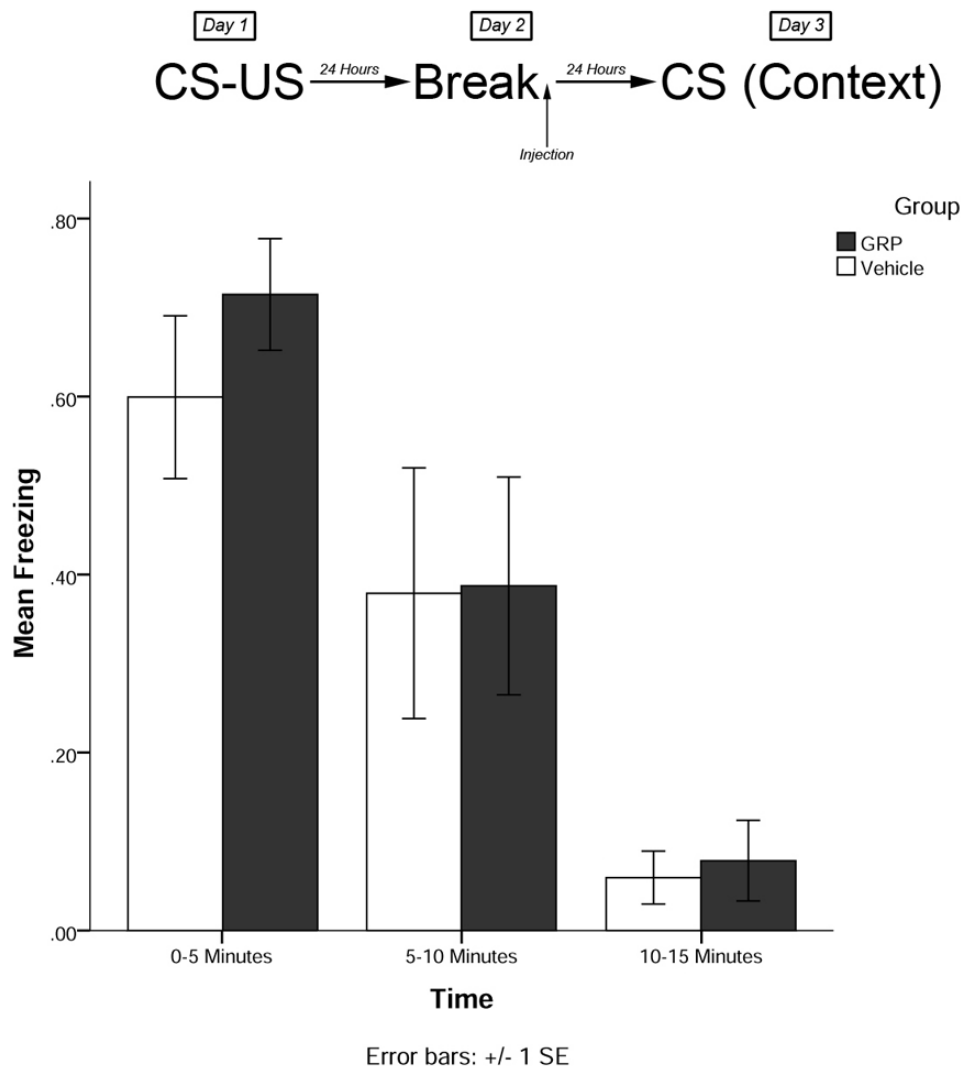


Figure 4. Peripheral GRP does not produce attenuation of the learned fear response when administered in the absence of memory trace reactivation.

Figure 4 shows the effects of peripheral GRP on reconsolidation when administered on Day 2 in the absence of memory reactivation. Two animals were excluded from the analyses. ANOVA revealed that GRP had no effect on fear-memory reconsolidation as compared to controls when reactivation of the fearful memory trace was absent, $F(1,12) = 0.21, p > 0.05$ (see Fig 4).

Experiment 4: Long-term effects of GRP on reconsolidation blockade

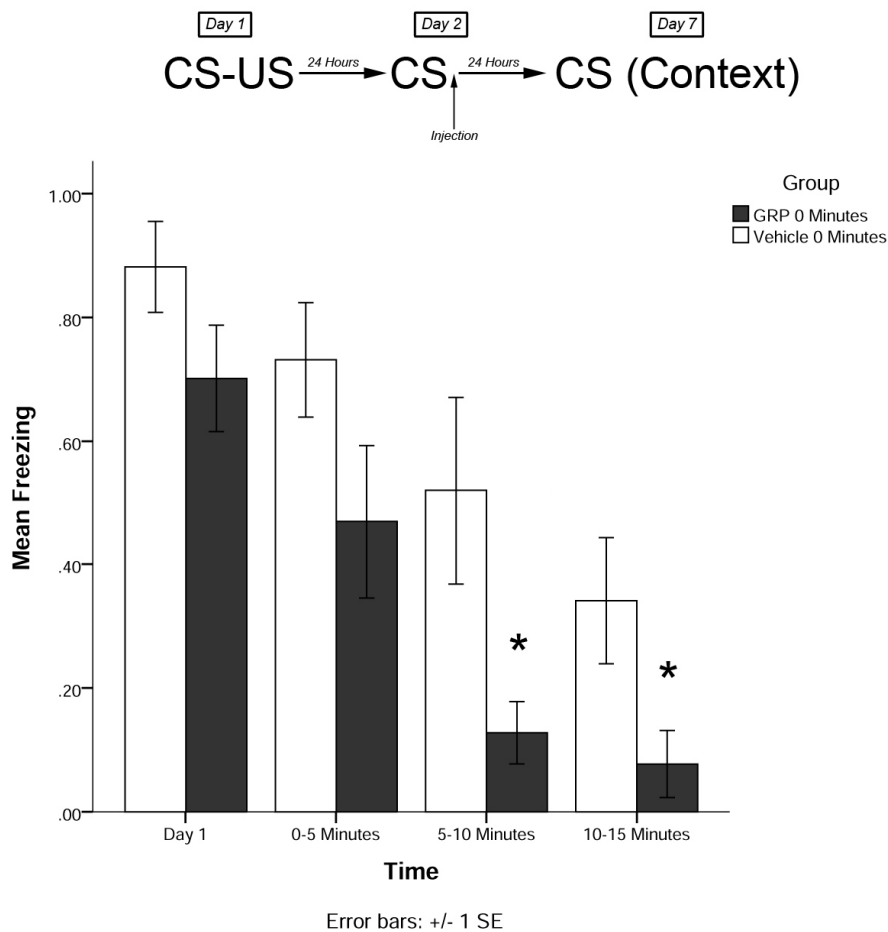


Figure 5. Attenuation of the learned fear response is persistent 5 days following treatment, suggesting the effect is not transient. $*p < 0.05$.

We also examined the long-term effects of peripheral GRP administration on reconsolidation blockade (see fig 5). Three animals were excluded from the analyses. ANOVA revealed a significant main effect of Treatment, $F(1,11) = 5.87, p < 0.05$ on levels of freezing. Follow up tests show that animals that had received GRP immediately post reactivation on Day 2 demonstrated significantly lower mean freezing at 5-10 min ($p < 0.05$) and 10-15 min ($p < 0.05$) time-blocks, versus controls when tested 5 days later suggesting that the effects of GRP on fear-memory reconsolidation are not transient, and persisted for at least 5 days post reactivation (see Fig 5).

2.5 Discussion

The aim of this research was to investigate the effects of systemically administered GRP on reconsolidation of learned-fear (when administered both immediately and at longer intervals following recall of a previously acquired fear memory), as well as to elucidate whether GRP acts via a GABAergic mechanism. The results support the notion that peripheral administration of GRP attenuates the reconsolidation of conditioned fear as GRP administered immediately following reactivation of the memory trace consistently produced attenuation of the conditioned fear response. Our findings also demonstrated that GRP produces no attenuation of the learned fear association in the absence of memory trace reactivation. This is in support of previous findings [6] which also suggest reconsolidation blockade requires memory trace reactivation.

However, our findings also indicated that when administered even at a short delay of 10 min following reactivation of the memory trace, GRP had no effect. Similarly, peripheral GRP had no significant effect on fear memory when administered at longer intervals of 30 or 60 min following memory trace reactivation. While previous research indicated that central drug administration 6 h following memory recall has no effect on memory [297], our results suggest

the window of opportunity for reconsolidation blockade using peripheral administration of GRP (which is more relevant for the development of potential treatments for fear-memory related disorders) is likely much shorter, making successful treatment strategies quite sensitive to the time-course of the reconsolidation window. Evidence suggests that peripherally administered bombesin, a closely related peptide and agonist of the same receptor, causes alterations in Fos-immunoreactivity in the brain 60-90 min post-administration [306]. Since GRP agonizes the same receptor is likely that peripheral GRP has similar effects; it may be the case that for compounds like Bombesin and/or GRP that may have a long latency to peak activity, even a short delay is sufficient to prevent effects on the reconsolidation process.

One of the limitations of our investigation is that it does not pinpoint the mechanism through which GRP exerts its effects. Although there is sufficient evidence to suggest peripherally administered Bombesin-like peptides have central effects [307], the precise mechanisms by which GRPR agonists act peripherally remains somewhat unknown. It is important to highlight that GRP has a very short biological half-life (~2.8 min in human subjects; [305]). In addition, as a 27-amino acid peptide, it is unlikely that GRP crosses the blood-brain barrier when administered peripherally. As a result, it is likely that central effects of peripherally administered GRP are triggered by an initial peripheral site of action which then affects central structures (i.e. by triggering central activity or release of other compounds). Central administration of GRP has been shown to exert effects on fear memory by both amygdala and hippocampal infusion [16,214,301]. As well, intra-cerebro-ventricular (i.c.v.) administration of GRP attenuates both fear-potentiated startle and conditioned emotional response [17]. Since the GRPR is highly distributed throughout both the hippocampus and basolateral amygdala [308,309] both are possible sites through which peripheral GRP might ultimately exert its effects

on fear memory. Peripheral bombesin also elicits Fos-like immunoreactivity in the anterior and posterior central nucleus of the amygdala [306].

There is some evidence that the peripheral mechanism of GRP may depend on brain-gut connectivity and/or vagus nerve signalling. Bombesin produces satiety when administered peripherally [310], and evidence suggest the effect is mediated centrally [311]. The satiety effects of systemic bombesin are also attenuated by a combination of spinal-visceral neural disconnection of the gastrointestinal tract and bilateral sub-diaphragmatic vagotomy [312]. This strongly suggests a peripheral site of action for bombesin which depends on brain-gut connectivity and the vagus nerve. This is also supported by our research using capsaicin (a neurotoxin that ablates afferent vagal/spinal fibers, leaving efferent neural projections intact) which demonstrated that neonatal capsaicin treatment for deafferentation blocked the effects of systemically administered - but not centrally administered - bombesin [313]. This suggests the central effects of peripheral BB are mediated by binding at a peripheral site, and central effects might result from transmission of signals via ascending projections. However, sub-diaphragmatic vagotomy or neural disconnection of the gastrointestinal tract alone do not block the satiety effects of bombesin suggesting multiple pathways may play a role [307]. As a result, it will be important in future research to seek to identify the site (or sites) at which peripherally administered GRP primarily exerts its effects. Blockade of peripheral GRP by central microinjection, for example, could help to determine whether peripheral administration triggers central release of compounds in the hippocampus or BLA. In addition, since centrally administered BB has similar effects on satiety to peripheral administration [313], it will be important to determine whether peripheral administration of BB or GRP agonists triggers central release of GRPR agonists.

In addition, we found that co-administration of the benzodiazepine receptor antagonist Flumazenil with GRP appeared to reverse the effects of GRP administered alone on memory reconsolidation suggesting that GRP may act via a GABAergic mechanism. These findings may be consistent with the network identified in the basolateral amygdala [213] whereby activation of GRPRs in the basolateral complex of the amygdala causes GABA release and the inhibition of amygdala principal neurons by inhibitory interneurons. However, the enhancement of memory consolidation by infusion of GRPR antagonist RC-3095 into the dorsal hippocampus is similarly blocked by selective GABA_A-receptor agonist muscimol [300], suggesting GABAergic networks in both the dorsal hippocampus and basolateral amygdala might play a role in the effects of peripheral GRP on fear memory reconsolidation. Future studies should aim to determine whether the effects of peripheral or central GRP are blocked by co-injection or pre-treatment with GABA_A antagonists Bicuculline or Muscimol. Fear-memory impairment produced by GRPR antagonism in the hippocampus is also reversed by systemic administration of a glucocorticoid receptor agonist dexamethasone [314]. Interactions of GRPRs with glucocorticoid receptors and stress-induced release of endogenous GRP have also been demonstrated, suggesting GRPRs likely interact with both GABA and glucocorticoid receptors in the mediation of fear memory at both the dorsal hippocampus and basolateral amygdala [314–317].

While the effective reversal of the effects of GRP by Flumazenil supports the contention that peripheral GRP exerts its effects via a GABAergic mechanism, it is worthy of note that the antagonist alone also had a significant effect on reconsolidation. Indeed, the effects of Flumazenil alone were slightly more pronounced than those produced by GRP. When administered alone, Flumazenil can act as a partial agonist [302,303,318]. Partial agonists behave as competitive antagonists when in the presence of another agonist of sufficient concentration,

but have the opposite effect (and instead behave like an agonist) when no other agent is present [319]. Partial agonists thus mimic the effects of an agonist when administered alone, but have the effect of an antagonist when co-administered with another compound. In this case, it may be that Flumazenil acts as a GABA_A-receptor agonist when administered alone, thus producing the same effects on fear memory as GRP. This effect is consistent with prior studies using Flumazenil [302,303,318]. This would be consistent with prior research suggesting that GABA agonist Midazolam administered post-reactivation reduces conditioned freezing on subsequent trials [281]; additionally, this study demonstrated that the effects of GABA agonists on reconsolidation are reversed by bicuculline, a GABA antagonist. Some research suggests that Flumazenil has an anxiolytic effect in humans when administered alone [320], suggesting that it may have therapeutic potential. Our research is limited in that it does not explore the mechanisms mediating this effect; however, some possible mechanisms can be inferred. For example, if it indeed behaves like a GABA_A-receptor agonist when administered alone, Flumazenil might act at both CA1 and the BLA to block reconsolidation. Future research should aim to replicate the effects on freezing using microinjection of Flumazenil at both CA1 and the BLA.

Similarly to GRP, we found no effect of Flumazenil when administered 30 minutes post-recall, which would suggest Flumazenil is similarly sensitive to the time-course of drug administration. Flumazenil has a short biological half-life [321] but disperses rapidly in the body and brain, maintaining antagonistic activity for 2-3 hours after administration [304]. Since the same limiting effect was found for both GRP and Flumazenil (even though Flumazenil fairly rapidly enters the brain), this would seem to further confirm that the window of opportunity for pharmacologic effects on reconsolidation by peripheral drug administration is likely quite short.

Thus, therapeutic potential of reconsolidation blockade paradigms might be limited by the pharmacokinetics of drugs administered.

Finally, our findings suggested that the effects of GRP were long-lasting, as the reduction in fear memory reconsolidation was still present when animals were tested 5 days following treatment. This is in contradiction to other findings which demonstrated that intra-hippocampal infusions of RC-3095 produced only transient effects on memory reconsolidation using an inhibitory avoidance task [301]. *In vitro* recording studies also suggested that GRP's ability to increase inhibitory activity of LA principal neurons was transient as peptide application resulted in short-term (but not long-term) effects on LTP [322]. It is somewhat surprising that an attenuation of fear memory reconsolidation was also evident with RC-3095 given this compound is considered a GRPR antagonist [301]. It should be noted however that prior research suggests that - like Flumazenil - RC-3095 has partial agonist qualities, and may produce the same effects expected of a GRPR agonist when administered alone [16,323]. Indeed, opposing effects of RC-3095 have also been observed with RC-3095 administered at different doses and also across studies [300,324]. It also should be considered that *in vitro* findings or those obtained with site-specific peptide administration are difficult to compare to peripheral GRP administration given that widespread activation of GRPR likely occurred with systemic delivery which may be contributing to the lasting effects on fear memory observed in the present investigation.

Conclusion

In sum, results for these studies suggest that both GRP and Flumazenil show some promise as agents able to attenuate fear memory reconsolidation which may have clinical relevance. Further investigation is needed however into the long-term efficacy of both peripheral and central GRP on reconsolidation blockade as well as on the time sensitive nature of drug

application during the reconsolidation window (which may limit therapeutic potential). Future research should aim to more extensively demonstrate the time-course of the reconsolidation window. Also, future studies should aim to pinpoint the mechanisms through which peripheral GRP exerts its effects and determine how brain-gut connectivity might play a role in the effects of GRP on fear-learning. Finally, future research should also seek to address the therapeutic potential of Flumazenil for reconsolidation blockade, as well as how other GABA_A BZD antagonists (e.g. Bicuculline) interact with GRPR agonists to elucidate the role of GABAergic mechanisms in the effects of peripheral GRP.

Funding

The experimental protocol for this study was approved by the University of Ottawa Animal Protocol Review Committee and conforms to the guidelines of the Canadian Council on Animal Care. None of the authors have any direct or indirect conflicts of interests of a financial or personal nature relevant to the present work.

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Chapter 3: Cannabidiol and the remainder of the plant extract modulate the effects of Δ^9 -Tetrahydrocannabinol on fear memory reconsolidation

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3.1 Abstract

Background: Δ^9 -Tetrahydrocannabinol (THC, a CB1 receptor agonist) and Cannabidiol (CBD, a non-competitive antagonist of endogenous CB1 and CB2 ligands) are two primary components of *Cannabis* species, and may modulate fear learning in mammals. The CB1 receptor is widely distributed throughout the cortex and some limbic regions typically associated with fear learning. Humans with posttraumatic disorder (PTSD) have widespread upregulation of CB1 receptor density and reduced availability of endogenous cannabinoid anandamide, suggesting a role for the endocannabinoid system in PTSD. Pharmacological blockade of memory reconsolidation following recall of a conditioned response modulates the expression of learned fear and may represent a viable target for the development of new treatments for PTSD. In this study, we focused on assessing the impact of the key compounds of the marijuana plant both singly and, more importantly, in concert on attenuation of learned fear. Specifically, we assessed the impact of THC, CBD and/or the remaining plant materials (post-extraction), on reconsolidation of learned fear.

Method: Male Sprague-Dawley rats received six 1.0 mA continuous foot shocks (contextual training). 24 h later, rats were re-exposed to the context. Immediately following memory retrieval (recall) rats received oral administration of low dose THC, high dose THC, CBD, CBD + low THC, CBD + high THC (as isolated phytochemicals and, in separate experiments, in combination with plant background material). Rodents were tested for freezing response context re-exposure at 24 h and 7 d following training.

Results: CBD alone, but not THC alone, significantly attenuated fear memory reconsolidation when administered immediately after recall. The effect persisted for at least 7 d. A combination of CBD and THC also attenuated the fear response. Plant background material also significantly attenuated reconsolidation of learned fear both on its own and in combination with THC and CBD. Finally, THC attenuated reconsolidation of learned fear only when co-administered with CBD or plant background material.

Conclusion: CBD may provide a novel treatment strategy for targeting fear-memories. Furthermore, plant background material also significantly attenuated the fear response. However, whereas THC alone had no significant effects, its effects were modulated by the addition of other compounds. Future research should investigate some of the other components present in the plant background material (such as terpenes) for their effects alone, or in combination with isolated pure cannabinoids, on fear learning.

3.2 Introduction

Δ^9 -Tetrahydrocannabinol (THC), the primary psychoactive component of *Cannabis* (*sativa*, *indica*, and *ruderalis*), has been reported to affect fear memory, expression, consolidation, and extinction [252,325–330]. In addition, Cannabidiol (or CBD), another component of the plant, has also been reported to impact fear memory. Medical cannabis (or marijuana for medical purposes, MMP) is widely used to self-medicate for a variety of medical conditions, including disorders rooted in fear learning such as post-traumatic stress disorder, PTSD (Lucas and Walsh, 2017). However, the effects of marijuana on fear memory reconsolidation have been only sparsely explored. Additionally, MMP is often utilized as whole plant material. However, the effects of combined doses of THC and CBD in varying concentrations, as well as the role of the remaining (non-THC, non-CBD) plant material and its interactions with THC and CBD, remain largely unexplored. Here, we aimed to identify whether combined THC and CBD could affect fear memory reconsolidation both in isolation and when combined at varying concentrations. In addition, since it is highly relevant for the use of whole plant material as MMP, we also sought to determine whether the effects of THC and CBD on reconsolidation are modulated by inclusion of the remaining plant material.

Behaviorally, in many respects CBD has been shown to produce effects which are opposite those of THC. One functional magnetic resonance imaging (fMRI) study found CBD and THC had opposite effects on regional activation in the hippocampus, amygdala, superior temporal cortex, and occipital cortex [221]. The same study found that pre-treatment with 5 mg CBD intravenously (IV) attenuated the severity of psychotic symptoms induced by THC.

There is some data from central studies to suggest that targeting the endocannabinoid (ECB) system may be a viable strategy for pharmacologically attenuating established fear memories.

While THC acts as an agonist for the CB1 and CB2 receptors, both of which have been implicated in fear-learning [219,220], the actions of CBD are complex. CBD exerts some of its effects indirectly by inhibiting the actions of endogenous CB1 and CB2 agonists. CBD has been shown to act as a potent antagonist for CB1 and CB2 ligands, while displaying low binding affinity for the CB1 receptor [253–255]. CBD has also been shown to act as an indirect agonist of 5-HT_{1A} receptors, and may exert some of its effects via this mechanism [331,332]. CB1 receptors are expressed in the hippocampus, basolateral and lateral amygdala, and medial prefrontal cortex (mPFC; [223]) – key regions implicated in fear learning - but are absent in the central and medial nuclei of the amygdala [231], regions involved in fear expression. Thus, CB1 likely affects fear expression via an indirect neuromodulatory mechanism. CB1 receptors are found on GABAergic neurons of the basolateral amygdala (BLA), and their activation dampens BLA inhibitory interneuron activity. This disinhibition increases output from BLA projections [333,334]. BLA stimulation induces long-term potentiation (LTP) along the BLA-prelimbic (PLC) pathway, and blockade of CB1 transmission prevents this [236]. The same study also demonstrated that pharmacological blockade of BLA-PLC CB1 signaling blocks encoding of fear learning. Thus, there is a strong theoretical framework for the notion that pharmacologically modulating the ECB system may allow for the attenuation of traumatic memories. It is possible that the blockade of CB1 agonists by CBD impedes BLA-PLC signaling, thereby exerting its effects on fear learning.

The distribution of central receptors in the ECB system have already been implicated in human PTSD. Evidence from positron emission tomography (PET) imaging suggests that among those with PTSD there is a widespread upregulation of CB1 receptor density in regions implicated in learned fear (the amygdala, hippocampus, orbitofrontal cortex, and anterior

cingulate; [224]. Combined with behavioral evidence of cannabinoid involvement in fear-learning, this data suggests the endocannabinoid system may be involved in the mediation of fear memories, and may be a strong target for the mitigation of some PTSD symptoms. Indeed, some research points to positive effects of oral THC in reduction of hyper-arousal and frequency of nightmares among those affected by PTSD [227].

Some findings have also suggested that THC and CBD may disrupt the reconsolidation of recalled fear memories [298,335], a novel therapeutic strategy that may have relevance for attenuating established memories of trauma. Reconsolidation blockade is the process by which the expression of formed memories is reduced by administration of drugs following recall (during the reconsolidation window; [13,55,336]. This procedure may offer new avenues for treatment of fear-based disorders that are resistant to extinction. Although several medicinal plants and isolated compounds have been reported to affect fear expression and reconsolidation in rodents [15,51,55,85,296,298,337], reconsolidation paradigms have had mixed success when translated in human studies [13,64,86]. Thus, new targets are needed for future translational studies with humans, and *Cannabis spp.* extracts may offer one such target.

Although anecdotal findings regarding CBD and THC are compelling, there is a dearth of information around the effectiveness of cannabinoids at blocking fear memory reconsolidation. There is some evidence to suggest that both CBD and THC may block fear memory reconsolidation [85,338]. This is curious, since THC and CBD typically produce opposite effects both centrally and peripherally. The effects of combined doses of CBD and THC on fear memory reconsolidation have also been sparsely explored [338], albeit at very low doses. It is important to assess this, as the consumption of marijuana would entail exposure to both the main cannabinoids simultaneously. In addition, there may be other active components present in the

plant material that may be biologically active and may modulate the effects of the key cannabinoids. However, the effects of other components of the plant in combination with cannabinoids (such as the terpenes) remain largely unexplored. In terms of relevance for human use of MMP, it is important to explore the effects of all components of the plant (since MMP, which is typically administered as whole plant material, does not solely consist of THC and CBD). It is also clear that the concentrations of THC and CBD can vary based on the specific species of the plant; it is therefore important to identify and standardize the concentrations of THC and CBD in administered extracts, and to verify the effects of the remainder of the plant extracts containing varying concentrations of THC and CBD.

Here, our experiments examined whether combined doses of THC and CBD would block fear memory reconsolidation, as well as whether the effects of the phytocannabinoids are modulated by the remaining plant background material (all remaining plant components following CBD/THC extraction). In order to simulate MMP preparations consisting of whole plant material (which is much more relevant for human medical cannabis use, which may contain other active non-cannabinoids affecting fear memory), we tested doses of isolated phytochemicals THC and CBD both alone, in combination with each other, and in combination with plant background material. In addition, in order to simulate the effects of varying concentrations of THC in plant material, we tested the effects of co-administration of CBD with both a low- and high-dose of THC (both with and without background material).

3.3 Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratories International, Inc.; 180-200 g on arrival) were pair housed and maintained on a 12-h light/dark cycle (lights on at 07:00-h).

Temperature was maintained at 23° C, and relative humidity at 37%. Throughout the duration of the study, animals had free access to food and water. All experiments were conducted in accordance with the guidelines established by the Canadian Council on Animal Care and approved by the University of Ottawa Animal Care Committee.

Drugs and Injections

Isolated compounds THC and CBD, as well as plant background material, were extracted from raw plant material of a *Cannabis Indica* and *Cannabis Sativa* hybrid variety ('Strawberry Kush'). Pure compounds and background material were provided by T. Durst (University of Ottawa, Ontario, Canada). Plant background material consisted of all other remaining plant components in the extracts following the isolation of THC and CBD. Due to the complexity of completely extracting all the THC and/or CBD, our background material contained less than 3 ± 0.5% THC and less than 0.6% of CBD. Animals were habituated to daily administration of oral almond oil (vehicle; intubation) for one week prior to the experiment. In conditions where the background material was co-administered with cannabinoids, the amount of background material was held constant at 30% of the total amount of compounds administered (i.e. treatment dose was 70% cannabinoids and 30% background material; the dose of background material (BM) was calculated as $\frac{BM}{(THC+CBD+BM)} = 0.3$). Our low doses of CBD and THC were comparable to moderate doses administered systemically in previous research [85,338]. Stern et al. (2015) observed strong effects on reconsolidation at 10mg/kg I.P. THC, but did not test at higher doses.

For experiments 1 and 2, rats were randomly assigned to one of 7 treatment groups. 1) 50 mg/kg THC + 21.5 mg/kg BM, 2) 50 mg/kg CBD + 21.5 mg/kg BM, 3) 5 mg/kg THC + 2 mg/kg BM, 4) 50 mg/kg THC + 50 mg/kg CBD + 43 mg/kg BM, 5) 50 mg/kg CBD + 5 mg/kg THC + 24 mg/kg BM, 6) 43 mg/kg BM, and 7) vehicle alone.

For experiments 3 and 4, in order to explore the effects of isolated cannabinoids in absence of the background material of the plant, rats were randomly assigned to 1 of 4 treatment groups: 1) 5 mg/kg THC, 2) 50 mg/kg CBD, 3) 50 mg/kg THC + 50 mg/kg CBD, 4) 43 mg/kg BM, or 5) Vehicle.

For experiment 5, rats were similarly randomly assigned to one of five treatment groups: 1) 5 mg/kg THC, 2) 50 mg/kg CBD, 3) 50 mg/kg THC + 50 mg/kg CBD, 4) 43 mg/kg BM, and 5) Vehicle.

Contextual fear conditioning

The conditioning chambers (Coulbourn Instruments) measured 31 cm x 25 cm x 30 cm. The front and back walls were made of clear acrylic, and the two side walls and top made of stainless steel. The floor was composed of 16 stainless steel rods (4 mm diameter spaced 1.4 cm apart) connected to Coulbourn precision regulated animal shockers, which delivered scrambled footshock (1.0 mA). Animals (N = 7-10/group) were randomly distributed into treatment groups. Subjects that failed to achieve a minimum baseline freezing level of 40% during re-exposure to the fearful context (memory recall; assessed on Day 2) were removed from the analyses. All experimental procedures were conducted in accordance with methods established by prior research [339].

Experimental Procedure

Experiment 1: Effects of plant extracts with background material on fear memory reconsolidation, short-term

Animals were exposed to six consecutive 1-s footshocks over the course of 11 min. Contextual conditioning was used (pairing of footshock with the conditioning chamber). 24-h later, animals were re-exposed to the context in which they received the footshock (conditioning

chamber) for a duration of 5 min, and freezing (total time spent in complete immobility) was measured (Day 2; recall). Cage placement and assignment to drug treatment groups was randomized and counterbalanced.

Immediately following the 5 min recall session, animals were administered drugs in one of the experimental conditions (low THC + BM, high THC + BM, CBD + BM, high CBD + low THC + BM, high CBD + high THC + BM, BM alone, and vehicle alone). 24 h later (Day 3; testing), animals were re-exposed to the conditioning chamber and freezing was measured over the course of 10 min. Freezing on Day 3 was scored in two 5-min time blocks (0-5 and 6-10 min). The timeline of procedures used for fear conditioning is illustrated in Fig 1.

Experiment 2: Effects of plant extracts with background material reconsolidation of fear memory, long-term

Using the same training procedures as Experiment 1, experiment 2 was conducted to test for long-term effects of isolated cannabinoids in combination with plant background material on fear memory reconsolidation.

Immediately following the 5 min recall session, animals were administered drugs in one of the experimental conditions (low THC + BM, high THC + BM, CBD + BM, high CBD + low THC + BM, high CBD + high THC + BM, BM alone, and vehicle alone). One week later (Day 10), animals were re-exposed to the conditioning chamber and freezing was measured over the course of 10 min. Freezing on Day 10 was scored in two 5-min time blocks (0-5 and 6-10 min). The timeline of procedures used for fear conditioning is illustrated in Fig 2.

Experiment 3: Effects of plant extracts without background material on fear memory reconsolidation, short-term

Using the same training procedures as Experiment 1, experiment 3 was conducted to test for short-term effects of isolated cannabinoids in the absence of plant background material on fear memory reconsolidation.

Immediately following the 5 min recall session, animals were administered drugs in one of the experimental conditions (low THC, high THC, CBD, CBD + THC, BM alone, and vehicle alone). 24 h later (Day 3; testing), animals were re-exposed to the conditioning chamber and freezing was measured over the course of 10 min. Freezing on Day 3 was scored in two 5-min time blocks (0-5 and 6-10 min). The timeline of procedures used for fear conditioning is illustrated in Fig 3.

Experiment 4: Effects of plant extracts without background material on fear memory reconsolidation, long-term

Using the same training procedures as Experiment 1, experiment 4 was conducted to test for long-term effects of isolated cannabinoids in the absence of plant background material on fear memory reconsolidation.

Immediately following the 5 min recall session, animals were administered drugs in one of the experimental conditions (low THC, high THC, CBD, CBD + THC, BM alone, and vehicle alone). One week later (Day 10), animals were re-exposed to the conditioning chamber and freezing was measured over the course of 10 min. Freezing on Day 10 was scored in two 5-min time blocks (0-5 and 6-10 min). The timeline of procedures used for fear conditioning is illustrated in Fig 4.

Experiment 5: Effects of plant extracts in the absence of memory recall (no-recall control conditions)

Experiment 5 was conducted as a control experiment to determine whether blockade of reconsolidation required reactivation of the memory trace. The same training and testing procedures as the other experiments were used, except that recall of the fearful memory trace on Day 2 was absent.

Animals in this experiment were administered drugs in one of five conditions (low THC, CBD, CBD + high THC, BM, or vehicle) administered on Day 2 in home cage (no recall). Animals were then exposed to the conditioning chamber on Day 3, and freezing was measured over the course of 10 min in two 5-min blocks (0-5 and 6-10 min). The timeline of procedures for this no-recall control is illustrated in Fig 5.

Statistical Analyses

All statistical analyses were conducted using IBM Statistics Package for the Social Sciences® (SPSS) 20. Data were analyzed by mixed-measures ANOVA, in which drug treatment was the between groups variable and time was the within groups variable. Greenhouse-Geisser correction was applied where the assumption of sphericity was violated. Follow-up comparisons of significant main effects and interaction effects were conducted using Bonferroni corrected t-tests, or Games-Howell post-hoc analysis where the assumption of homogeneity of variance was violated.

3.4 Results

Experiment 1

Figure 1 shows the effects of plant extracts administered immediately post recall on freezing behavior as measured during testing on Day 3. The mixed measures ANOVA revealed a significant main effect of treatment group on freezing behavior, $F(6,53) = 5.509, p < 0.001$.

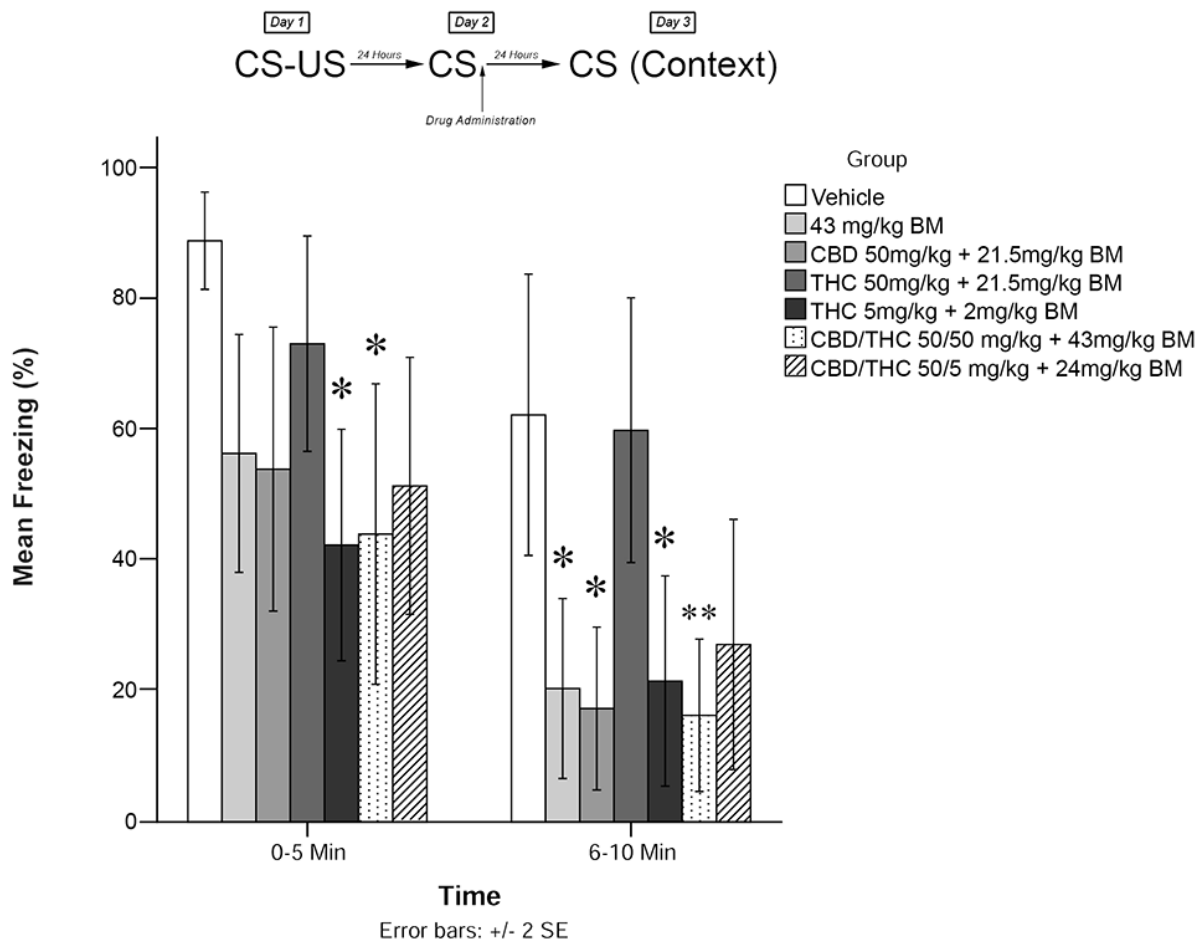


Figure 1. Cannabis extracts with BM (whole plant background material) significantly attenuate the reconsolidation of contextual learned fear 24 h after drug administration.

Follow-up analyses indicated that animals treated with either low THC (5 mg/kg + background material; $p < 0.05$) or CBD + high THC (50 mg/kg each + background material; $p < 0.05$) displayed significantly reduced freezing behavior during the first 5 min of testing. Animals that received low THC (5 mg/kg + BM; $p < 0.05$), CBD (50 mg/kg + BM; $p < 0.05$), or BM alone ($p < 0.05$) following memory recall on Day 2 also displayed significantly less Freezing than vehicle treated animals during the second 5 min bin of testing on Day 3. Animals that received CBD + high THC (50 mg CBD + 50 mg/kg THC + BM) $p < 0.01$ also displayed

significantly reduced freezing on day 3. This suggests that all groups except for high THC + BM (50 mg/kg; $p > 0.05$) and CBD + low THC (50 mg CBD + 5mg/kg THC + background material, $p > 0.05$) had a significant effect on freezing behavior when co-administered with plant background material.

Experiment 2

Figure 2 shows the effects of plant extracts administered immediately post recall, on freezing behavior on Day 10 (long-term). The mixed measures ANOVA revealed a significant main effect of treatment group on freezing behavior, $F(6,53) = 4.974, p < 0.001$.

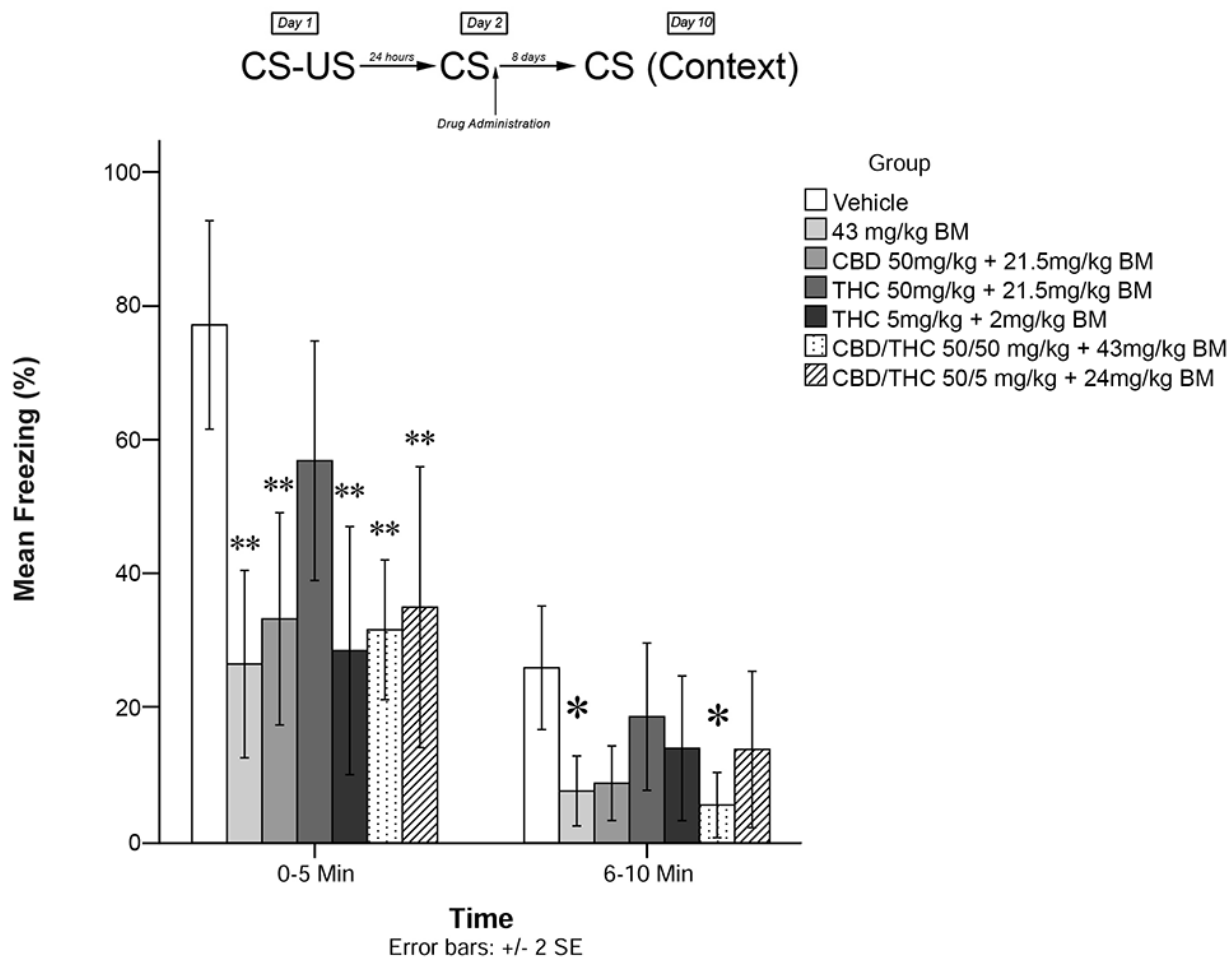


Figure 2. Cannabis extracts with BM significantly attenuated the reconsolidation of contextual learned fear; effect is present on testing day 10.

Follow-up analyses indicated that animals treated with either low dose of THC (5 mg/kg + BM; $p < 0.01$) or CBD + high THC (50 mg/kg each + BM; $p < 0.01$) displayed significantly reduced freezing behavior during the first 5 min of testing. Animals treated with low dose of THC (5 mg/kg + BM; $p < 0.01$), CBD (50 mg/kg + BM; $p < 0.01$), CBD + low THC (50 mg/kg CBD and 5 mg/kg THC + BM, $p < 0.01$), CBD + high THC (50 mg/kg each + BM, $p < 0.01$), or BM alone ($p < 0.01$) following memory recall on Day 2, displayed significantly less Freezing

than vehicle treated animals during the last 5 min of testing on Day 3. This suggests that all drug treatments except for the high dose of THC + BM(50 mg/kg; $p > 0.05$) had a significant effect on subsequent long-term freezing behavior when co-administered with plant background material.

During the last 5 min of testing, CBD + high THC (50 mg/kg each + BM) yielded significant reductions in freezing behavior ($p < 0.05$), as did background material ($p < 0.05$). All other group effects were non-significant during the last 5 min of testing.

Experiment 3

Figure 3 shows the results of experiment 3. The mixed measures ANOVA revealed a significant main effect of treatment group on freezing behavior, $F(4,40) = 7.517$, $p < 0.001$.

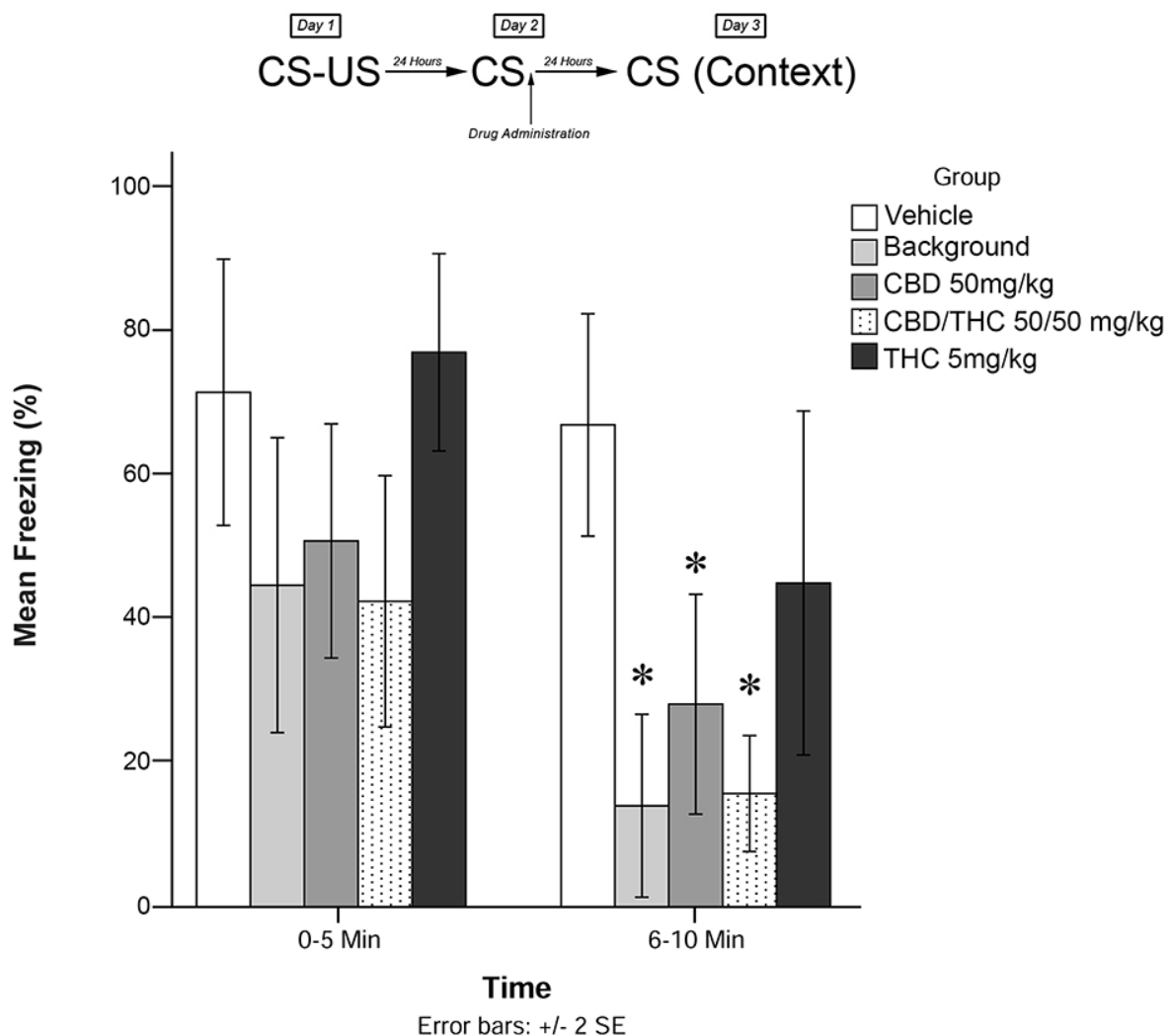


Figure 3. Isolated cannabinoids alone significantly attenuated reconsolidation of contextual learned fear 24 h after drug administration.

Follow-up analyses indicated that animals that received BM ($p < 0.05$) or CBD + high THC (50 mg/kg each; $p < 0.05$) displayed significantly reduced freezing behavior during the 6-10 min window of testing. Animals that received CBD (50 mg/kg; $p < 0.05$) following memory recall on Day 2 also displayed significantly less Freezing than vehicle treated animals during the last 5 min of testing on Day 3. This suggests that all drug treatments except for low-dose THC (5

mg/kg; $p > 0.05$) had a significant effect on subsequent freezing behavior when administered as pure compounds without plant background material.

Experiment 4

Figure 4 shows the results of experiment 4. The mixed measures ANOVA revealed a significant main effect of treatment group on freezing behavior, $F(4,40) = 6.670$, $p < 0.001$.

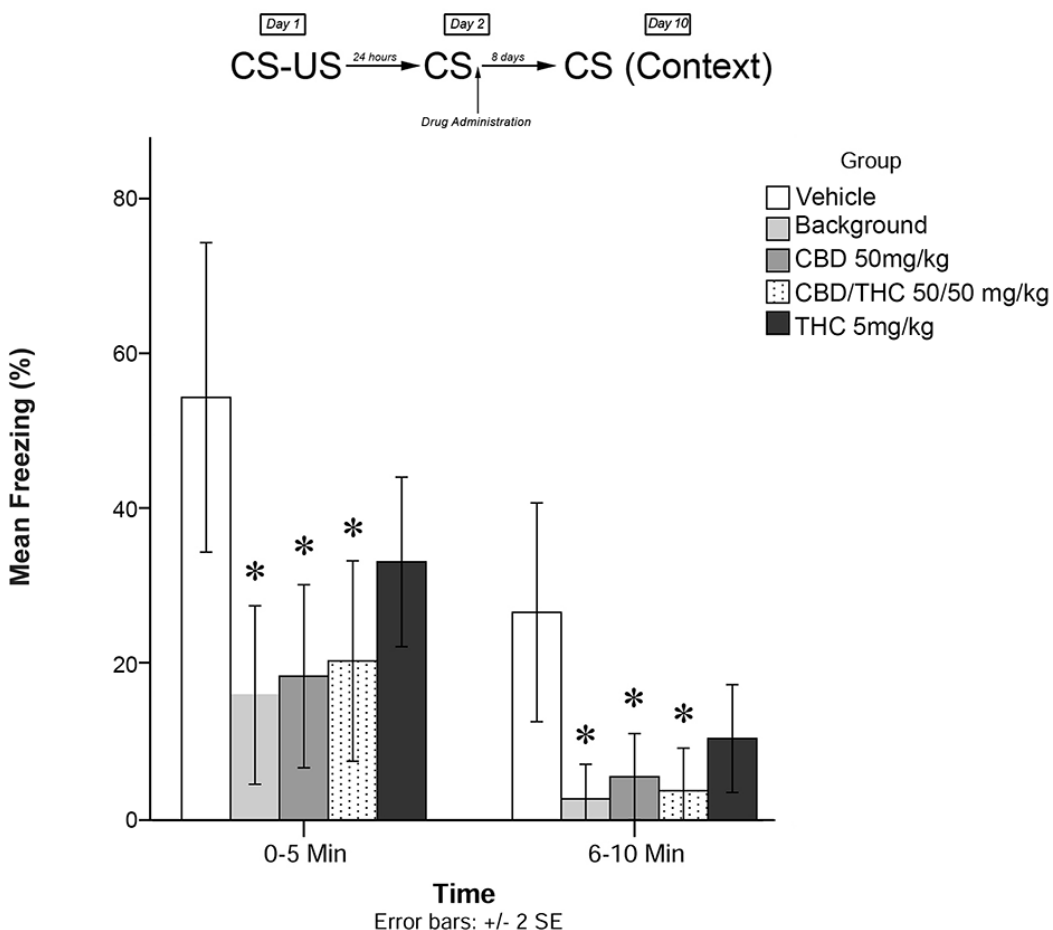


Figure 4. Isolated cannabinoids significantly attenuated the reconsolidation of contextual learned fear; effect is present on testing day 10.

Follow-up analyses indicated that animals that received the BM ($p < 0.05$) or CBD + high THC (50 mg/kg each; $p < 0.05$) displayed significantly reduced freezing behavior during the first

5 min and last 5 min of testing on Day 10. Animals that received oral CBD (50 mg/kg) following memory recall on Day 2, also displayed significantly less Freezing than control (vehicle treated) animals during both the first 5 min ($p < 0.05$) and last 5 min ($p < 0.05$) of testing on Day 10. This suggests that all drug treatments except for low-dose THC (5 mg/kg; $p > 0.05$) had a significant effect on subsequent long-term freezing behavior when administered as isolated compounds without plant background material.

During the last 5 min of testing, CBD + high THC (50 mg/kg each) yielded significant reductions in freezing behavior ($p < 0.05$), as did BM ($p < 0.05$). All other group effects were non-significant during the last 5 min of testing.

Experiment 5

Figure 5 shows the results of experiment 5. Analyses revealed no significant main effects of group for experiment 5, $F(4,40) = 0.919$, $p > 0.05$.

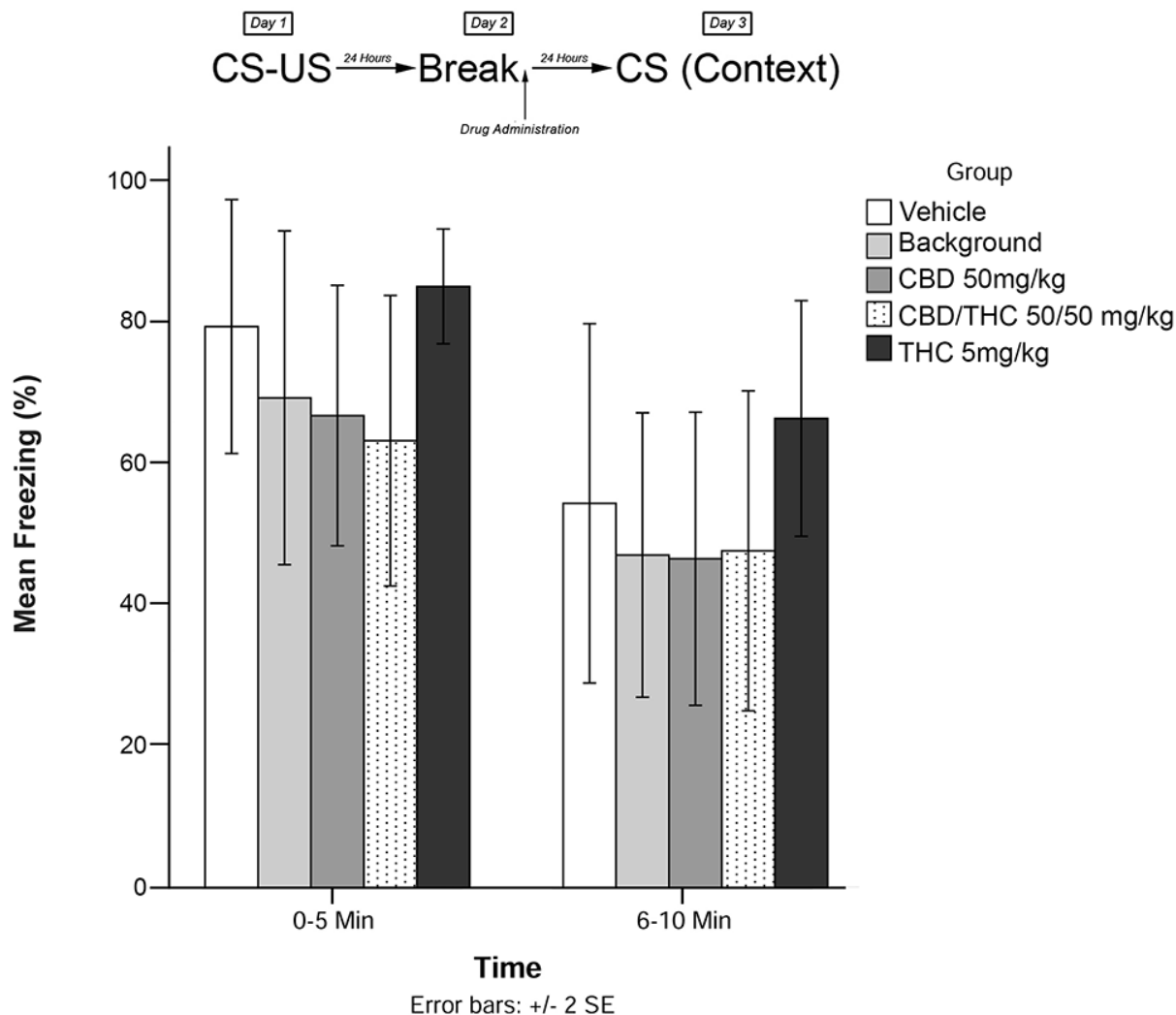


Figure 5. The individual Cannabis extracts on their own had no significant effects on reconsolidation of contextual learned fear 24 h after drug administration in the absence of fearful memory-trace recall.

3.5 Discussion

Our findings suggest that CBD can modulate reconsolidation of learned fear, potentially opening up new treatment avenues for fear-based disorders. Our results also demonstrated that THC at the doses used (the primary psychoactive component of the plant) had no discernible effects on its own, but when co-administered with CBD and/or whole plant background material,

it was effective in modulating the response (suggesting the effects of THC on fear learning are influenced by other components of the plant). Our studies also reveal an inverted “u” dose response, such that at low- and high-dose THC had opposite effects when co-administered with plant background material. Low-dose THC, but not high-dose, attenuated reconsolidation of learned fear, when co-administered with background material. However, these effects were dependent on mediation by co-administration of either CBD or background material. In contrast to previous work (Stern et al., 2015), we found no significant effects of isolated THC on reconsolidation of contextual learned fear. We also observed no attenuation of expression of the learned fear response when drugs were administered without recall (re-exposure to the conditioned stimulus), suggesting the effect was dependent upon recall of the fearful memory.

These findings partially support prior work which suggests the effects of THC are synergized by the addition of other compounds - either in combination with CBD or whole plant material [340–342]. Research suggests that the behavioral effects of THC are modified by co-administration with other compounds, increasing some potentially therapeutic effects while diminishing sedative and anxiogenic effects [343], and our findings would seem to partially confirm this. However, since background material significantly attenuated the reconsolidation of learned fear on its own, it is unclear whether this is a synergistic effect of THC administered with whole plant material. Since the effects of 5 mg/kg THC were not magnified by co-administration (and since the effect of background material persisted when administered without the addition of THC), it is possible that therapeutic effects resulted primarily from the background material alone rather than THC-background material synergism.

The effects of cannabinoids on fear learning might also be mediated by other factors. CB1 receptors have been shown to play a role in modulating the release of other

neurotransmitters such as acetylcholine and dopamine [344–346]. Knock-out mice lacking CB1 receptors on neurons expressing dopamine type-1 receptors (D1Rs) have enhanced expression of cued fear [346], and mice lacking CB1 receptors on neurons expressing D1Rs also exhibited deficits in safety learning in a step-down avoidance task in one study [347]. Similarly, the effects of CB1 activation on anxiety-like behavior in rodents is partially dependent upon GABAergic and glutamatergic factors [348]. It is therefore likely that the neuromodulatory effects of CB1 activation on a variety of neurochemical networks plays a complex role in the effects of cannabinoids on fear learning as well.

Since background material contained all remaining plant components, there are a number of molecules that could have had effects on reconsolidation of learned fear on their own. THC and CBD precursors cannabidiolic acid (CBDA) and tetrahydrocannabinolic acid (THCA) are decarboxylated to CBD and THC [349], and were present in the background material in small quantities. As a result, precursors in the background material can potentially be transformed to active cannabinoid molecules THC and CBD over time. The quantity of THC in our background material sample was $3 \pm 0.5\%$ and a minute quantity of CBD (less than 0.3%). The resulting dose of active THC may have been sub-anxiolytic on its own; however, it is likely that the effects of low-dose THC in the background material were synergized by the presence of other molecules (e.g. terpenoids). If this is the case, we would also anticipate a magnification of the behavioural effects of 5 mg/kg THC by co-administration with whole background material. In our experiments this was not the case, but a floor effect due to very low freezing levels among both conditions (background material alone and 5 mg/kg THC plus background material) may have masked this effect.

Another possibility is that other non-THC, non-CBD constituents of the plant modulated fear learning on their own. *Cannabis spp.* background material contains a number of terpenoid molecules, some of which have been shown to affect anxiety and fear learning. β -Caryophyllene (BCP), for example, is present in *Cannabis spp.* and has been shown to exert anxiolytic-like activity in rodents [286]. Anxiolytic effects of BCP are blocked by CB2 antagonist AM630 [286], but not by 5-HT_{1A} antagonist NAN-190 or the GABA_A Benzodiazepine partial agonist Flumazenil [285]. This suggests BCP may act through CB2 receptors to produce anxiolytic-like effects in rodents. However, the exact mechanism by which BCP exerts its effects remains unknown. In addition, the terpenoids present may vary significantly among different plant strains (in our case, a detailed analysis of the terpenoids present in the background material is not available). Clearly, further research is needed in this regard. Our experiments may also be limited by the fact that we did not conduct an in-depth dose-response of BM. In order to simulate MMP, dosages of BM were maintained at a constant 30% of total compounds administered for each group. It will be an important step for future studies to conduct dose-response experiments with BM both alone and in combination with THC and CBD. Future studies should also aim to explore the effects of isolated *Cannabis*-derived terpenes both alone and as synergists for THC and/or CBD. Finally, studies should also explore their effects with central microinjection at sites known to play a role in fear learning (e.g. BLA, CA1, mPFC), and attempt to block the effects with co-administration of antagonists.

With regards to humans, behavioral studies suggest that the response to marijuana in individuals self-medicating for PTSD varies with symptom severity. Wilkinson, Stefanovics, and Rosenheck [350] for example found that symptom severity and violent behavior are significantly worse among veterans with PTSD who self-medicated with marijuana. It may be the case that

those individuals with greater symptom severity were more likely to seek out alternate means to self-medicate. Indeed, veterans with PTSD are more likely to use marijuana and synthetic cannabis products than veterans without PTSD [351]. Further evidence suggests that individuals with PTSD with marijuana dependence have blunted emotional reactivity, supporting the notion that cannabinoids may affect fear expression [352]. Our results suggest that by using reconsolidation paradigms, prolonged treatment (or chronic use) may not be necessary in order to alleviate learned fear. Also, since CBD by itself was effective in our study, administration of CBD alone (i.e. a non-psychoactive component) may potentially be effective without exposing individuals to long-term treatment with psychoactive substances.

While these insights are valuable, the bulk of recent research on the effects of cannabinoids in humans with PTSD has observed the effects through the lens of self-medication (rather than clinical trial), and the lack of experimental manipulation of drug administration in studies utilizing raw plant material leaves open the question of whether differences in plant composition may have led to variability in results (and whether different components of the plant plants - e.g. THC, CBD, etc. – are present in differing ratios and hence differentially affect PTSD symptomology). Since our findings revealed an inverted-u shaped dose response for THC in combination with background material and CBD, it is important that (as we have done here) studies using plant-derived cannabinoids characterize the specific THC and CBD content of extracts and raw plant material. Our studies also demonstrated that administration of plant background material also exerts effects on reconsolidation of fear memory. This suggests THC and CBD are not the only fear memory modulating molecules contained within the plant. Although CBD and THC (when co-administered with other compounds) may modulate fear

learning, future research should be cautious to isolate and quantify the THC, CBD, and other compounds that may be contributing to the measured effects on behavior.

Karniol et al. examined the effects of CBD alone as early as 1974 [353], and found that oral CBD reduced THC-induced anxiety. The same group later demonstrated that CBD could block the effects of THC in normal, healthy participants [354]. More recently, synthetic cannabinoid Nabilone has been demonstrated to effectively reduce the frequency of nightmares in sufferers of PTSD [355,356]. Interestingly, oral THC has been shown to reduce amygdala activation in response to images of threat-related faces [357]; however, this contradicts earlier findings showing THC alone may be anxiogenic [353]. As a result, it is clear that our current understanding of the role of the endocannabinoid system in PTSD, anxiety, and learned fear is far from complete. Our findings seem to suggest that – at least at the doses used – THC by itself is not sufficient to modulate fear learning, but needs to be co-administered either with CBD, or with other compounds, to be effective. Since evidence suggests that PTSD is characterized by upregulation of CB1 receptors and reduced availability of anandamide, it may be the case that CB1 receptor activation by pharmacologic agents might serve to compensate for this receptor upregulation and restore the normal ‘tone’ of the endocannabinoid system over time. However, effects of acute versus chronic activation by pharmacological agents may not necessarily be the same. Future research should therefore aim to clarify the acute effects of CB1 agonists versus chronic use, as well as examining differences in effects among normal, healthy subjects versus those at risk of having altered endocannabinoid activity (e.g. sufferers of PTSD).

Conclusion

Both past and recent data cumulatively support the notion that CBD may impart anxiolytic action [358]. In addition, the action of THC in animal and human models of fear-

learning warrants further clinical research to elucidate whether cannabinoids may serve as a novel intervention(s) for fear-related disorders such as PTSD. It will be important for these trials to identify the other potentially non-psychoactive components of *Cannabis spp.* to determine if and how they mediate fear learning. It goes without saying that ongoing and future studies aimed at unraveling the mechanism(s) of action of THC and CBD are critically important to fully exploit the therapeutic potential of these pharmacologic targets. Finally, it would be interesting to better understand if and how various pharmacologically active components of *Cannabis spp.* may interact to modulate the signaling of relevant brain circuits (e.g. pathways linking cortex and the amygdala) to affect encoding, consolidation, and reconsolidation of learned fear.

Author Contribution Statement

AM wrote the paper, analyzed data, performed experiments, and contributed to study design. JJ and CC performed experiments. ZM and PK revised the paper and contributed to study design. TD produced plant extracts and pure compounds used in the experiments.

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Conflicts of Interest Statement

The authors declare no conflicts of interest of a financial or personal nature.

Chapter 4: Extract and active principle of the neotropical vine *Souroubea sympetala* Gilg. block fear-memory reconsolidation

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4.1 Abstract

Background: *Souroubea sympetala* Gilg. is a neotropical vine native to Central America, investigated as part of a targeted study of the plant family Marcgraviaceae. Our previous research showed that extract of *S. sympetala* leaf and small branch extract had anxiolytic effects in animals and acts as an agonist for the GABA_A receptor at the Benzodiazepine binding site. To date, the potential effects of *S. sympetala* and its constituents on reconsolidation have not been assessed. Reconsolidation, the process by which formed memories are rendered labile and susceptible to change, may offer a window of opportunity for pharmacological manipulation of learned fear. Here, we assessed the effects of *S. sympetala* crude extract and isolated phytochemicals (orally administered) on the reconsolidation of conditioned fear. In addition, we explored whether Betulin (BE), a closely related molecule to betulinic acid (BA, an active principle component of *S. sympetala*) has effects on reconsolidation of learned-fear and whether BE may synergize with BA to enhance attenuation of learned-fear.

Method: Male Sprague-Dawley rats received six 1.0 mA continuous foot-shocks (contextual training). 24h later, rats were re-exposed to the context (but in the absence of foot-shocks). Immediately following memory retrieval (recall) rats received oral administration of *S. sympetala* extract at various doses (8-75 mg/kg) or Diazepam (1 mg/kg). In separate experiments we compared the effects of BA (2 mg/kg), BE (2 mg/kg), and BA + BE (2 mg/kg BA + 2 mg/kg BE). The freezing response was assessed either 24 h later (day 3) or 5 d later (day 7). Effects of phytochemicals on fear-expression were also explored using the elevated plus maze paradigm.

Results: *S. sympetala* leaf extract significantly attenuated the reconsolidation of contextual fear at the 25 mg/kg and 75 mg/kg doses but not at the 8 mg/kg dose. Furthermore, BA + BE, but not BA or BE alone, attenuated the reconsolidation of learned fear and exerted an anxiolytic-like effect on fear expression.

Keywords: Reconsolidation, fear memory, *Souroubea sympetala*, *sin susto*, betulinic acid, betulin

4.2 Introduction

Fear-based disorders such as anxiety and post-traumatic stress disorder (PTSD) make up a significant portion of all diagnosed psychiatric conditions. A recent large-scale study found that among college students worldwide, major depression and anxious disorders are the most prevalent – with generalized anxiety and panic disorders constituting a combined 23.6% of all psychiatric conditions (with a 12-month prevalence rate for all mental conditions among the sampled population of over 30%; [359]). Despite that they are relatively commonplace, existing treatments for fear-based disorders are extremely limited. Pharmacological interventions for generalized anxiety are largely limited to Benzodiazepines, while serotonergic-noradrenergic reuptake inhibitors (SNRIs) are also in use for panic disorder.

Moreover, although PTSD affects a large percentage of individuals following exposure to trauma [1], there are no specific pharmacological treatments targeting PTSD. Rather, the current treatments instead aim to treat the co-morbid symptoms of anxiety and/or depression [4]. It is also of interest to note that more than half of individuals with fear-based disorders seek alternative medicine (approaches to the treatment of illness which fall outside of the scope of standard medical care; [287]). However, only 20% of those in the same study sought the guidance of a health care practitioner (i.e. a large percentage self-medicate; [287]). As a result, it is clear that new pharmacotherapies are needed to specifically address PTSD (as well as other fear-based disorders).

In an attempt to discover alternate treatment strategies, efforts have been under way to explore plant-based remedies. Indeed, plant extracts have been shown to exert anxiolytic-like effects [285,286] and in particular we found that a Canadian genotype *Rhodiola rosea* [15] may be useful for the development of novel therapeutic interventions for human disorders based in

fear-memory. As part of our collaborative research to find new anxiolytics, we targeted the poorly studied family of neotropical vines the Marcgraviaceae in Central America. In pre-clinical tests modelling anxiety-like symptoms in animals, extract of one of these vines, *Souroubea sympetala* Gilg. (Marcgraviaceae; *sin susto*) has shown promise as an effective anxiolytic. Betulinic acid (BA; the most prominent triterpenoid constituent of the plant) is one of the active molecules responsible for this effect (see figure 1; [19,20]).

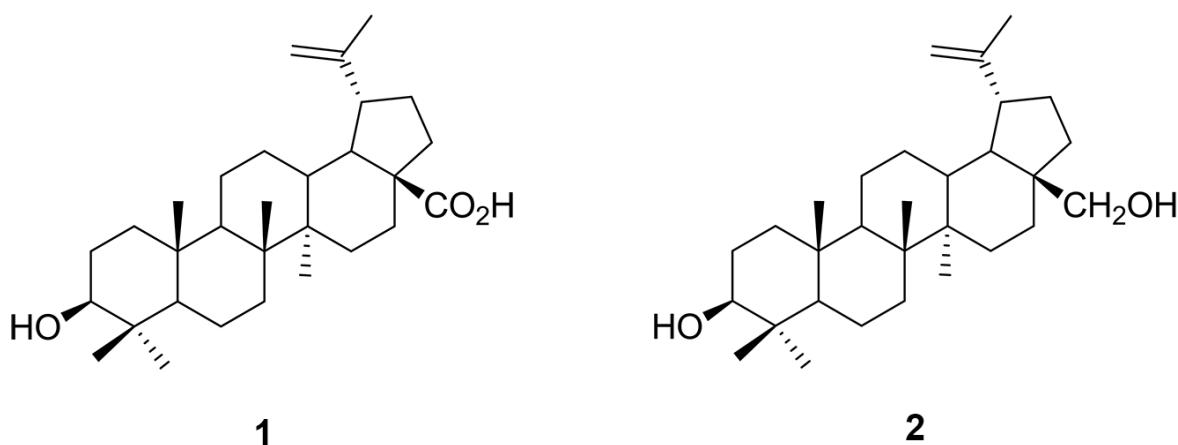


Figure 1. Chemical structure of (1) betulinic acid and (2) betulin.

Both *S. sympetala* leaf extract and betulinic acid reduced stressor-induced cortisol secretion in rainbow trout and canines [20,360,361]. Evidence also suggests *S. sympetala* and BA may exert their effects on fear-learning and expression by agonizing the Benzodiazepine (BZD) binding site of the GABA_A receptor [362]. This is in line with evidence suggesting that anxiolytic botanicals frequently possess agonistic qualities for the GABAergic system [21].

In contrast to traditional GABA_A BZD-receptor agonists such as diazepam, our previous research with *S. sympetala* leaf extract indicated that there were no withdrawal effects in rodents [363]. Thus, the plant might effectively modulate learned fear and/or anxiety with limited negative side effects typically associated with GABA_A BZD agonists. *S. sympetala* extracts and its combinations with another BA producing plant have been safe in 30 day feeding trials at elevated doses with dogs [360,364].

In addition to its experimentally demonstrated activity as an anxiolytic, *S. sympetala* may also possess memory-altering qualities that prove useful for the treatment of other human mood disorders. Reconsolidation blockade, a technique for pharmacologically altering formed memories, may provide an avenue for developing novel treatment approaches for alleviating traumatic memories which are resistant to extinction. Evidence suggests that following retrieval, memories may return to a labile state in which they are susceptible to manipulation [55]. During this period (the reconsolidation window), pharmacological disruption of memory re-stabilization can potentially diminish conditioned fear responses (and thus their expression). This technique has been used in preclinical research to help identify potential therapeutic targets [13,84,297,365]. Some, such as the β -adrenergic antagonist propranolol, xenon (which blocks NMDA receptors), and gastrin-releasing peptide (homologue of the amphibian peptide bombesin) show promise of attenuating learned-fear when administered during the reconsolidation window in rodents [78,297,365,366]. Although it has only been sparsely tested in humans, there is some evidence from translational studies to suggest that the blockade of reconsolidation may have practical applications. Propranolol, for example, reduces physiologic responses to traumatic imagery in sufferers of PTSD when administered post-recall [86]. However, findings are mixed, and subsequent follow-up studies have failed to replicate this

effect [64]. Thus, new targeted approaches are required to help identify viable treatments to attenuate fear memory reconsolidation in humans.

Since some phytochemicals have been shown to affect reconsolidation and expression of learned fear [15,85,338,366], phytotherapy may offer a novel approach to reconsolidation blockade as a treatment paradigm for fear-based disorders. Phytochemicals that affect consolidation and/or extinction of fear memory, as well as compounds acting through GABA that attenuate fear-expression, may have memory-altering properties that generalize to other processes such as reconsolidation (although reconsolidation and extinction are thought to be distinct processes at the cellular and molecular level; [57]). Research has demonstrated that reconsolidation of learned-fear can be altered by GABAergic neurotransmission in rodents following memory recall [296]. As possible GABA_A BZD-receptor agonists, *S. sympetala* extract and its active components might therefore be of use to the development of novel treatment approaches for fear-based disorders by affecting fear-memory.

Here we aimed to determine whether *S. sympetala* extract and its primary active component BA could block the reconsolidation of conditioned fear. In addition to BA and *S. sympetala* leaf and small branch extract (SIN), we also explored the effects of a more abundant but closely related molecule – Betulin (BE) – on reconsolidation of conditioned fear, and also a combination of the two (BA+BE) to determine whether the effects of BA are altered by the addition of BE. Previous work has shown that amyrins of similar chemical structure synergize with BA to enhance its effects [363]. We hypothesized that a similar effect may be observed with BE, given its very similar structure to BA (BA is structurally identical to BE, except for the exchange of the alcohol group with a carboxylic acid group). We also hypothesized that the leaf and small branch extract and BA would significantly attenuate reconsolidation of conditioned

fear in a dose-dependent manner, and that the effects of BA would be amplified by co-administration with BE.

Finally, in order to test for more general effects of isolated compounds from SIN on fear-expression and anxiety-like behavior, we explored the effects of isolated phytochemicals BE, BA, and a combination of the two using the elevated plus maze paradigm.

4.3 Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratories International, Inc.; 180-200g on arrival) were doubly housed and maintained on a 12-h light/dark cycle (lights on at 07:00-h). Temperature was maintained at 23° C, and relative humidity at 37%. Throughout the duration of the study, animals had free access to food and water. All experiments were conducted in accordance with the guidelines established by the Canadian Council on Animal Care and approved by the University of Ottawa Animal Care Committee.

Drugs and injections

Animals were habituated to administration of oral sweetened condensed milk (vehicle) one week prior to the beginning of the experiment. Since evidence suggests *S. sympetala* extract may act as a GABA_A BZD receptor agonist, Diazepam (Sandoz, Canada) was used as a positive control. Rats were randomly assigned to one of the treatment groups used throughout the experiments. Drug groups among all experiments consisted of 1) *S. Sympetala* leaf and small branch extract (SIN; 8-75 mg/kg), 2) Diazepam (Positive control; 1 mg/kg), 3) BA (2 mg/kg), 4) BE (2 mg/kg), 5) BA + BE (2 mg/kg BA + 2 mg/kg BE), and 6) vehicle.

Plant extract, isolated phytochemicals, and analysis

Souroubea sympetala Gilg. leaves and small branches were originally collected in Tortuguero, Costa Rica and propagated at a Universidad Nacional field in Sarapiquí. Samples were dried overnight in a commercial plant drier at 35° C. A Voucher specimen was identified by two researchers (MOR and PS) and deposited in the JVR herbarium, Universidad Nacional Costa Rica (voucher # 13231, in supplementary data). *S. sympetala* is an accepted name on the plant.list.org and tropicos.org. Plant material of *S. sympetala* was ground with a Wiley Mill (2mm mesh size). Samples were incubated with shaking in 1:20 (weight: volume) ethyl acetate (EtOAc) for 12–15 h at room temperature. The solvent was filtered (Whatman no. 1) and the filter cake re-extracted twice with half as much EtOAc (1:10 and 1:5). The total solvent from the three extractions were combined for an exhaustive extraction. The solvent was removed via rotary evaporation with a Yamato Rotary Evaporator RE50 (Yamato Scientific, Japan) at 40° C, lyophilized (Super Modulyo, Thermo Electron, USA) and stored in opaque glass vials at 4° C. Analysis of the plant was undertaken using a validated HPLC-MS method [367] which shows chromatograms for the analysis. The extract contained a mean (SE) of betulinic acid of 6.8 mg (0.2). Isolation, purification and spectroscopic identification of betulinic acid and betulin have been described previously [20] and was found to be greater than 95% pure by HPLC-MS.

Contextual fear conditioning

The conditioning chambers (Coulbourn Instruments) measured 31 cm x 25 cm x 30 cm. The front and back walls were made of clear acrylic, and the two sidewalls and top made of stainless steel. The floor comprised of 16 stainless steel bars (4 mm diameter spaced 1.4 cm apart) connected to Coulbourn precision regulated animal shockers, which delivered scrambled foot-shock (1.0 mA). Animals (N=7-10/group) were randomly distributed into treatment groups.

Subjects that failed to achieve a minimum baseline freezing level of 40% during recall (assessed on Day 2) were removed from the analyses.

Experimental Procedure

Experiment 1: Effects of plant extracts on reconsolidation blockade, short-term

Animals were exposed to 6 consecutive 1-s- foot-shocks over the course of 11 min. Contextual conditioning was used (pairing of foot-shock with the conditioning chamber). 24 h later animals were re-exposed to the context in which they received the foot-shock (conditioning chamber) for 5 min., and freezing (total time spent in complete immobility) was measured (Day 2; recall). Cage placement and assignment to drug treatment groups was randomized/counterbalanced.

Immediately after the 5 min recall session on Day 2, animals were administered drugs in one of five treatment groups: 8 mg/kg plant extract (low dose), 25 mg/kg plant extract (medium dose), 75 mg/kg plant extract (high dose), vehicle, and 1mg/kg diazepam (positive control). 24 h later (Day 3) animals were re-exposed to the conditioning chamber and freezing was measured over the course of 10 min in blocks of 0-5 and 6-10 min. The timeline of procedures for experiment 1 is illustrated in Figure 2.

Experiment 2: Long-term effects on reconsolidation blockade.

The same training procedures as Experiment 1 were utilized to test for long-term effects of plant compounds on fear memory reconsolidation blockade. Immediately after the 5 min recall session on Day 2, animals were administered drugs in one of five treatment groups: 8 mg/kg plant extract (low dose), 25 mg/kg plant extract (medium dose), 75 mg/kg plant extract (high dose), vehicle, and 1mg/kg diazepam (positive control). 5 d later (Day 7) animals were re-exposed to the conditioning chamber and freezing was measured over the course of 10 min. in

blocks of 0-5 and 6-10 min. The timeline of procedures for experiment 2 is illustrated in Figure 3.

Experiment 3: Short-term effects of plant extracts on reconsolidation blockade

The same training procedures as Experiment 1 were utilized to test for short-term effects of isolated plant compounds on fear memory reconsolidation blockade. Immediately after the 5 min recall session on Day 2, animals were administered drugs in one of four treatment groups: 2 mg/kg BE, 2 mg/kg BA, 2 mg/kg BE + 2 mg/kg BA, or Vehicle. 24 h later (Day 3) animals were re-exposed to the conditioning chamber and freezing was measured over the course of 10 min. in blocks of 0-5 and 6-10 min. The timeline of procedures for experiment 3 is illustrated in Figure 4.

Experiment 4: Long-term effects of plant extracts on reconsolidation blockade

The same training procedures as Experiment 1 were utilized to test for long-term effects of plant compounds on fear memory reconsolidation blockade. Immediately after the 5 min recall session on Day 2, animals were administered drugs in one of four treatment groups: 2 mg/kg BE, 2 mg/kg BA, 2 mg/kg BE + 2 mg/kg BA, or Vehicle. 5 d later (Day 7) animals were re-exposed to the conditioning chamber and freezing was measured over the course of 10 min. in blocks of 0-5 and 6-10 min. The timeline of procedures for experiment 4 is illustrated in Figure 5.

Experiment 5: Attenuation of the fear response requires reactivation of the fearful memory trace

In order to determine whether the effects were dependent upon memory trace reactivation, an experiment was performed using identical procedures to Experiment 1 and Experiment 3. In this experiment, however, animals were not re-exposed to the fearful stimulus on Day 2. The timeline of procedures for experiment 5 is illustrated in Figure 6.

Experiment 6: Elevated Plus Maze

The elevated plus maze (EPM), a paradigm used to characterize anxiety-like behavior, was conducted in accordance with methods as described by Cayer et al. [18]. The maze consisted of two open arms (50 x 10 cm) and two perpendicular arms enclosed by high walls (40 cm tall). The maze was placed 50 cm above the ground. Percentage of time spent in the open arms and number of unprotected head dips were measured.

Statistical Analyses

All statistical analyses were conducted using IBM Statistics Package for the Social Sciences® (SPSS) 20. Data was analyzed using mixed-measures ANOVA, in which drug treatment was the between groups variable and time was the within groups variable. Greenhouse-Geisser correction was applied where the assumption of sphericity is violated. Follow-up comparisons of significant main effects and interaction effects was conducted using Bonferroni corrected t-tests, or corrected post-hoc tests where the assumption of homogeneity of variance was violated.

4.4 Results

Experiment 1

The mixed-measures ANOVA revealed a significant main effect of Treatment on levels of freezing, $F(4,41) = 5.523, p < 0.01$. Levene's test revealed the assumption of equality of error variances was violated at 6-10 minutes, $p < 0.05$. There was also a significant time by group interaction effect, $F(4,41) = 4.485, p < 0.05$.

Post-hoc analyses further revealed that animals who received medium and high doses of plant extract immediately following memory recall on Day 2 displayed significantly less freezing than vehicle treated animals on Day 3 at 0-5 min ($p < 0.05$; see figure 2). Animals that received

high-dose plant extract or diazepam immediately following recall on Day 2, also displayed significantly less freezing than vehicle treated animals on day 3 at 6-10 min ($p < 0.05$).

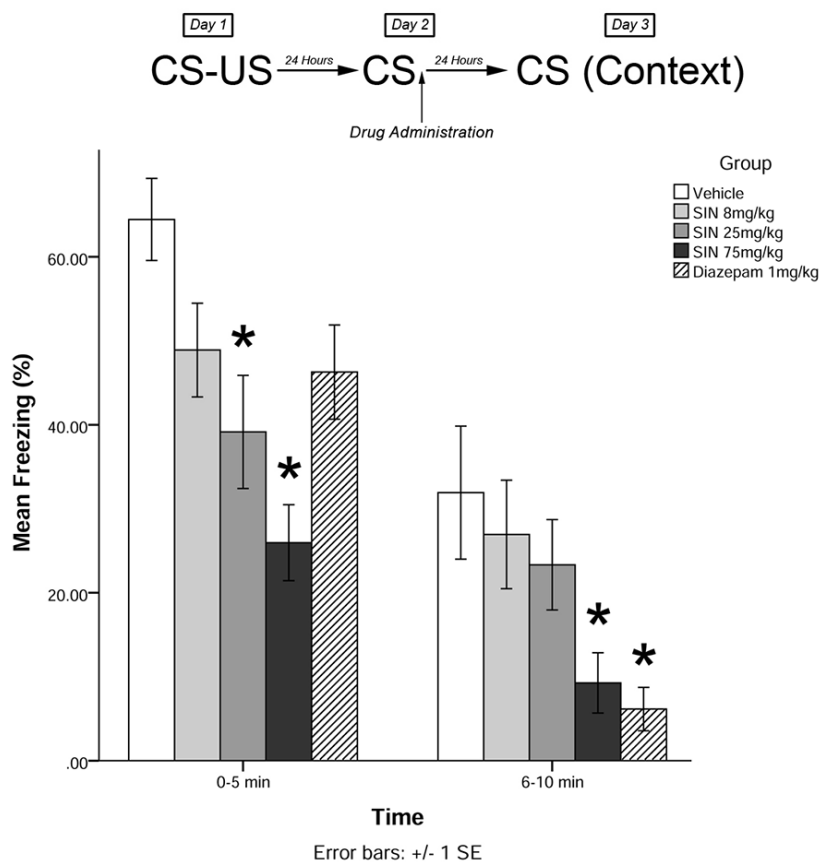


Figure 2. *S. sympetala* extracts attenuated the freezing response. 25mg/kg SIN (approximate BA content 0.37 $\mu\text{mol/kg}$) and 75mg/kg SIN (approximate BA content 1.12 $\mu\text{mol/kg}$), but not 8mg/kg SIN (approximate BA content 0.12 $\mu\text{mol/kg}$), attenuated freezing on Day 3 when administered immediately post-recall.

Experiment 2

The mixed-measures ANOVA revealed no significant main effects or interaction effects, $F(4,41) = 0.291, p > 0.05$ (see figure 3).

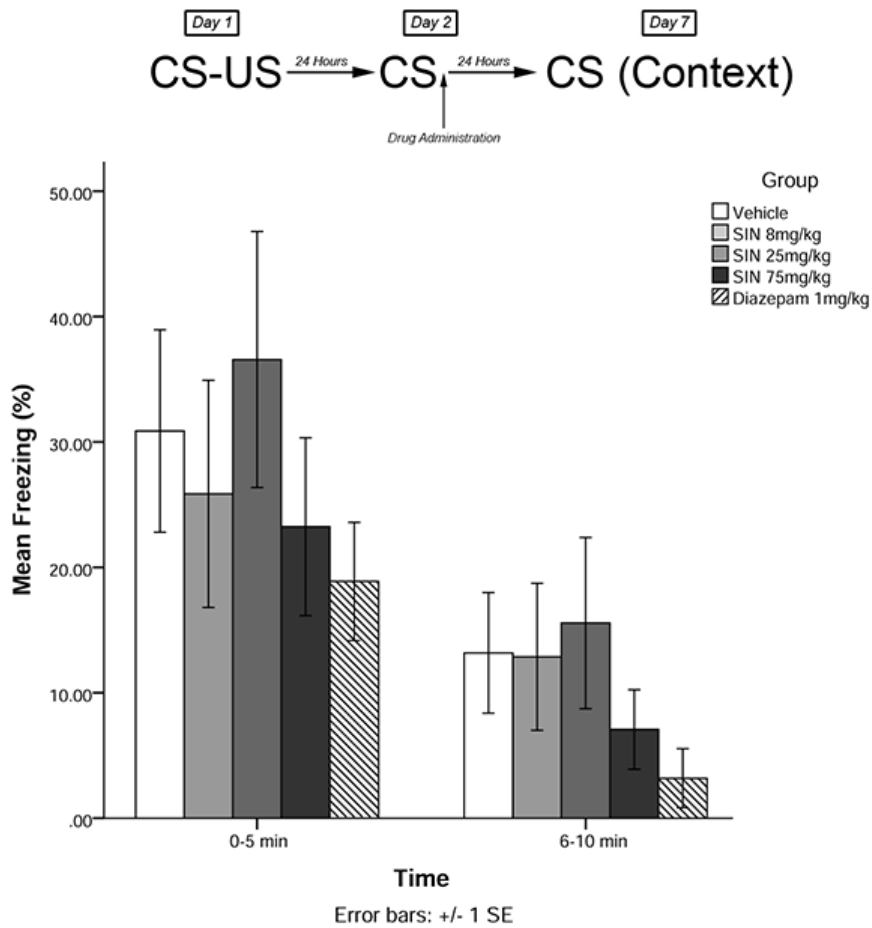


Figure 3. *S. sympetala* extract administered immediately following recall did not attenuate freezing on Day 7.

Experiment 3

The mixed-measures ANOVA revealed a significant time by group interaction effect, $F(4,40) = 5.227, p < 0.01$, and a significant main effect of group, $F(4,40) = 3.505, p < 0.05$. Levene's test revealed the assumption of equality of error variances was violated, $F(4,40) = 4.549, p < 0.05$. Games-howell corrected post-hoc analysis revealed that rodents that had received BA 2 mg/kg + BE 2 mg/kg exhibited significantly less freezing than vehicle treated animals ($p < 0.05$) at the 6-10 min interval (see figure 4). No other groups significantly differed from vehicle at either the 0-5 or 5-10 min interval (all $p > 0.05$).

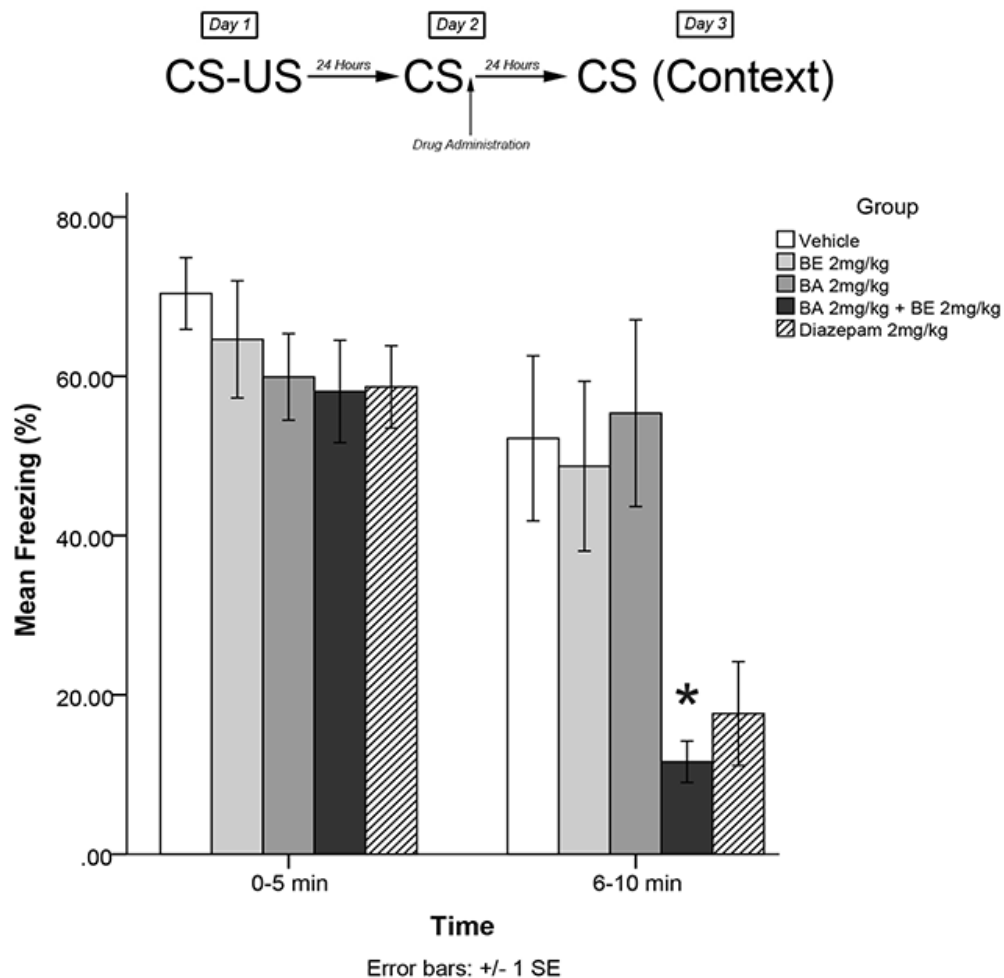


Figure 4. Isolated phytochemicals Betulinic Acid (BA) 2mg/kg (4.38 μ mol/kg) and Betulin (BE) 2mg/kg (4.52 μ mol/kg) attenuated reconsolidation of conditioned fear when co-administered, but not alone. The combined dose of BA + BE more closely resembles *S. Sympetala* extract.

Experiment 4

The mixed-measures ANOVA revealed no significant main effects or interaction effects, $F(4,40) = 0.952, p > 0.05$ (see figure 5).

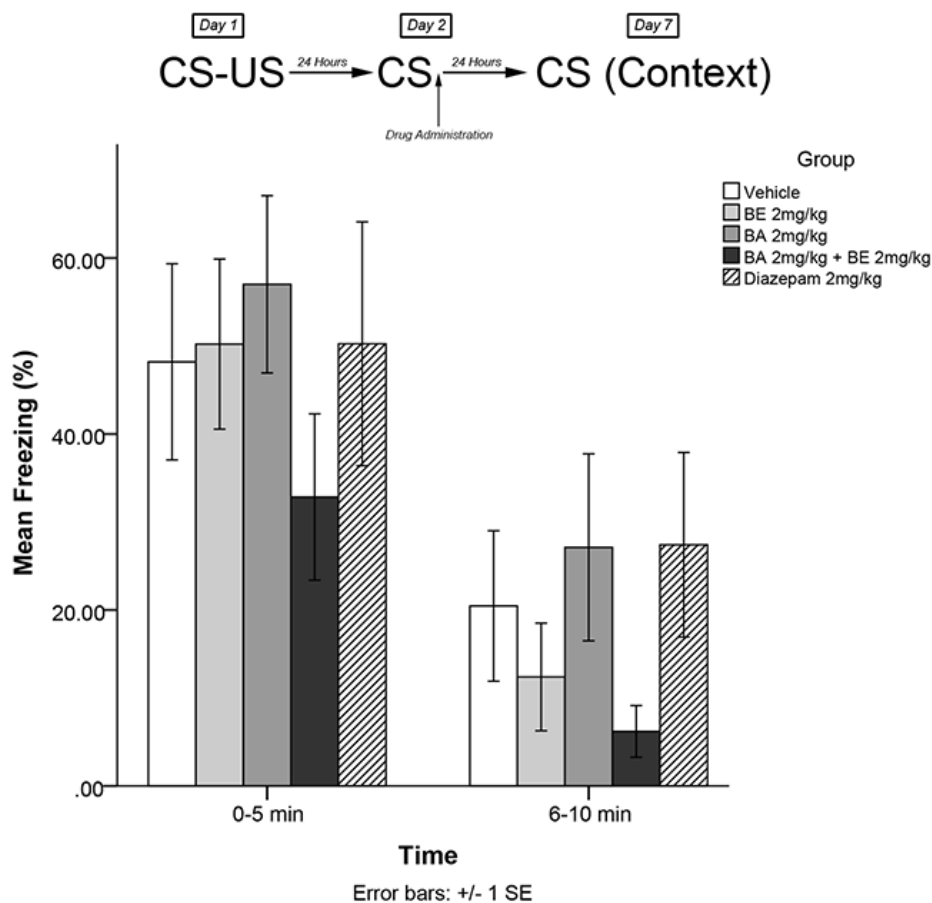


Figure 5. Isolated phytochemicals 2mg/kg (4.52 $\mu\text{mol/kg}$) BE and 2mg/kg (4.38 $\mu\text{mol/kg}$) BA did not attenuate freezing on Day 7.

Experiment 5

The mixed-measures ANOVA revealed no significant main effects or interaction effects, $F(2,27) = 0.062, p > 0.05$ (see figure 6).

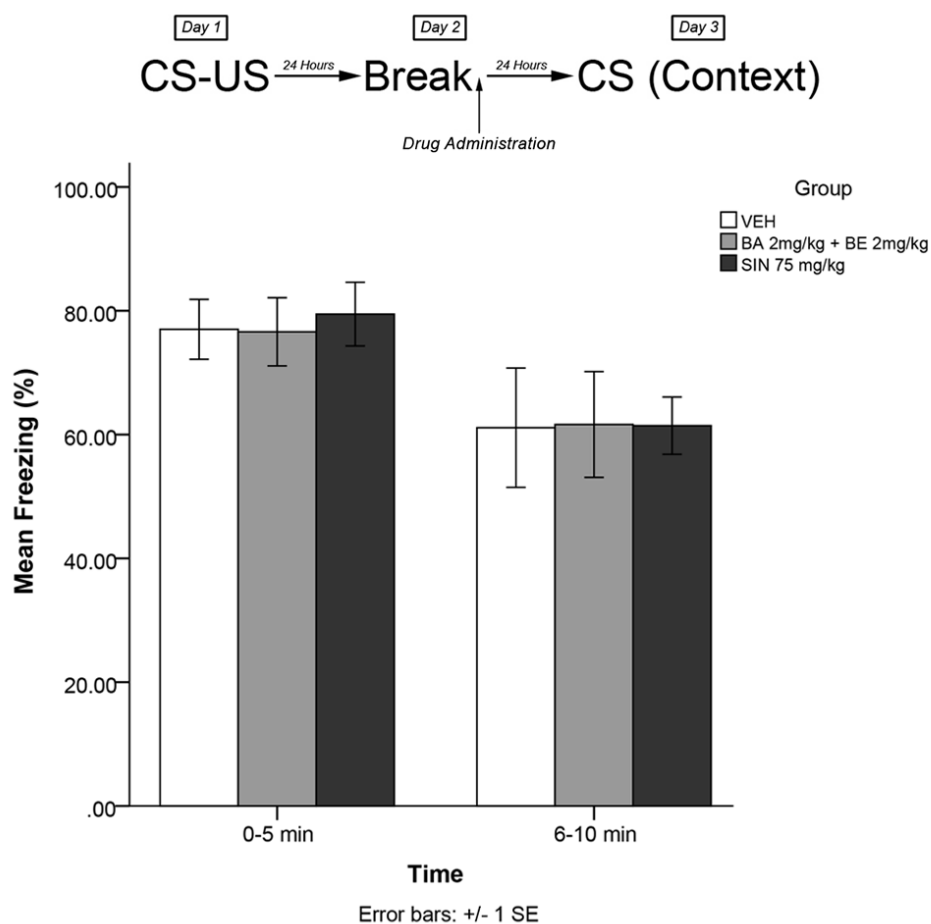


Figure 6. SIN 75mg/kg (approximate BA content 1.12 $\mu\text{mol/kg}$) and a combined dose of 2mg/kg (4.52 $\mu\text{mol/kg}$) BE + 2mg/kg (4.38 $\mu\text{mol/kg}$) BA did not produce attenuation of the learned fear response when administered in the absence of re-exposure to the fearful stimulus.

Experiment 6

A one-way ANOVA revealed a significant main effect of treatment group in the EPM paradigm on percentage of time spent in the open arms of the maze, $F(4,40) = 3.297, p < 0.05$. Levene's test indicated the assumption of equality of error variances was not violated, $p > 0.05$. Follow-up analyses indicated that rats treated with Diazepam ($p < 0.05$) and a combination of BA + BE (2 mg/kg each; $p < 0.05$) spent significantly more time in the open arms of the EPM than vehicle-treated animals (see figure 7). No other treatment groups significantly differed from vehicle.

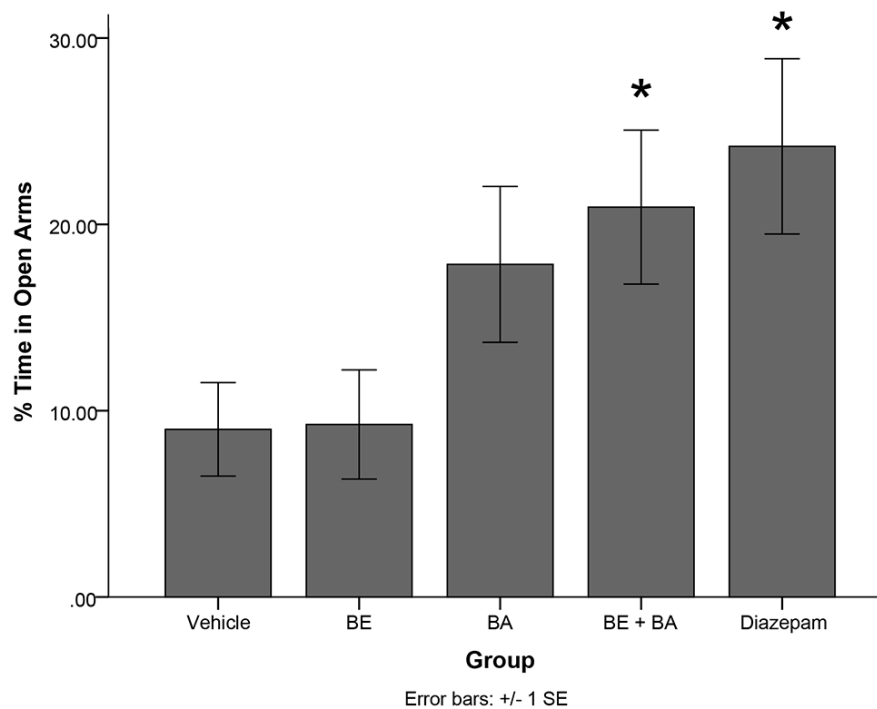


Figure 7. Rodents treated with 2mg/kg (4.52 μ mol/kg) BE + 2mg/kg (4.38 μ mol/kg) BA, as well as 1mg/kg (3.51 μ mol/kg) Diazepam, exhibited significantly more time in the open arms of the maze than rodents treated with vehicle.

One-way ANOVA also revealed a significant treatment effect on the number of unprotected head-dips, $F(4,40) = 5.084$, $p < 0.01$. Levene's test indicated the assumption of equality of error variances was violated, $p > 0.05$. Follow-up analyses indicated that rats treated with BA + BE ($p < 0.05$) and Diazepam ($p < 0.05$) had significantly more unprotected head-dips than vehicle-treated animals (see Figure 8). No other groups significantly differed from vehicle.

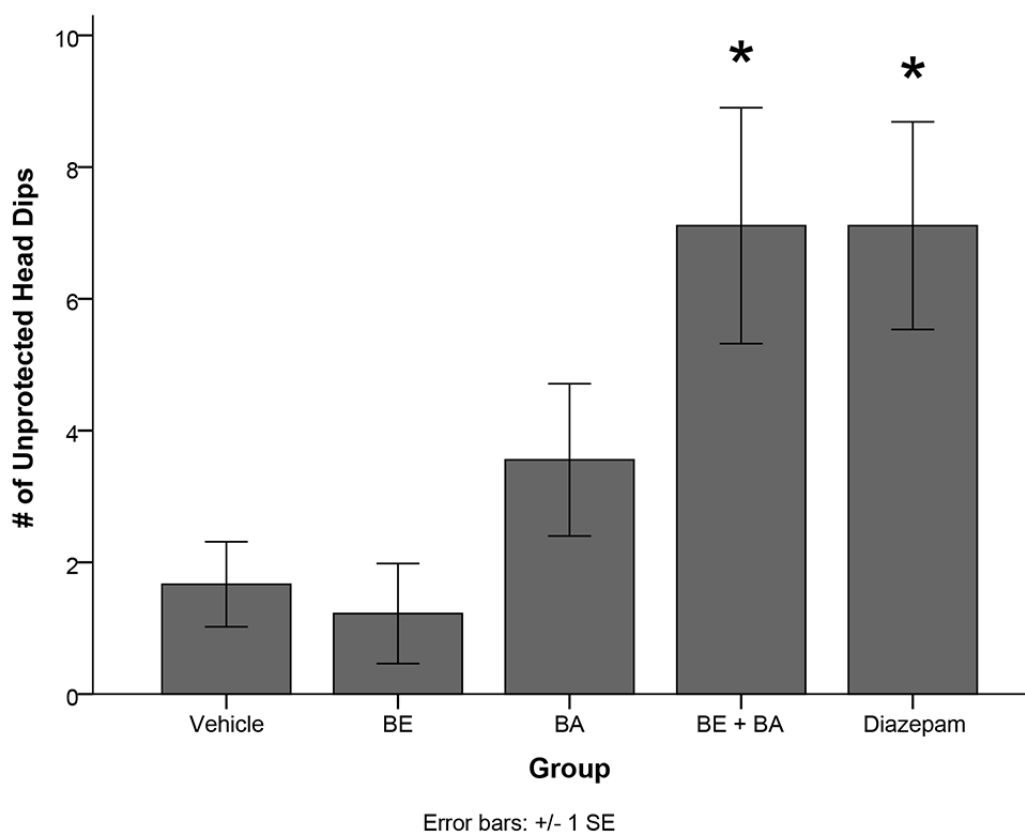


Figure 8. Rodents treated with 2mg/kg (4.52 μ mol/kg) BE + 2mg/kg (4.38 μ mol/kg) BA, as well as 1mg/kg (3.51 μ mol/kg) Diazepam, exhibited significantly more unprotected head dips than rodents treated with vehicle.

4.5 Discussion

Our findings suggest that *S. sympetala* leaf and small branch extract is effective in modulating reconsolidation of learned fear and fear expression. In Experiment 1, 25 mg/kg SIN and 75 mg/kg SIN (but not 8 mg/kg SIN) effectively attenuated the learned fear response as measured on Day 3 when administered immediately post-recall. This suggests that *S. sympetala* plant extract may have therapeutic potential for modulating reconsolidation of learned fear. However, there was no significant effect of SIN at day 7 (long-term testing), suggesting that the effects of the extract on learned fear may have been impermanent or that the learned fear responses underwent spontaneous recovery.

Our findings also demonstrated significant effects of 2 mg/kg BA + 2 mg/kg BE both on reconsolidation of learned fear responses and on fear expression in the EPM paradigm. However, similarly to what we observed with SIN, BA + BE was effective at attenuating reconsolidation of learned fear in the short-term - but not the long-term. Although it is possible the effects of SIN and BA + BE are not permanent, this would be inconsistent with previous work suggesting that memories targeted by reconsolidation blockade are not prone to reinstatement [55,78,296,365]. It is possible that a floor effect is responsible for this observation in both cases, since mean freezing levels at day 7 in both long-term experiments were lower overall for all groups in both of our long-term experiments. A reduction in freezing levels with the CER paradigm after 7 days is unfortunately normal; future work should therefore aim to determine whether the effects of SIN and its active principle components persist in the long term using measures that are less sensitive to drift over time and floor effects (e.g. fear-potentiated startle, which produces robust measurable effects even after longer periods) and. It would also be advantageous for future studies to include testing at a longer interval (i.e. Day 10 or 15).

In addition to highlighting that SIN and BA + BE may effectively modulate learned fear and fear expression, our findings also suggest that BE may indeed synergize the effects of BA – since both BA and BE alone were ineffective at attenuating the learned fear response or fear expression, but a combined dose yielded significant effects on both reconsolidation of learned fear (in Experiments 3) and fear-expression (in Experiment 6). The results of these experiments are in partial agreement with previous findings; for example, previously our group showed that BA exerted an anxiolytic effect when administered alone [20].

However, our prior work suggests that while BA may be an active principle of *S. sympetala* extracts, it is likely not the only active component. Thus, our combined BA + BE group may be the closest representation of the extract and the original leaf/small branch decoction *Sin Susto*, which contains both BA and a host of likely bioactive molecules.

Our findings here highlight the potential therapeutic benefit of *S. Sympetala*, betulinic acid (BA), and betulin (BE, as a possible synergist) for mediation of learned-fear and fear expression. However, further research is necessary to explore the nature of triterpenoid synergism. Future studies may benefit from a more extensive dose-response characterization of BA effects, to determine more precisely the effective dose-range of BA. In addition, although we have observed robust effects on behavior and memory, our experiments did not probe the central mechanisms mediating the effects of these compounds. Future studies should therefore aim to elucidate the central mechanisms by which *S. Sympetala* extracts might exert their effects. Since evidence suggests BA and *S. Sympetala* extract may act as GABA_A BZD-receptor agonists [362], it would be an important future step to determine whether the effects of orally administered SIN and/or BA + BE are mediated centrally and whether these effects can be blocked by a selective GABA_A BZD-binding site antagonist (such as flumazenil) as well as with site-specific

microinjection of those antagonists at brain regions where GABAergic transmission is known to play a role in learned fear (e.g. the basolateral complex of the amygdala; [148]).

Overall, our data suggests that *S. Sympetala* extracts and isolated phytochemicals may prove useful as pharmacological targets for the development of new and more effective treatments for human fear-based disorders such as PTSD. Furthermore, in contrast to the current mainline treatments for anxiety, some experiments suggest that BA produced no changes in weight-gain or locomotor activity, as well as no withdrawal effects on food intake, fecal output, and a variety of light-phase and dark-phase behaviors including scratching, grooming, resting, and exploring [363] while maintaining fulsome therapeutic effects on fear expression. Whether *S. sympetala* extracts produce withdrawal effects in humans after chronic administration, however, has yet to be explored. It will be a necessary and important step for future studies to determine whether – as suggested by its use in traditional native medicine - the effects of *S. sympetala* extracts translate well to studies with human participants.

Funding

The experimental protocol for this study was approved by the University of Ottawa Animal Protocol Review Committee and conforms to the guidelines of the Canadian Council on Animal Care. This research was supported by a discovery grant that was awarded to Z. Merali (application number 05388) from the Natural Sciences and Engineering Research Council of Canada (NSERC) and a Doctoral Research Award that was awarded to A. Murkar (application number 336752) from the Canadian Institutes of Health Research (CIHR).

Author Contribution Statement

AM wrote the paper, analyzed data, performed experiments, and contributed to study design. JJ and CC performed experiments. ZM revised the paper and contributed to study design.

TD and JTA produced plant extracts and pure compounds used in the experiments and contributed to study design.

Funding Disclosures Statement

This research was supported by grant support from the Canadian Institutes of Health Research (CIHR) that was awarded to Z. Merali and a Doctoral Research Award that was awarded to A. Murkar (application number 336752) from CIHR.

Conflicts of Interest Statement

AM, JJ, and CC declare no conflicts of interest. ZM, TD, PS and JA have direct involvement in Souroubea Botanicals Inc., which has brought the research to market in animal care field.

Chapter 5: General Discussion

5.1 Summary of Findings

The present findings suggest that these three naturally occurring products sourced from plants and animals may be significant mediators of the reconsolidation of fear-memory in rodents. First, we hypothesized that gastrin-releasing peptide would attenuate the reconsolidation of conditioned fear memory, and secondly that the effect would be reversed by co-administration of a GABA_A BZD antagonist Flumazenil. Our findings confirmed both of these hypotheses, suggesting (1) that GRP may be an effective mediator of learned fear, and (2) that the effects of GRP are likely mediated via a GABAergic mechanism (as Shumyatsky et al. had predicted in 2002) [213]. Since GABAergic transmission in the amygdala is highly involved in processing and expression of learned fear [113,281,368–372] and both traditional anti-anxiety medications and NHPs believed to exert anxiolysis tend to target this system [289,373–376], it is not surprising that the effects of GRP on learned fear should be similarly be mediated by GABA. However, our findings also suggested that the window of opportunity for pharmacologically targeting reconsolidation with GRP is quite short. In our study, GRP administered immediately post-retrieval yielded an attenuation of expression of conditioned fear in two separate experiments; however, when administered 30 minutes or longer following recall of the fearful context, no effect was observed. This contrasts with previous work, which has only explored the reconsolidation window with drugs administered immediately or several hours later [55].

There are two possibilities: firstly, the window of opportunity for pharmacological manipulation of reconsolidation may be much shorter than previously thought. Indeed, our findings would suggest that in order for medications to effectively yield reductions in subsequent fear expression, compounds must be administered without delay. While previous work has

assessed effects of drugs administered either immediately 4-6 h later [13,55], our findings suggest that the window for effective drug application is likely much shorter than that. Since there are no experiments as of yet examining the reconsolidation window at shorter time spans with other pharmaceuticals, it is possible that this is a feature of the time-frame of the reconsolidation window rather than GRP specifically (and thus might apply to other substances). Secondly, it may also be the case that because GRP is thought to bind first at a peripheral site and transmit signals centrally via the brain-gut axis and/or vagus nerve [307,312,313] the time-course of GRP binding peripherally may cause a delay before central effects appear. Therefore, the need to generate effects at central sites relevant to learned fear very quickly might limit the therapeutic potential of compounds such as GRP which very likely have a longer latency to peak activity in the brain. Most likely, it is some combination of these two factors that is responsible, although the window of opportunity for reconsolidation blockade using other compounds might be similarly short. As a result, those compounds with the greatest therapeutic potential for reconsolidation blockade may be those that exhibit the shortest latency to peak activity in the brain (e.g. Xenon, which – when inhaled – disperses and binds in the brain rapidly in humans) [78].

Our third and fourth hypotheses predicted that CBD and THC would similarly attenuate the reconsolidation of learned fear, leading to reduced expression of the freezing response at follow-up trials. Hypothesis 3 was partially supported by our findings. Interestingly, our findings were consistent with previous work suggesting that CBD may effectively block reconsolidation [85,337,338]. However, our findings also suggested that THC (the primary psychoactive component of the plant) had no such effects when administered alone. THC has generally been shown to exert anxiogenesis [353,377,378], while CBD has the opposite effect [379–385]. Our

findings are particularly relevant for human treatment (i.e. marijuana for medical purposes, or MMP), since they suggest that the consumption of psychoactive THC is unnecessary (since CBD, a non-psychoactive molecule in humans, had considerable effects on its own) in the reconsolidation blockade paradigm. Therefore, it may be feasible to treat fear-based disorders with medical marijuana without the use of long-term treatment with psychoactive substances (either using isolated CBD or low-THC strains of the plant).

Hypothesis 4 was also supported by our findings; we found that a combined dose of 50mg/kg THC + 50mg/kg CBD effectively attenuated reconsolidation of the learned fear response, suggesting that a combined dose of CBD and THC may be an effective modulator of fear memory. Interestingly some evidence suggests that the combination of THC with CBD yields effects which differ qualitatively in humans from those yielded by isolated phytochemicals: the addition of CBD may synergize with the THC, producing so-called “entourage effects” whereby the therapeutic effects of CBD are increased while the psychoactive and anxiogenic qualities of THC are reduced [343]. Entourage effects are also seen with other cannabinoids, including some endogenous cannabinoids such as 2-arachidonylglycerol (2-AG) [386–388]. Similar effects are also observed with other (non-cannabinoid) ligands for the TRPV1 receptor, to which some exogenous and endogenous cannabinoids are believed bind and possibly co-activate along with CB1 (and in some cases CB2) receptors [389–391]. Therefore, it is possible that the complex effects of co-administration of THC and CBD may be in part due to the entourage effects produced by receptor co-activation. The implications of this are two-fold: as our findings would seem to confirm, combined doses of THC and CBD may have therapeutic benefit, and also if the psychoactive effects of THC are indeed mitigated (and therapeutic effects

of cannabinoids enhanced) by the combination of THC with CBD in humans then low-THC preparations may be made safer and/or more efficacious in humans by the addition of CBD.

In terms of hypotheses 5 and 6, our findings in chapter 4 supported the idea that *S. sympetala* leaf extract may similarly mediate fear-learning in rodents. In addition, our findings supported that a combined dose of betulinic acid and betulin may yield significant effects on fear-memory reconsolidation. Thus, in support of previous work [289] our findings suggest that BA may indeed be one of the active principles responsible for the therapeutic effects of *S. sympetala*. However, while our findings found positive effects of BA + BE, we did not observe such an effect when BA was administered alone. This is in contradiction to previous work, which found that BA alone attenuated anxiety-like behavior in rodents [19].

Interestingly, our combined dose of betulinic acid and betulin may most closely recapitulate the constituents of the whole leaf extract, since *S. sympetala* leaf extract likely contains both BA and synergist molecules in the form of amyrins (which, as triterpenoids, are structurally similar to betulin) [363]. This suggests that although *S. sympetala* is thought to act as a GABA_A BZD binding site agonist, the therapeutic and/or memory-altering qualities of BA may be more complex and rely on synergism with other molecules present in the plant. Thus, our findings continue to suggest that the leaf extract from the South American vine (or a combination of its active principle BA plus a synergist like betulin or one of the closely-related amyrins, α -amyrin and β -amyrin) is the most effective in terms of anxiolytic and memory-altering properties.

5.2 Comparative therapeutic value of compounds

In terms of the comparative value of these products, one has to consider the benefits and drawbacks of each. While our findings suggest that GRP may be effective at modulating the

reconsolidation of learned fear, for example, the therapeutic benefit in humans might be limited by the fact that GRP likely acts peripherally, and in a manner that could delay central effects. Thus, while the effects were robust in rodents, it is unclear whether the delay in humans would prevent GRP from acting within the time-frame of the reconsolidation window. In addition, the time it takes for peripherally administered GRP to exert central effects (and the mechanism by which GRP exerts central effects) remains largely unknown.

As for the other compounds, the time to maximum serum concentration ($T_{\max}$) of THC is 60-188 minutes, while that of CBD is slightly longer (~230-260 min) [392–397] – although when inhaled THC induces psychoactive effects almost immediately. As a result, it may not be necessary for compounds to reach peak concentration before central effects are observed. PET imaging studies using fluorodeoxyglucose (FDG), a marker of tissue uptake of glucose in the brain and body, have also shown central changes in glucose uptake in the human cerebellum and frontal regions as soon as 30 minutes after receiving cannabinoids intravenously [398–400]. As a result, it is probable that the central effects of THC and CBD are more rapid than those of GRP. The $T_{\max}$ of betulinic acid in humans is unknown, although the time for other triterpenoids of similar structure (e.g. maslinic acid) to reach peak concentration in rodent serum is faster than THC or CBD (within ~30 minutes after oral and intravenous administration) [401]. However, whether the same is true of betulinic acid remains to be determined – although this is difficult to explore, since circulating levels of BA from preparations approved for veterinary use in canines is extremely low (below 0.02 $\mu\text{g/mL}$ at five times the recommended dose) and is below the threshold for detection at safe doses [402].

In addition to pharmacokinetic profile of the drug, other practical concerns may play a role in determining therapeutic potential. For example, although all three compounds (and some

combinations of their isolated active principles) appeared to successfully block reconsolidation in rodents, the safety and efficacy of these products in humans will play a large role in successful translation. THC, for example, yielded significant effects when combined with CBD and/or whole plant background material in our experiments. However THC is psychoactive in humans [403] and can exert other negative effects (for example rapid changes in progesterone following THC administration in nonhuman primates have been associated with pregnancy problems leading to significantly increased rates of stillbirth) [404]. In contrast, CBD is largely believed to be both safer and non-psychoactive in humans [405–409], and may be more practical for human clinical trials (especially considering that in our experiments CBD alone was equally effective as combined doses with THC). Several studies have shown no mutagenic or teratogenic (developmental) effects of CBD [410,411] or significant CNS alterations [412]. In addition, the lethal dose of CBD is high: the LD_{50} of CBD is 212mg/kg in nonhuman primates when given intravenously, and a dose of greater than 20x that was required to achieve noticeable intoxication when taken orally [413]. Also, CBD has been under investigation for its potential therapeutic benefits for a wide variety of other human conditions including inflammation and ischemia [414–420], psychosis [421–426], movement disorders such as Parkinson’s disease [427–431] and Huntington’s disorder [432–436], as well as epilepsy [437–444]. Therefore, because of its profile of relative safety and tolerability (as well as being non-psychoactive and extensively tested for other human conditions), oral CBD may prove to be one of the most attractive routes for future studies with human clinical trials for fear-based disorders.

S. sympetala, similarly, has a record suggesting it may be relatively safe for use in humans. Although there have been no formal clinical trials exploring *S. sympetala* leaf extract in humans, its long-term use in traditional native medicine by the Q’eqchi healers of Belize

suggests that it is very likely safe and tolerable in humans. In addition, some safety trials have been conducted in canines suggesting that preparations containing BA produced no significant adverse events at very high doses over 28 days [402]. Souroubea-Platanus was well tolerated in Beagles in a 28-day pilot study at 8x the recommended dose, producing significant reductions in plasma cortisol 1h post-administration without clinical signs of illness [360,364,402]. In the trial, all dogs exhibited normal behavior and there was no evidence of local or systemic adverse reactions. Two animals exhibited slightly reduced platelet count; this was attributed to infection with *Ehrlichia canis* likely contracted prior to the study onset (since all three animals in the study were previously strays) [364]. Although infection is not normally associated with platelet reduction, *Ehrlichia canis* is endemic to Costa Rica and is associated with increased platelet destruction [445,446]. A follow-up study was conducted in Canada with sixteen beagles and up to 5x the recommended dose for 28 days [402]. In the Canadian study, where *Ehrlichia canis* is not endemic, a decrease in platelets was not observed. There were also no other clinically significant adverse effects noted following treatment as determined by clinical observations, physical examinations, body weights, hematology, clinical biochemistry and urinalysis.

Perhaps most importantly is the fact that previous research with *S. sympetala* leaf extract yielded no withdrawal effects on food intake, locomotor activity, or fecal output in rodents [363]. As a result, it may offer a novel alternative to therapy with benzodiazepines (e.g. Diazepam) which are known to cause dependency. Thus, like CBD, *S. sympetala* is very promising in terms of therapeutic potential (indeed, a benzodiazepine which does not cause withdrawal upon rapid cessation of the drug could be a very important development in the search for safer and more effective pharmacotherapies for fear-based disorders). However, *S. Sympetala* has not been extensively explored in humans to the degree that cannabidiol has, and well-controlled human

safety trials are needed in order to establish the safety of both *S. sympetala* leaf extract and its constituent triterpenoids or related molecules.

5.3 Future Directions

5.3.1 The role of sex differences in PTSD

One area of interest that the current studies did not address is the role of sex differences in development of PTSD symptoms and response to interventions. While this thesis aimed to explore fear-learning as a model of PTSD, the experiments were conducted with male rodents. It has been widely reported that human females are more vulnerable to development of PTSD post-trauma than males [447], although results of studies exploring sex differences in trauma exposure have been mixed [448,449]. Evidence indicates that on the whole, studies consistently report that women and girls are much more likely to develop PTSD post-trauma than males despite that males are more likely to report a history of severely traumatic experiences [450]. This may be partly attributable to sex differences in types of assault – while males are more likely to experience accidents, physical assault, disasters, death, injury, and combat/war-related trauma, females are much more likely to experience sexual assault and childhood sexual abuse [450].

Unfortunately, sex-related trauma is difficult to model with rodents. Yet evidence suggests that in addition to differences in type of trauma, females may still be more likely to develop PTSD overall across different types of trauma exposure [9,450]. Despite this, very little research has explored sex differences in rodents with regards to fear-learning [451] (and even less-so with reconsolidation). This is partly due to the additional steps required (i.e. tracking of estrous cycles) and increased number of animals that would be required to adequately run comparative measures. Although it was not the target of this thesis, there is evidence in both humans and rodents that hormonal factors play a role in fear-learning and extinction in females

[452,453]. In the future, it will be a necessary and important step for researchers to conduct experiments with equal sized groups of male and female rats in order to determine whether the attenuation of fear-memory reconsolidation affects each sex equally.

5.3.2 *Cannabinoids*

Although here we have demonstrated efficacy of several natural health products at modulating reconsolidation of learned fear (and briefly, in the last study, fear expression), many questions remain. For example, it is clear that the mechanisms governing the therapeutic action of cannabinoids are extremely complex. Although the endocannabinoid system has been extensively explored (much more so than the bombesin and bombesin-like peptides or *S. sympetala*), our understanding of the role of CB1 in fear-learning is paradoxically still in its infancy. This is largely due to the fact that most CB1 agonists and antagonists, including both endogenous cannabinoids and synthetic compounds, are highly non-selective in nature (often resulting in contradicting findings).

The biphasic effects of some CB1 agonists may be attributed in part to effects on Transient Receptor Potential Vanilloid Type 1 receptors (TRPV₁; sometimes referred to as vanilloid or capsaicin receptors). While very low doses of CB1 agonists are often anxiolytic, moderate to high doses are anxiogenic. Intra-PLC administration of a CB1 agonist arachidonyl-2-chloroethylamide (ACEA) attenuated anxiety-like behaviour in rodents at low-doses in one study [454], for example. The same study demonstrated, however, that the effects of high-dose ACEA were discriminated by pre-treatment with either AM251 or a TRPV₁ antagonist (producing anxiogenesis when co-administered with CB1 antagonist AM251 but anxiolysis when co-administered with TRPV₁ antagonist 6-I-CPS). Thus, the biphasic effects of some CB1 agonists may be explained by co-activation of TRPV₁. Both receptor types are also expressed at

PL [454], and numerous studies have confirmed that the endogenous cannabinoid anandamide also binds to TRPV₁ [390,391]. Anandamide induces postsynaptic long-term depression (LTD) at DG neurons via these receptors [391], and TRPV₁-deficient mice demonstrate reduced fear-expression [455]. As a result, the effects of CB1 activation alone on fear-learning remains very unclear (since many agonists, including endogenous ligands like anandamide, are non-selective), and it is very likely that co-activation of multiple receptor types plays a significant role in their mediation of behavior.

The inconsistent results of CB1 agonists and antagonists on fear-learning and expression may also be partly attributable to the compounds acting at novel cannabinoid receptors. Much recent research has also lent itself to trying to address the inconsistent effects of CB1 agonism and antagonism, and research has suggested CB1 antagonist Rimonabant may exert agonist-like effects via a non-CB1, non-CB2 mechanism that is also separate from the Vanilloid receptor TRPV₁ [456]. This may in-part further explain the inconsistency of centrally administered compounds, and why in many cases CB1 antagonism experiments with various compounds that interact with CB1 receptors or ligands appear to exhibit opposing effects under different conditions. Indeed, research has noted that the orphan receptor GPR55 is a novel cannabinoid receptor [457] that responds to CP99540 (a potent CB1 agonist which is fully antagonized by rimonabant; [458]). GPR55 is sensitive to a wide variety of endogenous and synthetic cannabinoids and antagonists, including both anandamide and AM251 [459–461], and is most highly expressed in the adrenals and frontal cortex in rodents, as well as the hippocampus to a lesser degree. The distribution of GPR55 receptors throughout other regions of the brain known to play a role in mediating fear-learning and expression remains largely unknown, and further

experimentation is required in order to more definitively characterize the distribution of GPR55 in the rodent brain.

In addition, THC has been shown to act as an agonist for GPR18 receptors, and the effects of THC on GPR18 are antagonized by CBD [462]. Some evidence has also implicated GPR119 in cannabinoid functioning [463], and some have suggested oleoylethanolamide (OEA) may be the endogenous ligand for GPR119 [463,464] - although further research is required to confirm the activity of GPR119. At minimum, this suggests the presence of at least four cannabinoid receptors (CB1, CB2, GPR55, and GPR18), and possibly a fifth (GRP119; [464]) – with the central effects of the latter three remaining almost completely unexplored. As a result, whether these orphan receptors are expressed on neurons mediating learned-fear remains to be examined. Since research has yet to localize expression of most of these receptors in the rodent brain, this will be necessary in order to gain an understanding of their role in the CNS and also how cannabinoids mediate fear learning and expression.

As a result, future research into the role of cannabinoids could be improved in a number of ways: Firstly, (1) the use of more selective compounds will be necessary in order to fully understand the functional role of the central endocannabinoid system. Although more selective agonists are not yet available for CB1, AM281 (CB1 antagonist) is thought to be highly selective and does not interact with orphan cannabinoid receptor GPR55. Secondly (2) future studies should explore the effects of non-cannabinoid constituents of *Cannabis spp.* in more detail, since our findings suggested that plant background material exerted effects on reconsolidation when administered alone. Specifically, since β -caryophyllene is thought to possess anxiolytic qualities [285], the effects of this compound on reconsolidation should be explored when it is administered alone. Finally, (3) it will be an important step for future studies to examine the role

of plant background material alone on fear-learning and expression. Since our background material likely contained 3-5% THC and a lesser amount of CBD, it will be necessary to assess the effects of a purer background material that is entirely devoid of cannabinoids. Since it is extremely difficult to remove all THC and CBD from plant background material it will be necessary to build up an analogue from other known components of the plant background material in concentrations equivalent to those that are naturally occurring in the plant.

5.3.3 Bombesin-like peptides

In terms of bombesin-like peptides, there are several key areas that would benefit from being addressed by future studies exploring central mechanisms of fear-learning after peripheral administration of bombesin-like peptides. Since our experiments were behavioural in nature, these experiments fell outside of the scope of the current studies – but it will be a necessary and important step for future studies to conduct central studies in order to elucidate the mechanisms by which peripherally administered GRP exerts its effects.

Although research has shown the central effects of peripherally administered GRPR agonists largely relies on both the vagus nerve and the brain-gut axis [307,310,312,313], the central sites by which GRP exerts its effects remain unknown. If peripheral GRP transmits signals centrally via the vagus nerve or gut-brain axis, the possible central release of neurochemicals is likely not limited to GRPR agonists. Rather, peripheral binding could trigger the release of many different, or possibly multiple, neurochemicals in much the same way stress signals trigger a cascading release of several different neurohormones (indeed, since GRP is a gut hormone involved in the secretion of gastrin and plays a large role as a satiety peptide in rodents, a similar mechanism signalling information about feeding or digestion to central sites is likely). As a result, it would be very difficult to determine the central mechanisms that cascade

from peripherally administered GRP without first identifying central sites that become active following peripheral GRP binding. For future studies we therefore suggest (1) to examine a non-specific marker such as c-Fos expression following I.P. administration of GRP. Although this does not give an indication of the neurochemical mechanisms activated by GRP, it would allow pinpointing of the regions activated following peripheral GRPR agonist binding. Subsequently, follow-up studies can then (2) explore neurochemical alterations produced by I.P. administration of GRP in anesthetized rats using microdialysis or other techniques (such as qPCR) at sites of suspected activation or sites known to play a role in fear-learning (e.g. BLA, LA, mPFC, and hippocampus) in order to elucidate the central neurochemical signals associated with peripheral GRP activation.

5.3.4 *S. Sympetala*

Of the compounds assessed in this thesis, *S. sympetala* extracts and isolated phytochemicals may hold some of the most promise for eventual translation of therapeutic benefit to humans (since it has already seen use in native tradition with humans for what looks to be, according to local descriptions, a fear-based disorder). Interestingly, however, perhaps the most important feature is that – unlike traditional benzodiazepines – *S. sympetala* leaf extract does not appear to produce symptoms of biological dependency in studies with rodents [465]. However, these findings have yet to be replicated, and may not translate to humans.

Although *S. Sympetala* leaf extract has a history of use by traditional native healers, safety experiments have not yet been conducted in well-controlled clinical trials with humans. While the plant extract appears safe in canines [364,402] and is approved for use in veterinary medicine, clinical safety trials are necessary in humans. Future studies should attempt to first (1) establish the safety of *S. sympetala* and/or mixtures of betulinic acid and synergist molecule

betulin (or related amyryns) in human safety trials. Secondly, trials should (2) aim to determine whether the efficacy of plant extracts in terms of attenuating both fear-memory reconsolidation and fear expression translates to humans. Finally, it will be important to (3) determine whether or not *S. sympetala* does indeed fail to cause biological dependency. Insights from these studies could be invaluable in terms of developing safer treatments for anxiety-related conditions that have reduced potential for abuse.

Conclusion

Overall, the experiments presented in chapters 2, 3, and 4 of this thesis provide strong evidence for positive therapeutic effects of using of naturally occurring products as mediators of fear-learning and anxiety in rodents, with a focus on reconsolidation blockade as a paradigm that may have greater practical application in translational studies with humans than current therapeutic approaches. These studies together provide the pre-clinical groundwork necessary for future experiments with these NHPs, including translational studies with some of these products in Phase I and II clinical trials with humans. In terms of the immediate future, CBD and *S. Sympetala* leaf extract (and isolated phytochemicals) appear to hold the greatest promise in terms of possible translation of therapeutic benefit to humans. Whether the effects observed here will be successfully replicated with human participants, however, remains to be seen (since some products that fairly reliably affect reconsolidation of learned fear in rodent models, such as Propranolol, fail to successfully attenuate learned fear responses in human participants). Future studies are therefore required in order to first establish the safety of these natural products in human participants and also to determine whether the effects observed here in rodents can be successfully translated to pre-clinical proof-of-concept models in humans.

In terms of the practical applications of these experiments, future studies should first establish the safety and efficacy of these products in human Phase I and II trials. Some compounds, such as propranolol, have been tested in human trials – but the results of translational studies with reconsolidation blockade as a therapeutic paradigm have been somewhat unsuccessful [64]. The next logical step in this regard would be to begin testing other compounds using the paradigms established by previous work (for example the method utilized by Spring et al. in their human propranolol experiments [64]). Here, we have laid the necessary pre-clinical groundwork for such translational studies to begin.

6.0 References

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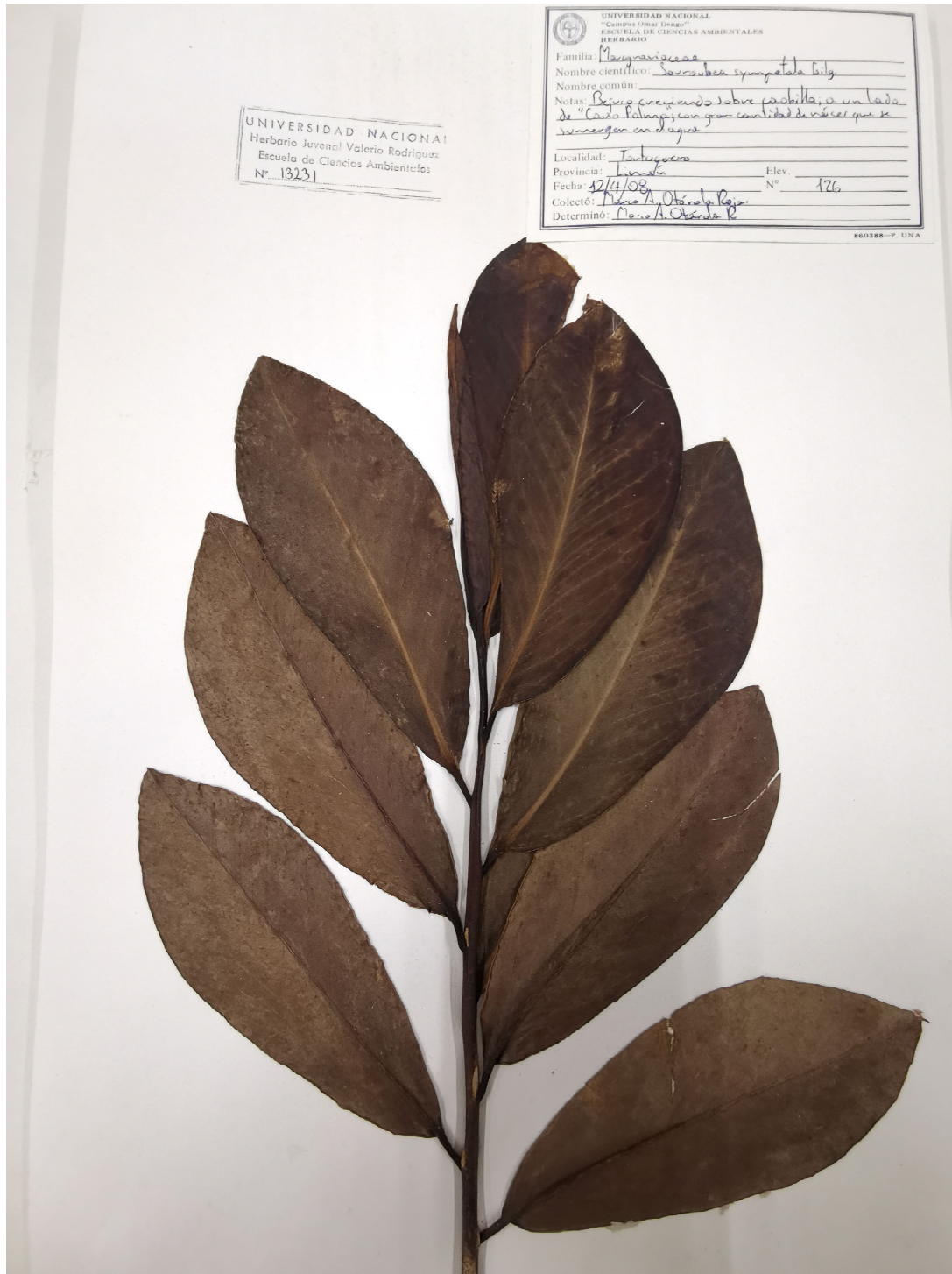
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7.0 Supplementary figures



Supplementary Figure 1. Souroubea sympetala Gilg. Voucher specimen deposited in the JVR herbarium, Universidad Nacional Costa Rica (voucher # 13231).