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Cannabidiol regulation of extinction and relapse of learned fear

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Abstract

Anxiety- and trauma-related disorders are chronic debilitating mental conditions, characterized by dysregulation of aversive memory processing and its impaired suppression. Current pharmacological and exposure-based psychotherapeutic approaches often produce inadequate responses, resulting in elevated rates of relapse. Cannabidiol (CBD), the non-psychotropic constituent of Cannabis Sativa, demonstrates a promising therapeutic potential for these disorders due to its modulating effects on the expression and extinction of learned fear.

In this thesis, the potential effects of systemic CBD on extinction and learned fear relapse over time were initially investigated, after developing a working protocol for spontaneous fear recovery. Rats underwent auditory fear conditioning (day 1), extinction training with CBD administered before or after the session (day 2), and drug-free tests of extinction recall (day 4) and spontaneous recovery (day 24). CBD administration before extinction training was found to acutely reduce the expression of contextual fear, without affecting auditory fear expression or extinction training. Although CBD did not affect extinction recall, it suppressed later spontaneous recovery of auditory fear.

Next, the pharmacological mechanisms underlying these CBD effects were investigated by examining the potential involvement of cannabinoid 1 receptor (CB1R) or 5-hydroxytryptamine 1A receptor (5-HT1AR) signaling, molecular targets through which CBD was found to elicit fear-alleviating and anxiolytic-like effects. After performing dose-response studies with the CB1R inverse agonist AM251 and the 5-HT1AR antagonist WAY100,635, these compounds were administered in combination

with CBD. However, when CBD was given alone in either study it failed to reproduce the previously observed effects, rendering it impossible to exclude the potential involvement of either transmission mechanism in mediating the effects of CBD on learned fear expression and spontaneous fear recovery.

Lastly, the effects of CBD on stress-induced impairments in extinction learning triggered by recent fear conditioning were examined by using an immediate extinction deficit (IED) protocol. Rats were administered with CBD before immediate or no extinction session, and the next day subjected to a drug-free extinction recall test. CBD enhanced recall of extinction to prevent the IED phenomenon, without interfering with the consolidation of learned fear memory.

Taken together, CBD induced long-term protection against fear relapse after successful extinction and alleviated stress-induced impairments in extinction learning. Because of the inability to reproduce the effects of CBD on fear relapse while investigating the involvement of CB1R or 5-HT1AR mediated signaling, this necessitates further studies to elucidate the exact pharmacological mechanisms underlying the CBD effects observed in this project. However, these findings add valuable insight into CBD's potential as a candidate therapeutic for the management of anxiety- and trauma-related disorders.

This thesis is dedicated to my family

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List of Abbreviations

2-AG	2-Arachidonoylglycerol
5-HT	5-Hydroxytryptamine/ Serotonin
5-HT1AR	Serotonin 1A Receptor
5-HT3R	Serotonin 3 Receptor
5-HTP	5-Hydroxytryptophan
AA-5-HT	N-Arachidonoyl-Serotonin
AC	Adenylyl Cyclase
ACC	Anterior Cingulate Cortex
ACx	Auditory Cortex
AEA	Anandamide
BA	Basal Amygdala
BBB	Blood Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
BLA	Basolateral Amygdala
BMA	Basal Medial Amygdala
BNST	Bed Nucleus Stria Terminalis
cAMP	Cyclic Adenosine Monophosphate
CB1R	Cannabinoid 1 Receptor
CB2R	Cannabinoid 2 Receptor
CBD	Cannabidiol
CBT	Cognitive Behavioural Therapy
CEA	Central Amygdala
CeL	Lateral Central Amygdala
CeM	Medial Central Amygdala
CNS	Central Nervous System
COX-2	Cyclooxygenase-2
CR	Conditioned Responses
CREB	cAMP-Response Element Binding Protein
CRF	Corticotropin-Releasing Factor
CRFR1	Corticotropin-Releasing Factor Receptor 1
CS	Conditioned Stimulus
CUS	Chronic Unpredictable Stress
D2R	Dopamine 2 Receptor
DA	Dopamine
dACC	Dorsal Anterior Cingulate Cortex
DAG	Diacylglycerol
DAGLα	Diacylglycerol Lipase α
dHPC	Dorsal Hippocampus
dlPAG	Dorsolateral Periaqueductal Gray
DRN	Dorsal Raphe Nuclei
DSM	Diagnostic and Statistical Manual of Mental Disorders
ECB	Endocannabinoid
EMT	Endocannabinoid Membrane Transporter
ENT	Equilibrative Nucleoside Transporter

EPM	Elevated Plus Maze
FAAH	Fatty Acid Hydrolase
FABP	Fatty Acid Binding Protein
FDA	Food and Drug Administration
FPS	Fear Potentiated Startle
GABA	γ-Aminobutyric Acid
GPCR	G-Protein Coupled Receptor
GPR55	G-Protein-Coupled Receptor 55
HPA	Hypothalamic-Pituitary-Adrenal
i.c.v	Intracerebroventricular
i.p.	Intraperitoneal
IED	Immediate Extinction Deficit
IL	Infralimbic Cortex
ITC	Intercalated Cells of Amygdala
ITI	Intertrial Interval
LA	Lateral Amygdala
LC	Locus Coeruleus
LC-NE	Locus Coeruleus-Norepinephrine
LOX	Lipooxygenase
LTD	Long-Term Depression
MAGL	Monoacylglycerol Lipase
MAOI	Monoamine Oxidase Inhibitors
MGm	Medial Geniculate Body
mPFC	Medial Prefrontal Cortex
MRN	Median Raphe Nuclei
MS	Multiple Sclerosis
mTOR	Mammalian Target of Rapamycin
NAc	Nucleus Accumbens
NAPE	N-Arachidonoyl Phosphatidylethanolamine
NAPE-PLC	N-Acyl Phosphatidylethanolamine Phospholipase C
NAPE-PLD	N-Acyl Phosphatidylethanolamine Phospholipase D
NE	Norepinephrine
PFC	Prefrontal Cortex
p-GSK3β	Glycogen Synthase Kinase 3β
PIN	Posterior Intralaminar Nucleus
PL	Prelimbic Cortex
PLA	Phospholipase
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
PTSD	Post-Traumatic Stress Disorder
PVN	Paraventricular Nucleus of Hypothalamus
RSC	Retrosplenial Cortex
s.c.	Subcutaneous
SERT	Serotonin Reuptake Transporter
SNRI	Serotonin Norepinephrine Reuptake Inhibitors

SSRI	Selective Serotonin Reuptake Inhibitors
TCA	Tricyclic Antidepressants
THC	Δ^9 Tetrahydrocannabinol
TRPV1	Transient Receptor Potential Cation Channel Subfamily Vanilloid Member 1
TSRD	Trauma And Stressor-Related Disorders
US	Unconditioned Stimulus
VGCCs	Voltage Gated Calcium Channels
vHPC	Ventral Hippocampus
vlPAG	Ventrolateral Periaqueductal Grey
VTA	Ventral Tegmental Area

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Chapter 1: General introduction

1.1 Introduction to anxiety- and trauma-related disorders

Anxiety and fear are adaptive responses to dangerous stimuli. Anxiety is considered as an inner conflict between approaching and passively avoiding a potential threat. On the other hand, fear is the emotional state induced by an imminent threat and manifested as defensive mobilization (Gray, 2000; Simon & Gorman, 2006; Bannerman et al., 2014). Depending on the stimulus nature, distinct neuronal mechanisms and subsequent defensive responses are activated. Innate fear is elicited by stimuli that are intrinsically appreciated as dangerous, while learned fear is induced by neutral stimuli that have been previously associated with innate threatening ones (Gross & Canteras, 2012).

A major characteristic of anxiety disorders is the misattribution of threat to benign conditions due to deficient suppression of fear. Anxiety disorders constitute the most prevalent group of psychiatric conditions, currently reaching a lifetime prevalence of 7.3% worldwide (Baxter et al., 2013). The latest revision of the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-V), along with the differences in survey methodological and sociodemographic factors, are thought to have contributed to broad cross-national prevalence variations (Stein et al., 2017). Currently, post-traumatic stress disorder (PTSD) along with acute stress disorder, adjustment disorder, reactive attachment disorder, disinhibited social engagement disorder, and persistent complex bereavement disorder have been excluded from the DSM-V spectrum of anxiety disorders and have been reassigned the trauma and stressor-related disorders (TSRDs), characterized by distinct

etiopathogenesis, onset, manifestation, severity, and relapse of symptomatology (Figure 1.1) (Pai et al., 2017). Similarly, obsessive-compulsive disorder has been removed from the anxiety disorders category and classified as obsessive-compulsive and related disorders along with body dysmorphic disorder, hoarding disorder, trichotillomania, and excoriation disorder (Abramowitz & Jacoby, 2014).

Anxiety- and trauma-related disorders constitute a significant socioeconomic burden in modern societies. They are associated with high utilization of healthcare resources and development of long-term disability interconnected with significant cognitive and emotional impairments that lead to social and occupational dysfunction (Bandelow & Michaelis, 2015). Therefore, the identification of environmental [e.g. female gender, racial maltreatment and discrimination, family history, adverse childhood experiences, temperamental vulnerabilities, low educational and household income level (Blanco et al., 2014)] and genetic [e.g. variations to glucocorticoid-related gene FKBP5, endocannabinoid degradation enzyme gene, serotonin transporter gene 5-HTTLPR, brain-derived neurotrophic factor (BDNF) Val66Met polymorphism (Daskalakis et al., 2016; Lazary et al., 2016; Craske et al., 2017; Hori et al., 2020)] risk factors related to developing these disorders have gained much attention as tools for their diagnosis, prognosis and effective therapeutic management. In particular for the development of PTSD, the peri-traumatic risk factors like pre-traumatic psychopathology, the extent of trauma-related losses and the emergence of acute stress disorder during the posttraumatic period are considered crucial (Stoddard Jr., 2018).

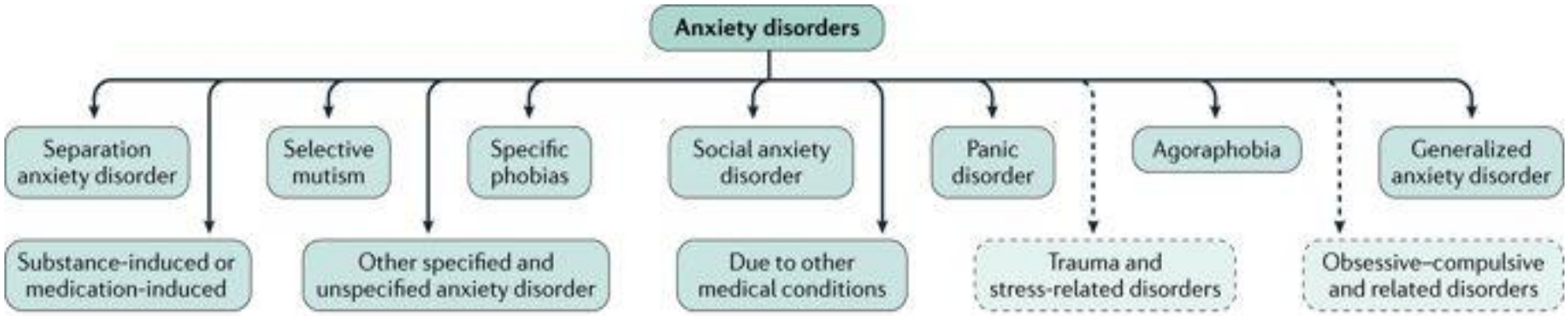

Figure 1.1 Revision of DSM-V in the anxiety disorders group (Craske et al., 2017). PTSD has been excluded from the broad spectrum of anxiety disorders and reassigned to trauma and stressor-related disorders (TSRDs).

Anxiety- and trauma-related disorders share common psychological issues like tension, excessive fear, apprehension, concentration and sleep disturbances, along with somatic symptoms like tachycardia, heart palpitations, tremor and sweating resulting from strong sympathetic stimulation, with panic attacks a representative feature triggered after a robust fear response (Craske et al., 2017; Papadakis, 2017). The severity of anxiety arousal and avoidance behavior have been suggested as strong predictors of long-term disability in anxiety disorders (Hendriks et al., 2016). The overlap of symptoms among and between anxiety, trauma and other mental disorders is often a challenge for the determination of differential diagnosis, as many of these may be concomitant or forerunners of other disorders. Symptomatology of medical conditions like asthma, hyperthyroidism, arrhythmia, epilepsy, etc. can mimic or contribute to the development of anxiety disorders (Craske et al., 2017), while it is not uncommon that self-medicated anxiety disorder may progress to alcohol or substance abuse (Stein et al., 2017). Therefore, detailed medical and psychiatric history with physical examination targeted to the onset, triggers, and course of symptoms are paramount in defining the right diagnosis and therapeutic strategy for the patient.

PTSD is a chronic debilitating mental condition, possibly developing due to poor adaptation after experiencing or witnessing an extremely traumatic event that has threatened someone's physical and/ or psychological integrity. Depending on the type of trauma, PTSD can have either immediate or delayed onset, occurring on average within six months, demonstrating a chronic or non-chronic course with an estimated mean duration of six years (Kessler et al., 2017; Schein et al., 2021). It is estimated that 70% of people worldwide will experience a traumatic event in their lifetime, but approximately only 4-10% of those will subsequently develop PTSD

(Benjet et al., 2016). The disorder is characterized by dysregulation in aversive memory processing that leads to impaired safety signal processing and suppression of fear (Dunsmoor & Paz, 2015). It is manifested as multidimensional cluster symptomatology of re-experiencing (e.g., intrusive memories, flashbacks, distressing dreams), avoidance behaviour to external reminders, hyperarousal and hypervigilance (e.g., irritability, exaggerated startle reactions, insomnia, concentration disturbances), dissociation phenomena (e.g., depersonalization, derealisation, fragmented thoughts) and negative alternation of cognition and emotion symptoms after the traumatic event (Norrholm & Ressler, 2009; Friedman et al., 2011; Stoddard Jr., 2018). Additionally, PTSD is linked with development of somatic and psychiatric co-morbidities, elevated rates of relapse after discontinuation of treatment, and increased risk of suicidal ideation (Goldstein et al., 2016; Tarrier & Gregg, 2004).

1.2 Current therapeutic approaches

Anxiety- and trauma-related disorders are characterized by under-diagnosis, a wide treatment gap and chronicity, rendering their management a real challenge (Wang et al., 2007; Stein et al., 2017). Current guidelines propose as first-step management the introduction of a psychotherapeutic method, commonly cognitive behavioral therapy (CBT), or an antidepressant like selective serotonin reuptake inhibitors (SSRIs). The appropriate therapy is determined by multiple factors, like the final diagnosis, patient age and co-morbidities, adverse effects, or interactions with concomitant medications (Bystritsky et al., 2013). Responding patients often continue with a maintenance regimen, while for non-responders a more flexible approach is considered based on Coordinated Anxiety Learning and Management (Roy-Byrne et al., 2010). More psychotherapy sessions are added, a trial with a different medication

is introduced, an adjunctive therapeutic regimen is designed with the combination of two methods, or more invasive techniques are applied for refractory anxiety.

Most psychotherapeutic methods are usually brief (≈ 8-12 sessions) and significantly reduce the duration and intensity of symptoms. These are based on the minimization of avoidance behaviour and the confrontation of the traumatic stimuli or situations. During these sessions, the patient will gradually learn to approach such stimuli under safe conditions, and cope with them with less distress and autonomic reactivity (Papadakis, 2017). CBT and relative treatment interventions, like exposure therapy, cognitive processing therapy, psychoeducation, breathing retraining, cognitive restructuring (Watkins et al., 2018) and eye movement desensitization and reprocessing are widely used and their efficacy has been assessed by a number of clinical trials (Craske et al., 2017). Noteworthy strategies have been proposed, like psychodynamic therapy, hypnotherapy, mindfulness-based cognitive therapy, stress management, acceptance and commitment therapy, habit-disruption approaches, retraining of avoidance tendencies (Bisson & Andrew, 2007; Arnaudova et al., 2017) and interpersonal therapy (Markowitz et al., 2014), which all require further evaluation in the clinical milieu.

Medications are used as an alternative to psychotherapy or as an adjunctive treatment. Numerous randomized controlled trials have supported the efficiency, safety, and minimal abusive profile of SSRIs and serotonin norepinephrine reuptake inhibitors (SNRIs), rendering them as first-line anxiolytics with a well-balanced risk benefit ratio (Murrough et al., 2015). To date, the only medications approved by Food and Drug Administration (FDA) for the management of PTSD are the SSRIs sertraline

and paroxetine (American Psychological, 2017). However, there is a considerable latency period of 2-6 weeks until the onset of SSRI/SNRI effectiveness, whereas their acute administration is often related to developing anxiogenic and physiological adverse effects like gastrointestinal disturbances, fluctuation in appetite and body weight, agitation, insomnia, headache, sexual dysfunction and increased suicidality rates in children and adolescents (Bridge et al., 2007; Bandelow et al., 2017). Low dosage commencement and progressive elevation have been shown to mitigate many of these side effects, while maximum benefit from these compounds is achieved upon appropriate dose selection and adherence to the treatment regimen (American Psychological, 2017). On the other hand, tricyclic antidepressants (TCA) and monoamine oxidase inhibitors (MAOIs), that are comparably as efficacious as SSRIs and SNRIs, have been restricted for use as second-line anxiolytics because of their adverse safety and tolerability profile (Murrough et al., 2015).

Benzodiazepines and barbiturates have been widely prescribed as anxiolytics in the past, as they have sedative properties by allosterically enhancing γ-aminobutyric acid (GABA) inhibitory neurotransmission. However, their prominent side effect profile, potential for tolerance, abuse, and physiological dependence, with potential fatal withdrawal symptoms upon abrupt discontinuation, dose-dependent anterograde amnesia, profound depression of central nervous system (CNS), especially after overdose or co-administration with ethanol or opioids, has limited their use to the management of acute anxiety states, control of panic attacks, and mitigation of anxiety symptoms until the onset of first-line anxiolytic effects (Bystritsky et al., 2013; Katzung, 2015; Bandelow et al., 2017). Interestingly, they are relatively contraindicated for patients already diagnosed with PTSD as they may worsen the

severity of symptoms and psychotherapy efficacy, resulting in development of co-morbid conditions, whereas benzodiazepines may potentiate the risk of developing PTSD in patients recently exposed to a traumatic event (Guina et al., 2015). Additionally, the GABA analogues pregabalin and gabapentin, well known for their anticonvulsant and mood-stabilizing effects, have attracted much interest for their potential anxiolytic properties, whereas buspirone, a serotonin (also called 5-hydroxytryptamine, 5-HT) 1A receptor (5-HT1AR) agonist, has been found to elicit some effectiveness in the management of generalized anxiety disorder (Murrough et al., 2015; Bandelow et al., 2017).

Lately, invasive techniques have attracted much attention and are reserved for treatment-refractory disorders, targeting brain areas implicated in fear and anxiety processing (see section 1.3 below). Electroconvulsive therapy, vagal nerve stimulation, repetitive transcranial magnetic stimulation, psychosurgery and deep brain stimulation have significant therapeutic effects, but have limited application due to short and long-term adverse cognitive, sensory and mood dysfunctions (Bystritsky et al., 2013).

Unfortunately, conventional medications are accompanied by adverse effects and shortcomings related to their efficacy, adequacy of response, and tolerability, while the available exposure-based therapies often induce temporary fear-suppressing effects, therefore limiting the successful management of anxiety- and trauma-related disorders. The fact that up to 40 % of patients relapse upon treatment discontinuation or fail to achieve complete remission renders crucial the development

of new pharmaceuticals that will be used as adjuncts to exposure therapy (Singewald et al., 2015).

1.3 Learned fear and the underlying neural circuitry

Pavlovian fear conditioning constitutes a widely used behavioural model in both animals and humans for the investigation of neurobiological mechanisms underlying the aetiology and symptomatology of anxiety- and trauma-related disorders. Fear conditioning is a form of associative learning, by which certain stimuli become predictive of threat. During fear acquisition, a neutral conditioned stimulus (CS) (e.g., tone, light, or environmental cues) is associated with an aversive unconditioned stimulus (US) (e.g., foot-shock). Repetition of such pairings induces behavioural and physiological reactions to the CS, called conditioned responses (CR) (Kaplan & Moore, 2011). In rodents, freezing (i.e., absence of movement apart from respiration), fear potentiated startle (FPS) (i.e., reflexive flinch reaction in response to an unpredictable CS), darting (i.e., escape-like response commonly exhibited by females as fast crossing of the chamber), ultrasonic distress vocalizations (i.e., 22 KHz vocalizations emitted predominantly by males during fear conditioning), and conditioned suppression (i.e., decrease in operant behaviour like lever press during CS presentation) constitute the most commonly measured behavioural CRs (Greville et al., 2013; Daldrup et al., 2015; Gruene et al., 2015; Tryon et al., 2021). In humans, skin conductance (i.e., electrical conductance induced by perspiration) and FPS (i.e., electromyographic recording of eyeblink reflex) are the mostly commonly quantified responses, while blood pressure, heart or respiratory rate alterations can also be monitored across both species (VanElzakker et al., 2014; Careaga et al., 2016).

The acquisition of the CS-US association and its subsequent consolidation into long-term fear memory are dynamic processes and mediated by excitatory and inhibitory interconnections within multiple brain regions and neuromodulatory circuits. The amygdala constitutes a critical location of fear learning and expression, being subdivided into two major areas, the basolateral (BLA) and central (CEA) amygdala, with each of them having distinct functions and neuronal composition. BLA is comprised predominately of glutamatergic spiny projection neurons, and to a lesser extent of GABA-ergic interneurons, consisting of lateral (LA), basal (BA) and basal medial (BMA) amygdala. CEA is subdivided to lateral (CeL) and medial (CeM) central amygdala, areas that are interconnected and composed of GABAergic medium spiny neurons (Tovote et al., 2015).

It is accepted that learned fear acquisition takes place in LA, which receives converging sensory information about the CS directly from auditory thalamus [i.e., medial subdivision of medial geniculate body (MGm) and posterior intralaminar nucleus (PIN)] and indirectly from ventral auditory cortex (ACx), while the US is relayed from somatosensory thalamus and cortex, stimulating synaptic plasticity and association of the CS-US (Figure 1.2) (Johansen et al., 2011; Luchkina & Bolshakov, 2019). LA is directly or indirectly connected through BA, BMA, and intercalated cell masses (ITC) of amygdala to CEA. This circuit is associated not only with the orchestration of CRs, but also with the modulation of associative plasticity. Notably, BA is reciprocally interconnected with ventral hippocampus (vHPC) and prefrontal cortex (PFC). Specifically, vHPC is responsible for encoding contextual representations (Kim & Cho, 2020), while PFC serves as a modulator of fear responses with prelimbic cortex (PL) and infralimbic cortex (IL) promoting and suppressing fear expression,

respectively (Bannerman et al., 2004; Tovote et al., 2015). CEA plays a significant role as a fear output, permitting fear learning through CeL activation, and regulation of fear responses through CeM, which sends projections to various brain regions (Johansen et al., 2011). Specifically, CeM is connected with bed nucleus of stria terminalis (BNST) and paraventricular nucleus of hypothalamus (PVN), which both stimulate stress hormone excretion, and with lateral hypothalamus, resulting in increased blood pressure, heart and respiration rate through alteration in autonomic activity (Johansen et al., 2011; Kim & Jung, 2006). In rodent models, depending on which of the efferent projections sent from the CEA to the ventrolateral periaqueductal grey (vlPAG) or substantia innominata are activated, then the fear responses can be respectively switched between those promoting freezing, FPS, vocalization, and analgesia, or those favouring arousal and risk assessment (Gross & Canteras, 2012; Bouton et al., 2020).

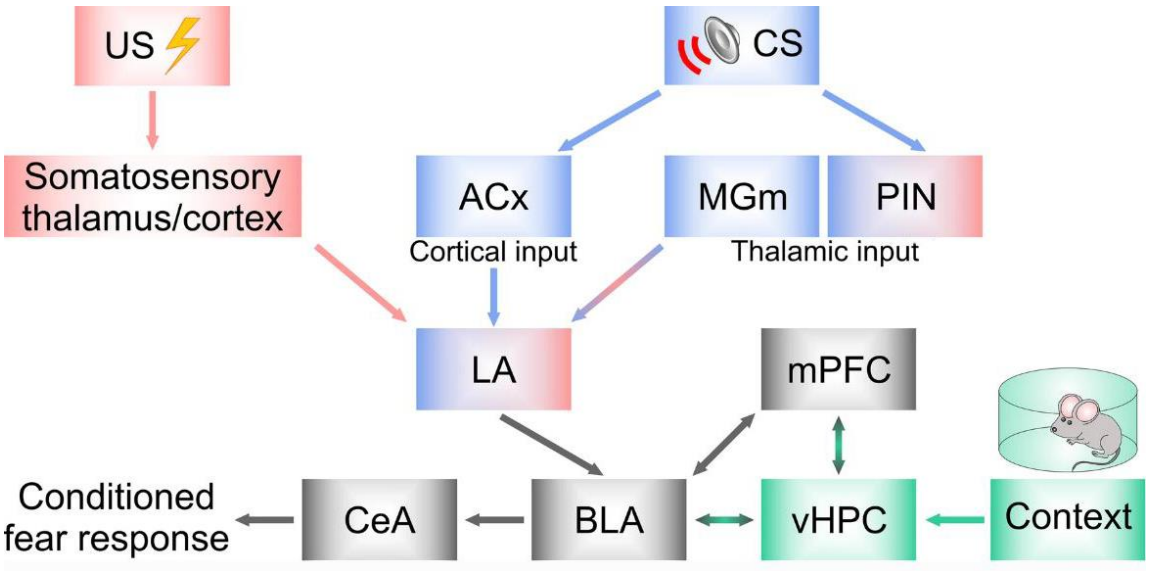

Figure 1.2 Diagram of circuitry underlying fear conditioning (Luchkina & Bolshakov, 2019)

CS=conditioned stimulus, US=unconditioned stimulus, ACx=auditory cortex, MGm=Medial geniculate body, PIN=Posterior intralaminar nucleus, LA=lateral amygdala, mPFC=medial prefrontal cortex, vHPC=ventral hippocampus, BLA=basolateral amygdala, CeA=central amygdala.

1.4 Extinction, conditions of fear relapse and underlying neural circuitry

Memory formation is a dynamic process and can enter into a labile state every time that it is reactivated. The retrieval of fear memory induced by brief re-exposure to the conditioned context or the CS without reinforcement by the US may favor reconsolidation, preserving the fear response (Lee et al., 2006). Reconsolidation can lead to memory updating through integration of new information into a previously consolidated memory, providing the opportunity to modify an undesired fear memory by updating its emotional valence (Haubrich et al., 2015). On the other hand, fear memory may gradually attenuate and extinguish upon prolonged conditioned context re-exposure or repeated presentation of the CS, and parallel omission of the US, resulting in suppression of the CR (Furini et al., 2014). This extinction of learned fear results from the violation of the original CS-US contingency (McNally & Westbrook, 2006). However extinction constitutes a fragile and less durable learning process compared to fear conditioning, and under specific circumstances the CR can re-emerge after extinction (Tsai & Gräff, 2014). For instance, an extinguished CR can be reinstated when the US is given unexpectedly in the absence of the CS. On the other hand, when the extinguished CS is delivered outside of the extinction context it can induce renewal of the CR, whereas spontaneous recovery occurs when fear to the CS returns merely because significant time has elapsed after extinction (Figure 1.3)(Goode & Maren, 2014). These relapse phenomena indicate that extinction is not simply a form of forgetting, erasing or eliminating of the fear conditioning memory, instead it generates a new inhibitory learning that competes with the excitatory fear memory trace (Myers & Davis, 2006).

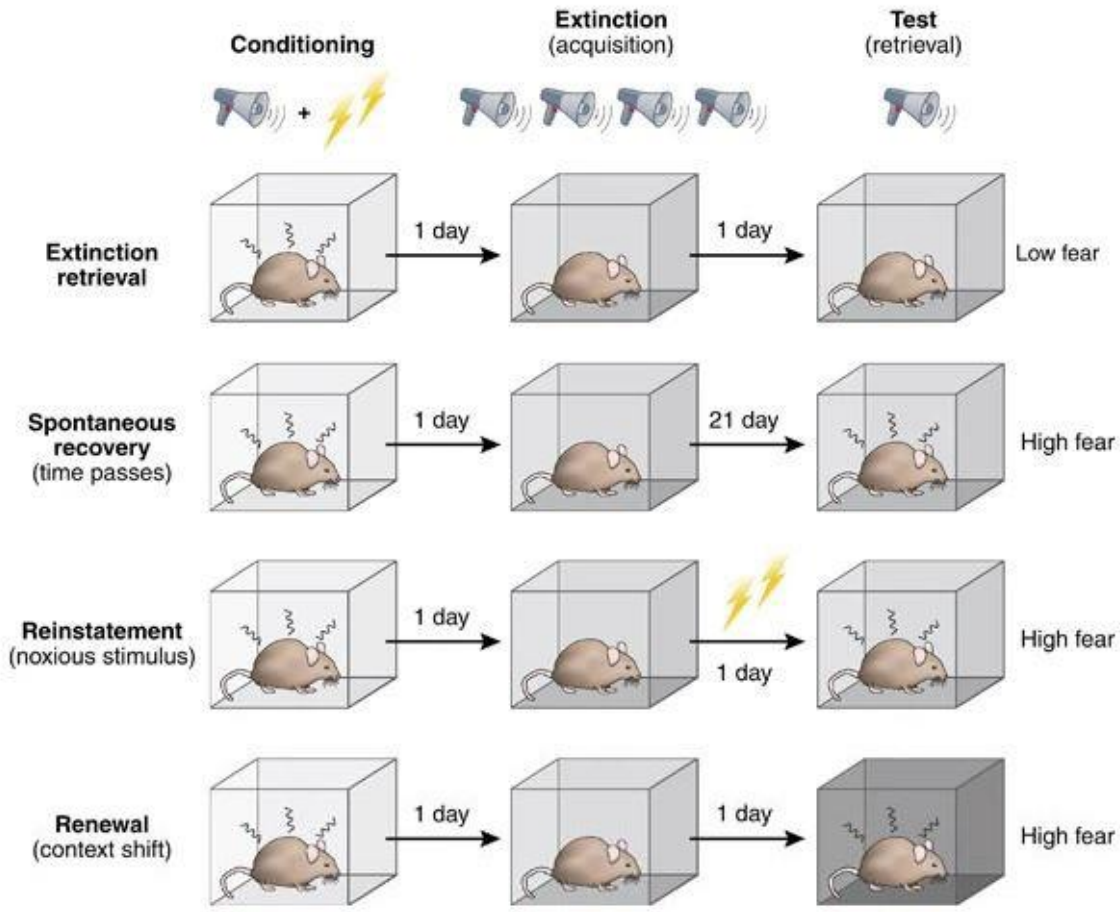

Figure 1.3 Conditions of fear relapse after extinction adapted after (Maren & Holmes, 2016)

It is not surprising that during extinction the same areas are recruited as for fear conditioning (Myers & Davis, 2006). It was found that amygdala and medial prefrontal cortex (mPFC) play crucial roles in extinction memory formation and maintenance, while hippocampus is critical for integrating contextual information and novelty, encoding contextual specificity, and regulating extinction expression (Corcoran & Maren, 2004; Orsini & Maren, 2012). PL facilitates fear expression by sending excitatory projections to BA, which subsequently innervates the CEA (Senn et al., 2014). In contrast, IL promotes fear extinction by sending efferent projections to BLA and the inhibitory intercalated cells of amygdala (Knapska et al., 2012), resulting in suppression of the CEA activity and fear responding (Figure 1.4)(Quirk et al., 2003).

However, it was recently reported that a unidirectional excitatory connection from PL to IL is crucial for extinction memory formation (Wang et al., 2022). Importantly, IL is also found to regulate extinction recall by integrating the CS with contextual information from hippocampus and inhibiting CEA-mediated fear responding (Quirk et al., 2003; Herry et al., 2010). It is well known that both extinction and its recall are characterized by contextual dependency, which is driven by hippocampus (Sierra-Mercado et al., 2011). Specifically, vHPC is responsible for establishing contextual representations during extinction learning and modulating fear renewal upon contextual change (Sevenster et al., 2018). Projections from vHPC directly to BLA or indirectly through PL are involved in fear expression and engaged upon fear renewal, while vHPC projections to IL are implicated in extinction recall (Marek et al., 2018).

Extinction is considered the basis for exposure therapy, which is used for the management of anxiety- and trauma-related disorders and therefore has considerable translational utility. From a clinical perspective, patients with PTSD demonstrate dysregulation of the hippocampus-prefrontal cortex-amygdala circuits. This finding is associated with impaired extinction retrieval due to overactivation of fear-generating areas like the amygdala and dorsal anterior cingulate cortex (dACC) (equivalent to rodent PL) and suppression of regions related to inhibition of conditioned fear like the ventromedial prefrontal cortex (vmPFC) (equivalent to rodent IL) and hippocampus (Pitman et al., 2012). However, extinction recall impairments may be not related to the loss of inhibitory regulation *per se* but rather to the loss of proper context engagement of this inhibitory mechanism, resulting in overgeneralization of fear memory (Maren & Holmes, 2016).

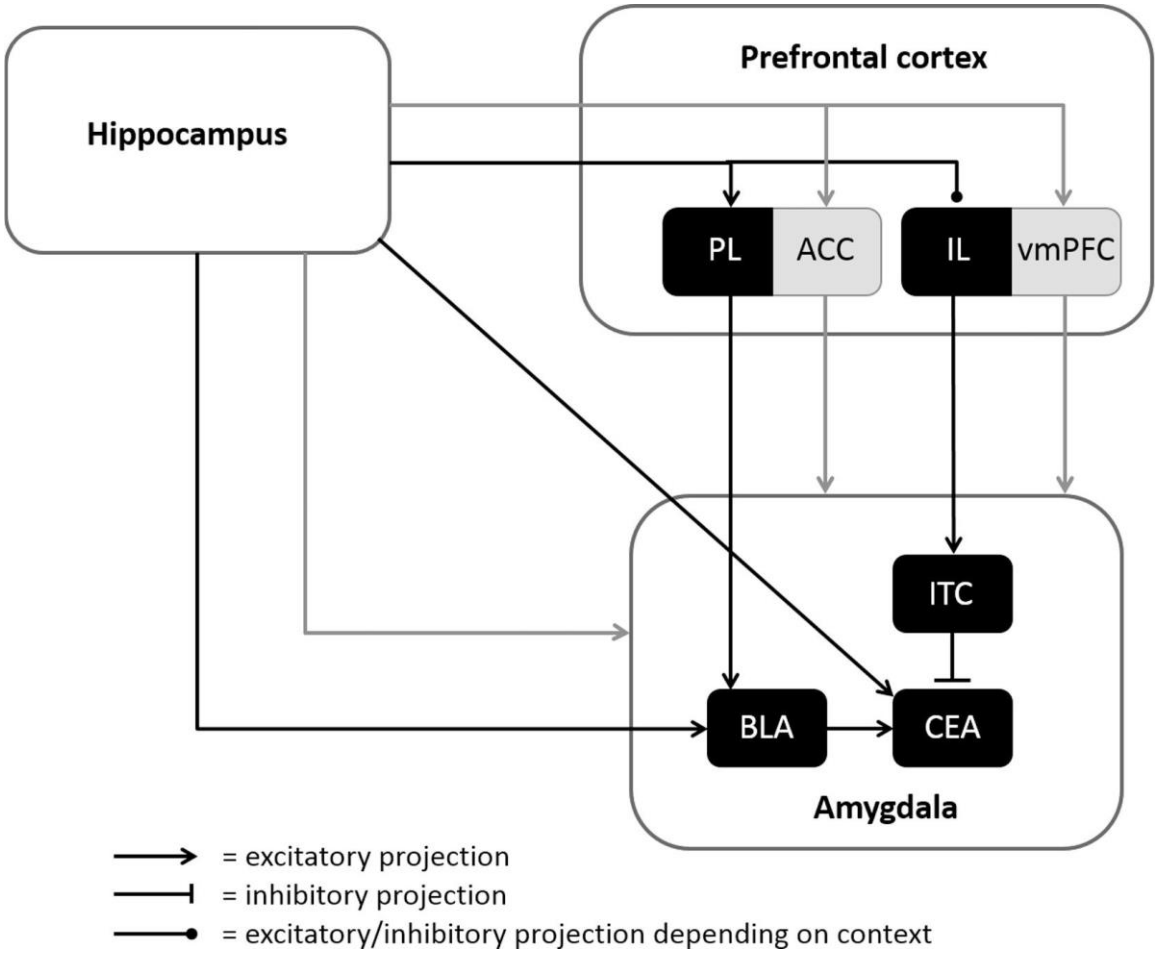

Figure 1.4 Fear expression and extinction circuitry (Pattwell et al., 2013).

PL=prelimbic cortex, ACC=anterior cingulate cortex, IL=infralimbic cortex, vmPFC=ventromedial prefrontal cortex, ITC=intercalated cells of amygdala, BLA=basalateral amygdala, CEA=central amygdala

1.5 Phytocannabinoids and their therapeutic actions

Cannabis sativa L. is one of the oldest plants used for its purported medicinal and recreational properties. It consists of more than 400 chemical compounds, more than 100 of which are pharmacologically active, called phytocannabinoids. Among them, the psychotropic Δ^9-tetrahydrocannabinol (Δ^9-THC) and the non-psychotropic cannabidiol (CBD) are the most studied and found in the greatest concentrations. Their isolation has led to the identification of specific transmembrane G-protein-coupled receptors (GPCRs) known as cannabinoid 1 (CB1R) and cannabinoid 2 (CB2R)

receptors by which they exert their effects, the discovery of endogenous ligands known as the endocannabinoids (ECBs) [e.g., anandamide (AEA) and 2-arachidonoylglycerol (2-AG)], and finally to the production of their synthetic analogues such as nabilone and dronabinol, altogether comprising the broad group of cannabinoids (Maurya & Velmurugan, 2018). THC and CBD are active compounds derived from the decarboxylation of their inactive acidic forms of the cannabis plant, tetrahydrocannabinolic acid and cannabidiolic acid, respectively, which are precursors of cannabigerolic acid. However, their composition as extracts depends on the variety of cannabis plant, in which other noteworthy phytocannabinoids exist, like (-) trans-Δ^9 tetrahydrocannabivarin, cannabigerol, cannabichromene, cannabicyclol and β-caryophyllene, with each having distinct pharmacodynamics properties (Ligresti et al., 2016).

The ECB system and its components (i.e., cannabinoid receptors, endogenous ligands and their synthesis and degradation enzymes) have been found to modulate many physiological processes, while its dysregulation is implicated in the development of both medical and psychiatric disorders. This is attributed to the widespread localization of CB1R and CB2R. Specifically, CB1R is the most abundant metabotropic receptor in the CNS and is distributed to a lesser extent in peripheral tissues. The highest levels are present in corticolimbic areas like the basal ganglia, especially in the substantia nigra and globus pallidus, hippocampus, BLA and PFC, while lower levels are found in brainstem and cerebellum. In contrast, CB2Rs predominate in peripheral immune tissues and cells like B-, T-, NK- and mast cells, monocytes, macrophages and microglia, with expression on the latter implicated in the inflammatory responses detected in neurodegenerative diseases (Pertwee, 1997; Maroon & Bost, 2018). Apart

from their expression by microglia, CB2Rs were identified in neurons of the frontal cortex, striatum, basal ganglia, ventral tegmental area (VTA), amygdala, hippocampus, while modulation of their activity was linked with anxiolytic, antidepressant, or antipsychotic-like effects (Navarrete et al., 2020). Additionally, cannabinoids were found to interact with other non-cannabinoid receptors like transient receptor potential vanilloid type 1 (TRPV1) channels, the orphan G-protein-coupled receptor 55 (GPR55), peroxisome proliferator-activated receptors (PPARγ) and 5-HT1AR (Ryberg et al., 2007; Pertwee, 2010).

Cannabinoids have attracted considerable interest as candidate therapeutics. Preclinical and clinical studies have revealed their beneficial properties as neuroprotective, anti-inflammatory, analgesic, anti-psychotic, antidepressant, anxiolytic, anticonvulsant, anti-emetic, anti-glaucoma, anti-cancer, gastro- and cardioprotective agents, by improving the symptomatology and decelerating the progression of numerous disorders (Fraguas-Sánchez & Torres-Suárez, 2018; Russo, 2018; Scherma et al., 2018). The neuroprotective effects of cannabinoids in neurodegenerative disorders like multiple sclerosis are exerted by CB1R and CB2R, with the former inducing anti-excitotoxic effects through suppression of glutamate transmission and calcium influx, while the latter provided immunomodulatory effects by controlling microglial activation and downregulation of pro-inflammatory cytokines and oxidative stress (Gowran et al., 2011; Sánchez & García-Merino, 2012). Of significance was the approval of nabiximols (Sativex) as an adjunctive treatment of spasticity and central neuropathic pain for MS and as a palliative treatment of intractable cancer-related pain. Noteworthy is the efficacy of cannabidiol oral extract (Epidiolex) for the management of treatment-resistant childhood epilepsies, Dravet

and Lennox-Gastaut Syndromes, and its approval by the FDA (Devinsky et al., 2017; Maroon & Bost, 2018). Additionally, the synthetic cannabinoid dronabinol has previously demonstrated enhancing effects on extinction learning and recall in humans, while improving the severity of symptoms linked with hyperarousal and sleep quality in PTSD patients (Rabinak et al., 2013; Roitman et al., 2014). Similar effects were also observed with nabilone administration (Jetly et al., 2015), rendering cannabinoid modulators potential candidates for the management of PTSD.

Finally, it is worth discussing the implication of endocannabinoid signaling dysregulation in the development of psychiatric disorders, like schizophrenia and depression, with an emphasis on anxiety disorders below. Clinical studies and animal models of schizophrenia revealed increased AEA levels in blood and cerebrospinal fluid, alteration of CB1R densities in the dorsolateral prefrontal cortex, ACC, insular cortex, pons and nucleus accumbens (NAc), along with a decrease in CB2R expression in mononuclear cells in peripheral blood (Scherma et al., 2018). Cannabis and CB1R agonist use, like Δ^9-THC and synthetic analogues such as WIN-55212 and CP-55940, is associated with an increased risk of development of schizophrenia in genetically predisposed people, exacerbation of psychotic symptoms in diagnosed patients, and induction of transient cognitive impairments, and positive and negative symptoms after acute administration in healthy individuals (D'Souza et al., 2004). On the other hand, pre-treatment with CBD blocked the psychotomimetic symptoms induced by Δ^9-THC in healthy volunteers (Bhattacharyya et al., 2010). However, administration of CBD has revealed that antipsychotic effects can be mediated through a supplementary mechanism, by partially agonizing the dopamine 2 receptors (D2Rs) (Seeman, 2016).

Additionally, evidence arising both from human and animal studies suggests that alterations in 2-AG concentrations in serum and depression-related brain areas, in parallel with changes in CB1R expression, play a crucial role in depression and bipolar disorder pathogenesis, while cannabis use is associated with improvement of mood-related symptoms. Nevertheless, high and regular doses of cannabis may constitute a risk factor for the development of new-onset depressive and mood disorders (Lev-Ran et al., 2014; Scherma et al., 2018). On the other hand, alterations in ECB signaling have been identified upon stress exposure. Specifically, increased levels of CB1R mRNA and protein expression were detected in BLA, and CA1 area of hippocampus in mice exposed to foot shock and reminders, while chronic stress was found to result in downregulation and loss of CB1Rs (Navarrete et al., 2020). On the other hand, elevated CB1R availability in the amygdala-hippocampal-cortico-striatal neural circuit after a positron emission tomography (PET) scan, accompanied by low plasma levels of AEA and cortisol, were identified in patients diagnosed with PTSD, suggesting that the upregulation of CB1R in response to low anandamide levels plays a critical role in the etiology of the disorder, while providing an important direction for the development of novel pharmaceuticals targeting the ECB system (Neumeister et al., 2013).

1.6 Cannabidiol's pharmacological properties and therapeutic potential

CBD has attracted considerable attention for its potential therapeutic effects in a wide range of neuropsychiatric disorders. Results from both clinical and preclinical studies support its promising anti-oxidative, anti-inflammatory, neuroprotective, and

anxiolytic-like properties against anxiety- and trauma-related disorders (Kerstin & Franjo, 2017).

CBD is a generally well-tolerated compound, with the only reported side effects being tiredness, diarrhea, alterations in appetite and weight, when administered for treatment of epilepsy and psychotic disorders, while neither psychomotor function nor vital signs have been adversely affected, demonstrating a more favorable safety profile compared to the commonly prescribed drugs for these disorders (Iffland & Grotenhermen, 2017). Nevertheless, *in vitro* and *in vivo* studies report some side effects related to cell viability alterations, decreased fertilization capacity, along with inhibition of enzymatically-induced hepatic metabolism and drug transporters, which need to be further investigated in human studies for potential long-term complications and interactions of CBD with other substances (Bergamaschi et al., 2011).

Because of its lipophilicity, CBD undergoes extensive first-pass metabolism and can have low bioavailability, depending on the route of administration and genetic polymorphisms of drug metabolizing enzymes, rendering its pharmacokinetics quite complex (Millar et al., 2018). Differences across species in affinity, capacity and duration of binding between CBD and its molecular targets are some of the crucial parameters controlling its effectiveness. Since polypharmacy has become integral to the management of anxiety- and trauma-related disorders and comorbid somatic conditions, drug interactions should be taken into consideration as many of the clinically prescribed medications are metabolized by the same isoforms of the cytochrome CYP450 family as CBD (Ujváry & Hanuš, 2016). Therefore, it is of crucial

importance to determine a thorough safety profile for CBD and co-administered substances, especially those with a narrow therapeutic window.

There is considerable evidence demonstrating that CBD induces both anxiolytic and modulatory effects on learned fear. Interestingly, it was found that CBD elicits acute reductions on learned fear expression, by facilitating 5-HT-ergic signalling (Campos & Guimarães, 2008; Gomes et al., 2011; Gomes et al., 2012; Fogaca et al., 2014; Marinho et al., 2015). Concomitantly, CBD produces sustained fear-suppressing effects by regulating several aspects of learned fear memory, disrupting either its consolidation (Stern et al., 2017; Raymundi et al., 2020) or reconsolidation (Stern et al., 2012; Bayer et al., 2022), and enhancing its extinction (Bitencourt et al., 2008; Do Monte et al., 2013). It is believed that CBD possibly facilitates endocannabinoid signaling indirectly through elevation of ECBs (Ligresti et al., 2016), however, the exact mechanism through which CBD mediates such effects is not fully elucidated. The extinction-enhancing effects of CBD may bear significant translational value, rendering CBD an interesting cannabinoid to explore its therapeutic potentials.

1.7 Pharmacology of the endocannabinoid system

The ECB system has multifunctional regulatory roles in various physiological processes, while it has attracted much attention for its involvement in the regulation of fear processing, rendering it a promising therapeutic target for the management of anxiety- and trauma-related disorder. It was suggested that several of the modulatory effects of CBD on learned fear memory, extinction and reconsolidation are possibly mediated indirectly through elevation in ECB levels and subsequent activation of

cannabinoid receptors. Therefore, it is very important to introduce the principal elements and mechanisms governing endocannabinoid signaling.

Major ECBs are AEA and 2-AG, while other endogenous ligands identified, like N-arachidonyldopamine, 2-arachidonylglyceryl ether and O-arachidonyl-ethanolamine, can also exert effects (Di Marzo & De Petrocellis, 2012). They are not stored in vesicles like classical neurotransmitters, instead they are synthesized on demand in the postsynaptic neuron in response to membrane depolarization, subsequent calcium influx through voltage gated calcium channels (VGCCs) and activation of metabotropic glutamatergic and cholinergic receptors (Figure 1.5). AEA synthesis starts from the membrane precursor N-arachidonoyl phosphatidylethanolamine (NAPE), either postsynaptically in BLA or presynaptically in hippocampus (Egertová et al., 2008). Production of AEA may follow four different enzymatic pathways, but the most significant is that involving NAPE hydrolysis by N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD). Another noteworthy pathway constitutes the cleavage of the NAPE phosphodiester bond by NAPE-phospholipase C (NAPE-PLC), followed by dephosphorylation of the resulting phosphoanandamide by phosphatase, liberating AEA (Liu et al., 2008). 2-AG derives from the hydrolysis of phosphatidyl-inositol bisphosphate by phospholipase Cβ and the subsequent hydrolysis of diacylglycerol (DAG) by diacylglycerol lipase α (DAGLα) (Tsuboi et al., 2018).

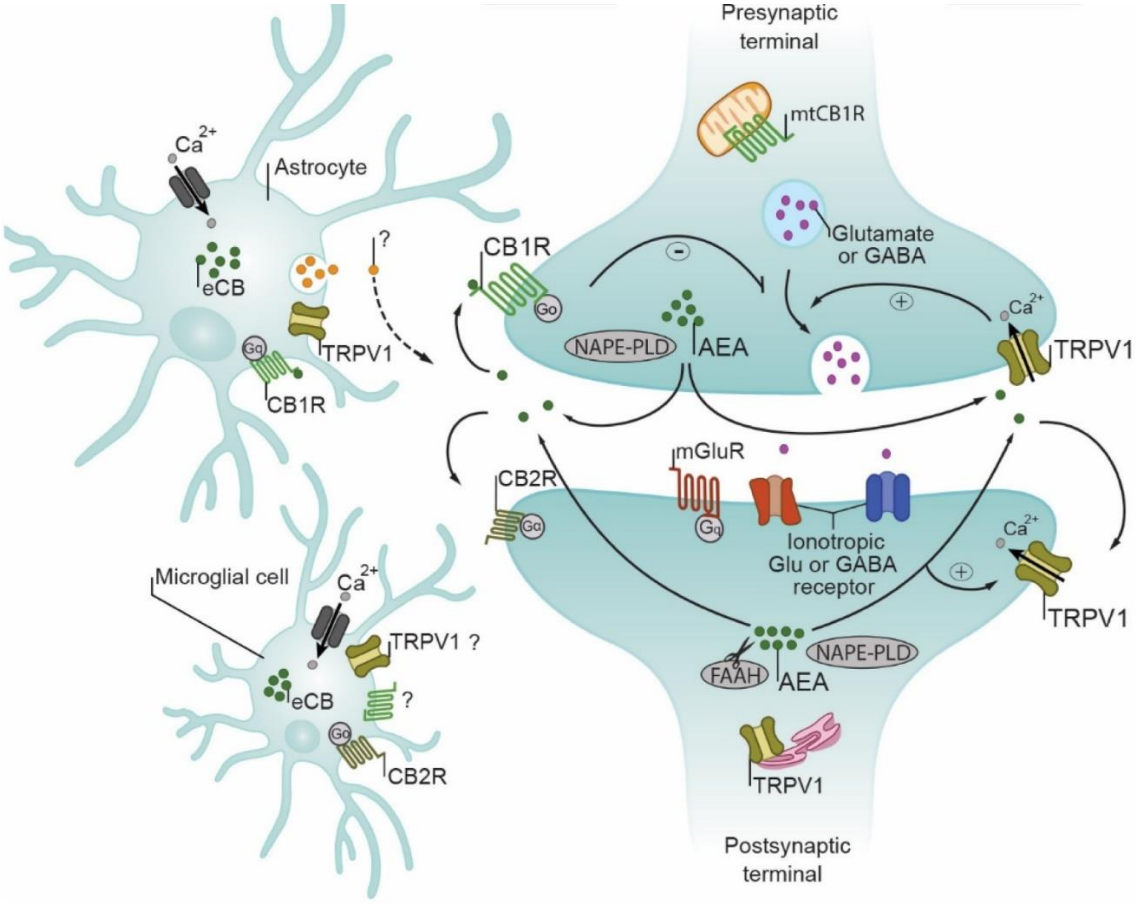

Figure 1.5 Schematic representation of retrograde, non-retrograde and neuron-astrocyte endocannabinoid signaling along with the components of the ECB system (Patel et al., 2017). ECBs are synthesized on demand in post-synaptic neuron and released in synaptic cleft. In retrograde signaling, ECBs activate pre-synaptic CB1Rs and suppress glutamate or GABA release. In non-retrograde signaling, ECBs stimulate post-synaptically expressed CB1Rs and TRPV1 channels, while in neuron-astrocyte signaling, ECBs stimulate CB1Rs that are expressed in adjacent astrocytes, leading to indirect modulation of synaptic function. ECB=endocannabinoid, AEA=anandamide, CB1R=cannabinoid 1 receptor, CB2R= Cannabinoid 2 receptor, TRPV1= transient receptor potential vanilloid type 1, FAAH=fatty acid amide hydrolase, NAPE-PLD= N-acyl phosphatidylethanolamine phospholipase D.

After their synthesis, ECBs are immediately released and travel across the synapse and bind to different receptors with distinct binding capacities and efficacies, mediating retrograde, non-retrograde and neuron-astrocyte signaling, playing a crucial role in the regulation of synaptic function. More specifically, 2-AG is a high efficacy agonist at both CB1Rs and CB2Rs, while AEA is a partial agonist with low efficacy at CB1Rs and even lower at CB2Rs, however it is a full agonist both at TRPV1 and PPARγ (Di Marzo & De Petrocellis, 2012). 2-AG mediates phasic signalling in the

ECB system, while AEA provides a tonic response, mitigating excessive neuronal excitability (Ahn et al., 2008). In retrograde signaling, ECBs activate CB1Rs found in the pre-synaptic terminals of excitatory or inhibitory neurons, exerting suppression of neurotransmitter release. Induction of short-term synaptic plasticity, in the form of either depolarization-induced suppression of excitation or depolarization-induced suppression of inhibition, is mediated by inhibiting presynaptic N and P/Q VGCCs and activating inward rectifier potassium channels. Long-term plasticity, in the form of either homosynaptic glutamatergic or heterosynaptic GABA-ergic long-term depression (LTD), is evoked through inhibition of adenylyl cyclase (AC) and subsequent downregulation of cyclic adenosine monophosphate (cAMP) production and protein kinase A activity (Lu & Mackie, 2016). Non-retrograde signaling occurs when AEA stimulates the post-synaptically co-expressed CB1Rs and TRPV1 channels. TRPV1 channel activation is crucial for the regulation of synaptic plasticity, as it induces postsynaptic LTD through stimulation of AMPA receptor endocytosis that leads to reduced glutamatergic signaling (Chavez et al., 2010; Ohno-Shosaku & Kano, 2014). Regarding neuron-astrocyte signaling, postsynaptically released ECBs target CB1Rs expressed in astrocytes adjacent to the synapse, resulting in gliotransmission and indirect modulation of presynaptic activity. Depending on the type of neuron that the astrocyte interacts with, this may result in either short-term plasticity or spike-timing-dependent LTD. The former occurs upon glutamate-mediated activation of mGluR1s expressed in presynaptic neurons, a process that enhances neurotransmitter release, while the latter results from activation of presynaptic NMDA receptors, which leads to reduced neurotransmitter release (Castillo et al., 2012).

The function of the ECB system is highly dependent on the maintenance of adequate endocannabinoid levels in synapses. Uptake of ECBs into the intracellular compartment and their subsequent enzymatic degradation, terminates their signaling activity (Ahn et al., 2008). Importantly, the exact mechanism through which ECBs cross cell membranes is not fully elucidated. Potential reuptake mechanisms may involve passive diffusion, transporter proteins, endocytosis, or combination of them (Baggelaar et al., 2018). It is believed that since ECBs are uncharged hydrophobic molecules, in contrast to other neurotransmitters, they do not require transmembrane transporters, an opinion that is still debated. To date, a putative endocannabinoid membrane transporter (EMT) has not been identified, and possibly AEA diffuses across the cellular membrane down the concentration gradient that is driven by the fatty acid amide hydrolase (FAAH) enzyme, which is the main degradative enzyme of AEA. Thereafter, fatty acid binding proteins (FABPs), by acting as intracellular carriers, shuttle AEA to FAAH for breakdown. Inhibition of FAAH or FABPs reduces the rate of AEA metabolism and therefore raises AEA levels available at the synapse (Deutsch, 2016). Although much less evidence exists regarding the uptake of 2-AG, Chicca and co-workers (2012) has previously suggested that there is a bidirectional membrane transporter that regulates the trafficking and metabolism of both ECBs.

FAAH maintains somato-dendritic localization at the membrane surface of Ca^{2+}-storing cytoplasmic organelles (i.e., endoplasmic reticulum) of principal cells, predominately distributed in the cerebellum Purkinje cells and the BLA while to a lesser extent in the CEA (Gulyas et al., 2004). It is responsible for the hydrolysis of AEA into its metabolites, arachidonic acid and ethanolamide, but also for the degradation

of other N-acylethanolamine type ECBs like N-palmitoyl ethanolamine and N-oleoyl ethanolamine (Gunduz-Cinar et al., 2013). Additionally, hydrolysis of AEA can be performed by N-acylethanoloamine hydrolyzing acid amidase resulting in the same metabolites, while oxidation constitutes another potential degradative pathway, mediated predominately by cyclooxygenase-2 (COX-2), producing prostaglandin E2–glycerol ester. Other noteworthy oxidative enzymes are CYP450 hydrolase, CYP450 epoxygenase and lipoxygenase (LOX) of the -5/ -8/ -11/ -12 and –15 subtypes that produce 20-HETE-EA, epoxytrienoic acid ethanolamides and 5-/ 8-/ 11-/ 12- HETE ethanolamides, respectively, as metabolites of AEA (Urquhart et al., 2015; Maccarrone, 2017).

The main degradative pathway of 2-AG is hydrolysis, through which it is converted into arachidonic acid and glycerol. This is primarily mediated by monoacylglycerol lipase (MAGL), which is distributed in granule cells, CA3 hippocampal pyramidal cells and in some interneurons, maintaining axono-terminal localization. The metabolism of 2-AG is also mediated to a lesser extent by alpha/beta domain-containing hydrolase 6, alpha/beta domain-containing hydrolase 12 and FAAH, located postsynaptically. Oxidation by COX-2 is another possible route of 2-AG metabolism, resulting in prostanoid glycerol ester formation, with prostaglandin E2 glycerol ester the most crucial for the enhancement of synaptic plasticity. However, LOX-12/ -15 and CYP450 epoxidase are additional oxidative enzymes responsible for 2-AG degradation into 12-/ 15-HETE-G and epoxytrienoic glycerol esters, respectively (Gulyas et al., 2004; Urquhart et al., 2015; Lu & Mackie, 2016; Tsuboi et al., 2018). Therefore, pharmacological manipulation of the ECB system at the level of cannabinoid receptors, or enzymes implicated in endocannabinoid biosynthesis or

degradation may comprise promising therapeutic targets for the treatment of anxiety and trauma-related disorders (Di Marzo, 2009). Selective inhibition of either FAAH or MAGL to elevate the levels of AEA or 2-AG is a very promising therapeutic approach with a more favorable adverse effect profile, with elevation of AEA levels by selective blockade of FAAH associated with less CB1Rs desensitization and behavioral tolerance in comparison to MAGL inhibition and 2-AG rise (Schlosburg et al., 2010).

1.8 Pharmacology of the serotonergic system

The ECB system was found to modulate the signaling of other neurotransmitter systems, with the serotonergic system attracting much attention not only due to its strong association with the regulation of anxiety, but also because CBD exerts acute effects on expression of innate and learned fear, by directly interacting with 5-HT1ARs. Therefore, before describing these interactions in the next sections, it will be useful to briefly introduce some key elements of serotonergic neurotransmission and circuitry.

5-HT is a widely distributed monoamine in neural and non-neural (e.g., gastrointestinal enterochromaffin cells, platelets) tissues, and plays a crucial role in various physiological processes by interacting with 14 receptor subtypes from seven families (i.e., 5-HT1, 2, 3, 4, 5, 6, 7). Six of them are G-protein coupled receptors, apart from 5-HT3 receptors (5-HT3Rs) that are ligand-gated ion channels. 5-HT can act locally in the synapse as a neurotransmitter or diffuse at extra-synaptic sites producing paracrine effects (De-Miguel & Trueta, 2005). In the brain, 5-HT-ergic neuronal cell bodies are restricted to the midbrain dorsal raphe nuclei (DRN) and median raphe nuclei (MRN), which synthesize, store, and release 5-HT through axonal projections to various cortical and limbic structures. The PFC, like other cortical areas, receives dense

convergent 5-HT-ergic innervation from both raphe nuclei, while descending projections to these nuclei regulate 5-HT release (Puig & Gulledge, 2011). Additionally, the DRN sends ascending projections to the amygdala, vHPC, lateral septum, and striatum, whereas the MRN innervates predominately the dHPC, hypothalamus, and medial septum (Hale & Lowry, 2011). Through this complex circuitry, 5-HT orchestrates a plethora of brain functions like perception, pain, mood, aggression, sleep, appetite, vomiting, temperature, and blood pressure, while impaired 5-HT-ergic signalling has been linked with the pathophysiology of several neuropsychiatric conditions like depression, anxiety, and migraine (Katzung, 2015).

Essential for the synthesis of 5-HT is the amino acid L-tryptophan (Figure 1.6), which is converted into 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase 1 (periphery) or 2 (brain) (Bader, 2020). Subsequently, 5-HTP is enzymatically transformed by aromatic amino acid decarboxylase into 5-HT, which is transported and stored in the vesicle with the help of vesicular monoamine transporter 2. 5-HT is released through exocytosis in response to depolarization of the presynaptic terminal and diffuses in the synaptic cleft to exert its effects after its binding to postsynaptic or presynaptic 5-HT receptors. Once neurotransmission is terminated, 5-HT is transferred across the presynaptic membrane by the serotonin reuptake transporter (SERT) and can be degraded by monoamine oxidase A and aldehyde dehydrogenase into 5-hydroxyindole acetic acid (Hensler, 2006).

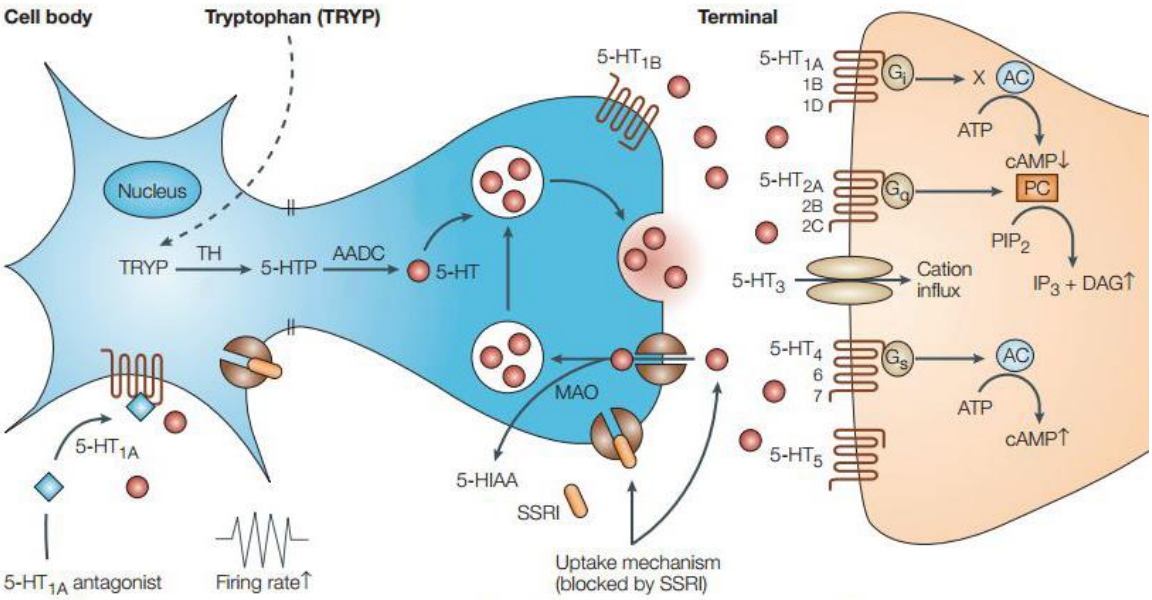

Figure 1.6 Schematic representation of processes and components involved in serotonergic signaling (Wong et al., 2005). TRYP=tryptophan, TH=tryptophan hydroxylase, 5-HTP=5-hydroxytryptophan, AADC=aromatic amino acid decarboxylase, 5-HT=5-hydroxytryptamine (serotonin), MAO=monoamine oxidase, 5-HIAA=5-hydroxyindole acetic acid, SSRI=serotonin reuptake inhibitor, ATP=adenosine triphosphate, AC=adenylyl cyclase, PC=Phospholipase C, DAG=diacylglycerol, PIP_2=phosphatidyl-inositol bisphosphate, IP_3=inositol triphosphate, cAMP=cyclic adenosine monophosphate.

Importantly, imbalanced 5-HT-ergic signalling was found to be implicated in the development of anxiety- and trauma-related disorders. Several studies have investigated the role of 5-HT1AR, 5-HT2AR, and 5-HT3R -mediated transmission in the modulation of fear learning and extinction processes (Homberg, 2012), while others have linked the presence of SERT gene polymorphisms with susceptibility to anxiety-like behaviours (Åhs et al., 2015). Interestingly, in response to stress or fear conditioning, increased neuronal excitability and 5-HT synthesis have been observed in the DRN, which is associated with elevated concentrations of 5-HT in both amygdala and PFC, areas that, along with the hippocampus, densely express 5-HT receptors (Krystal & Neumeister, 2009; Bauer, 2015). Specifically, 5-HT1ARs are inhibitory $Gi/_0$-protein coupled receptors that induce membrane hyperpolarization and inhibition of AC. Pre-synaptic 5-HT1ARs are distributed in the somatodendritic site of 5-HT-ergic

neurons in the raphe nuclei and, by acting as autoreceptors, inhibit neuronal firing and release of 5-HT from 5-HT-ergic terminals (Bockaert et al., 2006). 5-HT1ARs are also located postsynaptically in 5-HT-ergic synapses in pyramidal neurons and GABA-ergic interneurons of PFC and hippocampus, inhibiting neuronal excitability (Dong et al., 1998; Lladó-Pelfort et al., 2012; Lopez-Gil et al., 2010; Singewald et al., 2015). In contrast, 5-HT2ARs are excitatory G_q-protein coupled receptors that exert depolarizing effects, leading to enhancement of neuronal firing and presynaptic glutamate release (Stein et al., 2000; Singewald et al., 2015). 5-HT2AR agonism primarily stimulates phospholipase C that triggers the release of DAG and inositol triphosphate, which subsequently activate protein kinase C. 5-HT2ARs are widely expressed in the cerebral cortex (i.e., piriform, entorhinal), claustrum, olfactory bulb, basal ganglia (i.e., NAc, caudate nucleus), DRN, and BLA pyramidal and parvalbumin GABA-ergic interneurons, while showing both pre- and post-synaptic distribution (Singewald et al., 2015; Zhang & Stackman, 2015; da Silva Soares et al., 2019). On the other hand, 5-HT3Rs are excitatory ligand-gated ion channels, the activation of which leads to rapid depolarization by changing cation (Na^+, K^+, Ca^{2+}) conductance (Thompson & Lummis, 2006). Depending on their localization, either pre- or post-synaptic, 5-HT3Rs enhance neurotransmitter release (i.e., 5-HT, norepinephrine (NE), GABA, dopamine (DA), acetylcholine) or elicit excitatory postsynaptic potentials, respectively (Zhao et al., 2018). 5-HT3Rs have been identified in several areas in CNS including the NAc, raphe nucleus, substantia nigra, VTA, area postrema, nucleus tractus solitarius, entorhinal cortex, and GABA-ergic neurons of hippocampus and BLA (Cortes-Altamirano et al., 2018).

1.9 Interaction of endocannabinoid system with other neurotransmitters

As mentioned above, the ECB system interacts with other neurotransmitters to modulate their signaling in relation to the regulation of anxiety and learned fear, including glutamate, GABA, NE, and 5-HT. Bidirectional influences of the ECB system have been found, resulting in either anxiolytic or anxiogenic responses mediated by both augmentation and attenuation of endocannabinoid tone. This phenomenon is proposed to be greatly dependent on the localization, basal activation, and sensitivity of CB1Rs on GABA-ergic and glutamatergic neurons. More specifically, tonic activation with agonism of CB1Rs localized on GABA-ergic neurons, along with their high sensitivity to cannabinoids, was found to evoke anxiogenic effects through suppression of inhibition. On the other hand, phasic activation of CB1Rs localized on glutamatergic neurons and their lower sensitivity to cannabinoids are factors contributing to the development of anxiolytic effects by suppressing excitation after excess glutamate (Ruehle et al., 2012).

The ECB system plays a crucial role in regulating cognition and emotion by interacting with NE-ergic and 5-HT-ergic circuits. Cannabinoids directly or indirectly regulate activity of NE-ergic and 5-HT-ergic cells along with the release of their corresponding neurotransmitters, exerting complex effects from anti-depressive and anxiolytic effects to aversive behavior and disrupted attention. NE-ergic cells found predominately in locus coeruleus (LC) play a fundamental role in the control of cognition, vigilance, stress and selective attention. CB1Rs are mainly distributed postsynaptically on NE-ergic cells and, to a lesser extent, presynaptically at GABA-ergic and glutamatergic terminals arriving from prepositus hypoglossi and

paragigantocellular nucleus, respectively (Williams et al., 1991; Samuels & Szabadi, 2008). The firing of NE-ergic cells occurs in tonic or phasic modes. Tonic activation is associated with poor performance and attention, while phasic activation is linked with good task performance and focused attention. During basal conditions, GABA activates $GABA_A$Rs found at postsynaptic NE-ergic cells, decreasing their tonic activation. Glutamate activates AMPA receptors found postsynaptically to augment phasic activation of NE-ergic cells. Activation of CB1Rs leads to inhibition of GABA release and the subsequent increase in tonic activation of NE-ergic cells and, along with the enhancement of NMDA receptor-induced responses, is thought to account for the stimulatory effect. On the other hand, the CB1R-mediated inhibition of glutamate release leads to a decline in phasic activation of NE-ergic cells and to induction of an inhibitory effect (Ruehle et al., 2012; Mendiguren et al., 2018). Stimulatory effects on NE-ergic neuronal excitability resulting from a high dose of CB1R agonist leads to increased NE release and to a low phasic/ tonic activation ratio, which is linked to anxiogenesis and disruption of attention. Noteworthy are the mechanisms involved in the increase of NE levels, such as the enhancement of tyrosine hydroxylase activity, an enzyme involved in biosynthesis of NE, decrease in norepinephrine transporter activity, cannabinoid-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis, and the down-regulation of α_2-adrenergic receptors after chronic administration of CB1R agonists (Carvalho & Van Bockstaele, 2012).

The serotonergic and endocannabinoid systems act independently and also interact, modulating stress responses as both systems are implicated in the regulation of the HPA axis. ECBs are proposed to weaken HPA axis activation, facilitating 5-HT-

ergic signaling through 5-HT1ARs and 5-HT2ARs. It is suggested that the ECB system modulates the 5-HT-ergic system by influencing 5-HT release at the level of projection areas and by regulating the excitability of DRN neurons. This occurs because CB1Rs and the enzymes involved in ECB biosynthesis and metabolism are expressed in 5-HT-ergic neurons. The latter receive excitatory and inhibitory inputs from glutamatergic neurons in PFC and from local GABA-ergic interneurons, respectively. These neurons express presynaptic CB1Rs and their activation suppresses glutamate and GABA release. Therefore, ECBs exert indirect bidirectional modulation of 5-HT-ergic neurons by regulating their excitatory and inhibitory inputs. The balance between these inputs, along with the synaptic strength and the presynaptic CB1R will determine their excitability. The activation of CB1Rs by ECBs or exogenous agonists, or inhibitors of endocannabinoid degradative enzymes, lead to an increase in the firing activity of 5-HT-ergic neurons and 5-HT release, mediating antidepressant and anxiolytic effects (Haj-Dahmane & Shen, 2011; Geddes et al., 2016).

1.10 Endocannabinoid modulators as potential anxiolytics

As mentioned above, patients suffering from anxiety- and trauma-related disorders often do not respond to current pharmacological and psychotherapeutic treatments, showing limited responses with high relapse rates and impaired tolerance. Expression of ECB system components in brain areas implicated in emotional and cognitive processing of fearful stimuli has led to the idea of cannabinoids as potential therapeutic agents for these disorders, mainly by targeting the enhancement of aversive memory extinction and mitigation of stress responses after exposure to reminders (Singewald et al., 2015; Patel et al., 2017).

CB1R agonists have been found to mediate both anxiolytic and anxiogenic effects depending on the dose, the route of administration, the various sensitivities of CB1Rs expressed in different neuronal populations and the aversiveness of the testing model (Korem et al., 2016). AEA has anxiolytic-like effects with low doses through activation of CB1Rs, while anxiogenic effects appear with higher doses by stimulating TRPV1 receptors (Batista et al., 2015). Additionally, CB1R activation was found to enhance learned fear extinction, while opposing effects were exerted upon TRPV1 activation, leading to augmented fear expression (Moreira et al., 2012). Along with this, presynaptic and postsynaptic TRPV1 channel expression at brain areas implicated in anxiety like hippocampus, hypothalamus, BNST, and PAG, have rendered TRPV1 antagonists as emerging pharmacological agents in the treatment of anxiety disorders (Deng et al., 2016; Uliana et al., 2016). Agonism at TRPV1 in PAG, a region responsible for the control of coping strategies in response to fear conditioning, has anxiogenic-like effects, while TRPV1 blockade or activation of CB1Rs cause anxiolytic effects (Mascarenhas et al., 2013). Therefore, maintaining the balance between activation of TRPV1 and CB1R is crucial in controlling appropriate behavioural responses and aversive memory formation (Back & Carobrez, 2018). Interestingly, simultaneous blockade of FAAH and TRPV1 by N-arachidonoyl-serotonin (AA-5-HT) suppresses contextual fear memory retrieval by increasing AEA in the dorsal hippocampus, suggesting a potential therapeutic approach against traumatic memories (Gobira et al., 2017).

Another promising target for pharmacological intervention is the amplification of endocannabinoid signalling by blocking FAAH, MAGL and COX-2, or re-uptake mechanisms, which were found to have promising anxiolytic-like and fear-alleviating

properties. The investigation of MAGL inhibitors as potential therapeutics emanated from the fact that reduced circulating levels of 2-AG have been found in humans suffering from PTSD (Hill et al., 2013). JZL184, a MAGL inhibitor, was suggested to induce both antidepressant and anxiolytic-like effects through enhancement of synaptic plasticity and hippocampal neurogenesis (Zhang et al., 2015). However, contradictory findings have been identified regarding MAGL inhibitors as they potentially impair fear extinction, presumably through CB1R agonism at GABA-ergic interneurons (Llorente-Berzal et al., 2015). On the other hand, reduced AEA levels, resulting from stress-induced FAAH mobilization, are associated with increased BLA excitability, dendritic arborization and spinogenesis, along with exaggerated HPA-axis responses, which are all correlated with anxiety-like states (Hill et al., 2010). URB597, one of the most studied FAAH inhibitors, is proposed to reverse the aforementioned pathological findings and elicit anxiolytic-like effects in animal models of anxiety and PTSD by enhancing extinction through CB1R-mediated modulation of 5-HT-ergic and NE-ergic neurotransmission. This is interlinked with increased BDNF and modification of 5-HT1A and 5-HT2A/C receptor signalling in hippocampus and via astroglial CB1R-induced LTD in BLA (Bambico et al., 2016; Duan et al., 2017; Danandeh et al., 2018). It was found that systemic or central administration of URB597 into BLA or CA1 area of hippocampus before extinction suppressed fear expression across extinction sessions and later during spontaneous recovery. Simultaneously, URB597 prevented the alteration in ECB levels and metaplasticity within the BLA-CA1 induced by footshock and reminders in an inhibitory avoidance task, during which the innate preference of rodents to explore dark is suppressed following exposure to inescapable aversive stimulus (Ögren & Stiedl, 2010; Segev et al., 2018).

Analogous effects were observed after systemic URB597 administration in socially isolated rats subjected to inescapable foot-shocks. URB597 enhanced consolidation of extinction memory, resulting in long-lasting suppression of fear and normalization of social behavior, demonstrating better performance when compared to the MAGL inhibitor JZL184 or the CB1R agonist WIN55,212-2 (Morena et al., 2018). Remarkable at this point is the comparative study of JZ184 and the FAAH inhibitor PF-3845 with the dual FAAH/MAGL inhibitor JZL195. The two former agents are anxiolytic when administrated alone, while the latter has no effect, suggesting that the enhancement of endocannabinoid signalling caused by increased 2-AG or AEA levels alone has an anxiolytic-like effect, which does not arise after a simultaneous increase of both ECBs (Bedse et al., 2018). Another noteworthy mechanism of regulating anxiety states is via COX-2 inhibition mediated through two main mechanisms, the decrease in proinflammatory prostaglandins and increases in AEA and 2-AG levels by inhibiting their degradation. Both substrate selective COX-2 inhibitors, like LM-4131, and traditional ones, like Celecoxib, exert anxiolytic-like effects by reducing behavioural dysregulation after stress and expression of conditioned fear, without development of tolerance or impairment of locomotor activity (Hermanson et al., 2013; Gamble-George et al., 2016).

1.11 Molecular targets of cannabidiol

The possible mechanisms of action of CBD have been extensively investigated over the last decade. CBD binds with low affinity to CB1Rs and CB2Rs, acting as a negative allosteric modulator for the former and as a weak inverse agonist to the latter, properties that render it superior in relation to potential adverse side effects in

comparison with orthosteric modulators (Figure 1.7) (Laprairie et al., 2015; Ligresti et al., 2016). However, CBD can indirectly agonise CB1Rs and CB2Rs by increasing the levels of AEA through a decrease of FAAH-mediated degradation or inhibition of cellular uptake of AEA by targeting fatty acid binding proteins. CBD can also increase 2-AG levels, though to a lesser extent, by reducing MAGL-mediated degradation (De Petrocellis et al., 2011; Elmes et al., 2015). Interestingly, the neuroprotective, acute anxiolytic and antidepressant effects of CBD have been attributed to the agonism exerted at 5-HT1ARs in dorsal PAG, BNST and PFC (Campos et al., 2016; Russo et al., 2015; Zanelati et al., 2010). Like other cannabinoids, CBD was found to also interact with TRPV1 receptors, resulting in their activation and desensitization, which are associated with modulation of anxiety and nociception (Iannotti et al., 2014). Another noteworthy pharmacological target of CBD is the activation of PPARγ receptors, which act as a protective mechanism against reactive gliosis and neuronal damage, while eliciting positive effects on glucose and fatty acid metabolism (Esposito et al., 2011; Jadoon et al., 2016). CBD was also found to block the reuptake of adenosine and subsequently increase its levels through inhibition of the equilibrative nucleoside-transporter (ENT), thus inducing immunosuppressive effects by indirectly activating adenosine A2 receptors. Outside the CNS, CBD acts as a full agonist of adenosine A1 receptors, possibly exerting beneficial effects on cardiac arrythmias and myocardial ischemias (de Almeida & Devi, 2020). Furthermore, CBD, by partially agonising D2Rs, may elicit antipsychotic effects or be involved in emotional memory processing through D2Rs in vHPC (Campos et al., 2016; Ligresti et al., 2016; Seeman, 2016). CBD, by acting also as a negative allosteric modulator at μ- and δ-opioid receptors, may exert beneficial effects in reducing opioid and ethanol-seeking behavior, while

controlling drug abuse relapse and withdrawal symptoms. Lastly, CBD demonstrates modulatory effects on neuronal excitability by inhibiting Na^+ and Ca^{2+} channels, rendering a possible mechanism for CBD's antiepileptic effects (de Almeida & Devi, 2020).

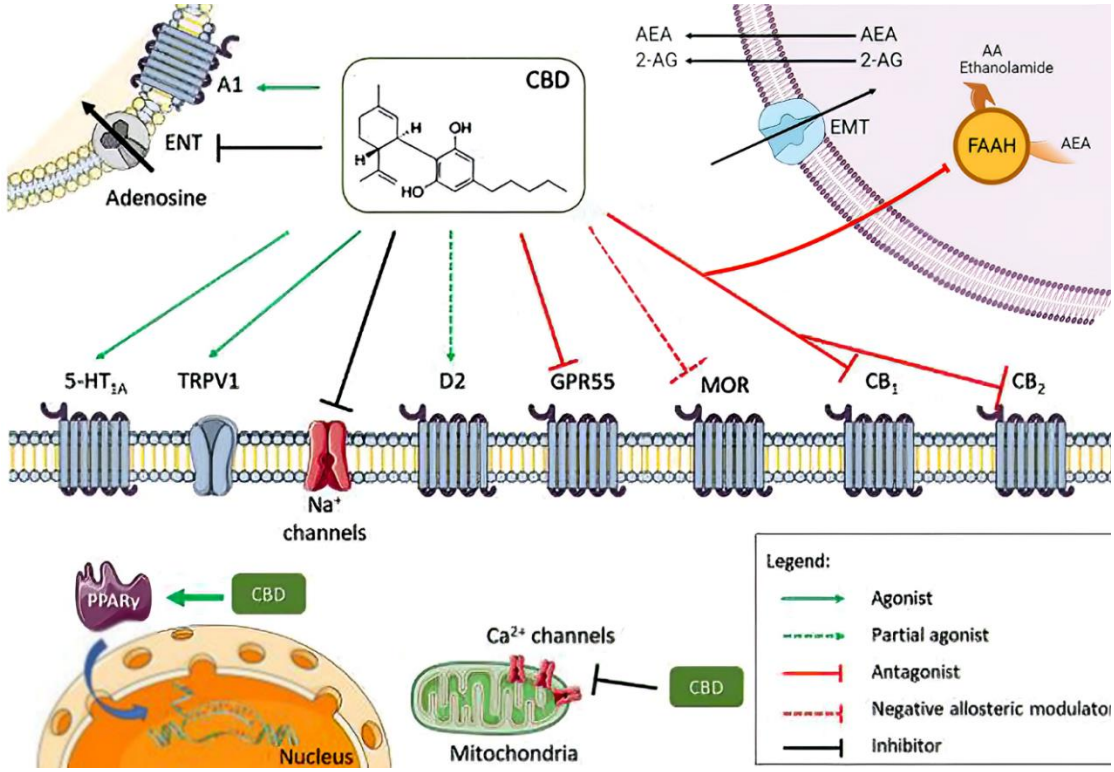

Figure 1.7 Molecular targets of CBD (adapted from de Almeida & Devi, 2020). 5-HT1A= serotonin 1A receptor, TRPV1= transient receptor potential cation channel subfamily V member 1, D2= dopamine 2 receptor, GPR55= G protein-coupled receptor 55, MOR=μ-opioid receptor, CB1/CB2=cannabinoid receptors, AEA-anandamide, 2-AG=2-Arachidonoylglycerol, AA=arachidonic acid, FAAH=fatty acid amide hydrolase, EMT=endocannabinoid membrane transporter, A1=adenosine 1 receptor, ENT= equilibrative nucleoside transporter, PPARγ=peroxisome proliferator-activated receptor gamma.

1.12 Cannabidiol's involvement in neuroplasticity and neuroprotection

Preclinical data have revealed that anxiety is associated with decreased hippocampal neurogenesis and stress-induced synaptic remodelling, in which reduced hippocampal volume is highly interlinked with overgeneralization of the aversive context (Fuchs et al., 2014; Levy-Gigi et al., 2015). These effects were reversed after chronic treatment

with SSRIs and a 10-week course of prolonged exposure therapy, in clinical studies (Rubin et al., 2016). Restoration of synaptic function can result from a combination of several phenomena, like promotion of dendritic remodelling, elevation of BDNF, increase in synaptic protein expression, like synapsin I/II, synaptophysin and post-synaptic density protein 95, and normalization of metabotropic glutamatergic receptors (Campos et al., 2017). Cannabinoids regulate neurogenesis via CB1R and CB2R activation by influencing proliferation, differentiation, survival, and migration of neural progenitor cells through induction of the phosphoinositide 3 kinase/ protein kinase B/ mammalian target of rapamycin (PI3K/Akt/mTOR) and mitogen-activated protein kinase kinase/mitogen activated protein kinase/cAMP-response element binding protein (MEK/MAPK/CREB) cascades. CREB, acting as a transcription factor, stimulates the production of BDNF, which is mandatory for hippocampal neural stem cell proliferation through its involvement in BDNF-tyrosine receptor kinase B signalling (Prenderville et al., 2015).

Preclinical studies using chronic unpredictable stress (CUS) and neurodegenerative models have revealed that the behavioural effects induced after acute or chronic CBD administration are mediated by facilitation of hippocampal neurogenesis, restoration of synaptic remodelling, and expression of intracellular protein glycogen synthase kinase 3β (p-GSK3β). In addition, reductions in FAAH activity and reactive microglial activation, along with modulation of autophagy cascades were found to induce neuroplastic and neuroprotective effects (Campos et al., 2017). The anxiolytic effects exerted by repeated CBD administration in the CUS model are attributed to neuroplastic changes induced by facilitation of CB1R and CB2R-mediated signalling (Fogaca et al., 2018). Chronic CBD administration was also

found to suppress proinflammatory responses by modulating intracellular cascades like Akt, extracellular-signal-regulated kinase 1/2, p-GSK3β, mTOR, through CB1R, CB2R and PPARγ-mediated activation (Campos et al., 2017).

1.13 Effectiveness of cannabidiol in learned fear

Preclinical studies have shown that CBD can affect every phase of the fear conditioning process by attenuating memory formation and retention, resulting in a reduction of conditioned fear behaviour. Resstel et al., (2006) showed that administration of CBD before fear retrieval testing acutely attenuated the expression of freezing behaviour and cardiovascular responses when rats were re-exposed to the conditioned aversive context. Lemos et al. (2010) confirmed the effectiveness of systemically administered CBD in attenuating contextual fear memory expression and further identified the involvement of PL and BNST through behavioural and c-fos immunoreactivity studies. Additionally, by directly infusing CBD into PL and IL, they found that the latter induced opposing effects by exaggerating fear responding. Two later studies revealed that the suppressing or enhancing effects induced by intra-PL or intra-IL CBD, respectively, on the contextual fear memory expression were mediated through a 5-HT1AR-dependent mechanism, given that pre-administration of the 5-HT1AR antagonist WAY100,635 reversed the effects of CBD (Fogaca et al., 2014; Marinho et al., 2015).

CBD was also found to attenuate aversive memory formation. Norris et al. (2016) demonstrated that intra-NAc shell CBD infusion dose-dependently blocked fear memory acquisition and prevented subsequent freezing behaviour by decreasing dopaminergic activity within the mesolimbic pathway through 5-HT1AR-mediated

signalling. However, few studies report conflicting effects on learned fear expression when CBD is administered before fear conditioning. Specifically, CBD administration before trace fear conditioning (i.e., during which CS is separated from US by short temporal gap) enhanced fear acquisition, while the following day augmented fear generalization in a novel context and impaired extinction of auditory fear memory (Uhernik et al., 2018). Analogous effects were observed after chronic administration of CBD for 14 days before fear conditioning, which led to increased expression of learned fear upon retrieval testing, indicating that CBD enhanced fear acquisition (ElBatsh et al., 2012). In contrast, administration of CBD for 21 days did not affect acquisition of conditioned fear in a transgenic mouse model of Alzheimer's disease (Cheng et al., 2014).

Noteworthy is that fear memory consolidation involves time-dependent synaptic reorganization, rendering the intervention time-window after acquisition narrow and specific. Rossignoli et al. (2017) demonstrated that bilateral intra-PFC infusion of CBD 5 hrs post-conditioning reduced the freezing behaviour 5 days later during fear retrieval test, while no effect was produced with CBD administration 0 hr after conditioning. This effect was associated with disruption in immediate early gene expression (i.e., c-fos and zif-268) in memory consolidation-specific regions (i.e., hippocampus, mPFC, midline thalamus) along with decreased DA-ergic signaling in PFC. Additionally, in a protocol of varying foot-shock intensity in rats, systemic administration of CBD was found to disrupt consolidation of both specific fear memories through reduction of fear expression and of more generalized ones through inhibition of fear generalization and disruption of extinction resistance, involving an AEA-mediated activation of CB1Rs and CB2Rs in dorsal hippocampus (Stern et al.,

2017). Since the principal concern in the treatment of PTSD is the relapse of symptomatology and return of fear, the identification of which memory stage interference could produce more efficient and long-lasting fear reduction, and which brain areas are implicated, have been the major focus of recent studies. Therapeutically intervening during acquisition or consolidation is considered controversial, as it should be applied as closely to the time of exposure to the traumatic event, which is not always feasible, while not all the individuals exposed to trauma will develop PTSD (Bernardy & Friedman, 2015; Bitencourt & Takahashi, 2018).

As mentioned above (section 1.4) extinction is a form of inhibitory learning that results in the formation of new memory trace that competes with and suppresses fear memory expression, involving predominately IL and ITC of amygdala. It is proposed that CBD may indirectly facilitate CB1R signalling through FAAH inhibition, resulting in decreased fear responsiveness to aversive memories by extinction enhancement and reconsolidation blockade (Chhatwal et al., 2005; Stern et al., 2012; Jurkus et al., 2016). Administration of CBD before extinction training was found to induce persistent freezing reduction by promoting the extinction of contextual fear. This response was reversed with pre-administration of the CB1R antagonist SR141716A but not altered by the TRPV1 antagonist capsazepine, suggesting that the facilitation of extinction was CB1R-dependent (Bitencourt et al., 2008). Interestingly, Song et al. (2016) found that CBD has bidirectional effectiveness in modulating contextual fear memory extinction, likely via the stress levels induced by conditioning rather than the strength of fear memory. It was found that CBD acutely reduced fear memory expression during extinction training and 24 hrs later at retention testing by enhancing its extinction after strong conditioning, while impairing extinction when conditioning was weaker.

CBD was found to mitigate both recent and older contextual fear memories by blocking their reconsolidation, leading to a prolonged freezing reduction and resistance to reinstatement and spontaneous recovery of fear. This effect was observed when CBD administration was restricted to a specific time-window of less than 6hrs after reactivation and was dependent on CB1R- rather than 5HT1AR-mediated signaling (Stern et al., 2012). In another preclinical study evaluating the effectiveness of THC alone and when co-administered with CBD, fear memory maintenance was attenuated in both conditions without interference of locomotor activity through disruption of contextual fear memory reconsolidation, resulting in reduction in the freezing response (Stern et al., 2015).

1.14 Cannabidiol: from preclinical to clinical studies

Data from preclinical studies highlight the effectiveness of CBD in attenuating the formation, disrupting the reconsolidation, enhancing the extinction of fear memory, and along with its favourable safety profile, have rendered necessary its evaluation in the clinical milieu. Recent clinical and case report studies in either healthy individuals or patients suffering from PTSD, and receiving CBD alone or in combination with THC, have revealed significant improvements in alleviation of symptoms (Passie et al., 2012; Greer et al., 2014). Interest attracts a case report of a ten-year old girl diagnosed with PTSD after experiencing partial relief and major adverse effects from previous pharmacotherapy. A maintenance dose of CBD oil trial relieved her anxiety symptoms and steadily improved her sleep quality (Shannon & Opila-Lehman, 2016). Encouraging results arise also from a retrospective, open label case series study, in which adult PTSD patients received oral formulations of CBD in combination with pharmacotherapy and psychotherapy for 8 consecutive weeks. CBD

demonstrated good tolerability in the majority of patients and reduced PTSD symptomatology based on a self-assessment questionnaire undertaken periodically every 4 weeks, while a subgroup suffering from nightmares presented significant improvement (Elms et al., 2019). On the other hand, evidence from a clinical study with healthy individuals revealed that CBD inhalation after extinction of visual fear memory resulted in attenuation of contextual fear expression during a retrieval session, suggesting that CBD facilitated the extinction memory consolidation. In contrast, pre-extinction CBD did not produce any effects on extinction acquisition or retrieval but demonstrated a trend-like reduction in reinstatement of the autonomic fear response (Das et al., 2013). In addition, a double-blind randomized trial has recently revealed that the CBD augmentation of therapist-assisted exposure therapy did not improve the overall treatment response of patients diagnosed with panic disorder with agoraphobia or social anxiety disorder, failing to enhance the learning and consolidation of extinction memory (Kwee et al., 2022). Another interesting outcome arises from a recent double-blind trial with adult PTSD patients receiving oral CBD formulation before the recall of previous traumatic experience. CBD reduced the cognitive impairments induced by the recall of aversive memory when compared to placebo, an effect that lasted for up to one week later, indicating that CBD disrupted fear reconsolidation. However, CBD failed to mitigate the increases in physiological or emotional responses (Bolsoni et al., 2022). These studies demonstrate promising evidence that CBD alone or in combination with exposure techniques can provide long-term alleviation in a diverse spectrum of symptoms associated with maladaptive traumatic memories, while encouraging further research for the exploration of

potential CBD effects in prevention from fear relapse and the understanding of its pharmacological and neurobiological underpinnings.

1.15 Aims of study

Given the supporting preclinical and clinical evidence regarding the beneficial effects of CBD in fear suppression, this thesis aimed to expand knowledge and investigate its effectiveness on modulation of extinction and relapse of auditory and contextual learned fear. The initial aim was to establish and validate a Pavlovian fear conditioning protocol that permitted extinction and subsequent return of learned fear memory under conditions of renewal, reinstatement, or spontaneous recovery. By using one such protocol, experiments aimed to investigate the potential effects of CBD in preventing fear relapse and determine whether this was achieved due to enhancement of acquisition or consolidation of extinction, given that both approaches have been found to promote extinction recall and reduce expression of learned fear. The next aim was to elucidate the pharmacological mechanisms underlying such effects of CBD. Experiments examined the potential involvement of CB1R- or 5-HT1AR-mediating signalling, since these constitute principal targets through which CBD elicits fear-alleviating and anxiolytic-like effects. Initially, dose-response studies were performed using CB1R or 5-HT1AR antagonists, which later were administered in combination with CBD. The last aim was to investigate whether CBD can ameliorate stress-induced impairments in extinction learning triggered by recent fear conditioning, using an immediate extinction deficit (IED) protocol.

Chapter 2. Validation studies of spontaneous recovery, reinstatement, and renewal of learned fear

2.1 Introduction

PTSD is a chronic debilitating mental condition that can develop after experiencing or witnessing an extreme traumatic event. It appears as a combination of symptoms like re-experiencing in response to trauma reminders, avoidance behavior, generalized hypervigilance, experiencing dissociation phenomena and presenting negative alteration of cognition and emotion (Friedman et al., 2011; Stoddard Jr, 2018). Despite the application of psychotherapeutic methods alone or in combination with antidepressants, which constitute standard management of PTSD, patients often reach inadequate remission or relapse after treatment discontinuation, rendering this disorder a long-term disability and demanding the development of new treatments (Murrough et al., 2015; Watkins et al., 2018).

The development and repurposing of compounds enhancing the extinction process has attracted an increased interest and researchers are focused on overcoming the great challenge of the simultaneous reduction of fear and its relapse (Singewald et al., 2015). Among them, CBD demonstrates promising therapeutic potential due to its beneficial effects on the disruption of fear memory consolidation (Stern et al., 2017), the attenuation of contextual fear memory expression (Lemos et al., 2010), the enhancement of extinction (Das et al., 2013; Do Monte et al., 2013), and the blockade of fear memory reconsolidation (Stern et al., 2012; Stern et al., 2015). However, further research is needed to identify whether CBD can elicit potential long-term effects on fear extinction and subsequently decrease the return

of contextual and auditory fear. The following validation studies aim to determine optimal parameters for establishing protocols of spontaneous recovery, reinstatement, and renewal of learned fear for assessing the effectiveness of CBD in extinction and prevention of fear relapse, and later to decipher the candidate mechanisms that mediate its effects. The design and parameters used are based on previous studies performed by our laboratory (Fenton et al., 2016; Jurkus et al., 2016) or modified protocols from studies found in the literature (King et al., 2018a; Vasquez et al., 2019).

2.2 Materials and methods

2.2.1 Subjects

Male Lister-Hooded rats (Charles River, UK), weighing 250-350 g were used in these validation experiments. Rats were group-housed in individually ventilated cages (3-4/ cage) in the Bio-Support Unit at the University of Nottingham, Sutton Bonington Campus. The animals were held under controlled temperature (~22°C), humidity (60%), and illumination (12h light/dark cycle, lights on at 8:00 am) conditions with a*d libitum* access to water and food, while wooden chew sticks and cardboard tunnels were placed in the home cages for environmental enrichment purposes. Behavioral testing was undertaken during the rats' light cycle, between 9:00 am and 5:00 pm, and approximately at the same time each day (± 1 hour). Rats were humanely culled with rising CO_2 concentration at the end of each experiment. All experimental procedures and animal care were conducted under the principles of refinement and reduction for the use of animals in preclinical research and performed under internal ethical approval and in accordance with the Animals (Scientific Procedures) Act 1986, UK (PPL

30/3230). The number of animals used for the following experiments was estimated based on power analysis and statistical power calculations from previous studies conducted in the lab investigating behavioural effects of systemically or centrally administered compounds on learned fear (Fenton et al., 2016; Jurkus et al., 2016; Stubbendorff et al., 2019). Data from approximately 8-12 animals per group will be needed to achieve statistically significant results, assuming a two-tailed hypothesis with significance level of $\alpha = 0.05$, moderate-to-large effect sizes with $f > 0.25$, and power > 0.8.

2.2.2 Apparatus

The behavioral procedures were conducted in four chambers with dimensions 30 x 24 x 30 cm, placed in sound attenuated boxes, containing a ventilation fan. The sidewalls were constructed of aluminum, whilst the ceiling, anterior and posterior walls were Perspex with a distinct pattern of dots or stripes (Figures 2.1 -2.3). The grid floor was made of 19 stainless steel bars of 0.5 cm diameter, placed 1.5 cm apart, and connected to an electric current generator (Med Associates, US), while a speaker was installed for tone emission. The shocks and tones were automatically delivered using MED-PC IV (Med Associates, US) software, whereas freezing behavior was digitally tracked by a camera located above the chamber and analyzed using ViewPoint software (ViewPoint Behavior Technology, France).

2.2.3 Experiment 1: Spontaneous recovery validation protocol

Rats underwent contextual habituation, auditory fear conditioning and extinction training, followed by extinction testing and spontaneous recovery, using a 24-day protocol (Figure 2.1), the design of which was based on parameters previously

used for studies in the lab (Fenton et al., 2014). On day 1, all animals (n=12) were habituated in two distinct contexts for a 10 min session, initially in context B and then in context A. Context B was defined as lights on, Perspex floor, and 40 % ethanol. The rats were gently handled before being transported within a transparent Perspex cage, containing a bedding material similar to their home cages, and introduced to the behavioral chambers with dotted black and white walls. Immediately after the end of the testing session, the rats were returned to their home cages in the same way as described above. Context A was designed as lights on, grid floor and 0.5 % acetic acid. Animals entered the chambers having black and white lines, transported with an opaque white plastic pot, containing post-surgical recovery bedding. On day 2, all rats were subjected to auditory fear conditioning in context A for a 25 min session. Rats were acclimatized for 2 min pre-CS interval, before receiving tone (CS) habituation, consisting of 5 CS presentations of 30 sec duration, 80 dB amplitude and 4 kHz frequency, with a 2 min inter-tone interval (ITI). Auditory fear conditioning commenced 2 min after the last tone presentation and consisted of 5 tone-shock (CS-US) pairings, with each tone (CS) lasting for 30 sec and co-terminating with an electric foot-shock (US) of 0.5 sec duration, current intensity I=0.4 mA, and ITI duration of 2 min. On day 3, all animals underwent fear extinction training in Context B during a session lasting 31 min and 30 sec. Initially, rats were tested during the 2 min pre-CS interval for their contextual fear memory, before being exposed to 30 CS presentations of 30 sec duration each and separated by ITIs of 30 sec. On day 4, the rats were submitted to a 4 min and 30 sec extinction testing session in Context B, consisting of a 2 min pre-CS period, before receiving 3 CSs separated by ITIs of 30 sec duration. On day 24 (i.e., 21 days after extinction training), all animals were returned

to Context B for spontaneous fear recovery testing and subjected to identical parameters as described above during the extinction testing on day 4. Immediately after the end of each session, the rats were removed and returned to their home cages, while the chambers were cleaned with the same solution as used for olfactory stimulus during behavioral testing, i.e., 40 % ethanol in context B or 0.5 % acetic acid in context A.

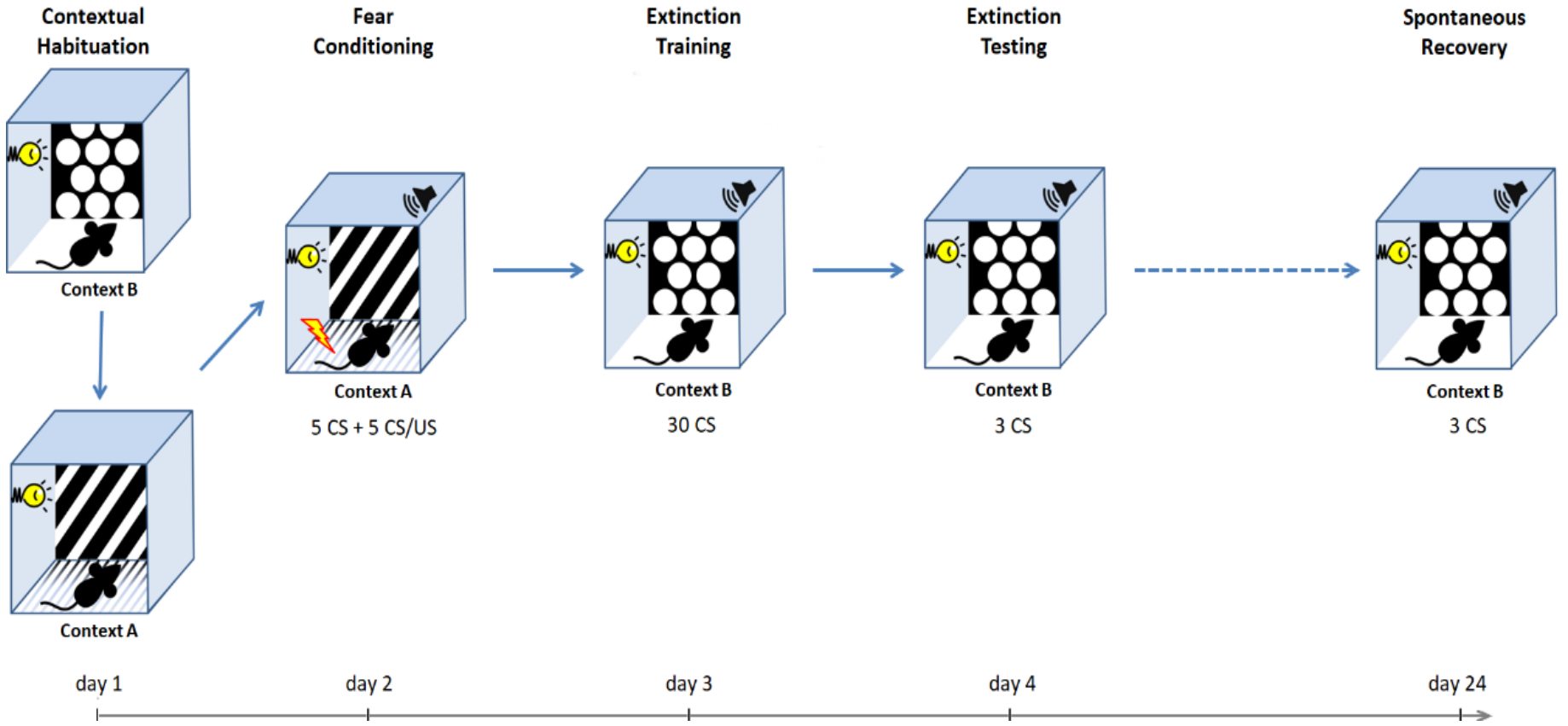

Figure 2.1: Design of spontaneous recovery protocol. On day 1, all animals were habituated in context B and then in Context A. On day 2, they underwent fear conditioning session with 5 CS + 5 CS-US pairings in Context A. On day 3, rats were subjected to extinction training receiving 30 CS presentations in Context B. On day 4, all rats were exposed to 3 CS for extinction testing, in Context B. All animals were returned to Context B 21 days after extinction training for a spontaneous recovery session, receiving 3 CS presentations.

2.2.4 Experiment 2: Reinstatement validation protocol

Using a 5-day protocol, all rats (n=20) underwent contextual habituation, auditory fear conditioning and extinction training on days 1, 2 and 3, respectively, as described above for Experiment 1, followed by reinstatement and extinction testing (Figure 2.2), the design of which was based on studies found in the literature (King et al., 2018a). Specifically, 24 hours after extinction training, the animals were randomly allocated into two numerically equal groups and subjected to a 3 min reinstatement session, in a modified version of Context B, where the Perspex floor was removed. The shock group (n=10 rats) was placed in the chambers and after 2 min received a single unpaired shock with 0.4 mA intensity and 0.5 sec duration, before being removed one minute later. In contrast, the animals of control group (n=10 rats) were introduced to the same context for 3 min without receiving any shock. On day 5, all rats underwent extinction testing in Context B, as previously described in Experiment 1.

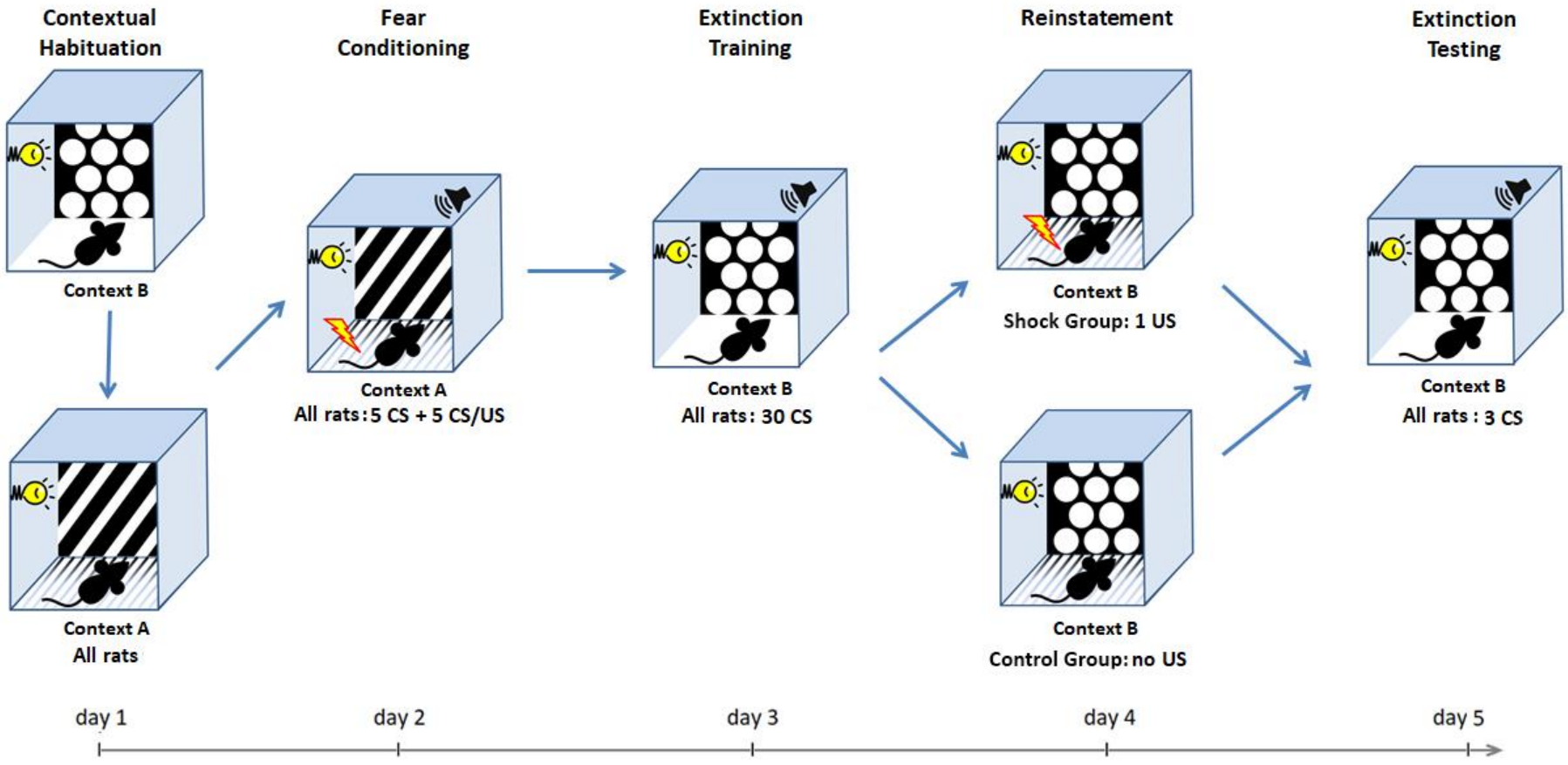

Figure 2.2: Design of Reinstatement Protocol. On day 1, all animals were habituated in context B and then in Context A. On day 2, they underwent fear conditioning session with 5 CS + 5 CS-US pairings in Context A. On day 3, rats were subjected to extinction training receiving 30 CS presentations in Context B. On day 4, rats were allocated into control and shock groups and either exposed to modified Context B or received an unreinforced US during reinstatement session. On day 5, all rats underwent extinction testing in Context B, receiving 3 CS presentations.

2.2.5 Experiment 3: Renewal validation protocol

All animals (n=10) underwent contextual habituation, auditory fear conditioning and extinction training, followed by renewal and extinction testing, using a 5-day protocol. The design of contextual alternation was based on the "ABA-scheme" (Vasquez et al., 2019), where fear conditioning and renewal testing were performed in the same context while extinction training and testing were conducted in a different one (Figure 2.3). In this experiment, Context A was designed as lights on, grid floor and 0.5 % acetic acid. In an attempt to make the context even more distinct from each other, a modified version of Context B was used, turning the lights off, but applying the Perspex floor and 40 % ethanol as described for other experiments. Similar to above, on day 1, all rats were habituated firstly in Context B and then in Context A. The next day, they underwent auditory fear conditioning in context A, receiving 5 CS presentations, followed by 5 CS-US pairings. On day 3, all animals were submitted to extinction training of 30 CS presentations in Context B. Across the fourth and fifth days of the protocol, rats were counterbalanced, and subjected initially either to renewal in Context A and then to extinction testing in context B, or vice versa, receiving 3 CS presentations on each session. The parameters used during this experiment, like electric current intensity, duration and/or number of pre-CS intervals, CS presentations, US-CS pairings, and ITI intervals, were identical to as described in Experiment 1 and 2.

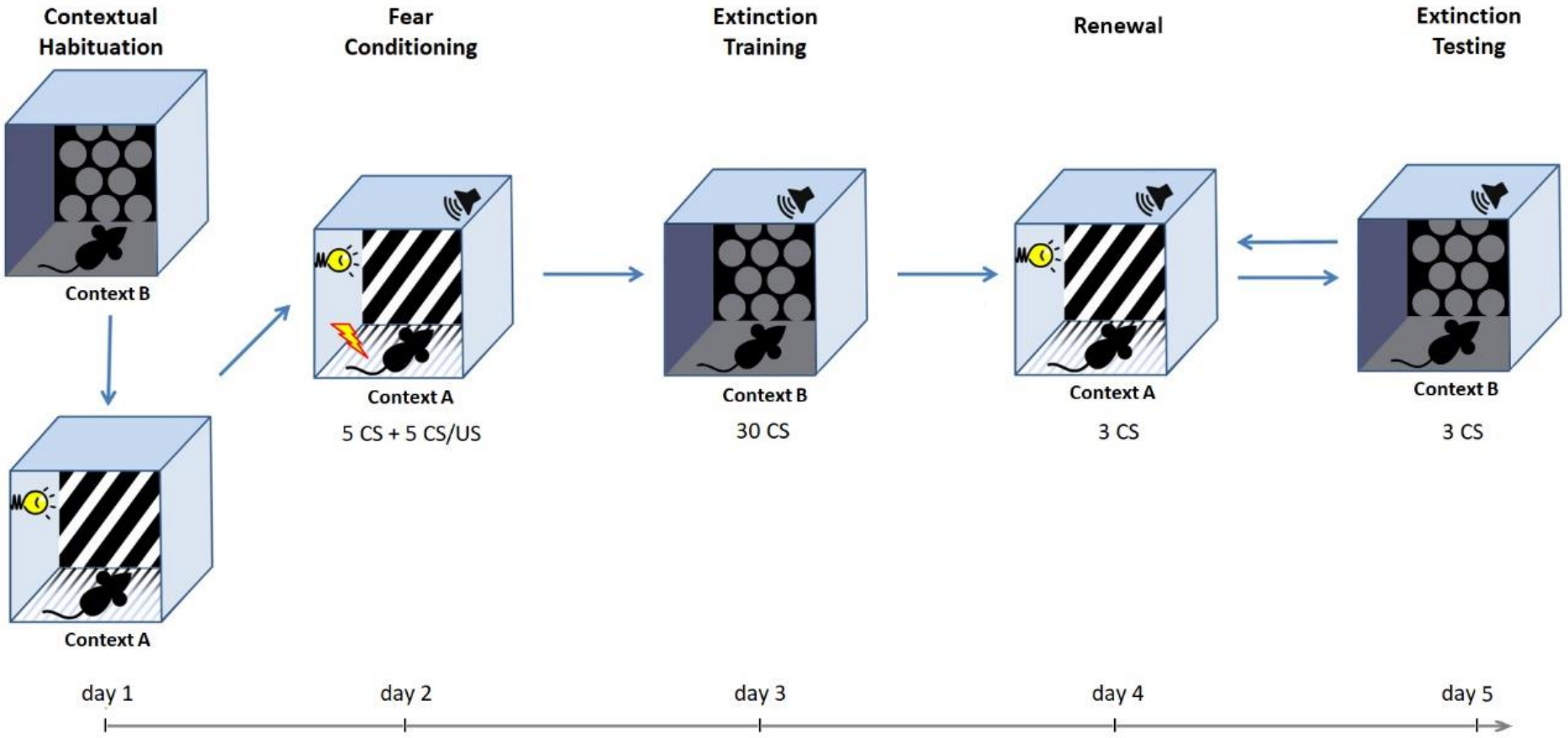

Figure 2.3: Design of Renewal Protocol. On day 1, all animals were habituated in context B and then in Context A. On day 2, they underwent fear conditioning session with 5 CS + 5 CS-US pairings in Context A. On day 3, rats were subjected to extinction training, receiving 30 CS presentations in Context B. Across days 4 and 5, rats were counterbalanced to renewal in Context A and extinction testing in Context B, receiving 3 CS presentations in each session.

2.3 Data analysis

Freezing behaviour was continuously recorded and automatically scored, using a video tracking software (Viewpoint, France) during each testing session, apart from contextual habituation, in all validation experiments. Freezing is defined as the absence of movement except for respiration (Fanselow, 1994) and was expressed as a percentage of freezing per pre-CS or CS intervals of 120 or 30 sec, respectively. All data are presented as mean ± SEM and statistical comparisons were performed using GraphPad Prism 9, while $p < 0.05$ was considered the level of statistical significance for all comparisons. Specifically, in spontaneous recovery validation experiment, fear conditioning was analyzed using one-way ANOVA, with trial as between-subject factor. The contextual fear memory during pre-CS interval of extinction training was presented separately as mean of freezing percentage. Auditory fear during the extinction training was expressed in blocks of 3 consecutive CS presentations, the freezing of which was averaged and then analyzed through one-way ANOVA, with block to be determined as between-subject factor. In order to identify whether there was fear return over time, contextual and auditory fear memory expression between extinction test and spontaneous recovery were compared, by using the freezing levels during the pre-CS intervals, and the mean of freezing levels during the 3 CS presentations, expressed as CS blocks. Statistical analysis was performed using two-way ANOVA, with cue and time as between- and within-subject factors, respectively.

In reinstatement validation experiment, contextual and auditory fear were expressed as percentage of freezing per pre-CS and CS intervals, respectively, as described above. Freezing differences during the CS-US pairings of fear conditioning were analyzed using two-way ANOVA, with group as between-subject and trial as

within-subject factors. Comparisons for the data of pre-CS interval during extinction training were performed through unpaired two-tailed t-tests, while two-way ANOVA was conducted for the CS blocks of extinction training, with group and block as the between- and within-subject factors, respectively. Finally, freezing differences between rats that were previously subjected to reinstatement or not, across pre-CS interval and CS block, were analyzed in single extinction test using two-way ANOVA, with group as the between-subject and cue as the within subject factors.

Likewise, to the first experiment, comparisons of freezing levels during fear conditioning and extinction training in renewal validation experiment were performed using one-way ANOVA, with trial or block to be determined as between-subject factor, respectively. In order to identify whether there was return of fear when the rats were tested outside the extinction context, the contextual and auditory fear memory expression between renewal and extinction test were compared, by using the freezing levels during the pre-CS intervals, and the mean of freezing levels during the 3 CS presentations, expressed as CS blocks. Statistical analysis was performed using two-way ANOVA, with cue and time as between- and within-subject factors, respectively.

In the following three experiments, Geisser-Greenhouse correction was applied to adjust for lack of sphericity, whilst Tukey's tests were conducted to compare every mean with every other mean where indicated.

2.4 Results

2.4.1 Experiment 1: Spontaneous recovery

The freezing levels during CS-US pairings in the fear conditioning session are presented in Figure 2.4.A. One-way ANOVA revealed a significant main effect of trial

($F_{(2.921, 32.13)}$ = 3.759, P = 0.0210), and increased freezing during trial 1 vs trials 2 and 3 (P < 0.05), indicating effective fear acquisition. Freezing during pre-CS interval before extinction training is shown in Figure 2.4.B, demonstrating low expression of contextual fear memory. Figure 2.4.C depicts the freezing levels across the CS blocks during extinction training session. One-way ANOVA revealed a significant main effect of block ($F_{(4.741, 52.15)}$ = 14.63, P < 0.0001), and decreased freezing during trial block 1 vs 5 (P < 0.05), 6, 7, 8 (P < 0.01), 9, 10 (P < 0.001), trial block 2 vs 4, 5, 6, 7, 8 (P < 0.01), 9, 10 (P < 0.001), and trial block 3 vs 10 (P < 0.05), suggesting reduction of auditory fear memory expression and an effective extinction memory acquisition. Contextual and auditory fear during pre-CS interval and CS block, respectively, across extinction and spontaneous recovery testing is presented in Figure 2.4.D. Two-way ANOVA revealed a significant main effect of time ($F_{(1, 11)}$ = 31.17, P=0.0002) and cue ($F_{(1, 11)}$ = 27.52, P=0.0003), but no time x cue interaction ($F_{(1, 11)}$ = 0.05462, P=0.8195). These results indicate that auditory fear is greater than the contextual fear at both time points. Simultaneously, contextual and auditory fear was increased during spontaneous recovery testing compared to extinction testing, suggesting that spontaneous recovery of fear occurred 21 days after extinction.

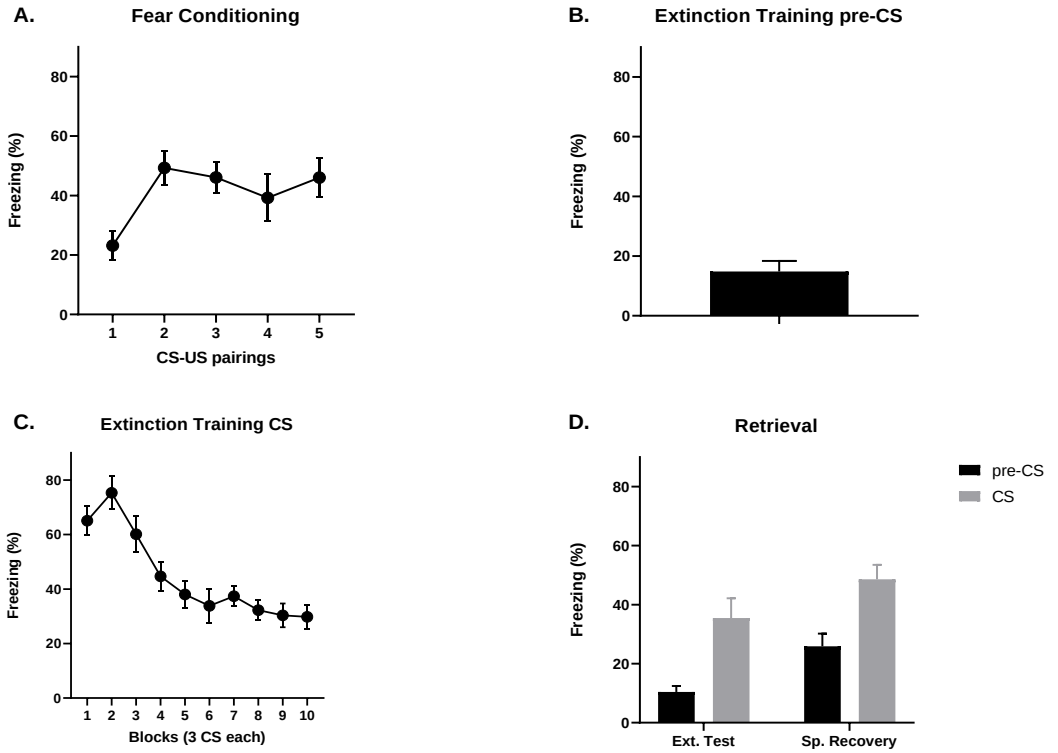

Figure 2.4: Spontaneous recovery of auditory and contextual fear 21 days after extinction training. (A) Freezing during fear conditioning (n=12 rats total). (B) Contextual fear expression during the pre-CS interval before extinction. (C) Reduction of auditory fear expression during extinction training, showing effective extinction memory acquisition. (D) The freezing levels were higher during spontaneous recovery session, indicating spontaneous recovery of auditory and contextual fear.

2.4.2 Experiment 2: Reinstatement

Freezing behaviour during fear conditioning in two groups is demonstrated in Figure 2.5.A. Two-way ANOVA revealed significant main effect of trial ($F_{(4, 72)}$ = 10.96, P < 0.0001), but no main effect of group ($F_{(1,18)}$ = 0.08392, P = 0.7754) or group × trial interaction ($F_{(4, 72)}$ = 1.97, P = 0.1083), suggesting similar patterns of fear conditioning between the animals allocated in Shock or Control groups. Levels of freezing during pre-CS interval before extinction training are shown in Figure 2.5.B. Unpaired two-tailed t-test revealed insignificant statistical difference in contextual fear memory (t = 2.022, P = 0.0583). Figure 2.5.C. depicts the freezing behaviour during the CS blocks in the extinction training session. Two-way ANOVA revealed significant main effect of block ($F_{(3.741, 67.34)}$ = 8.562, P<0.0001), but neither main group effect ($F_{(1, 18)}$ = 3.191,

P=0.0909) nor block x group interaction ($F_{(9, 162)}$ = 0.1764, P = 0.9962), indicating no reliable difference in auditory fear memory expression or extinction learning throughout the session. The following day, the Shock group of rats were subjected to reinstatement, while the controls were merely introduced to the same context without receiving any shock. 24 hours later, all rats underwent extinction testing and their freezing levels during pre-CS interval and CS block are shown in Figure 2.5.D. Two-way ANOVA revealed a significant main effect of cue ($F_{(1, 18)}$ = 21.16, P = 0.0002) but no main effect of group ($F_{(1, 18)}$ = 2.720, P=0.1164) or time x group interaction ($F_{(1, 18)}$ = 1.113, P=0.3055). This indicates higher auditory than contextual fear expression in both groups while failing to produce reinstatement of contextual and auditory learned fear.

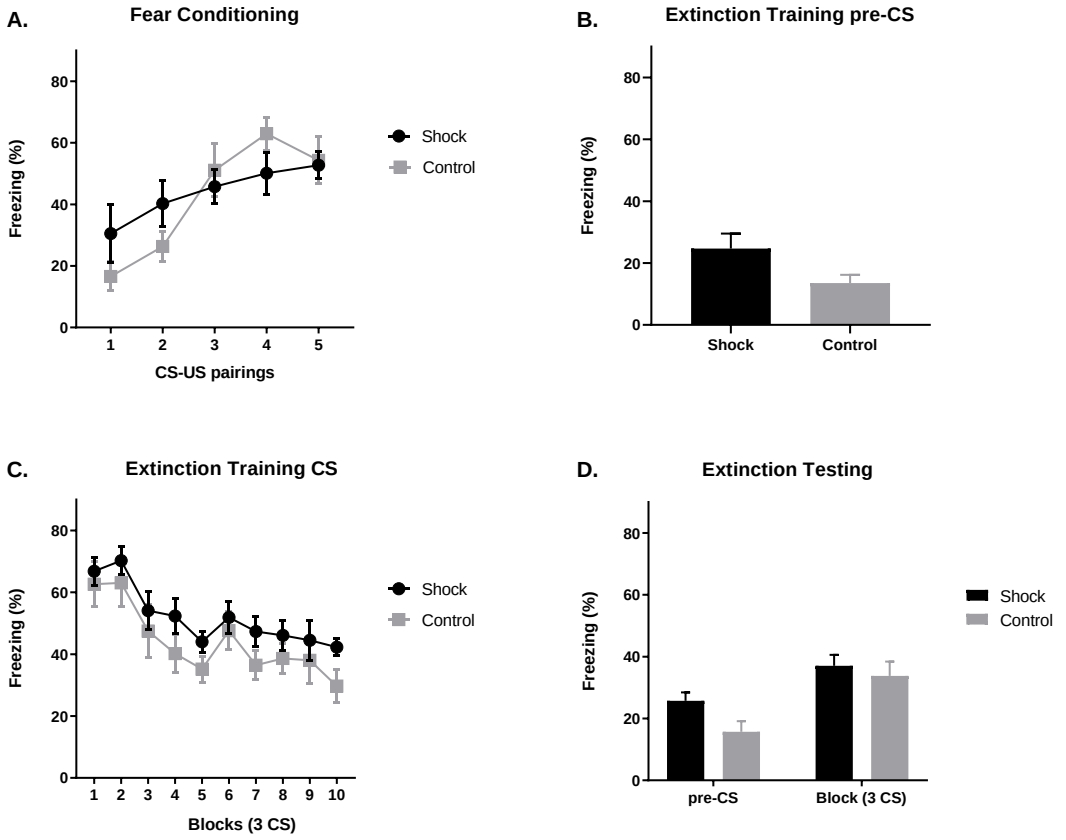

Figure 2.5: No evidence of contextual or auditory fear reinstatement with the use of a single unpaired foot-shock. (A) Auditory fear conditioning did not differ between the groups that later will or will not receive shock during the reinstatement session (n=10 rats/group). (B) No significant differences in contextual fear expression were found between the two groups during the pre-CS interval before extinction training. (C) No reliable differences were observed between two groups during extinction training. (D) No reliable differences in freezing were found during extinction test between the shock and the control groups.

2.4.3 Experiment 3: Renewal

Freezing during CS-US pairings in the fear conditioning is demonstrated in Figure 2.6.A. One-way ANOVA revealed a significant main effect of trial ($F_{(2.958, 26.62)}$ = 4.731, P = 0.0092), and increased freezing during trial 1 vs trial 5 (P < 0.01), suggesting an effective fear learning. Figure 2.6.B presents the freezing during the pre-CS interval, before extinction training. Figure 2.6.C. depicts the freezing behavior during the CS blocks in extinction training. Auditory fear expression levels were higher than expected towards the end of the session, while one-way ANOVA conduction revealed

insignificant main effect of block ($F_{(3.040, 27.36)}$ = 2.733, P = 0.0624). Freezing levels during the pre-CS interval and CS block of extinction and renewal test sessions are shown in Figure 2.6.D. Two-way ANOVA revealed significant main effects of time ($F_{(1, 9)}$ = 19.92, P = 0.0016) and cue ($F_{(1, 9)}$ = 6.824, P = 0.0282) but no time x cue interaction ($F_{(1, 9)}$ = 1.299, P = 0.2838), indicating lower freezing levels during renewal than extinction test, showing no evidence of contextual or auditory fear renewal.

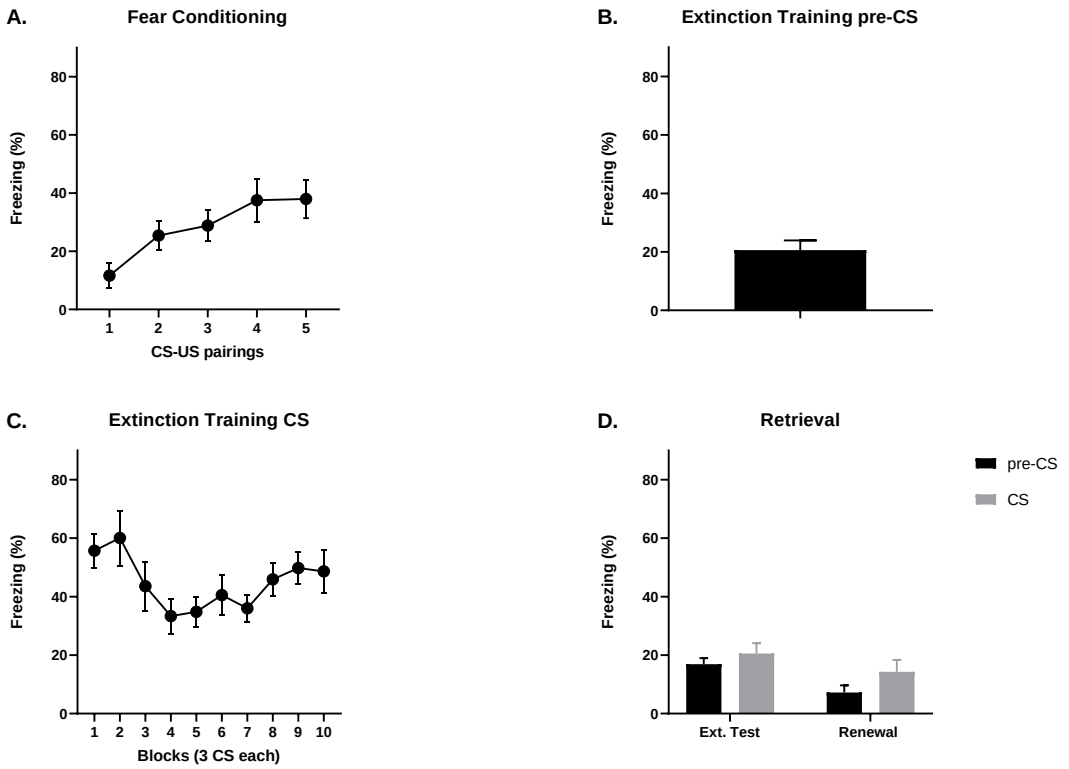

Figure 2.6. No evidence of contextual or auditory fear renewal with the use of 'ABA' paradigm. (A) Freezing during fear conditioning (n=10 rats total). (B) Contextual fear expression during the pre-CS interval before extinction. (C) Freezing during extinction training. (D) Auditory fear during CS blocks is higher than contextual fear during pre-CS intervals across the two retrieval points, with unexpected lower levels of freezing during renewal session.

2.5 Discussion

This study validated a number of experimental parameters for fear relapse paradigms of spontaneous recovery, reinstatement, and renewal that later will be used for the investigation of the modulatory effects of CBD in learned fear after

extinction. In Experiment 1, rats showed spontaneous recovery of auditory and contextual fear three weeks after successful extinction training. In contrast, despite the effective fear conditioning and its extinction during Experiment 2, the parameters used for the unreinforced US one day after extinction training did not result in reinstatement of fear. Additionally, in Experiment 3, tone presentations in conditioning context after extinction training did not result in fear renewal. These results point out not only the importance of the appropriate selection of conditioning and extinction parameters but also the circumstances under which the relapse phenomena will subsequently occur.

A challenge for the design of spontaneous recovery, reinstatement, and renewal of learned fear paradigms is to use robust fear conditioning that will produce adequately high fear responses, whose expression after extinction will be decreased but not completely abolished, so as to develop susceptibility for fear return and provide window for pharmacological intervention. For this reason, conditioning (e.g., number of CS-US pairings and foot-shock amplitude), extinction (e.g., days elapsed from fear acquisition, type of extinction, number of extinction sessions, CS presentation and their frequency), and fear return (e.g., days elapsed from extinction training for spontaneous recovery, number and intensity of unreinforced stimuli for reinstatement, and pattern of contextual alternation for renewal) parameters had to be carefully considered and determined.

The design and parameters used in the Experiment 1 were modified after their application in former studies of the lab (Fenton et al., 2016; Fenton et al., 2014). The present study maintained the same contextual alteration between fear conditioning (i.e., Context A), extinction and retrieval sessions (i.e., Context B), while the foot-shock

intensity and duration were decreased from 0.5mA and 1 sec in Fenton et al. (2014) study to 0.4 mA and 0.5 sec. Although the parameters were less aversive and the freezing levels were lower during fear conditioning in the present study, they were sufficient to induce similar auditory fear expression at the beginning of auditory extinction training session. Analogous freezing levels at an early stage during extinction were found in other studies investigating the fear relapse mechanisms, using the same foot-shock intensity (Cruz et al., 2014; King et al., 2018a and b). However, slightly different rates of extinction acquisition or fear expression at the end of the session may be attributed to the different number of CS-US pairing or US and ITI duration across the studies.

Several studies have shown that the magnitude of spontaneous recovery is influenced by several temporal factors, like the acquisition-extinction interval, the temporal distribution of extinction, and the extinction-test retention interval (Devenport, 1998; Rescorla, 2004a and b; Orinstein et al., 2010). Specifically, it was found that when the interval between conditioning and extinction is smaller, the spontaneous recovery magnitude is greater, which is often attributed to deficits in long-term extinction that are observed even with an extinction interval up to 6 hours post-conditioning (Chang & Maren, 2009). Several studies investigating the extinction and spontaneous recovery of auditory fear, have used a 24-hour acquisition-extinction interval that resulted during later spontaneous recovery in similar levels of freezing response coinciding with the current results (Cruz et al., 2014; Fenton et al., 2016; Migues et al., 2016; Martínez-Canabal et al., 2019).

Similarly, it was found that spaced intervals between extinction sessions or trials were more effective in suppressing spontaneous recovery, compared to

contiguous sessions or massed trials (Urcelay et al., 2009; Matsuda et al., 2014). Later an avoidance conditioning study added the observation that the temporal distribution of sessions left the extinction learning unaffected (Tapias-Espinosa et al., 2018). Common for the investigation of extinction and spontaneous recovery of auditory learned fear is the delivery of a single extinction session, while for the contextual fear multiple extinction sessions are frequently used (Matsuda et al., 2014; Kutlu et al., 2016; Kutlu et al., 2018; Tumolo et al., 2018). Similar to the present protocol, Martínez-Canabal et al. (2019) used intermediate length (i.e., 30 sec) ITI between extinction trials, resulting in levels of freezing during extinction and later spontaneous recovery comparable to the current results. Despite the identical extinction parameters used with Fenton et al. (2014 and 2016) studies, their results show faster rate of extinction acquisition and lower fear expression, especially towards the end of the session. The use of greater intensity and duration conditioning parameters may have affected fear expression, although a slower and not faster extinction would be anticipated. Two other factors that may have played a crucial role are the use of implanted animals (i.e., with electrodes) and the manually scored freezing. Noteworthy is that the freezing levels of the present study were analysed and presented in blocks of 3 CS trials instead of 2, and possibly this has altered the average percentage of the fear observed during each block. Encouraging for the validity of these results is that the freezing levels observed during the spontaneous recovery session are closely matching those of the corresponding pre-CS interval and first CS block of Fenton and co-workers (2014 and 2016) studies.

Various studies have evaluated the levels of fear return in relation to the time elapsed from extinction training and a common finding is that expansion of the

retention interval between extinction and test increases the probability and magnitude of spontaneous recovery (Quirk, 2002; Rescorla, 2004b). For this reason, the spontaneous recovery test was performed 21 days post-extinction instead of 15 days (Fenton et al., 2014). Although several studies have used a greater than 15 days retention interval and all of them have observed significant recovery of learned fear, the levels of freezing were higher than the present findings and largely varied. These variations are attributed to protocol differences across studies, such as the use of smaller number of trials (Cruz et al., 2014) or shorter intertrial intervals (Ponnusamy et al., 2016) during extinction training, longer retention interval between extinction and spontaneous recovery test in auditory learned fear protocols (Martínez-Canabal et al., 2019), or use of same context across all the testing days in the contextual learned fear protocols (Haubrich et al., 2017), thus impairing the retrieval of the context-dependent extinction (Orsini et al., 2011). Important for the effectiveness of the present protocol is that, despite the use of relatively less aversive fear conditioning, the appropriate combination of extinction parameters resulted in a significant return of auditory and contextual fear over time, suggesting as appropriate the application of these conditioning and extinction parameters for the validation of the following reinstatement and renewal protocols.

For the reinstatement protocol in Experiment 2, identical conditioning and extinction parameters were maintained as in Experiment 1, while rats were submitted to a single unreinforced US of 0.4 mA intensity, similar to King et al. (2018a) study. However, the shock group in the present study failed to show the anticipated high levels of freezing and, thus, fear reinstatement when compared to the control group.

King et al. (2018a) have also identified that individual differences in the extinction rate could differentially affect the return of fear, with the fast-extinguishing rats demonstrating reinstatement only under stronger relapse conditions, while individuals with a high anxiety trait have shown higher freezing across extinction training, test and reinstatement (Paula de Godoy et al., 2022), rendering individual differences an important factor for the variabilities observed across the protocols.

Although many studies have observed reinstatement of fear with a single unreinforced US, striking are the differences in foot-shock intensities. For instance, when compared to the present protocol, some studies delivered US with higher intensity range across both fear conditioning and reinstatement (Laurent & Westbrook, 2010; Shen et al., 2013; Hitora-Imamura et al., 2015; Chen et al., 2016), while others used a combination of higher US intensity during conditioning with lower unreinforced US intensity during reinstatement (Goode et al., 2015; Jo et al., 2020). Many of them have separately performed experiments to naïve rats only with the unreinforced US, in order to confirm that the US intensity does not elicit fear responses *per se*, but the return of fear observed after the US reminder derives from the original fear memory. Therefore, the variations in the results across the studies are more likely due to the differences in US intensities used during fear conditioning rather than the reinstatement. Of particular interest is a recent study by Wang et al. (2020), during which reinstatement of learned fear was observed, using an identical US reminder to the present study. The fact that their reinstatement session was performed in the same context as fear conditioning, while the reinstatement test in the extinction context, may have affected the return of learned fear. Similarly, altering the time of the unreinforced US delivery in relation to extinction acquisition may also

affect the magnitude of reinstated fear (Verma et al., 2019; Duran et al., 2022). Finally, evidence from previous studies indicates that using multiple US reminders leads to a more effective and robust reinstatement of auditory and contextual fear, an approach that could be applicable in future studies (Auchter et al., 2017; Chen et al., 2020; King et al., 2018a; Yang et al., 2012).

For the validation of the renewal protocol, in Experiment 3, the behavioural testing was performed in more distinguishable contexts, using a version of Context B under dark conditions, while keeping identical the context A along with the conditional and extinction parameters to those used in Experiments 1 and 2. Rats underwent fear conditioning in Context A, extinction training in Context B, and tested for renewal back in context A under strong renewal condition 'ABA', though they failed to produce the anticipated high levels of freezing when compared to moderate renewal conditions like 'ABC', or 'AAB' (Chen et al., 2017). Interesting is that several studies using either smaller (12-20 CSs) (Vasquez et al., 2019; Joo et al., 2020; Adkins et al., 2021; Li et al., 2021) or larger (40-45 CSs) (Goode et al., 2015; Wang et al., 2020; Shih & Chang, 2021) number of CS presentations compared to this experiment (30 CSs) during extinction training, observed effective extinction learning in Context B and renewal of learned fear when tested in Context A. Approaches like violating the expectancy related to frequency or intensity of aversive experience, overtraining extinction, retrieval cues before extinction, presentation of unpaired US during extinction, or performing CS extinction in multiple contexts have been attempted for maximizing extinction and have shown mitigation of fear renewal (Myers & Davis, 2006; Craske et al., 2014; Hermann et al., 2020; Lipp et al., 2021), while none of these have been applied in the present experiment. Overtraining extinction approaches used in other studies, such

as a massive number of trials during extinction or multiple extinction training sessions across consecutive days, resulted in the prevention of fear renewal in taste aversion and lick suppression paradigms (Denniston et al., 2003; Rosas et al., 2007; Laborda & Miller, 2013). Prevention of fear renewal in the present experiment might be also due to a potential "overtraining extinction" effect, even though this protocol does not include similarly massive numbers of CS trials. The fact that low levels of freezing were observed during extinction testing indicate that there was extinction encoding despite the high freezing levels during late stage of extinction. This come into alignment with a finding by Plendl & Wotjak (2010), that within-session extinction does not necessarily predict between-session extinction. Taking into account a potentially slower extinction rate in the rats of current experiment, a greater return of fear would be expected the next day especially under strong renewal condition (King et al., 2018a), but that comes in contrast with the low levels of freezing during renewal.

Recently, the modulatory effects of stress and cortisol in extinction and renewal of learned fear have attracted great research interest. Pre-extinction stress or cortisol administration in humans and post-conditioning corticosterone in rats were found to enhance the context-independent consolidation of extinction memory, resulting in suppression of fear renewal (Wang et al., 2014; Meir Drexler et al., 2020). In contrast, pre-retrieval manipulations were shown to elicit opposite effects (Kinner et al., 2016). Consequently, high stress in rats before or early during extinction training may have led to the lower freezing levels during extinction test and renewal in the present study. However, this was not verified due to the absence of behavioural testing (e.g., open field test, elevated plus maze (EPM) or corticosterone plasma detection before extinction training.

In conclusion, the parameters used in the validation study of spontaneous fear recovery demonstrate return of contextual and auditory fear. However, the parameters used for the reinstatement and renewal protocols did not result in a similar return of fear. This encourages the investigation of modulatory effects of CBD in extinction and spontaneous recovery of learned fear, while considering modification of the other protocols for their application in future studies.

Chapter 3. Effects of CBD administration before or after extinction on spontaneous recovery of learned fear

3.1 Introduction

Anxiety and trauma-related disorders are associated with persistent fear-related memories, while demonstrating dysfunctional extinction and impaired inhibition of fear responses triggered by specific situations or stimuli (Milad et al., 2009). Extinction learning, which is the integral component of psychotherapeutic methods for these disorders, may often be deficient. Even under successful completion of exposure therapy patients may experience only temporary benefits, resulting in return of symptoms with mere passage of time, commonly known as spontaneous recovery (Sartori & Singewald, 2017). A promising therapeutic approach is the administration of cognitive enhancing compounds either before or after exposure therapy, facilitating extinction learning or the consolidation of extinction memory, respectively (Singewald et al., 2015).

Among the novel extinction-enhancing therapeutics, CBD has attracted much attention due to its considerable anxiolytic and modulatory effects on innate and learned fear (Papagianni & Stevenson, 2019). Acute effects in animal models of conditioned fear were associated with attenuation in the expression of contextual fear when 10 mg/kg CBD was administered systemically (Resstel et al., 2006; Lemos et al., 2010; Jurkus et al., 2016). Similar effects were observed upon central infusion of 30 nmol CBD in PL (Lemos et al., 2010; Fogaca et al., 2014) and BNST (Gomes et al., 2012), but not after intra-IL infusion of 15 or 30 nmol CBD (Lemos et al., 2010; Marinho et al., 2015). Additionally, CBD was found to produce persistent fear reduction when

combined with fear memory reactivation, disrupting the fear memory reconsolidation. Specifically, when 10 mg/kg CBD was systemically administered after a brief retrieval session in the conditioning context, this resulted in long-lasting reduction of contextual fear (Gazarini et al., 2014). This effect was reproduced after direct infusion of 30 pmol into ACC or PL but not the IL (Bayer et al., 2022). On the other hand, systemic (i.e., 10 mg/kg) (Song et al., 2016), intra-IL (i.e., 0.4 μg/ 0.2 μl) (Do Monte et al., 2013), and intracerebroventricular (i.e., 2.0 μg/μl) (Bitencourt et al., 2008) CBD administration before extinction training in rodents, enhanced the acquisition of extinction memory, whereas inhalation of 32 mg CBD after an extinction session in healthy human volunteers (Das et al., 2013) facilitated the extinction consolidation, leading to later suppression of contextual fear expression. Therefore, these studies suggest that CBD used in conjunction with exposure techniques may comprise a promising therapeutic strategy with long-lasting suppression of maladaptive behaviours.

Despite the plethora of studies investigating the modulatory effects of CBD on contextual fear memory, little is known about its effectiveness in the regulation of cued fear. A study by Norris et al. (2016) revealed that direct infusion of CBD (i.e., 1, 10, and 100 ng/ 0.5 μl) into the shell of NAc dose-dependently impeded the formation of olfactory fear memory, whereas systemic CBD administration prior to extinction training resulted in the acute reduction of auditory (i.e., 20 mg/kg) and contextual (i.e., 5, 10, and 20 mg/kg) fear memory expression during extinction training (Jurkus et al., 2016). Despite the supporting evidence that CBD exerts enhancing effects on extinction, up to date, its effects on long-term extinction retention and subsequently on spontaneous recovery remain undetermined. Therefore, to investigate this issue

further, CBD was administered systemically either before or after extinction training. The hypothesis of this study is that enhanced extinction acquisition or consolidation would prevent the later spontaneous recovery of fear after extinction.

3.2 Materials and methods

3.2.1 Subjects

Male Lister-Hooded rats (Charles Rivers, UK), weighing 300-450 g and 250-350 g were used for Experiments 1 and 2, respectively. Rats were housed and behaviorally tested under the same conditions as described in previous chapter. All experimental procedures were performed with internal ethical approval and in accordance with the Animals (Scientific Procedures) Act 1986, UK (PPL: P6DA59444). The sample size of each treatment group was calculated based on the power analysis described in Chapter 2.

3.2.2 Drug preparation and administration

CBD (THC Pharm, Germany) at a dose of 10 or 20 mg/kg was suspended in vehicle (freshly prepared 98% sterile saline and 2% Tween 80) and administered intraperitoneally (1 mL/kg, i.p.) either 30 min before or immediately after the extinction training session. These doses were selected based on previous studies showing CBD modulation of learned fear processing (Resstel et al., 2006; Lemos et al., 2010; Stern et al., 2012; Jurkus et al., 2016; Song et al., 2016; Stern et al., 2017).

3.2.3 Experiment 1: Effects of pre-extinction CBD administration on extinction and spontaneous recovery of learned fear

The effects of systemic administration of CBD before extinction on modulation of extinction and spontaneous recovery of contextual and auditory learned fear were

investigated using a 24-day protocol, presented in Figure 3.1. The behavioural testing apparatus and recording software, along with the design and parameters used in the following experiments have been described in detail in Chapter 2. On day 1, animals were habituated in two distinct contexts, initially in context B and then in context A. On day 2, all rats were subjected to auditory fear conditioning in context A and acclimatized for a 2 min pre-CS interval, before being habituated to 5 CS presentations. Auditory fear conditioning consisted of 5 CS-US pairings. Each CS lasted 30 sec and co-terminated with a 0.5 sec foot-shock (US), of I=0.4 mA current intensity, and ITI duration of 2 min. On day 3, rats were randomly allocated into three groups of n=10 rats/group, that were administered 0 (i.e., vehicle), 10 or 20 mg/kg of CBD 30 min before undergoing fear extinction training in Context B. They were tested for their contextual fear memory during the 2 min pre-CS interval, before receiving 30 CS presentations of 30 sec duration each and separated by ITIs of 30 sec. On day 4, unexpectedly due to multiple power cuts in the laboratory, only n=16 rats were able to undergo extinction testing, while the rest (n=14 rats) were tested on day 5, instead. The rats returned to Context B for a 2 min pre-CS interval, before receiving 3 CSs of 30 sec duration and separated by ITIs of 30 sec. On day 24 (21 days after extinction training), all groups were subjected to spontaneous fear recovery testing in Context B, with procedure parameters identical to those used for extinction testing.

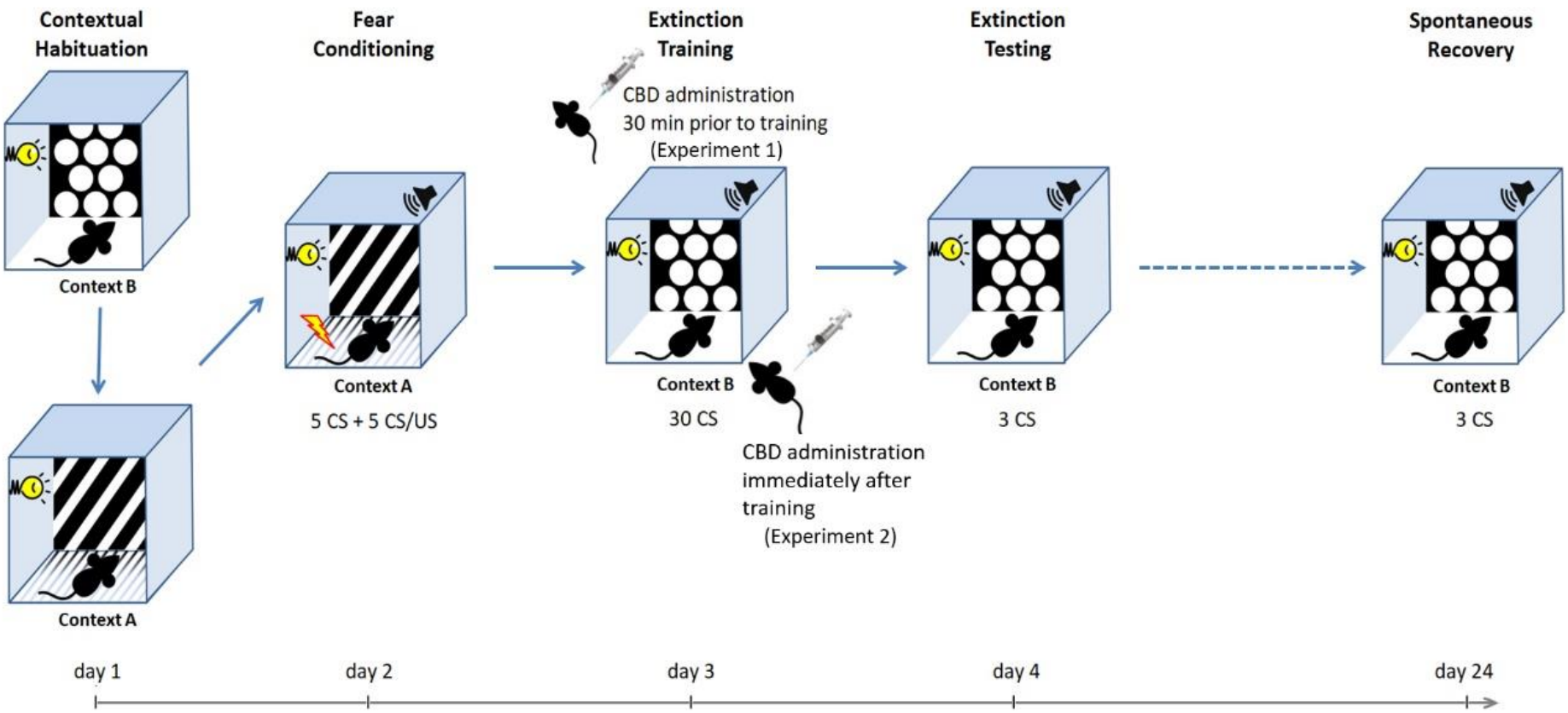

Figure 3.1 Schematic representation of the behavioral protocol used for investigating the effects of pre- and post- extinction CBD administration on spontaneous recovery of learned fear. On day 1, rats were habituated in context B and then in context A. On day 2, rats underwent fear conditioning with 5 CS + 5 CS-US pairings in context A. On day 3, rats were administered i.p. vehicle, 10, or 20mg/kg CBD 30 min prior to or immediately after extinction training, receiving 30 CS presentations in context B. On day 4, all groups were exposed to 3 CS for extinction testing, in context B, and 21 days after extinction training, they were returned for a spontaneous recovery session.

3.2.4 Experiment 2: Effects of post-extinction CBD administration on spontaneous recovery of learned fear

All rats underwent contextual habituation, auditory fear conditioning and extinction training on days 1, 2 and 3, respectively, as described above in Experiment 1. Rats were randomly allocated into three treatment groups administered 0 (n=10), 10 (n=9) or 20 (n=9) mg/kg of CBD immediately after the end of extinction training. On day 4 and 24, all rats were returned to Context B and subjected to extinction and spontaneous recovery testing, respectively, receiving 3 CS presentations, as described in Experiment 1, Chapter 2.

3.3 Data analysis

The duration of freezing behaviour was expressed as percentage of 120 sec for the pre-CS interval or 30 sec for each CS trial. Auditory fear during extinction training, extinction testing and spontaneous recovery testing sessions was expressed in CS blocks, defined by the average of freezing during 3 consecutive CS presentations. All data are presented as mean ± SEM and statistical analysis was performed using GraphPad Prism 9, while $p < 0.05$ was considered the level of statistical significance for all comparisons. In both experiments, fear conditioning was analyzed using repeated-measures or mixed model two-way ANOVA, with group as the between-subject factor and trial as the within-subject factor. Geisser-Greenhouse correction was applied to adjust for lack of sphericity. The pre-CS interval before extinction training was analyzed through one-way ANOVA, with treatment group as between-subject factor for both Experiment 1 and 2, during which CBD was administered either before or after extinction training, respectively. Bartlett's test was used to assess the

homogeneity of variance, while $p < 0.05$ was considered the level of assumption violation. Two-way ANOVA with Geisser-Greenhouse correction was conducted for the analysis of CS blocks during extinction training in Experiments 1 and 2 with treatment group as the between-subject factor and block as the within-subject factor. Finally, in both experiments, the freezing levels of pre-CS intervals and the CS blocks during extinction and spontaneous fear recovery testing were interpreted by using two-way ANOVA, with treatment and time defined as the between- and within-subject factors, respectively. The fact that the within-subjects factor was comprised of only two levels (i.e., extinction test vs spontaneous recovery) rendered inapplicable the sphericity assumptions. When ANOVA revealed significant main effect and interaction between independent variables, a Sidak's post-hoc test was conducted to perform multiple comparisons of limited pairs of means, comparing lower or higher dose of CBD against vehicle, but not against each other. Sidak method was preferred over Bonferroni for its higher power and used to compute adjusted P value for each individual test and confidence intervals (Lee & Lee, 2018).

3.4 Results

3.4.1 Experiment 1: Effects of pre-extinction CBD administration on extinction and spontaneous recovery of learned fear

The levels of freezing behavior during CS-US pairings in the fear conditioning session are presented in Figure 3.2.A. Two-way ANOVA revealed a significant main effect of trial ($F_{(3.403, 91.87)} = 5.867$, $P = 0.0006$) but no group effect ($F_{(2, 27)} = 0.9297$, $P= 0.4069$) and no trial x group interaction ($F_{(8, 108)} = 0.5067$, $P= 0.8490$). This indicates that there were no reliable differences between the groups to receive the different

doses of CBD prior to extinction training. Freezing during the pre-CS interval before extinction training is shown in Figure 3.2.B. One-way ANOVA revealed a significant main effect of treatment group ($F_{(2, 27)} = 3.421$, P = 0.0474). Post-hoc analysis revealed significantly lower freezing with CBD 10 mg/kg vs vehicle (P=0.0361), but no significant difference between vehicle and CBD 20 mg/kg (P=0.1361). These results indicate that the low dose of CBD reduced the expression of contextual fear memory before extinction training, when compared with vehicle. Freezing during the CS blocks in the extinction training session is shown in Figure 3.2.C. Two-way ANOVA revealed a significant main effect of block ($F_{(3.787, 102.2)} = 6.799$, P < 0.0001), but no treatment group effect ($F_{(2, 27)} = 1.517$, P = 0.2376) or treatment group x block interaction ($F_{(18, 243)} = 1.277$, P = 0.2035), suggesting that CBD had no effect on auditory fear memory expression or extinction learning. The effects of pre-extinction CBD administration on retrieval of contextual fear memory a day vs. 21 days after extinction training are depicted in Figure 3.2.D. To investigate this, two-way ANOVA was conducted on freezing during the pre-CS interval before extinction and spontaneous fear recovery testing, revealing a significant main effect of time ($F_{(1, 27)} = 9.920$, P = 0.0040), but no main effect of treatment group ($F_{(2, 27)} = 0.8713$, P = 0.4298) or treatment group x time interaction ($F_{(2, 27)} = 1.147$, P=0.3327). These results indicate that freezing during the pre-CS interval before spontaneous fear recovery is significantly increased across all groups when compared with extinction testing, suggesting increased contextual fear 21 days after extinction training but, without any effect of CBD. Finally, Figure 3.2.E shows the effects of CBD on return of auditory fear over time during the CS block of extinction and spontaneous recovery testing. Two-way ANOVA revealed no main effect of treatment group ($F_{(2, 27)} = 1.028$, P = 0.3712), but there was a significant effect

of time ($F_{(1, 27)}$ = 4.902, P = 0.0355) and treatment group x time interaction ($F_{(2, 27)}$ = 8.411, P = 0.0014). The post-hoc analysis showed that in the vehicle group freezing was significantly augmented during spontaneous fear recovery when compared with the extinction test (P = 0.0003). However, there was no reliable difference in freezing during spontaneous fear recovery versus extinction test with either 10 mg/kg (P > 0.9999) or 20 mg/kg CBD (P = 0.8465). During spontaneous recovery both CBD doses demonstrated significantly lower levels of freezing than vehicle as revealed by multiple comparisons of Vehicle vs. CBD 10mg/kg (P = 0.0252), Vehicle vs. CBD 20mg/kg (P = 0.0154) and CBD 10mg/kg vs. CBD 20mg/kg (P > 0.05). These results indicate that both doses of CBD prevented spontaneous recovery of auditory learned fear.

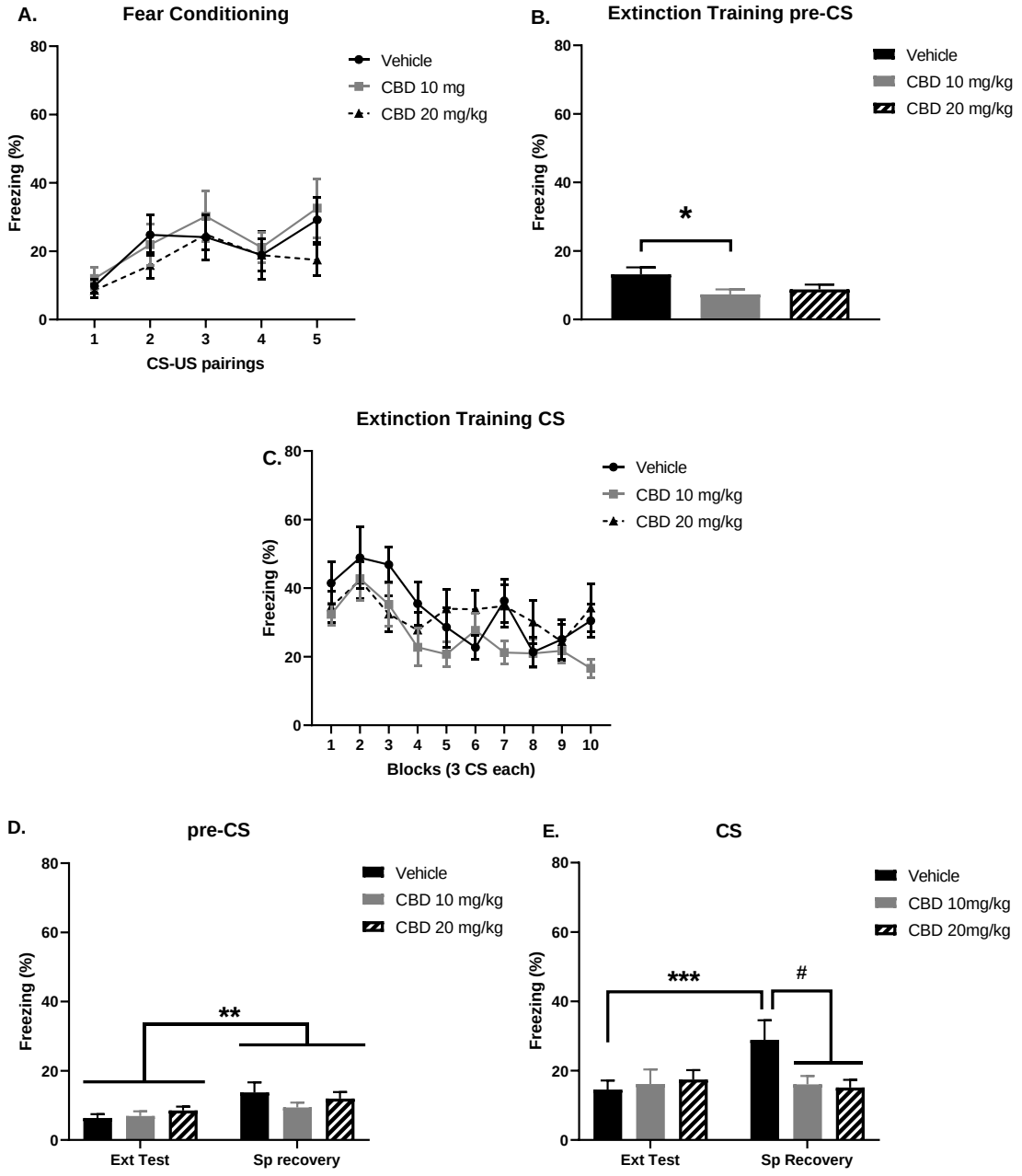

Figure 3.2 Pre-extinction CBD reduces the expression of contextual fear memory and prevents the spontaneous recovery of auditory fear after extinction. (A) Fear conditioning did not differ between the groups to receive different doses of CBD (n=10 rats/group). (B) CBD 10 mg/kg i.p. resulted in significantly less freezing during the pre-CS interval before extinction training, compared to vehicle (* $p < 0.05$). (C) No significant differences in freezing were found between the different groups during extinction training. (D) All groups demonstrated significantly more freezing during the pre-CS interval of spontaneous fear recovery compared to extinction test (** $p < 0.01$), but no reliable differences were observed between VEH and the two CBD doses during each time point. (E) The vehicle-treated group showed significantly higher levels of freezing during the CS block of spontaneous fear recovery (*** $p < 0.001$), while freezing in the CBD-treated groups did not differ between spontaneous fear recovery and extinction test. Importantly, vehicle-treated group showed significantly higher freezing than both CBD doses during spontaneous recovery (# $p < 0.05$). This indicates that both CBD doses suppresses spontaneous recovery of auditory fear 21 days after extinction training.

3.4.2 Experiment 2: Effects of post-extinction CBD administration on extinction and spontaneous recovery of learned fear

The levels of freezing behavior during CS-US pairings in the fear conditioning session are presented in Figure 3.3.A. Two-way ANOVA revealed a significant main effect of trial ($F_{(2.842, 71.04)} = 9.692$, $P < 0.0001$) but no group effect ($F_{(2, 25)} = 0.03336$, P=0.9672) or trial x group interaction ($F_{(8, 100)} = 0.6210$, P = 0.7584), indicating similar patterns of fear conditioning between the groups. Freezing during the pre-CS interval before extinction training is shown in Figure 3.3.B. One-way ANOVA revealed no main effect of treatment group ($F_{(2, 25)} = 0.7486$, P=0.4833). These results indicate that there was no reliable difference between the groups in the expression of contextual fear memory before extinction training. Freezing during the CS blocks in the extinction training session is shown in Figure 3.3.C. Two-way ANOVA revealed a significant main effect of block ($F_{(2.677, 66.93)} = 6.590$, P = 0.0009), but no treatment group effect ($F_{(2, 25)}$ =0.4888, P=0.6191) or treatment group x block interaction ($F_{(18, 225)} = 1.403$, P=0.1311), suggesting that there were no significant differences in auditory fear memory expression or extinction acquisition between the groups to receive the different doses of CBD after extinction training. The effects of post-extinction CBD administration on contextual and auditory fear during pre-CS interval and CS block, respectively, across extinction and spontaneous recovery testing are presented in Figure 3.3.D and E. To investigate the effect of CBD on contextual fear, two-way ANOVA was conducted on freezing during the pre-CS interval before extinction and spontaneous fear recovery testing, revealing a significant main effect of time ($F_{(1, 25)} = 20.12$, P=0.0001), but no main effect of treatment group ($F_{(2, 25)} = 0.2291$, P= 0.7969) or treatment group x time interaction ($F_{(2, 25)} = 0.8334$, P= 0.4463). These results indicate that freezing during the

pre-CS interval before spontaneous fear recovery is significantly increased in all groups when compared with extinction test, suggesting increased contextual fear 21 days after extinction training but, without any effect of CBD. To examine the effect of CBD on the return of auditory fear over time, two-way ANOVA was conducted on CS-block of extinction and spontaneous fear recovery testing. There was no main effect of treatment group ($F_{(2, 25)}$ = 0.7613, P=0.4776), time ($F_{(1, 25)}$ = 0.6014, P=0.4453) or treatment group x time interaction ($F_{(2, 25)}$ = 0.9942, P=0.3842), indicating that none of the treatment groups showed spontaneous recovery of auditory fear during the CS block, and no effect of post-extinction CBD was observed.

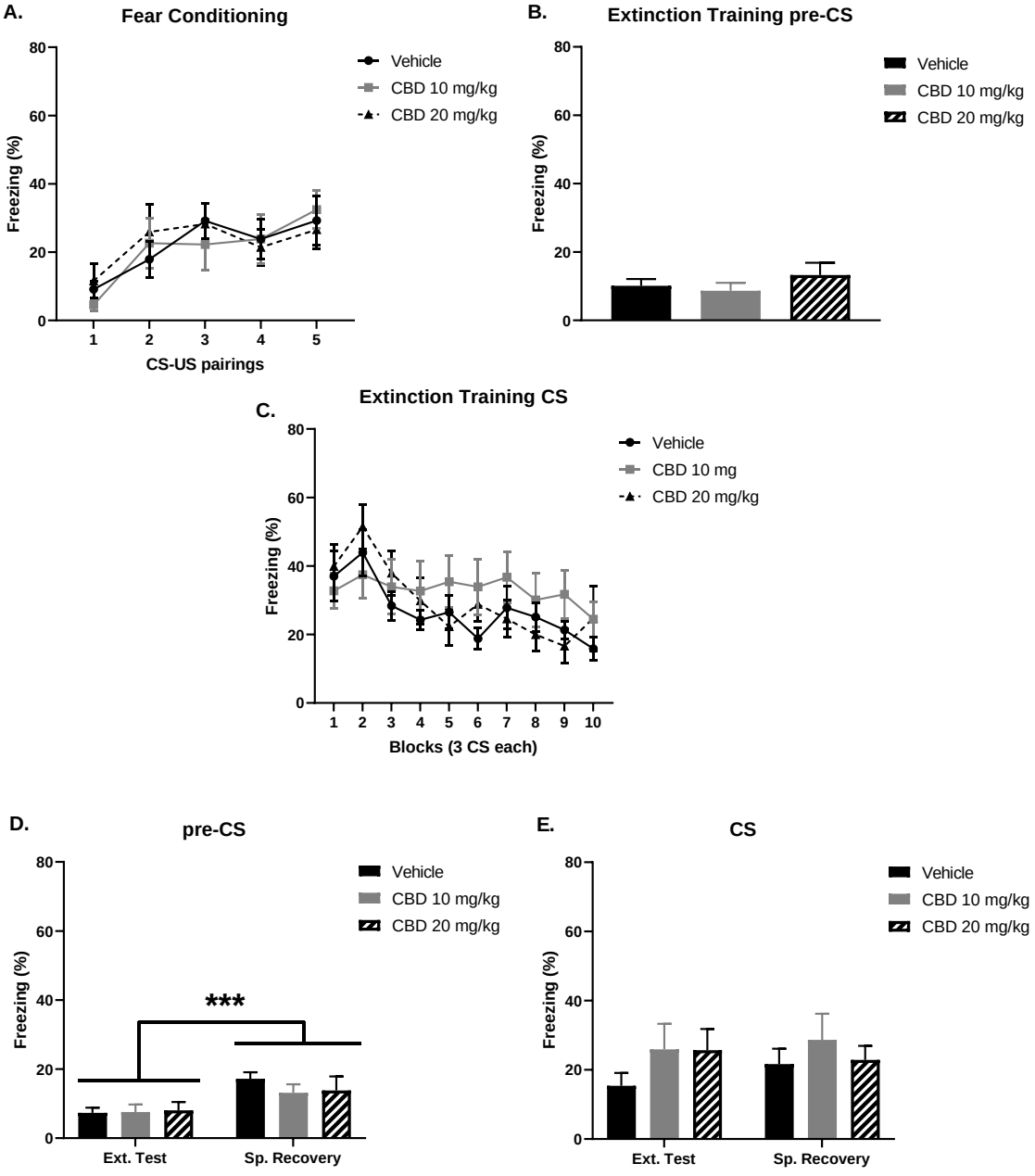

Figure 3.3 Post-extinction CBD did not produce any effects on extinction of learned fear or its extinction recall. (A) Fear conditioning, (B) contextual fear expression during pre-CS before extinction, and (C) auditory fear expression during CS blocks of extinction training did not differ between the groups to receive i.p. CBD 0, 10, or 20mg/kg after extinction training (Vehicle group: n=10 rats, CBD 10 mg/kg group: n=9 rats, CBD 20 mg/kg group: n=9 rats). (D) All groups demonstrated significantly more freezing during the pre-CS interval of spontaneous fear recovery compared to extinction test (*** $p < 0.001$), but no reliable differences were observed between groups during each time point, indicating no CBD effect on contextual fear expression. (E) Freezing levels did not significantly differ across the treatment groups or between the two time points during the CS block, indicating no CBD effect on auditory fear expression 1 and 21 days after extinction training and lack of spontaneous recovery.

3.5 Discussion

This study investigated the effects of pre- and post-extinction systemic CBD administration on the expression, extinction, and spontaneous recovery of contextual and auditory learned fear. In Experiment 1, intraperitoneal administration of CBD prior to auditory fear extinction reduced acutely the expression of contextual fear memory during the pre-CS interval before extinction training but did not induce any effects either on the expression or the extinction learning of auditory fear. Although pre-extinction CBD did not produce long-lasting effects on auditory or contextual fear when the rats were tested drug-free 24hrs later during the extinction test session, interestingly, both CBD doses prevented the spontaneous recovery of auditory but not contextual fear, 21 days after extinction training. In Experiment 2, CBD administration immediately after extinction training showed no long-term effects either on contextual or auditory fear memory during the drug-free extinction test session. Although, CBD failed to prevent the spontaneous recovery of contextual fear, there was no indication of spontaneous recovery of auditory learned fear in any of the treatment groups, when tested 21 days after extinction training. These results point out the importance of CBD administration time in relation to extinction training for the augmentation of extinction memory and the induction of a long-lasting effect in suppressing fear relapse over time, whilst stimulating discussion about possible experimental parameters that may alter such effects.

The result regarding the effect of the pre-extinction CBD on the reduction of contextual fear expression that was observed before extinction training agrees with the findings from previous studies investigating the effects of systemic CBD in

modulation of contextual fear, showing reduction in expression of fear memory when CBD was administered prior to behavioral testing (Resstel et al., 2006; Lemos et al., 2010). Similarly, a previous study by Jurkus et al. (2016) demonstrated reduction in baseline expression of contextual fear before auditory fear extinction training after i.p. administration of 10 mg/kg CBD. In contrast to the present results, the same study showed that 20 mg/kg CBD was effective to reduce acutely both contextual and auditory fear memory without affecting its extinction. The lack of CBD effect on auditory fear expression in the present study may be related to the weaker conditioning parameters when compared to those of Jurkus et al. (2016), during which foot-shock intensity used was 0.4 mA versus 0.5 mA, respectively. Although the milder foot-shock used here induced relatively low levels of freezing during CS-US pairings, fear conditioning led to a robust fear memory acquisition and consolidation as indicated by the high levels of freezing in the early stage of the auditory extinction training session the next day. Interestingly, it was found that the consolidation of extinction memory is dependent on the previous conditioning experience and it is triggered only upon a critical US duration threshold that will stimulate protein synthesis and lead to a sufficient difference in US mismatch magnitude between the experienced US during fear conditioning and the omitted US during retrieval (Stollhoff & Eisenhardt, 2009). The argument that the CBD effects on expression of learned fear may be dependent on the strength of fear conditioning is further supported by the CBD-induced bidirectional effects on contextual fear extinction. Specifically, after strong fear conditioning CBD induced acute and long-lasting reduction of contextual fear expression during extinction training and recall. In contrast, after weaker

conditioning, CBD resulted in no acute effects during extinction, while inducing higher contextual fear expression at the subsequent extinction recall test (Song et al., 2016).

Additionally, noteworthy are the discrepant findings associated with the effects of CBD on extinction and its recall. In this experiment, CBD did not produce either acute effects on extinction acquisition or extinction recall one day later, with the latter finding similarly observed by Jurkus et al. (2016). In contrast, previous studies have shown enhancement of contextual fear extinction with freezing reduction both during the extinction sessions and the next day during extinction recall testing, when CBD was administered systemically (Song et al., 2016), or infused intracerebroventricularly (Bitencourt et al., 2008) or directly into the infralimbic cortex (Do Monte et al., 2013) before each of the consecutive extinction sessions. Possibly, these beneficial effects of CBD on contextual fear extinction may be attributed to differences in the route of drug administration. A potential explanation might be that systemic CBD simultaneously affected multiple areas within the extinction circuit, thus mutually mitigating the effects of other areas, while infusions directly into brain areas related with extinction memory modulation may have exerted more targeted effects. Direct infusions may have also provided better drug bioavailability in brain parenchyma, by-passing the blood-brain-barrier (BBB) and hepatic metabolism when compared with systemic administration. On the other hand, the reduced freezing levels that were observed during extinction recall testing in Bitencourt et al. (2008) and Do Monte et al. (2013) studies may be attributed to the repeated CBD administration, given that CBD is highly hydrophobic and can be retained in lipid rich tissues like brain parenchyma (Long et al., 2012). However, this is unlikely to happen after a single administration of 10 mg/kg CBD in the present study. This is further

supported by pharmacokinetic data of Deiana et al. (2012) study, in which acute i.p. administration of 120 mg/kg CBD in rats demonstrated half-life in plasma $T_{1/2}$=8-10 hrs., whilst in brain $T_{1/2}$= 6-11 hrs., depending on the suspension vehicle used.

In addition to the previously mentioned bidirectional effects of CBD on contextual fear extinction depending on the fear conditioning averseness, both studies by Bitencourt et al. (2008) and Do Monte et al. (2013) used greater intensity of foot-shock when compared to the present study, suggesting that the extinction enhancement may have resulted by the stronger contextual fear (Song et al., 2016). Another striking difference in the protocols across the studies is the number of extinction sessions. Those investigating the contextual fear extinction frequently use multiple sessions, while a single extinction session is applied in studies investigating auditory fear (Matsuda et al., 2014). Regarding the current experiment and the Jurkus et al. (2016) study, it is unclear whether the single extinction session in conjunction with CBD was sufficient to decrease further the expression of contextual fear one day after the single auditory extinction training session. In contrast, repeated contextual extinction sessions delivered in three consecutive days and combined by prior CBD administration, might have acted synergistically to elicit a more robust extinction and subsequently a more effective recall of extinction memory. The fact that no sustained effects of CBD were observed on extinction retrieval in the present study may be attributed to a floor effect, in which the different treatment groups improve negligibly due to the low levels of freezing during the extinction test (Andrade, 2021). In support of this argument, despite the lack of CBD effect on extinction recall, CBD was found to suppress the later spontaneous recovery of auditory fear 21 days after extinction training. Based on current literature, this is the first report indicating that CBD

prevents the return of learned fear over time, presumably by enhancing the encoding of extinction training.

Noteworthy is a previous study by Stern et al. (2012) showing that CBD prevented the spontaneous recovery of contextual fear through disruption of fear memory reconsolidation. Nevertheless, this is an implausible mechanism through which CBD elicits its long-lasting effect in the present experiment, given that the duration of memory reactivation was much longer than that used in Stern et al. (2012) study (31 min and 30 sec vs 3 min), which would be expected to engage extinction rather than reconsolidation mechanisms (Cassini et al., 2017). This is indicated also by the suppression of freezing to low levels during extinction testing, instead of the higher freezing levels observed upon retrieval testing in the former study. Additionally, evidence from a clinical study in healthy individuals revealed that inhalation of 32 mg CBD before extinction training of visual fear memory did not produce any effects on extinction acquisition or retrieval, aligning to our findings and the Jurkus et al. (2016) study, although they demonstrated a trend towards a reduction in reinstatement of the autonomic fear response (Das et al., 2013). However, in the same study CBD administered after extinction training, resulted in attenuation of conditioned responding both during retrieval and reinstatement sessions, suggesting that these effects are mediated through facilitation of extinction memory consolidation. Based on this evidence, another aim was to investigate whether CBD administration after auditory fear extinction training would result in more effective suppression of spontaneous recovery, through promotion of extinction memory consolidation and facilitation of between-session retrieval of extinction.

However, to date there is no previous preclinical study that has investigated the effects of CBD on fear extinction consolidation.

In the second experiment, CBD administration after extinction training failed to affect extinction retention and long-term expression of contextual and auditory fear memory across the two extinction recall sessions. However, an unanticipated finding was the lack of spontaneous recovery of auditory fear across all the treatment groups. Interest attracts a study investigating the effect of selective FAAH inhibitor, AM3506, in the modulation of auditory fear extinction and its retention (Gunduz-Cinar et al., 2013). The systemic administration of AM3506 before extinction training enhanced extinction and resulted in the reduction of auditory fear expression 10 days later during a extinction test, an effect that was mediated through augmentation of anandamide levels in BLA and depended on CB1 receptor activation. However, AM3506 administration after extinction training failed to affect extinction recall, suggesting that the increase of AEA through inhibition of its hydrolysis is an insufficient mechanism to facilitate the extinction consolidation, a finding that partially agrees with our results. An important difference in the Gunduz-Cinar et al. (2013) study is that no extinction test was performed soon after extinction training in order to investigate the effects of AM3506 on extinction recall. Therefore, they could not directly assess the treatment effects over time on spontaneous recovery of auditory fear, rendering their finding not entirely comparable to the present results. Nevertheless, the use of massed extinction trials in their protocol may have exhibited greater susceptibility to spontaneous fear recovery than the spaced extinction trials used in the present study (Orinstein et al., 2010).

As mentioned above, none of the treatment groups exhibited spontaneous recovery of auditory learned fear in Experiment 2, despite using the same protocol parameters as in Experiment 1. A possible factor that may have played a role in this result is the age of rats used. A previous systematic review has highlighted the age of experimental animals as a crucial factor affecting the model traits of diseases. They observed variations in age ranges by 3-4 weeks not only between but also within studies, that were spanning critical ages of development like puberty. After hippocampal long-term potentiation, behavioural or ex-vivo studies was observed that the animals' age was erroneously interpreted, usually because animal body weight was considered as an accurate instead of approximate indicator of their age (McCutcheon & Marinelli, 2009). Despite the fact that the specific age of rats was undefined, there is an indication for age difference between the rats used in Experiment 1 and 2. The former ones remained at least 5 weeks longer in the premises until the start of the experiment due to welfare purposes related to reduction in the animal number used in the laboratory. It was found that adolescent rats present with impairments in retaining extinction of cued fear (Bisby et al., 2021), while juvenile male rats do not show spontaneous recovery of auditory fear (Park et al., 2017). Therefore, considering possible that if the rats in Experiment 2 underwent extinction training during adolescence, this might have affected their extinction memory retention and explain the similar levels of auditory fear across extinction recall and spontaneous recovery testing. Thus, it is possible that the present findings may be attributed to deficits in extinction retention rather than lack of spontaneous fear recovery. This observation renders age an important experimental factor to consider

when using animal models as disease traits or pharmacological efficacy in adult animals do not necessarily extrapolate to the younger ones.

In conclusion, the results of this study confirm previous findings regarding the mitigating effect of CBD acutely on expression of contextual fear memory, while also providing novel evidence that CBD can induce long-lasting effects by suppressing the spontaneous recovery of auditory fear when it is administered before extinction training. In contrast, CBD administration after extinction failed to produce any effect on extinction or its retention. These findings encourage the elucidation of pharmacological mechanisms through which CBD regulates the spontaneous recovery of learned fear, by performing pharmacological studies with either CB1 or 5-HT1A receptor antagonists (Do Monte et al., 2013; Fogaca et al., 2014), since such receptors were found to be implicated in the extinction enhancing and anxiolytic effects of CBD (Bitencourt & Takahashi, 2018).

Chapter 4. Investigation of CB1 receptor signalling as a potential pharmacological mechanism of CBD in its regulation of extinction and spontaneous recovery of learned fear

4.1 Introduction

As previously described, the diverse therapeutic potential of CBD is attributed to the multiple molecular targets that interact with it. Regarding its modulating effects on anxiety-like and learned fear behaviours, the involvement of not only endocannabinoid but also endovanilloid and serotonergic signalling has been reported by various preclinical studies. Specifically, the acute effects of CBD on the expression of innate and conditioned fear were 5-HT1AR-mediated, when it was centrally infused at low or intermediate doses (i.e., 15-30 nmol) in dPAG (Campos & Guimarães, 2008), PL (Fogaca et al., 2014), BNST (Gomes et al., 2012; Gomes et al., 2011), and IL (Marinho et al., 2015). In contrast, intra-dPAG administration of a higher CBD dose (i.e., 60 nmol) was found to be ineffective, due to activation of TRPV1 receptors (Campos & Guimaraes, 2009).

Several studies have reported that CBD induces sustained fear alleviating effects by either enhancing the extinction or disrupting the consolidation and reconsolidation of learned fear memory, during which engagement of cannabinoid receptors is indispensable. Systemic (i.e., 10mg/kg) or intrahippocampal (i.e., 30 pmol) CBD administration immediately after contextual fear conditioning was found to interfere with memory consolidation, through time-dependent activation of CB1 and CB2 receptors in dHPC (Stern et al., 2017; Raymundi et al., 2020). Additionally, systemic administration of 10 mg/kg CBD after retrieval resulted in long-lasting

suppression of contextual fear memory expression, through disruption of reconsolidation (Stern et al., 2012), an effect that was similarly observed when 30 pmol CBD was centrally infused into ACC or PL but not in the IL (Bayer et al., 2022). Systemic or intra-PFC pre-administration of 50 pmol CB1R antagonist/inverse agonist AM251, though, prevented the CBD-induced disrupting effects on fear memory reconsolidation. Additionally, it was found that intracerebroventricular (i.c.v) administration of 2 μg/μl CBD or intra-IL infusion of 0.4μg/0.2 μl CBD before extinction training resulted in a reduction of contextual fear expression through CB1R-mediated facilitation of extinction memory (Bitencourt et al., 2008; Do Monte et al., 2013). However, the fact that CBD demonstrates low binding affinity to CB1Rs and CB2Rs suggests that CBD exerts the above-described effects by indirect modulation of endocannabinoid signalling, through inhibition of degradative and re-uptake mechanisms of AEA (Ligresti et al., 2016).

Over the last decades, CB1R-mediated modulation in several aspects of learned fear processing was investigated. However, discrepancies in the findings were attributed to several factors, such as the behavioural task used, the direct or indirect activation of CB1Rs, as well as their localization within the fear circuitry, and the time of intervention. For instance, systemic administration of the CB1R agonist WIN55,212-2 or direct infusion of AEA into NAc core impaired the acquisition and reduced the expression of contextual fear, through a CB1R-dependent mechanism, leaving the auditory fear memory unaffected (Pamplona & Takahashi, 2006; Pedroza-Llinás et al., 2013). In contrast, CB1R activation in BLA, either directly by WIN 55,212-2 or indirectly by AM404 administration, an endocannabinoid reuptake inhibitor, potentiated the acquisition of olfactory fear memory (Tan et al., 2011). Similar contradictory findings

were observed also in CB1R-mediated regulation of fear memory consolidation. Post-conditioning CB1R activation in the hippocampus disrupted fear memory consolidation (Santana et al., 2016), while in retrosplenial cortex (RSC) it ameliorated the consolidation of contextual fear memory (Sachser et al., 2015).

On the other hand, CB1R-mediated signalling was found to modulate retrieval of fear memory as well. The elevation of AEA levels in dl-PAG or dHPC, either directly by infusing AEA in these areas or indirectly by inhibiting its hydrolysis/reuptake mechanisms, was found to inhibit the retrieval of contextual fear memory, through activation of CB1Rs (Resstel et al., 2008; Gobira et al., 2017). Similarly, post-reactivation infusion of the CB1R agonist CP55,940 in hippocampal CA1 area and IL or in RSC disrupted the reconsolidation of contextual fear memory, while CB1R activation in RSC facilitated extinction consolidation, providing long-lasting suppression of learned fear and preventing spontaneous fear recovery (Sachser et al., 2015; Santana et al., 2016). Importantly, systemic or intracranial administration of cannabinoids that facilitate endocannabinoid transmission was found to enhance the acquisition, consolidation, and retrieval of extinction memories, resulting in long-term suppression of contextual or cued learned fear. This can be mediated either by directly agonizing CB1Rs (e.g., WIN55212-2, HU210) (Pamplona et al., 2008; Lin et al., 2009; Ganon-Elazar & Akirav, 2013) or indirectly by increasing AEA levels through blockade of its reuptake (e.g., AM404) (Bitencourt et al., 2008) or degradation (e.g., URB597, AM3506) (Gunduz-Cinar et al., 2013; Morena et al., 2018; Segev et al., 2018).

On the other hand, pre-treatment with CB1R antagonists/inverse agonists or their sole administration was found to abolish the effects induced by the compounds

enhancing endocannabinoid signaling (Resstel et al., 2008; Gobira et al., 2017), or produce the exact opposite effects, respectively (Pamplona et al., 2006; Sachser et al., 2015). SR141716A, also known as rimonabant, and its analogue AM251 are the most used selective CB1R antagonists for the exploration of learned fear-related behaviours, due to their high selectivity and affinity to CB1Rs even at sub-nanomolar concentration range (Howlett et al., 2002; Raffa & Ward, 2012). On the other hand, under higher concentrations both compounds were found to act as inverse agonists and reduce the constitutive activity of CB1Rs or exert CB1R-independent actions, by antagonizing μ-opioid receptors (Seely et al., 2012), or agonizing GPR55 (Ryberg et al., 2007).

Noteworthy is that inconsistent findings are also observed across studies investigating the effects of CB1R antagonists/inverse agonists. Specifically, AM251 was found to enhance the acquisition of trace, cued or contextual fear conditioning (Reich et al., 2008; Sink et al., 2010) when administered before the conditioning session. In contrast, a study by Arenos et al. (2006) showed no effects of AM251 on the acquisition or consolidation of contextual fear memory. However, during the fear retention tests, AM251 decreased the expression of background contextual fear in the auditory conditioning task but not in the contextual one, while it resulted in enhanced expression of cued fear. Opposite findings were observed in CB1R-deficient mice, in which background contextual fear memory was enhanced under highly aversive conditions (Jacob et al., 2012), indicating that task aversiveness plays a crucial role. Additionally, when AM251 was directly infused into BLA immediately after the reactivation session, it disrupted the reconsolidation of auditory fear memory (Ratano et al., 2014). In contrast, intrahippocampal or intra-RSC infusion of AM251 enhanced

reconsolidation and impaired extinction, resulting in elevated conditioned responses upon extinction test and spontaneous recovery of contextual fear (de Oliveira Alvares et al., 2008; Sachser et al., 2015), suggesting that CB1R exert opposite effects in reconsolidation and extinction of learned fear. Similarly, impaired extinction was observed by either intra-mPFC or systemic AM251 administration in fear-potentiated startle or contextual fear conditioning protocols, respectively (Kuhnert et al., 2013; Laricchiuta et al., 2013). Comparable effects were described after systemic administration of CB1R antagonist rimonabant, impairing the extinction of contextual and cued fear (Marsicano et al., 2002; Chhatwal et al., 2005; Pamplona et al., 2006; Pamplona et al., 2008; Chhatwal et al., 2009;). The effects of rimonabant have been also investigated under a protocol of low preincubated fear conditioning, in which animals received 100 CS-US pairings over 10 days and demonstrated lower fear expression 2 days after the last fear conditioning session and higher fear when tested for its retention 30 days later (Pickens et al., 2009). Interestingly, rimonabant was shown to interfere with the between-session extinction and impair its retention on low preincubated fear conditioning (Pickens & Theberge, 2014), but disrupted the within-session extinction in the auditory fear conditioning and the passive avoidance task (Niyuhire et al., 2007), indicating that CB1R modulation of extinction is specific to the behavioral task.

In the previous chapter, it was described that CBD administration before extinction training acutely reduced the expression of contextual fear memory and elicited a long-lasting effect in preventing spontaneous recovery of auditory fear 21 days after extinction. This chapter aims to extend the previous findings and investigate whether CB1R signaling is implicated in CBD's regulation of fear extinction and

spontaneous fear recovery. In Experiment 1, a dose-response study of the CB1R antagonist/inverse agonist AM251 was performed to determine an appropriate dose that would not elicit any effects on its own, in order to investigate in Experiment 2 its potential interference with the previously described effects of CBD.

4.2 Materials and methods

4.2.1 Subjects

Male Lister-Hooded rats (Envigo, UK), weighing 280-340 g were used for Experiments 1 and 2. Rats were group-housed and behaviourally tested under the same conditions as described in previous chapters. All experimental procedures were performed with internal ethical approval and in accordance with the Animals (Scientific Procedures) Act 1986, UK (PPL: P6DA59444). The sample size of each treatment group was calculated based on the power analysis described in Chapter 2.

4.2.2 Drug preparation and administration

AM251, a CB1R antagonist/inverse agonist, (APExBIO Technology, USA) at a dose of 1 or 3 mg/kg was used in Experiment 1, while 1 mg/kg AM251 was administered in combination with 10 mg/kg CBD (THC Pharm, Germany) in Experiment 2. Both compounds were suspended in a freshly prepared vehicle made of 95 % sterile saline and 5 % Tween 80 and administered intraperitoneally before the extinction session at a volume of 1 ml/kg. The doses of AM251 were selected based on previous studies investigating their effects on learned fear processing (Arenos et al., 2006; Stern et al., 2012; Stern et al., 2017). The CBD dose was selected as the lower effective dose modulating spontaneous fear recovery in the previous experiment in Chapter 3. None

of the selected doses were found to affect baseline locomotion or sensitivity to the US footshock in a previous study (Reich et al., 2008).

4.2.3 Experiment 1: Dose-response effects of pre-extinction AM251 administration on extinction and spontaneous recovery of learned fear

The effects of systemic administration of different AM251 doses before extinction on modulation of extinction and spontaneous recovery of contextual and auditory learned fear were investigated using a 24-day protocol, presented in Figure 4.1. The behavioral testing apparatus and recording software, along with the design and parameters used in the following experiments have been described in detail in Chapters 2 and 3. On day 1, all rats underwent contextual habituation into two distinct contexts, A and B. The next day, they were subjected to auditory fear conditioning in context A, receiving 5 tone presentations (30 s, 4 kHz, 80 dB, ITI=2 min), followed by 5 tone-footshock pairings (0.5 s, I=0.4 mA, US co-terminated with CS). On day 3, rats were randomly allocated into three treatment groups (n=10/group) and administered with 0, 1, or 3 mg/kg AM251 30 minutes before receiving extinction training in Context B, which consisted of 30 tone presentations (30 sec, ITI=30sec). On days 4 and 24, all rats returned to the extinction context and were subjected to extinction and spontaneous recovery testing, respectively, receiving 3 tone presentations.

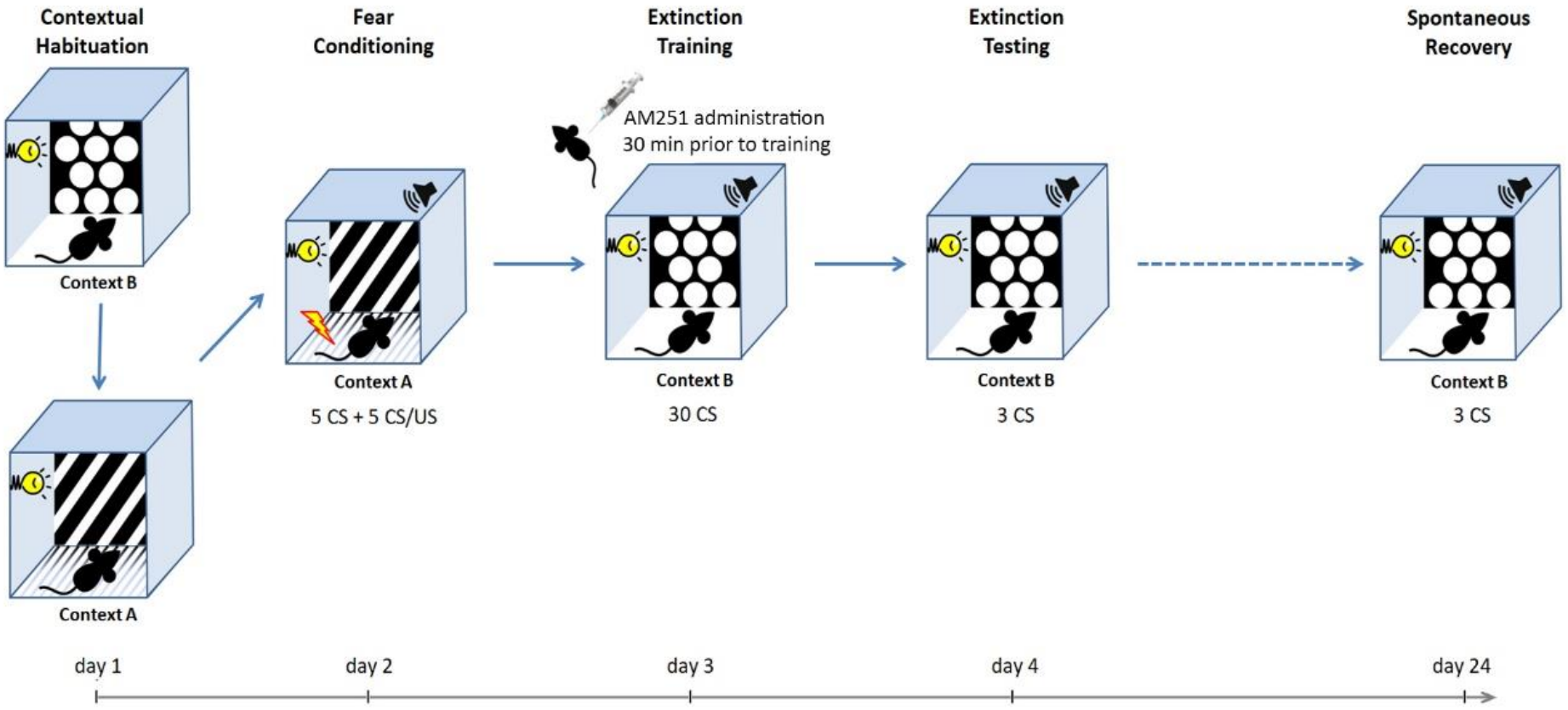

Figure 4.1 Schematic representation of dose-response study of the CB1R antagonist/ inverse agonist AM251. On day 1, rats were habituated in context B and then in context A. On day 2, rats underwent fear conditioning with 5 CS followed by 5 CS-US pairings in context A. On day 3, all rats were administered i.p. vehicle, 1, or 3 mg/kg AM251 30 min before extinction training, and received 30 CS presentations in context B. On day 4, all groups were exposed to 3 CS for extinction testing, in context B. 21 days after extinction training, they were returned for a spontaneous recovery session, receiving 3 CS presentations.

4.2.4 Experiment 2: Effect of AM251 pre-treatment on CBD regulation of extinction and spontaneous recovery of learned fear

All rats underwent contextual habituation and auditory fear conditioning on days 1 and 2, respectively as described above in Experiment 1. On day 3, rats were randomly allocated into four treatment groups (n=9/group), Vehicle + Vehicle, AM251 + Vehicle, Vehicle + CBD, and AM251 + CBD (Figure 4.2). Rats received their pre-treatment (Vehicle or AM251) and treatment (Vehicle or CBD) with a 30 min-interval between them, while treatment was administered 30 min before the extinction training. On days 4 and 24, all rats were subjected to sessions of extinction and spontaneous recovery testing, respectively, as described in Experiment 1.

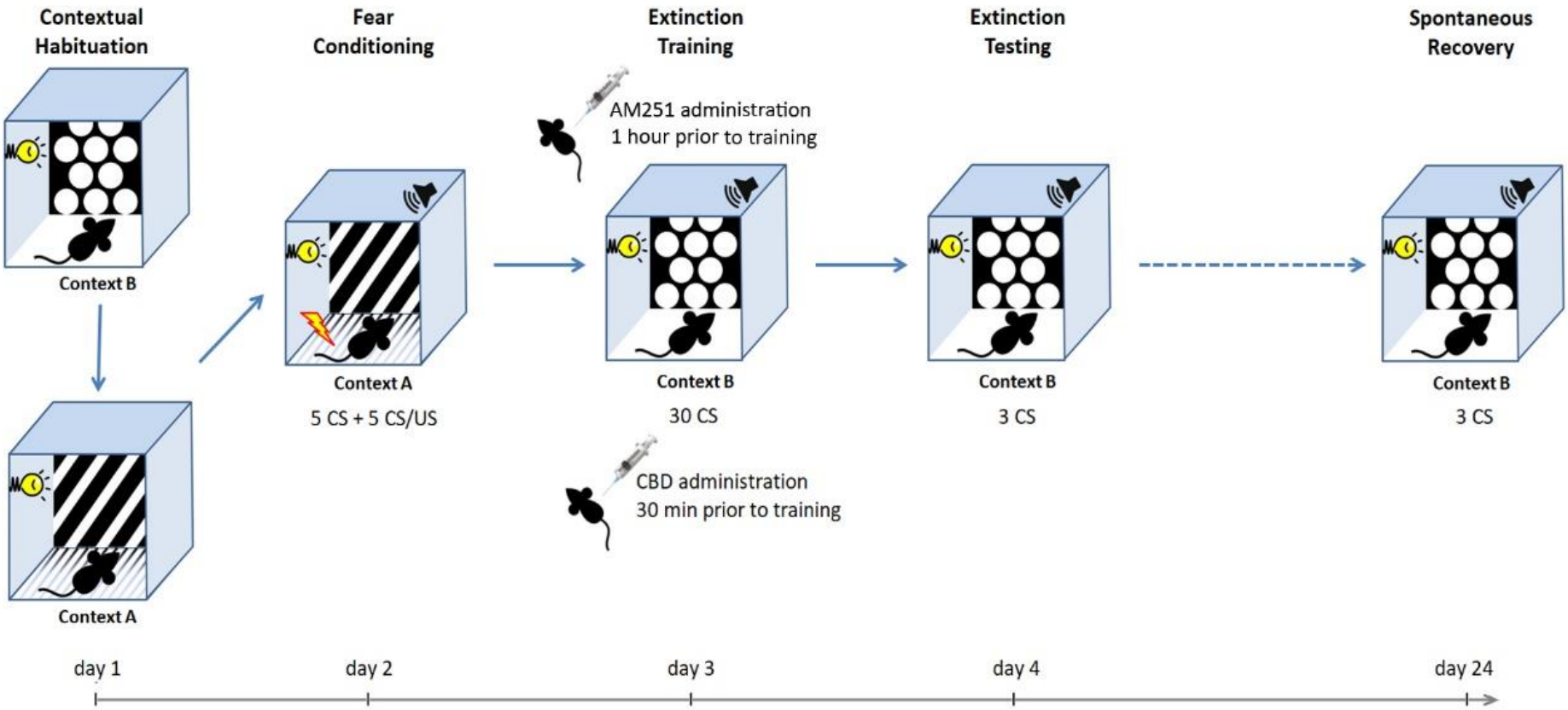

Figure 4.2 Protocol used for investigating the effect of AM251 pre-treatment on the CBD regulation of extinction and spontaneous recovery. On day 1, rats were habituated in context B and then in context A. On day 2, rats underwent fear conditioning with 5 CS followed by 5 CS-US pairings in context A. On day 3, rats were randomly allocated into 4 different groups, receiving i.p. injection of either vehicle or 1 mg/kg AM251 60 min before extinction training, and vehicle or 10 mg/kg CBD 30 min before extinction training. Rats were subjected to 30 CS presentations in context B. On day 4, all groups were exposed to 3 CS for extinction testing, in context B. 21 days after extinction training, they were returned for a spontaneous recovery session.

4.3. Data analysis

The analysis of the experimental data in this chapter was performed in a similar way as in the previous chapters. Specifically, the duration of freezing behavior was expressed as the percentage of 120 sec for the pre-CS interval or 30 sec for each CS trial. Auditory fear during extinction training, extinction testing, and spontaneous recovery testing sessions was expressed in CS blocks, defined by the average of freezing during 3 consecutive CS presentations. All data are presented as mean ± SEM and statistical analysis was performed using GraphPad Prism 9, while $p < 0.05$ was considered the level of statistical significance for all comparisons. In both experiments, fear conditioning was analyzed using repeated-measures or mixed-model two-way ANOVA, with group and trial being designated as the between- and within-subject factors, respectively. Geisser-Greenhouse correction was applied to adjust for the lack of sphericity. The pre-CS interval before extinction training was analyzed through one-way ANOVA, with dose or treatment defined as the between-subject factor for Experiments 1 and 2, respectively. Bartlett's test was used to assess the homogeneity of variance, while $p < 0.05$ was considered the level of assumption violation. Two-way ANOVA with Geisser-Greenhouse correction was conducted for the analysis of CS blocks during extinction training in Experiments 1 and 2 with dose or treatment as the between-subject factor, respectively, and block as the within-subject factor. Freezing levels during the pre-CS interval of the extinction test and spontaneous recovery were analyzed using two-way ANOVA, with dose or treatment as the between-subject factor for Experiments 1 and 2, respectively, whereas time was defined as the within-subject factor. The fact that the within-subjects factor was comprised of only two levels (e.g., extinction test vs spontaneous recovery) rendered inapplicable the

sphericity assumptions. The freezing differences during the CS block of extinction and spontaneous fear recovery testing were analyzed similarly as above. Sidak's post-hoc test for multiple comparisons was applied when ANOVA revealed a significant main effect or interaction between independent variables. Limited pairwise comparisons were performed between the means of the lower or higher dose of AM251 and the vehicle in the dose-response study (Experiment 1). In contrast, the means of each treatment group were fully pairwise compared against the means of each other group in the AM251-CBD combination study (Experiment 2). Sidak method was preferred over Bonferroni for its higher power and used to compute adjusted P value for each individual test and confidence intervals (Lee & Lee, 2018).

4.4 Results

4.4.1 Experiment 1: Dose-response effects of pre-extinction AM251 administration on extinction and spontaneous recovery of learned fear

The effects of pre-extinction training AM251 administration on learned fear expression, extinction training, extinction recall, and later spontaneous recovery are depicted in Figure 4.3. The levels of freezing behavior during the CS-US pairings in the fear conditioning session are shown in Figure 4.3.A. Two-way ANOVA revealed significant main effects of trial ($F_{(3.053, 82.42)} = 34.94$, $P < 0.0001$) and group ($F_{(2, 27)} = 3.776$, $P = 0.0358$) but no trial x group interaction ($F_{(8, 108)} = 1.887$, $P = 0.0693$). However, after performing post-hoc analysis, no reliable differences in freezing were identified between the groups ($P > 0.05$) to receive the different doses of AM251 before extinction training. Freezing during the pre-CS interval before extinction training is shown in Figure 4.3.B. One-way ANOVA found no main effect of dose ($F_{(2,}$

$_{27)}$ = 1.339, P = 0.2791), indicating that neither of the AM251 doses affected the baseline expression of contextual fear memory before extinction training. Freezing during the CS blocks in the extinction training session is presented in Figure 4.3.C. Two-way ANOVA revealed a significant main effect of block ($F_{(2.396,\ 64.68)}$ = 26.05, P < 0.0001), but no effect of dose ($F_{(2,\ 27)}$ = 0.7305, P = 0.4909) or block x dose interaction ($F_{(18,\ 243)}$ = 1.141, P = 0.3128), indicating that AM251 induced no effect on auditory fear memory expression or extinction learning. The effects of the pre-extinction AM251 administration on contextual and auditory fear during the pre-CS interval and CS block, respectively, across extinction recall and spontaneous recovery testing are presented in Figure 4.3.D and E. To investigate the effect of AM251 on contextual fear, two-way ANOVA was conducted on freezing during the pre-CS interval before extinction and spontaneous fear recovery testing, revealing a significant main effect of time ($F_{(1,\ 27)}$ = 26.53, P < 0.0001), but no dose effect ($F_{(2,\ 27)}$ = 0.6753, P = 0.5174) or time x dose interaction ($F_{(2,\ 27)}$ = 1.742, P = 0.1943). This indicates that freezing during the pre-CS interval of spontaneous recovery testing was significantly increased in all groups when compared with extinction testing, suggesting spontaneous recovery of contextual fear 21 days after extinction training, without any reliable lasting effect of AM251 during either test. To determine the effect of AM251 on the return of auditory fear over time, two-way ANOVA was conducted on CS-block of extinction and spontaneous fear recovery testing. There was no main effect of time ($F_{(1,\ 27)}$ = 1.191, P = 0.2849), dose ($F_{(2,\ 27)}$ = 0.9623, P = 0.3947) or time x dose interaction ($F_{(2,\ 27)}$ = 0.0009292, P = 0.9991). This indicates that no reliable differences in freezing behavior were observed during the CS presentations across the extinction recall and spontaneous recovery testing, suggesting that none of the AM251 dose groups

showed spontaneous recovery of auditory fear, while AM251 failed to produce any lasting effect in either test.

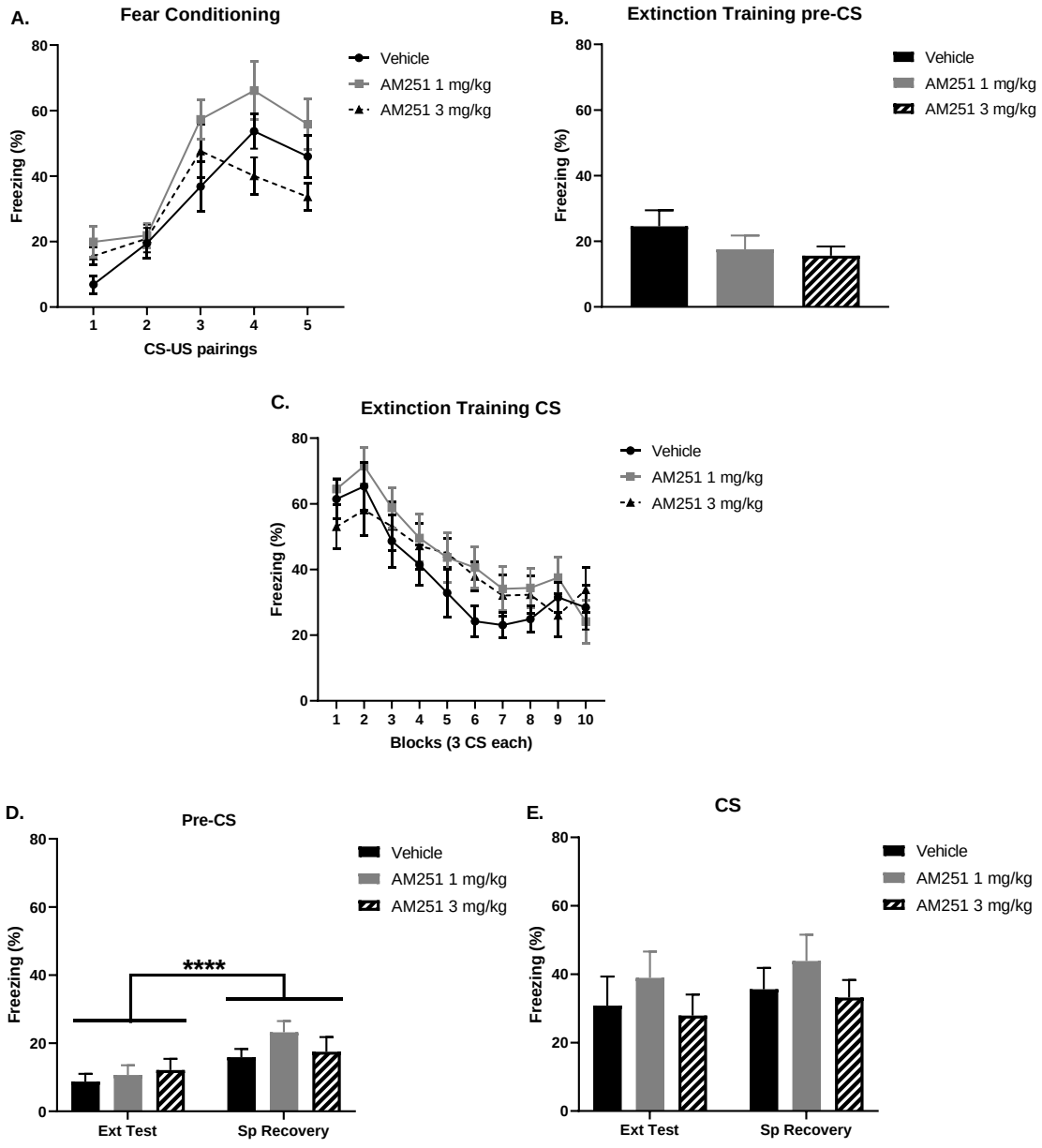

Figure 4.3 AM251 did not affect expression, extinction or spontaneous recovery of learned fear. (A) Fear conditioning did not differ between the groups to receive i.p. different doses of AM251 (n=10 rats/group). (B) AM251 did not affect freezing during the pre-CS interval before extinction training. (C) No reliable differences in freezing were found between the different groups during extinction training. (D) All groups demonstrated significantly higher freezing during the pre-CS interval of spontaneous fear recovery compared to extinction recall test (**** $p < 0.0001$), but no effect on freezing was observed by AM251. (E) Freezing levels did not significantly differ across the treatment groups or between the two time points during the CS block, indicating no AM251 effect on auditory fear expression 1 and 21 days after extinction training and lack of spontaneous recovery.

4.4.2 Experiment 2: Effect of AM251 pre-treatment on CBD regulation of extinction and spontaneous recovery of learned fear

To investigate the hypothesis that CBD prevents spontaneous recovery of auditory fear through activation of CB1Rs, AM251 (1 mg/kg) was administered in combination with CBD (10 mg/kg), to determine whether AM251 can inhibit this beneficial effect of CBD. The dose of AM251 was selected based on the results from Experiment 1, revealing that AM251 alone did not elicit any changes in the freezing behavior. The effects of the different treatments on learned fear expression, extinction training, extinction recall, and later spontaneous recovery are depicted in Figure 4.4. The levels of freezing behavior during the CS-US pairings in the fear conditioning session are shown in Figure 4.4.A. Two-way ANOVA revealed significant main effect of trial ($F_{(3.218, 103.0)} = 26.56$, $P < 0.0001$), but no group effect ($F_{(3, 32)} = 0.9701$, $P = 0.4189$), or trial x group interaction ($F_{(12, 128)} = 1.384$, $P = 0.1814$), indicating that there were no reliable differences between the groups to receive the different treatment combinations prior to extinction training. Freezing during the pre-CS interval before extinction training is shown in Figure 4.4.B. One-way ANOVA revealed no significant main effect of treatment ($F_{(3, 32)} = 0.5822$, $P = 0.6310$), indicating that treatment with AM251 or CBD alone, or their combination, did not affect the baseline expression of contextual fear before extinction training. Freezing during the CS blocks in the extinction training session is presented in Figure 4.4.C. Two-way ANOVA revealed a significant main effect of block ($F_{(2.820, 90.25)} = 21.88$, $P < 0.0001$), but no effect of treatment ($F_{(3, 32)} = 1.920$, $P = 0.1462$) or block x treatment interaction ($F_{(27, 288)} = 1.076$, $P = 0.3682$), indicating that none of the treatments affected auditory fear memory expression or extinction learning. The effects of pre-extinction administration

of AM251, CBD, or their combination on contextual and auditory fear during the pre-CS interval and the CS block, respectively, across the extinction recall and spontaneous recovery testing, are presented in Figures 4.4.D and E. To investigate their effect on contextual fear, two-way ANOVA was conducted on freezing levels during the pre-CS interval before extinction and spontaneous fear recovery testing, revealing a significant main effect of time ($F_{(1, 32)} = 50.51$, $P < 0.0001$), but no effect of treatment ($F_{(3, 32)} = 0.1628$, $P = 0.9206$), or time x treatment interaction ($F_{(3, 32)} = 0.3381$, $P = 0.7979$). This indicates that the freezing levels were significantly increased in all treatment groups during spontaneous recovery when compared to extinction testing, suggesting spontaneous recovery of contextual fear 21 days after extinction training, which was not affected by any of the treatments. To determine the effects of AM251 or CBD alone or their combination on the return of auditory fear over time, two-way ANOVA was conducted on CS-block of extinction and spontaneous fear recovery testing. There was a significant main effect of time ($F_{(1, 32)} = 5.346$, $P = 0.0274$), but no effect of treatment ($F_{(3, 32)} = 0.6078$, $P = 0.6148$), or time x treatment interaction ($F_{(3, 32)} = 0.5754$, $P = 0.6354$). This indicates elevated freezing during the CS presentations of spontaneous recovery, compared to extinction testing and suggests that spontaneous recovery of auditory fear occurred in all treatment groups, while neither AM251 nor CBD produced any lasting effect in either test, and freezing remained unaffected by the administration of AM251 before CBD.

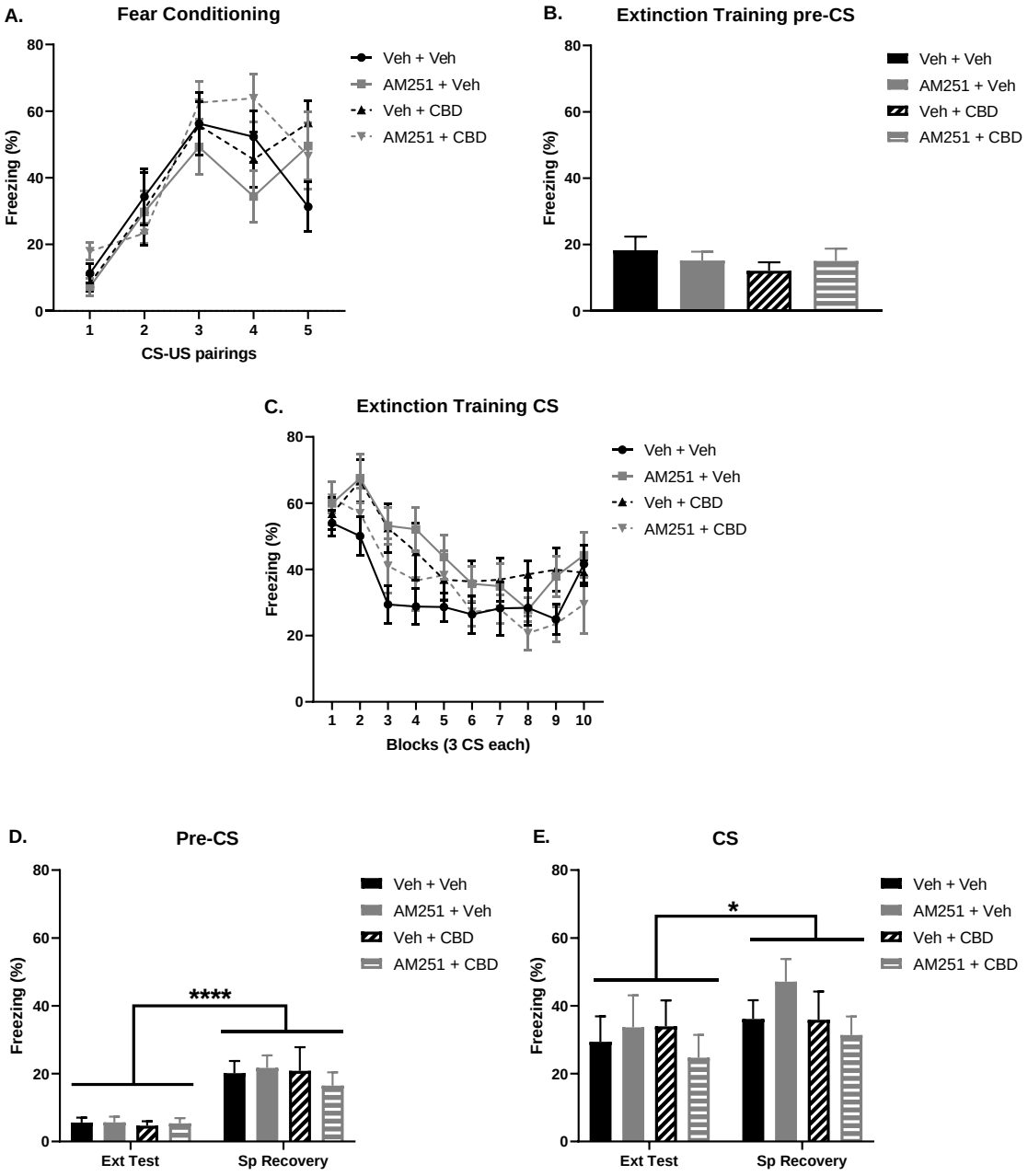

Figure 4.4: AM251, CBD, or their combination had no effect on the expression of learned fear, extinction, or spontaneous recovery. (A) Fear conditioning did not differ between the groups to receive i.p. AM251 (1 mg/kg), CBD (10 mg/kg) or their combination (n=9 rats/group). (B) None of the treatments affected freezing during the pre-CS interval before extinction training. (C) No reliable differences in tone-induced freezing were found between the treatment groups during extinction training. (D) All groups demonstrated significantly higher freezing during the pre-CS interval of spontaneous fear recovery compared to extinction recall test (**** $p < 0.0001$), but no effect of AM251, CBD, or their combination was observed during either time point. (E) All treatment groups showed significantly higher tone-induced freezing during spontaneous recovery testing than the extinction recall test (* $p < 0.05$), but AM251, CBD, or their combination did not have any reliable effect.

4.5 Discussion

This study investigated the possible role of CB1R signaling in mediating the acute reduction of contextual fear expression and the long-lasting suppression of spontaneous recovery of auditory fear induced by pre-extinction CBD administration. In Experiment 1, a dose-response study with AM251 was performed to identify the dose that did not elicit effects *per se* but could potentially abolish the CBD effects observed in Chapter 3. None of the AM251 doses used affected the expression of learned fear, extinction learning, or had long-lasting effects on extinction recall the next day. Although AM251 had no effects on the spontaneous recovery of contextual fear, none of the treatment groups showed spontaneous recovery of auditory fear. In Experiment 2, CBD and the low dose of AM251 were administered either in combination or with their vehicle counterpart. None of the treatment groups affected the baseline expression of contextual fear memory, while CBD-treated animals failed to reproduce the previous findings. Neither the expression of auditory fear memory nor extinction learning was affected by the different treatment groups. Similarly, none of the treatment groups elicited any long-lasting effect on extinction recall one day later during the extinction test or 21 days later upon spontaneous fear recovery testing. Importantly, CBD failed to suppress the spontaneous recovery of auditory fear, as demonstrated in the previous chapter, while the pretreatment with AM251 failed to induce any effects either. Due to the lack of reproducibility of the CBD effects in the current study, it is unclear whether CB1R signalling is involved in CBD's fear-suppressing effects shown in the previous chapter. Nevertheless, the present findings will be compared with relevant studies from the literature and the possible factors that may have contributed to this irreproducibility will be discussed in detail below.

In Experiment 1, the fact that the administration of 1 or 3 mg/kg AM251 before extinction did not elicit any effects on the expression of contextual or auditory learned fear is not an unexpected finding. In a study by Gobira et al. (2017) i.p. administration of 1 mg/kg AM251 was found to abolish the effects of systemically injected AA-5-HT, a dual FAAH and TRPV1 blocker, that inhibited the contextual fear memory retrieval, when administered before the retrieval test. However, in a dose-response study, none of the AM251 doses (0.3, 1, or 3 mg/kg) affected fear memory expression when administered alone. In contrast, in another study, the i.p. administration of 3 mg/kg before the fear retrieval tests decreased the expression of background contextual fear but increased cued fear expression (Arenos et al., 2006). The lack of effects in the present study might be attributed to the lower foot-shock intensity in comparison to theirs (0.4 vs 1.0 mA) and the lower volume of drug administration (V=1 vs 3 mL/kg) applied. In addition, AM251 did not produce any acute effects during extinction training or long-lasting effects on extinction recall the next day, which is contradicted by other studies using either AM251 or rimonabant. Noteworthy is that other studies have shown impairments in the extinction encoding after contextual or trace conditioning, only under higher doses of AM251 (i.e., 5 or 6 mg/kg) (Reich et al., 2008; Laricchiuta et al., 2013). Similarly, dose-dependent effects were observed with rimonabant, disrupting the extinction of contextual and cued fear with doses usually greater than 1 mg/kg, while 0.2 mg/kg only prevented the extinction enhancing effects of systemically or centrally administered cannabinoids (e.g., WIN 55212-2, AM404, CBD) (Marsicano et al., 2002; Chhatwal et al., 2005; Pamplona et al., 2006; Niyuhire et al., 2007; Bitencourt et al., 2008; Do Monte et al., 2013).

Interestingly, a study by Sachser et al. (2015) has shown that intra-RSC infusion of AM251 did not elicit within-extinction effects, and instead resulted in between-extinction impairments, suggesting that AM251 blocked extinction consolidation. This was observed as an increased expression of contextual fear upon extinction test and later during spontaneous recovery sessions, a finding that did not resemble the present results. This possibly indicates that the route of drug administration along with the differential modulation of contextual and cued fear conditioning by CB1Rs might play a role in the discrepancies between the studies. Although AM251 did not induce any long-lasting effects on the spontaneous recovery of contextual fear in this experiment, the lack of spontaneous recovery of auditory fear in all treatment groups is difficult to interpret. A possible explanation might be that the rats used came from a different breeding company compared to those used in the previous chapter. Previous studies have observed differences in anxiety-like behaviours between rodents of the same strain obtained from different vendors, although conditioned fear responses either did not reveal any significant differences or were not assessed (Tsuda et al., 2020; Ericsson et al., 2021). Noteworthy is that the freezing levels throughout this experiment were higher than those observed in the previous chapters. Possibly, the rats might present poor between-session extinction recall of cued fear, thus reaching a maximal tone-induced fear responding (i.e., ceiling effect) across both extinction and spontaneous recovery testing.

Despite not finding spontaneous fear recovery in any of the groups in Experiment 1, the low dose of AM251 was selected to proceed with the AM251-CBD combination study. This was supported by previous studies showing that this dose (i.e., AM251 1mg/kg) was sufficient to abolish the reconsolidation blockade induced

by CBD (Stern et al., 2012; Stern et al., 2017) or to prevent anxiolytic-like effects of URB597, a FAAH inhibitor (Haller et al., 2009), but not to elicit behavioural effects on its own. A lower dose of 0.3 mg/kg AM251 has also been used to block the effects of URB597 or WIN 55212-2 on extinction memory or acoustic startle response (Fidelman et al., 2018; Segev et al., 2018). However, there is no previous evidence of using this dose of AM251 to investigate the potential involvement of CB1Rs in CBD's extinction-facilitating effects.

In Experiment 2, as mentioned above, none of the treatments CBD or AM251 alone, or their combination, administered before extinction training affected the expression of learned fear, extinction learning, extinction recall, or spontaneous fear recovery when compared to the vehicle-treated group. As expected, AM251 + Veh showed no effects since this dose (1mg/kg) was validated and selected for not eliciting any effect by itself, but for solely interfering with CBD-induced effects. Here AM251-treated animals demonstrated spontaneous recovery of auditory fear in contrast to Experiment 1, but they did not show any differences in freezing in comparison to the other treatment groups.

Striking is that CBD administration (i.e., Veh + CBD) in Experiment 2 did not acutely reduce the baseline expression of contextual fear or suppress the spontaneous recovery of auditory fear as previously observed in Chapter 2. One factor that needs to be considered is the increase in Tween 80 concentration of the vehicle in which CBD was suspended, from 2% to 5%. This was decided in order to use the same vehicle both for AM251 and CBD suspension, given that this method had previously been applied in other studies (Stern et al., 2012; Stern et al., 2017). However, this change might have affected the micellization process and thus the penetration of CBD through

the BBB (Bruni et al., 2018). A previous study revealed that an increase in Tween 80 concentration was found to decrease the droplet size of the nanoparticle during the drug formulation process. As the particle size plays a crucial role in the rate of endocytosis across the BBB, smaller particles demonstrated enhanced BBB penetration and improved drug delivery to the brain parenchyma, along with increased pharmacological effects (Yadav et al., 2017). Additionally, other groups observed higher absorption, plasma, and brain parenchyma levels of *per os* administered compounds, that have been suspended in higher Tween 80 concentration solutions, demonstrating as well a greater area under the curve (AUC) (i.e., definite integral of the drug plasma concentration over time) and Cmax (i.e., maximum drug concentration in a compartment) (Azmin et al., 1982; Zhang et al., 2003). Therefore, the suspension of CBD in a vehicle solution with a higher concentration of Tween 80 might have resulted in greater CBD bioavailability in the extinction brain-related areas, inducing possibly a more robust inhibition of AEA reuptake and degradation mechanisms. Increasing AEA pools in the synapse, CBD may have indirectly stimulated both CB1 and TRPV1 receptors, or activated other non-specific targets, resulting in the neutralization of previously observed effects (Moreira et al., 2012; Aguiar et al., 2014).

Despite the lack of CBD effects in this experiment, we could speculate that the pre-administration of AM251 would have blocked the CB1R signalling, leaving intact the TRPV1 receptors and thus increasing conditioning responses (Uliana et al., 2016). However, AM251 + CBD treated animals did not show any effects on learned fear, extinction, or spontaneous recovery when compared with the other treatment groups. It was found that i.c.v or intra-IL CBD infusion facilitated the extinction of

contextual fear memory and produced a long-lasting reduction of freezing during the extinction recall through a CB1R-dependent manner (Bitencourt et al., 2008; Do Monte et al., 2013). In the previous chapter, it was discussed that the protocol aversiveness (i.e., 0.4 vs 1.5mA), route of CBD administration (i.e., systemic vs i.c.v or intra-IL), and the type of fear conditioning (i.e., auditory vs contextual) might have played a critical role in the lack of CBD effect on extinction facilitation observed in the present study, possibly because CB1R signaling differentially modulated fear retrieval and extinction processes. Indeed, there is evidence from studies abolishing CB1R signaling either through genetic silencing or pharmacological blockade that increased contextual fear memory was observed only with stronger footshock intensity (Lin et al., 2011; Jacob et al., 2012), while no effects were produced with weaker footshock (Suzuki et al., 2004). This suggests that a critical threshold of aversiveness should be reached for the endocannabinoid system to get activated. Also, supporting this idea are the bidirectional effects induced by CBD on contextual fear extinction upon stronger or weaker fear conditioning parameters (Song et al., 2016). Additionally, it was observed that CB1R signaling differentially modulated the contextual versus auditory fear conditioning and the processing of background (i.e., tone is principally associated with US) versus foreground (i.e., contextual stimuli only associated with US) contextual fear memory (Arenos et al., 2006; Sink et al., 2010). Therefore, in the present studies, CBD may have differentially regulated the extinction and spontaneous recovery of learned fear, possibly because indirect CB1R activation elicited different behavioral responses under these specific experimental parameters, or such a mechanism was partially involved in these effects, or CBD has acted by engaging an entirely different pharmacological mechanism.

Although CB1R signaling was found to be implicated in CBD-mediated modulation of extinction and reconsolidation of learned fear (Bitencourt et al., 2008; Stern et al., 2012; Do Monte et al., 2013; Bayer et al., 2022), it is worth mentioning that its effects on acquisition and consolidation are governed by other mechanisms of action as well. Specifically, direct infusion of CBD in the shell of NAc before fear conditioning resulted in disruption of auditory fear memory acquisition, through 5-HT1AR-mediated signaling (Norris et al., 2016). Additionally, systemic or central infusion in dHPC of CBD immediately after fear conditioning, induced impairment in contextual fear memory consolidation, involving concomitant CB1R and CB2R activation. In contrast, the same effect induced by CBD when administered one hour after conditioning was mediated by PPARγ signaling in dHPC, indicating that CBD's effects on learned fear memory consolidation rely on a time-dependent engagement of CB1, CB2, and PPARγ receptors (Stern et al., 2017; Raymundi et al., 2020). Possibly, through a similar mechanism, CBD may time-dependently modulate extinction encoding and consolidation, activating distinct types of receptors, that remains to be elucidated.

In conclusion, the results of this study failed to reproduce the previously observed effects of CBD on acute reduction of contextual fear expression and long-lasting suppression of spontaneous recovery of auditory fear, while the pre-treatment with AM251 left unaltered the conditioned responses mediated by CBD. Due to the results of this study, the possibility of indirect CB1R activation mediating, at least in part, the effects of CBD cannot be excluded. Therefore, future studies could involve the repetition of AM251-CBD experiment under as identical as possible experimental conditions to those of Chapter 3 (i.e., using the same vehicle for CBD), and thereafter,

the exploration of other pharmacological targets either by dually inhibiting CB1 and CB2 receptors, or antagonizing PPARγ, and 5-HT1ARs.

Chapter 5. Investigation of 5-HT1AR signalling as a potential pharmacological mechanism of CBD regulation of extinction and spontaneous recovery of learned fear

5.1 Introduction

As mentioned earlier, CBD's diverse therapeutic effects are attributed to its multifaceted mechanisms of action, engaging signaling pathways that are not only restricted within the endocannabinoid system. CBD was found also to modulate serotonergic neurotransmission by acting as an agonist or allosterically interacting with 5-HT1ARs (Russo et al., 2005; Rock et al., 2012). Facilitation of 5-HT1AR-mediated signaling is associated with CBD's antidepressant (Zanelati et al., 2010; Linge et al., 2016; Sartim et al., 2016) anti-aggressive (Hartmann et al., 2019), and panicolytic (Soares Vde et al., 2010; Campos et al., 2013) properties. Additionally, CBD has attracted much attention for its 5-HT1AR-dependent anxiolytic-like effects that were observed after systemic (Moreira et al., 2006; Resstel et al., 2009), and central administration (i.e., PAG and BNST) in the anxiety models of EPM and Vogel conflict test (Campos & Guimarães, 2008; Gomes et al., 2011). Noteworthy is that EPM is based on the animal's innate conflict between motivation to explore and avoidance of open spaces that may expose it to danger, whilst Vogel conflict test is based on the approach-avoidance conflict between the animal's intense motivation to drink after a long period of water deprivation and the footshock delivery after a fixed number of licks (Hoffman, 2016).

As outlined above, several studies have demonstrated that CBD induces sustained fear-alleviating effects through facilitation of extinction (Bitencourt et al., 2008; Do Monte et al., 2013) or disruption of the consolidation (Stern et al., 2017;

Raymundi et al., 2020) and reconsolidation (Stern et al., 2012; Bayer et al., 2022) of learned fear, by indirectly activating CB1Rs. In contrast, CBD was found to acutely produce anxiolytic responses in models of innate and learned fear, through a 5-HT1AR-dependent mechanism, only upon administration in specific brain areas within the circuitry. Specifically, intra-BNST infusion of CBD before the EPM and Vogel conflict test (Gomes et al., 2011) or re-exposure to a conditioning context (Gomes et al., 2012) resulted in anxiolytic-like responses, by increasing the number of the open arm explorations and the footshock-associated licks, or suppressing the expression of contextual learned fear, respectively. Interestingly, opposing effects were observed in the EPM after direct infusion of CBD in IL and PL, inducing anxiolytic and anxiogenic-like responses, respectively (Fogaca et al., 2014; Marinho et al., 2015). However, these effects were reversed when restraint stress was applied a day before the EPM test. Analogous effects were reported when CBD was directly infused in PL, but not IL, before re-exposure to the conditioning context, leading to suppression of contextual fear memory expression. The anxiogenic or anxiolytic effects induced by intra-PL CBD infusion on the expression of innate and learned fear, respectively, were mediated through 5-HT1AR-dependent mechanisms, since pre-administration with the 5-HT1AR antagonist WAY100,635 blocked the effects of CBD. Importantly, the opposing effects observed after intra-IL CBD infusion were also mediated by activation of 5-HT1ARs. Additionally, a study by Norris et al. (2016) revealed that 5-HT1AR-mediated signaling was responsible for the disrupting effects of CBD on the acquisition of olfactory fear memory after direct infusion into shell of NAc, as this effect of CBD was abolished by 5-HT1AR antagonist pre-treatment. Although a recent study has revealed that intra-dHPC CBD administration impaired contextual fear memory consolidation through a

time-dependent activation of CB1 and CB2 receptors, this effect was also partially mediated by 5-HT1AR signaling when CBD was administered immediately after conditioning (Raymundi et al., 2020). Additionally, a study by Stern et al. (2012) has revealed that CBD provided lasting suppression of reinstatement and spontaneous recovery of contextual fear by disrupting reconsolidation of learned fear memory, through activation of CB1Rs, rather than 5-HT1ARs. To date, there is no evidence from previous studies that 5-HT1ARs are involved in the facilitatory effects of CBD on extinction, rendering this an interesting question to be investigated.

Although this chapter is centered around the effects of 5-HT1AR-mediated signaling on the expression of learned fear memory, and its extinction, it is worth briefly mentioning the roles of 5-HT2A and 5-HT3 receptors. A study by Zhang et al. (2013) has shown that systemic administration of the 5-HT2AR agonist TCB-2 (i.e., 1.0 mg/kg, i.p.) after fear conditioning enhanced consolidation of both contextual and cued fear memory, while fear acquisition and retrieval were unaffected when TCB-2 was administered either before conditioning or before retrieval testing. Additionally, pre-extinction TCB-2 administration facilitated the acquisition of extinction memory after trace and delay conditioning (Zhang et al., 2013). Regarding 5-HT3Rs, mice overexpressing these receptors showed enhancement in contextual fear conditioning (Harrell & Allan, 2003). Conversely, antagonism of 5-HT3Rs through i.p. administration of tropisetron (i.e., 0.01-0.1 mg/kg) and ondansetron (i.e., 0.001-1.0 mg/kg) led to impaired expression of contextual learned fear (Yoshioka et al., 1995) and potentiated startle (Nevins & Anthony, 1994), respectively, while another 5-HT3R antagonist granisetron (i.e., 0.5 or 1.0 mg/kg, i.p.) enhanced extinction consolidation of cued and contextual fear memory (Park & Williams, 2012).

Over recent decades, several studies have investigated the role of 5-HT1AR-mediated signaling in the modulation of learned fear processing, while trying to disambiguate the differential involvement of 5-HT1A autoreceptors and postsynaptic receptors, distributed in the raphe nuclei or projection forebrain areas, respectively. Stiedl et al. (2000) approached this issue by performing subcutaneous (i.e., 0.1–1.0 mg/kg) and intra-hippocampal (i.e., 5.0 μg) administration of the 5-HT1AR agonist 8-OH-DPAT. Both methods interfered with the acquisition of fear conditioning. However, intrahippocampal infusion of 0.5 μg WAY100,635 reversed only the impairment induced by the intrahippocampal, and not by subcutaneous 8-OH-DPAT administration indicating that these inhibitory effects were mediated through post-synaptic 5-HT1AR activation. Similarly, a study by Youn et al. (2009) revealed that subcutaneous administration of 0.01–0.5 mg/kg 8-OH-DPAT impaired both contextual and cued fear memory acquisition in a dose-dependent manner. In contrast, S15535 (i.e., 0.01–5.0 mg/kg), a selective presynaptic 5-HT1AR agonist with lower efficacy for postsynaptic 5-HT1ARs, predominantly impaired contextual fear and produced weaker effects than those exerted by 8-OH-DPAT. This suggests that the impairments observed in the fear acquisition were mediated by post-synaptic 5-HT1ARs (Youn et al., 2009). In contrast, a recent study revealed that systemic administration of both 8-OH-DPAT (i.e., 0.03 mg/kg, s.c) and the selective 5-HT1A autoreceptor agonist F13714 (i.e., 0.16 mg/kg, i.p.) impaired cued fear acquisition, but reduction of both cued and contextual fear expression in the FPS test was presented upon administration of 0.1–0.3 mg/kg 8-OH-DPAT and 0.04–0.16 mg/kg F13714 (Zhao et al., 2019). Whereas i.p. administration of either 0.04 or 0.16 mg/kg F15599, a selective agonist of cortical post-synaptic 5-HT1A heteroreceptors, did not induce any effects either on acquisition or

expression of conditioning fear, indicating that these processes are regulated by 5-HT1A autoreceptors, rather than heteroreceptors (Zhao et al., 2019). Another study has also demonstrated the involvement of 5-HT1AR activation in inhibition of auditory learned fear acquisition that was induced by s.c. administration of 0.5 mg/kg 8-OH-DPAT. This was indicated by the attenuation of conditioned-induced tachycardia, an effect that was abolished by pretreatment with the 5-HT1AR antagonist WAY100,635 (i.e., 0.03 mg/kg, s.c.) (Youn et al., 2013). All these studies indicate that 5-HT1AR activation leads to impairments in both contextual and cued fear memory acquisition.

In addition, there is extended evidence supporting that 5-HT1AR activation leads to a reduction of learned fear expression, with postsynaptic 5-HT1ARs contributing to a greater extent compared to autoreceptors (Homberg, 2012). Specifically, it was found that intra-CEA infusion of flesinoxan (i.e., 5 –20 µg/ 1 µl and 40 µg/ 2 µl) a selective 5-HT1AR agonist, dose-dependently inhibited fear-potentiated startle, while intra-DRN or intra-MRN infusion failed to elicit any effects, indicating that flesinoxan-induced inhibitory effects were dependent on activation of 5-HT1AR found in CEA, rather than autoreceptors distributed in raphe nuclei (Groenink et al., 2000). Another study revealed that intra-dHPC and intra-amygdala flesinoxan (i.e., 3 µg/ 0.5 µl) infusion reduced the expression of contextual fear, through activation of 5-HT1ARs expressed post-synaptically in these areas (Li et al., 2006). A study by Almada et al. (2009) has also demonstrated a greater contribution of post-synaptic 5-HT1ARs compared to autoreceptors in the modulation of learned fear expression, as the intra-dlHPC infusion of 1 nmol/0.2 µl 8-OH-DPAT led to reduction in both contextual-induced freezing and fear-potentiated startle responses, while intra-MRN infusion of the same dose reduced only freezing behavior. It is worth mentioning that

compounds activating or antagonizing 5-HT1ARs were found not only to elicit effects on their own on learned fear but also to modulate SSRI-mediated responses. Specifically, subcutaneous administration of a sub-effective dose of WAY100,635 (i.e., 0.15mg/kg) potentiated the reduction of contextual fear expression induced by 3 mg/kg citalopram, possibly by further increasing the 5-HT levels through inhibition of presynaptic 5-HT1AR autoreceptors (Muraki et al., 2008). Analogous additive effects on the reduction of contextual-induced freezing were demonstrated by co-administration of the selective 5-HT1AR agonist flesixosan (i.e., 0.3 mg/kg, s.c.) and the SSRI fluvoxamine (i.e., 30 mg/kg) (Li et al., 2001). Therefore, the above-mentioned studies provide supportive evidence that the activation of 5-HT1AR results in suppression of learned fear expression, while indicating that the contribution of post-synaptic 5-HT1ARs in these inhibitory effects is greater than autoreceptors.

On the other hand, there is limited evidence regarding the effects of 5-HT1AR-mediated signaling on the modulation of learned fear extinction. A study by Nachtigall et al. (2019) revealed that hippocampal 5-HT1ARs influence the novelty-induced enhancement of contextual fear extinction while demonstrating that DA-ergic and NE-ergic transmission have an integral role in this process. Additionally, it was found that systemic administration of a partial 5-HT1AR agonist tandospirone (i.e., 5 mg/kg, i.p.) facilitated the acquisition and recall of contextual fear extinction in a juvenile stress exposure model and restored the synaptic function in CA1 and mPFC (Saito et al., 2013). Interestingly, a study by Pereyra et al. (2021) has demonstrated that amygdalar 5-HT1AR display a modulatory role in the extinction of reward-driven learning, given that intra-BLA infusion of 8 nmol/0.2 µl 8-OH-DPAT impaired extinction, while 0.37 nmol/µl WAY100,635 resulted in opposite effects. The fact that the BLA neuronal

populations responsible for the formation and storage of fear extinction memory are overlapping with those driving reward behavior (Zhang et al., 2020), raises the possibility that 5-HT1AR expressed in BLA may modulate fear extinction as well.

The 5-HT1AR antagonist WAY100,635 has been widely used over the recent decades in plethora of preclinical studies, comprising an invaluable tool for the investigation of pharmacological and physiological function of 5-HT1ARs in learned fear processing and anxiety, whilst it has been used in several human studies as radiotracer in PET studies for determining the distribution of 5-HT1ARs. This was attributed to the compound's high potency and selectivity to both presynaptic and postsynaptic 5-HT1ARs, with 100-fold selectivity over other receptors in CNS (Fletcher et al., 1996). However, a later study by Chemel et al. (2006) revealed that both WAY100,635 and its metabolite WAY100,634 can also act as full agonists at human D4.2Rs, demonstrating high affinity.

The above-mentioned studies provide supporting evidence to investigate further the role of 5-HT1AR agonists in the extinction of learned fear and reveal any potential benefit in prevention of fear relapse. The fact that CBD demonstrates affinity for the 5-HT1AR along with the previous observations that it reduced acutely the expression of contextual fear and suppressed the spontaneous recovery of auditory fear, encouraged the investigation of potential involvement of 5-HT1ARs on these pharmacological effects. In Experiment 1, a dose-response study of the 5-HT1AR antagonist WAY100,635 was performed to determine the appropriate dose that does not elicit effects on its own on learned fear expression, extinction, or spontaneous recovery. In Experiment 2, CBD or the sub-effective dose of WAY100,635 was

administered alone or in combination to investigate whether WAY100,635 could abolish the previously described effects of CBD.

5.2 Materials and methods

5.2.1 Subjects

Male Lister-Hooded rats (Charles Rivers, UK), weighing 290-380 g were used for Experiments 1 and 2. Rats were group-housed and behaviourally tested under the same conditions as described in previous chapters, while the sample size of each treatment group was calculated based on the power analysis described earlier in Chapter 2. All experimental procedures were performed with internal ethical approval and in accordance with the Animals (Scientific Procedures) Act 1986, UK (PPL: P6DA59444).

5.2.2 Drug preparation and administration

In Experiment 1, WAY100,635 maleate (Selleck Chemicals, US) at a dose of 0.3 or 1.0 mg/kg was suspended in sterile saline and administered 30 min before the extinction training session. The doses of WAY100,635 were selected based on previous studies investigating its effects on learned fear processing (Youn et al., 2009; Zhao et al., 2019). In Experiment 2, rats were pre-treated with the low dose of WAY100,635 (0.3 mg/kg) administered 60 mins before the extinction session. This dose was selected after Experiment 1 showed that it was not effective to elicit effects in freezing behaviour on its own. In the same experiment, 10 mg/kg CBD was suspended in a vehicle made of 98 % sterile saline and 2 % Tween 80 and administered 30 min before extinction. This CBD dose was selected as the lowest effective dose to modulate spontaneous fear recovery in the previous experiment in Chapter 3. The

compounds were freshly prepared the morning of extinction training sessions and administered intraperitoneally at a volume of 1 ml/kg.

5.2.3 Experiment 1: Dose-response effects of pre-extinction WAY100,635 administration on extinction and spontaneous recovery of learned fear

The effects of different WAY100,635 doses administered before extinction training on modulation of extinction and spontaneous recovery of contextual and auditory fear were investigated performing a 24-day behavioural protocol (Figure 5.1), that was identical to Experiment 1, in Chapter 4. The behavioural testing apparatus and recording software, along with the experimental design and parameters used have been described in detail in Chapters 2 and 3. On day 1, all rats underwent contextual habituation in two distinct contexts, and 24 hrs later were subjected to auditory fear conditioning in Context A. On day 3, rats were randomly allocated into three treatment groups (n=10/group) and administered with 0, 0.3, or 1.0 mg/kg WAY100,635 30 minutes before receiving extinction training in Context B. On days 4 and 24, all rats returned to the extinction context and were submitted to extinction recall and spontaneous recovery testing, respectively.

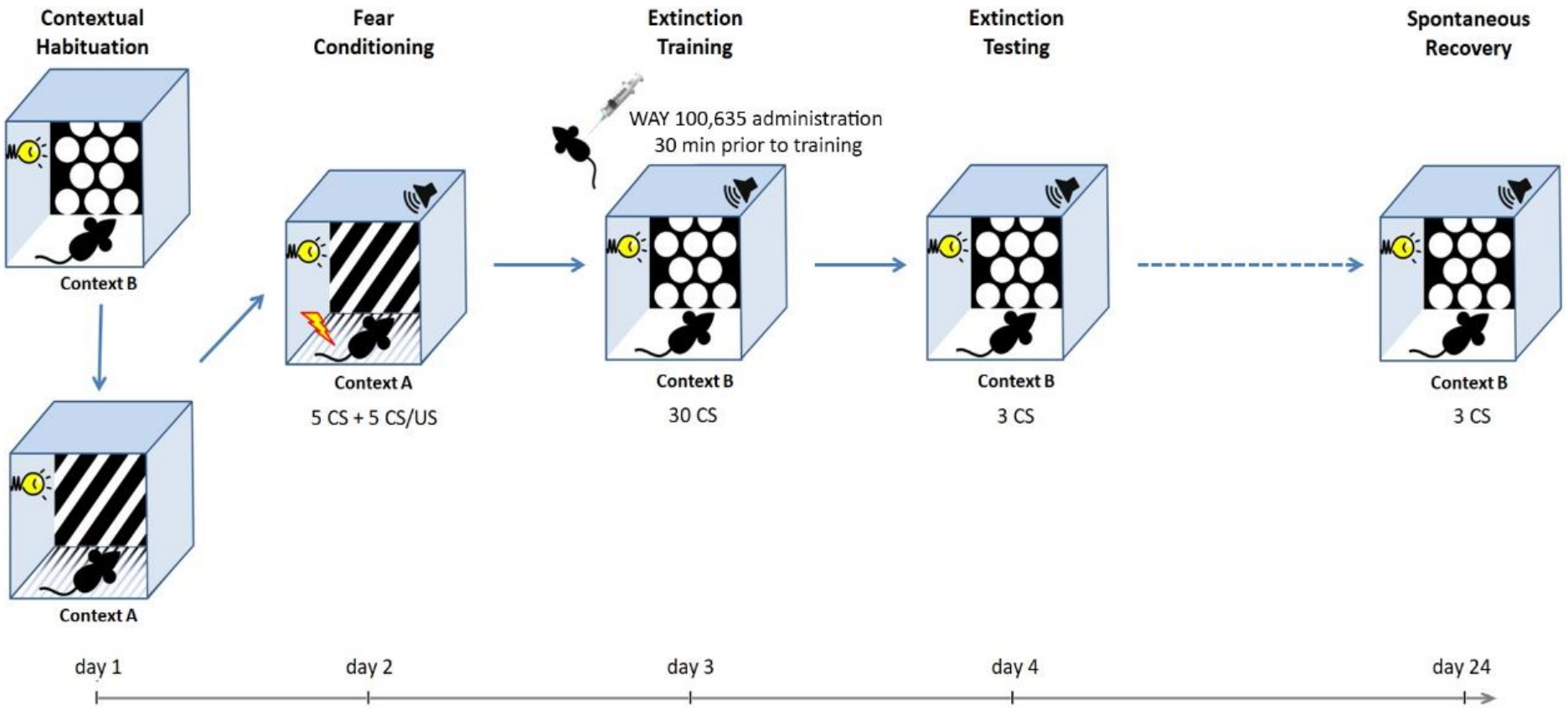

Figure 5.1 Schematic representation of dose-response study of the 5-HT1AR antagonist WAY100,635. On day 1, rats were habituated to context B and then context A. On day 2, rats underwent fear conditioning with 5 CS followed by 5 CS-US pairings in context A. On day 3, all rats were administered i.p. saline, 0.3, or 1 mg/kg WAY100,635 30 min before extinction training, and received 30 CS presentations in context B. On day 4, all groups were exposed to 3 CS for extinction testing, in context B. 21 days after extinction training, they were returned for a spontaneous recovery session, receiving 3 CS presentations.

5.2.4 Experiment 2: Effect of WAY100,635 pre-treatment on CBD regulation of extinction and spontaneous recovery of learned fear

All rats were subjected to the same behavioral procedures as described in Experiment 2 of Chapter 4, undergoing contextual habituation and auditory fear conditioning on days 1 and 2, respectively. On day 3, rats were randomly allocated to four treatment groups (n=10/ group): Saline + Vehicle, WAY100,635 + Vehicle, Saline + CBD, and WAY100,635 + CBD. Rats received their pre-treatment (Saline or WAY100,635) and treatment (Vehicle or CBD) with a 30 min-interval between them, with the latter administered 30 min before the extinction training (Figure 5.2). Similarly to as described above, extinction recall and spontaneous recovery testing were delivered on days 4 and 24, respectively.

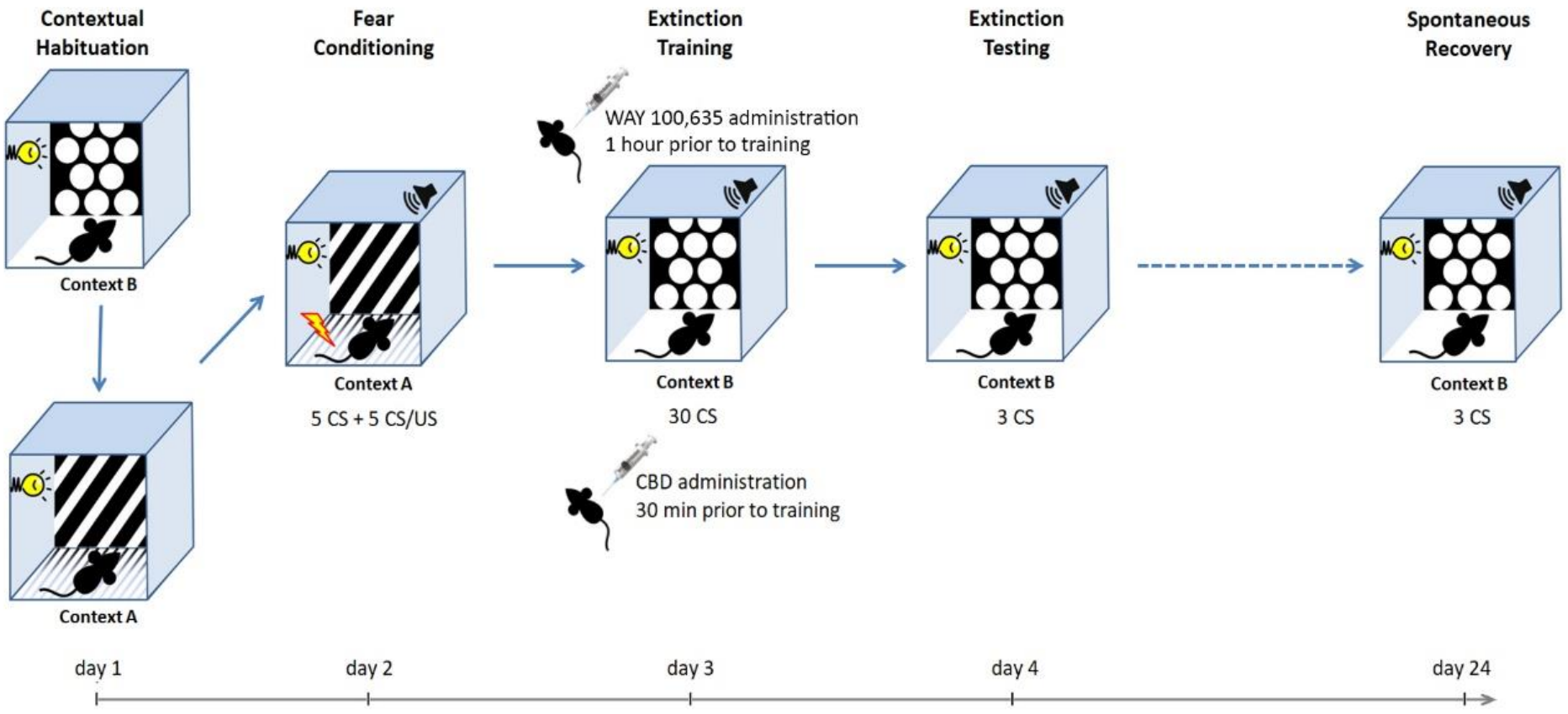

Figure 5.2 Schematic representation of protocol used for investigating the effect of WAY100,635 pre-treatment on the CBD regulation of extinction and spontaneous recovery of learned fear. On day 1, rats were habituated to context B and then context A. On day 2, rats underwent fear conditioning with 5 CS followed by 5 CS-US pairings in context A. On day 3, rats were randomly allocated to 4 different groups, receiving i.p. either saline or 0.3 mg/kg WAY100,635 60 min before extinction training, and vehicle or 10 mg/kg CBD 30 min before extinction training. Rats were subjected to 30 CS presentations in context B. On day 4, all groups were exposed to 3 CS for extinction testing, in context B. 21 days after extinction training, they were returned for a spontaneous recovery session.

5.3 Data analysis

Freezing behavior was expressed and analyzed similarly as described in Chapters 3 and 4. However, due to a failure in the automatic scoring software, some of the recordings ended up interrupted, resulting in loss of data and, thus, exclusion of these animals from the group analysis (n=7-10/group). The cumulative duration of freezing was expressed as the percentage of 120 sec for the pre-CS interval or 30 sec for each CS trial. Auditory fear during extinction training, extinction recall testing, and spontaneous recovery testing was calculated in CS blocks, defined by the average freezing percentage during 3 consecutive CS presentations. Fear conditioning was analyzed using repeated-measures or mixed-model two-way ANOVA, with group and trial being designated as the between- and within-subject factors, respectively. Geisser-Greenhouse correction was applied to adjust for the lack of sphericity. To compare baseline contextual fear at the start of extinction training, the pre-CS interval was analyzed through one-way ANOVA, with dose or treatment defined as the between-subject factor for Experiments 1 and 2, respectively. Bartlett's test was used to assess the homogeneity of variance, while $p < 0.05$ was considered the level of assumption violation. Two-way ANOVA with Geisser-Greenhouse correction was conducted for the analysis of CS blocks during extinction training in Experiments 1 and 2 with dose or treatment as the between-subject factor, respectively, and block as the within-subject factor. Freezing levels during the pre-CS interval of the extinction test and spontaneous recovery were analyzed using two-way ANOVA, with dose or treatment as the between-subject factor for Experiments 1 and 2, respectively, whereas time was defined as the within-subject factor. The fact that the within-subjects factor was comprised of only two levels (e.g., extinction test vs spontaneous

recovery) rendered inapplicable the sphericity assumptions. Differences in tone-induced freezing during CS blocks of extinction recall and spontaneous fear recovery testing were analyzed similarly as above. All data are presented as mean ± SEM and statistical analysis was performed using GraphPad Prism 9, while $p < 0.05$ was considered the level of statistical significance for all comparisons. Sidak's post-hoc tests were applied where indicated. In the dose-response study (Experiment 1), limited pairwise comparisons were performed between the means of the lower or higher dose of WAY100,635 and saline. Whereas, in the WAY100,635-CBD combination study (Experiment 2), the means of each treatment group were fully pairwise compared against the means of each other group.

5.4 Results

5.4.1 Experiment 1: Dose-response effects of pre-extinction WAY100,635 administration on extinction and spontaneous recovery of learned fear

The effects of pre-extinction training WAY100,635 administration on learned fear expression, extinction training, extinction recall, and later spontaneous recovery are depicted in Figure 5.3. As previously stated, due to interruption of experimental recordings, statistical analysis was performed in data from n=9 rats in Saline group, n=9 rats in WAY 0.3 mg/kg group, and n=8 rats in WAY 1.0 mg/kg group. The freezing levels during the CS-US pairings in the fear conditioning session are shown in Figure 5.3.A. Two-way ANOVA revealed a significant main effect of trial ($F_{(2.936, 67.53)} = 7.592$, $P = 0.0002$), but no effect of group ($F_{(2, 23)} = 0.4839$, $P = 0.6225$) or trial x group interaction ($F_{(8, 92)} = 0.5359$, $P = 0.8266$), indicating that there were no reliable differences between the groups to receive the different WAY100,635 doses before

extinction training. Freezing during the pre-CS interval before extinction training is presented in Figure 5.3.B. One-way ANOVA revealed a significant main effect of dose ($F_{(2, 23)}$ = 3.704, P = 0.0403). Post-hoc analysis demonstrated significantly lower freezing with WAY100,635 1.0 mg/kg vs saline (P = 0.0227), but no reliable difference between saline and WAY100,635 0.3 mg/kg (P = 0.3487). These results indicate that the high dose of WAY100,635 reduced the baseline expression of contextual fear memory before extinction training when compared with the vehicle. Tone-induced freezing during the CS blocks in the extinction training session is shown in Figure 5.3.C. Two-way ANOVA revealed a significant main effect of block ($F_{(2.160, 49.67)}$ = 14.72, P < 0.0001), but no effect of dose ($F_{(2, 23)}$ = 1.323, P = 0.2858) or block x dose interaction ($F_{(18, 207)}$ = 0.9998, P = 0.4613), indicating that WAY100,635 did not affect auditory fear memory expression or extinction learning. The effects of pre-extinction WAY100,635 on contextual fear during the pre-CS interval across extinction recall and spontaneous recovery testing are presented in Figure 5.3.D. Two-way ANOVA revealed a significant main effect of time ($F_{(1, 23)}$ = 26.78, P < 0.0001) but no effect of dose ($F_{(2, 23)}$ = 1.208, P = 0.3172) or time x dose interaction ($F_{(2, 23)}$ = 2.412, P = 0.1119). This indicates that freezing during the pre-CS interval of spontaneous recovery testing was significantly increased in all groups when compared with extinction recall testing, indicating spontaneous recovery of contextual fear 21 days after extinction training, without any lasting effect of WAY100,635 during either test. To investigate the effect of WAY100,635 on the return of auditory fear over time, two-way ANOVA was conducted on CS block of extinction recall and spontaneous recovery testing, demonstrating a significant main effect of time ($F_{(1, 23)}$ = 14.46, P = 0.0009) but no effect of dose ($F_{(2, 23)}$ = 0.5318, P = 0.5946) or time x dose interaction ($F_{(2, 23)}$ = 0.4844, P = 0.6222). This

indicates that tone-induced freezing during the CS block of spontaneous recovery was elevated in comparison to extinction recall testing (Figure 5.3.E) and indicates that auditory fear spontaneously recovered in all groups, regardless of WAY100,635 treatment.

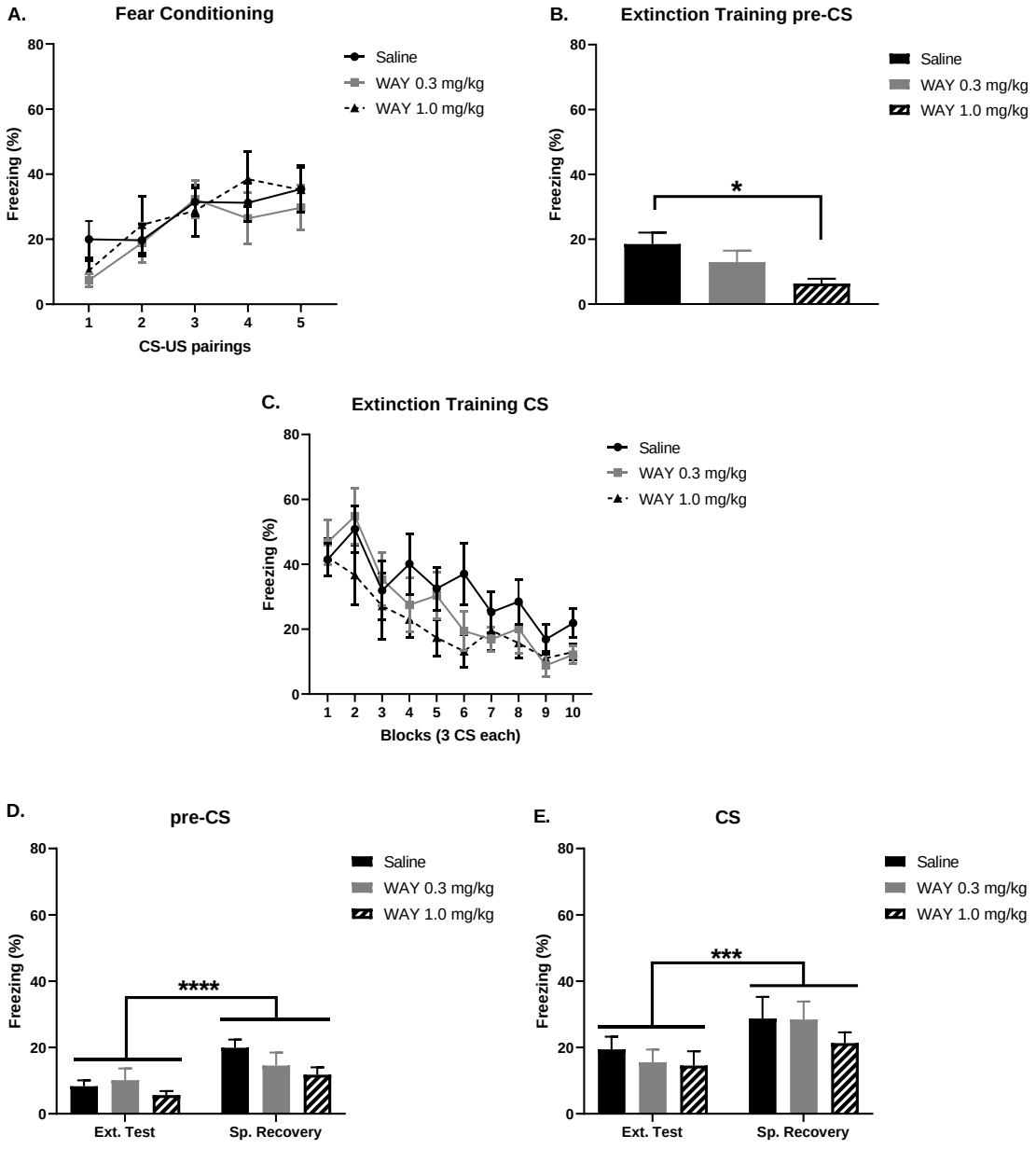

Figure 5.3 Pre-extinction high dose of WAY100,635 reduces the expression of contextual fear. (A) Fear conditioning did not differ between the groups (Saline group: n=9, WAY 0.3 mg/kg group: n=9, WAY 1.0 mg/kg group: n=8) to receive different doses of WAY100,635. (B) WAY100,635 1.0 mg/kg resulted in significantly less freezing during the pre-CS interval before extinction, compared to the saline-treated group (* $p < 0.05$). (C) No reliable differences in freezing were found between the different groups during extinction training. (D) All groups demonstrated significantly higher freezing during the pre-CS interval of spontaneous fear recovery compared to the extinction recall test (**** $p < 0.0001$), but no effect on freezing was observed with WAY100,635. (E) All treatment groups showed significantly higher tone-induced freezing during spontaneous recovery testing than the extinction recall test (*** $p < 0.001$), but WAY100,635 did not induce any significant effect.

5.4.2 Experiment 2: Effect of WAY100,635 pre-treatment on CBD regulation of extinction and spontaneous recovery of learned fear

To investigate the hypothesis that CBD prevents spontaneous recovery of auditory fear in 5-HT1A receptor-dependent manner, WAY100,635 (0.3 mg/kg) was administered in combination with CBD (10 mg/kg), to determine whether WAY100,635 can inhibit this beneficial effect of CBD. The low dose of WAY100,635 was selected based on the results from Experiment 1, where on its own it did not elicit any changes in freezing behaviour. The effects of the different treatment combinations on learned fear expression, extinction training, extinction recall, and later spontaneous recovery are depicted in Figure. 5.4. Freezing levels from a reduced number of animals per treatment group (i.e., n=8 in Sal + Veh, n=8 in WAY + Veh, n=7 in Sal + CBD, and n=8 in WAY + CBD) were statistically analyzed, due to multiple interruptions in the behavioural recording during this experiment. Tone-induced freezing during the CS-US pairings with fear conditioning is shown in Figure 5.4.A. Two-way ANOVA revealed a significant main effect of trial ($F_{(2.869, 77.47)} = 14.60$, $P < 0.0001$), but no effect of group ($F_{(3, 27)} = 1.082$, $P = 0.3733$) or trial x group interaction ($F_{(12, 108)} = 0.6743$, $P = 0.7727$), indicating that no reliable differences were observed between the groups to receive the different treatment combinations before extinction training. Freezing levels during the pre-CS interval before extinction training are demonstrated in Figure 5.4.B. One-way ANOVA revealed no significant main effect of treatment ($F_{(3, 27)} = 0.6748$, $P = 0.5750$), indicating that treatment with WAY100,635 or CBD alone, or their combination, did not influence the baseline expression of contextual fear before extinction training. Figure 5.4.C depicts the tone-induced freezing during the extinction training. Two-way ANOVA revealed a significant main effect of block ($F_{(2.797,}$

$_{75.53)}$ = 17.17, P < 0.0001), but no effect of treatment ($F_{(3, 27)}$ = 1.110, P = 0.3624) or block x treatment interaction ($F_{(27, 243)}$ = 0.8820, P = 0.6377), indicating that none of the treatments affected auditory fear memory expression or extinction learning. Figure 5.4.D presents the effects of pre-extinction administration of WAY100,635, CBD, or their combination on contextual fear memory across extinction recall and spontaneous recovery testing. Two-way ANOVA was conducted on freezing levels during the pre-CS interval before either test, revealing a significant main effect of time ($F_{(1, 27)}$ = 13.01, P = 0.0012), but no treatment effect ($F_{(3, 27)}$ = 0.5490, P = 0.6531), or time x treatment interaction ($F_{(3, 27)}$ = 0.7801, P = 0.5154). This indicates that the freezing levels were significantly elevated in all treatment groups during spontaneous recovery in comparison with extinction recall testing, indicating that contextual fear memory spontaneously recovered 21 days after extinction training and was not affected by any of the treatments. The effects of different treatment combinations on the return of auditory fear over time are shown in Figure 5.4.E. Two-way ANOVA was conducted on tone-induced freezing during extinction recall and spontaneous fear recovery testing, revealing a significant main effect of time ($F_{(1, 27)}$ = 9.199, P = 0.0053), but no effect of treatment ($F_{(3, 27)}$ = 1.185, P = 0.3340), or time x treatment interaction ($F_{(3, 27)}$ = 0.2546, P = 0.8574). These results indicate that tone-induced freezing was higher during spontaneous recovery than extinction recall testing and suggest that spontaneous recovery of auditory fear occurred in all treatment groups. Neither WAY100,635 nor CBD alone induced any lasting effects in either testing session, while freezing was not affected by the pretreatment of WAY100,635 before CBD administration.

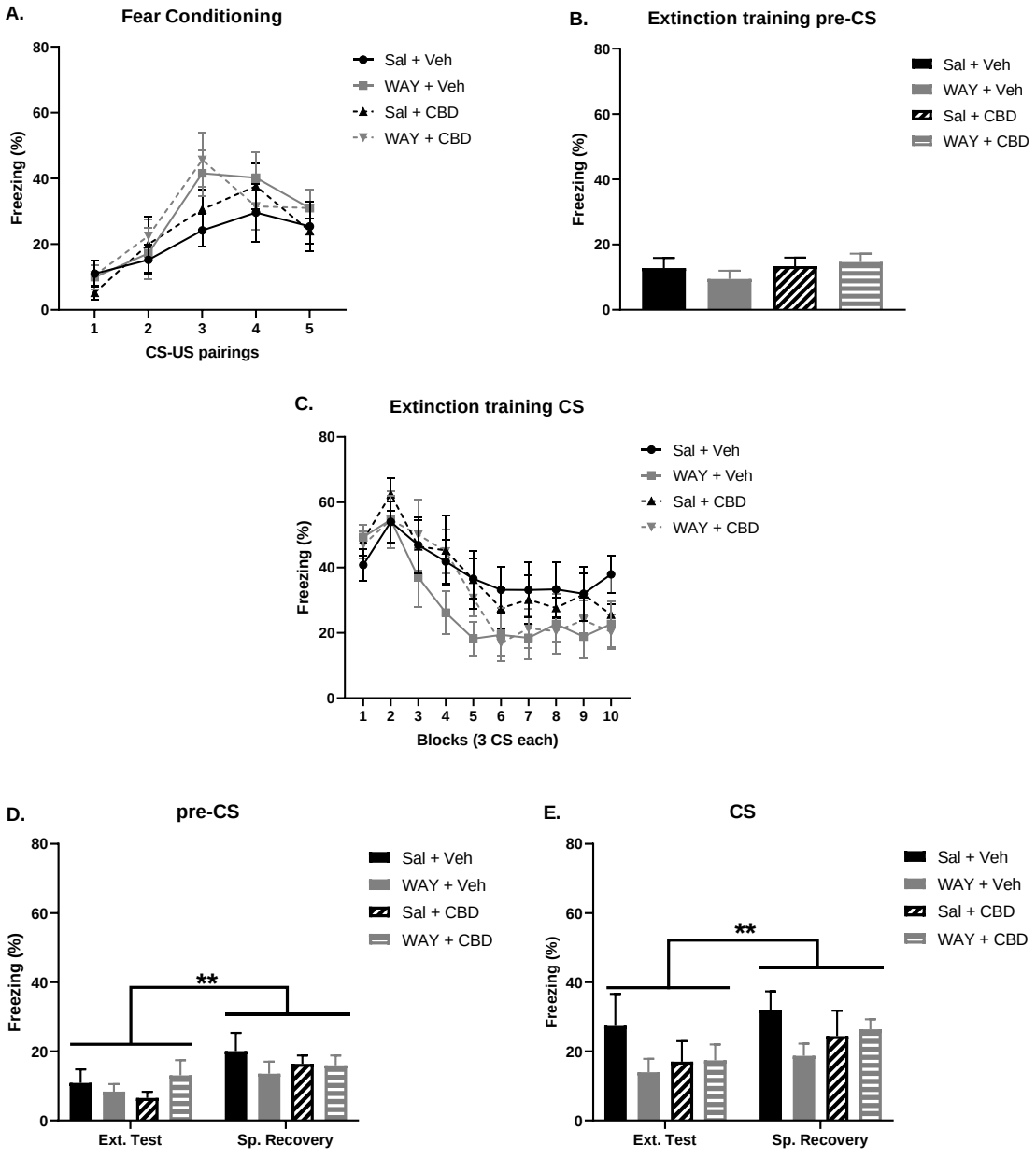

Figure 5.4: WAY100,635, CBD, or their combination did not affect the expression of learned fear, extinction, or spontaneous recovery. (A) Fear conditioning did not differ between the groups to receive WAY100,635 (0,3 mg/kg), CBD (10 mg/kg), or their combination. (B) None of the treatments (Sal + Veh group: n=8, WAY + Veh group: n=8, Sal + CBD group: n=7, WAY + CBD group: n=8) affected freezing during the pre-CS interval before extinction training. (C) No reliable differences in tone-induced freezing were found between the treatment groups during extinction training. (D) All groups demonstrated significantly higher freezing during the pre-CS interval of spontaneous fear recovery compared to the extinction recall test, but no effect of WAY100,635, CBD, or their combination was observed during either time point (** $p < 0.01$). (E) All treatment groups showed significantly higher tone-induced freezing during spontaneous recovery than the extinction recall test (** $p < 0.01$), but WAY100,635, CBD, or their combination did not have any effect.

5.5 Discussion

This study investigated the potential involvement of 5-HT1AR signaling in the reduction of contextual fear expression and the lasting suppression of spontaneous recovery of auditory fear induced by the systemic administration of CBD before extinction training. A dose-response study with the 5-HT1AR antagonist WAY100,635 was carried out in Experiment 1 to determine a dose that did not produce any effect on its own but could potentially reverse the previously observed effects of CBD. The high dose of WAY100,635 administered systemically before extinction training acutely reduced the baseline expression of contextual fear memory, leaving auditory fear expression and extinction learning unaffected. Additionally, none of the WAY100,635 doses induced any lasting effects on extinction recall when rats were tested drug-free the following day during the extinction test or 21 days later upon spontaneous fear recovery testing. Next, in Experiment 2, CBD and the low dose of WAY100,635 were administered in combination or with their vehicle/saline counterpart before extinction training. None of the treatment combinations affected learned fear expression, its extinction, or its recall during the extinction test and spontaneous recovery sessions. Importantly, in the current study CBD failed to reproduce the previous effects observed in Chapter 3 in reducing baseline expression of contextual fear and suppression of the spontaneous recovery of auditory fear, while pre-treatment with WAY100,635 failed to induce any effects either. Therefore, the lack of reproducibility of these findings rendered it impossible to exclude the potential involvement of 5-HT1AR transmission in CBD's fear-suppressing effects.

In Experiment 1, the finding that the administration of 0.3 mg/kg WAY100,635 before extinction did not exert any effects on learned fear expression is not

unexpected. This dose has been used in previous studies for blocking the inhibitory effects of 5-HT1AR agonist 8-OH-DPAT on acquisition (Stiedl et al., 2000) or expression of learned fear (Youn et al., 2009), without producing any effects on its own. Importantly, systemic administration of 0.3mg/kg WAY100,635 was found to be devoid of any effects by itself, while such a dose was able to reverse the CBD-induced anti-aggressive (Hartmann et al., 2019) and anti-depressant effects (Linge et al., 2016) in resident-intruder and open field tests, respectively. On the other hand, 1 mg/kg WAY100,635 in the present study resulted in the reduction of baseline expression of contextual fear, without affecting auditory fear expression. Noteworthy is that quite diverse findings have been reported with the higher doses of WAY100,635. For instance, previous studies have demonstrated that a dose range from 0.03 - 3 mg/kg of WAY100,635 did not induce any effect by itself in either conditioned emotional response or resident-intruder test (Stanhope & Dourish, 1996; Hartmann et al., 2019). However, a study by Madjid et al. (2006) revealed that doses of 1-3 mg/kg WAY100,635 administered before passive avoidance training led to enhanced aversive memory retention during the test session. In contrast, WAY100635 (0.03–1.0 mg/kg) failed to influence the acquisition of fear conditioning but reduced overall fear expression during FPS test (Zhao et al., 2019). Possibly, these discrepancies might be attributed to the different behavioural models used across the studies, and to the property of WAY100,635 in antagonizing both 5-HT1A autoreceptors and post-synaptic receptors at higher doses (Kakui et al., 2009).

Additionally, neither of the WAY100,635 doses administered before extinction training elicited any effects on extinction learning, extinction test, or spontaneous recovery of learned fear when compared to saline. There is very limited evidence from

the literature regarding the involvement of 5-HT1AR signalling in extinction. Solely, a study by Pereyra et al. (2021) has recently revealed the facilitatory effects of intra-BLA infusion of 0.37 nmol/0.2 µl WAY100,635 in the extinction of reward-driven learning. Whereas a study by Nachtigall et al. (2019) infused 1.25 µg/µl NAN-190, a 5-HT1AR antagonist, bilaterally into CA1 to investigate the involvement of 5-HT1AR signalling in the enhancement of fear extinction by novelty. NAN-190 did not affect extinction consolidation on its own but reversed the impairments on extinction induced by 8-OH-DPAT (i.e., 6.25 µg/ 1 µl). However, neither of these studies is directly comparable to the present one due to the differences in the 5-HT1AR antagonist used, the route of drug administration, and the behavioural model. Thus, for the following CBD-WAY100,635 combination study, the low dose of WAY100,635 (i.e., 0.3 mg/kg) was selected, that according to the present dose-response study did not produce any effects across the behavioral sessions.

In Experiment 2, as mentioned above, administration of CBD or WAY100,635 alone, or their combination, before extinction training failed to have any effects on the expression of learned fear, extinction learning, extinction recall, or spontaneous fear recovery, when compared to vehicle and saline. The fact that WAY + Veh produced no effects is an anticipated finding as this dose of WAY100,635 (i.e., 0.3mg/kg) was validated and selected for not exerting any effect on its own, but for preventing CBD-induced effects. Striking is that the administration of 10 mg/kg CBD (i.e., Sal + CBD) before extinction training did not induce either acute reduction of baseline expression of contextual fear or long-term suppression of spontaneous recovery of auditory fear, in contrast to the findings of Chapter 2. It is unclear what has caused the irreproducibility of CBD effects in this experiment, given that all the

previously identified variations in the experimental factors have been currently eliminated. Here, the same vehicle has been used for the suspension of CBD as in Chapter 2, while the rats were obtained from the same breeding company. It is important to mention that due to recording interruptions in some of the behavioural sessions, at least 2 animals per treatment group were left out from the analysis (n=8 in Sal + Veh, n=8 in WAY + Veh, n=7 in Sal + CBD, and n=8 in WAY + CBD). This decrease in sample size along with some extreme freezing values that were exhibited by rats within Sal + Veh and Sal + CBD groups may have led to the wider inferential error bars observed, especially during the CS interval of extinction test and spontaneous recovery session, providing less reliable treatment effects (Cumming et al., 2007).

Despite the lack of CBD effects in this experiment, the fact that pre-treatment of WAY100,635 did not induce any alterations in freezing behaviour may be attributed to other factors such as the route of WAY100,635 administration. Specifically, it was found that CBD infused directly into PL (Fogaca et al., 2014) or BNST (Gomes et al., 2012), but not in IL (Marinho et al., 2015), before the fear retrieval test resulted in reduction of contextual fear expression, effects that were reversed by local WAY100,635 infusion. This suggests that the 5-HT1AR-mediated fear suppressing effects of CBD were limited only to these specific areas.

Although most of the studies have shown that the acute effects of CBD on the reduction of learned fear expression are mediated through 5-HT1AR signaling, CBD's enhancing effects on extinction through this pathway have not been yet investigated. Interestingly, a previous study has revealed that the disrupting effects of CBD on fear memory reconsolidation were reversed by the pre-administration of AM251 but not

WAY100,635, indicating that these effects were mediated through CB1R-, rather than a 5-HT1AR-dependent mechanism (Stern et al., 2012). Given that both reconsolidation and extinction require reactivation of previously acquired learned fear memory, this raises the question of whether 5-HT1AR transmission is implicated in CBD's effects on extinction. Worth mentioning is that intra-PL and -IL infusion of CBD exerted anti-depressant-like effects in the forced swim test through activation of both CB1 and 5-HT1A receptor signalling. It was suggested that CBD possibly mediated these effects by modulating serotoninergic transmission in PFC through indirect activation of CB1Rs (Sartim et al., 2016). Similarly, another study revealed that systemic CBD administration induced anti-aggressive effects in a resident-intruder test, through a CB1R- and 5-HT1AR-dependent manner (Hartmann et al., 2019).

Regarding the short- and long-term alleviating effects of CBD on learned fear, Alexander & Vasefi (2021) have recently put forward a model through which CBD acts within the cortico-raphe circuit. It was suggested that acute CBD administration activates 5-HT1AR expressed on DRN serotonergic neurons projecting to BLA, thus suppressing serotonin release in DRN-BLA synapses and limiting overexcitation of BLA. Simultaneously, CBD-induced elevation of AEA at DRN serotonergic neurons retrogradely activates presynaptic CB1R of the BLA glutamatergic neurons projecting to DRN, thus inhibiting glutamate release to DRN and reducing its firing. This CBD-mediated reduction of DRN-BLA excitability constrains the disparity between BLA overactivation and PFC hypoactivation, enabling PFC to restore its inhibitory role on BLA excitability. In this way, CBD interferes with learned fear memory formation and reduces its expression upon re-experiencing of conditioned stimuli. In contrast, facilitation of extinction memory formation is induced after chronic CBD

administration. This is enabled after desensitization of 5-HT1AR on DRN serotonergic neurons, leading to enhancement of serotonin release in PFC, and thus to restoration of PFC activity. In parallel, CBD-induced stabilization of AEA levels in DRN results in activation of CB1Rs on GABAergic interneurons, suppressing their inhibitory control, while permitting PFC glutamatergic innervation of DRN. However, in the present study, it is unlikely that CBD would engage the second mechanism described above, given that a single CBD administration cannot induce desensitization of 5-HT1ARs. Instead, it is more plausible for CBD to suppress BLA overexcitability through blockade of 5-HT1AR on DRN serotonergic neurons, leading to restoration in the balance between PFC and BLA activities. Although all the mechanisms proposed above remain to be determined, we need to consider the possibility that CBD may induce long-lasting fear-suppressing effects by engaging both CB1R and 5-HT1AR-dependent pathways.

Conclusively, in this study it was initially observed that high dose of WAY100,635 administered before extinction acutely reduced the baseline expression of contextual fear memory, a result that could be compared with previous findings demonstrating an overall reduction of fear expression induced by WAY100,635 in the FPS test (Zhao et al., 2019). Next, in the WAY100,635-CBD combination study, CBD alone failed to reproduce the previously observed effects on suppression of spontaneous fear recovery. Due to these results, it is impossible to exclude the potential involvement of 5-HT1AR signaling, at least in part, in the CBD-mediated suppression of learned fear. For this reason, a repetition of the WAY100,635-CBD experiment is recommended for the future studies, while improving the experiment's statistical power by increasing the animal number per treatment group.

Chapter 6: Effects of pre-extinction CBD administration on immediate extinction and extinction recall of learned fear

6.1 Introduction

Inconsistencies in the CBD-mediated modulation of extinction learning and retrieval have not only been observed across the present experiments but also between other studies in the field, an issue that was thoroughly discussed in the previous chapters. The level of aversion and stress evoked by the relatively mild fear conditioning in the current protocol may have resulted in an aberrant or lack of engagement of endocannabinoid signaling in some experiments. In parallel, this mild conditioning may have also led to those relatively low freezing levels observed across all the treatment groups, especially during the late phase of extinction training, upon the extinction test, and spontaneous recovery sessions. Therefore, the inconsistent effects of CBD in modulating learned fear could be attributed to a floor effect in conditioned responding. To address this issue, it would be particularly interesting to investigate the effects of CBD under a more aversive learned fear protocol that is resistant to fear extinction.

Performing extinction training immediately after fear conditioning or within the fear memory consolidation window (i.e., 15 min up to 6 hours post-conditioning) has been linked to impairments in long-term retention of extinction memory despite the within-extinction session suppression of conditioned freezing, a phenomenon described as the immediate extinction deficit (IED) (Maren & Chang, 2006; Maren, 2014). Chang & Maren (2009) described that immediate extinction leads to a context-independent and short-lasting suppression of fear, that completely relapses upon

extinction retrieval testing the following day, thus indicating a habituation process rather than extinction. In contrast, standard delayed extinction (i.e., delivered 24 hours after conditioning) results in successful extinction retention, demonstrating context-dependent and long-lasting fear suppression. It appears that the IED results from the high levels of arousal and stress induced by the experience of the conditioning procedure (Maren, 2022). Weakening the fear conditioning parameters was found to abolish the IED, whereas delivering an unsignalled US right before a delayed extinction session was found to impair its later retrieval (Maren & Chang, 2006). Interestingly, the IED was also observed in appetitive conditioning tasks, where no noxious stimuli were presented (Woods & Bouton, 2008). This might be explained by the overlapping of neural circuits governing fear and appetitive conditioning procedures (Goode & Maren, 2019). Of particular importance is that appetitive conditioning and extinction may represent stressful events in food-deprived animals, triggering hyperarousal (Mingote et al., 2004). Additionally, IED was observed in absence of emotional arousal in human participants subjected to a predictive learning task. This effect was detected only when a stimulus did not undergo contextual change between the acquisition and extinction phases. In contrast, IED was abolished when a contextual change occurred, indicating that contextual processing plays a critical role in the modulation of IED occurrence (Merz & Wolf, 2019).

However, other factors like the interoceptive context in which conditioning and immediate extinction take place or the temporal gap between these two events, can serve as potent contexts that may influence the IED (Maren et al., 2013). Possibly, when extinction trials are immediately delivered after fear conditioning, extinction encoding is realized within the same interoceptive context induced by the US. Such

context is no longer available upon the extinction recall, serving thus as a change in the interoceptive context that leads to renewal of conditioned responses (Maren, 2022). However, even after matching the conditioning-induced arousal state during the extinction recall test by delivering immediately before that a conditioning session with a distinct CS, this was not enough to rescue IED (Woods & Bouton, 2008). On the other hand, a human study by Dunsmoor et al. (2018) suggested that the short temporal interval separating fear conditioning and immediate extinction may act as an event boundary, promoting the consolidation and retrieval of fear memory, while protecting its interference by extinction memory. In contrast, a later animal study revealed that the elimination of the event boundary did not prevent the IED, as the delivery of both continuous (i.e., immediately) and segmented extinction (i.e., 15 min) sessions after fear conditioning led to an equally robust IED in comparison to the non-extinction group during the extinction recall test (Totty et al., 2019). This indicated that IED is not dependent on a temporal gap, but rather on the stress and arousal state associated with the conditioning.

Although the exact mechanisms underlying IED are not fully elucidated, stress was found to modulate the neural activity within the extinction circuitry, through coordinated activation of the locus coeruleus-norepinephrine (LC-NE) and corticotropin-releasing factor (CRF) systems. The pharmacological inhibition of these stress-sensitive neuromodulatory and neurohormonal systems was found to prevent IED and contributed to an in-depth understanding of its etiology (Hollis et al., 2016; Maren & Holmes, 2016; Giustino et al., 2017; Jo et al., 2020). The LC-NE system is thought to provide a fine-tuning between learned fear encoding and its extinction. Under low arousal levels, LC favors PFC function and downregulates the amygdala,

enhancing the extinction of learned fear memory. In contrast, under high-stress levels, such as immediately after conditioning, LC promotes fear learning at expense of extinction by enhancing amygdala function and simultaneously suppressing PFC activity (Giustino & Maren, 2018). Several studies revealed that acute stress can induce synaptic remodeling and excitability alterations in IL, a critical structure for extinction acquisition and expression (Wilber et al., 2011; Nava et al., 2017). Noteworthy is that upon basal stress levels during delayed extinction, IL is recruited and suppresses BLA excitability, by stimulating IL excitatory projections to inhibitory interneurons in BLA (Bloodgood et al., 2018). In contrast, upon immediate extinction, PFC demonstrates compromised activity as observed by the sustained decreases in c-Fos expression (Singh et al., 2018) and spontaneous single-unit firing in IL (Chang et al., 2010). Electrical stimulation of IL with concomitant CS delivery or systemic administration of the β-adrenergic blocker propranolol (i.e.,10 mg/kg, i.p.) was found to mitigate the IED and restore the conditioning-induced impairments in IL activity (Kim et al., 2010; Fitzgerald et al., 2015). This suggests that IED may result after the suppression of IL activity mediated by the excessive stress and NE-ergic stimulation triggered by the recent fear conditioning.

However, a later study by Giustino et al. (2017) added new insight into the neural circuitry underlying IED. Although the systemic administration of propranol was found to prevent IED through enhancement of extinction retention (Fitzgerald et al., 2015), this effect was reproduced only after 5 μg/μl intra-BLA, and not 10 μg/μl intra-IL propranolol infusion (Giustino et al., 2017), suggesting that possibly the stress-induced elevated NE-ergic activity in BLA, and not IL, was responsible for the IED. The fact that acute stress results in elevation of BLA principal neurons' intrinsic excitability

(Hetzel & Rosenkranz, 2014), while BLA receives dense projections from LC, stimulated further research investigating their roles in IED. A study by Giustino et al. (2020) revealed that spontaneous firing activity in BLA was increased during and/or immediately after fear conditioning, which was restored by systemic propranolol (i.e., 10 mg/kg). Although weaker conditioning did not elicit either robust increases in BLA firing or extinction impairment, prior LC chemogenetic activation in association with weaker conditioning was sufficient to produce IED. Taken together, these findings indicated that the high-stress levels induced by the conditioning experience activate LC-NE afferents projecting to BLA, leading to its sustained overactivation. Possibly, BLA by stimulating IL inhibitory interneurons, may lead to feed-forward inhibition of IL principal cells, and thus to suppression of IL firing and promotion of the IED.

In addition to the LC-NE system, CRF-expressing neurons in the CEA were suggested to also be involved in the modulation of IED. Anatomical studies revealed that LC does not only send potent projections to BLA but also receives efferents from CEA (Van Bockstaele et al., 2001). Fear conditioning was found to increase the CEA neuronal activity (Valentino & Van Bockstaele, 2008), while optogenetic stimulation of the CEA-CRF terminals reaching LC resulted in a high-tonic firing of LC-NE neurons, producing robust anxiogenic-like responses in a CRF-mediated manner, as these effects were reversed after systemic or local CRFR1 receptor blockade (McCall et al., 2015). Recently, a study by Jo et al. (2020) demonstrated the role of CEA-CRF neurons in IED. Specifically, they observed an elevated activation in the CEA-CRF neurons both during immediate extinction training and later upon the extinction recall test when compared to the delayed extinction group. Importantly, inhibition of the CEA-CRF neurons resulted in the prevention of extinction deficits when extinction was

delivered immediately after conditioning, while their activation after delayed extinction resulted in reinstatement of previously extinguished responses. Collectively, these findings suggest that the CEA-CRF system contributes to the development of extinction deficit by potentiating the increased LC firing activity induced by the recent conditioning, leading to excessive NE-mediated BLA activation and subsequent IL suppression (Maren, 2022). Although this represents an indirect mechanism through which CRF modulates BLA excitability, it was suggested that stress-induced increases in CRF levels also directly overstimulate BLA through activation of abundantly expressed CRFR1 receptors (Rainnie et al., 2004; Korosi & Baram, 2008). Interestingly, intra-BLA infusion of the CRF1R antagonist NBI30775 (i.e., 1 or 10 μg/ 0.5 μl) before immediate extinction session enhanced the recall of extinction (Hollis et al., 2016), while intra-BLA administration of 1 μg/ 0.5 μl CRF_{6-33}, a CRF agonist, before standard delayed extinction led to opposite effects (Abiri et al., 2014), indicating that CRFR1 receptor activation plays a crucial role in stress-induced increased BLA firing and contributes to IED effect.

Evidence from several studies indicates that the ECB system plays a crucial role in the modulation of NE and CRF signaling within the stress and fear extinction circuitry (Gazarini et al., 2022; Warren et al., 2022), suggesting the plausibility for its role in the regulation of stress-induced extinction learning impairments. After a thorough search on the relevant literature, the effects of cannabinoids have not been investigated up to now in the IED model, which has significant translational value for the development of pharmacological treatments for PTSD. Therefore, in the following experiment, CBD was systemically administered before the immediate extinction session to determine its effects on extinction recall, while non-extinction control groups were used to assess

whether CBD elicited any of its effects by engaging fear memory consolidation or extinction processes.

6.2 Materials and methods

6.2.1 Subjects

Male Lister-Hooded rats (Charles Rivers, UK), weighing 300-350 g were used for this experiment. Rats were housed and behaviourally tested under the same conditions as described in previous chapters, while the sample size of each treatment group was calculated based on the power analysis described earlier in detail. All experimental procedures were performed with internal ethical approval and in accordance with the Animals (Scientific Procedures) Act 1986, UK (PPL: P6DA59444).

6.2.2 Drug preparation and administration

CBD (Noramco, US) at a dose of 10 mg/kg was suspended in a freshly prepared vehicle, consisting of 98 % sterile saline and 2 % Tween, and administered intraperitoneally at a volume of 1 mg/kg immediately after the end of the fear conditioning session and 30 min before the immediate extinction training session. This dose was selected based on previous studies in the literature (Resstel et al., 2006; Lemos et al., 2010; Stern et al., 2012; Jurkus et al., 2016; Song et al., 2016; Stern et al., 2017) and the results from Experiment 1 in Chapter 3, demonstrating CBD modulation of spontaneous fear recovery after extinction.

6.2.3 Experimental procedures

To determine the effects of systemic pre-extinction CBD administration on the amelioration of immediate extinction deficit phenomenon, a 2-day protocol was used,

presented in Figure 6.1. The behavioural testing apparatus and recording software used have been described in detail in Chapter 2, while the experimental design and parameters were adapted from previous studies in the field (Maren & Chang, 2006; Chang & Maren, 2009) and validated in the lab. On day 1, all rats were subjected to auditory fear conditioning in context A for a 12 min and 30-sec session. Rats were acclimatized for a 2 min pre-CS interval before receiving 5 tone-shock (CS-US) pairings (CS=30 sec, US=1 sec co-terminated with CS, I=0.5 mA, ITI=2 min). Rats were randomly allocated to four groups (n=10 rats/group) as follows: receiving vehicle with immediate extinction, CBD with immediate extinction, vehicle with immediate no extinction, and CBD with immediate no extinction. Immediately after the end of fear conditioning, rats were administered either CBD or vehicle and then were returned to their home cages. Immediate Extinction or No Extinction sessions were performed in context B 30 min after the conditioning session, lasting for 46 min and 30 sec. The rats in the immediate extinction groups were tested during the 2 min pre-CS interval for their baseline contextual fear memory, before receiving 45 CS presentations (CS=30 sec, ITI=30 sec). In contrast, rats of the immediate no extinction group were exposed solely to Context B without receiving any CS presentations. The following day, all rats were returned to context B for a drug-free extinction recall testing session of 11 min and 30-sec duration, consisting of a 2 min pre-CS period before receiving 10 CS presentations (CS=30 sec, ITI=30 sec).

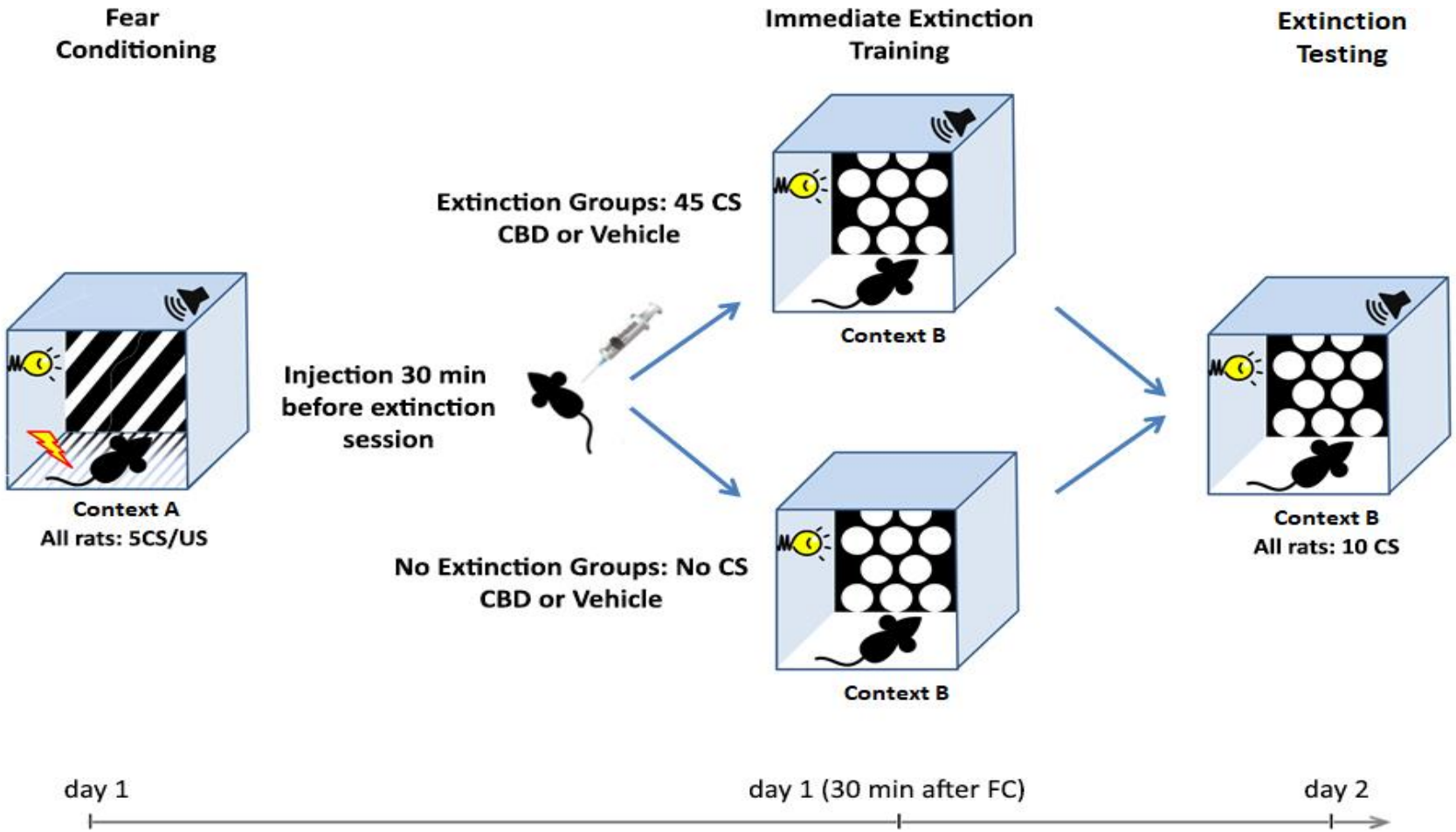

Figure 6.1 Schematic representation of the IED protocol with CBD administration: On day 1, all rats underwent fear conditioning with 5 CS-US pairings in context A. Immediately after fear conditioning, rats were administered i.p. Vehicle or 10 mg/kg CBD. 30 mins later, half of the rats from each treatment group underwent either immediate extinction training with 45 CS presentations in Context B or exposed for an equal amount of time to the same context without receiving any CS. On day 2, all rats returned to Context B and were exposed to 10 CS for extinction recall testing.

6.3 Data analysis

The duration of freezing behavior was expressed as the percentage of 120 sec for the pre-CS interval or 30 sec for each CS trial. Auditory fear during extinction training or extinction recall testing was calculated in CS blocks, defined by the average freezing percentage during 5 or 10 consecutive CS presentations, respectively. Freezing differences during fear conditioning were analyzed using two-way ANOVA, with group designated as the between-subject factor and trial as the within-subject factor, whilst Geisser-Greenhouse correction was applied to adjust for the lack of sphericity. For the evaluation of the acute effects of CBD on baseline contextual fear, the freezing levels from the two CBD-treated groups (i.e., immediate extinction and immediate no extinction) and two vehicle-treated groups (i.e., immediate extinction and immediate no extinction) were combined during the pre-CS interval before extinction. For the investigation of acute CBD effects on auditory fear expression, the freezing levels of the CBD and vehicle immediate extinction groups were compared during the first CS block at the start of extinction. Differences in both context- and tone-induced freezing were similarly analyzed using two-tailed unpaired t-tests. *A priori* F tests were performed to determine whether the variance between groups was not significantly different for permitting the application of a parametric t-test, while $p < 0.05$ was set the level of assumption violation. Two-way ANOVA with Geisser-Greenhouse correction was conducted for the freezing analysis of the CS blocks during the immediate extinction session, with treatment and block as the between-, and within-subject factors, respectively. Freezing levels during the pre-CS interval of the extinction recall test were analyzed using one-way ANOVA, with treatment defined as the between-subject factor. Bartlett's test was used to assess the homogeneity of

variance, while $p < 0.05$ was considered the level of assumption violation. Finally, the tone-induced freezing during the extinction recall test was analyzed by comparing either the differences in average freezing during the CS block or each individual CS presentation across the different treatment groups. For the former analysis, one-way ANOVA was applied, with treatment as the between-subject factor, while for the latter analysis, two-way ANOVA was used, with treatment and trial being defined as the between- and within-subject factors, respectively. All data are presented as mean ± SEM and statistical analysis was performed using GraphPad Prism 9, while $p < 0.05$ was considered the level of statistical significance for all comparisons. Newman-Keuls or fully pairwise Sidak's posthoc analyses were applied for multiple comparisons where indicated.

6.4 Results

The effects of CBD administration before the immediate extinction training on the expression of learned fear, immediate extinction, and extinction recall are depicted in Figure 6.2. The levels of freezing behavior during the CS-US pairings in the fear conditioning session are shown in Figure 6.2.A. Two-way ANOVA revealed a significant main effect of trial ($F_{(3.137, 112.9)} = 20.81$, $P < 0.0001$) but no main effect of group ($F_{(3, 36)} = 0.7383$, $P = 0.5361$) or trial x group interaction ($F_{(12, 144)} = 0.6628$, $P = 0.7846$), indicating that there were no reliable differences between the groups. Freezing during the pre-CS interval before immediate extinction is shown in Figure 6.2.B. An unpaired two-tailed t-test revealed no significant difference ($t_{(38)} = 0.1578$, $P = 0.8754$) between the vehicle (immediate extinction and immediate no extinction) and CBD (immediate extinction and immediate no extinction) treated groups, indicating that CBD did not affect the baseline contextual fear expression. Freezing

during the first CS block of immediate extinction is shown in Figure 6.2.C. An unpaired two-tailed t-test revealed a significant statistical difference ($t_{(18)} = 3.10$, $P = 0.0062$) between the vehicle and CBD immediate extinction groups, indicating that CBD reduced the expression of auditory learned fear acutely when compared with the vehicle. Tone-induced freezing during the CS blocks of immediate extinction is presented in Figure 6.2.D. Two-way ANOVA revealed a significant main effect of block ($F_{(3.119, 56.14)} = 8.623$, $P < 0.0001$), but no treatment effect ($F_{(1, 18)} = 3.718$, $P = 0.0697$), or treatment x block interaction ($F_{(8, 144)} = 0.9594$, $P = 0.4702$), suggesting that CBD had no reliable effect on extinction learning. The freezing levels during the pre-CS interval before the extinction recall test are depicted in Figure 6.2.E. One-way ANOVA revealed a significant main effect of treatment ($F_{(3, 36)} = 5.116$, $P = 0.0047$), with posthoc analysis demonstrating significantly higher freezing in vehicle immediate extinction over the other groups ($P < 0.05$). These results indicate that the vehicle immediate extinction group resulted in higher baseline expression of contextual fear before the extinction recall test. The average freezing of 10 CS presentation during the extinction recall is presented in Figure 6.2, while differences across treatment groups were analyzed performing an *a priori* confirmatory one-way ANOVA, which revealed a significant main effect of treatment ($F_{(3, 36)} = 8.028$, $P = 0.0003$). Posthoc analysis revealed significantly lower levels of freezing with CBD immediate extinction when compared with the other groups ($P < 0.01$), whilst no reliable differences were identified between any of the other groups. This indicates that CBD immediate extinction resulted in enhancement of extinction recall, an effect that was further explored by analyzing each CS presentation separately across the different treatment groups, as depicted in Figure. 6.2.G. Two-way ANOVA revealed a significant main

effect of treatment ($F_{(3, 36)}$ = 8.028, P = 0.0003), trial ($F_{(9, 324)}$ = 27.30, P < 0.0001), and a treatment x trial interaction ($F_{(27, 324)}$ = 6.736, P < 0.0001). Posthoc analysis demonstrated significantly lower freezing levels with the CBD immediate extinction group during the CS presentations 4 - 7 and 10 (P < 0.05), when compared to vehicle immediate extinction and the two immediate no extinction groups. Significantly lower freezing was also observed with the vehicle immediate extinction group during the CS presentations 7-10 (P < 0.05) in comparison to the vehicle and CBD immediate no extinction groups. Additionally, there were no reliable differences in freezing between the two immediate no extinction groups throughout the extinction recall test. These results suggest that CBD combined with immediate extinction facilitated extinction encoding to prevent the IED, in comparison to vehicle combined with immediate extinction. Additionally, they indicate that extinction is a prerequisite for CBD effectiveness and exclude the possibility that CBD acts by impairing the consolidation of learned fear memory, as CBD administration without extinction did not induce any effect. Finally, the fact that vehicle immediate extinction demonstrated a freezing reduction during the late phase of the extinction recall test suggests that immediate extinction exerted a beneficial effect on learned fear suppression, when compared to the vehicle and CBD treated groups not receiving extinction.

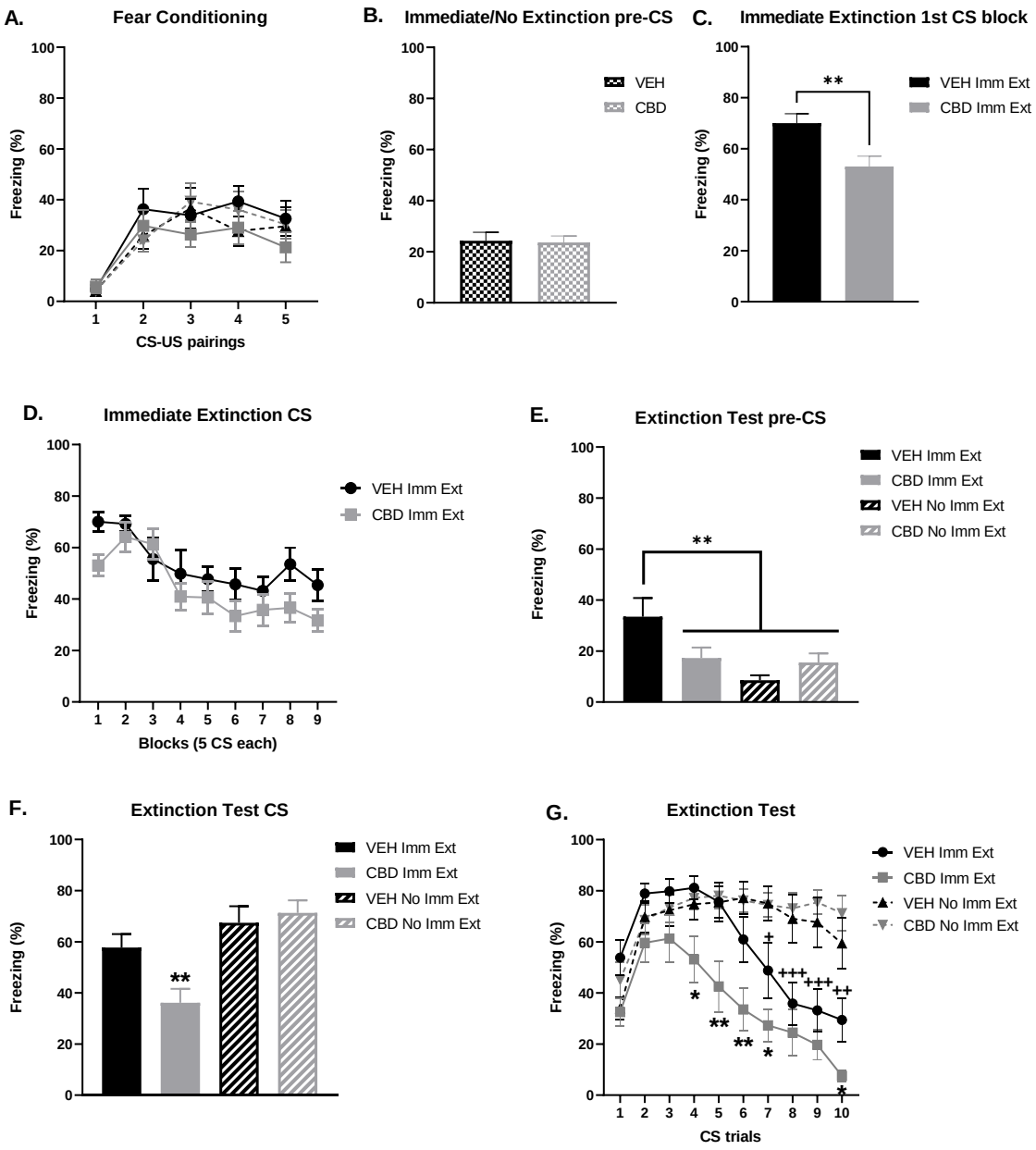

Figure 6.2 Cannabidiol administration before immediate extinction prevents the IED. (A) Fear conditioning did not differ between the groups (n=10 rats /group) to receive i.p. vehicle or 10 mg/kg CBD before immediate extinction (VEH Imm Ext, CBD Imm Ext) or no immediate extinction (VEH No Imm Ext, CBD No Imm Ext). (B) CBD had no effect on baseline contextual fear expression during the pre-CS interval before immediate or no extinction. (C) CBD Imm Ext resulted in significantly less freezing compared to VEH Imm Ext in the first CS block at the start of immediate extinction (** $p < 0.01$). (D) CBD had no effect on tone-induced freezing during immediate extinction training. (E) VEH Imm Ext showed significantly higher levels of freezing during the pre-CS interval of extinction recall testing, compared to the other groups (** $p < 0.01$). (F) CBD Imm Ext demonstrated significantly decreased levels of tone-induced freezing during the extinction recall testing, compared with the other groups (** $p < 0.01$). (G) CBD Imm Ext showed significantly lower freezing in response to tones 4-7 and 10 during the extinction recall testing, when compared to other groups (* $p < 0.05$, ** $P < 0.01$). VEH Imm Ext resulted in decreased freezing in response to tones 7-10, when compared to VEH and CBD No Imm Ext groups (++ $p < 0.01$, +++ $p < 0.001$).

6.5 Discussion

This experiment investigated the effects of pre-immediate extinction CBD administration on the extinction learning impairments induced by high-stress levels associated with the recent fear conditioning. Specifically, CBD did not affect the baseline expression of contextual fear during the interval before the CS trials of immediate extinction. However, particularly interesting is that CBD reduced freezing levels during the first CS-block of extinction training when compared to the vehicle, indicating an acute suppression of auditory fear. Although CBD did not influence the extinction learning, it led to the suppression of both baseline contextual and auditory fear expression when rats were tested drug-free the following day, indicating that CBD enhanced recall of extinction to prevent the IED phenomenon. These long-term beneficial effects of CBD in ameliorating the stress-induced impairments of extinction demonstrate a novel finding in the field.

The fact that the administration of CBD immediately after conditioning did not show any effects in the absence of extinction indicates that CBD required extinction training to prevent IED and excludes the possibility that CBD elicited its effects by interfering with the consolidation of learned fear memory. Likewise Song et al. (2016) observed extinction enhancement of a stronger contextual fear memory only when CBD was given in conjunction with extinction training, and not without it. It is worth highlighting that immediate extinction groups in the present study demonstrated extinction recall during later phases of testing, indicating that extinction encoding is impaired and not obliterated by immediate extinction (Chang & Maren, 2009).

As mentioned earlier, weakening the aversiveness of the conditioning protocol or administering antagonists of stress mediators were found to rescue IED. Particularly interesting are two previous studies in which intra-BLA infusion of a β-receptor (Giustino et al., 2017) or CRFR1 (Hollis et al., 2016) antagonist prevented IED. These compounds, similarly to CBD, did not induce within-extinction session reductions in freezing, but only upon the drug-free extinction recall testing. Although the exact mechanisms through which CBD regulates immediate extinction remain to be investigated, the above observation suggests the possibility that CBD may act by modulating the NE-ergic and CRF signalling, possibly through elevation of the endocannabinoid levels.

This is supported by the fact that endocannabinoid and noradrenergic interactions were identified in various brain areas governing both innate and learned fear processes. The LC-NE afferent projection to mPFC plays a particularly important role in successful extinction learning (Hugues et al., 2007). CB1Rs are localized pre- and post-synaptically in both LC and mPFC, modulating their excitability and NE release (Gobbi et al., 2005; Mendiguren & Pineda, 2006). Specifically, it was found that CB1Rs located either post-synaptically on LC-NE neurons or pre-synaptically at glutamatergic terminals projecting to LC-NE neurons modulate their basal activity (Mendiguren & Pineda, 2006). Additionally, CB1Rs expressed either pre- or post-synaptically at LC-NE neurons projecting to mPFC were suggested to regulate NE release and mPFC firing, respectively (Oropeza et al., 2007; Reyes et al., 2015). Based on the above observations, Warren et al. (2022) have recently suggested that the ECB

and NE systems have synergistic roles in regulating extinction, as CB1Rs when activated facilitate extinction, while simultaneously increasing the spontaneous firing of LC-NE neurons and NE efflux from afferent projections to mPFC, which is also linked with potentiation of extinction learning. Furthermore, it is worth noting that the ECB system is also engaged in high-stress states to modulate NE signaling within BLA (Atsak et al., 2015; Bedse et al., 2015).

Another potential mechanism through which CBD may regulate immediate extinction is through ECB-mediated modulation of CRF system, which is well known for controlling stress-related responses through HPA axis and amygdala activation (Steiner & Wotjak, 2008; Ganon-Elazar & Akirav, 2013). Supporting evidence indicates that a large percentage of CRF neurons within BLA and CEA express CB1Rs (Cota et al., 2007), while significantly higher mRNA levels of CRF, CRF1R, and CB1R were identified in rats exposed to aversive events (Aisenberg et al., 2017). A study by Gray et al. (2015) has suggested that under low-stress conditions, AEA-mediated activation of presynaptic CB1Rs located in glutamatergic neurons projecting to BLA inhibits the excessive efflux of glutamate and therefore the BLA hyperactivation. In contrast, under high stress, elevations in CRF release within BLA induce CRF1R-dependent activation of FAAH, depleting the AEA pools within glutamatergic BLA synapses. This loss of AEA-mediated tonic inhibition results in enhanced release of glutamate and subsequent overactivation of BLA principal neurons, thus initiating stress-related responses. The fact that FAAH inhibition reverses the stress-induced effects indicates that possibly CBD can exert similar effects by increasing AEA levels through inhibition of its enzymatic degradation by FAAH and transporter-mediated reuptake (Bisogno & Maccarrone, 2013). Another study revealed that CBD restrains the stress-induced

elevations of CRF expression in amygdala (Viudez-Martínez et al., 2018), indicating that CBD may act by modulating CRF in BLA. Additionally, compelling evidence of CRF modulation by the ECB system at the LC level is provided by a previous study revealing that CEA inhibitory and excitatory afferents projecting to LC were found to co-express both CRF and CB1 receptors (Wyrofsky et al., 2017). This raises the possibility that CBD could induce stress-alleviating effects by modulating CEA-CRF afferents to LC through indirect activation of CB1Rs.

In conclusion, the results of this study demonstrate novel evidence that CBD can alleviate impairments in extinction learning that accompany immediate extinction as a result of excessive stress induced by recent conditioning. These findings encourage the elucidation of pharmacological and neural mechanisms underpinning CBD effects on the regulation of immediate extinction, focusing on its potential interaction with the ECB, NE, and/or CRF systems, that appear dysregulated after exposure to an aversive event (Gazarini et al., 2022). Unraveling such mechanisms could stimulate further research at a clinical level, determining the potential efficacy of CBD in achieving long-term remission from anxiety and fear-related symptoms when combined with exposure therapy.

Chapter 7. General discussion

This thesis aimed to investigate the effectiveness of CBD on the regulation of extinction and relapse of learned fear and to determine the underlying pharmacological mechanisms. In Chapter 2, validation studies were initially performed to establish a fear relapse protocol, which was used in Chapter 3 to assess the effects of CBD on extinction and spontaneous recovery of contextual and auditory fear. CBD administration before extinction acutely reduced the expression of contextual fear memory and prevented the spontaneous recovery of auditory fear. To elucidate on the pharmacological mechanisms behind the CBD-mediated effects, the involvement of CB1R and 5-HT1AR signalling was examined in Chapters 4 and 5, respectively, by administrating CBD in combination with either the CB1R or the 5-HT1AR antagonist. The results of these experiments did not replicate the previously observed effects of CBD on spontaneous fear recovery, rendering inconclusive the involvement of either signalling mechanisms. Lastly, Chapter 6 aimed to investigate the effects of CBD on the extinction learning impairments induced by stress following recent fear conditioning, using the IED protocol previously established in the lab. CBD enhanced recall of extinction to prevent the IED phenomenon, without interfering with the consolidation of learned fear memory. Below follows a more in-depth explanation of the findings from each chapter, possible refinements and suggestions for future research directions.

7.1 Validation studies of spontaneous recovery, reinstatement, and renewal of learned fear

In the first validation experiment, spontaneous recovery of contextual and auditory fear was observed 21 days after successful extinction. Despite the use of identical parameters during fear conditioning and extinction training, in the second experiment, delivering a single unreinforced stimulus one day after extinction failed to reinstate fear responses. Similarly, renewal of learned fear was not observed after delivery of tone presentations in the conditioning context a day after extinction training in a distinct context. Despite the use of a relatively weak fear conditioning, this was sufficient to induce high levels of tone-induced freezing at the beginning of extinction training, which resulted in effective extinction. Particularly important for the robustness of fear return is the elapsed retention interval from extinction training until the test.

The fact that the animals did not exhibit either reinstatement or renewal of learned fear may be attributed to the combination of experimental parameters used. Regarding the reinstatement paradigm, possibly the degree of aversiveness induced by the single unreinforced stimulus was not sufficient to reinstate fear, compared with the multiple stimuli delivered in other studies (King et al., 2018a). Noteworthy is that US intensity used during the fear conditioning plays a crucial role in the robustness of fear renewal and reinstatement of learned fear, which in the present experiments was relatively weak. In the validation of renewal protocol, unexpected was the lack of fear return despite animals being tested after strong ‘ABA’ renewal conditions. The variability in light-dark conditions between contexts A and B, apart from the other

contextual cues, may have played an important, though underappreciated role in the modulation of emotional responses. Warthen et al. (2011) have shown that light enhances freezing behaviour in mice during both acquisition and recall of learned fear, specifically in response to the acoustic and not contextual conditioned stimuli. Importantly, alteration in lighting conditions between fear conditioning and recall testing can acutely modulate the freezing response, manifested as decreased freezing upon light removal during testing or enhancement when light is added. When trying to translate this evidence to the present protocol, somebody would anticipate higher levels of freezing during the renewal session given that it took place in light conditions. However, it is not known how extinction learning may be affected by dark conditions.

Additionally, the stress experienced before fear conditioning, extinction or extinction test critically influences the effective encoding and recall of extinction. Specifically, exposure to stress before fear conditioning enhances its consolidation and induces deficits in extinction learning (Maldonado et al., 2011; Bender et al., 2018). On the other hand, stress before extinction training was found to enhance its consolidation in a context-independent manner, thus preventing renewal upon contextual change, while exposure to stress prior extinction test impedes its recall therefore promoting relapse (Meir Drexler et al., 2020).

7.2 Suggestions and refinements for future validation studies

The procedural parameters validated for the spontaneous fear recovery protocol, demonstrating the return of both contextual and auditory fear, encouraged the investigation of the modulatory effects of CBD on extinction and the relapse condition of spontaneous recovery. For future studies it would be particularly

interesting to investigate the efficacy of CBD upon reinstatement and renewal conditions. A promising approach to ensure a more robust reinstatement protocol is to increase the number of unreinforced US stimuli applied after extinction training (Yang et al., 2012). On the other hand, the renewal protocol can be modified by presenting rats in the same contexts A and B as in other relapse paradigms but both under "lights-on" conditions, while maintaining the same "ABA" contextual alteration (Khoo et al., 2020). In the case that these modifications fail to reinstate or renew fear, increasing the intensity and/ or duration of US stimulus during fear conditioning could be another potentially effective approach as indicated by previous studies in the field (Jo et al., 2020; Li et al., 2021). According to Lissek et al. (2006) and Lonsdorf & Merz (2017) stronger experimental conditions may also elicit more uniform fear responding, diminishing the inter-individual differences between the experimental subjects, that are theoretically manifested upon weaker conditions.

7.3 Effects of CBD in the regulation of extinction and spontaneous fear recovery

Based on the enhancing effects of CBD on extinction that were previously observed (Bitencourt et al., 2008; Do Monte et al., 2013; Song et al., 2016), this study aimed to investigate its potential long-term effects on extinction retention and prevention of spontaneous fear recovery, using the above-mentioned validated spontaneous fear recovery protocol. CBD administration before extinction training resulted in an acute reduction in baseline expression of contextual fear memory, a finding that agrees with previous studies (Resstel et al., 2006; Lemos et al., 2010; Jurkus et al., 2016). Notably, the present study provided novel evidence that CBD can produce long-term fear-suppressing effects by inhibiting spontaneous recovery of

auditory fear 21 days after extinction training, presumably through facilitation of extinction encoding. In contrast, CBD administration after extinction training failed to exert any long-term effect either during extinction recall or spontaneous recovery testing, while unexpectedly none of the treatment groups exhibited spontaneous recovery of auditory fear.

The fact that pre-extinction CBD failed to reduce auditory fear expression or enhance extinction learning or its recall contradicts previous findings in the field (Bitencourt et al., 2008; Do Monte et al., 2013; Jurkus et al., 2016). Such discrepancies may be attributed to the differences in the aversiveness of fear conditioning, as CBD has previously demonstrated acute and long-lasting reduction of contextual fear expression during extinction training and recall, respectively, but only upon strong and not weak fear conditioning (Song et al., 2016). Additionally, another crucial factor might be the route of CBD administration. Direct brain infusions may provide better bioavailability and more targeted effects compared to the intraperitoneal administration, in which CBD undergoes extensive first-pass metabolism, leading to low concentration particularly in brain tissue (Lust et al., 2022). On the other hand, there is no evidence from preclinical studies regarding the effects of post-extinction CBD that can be compared with the present study. However, administration of CBD in healthy human participants after extinction training suppressed conditioned responding during retrieval through enhancement of extinction consolidation (Das et al., 2013).

Particularly striking in the second experiment was that none of the treatment groups demonstrated spontaneous recovery of auditory fear. This raised the

possibility that this finding may be attributed to the younger age of the rats used in this experiment compared to previous ones, although their exact age was not specified. Indeed, several studies have demonstrated age-dependent differences in extinction learning and retention. For instance, juvenile male rats do not exhibit renewal, reinstatement, and spontaneous recovery of learned fear (Park et al., 2017). In contrast, adolescent male rats demonstrate impaired extinction retention compared to juvenile or older rats, while no age-related differences are observed in extinction learning (McCallum et al., 2010). This might explain that the present findings were induced due to the impairments in extinction retention rather than to lack of fear relapse. All these observations provide significant translational insights that deepen the understanding regarding the roles of age- and sex in the predisposition, development, and pharmacological management of PTSD.

7.4 Suggested refinements for future studies investigating the effects of CBD on extinction and spontaneous recovery of learned fear

For the future studies, it is recommended to use animals as identical as possible in developmental stage, so to avoid age-dependent discrepancies in pharmacological and behavioural effects. An interesting topic from both the preclinical and clinical perspectives will be to explore the therapeutic potential of CBD in modulation of extinction and learned fear relapse in rats of different age and/ or sex groups, given that females are twice as likely to experience PTSD than males, a difference that begins to emerge at adolescence (Garza & Jovanovic, 2017). A recent study revealed that systemic or intra-dHPC CBD infusion impaired fear memory consolidation in adult female rats (Franzen et al., 2022), but CBD effects on

modulation of extinction in female rats remain to be investigated. Given the fact that females display more active defensive behaviours compared to males, it is crucial for such studies to implement additional quantitative measures of conditioned responses like darting (i.e., escape-like response exhibited as fast crossing of the chamber) to avoid misinterpretation of female's low freezing as poor conditioning or extinction learning (Gruene et al., 2015).

The findings of the present study encourage the investigation in future studies of the neural mechanisms underpinning these long-lasting fear-suppressing effects induced by pre-extinction CBD. Combining behavioural testing with *in vivo* electrophysiological recordings from areas regulating extinction learning and retention (e.g., IL, PL, BLA, vHPC or dHPC CA1/CA3) will help to elucidate how CBD modulates neuronal activity to induce its effects (Goonawardena et al., 2011). It would be also interesting to identify the neural circuitry involved in CBD-mediated effects by culling the animals either after extinction or spontaneous recovery testing and performing *ex vivo* sampling and immunohistochemistry for expression of c-Fos, Arc or Zif268, immediate early genes that were previously associated with neuronal activation and synaptic plasticity related to extinction and fear relapse (Knapska & Maren, 2009; Gallo et al., 2018). Another potential approach is to infuse CBD directly into the brain areas mentioned above to replicate the effects induced by systemic CBD administration. The principal aim of this thesis was to investigate the potential involvement of CB1R or 5-HT1AR signalling on CBD-induced pharmacological effects, however results of these studies will be further discussed below.

7.5 Possible pharmacological mechanisms involved in CBD regulation of extinction and spontaneous fear recovery

Evidence from previous studies reveals that CBD acutely reduces conditioned fear expression through a 5-HT1AR-dependent mechanism (Gomes et al., 2012; Fogaca et al., 2014; Marinho et al., 2015). On the other hand, CBD produces sustained fear alleviation by enhancing the extinction (Bitencourt et al., 2008; Do Monte et al., 2013) or disrupting the consolidation (Stern et al., 2017; Raymundi et al., 2020) and reconsolidation of learned fear memory (Stern et al., 2012; Bayer et al., 2022), in a CB1R-dependent manner. Based on this encouraging evidence, the present study aimed to explore whether these two signalling pathways are involved in the long-lasting suppression of spontaneous fear recovery induced by CBD.

Initially in Chapter 4 a dose-response experiment was performed using the CB1R inverse agonist AM251 to identify the dose that could abolish the previously observed CBD effects, without eliciting an effect by itself. AM251 did not affect expression of learned fear, extinction learning or recall, however none of the treatment groups displayed spontaneous recovery of auditory fear. For the combination study with CBD, the low dose of AM251 was selected. However, none of the treatments affected fear responses across the different sessions. Importantly, the administration of CBD alone failed to replicate the previous effects in suppressing spontaneous recovery. Next, Chapter 5 aimed to investigate the potential involvement of 5-HT1AR-signalling in mediating CBD's effects. The dose-response study for the 5-HT1AR antagonist WAY100635 revealed that the high dose reduced the baseline expression of contextual fear, rendering it inappropriate for the combination study

with CBD. However, none of the treatment combinations affected freezing behaviour in any of these sessions, while CBD was incapable of replicating any of the previously observed effects. The lack of reproducibility in these two studies rendered it impossible to exclude the potential involvement of CB1R or 5-HT1AR-mediated signaling in CBD's acute and long-lasting fear-suppressing effects.

As mentioned above, the irreproducibility of CBD effects observed across the experiments may be attributed to the age of the rats used, which was not determined in the present studies. Although it is inconclusive whether ECB or 5-HT-ergic signaling is involved in the previously observed fear suppressing effects of CBD, it is worth discussing the age-dependent adaptations that have been identified in these two neurotransmitter systems. Regarding ECB signaling, 2-AG levels rise during perinatal period and then decline at early postnatal dates, reaching the adult concentrations. In contrast, AEA gradually increases from early postnatal stage reaching maximum levels around adolescence which is associated with increased expression of NAPE-PLD (i.e., responsible for AEA synthesis) in the PAG (Kwok et al., 2017). Robust changes in CB1R expression occur in prefrontal and limbic areas, reaching its highest expression during adolescence and then decreases by adulthood. Such changes contribute to the maturation of corticolimbic circuitry, maintaining balance between excitatory and inhibitory neurotransmission and thus playing a critical role in cognitive and behavioural processing (Meyer et al., 2018). However, stress during early life or adolescence can interfere with this developmental process, leading to deficits in hippocampal function and downregulation of CB1R expression later in life, increasing the vulnerability to psychopathology (Goldstein Ferber et al., 2021). Possibly, fear conditioning may exert analogous effects in the hippocampus of adolescent rats,

making them more vulnerable to fear generalization or unsuccessful recall of extinction when tested several days after extinction training. On the other hand, it was found a decrease in 2-AG and DAGLa (i.e., responsible for synthesis of 2-AG) levels in hippocampus and putamen, while reductions in AEA were observed in the putamen, PFC, and cingulate cortex, strongly affecting mice of middle-age. This was associated with a transient compensatory increase in CB1R expression, followed by a steep decline in both hippocampus and limbic forebrain, that was associated with learning and memory impairments (Nidadavolu et al., 2022).

Age specific-differences have been described as well in 5-HT-ergic transmission. Both human and rodent studies demonstrate an age-dependent increase of SERT levels in the DRN and lateral septum, which is followed by a gradual decrease in middle age (Ulloa et al., 2014). Significantly larger declines with age were observed in the expression of 5-HT2ARs, compared to post-synaptic 5-HT1ARs, while pre-synaptic 5-HT1ARs in raphe nuclei were preserved (Karrer et al., 2019). On the other hand, exposure to stress early in life or during puberty was found to induce persistent alterations in the 5-HT system e.g., increased 5-HT1AR expression in amygdala but reduced in DRN or hippocampus, and reduced SERT expression in DRN, within brain areas associated with the development of stress-related disorders (Matsuzaki et al., 2011; Bravo et al., 2014). Therefore, across the different studies, it is important to select rats at the same stage of development, to avoid unexpected behavioural and pharmacological responses that may be driven by the age-dependent alterations in the ECB and 5-HT-ergic signaling.

The fact that these studies failed to reproduce the previously observed fear-suppressing effects of CBD may be attributed to differences in animals' personality traits, that can be promoted especially under weak experimental situations. Often the existence of potentially unrecognized subpopulations within a group (i.e., rats competent vs resistant to extinction) may mask important patterns of behavioral responding (i.e., suppression or relapse of conditioned fear) and lead thus to non-significant statistical effects between the different groups (Lonsdorf & Merz, 2017). Therefore, particularly challenging can be the interpretation of the present results when considering the influence of temperamental factors in a study with a decreased sample size (i.e., WAY100,635-CBD study) in combination with the previously described bidirectional effects of CBD on extinction memory depending on the aversity of fear conditioning (Song et al., 2016).

Alterations in the process of CBD formulation may also result in an inability to replicate previous findings. Specifically, in the AM251-CBD combination study, CBD was suspended in a vehicle made of a greater concentration of Tween 80, when compared to the study reported in Chapter 3. As described above, alterations in surfactant concentration within a vehicle solution can affect the micellization process during the drug formulation, altering its pharmacokinetic properties (Yadav et al., 2017). Other parameters like temperature, surfactant type, pressure, solvent, sonication, etc., were found also to influence the process of micellization through which CBD formulation is developed (Mohajeri & Noudeh, 2012; Mohd Yusof, 2021). Although these parameters generally are not meticulously monitored or described in different studies, any changes could influence the physicochemical properties of the drug, and therefore its effectiveness (Bruni et al., 2018). A possible solution to this

issue would be to introduce a quality control checkpoint during the drug preparation for example, to monitor the size of the CBD micelles with dynamic light scattering (Sutherland et al., 2009).

7.6 Future studies for the elucidation of pharmacological mechanisms involved in CBD regulation of extinction and spontaneous recovery of learned fear

A principal aim for the future is to determine the potential involvement of CB1R or 5-HT1AR signaling in the suppression of spontaneous fear recovery induced by CBD. This could be achieved by repeating the AM251-CBD and WAY100,635-CBD experiments under as identical as possible experimental conditions to Chapter 3, like using rats of the same age, or vehicle of same Tween 80 concentration for CBD.

In case that CB1R-signalling is involved in mediating the effects of CBD, it would be interesting to characterize the fluctuations of AEA and 2-AG within the extinction circuitry, to further examine the role of CB1Rs. *Ex-vivo* sampling can be performed in discrete brain areas like dHPC, vHPC, BLA, IL, and PL after completion of extinction training, extinction testing or spontaneous recovery testing to undergo later quantification for endocannabinoid levels through liquid chromatography-mass spectrometry. This would provide significant information about both the pharmacological and neural targets related to these effects of CBD. For instance, elevated AEA levels would suggest CBD-mediated inhibition of either reuptake (i.e., FABPs) or degradation (i.e., FAAH, COX-2) of AEA, while elevated 2-AG levels would suggest MAGL inhibition. On the other hand, elevation of either ECB alone would narrow down the receptors that may be involved, given that AEA activates CB1Rs,

CB2Rs, TRPV1, PPARγ, and SK channels, while 2-AG only activates CB1Rs and CB2Rs (Patel et al., 2017).

The fact that CB1Rs, CB2Rs and PPARγRs in the dHPC were previously found to time-dependently modulate CBD-induced disruption of fear memory consolidation makes it interesting to explore their involvement in extinction and spontaneous recovery of learned fear (Stern et al., 2017; Raymundi et al., 2020). On the other hand, it is less likely that TRPV1 is implicated in the fear-attenuating effects induced by CBD in the present study. This is supported by a previous study revealing that both CBD and AM404, compounds that similarly elevate AEA and stimulate TRPV1, enhanced contextual fear extinction through CB1R-, rather TRPV1-mediated signalling (Bitencourt et al., 2008). Additionally, activation of TRPV1 receptors was found to impair extinction of cued fear, while their blockade produced facilitating effects on contextual fear extinction (Laricchiuta et al., 2013; Morena et al., 2021). Another interesting pathway to explore is whether CBD can potentially attenuate learned fear through inhibition of COX-2-mediated degradation of AEA. Evidence from a previous study revealed that both traditional (i.e., celecoxib and lumiracoxib) and substrate selective (i.e., LM-4131) COX-2 inhibitors induced anxiolytic-like effects in the EPM and novelty-induce hypophagia assay after exposure to foot-shock stress. These effects were mediated through a SK-dependent mechanism, as pre-administration of SK-inhibitor, but not CB1R, CB2R or TRPV1 antagonist, blocked such effects (Gamble-George et al., 2016). Additionally, the fact that SK channels are activated by AEA (Wang et al., 2011) and have been identified on pyramidal neurons of extinction-related areas like, BLA, PFC, and hippocampus (Faber, 2010; Hermann et al., 2020),

raises the possibility that CBD may indirectly modulate SK signaling through inhibition of COX-2-mediated inactivation of AEA.

As already mentioned, 5-HT1AR-mediated signaling attracts significant attention as a possible pharmacological mechanism underlying the CBD-induced effects on suppression of spontaneous fear recovery. This is because CBD was previously found to interfere with learned fear acquisition and expression in 5-HT1AR-dependent manner (Gomes et al., 2012; Fogaca et al., 2014; Norris et al., 2016). Interestingly, chronic fluoxetine applied in combination with extinction training resulted in prevention of spontaneous fear recovery (Karpova et al., 2011), although the involvement of 5-HT1AR has not yet been determined. Given this evidence, it is worth repeating the previous WAY100,635-CBD combination study, and thereafter identifying the neural substrates and the specific 5-HT1ARs (i.e., pre- or post-synaptic) that underpin the effects of systemically administered CBD. This could be approached by reproducing systemic effects after direct CBD infusion into areas of the corticoraphe circuit like DRN, BLA, or PFC, that may govern the effects of CBD on reduction of learned fear expression and facilitation of extinction. Additionally, prior injection or infusion with 5-HT1AR antagonists into BLA or PFC would provide insight about the pharmacological mechanism underlying CBD effects (Alexander & Vasefi, 2021).

7.7 Effects of CBD on immediate extinction and extinction recall of learned fear

The inconsistencies observed across the present studies in the CBD-mediated modulation of extinction learning and its recall triggered the exploration of CBD effects under more aversive experimental conditions, given that the level of stress

regulates not only the vulnerability for developing maladaptive fear memories but also the responsiveness to extinction and pharmacological interventions. To achieve this, the effects of CBD were investigated on stress-induced extinction learning impairments following recent fear conditioning, by using the IED protocol. It was observed that CBD administration before immediate extinction acutely reduced auditory fear expression but did not influence either baseline expression of contextual fear or extinction learning. The following day, CBD resulted in suppression of both contextual and auditory fear expression, indicating an enhanced extinction recall that prevented the IED phenomenon. This novel ameliorating effect of CBD required extinction training and did not involve any interference in fear memory consolidation. After a thorough search on the relevant literature, CBD is one of the first cannabinoids investigated for its effectiveness in the IED model, but the pharmacological and neural mechanisms underlying its effects remain to be determined.

7.8 Future directions towards investigation of pharmacological and neural mechanisms underlying CBD effects on IED

As earlier described, it is suggested that the IED involves excessive activation of BLA induced by the enhanced NE-ergic and CRF signaling associated with high stress levels after recent fear conditioning. BLA subsequently may stimulate IL inhibitory interneurons, leading to suppression of IL firing (Maren, 2022). The fact that the ECB system was found to modulate NE-ergic and CRF signaling within the stress and fear extinction circuitry (Gazarini et al., 2022; Warren et al., 2022) raises the possibility that it may be also involved in regulation of stress-induced impairments in extinction learning, although the exact mechanisms need to be explored. A potential mechanism

through which CBD may ameliorate extinction resistance could involve the modulation of the NE-ergic and/or CRF signaling through indirect activation of CB1 receptors. An initial approach would be to identify the circuit which systemic CBD modulates. *In vivo* microdialysis for the quantification of NE levels can be performed at IL and BLA, the principal sites involved in the IED to which LC-NE neurons project. This would provide insight into whether CBD acts to restore NE-ergic stimulation of IL or to dampen the NE-mediated overstimulation of BLA. Therefore, possible elevated NE levels at IL could indicate that CBD, by indirectly activating CB1Rs, may enhance the efflux of NE at IL, or increase the activity of LC-NE neurons projecting to IL, resulting in increased NE release post-synaptically in IL (Warren et al., 2022). However, these two mechanisms require further exploration. On the other hand, decreased NE levels at BLA could indicate that CBD may inhibit overstimulation of LC-NE by activating presynaptic CB1Rs located at the CEA-CRF neurons projecting to LC. This would restrict the CRF release and prevent therefore the CRFR1-mediated stimulation of LC-NE neurons projecting to BLA, thus dampening excessive BLA firing (Wyrofsky et al., 2017; Maren, 2022). Further studies administering CBD directly into the LC, BLA or PFC, or combining that with CB1R antagonist pre-treatment, would provide additional information about the involvement of these areas in immediate extinction processes. Finally, it would be interesting to investigate potential long-term effects of CBD when combined with immediate extinction in prevention of fear relapse conditions, while incorporating also female gender rats in future studies, given that male rats have previously exhibited greater levels of fear renewal than females in the aftermath of immediate extinction (Binette et al., 2022).

7.9 Conclusion

The results of this thesis confirm previous findings regarding the acute effects of CBD in reducing learned fear memory expression, while demonstrating novel evidence that CBD can provide long-term protection against fear relapse after successful extinction and alleviate stress-induced impairments in extinction learning. Although it was inconclusive whether CB1R or 5-HT1AR signaling is involved in CBD-induced suppression of spontaneous fear recovery, the potential pharmacological and neural mechanisms underlying its effects were still discussed, while reevaluating possible experimental factors that may have influenced the experimental reproducibility. Determining CBD's effects and mechanisms of action in both sexes will provide valuable insight about its adequacy as a candidate therapeutic and potential adjunct to exposure therapy in achieving lasting remission from trauma and stressor-related symptoms.

8. References:

Abiri, D., Douglas, C. E., Calakos, K. C., Barbayannis, G., Roberts, A., & Bauer, E. P. (2014). Fear extinction learning can be impaired or enhanced by modulation of the CRF system in the basolateral nucleus of the amygdala. *Behav Brain Res*, *271*, 234-239. https://doi.org/10.1016/j.bbr.2014.06.021

Abramowitz, J. S., & Jacoby, R. J. (2014). Obsessive-Compulsive Disorder in the DSM-5 [https://doi.org/10.1111/cpsp.12076]. *Clinical Psychology: Science and Practice*, *21*(3), 221-235. https://doi.org/https://doi.org/10.1111/cpsp.12076

Adkins, J. M., Lynch, J., Gray, M., & Jasnow, A. M. (2021). Presynaptic GABAB receptor inhibition sex dependently enhances fear extinction and attenuates fear renewal. *Psychopharmacology*, *238*(8), 2059-2071. https://doi.org/10.1007/s00213-021-05831-w

Aguiar, D. C., Moreira, F. A., Terzian, A. L., Fogaca, M. V., Lisboa, S. F., Wotjak, C. T., & Guimaraes, F. S. (2014). Modulation of defensive behavior by Transient Receptor Potential Vanilloid Type-1 (TRPV1) channels. *Neurosci Biobehav Rev*, *46 Pt 3*, 418-428. https://doi.org/10.1016/j.neubiorev.2014.03.026

Ahn, K., McKinney, M. K., & Cravatt, B. F. (2008). Enzymatic pathways that regulate endocannabinoid signaling in the nervous system. *Chemical reviews*, *108*(5), 1687-1707. https://doi.org/10.1021/cr0782067

Aisenberg, N., Serova, L., Sabban, E. L., & Akirav, I. (2017). The effects of enhancing endocannabinoid signaling and blocking corticotrophin releasing factor receptor in the amygdala and hippocampus on the consolidation of a stressful event. *Eur Neuropsychopharmacol*, *27*(9), 913-927. https://doi.org/10.1016/j.euroneuro.2017.06.006

Alexander, C., & Vasefi, M. (2021). Cannabidiol and the corticoraphe circuit in post-traumatic stress disorder. *IBRO Neuroscience Reports*, *11*, 88-102. https://doi.org/https://doi.org/10.1016/j.ibneur.2021.08.001

Almada, R. C., Borelli, K. G., Albrechet-Souza, L., & Brandão, M. L. (2009). Serotonergic mechanisms of the median raphe nucleus–dorsal hippocampus in conditioned fear: Output circuit involves the prefrontal cortex and amygdala. *Behavioural Brain Research*, *203*(2), 279-287. https://doi.org/https://doi.org/10.1016/j.bbr.2009.05.017

American Psychological, A. (2017). Clinical practice guideline for the treatment of posttraumatic stress disorder (PTSD) in adults.

Andrade, C. (2021). The Ceiling Effect, the Floor Effect, and the Importance of Active and Placebo Control Arms in Randomized Controlled Trials of an Investigational Drug. *Indian Journal of Psychological Medicine*, *43*(4), 360-361. https://doi.org/10.1177/02537176211021280

Arenos, J. D., Musty, R. E., & Bucci, D. J. (2006). Blockade of cannabinoid CB1 receptors alters contextual learning and memory. *European Journal of Pharmacology*, *539*(3), 177-183. https://doi.org/https://doi.org/10.1016/j.ejphar.2006.04.017

Arnaudova, I., Kindt, M., Fanselow, M., & Beckers, T. (2017). Pathways towards the proliferation of avoidance in anxiety and implications for treatment. *Behaviour Research and Therapy*, *96*, 3-13. https://doi.org/https://doi.org/10.1016/j.brat.2017.04.004

Atsak, P., Hauer, D., Campolongo, P., Schelling, G., Fornari, R. V., & Roozendaal, B. (2015). Endocannabinoid signaling within the basolateral amygdala integrates multiple stress hormone effects on memory consolidation. *Neuropsychopharmacology*, *40*(6), 1485-1494. https://doi.org/10.1038/npp.2014.334

Auchter, A. M., Shumake, J., Gonzalez-Lima, F., & Monfils, M. H. (2017). Preventing the return of fear using reconsolidation updating and methylene blue is differentially dependent

on extinction learning. *Scientific Reports*, *7*(1), 46071. https://doi.org/10.1038/srep46071

Azmin, M. N., Stuart, J. F., Calman, K. C., & Florence, A. T. (1982). Effects of polysorbate 80 on the absorption and distribution of oral methotrexate (MTX) in mice. *Cancer Chemother Pharmacol*, *9*(3), 161-164. https://doi.org/10.1007/bf00257745

Back, F. P., & Carobrez, A. P. (2018). Periaqueductal gray glutamatergic, cannabinoid and vanilloid receptor interplay in defensive behavior and aversive memory formation. *Neuropharmacology*, *135*, 399-411. https://doi.org/10.1016/j.neuropharm.2018.03.032

Bader, M. (2020). Inhibition of serotonin synthesis: A novel therapeutic paradigm. *Pharmacology & Therapeutics*, *205*, 107423. https://doi.org/https://doi.org/10.1016/j.pharmthera.2019.107423

Baggelaar, M. P., Maccarrone, M., & van der Stelt, M. (2018). 2-Arachidonoylglycerol: A signaling lipid with manifold actions in the brain. *Progress in Lipid Research*, *71*, 1-17. https://doi.org/https://doi.org/10.1016/j.plipres.2018.05.002

Bambico, F. R., Duranti, A., Nobrega, J. N., & Gobbi, G. (2016). The fatty acid amide hydrolase inhibitor URB597 modulates serotonin-dependent emotional behaviour, and serotonin1A and serotonin2A/C activity in the hippocampus. *Eur Neuropsychopharmacol*, *26*(3), 578-590. https://doi.org/10.1016/j.euroneuro.2015.12.027

Bandelow, B., & Michaelis, S. (2015). Epidemiology of anxiety disorders in the 21st century. *Dialogues in clinical neuroscience*, *17*(3), 327-335.

Bandelow, B., Michaelis, S., & Wedekind, D. (2017). Treatment of anxiety disorders. *Dialogues in clinical neuroscience*, *19*(2), 93-107.

Bannerman, D. M., Rawlins, J. N. P., McHugh, S. B., Deacon, R. M. J., Yee, B. K., Bast, T., . . . Feldon, J. (2004). Regional dissociations within the hippocampus—memory and anxiety. *Neuroscience & Biobehavioral Reviews*, *28*(3), 273-283. https://doi.org/https://doi.org/10.1016/j.neubiorev.2004.03.004

Bannerman, D. M., Sprengel, R., Sanderson, D. J., McHugh, S. B., Rawlins, J. N., Monyer, H., & Seeburg, P. H. (2014). Hippocampal synaptic plasticity, spatial memory and anxiety. *Nat Rev Neurosci*, *15*(3), 181-192. https://doi.org/10.1038/nrn3677

Batista, P. A., Fogaca, M. V., & Guimaraes, F. S. (2015). The endocannabinoid, endovanilloid and nitrergic systems could interact in the rat dorsolateral periaqueductal gray matter to control anxiety-like behaviors. *Behav Brain Res*, *293*, 182-188. https://doi.org/10.1016/j.bbr.2015.07.019

Bauer, E. P. (2015). Serotonin in fear conditioning processes. *Behavioural Brain Research*, *277*, 68-77. https://doi.org/https://doi.org/10.1016/j.bbr.2014.07.028

Baxter, A. J., Scott, K. M., Vos, T., & Whiteford, H. A. (2013). Global prevalence of anxiety disorders: a systematic review and meta-regression. *Psychol Med*, *43*(5), 897-910. https://doi.org/10.1017/s003329171200147x

Bayer, H., Stern, C. A. J., Troyner, F., Gazarini, L., Guimarães, F. S., & Bertoglio, L. J. (2022). Medial prefrontal cortex mechanisms of cannabidiol-induced aversive memory reconsolidation impairments. *Neuropharmacology*, *205*, 108913. https://doi.org/https://doi.org/10.1016/j.neuropharm.2021.108913

Bedse, G., Bluett, R. J., Patrick, T. A., Romness, N. K., Gaulden, A. D., Kingsley, P. J., . . . Patel, S. (2018). Therapeutic endocannabinoid augmentation for mood and anxiety disorders: comparative profiling of FAAH, MAGL and dual inhibitors. *Transl Psychiatry*, *8*(1), 92. https://doi.org/10.1038/s41398-018-0141-7

Bedse, G., Romano, A., Tempesta, B., Lavecchia, M. A., Pace, L., Bellomo, A., . . . Gaetani, S. (2015). Inhibition of anandamide hydrolysis enhances noradrenergic and GABAergic transmission in the prefrontal cortex and basolateral amygdala of rats subjected to

acute swim stress [https://doi.org/10.1002/jnr.23539]. *Journal of Neuroscience Research*, *93*(5), 777-787. https://doi.org/https://doi.org/10.1002/jnr.23539

Bender, C. L., Giachero, M., Comas-Mutis, R., Molina, V. A., & Calfa, G. D. (2018). Stress influences the dynamics of hippocampal structural remodeling associated with fear memory extinction. *Neurobiology of Learning and Memory*, *155*, 412-421. https://doi.org/https://doi.org/10.1016/j.nlm.2018.09.002

Benjet, C., Bromet, E., Karam, E. G., Kessler, R. C., McLaughlin, K. A., Ruscio, A. M., . . . Koenen, K. C. (2016). The epidemiology of traumatic event exposure worldwide: results from the World Mental Health Survey Consortium. *Psychological Medicine*, *46*(2), 327-343. https://doi.org/10.1017/S0033291715001981

Bergamaschi, M. M., Queiroz, R. H., Chagas, M. H., de Oliveira, D. C., De Martinis, B. S., Kapczinski, F., . . . Crippa, J. A. (2011). Cannabidiol reduces the anxiety induced by simulated public speaking in treatment-naive social phobia patients. *Neuropsychopharmacology*, *36*(6), 1219-1226. https://doi.org/10.1038/npp.2011.6

Bernardy, N. C., & Friedman, M. J. (2015). Psychopharmacological strategies in the management of posttraumatic stress disorder (PTSD): what have we learned? *Curr Psychiatry Rep*, *17*(4), 564. https://doi.org/10.1007/s11920-015-0564-2

Bhattacharyya, S., Morrison, P. D., Fusar-Poli, P., Martin-Santos, R., Borgwardt, S., Winton-Brown, T., . . . McGuire, P. K. (2010). Opposite effects of delta-9-tetrahydrocannabinol and cannabidiol on human brain function and psychopathology. *Neuropsychopharmacology*, *35*(3), 764-774. https://doi.org/10.1038/npp.2009.184

Bifulco, M., Grimaldi, C., Gazzerro, P., Pisanti, S., & Santoro, A. (2007). Rimonabant: Just an Antiobesity Drug? Current Evidence on Its Pleiotropic Effects. *Molecular Pharmacology*, *71*(6), 1445. https://doi.org/10.1124/mol.106.033118

Binette, A. N., Totty, M. S., & Maren, S. (2022). Sex differences in the immediate extinction deficit and renewal of extinguished fear in rats. *PLOS ONE*, *17*(6), e0264797. https://doi.org/10.1371/journal.pone.0264797

Bisby, M. A., Stylianakis, A. A., Baker, K. D., & Richardson, R. (2021). Fear extinction learning and retention during adolescence in rats and mice: A systematic review. *Neuroscience & Biobehavioral Reviews*, *131*, 1264-1274. https://doi.org/https://doi.org/10.1016/j.neubiorev.2021.10.044

Bisogno, T., & Maccarrone, M. (2013). Latest advances in the discovery of fatty acid amide hydrolase inhibitors. *Expert Opin Drug Discov*, *8*(5), 509-522. https://doi.org/10.1517/17460441.2013.780021

Bisson, J., & Andrew, M. (2007). Psychological treatment of post-traumatic stress disorder (PTSD). *Cochrane Database Syst Rev*(3), Cd003388. https://doi.org/10.1002/14651858.CD003388.pub3

Bitencourt, R. M., Pamplona, F. A., & Takahashi, R. N. (2008). Facilitation of contextual fear memory extinction and anti-anxiogenic effects of AM404 and cannabidiol in conditioned rats. *Eur Neuropsychopharmacol*, *18*(12), 849-859. https://doi.org/10.1016/j.euroneuro.2008.07.001

Bitencourt, R. M., & Takahashi, R. N. (2018). Cannabidiol as a Therapeutic Alternative for Post-traumatic Stress Disorder: From Bench Research to Confirmation in Human Trials. *Front Neurosci*, *12*, 502. https://doi.org/10.3389/fnins.2018.00502

Blanco, C., Rubio, J., Wall, M., Wang, S., Jiu, C. J., & Kendler, K. S. (2014). RISK FACTORS FOR ANXIETY DISORDERS: COMMON AND SPECIFIC EFFECTS IN A NATIONAL SAMPLE. *Depression and Anxiety*, *31*(9), 756-764. https://doi.org/doi:10.1002/da.22247

Bloodgood, D. W., Sugam, J. A., Holmes, A., & Kash, T. L. (2018). Fear extinction requires infralimbic cortex projections to the basolateral amygdala. *Translational Psychiatry*, *8*(1), 60. https://doi.org/10.1038/s41398-018-0106-x

Bockaert, J., Claeysen, S., Bécamel, C., Dumuis, A., & Marin, P. (2006). Neuronal 5-HT metabotropic receptors: fine-tuning of their structure, signaling, and roles in synaptic modulation. *Cell and Tissue Research*, *326*(2), 553-572. https://doi.org/10.1007/s00441-006-0286-1

Bogáthy, E., Papp, N., Vas, S., Bagdy, G., & Tóthfalusi, L. (2019). AM-251, A Cannabinoid Antagonist, Modifies the Dynamics of Sleep-Wake Cycles in Rats. *Front Pharmacol*, *10*, 831. https://doi.org/10.3389/fphar.2019.00831

Bolsoni, L. M., Crippa, J. A. S., Hallak, J. E. C., Guimarães, F. S., & Zuardi, A. W. (2022). Effects of cannabidiol on symptoms induced by the recall of traumatic events in patients with posttraumatic stress disorder. *Psychopharmacology*, *239*(5), 1499-1507. https://doi.org/10.1007/s00213-021-06043-y

Bouton, M. E., Maren, S., & McNally, G. P. (2020). Behavioral and neurobiological mechanisms of pavlovian and instrumental extinction learning. *Physiological Reviews*, *101*(2), 611-681. https://doi.org/10.1152/physrev.00016.2020

Bravo, J. A., Dinan, T. G., & Cryan, J. F. (2014). Early-life stress induces persistent alterations in 5-HT1A receptor and serotonin transporter mRNA expression in the adult rat brain. *Front Mol Neurosci*, *7*, 24. https://doi.org/10.3389/fnmol.2014.00024

Bridge, J. A., Iyengar, S., Salary, C. B., Barbe, R. P., Birmaher, B., Pincus, H. A., . . . Brent, D. A. (2007). Clinical response and risk for reported suicidal ideation and suicide attempts in pediatric antidepressant treatment: a meta-analysis of randomized controlled trials. *Jama*, *297*(15), 1683-1696. https://doi.org/10.1001/jama.297.15.1683

Bruni, N., Della Pepa, C., Oliaro-Bosso, S., Pessione, E., Gastaldi, D., & Dosio, F. (2018). Cannabinoid Delivery Systems for Pain and Inflammation Treatment. *Molecules*, *23*(10). https://doi.org/10.3390/molecules23102478

Bystritsky, A., Khalsa, S. S., Cameron, M. E., & Schiffman, J. (2013). Current diagnosis and treatment of anxiety disorders. *P & T : a peer-reviewed journal for formulary management*, *38*(1), 30-57.

Campos, A. C., de Paula Soares, V., Carvalho, M. C., Ferreira, F. R., Vicente, M. A., Brandão, M. L., . . . Guimarães, F. S. (2013). Involvement of serotonin-mediated neurotransmission in the dorsal periaqueductal gray matter on cannabidiol chronic effects in panic-like responses in rats. *Psychopharmacology (Berl)*, *226*(1), 13-24. https://doi.org/10.1007/s00213-012-2878-7

Campos, A. C., Fogaca, M. V., Scarante, F. F., Joca, S. R. L., Sales, A. J., Gomes, F. V., . . . Guimaraes, F. S. (2017). Plastic and Neuroprotective Mechanisms Involved in the Therapeutic Effects of Cannabidiol in Psychiatric Disorders. *Front Pharmacol*, *8*, 269. https://doi.org/10.3389/fphar.2017.00269

Campos, A. C., Fogaça, M. V., Sonego, A. B., & Guimarães, F. S. (2016). Cannabidiol, neuroprotection and neuropsychiatric disorders. *Pharmacological Research*, *112*, 119-127. https://doi.org/https://doi.org/10.1016/j.phrs.2016.01.033

Campos, A. C., & Guimaraes, F. S. (2009). Evidence for a potential role for TRPV1 receptors in the dorsolateral periaqueductal gray in the attenuation of the anxiolytic effects of cannabinoids. *Prog Neuropsychopharmacol Biol Psychiatry*, *33*(8), 1517-1521. https://doi.org/10.1016/j.pnpbp.2009.08.017

Campos, A. C., & Guimarães, F. S. (2008). Involvement of 5HT1A receptors in the anxiolytic-like effects of cannabidiol injected into the dorsolateral periaqueductal gray of rats. *Psychopharmacology*, *199*(2), 223. https://doi.org/10.1007/s00213-008-1168-x

Careaga, M. B. L., Girardi, C. E. N., & Suchecki, D. (2016). Understanding posttraumatic stress disorder through fear conditioning, extinction and reconsolidation. *Neuroscience & Biobehavioral Reviews*, *71*, 48-57. https://doi.org/https://doi.org/10.1016/j.neubiorev.2016.08.023

Carvalho, A. F., & Van Bockstaele, E. J. (2012). Cannabinoid modulation of noradrenergic circuits: Implications for psychiatric disorders. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *38*(1), 59-67. https://doi.org/https://doi.org/10.1016/j.pnpbp.2012.01.008

Cassini, L. F., Flavell, C. R., Amaral, O. B., & Lee, J. L. C. (2017). On the transition from reconsolidation to extinction of contextual fear memories. *Learn Mem*, *24*(9), 392-399. https://doi.org/10.1101/lm.045724.117

Castillo, Pablo E., Younts, Thomas J., Chávez, Andrés E., & Hashimotodani, Y. (2012). Endocannabinoid Signaling and Synaptic Function. *Neuron*, *76*(1), 70-81. https://doi.org/https://doi.org/10.1016/j.neuron.2012.09.020

Chang, C.-h., Berke, J. D., & Maren, S. (2010). Single-Unit Activity in the Medial Prefrontal Cortex during Immediate and Delayed Extinction of Fear in Rats. *PLOS ONE*, *5*(8), e11971. https://doi.org/10.1371/journal.pone.0011971

Chang, C.-h., & Maren, S. (2009). Early extinction after fear conditioning yields a context-independent and short-term suppression of conditional freezing in rats. *Learn Mem*, *16*(1), 62-68. https://doi.org/10.1101/lm.1085009

Chavez, A. E., Chiu, C. Q., & Castillo, P. E. (2010). TRPV1 activation by endogenous anandamide triggers postsynaptic long-term depression in dentate gyrus. *Nat Neurosci*, *13*(12), 1511-1518. https://doi.org/10.1038/nn.2684

Chemel, B. R., Roth, B. L., Armbruster, B., Watts, V. J., & Nichols, D. E. (2006). WAY-100635 is a potent dopamine D4 receptor agonist. *Psychopharmacology*, *188*(2), 244-251. https://doi.org/10.1007/s00213-006-0490-4

Chen, F.-F., Wang, C.-M., Chen, H.-S., Wang, J., Han, Q.-Q., Cao, Y., . . . Wu, P.-F. (2020). SKF83959, an agonist of phosphatidylinositol-linked dopamine receptors, prevents renewal of extinguished conditioned fear and facilitates extinction. *Brain Research*, *1749*, 147136. https://doi.org/https://doi.org/10.1016/j.brainres.2020.147136

Chen, N. A., Ganella, D. E., Bathgate, R. A. D., Chen, A., Lawrence, A. J., & Kim, J. H. (2016). Knockdown of corticotropin-releasing factor 1 receptors in the ventral tegmental area enhances conditioned fear. *European Neuropsychopharmacology*, *26*(9), 1533-1540. https://doi.org/https://doi.org/10.1016/j.euroneuro.2016.06.002

Chen, W., Wang, Y., Wang, X., & Li, H. (2017). Neural circuits involved in the renewal of extinguished fear. *IUBMB Life*, *69*(7), 470-478. https://doi.org/10.1002/iub.1636

Cheng, D., Low, J. K., Logge, W., Garner, B., & Karl, T. (2014). Chronic cannabidiol treatment improves social and object recognition in double transgenic APPswe/PS1E9 mice. *Psychopharmacology (Berl)*, *231*(15), 3009-3017. https://doi.org/10.1007/s00213-014-3478-5

Chhatwal, J. P., Davis, M., Maguschak, K. A., & Ressler, K. J. (2005). Enhancing cannabinoid neurotransmission augments the extinction of conditioned fear. *Neuropsychopharmacology*, *30*(3), 516-524. https://doi.org/10.1038/sj.npp.1300655

Chhatwal, J. P., Gutman, A. R., Maguschak, K. A., Bowser, M. E., Yang, Y., Davis, M., & Ressler, K. J. (2009). Functional interactions between endocannabinoid and CCK neurotransmitter systems may be critical for extinction learning. *Neuropsychopharmacology*, *34*(2), 509-521. https://doi.org/10.1038/npp.2008.97

Chicca, A., Marazzi, J., Nicolussi, S., & Gertsch, J. (2012). Evidence for bidirectional endocannabinoid transport across cell membranes. *J Biol Chem*, *287*(41), 34660-34682. https://doi.org/10.1074/jbc.M112.373241

Corcoran, K. A., & Maren, S. (2004). Factors regulating the effects of hippocampal inactivation on renewal of conditional fear after extinction. *Learn Mem*, *11*(5), 598-603. https://doi.org/10.1101/lm.78704

Cortes-Altamirano, J. L., Olmos-Hernandez, A., Jaime, H. B., Carrillo-Mora, P., Bandala, C., Reyes-Long, S., & Alfaro-Rodríguez, A. (2018). Review: 5-HT1, 5-HT2, 5-HT3 and 5-HT7

Receptors and their Role in the Modulation of Pain Response in the Central Nervous System. *Curr Neuropharmacol*, *16*(2), 210-221. https://doi.org/10.2174/1570159x15666170911121027

Cota, D., Steiner, M.-A., Marsicano, G., Cervino, C., Herman, J. P., Grübler, Y., . . . Pagotto, U. (2007). Requirement of Cannabinoid Receptor Type 1 for the Basal Modulation of Hypothalamic-Pituitary-Adrenal Axis Function. *Endocrinology*, *148*(4), 1574-1581. https://doi.org/10.1210/en.2005-1649

Craske, M. G., Stein, M. B., Eley, T. C., Milad, M. R., Holmes, A., Rapee, R. M., & Wittchen, H. U. (2017). Anxiety disorders. *Nat Rev Dis Primers*, *3*, 17024. https://doi.org/10.1038/nrdp.2017.24

Craske, M. G., Treanor, M., Conway, C. C., Zbozinek, T., & Vervliet, B. (2014). Maximizing exposure therapy: An inhibitory learning approach. *Behaviour Research and Therapy*, *58*, 10-23. https://doi.org/https://doi.org/10.1016/j.brat.2014.04.006

Cruz, E., López, A. V., & Porter, J. T. (2014). Spontaneous Recovery of Fear Reverses Extinction-Induced Excitability of Infralimbic Neurons. *PLoS One*, *9*(8), e103596. https://doi.org/10.1371/journal.pone.0103596

Cumming, G., Fidler, F., & Vaux, D. L. (2007). Error bars in experimental biology. *J Cell Biol*, *177*(1), 7-11. https://doi.org/10.1083/jcb.200611141

D'Souza, D. C., Perry, E., MacDougall, L., Ammerman, Y., Cooper, T., Wu, Y. T., . . . Krystal, J. H. (2004). The psychotomimetic effects of intravenous delta-9-tetrahydrocannabinol in healthy individuals: implications for psychosis. *Neuropsychopharmacology*, *29*(8), 1558-1572. https://doi.org/10.1038/sj.npp.1300496

da Silva Soares, R., Falconi-Sobrinho, L. L., dos Anjos-Garcia, T., & Coimbra, N. C. (2019). 5-Hydroxytryptamine 2A receptors of the dorsal raphe nucleus modulate panic-like behaviours and mediate fear-induced antinociception elicited by neuronal activation in the central nucleus of the inferior colliculus. *Behavioural Brain Research*, *357-358*, 71-81. https://doi.org/https://doi.org/10.1016/j.bbr.2017.07.016

Daldrup, T., Remmes, J., Lesting, J., Gaburro, S., Fendt, M., Meuth, P., . . . Seidenbecher, T. (2015). Expression of freezing and fear-potentiated startle during sustained fear in mice. *Genes Brain Behav*, *14*(3), 281-291. https://doi.org/10.1111/gbb.12211

Danandeh, A., Vozella, V., Lim, J., Oveisi, F., Ramirez, G. L., Mears, D., . . . Piomelli, D. (2018). Effects of fatty acid amide hydrolase inhibitor URB597 in a rat model of trauma-induced long-term anxiety. *Psychopharmacology (Berl)*, *235*(11), 3211-3221. https://doi.org/10.1007/s00213-018-5020-7

Das, R. K., Kamboj, S. K., Ramadas, M., Yogan, K., Gupta, V., Redman, E., . . . Morgan, C. J. (2013). Cannabidiol enhances consolidation of explicit fear extinction in humans. *Psychopharmacology (Berl)*, *226*(4), 781-792. https://doi.org/10.1007/s00213-012-2955-y

Daskalakis, N. P., Cohen, H., Nievergelt, C. M., Baker, D. G., Buxbaum, J. D., Russo, S. J., & Yehuda, R. (2016). New translational perspectives for blood-based biomarkers of PTSD: From glucocorticoid to immune mediators of stress susceptibility. *Exp Neurol*, *284*(Pt B), 133-140. https://doi.org/10.1016/j.expneurol.2016.07.024

de Almeida, D. L., & Devi, L. A. (2020). Diversity of molecular targets and signaling pathways for CBD [https://doi.org/10.1002/prp2.682]. *Pharmacology Research & Perspectives*, *8*(6), e00682. https://doi.org/https://doi.org/10.1002/prp2.682

de Oliveira Alvares, L., Pasqualini Genro, B., Diehl, F., Molina, V. A., & Quillfeldt, J. A. (2008). Opposite action of hippocampal CB1 receptors in memory reconsolidation and extinction. *Neuroscience*, *154*(4), 1648-1655. https://doi.org/https://doi.org/10.1016/j.neuroscience.2008.05.005

De Petrocellis, L., Ligresti, A., Moriello, A. S., Allara, M., Bisogno, T., Petrosino, S., . . . Di Marzo, V. (2011). Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP

channels and endocannabinoid metabolic enzymes. *Br J Pharmacol*, *163*(7), 1479-1494. https://doi.org/10.1111/j.1476-5381.2010.01166.x

De-Miguel, F. F., & Trueta, C. (2005). Synaptic and Extrasynaptic Secretion of Serotonin. *Cellular and Molecular Neurobiology*, *25*(2), 297-312. https://doi.org/10.1007/s10571-005-3061-z

Deiana, S., Watanabe, A., Yamasaki, Y., Amada, N., Arthur, M., Fleming, S., . . . Riedel, G. (2012). Plasma and brain pharmacokinetic profile of cannabidiol (CBD), cannabidivarine (CBDV), Δ9-tetrahydrocannabivarin (THCV) and cannabigerol (CBG) in rats and mice following oral and intraperitoneal administration and CBD action on obsessive–compulsive behaviour. *Psychopharmacology*, *219*(3), 859-873. https://doi.org/10.1007/s00213-011-2415-0

Deng, H., Xiao, X., & Wang, Z. (2016). Periaqueductal Gray Neuronal Activities Underlie Different Aspects of Defensive Behaviors. *The Journal of Neuroscience*, *36*(29), 7580. https://doi.org/10.1523/JNEUROSCI.4425-15.2016

Denniston, J. C., Chang, R. C., & Miller, R. R. (2003). Massive extinction treatment attenuates the renewal effect. *Learning and Motivation*, *34*(1), 68-86. https://doi.org/https://doi.org/10.1016/S0023-9690(02)00508-8

Deutsch, D. G. (2016). A Personal Retrospective: Elevating Anandamide (AEA) by Targeting Fatty Acid Amide Hydrolase (FAAH) and the Fatty Acid Binding Proteins (FABPs) [Perspective]. *Frontiers in Pharmacology*, *7*.

Devenport, L. D. (1998). Spontaneous recovery without interference: Why remembering is adaptive. *Animal Learning & Behavior*, *26*(2), 172-181. https://doi.org/10.3758/BF03199210

Devinsky, O., Cross, J. H., Laux, L., Marsh, E., Miller, I., Nabbout, R., . . . Wright, S. (2017). Trial of Cannabidiol for Drug-Resistant Seizures in the Dravet Syndrome. *N Engl J Med*, *376*(21), 2011-2020. https://doi.org/10.1056/NEJMoa1611618

Di Marzo, V. (2009). The endocannabinoid system: Its general strategy of action, tools for its pharmacological manipulation and potential therapeutic exploitation. *Pharmacological Research*, *60*(2), 77-84. https://doi.org/https://doi.org/10.1016/j.phrs.2009.02.010

Di Marzo, V., & De Petrocellis, L. (2012). Why do cannabinoid receptors have more than one endogenous ligand? *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *367*(1607), 3216-3228. https://doi.org/10.1098/rstb.2011.0382

Do Monte, F. H., Souza, R. R., Bitencourt, R. M., Kroon, J. A., & Takahashi, R. N. (2013). Infusion of cannabidiol into infralimbic cortex facilitates fear extinction via CB1 receptors. *Behav Brain Res*, *250*, 23-27. https://doi.org/10.1016/j.bbr.2013.04.045

Dong, J., de Montigny, C., & Blier, P. (1998). Full Agonistic Properties of Bay x 3702 on Presynaptic and Postsynaptic 5-HT_{1A} Receptors Electrophysiological Studies in the Rat Hippocampus and Dorsal Raphe. *Journal of Pharmacology and Experimental Therapeutics*, *286*(3), 1239.

Duan, T., Gu, N., Wang, Y., Wang, F., Zhu, J., Fang, Y., . . . Zhang, X. (2017). Fatty acid amide hydrolase inhibitors produce rapid anti-anxiety responses through amygdala long-term depression in male rodents. *J Psychiatry Neurosci*, *42*(4), 230-241.

Dunsmoor, J. E., Kroes, M. C. W., Moscatelli, C. M., Evans, M. D., Davachi, L., & Phelps, E. A. (2018). Event segmentation protects emotional memories from competing experiences encoded close in time. *Nature Human Behaviour*, *2*(4), 291-299. https://doi.org/10.1038/s41562-018-0317-4

Dunsmoor, J. E., & Paz, R. (2015). Fear Generalization and Anxiety: Behavioral and Neural Mechanisms. *Biol Psychiatry*, *78*(5), 336-343. https://doi.org/10.1016/j.biopsych.2015.04.010

Duran, J. M., Sierra, R. O., Corredor, K., & Cardenas, F. P. (2022). Cathodal transcranial direct current stimulation on the prefrontal cortex applied after reactivation attenuates fear memories and prevent reinstatement after extinction. *Journal of Psychiatric Research*, *145*, 213-221. https://doi.org/https://doi.org/10.1016/j.jpsychires.2021.12.025

Egertová, M., M Simon, G., F Cravatt, B., & Elphick, M. (2008). *Localization ofN-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) expression in mouse brain: A new perspective onN-acylethanolamines as neural signaling molecules* (Vol. 506). https://doi.org/10.1002/cne.21568

ElBatsh, M. M., Assareh, N., Marsden, C. A., & Kendall, D. A. (2012). Anxiogenic-like effects of chronic cannabidiol administration in rats. *Psychopharmacology (Berl)*, *221*(2), 239-247. https://doi.org/10.1007/s00213-011-2566-z

Elmes, M. W., Kaczocha, M., Berger, W. T., Leung, K., Ralph, B. P., Wang, L., . . . Deutsch, D. G. (2015). Fatty acid-binding proteins (FABPs) are intracellular carriers for Delta9-tetrahydrocannabinol (THC) and cannabidiol (CBD). *J Biol Chem*, *290*(14), 8711-8721. https://doi.org/10.1074/jbc.M114.618447

Elms, L., Shannon, S., Hughes, S., & Lewis, N. (2019). Cannabidiol in the Treatment of Post-Traumatic Stress Disorder: A Case Series. *J Altern Complement Med*, *25*(4), 392-397. https://doi.org/10.1089/acm.2018.0437

Ericsson, A. C., Hart, M. L., Kwan, J., Lanoue, L., Bower, L. R., Araiza, R., . . . Franklin, C. L. (2021). Supplier-origin mouse microbiomes significantly influence locomotor and anxiety-related behavior, body morphology, and metabolism. *Communications Biology*, *4*(1), 716. https://doi.org/10.1038/s42003-021-02249-0

Esposito, G., Scuderi, C., Valenza, M., Togna, G. I., Latina, V., De Filippis, D., . . . Steardo, L. (2011). Cannabidiol reduces Aβ-induced neuroinflammation and promotes hippocampal neurogenesis through PPARγ involvement. *PLoS One*, *6*(12), e28668. https://doi.org/10.1371/journal.pone.0028668

Faber, E. S. (2010). Functional interplay between NMDA receptors, SK channels and voltage-gated Ca2+ channels regulates synaptic excitability in the medial prefrontal cortex. *J Physiol*, *588*(Pt 8), 1281-1292. https://doi.org/10.1113/jphysiol.2009.185645

Fanselow, M. S. (1994). Neural organization of the defensive behavior system responsible for fear. *Psychon Bull Rev*, *1*(4), 429-438. https://doi.org/10.3758/bf03210947

Fenton, G. E., Halliday, D. M., Mason, R., Bredy, T. W., & Stevenson, C. W. (2016). Sex differences in learned fear expression and extinction involve altered gamma oscillations in medial prefrontal cortex. *Neurobiol Learn Mem*, *135*, 66-72. https://doi.org/https://doi.org/10.1016/j.nlm.2016.06.019

Fenton, G. E., Pollard, A. K., Halliday, D. M., Mason, R., Bredy, T. W., & Stevenson, C. W. (2014). Persistent prelimbic cortex activity contributes to enhanced learned fear expression in females. *Learn Mem*, *21*(2), 55-60. https://doi.org/10.1101/lm.033514.113

Fidelman, S., Mizrachi Zer-Aviv, T., Lange, R., Hillard, C. J., & Akirav, I. (2018). Chronic treatment with URB597 ameliorates post-stress symptoms in a rat model of PTSD. *European Neuropsychopharmacology*, *28*(5), 630-642. https://doi.org/https://doi.org/10.1016/j.euroneuro.2018.02.004

Fitzgerald, P. J., Giustino, T. F., Seemann, J. R., & Maren, S. (2015). Noradrenergic blockade stabilizes prefrontal activity and enables fear extinction under stress. *Proc Natl Acad Sci U S A*, *112*(28), E3729-3737. https://doi.org/10.1073/pnas.1500682112

Fletcher, A., Forster, E. A., Bill, D. J., Brown, G., Cliffe, I. A., Hartley, J. E., . . . Dourish, C. T. (1996). Electrophysiological, biochemical, neurohormonal and behavioural studies with WAY-100635, a potent, selective and silent 5-HT1A receptor antagonist. *Behav Brain Res*, *73*(1-2), 337-353. https://doi.org/10.1016/0166-4328(96)00118-0

Fogaca, M. V., Campos, A. C., Coelho, L. D., Duman, R. S., & Guimaraes, F. S. (2018). The anxiolytic effects of cannabidiol in chronically stressed mice are mediated by the

endocannabinoid system: Role of neurogenesis and dendritic remodeling. *Neuropharmacology*, *135*, 22-33. https://doi.org/10.1016/j.neuropharm.2018.03.001

Fogaca, M. V., Reis, F. M., Campos, A. C., & Guimaraes, F. S. (2014). Effects of intra-prelimbic prefrontal cortex injection of cannabidiol on anxiety-like behavior: involvement of 5HT1A receptors and previous stressful experience. *Eur Neuropsychopharmacol*, *24*(3), 410-419. https://doi.org/10.1016/j.euroneuro.2013.10.012

Fraguas-Sánchez, A. I., & Torres-Suárez, A. I. (2018). Medical Use of Cannabinoids [journal article]. *Drugs*, *78*(16), 1665-1703. https://doi.org/10.1007/s40265-018-0996-1

Franzen, J. M., Vanz, F., Werle, I., Guimarães, F. S., & Bertoglio, L. J. (2022). Cannabidiol impairs fear memory reconsolidation in female rats through dorsal hippocampus CB1 but not CB2 receptor interaction. *European Neuropsychopharmacology*, *64*, 7-18. https://doi.org/https://doi.org/10.1016/j.euroneuro.2022.08.002

Friedman, M. J., Resick, P. A., Bryant, R. A., Strain, J., Horowitz, M., & Spiegel, D. (2011). Classification of trauma and stressor-related disorders in DSM-5. *Depress Anxiety*, *28*(9), 737-749. https://doi.org/10.1002/da.20845

Fuchs, E., Fl, #xfc, & gge, G. (2014). Adult Neuroplasticity: More Than 40 Years of Research. *Neural Plasticity*, *2014*, 10, Article 541870. https://doi.org/10.1155/2014/541870

Furini, C., Myskiw, J., & Izquierdo, I. (2014). The learning of fear extinction. *Neuroscience & Biobehavioral Reviews*, *47*, 670-683. https://doi.org/https://doi.org/10.1016/j.neubiorev.2014.10.016

Gallo, F. T., Katche, C., Morici, J. F., Medina, J. H., & Weisstaub, N. V. (2018). Immediate Early Genes, Memory and Psychiatric Disorders: Focus on c-Fos, Egr1 and Arc. *Front Behav Neurosci*, *12*, 79. https://doi.org/10.3389/fnbeh.2018.00079

Gamble-George, J. C., Baldi, R., Halladay, L., Kocharian, A., Hartley, N., Silva, C. G., . . . Patel, S. (2016). Cyclooxygenase-2 inhibition reduces stress-induced affective pathology. *Elife*, *5*. https://doi.org/10.7554/eLife.14137

Ganon-Elazar, E., & Akirav, I. (2013). Cannabinoids and traumatic stress modulation of contextual fear extinction and GR expression in the amygdala-hippocampal-prefrontal circuit. *Psychoneuroendocrinology*, *38*(9), 1675-1687. https://doi.org/https://doi.org/10.1016/j.psyneuen.2013.01.014

Garza, K., & Jovanovic, T. (2017). Impact of Gender on Child and Adolescent PTSD. *Current Psychiatry Reports*, *19*(11), 87. https://doi.org/10.1007/s11920-017-0830-6

Gazarini, L., Stern, C. A., Piornedo, R. R., Takahashi, R. N., & Bertoglio, L. J. (2014). PTSD-like memory generated through enhanced noradrenergic activity is mitigated by a dual step pharmacological intervention targeting its reconsolidation. *Int J Neuropsychopharmacol*, *18*(1). https://doi.org/10.1093/ijnp/pyu026

Gazarini, L., Stern, C. A., Takahashi, R. N., & Bertoglio, L. J. (2022). Interactions of Noradrenergic, Glucocorticoid and Endocannabinoid Systems Intensify and Generalize Fear Memory Traces. *Neuroscience*, *497*, 118-133. https://doi.org/10.1016/j.neuroscience.2021.09.012

Geddes, S. D., Assadzada, S., Lemelin, D., Sokolovski, A., Bergeron, R., Haj-Dahmane, S., & Beique, J. C. (2016). Target-specific modulation of the descending prefrontal cortex inputs to the dorsal raphe nucleus by cannabinoids. *Proc Natl Acad Sci U S A*, *113*(19), 5429-5434. https://doi.org/10.1073/pnas.1522754113

Giustino, T. F., & Maren, S. (2018). Noradrenergic Modulation of Fear Conditioning and Extinction [10.3389/fnbeh.2018.00043]. *Frontiers in Behavioral Neuroscience*, *12*, 43.

Giustino, T. F., Ramanathan, K. R., Totty, M. S., Miles, O. W., & Maren, S. (2020). Locus Coeruleus Norepinephrine Drives Stress-Induced Increases in Basolateral Amygdala Firing and Impairs Extinction Learning. *J Neurosci*, *40*(4), 907-916. https://doi.org/10.1523/jneurosci.1092-19.2019

Giustino, T. F., Seemann, J. R., Acca, G. M., Goode, T. D., Fitzgerald, P. J., & Maren, S. (2017). β-Adrenoceptor Blockade in the Basolateral Amygdala, But Not the Medial Prefrontal Cortex, Rescues the Immediate Extinction Deficit. *Neuropsychopharmacology*, *42*(13), 2537-2544. https://doi.org/10.1038/npp.2017.89

Gobbi, G., Bambico, F. R., Mangieri, R., Bortolato, M., Campolongo, P., Solinas, M., . . . Piomelli, D. (2005). Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(51), 18620. https://doi.org/10.1073/pnas.0509591102

Gobira, P. H., Lima, I. V., Batista, L. A., de Oliveira, A. C., Resstel, L. B., Wotjak, C. T., . . . Moreira, F. A. (2017). N-arachidonoyl-serotonin, a dual FAAH and TRPV1 blocker, inhibits the retrieval of contextual fear memory: Role of the cannabinoid CB1 receptor in the dorsal hippocampus. *J Psychopharmacol*, *31*(6), 750-756. https://doi.org/10.1177/0269881117691567

Goldstein Ferber, S., Trezza, V., & Weller, A. (2021). Early life stress and development of the endocannabinoid system: A bidirectional process in programming future coping. *Dev Psychobiol*, *63*(2), 143-152. https://doi.org/10.1002/dev.21944

Goldstein, R. B., Smith, S. M., Chou, S. P., Saha, T. D., Jung, J., Zhang, H., . . . Grant, B. F. (2016). The epidemiology of DSM-5 posttraumatic stress disorder in the United States: results from the National Epidemiologic Survey on Alcohol and Related Conditions-III [journal article]. *Social Psychiatry and Psychiatric Epidemiology*, *51*(8), 1137-1148. https://doi.org/10.1007/s00127-016-1208-5

Gomes, F. V., Reis, D. G., Alves, F. H., Correa, F. M., Guimaraes, F. S., & Resstel, L. B. (2012). Cannabidiol injected into the bed nucleus of the stria terminalis reduces the expression of contextual fear conditioning via 5-HT1A receptors. *J Psychopharmacol*, *26*(1), 104-113. https://doi.org/10.1177/0269881110389095

Gomes, F. V., Resstel, L. B., & Guimaraes, F. S. (2011). The anxiolytic-like effects of cannabidiol injected into the bed nucleus of the stria terminalis are mediated by 5-HT1A receptors. *Psychopharmacology (Berl)*, *213*(2-3), 465-473. https://doi.org/10.1007/s00213-010-2036-z

Goode, T. D., Kim, J. J., & Maren, S. (2015). Reversible Inactivation of the Bed Nucleus of the Stria Terminalis Prevents Reinstatement But Not Renewal of Extinguished Fear. *eneuro*, *2*(3), ENEURO.0037-0015.2015. https://doi.org/10.1523/ENEURO.0037-15.2015

Goode, T. D., & Maren, S. (2014). Animal models of fear relapse. *ILAR journal*, *55*(2), 246-258. https://doi.org/10.1093/ilar/ilu008

Goode, T. D., & Maren, S. (2019). Common neurocircuitry mediating drug and fear relapse in preclinical models. *Psychopharmacology (Berl)*, *236*(1), 415-437. https://doi.org/10.1007/s00213-018-5024-3

Goonawardena, A. V., Sesay, J., Sexton, C. A., Riedel, G., & Hampson, R. E. (2011). Pharmacological elevation of anandamide impairs short-term memory by altering the neurophysiology in the hippocampus. *Neuropharmacology*, *61*(5), 1016-1025. https://doi.org/https://doi.org/10.1016/j.neuropharm.2011.07.003

Gowran, A., Noonan, J., & Campbell, V. A. (2011). The Multiplicity of Action of Cannabinoids: Implications for Treating Neurodegeneration. *CNS Neuroscience & Therapeutics*, *17*(6), 637-644. https://doi.org/doi:10.1111/j.1755-5949.2010.00195.x

Gray, J. A. M., N. (2000). *The Neuropsychology of Anxiety: An Enquiry into the Functions of the Septo-Hippocampal System* (Second ed.). Oxford University Press.

Gray, J. M., Vecchiarelli, H. A., Morena, M., Lee, T. T., Hermanson, D. J., Kim, A. B., . . . Hill, M. N. (2015). Corticotropin-releasing hormone drives anandamide hydrolysis in the

amygdala to promote anxiety. *J Neurosci*, *35*(9), 3879-3892. https://doi.org/10.1523/jneurosci.2737-14.2015

Greer, G. R., Grob, C. S., & Halberstadt, A. L. (2014). PTSD symptom reports of patients evaluated for the New Mexico Medical Cannabis Program. *J Psychoactive Drugs*, *46*(1), 73-77. https://doi.org/10.1080/02791072.2013.873843

Greville, W. J., Newton, P. M., Roche, B., & Dymond, S. (2013). Conditioned suppression in a virtual environment. *Computers in Human Behavior*, *29*(3), 552-558. https://doi.org/https://doi.org/10.1016/j.chb.2012.11.016

Groenink, L., Joordens, R. J. E., Hijzen, T. H., Dirks, A., & Olivier, B. (2000). Infusion of flesinoxan into the amygdala blocks the fear-potentiated startle. *NeuroReport*, *11*(10).

Gross, C. T., & Canteras, N. S. (2012). The many paths to fear [Perspective]. *Nature Reviews Neuroscience*, *13*, 651. https://doi.org/10.1038/nrn3301

Gruene, T. M., Flick, K., Stefano, A., Shea, S. D., & Shansky, R. M. (2015). Sexually divergent expression of active and passive conditioned fear responses in rats. *Elife*, *4*. https://doi.org/10.7554/eLife.11352

Guina, J., Rossetter, S. R., De, R. B., Nahhas, R. W., & Welton, R. S. (2015). Benzodiazepines for PTSD: A Systematic Review and Meta-Analysis. *J Psychiatr Pract*, *21*(4), 281-303. https://doi.org/10.1097/pra.0000000000000091

Gulyas, A. I., Cravatt, B. F., Bracey, M. H., Dinh, T. P., Piomelli, D., Boscia, F., & Freund, T. F. (2004). Segregation of two endocannabinoid-hydrolyzing enzymes into pre- and postsynaptic compartments in the rat hippocampus, cerebellum and amygdala. *Eur J Neurosci*, *20*(2), 441-458. https://doi.org/10.1111/j.1460-9568.2004.03428.x

Gunduz-Cinar, O., MacPherson, K. P., Cinar, R., Gamble-George, J., Sugden, K., Williams, B., . . . Holmes, A. (2013). Convergent translational evidence of a role for anandamide in amygdala-mediated fear extinction, threat processing and stress-reactivity. *Mol Psychiatry*, *18*(7), 813-823. https://doi.org/10.1038/mp.2012.72

Haj-Dahmane, S., & Shen, R.-Y. (2011). Modulation of the serotonin system by endocannabinoid signaling. *Neuropharmacology*, *61*(3), 414-420. https://doi.org/https://doi.org/10.1016/j.neuropharm.2011.02.016

Hale, M. W., & Lowry, C. A. (2011). Functional topography of midbrain and pontine serotonergic systems: implications for synaptic regulation of serotonergic circuits. *Psychopharmacology*, *213*(2), 243-264. https://doi.org/10.1007/s00213-010-2089-z

Haller, J., Barna, I., Barsvari, B., Gyimesi Pelczer, K., Yasar, S., Panlilio, L. V., & Goldberg, S. (2009). Interactions between environmental aversiveness and the anxiolytic effects of enhanced cannabinoid signaling by FAAH inhibition in rats. *Psychopharmacology*, *204*(4), 607-616. https://doi.org/10.1007/s00213-009-1494-7

Harrell, A. V., & Allan, A. M. (2003). Improvements in hippocampal-dependent learning and decremental attention in 5-HT(3) receptor overexpressing mice. *Learn Mem*, *10*(5), 410-419. https://doi.org/10.1101/lm.56103

Hartmann, A., Lisboa, S. F., Sonego, A. B., Coutinho, D., Gomes, F. V., & Guimarães, F. S. (2019). Cannabidiol attenuates aggressive behavior induced by social isolation in mice: Involvement of 5-HT1A and CB1 receptors. *Prog Neuropsychopharmacol Biol Psychiatry*, *94*, 109637. https://doi.org/10.1016/j.pnpbp.2019.109637

Haubrich, J., Crestani, A. P., Cassini, L. F., Santana, F., Sierra, R. O., Alvares, L. d. O., & Quillfeldt, J. A. (2015). Reconsolidation allows fear memory to be updated to a less aversive level through the incorporation of appetitive information. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *40*(2), 315-326. https://doi.org/10.1038/npp.2014.174

Haubrich, J., Machado, A., Boos, F. Z., Crestani, A. P., Sierra, R. O., Alvares, L. d. O., & Quillfeldt, J. A. (2017). Enhancement of extinction memory by pharmacological and behavioral

interventions targeted to its reactivation. *Sci Rep*, *7*(1), 10960. https://doi.org/10.1038/s41598-017-11261-6

Hendriks, S. M., Spijker, J., Licht, C. M. M., Hardeveld, F., de Graaf, R., Batelaan, N. M., . . . Beekman, A. T. F. (2016). Long-term disability in anxiety disorders [journal article]. *BMC Psychiatry*, *16*(1), 248. https://doi.org/10.1186/s12888-016-0946-y

Hensler, J. G. (2006). Serotonergic modulation of the limbic system. *Neuroscience & Biobehavioral Reviews*, *30*(2), 203-214. https://doi.org/https://doi.org/10.1016/j.neubiorev.2005.06.007

Hermann, A., Stark, R., Müller, E. A., Kruse, O., Wolf, O. T., & Merz, C. J. (2020). Multiple extinction contexts modulate the neural correlates of context-dependent extinction learning and retrieval. *Neurobiology of Learning and Memory*, *168*, 107150. https://doi.org/https://doi.org/10.1016/j.nlm.2019.107150

Hermanson, D. J., Hartley, N. D., Gamble-George, J., Brown, N., Shonesy, B. C., Kingsley, P. J., . . . Patel, S. (2013). Substrate-selective COX-2 inhibition decreases anxiety via endocannabinoid activation. *Nat Neurosci*, *16*(9), 1291-1298. https://doi.org/10.1038/nn.3480

Herry, C., Ferraguti, F., Singewald, N., Letzkus, J. J., Ehrlich, I., & Lüthi, A. (2010). Neuronal circuits of fear extinction [https://doi.org/10.1111/j.1460-9568.2010.07101.x]. *European Journal of Neuroscience*, *31*(4), 599-612. https://doi.org/https://doi.org/10.1111/j.1460-9568.2010.07101.x

Hetzel, A., & Rosenkranz, J. A. (2014). Distinct effects of repeated restraint stress on basolateral amygdala neuronal membrane properties in resilient adolescent and adult rats. *Neuropsychopharmacology*, *39*(9), 2114-2130. https://doi.org/10.1038/npp.2014.60

Hill, M. N., Bierer, L. M., Makotkine, I., Golier, J. A., Galea, S., McEwen, B. S., . . . Yehuda, R. (2013). Reductions in circulating endocannabinoid levels in individuals with post-traumatic stress disorder following exposure to the World Trade Center attacks. *Psychoneuroendocrinology*, *38*(12), 2952-2961. https://doi.org/10.1016/j.psyneuen.2013.08.004

Hill, M. N., Patel, S., Campolongo, P., Tasker, J. G., Wotjak, C. T., & Bains, J. S. (2010). Functional interactions between stress and the endocannabinoid system: from synaptic signaling to behavioral output. *J Neurosci*, *30*(45), 14980-14986. https://doi.org/10.1523/jneurosci.4283-10.2010

Hitora-Imamura, N., Miura, Y., Teshirogi, C., Ikegaya, Y., Matsuki, N., & Nomura, H. (2015). Prefrontal dopamine regulates fear reinstatement through the downregulation of extinction circuits. *Elife*, *4*. https://doi.org/10.7554/eLife.08274

Hoffman, K. L. (2016). 3 - Modeling disorders of fear and anxiety in animals. In K. L. Hoffman (Ed.), *Modeling Neuropsychiatric Disorders in Laboratory Animals* (pp. 87-160). Woodhead Publishing. https://doi.org/https://doi.org/10.1016/B978-0-08-100099-1.00003-0

Hollis, F., Sevelinges, Y., Grosse, J., Zanoletti, O., & Sandi, C. (2016). Involvement of CRFR₁ in the Basolateral Amygdala in the Immediate Fear Extinction Deficit. *eneuro*, *3*(5), ENEURO.0084-0016.2016. https://doi.org/10.1523/ENEURO.0084-16.2016

Homberg, J. R. (2012). Serotonergic Modulation of Conditioned Fear. *Scientifica*, *2012*, 821549. https://doi.org/10.6064/2012/821549

Hori, H., Itoh, M., Yoshida, F., Lin, M., Niwa, M., Hakamata, Y., . . . Kim, Y. (2020). The BDNF Val66Met polymorphism affects negative memory bias in civilian women with PTSD. *Scientific Reports*, *10*(1), 3151. https://doi.org/10.1038/s41598-020-60096-1

Howlett, A. C., Barth, F., Bonner, T. I., Cabral, G., Casellas, P., Devane, W. A., . . . Pertwee, R. G. (2002). International Union of Pharmacology. XXVII. Classification of Cannabinoid Receptors. *Pharmacological Reviews*, *54*(2), 161. https://doi.org/10.1124/pr.54.2.161

Hugues, S., Garcia, R., & Léna, I. (2007). Time course of extracellular catecholamine and glutamate levels in the rat medial prefrontal cortex during and after extinction of conditioned fear. *Synapse*, *61*(11), 933-937. https://doi.org/10.1002/syn.20448

Iannotti, F. A., Hill, C. L., Leo, A., Alhusaini, A., Soubrane, C., Mazzarella, E., . . . Stephens, G. J. (2014). Nonpsychotropic plant cannabinoids, cannabidivarin (CBDV) and cannabidiol (CBD), activate and desensitize transient receptor potential vanilloid 1 (TRPV1) channels in vitro: potential for the treatment of neuronal hyperexcitability. *ACS Chem Neurosci*, *5*(11), 1131-1141. https://doi.org/10.1021/cn5000524

Iffland, K., & Grotenhermen, F. (2017). An Update on Safety and Side Effects of Cannabidiol: A Review of Clinical Data and Relevant Animal Studies. *Cannabis Cannabinoid Res*, *2*(1), 139-154. https://doi.org/10.1089/can.2016.0034

Jacob, W., Marsch, R., Marsicano, G., Lutz, B., & Wotjak, C. T. (2012). Cannabinoid CB1 receptor deficiency increases contextual fear memory under highly aversive conditions and long-term potentiation in vivo. *Neurobiology of Learning and Memory*, *98*(1), 47-55. https://doi.org/https://doi.org/10.1016/j.nlm.2012.04.008

Jadoon, K. A., Ratcliffe, S. H., Barrett, D. A., Thomas, E. L., Stott, C., Bell, J. D., . . . Tan, G. D. (2016). Efficacy and Safety of Cannabidiol and Tetrahydrocannabivarin on Glycemic and Lipid Parameters in Patients With Type 2 Diabetes: A Randomized, Double-Blind, Placebo-Controlled, Parallel Group Pilot Study. *Diabetes Care*, *39*(10), 1777-1786. https://doi.org/10.2337/dc16-0650

Jetly, R., Heber, A., Fraser, G., & Boisvert, D. (2015). The efficacy of nabilone, a synthetic cannabinoid, in the treatment of PTSD-associated nightmares: A preliminary randomized, double-blind, placebo-controlled cross-over design study. *Psychoneuroendocrinology*, *51*, 585-588. https://doi.org/https://doi.org/10.1016/j.psyneuen.2014.11.002

Jo, Y. S., Namboodiri, V. M. K., Stuber, G. D., & Zweifel, L. S. (2020). Persistent activation of central amygdala CRF neurons helps drive the immediate fear extinction deficit. *Nature Communications*, *11*(1), 422. https://doi.org/10.1038/s41467-020-14393-y

Johansen, J. P., Cain, C. K., Ostroff, L. E., & LeDoux, J. E. (2011). Molecular mechanisms of fear learning and memory. *Cell*, *147*(3), 509-524. https://doi.org/10.1016/j.cell.2011.10.009

Joo, B., Koo, J. W., & Lee, S. (2020). Posterior parietal cortex mediates fear renewal in a novel context. *Molecular Brain*, *13*(1), 16. https://doi.org/10.1186/s13041-020-0556-y

Jurkus, R., Day, H. L. L., Guimarães, F. S., Lee, J. L. C., Bertoglio, L. J., & Stevenson, C. W. (2016). Cannabidiol Regulation of Learned Fear: Implications for Treating Anxiety-Related Disorders. *Frontiers in pharmacology*, *7*, 454-454. https://doi.org/10.3389/fphar.2016.00454

Kakui, N., Yokoyama, F., Yamauchi, M., Kitamura, K., Imanishi, T., Inoue, T., & Koyama, T. (2009). Anxiolytic-like profile of mirtazapine in rat conditioned fear stress model: Functional significance of 5-hydroxytryptamine 1A receptor and α1-adrenergic receptor. *Pharmacology Biochemistry and Behavior*, *92*(3), 393-398. https://doi.org/https://doi.org/10.1016/j.pbb.2008.12.022

Kaplan, G. B., & Moore, K. A. (2011). The use of cognitive enhancers in animal models of fear extinction. *Pharmacology Biochemistry and Behavior*, *99*(2), 217-228. https://doi.org/https://doi.org/10.1016/j.pbb.2011.01.009

Karpova, N. N., Pickenhagen, A., Lindholm, J., Tiraboschi, E., Kulesskaya, N., Agústsdóttir, A., . . . Castrén, E. (2011). Fear erasure in mice requires synergy between antidepressant

drugs and extinction training. *Science*, *334*(6063), 1731-1734. https://doi.org/10.1126/science.1214592

Karrer, T. M., McLaughlin, C. L., Guaglianone, C. P., & Samanez-Larkin, G. R. (2019). Reduced serotonin receptors and transporters in normal aging adults: a meta-analysis of PET and SPECT imaging studies. *Neurobiol Aging*, *80*, 1-10. https://doi.org/10.1016/j.neurobiolaging.2019.03.021

Katzung, B. G., Trevor, A. J. (2015). *Basic & Clinical Pharmacology* (Vol. 13th Edition). Mc Graw Hill Education.

Kerstin, I., & Franjo, G. (2017). An Update on Safety and Side Effects of Cannabidiol: A Review of Clinical Data and Relevant Animal Studies. *Cannabis and Cannabinoid Research*, *2*(1), 139-154. https://doi.org/10.1089/can.2016.0034

Kessler, R. C., Aguilar-Gaxiola, S., Alonso, J., Benjet, C., Bromet, E. J., Cardoso, G., . . . Koenen, K. C. (2017). Trauma and PTSD in the WHO World Mental Health Surveys. *European Journal of Psychotraumatology*, *8*(sup5), 1353383. https://doi.org/10.1080/20008198.2017.1353383

Khoo, S. Y., Sciascia, J. M., Brown, A., & Chaudhri, N. (2020). Comparing ABA, AAB, and ABC Renewal of Appetitive Pavlovian Conditioned Responding in Alcohol- and Sucrose-Trained Male Rats. *Front Behav Neurosci*, *14*, 5. https://doi.org/10.3389/fnbeh.2020.00005

Kim, J. J., & Jung, M. W. (2006). Neural circuits and mechanisms involved in Pavlovian fear conditioning: A critical review. *Neuroscience & Biobehavioral Reviews*, *30*(2), 188-202. https://doi.org/https://doi.org/10.1016/j.neubiorev.2005.06.005

Kim, S. C., Jo, Y. S., Kim, I. H., Kim, H., & Choi, J.-S. (2010). Lack of Medial Prefrontal Cortex Activation Underlies the Immediate Extinction Deficit. *The Journal of Neuroscience*, *30*(3), 832. https://doi.org/10.1523/JNEUROSCI.4145-09.2010

Kim, W. B., & Cho, J.-H. (2020). Encoding of contextual fear memory in hippocampal–amygdala circuit. *Nature Communications*, *11*(1), 1382. https://doi.org/10.1038/s41467-020-15121-2

King, G., Graham, B. M., & Richardson, R. (2018a). Individual differences in fear relapse. *Behaviour Research and Therapy*, *100*, 37-43. https://doi.org/https://doi.org/10.1016/j.brat.2017.11.003

King, G., Graham, B. M., & Richardson, R. (2018b). Effects of d-cycloserine on individual differences in relapse of fear. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *84*, 115-121. https://doi.org/https://doi.org/10.1016/j.pnpbp.2018.02.005

Kinner, V. L., Merz, C. J., Lissek, S., & Wolf, O. T. (2016). Cortisol disrupts the neural correlates of extinction recall. *NeuroImage*, *133*, 233-243. https://doi.org/https://doi.org/10.1016/j.neuroimage.2016.03.005

Knapska, E., Macias, M., Mikosz, M., Nowak, A., Owczarek, D., Wawrzyniak, M., . . . Kaczmarek, L. (2012). Functional anatomy of neural circuits regulating fear and extinction. *Proc Natl Acad Sci U S A*, *109*(42), 17093-17098. https://doi.org/10.1073/pnas.1202087109

Knapska, E., & Maren, S. (2009). Reciprocal patterns of c-Fos expression in the medial prefrontal cortex and amygdala after extinction and renewal of conditioned fear. *Learn Mem*, *16*(8), 486-493. https://doi.org/10.1101/lm.1463909

Korem, N., Zer-Aviv, T. M., Ganon-Elazar, E., Abush, H., & Akirav, I. (2016). Targeting the endocannabinoid system to treat anxiety-related disorders. *J Basic Clin Physiol Pharmacol*, *27*(3), 193-202. https://doi.org/10.1515/jbcpp-2015-0058

Korosi, A., & Baram, T. Z. (2008). The central corticotropin releasing factor system during development and adulthood. *European Journal of Pharmacology*, *583*(2), 204-214. https://doi.org/https://doi.org/10.1016/j.ejphar.2007.11.066

Krystal, J. H., & Neumeister, A. (2009). Noradrenergic and serotonergic mechanisms in the neurobiology of posttraumatic stress disorder and resilience. *Brain Research*, *1293*, 13-23. https://doi.org/https://doi.org/10.1016/j.brainres.2009.03.044

Kuhnert, S., Meyer, C., & Koch, M. (2013). Involvement of cannabinoid receptors in the amygdala and prefrontal cortex of rats in fear learning, consolidation, retrieval and extinction. *Behav Brain Res*, *250*, 274-284. https://doi.org/10.1016/j.bbr.2013.05.002

Kutlu, M. G., Tumolo, J. M., Cann, C., & Gould, T. J. (2018). Differential effects of α4β2 nicotinic receptor antagonists and partial-agonists on contextual fear extinction in male C57BL/6 mice. *Psychopharmacology (Berl)*, *235*(4), 1211-1219. https://doi.org/10.1007/s00213-018-4837-4

Kutlu, M. G., Tumolo, J. M., Holliday, E., Garrett, B., & Gould, T. J. (2016). Acute nicotine enhances spontaneous recovery of contextual fear and changes c-fos early gene expression in infralimbic cortex, hippocampus, and amygdala. *Learn Mem*, *23*(8), 405-414. https://doi.org/10.1101/lm.042655.116

Kwee, C. M. B., Baas, J. M. P., van der Flier, F. E., Groenink, L., Duits, P., Eikelenboom, M., . . . Cath, D. C. (2022). Cannabidiol enhancement of exposure therapy in treatment refractory patients with social anxiety disorder and panic disorder with agoraphobia: A randomised controlled trial. *European Neuropsychopharmacology*, *59*, 58-67. https://doi.org/https://doi.org/10.1016/j.euroneuro.2022.04.003

Kwok, C. H., Devonshire, I. M., Imraish, A., Greenspon, C. M., Lockwood, S., Fielden, C., . . . Hathway, G. J. (2017). Age-dependent plasticity in endocannabinoid modulation of pain processing through postnatal development. *Pain*, *158*(11), 2222-2232. https://doi.org/10.1097/j.pain.0000000000001027

Laborda, M. A., & Miller, R. R. (2013). Preventing Return of Fear in an Animal Model of Anxiety: Additive Effects of Massive Extinction and Extinction in Multiple Contexts. *Behavior Therapy*, *44*(2), 249-261. https://doi.org/https://doi.org/10.1016/j.beth.2012.11.001

Laprairie, R. B., Bagher, A. M., Kelly, M. E., & Denovan-Wright, E. M. (2015). Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. *Br J Pharmacol*, *172*(20), 4790-4805. https://doi.org/10.1111/bph.13250

Laricchiuta, D., Centonze, D., & Petrosini, L. (2013). Effects of endocannabinoid and endovanilloid systems on aversive memory extinction. *Behav Brain Res*, *256*, 101-107. https://doi.org/10.1016/j.bbr.2013.08.010

Laurent, V., & Westbrook, R. F. (2010). Role of the basolateral amygdala in the reinstatement and extinction of fear responses to a previously extinguished conditioned stimulus. *Learning & Memory*, *17*(2), 86-96. https://doi.org/10.1101/lm.1655010

Lazary, J., Eszlari, N., Juhasz, G., & Bagdy, G. (2016). Genetically reduced FAAH activity may be a risk for the development of anxiety and depression in persons with repetitive childhood trauma. *Eur Neuropsychopharmacol*, *26*(6), 1020-1028. https://doi.org/10.1016/j.euroneuro.2016.03.003

Lee, J. L. C., Milton, A. L., & Everitt, B. J. (2006). Reconsolidation and Extinction of Conditioned Fear: Inhibition and Potentiation. *The Journal of Neuroscience*, *26*(39), 10051-10056. https://doi.org/10.1523/jneurosci.2466-06.2006

Lee, S., & Lee, D. K. (2018). What is the proper way to apply the multiple comparison test? *Korean J Anesthesiol*, *71*(5), 353-360. https://doi.org/10.4097/kja.d.18.00242

Lemos, J. I., Resstel, L. B., & Guimaraes, F. S. (2010). Involvement of the prelimbic prefrontal cortex on cannabidiol-induced attenuation of contextual conditioned fear in rats. *Behav Brain Res*, *207*(1), 105-111. https://doi.org/10.1016/j.bbr.2009.09.045

Lev-Ran, S., Roerecke, M., Le Foll, B., George, T. P., McKenzie, K., & Rehm, J. (2014). The association between cannabis use and depression: a systematic review and meta-analysis of longitudinal studies. *Psychol Med*, *44*(4), 797-810. https://doi.org/10.1017/s0033291713001438

Levy-Gigi, E., Szabo, C., Richter-Levin, G., & Keri, S. (2015). Reduced hippocampal volume is associated with overgeneralization of negative context in individuals with PTSD. *Neuropsychology*, *29*(1), 151-161. https://doi.org/10.1037/neu0000131

Li, W.-G., Wu, Y.-J., Gu, X., Fan, H.-R., Wang, Q., Zhu, J.-J., . . . Xu, T.-L. (2021). Input associativity underlies fear memory renewal. *National science review*, *8*(9), nwab004-nwab004. https://doi.org/10.1093/nsr/nwab004

Li, X., Inoue, T., Abekawa, T., Weng, S., Nakagawa, S., Izumi, T., & Koyama, T. (2006). 5-HT1A receptor agonist affects fear conditioning through stimulations of the postsynaptic 5-HT1A receptors in the hippocampus and amygdala. *European Journal of Pharmacology*, *532*(1), 74-80. https://doi.org/https://doi.org/10.1016/j.ejphar.2005.12.008

Li, X. B., Inoue, T., Hashimoto, S., & Koyama, T. (2001). Effect of chronic administration of flesinoxan and fluvoxamine on freezing behavior induced by conditioned fear. *European Journal of Pharmacology*, *425*(1), 43-50. https://doi.org/https://doi.org/10.1016/S0014-2999(01)01159-1

Ligresti, A., Petrocellis, L. D., & Marzo, V. D. (2016). From Phytocannabinoids to Cannabinoid Receptors and Endocannabinoids: Pleiotropic Physiological and Pathological Roles Through Complex Pharmacology. *Physiological Reviews*, *96*(4), 1593-1659. https://doi.org/10.1152/physrev.00002.2016

Lin, H.-C., Mao, S.-C., Su, C.-L., & Gean, P.-W. (2009). The Role of Prefrontal Cortex CB1 Receptors in the Modulation of Fear Memory. *Cerebral Cortex*, *19*(1), 165-175. https://doi.org/10.1093/cercor/bhn075

Lin, Q.-S., Yang, Q., Liu, D.-D., Sun, Z., Dang, H., Liang, J., . . . Li, S.-T. (2011). Hippocampal endocannabinoids play an important role in induction of long-term potentiation and regulation of contextual fear memory formation. *Brain Research Bulletin*, *86*(3), 139-145. https://doi.org/https://doi.org/10.1016/j.brainresbull.2011.07.011

Linge, R., Jiménez-Sánchez, L., Campa, L., Pilar-Cuéllar, F., Vidal, R., Pazos, A., . . . Díaz, Á. (2016). Cannabidiol induces rapid-acting antidepressant-like effects and enhances cortical 5-HT/glutamate neurotransmission: role of 5-HT1A receptors. *Neuropharmacology*, *103*, 16-26. https://doi.org/10.1016/j.neuropharm.2015.12.017

Lipp, O. V., Ryan, K. M., Luck, C. C., Craske, M. G., & Waters, A. M. (2021). Presentation of unpaired unconditional stimuli during extinction reduces renewal of conditional fear and slows re-acquisition. *Psychophysiology*, *58*(10), e13899. https://doi.org/10.1111/psyp.13899

Lissek, S., Pine, D. S., & Grillon, C. (2006). The strong situation: A potential impediment to studying the psychobiology and pharmacology of anxiety disorders. *Biological Psychology*, *72*(3), 265-270. https://doi.org/https://doi.org/10.1016/j.biopsycho.2005.11.004

Liu, J., Wang, L., Harvey-White, J., Huang, B. X., Kim, H. Y., Luquet, S., . . . Kunos, G. (2008). Multiple pathways involved in the biosynthesis of anandamide. *Neuropharmacology*, *54*(1), 1-7. https://doi.org/10.1016/j.neuropharm.2007.05.020

Lladó-Pelfort, L., Santana, N., Ghisi, V., Artigas, F., & Celada, P. (2012). 5-HT1A Receptor Agonists Enhance Pyramidal Cell Firing in Prefrontal Cortex Through a Preferential Action on GABA Interneurons. *Cerebral Cortex*, *22*(7), 1487-1497. https://doi.org/10.1093/cercor/bhr220

Llorente-Berzal, A., Terzian, A. L., di Marzo, V., Micale, V., Viveros, M. P., & Wotjak, C. T. (2015). 2-AG promotes the expression of conditioned fear via cannabinoid receptor type 1 on GABAergic neurons. *Psychopharmacology (Berl)*, *232*(15), 2811-2825. https://doi.org/10.1007/s00213-015-3917-y

Long, L. E., Chesworth, R., Huang, X.-F., Wong, A., Spiro, A., McGregor, I. S., . . . Karl, T. (2012). Distinct Neurobehavioural Effects of Cannabidiol in Transmembrane Domain

Neuregulin 1 Mutant Mice. *PLOS ONE*, *7*(4), e34129. https://doi.org/10.1371/journal.pone.0034129

Lonsdorf, T. B., & Merz, C. J. (2017). More than just noise: Inter-individual differences in fear acquisition, extinction and return of fear in humans - Biological, experiential, temperamental factors, and methodological pitfalls. *Neuroscience & Biobehavioral Reviews*, *80*, 703-728. https://doi.org/https://doi.org/10.1016/j.neubiorev.2017.07.007

Lopez-Gil, X., Artigas, F., & Adell, A. (2010). Unraveling Monoamine Receptors Involved in the Action of Typical and Atypical Antipsychotics on Glutamatergic and Serotonergic Transmission in Prefrontal Cortex. *Current Pharmaceutical Design*, *16*(5), 502-515. https://doi.org/10.2174/138161210790361416

Lu, H.-C., & Mackie, K. (2016). An Introduction to the Endogenous Cannabinoid System. *Biol Psychiatry*, *79*(7), 516-525. https://doi.org/https://doi.org/10.1016/j.biopsych.2015.07.028

Luchkina, N. V., & Bolshakov, V. Y. (2019). Mechanisms of fear learning and extinction: synaptic plasticity–fear memory connection. *Psychopharmacology*, *236*(1), 163-182. https://doi.org/10.1007/s00213-018-5104-4

Lust, C. A. C., Lin, X., Rock, E. M., Limebeer, C. L., Parker, L. A., & Ma, D. W. L. (2022). Short communication: Tissue distribution of major cannabinoids following intraperitoneal injection in male rats. *PLoS One*, *17*(1), e0262633. https://doi.org/10.1371/journal.pone.0262633

Maccarrone, M. (2017). Metabolism of the Endocannabinoid Anandamide: Open Questions after 25 Years. *Frontiers in molecular neuroscience*, *10*, 166-166. https://doi.org/10.3389/fnmol.2017.00166

Madjid, N., Tottie, E. E., Lüttgen, M., Meister, B., Sandin, J., Kuzmin, A., . . . Ögren, S. O. (2006). 5-Hydroxytryptamine 1A Receptor Blockade Facilitates Aversive Learning in Mice: Interactions with Cholinergic and Glutamatergic Mechanisms. *Journal of Pharmacology and Experimental Therapeutics*, *316*(2), 581. https://doi.org/10.1124/jpet.105.092262

Maldonado, N. M., Martijena, I. D., & Molina, V. A. (2011). Facilitating influence of stress on the consolidation of fear memory induced by a weak training: Reversal by midazolam pretreatment. *Behavioural Brain Research*, *225*(1), 77-84. https://doi.org/https://doi.org/10.1016/j.bbr.2011.06.035

Marek, R., Jin, J., Goode, T. D., Giustino, T. F., Wang, Q., Acca, G. M., . . . Sah, P. (2018). Hippocampus-driven feed-forward inhibition of the prefrontal cortex mediates relapse of extinguished fear. *Nature Neuroscience*, *21*(3), 384-392. https://doi.org/10.1038/s41593-018-0073-9

Maren, S. (2014). Nature and causes of the immediate extinction deficit: A brief review. *Neurobiology of Learning and Memory*, *113*, 19-24. https://doi.org/https://doi.org/10.1016/j.nlm.2013.10.012

Maren, S. (2022). Unrelenting Fear Under Stress: Neural Circuits and Mechanisms for the Immediate Extinction Deficit. *Front Syst Neurosci*, *16*, 888461. https://doi.org/10.3389/fnsys.2022.888461

Maren, S., & Chang, C. H. (2006). Recent fear is resistant to extinction. *Proc Natl Acad Sci U S A*, *103*(47), 18020-18025. https://doi.org/10.1073/pnas.0608398103

Maren, S., & Holmes, A. (2016). Stress and Fear Extinction. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *41*(1), 58-79. https://doi.org/10.1038/npp.2015.180

Maren, S., Phan, K. L., & Liberzon, I. (2013). The contextual brain: implications for fear conditioning, extinction and psychopathology. *Nat Rev Neurosci*, *14*(6), 417-428. https://doi.org/10.1038/nrn3492

Marinho, A. L., Vila-Verde, C., Fogaca, M. V., & Guimaraes, F. S. (2015). Effects of intra-infralimbic prefrontal cortex injections of cannabidiol in the modulation of emotional behaviors in rats: contribution of 5HT(1)A receptors and stressful experiences. *Behav Brain Res*, *286*, 49-56. https://doi.org/10.1016/j.bbr.2015.02.023

Markowitz, J. C., Lipsitz, J., & Milrod, B. L. (2014). Critical review of outcome research on interpersonal psychotherapy for anxiety disorders. *Depression and Anxiety*, *31*(4), 316-325. https://doi.org/10.1002/da.22238

Maroon, J., & Bost, J. (2018). Review of the neurological benefits of phytocannabinoids. *Surgical neurology international*, *9*, 91-91. https://doi.org/10.4103/sni.sni_45_18

Marsicano, G., Wotjak, C. T., Azad, S. C., Bisogno, T., Rammes, G., Cascio, M. G., . . . Lutz, B. (2002). The endogenous cannabinoid system controls extinction of aversive memories. *Nature*, *418*(6897), 530-534. https://doi.org/10.1038/nature00839

Martínez-Canabal, A., López-Oropeza, G., Gaona-Gamboa, A., Ballesteros-Zebadua, P., de la Cruz, O. G., Moreno-Jimenez, S., & Sotres-Bayon, F. (2019). Hippocampal neurogenesis regulates recovery of defensive responses by recruiting threat- and extinction-signalling brain networks. *Scientific Reports*, *9*(1), 2939. https://doi.org/10.1038/s41598-019-39136-y

Mascarenhas, D. C., Gomes, K. S., & Nunes-de-Souza, R. L. (2013). Anxiogenic-like effect induced by TRPV1 receptor activation within the dorsal periaqueductal gray matter in mice. *Behav Brain Res*, *250*, 308-315. https://doi.org/10.1016/j.bbr.2013.05.023

Matsuda, S., Matsuzawa, D., Ishii, D., Tomizawa, H., & Shimizu, E. (2014). Effects of memory age and interval of fear extinction sessions on contextual fear extinction. *Neurosci Lett*, *578*, 139-142. https://doi.org/https://doi.org/10.1016/j.neulet.2014.06.054

Matsuzaki, H., Izumi, T., Horinouchi, T., Boku, S., Inoue, T., Yamaguchi, T., . . . Yoshioka, M. (2011). Juvenile stress attenuates the dorsal hippocampal postsynaptic 5-HT1A receptor function in adult rats. *Psychopharmacology*, *214*(1), 329-337. https://doi.org/10.1007/s00213-010-1987-4

Maurya, N., & Velmurugan, B. K. (2018). Therapeutic applications of cannabinoids. *Chemico-Biological Interactions*, *293*, 77-88. https://doi.org/https://doi.org/10.1016/j.cbi.2018.07.018

McCall, J. G., Al-Hasani, R., Siuda, E. R., Hong, D. Y., Norris, A. J., Ford, C. P., & Bruchas, M. R. (2015). CRH Engagement of the Locus Coeruleus Noradrenergic System Mediates Stress-Induced Anxiety. *Neuron*, *87*(3), 605-620. https://doi.org/10.1016/j.neuron.2015.07.002

McCallum, J., Kim, J. H., & Richardson, R. (2010). Impaired extinction retention in adolescent rats: effects of D-cycloserine. *Neuropsychopharmacology*, *35*(10), 2134-2142. https://doi.org/10.1038/npp.2010.92

McCutcheon, J. E., & Marinelli, M. (2009). Age matters. *Eur J Neurosci*, *29*(5), 997-1014. https://doi.org/10.1111/j.1460-9568.2009.06648.x

McLaughlin, P. J., Winston, K., Swezey, L., Wisniecki, A., Aberman, J., Tardif, D. J., . . . Salamone, J. D. (2003). The cannabinoid CB1 antagonists SR 141716A and AM 251 suppress food intake and food-reinforced behavior in a variety of tasks in rats. *Behav Pharmacol*, *14*(8), 583-588. https://doi.org/10.1097/00008877-200312000-00002

McNally, G. P., & Westbrook, R. F. (2006). Predicting danger: the nature, consequences, and neural mechanisms of predictive fear learning. *Learn Mem*, *13*(3), 245-253. https://doi.org/10.1101/lm.196606

Meir Drexler, S., Merz, C. J., Jentsch, V. L., & Wolf, O. T. (2020). Stress modulation of fear and extinction in psychopathology and treatment. *Neuroforum*, *26*(3), 133-141. https://doi.org/doi:10.1515/nf-2020-0018

Mendiguren, A., Aostri, E., & Pineda, J. (2018). Regulation of noradrenergic and serotonergic systems by cannabinoids: relevance to cannabinoid-induced effects. *Life Sci*, *192*, 115-127. https://doi.org/10.1016/j.lfs.2017.11.029

Mendiguren, A., & Pineda, J. (2006). Systemic effect of cannabinoids on the spontaneous firing rate of locus coeruleus neurons in rats. *Eur J Pharmacol*, *534*(1-3), 83-88. https://doi.org/10.1016/j.ejphar.2006.01.002

Merz, C. J., & Wolf, O. T. (2019). The immediate extinction deficit occurs in a nonemotional learning paradigm. *Learn Mem*, *26*(2), 39-45. https://doi.org/10.1101/lm.048223.118

Meyer, H. C., Lee, F. S., & Gee, D. G. (2018). The Role of the Endocannabinoid System and Genetic Variation in Adolescent Brain Development. *Neuropsychopharmacology*, *43*(1), 21-33. https://doi.org/10.1038/npp.2017.143

Migues, P. V., Liu, L., Archbold, G. E. B., Einarsson, E. Ö., Wong, J., Bonasia, K., . . . Hardt, O. (2016). Blocking Synaptic Removal of GluA2-Containing AMPA Receptors Prevents the Natural Forgetting of Long-Term Memories. *The Journal of Neuroscience*, *36*(12), 3481. https://doi.org/10.1523/JNEUROSCI.3333-15.2016

Milad, M. R., Pitman, R. K., Ellis, C. B., Gold, A. L., Shin, L. M., Lasko, N. B., . . . Rauch, S. L. (2009). Neurobiological basis of failure to recall extinction memory in posttraumatic stress disorder. *Biol Psychiatry*, *66*(12), 1075-1082. https://doi.org/10.1016/j.biopsych.2009.06.026

Millar, S. A., Stone, N. L., Yates, A. S., & O'Sullivan, S. E. (2018). A Systematic Review on the Pharmacokinetics of Cannabidiol in Humans [Original Research]. *Frontiers in Pharmacology*, *9*(1365). https://doi.org/10.3389/fphar.2018.01365

Mingote, S., de Bruin, J. P., & Feenstra, M. G. (2004). Noradrenaline and dopamine efflux in the prefrontal cortex in relation to appetitive classical conditioning. *J Neurosci*, *24*(10), 2475-2480. https://doi.org/10.1523/jneurosci.4547-03.2004

Mohajeri, E., & Noudeh, G. D. (2012). Effect of Temperature on the Critical Micelle Concentration and Micellization Thermodynamic of Nonionic Surfactants: Polyoxyethylene Sorbitan Fatty Acid Esters. *E-Journal of Chemistry*, *9*, 961739. https://doi.org/10.1155/2012/961739

Mohd Yusof, N. S. (2021). The effect of sonication on the ion exchange constant, KXBr of CTABr/chlorobenzoates micellar systems. *Ultrasonics Sonochemistry*, *71*, 105360. https://doi.org/https://doi.org/10.1016/j.ultsonch.2020.105360

Moreira, F. A., Aguiar, D. C., & Guimarães, F. S. (2006). Anxiolytic-like effect of cannabidiol in the rat Vogel conflict test. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *30*(8), 1466-1471. https://doi.org/https://doi.org/10.1016/j.pnpbp.2006.06.004

Moreira, F. A., Aguiar, D. C., Terzian, A. L. B., Guimarães, F. S., & Wotjak, C. T. (2012). Cannabinoid type 1 receptors and transient receptor potential vanilloid type 1 channels in fear and anxiety—two sides of one coin? *Neuroscience*, *204*, 186-192. https://doi.org/https://doi.org/10.1016/j.neuroscience.2011.08.046

Morena, M., Berardi, A., Colucci, P., Palmery, M., Trezza, V., Hill, M. N., & Campolongo, P. (2018). Enhancing Endocannabinoid Neurotransmission Augments The Efficacy of Extinction Training and Ameliorates Traumatic Stress-Induced Behavioral Alterations in Rats. *Neuropsychopharmacology*, *43*(6), 1284-1296. https://doi.org/10.1038/npp.2017.305

Morena, M., Nastase, A. S., Santori, A., Cravatt, B. F., Shansky, R. M., & Hill, M. N. (2021). Sex-dependent effects of endocannabinoid modulation of conditioned fear extinction in rats [https://doi.org/10.1111/bph.15341]. *British Journal of Pharmacology*, *178*(4), 983-996. https://doi.org/https://doi.org/10.1111/bph.15341

Muraki, I., Inoue, T., & Koyama, T. (2008). Effect of co-administration of the selective 5-HT1A receptor antagonist WAY 100,635 and selective 5-HT1B/1D receptor antagonist GR

127,935 on anxiolytic effect of citalopram in conditioned fear stress in the rat. *Eur J Pharmacol*, *586*(1-3), 171-178. https://doi.org/10.1016/j.ejphar.2008.01.040

Murrough, J. W., Yaqubi, S., Sayed, S., & Charney, D. S. (2015). Emerging drugs for the treatment of anxiety. *Expert Opin Emerg Drugs*, *20*(3), 393-406. https://doi.org/10.1517/14728214.2015.1049996

Myers, K. M., & Davis, M. (2006). Mechanisms of fear extinction [Feature Review]. *Molecular Psychiatry*, *12*, 120. https://doi.org/10.1038/sj.mp.4001939

Nachtigall, E. G., Furini, C. R. G., Behling, J. A. K., Farias, C. P., Izquierdo, I., & Myskiw, J. C. (2019). Facilitation of fear extinction by novelty is modulated by β-adrenergic and 5-HT(1A) serotoninergic receptors in hippocampus. *Neurobiol Learn Mem*, *166*, 107101. https://doi.org/10.1016/j.nlm.2019.107101

Nava, N., Treccani, G., Alabsi, A., Kaastrup Mueller, H., Elfving, B., Popoli, M., . . . Nyengaard, J. R. (2017). Temporal Dynamics of Acute Stress-Induced Dendritic Remodeling in Medial Prefrontal Cortex and the Protective Effect of Desipramine. *Cereb Cortex*, *27*(1), 694-705. https://doi.org/10.1093/cercor/bhv254

Navarrete, F., García-Gutiérrez, M. S., Jurado-Barba, R., Rubio, G., Gasparyan, A., Austrich-Olivares, A., & Manzanares, J. (2020). Endocannabinoid System Components as Potential Biomarkers in Psychiatry [Review]. *Frontiers in Psychiatry*, *11*.

Neumeister, A., Normandin, M. D., Pietrzak, R. H., Piomelli, D., Zheng, M. Q., Gujarro-Anton, A., . . . Huang, Y. (2013). Elevated brain cannabinoid CB1 receptor availability in post-traumatic stress disorder: a positron emission tomography study. *Mol Psychiatry*, *18*(9), 1034-1040. https://doi.org/10.1038/mp.2013.61

Nevins, M. E., & Anthony, E. W. (1994). Antagonists at the serotonin-3 receptor can reduce the fear-potentiated startle response in the rat: evidence for different types of anxiolytic activity? *Journal of Pharmacology and Experimental Therapeutics*, *268*(1), 248.

Nidadavolu, P., Bilkei-Gorzo, A., Effah, F., Leidmaa, E., Schürmann, B., Berger, M., . . . Bailey, A. (2022). Dynamic Changes in the Endocannabinoid System during the Aging Process: Focus on the Middle-Age Crisis. *Int J Mol Sci*, *23*(18). https://doi.org/10.3390/ijms231810254

Niyuhire, F., Varvel, S. A., Thorpe, A. J., Stokes, R. J., Wiley, J. L., & Lichtman, A. H. (2007). The disruptive effects of the CB1 receptor antagonist rimonabant on extinction learning in mice are task-specific. *Psychopharmacology*, *191*(2), 223-231. https://doi.org/10.1007/s00213-006-0650-6

Norrholm, S. D., & Ressler, K. J. (2009). Genetics of anxiety and trauma-related disorders. *Neuroscience*, *164*(1), 272-287. https://doi.org/10.1016/j.neuroscience.2009.06.036

Norris, C., Loureiro, M., Kramar, C., Zunder, J., Renard, J., Rushlow, W., & Laviolette, S. R. (2016). Cannabidiol Modulates Fear Memory Formation Through Interactions with Serotonergic Transmission in the Mesolimbic System. *Neuropsychopharmacology*, *41*(12), 2839-2850. https://doi.org/10.1038/npp.2016.93

Ohno-Shosaku, T., & Kano, M. (2014). Endocannabinoid-mediated retrograde modulation of synaptic transmission. *Curr Opin Neurobiol*, *29*, 1-8. https://doi.org/10.1016/j.conb.2014.03.017

Orinstein, A. J., Urcelay, G. P., & Miller, R. R. (2010). Expanding the Intertrial Interval During Extinction: Response Cessation and Recovery. *Behavior Therapy*, *41*(1), 14-29. https://doi.org/https://doi.org/10.1016/j.beth.2008.11.001

Oropeza, V. C., Mackie, K., & Van Bockstaele, E. J. (2007). Cannabinoid receptors are localized to noradrenergic axon terminals in the rat frontal cortex. *Brain Res*, *1127*(1), 36-44. https://doi.org/10.1016/j.brainres.2006.09.110

Orsini, C. A., Kim, J. H., Knapska, E., & Maren, S. (2011). Hippocampal and prefrontal projections to the basal amygdala mediate contextual regulation of fear after

extinction. *J Neurosci*, *31*(47), 17269-17277. https://doi.org/10.1523/jneurosci.4095-11.2011

Orsini, C. A., & Maren, S. (2012). Neural and cellular mechanisms of fear and extinction memory formation. *Neuroscience & Biobehavioral Reviews*, *36*(7), 1773-1802. https://doi.org/https://doi.org/10.1016/j.neubiorev.2011.12.014

Pai, A., Suris, A. M., & North, C. S. (2017). Posttraumatic Stress Disorder in the DSM-5: Controversy, Change, and Conceptual Considerations. *Behavioral sciences (Basel, Switzerland)*, *7*(1), 7. https://doi.org/10.3390/bs7010007

Pamplona, F. A., Bitencourt, R. M., & Takahashi, R. N. (2008). Short- and long-term effects of cannabinoids on the extinction of contextual fear memory in rats. *Neurobiol Learn Mem*, *90*(1), 290-293. https://doi.org/10.1016/j.nlm.2008.04.003

Pamplona, F. A., Prediger, R. D. S., Pandolfo, P., & Takahashi, R. N. (2006). The cannabinoid receptor agonist WIN 55,212-2 facilitates the extinction of contextual fear memory and spatial memory in rats. *Psychopharmacology*, *188*(4), 641-649. https://doi.org/10.1007/s00213-006-0514-0

Pamplona, F. A., & Takahashi, R. N. (2006). WIN 55212-2 impairs contextual fear conditioning through the activation of CB1 cannabinoid receptors. *Neuroscience Letters*, *397*(1), 88-92. https://doi.org/https://doi.org/10.1016/j.neulet.2005.12.026

Papadakis, M. A., McPhee, S. J & Rabow, M. W. (2017). *Current Medical Diagnosis & Treatment*. McGraw-Hill Education.

Papagianni, E. P., & Stevenson, C. W. (2019). Cannabinoid Regulation of Fear and Anxiety: an Update [journal article]. *Current Psychiatry Reports*, *21*(6), 38. https://doi.org/10.1007/s11920-019-1026-z

Park, C. H. J., Ganella, D. E., & Kim, J. H. (2017). Juvenile female rats, but not male rats, show renewal, reinstatement, and spontaneous recovery following extinction of conditioned fear. *Learning & memory (Cold Spring Harbor, N.Y.)*, *24*(12), 630-636. https://doi.org/10.1101/lm.045831.117

Park, S. M., & Williams, C. L. (2012). Contribution of serotonin type 3 receptors in the successful extinction of cued or contextual fear conditioned responses: interactions with GABAergic signaling. *23*(5-6), 555-569. https://doi.org/doi:10.1515/revneuro-2012-0052 (Reviews in the Neurosciences)

Passie, T., Emrich, H. M., Karst, M., Brandt, S. D., & Halpern, J. H. (2012). Mitigation of post-traumatic stress symptoms by Cannabis resin: a review of the clinical and neurobiological evidence. *Drug Test Anal*, *4*(7-8), 649-659. https://doi.org/10.1002/dta.1377

Patel, S., Hill, M. N., Cheer, J. F., Wotjak, C. T., & Holmes, A. (2017). The endocannabinoid system as a target for novel anxiolytic drugs. *Neurosci Biobehav Rev*, *76*(Pt A), 56-66. https://doi.org/10.1016/j.neubiorev.2016.12.033

Pattwell, S. S., Lee, F. S., & Casey, B. J. (2013). Fear learning and memory across adolescent development: Hormones and Behavior Special Issue: Puberty and Adolescence. *Hormones and Behavior*, *64*(2), 380-389. https://doi.org/https://doi.org/10.1016/j.yhbeh.2013.01.016

Paula de Godoy, A., Verginia de Medeiros, M., Pasquini de Souza, C., & Jacson Martynhak, B. (2022). High trait anxiety in mice is associated with impaired extinction in the contextual fear conditioning paradigm. *Neurobiology of Learning and Memory*, *190*, 107602. https://doi.org/https://doi.org/10.1016/j.nlm.2022.107602

Pedroza-Llinás, R., Méndez-Díaz, M., Ruiz-Contreras, A. E., & Prospéro-García, Ó. (2013). CB1 receptor activation in the nucleus accumbens core impairs contextual fear learning. *Behavioural Brain Research*, *237*, 141-147. https://doi.org/https://doi.org/10.1016/j.bbr.2012.09.032

Pereyra, A. E., Mininni, C. J., & Zanutto, B. S. (2021). Serotonergic modulation of basolateral amygdala nucleus in the extinction of reward-driven learning: The role of 5-HT bioavailability and 5-HT1A receptor. *Behavioural Brain Research*, *404*, 113161. https://doi.org/https://doi.org/10.1016/j.bbr.2021.113161

Pertwee, R. G. (1997). Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther*, *74*(2), 129-180. https://doi.org/https://doi.org/10.1016/S0163-7258(97)82001-3

Pertwee, R. G. (2010). Receptors and channels targeted by synthetic cannabinoid receptor agonists and antagonists. *Current medicinal chemistry*, *17*(14), 1360-1381.

Pickens, C. L., Golden, S. A., Adams-Deutsch, T., Nair, S. G., & Shaham, Y. (2009). Long-lasting incubation of conditioned fear in rats. *Biol Psychiatry*, *65*(10), 881-886. https://doi.org/10.1016/j.biopsych.2008.12.010

Pickens, C. L., & Theberge, F. R. (2014). Blockade of CB1 receptors prevents retention of extinction but does not increase low preincubated conditioned fear in the fear incubation procedure. *Behavioural pharmacology*, *25*(1), 23-31. https://doi.org/10.1097/FBP.0000000000000020

Pitman, R. K., Rasmusson, A. M., Koenen, K. C., Shin, L. M., Orr, S. P., Gilbertson, M. W., . . . Liberzon, I. (2012). Biological studies of post-traumatic stress disorder. *Nat Rev Neurosci*, *13*(11), 769-787. https://doi.org/10.1038/nrn3339

Plendl, W., & Wotjak, C. T. (2010). Dissociation of within- and between-Session Extinction of Conditioned Fear. *The Journal of Neuroscience*, *30*(14), 4990. https://doi.org/10.1523/JNEUROSCI.6038-09.2010

Ponnusamy, R., Zhuravka, I., Poulos, A. M., Shobe, J., Merjanian, M., Huang, J., . . . Fanselow, M. S. (2016). Retrieval and Reconsolidation Accounts of Fear Extinction [Original Research]. *Frontiers in Behavioral Neuroscience*, *10*.

Prenderville, J. A., Kelly, A. M., & Downer, E. J. (2015). The role of cannabinoids in adult neurogenesis. *Br J Pharmacol*, *172*(16), 3950-3963. https://doi.org/10.1111/bph.13186

Puig, M. V., & Gulledge, A. T. (2011). Serotonin and Prefrontal Cortex Function: Neurons, Networks, and Circuits. *Molecular Neurobiology*, *44*(3), 449-464. https://doi.org/10.1007/s12035-011-8214-0

Quirk, G. J. (2002). Memory for extinction of conditioned fear is long-lasting and persists following spontaneous recovery. *Learn Mem*, *9*(6), 402-407. https://doi.org/10.1101/lm.49602

Quirk, G. J., Likhtik, E., Pelletier, J. G., & Paré, D. (2003). Stimulation of medial prefrontal cortex decreases the responsiveness of central amygdala output neurons. *J Neurosci*, *23*(25), 8800-8807. https://doi.org/10.1523/jneurosci.23-25-08800.2003

R. Wyrofsky, R., Reyes, B. A. S., & Van Bockstaele, E. J. (2017). Co-localization of the cannabinoid type 1 receptor with corticotropin-releasing factor-containing afferents in the noradrenergic nucleus locus coeruleus: implications for the cognitive limb of the stress response. *Brain Structure and Function*, *222*(7), 3007-3023. https://doi.org/10.1007/s00429-017-1381-7

Rabinak, C. A., Angstadt, M., Sripada, C. S., Abelson, J. L., Liberzon, I., Milad, M. R., & Phan, K. L. (2013). Cannabinoid facilitation of fear extinction memory recall in humans. *Neuropharmacology*, *64*, 396-402. https://doi.org/https://doi.org/10.1016/j.neuropharm.2012.06.063

Raffa, R. B., & Ward, S. J. (2012). CB1-independent mechanisms of Δ9-THCV, AM251 and SR141716 (rimonabant) [https://doi.org/10.1111/j.1365-2710.2011.01284.x]. *Journal of Clinical Pharmacy and Therapeutics*, *37*(3), 260-265. https://doi.org/https://doi.org/10.1111/j.1365-2710.2011.01284.x

Rainnie, D. G., Bergeron, R., Sajdyk, T. J., Patil, M., Gehlert, D. R., & Shekhar, A. (2004). Corticotrophin releasing factor-induced synaptic plasticity in the amygdala translates

stress into emotional disorders. *J Neurosci*, *24*(14), 3471-3479. https://doi.org/10.1523/jneurosci.5740-03.2004

Ratano, P., Everitt, B. J., & Milton, A. L. (2014). The CB1 receptor antagonist AM251 impairs reconsolidation of pavlovian fear memory in the rat basolateral amygdala. *Neuropsychopharmacology*, *39*(11), 2529-2537. https://doi.org/10.1038/npp.2014.103

Raymundi, A. M., da Silva, T. R., Zampronio, A. R., Guimarães, F. S., Bertoglio, L. J., & Stern, C. A. J. (2020). A time-dependent contribution of hippocampal CB(1) , CB(2) and PPARγ receptors to cannabidiol-induced disruption of fear memory consolidation. *Br J Pharmacol*, *177*(4), 945-957. https://doi.org/10.1111/bph.14895

Reich, C. G., Mohammadi, M. H., & Alger, B. E. (2008). Endocannabinoid modulation of fear responses: learning and state-dependent performance effects. *Journal of psychopharmacology (Oxford, England)*, *22*(7), 769-777. https://doi.org/10.1177/0269881107083999

Rescorla, R. A. (2004a). Spontaneous recovery. *Learn Mem*, *11*(5), 501-509. https://doi.org/10.1101/lm.77504

Rescorla, R. A. (2004b). Spontaneous recovery varies inversely with the training-extinction interval. *Animal Learning & Behavior*, *32*(4), 401-408. https://doi.org/10.3758/BF03196037

Resstel, L. B., Joca, S. R., Moreira, F. A., Correa, F. M., & Guimaraes, F. S. (2006). Effects of cannabidiol and diazepam on behavioral and cardiovascular responses induced by contextual conditioned fear in rats. *Behav Brain Res*, *172*(2), 294-298. https://doi.org/10.1016/j.bbr.2006.05.016

Resstel, L. B. M., Lisboa, S. F., Aguiar, D. C., Corrêa, F. M. A., & Guimarães, F. S. (2008). Activation of CB1 cannabinoid receptors in the dorsolateral periaqueductal gray reduces the expression of contextual fear conditioning in rats. *Psychopharmacology*, *198*(3), 405-411. https://doi.org/10.1007/s00213-008-1156-1

Resstel, L. B. M., Tavares, R. F., Lisboa, S. F. S., Joca, S. R. L., Corrêa, F. M. A., & Guimarães, F. S. (2009). 5-HT1A receptors are involved in the cannabidiol-induced attenuation of behavioural and cardiovascular responses to acute restraint stress in rats [https://doi.org/10.1111/j.1476-5381.2008.00046.x]. *British Journal of Pharmacology*, *156*(1), 181-188. https://doi.org/https://doi.org/10.1111/j.1476-5381.2008.00046.x

Reyes, B. A., Heldt, N. A., Mackie, K., & Van Bockstaele, E. J. (2015). Ultrastructural evidence for synaptic contacts between cortical noradrenergic afferents and endocannabinoid-synthesizing post-synaptic neurons. *Neuroscience*, *303*, 323-337. https://doi.org/10.1016/j.neuroscience.2015.07.009

Rock, E. M., Bolognini, D., Limebeer, C. L., Cascio, M. G., Anavi-Goffer, S., Fletcher, P. J., . . . Parker, L. A. (2012). Cannabidiol, a non-psychotropic component of cannabis, attenuates vomiting and nausea-like behaviour via indirect agonism of 5-HT1A somatodendritic autoreceptors in the dorsal raphe nucleus [https://doi.org/10.1111/j.1476-5381.2011.01621.x]. *British Journal of Pharmacology*, *165*(8), 2620-2634. https://doi.org/https://doi.org/10.1111/j.1476-5381.2011.01621.x

Roitman, P., Mechoulam, R., Cooper-Kazaz, R., & Shalev, A. (2014). Preliminary, Open-Label, Pilot Study of Add-On Oral Δ9-Tetrahydrocannabinol in Chronic Post-Traumatic Stress Disorder. *Clinical Drug Investigation*, *34*(8), 587-591. https://doi.org/10.1007/s40261-014-0212-3

Rosas, J. M., García-Gutiérrez, A., & Callejas-Aguilera, J. E. (2007). AAB and ABA renewal as a function of the number of extinction trials in conditioned taste aversion. *Psicológica*, *28*(2), 129-150.

Rossignoli, M. T., Lopes-Aguiar, C., Ruggiero, R. N., Do Val da Silva, R. A., Bueno-Junior, L. S., Kandratavicius, L., . . . Romcy-Pereira, R. N. (2017). Selective post-training time window for memory consolidation interference of cannabidiol into the prefrontal cortex: Reduced dopaminergic modulation and immediate gene expression in limbic circuits. *Neuroscience*, *350*, 85-93. https://doi.org/https://doi.org/10.1016/j.neuroscience.2017.03.019

Roy-Byrne, P., Craske, M. G., Sullivan, G., & et al. (2010). Delivery of evidence-based treatment for multiple anxiety disorders in primary care: A randomized controlled trial. *JAMA*, *303*(19), 1921-1928. https://doi.org/10.1001/jama.2010.608

Rubin, M., Shvil, E., Papini, S., Chhetry, B. T., Helpman, L., Markowitz, J. C., . . . Neria, Y. (2016). Greater hippocampal volume is associated with PTSD treatment response. *Psychiatry research. Neuroimaging*, *252*, 36-39. https://doi.org/10.1016/j.pscychresns.2016.05.001

Ruehle, S., Rey, A. A., Remmers, F., & Lutz, B. (2012). The endocannabinoid system in anxiety, fear memory and habituation. *J Psychopharmacol*, *26*(1), 23-39. https://doi.org/10.1177/0269881111408958

Russo, E. B. (2018). Cannabis Therapeutics and the Future of Neurology [Perspective]. *Frontiers in Integrative Neuroscience*, *12*(51). https://doi.org/10.3389/fnint.2018.00051

Russo, E. B., Burnett, A., Hall, B., & Parker, K. K. (2005). Agonistic properties of cannabidiol at 5-HT1a receptors. *Neurochem Res*, *30*(8), 1037-1043. https://doi.org/10.1007/s11064-005-6978-1

Russo, M., Calabr, #xf2, , R. S., Naro, A., Sessa, E., . . . Bramanti, P. (2015). Sativex in the Management of Multiple Sclerosis-Related Spasticity: Role of the Corticospinal Modulation. *Neural Plasticity*, *2015*, 6, Article 656582. https://doi.org/10.1155/2015/656582

Ryberg, E., Larsson, N., Sjogren, S., Hjorth, S., Hermansson, N. O., Leonova, J., . . . Greasley, P. J. (2007). The orphan receptor GPR55 is a novel cannabinoid receptor. *Br J Pharmacol*, *152*(7), 1092-1101. https://doi.org/10.1038/sj.bjp.0707460

Sachser, R. M., Crestani, A. P., Quillfeldt, J. A., Mello, E. S. T., & de Oliveira Alvares, L. (2015). The cannabinoid system in the retrosplenial cortex modulates fear memory consolidation, reconsolidation, and extinction. *Learn Mem*, *22*(12), 584-588. https://doi.org/10.1101/lm.039891.115

Saito, Y., Matsumoto, M., Yanagawa, Y., Hiraide, S., Inoue, S., Kubo, Y., . . . Togashi, H. (2013). Facilitation of fear extinction by the 5-HT1A receptor agonist tandospirone: Possible involvement of dopaminergic modulation [https://doi.org/10.1002/syn.21621]. *Synapse*, *67*(4), 161-170. https://doi.org/https://doi.org/10.1002/syn.21621

Samuels, E. R., & Szabadi, E. (2008). Functional Neuroanatomy of the Noradrenergic Locus Coeruleus: Its Roles in the Regulation of Arousal and Autonomic Function Part I: Principles of Functional Organisation. *Current Neuropharmacology*, *6*(3), 235-253.

Santana, F., Sierra, R. O., Haubrich, J., Crestani, A. P., Duran, J. M., de Freitas Cassini, L., . . . Quillfeldt, J. A. (2016). Involvement of the infralimbic cortex and CA1 hippocampal area in reconsolidation of a contextual fear memory through CB1 receptors: Effects of CP55,940. *Neurobiology of Learning and Memory*, *127*, 42-47. https://doi.org/https://doi.org/10.1016/j.nlm.2015.11.016

Sartim, A. G., Guimarães, F. S., & Joca, S. R. (2016). Antidepressant-like effect of cannabidiol injection into the ventral medial prefrontal cortex-Possible involvement of 5-HT1A and CB1 receptors. *Behav Brain Res*, *303*, 218-227. https://doi.org/10.1016/j.bbr.2016.01.033

Sartori, S. B., & Singewald, N. (2017). New pharmacological strategies for augmenting extinction learning in anxiety disorders. *e-Neuroforum*, *23*(4), A145-A156. https://doi.org/doi:10.1515/nf-2017-A011

Schein, J., Houle, C., Urganus, A., Cloutier, M., Patterson-Lomba, O., Wang, Y., . . . Davis, L. L. (2021). Prevalence of post-traumatic stress disorder in the United States: a systematic literature review. *Current Medical Research and Opinion*, *37*(12), 2151-2161. https://doi.org/10.1080/03007995.2021.1978417

Scherma, M., Masia, P., Deidda, M., Fratta, W., Tanda, G., & Fadda, P. (2018). New Perspectives on the Use of Cannabis in the Treatment of Psychiatric Disorders. *Medicines (Basel)*, *5*(4). https://doi.org/10.3390/medicines5040107

Schlosburg, J. E., Blankman, J. L., Long, J. Z., Nomura, D. K., Pan, B., Kinsey, S. G., . . . Cravatt, B. F. (2010). Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system [Article]. *Nat Neurosci*, *13*, 1113. https://doi.org/10.1038/nn.2616

Seely, K. A., Brents, L. K., Franks, L. N., Rajasekaran, M., Zimmerman, S. M., Fantegrossi, W. E., & Prather, P. L. (2012). AM-251 and rimonabant act as direct antagonists at mu-opioid receptors: Implications for opioid/cannabinoid interaction studies. *Neuropharmacology*, *63*(5), 905-915. https://doi.org/https://doi.org/10.1016/j.neuropharm.2012.06.046

Seeman, P. (2016). Cannabidiol is a partial agonist at dopamine D2High receptors, predicting its antipsychotic clinical dose. *Transl Psychiatry*, *6*(10), e920. https://doi.org/10.1038/tp.2016.195

Segev, A., Korem, N., Mizrachi Zer-Aviv, T., Abush, H., Lange, R., Sauber, G., . . . Akirav, I. (2018). Role of endocannabinoids in the hippocampus and amygdala in emotional memory and plasticity. *Neuropsychopharmacology*, *43*(10), 2017-2027. https://doi.org/10.1038/s41386-018-0135-4

Senn, V., Wolff, S. B., Herry, C., Grenier, F., Ehrlich, I., Gründemann, J., . . . Lüthi, A. (2014). Long-range connectivity defines behavioral specificity of amygdala neurons. *Neuron*, *81*(2), 428-437. https://doi.org/10.1016/j.neuron.2013.11.006

Sevenster, D., Visser, R. M., & D'Hooge, R. (2018). A translational perspective on neural circuits of fear extinction: Current promises and challenges. *Neurobiology of Learning and Memory*, *155*, 113-126. https://doi.org/https://doi.org/10.1016/j.nlm.2018.07.002

Shannon, S., & Opila-Lehman, J. (2016). Effectiveness of Cannabidiol Oil for Pediatric Anxiety and Insomnia as Part of Posttraumatic Stress Disorder: A Case Report. *Perm J*, *20*(4), 108-111. https://doi.org/10.7812/tpp/16-005

Shen, H., Igarashi, H., Imamura, N., Matsuki, N., & Nomura, H. (2013). N-methyl-D-aspartate receptors and protein synthesis are necessary for reinstatement of conditioned fear. *NeuroReport*, *24*(14), 763-767. https://doi.org/10.1097/WNR.0b013e328363b36c

Shih, C. W., & Chang, C. H. (2021). Medial or lateral orbitofrontal cortex activation during fear extinction differentially regulates fear renewal. *Behav Brain Res*, *412*, 113412. https://doi.org/10.1016/j.bbr.2021.113412

Sierra-Mercado, D., Padilla-Coreano, N., & Quirk, G. J. (2011). Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. *Neuropsychopharmacology*, *36*(2), 529-538. https://doi.org/10.1038/npp.2010.184

Simon, A. B., & Gorman, J. M. (2006). Advances in the treatment of anxiety: targeting glutamate. *NeuroRx*, *3*(1), 57-68. https://doi.org/10.1016/j.nurx.2005.12.005

Singewald, N., Schmuckermair, C., Whittle, N., Holmes, A., & Ressler, K. J. (2015). Pharmacology of cognitive enhancers for exposure-based therapy of fear, anxiety and

trauma-related disorders. *Pharmacol Ther*, *149*, 150-190. https://doi.org/10.1016/j.pharmthera.2014.12.004

Singh, S., Siddiqui, S. A., Tripathy, S., Kumar, S., Saha, S., Ugale, R., . . . Prakash, A. (2018). Decreased level of histone acetylation in the infralimbic prefrontal cortex following immediate extinction may result in deficit of extinction memory. *Brain Res Bull*, *140*, 355-364. https://doi.org/10.1016/j.brainresbull.2018.06.004

Sink, K. S., Segovia, K. N., Collins, L. E., Markus, E. J., Vemuri, V. K., Makriyannis, A., & Salamone, J. D. (2010). The CB1 inverse agonist AM251, but not the CB1 antagonist AM4113, enhances retention of contextual fear conditioning in rats. *Pharmacology Biochemistry and Behavior*, *95*(4), 479-484. https://doi.org/https://doi.org/10.1016/j.pbb.2010.03.011

Soares Vde, P., Campos, A. C., Bortoli, V. C., Zangrossi, H., Jr., Guimarães, F. S., & Zuardi, A. W. (2010). Intra-dorsal periaqueductal gray administration of cannabidiol blocks panic-like response by activating 5-HT1A receptors. *Behav Brain Res*, *213*(2), 225-229. https://doi.org/10.1016/j.bbr.2010.05.004

Song, C., Stevenson, C. W., Guimaraes, F. S., & Lee, J. L. (2016). Bidirectional Effects of Cannabidiol on Contextual Fear Memory Extinction. *Front Pharmacol*, *7*, 493. https://doi.org/10.3389/fphar.2016.00493

Stanhope, K. J., & Dourish, C. T. (1996). Effects of 5-HT1A receptor agonists, partial agonists and a silent antagonist on the performance of the conditioned emotional response test in the rat. *Psychopharmacology (Berl)*, *128*(3), 293-303. https://doi.org/10.1007/s002130050137

Stein, C., Davidowa, H., & Albrecht, D. (2000). 5-HT1A receptor-mediated inhibition and 5-HT2 as well as 5-HT3 receptor-mediated excitation in different subdivisions of the rat amygdala [https://doi.org/10.1002/1098-2396(20001201)38:3<328::AID-SYN12>3.0.CO;2-T]. *Synapse*, *38*(3), 328-337. https://doi.org/https://doi.org/10.1002/1098-2396(20001201)38:3<328::AID-SYN12>3.0.CO;2-T

Stein, D. J., Scott, K. M., de Jonge, P., & Kessler, R. C. (2017). Epidemiology of anxiety disorders: from surveys to nosology and back. *Dialogues in clinical neuroscience*, *19*(2), 127-136.

Steiner, M. A., & Wotjak, C. T. (2008). Role of the endocannabinoid system in regulation of the hypothalamic-pituitary-adrenocortical axis. In I. D. Neumann & R. Landgraf (Eds.), *Progress in Brain Research* (Vol. 170, pp. 397-432). Elsevier. https://doi.org/https://doi.org/10.1016/S0079-6123(08)00433-0

Stern, C. A., Gazarini, L., Takahashi, R. N., Guimaraes, F. S., & Bertoglio, L. J. (2012). On disruption of fear memory by reconsolidation blockade: evidence from cannabidiol treatment. *Neuropsychopharmacology*, *37*(9), 2132-2142. https://doi.org/10.1038/npp.2012.63

Stern, C. A. J., da Silva, T. R., Raymundi, A. M., de Souza, C. P., Hiroaki-Sato, V. A., Kato, L., . . . Bertoglio, L. J. (2017). Cannabidiol disrupts the consolidation of specific and generalized fear memories via dorsal hippocampus CB1 and CB2 receptors. *Neuropharmacology*, *125*, 220-230. https://doi.org/https://doi.org/10.1016/j.neuropharm.2017.07.024

Stern, C. A. J., Gazarini, L., Vanvossen, A. C., Zuardi, A. W., Galve-Roperh, I., Guimaraes, F. S., . . . Bertoglio, L. J. (2015). Δ9-Tetrahydrocannabinol alone and combined with cannabidiol mitigate fear memory through reconsolidation disruption. *European Neuropsychopharmacology*, *25*(6), 958-965. https://doi.org/https://doi.org/10.1016/j.euroneuro.2015.02.001

Stiedl, O., Misane, I., Spiess, J., & Ogren, S. O. (2000). Involvement of the 5-HT1A receptors in classical fear conditioning in C57BL/6J mice. *J Neurosci*, *20*(22), 8515-8527. https://doi.org/10.1523/jneurosci.20-22-08515.2000

Stoddard JR. F.J. , Milad M.R & Ursano R.J. (2018). *Trauma- and Stressor- Related Disorders*. Oxford University Press.

Stollhoff, N., & Eisenhardt, D. (2009). Consolidation of an Extinction Memory Depends on the Unconditioned Stimulus Magnitude Previously Experienced during Training. *The Journal of Neuroscience*, *29*(30), 9644. https://doi.org/10.1523/JNEUROSCI.0495-09.2009

Stubbendorff, C., Hale, E., Cassaday, H. J., Bast, T., & Stevenson, C. W. (2019). Dopamine D1-like receptors in the dorsomedial prefrontal cortex regulate contextual fear conditioning. *Psychopharmacology*, *236*(6), 1771-1782. https://doi.org/10.1007/s00213-018-5162-7

Sutherland, E., Mercer, S. M., Everist, M., & Leaist, D. G. (2009). Diffusion in Solutions of Micelles. What Does Dynamic Light Scattering Measure? *Journal of Chemical & Engineering Data*, *54*(2), 272-278. https://doi.org/10.1021/je800284g

Suzuki, A., Josselyn, S. A., Frankland, P. W., Masushige, S., Silva, A. J., & Kida, S. (2004). Memory reconsolidation and extinction have distinct temporal and biochemical signatures. *J Neurosci*, *24*(20), 4787-4795. https://doi.org/10.1523/jneurosci.5491-03.2004

Sánchez, A. J., & García-Merino, A. (2012). Neuroprotective agents: Cannabinoids. *Clinical Immunology*, *142*(1), 57-67. https://doi.org/https://doi.org/10.1016/j.clim.2011.02.010

Tan, H., Lauzon, N. M., Bishop, S. F., Chi, N., Bechard, M., & Laviolette, S. R. (2011). Cannabinoid Transmission in the Basolateral Amygdala Modulates Fear Memory Formation via Functional Inputs to the Prelimbic Cortex. *The Journal of Neuroscience*, *31*(14), 5300. https://doi.org/10.1523/JNEUROSCI.4718-10.2011

Tapias-Espinosa, C., Kádár, E., & Segura-Torres, P. (2018). Spaced sessions of avoidance extinction reduce spontaneous recovery and promote infralimbic cortex activation. *Behav Brain Res*, *336*, 59-66. https://doi.org/https://doi.org/10.1016/j.bbr.2017.08.025

Tarrier, N., & Gregg, L. (2004). Suicide risk in civilian PTSD patients--predictors of suicidal ideation, planning and attempts. *Soc Psychiatry Psychiatr Epidemiol*, *39*(8), 655-661. https://doi.org/10.1007/s00127-004-0799-4

Thompson, A. J., & Lummis, S. C. (2006). 5-HT3 receptors. *Curr Pharm Des*, *12*(28), 3615-3630. https://doi.org/10.2174/138161206778522029

Totty, M. S., Payne, M. R., & Maren, S. (2019). Event boundaries do not cause the immediate extinction deficit after Pavlovian fear conditioning in rats. *Scientific Reports*, *9*(1), 9459. https://doi.org/10.1038/s41598-019-46010-4

Tovote, P., Fadok, J. P., & Luthi, A. (2015). Neuronal circuits for fear and anxiety. *Nat Rev Neurosci*, *16*(6), 317-331. https://doi.org/10.1038/nrn3945

Tryon, S. C., Sakamoto, I. M., Kellis, D. M., Kaigler, K. F., & Wilson, M. A. (2021). Individual Differences in Conditioned Fear and Extinction in Female Rats. *Front Behav Neurosci*, *15*, 740313. https://doi.org/10.3389/fnbeh.2021.740313

Tsai, L.-H., & Gräff, J. (2014). On the resilience of remote traumatic memories against exposure therapy-mediated attenuation [https://doi.org/10.15252/embr.201438913]. *EMBO reports*, *15*(8), 853-861. https://doi.org/https://doi.org/10.15252/embr.201438913

Tsuboi, K., Uyama, T., Okamoto, Y., & Ueda, N. (2018). Endocannabinoids and related N-acylethanolamines: biological activities and metabolism. *Inflammation and regeneration*, *38*, 28-28. https://doi.org/10.1186/s41232-018-0086-5

Tsuda, M. C., Mahdi, S., Namchuk, A., Wu, T. J., & Lucki, I. (2020). Vendor differences in anxiety-like behaviors in female and male Sprague Dawley rats. *Physiology & Behavior*, *227*, 113131. https://doi.org/https://doi.org/10.1016/j.physbeh.2020.113131

Tumolo, J. M., Kutlu, M. G., & Gould, T. J. (2018). Chronic nicotine differentially alters spontaneous recovery of contextual fear in male and female mice. *Behav Brain Res*, *341*, 176-180. https://doi.org/https://doi.org/10.1016/j.bbr.2018.01.002

Uhernik, A. L., Montoya, Z. T., Balkissoon, C. D., & Smith, J. P. (2018). Learning and memory is modulated by cannabidiol when administered during trace fear-conditioning. *Neurobiol Learn Mem*, *149*, 68-76. https://doi.org/10.1016/j.nlm.2018.02.009

Ujváry, I., & Hanuš, L. (2016). Human Metabolites of Cannabidiol: A Review on Their Formation, Biological Activity, and Relevance in Therapy. *Cannabis and cannabinoid research*, *1*(1), 90-101. https://doi.org/10.1089/can.2015.0012

Uliana, D. L., Hott, S. C., Lisboa, S. F., & Resstel, L. B. (2016). Dorsolateral periaqueductal gray matter CB1 and TRPV1 receptors exert opposite modulation on expression of contextual fear conditioning. *Neuropharmacology*, *103*, 257-269. https://doi.org/10.1016/j.neuropharm.2015.12.020

Ulloa, R.-E., Díaz-Valderrama, A., Herrera-Pérez, J., León-Olea, M., & Martínez-Mota, L. (2014). Age differences in the impact of forced swimming test on serotonin transporter levels in lateral septum and dorsal raphe. *Behavioral and Brain Functions*, *10*(1), 3. https://doi.org/10.1186/1744-9081-10-3

Urcelay, G. P., Wheeler, D. S., & Miller, R. R. (2009). Spacing extinction trials alleviates renewal and spontaneous recovery. *Learning & Behavior*, *37*(1), 60-73. https://doi.org/10.3758/LB.37.1.60

Urquhart, P., Nicolaou, A., & Woodward, D. F. (2015). Endocannabinoids and their oxygenation by cyclo-oxygenases, lipoxygenases and other oxygenases. *Biochim Biophys Acta*, *1851*(4), 366-376. https://doi.org/10.1016/j.bbalip.2014.12.015

Valentino, R. J., & Van Bockstaele, E. (2008). Convergent regulation of locus coeruleus activity as an adaptive response to stress. *Eur J Pharmacol*, *583*(2-3), 194-203. https://doi.org/10.1016/j.ejphar.2007.11.062

Van Bockstaele, E. J., Bajic, D., Proudfit, H., & Valentino, R. J. (2001). Topographic architecture of stress-related pathways targeting the noradrenergic locus coeruleus. *Physiol Behav*, *73*(3), 273-283. https://doi.org/10.1016/s0031-9384(01)00448-6

VanElzakker, M. B., Kathryn Dahlgren, M., Caroline Davis, F., Dubois, S., & Shin, L. M. (2014). From Pavlov to PTSD: The extinction of conditioned fear in rodents, humans, and anxiety disorders. *Neurobiology of Learning and Memory*, *113*, 3-18. https://doi.org/https://doi.org/10.1016/j.nlm.2013.11.014

Vasquez, J. H., Leong, K. C., Gagliardi, C. M., Harland, B., Apicella, A. j., & Muzzio, I. A. (2019). Pathway specific activation of ventral hippocampal cells projecting to the prelimbic cortex diminishes fear renewal. *Neurobiology of Learning and Memory*, *161*, 63-71. https://doi.org/https://doi.org/10.1016/j.nlm.2019.03.003

Verma, D., Jamil, S., Tasan, R. O., Lange, M. D., & Pape, H.-C. (2019). Single stimulation of Y2 receptors in BNSTav facilitates extinction and dampens reinstatement of fear. *Psychopharmacology*, *236*(1), 281-291. https://doi.org/10.1007/s00213-018-5080-8

Viudez-Martínez, A., García-Gutiérrez, M. S., & Manzanares, J. (2018). Cannabidiol regulates the expression of hypothalamus-pituitary-adrenal axis-related genes in response to acute restraint stress. *J Psychopharmacol*, *32*(12), 1379-1384. https://doi.org/10.1177/0269881118805495

Wang, C., Stratton, P. G., Sah, P., & Marek, R. (2022). Theta coupling within the medial prefrontal cortex regulates fear extinction and renewal. *iScience*, *25*(10), 105036. https://doi.org/10.1016/j.isci.2022.105036

Wang, C.-M., Zhang, Y.-F., Lin, Z.-Q., Cai, Y.-F., Fu, X.-Y., & Lin, Z.-H. (2020). Pre-extinction activation of hippocampal AMPK prevents fear renewal in mice. *Pharmacological Research*, *161*, 105099. https://doi.org/https://doi.org/10.1016/j.phrs.2020.105099

Wang, H., Xing, X., Liang, J., Bai, Y., Lui, Z., & Zheng, X. (2014). High-dose corticosterone after fear conditioning selectively suppresses fear renewal by reducing anxiety-like response. *Pharmacology Biochemistry and Behavior*, *124*, 188-195. https://doi.org/https://doi.org/10.1016/j.pbb.2014.06.003

Wang, P. S., Angermeyer, M., Borges, G., Bruffaerts, R., Tat Chiu, W., De Girolamo, G., . . . Ustün, T. B. (2007). Delay and failure in treatment seeking after first onset of mental disorders in the World Health Organization's World Mental Health Survey Initiative. *World psychiatry : official journal of the World Psychiatric Association (WPA)*, *6*(3), 177-185.

Wang, W., Zhang, K., Yan, S., Li, A., Hu, X., Zhang, L., & Liu, C. (2011). Enhancement of apamin-sensitive medium afterhyperpolarization current by anandamide and its role in excitability control in cultured hippocampal neurons. *Neuropharmacology*, *60*(6), 901-909. https://doi.org/10.1016/j.neuropharm.2011.01.027

Warren, W. G., Papagianni, E. P., Stevenson, C. W., & Stubbendorff, C. (2022). In it together? The case for endocannabinoid–noradrenergic interactions in fear extinction [https://doi.org/10.1111/ejn.15200]. *European Journal of Neuroscience*, *55*(4), 952-970. https://doi.org/https://doi.org/10.1111/ejn.15200

Warthen, D. M., Wiltgen, B. J., & Provencio, I. (2011). Light enhances learned fear. *Proc Natl Acad Sci U S A*, *108*(33), 13788-13793. https://doi.org/10.1073/pnas.1103214108

Watkins, L. E., Sprang, K. R., & Rothbaum, B. O. (2018). Treating PTSD: A Review of Evidence-Based Psychotherapy Interventions [Review]. *Frontiers in Behavioral Neuroscience*, *12*(258). https://doi.org/10.3389/fnbeh.2018.00258

Wilber, A. A., Walker, A. G., Southwood, C. J., Farrell, M. R., Lin, G. L., Rebec, G. V., & Wellman, C. L. (2011). Chronic stress alters neural activity in medial prefrontal cortex during retrieval of extinction. *Neuroscience*, *174*, 115-131. https://doi.org/https://doi.org/10.1016/j.neuroscience.2010.10.070

Williams, J. T., Bobker, D. H., & Harris, G. C. (1991). Chapter 11 - Synaptic potentials in locus coeruleus neurons in brain slices. In C. D. Barnes & O. Pompeiano (Eds.), *Progress in Brain Research* (Vol. 88, pp. 167-172). Elsevier. https://doi.org/https://doi.org/10.1016/S0079-6123(08)63806-6

Wong, D. T., Perry, K. W., & Bymaster, F. P. (2005). The Discovery of Fluoxetine Hydrochloride (Prozac). *Nature Reviews Drug Discovery*, *4*(9), 764-774. https://doi.org/10.1038/nrd1821

Woods, A. M., & Bouton, M. E. (2008). Immediate extinction causes a less durable loss of performance than delayed extinction following either fear or appetitive conditioning. *Learn Mem*, *15*(12), 909-920. https://doi.org/10.1101/lm.1078508

Yadav, M., Parle, M., Sharma, N., Dhingra, S., Raina, N., & Jindal, D. K. (2017). Brain targeted oral delivery of doxycycline hydrochloride encapsulated Tween 80 coated chitosan nanoparticles against ketamine induced psychosis: behavioral, biochemical, neurochemical and histological alterations in mice. *Drug Delivery*, *24*(1), 1429-1440. https://doi.org/10.1080/10717544.2017.1377315

Yang, C.-h., Shi, H.-s., Zhu, W.-l., Wu, P., Sun, L.-l., Si, J.-j., . . . Yang, J.-l. (2012). Venlafaxine facilitates between-session extinction and prevents reinstatement of auditory-cue conditioned fear. *Behavioural Brain Research*, *230*(1), 268-273. https://doi.org/https://doi.org/10.1016/j.bbr.2012.02.023

Yoshioka, M., Matsumoto, M., Togashi, H., & Saito, H. (1995). Effects of conditioned fear stress on 5-HT release in the rat prefrontal cortex. *Pharmacology Biochemistry and Behavior*, *51*(2), 515-519. https://doi.org/https://doi.org/10.1016/0091-3057(95)00045-X

Youn, J., Hager, T., Misane, I., Pieneman, A. W., Jansen, R. F., Ogren, S. O., . . . Stiedl, O. (2013). Central 5-HT1A receptor-mediated modulation of heart rate dynamics and its

adjustment by conditioned and unconditioned fear in mice. *Br J Pharmacol*, *170*(4), 859-870. https://doi.org/10.1111/bph.12325

Youn, J., Misane, I., Eriksson, T. M., Millan, M. J., Ogren, S. O., Verhage, M., & Stiedl, O. (2009). Bidirectional modulation of classical fear conditioning in mice by 5-HT(1A) receptor ligands with contrasting intrinsic activities. *Neuropharmacology*, *57*(5-6), 567-576. https://doi.org/10.1016/j.neuropharm.2009.07.011

Zanelati, T. V., Biojone, C., Moreira, F. A., Guimaraes, F. S., & Joca, S. R. (2010). Antidepressant-like effects of cannabidiol in mice: possible involvement of 5-HT1A receptors. *Br J Pharmacol*, *159*(1), 122-128. https://doi.org/10.1111/j.1476-5381.2009.00521.x

Zhang, G., & Stackman, R. W. (2015). The role of serotonin 5-HT2A receptors in memory and cognition [Review]. *Frontiers in Pharmacology*, *6*.

Zhang, G., Ásgeirsdóttir, H. N., Cohen, S. J., Munchow, A. H., Barrera, M. P., & Stackman, R. W. (2013). Stimulation of serotonin 2A receptors facilitates consolidation and extinction of fear memory in C57BL/6J mice. *Neuropharmacology*, *64*, 403-413. https://doi.org/https://doi.org/10.1016/j.neuropharm.2012.06.007

Zhang, H., Yao, M., Morrison, R. A., & Chong, S. (2003). Commonly used surfactant, Tween 80, improves absorption of P-glycoprotein substrate, digoxin, in rats. *Archives of Pharmacal Research*, *26*(9), 768-772. https://doi.org/10.1007/BF02976689

Zhang, X., Kim, J., & Tonegawa, S. (2020). Amygdala Reward Neurons Form and Store Fear Extinction Memory. *Neuron*, *105*(6), 1077-1093.e1077. https://doi.org/https://doi.org/10.1016/j.neuron.2019.12.025

Zhang, Z., Wang, W., Zhong, P., Liu, S. J., Long, J. Z., Zhao, L., . . . Liu, Q. S. (2015). Blockade of 2-arachidonoylglycerol hydrolysis produces antidepressant-like effects and enhances adult hippocampal neurogenesis and synaptic plasticity. *Hippocampus*, *25*(1), 16-26. https://doi.org/10.1002/hipo.22344

Zhao, H., Lin, Y., Chen, S., Li, X., & Huo, H. (2018). 5-HT3 Receptors: A Potential Therapeutic Target for Epilepsy. *Curr Neuropharmacol*, *16*(1), 29-36. https://doi.org/10.2174/1570159x15666170508170412

Zhao, Y., Bijlsma, E. Y., ter Heegde, F., Verdouw, M. P., Garssen, J., Newman-Tancredi, A., & Groenink, L. (2019). Activation of somatodendritic 5-HT1A autoreceptors reduces the acquisition and expression of cued fear in the rat fear-potentiated startle test. *Psychopharmacology*, *236*(4), 1171-1185. https://doi.org/10.1007/s00213-018-5124-0

Zuideveld, K. P., Treijtel, N., Maas, H. J., Gubbens-Stibbe, J. M., Peletier, L. A., van Der Graaf, P. H., & Danhof, M. (2002). A competitive interaction model predicts the effect of WAY-100,635 on the time course of R-(+)-8-hydroxy-2-(di-n-propylamino)tetralin-induced hypothermia. *J Pharmacol Exp Ther*, *300*(1), 330-338. https://doi.org/10.1124/jpet.300.1.330

Åhs, F., Frick, A., Furmark, T., & Fredrikson, M. (2015). Human serotonin transporter availability predicts fear conditioning. *International Journal of Psychophysiology*, *98*(3, Part 2), 515-519. https://doi.org/https://doi.org/10.1016/j.ijpsycho.2014.12.002

Ögren, S. O., & Stiedl, O. (2010). Passive Avoidance. In I. P. Stolerman (Ed.), *Encyclopedia of Psychopharmacology* (pp. 960-967). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-540-68706-1_160