Endocannabinoid Regulation of Learned Fear Inhibition

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Abstract

Post-traumatic stress disorder and other anxiety-related disorders are characterised by impaired extinction and symptom relapse. Preclinical research into fear and extinction learning provides a translatable model for clinical settings. The endocannabinoid system is involved in extinction learning. However, research regarding endocannabinoid metabolism inhibition in extinction impairment and fear relapse is lacking. Spontaneous fear recovery, the return of learned fear over time after successful extinction, models fear relapse. Immediate extinction following fear conditioning impairs later extinction recall. Male Lister Hooded rats underwent Pavlovian fear conditioning in paradigms of spontaneous fear recovery or immediate extinction. The endocannabinoid metabolism inhibitors, URB597 or JZL184 (which increase the levels of anandamide and 2-arachidonoylglycerol, respectively) were administered intraperitoneally before or after extinction training depending on the paradigm. A positive control experiment used the β-adrenergic receptor antagonist, propranolol, in the immediate extinction paradigm. Neither URB597 nor JZL184 prevented fear relapse, nor did they rescue extinction impairments. A high dose of JZL184 acutely impaired immediate extinction acquisition. Propranolol acutely reduced baseline freezing prior to extinction training, facilitated immediate extinction acquisition, but did not rescue extinction impairment. These results suggest that endocannabinoid metabolism inhibition may not affect fear relapse and extinction impairment within the current paradigms. However, factors such as drug timing (e.g., when treatment occurred in relation to behavioural training or testing) and rat characteristics (e.g., strain, sex, and age) may have affected the results. Future research should address these potential confounds to assess the potential benefit of endocannabinoid metabolism inhibition in fear relapse and impaired extinction.

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Abbreviations

- 2-AG - 2-arachidonoylglycerol
- 5-HT - serotonin
- AEA - anandamide
- AMG - amygdala
- ANOVA - analysis of variance
- AR - adrenoreceptor
- BA - basal amygdala
- BLA - basolateral amygdala
- BNST - bed nucleus of the stria terminalis
- BZD - benzodiazepine
- Ca^{2+} - calcium
- cAMP - cyclic adenosine monophosphate
- CB1R - cannabinoid receptor type one
- $CB1R^{-/-}$ - cannabinoid receptor type one deficient
- CB2R - cannabinoid receptor type two
- CBD - cannabidiol
- CBT - cognitive behavioural therapy
- CCK - cholecystokinin
- CeA - central amygdala
- CFR - conditioned fear response
- CNS - central nervous system
- CRF - corticotropin-releasing factor

- CRFR1 - corticotropin-releasing factor receptor type 1
- CRFR2 - corticotropin-releasing factor receptor type 2
- CS - conditioned stimulus
- DβH - dopamine-β-hydroxylase
- DAGL - diacylglycerol lipase
- dHPC - dorsal hippocampus
- eCB - endocannabinoid
- ERK - extracellular signal-regulated kinase
- FAAH - fatty acid amide hydrolase
- GABA - gamma-aminobutyric acid
- GAD - generalised anxiety disorder
- GI - gastro-intestinal
- Glu - glutamate
- GPCR - G-protein coupled receptor
- HPA - hypothalamic-pituitary-adrenal axis
- HPC - hippocampus
- IA - inhibitory avoidance
- IED - immediate extinction deficit
- IL - infralimbic cortex
- IPSC - inhibitory post-synaptic current
- ITI - inter-trial interval
- KO - knockout
- LC - locus coeruleus

- LTD - long-term depression
- LTP - long-term potentiation
- MAGL - monoacylglycerol lipase
- MDMA - 3,4-methylenedioxymethamphetamine
- mPFC - medial prefrontal cortex
- NA - noradrenaline
- NET - noradrenaline transporter
- NMDA - *N*-methyl-D-aspartate
- OCD - obsessive-compulsive disorder
- PAG - periaqueductal grey
- PD - panic disorder
- PEG - polyethylene glycol
- PFC - prefrontal cortex
- PL - prelimbic cortex
- PTSD - post-traumatic stress disorder
- PVN - paraventricular nucleus
- RSC - retrosplenial cortex
- SAD - social anxiety disorder
- SNRI - selective noradrenaline reuptake inhibitor
- SSRI - selective serotonin reuptake inhibitor
- THC - Δ9-tetrahydrocannabinol
- TRPV1 - transient receptor potential vanilloid type 1
- US - unconditioned stimulus

- vHPC - ventral hippocampus

1. General Introduction

1.1. Anxiety Disorders

1.1.1. Overview

Anxiety disorders are characterised by excessive and persistent fear and/or anxiety. Anxiety is an emotional response evoked by the anticipation of a perceived threat, whilst fear results from imminent threat or danger. The disorders manifest as a maladaptive emotional response to a perceived threat in the external (e.g., social settings) and/or internal (e.g., somatic symptoms) environment. Anxiety disorders include generalised anxiety disorder (GAD), social anxiety disorder (SAD), panic disorder (PD), phobias, agoraphobia, and separation anxiety. Whilst related to anxiety disorders, post-traumatic stress disorder (PTSD) and obsessive-compulsive disorder (OCD) are now classified as distinct conditions in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (American Psychiatric Association, 2013). Over 50% of anxiety disorder patients present with comorbid anxiety disorders (e.g., PD, SAD, and agoraphobia), whilst comorbidity with depression, substance abuse, and other mental health conditions is also common (Alonso et al., 2004; Kessler et al., 2005; Onaemo et al., 2021; Simon et al., 2004; Weber et al., 2021). All anxiety-related disorders are characterised by psychosocial dysfunction and poor quality of life. Avoidance behaviours are commonly associated with anxiety disorders, ranging from mild (e.g., avoidance of performance situations such as public speaking) to severe (e.g., withdrawal from all social situations). Occasionally, patients may suffer from panic attacks as the result of a transient, but substantial, fear response (Sheehan, 1982). Psychological symptoms can include fear/anxiety, irritability, restlessness, and sleep disturbances, whilst somatic symptoms include motor restlessness and sympathetic hyperactivity (e.g., gastro-intestinal [GI] disturbances, heart palpitations, and nausea; Kogan et al., 2016). Such symptomology explains why anxiety disorders are recognised as a principal cause of disability (Haro et al., 2014; Wittchen et al., 2011).

Lifetime prevalence rates suggest 25%-34% of individuals will suffer, or have suffered, from an anxiety-related disorder (inclusive of PTSD and OCD; Kessler et al., 2007, 2012). Additionally, global prevalence rates suggest that around 14% will suffer from an anxiety-related disorder within a given year (Baxter et al., 2013). During the coronavirus pandemic, an estimated 46% of 25,557 individuals (across eight countries) experienced anxiety symptoms (da Silva et al., 2021). The high prevalence of anxiety-related disorders, coupled with their debilitative nature, can lead to a significant socio-economic burden. Lost work productivity and healthcare form but some of the economic expenses, whilst strained interpersonal relationships and withdrawal may contribute to social costs. In 2010, over 60 million Europeans suffered from an anxiety-related disorder (Wittchen et al., 2011), whilst the estimated cost to the continent was over 74 billion euros (Haro et al., 2014). Effective treatment of these disorders is therefore essential, not only for the patient but society at large.

1.1.2. Psychological Treatment of Anxiety Disorders

Current psychological treatments include, but are not limited to, cognitive behavioural therapy (CBT), interpersonal psychotherapy, and mindfulness/ acceptance-based therapies. Of these, CBT is most efficacious and cost-effective. It is currently a first-line treatment in multiple countries (Bandelow et al., 2015; Cuijpers et al., 2014; Katzman et al., 2014; National Institute for Health and Care Excellence, 2014). CBT, a short-term talking therapy, focuses on challenging maladaptive thought processes and behavioural patterns to reduce psychological suffering (Hofmann et al., 2013). CBT offers health benefits in children, adolescents, adults, and elderly patients. However, its therapeutic effects vary across different anxiety-related disorders. Randomised placebo-controlled trials show that CBT is largely beneficial for OCD, GAD, and acute stress disorder, but only moderately effective for PTSD, SAD, and PD (Carpenter et al., 2018). Around 50% of patients experience symptom amelioration to within a normative range after CBT therapy (Loerinc et al., 2015). However, 23.8% experience relapse of anxiety symptoms following CBT (Lorimer et al., 2021).

Exposure therapy, a derivative of CBT, is a mainstay treatment for specific phobia and PTSD (Choy et al., 2007; Hofmann and Smits, 2008; Nemeroff et al., 2006). Within exposure therapy, patients are repeatedly confronted with fear-inducing yet safe aversive stimuli. The interventions may be brief or prolonged and gradual or intense (Meuret et al., 2012). Exposure therapy promotes the extinction of traumatic memories, a process by which the maladaptive emotional response to the memory is reduced. Despite exposure therapy's proven efficacy, relapse poses a major challenge to long-term clinical outcomes. The return of fear is characterised as the reappearance of a diminished or extinguished fear, affecting patients who have not been re-exposed to the initial fear stimulus (Rachman, 1979). A significant number of patients do not experience symptom amelioration after exposure therapy (Arch and Craske, 2009). The therapeutic alliance between patient and therapist is also a crucial prognostic variable (Buchholz and Abramowitz, 2020). Anxiety disorder patients commonly show deficient inhibitory neural regulation, which is required for extinction learning (Craske et al., 2008). As such, these deficits not only beget patients' poor response rates to exposure therapy but may also underlie the symptomatic development of fear and anxiety (Indovina et al., 2011).

1.1.3. Pharmacological Treatment of Anxiety Disorders

Pharmacological treatments are used alone or in conjunction with psychological interventions. Treating both psychological and somatic symptoms, current therapeutics target the putative neuropharmacological underpinnings of anxiety-related disorder pathophysiology, e.g., gamma-aminobutyric acid (GABA)-ergic, serotonergic, and adrenergic systems (Griebel and Holmes, 2013; Murrough et al., 2015). Anti-depressants, such as selective serotonin reuptake inhibitors (SSRIs), are the first-line pharmacological treatment for anxiety-related disorders due to their favourable safety profile, low abuse potential, and efficacious performance in randomised controlled trials (Ravindran and Stein, 2010). SSRIs are most commonly prescribed, with selective noradrenaline reuptake inhibitors (SNRIs) favoured in the absence of SSRI efficacy. However, SSRIs and SNRIs are not without their drawbacks. Delayed therapeutic effects and an array of transient side-effects (e.g., worsened

anxiety, GI disturbances, jitteriness, insomnia, and headache; Ferguson, 2001) mandate research for better treatment options. Tricyclic anti-depressants and monoamine oxidase inhibitors are also available; however, use of these drugs is receding due to their safety risk and poor tolerability (Sadock et al., 2017).

Benzodiazepines (BZDs) may be used to treat anxiety-related disorders (Baldwin et al., 2014; Katzman et al., 2014). Whilst efficacious, BZDs are associated with several adverse effects including drowsiness, dizziness, anterograde amnesia, tolerance, abuse potential, and overdose (Bachhuber et al., 2016; Ballenger, 2000; Lader, 1999). Not only this, but withdrawal from BZDs is also dangerous and can lead to severe, protracted symptoms such as insomnia, GI irritation, suicidal ideation, and seizures (Chouinard, 2004). Due to such issues, the use of BZDs is now limited to the short-term management of acute anxiety (Bystritsky et al., 2013). BZDs may exacerbate PTSD symptoms and decrease the efficacy of adjunct psychological treatments (Guina et al., 2015). However, a meta-analysis found that BZDs are of equivalent safety and efficacy to anti-depressants in generalised anxiety disorder patients that respond to an 8-week BZD treatment programme (Shinfuku et al., 2019). These data suggest a differential response of BZDs in the various anxiety disorders.

Less common treatments include anti-epileptic drugs (e.g., pregabalin, gabapentin), atypical antipsychotics (e.g., risperidone, quetiapine), serotonin$_{1a}$ (5-HT$_{1A}$) receptor agonists (e.g., buspirone), β-adrenergic blockers (e.g., propranolol), and peptide hormones (e.g., oxytocin). These drugs show selective efficacy between the different anxiety-related disorders. For example, buspirone, pregabalin, and quetiapine ameliorate GAD symptoms (Baldwin et al., 2015; Sadock et al., 2017), whilst propranolol and oxytocin are more efficacious when improving specific aspects of SAD (Guastella et al., 2009; Steenen et al., 2016). However, serious side-effects are still of great concern. For example, atypical antipsychotics are commonly associated with metabolic syndrome and rapid weight gain (Brecher and Geller, 1997).

Research into 3,4-methylenedioxymethamphetamine (MDMA; monoamine uptake inhibitor and receptor agonist) assisted psychotherapy for PTSD has emerged

in recent years, with a meta-analysis confirming its efficacy compared to prolonged exposure therapy alone (Amoroso and Workman, 2016). A recent phase three clinical trial found MDMA to be extremely efficacious and well tolerated in patients with severe PTSD (Mitchell et al., 2021). However, MDMA consumption may lead to deleterious neurotoxic effects (Moratalla et al., 2017; Parrott, 2013, 2014). Ketamine (*N*-methyl-D-aspartate (NMDA) antagonist) has also been investigated as a potential treatment for PTSD. However, there is little evidence of ketamine's therapeutic effect when used as a stand-alone treatment in comorbid depression and PTSD or as an adjunct to psychotherapy in PTSD alone (Varker et al., 2021).

Overall, the evidence suggests that whilst current treatment options are efficacious in the treatment of anxiety disorders, they are not without their drawbacks. For example, relapse is commonplace in psychotherapy, whilst side-effect profiles of pharmacological treatments are far from favourable. Safer and more tolerable pharmacological interventions are required for the treatment of anxiety-related disorders. With this, there may be potential for combined pharmacological and psychological treatments to yield more efficacious results than either one alone.

1.2. Animal Models of Learned Fear

1.2.1. Fear Conditioning

As mentioned above, anxiety is evoked by perceived or anticipated threat, whilst fear results from imminent threat or danger. This discrepancy underpins anxiety's complex pathophysiology and leads to difficulties when trying to induce a consistent anxiety response under experimental conditions. Despite this, the neural circuitry underpinning fear and anxiety is comparable, with research into fear providing a sound base for the further understanding of anxiety (Tovote et al., 2015).

Pavlovian fear conditioning is used to study learned fear in both humans and animals. The cornerstone of fear conditioning is the pairing of a neutral, conditioned stimulus (CS; e.g., tone or context) with a noxious, unconditioned stimulus (US; e.g., footshock). Through this process, a conditioned fear response (CFR) to the CS is induced, leaving a robust behavioural output characterised by heightened defensive

behaviour (e.g., freezing), elevated glucocorticoid levels, and sympathetic activation (Fanselow, 1980; Fendt and Fanselow, 1999; LeDoux, 2000; Maren, 2001). Typical fear acquisition training involves repeated exposure to multiple CS-US pairings, leading to a gradual increase in the CFR. In the hours following acquisition, the memory trace is stabilised through consolidation. This process is mediated by late-phase long-term potentiation (LTP), a form of synaptic plasticity that underlies long-term memory (Bramham and Messaoudi, 2005). As the CS takes on a negative valence through the associative learning process, subsequent exposure to the CS alone initially induces retrieval of the consolidated fear memory, resulting in behavioural expression of the CFR.

In rodents, the neural pathway that underpins auditory fear conditioning begins with integration of the footshock and tone stimuli in the lateral amygdala (AMG); a crucial site for synaptic plasticity and NMDA-dependent LTP during fear acquisition (LeDoux, 2000; Maren, 2001). This signal is then relayed to the central amygdala (CeA) via excitatory neurons in the basolateral amygdala (BLA; Janak and Tye, 2015). The BLA neurons are activated via excitatory inputs from the prelimbic cortex (PL) of the medial prefrontal cortex (mPFC). Activation of PL neurons is significantly correlated with cued fear expression (Anglada-Figueroa and Quirk, 2005; Gilmartin and McEchron, 2005; Giustino et al., 2016a). Ultimately, projections from the CeA to the periaqueductal grey (PAG), hypothalamus, and brainstem regulate activity in the latter brain areas, which then mediate the behavioural and physiological components of the CFR (Carrive et al., 1997, 1999; LeDoux et al., 1988). During contextual fear conditioning, synaptic plasticity in the basal amygdala (BA) encodes the association between the context and US. The contextual fear memory is subsequently processed and regulated through the dorsal hippocampus (dHPC), which projects to the BLA and mPFC via the ventral hippocampus (vHPC; Maren et al., 2013).

1.2.2. Fear Extinction

Extinction is a form of inhibitory learning that suppresses the CFR associated with the aversive memory (Abramowitz, 2013; Hofmann, 2008; Morrison and Ressler, 2014).

In a Pavlovian paradigm, fear extinction training dissociates the aversive qualities of the US from the CS. This is achieved by repeated exposure to the CS alone sometime after conditioning. Typical examples of fear extinction training include repeatedly playing a tone that has previously been paired with a footshock, or repeated or prolonged exposure to a context in which fear conditioning has taken place (Maren et al., 2013). With successful extinction, the CS no longer predicts the onset of the US, as a new extinction memory is formed alongside the existing fear memory (Bouton, 1993). After extinction training, the extinction memory is consolidated in a process that lasts for several hours. This involves numerous molecular processes that serve to strengthen and stabilise the memory, ensuring the subject learns that the CS is no longer aversive (Schafe et al., 2001). This process underpins a form of inhibitory learning in which the newly acquired extinction memory competes with the fear memory to control behaviour. The CS resumes a neutral valence, resulting in a dampened CFR (Chang et al., 2009; Pavlov, 1927). However, fear extinction not only requires the successful acquisition of the extinction memory but also its long-term retention and recall.

Much of the neural circuitry involved in fear extinction is shared with fear conditioning. Extinction in rodents involves the recruitment of the infralimbic cortex (IL; Sotres-Bayon and Quirk, 2010). IL projections to the AMG and hippocampus (HPC) are implicated in extinction acquisition and expression (Bloodgood et al., 2018; Bukalo et al., 2015; Do-Monte et al., 2015; Likhtik et al., 2005, 2008; Ramanathan and Maren, 2019; Ramanathan et al., 2018). The IL inhibits the CFR by recruiting GABAergic interneurons in the AMG known as the intercalated cells (Hagihara et al., 2021). These inhibitory neurons project to the medial division of the CeA, reducing CeA output to downstream targets and dampening fear expression. The vHPC also encodes the extinction context and inhibits the PL via inhibitory GABA neurons, thus preventing BLA activation and the subsequent CFR (Sierra-Mercado et al., 2011; Wotjak and Pape, 2013). However, vHPC gating of the PL-BLA circuitry induces fear expression (Jin and Maren, 2015; Kim and Cho, 2017; Orsini et al., 2011; Sotres-Bayon et al., 2012). Glu synaptic plasticity in the BLA underpins extinction learning as NMDA antagonism and mitogen-activated protein kinase / extracellular-signal regulated

kinase (ERK) deactivation in the BLA can prevent or attenuate extinction learning (Falls et al., 1992; Herry et al., 2006, Zimmerman and Maren, 2010). Given that the corresponding brain circuitry is active during human fear extinction (Delamater and Westbrook, 2014; Milad and Quirk, 2012), extinction training in rodents serves as a translational model for exposure therapy in humans (Bouton, 1988; Hofmann, 2007).

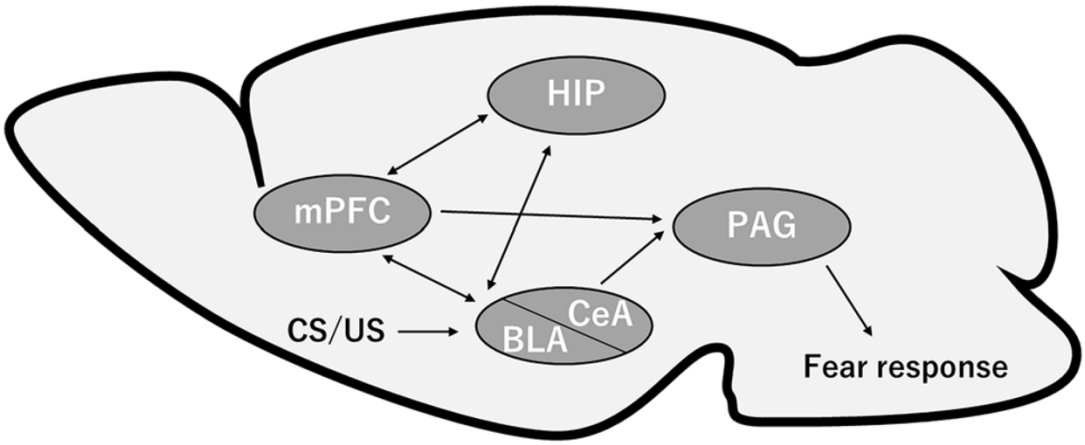

Figure 1.1. Brain regions involved in fear and extinction learning in the rat brain. The conditioned and unconditioned stimuli are processed in the basolateral amydala. This integrated information is then relayed to the central amygdala, which signals to the periaqueductal grey to elicit a fear response. The medial prefrontal cortex and hippocampus modulate the association between conditioned and unconditioned stimuli and aid in eliciting fear promotion or extinction. CS, conditioned stimulus; BLA, basolateral amygdala; CeA, central amygdala; HIP, hippocampus; mPFC, medial prefrontal cortex; PAG, periaqueductal grey; US, unconditioned stimulus. Mizuno and Matsuda, 2021.

1.2.3. Extinction Retention

Fear-related symptoms are susceptible to relapse after extinction-based therapies (Bouton, 1988; Rachman, 1979). Manifesting in distinct forms, these relapse phenomena are a hindrance to long-term clinical outcomes. For patients, relapse can be distressing. Fear and anxiety, once thought to be eradicated, may return in an insidious or immediate fashion under certain circumstances. With this, research into relapse prevention is important.

There are many ways in which a patient may experience relapse following an extinction-based therapy. Renewal occurs when a previously extinguished CS is encountered in a novel context (i.e., not the context in which the extinction training took place; Bouton, 2004; Vervliet et al., 2013). Reinstatement happens when the US (of equal, greater, or lesser amplitude than that used in fear conditioning) is re-encountered in any context (Bouton and Bolles, 1979). Aside from relapse, reacquisition is an active learning process that occurs following more conditioning trials after extinction training (Bouton, 2002).

The return of fear over time following extinction is called spontaneous fear recovery. This occurs after a period of non-reinforcement, i.e., when neither the CS nor the US are re-encountered. Spontaneous recovery is induced via CS presentations sometime after extinction training (e.g., 1-3 weeks later). The recovery of the CFR occurs because the initial fear conditioning memory remains intact following extinction training (Quirk, 2002; Rescorla, 2004). Sustained gamma oscillations in the BLA during extinction training predict the amount of later spontaneous recovery (Courtin et al., 2014). The enhancement of HPC neurogenesis following extinction training prevents spontaneous fear recovery, whilst ablating neurogenesis in the HPC after extinction training enhances spontaneous fear recovery (Martínez-Canabal et al., 2019). In a passive avoidance paradigm, a fear-based behavioural paradigm that measures the time it takes for an animal to re-enter a previously conditioned context, the AMG, piriform cortex, and entorhinal cortex show evidence of activation following spontaneous recovery (Huang et al., 2013). Animals with IL/PL lesions show

impaired spontaneous recovery, despite having normal patterns of fear conditioning and extinction (Zelinski et al., 2010).

Pavlov (1927) showed that larger time periods between appetitive extinction training and retention testing mediated greater spontaneous recovery. However, Rescorla (2004) found that shorter time periods between appetitive extinction training and retention testing induced more spontaneous recovery. Spontaneous recovery is similar to renewal given that recovery of the conditioned response occurs following a change in temporal context (Bouton, 1993). Re-encountering the extinction context prior to spontaneous recovery testing dampens relapse in both renewal and spontaneous recovery paradigms (Brooks and Bouton, 1993, 1994). Whilst spontaneous recovery is negated by repeated extinction (Rescorla, 2004), it is unlikely that all clinical patients will successfully adhere to long-term therapeutic treatments. Therefore, interventions that can suppress spontaneous recovery are essential.

1.2.4. Immediate Extinction

In fear extinction protocols, it is typical for extinction training to take place at least 24 hrs after fear conditioning (i.e., delayed extinction). However, extinction training may be conducted immediately following fear conditioning (e.g., 1 min to 6 hrs after). In some cases, such paradigms induce a phenomenon known as the immediate extinction deficit (IED; Chang and Maren, 2009; Maren and Chang, 2006; Totty et al., 2019). The IED is characterised by an inability to retain extinction memory despite a reduction of the CFR during extinction training. This results in an elevated CFR during an extinction retention test compared to subjects that undergo delayed extinction training. PTSD is associated with deficient extinction (Milad et al., 2009). Therefore, it may be of translational relevance to implement a paradigm such as the IED to model deficient extinction in a preclinical model. Using such a paradigm allows for pharmacological manipulation of deficient extinction with existing or novel treatments.

Research suggests that this deficit in extinction retention is caused by the level of emotional arousal or stress at the time of extinction training (Fitzgerald et al., 2015; Giustino et al., 2017, 2020; Kim et al., 2010; Maren and Chang, 2006; Totty et al., 2019). The IED involves the monoamine neurotransmitter noradrenaline (NA) and corticotropin-releasing factor (CRF; Maren, 2022), a neuropeptide that is involved in regulating neuroendocrine and behavioural stress responses (Deussing and Chen, 2018). Acute or chronic stress impairs delayed extinction (Chauveau et al., 2012; Hartley et al., 2014; Izquierdo et al., 2006; Knox et al., 2012; Maren and Holmes, 2016; Maroun et al., 2013; Miracle et al., 2006). For example, the delivery of unsignalled footshocks prior to delayed extinction training impairs extinction retention. This suggests high arousal at the time of extinction training inhibits later extinction retention (Maren and Chang et al., 2006). However, the IED can be induced under low levels of pre-extinction fear as evidenced by low baseline freezing prior to extinction training (Chang and Maren, 2011; Kim et al., 2010). It is important to note that the absence of fearful behaviour does not imply the absence of stress.

The IED can also be implemented in appetitive conditioning paradigms that do not induce a CFR (Merz and Wolf, 2019; Rescorla, 2004; Woods and Bouton, 2008). The neural circuitry underpinning the extinction of appetitive responding is analogous to that involved in the extinction of conditioned fear (Goode and Maren, 2019; Quirk and Mueller, 2008). Appetitive paradigms may induce some degree of stress, which is evidenced by NA efflux in the rat IL during the conditioning and extinction of appetitive cues (Mingote et al., 2004). However, humans express the IED in a non-emotional learning paradigm when acquisition and extinction are conducted in the same context (Merz and Wolf, 2019).

Various groups have implemented immediate extinction protocols, often with varying results. Myers et al. (2006) reported that when extinction training was conducted 10 mins after fear conditioning it prevented reinstatement, renewal, and spontaneous recovery of the fear memory. The authors posited that immediate fear extinction ‘erased’ the fear memory, or at least disrupted its consolidation. However, data from the Myers et al. (2006) paper suggest that the animals never established

an extinction memory, therefore there was no opportunity for fear relapse (Maren, 2022). Schiller et al. (2008) assessed whether short conditioning-extinction intervals could disrupt the reinstatement and spontaneous recovery of fear in rats and humans. The authors found that both delayed and immediate protocols showed successful extinction retention (i.e., failed to induce the IED). They also found that immediate extinction did not prevent reinstatement or spontaneous recovery of the fear memory, as shown by Myers et al. (2006). In a contextual fear conditioning paradigm, Archbold et al. (2010) failed to induce the IED. Ponnusamy et al. (2016) found that immediate extinction reduced cued freezing in extinction retention tests when compared to no extinction. The authors suggested that the use of vastly distinct conditioning and extinction contexts may have ameliorated the arousal state prior to extinction training, thus mitigating the IED.

The discrepancy between the results of these different groups may be explained by the acute level of fear induced by the conditioning procedure. For example, weak and single shock conditioning parameters are not sufficient to induce the IED (Maren and Chang, 2006). This is due to the rats experiencing a lower level of emotional arousal prior to the extinction training session. It appears that the IED, in certain circumstances, may require stress and emotional arousal prior to extinction training. This heightened arousal state prior to extinction training may encode the extinction memory independently of the context. In such cases, the animal is unable to retrieve the extinction memory as it has not been paired with the extinction context, resulting in expression of the IED (Maren, 2014). Despite this assumption, Johnson et al. (2010) showed that immediate extinction facilitated greater fear suppression in an extinction test conducted 7 days after extinction training (when compared to delayed extinction). Johnson et al. (2010) also found that immediate extinction induced greater fear expression in a retention test 48 hrs after extinction training (compared to delayed extinction). This suggests that immediate extinction may form a context-dependent extinction memory that can be incubated, and that the severity of the IED may also be influenced by temporal proximity to the original conditioning event.

The IED is underpinned by aberrant neuronal signalling in the mPFC. Following extinction training, immediate extinction lowers c-Fos expression (an indirect marker of recent neural activity) in the IL and PL when compared to delayed extinction (Singh et al., 2018), whilst mPFC c-Fos levels are comparable between immediate extinction and no extinction groups (Kim et al., 2010). Single-unit bursting in the IL is significantly lower during immediate extinction training when compared to delayed extinction training (Chang et al., 2010). IL firing during extinction retention testing is positively correlated to the level of fear expression, with immediate extinction inducing high levels of IL activity during the retention test (Chang et al., 2010). Electrical and pharmacological stimulation of the IL during immediate extinction training rescues the IED and facilitates re-extinction, respectively (Chang and Maren, 2011; Kim et al., 2010). However, in a contextual paradigm, immediate extinction elevates c-Fos levels in the IL and PL compared to delayed extinction (Stafford et al., 2013).

Overall, the evidence suggests that the mPFC is fundamental in mediating the IED. Aversive stimuli impair mPFC activity (Akirav and Maroun, 2007; Arnsten, 2009; McEwen and Morrison, 2013). For example, chronic stress can induce dendritic retraction in mPFC neurons, which are correlated with lasting extinction impairments (Izquierdo et al., 2006; Miracle et al., 2006; Wilber et al., 2011). AMG hyperactivity during fear dampens and impairs mPFC and HPC activity (Dilgen et al., 2013; Garcia et al., 1999; Kim et al., 2001). It may be that heightened AMG activity induced by aversive CSs inhibits HPC-mPFC circuitry during extinction training (Goosens et al., 2003; Maren, 2000; Quirk et al., 1995), thus interfering with adaptive extinction encoding and later retrieval of the extinction memory (Maren, 2014).

1.3. Endocannabinoid System

1.3.1. Overview

Endocannabinoid (eCB)-based interventions may be a promising line of research for the treatment of anxiety-related disorders. To better understand the target sites for novel treatments, eCB pharmacology is here described in detail. The eCB system is ubiquitously distributed throughout the central nervous system (CNS; Marsicano and

Kuner, 2008). Its endogenous ligands and receptors modulate synaptic transmission (Castillo et al., 2012; Ohno-Shosaku and Kano, 2014). The eCB system plays a role in numerous behavioural and neurophysiological processes (e.g., stress, appetite, memory, and sleep regulation). Clinically approved use of cannabinoid-based treatments can be found in indications such as multiple-sclerosis, nausea, pain, and cancer-induced spasticity (Ligresti et al., 2016).

1.3.2. Endocannabinoid Receptors

The eCB system was discovered following investigations into the action of the phytocannabinoid ∆9-tetrahydrocannabinol (THC). In 1990, an orphan G-protein-coupled receptor (GPCR) from rat cerebral cortex, later known as cannabinoid receptor type one (CB1R), was shown to mediate THC's pharmacological effects (Matsuda et al., 1990). Some years after, another orphan GPCR was cloned from human immune tissue and named cannabinoid receptor type two (CB2R; Munro et al., 1993). At the time of discovery, these receptors were thought to be expressed in distinct anatomical locations, with 'central' CB1Rs and 'peripheral' CB2Rs. Since then, research has shown that both receptors are expressed centrally and peripherally, albeit at different levels (Onaivi, 2006; Pagotto et al., 2005). The eCB system also utilises non-cannabinoid receptors to mediate its effects. Of note, the non-selective cation channel, transient receptor potential vanilloid type one (TRPV1) is expressed near CB1Rs and CB2Rs (Anand et al., 2008; Cristino et al., 2006), and is activated by eCB ligands (Smart and Jerman, 2000). In this way, cannabinoid and non-cannabinoid receptors synergistically modulate synaptic transmission pathways (Di Marzo and Cristino, 2008).

CB1R and CB2R belong to the rhodopsin-like subfamily of GPCRs. As such, both receptors exert their primary effects via $G_{i/o}$ protein signalling, causing inhibition of adenylyl cyclase and activation of several mitogen-activated protein kinase cascades (Bayewitch et al., 1995; Bouaboula et al., 1995, 1996). CB1Rs effectively modulate voltage-gated ion channels, namely inhibiting N- and P/Q-type calcium (Ca^{2+}) channels and activating inwardly rectifying potassium channels (Mackie et al., 1993, 1995; Shen and Thayer, 1998; Sugiura et al., 1997). They also signal indirectly

via G_s and G_q proteins, which mediate cyclic adenosine monophosphate (cAMP) stimulation and increase intracellular Ca^{2+}, respectively (Glass and Felder, 1997; Lauckner et al., 2005). Both receptors control synaptic plasticity via phosphorylation of key amino acid residues in the signal transduction pathways (Howlett, 2005).

CB1Rs are abundantly expressed in corticolimbic areas related to cognition, behaviour, and emotional memory, e.g., AMG, prefrontal cortex (PFC), and HPC (McPartland et al., 2007). Whilst characterisation of corticolimbic CB1Rs is further advanced, central CB2Rs are extant. However, their function within the CNS is less understood. With all things considered, the localisation and multimodal signalling pathways of CB1Rs and CB2Rs implicates them in the putative neural circuits underpinning learned fear processing and anxiety-related disorders.

1.3.3. Endocannabinoid Ligands

Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are endogenous ligands that bind to cannabinoid receptors. Whilst both eCBs are distributed ubiquitously throughout the body (i.e., centrally and peripherally), 2-AG is far more abundant in the brain than AEA (Bisogno et al., 1999). Both AEA and 2-AG are synthesised *de novo* and 'on demand', which is usually preceded by Ca^{2+} influx at the post-synaptic terminal (Cadas et al., 1997). AEA is a partial agonist of CB1R and CB2R, and a full agonist of TRPV1 (Felder et al., 1995; Smart et al., 2000). Biosynthesis of AEA begins with its precursor, N-arachidonoyl-phosphatidylethanolamine, which can be converted into AEA via four distinct pathways (Ligresti et al., 2016). 2-AG, a full agonist of both cannabinoid receptors (Sugiura et al., 1995), is produced from arachidonic acid-containing diacylglycerols via one of two enzymes, diacylglycerol lipase α or β (DAGLα or DAGLβ; Bisogno et al., 2003). Once synthesised and active, both ligands are transported via extracellular membrane vesicles known as exosomes (Gabrielli et al., 2015).

AEA and 2-AG function as retrograde synaptic messengers, travelling from the post-synaptic terminal to activate pre-synaptic cannabinoid receptors (Wilson and Nicoll, 2001). The multimodal signalling of these neurotransmitters underlies both

short-term (e.g., depolarisation-induced inhibition of excitatory or inhibitory currents) and long-term (e.g., long-term depression [LTD] of excitatory and inhibitory transmission) synaptic plasticity. 2-AG-mediated activation of central CB1Rs initiates a negative feedback loop for modulatory control of neurotransmitter release (Mackie, 2008). For example, a rise in glutamate (Glu) signalling precipitates an influx of Ca^{2+} into the post-synaptic terminal via $G_{q/11}$-coupled GPCRs. This influx stimulates DAGLα activity, thus increasing 2-AG tone. As 2-AG activates the CB1Rs, Ca^{2+} channel activity is attenuated in the pre-synaptic terminal, ultimately attenuating Glu transmission, and negating the Glu activation of G_{q-11}-coupled GPCRs (Kano et al., 2009; Ligresti et al., 2016; Maejima et al., 2001). CB1Rs inhibit the release of several neurotransmitters into the neighbouring synaptic cleft (e.g., Glu, GABA, acetylcholine, and monoamines; Cadogan et al., 1997; Gifford and Ashby, 1996; Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). Whilst AEA acts as a retrograde messenger in some instances (Kim and Alger, 2010), its function as an endovanilloid underlies much of its pharmacological effect. In the PAG, AEA-mediated TRPV1 activation serves to increase local Glu synaptic transmission (Kawahara et al., 2011). However, in other brain areas, post-synaptic TRPV1 activation induces LTD through a reduction in excitatory signalling (Chávez et al., 2010; Grueter et al., 2010).

1.3.4. Endocannabinoid Degradation and Transport

Fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) primarily mediate the breakdown of AEA and 2-AG, respectively (Blankman et al., 2007; Cravatt et al., 1996; Dinh et al., 2002; Kurahashi et al., 1997). AEA is broken down into arachidonic acid and ethanolamine, whilst 2-AG is broken down into arachidonic acid and monoacylglycerol. The localisation of FAAH and MAGL supports the assumed mechanisms of eCB signalling, as post-synaptic FAAH inhibits AEA-mediated TRPV1 activation and pre-synaptic MAGL halts retrograde 2-AG signalling. Subsidiary enzymes (e.g., cyclooxygenase-2, lipoxygenase, and cytochrome P450) also facilitate eCB degradation (Bornheim et al., 1995; Hampson et al., 1995; Kozak and Marnett, 2002).

Currently, the mechanisms of eCB membrane transport are unclear, with contrasting models being proposed. Primarily, a simple diffusion model suggested that AEA crosses the plasma membrane due to an inward concentration gradient caused by the metabolism of intracellular AEA (Deutsch et al., 2001). However, evidence that FAAH deficient mice exhibit quick and saturable AEA uptake opposes this idea (Ligresti et al., 2004). More recently, an integrated view on eCB uptake has been proposed. It suggests that AEA amasses at specific sites on the plasma membrane. From here, AEA binds to one or more specific membrane transporter proteins, allowing it to be bi-directionally translocated through the cell membrane. The AEA is then collected by intracellular transporters that translocate the eCB to its target destination (Maccarrone et al., 2010). Whilst AEA uptake is better characterised, evidence suggests that AEA intracellular transporters and uptake mechanisms also mediate 2-AG translocation (Chicca et al., 2012; Nicolussi et al., 2014). Taken as a whole, the eCB system provides a plethora of therapeutic targets, including receptors, metabolic pathways, and transportation. Its capacity to modulate neurotransmitter release makes it a prime candidate to remediate the aberrant neuronal signalling implicated in anxiety-related disorders.

1.4. Endocannabinoid-Based Interventions in Animal Anxiety Models

1.4.1. Pharmacological Interventions

The timing of pharmacological interventions during fear conditioning determines which memory formation process is affected. For example, drug administration prior to conditioning may affect memory acquisition (Barros et al., 2005), whilst interventions immediately following conditioning could impact memory consolidation (Fiorenza et al., 2012). However, considering the unpredictable nature of traumatic events, it may be difficult to implement fear memory encoding interventions in real-life scenarios. Currently, there are no established prognostic tools to assess who will develop an anxiety-related disorder following exposure to trauma. As such, it is unknown which individuals require treatment at an early stage. Complications such as informed consent, medical history, and the patient's recollective capacity make it difficult to develop standardised treatment protocols. It

is estimated that between 20-33% of people who are exposed to severe trauma go on to develop PTSD (NHS, 2022; PTSD UK, 2022).

Extinction memory can be manipulated through drug administration prior to or after extinction training, as well as before extinction testing (Quirk and Mueller, 2008). Given the difficulty in targeting trauma memory encoding, it is more likely that clinical patients will present with an established anxiety-related disorder. With all things considered, studies targeting extinction are more applicable to the clinical setting. Here, research investigating eCB regulation of learned fear inhibition through extinction is presented.

1.4.2. Cannabinoid Receptor Agonists

WIN 55,212-2 (CB1R and CB2R agonist) can enhance the extinction of aversive memories in animal models. In rodents, WIN 55,212-2 enhances the extinction of short- and long-term contextual fear memories when compared to vehicle controls (Pamplona et al., 2006, 2008). WIN 55,212-2 also enhances fear extinction in other behavioural models (e.g., fear-potentiated startle; Abush and Akirav, 2010; Lin et al., 2009). However, numerous studies have also reported no effect of the drug. Chhatwal et al. (2005) and Lin et al. (2008) found that high doses of WIN 55,212-2, given acutely or repeatedly, had no effect on extinction. Bisby et al. (2020) found that WIN 55,212-2 given prior to extinction training enhanced extinction retention in adult rats but hindered within-session extinction and had no effect on extinction retention in adolescents. In a contextual fear conditioning paradigm that incorporated concurrent social isolation, Morena et al. (2018) showed that a high dose of WIN 55,212-2 given post-extinction training facilitated extinction memory consolidation; however, the same dose showed no effect when given pre-extinction training. Mizuno et al. (2022) found that WIN 55,212-2 given before extinction training enhanced fear memory recall at the start of the extinction session and impaired within-session extinction in both male and female mice. The discrepancy between WIN 55,212-2's purported effects may be due to a dose response curve, its divergent action on extinction acquisition and consolidation, or the extinction/animal model in which it is tested. Pamplona et al. (2006) showed that CB1R antagonism prevented

low doses of WIN 55,212-2 from enhancing extinction. However, it may be that higher doses indiscriminately activate CB1Rs (and CB2Rs) in otherwise inactive synaptic pathways, thus leading to non-specific cannabinoid signalling. In addition to this, WIN 55,212-2 dose-dependently affects Glu transmission in the rat cerebral cortex. Ferraro et al. (2001) found that an intermediate dose (1 mg/kg) increased dialysate Glu levels in the awake rat, whilst low and high doses (0.1 and 2 mg/kg) showed no effect.

AM404 is an indirect agonist that mediates its effects via eCB uptake inhibition and potent TRPV1 agonism (Zygmunt et al., 2000). In contextual fear conditioning paradigms, AM404 enhances short- and long-term extinction (Bitencourt et al., 2008, 2014; Pamplona et al., 2006). As with WIN 55,212-2, these benefits translate into other extinction models (Abush and Akirav, 2013; Chhatwal et al., 2005). Co-administration of CB1R antagonists negates the drug's action, whilst TRPV1 antagonism fails to block its effect (Bitencourt et al., 2008; Chhatwal et al., 2005). AM404 also reduces fear expression in mice. However, when given in conjunction with CB1R or TRPV1 antagonists separately, this effect is negated (Llorente-Berzal et al., 2015). These results may indicate a differential role for TRPV1 receptors in fear expression and extinction. Additionally, AEA exerts distinct effects at higher (anxiogenic) and lower (anxiolytic) doses. This may be due to concurrent activation of TRPV1 channels at higher doses, a receptor that opposes the effects of CB1R (Aguiar et al., 2014; Uliana et al., 2016). Given this, further interventions may seek to optimally activate each of these receptors to better facilitate anxiolysis and extinction.

It is important to consider that CB1R agonists can impart psychotropic effects (Akram et al., 2019). These may become problematic in a clinical setting if patients are unable to tolerate such alterations to mood, behaviour, thoughts, and perception. Drugs that are full agonists of CB1R induce more side-effects than THC (partial CB1R agonist), and may be linked to psychosis (Tikka and D'Souza, 2019). Additionally, agonism of CB1Rs alone may not provide the holistic action required to successfully ameliorate behavioural deficits caused by aberrant fear memories.

1.4.3. Cannabinoid Receptor Antagonists

In auditory and contextual fear conditioning paradigms, systemic administration of CB1R antagonists impairs short- and long-term extinction (Chhatwal et al., 2005; Finn et al., 2004; Marsicano et al., 2002; Mizuno et al., 2022; Pamplona et al., 2006; Pickens and Theberge, 2014; Suzuki et al., 2004). This highlights the importance of CB1R's role in the process of extinction.

Genetic ablation of CB1R corroborates the findings from antagonist-based extinction studies. Seminal research by Marsicano et al. (2002) showed that CB1R-deficient ($CB1R^{-/-}$) mice failed to extinguish auditory-cued fear memories. This effect was borne from disparate kinase and phosphatase activity in the BLA, vHPC, dHPC, and mPFC of $CB1R^{-/-}$ mice compared to wild-types (Cannich et al., 2004). CB1Rs expressed on Glu and GABA neurons may be differentially implicated in fear expression and extinction. Llorente-Berzal et al. (2015) re-exposed Glu-$CB1R^{-/-}$ and GABA-$CB1R^{-/-}$ mice to a tone that had previously been paired with a single shock. During this 3 min re-exposure, Glu-$CB1R^{-/-}$ mice showed heightened fear expression, whilst GABA-$CB1R^{-/-}$ animals showed reduced fear during a second extinction training session. Acute attenuation of extinction has been shown in Glu-$CB1R^{-/-}$ mice (Kamprath et al., 2009), with no effect seen in the GABA-$CB1R^{-/-}$ type (Dubreucq et al., 2012). However, Ruehle et al. (2013) showed that restoration of CB1R expression in dorsal telencephalic neurons negated the anxiogenic effects of Glu-CB1R ablation, but did not affect extinction impairment. Despite this, evidence suggests that eCB-mediated extinction may be more dependent on Glu-CB1Rs than GABA-CB1Rs.

Successful extinction, and its enhancement, requires functional CB1Rs (Bitencourt et al., 2008; Chhatwal et al., 2005; Marsicano et al., 2002; Pamplona et al., 2006). However, due to the complexity of the eCB system, it is unlikely that these receptors are solely responsible for the effect. When compared to vehicle, ACEA, a selective CB1R agonist with low affinity for CB2R, showed no extinction enhancement of auditory-cued fear memories in male and female rats (Simone et al., 2015a, 2015b). However, compounds that activate both CB1Rs and CB2Rs (e.g., WIN 55,212-

2) and increase AEA tone (e.g., URB597, AM3506) show promising results. With this, research into non-CB1R ligands is warranted.

1.4.4. Inhibition of Enzymes Mediating Endocannabinoid Metabolism

URB597 and AM3506 are drugs that inhibit FAAH activity (Bergman et al., 2011; Piomelli et al., 2006). URB597 facilitates both the acquisition and consolidation of contextual extinction memories (Laricchiuta et al., 2013; Morena et al., 2018). URB597 also reduces fear expression of contextual and auditory-cued fear memories (Bowers and Ressler, 2015; Lisboa et al., 2015). Gunduz-Cinar et al. (2013a) found that systemic administration of AM3506 enhanced the long-term extinction of auditory-cued fear memories, which was driven by AEA-mediated CB1R activation. Morena et al. (2021) found that FAAH inhibition had no effect on male rats; however, enhanced AEA signalling at TRPV1 channels impaired within-session extinction and its later retention in females. Mice with genetically dampened FAAH activity also show enhanced extinction (Dincheva et al., 2015).

JZL184 inhibits MAGL, thus elevating 2-AG tone (Long et al., 2009a). Fewer studies have assessed 2-AG's role in the extinction of aversive memories, with mixed results in those that have. In traumatised rats, Morena et al. (2018) found that lower doses of JZL184 (0.5-1 mg/kg) given before extinction training reduced global freezing in repeated tests of extinction training and retention, whilst post-extinction training administration had no effect on extinction consolidation. However, Hartley et al. (2016) showed that a higher dose of JZL184 (8 mg/kg) impaired short-term extinction of auditory-cued fear memories. Morena et al. (2021) found that MAGL inhibition, induced via MJN110 treatment, did not affect fear expression or extinction in male rats. However, in females, 2-AG upregulation increased darting behaviour, an active fear response characterised by short but rapid bursts of movement, and reduced freezing. In female mice, Mizuno et al. (2022) found that JZL184 increased fear expression at the start of fear extinction in a CB1R-dependent but not CB2R-dependent manner. The authors also showed that JZL184 impaired within-session extinction in both sexes. Llorente-Berzal et al. (2015) showed that systemically administered JZL184 (4 mg/kg) acutely enhanced freezing upon re-exposure to a CS

via activation of CB1R-containing GABA interneurons. However, Bedse et al. (2018) found that JZL184 was anxiolytic when given in a restraint stress-induced anxiety model. Cavener et al. (2018) showed that dampened 2-AG signalling brought about via DAGL inhibition impaired auditory-cued extinction. Taken together, the role of 2-AG in fear extinction remains unclear and therefore requires more research.

1.4.5. Mechanisms of Endocannabinoid-Based Modulation of Fear Extinction

Human data implicates the eCB system in the pathology of anxiety disorders and PTSD. Variants in eCB-related genes are associated with anxiety disorders (Demers et al., 2016; Dincheva et al., 2015; Gee et al., 2016; Lazary et al., 2009, 2016; Lester et al., 2017). A case study presenting a woman with a history of painless injuries and an inability to feel fear or anxiety reported microdeletion in a FAAH pseudogene, along with a functional single-nucleotide polymorphism in the FAAH gene that hindered its expression and subsequent action (Habib et al., 2019). PTSD patients present with elevated or reduced eCB levels in the plasma (Hauer et al., 2013; Hill et al., 2013b), as well as elevated CB1R availability (Neumeister et al., 2013). Rabinak et al. (2013) found that dronabinol, a psychoactive enantiomer of THC, enhanced cued fear retrieval when given prior to extinction training. The authors later determined that this was mediated by elevated PFC and HPC activation, along with PFC-AMG interactions, at the time of extinction retrieval (Rabinak et al., 2014, 2018). Finally, Mayo et al. (2020b) found that FAAH inhibition in healthy volunteers potentiated extinction memory recall in a retention test. FAAH inhibition also reduced autonomic stress reactivity (Mayo et al., 2020b).

The BLA is heavily implicated in the extinction of aversive memories (Falls et al., 1992). The BLA also has a high concentration of CB1Rs expressed on local GABA interneurons (Katona et al., 2001). During extinction training, eCB signalling in the BLA increases following CS presentation, thus stimulating CB1R-dependent LTD of GABA-mediated inhibitory post-synaptic currents (IPSCs, Marsicano et al., 2002). The LTD is driven by adenylyl cyclase inhibition, lowered cAMP levels, reduced protein kinase A action, and increased calcineurin activity, a protein that is implicated in fear extinction. This culminates in increased dephosphorylation of proteins, specifically

those required for neurotransmitter release (Castillo et al., 2012; Chevaleyre et al., 2007; Kano, 2014; Ohno-Shosaku et al., 2012). Genetic ablation or antagonism of CB1R prior to extinction training abolishes this effect (Marsicano et al., 2002). Infusion of rimonabant (CB1R inverse agonist) into the right BLA inhibits the short-term extinction of contextual fear memories (Roche et al., 2007). However, Roche et al. (2010) found that bilateral infusion of rimonabant into the BLA had no effect on short-term, within trial extinction. Kuhnert et al. (2013) also found that infusion of AM251 (CB1R inverse agonist) into the BLA did not impede extinction. AEA is likely the key mediator of CB1R-induced LTD as systemic injection of AM3506 prior to extinction training augments AEA signalling in the BLA. Administration of AM3506 to AMG slices also potentiates LTD of IPSCs (Gunduz-Cinar et al., 2013a). However, Morena et al. (2019) used a viral vector that quickly and transiently overexpressed FAAH in pyramidal neurons of the BLA in male rats. When induced after conditioning, they found that FAAH overexpression counterintuitively impaired fear memory expression/retrieval in later extinction training and retention tests. This effect was reversed by intra-BLA administration of URB597 or bicuculline (GABAa antagonist) prior to extinction training. These results suggest that AEA tone may have a dichotomous relationship with inhibitory/excitatory circuits during fear conditioning depending on the brain region in question. Finally, Hartley et al. (2016) found that infusion of JZL184 into the BLA, but not the CeA, attenuated long-term extinction. Taken together, these results suggest a complex role of AEA, 2-AG, and CB1Rs in the BLA.

Cholecystokinin (CCK) is a peptide expressed by GABAergic interneurons containing CB1Rs (Belcheva et al., 1994; McDonald and Mascagni, 2001). CB1Rs are colocalised with CCK2, a CCK receptor that is expressed in the BLA (Bowers and Ressler, 2015). In a fear-potentiated startle paradigm, CCK mediates an anxiogenic effect via CCK2 receptors in the BLA (Josselyn et al., 1995). This has implications for extinction as intracerebroventricular infusion of a CCK2 agonist impairs extinction retrieval (Chhatwal et al., 2009). Interestingly, systemic and intra-AMG administration of a CCK2 antagonist partially restores CB1R antagonist-mediated extinction impairment. Post-extinction, enhanced Akt phosphorylation seen in

control animals is abolished with CB1R antagonism. However, co-administration of CB1R and CCK2 antagonists returns phosphorylation levels to that of vehicle-treated animals (Chhatwal et al., 2009). Bowers and Ressler (2015) found that CB1R antagonism increased fear expression in wild-type mice but had no effect in CCK2-knockout (KO) mice. Beinfeld and Connolly (2001) showed that CB1R activation inhibited CCK release in rat HPC slices. It is therefore theorised that CB1R activation not only mediates LTD of GABA transmission but simultaneously attenuates CCK signalling. This is supported by the proliferation of CB1R- and CCK- positive interneurons around BLA fear neurons following fear extinction (Trouche et al., 2013). Despite this, optogenetic activation of CCK interneurons in the BA during extinction acquisition mediates a reduction in the CFR at a later extinction test (Rovira-Esteban et al., 2019). Taken together, these findings heavily implicate BLA CB1R/CCK GABA interneurons in the successful extinction of aversive memories.

As mentioned above, the IL is another regulator of extinction (Milad and Quirk, 2002). Intra-IL infusion of WIN 55,212-2 and FAAH inhibitors enhances extinction performance (Lin et al., 2009). IL inactivation or lesion impairs later extinction retrieval (Do-Monte et al., 2015; Laurent and Westbrook., 2009; Quirk et al. 2000; Sierra-Mercado et al., 2011). Furthermore, CB1R antagonism in the IL attenuates extinction (Kuhnert et al., 2013). Research suggests that the majority of CB1Rs in the PFC are expressed on GABAergic neurons (Marsicano and Lutz, 1999). Importantly, the IL also has efferent Glu projections synapsing at the BLA (Vertes, 2004). It is therefore possible that increased CB1R activity in the IL disinhibits this Glu signalling pathway. This could result in elevated excitatory tone in the BLA, thus upregulating eCB transmission and initiating the LTD required for successful extinction.

As mentioned above, the HPC regulates extinction encoding (Corcoran et al., 2005). AM251 infused into the retrosplenial cortex (RSC; Sachser et al., 2015) and the dHPC (de Oliveira Alvares et al., 2008) impedes long-term contextual fear memory extinction and its consolidation, respectively. AM251 infused into the dHPC inhibits long-term extinction (Abush and Akirav, 2013). It is speculated that activation of

CB1Rs in inhibitory networks leads to disinhibition of Glu projections from the HPC (de Oliveira Alvares et al., 2008; Ohno-Shosaku and Kano, 2001). From here, LTP strengthens the memory trace, a process that drives memory formation (Bliss and Collingridge, 1993). Additionally, eCB tone is linked to the scaling of synaptic plasticity in the HPC. For example, lowered eCB activity facilitates increased activity of GABA circuits, thus allowing homeostatic tuning via inhibitory signalling pathways (Kim and Alger, 2010). CB1R activation in dHPC mediates upregulation of ERK and calcineurin (Cestari et al., 2014; Lin et al., 2003; Merlo et al., 2014). Cannich et al. (2004) found that CB1R-KO mice showed reduced ERK in the BLA and mPFC, and reduced calcineurin in the BLA, mPFC, dHPC, and vHPC following extinction training, compared to their wild-type counterparts. These data suggest that CB1R activation regulates localised ERK and calcineurin signalling pathways during fear extinction (Cannich et al., 2004). Aside from these brain areas, infusion of CP 55,940 (CB1R and CB2R agonist) into the RSC has been shown to impair fear memory expression and prevent spontaneous recovery (Sachser et al., 2015). Taken together, eCB signalling in the BLA, IL and HPC has been shown to mediate cued and contextual fear extinction.

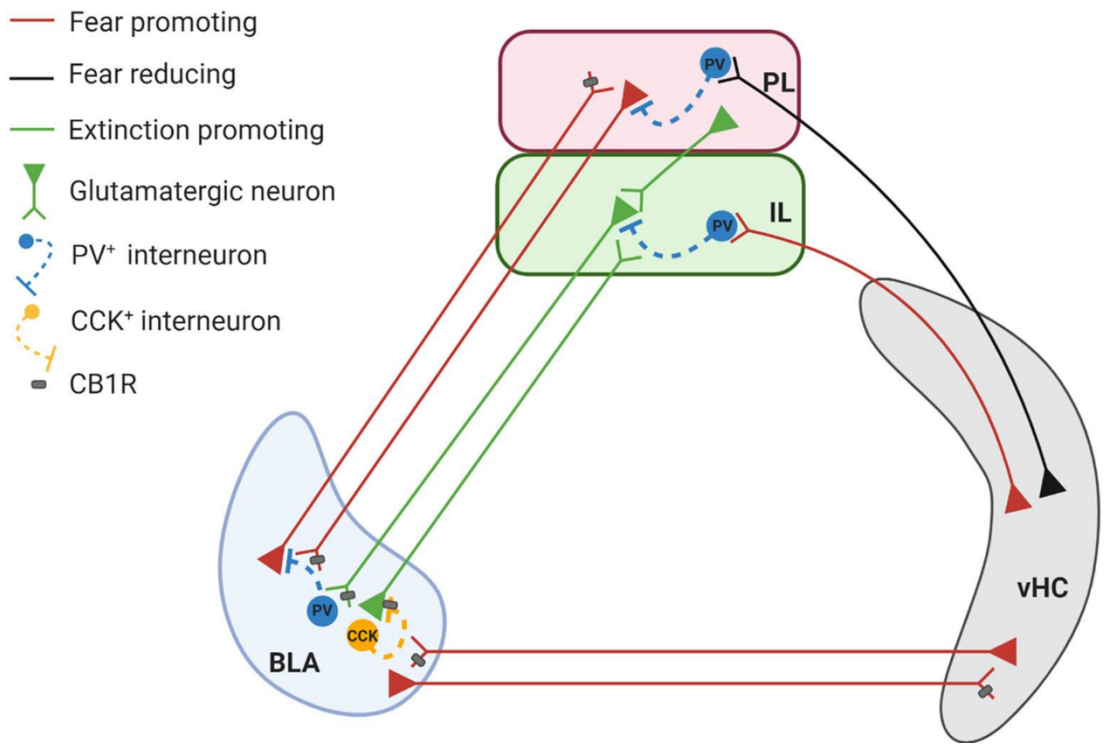

Figure 1.2. Cannabinoid receptor type-1 localisation in fear and extinction learning brain circuitry. Cannabinoid receptor type-1 is well placed to modulate excitatory and inhibitory projections to and from the basolateral amygdala, prelimbic cortex, infralimic cortex and ventral hippocampus. Cannabinoid receptor type-1 is well placed to modulate inhibitory cholecystokinin interneurons in the basolateral amygdala. BLA, basolateral amygdala; CB1R, cannabinoid receptor type-1; CCK, cholecystokinin; IL, infralimbic cortex; PL, prelimbic cortex; PV; parvalbumin; vHC; ventral hippocampus. Gunduz-Cinar, 2021.

1.5. Noradrenergic System

Anxiety disorders and PTSD are underpinned by aberrant NA signalling (Delahanty et al., 2005; Giustino and Maren, 2018). NA signalling also regulates the retrieval and extinction of conditioned fear memories (Atsak et al., 2012; Cain et al., 2004; Do-Monte et al., 2010). NA released by the locus coeruleus (LC) innervates several brain regions associated with fear and extinction processing, including HPC, mPFC, and AMG (Giustino and Maren, 2018; Schwarz and Luo, 2015; Schwarz et al., 2015). NA exerts its effects through a variety of G-protein coupled adrenoreceptors (ARs), which are highly expressed in the aforementioned brain areas (Day et al., 1997; McCune et al., 1993; Rainbow et al., 1984). ARs are separated into two distinct classes, α (α1 and α2) and β (β1, β2, and β3). All ARs can be expressed at the post-synaptic terminal, whilst α2 and all β-receptors may also be localised at the pre-synaptic terminal. Activation of α1 and β-receptors induces an excitatory effect on neural activity, whilst α2 activation mediates an inhibitory effect. At the pre-synaptic terminal, α2 receptors act as autoreceptors that modulate NA efflux (Giustino and Maren, 2018; MacDonald et al., 1997; Marshall et al., 1999; Ordway et al., 1987). NA signalling is further regulated by the NA transporter (NET), which sequesters NA into the pre-synaptic terminal where it is metabolised by monoamine oxidase. Catechol-O-methyltransferase also metabolises synaptic NA (Hussain et al., 2022).

1.5.1. Noradrenergic Signalling in Fear Extinction

Several systemic pharmacological interventions have been conducted to assess AR contribution to learned fear and its extinction. Ouyang and Thomas (2005) found that mice deficient in NA exhibited impaired contextual fear extinction. This was remediated by xamoterol (β-AR agonist) and further impaired with propranolol (β-AR antagonist). Bernardi and Lattal (2010) showed that repeated administration of prazosin (α1-AR antagonist) following extinction training impaired extinction of contextual fear. However, Lucas et al. (2019) found prazosin did not affect cued extinction. Cain et al. (2004) and Morris and Bouton (2007) found that pre-extinction, but not post-extinction, administration of yohimbine (α2-AR antagonist) enhanced extinction memory in a later extinction test. This effect was mediated by the

inhibition of pre-synaptic autoreceptors, thus resulting in greater NA release. Despite this, Mueller et al. (2009) showed that pre-extinction injections of yohimbine acutely reduced cued fear expression with no later effect on extinction retention. Do-Monte et al. (2010) found that isoproterenol (β-AR agonist) augmented extinction memory consolidation if administered after contextual extinction training. Administration of propranolol before and after extinction training impairs cued and contextual extinction memory (Cain et al., 2004; Do-Monte et al., 2010; Fitzgerald et al., 2015). Propranolol impairs cued fear expression when administered prior to extinction training and extinction testing (Rodriguez-Romaguera et al., 2009; Santos et al., 2021). Taken together, it appears that NA activation of β-ARs is crucial for successful extinction encoding.

There are several brain areas involved in NA regulation of fear extinction. Hugues et al. (2007) found that extinction training increased NA signalling in the mPFC. Uematsu et al. (2017) found that projections from the LC to the mPFC are required for adaptive extinction encoding. Do-Monte et al. (2010) found that mPFC injections of atenolol (β-AR antagonist) prior to contextual extinction training impaired extinction. Mueller et al. (2008) showed that IL infusions of propranolol prior to cued extinction training impaired extinction. The authors found that NA enhanced IL activity *in vitro*, which was dependent on β-AR activity. This suggests that under basal conditions fear conditioning induces emotional arousal that paradoxically promotes later fear extinction. Chai et al. (2014) found that NA enhanced long-term extinction retention when infused into the dHPC either immediately or 12 hrs following contextual extinction training. This effect was also shown to be β-AR-dependent. Furini et al. (2017) enhanced the effects of weak extinction training by infusing methylphenidate (dopamine/NA re-uptake inhibitor) into the dHPC; this effect was impaired by timolol (β-AR antagonist). Ouyang and Thomas (2005) showed that atenolol infused into the dHPC post-extinction hindered extinction retrieval in a later extinction test. Berlau and McGaugh (2006) found that NA infused into the BLA post-extinction augmented contextual extinction, whilst propranolol infused into the same area showed no effect. Whilst these results suggest NA may enhance extinction, Fiorenza et al. (2012) found that timolol

augmented contextual fear extinction when infused into the mPFC and BLA after contextual extinction training. The authors also showed no effect when infusing NA into the dHPC and BLA but found NA impaired extinction when infused into the mPFC.

Considered together, research suggests that adaptive NA signalling is required for the successful extinction of learned fear. However, discrepant results regarding the central infusion of NA into extinction-related brain areas may be attributed to the timing of administration (e.g., pre- or post-extinction training) or the level of NA signalling. For example, NA's effect on learning and memory occurs in a non-linear (inverted U) fashion (Gold et al., 1975; Snyder et al., 2012). When central NA tone is below or above an adaptive level it can impair extinction (Arnsten, 2009; Fitzgerald et al., 2015; Giustino and Maren, 2018). In immediate extinction, when LC-NA signalling is excessively high, the fear memory is favourably encoded instead of the extinction memory. Conversely, when the LC-NA system is moderately engaged under low levels of stress, extinction encoding is favoured (Giustino et al., 2016b; Giustino and Maren, 2018).

1.5.2. Noradrenergic Signalling and the Immediate Extinction Deficit

As alluded to above, the NA system is heavily implicated in the IED. Propranolol can remediate the IED when given prior to extinction training (Fitzgerald et al., 2015; Giustino et al., 2017, 2020). Fitzgerald et al. (2015) found that rats treated with propranolol showed significantly less fear compared to vehicle counterparts in a drug-free extinction test conducted 24 hrs after extinction training. Giustino et al. (2017) reported that intra-BLA but not intra-mPFC infusions of propranolol attenuated cued freezing during an extinction test conducted 24 hrs after immediate extinction training. In both studies, propranolol's effect was not caused by an impaired ability to consolidate the fear memory. A control group that received propranolol and no extinction training showed elevated CS-induced freezing in the extinction test, comparable to that seen in the vehicle group. Giustino et al. (2020) assessed the neural underpinnings of the IED. The authors found that fear conditioning enhanced spontaneous neural activity in the BLA for up to 1 hr after the session; this effect was nullified by systemic administration of propranolol. Weak

footshocks mediated smaller increases in BLA activity and failed to induce the IED. However, chemogenetic activation of the LC prior to weak fear conditioning increased the CFR and BLA activity, and induced the IED. Interestingly, intra-BLA infusions of propranolol prior to extinction training prevented this chemogenetic-mediated induction of the IED. This evidence suggests that sufficiently strong fear stimuli activate NA projections from the LC to the BLA. This elevation in BLA firing serves to augment consolidation of the fear memory. Given that BLA activation of the IL induces feed-forward inhibition (Dilgen et al., 2013; Floresco and Tse, 2007; McGarry and Carter, 2016), it is likely that excessive BLA activity inhibits IL, thus impairing extinction.

1.5.3. Modulation of Noradrenaline Transmission by the Endocannabinoid System

There is evidence to suggest that the eCB system modulates NA signalling in brain areas related to fear extinction (Warren et al., 2021). CB1Rs are abundant in the LC (Herkenham et al., 1991; Matsuda et al., 1993). Within the LC, CB1Rs are colocalised with tyrosine hydroxylase, an enzyme required for NA biosynthesis (Scavone et al., 2010). In the mPFC, CB1Rs are colocalised with dopamine-β-hydroxylase (DβH; the enzyme that converts dopamine to NA), NET, and α2-ARs within pre-synaptic neurons (Cathel et al., 2014; Oropeza et al., 2007; Reyes et al., 2009; Richter et al., 2012). Post-synaptic, DAGL-expressing neurons in the mPFC are activated by NA afferents containing DβH, NET, and CB1Rs (Reyes et al., 2015). It is therefore possible that 2-AG modulates NA signalling via presynaptic CB1Rs localised on mPFC afferents. In postsynaptic mPFC neurons, CB1Rs colocalise with α2-ARs (Cathel et al., 2014; Reyes et al., 2017). This evidence suggests the eCB system is well placed to modulate NA signalling in the LC and mPFC, both areas that have been implicated in fear extinction and the IED.

WIN 55,212-2, CP 55,940, and URB597 activate LC neurons in a CB1R-dependent manner (Gobbi et al., 2005; Mendiguren and Pineda, 2006; Muntoni et al., 2006; Oropeza et al., 2005). Systemic infusion of rimonabant attenuates the firing rate of LC neurons, which suggests that CB1Rs regulate LC activity (Muntoni et al., 2006). In the mPFC, WIN 55,212-2 stimulates NA release in a CB1R-dependent

manner, both *in vivo* and *in vitro* (Oropeza et al., 2005; Page et al., 2007, 2008). WIN 55,212-2 desensitises presynaptic α2-ARs in the mPFC, which may contribute to increased NA efflux (Cathel et al., 2014). Despite this, WIN 55,212-2 inhibits electrically-induced NA release in mPFC slices (Richter et al., 2012). High, but not low, doses of rimonabant increase NA signalling, suggesting that CB1R inhibition mediates greater NA release (Need et al., 2006; Tzavara et al., 2003). Evidence for eCB modulation of NA signalling in the dHPC has shown mixed results. WIN 55,212-2 inhibits electrically-induced NA efflux in dHPC slices in a CB1R-dependent manner (Kathmann et al., 1999; Schlicker et al., 1997), whilst CP 55,940 and rimonabant have no effect (Gifford et al. 1997). Despite this, WIN 55,212-2 and rimonabant have no effect on electrically evoked NA release in wild-type and CB1R-deficient mice (Kathmann et al. 2001). *Ex vivo* assessments of the dHPC show that AEA and WIN 55,212-2 increase NA levels, whilst UCM707 (an eCB transport inhibitor) has no effect (de Lago et al., 2007; Hao et al., 2000; Moranta et al., 2004, 2006). URB597 potentiates NA efflux in the BLA under stressful conditions (Bedse et al., 2015).

Whilst more work is required to understand eCB modulation of NA signalling, there is evidence to suggest a link between the systems in brain areas that are relevant to fear learning and memory. Nasehi et al. (2016, 2018) found that systemic injection of atenolol and yohimbine enhanced the impairment of fear acquisition induced by ACPA (CB1R agonist). Xameterol (β-AR partial agonist) reduced the effect of ACPA on fear acquisition impairment, whilst clonidine (α2-AR agonist) had no effect. Atsak et al. (2012) found that WIN55-212,2-induced impairment of fear memory retrieval was mitigated by co-infusion of propranolol when both drugs were administered into the dHPC. These results suggest a synergistic relationship between the eCB and NA systems in fear learning and memory. Given the evidence of eCB and NA signalling in the mPFC and other brain areas related to fear and extinction learning, it is possible that these two neurotransmitter systems co-dependently modulate fear extinction.

1.6. Aims and Objectives

The evidence reviewed here suggests that eCB-based treatments can enhance the extinction of aversive fear memories. Due to its ubiquitous distribution, systemic interventions based on modulating eCB transmission can manipulate neurotransmitter signalling in brain areas implicated in anxiety-related disorders. Ultimately, these treatments could be used alone, or in conjunction with existing pharmacological and psychological treatments, to enhance extinction and prevent fear relapse. Given the problems associated with current first-line treatments, there is reason to investigate novel pharmacological interventions for anxiety-related disorders. Whilst pre-clinical research seems promising, there is much to be elucidated regarding eCB regulation of fear extinction. There is a paucity of research exploring how eCB transmission might regulate relapse and extinction resistance. This thesis aims to investigate whether eCB metabolism inhibition could attenuate relapse in a spontaneous recovery paradigm, and whether it could remediate deficient extinction in the IED paradigm. URB597 and JZL184 were systemically administered to induce FAAH and MAGL inhibition, respectively. All studies used male Lister Hooded rats. Freezing behaviour was used as a measure of the CFR.

<u>URB597 Modulation of Spontaneous Fear Recovery</u>

- Test rats in a spontaneous recovery paradigm using fear conditioning, delayed extinction training (1 day after fear conditioning), and extinction testing (1 and 21 days after extinction training)
- Determine how URB597 (0.1, 0.3, 1 mg/kg) affects extinction testing and spontaneous recovery when given before or after extinction training

<u>JZL184 Modulation of Spontaneous Fear Recovery</u>

- Test rats in a spontaneous recovery paradigm using fear conditioning, delayed extinction training (1 day after fear conditioning), and extinction testing (1 and 21 days after extinction training)

- Determine how JZL184 (1, 3, 10 mg/kg) affects extinction testing and spontaneous recovery when given before or after extinction training

Immediate Extinction Validation and Propranolol Effects

- Validate the IED paradigm using fear conditioning, immediate extinction training (30 mins after fear conditioning), and extinction testing (1 day after extinction training)
- Validate the use of systemic propranolol (10 mg/kg) within the IED paradigm when given immediately after fear conditioning

URB597 and JZL184 Modulation of Immediate Extinction

- Determine how URB597 (0.3 mg/kg) and JZL184 (1, 10 mg/kg) affects immediate extinction training and the IED when given immediately after fear conditioning

1.7. Hypotheses

URB597 Modulation of Spontaneous Fear Recovery

- URB597 (0.1, 0.3, 1 mg/kg) was expected to enhance extinction encoding and suppress spontaneous recovery when administered prior to and after extinction training

JZL184 Modulation of Spontaneous Fear Recovery

- Low doses of JZL184 (1, 3 mg/kg) were expected to enhance extinction encoding and suppress spontaneous recovery when administered prior to and after extinction training
- The high dose of JZL184 (10 mg/kg) was expected to impair extinction encoding and exacerbate spontaneous recovery when administered prior to and after extinction training

Immediate Extinction Validation and Propranolol Effects

- Immediate extinction was expected to induce higher levels of freezing during the extinction retrieval test when compared to delayed extinction
- Propranolol was expected to reduce freezing during extinction training and extinction testing compared to vehicle

URB597 and JZL184 Modulation of Immediate Extinction

- URB597 (0.3 mg/kg) was expected to reduce freezing during extinction training and recall testing, and rescue the IED
- The low dose of JZL184 (1 mg/kg) was expected to reduce freezing during extinction training and recall testing, and rescue the IED
- The high dose of JZL184 (10 mg/kg) was expected to increase freezing during extinction training and recall testing, and exacerbate the IED

2. URB597 Modulation of Spontaneous Fear Recovery

2.1. Introduction

Short-term extinction retention can be tested after extinction training (e.g., 1-2 days) by presenting CSs alone in the extinction context. After successful fear extinction, subjects typically show reduced fearful behaviour when presented with the CS. Despite this, extinction memory is prone to relapse. Defined as the prolonged return of fear over time, spontaneous recovery occurs following a period of non-reinforcement where neither the CS nor US are re-encountered (Rescorla, 2004). In the extinction context, presentation of CSs alone sometime after extinction training (e.g., weeks or months) induces a fearful conditioned response like that seen prior to extinction training. Repeated extinction training can postpone spontaneous recovery (Rescorla, 2004). However, from a translational approach it is both desirable and economical to suppress spontaneous fear recovery with more efficacious extinction training than to have subjects engage with repeated extinction sessions.

The neural circuitry underpinning PTSD is linked to that implicated in fear conditioning and extinction (e.g., AMG, IL, PL, and HPC; Milad and Quirk, 2012). This convergence of neural substrates means that fear conditioning and extinction can be used to investigate the pathology of PTSD. Given that the underlying psychological processes during fear learning are comparable in rats, it is possible to translate findings from pre-clinical fear conditioning models to humans. Considering the discussion from the previous chapter, novel pharmacological and psychological approaches are required to ameliorate the extinction deficits commonly seen in PTSD. Preclinical work in rodents provides a valid model to investigate this further.

As previously discussed, experimental evidence implicates the eCB system in fear and extinction learning. eCBs can be manipulated during fear and extinction learning through inhibition of eCB degradation. URB597 and AM3506 are drugs that prevent the hydrolysis of AEA via FAAH inhibition, which results in increased AEA tone and signalling. AEA upregulation augments extinction learning in contextual and auditory-cued fear paradigms, as well as in inhibitory avoidance (IA; a paradigm

assessing the latency at which rats step from a safe area into an area previously associated with a footshock) experiments (Laricchiuta et al., 2013; Fidelman et al., 2018; Morena et al., 2018; Segev et al., 2018). AEA upregulation reduces fear expression in auditory-cued and contextual paradigms (Bowers and Ressler, 2015; Lisboa et al., 2015; Llorente-Berzal et al., 2015). Gunduz-Cinar et al. (2013a) showed that CB1R blockade in the BLA prevented the suppression of freezing mediated by systemic AM3506. The effect was then fully recapitulated following intra-AMG infusion of AM3506. However, the role of AEA signalling during fear and extinction learning is complex. Morena et al. (2019) showed that over-expression of FAAH in the BLA after conditioning counterintuitively reduced fear expression. Morena et al. (2021) showed that AEA upregulation had no effect on fear extinction or expression in male rats. Assessing rats that showed weak extinction (i.e., rats showing >70% freezing at the end of extinction training and testing), Vimalanathan et al. (2020) found no effect of URB597 on long-term extinction recall when given 1 hr before recall testing or when administered daily for 2 weeks prior to recall testing. However, URB597 diminished anxiety-related behaviours (Vimalanathan et al., 2020). With all things considered, further research is required to understand the intricate role of AEA signalling during fear and extinction learning.

2.1.1. Aims and Hypotheses

No studies have previously assessed the effect of AEA upregulation on spontaneous fear recovery. In the current chapter, FAAH was pharmacologically inhibited in a rat model of fear conditioning, extinction learning/retention, and spontaneous recovery. Separate cohorts of rats were systemically injected with various doses of URB597 before or after extinction training to manipulate the acquisition or consolidation of extinction memory, respectively. The effect of URB597 on extinction encoding was assessed in further tests of extinction retention and spontaneous fear recovery. URB597 given before extinction training was expected to enhance extinction acquisition. This was expected to manifest as reduced freezing in extinction training, testing, and spontaneous recovery. URB597 given after extinction training was

expected to enhance extinction consolidation. This was expected to manifest as reduced freezing in extinction testing and spontaneous recovery.

2.2. Methods

2.2.1. Animals

Male Lister Hooded rats (Charles River, UK) weighing 200-300g upon delivery were used in the experiments. Individually ventilated cages held four rats per cage with a 12 hr light/dark cycle (lights on at 07:00). Food and water were provided *ad libitum,* with care given in line with the principles of laboratory animal care. All procedures were conducted between 09:00-15:00, with animals tested at approximately the same time each day. These procedures had ethical approval from the institution's ethics committee and adhered to the UK Animals (Scientific Procedures) Act 1986 (Home Office Project Licences 30/3230 and P6DA59444).

2.2.2. Drugs

URB597 (0.1, 0.3, and 1 mg/kg; Sigma-Aldrich, UK) was suspended in 5% polyethylene glycol (PEG; Fluka Chemicals, Switzerland), 5% Tween 80 (Sigma-Aldrich), and 90% saline. These doses were selected due to their efficacy in previous fear memory research (Gobira et al., 2017; Laricchiuta et al., 2013; Morena et al., 2018). All injections were administered intraperitoneally at 1 ml/kg. Vehicle-treated controls received injections of 5% PEG, 5% Tween 80, and 90% saline. All studies included 12 rats per treatment group at the start of the experiment.

2.2.3. Apparatus and Materials

Four conditioning boxes (L:30cm, W:24cm, H:30cm; Med Associates, US) containing a metal grid floor (19 horizontal bars; 0.5cm diameter, 1.5cm spacing), mounted speaker (Jupiter 500, Black Star, UK) and electric shock generator (Med Associates) were used for the testing procedures. Each box had two opposing metal walls and two walls containing a Perspex-sealed pattern, e.g., black and white stripes or spots. The boxes were illuminated by the house light that became active at the start of the

session. Cameras above the conditioning boxes recorded rat activity. Videos were captured using the PhenoRack system (ViewPoint Life Sciences Inc, France). Stimulus presentation, including electric shocks, light flashes, and tones, were controlled by a computer running MED-PC V software (Med Associates). Two distinct contexts were used to reduce the effect of contextual fear memory on freezing behaviour. Context A (fear conditioning) involved: transporting the rats in small, opaque tubs with paper bedding; leaving the metal grids uncovered; and using acetic acid (0.5%) as the olfactory cue. Context B (extinction) involved: transporting the rats in large, transparent cages with sawdust bedding; covering the metal grids with white Perspex sheets; and using ethanol (40%) as the olfactory cue. Rats were also exposed to different wall patterns (i.e., spots vs stripes) in the two contexts. The CSs were tones (30 secs, 80dB, 4kHz) accompanied by a flash of light (50ms) upon tone onset. The US were footshocks (0.4mA, 0.5s; co-terminating with a CS) delivered via the metal floor grid.

2.2.4. Behavioural Testing

Habituation

Day 0 – Rats were placed in the conditioning boxes for 10 mins per context. All rats experienced context B first and then context A. A schematic representation of the experiments is shown below (see Figs 2.1a and 2.2a).

Fear Conditioning

Day 1 – Rats were placed in context A and left for 2 mins. Five CSs (120s inter-trial interval [ITI]) were presented alone, allowing the rats to become habituated to the stimulus. Two mins following the last CS, five CS-US pairings were presented. Animals were returned to their home cages immediately following the last CS-US pairing. Total procedure time was 25 mins.

Extinction Training

Day 2 – Thirty mins prior to testing or immediately following the extinction training protocol, rats were injected with one dose (see above) of URB597 or vehicle. Rats were placed in context B and left for the 2 min baseline period. Thirty CSs (30 secs ITI) were then presented alone. Animals were immediately returned to their home cages after the last CS. Total procedure time was 31 mins, 30 secs.

Extinction Testing

Day 3 – Rats were placed in context B and left for the 2 min baseline period. Three CSs (30 secs ITI) were presented alone. Animals were returned to their home cages after the last CS. This procedure lasted 4 mins, 30 secs and the animals were tested drug-free.

Spontaneous Fear Recovery Testing

Day 23 – This procedure was the same as that described in extinction testing above.

2.2.5. Data Analysis and Statistics

Freezing, defined as the absence of all movement except that in relation to respiration (Fanselow et al., 1980), was used as a behavioural indicator of fear. Due to issues with the automated scoring system, a trained observer, blinded to the treatment groups, manually scored behaviour of the rats that were injected before extinction training. Freezing was assessed in 3 sec intervals following CS presentation. Freezing for 2 secs or more within the predetermined interval denoted freezing behaviour. The total amount of freezing was subsequently expressed as a percentage of each 30 sec tone. Baseline freezing during the pre-CS period was calculated as a percentage of freezing in the first 2 mins before the onset of the first CS during the extinction training, extinction testing, and spontaneous fear recovery sessions. Freezing for rats that received injections after extinction training was scored automatically using ViewPoint software (ViewPoint Behaviour Technology, France; Papagianni et al., 2022). Whilst manual and automated scoring were not compared

in the current analyses, automated scoring was validated to match manual scoring. *n* numbers less than 12 (stated in figure legends) were caused by the unexpected corruption of numerous video files. If a video was found to be corrupted, the freezing data for that rat could not be recovered.

No data were recorded during contextual habituation. Prior to analysis, data sets were assessed for normal distribution. In the event that data sets were not normally distributed, as determined by a *P* value of < 0.05 in the Shapiro-Wilk test, data were log transformed (Y = Log[Y]) to ensure normality before being analysed. For fear conditioning, a two-way repeated measures analysis of variance (ANOVA) was used to assess freezing during the CS-US presentations. In this analysis, treatment formed the between-subject factor and the CS-US pairings (i.e., trial) formed the within-subject factor. For extinction training, baseline freezing was analysed using a one-way ANOVA; this assessed the between-subject factor of treatment. Freezing during the CS presentations of extinction training was assessed with a two-way repeated measures ANOVA in which treatment formed the between-subject factor and the averaged CS presentations (i.e., trial) formed the within-subject factor. Each three successive CSs (e.g., tones 1-3, 4-6 etc.) were averaged to reduce variability. Baseline freezing from extinction testing and spontaneous recovery were analysed together in a two-way repeated measures ANOVA. Treatment formed the between-subject factor and the experimental session formed the within-subject factor. Similarly, freezing during the CS presentations of extinction testing and spontaneous recovery were compared with a two-way repeated measures ANOVA in which treatment formed the between-subject factor and the experimental session formed the within-subject factor. The three CSs (e.g., tones 1-3) were averaged to reduce variability. All data are presented as mean + SEM, with the significance threshold set to $p < 0.05$. The Greenhouse-Geisser correction was applied to all repeated measures ANOVAs to adjust for the data's lack of sphericity.

2.3. Results

2.3.1. Experiment 1 – URB597 Given Pre-Extinction Training

URB597 did not affect extinction encoding or spontaneous fear recovery when administered before extinction training

During fear conditioning, freezing was low for the first CS-US pairing and proceeded to increase with subsequent presentations (Fig. 2.1b). A two-way ANOVA showed a main effect of trial ($F(3.486, 129.0) = 16.91$, $p < 0.0001$), indicating that there was a significant increase in freezing as more CS-US pairings were presented across all groups. There was no significant effect of treatment ($F(3, 37) = 0.2615$, $p = 0.8527$), showing that there were no differences in freezing between the treatment groups. Lastly, there was no significant trial x treatment interaction ($F(12, 148) = 0.9510$, $p = 0.4983$), indicating that there was no effect of treatment at specific CS-US pairings. These data indicate all animals showed comparable levels of freezing before being given their pre-allocated drug treatment the next day.

During extinction training, freezing during the 2 min baseline period was low (Fig. 2.1c). A one-way ANOVA of baseline freezing showed no significant effect of treatment ($F(3, 38) = 1.172$, $p = 0.3332$), indicating that freezing was comparable between the treatment groups during baseline. In the first tone block, freezing increased to its peak value in all groups (Fig. 2.1d). As more CSs were presented, freezing was steadily reduced until it plateaued at a low level. A two-way ANOVA of the extinction training data showed a main effect of trial ($F(4.835, 183.7) = 12.79$, $p < 0.0001$), indicating significant differences in freezing between early and late extinction. There was no main effect of treatment ($F(3, 38) = 2.652$, $p = 0.0625$), nor a trial x treatment interaction ($F(27, 342) = 1.149$, $p = 0.2808$), showing that URB597 had no effect on freezing during extinction training.

Baseline freezing was then compared between extinction testing and spontaneous recovery sessions (Fig. 2.1e). Baseline freezing was higher during spontaneous recovery than during extinction testing. A two-way ANOVA found a main effect of session ($F(1, 42) = 14.46$, $p = 0.0005$), indicating that this difference in

baseline freezing was significant. However, there was no effect of treatment ($F(3, 42) = 0.07576$, $p = 0.9727$), nor a session x treatment interaction ($F(3, 42) = 2.465$, $p = 0.0755$). These results show there was spontaneous recovery of baseline fear across all animals, with no effect of URB597 given before extinction training on baseline fear during extinction testing or spontaneous recovery.

Freezing levels during CS presentation were compared between extinction testing and spontaneous recovery (Fig. 2.1f). Freezing was higher in the spontaneous recovery session than in the extinction testing session. A two-way ANOVA revealed a main effect of session ($F(1, 42) = 14.67$, $p = 0.0004$), showing there was a significant increase in freezing during spontaneous recovery, compared to extinction testing across groups. The analysis did not show a main effect of treatment ($F(3, 42) = 0.5734$, $p = 0.6357$), nor a session x treatment interaction ($F(3, 42) = 1.026$, $p = 0.3908$), indicating there was no effect of URB597 given before extinction training on extinction testing or spontaneous recovery.

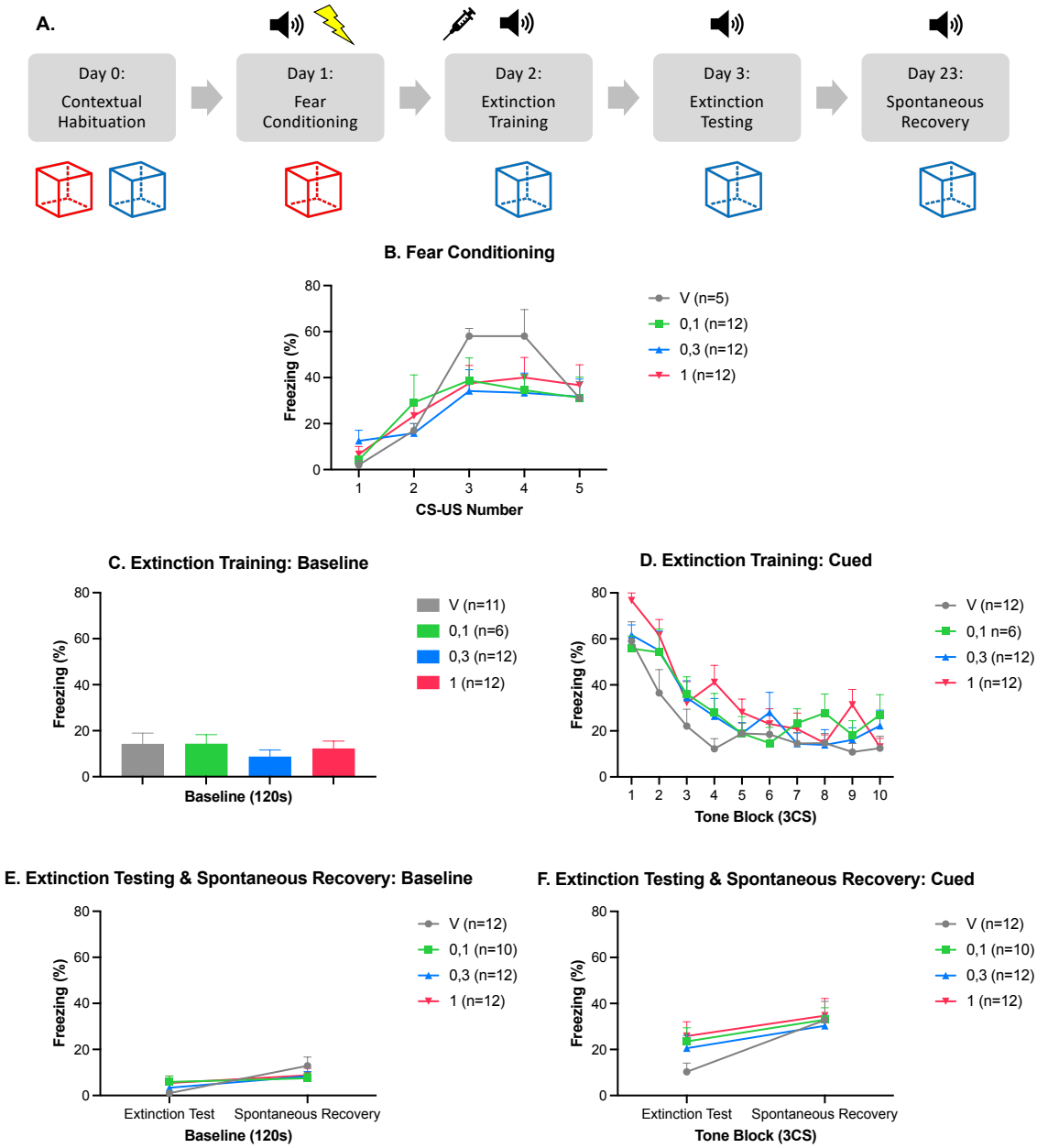

Figure 2.1. URB597 given pre-extinction training in a paradigm of spontaneous fear recovery. (A) Schematic representation of the experimental protocol. Red cubes indicate testing in context A, blue cubes represent testing in context B. Speakers indicate conditioned stimulus (CS) presentation, lightning indicates footshock presentation, syringe indicates injection. Fear conditioning was conducted drug-free. URB597 was injected 30 mins before extinction training. (B, D, F) Graphs showing the percentage of time rats spent freezing during the CS. (C, E) Graphs showing the percentage of time rats spent freezing during the 2-min baseline period prior to CS presentation.

V = vehicle, 0.1 = 0.1 mg/kg, 0.3 = 0.3 mg/kg, 1 = 1 mg/kg. Data are expressed as mean + SEM.

2.3.2. Experiment 2 – URB597 Given Post-Extinction Training

URB597 did not affect extinction encoding or spontaneous fear recovery when administered after extinction training

In fear conditioning, freezing was low for the first CS-US pairing and then steadily increased as more pairings were presented (Fig. 2.2b). A two-way ANOVA found a main effect of trial ($F (3.177, 139.8) = 14.68$, $p < 0.0001$), showing there was a significant increase in freezing as rats experienced more CS-US pairings. There was no significant effect of treatment ($F (3, 44) = 1.790$, $p = 0.1630$), nor a significant trial x treatment interaction ($F (12, 176) = 1.245$, $p = 0.2560$). These results indicate there were no significant differences in the conditioning of animals that were later separated into their pre-allocated treatment groups.

In extinction training, freezing during the 2 min baseline period was low (Fig. 2.2c). A one-way ANOVA of baseline freezing found no significant effect of treatment ($F (3, 44) = 2.027$, $p = 0.1239$). This indicates that freezing was comparable between the treatment groups during baseline. In all groups, freezing increased to its peak value upon presentation of the first tone block (Fig. 2.2d). As more CSs were presented, freezing steadily reduced until it gradually reached a plateau. A two-way ANOVA of the extinction training data showed a main effect of trial ($F (4.215, 185.4) = 7.537$, $p < 0.0001$), showing significant differences in freezing between early and late extinction. There was no main effect of treatment ($F (3, 44) = 0.1391$, $p = 0.9361$), nor a trial x treatment interaction ($F (27, 396) = 1.034$, $p = 0.4202$), indicating that there were no differences between treatment groups before receiving URB597 after extinction training.

Comparing baseline freezing between extinction testing and spontaneous recovery sessions (Fig. 2.2e), results showed that baseline freezing was higher in the spontaneous recovery session than extinction testing across all groups. A two-way ANOVA showed a main effect of session ($F (1, 44) = 11.05$, $p = 0.0018$), indicating that there was a significant difference in baseline freezing across all groups. There was no effect of treatment ($F (3, 44) = 0.8543$, $p = 0.4719$), nor a session x treatment

interaction (F (3, 44) = 0.6020, p = 0.6172), showing that URB597 after extinction training did not affect freezing.

Freezing levels during CS presentation were compared between extinction testing and spontaneous recovery (Fig. 2.2f). Freezing during the spontaneous recovery session was higher in comparison to the extinction testing session across all groups. A two-way ANOVA showed a main effect of session (F (1, 44) = 6.371, p = 0.0153), indicating there was a significant increase in freezing during spontaneous recovery testing, compared to extinction testing across groups. There was no main effect of treatment (F (3, 44) = 0.3139, p = 0.8152), nor a session x treatment interaction (F (3, 44) = 0.03901, p = 0.9896), indicating that URB597 after extinction training imparted no effect on extinction testing or spontaneous recovery.

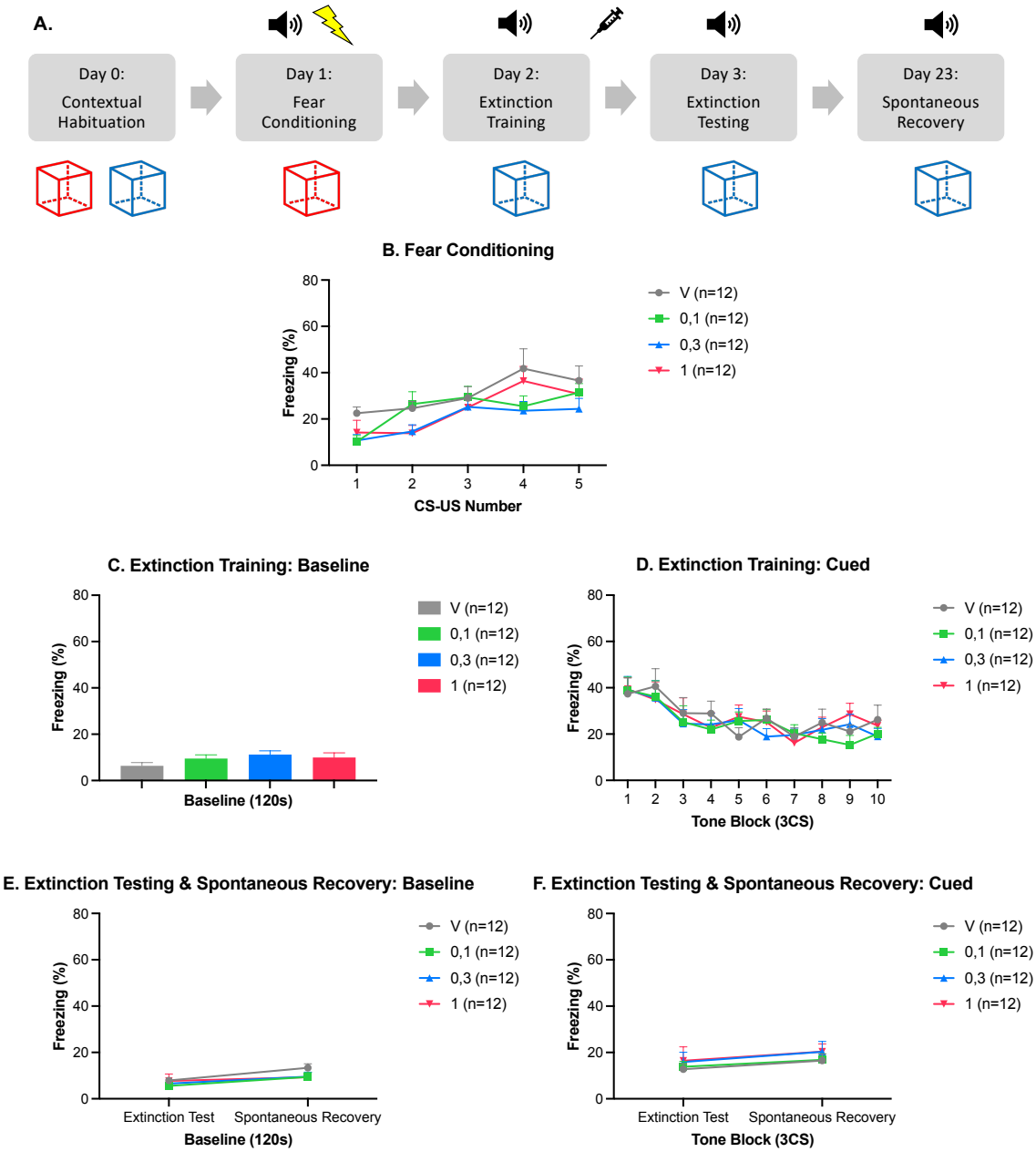

Figure 2.2. URB597 given post-extinction training in a paradigm of spontaneous fear recovery. (A) Schematic representation of the experimental protocol. Red cubes indicate testing in context A, blue cubes represent testing in context B. Speakers indicate conditioned stimulus (CS) presentation, lightning indicates footshock presentation, syringe indicates injection. Fear conditioning and extinction training were conducted drug-free. URB597 was injected immediately after extinction training. (B, D, F) Graphs showing the percentage of time rats spent freezing during the CS. (C, E) Graphs showing the percentage of time rats spent freezing during the 2-min baseline period prior to CS presentation.

V = vehicle, 0.1 = 0.1 mg/kg, 0.3 = 0.3 mg/kg, 1 = 1 mg/kg. Data are expressed as mean + SEM.

2.4. Discussion

This chapter assessed if AEA upregulation, via inhibition of its metabolism, prior to or after extinction training affected extinction encoding and spontaneous recovery in an auditory-cued fear paradigm. URB597 administered before or after extinction was expected to augment acquisition and/or consolidation of the extinction memory. Fear conditioning, extinction encoding, and spontaneous recovery were successfully implemented across the treatment cohorts. In spontaneous recovery, all groups showed significant increases in baseline and cued fear. However, URB597 had no effect on extinction memory when given before or after extinction training. In tests of extinction retention and spontaneous recovery, there were no differences in freezing between drug treatment groups.

Pharmacological upregulation of AEA tone enhances extinction memory by inhibiting fear expression (Bitencourt et al., 2008; Bowers and Ressler, 2015; Chhatwal et al., 2005; Gunduz-Cinar et al., 2013a; Pamplona et al., 2008). This is likely driven by activation of CB1Rs expressed on Glu receptors in the forebrain (Llorente-Berzal et al., 2015). However, as in the current chapter, others have shown that FAAH inhibition in male rodents prior to extinction training does not affect extinction encoding under low stress conditions (Hartley et al., 2016; Morena et al., 2021; Mizuno et al., 2022). Whilst these divergent results may appear counter-intuitive, they may be explained by procedural differences. For example, Gunduz-Cinar et al. (2013a) used inbred 129S1/Sv1mJ mice in a model of impaired fear extinction, whilst also opting to use AM3506 instead of URB597. Bowers and Ressler (2015) used C57BL/6J mice in their auditory-cued paradigm. Further treatment discrepancies are seen in studies that used AM404 to manipulate AEA tone (Bitencourt et al., 2008; Chhatwal et al., 2005; Pamplona et al., 2008). Not only does AM404 utilise an alternative mechanism to upregulate tonic AEA levels (reuptake inhibition vs FAAH inhibition), it also enhances 2-AG and TRPV1 signalling (Hájos et al., 2004; Wiskerke et al., 2012; Zygmunt et al., 2000). Differences in shock parameters may also help to explain the lack of URB597's effect within the current paradigm. Bowers and Ressler (2015) demonstrated that administration of URB597 prior to auditory-cued

extinction training reduced freezing during extinction yet did not enhance extinction retention. However, shock parameters used by Bowers and Ressler (2015; 1mA, 0.5s) were more intense than those used in the current chapter (0.4mA, 0.5s). Finally, it could be that repeated doses of URB597 are required to enhance extinction retention or prevent spontaneous recovery rather than the single dose used here. For example, repeated administration of URB597 enhances extinction learning in male rats and mice, as well as in male and female humans (Mayo et al., 2020b; Morena et al., 2018). Despite this, Vimalanathan et al. (2020) found that repeated daily doses of URB597 for 2 weeks prior to long-term extinction recall did not reduce freezing expression during the extinction test. Taken together, it appears that studies assessing the effects of FAAH inhibition on extinction memory are heterogenous in their methodological approaches. This may explain why there is such disparity between the previous and present results acquired.

Stress prior to extinction greatly impacts extinction retention. Maren and Chang (2006) showed that increasing stress prior to extinction training (via an immediate extinction protocol or unsignalled footshocks) did not affect extinction acquisition but instead impaired extinction retention in tests 1 and 7 days after extinction training. This may be due to inadequate consolidation of the extinction memory or failure to later retrieve the inhibitory memory. AEA signalling is implicated in stress (Dlugos et al., 2012; Gunduz-Cinar et al., 2013b; Mayo et al., 2018). FAAH inhibition can exert anxiolytic effects following exposure to stress but is relatively ineffective under basal conditions (Bluett et al., 2014; Haller et al., 2009; Hill et al., 2013a; Kathuria et al., 2003). Morena et al. (2018) showed that repeated post-extinction injections of URB597 enhanced extinction consolidation, retention, and social interaction in rats that were socially isolated prior to and throughout behavioural testing. Rats given URB597 prior to extinction training did not show a reduction in freezing. Morena et al. (2018) also showed that their fear and extinction procedure, coupled with social isolation, caused a reduction in hippocampal AEA tone. The authors postulated that repeated administration of URB597 post-extinction training ameliorated the deficit in hippocampal AEA signalling, thus restoring freezing behaviour to a level comparable to rats who did not undergo fear

conditioning. Mice exposed to social defeat stress prior to fear conditioning show impaired extinction retention, which is restored by URB597 (Laricchiuta et al., 2013). Zer-Aviv and Akirav (2016) subjected rats to a prolonged stress model of PTSD that included restraint, forced swim, and sedation. The authors found that male rats given URB597 after stress showed enhanced extinction in a CB1R-dependent fashion. This effect was thought to be mediated by prevention of both LTP in the CA1 region of the HPC and CB1R upregulation in the AMG, PFC, and HPC. Considering these results, it may be that both fear conditioning and extinction training in the current chapter took place under lower stress conditions. This was reflected in the low baseline freezing recorded before each session and low freezing recorded during extinction testing. Other studies that incorporated prior stress showed relatively high levels of freezing in the controls during extinction recall testing (Laricchiuta et al., 2013; Zer-Aviv and Akirav, 2016). It is likely that extinction training in the current chapter was successful in creating a robust extinction memory that was acquired, consolidated, and retrieved in an adaptive fashion. This was evidenced by low freezing at the end of extinction training and during extinction testing. This may mean that in the absence of aberrant neurotransmission, URB597 was unable to impart a remedial effect on extinction training and short-term recall of the extinction memory. Issues regarding the timing of drug administration prior to extinction training and the specificity of URB597's pharmacodynamics are considered in the general discussion.

2.4.1. Conclusion

URB597 given before or after delayed extinction training had no effect on extinction memory consolidation, or on later spontaneous fear recovery. The parameters used in the current experiment were successful in implementing fear conditioning, extinction training/retention, and spontaneous recovery. However, extinction memory was likely formed under basal stress conditions. Therefore, the extinction memory may have been functional and adaptive. URB597 may not have been able to impart a beneficial effect during extinction training or recall due to the absence of aberrant neurotransmission. Future research will assess whether URB597 can ameliorate stress-related impairments of extinction memory. This will be achieved

through an immediate extinction protocol with more severe conditioning parameters.

3. JZL184 Modulation of Spontaneous Fear Recovery

3.1. Introduction

The previous chapter found that URB597 did not affect extinction encoding or spontaneous fear recovery when given before or after extinction training. The current chapter assessed whether 2-AG upregulation induced by JZL184 (MAGL inhibitor) could prevent fear relapse in the same paradigm.

2-AG is the primary eCB involved in retrograde synaptic transmission. 2-AG-mediated agonism of central CB1R modulates neurotransmission in a homeostatic manner (Castillo et al., 2012). 2-AG tone can be manipulated through pharmacological inhibition or genetic knock-out of DAGLα, the enzyme required for 2-AG synthesis. Jenniches et al. (2016) found that DAGLα-KO mice showed an increased CFR throughout multiple extinction training sessions, and an impaired ability to extinguish compared to wild-type mice. Cavener et al. (2018) found that DAGLα-KO mice, as well as those given DO34 (DAGLα inhibitor) prior to extinction training, showed elevated freezing during two extinction sessions and an extinction retention test compared to wild-type and vehicle mice, respectively. These results indicate the importance of 2-AG signalling in the formation of fear extinction memories.

2-AG tone can also be upregulated through the inhibition of its degrading enzyme MAGL. JZL184 selectively enhances 2-AG-mediated activation of central CB1Rs without a concomitant increase in AEA tone (Long et al., 2009a). Systemic administration of JZL184 evokes canonical cannabinoid behavioural effects, e.g., analgesia, hypothermia, and hypomotility (Long et al., 2009a). JZL184 and other MAGL inhibitors have been used to modulate 2-AG tone within fear learning and extinction paradigms. Busquets-Garcia et al. (2011) found that JZL184 given after contextual fear conditioning had no effect on fear memory consolidation. Llorente-Berzal et al. (2015) reported that JZL184 (4 and 8 mg/kg) enhanced cued fear expression in mice via CB1R signalling on GABAergic neurons. Rea et al. (2014) infused 2-AG into rat vHPC and showed significantly reduced contextual fear

expression compared to vehicle, with 2-AG's effect being blocked by pre-administration of rimonabant (CB1R inverse agonist). In mice, Hartley et al. (2016) showed that a higher dose of JZL184 (8 mg/kg) impaired cued extinction in a CB1R-dependent manner when given before extinction training compared to vehicle. A lower dose (2 mg/kg) neither impaired nor enhanced extinction training. Using an alternative MAGL inhibitor, Morena et al. (2021) found that MJN110 exerted no effect on cued freezing in male rats when given before extinction training compared to vehicle. However, in female rats, MJN110 reduced freezing and increased darting behaviours in a CB1R-dependent manner. Morena et al. (2018) subjected socially isolated rats to contextual fear conditioning. When given before multiple extinction sessions, low doses of JZL184 (0.5 and 1 mg/kg) reduced global levels of freezing throughout the extinction sessions and a drug-free retention session compared to vehicle. Morena et al. (2018) found no effect of JZL184 when given after extinction training. Evidence from IA studies suggests that post-training administration of the drug can enhance performance in an IA retention test (Ratano et al., 2018). Collectively, these results show disparate findings. However, the divergent effects may be attributable to differences in species, dosage of a given MAGL inhibitor, or the type of conditioning implemented (e.g., cued or contextual).

3.1.1. Aims and Hypotheses

Research considering the role of MAGL in extinction is limited in comparison to CB1R and FAAH. The current chapter sought to clarify the effect of 2-AG upregulation in extinction memory encoding, its subsequent retention, and spontaneous fear recovery. JZL184 was administered at various doses before or after fear extinction training, with tests of extinction retention and spontaneous fear recovery taking place 1 and 21 days after training, respectively. No research has yet assessed the effects of JZL184 in fear relapse via spontaneous recovery. However, previous results suggest that low doses of JZL184 may reduce freezing during extinction training and testing, whilst higher doses seem to impair extinction. It was expected that lower doses of JZL184 would reduce freezing in extinction training, testing, and spontaneous recovery when given before extinction, whilst reducing freezing in

extinction testing and spontaneous recovery when given after. Higher doses of JZL184 were expected to impair the acquisition or consolidation of extinction memory, thus enhancing freezing.

3.2. Methods

3.2.1. Animals

Male Lister Hooded rats (Charles River, UK) weighing 200-300g upon delivery were used in the experiments. Husbandry protocol and ethics are as described in section 2.2.1.

3.2.2. Drugs

JZL184 (1, 3, and 10 mg/kg; Sigma-Aldrich, UK) was dissolved in 5% PEG (Fluka Chemicals, Switzerland), 5% Tween 80 (Sigma-Aldrich), and 90% saline. The doses selected have been shown to induce MAGL inhibition without concurrent FAAH inhibition (Busquets-Garcia et al., 2011; Long et al., 2009a). Similar doses have also previously shown an effect in other fear learning paradigms (Hartley et al., 2016; Llorente-Berzal et al., 2015; Morena et al., 2018). All injections were administered intraperitoneally at 1 ml/kg. Vehicle-treated controls received injections of 5% PEG, 5% Tween 80, and 90% saline. All studies included 12 rats per treatment group at the start of the experiment.

3.2.3. Apparatus and Materials

All apparatus, materials, and conditioning parameters used were the same as those described in section 2.2.3.

3.2.4. Behavioural Testing

Behavioural testing was the same as the previous chapter (see section 2.2.4.). A schematic representation of the experiments is shown below (see Figs 3.1a and 3.2a).

3.2.5. Data Analysis and Statistics

Freezing was used as a behavioural indicator of fear. Analysis techniques used were the same as section 2.2.5. In experiment 2, n = 11 during fear conditioning. This loss of data was caused by a crash in the computer software after conditioning had been completed. Fear conditioning data was omitted for this cage; however, data from these rats were included in the analysis of the other sessions thereafter (n = 12).

3.3. Results

3.3.1. Experiment 1 – JZL184 Given Pre-Extinction Training

JZL184 did not affect extinction encoding or spontaneous fear recovery when administered before extinction training

When undergoing fear conditioning, freezing was low for the first CS-US pairing and proceeded to increase as more CS-US pairings were presented (Fig. 3.1b). A two-way ANOVA showed a main effect of trial ($F(3.057, 134.5) = 7.223$, $p = 0.0001$); this shows that the freezing in all groups significantly increased as more CS-US pairings were presented. There was no significant effect of treatment ($F(3, 44) = 0.9217$, $p = 0.4382$), indicating that there were no significant differences in freezing between the treatment groups. There was no significant trial x treatment interaction ($F(12, 176) = 1.042$, $p = 0.4123$), indicating that the treatment groups showed no difference in freezing at specific CS-US pairings. These results show that there were no significant differences between the cohorts before being given their pre-allocated drug treatment the next day.

During extinction training, baseline freezing during the 2 min pre-CS period showed that freezing was low with no differences between treatment groups (Fig. 3.1c). A one-way ANOVA of baseline freezing showed no significant effect of treatment ($F(3, 38) = 0.2707$, $p = 0.8461$). Freezing increased to its peak value in all groups following presentation of the first tone block (Fig. 3.1d). Freezing was steadily reduced as more CSs were presented until it plateaued at a low level after around 12-to-15 CSs presentations. A two-way ANOVA on the extinction training data showed a

main effect of trial (F (3.884, 147.6) = 22.10, p <0.0001), indicating that freezing was significantly decreased later in the extinction training protocol. There was no main effect of treatment (F (3, 38) = 0.6477, p = 0.5892), nor a trial x treatment interaction (F (27, 342) = 1.245, p = 0.1899). These results show that JZL184 had no effect on freezing during extinction training.

Baseline freezing during extinction testing and spontaneous recovery sessions were compared (Fig. 3.1e). Baseline freezing was significantly higher during spontaneous recovery than during extinction testing across all groups. A two-way ANOVA found a main effect of session (F (1, 44) = 10.59, p = 0.0022). However, there was no effect of treatment (F (3, 44) = 0.8295, p = 0.4848), nor a session x treatment interaction (F (3, 44) = 1.576, p =0.2088). These results show there was spontaneous recovery of baseline fear across all treatment groups; however, there was no effect of JZL184 given before extinction training on baseline fear during extinction testing or spontaneous recovery.

Freezing levels during CS presentation were compared between extinction testing and spontaneous recovery (Fig. 3.1f). Freezing was higher in the spontaneous recovery session than in the extinction testing session across groups. A two-way ANOVA revealed a main effect of session (F (1, 44) = 21.99, p <0.0001), showing there was a significant increase in freezing during spontaneous recovery, compared to extinction testing. The analysis did not show a main effect of treatment (F (3, 44) = 0.08238, p = 0.9693), nor a session x treatment interaction (F (3, 44) = 0.4320, p = 0.7311), indicating there was no effect of JZL184 given before extinction training on extinction testing or spontaneous recovery.

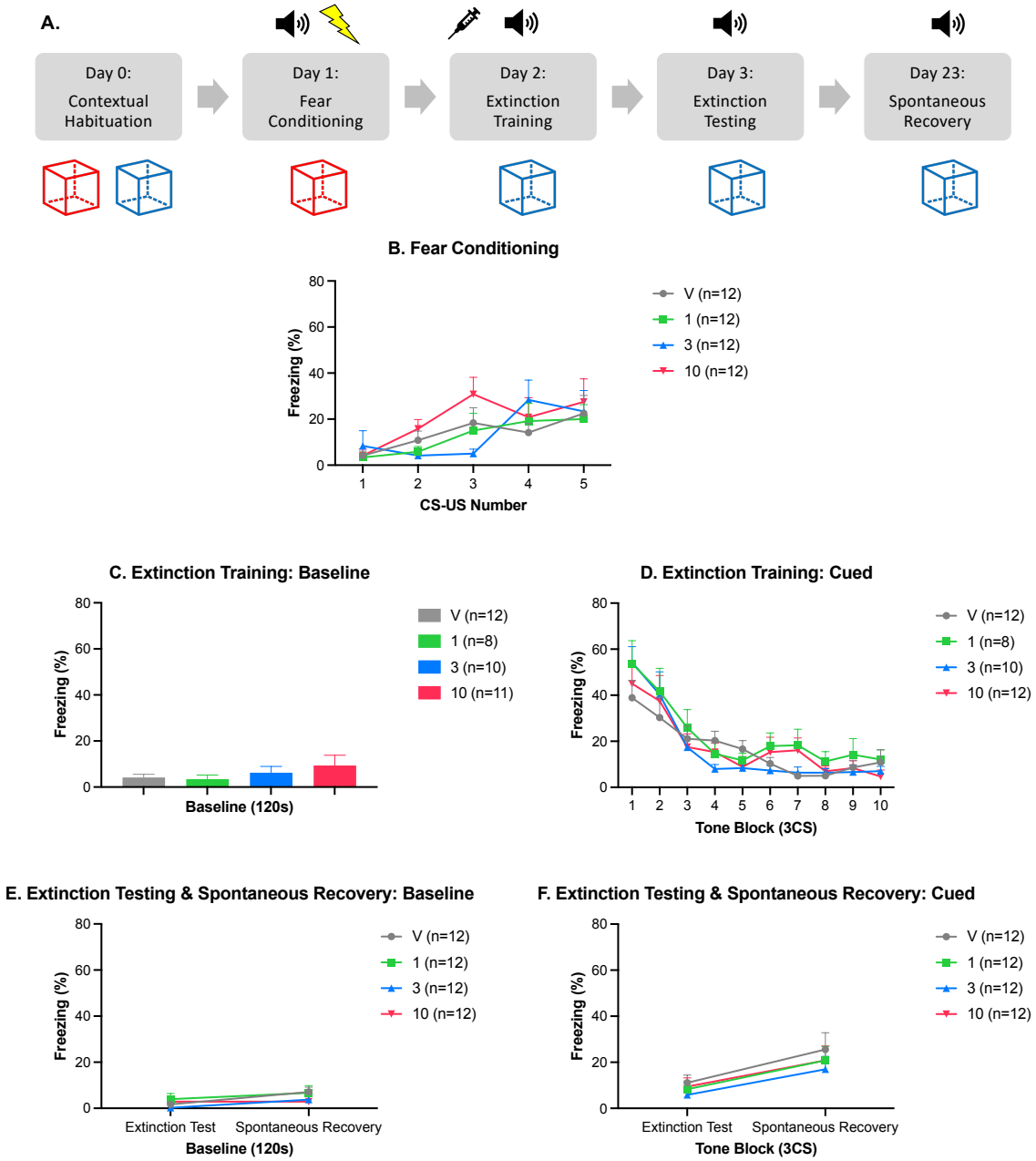

Figure 3.1. JZL184 given pre-extinction training in a paradigm of spontaneous fear recovery. (A) Schematic representation of the experimental protocol. Red cubes indicate testing in context A, blue cubes represent testing in context B. Speakers indicate conditioned stimulus (CS) presentation, lightning indicates footshock presentation, syringe indicates injection. Fear conditioning was conducted drug-free. JZL184 was injected 30 mins before extinction training. (B, D, F) Graphs showing the percentage of time rats spent freezing during the CS. (C, E) Graphs showing the percentage of time rats spent freezing during the 2-min baseline period prior to CS presentation.

V = vehicle, 1 = 1 mg/kg, 3 = 3 mg/kg, 10 = 10 mg/kg. Data are expressed as mean + SEM.

3.3.2. Experiment 2 – JZL184 Given Post-Extinction Training

JZL184 did not affect extinction encoding or spontaneous fear recovery when administered after extinction training

In fear conditioning, freezing was low for the first CS-US pairing and then steadily increased as more pairings were presented (Fig. 3.2b). Using a two-way ANOVA, we found a main effect of trial ($F (2.491, 99.63) = 35.94$, $p < 0.0001$), showing there was a significant increase in freezing as more CS-US pairings were presented. There was no significant effect of treatment ($F (3, 40) = 0.1408$, $p = 0.9349$), nor a significant trial x treatment interaction ($F (12, 160) = 1.037$, $p = 0.4178$). These results indicate there were no significant differences in the conditioning of animals that were later separated into pre-allocated treatment groups.

In extinction training, freezing during the 2 min pre-CS baseline period was low (Fig. 3.2c). A one-way ANOVA of baseline freezing found no significant effect of treatment ($F (3, 44) = 1.124$, $p = 0.3496$). This indicates that freezing was comparable between the treatment groups during baseline. In all groups, freezing increased upon presentation of the first tone block (Fig. 3.2d). As more CSs were presented, freezing steadily reduced until it gradually reached a plateau. A two-way ANOVA of the extinction training data showed a main effect of trial ($F (3.687, 162.2) = 29.25$, $p < 0.0001$), showing significant differences in freezing between early and late extinction. There was no main effect of treatment ($F (3, 44) = 1.573$, $p = 0.2094$), nor a trial x treatment interaction ($F (27, 396) = 1.033$, $p = 0.4223$), indicating that there were no differences between treatment groups before receiving a post-extinction training injection of JZL184.

Comparing baseline freezing between extinction testing and spontaneous recovery sessions (Fig. 3.2e), results showed that baseline freezing was higher in the spontaneous recovery session than extinction testing. A two-way ANOVA showed a main effect of session ($F (1, 44) = 61.99$, $p < 0.0001$), indicating that there was a significant difference in baseline freezing. There was no effect of treatment ($F (3, 44)$

= 0.7337, p = 0.5375), nor a session x treatment interaction (F (3, 44) = 0.5595, p = 0.6446), showing that JZL184 did not affect baseline freezing.

Freezing levels during CS presentation were compared between extinction testing and spontaneous recovery (Fig. 3.2f). Freezing during the spontaneous recovery session was higher in comparison to the extinction testing session across all groups. A two-way ANOVA showed a main effect of session (F (1, 44) = 12.65, p = 0.0009), indicating there was a significant increase in freezing during spontaneous recovery testing, compared to extinction testing. There was no main effect of treatment (F (3, 44) = 0.8342, p = 0.4823), nor session x treatment interaction (F (3, 44) = 0.2110, p = 0.8883), indicating that JZL184 imparted no effect on extinction testing or spontaneous recovery.

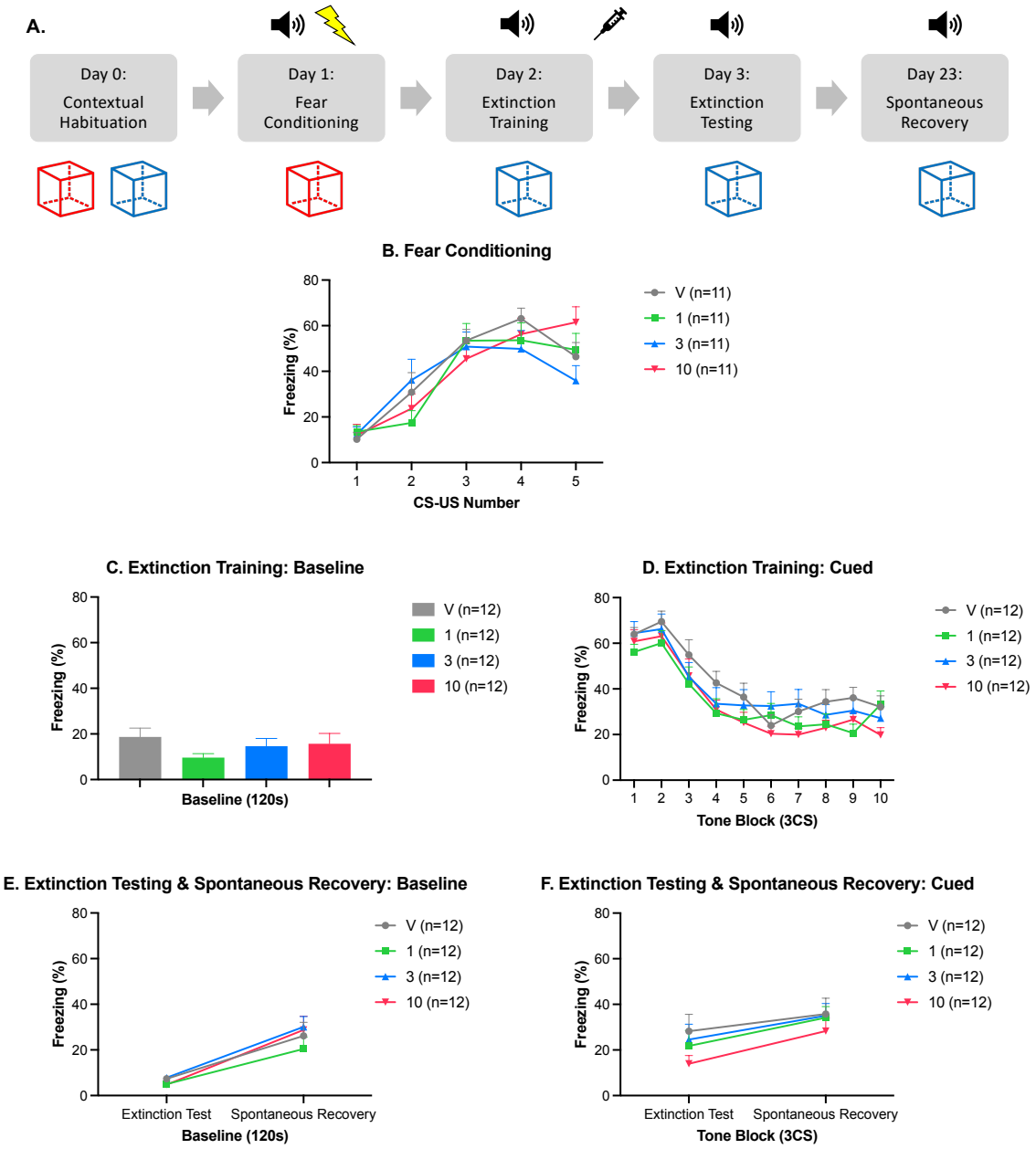

Figure 3.2. JZL184 given post-extinction training in a paradigm of spontaneous fear recovery. (A) Schematic representation of the experimental protocol. Red cubes indicate testing in context A, blue cubes represent testing in context B. Speakers indicate conditioned stimulus (CS) presentation, lightning indicates footshock presentation, syringe indicates injection. Fear conditioning and extinction training were conducted drug-free. JZL184 was injected immediately after extinction training. (B, D, F) Graphs showing the percentage of time rats spent freezing during the CS. (C, E) Graphs showing the percentage of time rats spent freezing during the 2-min baseline period prior to CS presentation.

V = vehicle, 1 = 1 mg/kg, 3 = 3 mg/kg, 10 = 10 mg/kg. Data are expressed as mean + SEM.

3.4. Discussion

This chapter sought to investigate the role of 2-AG in extinction encoding and spontaneous fear recovery. The low doses of JZL184 (1 and 3 mg/kg) administered before or after extinction training were expected to enhance extinction and attenuate spontaneous fear recovery, whilst the high dose (10 mg/kg) was expected to impair extinction and augment spontaneous recovery. Collectively, the rats showed successful fear conditioning, extinction encoding, and spontaneous recovery of both baseline and cued fear. JZL184 had no effect on freezing when administered before or after extinction training, regardless of dose. Freezing in the JZL184 groups during extinction retention and spontaneous recovery testing showed no difference when compared to vehicle.

The role of 2-AG signalling in the extinction of learned fear memory is complex. Jenniches et al. (2016) and Cavener et al. (2018) showed that DAGLα-KO mice exhibited impaired cued extinction learning. Cavener et al. (2018) also showed similar results in mice treated with DO34. Rea et al. (2014) reported that intracerebral infusion of 2-AG into the ventral HPC enhanced contextual fear extinction. Whilst these results are in accordance with one another, the literature surrounding the effect of MAGL inhibition on extinction memory is less conclusive. In socially isolated rats, Morena et al. (2018) found that low doses of JZL184 (0.5, 1 mg/kg) given prior to repeated extinction training sessions induced a global reduction of freezing throughout repeated extinction training sessions, and during extinction testing. However, Hartley et al. (2016) and Mizuno et al. (2022) showed that higher doses of JZL184 impaired extinction learning. Using a different MAGL inhibitor, Morena et al. (2021) found that 2-AG upregulation had no effect on cued extinction learning in male rats but increased the expression of active fear in female rats. Considering the methodological differences between previous research studies, it is difficult to draw conclusions on the effect of JZL184. One possibility is that the drug elicits a bell-shaped dose-response curve, which may be similar to WIN 55-212,2 (Chhatwal et al., 2005; Lin et al., 2008; Pamplona et al., 2006). For example, lower doses could reduce fear expression, whilst higher doses may augment fear expression and impair

extinction. This may be due to excessive CB1R agonism given that JZL184-mediated enhancement of fear is CB1R-dependent (Hartley et al. 2016; Llorente-Berzal et al. 2015). Another distinction is the type of conditioning used. JZL184 and 2-AG based-treatments show positive pharmacological effects in contextual fear conditioning paradigms (Morena et al., 2018; Rea et al., 2014). It is possible that JZL184 elicits a divergent response when used in cued or contextual paradigms depending on its action in different brain areas. This is considered further in the general discussion.

The current chapter shows that MAGL inhibition did not modulate freezing in male rats, a finding that is corroborated by Morena et al. (2021). Despite this, Morena et al. (2018) found that low doses of JZL184 (0.5 and 1 mg/kg) globally reduced freezing levels during contextual extinction training and testing in socially isolated rats. This effect was seen when JZL184 was given before repeated extinction training sessions. Mizuno et al. (2022) also administered JZL184 (4 and 8 mg/kg) before repeated contextual extinction sessions. The authors found no acute effect of JZL184 in the first extinction session but saw elevated freezing in subsequent sessions. It is possible that chronic administration of JZL184, combined with repeated extinction sessions, is required to modulate freezing behaviour, likely through the gradual upregulation of 2-AG tone. However, others have shown that a single dose of JZL184 (8 mg/kg) can elevate freezing during auditory-cued extinction training that used strong footshocks (0.7 mA, 2s; Hartley et al., 2016). It is important to note that Hartley et al. (2016) used mice whilst Morena et al. (2018) and Mizuno et al. (2022) used rats. This suggests that multiple doses of JZL184 may be required to alter fear-related behaviour in rats. Taken together, it appears that the doses selected for the current chapter were similar to those that have shown an effect in other extinction paradigms. However, discrepancies between the conditioning modality, footshock intensity, and number of extinction training sessions may explain the lack of JZL184's effect in the current chapter.

As mentioned in the discussion of chapter 2, JZL184 may have been unable to impart an effect on extinction training and short-term extinction recall due to the weak conditioning and adaptive extinction that was acquired. The potential for any

therapeutic effects of the drug may have been negated by a 'floor effect'. Data show that fear conditioning, extinction learning, and short-term retention all occurred in an adaptive fashion. It is possible that in the absence of aberrant neurotransmission, extinction could not be enhanced further by JZL184 administration. Issues regarding the timing of drug administration prior to extinction training and the specificity of JZL184's pharmacodynamics are considered in the general discussion.

3.4.1. Conclusion

The current chapter found no effect of JZL184 on extinction encoding or spontaneous fear recovery. Interpretation of these results is difficult given the limited research that has assessed the effect of MAGL inhibition on extinction learning and retention. Between these studies, the methods are somewhat heterogenous. Differences in species, drug, dosage, and drug administration timing may all contribute to the disparate outcomes in which extinction learning has been enhanced, impaired, or not affected. Further study of MAGL inhibition in the modulation of extinction learning is required to determine the conditions in which extinction is impaired or enhanced.

4. Immediate Extinction Validation and Propranolol Effects

4.1. Introduction

In the previous chapters, URB597 and JZL184 did not affect extinction encoding or spontaneous fear recovery when extinction learning and retention occurred in an adaptive fashion. Despite showing spontaneous recovery, freezing in all treatment groups was relatively low at the end of extinction training, during extinction testing, and during the spontaneous recovery session. It could be that URB597 and JZL184 were unable to modulate behaviour due to a floor effect on freezing levels. In this case, it is possible that extinction could not be pharmacologically enhanced due to the absence of aberrant neuronal transmission. To address this, an alternative protocol was used that sought to induce a deficit in extinction. This chapter is concerned with the validation of this model and the suitability of its use for future investigations.

The IED is a phenomenon that occurs when fear extinction is conducted immediately following conditioning (e.g., 1 min to 6 hrs after conditioning; Chang and Maren, 2009; Maren and Chang, 2006; Totty et al., 2019). The IED is characterised by an inability to retain extinction memory despite a reduction of the CFR during extinction training. The IED may provide a translatable preclinical model of extinction resistance, which is a feature of anxiety-related disorders (Michael et al., 2007; Milad et al., 2009, 2013; Milad and Quirk, 2012). Research suggests that this deficit in extinction retention is caused by the level of emotional arousal at the time of extinction training (Fitzgerald et al., 2015; Giustino et al., 2017; Kim et al., 2010; Maren and Chang, 2006; Totty et al., 2019). Fitzgerald et al. (2015) found that systemic administration of propranolol, a non-selective β-AR antagonist, prior to immediate extinction training remediated the IED. Wang et al. (2021) showed that whilst systemic propranolol given prior to immediate extinction training did not rescue the IED, it did remedy an impaired ability to re-extinguish fear. These findings are likely explained by propranolol-mediated dampening of shock-induced NA signalling prior to immediate extinction training. Stress induces BLA hyperexcitability (Sharp, 2017). Giustino et al. (2017) found that intra-BLA, but not intra-mPFC infusion

of propranolol rescued the IED. CRF antagonism within the BLA can facilitate extinction retention after immediate extinction (Hollis et al., 2016). Jo et al. (2020) showed that inhibition of CRF neurons in the CeA rescued the IED. Research suggests that deficits in extinction learning are mediated by stress-induced NA signalling in the AMG, which is thought to be driven by excessive NA release from the LC. Giustino et al. (2020) showed that chemogenetic activation of the LC prior to a weak conditioning protocol (i.e., a protocol in which rats were immune to the IED) induced the IED. This effect was blocked by intra-BLA infusions of propranolol. With all things considered, current evidence suggests that the IED is caused by shock-induced stress that promotes elevated NA release, resulting in BLA hyperactivity. This activity in the BLA is then thought to inhibit the IL function required for successful encoding and later retention of extinction memory (Giustino et al., 2020).

4.1.1. Aims and Hypotheses

The IED protocol offers a convenient model to induce aberrant neuronal signalling within a fear conditioning and extinction paradigm. Experiment 1 was conducted to validate this model for further use in pharmacological experiments. The parameters from canonical IED studies were used as the basis of the experiment (Chang and Maren, 2009; Maren and Chang, 2006). Shock parameters were increased compared to the previous chapters to ensure that the IED was induced. Immediate extinction training took place 30 mins after fear conditioning, whilst delayed extinction took place 24 hrs later. Extinction retention tests were implemented 1 day after the respective extinction training sessions. It was expected that immediate extinction training would cause a deficit in extinction retention. The immediate extinction group were expected to show higher freezing during the extinction test when compared to delayed extinction. Freezing levels in the immediate extinction group were not expected to differ from no extinction control groups that were also included.

Experiment 2 assessed whether propranolol could rescue the IED. Rats were given either propranolol or vehicle and underwent either immediate or no extinction training. An extinction retention test was conducted 1 day after extinction training. Given that propranolol can reduce freezing during extinction training and rescue the

IED (Fitzgerald et al., 2015; Giustino et al., 2017, 2020), it was expected that propranolol would reduce baseline and cued freezing during immediate extinction training compared to vehicle. It was also expected that propranolol given prior to immediate extinction training would reduce freezing in the extinction retention test compared to vehicle. No extinction controls were included to demonstrate that propranolol's effect on freezing behaviour required extinction and was not caused by impaired fear memory consolidation.

4.2. Methods – Experiment 1: Procedure Validation

4.2.1. Animals

Experiment 1 used 40 male Lister Hooded rats (Charles River, UK) weighing 200-300g upon delivery. Rats were split into either immediate, no immediate, delayed, or no delayed extinction groups (*n* = 10 per group). Husbandry and ethics were the same as those described in section 2.2.1.

4.2.2. Apparatus and Materials

The apparatus and materials were the same as described in section 2.2.3. The methodology was adapted to match previous studies that have successfully induced the IED, e.g., Chang and Maren (2009) and Maren and Chang (2006).

4.2.3. Behavioural Testing

Fear Conditioning

Day 1 – Rats were placed in context A and left for 2 mins. Five CS-US pairings were then presented with an ITI of 2 mins. The shock parameters for the US (0.5mA, 1s) were stronger than in chapters 2 and 3. CS characteristics remained unchanged. Procedure time was 12 mins, 30 secs. Animals were returned to their home cages immediately following the last CS-US pairing. A schematic representation of the experiment is shown below (see Fig 4.1a).

Extinction Training

Day 1 or 2 – Immediate (day 1) and delayed (day 2) extinction groups underwent extinction training either 30 mins or 24 hrs after fear conditioning, respectively. Rats were placed in context B and left for a 2 min baseline period. Following this, 45 CSs (30 secs ITI) were then presented alone. Rats in the no immediate and no delayed extinction groups were placed in context B either 30 mins or 24 hrs after fear conditioning, respectively. The first 2 mins of freezing activity were recorded before animals were left to remain in the context without experiencing any CSs. The total procedure time for both extinction and no extinction groups was 46 mins, 30 secs Animals were immediately returned to their home cages after the last CS.

Extinction Testing

Day 2 or 3 – All groups underwent extinction testing 24 hrs after their respective extinction training sessions. Rats were placed in context B and left for the 2 min baseline period. Five CSs (30 secs ITI) were presented alone. This procedure lasted 6 mins, 30 secs. Animals were returned to their home cages after the last CS.

4.2.4. Data Analysis and Statistics

Freezing was scored using ViewPoint software (ViewPoint Behaviour Technology, France). Prior to analysis, data sets were assessed for normal distribution. In the event that data sets were not normally distributed, as determined by a p value of < 0.05 in the Shapiro-Wilk test, data were log transformed ($Y = \text{Log}[Y]$) to ensure normality before being analysed. For fear conditioning, a two-way repeated measures ANOVA was used to assess freezing during the CS-US presentations. In this analysis, extinction protocol (immediate extinction, no immediate extinction, delayed extinction, and no delayed extinction) formed the between-subject factor and the CS-US pairings (i.e., trial) formed the within-subject factor. To compare the effects of extinction recency, baseline freezing of the two immediate groups (immediate and no immediate extinction) and the two delayed groups (delayed and no delayed extinction) were combined and analysed using an unpaired t-test. Freezing during the CS presentations of extinction training was assessed with a two-

way repeated measures ANOVA in which extinction protocol formed the between-subject factor and the CS presentations (i.e., trial) formed the within-subject factor. In the extinction training session, each five successive CSs (e.g., tones 1-5, 6-10 etc.) were averaged to reduce variability. No extinction training data were recorded for rats that underwent no immediate extinction after the initial baseline period. Baseline freezing from the extinction test was analysed with a one-way ANOVA, where extinction protocol formed the between-subject factor. Freezing during the CS presentations of the extinction test was analysed with a two-way repeated measures ANOVA in which extinction protocol formed the between-subject factor and the CS presentations (i.e., trial) formed the within-subject factor. Post-hoc tests were conducted using Tukey's multiple comparisons tests. All data are presented as mean + SEM, with the significance threshold set to $p < 0.05$. The Greenhouse-Geisser correction was applied to all repeated measures ANOVAs to adjust for the data's lack of sphericity.

4.3. Methods: Experiment 2 – Propranolol Positive Control

4.3.1. Animals

Experiment 2 also used 40 male Lister Hooded rats (Charles River, UK) weighing 200-300g upon delivery. Husbandry and ethics were the same as those described in section 2.2.1.

4.3.2. Drugs

DL-Propranolol hydrochloride (10 mg/kg, Fisher Scientific, UK) was dissolved in saline. This dose was chosen due to positive results being shown in several IED studies (Fitzgerald et al., 2015; Giustino et al., 2017, 2020; Wang et al., 2021). Rats were split into either immediate vehicle, immediate drug, no immediate vehicle, or no immediate drug groups (n = 10 per group). All injections were administered intraperitoneally at 1 ml/kg. Vehicle-treated controls received injections of saline alone.

4.3.3. Apparatus and Materials

The apparatus and materials were the same as described in section 2.2.3.

4.3.4. Behavioural Testing

Fear Conditioning

Day 1 – Fear conditioning parameters were the same as described in section 4.2.3. Rats were injected with either drug or vehicle immediately after the last CS-US pairing and subsequently returned to their home cage. A schematic representation of the experiment is shown below (see Fig 4.2a).

Extinction Training

Day 1 – Rats underwent immediate extinction or no extinction training 30 mins after injection. Rats were placed in context B and left for a 2 min baseline period. Following this, 45 CSs (30 secs ITI) were then presented alone. Rats in the no extinction group were placed in context B for 30 mins after fear conditioning. The first 2 mins of freezing activity were recorded before animals were left to remain in the context without experiencing any CSs. The total procedure time for both the extinction and no extinction groups was 46 mins, 30 secs. Animals were immediately returned to their home cages after the last CS.

Extinction Testing

Day 2 - All groups underwent extinction testing 24 hrs after extinction training. Rats were placed in context B and left for the 2 min baseline period. Ten CSs (30 secs ITI) were presented alone. The additional five CSs used here were included to assess for any drug effects that may have occurred later during extinction testing. This procedure lasted 6 mins, 30 secs. Animals were returned to their home cages after the last CS.

4.3.5. Data Analysis and Statistics

Freezing was scored using ViewPoint software (ViewPoint Behaviour Technology, France). Prior to analysis, data sets were assessed for normal distribution. In the event that data sets were not normally distributed, as determined by a p value of < 0.05 in the Shapiro-Wilk test, data were log transformed (Y = Log[Y]) to ensure normality before being analysed. For fear conditioning, a two-way repeated measures ANOVA was used to assess freezing during the CS-US presentations. In this analysis, protocol (immediate vehicle, immediate drug, no immediate vehicle, and no immediate drug) formed the between-subject factor and the CS-US pairings (i.e., trial) formed the within-subject factor. To assess the between-subject factor of drug treatment on immediate extinction training baseline, pre-CS freezing from the two drug (extinction and no extinction) and two vehicle (extinction and no extinction) groups were combined. These groups were then analysed using an unpaired t-test. Freezing during the CS presentations of extinction training was assessed with a two-way repeated measures ANOVA in which protocol formed the between-subject factor and the CS presentations (i.e., trial) formed the within-subject factor. In the extinction training session, each five successive CSs (e.g., tones 1-5, 6-10 etc.) were averaged to reduce variability. No extinction training data were recorded for rats that underwent no immediate extinction after the initial baseline period. Baseline freezing from the extinction test was analysed with a one-way ANOVA, where protocol formed the between-subject factor. Freezing during the CS presentations of the extinction test was analysed with a two-way repeated measures ANOVA in which protocol formed the between-subject factor and the CS presentations (i.e., trial) formed the within-subject factor. Each two successive CSs (e.g., tones 1-2, 3-4) were averaged to reduce variability. Post-hoc tests were conducted using Tukey's multiple comparisons tests. All data are presented as mean + SEM, with the significance threshold set to $p < 0.05$. The Greenhouse-Geisser correction was applied to all repeated measures ANOVAs to adjust for the data's lack of sphericity.

4.4. Results

4.4.1. Experiment 1: Procedure Validation

Delayed extinction reduced freezing during extinction retention testing more rapidly than immediate extinction

During fear conditioning (Fig. 4.1b), a two-way ANOVA showed a main effect of trial ($F (3.349, 120.6) = 102.4$, $p < 0.0001$). There was no significant effect of extinction protocol ($F (3, 36) = 0.2143$, $p = 0.8858$), showing that there were no differences in freezing between the groups. Lastly, there was no significant trial x extinction protocol interaction ($F (12, 144) = 0.2605$, $p = 0.9940$), indicating that there was no effect of extinction protocol at specific CS-US pairings. These data indicate all animals showed comparable levels of freezing before being assigned to their extinction protocol groups.

During extinction training (Fig. 4.1c), a two-tailed, unpaired t-test of baseline freezing showed no significant effect of extinction recency ($t (38) = 0.6851$, $p = 0.4974$), indicating that freezing was comparable between groups during baseline. A two-way ANOVA of the extinction training data (Fig. 4.1d) showed a main effect of trial ($F (2.781, 50.06) = 19.06$, $p < 0.0001$), this indicates significant differences in freezing between early and late extinction where higher freezing was seen at the start and lower freezing at the end of extinction training. There was no main effect of extinction protocol ($F (1, 18) = 0.4472$, $p = 0.5122$), nor a trial x extinction protocol interaction ($F (8, 144) = 0.4150$, $p = 0.9105$). These results show that there was no difference in extinction learning between rats that underwent immediate or delayed extinction training.

During the extinction test (Fig. 4.1e), a one-way ANOVA of baseline freezing found a significant effect of extinction protocol ($F (3, 36) = 4.060$, $p = 0.0139$). Post-hoc tests revealed significantly lower freezing in the no delayed extinction group compared to the immediate extinction group ($p < 0.01$). Upon presentation of the first CS, all groups showed moderate freezing, which increased until the third CS presentation. For the fourth and fifth CS presentations, the delayed extinction group

showed a reduction in freezing, whilst the other groups maintained high levels of freezing (Fig. 4.1f). A two-way ANOVA revealed a significant main effect of trial ($F(2.455, 88.37) = 26.50$, $p < 0.0001$) showing that CS-induced freezing was significantly different over the course of the session in all groups. The analysis also showed a significant main effect of extinction protocol ($F(3, 36) = 4.410$, $p = 0.0097$), and a trial x extinction protocol interaction ($F(12, 144) = 11.54$, $p < 0.0001$). Post-hoc tests showed that during the first trial, the immediate ($p < 0.01$) and delayed ($p < 0.05$) group froze more than the no immediate group. On the fifth ($p < 0.05$) trial, the delayed extinction group froze significantly less than all other groups. These results suggest that the delayed group exhibited extinction retention. In contrast, the immediate extinction group showed no reduction in freezing within the session, indicating aberrant retention of the extinction memory.

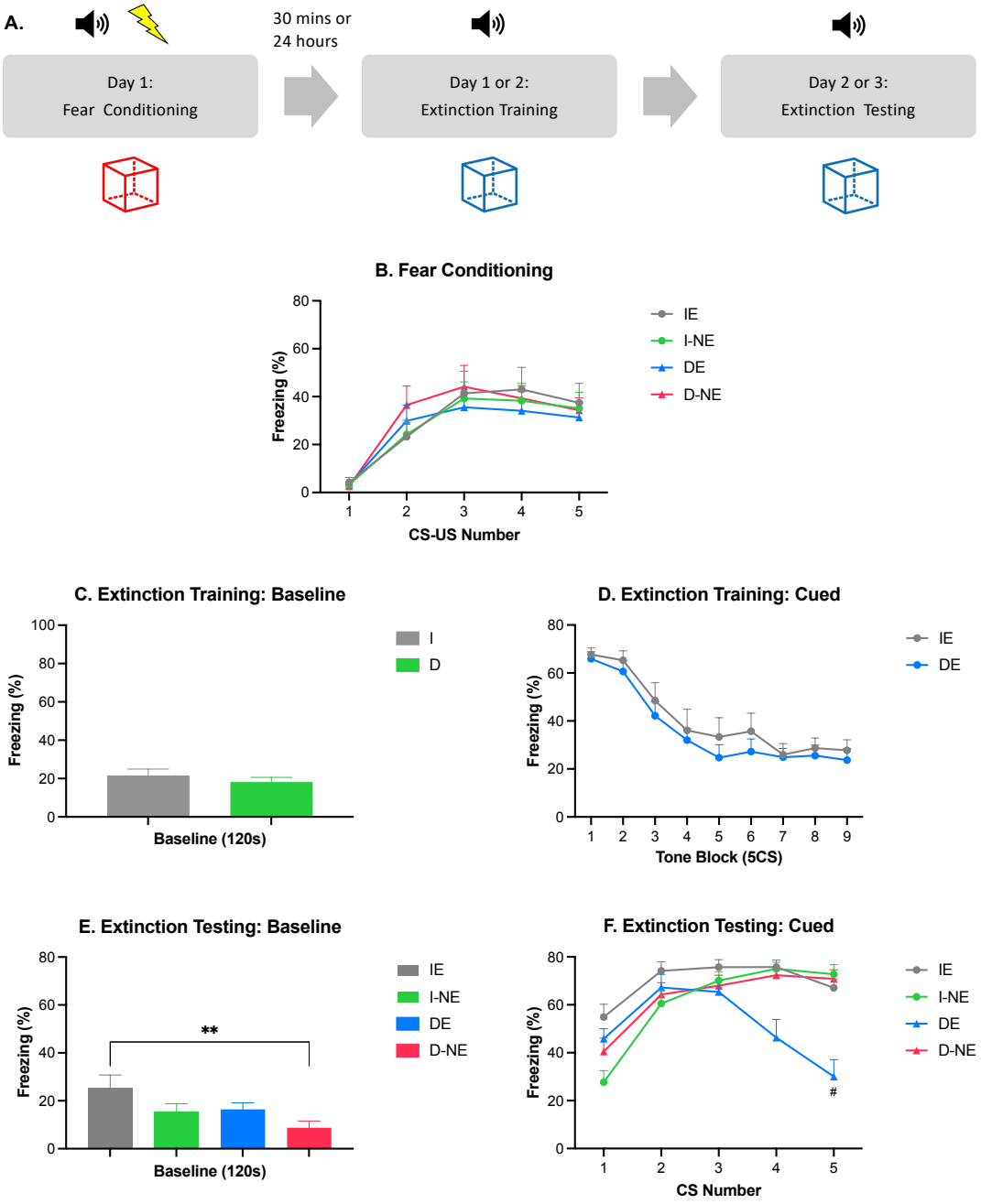

Figure 4.1. Validation of the immediate extinction deficit paradigm. (A) Schematic representation of the experimental protocol. Red cubes indicate testing in context A, blue cubes represent testing in context B. Speakers indicate conditioned stimulus (CS) presentation, lightning indicates footshock presentation. (B, D, F) Graphs showing the percentage of time rats spent freezing during the CS. (C, E) Graphs showing the percentage of time rats spent freezing during the 2-min baseline period prior to CS presentation.

*IE = immediate extinction, I-NE = immediate no extinction, DE = delayed extinction, D-NE = delayed no extinction. I = immediate and no immediate extinction combined, D = delayed and no delayed extinction combined. Data are expressed as mean + SEM. ** = $p < 0.01$ immediate extinction vs. delayed no extinction. # = $p < 0.05$ delayed extinction vs immediate extinction.*

4.4.2. Experiment 2: Propranolol Positive Control

Propranolol given immediately after fear conditioning lowered freezing during extinction training baseline and in response to the CS, did not affect freezing during extinction testing, and had no effect on fear memory consolidation

During fear conditioning (Fig. 4.2b), a two-way ANOVA showed a main effect of trial ($F(3.202, 115.3) = 42.62$, $p < 0.0001$). There was no significant effect of protocol ($F(3, 36) = 1.394$, $p = 0.2603$), nor trial x protocol interaction ($F(12, 144) = 0.6717$, $p = 0.7763$), indicating that there were no group differences in freezing during conditioning. These data indicate all animals showed comparable levels of freezing before being assigned to their protocol groups.

To assess the effect of propranolol on freezing during extinction training baseline, rats were grouped into vehicle and propranolol groups regardless of whether they would go on to experience immediate or no immediate extinction training. During the baseline period (Fig. 4.2c), a two-tailed, unpaired t-test of baseline freezing showed a significant effect of drug treatment ($t(37) = 6.869$, $p < 0.0001$) indicating that freezing was significantly lower in the propranolol treated rats compared to their vehicle counterparts.

Freezing data were obtained in response to CS presentations during extinction training for the vehicle and propranolol groups that underwent immediate extinction (Fig. 4.2d). Both vehicle and propranolol groups exhibited peak freezing behaviour in the first block of five CSs. As more CSs were presented, freezing was steadily reduced until it plateaued at a low level. Aside from the first two blocks, freezing in the propranolol group was consistently lower than that of the vehicle. A two-way ANOVA of the extinction training data showed a main effect of trial ($F(3.966, 67.41) = 16.32$, $p < 0.0001$). This indicates significant differences in freezing between early and late extinction, where higher freezing was seen at the start and lower freezing at the end of extinction training. There was a main effect of drug treatment ($F(1, 17) = 10.41$, $p = 0.0050$), and a trial x drug treatment interaction ($F(8, 136) = 3.381$, $p = 0.0014$). Despite the significant interaction, post-hoc testing

revealed no differences between vehicle and propranolol rats at specific CSs. These results illustrate the acute effect of propranolol in reducing freezing behaviour during extinction training.

During the extinction test (Fig. 4.2e), a one-way ANOVA of baseline freezing found no significant effect of protocol ($F (3, 36) = 0.8180$, $p = 0.4925$). All groups showed moderate freezing during the first block of two CSs, which then increased sharply during the second block. Following this, the immediate vehicle and propranolol group showed a gradual reduction in freezing (Fig. 4.2f). In accordance with experiment 1, the no extinction groups maintained high freezing levels throughout the test. A two-way ANOVA of CS-induced freezing showed a main effect of trial ($F (2.146, 77.26) = 29.87$, $p < 0.0001$), indicating a significant change in freezing as CSs were presented. The analysis showed a main effect of protocol ($F (3, 36) = 4.799$, $p = 0.0065$), and a significant trial x protocol interaction ($F (12, 144) = 5.229$, $p < 0.0001$).

Post-hoc tests revealed that during the last CS block, the no immediate vehicle group froze significantly more than the immediate extinction vehicle group ($p < 0.05$). During the last three CS blocks, the no immediate extinction propranolol group froze significantly more than the immediate extinction vehicle group (blocks 3 and 4: $p < 0.05$; block 5: $p < 0.01$). Overall, these results suggest that propranolol did not rescue the IED.

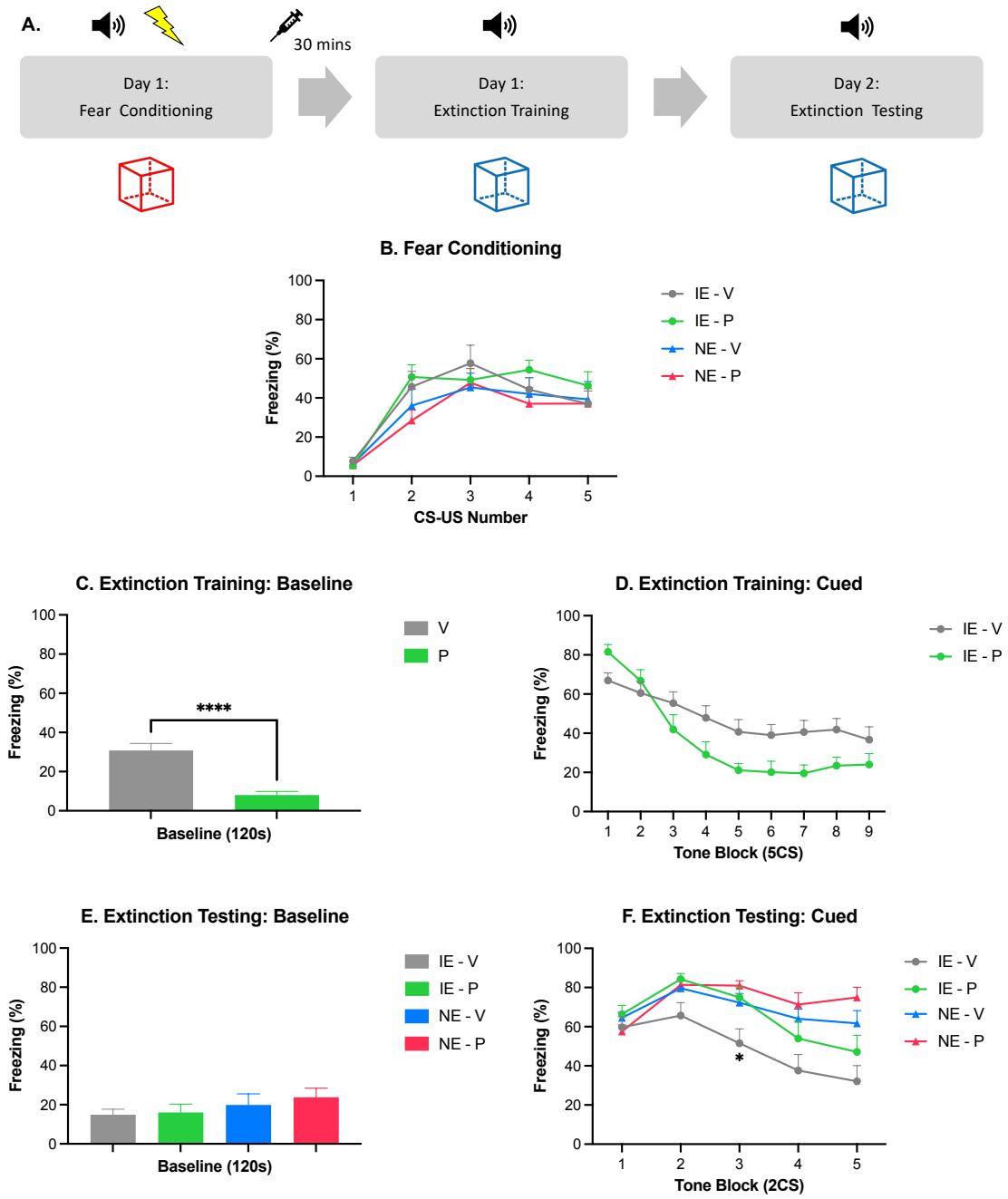

Figure 4.2. Propranolol (10 mg/kg) given immediately after fear conditioning in an immediate extinction paradigm. (A) Schematic representation of the experimental protocol. Red cubes indicate testing in context A, blue cubes represent testing in context B. Speakers indicate conditioned stimulus (CS) presentation, lightning indicates footshock presentation, syringe indicates injection. Fear conditioning was conducted drug-free. (B, D, F) Graphs showing the percentage of time rats spent freezing during the CS. (C, E) Graphs showing the percentage of time rats spent freezing during the 2-min baseline period prior to CS presentation.

*IE - V = immediate extinction vehicle, IE – P = immediate extinction propranolol, NE – V = no extinction vehicle, NE – P = no extinction propranolol. V = immediate and no immediate extinction vehicle combined, P = immediate and no immediate propranolol combined. Data are expressed as mean + SEM. **** = p<0.0001 vehicle combined vs propranolol combined.*

4.5. Discussion

This chapter aimed to validate the IED protocol and assess the effect of propranolol as a positive control. In experiment 1, immediate extinction training caused high levels of freezing during the extinction test, comparable to that of the no extinction controls. Delayed extinction training caused a rapid reduction of freezing during the extinction test when compared to all other groups. In experiment 2, propranolol acutely reduced baseline and cued freezing during immediate extinction training when compared to vehicle. In the extinction test, propranolol given prior to immediate extinction training did not affect freezing. Whilst propranolol significantly reduced freezing during extinction training, it did not rescue the IED.

In experiment 1, the immediate and delayed extinction groups showed a comparable reduction in freezing throughout extinction training. A number of studies found that immediate extinction induced less freezing at the start of the extinction training session, and caused a more rapid reduction in freezing, compared to delayed extinction (Kim and Richardson, 2009; Maren and Chang, 2006; Schiller et al., 2008; Woods and Bouton, 2008). Despite this, both immediate and delayed extinction showed equivalent levels of freezing by the end of extinction training. In studies where the immediate extinction group froze less than the delayed extinction group during extinction training, the IED was not induced (i.e., the immediate extinction group successfully retained the extinction memory). For example, this occurred where Maren and Chang (2006) attempted to induce the IED with a single shock, whilst Kim and Richardson (2009) and Schiller et al. (2008) used only three shocks in their conditioning protocol. It is possible that these studies did not induce sufficient pre-extinction stress to later induce the IED. Like others that have induced the IED (Chang and Maren, 2009; Johnson et al., 2010; Kim et al., 2010; Maren and Chang, 2006; Singh et al., 2018; Wang et al., 2021), the current results show that there was equivalent extinction learning between the immediate and delayed group. During the extinction test, baseline freezing in the immediate extinction group was significantly higher than the delayed no extinction group. One explanation may be that the combination of fear conditioning-induced stress and tone-induced fear at the start of

immediate extinction may have formed an association between the fearful state and the novel context, thus resulting in a form of contextual fear conditioning (Bouton et al., 2006). During the extinction test, the delayed extinction group showed a reduction in freezing, whilst the other groups maintained high levels of freezing. Freezing data from other immediate extinction studies typically show averaged CS data for the extinction retention test (e.g., averages of five CSs). However, during their extinction retention test, Maren and Chang (2006) showed a similar pattern of freezing in the delayed extinction group where freezing increased during the first three CSs and subsequently reduced during the fourth and fifth CS. In the current chapter, delayed extinction retrieval clearly differed from the immediate and no extinction groups during the fifth CS presentation. This nuanced effect may have been masked if all five CSs were averaged together. Overall, it is clear that the delayed group showed better extinction retention than the immediate and no extinction groups.

The current results pose an interesting question as to the nature of the IED. The IED is likely caused by aberrant extinction retrieval as opposed to a complete failure of extinction encoding (Maren, 2014). Evidence suggests there is at least partial encoding of the extinction memory in immediate extinction rats. As mentioned above, studies have shown comparable reductions in freezing between immediate and delayed cohorts during the extinction training session. However, Chang and Maren (2009) suggested that this reduction in freezing during extinction training may be due to short-term habituation as opposed to successful extinction acquisition. Chang and Maren (2009) found that immediate extinction caused a short-lasting, context independent loss of fear and delayed extinction resulted in long-lasting, context dependent suppression of fear. These short-term, context independent reductions in fear are typical of habituation (Bouton, 2004). Despite this, Chang and Maren (2011) showed that immediate extinction rats demonstrated savings of extinction in a later re-extinction session. In experiment 2's extinction retention test, the immediate extinction vehicle group showed a significant reduction in freezing compared to both no extinction groups at the last tone block. This suggests that the immediate extinction vehicle group showed savings of extinction. In an

extinction test conducted 48 hrs after immediate or delayed extinction training, Johnson et al. (2010) found that the delayed cohort were less susceptible to spontaneous recovery (i.e., delayed extinction resulted in less freezing than immediate extinction). However, when extinction testing was conducted 7 days after immediate or delayed extinction training, the immediate group were less susceptible to spontaneous recovery (i.e., immediate extinction resulted in less freezing than delayed extinction). This suggests that short-term extinction recall was impaired, whilst long-term recall was enhanced by immediate extinction. Johnson et al. (2010) posited that immediate extinction may therefore have more beneficial long-term effects after the extinction memory has been 'incubated'. Singh et al. (2018) showed c-Fos expression in the IL and PL was significantly lower in an immediate extinction group compared to no extinction controls; however, there was some engagement of the extinction circuitry during extinction training. Overall, it appears that some extinction encoding was achieved during the current immediate extinction session; however, an inability to retrieve the extinction memory resulted in sustained freezing during the extinction retention test.

In experiment 2, propranolol acutely reduced baseline and cued freezing during immediate extinction training compared to the immediate vehicle group. There are mixed findings regarding the acute effects of propranolol during immediate extinction training. In accordance with the current results, Fitzgerald et al. (2015) showed that systemic administration of propranolol in rats acutely reduced baseline and CS-induced freezing during immediate extinction. The authors attributed this to propranolol's ability to acutely stabilise neural activity in the PL and IL. However, Wang et al. (2021) showed no acute effects of systemic propranolol during immediate cued extinction. Despite using identical conditioning and extinction parameters to those reported by Fitzgerald et al. (2015), Giustino et al. (2017) found that intracranial infusion of propranolol into the BLA or PFC did not acutely affect freezing during immediate extinction training. Giustino et al. (2020) also showed no acute effect of propranolol on freezing when infused into the BLA prior to immediate extinction training. These results suggest that the acute, systemic effects of propranolol on extinction training may be mediated through other brain areas. In a

contextual paradigm, Santos et al. (2021) found that systemically administered propranolol did not affect freezing during context re-exposure immediately following fear conditioning. Santos et al. (2021) instead found that propranolol reduced freezing during a delayed context re-exposure, 5 days after fear conditioning. Systemic propranolol given immediately prior to delayed extinction training enhances extinction in rabbits and reduces fear expression in rats (Burhans et al., 2018; Rodriguez-Romaguera et al., 2009). However, others have shown that intra-IL and systemic infusions of propranolol had no effect on fear expression in delayed extinction paradigms (Fitzgerald et al., 2015; Mueller et al., 2008). Considering these results, it is clear there is discordance in the literature surrounding the acute effects of propranolol on fear expression and within-session extinction. These disparate findings might be due to the fear conditioning modality (i.e., cued vs contextual), route of administration, and the temporal gap between conditioning and extinction. Whilst no tests on locomotor activity were conducted in the current chapter, previous research has shown that propranolol does not affect spontaneous locomotion (Rodriguez-Romaguera et al., 2009).

Propranolol given prior to immediate extinction training did not rescue the IED, a result that may be contrary to previous literature. Fitzgerald et al. (2015) found that systemic injection of propranolol prior to immediate extinction training rescued the IED. This was demonstrated by reduced freezing during the first 5 CSs of the extinction retention test compared to vehicle. The authors posited that the administration of propranolol immediately after fear conditioning attenuated the footshock-induced increase in NA signalling resulting in adaptive extinction acquisition that was well retained. Giustino et al. (2017, 2020) found that intra-BLA infusions of propranolol rescued the IED. However, Wang et al. (2021) reported no effect of propranolol on the IED when administered systemically. Propranolol may have no effect or may even be detrimental to extinction retention when used in a delayed paradigm where NA levels are lower than those seen in IED paradigms (Burhans et al., 2018; Fitzgeral et al., 2015; Giustino et al., 2017; Mueller et al., 2008; Rodriguez-Romaguera et al., 2009). In the current paradigm, weaker footshocks (e.g., 0.5mA, 1s) may not have increased NA signalling to the same level as other IED

studies that used stronger footshocks (e.g., 1mA, 2s; Fitzgerald et al., 2015; Giustino et al. 2017, 2020). Giustino et al. (2020) showed that propranolol rescued the IED after a footshock matching the current chapter's parameters. However, this weaker shock was augmented by chemogenetic enhancement of LC output, which induced greater NA signalling in the BLA. In the current chapter, it is possible that NA signalling was not increased to the same level as previous studies that show propranolol can rescue the IED (Fitzgerald et al., 2015; Giustino et al. 2017, 2020). It may be that the current IED was NA-independent.

NA signalling is not the only neurochemical mediator of the IED. Jo et al. (2020) found that silencing or attenuating CRF release from CeA neurons diminished the IED. Additionally, activating these cells during a normally successful delayed extinction paradigm induced extinction retention deficits. Hollis et al. (2016) administered a CRF receptor antagonist in the BLA prior to immediate extinction training. The authors found that the antagonist dose-dependently ameliorated the IED without affecting the fear conditioning memory. Hollis et al. (2016) showed that CRF modulated AMPA-Glu receptors, suggesting that reduced local plasticity in the BLA may underpin the IED. Footshock induces BLA excitability (Giustino et al., 2020), which may be mediated by CRF (Maren, 2022). Considering this, it is possible that the IED shown in the current chapter was induced by conjunctive neuronal signalling pathways (e.g., NA and CRF). The weighted contribution of these pathways towards the IED is not known. However, it is possible that in the current paradigm, there was less involvement of the NA system when compared to other studies that have used stronger footshocks (Fitzgerald et al., 2015; Giustino et al., 2017, 2020). Therefore, the administration of propranolol may have had no effect on an NA-independent IED, whilst also having no effect on CRF signalling. This may account for propranolol's inability to rescue the IED.

4.5.1. Conclusion

The current chapter successfully validated the IED paradigm for future pharmacological studies. Immediate extinction impaired extinction retention as evidenced by sustained freezing to the CSs in the extinction test. Propranolol was

successfully implemented as a positive control, demonstrating acute effects. However, it was not able to rescue the IED. It is possible that the weaker footshocks used in the current chapter did not increase NA to same level as those that have shown the remedial effects of propranolol (Fitzgerald et al., 2015; Giustino et al., 2017, 2020). In the current paradigm, the IED may have been mediated by other neuronal signalling pathways (e.g., CRF). Further experiments will look to assess whether the eCB metabolism inhibitors URB597 and JZL184 can rescue the IED.

5. URB597 and JZL184 Modulation of Immediate Extinction

5.1. Introduction

The previous chapter validated the IED paradigm. Propranolol reduced baseline and cued freezing during extinction training but did not rescue the IED. The current chapter assessed whether the eCB metabolism inhibitors URB597 and JZL184 could rescue the IED.

As previously discussed, the current IED may have been mediated by CRF signalling instead of NA. CRF signalling in the AMG is involved in the IED (Hollis et al., 2016; Jo et al., 2020; Maren, 2022). Previous studies have shown a functional link between the eCB and CRF systems (Wyrofsky et al. 2019). CB1Rs and CRF are colocalised in the piriform cortex, lateral hypothalamus, paraventricular nucleus (PVN), and LC (Ruat et al., 2021; Wyrofsky et al., 2017). *In situ* studies have shown that repeated administration of CB1R agonists upregulates CRF expression in hypothalamic tissue (Corchero et al., 1999a, b). In a typical stress response, glucocorticoids increase eCB levels in the hypothalamus, which in turn suppresses Glu transmission to CRF-releasing neurons in the PVN (Hill and McEwen, 2009; Hill and Tasker, 2012; Patel et al., 2004). In the mPFC, glucocorticoids stimulate DAGL to synthesise 2-AG, which through the activation of pre-synaptic GABAergic interneurons disinhibits excitation of GABA neurons in the bed nucleus of the stria terminalis (BNST), thus suppressing the PVN and attenuating CRF release (Hill et al., 2011). In the BLA, tonic AEA transmission suppresses the hypothalamic-pituitary-adrenal (HPA) axis (Hill and McEwen, 2009). In the event of an acute stressor, AEA signalling is reduced due to elevated FAAH activity, which increases HPA activity (Gray et al., 2015; Hill et al., 2009; Hill et al., 2010b). In the LC, CB1Rs modulate CRF release, particularly on AMG afferents (Wyrofsky et al. 2017). This may have implications for the modulation of NA signalling mediated by CRF (Curtis et al., 1997).

Selective CB1R-KO on CRF neurons enhances acoustic startle response in male mice (but not females), whilst having no effect on fear memory retrieval (Ruat et al., 2021). CB1R antagonism remediates behavioural anxiety induced by

coadministration of exogenous CRF (Kupferschmidt et al., 2012). URB597 can ameliorate forced swim-induced elevation of HPA activity, whilst reducing CRF mRNA expression in the PVN (Bedse et al., 2015). Restraint stress and infusion of CRF into the BLA reduces AEA signalling through enhanced FAAH activity, which is mediated by CRF receptor type 1 (CRFR1) but not CRF receptor type 2 (CRFR2; Gray et al., 2015). Coadministration of CRF and FAAH into the BLA remediates CRF-mediated anxiety behaviours in an elevated plus maze model (Gray et al., 2015). However, Kamprath et al. (2009) found that rimonabant induced a sustained fear response to a novel tone played immediately after footshock in CRFR1-KO, CRFR2-KO, and wild-type mice. The authors suggested that CB1R-mediated fear adaptation was CRFR independent. Overall, there is evidence to suggest that upregulation of eCBs, particularly AEA in the BLA (Gray et al., 2015; Hill and McEwen, 2009, Hill et al., 2010b), may be beneficial in a CRF-dependent IED paradigm.

Aims and Hypotheses

To date, no studies have assessed pharmacological regulation of the IED beyond NA and CRF signalling. The current chapter utilised the experimental parameters previously shown to induce the IED. URB597 (0.3 mg/kg) and JZL184 (1 and 10 mg/kg) were systemically injected immediately following fear conditioning. In chapter 2, URB597 did not affect freezing when shock parameters were mild (0.4mA, 0.5s). Under those conditions, it was thought that stress was low and adaptive fear extinction was acquired. Other research has shown that URB597 reduced freezing when footshocks were strong (0.5mA, 1s; Bowers and Ressler, 2015) or in those that induced prior or concurrent stress (Laricchiuta et al., 2013; Morena et al., 2018; Zer-Aviv and Akirav, 2016). URB597 favourably modulates CRF signalling in models of acute stress (Bedse et al., 2015; Gray et al., 2015). It was therefore expected that URB597 would rescue the IED given that the paradigm utilised higher shock parameters (0.5mA, 1s), and involves stress-induced extinction impairment (Maren, 2022).

Low and high doses of JZL184 have been shown to reduce and enhance freezing during extinction training, respectively, albeit in heterogenous extinction

paradigms (Hartley et al., 2016; Morena et al., 2018; Mizuno et al., 2022). During a stress response, stimulation of DAGL in the mPFC by glucocorticoids causes 2-AG mediated suppression of PVN and reduced CRF release (Hill et al., 2011). These findings suggest 2-AG may be able to modulate CRF neurotransmission. Given that low doses of JZL184 reduce global freezing during repeated extinction training sessions in socially isolated rats (Morena et al., 2018), it was expected that low doses of JZL184 would beneficially regulate CRF signalling prior to immediate extinction training. The low dose of JZL184 was expected to reduce freezing during extinction training and testing. However, previous studies have shown that a high dose of JZL184 disrupts extinction (Hartley et al., 2016; Mizuno et al., 2022). Therefore, the high dose of JZL184 was expected to increase freezing during extinction training and exacerbate the IED. No extinction controls were included to demonstrate that the drugs' effects on freezing behaviour required extinction, and were not caused by impaired fear memory consolidation.

5.2. Methods

5.2.1. Animals

All experiments used 40 male Lister Hooded rats each (Charles River, UK) weighing 200-300g upon delivery. Husbandry and ethics were the same as those described in section 2.2.1.

5.2.2. Drugs

URB597 (0.3 mg/kg) and JZL184 (1 and 10 mg/kg) were dissolved in 5% PEG (Fluka Chemicals, Switzerland), 5% Tween 80 (Sigma-Aldrich), and 90% saline. All injections were administered intraperitoneally at 1 ml/kg. Vehicle-treated controls received injections of 5% PEG, 5% Tween 80, and 90% saline. Rats were split into immediate vehicle, immediate drug, no immediate vehicle, or no immediate drug groups (n = 10 per group).

5.2.3. Apparatus and Materials

The apparatus and materials were the same as described in 2.2.3. Shock parameters were maintained at 0.5mA, 1s.

5.2.4. Behavioural Testing

Behavioural testing was the same as described in section 4.3.4. A schematic representation of the experiments is shown below (see Figs 5.1a, 5.2a, 5.3a).

5.2.5. Data Analysis and Statistics

Data analysis was the same as described in section 4.3.5. Where applicable, post-hoc tests were conducted using Tukey's multiple comparisons tests (unless stated as having used Šídák's).

5.3. Results

5.3.1. Experiment 1: URB597

URB597 showed no acute effect on extinction encoding, and did not rescue the IED

During fear conditioning (Fig. 5.1b), a two-way ANOVA showed a main effect of trial ($F(2.878, 103.6) = 95.62$, $p < 0.0001$), indicating that there was a significant increase in freezing as more CS-US pairings were presented across all groups. There was no significant effect of treatment ($F(3, 36) = 8015$, $p = 0.5013$), showing that there were no differences in freezing between the extinction protocol groups. Lastly, there was no significant trial x extinction protocol interaction ($F(12, 144) = 0.8933$, $p = 0.5554$), indicating that there was no effect of extinction protocol at specific CS-US pairings. These data suggest all groups showed comparable levels of freezing before being treated with URB597.

To assess the effect of URB597 on freezing during the baseline period of the extinction training session, all rats from the immediate and no extinction groups were categorised as vehicle or URB597. During this time, rats treated with URB597 showed

comparable freezing levels to those treated with vehicle (Fig. 5.1c). A two-tailed, unpaired t-test of baseline freezing showed no significant effect of drug treatment ($t(38) = 0.1824$, $p = 0.8562$) indicating that freezing was equivalent between URB597 treated rats and their vehicle counterparts.

In the immediate extinction training session, freezing increased to its peak value in both groups after presentation of the first five CSs (Fig. 5.1d). As more CSs were presented, freezing was steadily reduced until it plateaued at a low level. A two-way ANOVA on the extinction training data showed a main effect of trial ($F(4.415, 79.98) = 20.50$, $p < 0.0001$). This indicates significant differences in freezing between early and late extinction, where higher freezing was seen at the start and lower freezing at the end of extinction training. There was no main effect of extinction protocol ($F(1, 18) = 0.04809$, $p = 0.8289$), nor a trial x extinction protocol interaction ($F(8, 144) = 0.4945$, $p = 0.8587$). These results show that URB597 had no effect on freezing during extinction training.

During the extinction test, freezing during the baseline period was low (Fig. 5.1e). A one-way ANOVA of baseline freezing found no significant effect of extinction protocol ($F(3, 36) = 2.132$, $p = 0.1132$). All groups showed moderate freezing during the first CS block; freezing then increased sharply during the second CS block. The immediate vehicle and immediate URB597 groups showed a steady reduction in freezing as more CSs were presented, whereas the other groups maintained high freezing until the end of the extinction test (Fig. 5.1f). A two-way ANOVA of freezing during the extinction test showed a main effect of the trial ($F(1.832, 65.94) = 19.31$, $p < 0.0001$) indicating a significant change in freezing as CSs were presented. The analysis showed a main effect of extinction protocol ($F(3, 36) = 6.481$, $p = 0.0013$), and a significant trial x extinction protocol interaction ($F(12, 144) = 6.838$, $p < 0.0001$). These results indicate there was a significant difference in freezing between the extinction protocol groups, and that there were significant differences in freezing between the groups at specific CS presentations.

Post-hoc tests revealed that during the fifth ($p < 0.05$) CS block the immediate extinction URB597 group froze significantly less than the no extinction URB597 group.

During the fourth ($p < 0.05$) and fifth ($p < 0.05$) CS presentations the immediate extinction vehicle group froze less than the no extinction vehicle group. During the third ($p < 0.05$) CS block the immediate extinction URB597 group froze significantly less than the no extinction vehicle group. During the fourth ($p < 0.01$) and fifth ($p < 0.01$) CS blocks the immediate extinction vehicle group froze significantly less than the no extinction URB597 group. There were no differences between the immediate extinction vehicle and immediate URB597 groups at any point. These results suggest URB597 did not rescue the IED. Significantly reduced freezing in the immediate extinction groups compared to the no extinction groups at the end of extinction testing indicate savings of extinction. Freezing was equivalent between the no extinction URB597 and vehicle groups indicating there was no effect of the drug on fear memory consolidation.

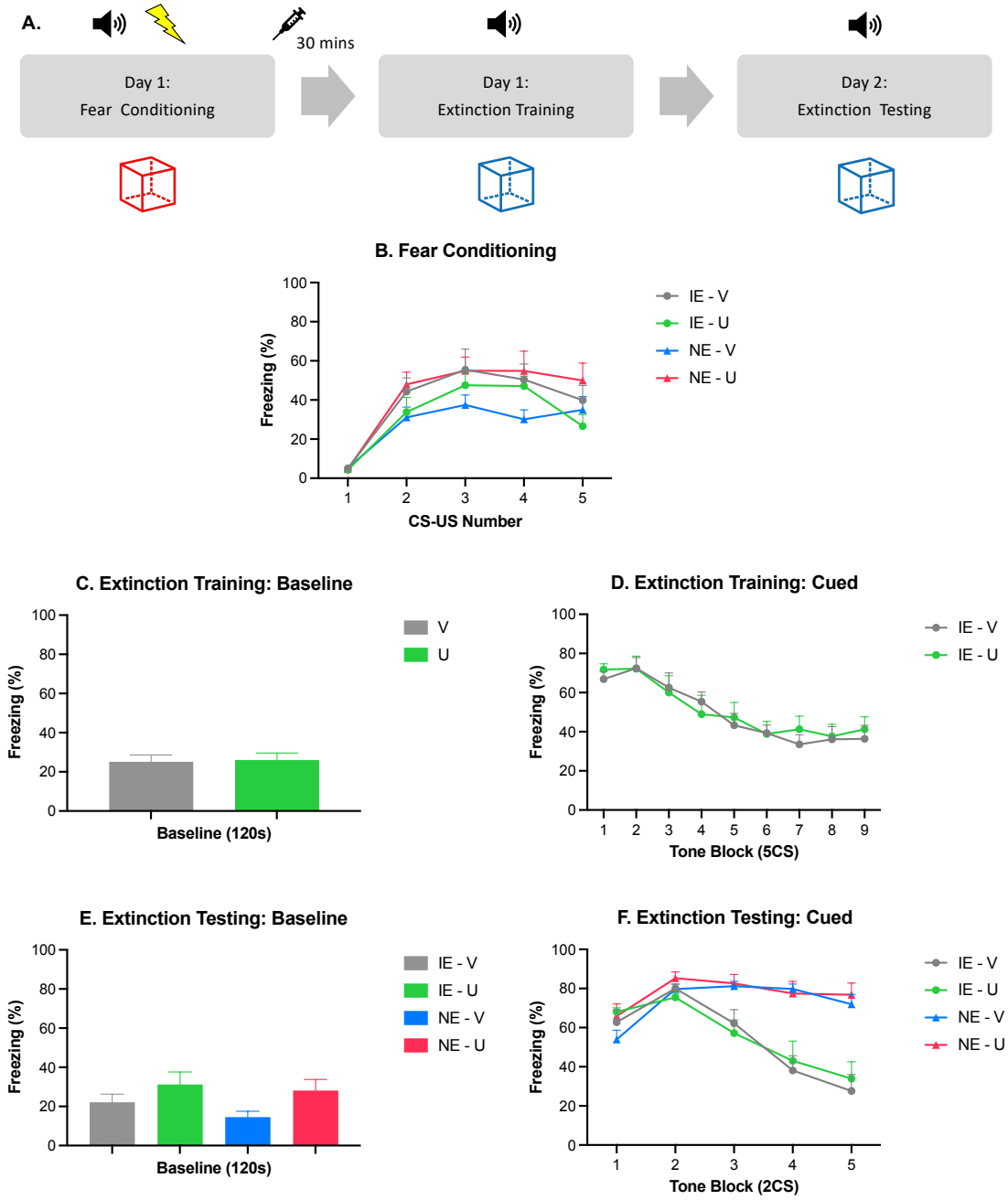

Figure 5.1. URB597 (0.3 mg/kg) given immediately after fear conditioning in an immediate extinction paradigm. (A) Schematic representation of the experimental protocol. Red cubes indicate testing in context A, blue cubes represent testing in context B. Speakers indicate conditioned stimulus (CS) presentation, lightning indicates footshock presentation, syringe indicates injection. Fear conditioning was conducted drug-free. (B, D, F) Graphs showing the percentage of time rats spent freezing during the CS. (C, E) Graphs showing the percentage of time rats spent freezing during the 2-min baseline period prior to CS presentation.

IE - V = immediate extinction vehicle, IE – U = immediate extinction URB597, NE – V = no extinction vehicle, NE – U = no extinction URB597. V = immediate extinction vehicle and no extinction vehicle combined, U = immediate extinction URB597 and no extinction URB597 combined. Data are expressed as mean + SEM.

5.3.2. Experiment 2: JZL184 (1 mg/kg)

A low dose of JZL184 showed no acute effect on extinction encoding, and did not rescue the IED

During fear conditioning (Fig. 5.2b), a two-way ANOVA showed a main effect of trial (F (2.942, 105.9) = 49.62, p <0.0001), indicating that there was a significant increase in freezing as more CS-US pairings were presented across all groups. There was no significant effect of treatment (F (3, 36) = 0.7918, p = 0.5065), showing that there were no differences in freezing between the extinction protocol groups. Lastly, there was no significant trial x extinction protocol interaction (F (12, 144) = 0.5591, p = 0.8716), indicating that there was no effect of extinction protocol at specific CS-US pairings. These data indicate all groups showed comparable levels of freezing before being treated with a low dose of JZL184.

To assess the effect of JZL184 on freezing during the baseline session, all immediate and no extinction rats were categorised as vehicle or JZL184. During this time, rats treated with JZL184 showed comparable freezing levels to those treated with vehicle (Fig. 5.2c). A two-tailed, unpaired t-test of baseline freezing showed no significant effect of drug treatment (t (38) = 1.138, p = 0.2623), indicating that freezing was equivalent between the JZL184 and vehicle groups.

In the immediate extinction training session, freezing was high in both groups during the first two CS blocks (Fig. 5.2d). As more CSs were presented, freezing was steadily reduced throughout the course of the session in both groups. A two-way ANOVA of the extinction training data showed a main effect of trial (F (3.588, 64.58) = 30.70, p <0.0001). This indicates significant differences in freezing between early and late extinction, where higher freezing was seen at the start and lower freezing at the end of extinction training. There was no main effect of extinction protocol (F (1, 18) = 0.6039, p = 0.4472), nor a trial x extinction protocol interaction (F (8, 144) = 1.653, p = 0.1149), these results show that the low dose of JZL184 had no effect on freezing during extinction training.

During the extinction test, freezing during the baseline period was low in all groups except in the immediate vehicle group (Fig. 5.2e). A one-way ANOVA of baseline freezing found no significant effect of extinction protocol ($F (3, 36) = 1.848$, $p = 0.1560$). All groups showed moderate freezing during the first CS block; freezing then increased sharply during the second CS block in the no extinction groups, whilst both immediate groups showed consistent freezing. The immediate vehicle and immediate JZL184 groups then showed a steady reduction in freezing as more CSs were presented, whereas the other groups maintained high freezing until the end of the extinction test (Fig. 5.2f). A two-way ANOVA of freezing during the extinction test showed a main effect of trial ($F (2.100, 75.61) = 33.58$, $p < 0.0001$), indicating a significant change in freezing as CSs were presented. The analysis showed a main effect of extinction protocol ($F (3, 36) = 11.28$, $p < 0.0001$), and a significant trial x extinction protocol interaction ($F (12, 144) = 14.34$, $p < 0.0001$). These results indicate there was a significant difference in freezing between the extinction protocol groups, and that there were significant differences in freezing between the groups at specific CS blocks.

Post-hoc tests revealed that during the second ($p < 0.01$), third ($p < 0.01$), fourth ($p < 0.0001$), and fifth ($p < 0.001$) CS blocks the immediate extinction JZL184 group froze significantly less than the no extinction JZL184 group. During the third ($p < 0.05$), fourth ($p < 0.05$), and fifth ($p < 0.01$) CS presentations the immediate extinction vehicle group froze less than the no extinction vehicle group. During the third ($p < 0.01$), fourth ($p < 0.0001$), and fifth ($p < 0.0001$) CS blocks the immediate extinction JZL184 group froze significantly less than the no extinction vehicle group. During the third (p 0.05), fourth (p 0.05), and fifth (p 0.05) CS blocks the immediate extinction vehicle group froze significantly less than the no extinction JZL184 group. There were no differences between the immediate vehicle and immediate JZL184 groups at any point. These results suggest that a low dose of JZL184 did not rescue the IED. Significantly reduced freezing in the immediate extinction groups compared to the no extinction groups at the end of extinction testing indicate savings of extinction. Freezing was equivalent between the no extinction JZL184 and vehicle groups indicating there was no effect of the drug on fear memory consolidation.

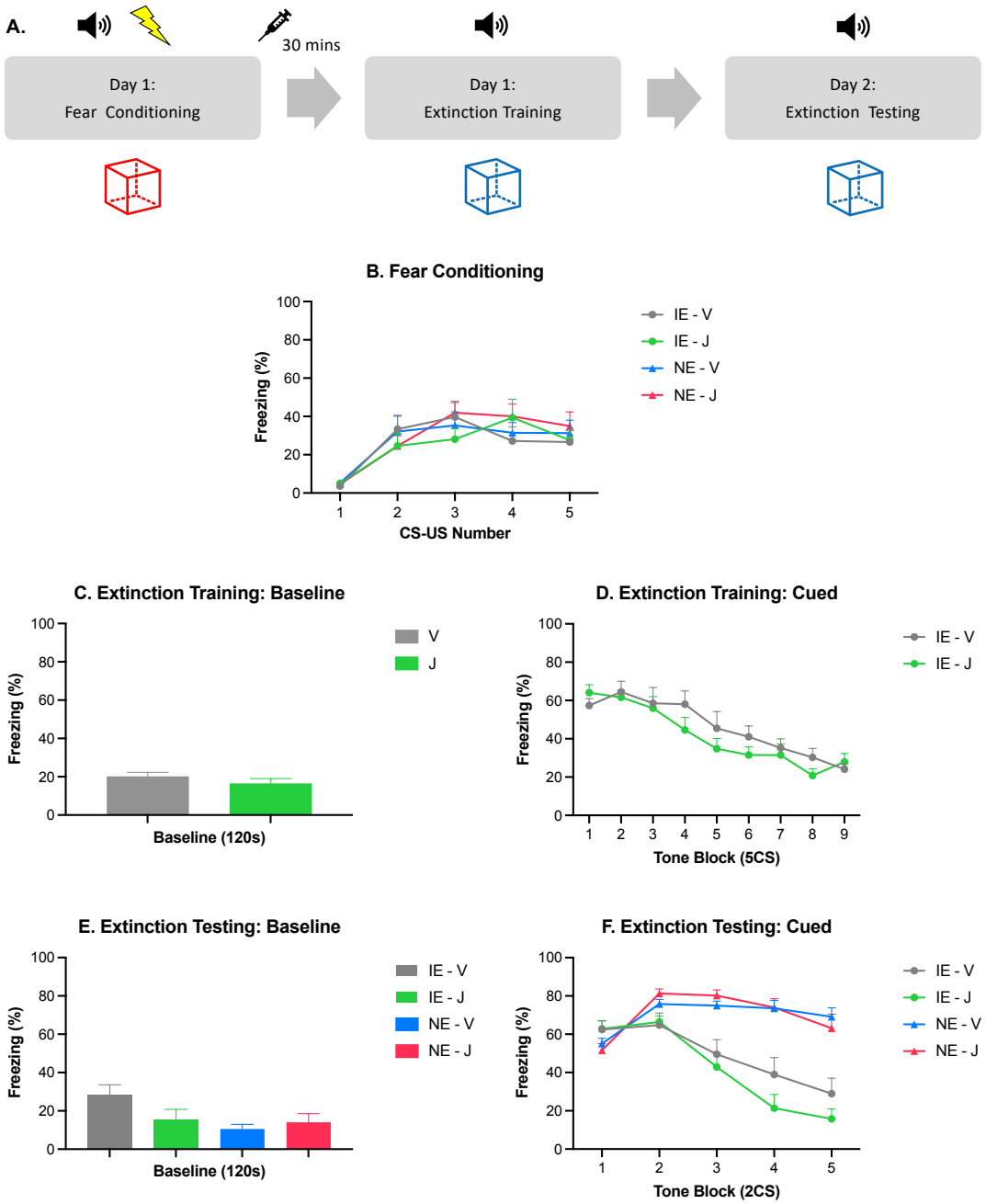

Figure 5.2. Low dose of JZL184 (1 mg/kg) given immediately after fear conditioning in an immediate extinction paradigm. (A) Schematic representation of the experimental protocol. Red cubes indicate testing in context A, blue cubes represent testing in context B. Speakers indicate conditioned stimulus (CS) presentation, lightning indicates footshock presentation, syringe indicates injection. Fear conditioning was conducted drug-free. (B, D, F) Graphs showing the percentage of time rats spent freezing during the CS. (C, E) Graphs showing the percentage of time rats spent freezing during the 2-min baseline period prior to CS presentation.

IE - V = immediate extinction vehicle, IE – J = immediate extinction JZL184, NE – V = no extinction vehicle, NE – J = no extinction JZL184. V = immediate extinction vehicle and no extinction vehicle combined, J = immediate extinction JZL184 and no extinction JZL184 combined. Data are expressed as mean + SEM.

5.3.3. Experiment 3: JZL184 (10 mg/kg)

A high dose of JZL184 acutely impaired extinction acquisition during extinction training but did not rescue the IED

During fear conditioning (Fig. 5.3b), a two-way ANOVA showed a main effect of trial ($F(3.650, 131.4) = 20.18$, $p < 0.0001$), indicating that there was a significant increase in freezing as more CS-US pairings were presented across all groups. There was no significant effect of treatment ($F(3, 36) = 1.541$, $p = 0.2206$), showing that there were no differences in freezing between the extinction protocol groups. Lastly, there was no significant trial x extinction protocol interaction ($F(12, 144) = 1.239$, $p = 0.2623$), indicating that there was no effect of extinction protocol at specific CS-US pairings. These data indicate all groups showed comparable levels of freezing before being treated with a high dose JZL184.

To assess the effect of JZL184 on freezing during the baseline session, all immediate and no extinction rats were categorised as vehicle or JZL184. During this time, rats treated with JZL184 showed comparable freezing levels to those treated with vehicle (Fig. 5.3c). A two-tailed, unpaired t-test of baseline freezing showed a trend towards a significant effect of drug treatment ($t(38) = 1.970$, $p = 0.0562$), indicating that JZL184 may have elevated freezing compared to the vehicle groups.

In the immediate extinction training session, freezing was moderate in both groups during the first two blocks of five CSs (Fig. 5.3d). As more CSs were presented, freezing was steadily reduced throughout the course of the session in both groups. However, freezing was further reduced in the immediate vehicle group compared to the immediate JZL184 group. A two-way ANOVA on the extinction training data showed a main effect of trial ($F(4.820, 86.77) = 10.25$, $p < 0.0001$) This indicates significant differences in freezing between early and late extinction, where higher freezing was seen at the start and lower freezing at the end of extinction training. There was a significant main effect of extinction protocol ($F(1, 18) = 4.695$, $p = 0.0439$), and a trial x extinction protocol interaction ($F(8, 144) = 2.717$, $p = 0.081$).

These results show that the high dose of JZL184 acutely increased freezing during extinction training.

During the extinction test, freezing during the baseline period was higher in the JZL184 groups than the vehicle groups (Fig. 5.3e). A one-way ANOVA of baseline freezing found a significant effect of extinction protocol ($F(3, 36) = 3.163$, $p = 0.0361$). All groups showed moderate freezing during the first CS block; freezing then increased sharply during the second CS block in all groups. The immediate vehicle and immediate JZL184 groups then showed a steady reduction in freezing as more CSs were presented, whereas the other groups maintained high freezing until the end of the extinction test (Fig. 5.3f). A two-way ANOVA of freezing during the extinction test showed a main effect of trial ($F(2.476, 89.14) = 37.40$, $p < 0.0001$), indicating a significant change in freezing as CSs were presented. The analysis showed a main effect of extinction protocol ($F(3, 36) = 8.430$, $p = 0.0002$), and a significant trial x extinction protocol interaction ($F(12, 144) = 11.71$, $p < 0.0001$). These results indicate there was a significant difference in freezing between the extinction protocol groups, and that there were significant differences in freezing between the groups at specific CS blocks.

Post-hoc tests revealed that during the third ($p < 0.05$), fourth ($p < 0.01$), and fifth ($p < 0.01$) CS blocks the immediate extinction JZL184 group froze significantly less than the no extinction JZL184 group. During the third ($p < 0.05$), fourth ($p < 0.01$), and fifth ($p < 0.01$) CS presentations the immediate extinction vehicle group froze less than the no extinction vehicle group. During the third ($p < 0.05$), fourth ($p < 0.0001$), and fifth ($p < 0.0001$) CS blocks the immediate extinction vehicle group froze significantly less than the no extinction JZL184 group. There were no differences between the immediate vehicle and immediate JZL184 groups at any point. These results suggest a high dose of JZL184 did not rescue the IED. Significantly reduced freezing in the immediate extinction groups compared to the no extinction groups at the end of extinction testing indicate savings of extinction. Freezing was equivalent between the no extinction JZL184 and vehicle groups, indicating there was no effect of the drug on fear memory consolidation.

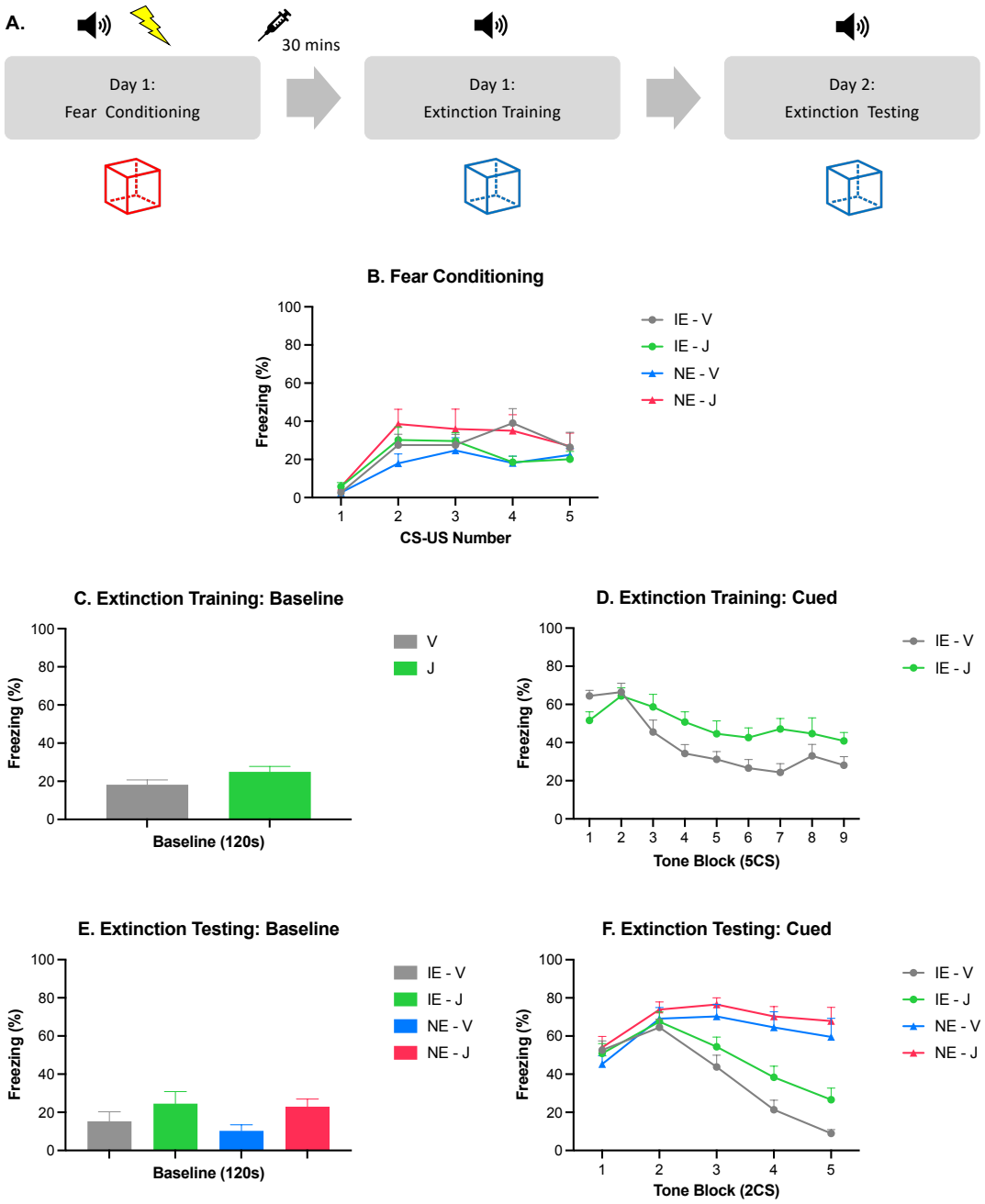

Figure 5.3. High dose of JZL184 (10 mg/kg) given immediately after fear conditioning in an immediate extinction paradigm. (A) Schematic representation of the experimental protocol. Red cubes indicate testing in context A, blue cubes represent testing in context B. Speakers indicate conditioned stimulus (CS) presentation, lightning indicates footshock presentation, syringe indicates injection. Fear conditioning was conducted drug-free. (B, D, F) Graphs showing the percentage of time rats spent freezing during the CS. (C, E) Graphs showing the percentage of time rats spent freezing during the 2-min baseline period prior to CS presentation.

*IE - V = immediate extinction vehicle, IE – J = immediate extinction JZL184, NE – V = no extinction vehicle, NE – J = no extinction JZL184. V = immediate extinction vehicle and no extinction vehicle combined, J = immediate extinction JZL184 and no extinction JZL184 combined. Data are expressed as mean + SEM. * $p < 0.05$ immediate extinction JZL184 vs immediate extinction vehicle.*

5.4. Discussion

The current chapter investigated whether eCB metabolism inhibition could rescue the IED. URB597 was expected to enhance immediate extinction encoding. The low and high doses of JZL184 were expected to remediate and exacerbate fear extinction, respectively. URB597 and the low dose of JZL184 had no effect on freezing throughout immediate extinction training and extinction testing when compared to vehicle. The high dose of JZL184 increased freezing during extinction training baseline, extinction training, and extinction testing baseline but had no effect during extinction testing. Neither drug influenced fear memory consolidation as freezing was equivalent between the no extinction drug groups and their vehicle counterparts.

URB597 did not reduce freezing during immediate extinction training or rescue the IED. NA signalling is a fundamental driver of the IED (Maren, 2022). Evidence suggests that URB597 can modulate NA signalling. Single and multiple injections of URB597 (0.1 mg/kg) increase the spontaneous activity of NA-releasing LC neurons, without affecting NA efflux in the mPFC (Gobbi et al., 2005). A low (0.1 mg/kg), but not a high (0.3 mg/kg), dose of URB597 given prior to forced swim stress increases NA levels in the PFC and BLA, despite neither URB597 nor forced swim increasing NA levels individually (Bedse et al., 2015). In male rats, URB597 rescues deficient NA signalling in the mPFC, HPC, and adrenal medulla in a chronic unpredictable stress paradigm (Ferizovic et al. 2022; Jankovic et al., 2020). However, in the previous chapter, propranolol did not rescue the IED. Therefore, it is possible that the IED shown in the current thesis was NA-independent. Whilst URB597 has been shown to modulate NA signalling, it is possible that this would have no remedial effect in the current paradigm.

FAAH inhibition is relatively ineffective under basal conditions but can exert anxiolytic effects following exposure to stress (Bluett et al., 2014; Haller et al., 2009; Hill et al., 2013a; Kathuria et al., 2003). URB597 is beneficial in rats that have experienced stress prior to or alongside fear and extinction paradigms. Zer-Aviv and Akirav (2016) found that URB597, given 2 hrs after stress exposure, augmented

extinction training when conducted 1 week after injection, whilst showing no effect in rats with no prior stress. Morena et al. (2018) found that URB597 enhanced extinction consolidation and retention in rats that were socially isolated prior to and throughout behavioural testing. Laricchiuta et al. (2013) found that mice exposed to social defeat stress prior to fear conditioning showed impaired extinction retention, which was restored by URB597. However, in the absence of stress, URB597 fails to acutely enhance delayed extinction training and later recall in males (Morena et al., 2021; Mizuno et al., 2022; Vimalanathan et al., 2020). Acute stress CRFR1-dependently reduces AEA signalling in the AMG through enhanced FAAH activity (Gray et al., 2015; Hill and McEwen, 2009; Hill et al., 2010b). URB597 ameliorates stress and anxiety behaviours through a reduction in CRF and corticosterone signalling (Bedse et al., 2015; Gray et al., 2015). However, whilst restraint and forced swim models reduce AEA signalling in the AMG, footshock acutely increases AEA levels in the mPFC, AMG, HPC, and PAG (Hohmann et al., 2005; Morena et al., 2014). Therefore, it is possible that in the IED paradigm, where stress was induced by previous footshock, AEA activity may have already been elevated. This may have prevented URB597 from optimally regulating AEA throughout the brain, which may explain its negative result in the current paradigm.

The high dose of JZL184 elevated freezing acutely during immediate extinction training baseline and during CS presentations, as well as chronically during extinction testing baseline. MAGL-KO mice show impaired extinction memory (Kishimoto et al., 2015). Llorente-Berzal et al. (2015) found that a high dose of JZL184 increased fear expression due to CB1R-activation on GABAergic neurons. Studies have shown a high dose of JZL184 can impair cued and contextual fear extinction (Hartley et al., 2016; Mizuno et al., 2022), whilst Morena et al. (2021) found that elevating 2-AG levels with MJN110 had no effect on freezing in males but enhanced darting behaviour in females during extinction training. Hartley et al. (2016) showed that JZL184 infusions into the BLA, but not CeA, prior to extinction training impaired extinction in male mice. In the current chapter, it is possible that the high dose of JZL184 increased freezing through excessive activation of CB1Rs in the BLA.

Corticosterone may mediate 2-AG's response to stress (Morena et al., 2016). Stress increases corticosterone levels along with 2-AG (Di et al., 2005; Hill et al., 2010a), particularly in the PFC (Roberts et al., 2012). Cordero et al. (2003) showed that corticosterone levels are elevated immediately after fear conditioning. In the current thesis, it is possible that fear conditioning elevated corticosterone and subsequently increased 2-AG levels prior to immediate extinction training. This stress-induced elevation in 2-AG signalling may have augmented the impairing effect of JZL184 (10 mg/kg) on immediate extinction baseline and acquisition. The fact that JZL184 increased freezing levels later during extinction training may also be indicative of this putative mechanism. Research has shown it can take between 20-60 mins for corticosterone levels to increase in the brain (Bouchez et al., 2012; Dominguez et al., 2014; Heinzmann et al., 2010). There is also a temporal lag in 2-AG's response given that 2-AG content is not altered 15 mins and 30 mins after a swim-stress and social defeat procedure, respectively (Dubreucq et al., 2012; McLaughlin et al., 2012). However, increased 2-AG levels following restraint stress have been shown after 60 mins (Hill et al., 2011). Hartley et al. (2016) demonstrated an acute effect of JZL184 1 hr after administration. In the current IED paradigm, JZL184 acutely increased freezing in extinction training baseline and during CS presentation. Taken together, it is possible that 2-AG levels became elevated due to the response of 2-AG to stress and JZL184 injection. This may have caused the significant difference in freezing between the JZL184 and vehicle groups.

Neither dose of JZL184 rescued the IED. There are currently no studies that have assessed the effect of JZL184 on NA or CRF signalling. Prefrontal afferents containing DβH, NET, and CB1Rs are colocalised with somatodendritic processes containing DAGL, and CB1Rs are co-expressed with α2-ARs (Cathel et al., 2014; Reyes et al., 2015, 2017). Whilst the precursor and target of 2-AG are well placed to modulate NA signalling, there is no evidence to show that JZL184 alters NA signalling. Therefore, if JZL184 were able to manipulate NA signalling, this may not be beneficial if the current IED was NA-independent. There are also no studies that have assessed the effect of JZL184 on CRF signalling. Acute stress increases 2-AG levels in the mPFC, which activates GABAergic interneurons and disinhibits excitatory activation of GABA

neurons in the BNST, thus attenuating PVN activity and reducing CRF release (Hill et al., 2011). Whilst 2-AG is known to regulate the HPA axis in certain models of stress (Hill and McEwen, 2009; Hill and Tasker, 2012; Patel et al., 2004), it is possible that footshock stress is characterised by a distinct eCB profile (Hohmann et al., 2005). For example, 2-AG levels are not affected by aversive IA training involving footshocks (Morena et al., 2014). Therefore, in a paradigm that used footshock-induced stress, it is unclear whether JZL184 modulation of 2-AG would be able to remediate the IED.

5.4.1. Conclusion

The current chapter assessed whether eCB metabolism inhibition could enhance immediate extinction encoding and rescue the IED. The high dose of JZL184 increased freezing both acutely and chronically, which may have been due to excessive 2-AG signalling in the BLA. Neither URB597 nor high or low doses of JZL184 rescued the IED. Considering previous studies, URB597 may not enhance extinction encoding in male rats that have not experienced stress prior to fear conditioning. Given that propranolol did not rescue the IED in the previous chapter, it is possible that the current paradigm did not increase NA to the same levels shown in previous IED experiments (Fitzgerald et al., 2015; Giustino et al., 2017, 2020). It is possible that CRF signalling contributed to the IED (Hollis et al., 2016; Jo et al., 2020). Whilst URB597 can modulate NA and CRF signalling, and 2-AG is well placed to modulate NA and CRF signalling, it is possible that footshock stress is characterised by a distinct eCB profile when compared to other stress models. This may have inhibited the drugs' capacity to rescue the IED. Issues around the timing of drug administration, brain eCB levels, and the specificity of URB597's and JZL184's pharmacodynamics are considered further in the general discussion.

6. General Discussion

This thesis aimed to determine whether eCB metabolism inhibition could enhance fear extinction, attenuate spontaneous fear recovery, and rescue the IED. Chapter 2 assessed whether three doses of URB597 could enhance extinction encoding and spontaneous recovery testing. None of the doses affected freezing throughout the experiment when given before or after extinction training. In chapter 3, three doses of JZL184 were used in the extinction and spontaneous recovery paradigm. None of the doses affected freezing throughout the experiment when given before or after extinction training. In chapter 4, experiment 1 successfully validated the experimental protocol required to induce the IED. During the extinction retention test, delayed extinction training caused a greater reduction in freezing compared to immediate extinction training at the fifth CS. In chapter 4, experiment 2, propranolol was administered immediately following fear conditioning in the IED paradigm. This positive control was included to validate the use of pharmacological interventions in the paradigm. Propranolol reduced freezing during baseline and cued extinction training but did not rescue the IED. Finally, chapter 5 assessed whether eCB metabolism inhibition could modulate freezing behaviour within the IED paradigm. In experiment 1, URB597 was administered immediately after fear conditioning. In experiment 2 and 3, high and low doses of JZL184 were administered immediately after fear conditioning, respectively. URB597, and the low dose of JZL184, had no effect on freezing, whilst the high dose of JZL184 increased freezing during extinction training baseline, extinction training, and extinction testing baseline. None of the drugs affected extinction recall and or rescued the IED.

6.1. Timing of Drug Administration and Brain Endocannabinoid Levels

URB597 did not affect freezing in the spontaneous recovery or IED paradigms. As previously discussed, URB597 enhances extinction in mice with strong footshock parameters (Bowers and Ressler, 2015; Gunduz-Cinar et al., 2013a; Laricchiuta et al., 2013; Lisboa et al., 2015; Llorente-Berzal et al., 2015), and rats with prior or concurrent trauma (Morena et al., 2018; Zer-Aviv and Akirav, 2016). In the current thesis, shock parameters in the spontaneous recovery and IED paradigms were lower

than these studies. Despite this, it is important to consider methodological factors that may have affected the drug's performance.

Gobbi et al. (2005) found that a single dose of URB597 did not increase AEA levels in HPC and PFC until 2 hrs after administration. Other studies have shown that URB597 elevated brain levels of AEA 1-2 hrs after administration, despite FAAH activity being inhibited within 15 mins (Greco et al., 2020; Kathuria et al., 2003; Kinsey et al., 2009; Lomazzo et al., 2015; Smaga et al., 2014). It should be noted that AEA was measured at one time point in these studies, as opposed to Gobbi et al. (2005) where measurements were taken every 30 mins from separate cohorts. A single dose of URB597 increases brain AEA levels within 30 mins of administration (Fegley et al., 2005), particularly in the HPC and cortex (Basavarajappa et al., 2014). It is important to note that the vehicle used by Fegley et al. (2005) was the same as that used in the current thesis (90% saline, 5% Tween 80, and 5% PEG). One key discrepancy between the Gobbi et al. (2005) and Fegley et al. (2005) studies is that a lower dose (0.1 mg/kg) was used in the former, whilst a higher dose (0.3 mg/kg) was used in the latter. This may indicate that the lower dose of URB597 used in chapter 2 (0.1 mg/kg) was unable to modulate AEA levels prior to extinction training, thus accounting for its lack of effect when given prior to extinction training. Despite this, URB597 (0.3 mg/kg) given 30-40 mins before testing alters behaviour in non-aversive memory and social paradigms (Hasanein and Far, 2015; Hlavacova et al., 2015; Warren et al., 2022). URB597 (0.3 and 1 mg/kg) given 30 mins prior to testing reduces freezing behaviour during extinction training and impairs fear memory retrieval (Bowers and Ressler, 2015; Gobira et al., 2017; Laricchiuta et al., 2013). The evidence presented above suggests that the timing of URB597 administration may have prevented the low dose from modulating freezing behaviour when given prior to extinction training. However, other studies have shown that higher doses of URB597 can modulate behaviour within 30 mins of administration. It is important to note that the latency between drug administration and elevated eCB levels does not account for the lack of drug effect when given after extinction training, nor does it account for the lack of effects on extinction consolidation.

Footshock acutely increases AEA levels in the mPFC, AMG, HPC, and PAG (Hohmann et al., 2005; Morena et al., 2014). As previously discussed, this has implications for the current thesis as footshock-induced stress prior to immediate extinction may have elevated AEA at the point of testing. This may have prevented further modulation of AEA by URB597, which could explain the lack of an effect in the IED paradigm. However, Bluett et al. (2014) found that AEA was reduced 24 hrs after footshock due to elevated FAAH activity. FAAH activity in the brain is acutely increased following a stressful event (e.g., restraint, forced swim, and social defeat; Gray et al., 2015; Hill and McEwen, 2009; Navarria et al., 2014). Consequently, this leads to a stress-induced reduction of AEA levels in brain regions relevant to fear conditioning and extinction, e.g., HPC, AMG, and in specific cases the mPFC (Dubreucq et al., 2012; Gray et al., 2015; Hill and McEwen, 2009; McLaughlin et al., 2012; Patel et al., 2005; Rademacher et al., 2008; Wang et al, 2012). In the spontaneous recovery paradigm, extinction training took place 24 hrs after fear conditioning. It is possible that the fear conditioning may have elevated FAAH activity during delayed extinction training. In this case, URB597 may have been unable to sufficiently inhibit FAAH, thus failing to increase AEA levels to an optimal level that may have enhanced extinction encoding and prevented fear relapse.

In vivo, JZL184 more potently inhibits MAGL in mice than in rats (Long et al., 2009b; Pan et al., 2009). Through *ex vivo* assays in mice, Long et al. (2009a) reported that a high dose of JZL184 (16 mg/kg) significantly inhibited MAGL activity, and increased brain 2-AG levels within 30 mins of administration, persisting for around 24 hrs. Kinsey et al. (2009) found that JZL184 (16 mg/kg) elevated brain 2-AG levels 2 hrs after administration in mice. Lomazzo et al. (2015) found that JZL184 (8 mg/kg) elevated 2-AG across several brain areas (including PFC and HPC) within 1.5 hrs of administration. Long et al. (2009b) found that mice treated with JZL184 (4, 16, and 40 mg/kg) showed elevated brain 2-AG levels 4 hrs after injection. Microdialysis shows that JZL184 does not upregulate eCB levels in rat nucleus accumbens (Wiskerke et al., 2012). Other rat studies have shown that JZL184 has no effect on 2-AG levels when administered locally or systemically (Kerr et al., 2013, Woodhams et al., 2012). However, Ivy et al. (2020) found that JZL184 (16 mg/kg) increased rat HPC

2-AG levels within 4 hrs of administration. The authors found no significant increase in 2-AG with 4 and 8 mg/kg doses. However, it should be noted that Ivy et al. (2020) presented no data prior to the samples taken at 4 hrs, therefore it is possible that increases in 2-AG levels were missed at time points more temporally relevant to the current thesis. Given that the current thesis does not have data to demonstrate significant MAGL inhibition or 2-AG elevation at the time of behavioural testing, it may be that doses selected were not sufficient to upregulate levels of 2-AG at relevant timepoints. Alternatively, it is possible that JZL184 does not increase central 2-AG levels in rats, which may have confounded use of this drug. However, given that the high dose of JZL184 showed a trend towards a significant result in the immediate extinction training baseline test, JZL184 may have penetrated the blood brain barrier to mediate an acute behavioural effect.

6.2. URB597 or JZL184 Target Specificity and State-Dependency

The current thesis successfully induced spontaneous fear recovery and the IED in all groups. However, URB597 or JZL184 did not attenuate nor prevent fear relapse or the IED, regardless of the doses given. Research conducted in the same lab, using an identical paradigm, found that cannabidiol (CBD) prevented spontaneous fear recovery and rescued the IED when administered 30 mins prior to extinction training (Papagianni et al., 2022). This finding shows that pharmacological remediation of fear relapse and extinction impairment is possible within this thesis' paradigm. Sachser et al. (2015) also found that infusion of CP 55,940 (CB1R and CB2R agonist) into the RSC prevented spontaneous recovery. When considering the action of the drugs used in the current thesis, both URB597 and JZL184 are known to be highly selective for their respective targets. For example, URB597 does not increase brain levels of 2-AG, whilst JZL184 has no effect on AEA (Fegley et al., 2005; Gobbi et al., 2005; Kinsey et al., 2009; Lomazzo et al., 2015). It is therefore possible that the isolated mechanisms of URB597 and JZL184 are insufficient to prevent spontaneous fear recovery and rescue the IED. Instead, it is possible that a combination of elevated eCB tone and pharmacological manipulation of related receptors may be required to prevent the relapse of extinction memory. For example, it may be that dual inhibition of FAAH

and MAGL is required to affect freezing behaviour in the current paradigms. However, there is a paucity of research on the effect of dual FAAH and MAGL inhibition on conditioned fear extinction.

When the internal drug state is similar during memory acquisition and retrieval, memory retrieval can be augmented through state-dependency (Overton, 1964). This effect has been shown in learned fear extinction (Bouton et al., 1990; Briggs and Olson, 2013). Bouton et al. (1990) found that fear extinction memory acquired in a certain drug state was specific to that particular drug state. This is similar to the context-dependent nature of extinction memory. For example, Bouton and Bolles (1979) showed that fear extinction memories were specific to the physical context in which they were acquired. This may be relevant to the current experiments that administered drugs prior to extinction training. In these experiments, drugs were only administered once, suggesting that rats were in separate drug contexts during extinction training and testing. This may have prevented the retrieval of extinction memory. It is also possible that drug administration prior to extinction retrieval may have aligned the interoceptive drug states between extinction training and testing, thus facilitating extinction memory retrieval. However, as discussed above, the timing of drug administration prior to extinction training may have been too short to induce a drug state during extinction acquisition. In such a scenario, state dependency would not contribute to the lack of drug effects on extinction retention and fear relapse. State-dependency would not have been a factor in experiments where drugs were given after extinction training. Studies that have shown pharmacological modulation of extinction encoding with URB597 and JZL184 were not state-dependent (Bowers and Ressler et al., 2015; Gunduz-Cinar et al., 2013a; Hartley et al., 2016; Laricchiuta et al., 2013; Mizuno et al., 2022; Morena et al., 2018, 2021). Considered together, it seems unlikely that state-dependency contributed to the lack of URB597's and JZL184's effect on extinction encoding.

6.3. JZL184 Efficacy in Contextual Fear Conditioning Paradigms

In the current thesis, JZL184 imparted no beneficial effect on auditory-cued extinction. It is possible that the beneficial effects of JZL184 may be context

dependent. Rea et al. (2014) found that bilateral infusion of 2-AG into the vHPC reduced contextual fear expression in a CB1R-dependent manner. In socially isolated rats, Morena et al. (2018) showed that low doses of JZL184 (0.5 and 1 mg/kg) given before repeated extinction training sessions reduced contextual freezing during extinction training and recall. These findings suggest that sufficient elevation of 2-AG signalling can enhance delayed contextual extinction encoding. Atsak et al. (2012) showed that corticosterone injection prior to fear memory retrieval impaired contextual but not auditory-cued fear memory retrieval in a CB1R-dependent manner. The authors found that the same impairing dose of corticosterone increased 2-AG levels in the HPC. Atsak et al. (2018) found that elevating HPC levels of 2-AG with intracerebral infusion of JZL184 reduced fear expression in animals exposed to early life stress. Away from fear conditioning, Sciolino et al. (2011) found that JZL184 imparted anxiolytic effects in a context-dependent manner. Taken together, it is possible that elevation of 2-AG is remedial in contextual settings, which may involve 2-AG signalling in the HPC. The HPC plays an integral role in the extinction of contextual fear (Ji and Maren, 2007). CB1Rs are highly expressed in the HPC compared to other brain areas (Hu and Mackie, 2015), thus providing the theoretical basis for 2-AG mediated effects on contextual memory. This may explain why JZL184 was unable to impart a remedial effect within the current auditory-cued paradigm. However, Zhang et al. (2017) found that NMDA receptor blockade in the vHPC impaired auditory-cued extinction encoding. This suggests that vHPC function is still involved in auditory-cued fear extinction.

6.4. Strain Differences

The current thesis used pigmented Lister Hooded rats in all studies. Rodent studies that have assessed the effects of FAAH and MAGL inhibition on fear extinction have used albino Sprague-Dawley or Wistar rats (Balogh et al., 2016, 2019; Morena et al., 2018, 2019, 2021; Zer-Aviv and Akirav, 2016). Lister Hooded rats show less anxiety in an elevated plus maze test compared to Sprague-Dawley rats (McDermott and Kelly, 2008). This is characterised by increased open arm entries, increased time spent in the open arms and greater distance travelled in the open arms. Administration of

diazepam increases open arm entries in Sprague-Dawley rats (compared to drug-free baseline) but has no effect on Lister Hooded rats. Lister Hooded rats show heightened locomotor activity during the first 4 hrs of the light-on cycle compared to Sprague-Dawley rats (McDermott and Kelly, 2008). In the current thesis, this was the time at which experiments were conducted. It is possible that this elevation in baseline locomotion may have obfuscated nuanced drug effects on freezing. Taken together, it may be that the Sprague-Dawley strain is better suited to assess anxiolytic drug effects that depend on locomotor activity. Alternatively, it is possible that testing Lister Hooded rats in the afternoon or with a reverse light cycle (e.g., lights off at 07:00) may have highlighted subtle drug effects.

Rat strains show divergent behavioural responses to cannabinoid-based therapies. Pigmented rats (Long-Evans and Lister Hooded) acquire and retain a stable routine of WIN 55,212-2 self-administration (Deiana et al., 2007; Fattore et al., 2007), whilst Sprague-Dawley rats do not (Deiana et al., 2007). In an object location task, CP55,940 (CB1R and CB2R agonist) impairs short-term spatial memory in Wistar (albino) rats but has no effect on Lister Hooded rats (Renard et al., 2013). These results suggest there may be some degree of disparity between the eCB systems in pigmented and albino rats. Other studies have shown differential CB1R expression between albino strains (Brand et al., 2012; Coria et al., 2014), as well as differences in c-Fos expression and behavioural responses following CP55,940 administration (Arnold et al., 2001, 2010). In the current thesis, a high dose of JZL184 elevated freezing during immediate extinction training. Rea et al. (2014) showed that elevating 2-AG via microinfusion into the vHPC reduced contextual fear expression in Lister Hooded rats. Importantly, Papagianni et al. (2022) found that CBD prevented spontaneous recovery in Lister Hooded rats when using an identical paradigm to the current thesis. This suggests that the fear response of Lister Hooded rats can be modified following systemic cannabinoid administration. As this thesis did not compare rodent strains, it cannot be concluded whether use of Lister Hooded rats affected the current results or not.

6.5. Sex Differences

The current thesis used male rats. Drugs that target the eCB system show varied responses between sexes. Females are often more sensitive to cannabinoids than males (Craft, 2005). In Long Evans and Lister Hooded rats, females more rapidly develop higher rates of WIN 55,212-2 self-administration than males (Fattore et al., 2007). Studies have shown sex differences in the freezing response to eCB-based treatments. Mizuno et al. (2022) found that WIN 55,212-2, rimonabant, and JZL184 impaired extinction in both sexes. However, URB597 augmented extinction in females that were in the dioestrus phase during the first extinction training session. The drug had no effect on males. Morena et al. (2021) found that inhibition of AEA and 2-AG hydrolysis had no effect on fear expression or extinction in males. In females, increased AEA signalling at TRPV1 receptors impaired extinction, whilst increased 2-AG signalling at CB1Rs augmented darting behaviour in lieu of freezing. Cavener et al. (2018) found no differences in freezing between male and female $DAGL^{-/-}$ mice. A week after stress and URB597 injection, Zer-Aviv and Akirav (2016) conducted fear conditioning and three extinction training sessions on consecutive days. In males, URB597 remediated extinction impairments in all three extinction training sessions, whilst females only showed enhanced extinction in the first two training sessions. This was thought to be due to sex differences in the response to URB597 given after severe stress.

Sex differences in response to eCB-based treatments may be due to hormonal influences (e.g., the oestrus cycle; Ney et al., 2018; Mizuno et al., 2022). Additionally, sex hormones are thought to underpin cued extinction resistance in females, as they show higher levels of fear expression during extinction training and testing than males (Day and Stevenson, 2020). Sex differences may also be attributed to differences in neural activity (McCarthy et al., 2015). Zer-Aviv and Akirav (2016) showed sex differences in URB597's effects on HPC plasticity following severe stress. In males, URB597 normalised stress-induced upregulation of CB1Rs in the AMG, PFC, and HPC. However, normalisation in the HPC was not seen in females. This discrepancy in neuroplasticity may have accounted for the lack of URB597's effect in

females during the third extinction session. Following drug-free extinction training, Morena et al. (2021) found that male rats showed elevated and reduced AEA levels in the AMG and PAG compared to no extinction males, respectively. However, extinction and no extinction females showed no difference in brain eCB levels following drug-free extinction training (Morena et al., 2021). Llorente-Berzal et al. (2022) found that females showed greater CB1R expression in the PAG. With all this considered, it is possible that the drugs used in the current paradigms could have modulated freezing in females. It may be that the drugs were unable to impart an effect due to the use of male rats in the current thesis. Despite this, FAAH and MAGL inhibition have been shown to modulate male freezing behaviour in other extinction paradigms (Balogh et al., 2016; Bowers et al., 2015; Gunduz-Cinar et al., 2013a; Gobira et al., 2017; Hartley et al., 2016; Laricchiuta et al., 2013; Morena et al., 2018, 2019; Zer-Aviv and Akirav, 2016). However, given the methodological differences between these studies, it is difficult to attribute the divergent results to a single characteristic, e.g., sex.

6.6. Age Differences

In the current thesis, all rats weighed between 200-300g upon delivery. Whilst their specific ages were not known, growth charts from the supplier suggest that rats were between 7 and 11 weeks old when arriving at the lab (Charles River, UK). Rats were left for one week after arrival suggesting they were between 8-12 weeks old when tested. Rats at this age are late adolescents. Juvenile and adult rodents show differential responses to cannabinoid-based drugs (Gorey et al., 2019). THC and other synthetic CB1R agonists more severely impair spatial and nonspatial learning, and increase anxiety in adolescents compared to adults (Abboussi et al., 2014; Cha et al., 2006, 2007; Moore et al., 2010; O'Shea et al., 2004; Schneider and Koch, 2003). However, Schramm-Sapyta et al. (2007) found that in adults, THC was more aversive, more anxiogenic, and reduced locomotor activity when compared to adolescents. CBD has more potent antidepressant and anorexigenic-like effects in adult rats, as opposed to adolescents (Bis-Humbert et al., 2020). There is no research comparing the effects of URB597 or JZL184 between adolescent and adult rats. However, studies

have demonstrated age differences in behavioural responses to other cannabinoid-based drugs. Taken together, it is possible that the age range of the rats confounded the results in the current thesis. It may be that URB597 and JZL184 do not impart effects in a younger cohort, as opposed to more mature adults.

6.7. Freezing Variability

In the current thesis, freezing at the end of fear conditioning was relatively low compared to that seen at the start of extinction training. For example, freezing during fear conditioning plateaued at around 40% by the third CS-US pairing, whilst freezing could reach over 80% at the start of extinction training. High fear expression at the start of extinction training is indicative of successful fear memory encoding. However, this does not explain why freezing was low during fear conditioning. Animals show divergent behavioural responses to fear depending on the level of threat they perceive (Bolles and Fanselow, 1980). The predatory-imminence continuum provides a model for the range of behaviours animals display dependent on the threat level (Fanselow and Lester, 1988). In this model, prey animals (such as rats) show passive or active fear behaviours dependent on the predatory imminence. After a threat has been perceived, prey animals are likely to engage in passive fear behaviours (e.g., freezing) that reduce the chance of being noticed by a predator. However, as predatory imminence increases prey animals begin to show more active fear behaviour (e.g., darting and flight). In the context of the current thesis, it is possible that rats exhibited more active fear behaviours when experiencing CS-US pairings. It may be that the physical sensation of footshocks increased the level of perceived threat, meaning rats attempted to escape the cage. This active behaviour would have resulted in less freezing. During extinction training, where no footshocks were used, it is possible that the reduction in perceived threat caused an increase in passive fear behaviour, which is in accordance with the elevated freezing levels recorded during the first tone block. Taken together, it appears that the low freezing levels seen during fear conditioning may be explained by active fear behaviour. However, it is unlikely that the active fear behaviour elicited during fear conditioning was related to the lack of drug effects.

In both chapters 2 and 3, freezing levels were disparate between experiment 1 (pre-extinction drug administration) and experiment 2 (post-extinction drug administration). For example, in chapter 2, fear expression at the start of extinction training was around 60-80% in experiment 1 and around 40% in experiment 2. Likewise, in chapter 3, experiment 1 showed around 20% freezing during spontaneous recovery testing, whilst experiment 2 showed around 35%. Whilst no statistical comparisons were made between these data sets, it is important to consider why freezing may have differed so greatly. The primary difference in both chapters is that experiment 1 was scored manually, whilst experiment 2 was scored automatically. It is possible that these scoring modalities have varying degrees of sensitivity to freezing behaviour. It may be that one modality scored freezing behaviour more or less frequently than the other. Another explanation for this discrepancy in freezing may be due to batch differences in the rats that took part in each experiment. Whilst all rats were bought from the same supplier, there may be inherent variability between each batch of animals. However, it is unlikely that batch differences in laboratory animals is related to the lack of drug effects.

JZL184 (10 mg/kg) increased freezing during extinction training baseline, extinction training, and extinction testing baseline in the IED paradigm but had no effect in the spontaneous recovery paradigm. Differences between these studies include the increase in footshock intensity in the IED paradigm (0.5 mA, 1 s), compared to the spontaneous recovery paradigm (0.4 mA, 0.5 s), and the interval between fear conditioning and extinction training (30 mins vs. 24 hrs, respectively). Comparing footshock intensity to other studies that have shown JZL184-mediated increases in freezing during extinction training, Hartley et al. (2016) and Mizuno et al. (2022) used stronger parameters than those used in either of the current chapters (0.7 mA, 2 s and 0.75 mA, 2 s, respectively). This suggests that more severe conditioning parameters may be required to see the disruptive effects of JZL184.

6.8. Limitations and Future Study

Interpretation of the current results is limited by several methodological factors. The first limitation is that brain eCB levels were not assessed following URB597 or JZL184

administration. It is important to understand the time scale in which the drugs imparted their effects on eCB signalling and behaviour. In the current thesis, it was not possible to determine whether AEA or 2-AG levels were elevated within the 30 mins prior to extinction training. As discussed above, this may explain why the low dose of URB597 and JZL184 failed to modulate freezing behaviour. Future study could address this by using cohorts that are culled 30 mins, 1 hr, and 2 hrs post-drug administration, with brain tissue collected and stored for later assessment of eCB levels. A similar limitation is that brain eCB levels were not assessed at relevant time points. During fear memory retrieval, CB1Rs are differentially recruited depending on the footshock intensity. Activation of these receptors is thought to suppress fear retrieval following strong fear conditioning (Mizuno and Matsuda, 2021). It would have been useful to know how the different shock intensities used in the current thesis affected CB1R signalling, and the state of eCB signalling at relevant moments. This may have guided more specific targeting of the eCB system at time points where eCB signalling was dysregulated. Vehicle-treated cohorts would have to undergo fear conditioning and/or extinction training that is identical to the main experiment. This would then account for any effect of fear conditioning/extinction/injection on eCB transmission, making the results obtained from brain eCB analysis more relevant. Further drug-free cohorts could be culled prior to and after spontaneous recovery testing or immediate extinction recall testing to provide an insight into the state of eCB neurotransmission at relevant time points.

A further limitation of the current thesis was that footshock intensity could not be increased beyond the project licence constraints (0.5 mA, 1 s). Studies that have assessed pharmacological manipulation of the IED used more intense footshocks (Fitzgerald et al., 2015; Giustino et al., 2017, 2020; Wang et al., 2021). As discussed previously, footshock intensity in the current thesis may have limited the increase in LC-NA signalling compared to that shown by other groups. Given that propranolol did not rescue the IED suggests NA signalling may not have mediated the current IED. It is possible that CRF signalling may have contributed to the IED in the current paradigm (Hollis et al., 2016; Jo et al., 2020; Maren, 2022). Given the constraints imposed by the current project licence, it may not be possible to increase

footshock intensity in future studies unless an amendment to the project licence allows for the use of stronger footshocks. However, future IED studies should be conducted with stronger conditioning parameters that match those seen in the literature. More intense footshocks may induce aberrant NA and CRF signalling during immediate extinction training, thus providing the putative conditions required for pharmacological remediation. However, Papagianni et al. (2022) found that CBD rescued the IED in an identical paradigm to that used in the current thesis. This suggests that CBD restored extinction retention by remediating the aberrant neuronal signalling. Due to URB597's and JZL184's target specificity, it is possible that the drugs were unable to rescue the IED. With this in mind, future study could assess whether dual FAAH and MAGL inhibition can prevent fear relapse and rescue the IED.

In the spontaneous recovery paradigm, extinction acquisition and short-term retention were likely adaptive given that fear conditioning was relatively mild. However, the footshock parameters were previously validated to keep freezing low during short-term extinction testing so that rats showed higher freezing during later spontaneous recovery (Papagianni et al., 2022). This was successfully implemented given that all groups showed spontaneous recovery. Given that CBD prevents spontaneous recovery in an identical paradigm (Papagianni et al., 2022), alterations to footshock intensity should not be required for future study. One aspect that could be altered is the number of extinction sessions and drug injections. Morena et al. (2018) showed that repeated administration of URB597 after multiple contextual extinction sessions enhanced extinction training and retention, whilst JZL184 (0.5 and 1 mg/kg) repeatedly given before extinction training reduced global levels of freezing throughout extinction training and retention. Mizuno et al. (2022) found that JZL184 required multiple extinction sessions to elevate freezing levels during extinction training. Mayo et al. (2020b) found repeated administration of a FAAH inhibitor enhanced extinction in humans. Future study could assess URB597's and JZL184's effects in a spontaneous recovery paradigm that uses weaker, repeated extinction. For example, shorter extinction training sessions (e.g., 15 CS instead of 30 CS) with drugs given before or after each session. Such a model would assess whether URB597 or JZL184 can facilitate weaker extinction over a longer period. This could account for

any temporal delay in the drugs' effects. In the current thesis, the effects of repeated drug administration would have been missed given that only one injection was given. It is important to note that spontaneous recovery can still be assessed after multiple extinction sessions (Matsuda et al., 2015).

During immediate extinction training, the high dose of JZL184 acutely and chronically increased freezing. A limitation of the current thesis is that the mechanism of this effect was not determined. Following a positive pharmacological result, it is commonplace to administer receptor antagonists alongside the test drug, thus preventing the test drug from activating a specific receptor. If the change in behaviour is not induced with concurrent drug/antagonist administration, it likely means that the antagonised receptor mediated the drug's effect. In the context of the current results, future study should administer a CB1R antagonist (e.g., AM251) alongside JZL184. This would highlight whether JZL184's effect on freezing behaviour was CB1R-dependent. A CB2R antagonist may also be considered to determine the involvement of CB2Rs in mediating JZL184's effect on freezing. A separate antagonist-only cohort would have to be included to account for any antagonist mediated effects on freezing behaviour.

As previously discussed, JZL184 may be more effective in a contextual paradigm given that CB1Rs are highly expressed in the HPC. Future study should assess the effect of JZL184 in a contextual paradigm. For example, it may be useful to establish a contextual IED paradigm as Archbold et al. (2010) failed to establish the IED in a contextual paradigm. Most pharmacology-based research on the IED has been auditory-cued (Fitzgerald et al., 2015; Giustino et al., 2017, 2020; Hollis et al., 2016; Wang et al., 2021). However, Santos et al. (2021) found that propranolol did not affect freezing when given before immediate contextual extinction. It may be that JZL184 is more effective in a contextual setting given that it can reduce global freezing during contextual extinction training sessions, and reduce contextual fear expression (Atsak et al., 2018; Morena et al., 2018). Additionally, a contextual-based spontaneous recovery paradigm may be more suited to the investigation of JZL184's effects on extinction (Matsuda et al., 2014, 2015).

A limitation of the current thesis is that experiments were conducted using male, Lister Hooded rats of equivalent age. Female rats are more sensitive to eCB-based treatments (Craft, 2005). This, coupled with their cued extinction resistance (Day and Stevenson, 2020), makes them an interesting model for the investigation of potential extinction enhancing eCB-based drugs. Future study should include male and female cohorts to assess sex differences in freezing behaviour following eCB-based treatments. In these studies, it would be important to consider the oestrous cycle and how drug effects might be modulated by the cycle phase. To assess variability across rat strains, future study should compare Lister Hooded and Sprague-Dawley rats in the spontaneous recovery and IED paradigms. Sprague-Dawley rats show reduced baseline locomotor activity compared to Lister Hooded rats in the hours following the lights-on period (McDermott and Kelly, 2008). By using a model with lower baseline locomotion, more nuanced effects on freezing may be highlighted. To account for differential responses between adults and adolescents in response to cannabinoid-based drugs, future study should consider including extra cohorts of older adult rats. This would allow for evaluation of any age effect on eCB signalling and drug response. Taken together, future study should compare variability between sex, strain, and age.

6.9. Translation of Results

As discussed previously, fear symptomology in PTSD is caused by an impaired ability to extinguish fear memories (Zuj et al., 2016). Exposure therapy is the most efficacious treatment for PTSD (Furini et al., 2014; Zuj and Norrholm, 2019); however, patients are prone to relapse. Fear extinction in rodent models is considered the theoretical basis of exposure therapy in humans. In human extinction studies, aversive stimuli usually include electric shocks or loud noises, whilst the CFR can be inferred through skin conductivity or perceived threat scales. Due to the similarities between rodent and human extinction studies, results from pre-clinical studies can be translated to human paradigms (Milad and Quirk, 2012).

Animal research has shown that the eCB system, particularly CB1R activation, is involved in fear extinction (Hill et al., 2018). In healthy humans, genetic variation in

the FAAH gene (C385A allele carriers of rs324420) can enhance fear extinction through enhanced AEA signalling (Dincheva et al., 2015; Mayo et al., 2020a; Ney et al., 2021). C385A allele carriers show differences in AEA response and AMG reactivity to extinction-based tasks despite no genotype effect on extinction behaviour (Spohrs et al., 2021; Zabik et al., 2021). Heitland et al. (2012) found that a polymorphism in the CB1R gene (rs1049353) of healthy humans was implicated in weaker fear extinction. Ney et al. (2021) showed that CB1R (rs2180619 and rs1049353) and FAAH (rs324420) polymorphisms were associated with poor extinction in PTSD patients. THC and PF-04,457,845 (FAAH inhibitor), but not dronabinol (partial CB1R and CB2R agonist), improve physiological measures of extinction in healthy humans (Hammoud et al., 2019; Klumpers et al., 2012; Mayo et al., 2020b; Rabinak et al., 2014). Behavioural interventions that increase AEA levels (e.g., aerobic exercise) can improve cognitive measures of extinction in healthy humans and women with PTSD (Crombie et al., 2021a, b, c). Overall, the eCB system is implicated in human extinction paradigms, as well as PTSD. Given that PTSD is characterised by impaired extinction and relapse, the models used in the current thesis are of high translational value.

However, there are limitations to the translational value of the current results. Ethical constraints in animal studies are far less than those imposed in human research. For example, stronger fear conditioning that triggers defensive responding essential for survival can be implemented in animals. This contrasts the aversive stimuli used in human studies that are not perceived as life-threatening. This means that nuanced effects of therapeutic treatments may not be shown in human studies. The doses of novel pharmacological treatments are less regulated in animal studies. This means that stronger drug effects can be shown in animal studies, with less initial focus on toxicity and side-effects. Finally, there may be issues with the validity of clinical extinction studies and their relevance to PTSD symptoms (Ney et al., 2022). For example, human extinction studies are often underpowered, contain unstandardised statistical analyses, and may not model learning deficits seen in PTSD (Duits et al., 2015; Pöhlchen et al., 2020; Ney et al., 2020). All things considered, the current thesis investigated the effect of eCB metabolism inhibitors in a homogenous

animal model of fear relapse and impaired extinction. Translation of these results to a heterogeneous human population should be done with caution.

6.10. Conclusion

At first glance, the current results suggest that eCB metabolism inhibition may not be a useful treatment for extinction relapse and impairment. However, there were several confounding factors that may have influenced this outcome. Differences in eCB signalling between rat strain, sex, and age may have contributed to the lack of drug effects. Footshocks at the weaker intensities may have failed to induce the aberrant neuronal transmission required to URB597 and JZL184 to show a drug effect. Both drugs show high selectivity for their respective enzymatic targets, thus it is possible that the isolated action of each drug was inadequate for the amelioration of extinction deficits and relapse. The auditory-cued conditioning modality may have obscured the effects of JZL184, which appears to show greater efficacy in contextual conditioning paradigms. Despite the negative results in the current thesis, research into the therapeutic effects of eCB-based research into PTSD and anxiety disorders shows promise and should be further pursued.

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Appendices

Appendix 1 – Placement Report

I undertook my professional internship for postgraduate students (PIPS) placement with CanPharmaConsulting between 3rd November 2020 to 12th February 2021. During my time there, I worked as an assistant to a consultant who liaised with multiple pharmaceutical companies. Some of these companies were well established in the industry, whilst others were small start-up businesses that outsourced their research. The type of work varied with each company, however most tasks involved extensive background research into medical indications and drugs that the companies were targeting. Typically, I would collate background literature into a spreadsheet and then create a comprehensive document that provided as much information as possible. I helped to create PowerPoint slides that would go on to be used as investor decks, which would provide information on the investment opportunity each company offered. I was involved in designing future preclinical and clinical trials for companies who had an interest in drug discovery. During my time on the placement, I formed a good relationship with my supervisor. This made my time on the placement enjoyable and made me feel supported.

Before the placement, I was interested to see how the pharmaceutical industry operated, particularly how financial decisions are made about which drugs are viable. I wanted to learn more about the pathway from drug discovery to reaching the marketplace with a final product. Lastly, I was keen to learn what kind of jobs were available for researchers like myself given that I had only had experienced academia prior to starting the placement. Upon completing the placement, I have since discovered that pharmaceutical companies are often faced with tough decisions about whether a product is efficacious enough to continue its pursuit. For example, medications that only need to be taken once are not as sought after as medications that would need to be taken in repeated doses. Through studying investor decks, I was able to see how companies planned to take their novel drugs from concept to a final product. I also found out that companies who receive sufficient funding will look to hire researchers to conduct their preclinical and clinical trials.

I improved two major research skills as a result of my placement. The first was the ability to thoroughly sift through academic literature to find relevant information about a topic. I learnt how to compile the literature into segmented spreadsheets, thus allowing me to find and reference information quickly and efficiently. The second skill was an improvement to the style of my academic writing. It was quickly pointed out that I was adding too many words such as ‘moreover’ and ‘furthermore’. I now feel I am able to write quickly in a concise manner without the need for superfluous language.

After completing my placement, I realised that I would like to pursue a career within the pharmaceutical industry. I find the concept of working with novel therapeutics an interesting and fulfilling endeavour. I would particularly like to be a researcher for preclinical and/or clinical trials specific to psychopharmacology and mental health.

Appendix 2 – Impact Statement

Manipulation of the endocannabinoid system can impact the efficacy of fear extinction learning and retention. Extinction training in rats is a translatable model for exposure therapy in human PTSD treatment. Ultimately, this research aims to further understanding of fear memory and extinction in order to augment the treatment of PTSD in human patients. Anxiety and trauma-related psychiatric issues cause a significant socio-economic burden. The current medical treatments available for PTSD patients are often off-label medications for anxiety and depression. It is commonly known that these drugs offer variable success and can also incur severe side-effects for the end user. Furthering the research of novel, evidence-based treatments for PTSD will have a positive impact on socio-economic factors in the greater society. The current research aims to further the understanding of a novel treatment for PTSD that may be more efficacious and offer a more favourable side effect profile. The research investigated whether drugs that enhance the levels of endocannabinoids in the brain could help to prevent fear relapse or impairments in extinction. Overall, this thesis offers useful information on the inhibition of endocannabinoid metabolism within multiple extinction paradigms. This information will help further understanding of the use of endocannabinoid treatments in PTSD.