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Endocannabinoid modulating drugs improve anxiety but not the expression of conditioned fear in a rodent model of post-traumatic stress disorder

Akshayan Vimalanathan[a,b,1], Darryl C. Gidyk[a,1], Mustansir Diwan[a], Flavia V. Gouveia[a], Nir Lipsman[a], Peter Giacobbe[a], José N. Nobrega[b], Clement Hamani[a,b,*]

[a] Harquail Centre for Neuromodulation, Sunnybrook Health Sciences Centre, Sunnybrook Research Institute, Toronto, ON, M4N 3M5, Canada
[b] Behavioural Neurobiology Laboratory, Research Imaging Centre, Centre for Addiction and Mental Health, 250 College Street, Toronto, ON, M5T 1R8, Canada

HIGHLIGHTS

- 25-30% of rats exhibit impaired extinction learning and long-term anxiety following fear conditioning.
- Acute injections of endocannabinoid agonists reduced anxiety-type behaviour in these animals.
- In animals presenting normal extinction and anxiety-type responses, inverse agonists of CB1 were anxiogenic.
- Neither of these drugs affected fear-type responses.
- The chronic administration of drugs that modulate the endocannabinoid system did not alter behavioral responses.

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ABSTRACT

The endocannabinoid (eCB) system is a potential target for the treatment of symptoms of post-traumatic stress disorder (PTSD). Similar to clinical PTSD, approximately 25–30% of rats that undergo cued fear conditioning exhibit impaired extinction learning. In addition to extinction-resistant fear, these "weak extinction" (WE) rats show persistent anxiety-like behaviors. The goal of the present study was to test the hypothesis that behavioural differences between WE animals and those presenting normal extinction patterns (strong extinction; SE) could be mediated by the eCB system. Rats undergoing fear conditioning/extinction and fear recall sessions were initially segregated in weak and strong-extinction groups. Two weeks later, animals underwent a fear recall session followed by a novelty-suppressed feeding (NSF) test. In acute experiments, WE rats were injected with either the fatty acid amide hydrolase (FAAH) inhibitor URB597 or the CB1 agonist WIN55,212-2 1 h prior to long-term recall and NSF testing. SE animals were injected with the inverse CB_1 receptor agonist AM251. In chronic experiments, WE and SE rats were given daily injections of URB597 or AM251 between short and long-term recall sessions. We found that acute administration of WIN55,212-2 but not URB597 reduced anxiety-like behaviour in WE rats. In contrast, AM251 was anxiogenic in SE animals. Neither treatment was effective in altering freezing expression during fear recall. The chronic administration of AM251 to SE or URB597 to WE did not alter fear or anxiety-like behaviour or changed the expression of FAAH and CB_1. Together, these results suggest that systemic manipulations of the eCB system may alter anxiety-like behaviour but not the behavioural expression of an extinction-resistant associative fear memory.

1. Introduction

Post-traumatic stress disorder (PTSD) is a debilitating psychiatric illness that manifests in 20–30% of the individuals exposed to a traumatic event (American Psychiatric Association, 2013; Kessler et al., 1995). In addition to being common, PTSD is refractory in 10–30% of

Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, anandamide; AMY, amygdala; BLA, basolateral amygdala; CB_1, CB_2. type-1 and type-2 cannabinoid receptors; eCBs, endocannabinoids; FAAH, fatty acid amide hydrolase; HPC, hippocampus; MAGL, monoacylglycerol lipase; mPFC, medial prefrontal cortex; NSF, novelty-suppressed feeding; PTSD, post-traumatic stress disorder; SE, strong extinction; WE, weak extinction

* Corresponding author. Sunnybrook Research Institute, 2075 Bayview Av, S126, Toronto, ON, M4N3M5, Canada.
E-mail address: clement.hamani@sunnybrook.ca (C. Hamani).

[1] Both authors have equally contributed to the execution of this work.

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patients who receive conventional pharmacological treatments and/or psychotherapy (Watts et al., 2013). This highlights the need for a broader set of treatment options for treatment-resistant PTSD to improve patient outcomes and reduce negative effects on families, caregivers, and society (Howlett and Stein, 2016; Kessler, 2000).

The endocannabinoid system is a potential target for the treatment of PTSD symptoms. Endocannabinoids (eCBs) are retrograde neurotransmitters produced through the cleavage of membrane phospholipids (Piomelli, 2003). These include anandamide (AEA) and 2-arachidonoylglycerol (2-AG), which are synthesized *de novo* in post-synaptic terminals and migrate through the synaptic cleft to act on presynaptic type-1 and type-2 cannabinoid receptors (CB_1, CB_2) (Ganon-Elazar and Akirav, 2009; Gunduz-Cinar et al., 2013a; Viveros et al., 2005). In the central nervous system, eCBs bind mainly to CB_1 receptors and inhibit Ca^{2+} influx into pre-synaptic terminals, thereby decreasing neurotransmitter release (Viveros et al., 2005). Overall, the net effect of eCBs depend on the cell populations expressing its receptors (Gunduz-Cinar et al., 2013a). When binding to glutamatergic terminals, eCBs reduce glutamate release and firing of the target cells (Diana and Marty, 2004; Kodirov et al., 2010; Ohno-Shosaku et al., 2002). Similarly, eCBs binding to GABAergic terminals induce excitation by decreasing the release of GABA (Diana and Marty, 2004; Ohno-Shosaku et al., 2002; Zhu and Lovinger, 2005).

The eCB system is implicated in various physiological processes, including the modulation of memory and motor function (Piomelli, 2003), as well as stress, anxiety, and fear (Lutz, 2007; Riebe and Wotjak, 2011). For example, eCB levels are increased in key brain regions required for normal extinction learning in rodents (Gunduz-Cinar et al., 2013b). The two well-described eCBs, AEA and 2-AG, are hydrolyzed by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (Patel et al., 2017). Whereas selective knockout of CB_1 receptors in mice and CB_1 antagonism cause deficits in extinction and increased anxiety-like behaviour, inhibition of FAAH has been reported to enhance extinction (Marsicano et al., 2002; Segev et al., 2018; Varvel et al., 2007). Importantly, CB_1 receptors are ubiquitous in the affective memory neurocircuitry, which includes the hippocampus (HPC), amygdala (AMY), and medial prefrontal cortex (mPFC) (Gouveia et al., 2019; Piomelli, 2003). Because the systemic administration of FAAH inhibitors (e.g., URB597) has the potential to attenuate fear responses through anxiolytic effects in rodents, it is likely that eCB system function may be leveraged to improve PTSD-like symptoms in preclinical models and provide unique therapeutic options to patients in the future (Fidelman et al., 2018; Ganon-Elazar and Akirav, 2009; Gunduz-Cinar et al., 2013b; Kuhnert et al., 2013; Shoshan and Akirav, 2017).

Various animal models have been used to study acute and chronic forms of fear and stress (Adamec, 1997; Adamec et al., 2010; Cohen et al., 2003; Milad et al., 2006; Pynoos et al., 1996; Reznikov et al., 2016; Richter-Levin, 1998; Stam et al., 2000; Ursano et al., 2008). We and others have found that approximately 25–30% of rats that undergo cued fear conditioning fail to extinguish fear responses and exhibit impaired extinction learning (Cohen et al., 2003, 2004; Reznikov et al., 2015, 2016, 2018). In addition to extinction-resistant fear, these “weak extinction” (WE) rats show persistent anxiety-like behaviors in standard tests, including novelty-suppressed feeding (NSF) (Reznikov et al., 2015, 2018). Such results are noteworthy due to the similarity with the proportion of individuals that develop PTSD following a traumatic event (Kessler et al., 2005). This is in line with earlier work demonstrating the utility of employing exclusion/inclusion criteria for preclinical models of PTSD (Cohen et al., 2004).

A recent preclinical study has reported positive effects when eCB levels are increased 2 h after trauma, prior to, or during extinction both acutely and chronically via local FAAH inhibition or CB_1/CB_2 antagonism in the hippocampus or basolateral amygdala (BLA) (Fidelman et al., 2018). Interestingly, it has been suggested that chronic and direct agonism of CB_1/CB_2 receptors with WIN55,212-2 before trauma may lead to downregulation of eCB signalling and thus represent a counterproductive strategy when attempting to leverage the eCB system to treat PTSD-like symptoms and anxiety (Sbarski and Akirav, 2018).

The goal of the present study was to test the hypothesis that behavioural differences between WE and animals presenting normal extinction patterns (strong extinction; SE) may be mediated in part by the eCB system. Specifically, we tested whether acute inhibition of FAAH hydrolysis with URB597 ameliorates fear and anxiety responses in WE rats and whether inverse agonism of CB_1 receptors using AM251 induces fear and anxiety in SE animals. In addition, we tested if the CB_1/CB_2 agonist WIN55,212-2 could effectively reduce fear and anxiety in the WE phenotype.

2. Materials and methods

Protocols were approved by the Animal Care Committee of the Centre for Addiction and Mental Health and are in accordance with the Canadian Council on Animal Care (CCAC) guidelines. Adult male Sprague-Dawley rats (250–300 g; Charles River, Quebec) were used.

2.1. Behavioural tests

On day 1, rats were presented with six conditioned stimuli (CS; 30sec, 85 dB, 4 kHz auditory tones), each co-terminating with a footshock (unconditioned stimulus, US; 0.8 mA, 0.5s) (Reznikov et al., 2015, 2018). The intertrial interval was pseudo-randomly varied, averaging 3 min. On day 2, rats underwent extinction training consisting of 12 presentations of CS in the absence of shocks. On day 3, rats were exposed to 3 presentations of the CS alone to test for extinction recall. All trials were recorded with a video camera for offline scoring by a blind observer. Percentage of freezing was calculated as freezing time during tone presentation/time of tone presentation*100.

Segregation of subgroups was based on freezing scores during the last 2 extinction blocks and the recall block (Reznikov et al., 2015). Weak and strong extinction animals were defined as those showing average freezing $> 70\%$ and $< 30\%$, respectively (Reznikov et al., 2015).

Long-term extinction recall. Long-term recall sessions were conducted two weeks after short-term recall trials. These consisted of re-exposing the rats to 3 presentation of the CS alone.

Novelty Suppressed Feeding. Two days after long-term recall, rats were placed in a Plexiglas cage (MED Associates, St. Albans, VT) lined with black card material on all sides and bottom, which contained a white platform with a previously habituated treat on top (Froot Loops, Kellogg's ®) (Reznikov et al., 2018). Latency to begin consuming the food was measured by a blind observer (sniffing or simply touching the food was not scored). After the test, animals received the same treat in their home cage. All consumed the food reward in less than 30 s.

2.2. Drug administration

URB597 (0.3 mg/kg or 0.6 mg/kg; Cayman Chemical, Ann Arbor, MI), AM251 (3 mg/kg or 6 mg/kg; Cayman Chemical, Ann Arbor, MI) and WIN,55212-2 (2 mg/kg, Ann Arbor, MI) were initially diluted in dimethyl sulfoxide (DMSO). Solutions containing 5% polyethyleneglycol, 5% Tween-80, and DMSO (3% for URB597; 15% for AM251 and 4.2% for WIN,55212-2) diluted in saline were prepared. Doses of URB597, AM251 and WIN,55212-2 were selected based on efficacy shown by others (Haller et al., 2004; Kathuria et al., 2003).

During acute experiments, drugs were injected 1 h prior to long-term recall and novelty supressed feeding. Chronic treatment consisted of daily drug administration for 14 days between short and long-term recall. In these experiments, no drug was given to the animals on behavioural testing days.

2.3. Statistical analyses

A mixed design ANOVA was used to compare fear conditioning/extinction results. Student's t-test was used for the analysis of the first recall session between weak and strong extinction rats. One-way ANOVA (LSD post hoc) was used to compare data across pharmacological groups, since different drugs were used to treat WE and SE animals and since we were mainly interested in comparing the results of drug-treated groups and their respective controls. Results in the text and figures are expressed as means and standard errors.

3. Results

3.1. URB597 and AM251

As in our previous reports (Reznikov et al., 2015, 2018), freezing scores during the last extinction trials and short-term recall were used to subdivide animals in WE and SE. Conditioning and extinction data in these two subpopulations (WE n = 14; SE n = 16) could only be assessed retrospectively. During conditioning (C1-3), a significant time effect [$F(2,56) = 268.7$; $p < 0.001$] but no group effect [$F(1,28) = 0.50$; $p = 0.49$] or time × group interaction [$F(2,56) = 0.41$; $p = 0.66$] were noted (Fig. 1A). During extinction (E1-6), we found significant group [$F(1,28) = 48.22$; $p < 0.001$] and time effects [$F(5,140) = 16,60$; $p < 0.001$] but no group × time interaction [$F(5,140) = 0.48$; $p = 0.79$] (Fig. 1A). Significant differences between WE and SE animals were also observed during short-term extinction recall (R1; $p < 0.001$) (Fig. 1A). Long-term recall (R2) was conducted two weeks later, 1 h after drug administration. WE rats were injected with URB597 0.3 mg/kg i.p. (n = 7), while SE rats received AM251 3 mg/kg i.p. (n = 8). WE (n = 7) or SE (n = 8) controls received respective vehicle injections. Significant ANOVA results were found [$F(3,26) = 5.99$; $p = 0.003$] due to differences between SE and WE groups, with no differences being recorded when WE URB597 and SE AM251 injected animals were compared to their respective controls (Fig. 1B). In the novelty suppressed feeding test, in addition to significant differences across groups [$F(3,26) = 3.16.$; $p = 0.04$] SE AM251 rats had a higher latency to feed compared to vehicle-treated SE controls ($p = 0.05$; Fig. 1C).

To test whether the above described behavioural effects could be enhanced by increasing the treatment dose, different groups of rats (WE n = 13; SE n = 15) were administered with a double dose of URB597 (0.6 mg/kg) or AM251 (6 mg/kg) 1 h prior to long-term recall and novelty suppressed feeding. In this new batch, a significant time effect [$F(2,52) = 242.2$; $p < 0.001$] but no group effect [$F(1,26) = 3.37$; $p = 0.07$] or time × group interaction [$F(2,52) = 1.03$; $p = 0.37$] were observed during conditioning (Fig. 2A). While small differences were found at C1, WE and SE had equal levels of freezing reaching almost 30 s in the last conditioning sessions. During extinction, significant group effect [$F(1,26) = 95.49$; $p < 0.001$], time effect [$F(5,130) = 22.60$; $p < 0.001$] and group × time interaction [$F(5,130) = 7.68$; $p < 0.001$] were observed (Fig. 2A). Significant group differences were also present during short-term recall ($p < 0.001$) (Fig. 2A).

During long-term recall, significant results were noted [$F(3,24) = 10.91$; $p = 0.0001$] largely due to differences between SE vs. WE groups but not between our groups of interest (WE n = 6 vs. WE URB597 n = 7; SE n = 8 vs. SE AM251 n = 7; Fig. 2B). In the novelty suppressed feeding test, no differences were found across groups [$F(3,24) = 1.25.$; $p = 0.25$], despite a substantial (28%) increase in the latency to feed in SE AM251 animals compared to vehicle-treated SE controls ($p = 0.09$; Fig. 2C).

3.2. WIN 55,212-2

In the experiments described above we found that blocking CB1 receptors in SE rats induced an anxiogenic effect, with no behavioural changes observed in WE animals treated with blockers of FAAH activity. Based on these findings, we decided to test in a different batch of rats (WE n = 13; SE n = 16) whether the CB1 agonist WIN55,212-2 (2 mg/kg i.p.) injected 1 h prior to behavioural testing could ameliorate anxiety in WE animals.

During fear conditioning, we found significant effects of time [$F(2,54) = 154.1$; $p < 0.001$], group [$F(1,27) = 13.65$; $p = 0.001$], and time × group interaction [$F(2,54) = 4.379$; $p = 0.02$] (Fig. 3A). The Group effect was largely due to differences in C2 sessions with no differences in freezing between WE and SE being recorded in the last conditioning sessions. During extinction, significant group [F

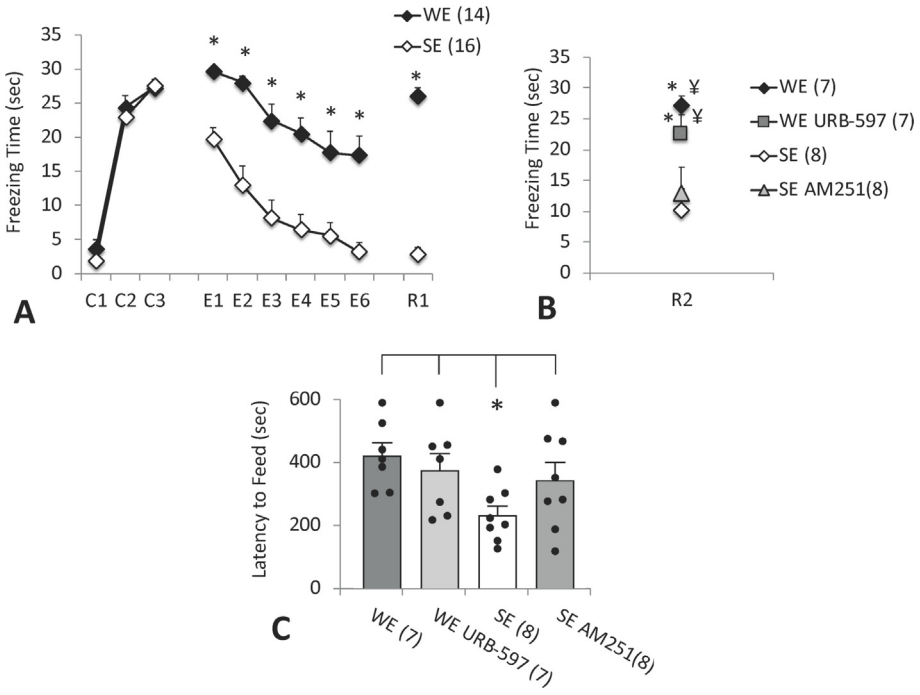

Fig. 1. Acute injections of URB597 (0.3 mg/kg) to weak extinction rats (WE) and AM251 (3 mg/kg) to strong extinction rats (SE). (A) Freezing behaviour expressed across conditioning (C1–C3, day 1), extinction (E1-E6, day 2), and short-term recall (R1, day 3) sessions. WE animals displayed more freezing than SE animals during the entire extinction trial block (E1-E6) and short-term recall. (B) During long-term recall, WE groups displayed significantly more freezing than SE groups with no significant differences being recorded between treatment and control groups (WE vs. WE-URB597 and SE vs. SE-AM251). (C) A significant group effect was also observed in the novelty supressed feeding, with vehicle-treated SE controls displaying a lower latency to feed compared to all other treatment groups. In comparison to vehicle-treated SE rats, SE-AM251 rats had a higher latency to feed, suggesting a significant treatment effect. Values are means with error bars as ± SEM. Numbers in parentheses represent animals per group. $p < 0.05$. In B * represents significant values between WE/WE URB597 and SE rats. ¥ represents significant differences between WE/WE URB597 and SE AM251 rats.

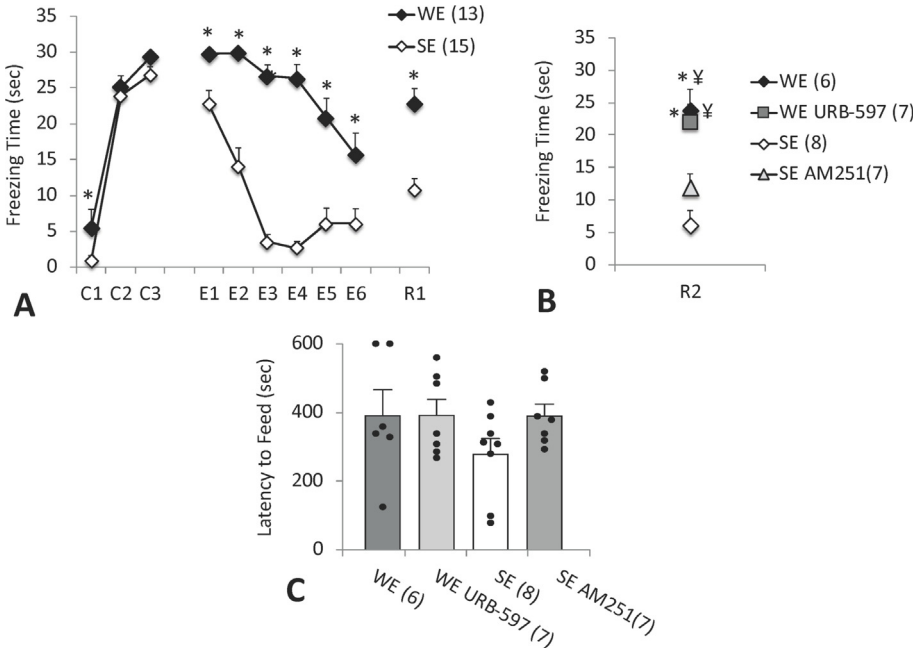

Fig. 2. Acute injections of URB597 (0.6 mg/kg) to weak extinction rats (WE) and AM251 (6 mg/kg) to strong extinction rats (SE). (A) Freezing behaviour expressed across conditioning (C1–C3, day 1), extinction (E1-E6, day 2), and short-term recall (R1, day 3) sessions. WE animals displayed more freezing than SE animals during the entire extinction trial block (E1-E6) and short-term recall. (B) During long-term recall, WE groups had significantly more freezing than SE groups with no significant differences being recorded between treatment and control groups (WE vs. WE-URB597 and SE vs. SE-AM251). (C) No significant group effect was observed in the novelty supressed feeding. Values are means with error bars as ± SEM. Numbers in parentheses represent animals per group. $p < 0.05$. In B * represents significant values between WE/WE URB597 and SE rats. ¥ represents significant differences between WE/WE URB597 and SE AM251 rats.

(1,27) = 356.3; $p < 0.001$] and time effect [F(5,135) = 14.92; $p < 0.001$], as well as a group × time interaction [F(5,135) = 9.79; $p < 0.001$] were observed (Fig. 3A). Differences between WE and SE animals were also found during short-term extinction recall ($p < 0.001$) (Fig. 3A).

WIN,55212-2 (n = 6) or vehicle (n = 7) were injected to WE prior to extinction recall and NSF. SE rats (n = 16) were only treated with vehicle. One-way ANOVA revealed significant differences across groups

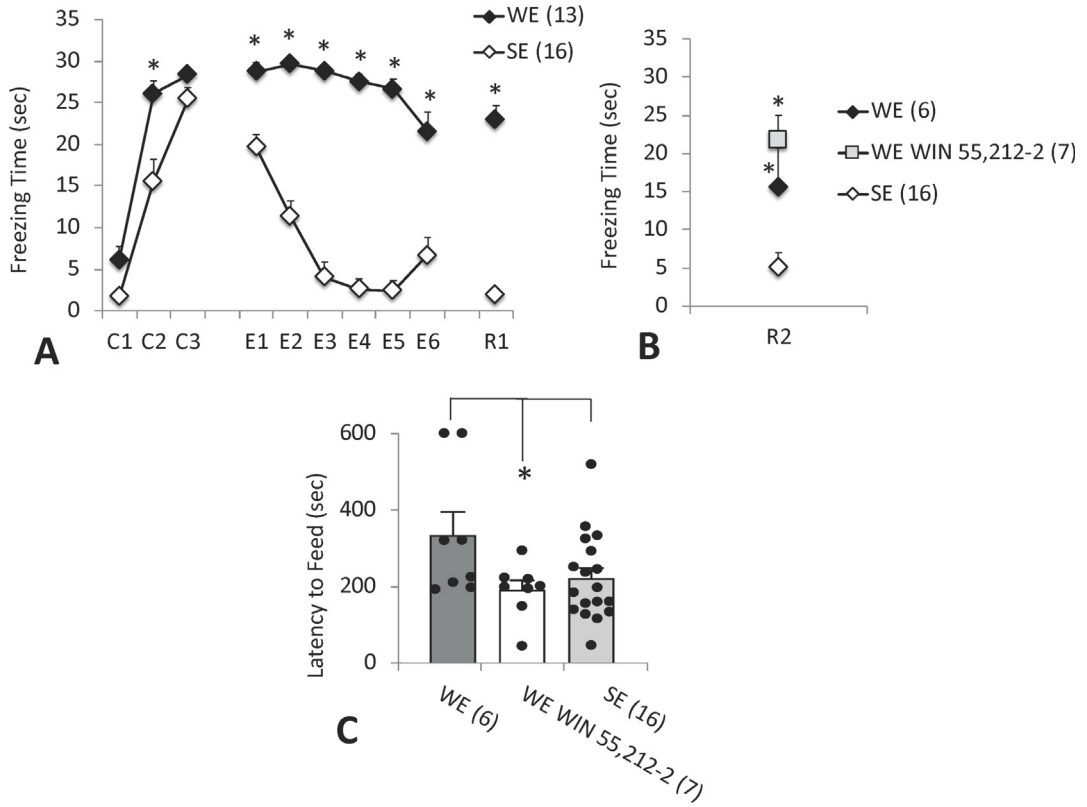

Fig. 3. Acute injections of WIN,55212-2 (2 mg/kg) to weak extinction rats (WE). (A) Freezing behaviour expressed across conditioning (C1–C3, day 1), extinction (E1-E6, day 2), and short-term recall (R1, day 3) sessions. WE animals displayed more freezing than SE animals during the entire extinction trial block (E1-E6) and short-term recall. (B) During long-term recall, WE groups displayed significantly more freezing than SE groups with no significant differences being recorded between treatment and control groups (WE vs. WE- WIN,55212-2). (C) A significant group effect was also observed in the novelty supressed feeding. Latency to feed in WE was significantly higher than in either SE and WE WIN,55212-2 animals. Values are means with error bars as ± SEM. Numbers in parentheses represent animals per group. $p < 0.05$. In B * represents significant values between WE/WE URB597 and SE rats. ¥ represents significant differences between WE/WE URB597 and SE AM251 rats.

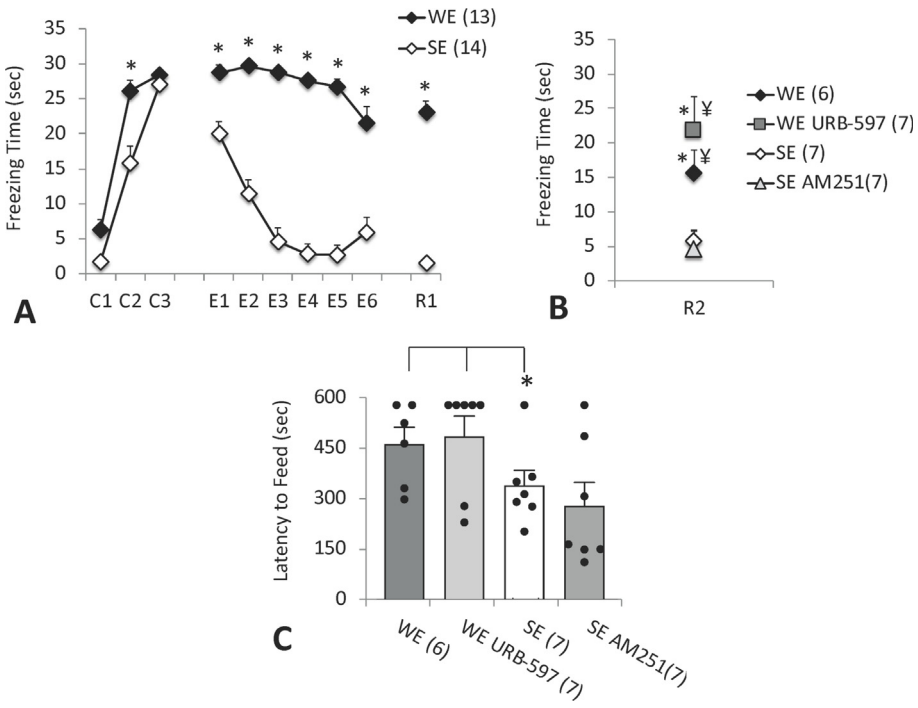

Fig. 4. Chronic injections of URB597 (0.3 mg/kg) to weak extinction rats (WE) and AM251 (3 mg/kg) to strong extinction rats (SE). (A) Freezing behaviour expressed across conditioning (C1–C3, day 1), extinction (E1-E6, day 2), and short-term recall (R1, day 3) sessions. WE animals displayed more freezing than SE animals during the entire extinction trial block (E1-E6) and short-term recall. (B) During long-term recall, WE groups displayed significantly more freezing than SE groups with no significant differences being recorded between treatment and control groups (WE vs. WE-URB597 and SE vs. SE-AM251). (C) A significant group effect was also observed in the novelty supressed feeding, with vehicle-treated SE controls displaying a lower latency to feed compared to all other treatment groups. No differences were found in animals receiving drugs and their respective controls. Values are means with error bars as ± SEM. Numbers in parentheses represent animals per group. $p < 0.05$. In B * represents significant values between WE/WE URB597 and SE rats. ¥ represents significant differences between WE/WE URB597 and SE AM251 rats.

[F(2,26) = 12.41; p = 0.0002], largely due to differences between SE and WE animals (Fig. 3B). No differences were found between WE rats given WIN,55212-2 or vehicle. As for the novelty suppressed feeding, we recorded significant group differences [F(2,31) = 3.23.; p = 0.05], including a reduction in the latency to feed in rats given WIN,55212-2 compared to WE controls (p = 0.03; Fig. 3C).

3.3. Chronic URB597 and AM251

We next tested whether the chronic administration of URB597 or AM251 could alter fear and anxiety responses. During conditioning, we found a significant time effect [F(2,50) = 205.2; p < 0.001], group effect [F(1,25) = 11.90; p = 0.002] and time × group interaction [F (2,50) = 7.31; p = 0.002] (WE n = 13; SE n = 14) (Fig. 4A). Once again, differences between groups were observed at C2, with similar freezing scores being recorded in the end of the trial (C3). During extinction, significant group [F(1,25) = 309.1; p < 0.001], time [F (5,125) = 15.52; p < 0.001] and group × time interaction [F (5,125) = 9.34; p < 0.001] were recorded (Fig. 4A). Also significant were short-term recall differences between WE and SE animals (p < 0.001) (Fig. 4A). After short-term recall, WE and SE rats were given daily i.p. injections of URB597 0.3 mg/kg i.p. (n = 7) or AM251 3 mg/kg i.p. (n = 7) for 14 days while controls were injected with vehicle (WE n = 6; SE n = 7). On the following day, animals underwent long-term recall, followed two days later by novelty suppressed feeding. No drug was administered on the days of testing. Significant results during long-term recall [F(3,23) = 7.07; p = 0.002] were largely due to differences between SE and WE groups (Fig. 4B). Similar freezing time was observed when WE vs. WE URB597 or SE vs. SE AM251 were compared. In the novelty suppressed feeding, significant results [F(3,23) = 2.97; p = 0.05] were also due to WE vs. SE comparisons with no significant differences being recorded between drug and vehicle treatment groups (Fig. 4C).

4. Discussion

The main findings of this study are that the modulation of the eCB system significantly altered anxiety-like behaviour but had no detectable effect on conditioned fear in WE animals. We found that the CB_1 agonist WIN55,212-2 but not the FAAH inhibitor URB597 decreased latency to feed during NSF testing, while CB_1 antagonism with AM251 had an anxiogenic effect on SE rats. In contrast, neither WIN55,212-2 nor URB597 administered 1 h before long-term extinction recall attenuated fear in WE rats. Together these results suggest that systemic manipulations of the eCB system, as conducted in our study, may alter anxiety-like behaviour but not the behavioural expression of an extinction-resistant CS-US associative fear memory.

Our findings are in contrast to other studies in the field. Intracerebral (e.g. HPC, basolateral amygdala) and the systemic administration of CB_1/CB_2 agonists have been reported to decrease inhibitory avoidance and improve extinction in rats exposed to a single foot shock and situational reminders of the traumatic context (Fidelman et al., 2018; Segev et al., 2018; Shoshan and Akirav, 2017; Shoshan et al., 2017). In those studies, rats were treated with eCB agonists after a version of contextual fear conditioning prior to situational reminders (which increase fear) or extinction, rather than after unsuccessful extinction learning, as in our study. It is possible that CB_1/CB_2 agonists or FAAH inhibitors might be effective in preventing the WE phenotype if administered after conditioning/before extinction. However, disrupting memory consolidation is unlike to ameliorate exaggerated behavioural responses due to an existing traumatic memory, which was the primary goal of our study. At present, more work is required to determine precisely how the neurobiology of contextual fear memory (e.g. (Segev et al., 2018)) and cued fear memory (present study) differ with regard to extinction and behavioural models of PTSD. One plausible explanation between the contrasting findings of our study and others is that our data may have been driven by the selection of animals with important extinction deficits.

From a circuitry perspective, functional networks with substantial CB_1 expression (e.g., mPFC, HPC, and AMY) (Piomelli, 2003) make important contributions to fear memory, extinction, and anxiety-like behaviours (Maren et al., 2013; Tovote et al., 2015). This is particularly relevant to our findings, as it suggests an unequal involvement of the eCB system across microcircuits mediating fear behaviours, rather than a general role of eCBs in the modulation of fear. Moreover, due to the ubiquity of CB_1 receptors in the central nervous system, their complex neurophysiological role and the secondary effects on neuroendocrine and neurotransmitter function (Gaetani et al., 2009; Hill et al., 2010,

2018; Ramikie and Patel, 2012), it is likely that the systemic modulation of eCB activity may simultaneously affect circuits involved in different aspects of affective processing in less predictable ways than stereotactic infusions into specific structures. Forebrain CB_1 receptors are primarily found on GABAergic axon terminals (Freund et al., 2003), but eCBs also exert inhibitory effects on glutamatergic neurotransmission that are GABA-independent (Hajos et al., 2001). Additionally, it is known that different stages of fear learning, fear memory expression, and extinction involve overlapping, yet distinct microcircuits within the limbic system (Gouveia et al., 2019; Ramanathan et al., 2018; Tovote et al., 2015). If CB_1 agonists infused into the BLA following fear conditioning interrupt consolidation of a CS-US association, it is unclear why the same infusion would facilitate rather than inhibit extinction, which involves the formation a CS–US^- association in BLA (Sotres-Bayon et al., 2004). Indeed, multiple lines of evidence suggest that suppression of neuronal activity in the BLA impairs the acquisition and consolidation of extinction learning (for a recent review see (Lingawi et al., 2019)). Therefore, it might be unreasonable to expect uniform effects of eCB antagonists across the many structures involved in various fear/anxiety-like behaviours, especially the distinct amygdala microcircuits involved in different stages of fear learning/memory and extinction. In addition to brain sites, the dose of injection seems to be fairly important for the effects and mechanisms of eCB activity. At low and high doses, CB_1-mediated anxiolytic and anxiogenic-type responses in transgenic mice seem to be respectively mediated by glutamate and GABA signaling (Rey et al., 2012).

One mechanism underlying the post-stressor amelioration of anxiety-like behaviours by CB_1 agonists may involve the normalization of glutamate and GABA signaling from BLA to central amygdala (CeA) (Lingawi et al., 2019). For example, chronic restraint stress can decrease GABA signaling in BLA, whereas acute stress has the opposite effect in the CeA (Ramikie et al., 2014). In our study, it is possible that additional structures classically involved in mechanisms of anxiety that are not a formal component of the extinction neurocircuitry, such as the bed nucleus of the stria terminalis, might have been affected by eCB neuromodulation (Puente et al., 2010).

In a second set of experiments we tested whether the chronic administration of URB597 inhibitors to WE rats and AM251 to SE animals would improve or impair fear and anxiety-type responses. This was not supported, as chronic treatment with agents that modulate behavioural expression when administered acutely did not alter fear or anxiety-like responses in WE and SE animals. In these experiments, drugs were not injected on the day of behavioural testing to rule out potential confounding effects of acute interventions. We reasoned that the chronic regimen could alter eCB transmission at long-term, which would be apparent through changes in FAAH and CB_1 expression. Once again, no significant differences were noted when WE animals receiving URB597 or SE animals given AM251 were compared to their respective controls.

Our study is not without caveats that need to be discussed. In our experiments we chose to use synthetic drugs that modulate CB_1 receptors and FAAH. These were selected because CB_1 receptors are prominent in the brain and FAAH inhibitors are being tested in clinical trials (Hu and Mackie, 2015; Mallet et al., 2016). It is possible that different results might have been observed should we have selected drugs that act on CB2 receptors, block DGL activity or phytocannabinoids (e.g. cannabidiol or tetrahydrocannabinol; THC).

A second caveat is that we have only tested the effects of chronic FAAH inhibitor injections in WE animals. We opted to use these agents instead of WIN55,212-2 with the reasoning that URB597 would increase AEA, modulate the eCB system, induce anxiolytic effects and reduce freezing. In contrast, by acting on CB_1 receptors WIN55,212-2 would not reflect a state of enhanced cannabinoid levels. Since chronic AM251 injections did not increase anxiety in SE animals, we suspect no effects would have been observed following chronic WIN55,212-2 administration in WE. This, however, needs to be confirmed.

As the main goal of our study was to test the hypothesis that behavioural differences between WE and SE animals were mediated in part by the eCB system, we did not include naïve animals. Though this would not have helped to address our question, it would have allowed us to study whether fear conditioning/extinction induced changes in FAAH and CB1, as previously described (Fidelman et al., 2018).

Finally, one potential confounder in experiments measuring freezing is the effect of drugs on locomotion. We find this to be unlikely in our study for several reasons. In previous reports we have shown no differences in locomotion between SE and WE rats (Reznikov et al., 2015, 2018). In the current study, freezing was equally low when SE and SE AM251 rats were compared. Moreover, no differences were noted between URB597 and vehicle treated WE rats in the NSF test. This suggests that locomotor activity was not a major factor in our findings when drug and vehicle treated WE and SE animals were compared.

In the clinic, THC has been shown to improve fear extinction recall in healthy individuals (Rabinak et al., 2013). In PTSD, both nabilone and THC have been shown to improve nightmares, sleep quality, and hyperarousal (Cameron et al., 2014; Jetly et al., 2015; Ney et al., 2019; Roitman et al., 2014). FAAH inhibitors are also being clinically investigated but the side effect profile of some of these compounds seems to be substantial (Mallet et al., 2016). Though the translation of findings from animal models to humans has to be considered with a great degree of caution, our data suggest some potentially interesting conclusions. First, eCB may be more effective for treating anxiety symptoms than helping with fear extinction. Second, these agents may be more effective when given acutely, rather than in a chronic fashion. That said, our study has only tested the long-term effects of eCB system modulating agents. A recent study has shown that URB597 and WIN55,212-2 reduced long-term retention of extinction learning when administered before and after extinction sessions in a paradigm in which three spaced extinction sessions were conducted one week after trauma (Morena et al., 2018). It is possible that different regimens of drug administration in the model used in our study might yield different results.

Once again, we note that the above-made inferences were made based on male rodents receiving specific synthetic cannabinoids. Further research needs to be conducted to ascertain whether similar findings occur in females and humans treated with other agents or phytocannabinoids.

CRediT authorship contribution statement

Akshayan Vimalanathan: Conceptualization, Methodology, Validation, Writing - original draft. **Darryl C. Gidyk:** Conceptualization, Methodology, Validation, Writing - original draft. **Mustansir Diwan:** Validation. **Flavia V. Gouveia:** Validation. **Nir Lipsman:** Writing - review & editing. **Peter Giacobbe:** Writing - review & editing. **José N. Nobrega:** Writing - review & editing. **Clement Hamani:** Conceptualization, Methodology, Validation, Formal analysis, Writing - review & editing, Supervision.

Declaration of competing interest

The authors report no conflicts of interest related to this topic.

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