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FAAH inhibitor, URB-597, promotes extinction and CB_1 antagonist, SR141716, inhibits extinction of conditioned aversion produced by naloxone-precipitated morphine withdrawal, but not extinction of conditioned preference produced by morphine in rats

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ABSTRACT

Converging evidence suggests that the endogenous cannabinoid (eCB) system is involved in extinction of learned behaviours. Using operant and classical conditioning procedures, the potential of the fatty acid amide (FAAH) inhibitor, URB-597, and the CB_1 antagonist/inverse agonist, SR141716, to promote and inhibit (respectively) extinction of learned responses previously motivated by either rewarding or aversive stimuli was investigated. In the operant conditioning procedure (Expt. 1), rats previously trained to lever press for sucrose reward were administered URB-597 (0.3 mg/kg) or the CB_1 antagonist/inverse agonist SR141716 (2.5 mg/kg) prior to each of three extinction trials. In the conditioned floor preference procedure (Expts 2a–d), rats trained to associate morphine with one of two distinctive floors were administered one of several doses of the CB_1 antagonist/inverse agonist, AM-251 (Expt 2a) or URB-597 (Expt 2b and 2d) prior to each extinction/test trial wherein a choice of both floors was presented and prior to forced exposure to each floor (Expt 2c). In the conditioned floor aversion procedure (Expt. 3), rats trained to associate a naloxone-precipitated morphine withdrawal with a floor cue were administered URB-597 or SR141716 prior to each of 24 extinction/testing trials. URB-597 did not promote and SR141716 did not reduce extinction rates for sucrose reward-induced operant responding (Expt. 1) or morphine-induced conditioned floor preference (Expts. 2a–d). In contrast, URB-597 facilitated, whereas SR141716 impaired, extinction of the conditioned floor aversion (Expt. 3). These data support previous reports that the eCB system selectively facilitates extinction of aversive memories. URB-597 may prove useful in targeting extinction of aversively motivated behaviours.

1. Introduction

Considerable evidence indicates that the endogenous cannabinoid (eCB) system is involved in extinction learning of aversively motivated learned behaviors (Marsicano et al., 2002; Varvel and Lichtman, 2002). Marsicano et al. (2002) reported that CB_1 knockout mice and wild-type mice administered the CB_1 inverse agonist/antagonist, SR141716 (Rimonabant), showed impaired extinction in classical auditory fear-conditioning tests, with unaffected memory acquisition and consolidation. Using the Morris water maze task, Varvel and Lichtman (2002) reported that CB_1 knockout mice and wild-type mice exhibited identical acquisition rates in learning to swim to a fixed platform; however, the CB_1-deficient mice demonstrated considerable deficits during a reversal task in which the location of the hidden platform was moved to the opposite side of the tank. Since animals deficient in CB_1 receptor activity show impairments in suppressing previously learned behaviors, cannabinoid agonists would be expected to facilitate extinction of learned behaviors in non-deficient animals. Indeed, Pamplona et al. (2006) recently reported that the potent cannabinoid receptor agonist WIN 55,212-2 facilitated extinction of contextual fear memory and spatial memory in rats, whereas an ethyl derivative of SR141716 significantly impaired extinction. These results suggest that the eCB system modulates extinction of aversively motivated learned responses.

With the recent discovery of drugs that enhance endogenous cannabinoids by blocking their reuptake (e.g., AM404) or by inhibiting the enzyme (fatty acid amide hydrolase [FAAH]) that deactivates anandamide (e.g., OL135 and URB-597), the effect of prolonging anandamide's activity during extinction learning has also been investigated (Chhatwal et al., 2005; Varvel et al., 2007). Chhatwal et al. (2005) reported that pretreatment with AM404 selectively facilitated extinction of fear potentiated startle in rats (Chhatwal et al., 2005), an effect that was reversed by SR141716 pretreatment. Varvel et al. (2007) reported that mice deficient in FAAH, either by genetic deletion (FAAH $^{-/-}$) or by pharmacological inhibition with OL135,

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displayed both faster acquisition and extinction of spatial memory tested in the Morris water maze; SR141716 reversed the effect of OL135 during both task phases.

In fact, recent evidence suggests that the eCB system may not regulate extinction of reward-based learning (e.g., Lutz, 2007). Neither CB_1-deficient mice (Hölter et al., 2005) nor wild-type mice treated with SR141716 (Niyuhire et al., 2007) displayed a deficit in extinction of operant responding reinforced with food. In comparison, SR141716-treated mice displayed impaired extinction of conditioned freezing and passive avoidance (Niyuhire et al., 2007). Most recently, SR141716 disrupted extinction learning in an aversive, but not appetitive, Barnes maze conditioning task (Harloe et al., 2008). Therefore, unlike the recent evidence that the partial N-methyl-D-aspartate (NMDA) glutamate receptor agonist, D-cyloserine promotes extinction of both positive and aversive based learning (e.g. Paolone et al., 2009), the eCB system may be more selective for aversive memories. Further research on the role of the eCB system in the extinction of other forms of reward-based learning and aversion learning is warranted.

The present experiments evaluated the potential of FAAH inhibition (by URB-597) and CB_1 inverse agonism/antagonism (by SR141716 [Experiments 1 and 3] and AM-251 [Experiment 2a]) to facilitate and inhibit (respectively) extinction of operant responding for sucrose (Experiment 1), morphine-induced conditioned floor preference (Experiments 2a–d) and naloxone-precipitated morphine withdrawal-induced conditioned floor aversion (Experiment 3). The effect of endocannabinoid manipulation on extinction of a conditioned floor preference (Experiments 2a-d) was evaluated in four sub-experiments. In Experiments 2a and 2b various doses of AM-251 (0, 1, 3 or 8 mg/kg) and URB-597 (0.0, 0.03, 0.1 and 0.3 mg/kg) failed to modify extinction of morphine conditioned floor preferences when administered prior to each extinction/test trial. In Experiment 2c, URB-597 also failed to modify extinction of morphine conditioned floor preferences when administered prior to extinction trials with forced confinement to each of the floors. In each of Experiments 2a–c, the conditioned preference among the vehicle treated rats extinguished rapidly; conditioned preferences are less resistant to extinction than are conditioned aversions (e.g., Parker and McDonald, 2000). Therefore, Experiment 2d, evaluated the potential of 0.3 mg/kg URB-597 to promote extinction of a more robust conditioned floor preference according to a procedure recently described by Paolone et al. (2009). Again, URB-597 failed to promote extinction of a conditioned preference.

Experiment 3 evaluated the potential of URB-597 and SR141716 to modify the rate of extinction of a conditioned floor aversion. Although naloxone alone produces only a weak conditioned place aversion (Mucha and Iverson, 1984; Parker and Rennie, 1992), it produces a profound conditioned place aversion when preceded by an injection of morphine (Parker and Joshi, 1998). In fact, naloxone-precipitated morphine withdrawal-induced conditioned place aversions occur even when naloxone (1 mg/kg) is administered 24–48 h after an injection of morphine (20 mg/kg) in a single conditioning trial (Parker et al., 2002). If the eCB system selectively facilitates extinction of aversively motivated learned responses, then it should more effectively enhance the speed of extinction of the conditioned floor aversion than the conditioned preference or operant responding for sucrose.

2. Materials and Method

2.1. Subjects

The subjects in Experiment 1 and 2a were Sprague–Dawley rats and in Experiments 2b–d, and 3 were male Long–Evans rats. The change in the strain to Long–Evans rats was aimed at producing a robust conditioned preference/aversion (e.g., Paolone et al., 2009). The animals were group-housed in shoebox cages in the colony room at an ambient temperature of 21 °C with a 12/12 light dark schedule (lights off at 8 AM) and were maintained on an *ad libitum* schedule of food (except Experiment 1 in which they were maintained at 85% body weight) and water. All procedures adhered to the guidelines of the Canadian Council of Animal Care and were approved by the Animal Care Committee of University of Guelph.

2.2. Drugs

Morphine (Sigma) was prepared in physiological saline (Sigma) at concentrations of either 10 (Expts 2a–d) or 20 (Expt. 3) mg/ml and administered subcutaneously (s.c.) in a volume of 1 ml/kg at 10 min prior to conditioning. Naloxone (Sigma) was prepared in physiological saline in a concentration of 1 mg/ml and administered (s.c.) in a volume of 1 ml/kg at 10 min prior to conditioning (Expt. 3). URB-597 (Cayman Chemicals) was prepared in 2-hydroxypropyl-β-cyclodextrin (2-HPBCD, 45%; Sigma) at a concentration of 0.3 mg/kg (Expts. 1, 2d and 3) administrated at a volume of 1 ml/kg intraperitoneally (i.p.) at 2 h prior to extinction testing. Previous work has shown that 0.3 mg/kg URB-597 produces neither a place preference nor a place aversion (Gobbi et al., 2005), although it augments the hypothermic effects of exogenously administered anandamide (Fegley et al., 2005), produces anxiolytic effects (Kathuria et al., 2003; Scherma et al., 2007), antidepressant effects (Gobbi et al., 2005), and anti-nausea effects (Cross-Mellor et al., 2007; Rock et al., 2008). URB-597 has been shown to produce a slow and reliable accumulation of anandamide in the brain with a maximal effect at 2 h post-injection (Fegley et al., 2005; Gobbi et al., 2005; Kathuria et al., 2003). Experiments 2b–c employed various doses of URB-597 as indicated. SR141716 (Sequoia Research, UK) was prepared in 45% 2-HPBCD at a concentration of 1 mg/ml and administered (i.p.) in a volume of 2.5 ml/kg at 30 min prior to extinction testing (Expts. 1 and 3). AM-251 (Cayman Chemicals; Expt 2a) was prepared in a vehicle of 1 ml ethanol/1 ml Cremaphor (Sigma)/18 ml saline at concentrations of 1, 3 and 8 mg/ml and administered at a volume of 1 ml/kg.

2.3. Apparatus

In Experiment 1, 16 Plexiglas operant chambers (model ENV-008CT, Med Associates, Lafayette, IN) were used, each enclosed in larger sound-attenuating plywood chambers model ENV-018M, Med Associates). Each operant box had a retractable lever located 8 cm above the floor of the box and 5 cm from the magazine feeder. Two lights (28 V) were on during testing, one located on the same wall as the lever, the other on the opposing wall. Each chamber was also equipped with a hopper mounted on the exterior of the operant chamber that would deliver sucrose pellets (45 mg dustless Precision Pellets; Bio-Serv, Frenchtown, NJ) into the magazine feeder.

In Experiments 2 and 3, the conditioning apparatus used consisted of a black Plexiglas rectangular box ($60\times25\times25$ cm^3) with a wire-mesh lid (as previously described in Parker et al., 2004). During conditioning trials, the tactile cues on both sides of the box were identical. During pretest and choice tests, one side of the chamber had a metal hole floor and the other side a metal bar floor (counterbalanced), and the intersection of the two floors was defined as a neutral zone (9×25 cm). The amount of time (sec) each rat spent on each of the floors was recorded and subsequently analyzed by the Noldus Etho-Vision videotaping system (Noldus Information Technology, Sterling, VA). Pretests did not indicate a significant difference between seconds spent on the metal hole or bar floors indicating that the apparatus provides an unbiased test of conditioned preference and aversion.

2.4. Procedure

2.4.1. Experiment 1: Effect of URB-597 and SR141716 on extinction of operant responding motivated by sucrose

The rats had been extensively trained to lever press for sucrose as part of a previous experiment in which some animals received cocaine. The previous drug groups were counterbalanced across treatment groups; an analysis showed that there was no effect of the counterbalancing variable on the behavior reported here. A stable baseline of reinforced responding was established over 5 days of testing on a fixed ratio 25 (1 pellet for every 25 responses) schedule of reinforcement. Each day, rats received one 1 h long session consisting of 5 min acclimatization to the chamber without the lights or levers present, followed by 1 h of testing starting with house and key lights turning on and insertion of the lever.

Beginning the following day, rats (matched by baseline responding) were administered (i.p.) URB-597 ($n=14$; 0.3 mg/kg) at 2 h or Vehicle ($n=13$) at 30 min or SR141716 ($n=13$; 2.5 mg/kg) at 30 min, prior to each extinction trial. Extinction conditions were identical to baseline but no sucrose was delivered. Each animal received 3 extinction sessions over 3 days with each session 1 h long.

2.4.2. Experiment 2: Effect of AM-251 or URB-597 on extinction of morphine-induced conditioned preference

2.4.2.1. Experiment 2a. All rats were administered a 10 min pretest and the amount of time spent on each floor was measured. They were assigned to groups matched on the basis of their pretest score. The rats received 4 cycles of conditioning trials. During conditioning cycles all rats were injected with morphine or saline 10 min prior to being placed into the box with a distinctive floor for 30 min. Thus, each conditioning cycle consisted of one morphine trial and one saline trial separated by 24 h. Each of the cycles of conditioning was separated by 48–72 h. The order of the morphine trial within a cycle and the floor paired with morphine were counterbalanced. Forty-eight h after the fourth conditioning cycle, the rats were given a 15-min test. The rats were given two additional conditioning trial cycles following the test. Forty-eight h after the sixth conditioning cycle, the rats were given repeated 15-min extinction choice test trials, each separated by 24 h. Thirty min prior to each extinction trial, the rats ($n=10$/group) were injected (ip) with vehicle (1 ml/kg), or 1, 3 or 8 mg/kg AM-251. The trials continued until the conditioned preference had extinguished; that is there was no significant difference in preference for the morphine-paired floor and the saline-paired floor.

2.4.2.2. Experiment 2b. The rats were treated as in Experiment 2a during conditioning except that they received 4 conditioning trial cycles. The 10-min extinction/test trials began 72 h after the final conditioning cycle. Two hours prior to each extinction/test trial, the rats were injected (1 ml/kg, ip) with vehicle ($n=18$), 0.03 mg/kg URB-597 ($n=18$), 0.1 mg/kg URB-597 ($n=15$), 0.3 mg/kg URB-597 ($n=18$). Every 48 h, the rats continued to receive this treatment until the conditioned preference had extinguished.

2.4.2.3. Experiment 2c. The conditioning procedure for Experiment 2c was identical to that of 2b, except the rats received 6 conditioning trial cycles. Unlike Experiments 2a and 2b, rats were given forced exposure to the floor cues during extinction training, rather than extinction by testing. Forty-eight h following the final conditioning trial, the rats received the extinction manipulation in one of 4 conditions: No Extinction ($n=10$), Vehicle ($n=9$), 0.1 mg/kg URB-597 ($n=10$) or 0.3 mg/kg URB-597 ($n=10$). Rats in the No Extinction group remained in their home cage during the extinction manipulation. Rats in the remaining groups were injected with the appropriate solution 2 h prior to the extinction manipulation on each trial. The extinction manipulation included a cycle of two trials, during which rats were treated exactly as they were during the conditioning trials, but received saline injections on both trials. In a counterbalanced order, on one trial the rats were placed on the morphine-paired floor for 30 min and on the other trial they were placed on the saline-paired floor for 30 min.

Forty-eight h after each cycle of extinction training, the rats were given a 20 min drug-free preference test trial; they were injected with saline and placed in the test chamber and the preference for the morphine or the saline floor was assessed. This procedure of extinction/testing continued until the groups no long demonstrated a conditioned preference.

2.4.2.4. Experiment 2d. In Experiment 2d the procedures of conditioning and testing were modified to ensure a robust conditioned preference that was resistant to extinction by using some procedures described by Paolone et al. (2009). The rats received 8 cycles of conditioning trials in a similar manner as Experiments 2a–c, however, only those rats which developed a preference continued to extinction testing. To test for the initial strength of the conditioned preference, all rats received a 10 min drug-free choice test 24 h after the last conditioning trial. The rats were considered not to have developed a conditioned preference if the difference in time spent on the morphine-paired floor and the saline-paired floor was less than 45 s and were then removed from the experiment (e.g., Paolone et al., 2009). The extinction trials began 96 h after this test. On each of 10 extinction trials separated by 72–96 h to attenuate the development of extinction (e.g., Mueller et al., 2002; Paolone et al., 2009), the rats were injected (ip.) with 0.3 mg/kg URB-597 ($n=8$) or Vehicle ($n=9$) 2 h prior to a 10 min test trial. One week after the final extinction test trial, the rats received a test trial following an injection of saline and on the following day a test trial following an injection of morphine (2.5 mg/kg) in a reinstatement test. This procedure evaluated the effect of prior treatment with URB-597 during extinction training on subsequent morphine-induced reinstatement of the floor preference.

2.4.3. Experiment 3: Effect of URB-597 and SR141716 on naloxone-precipitated morphine withdrawal-induced conditioned aversion

The rats were assigned to groups matched on the basis of a 20 min pretest score. They were given two conditioning trial cycles (separated by 72 h), each consisting of a 3 day schedule separated by 24 h (as previously described by Parker and Joshi, 1998). For each conditioning cycle, on Day 1 the rats were injected with saline (s.c.) at 10 min prior to placement in the chamber with a distinctive floor for 30 min. On Day 2, they were given an injection (s.c.) of 20 mg/kg morphine in their home cages. On Day 3, they were given an injection (s.c.) of naloxone (1 mg/kg) at 10 min prior to placement in the chamber with the opposite distinctive floor (as on Day 1) for 30 min. Naloxone-alone controls were not included in the present experiment because previous work (e.g., McDonald et al., 1997; Parker and Joshi, 1998; Parker et al., 2002) demonstrated that the conditioned aversion produced by naloxone alone is greatly strengthened by pretreatment with morphine 24–48 h prior to the conditioning trial. For all extinction testing trials, which began 72 h after the final conditioning cycle, rats were administered (i.p.) 0.3 mg/kg URB-597 ($n=8$) at 2 h, or vehicle ($n=8$) at 30 min or 2.5 mg/kg SR141716 ($n=8$) at 30 min, prior to a test trial which lasted for 20 min.

A 20 min drug-free conditioned aversion test was administered 72 h following the final extinction trial. One week later, the potential of naloxone-precipitated morphine withdrawal to reinstate the extinguished floor aversion was evaluated. On Day 1, rats were injected (s.c.) with 1 ml/kg saline 10 min prior to a 20 min choice test. On Day 2, they were injected (s.c.) with 20 mg/kg morphine in their home cage. On Day 3, the rats were injected (s.c.) with 1 mg/kg naloxone 10 min prior to a choice test to determine the effect of prior extinction treatments on reinstatement of the conditioned aversion.

2.5. Data Analysis

In Experiment 1, the number of responses during each 10 min interval on each of 3 extinction days were entered into a mixed factors ANOVA with the extinction treatment as a between groups factor and 10-min interval and extinction day as a within group factor. In Experiment 2 the mean seconds spent on the drug-paired floor and on the saline-paired floor during extinction by repeated test trials (Expts 2a,b, d) or during each preference test that followed forced extinction (Expt 2c) were entered into a mixed factors ANOVA with extinction treatment as the between groups factor and the floor and trial (Expts 2a, b and d) as within groups factors. Because conditioned aversions are more resistant to extinction than conditioned preferences (e.g., Parker and McDonald, 2000), in Experiment 3 the data for the 24 extinction trials was analyzed as blocks of 4 trials in 2×6 (floors×blocks of trials) repeated measures ANOVAs for each extinction condition. The data for the reinstatement trials were analyzed as mixed factors ANOVAs for each trial. Bonferroni post hoc comparison tests were conducted as appropriate. In each experiment, the time spent in the intersecting center zone was also evaluated revealing no differences among groups in Experiments 2 and 3. Statistical significance was defined as $p<0.05$.

3. Results

3.1. Experiment 1: Effect of URB-597 and SR141716 on extinction of operant responding for sucrose reward

URB-597 pretreatment during extinction training did not modify the rate of extinction of operant responding for sucrose reward. In comparison, SR141716 pretreatment suppressed operant responding during extinction. Fig. 1 presents the mean number of responses across six 10-min intervals on each of three test trials (separated by 48 h). The mixed factors ANOVA revealed significant effects of drug, $F(2, 37)=7.8$; $p<0.001$, day, $F(2, 74)=103.7$; $p<0.001$, interval, $F(5, 185)=176.3$; $p<0.001$, drug by day $F(4, 74)=3.9$; $p<0.01$; drug by interval, $F(10, 185)=3.45$; $p<0.01$; day by interval, $F(10, 370)=34.6$; $p<0.001$ and a drug by day by interval interaction, $F(20, 370)=2.0$; $p<0.01$). The triple interaction was analyzed as separate drug by interval mixed factors ANOVAs for each Day. On Day1 there was a significant effect of drug, $F(2, 37)=6.9$; $p<0.01$, interval, $F(10, 185)=3.8$; $p<0.001$ and a drug by interval interaction, $F(5, 185)=133.9$; $p<0.001$. Overall, SR141716 suppressed operant responding relative to URB-597 and Vehicle (p's<0.05) which was most pronounced during the first 10 min of responding (p's<0.05).

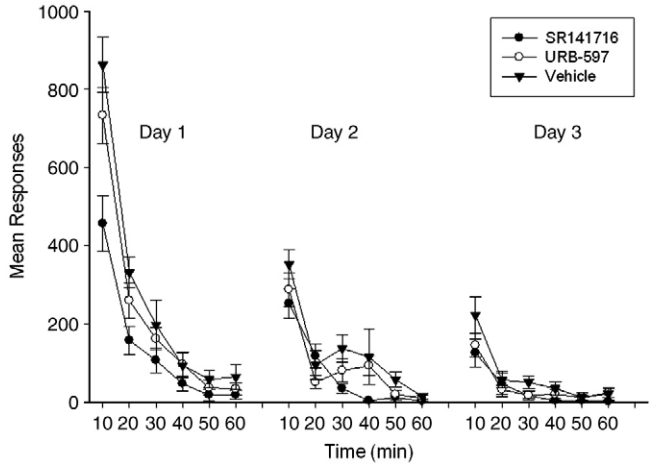

Fig. 1. Experiment 1. Mean (+sem) number of lever-pressing responses for sucrose pellet during extinction for rats pretreated with Vehicle ($n=13$), 0.3 mg/kg URB-597 ($n=14$) or 2.5 mg/kg SR141716 ($n=13$).

On Days 2 and 3 there was a significant effect of drug (p's<0.025) with SR141716 showing greater suppression overall than vehicle ($p<0.05$) and Intervals (p's<0.001) with responding decreasing across the intervals. The operant responding extinguished within the session and between sessions, but the speed was not affected by pretreatment with URB-597.

3.2. Experiment 2: Effect of AM-251 and URB-597 on extinction of morphine-induced conditioned preference

3.2.1. Experiment 2a

AM-251 did not modify the progression of extinction of a morphine-induced conditioned preference in Experiment 1. Fig. 2 presents the mean (+sem) seconds that the rats spent on the morphine-paired floor and on the saline-paired floor following the pretreatment with the vehicle or one of several doses of AM-251 during each extinction/test trial. The mixed factors ANOVA revealed only a significant effect of trial, $F(2, 72)=3.5$; $p<.05$ and a significant trial by floor interaction, $F(2, 72)=4.2$; $p<.025$, but no effects of pretreatment drug. Across all pretreatment drug conditions, the rats displayed a significant preference for the morphine-paired floor only on the first ($p<.01$) and the second ($p<.05$) extinction trial, but not on the third. Additionally, on the drug-free test after 4 conditioning trials, the rats spent significantly ($p<.001$) more time on the morphine-paired floor (Mean = 498 s) than on the saline-paired floor (Mean = 330 s).

3.2.2. Experiment 2b

The FAAH inhibitor, URB-597, did not promote the extinction of a morphine-induced conditioned preference. Fig. 3 presents the mean (+sem) seconds that the rats spent on the morphine- and the saline-paired floors during each extinction/test trial following 4 cycles of conditioning trials. The mixed factors ANOVA revealed significant

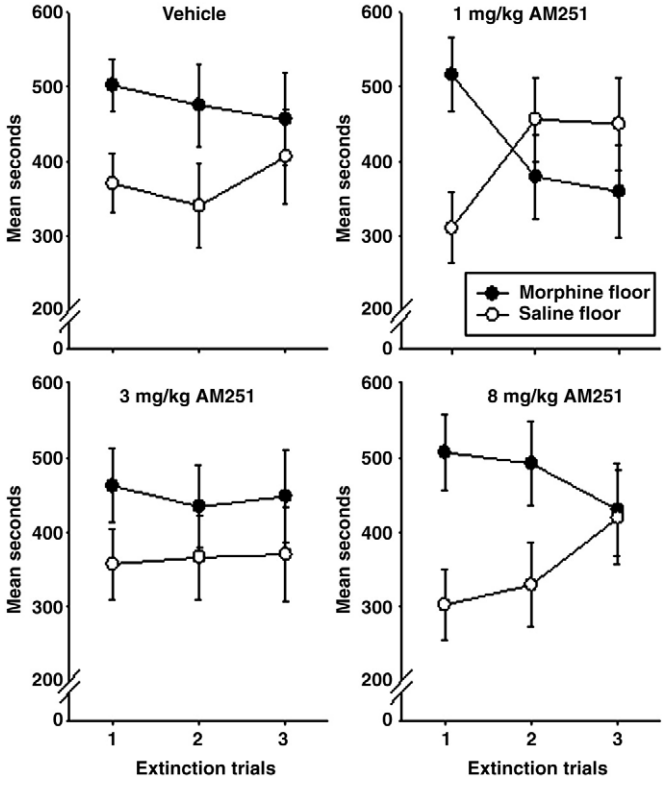

Fig. 2. Experiment 2a: Mean (+sem) seconds spent on the morphine-paired floor and on the saline-paired floor following 6 conditioning trials during each the 15-min extinction/test trial during which rats were administered 30 min following pretreatment with vehicle ($n=10$), 1 mg/kg AM-251 ($n=10$), 3 mg/kg AM-251 ($n=10$), or 8 mg/kg AM-251 ($n=10$).

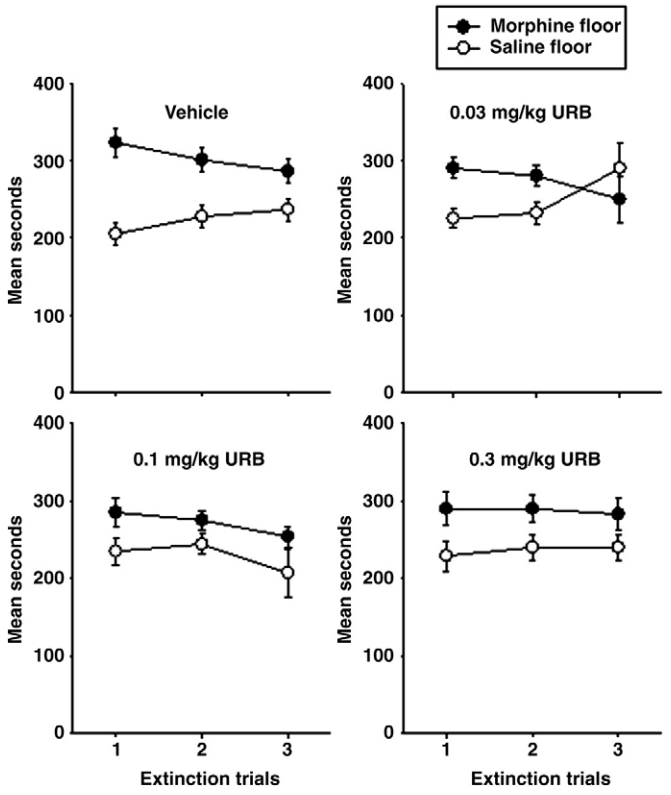

Fig. 3. Experiment 2b: Mean (+sem) seconds spent on the morphine-paired floor and on the saline-paired floor during each extinction/test trial following 4 cycles of conditioning. Two hours prior to each 10-min extinction/test trial, rats were administered Vehicle ($n=18$), 0.03 mg/kg URB-597 ($n=18$), 0.10 URB-597 ($n=15$) or 0.30 URB-597 ($n=18$).

effects of conditioning floor, $F(1, 130)=13.3$; $p<.001$, and an extinction drug by conditioning floor by trial interaction, $F(6, 130)=2.3$; $p<.05$. To evaluate the 3 way interaction, the data for each extinction trial was entered into a 4 by 2 repeated measures ANOVA. These analyses revealed a significant effect of conditioning floor on extinction trial 1, $F(1, 65)=15.9$; $p<.001$, and trial 2, $F(1,65)=11.3$; $p<.001$, but not on trial 3. The extinction drug by floor effect was not significant on any trial, largest $F(3, 65)=1.9$. Since the conditioned preference extinguished after only two test trials, it is conceivable that the rats displayed within session extinction across the 10 min trials. To assess changes in the preference among the groups within the session, the mean number of seconds spent in the drug-paired floor and the saline-paired floor during the first 5 min and the second 5 min of the test were included as an additional factor in the 4 (drug) by 2 (floor) mixed factor ANOVA for each extinction/test session. These additional analyses revealed the same pattern as the overall analyses for each trial; that is only the drug floor effect was significant and the strength of the floor preference did not change across test time or pretreatment group.

3.2.3. Experiment 2c

URB-597 also did not promote extinction of a morphine-induced conditioned preference when administered during confinement on the morphine-paired and saline-paired floor. As is depicted in Fig. 4, there was only a significant effect of floor following one cycle of extinction training, $F(1, 35)=10.4$; $p<0.01$, and following two cycles of extinction training, $F(1, 35)=5.2$; $p<0.05$, but not following three cycles of extinction training. There were no significant interactions on any test trial.

3.2.4. Experiment 2d

A more robust conditioned preference was produced in Experiment 2d than Experiments 2a–c, but URB 597 did not promote extinction of a morphine-induced conditioned preference across 10

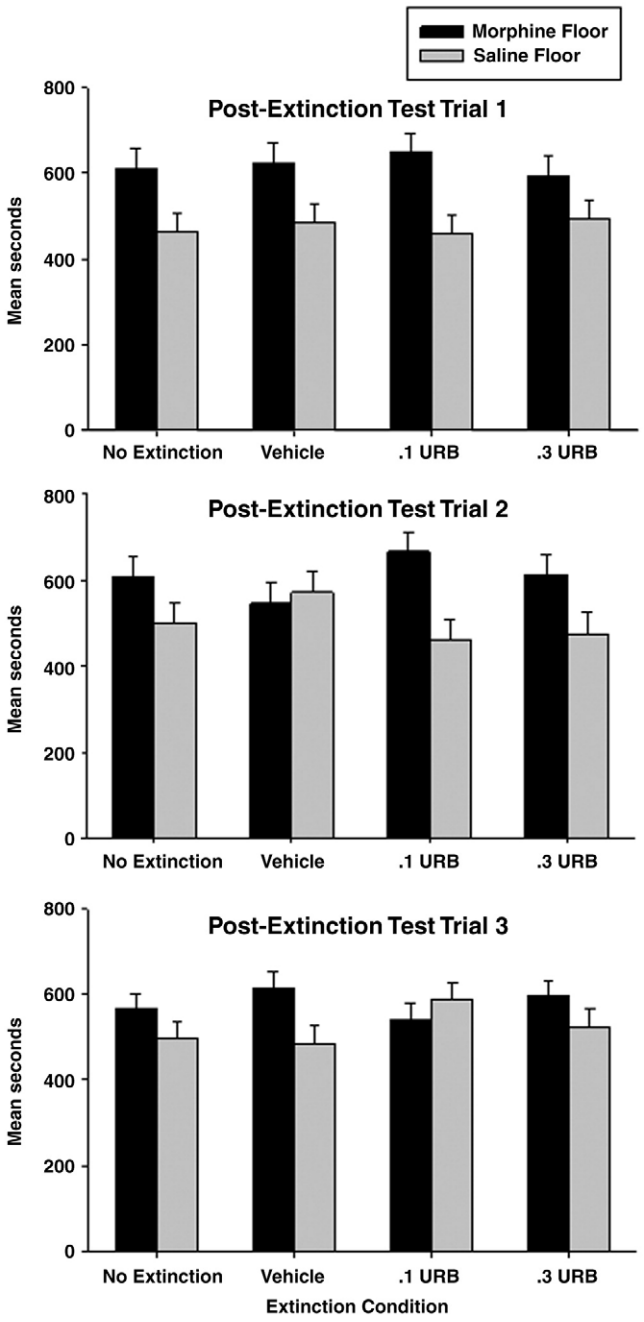

Fig. 4. Experiment 2c: Mean (+sem) seconds spent on the morphine-paired floor and on the saline-paired floor during each 20-min test trial that followed an extinction cycle during which rats were confined on the morphine-paired floor on one day and on the saline-paired floor on the other day. Two hours prior to each extinction day, rats were injected with Vehicle ($n=9$), 0.1 mg/kg URB-597 ($n=10$) or 0.3 mg/kg URB-597 ($n=10$). An additional group of rats (No Extinction; $n=10$) remained in their home cage during the extinction sessions.

extinction trials. Following 8 conditioning trial cycles, rats displayed an overall preference for the morphine-paired floor, $t(17)=8.6$; $p<0.001$ in the initial preference test. Fig. 5 displays the mean (+sem) seconds spent on the morphine-paired floor and the saline-paired floor for the rats pretreated with Vehicle or URB-597 at 2 h prior to each extinction trial. The mixed factor analysis revealed only a significant effect of conditioning floor, $F(1, 135)=9.4$; $p<0.01$, and a significant conditioning floor by trial interaction, $F(9, 135)=2.0$; $p<0.05$, but no effect of extinction treatment. Rats displayed significantly ($p<0.05$) greater preference for the morphine-paired

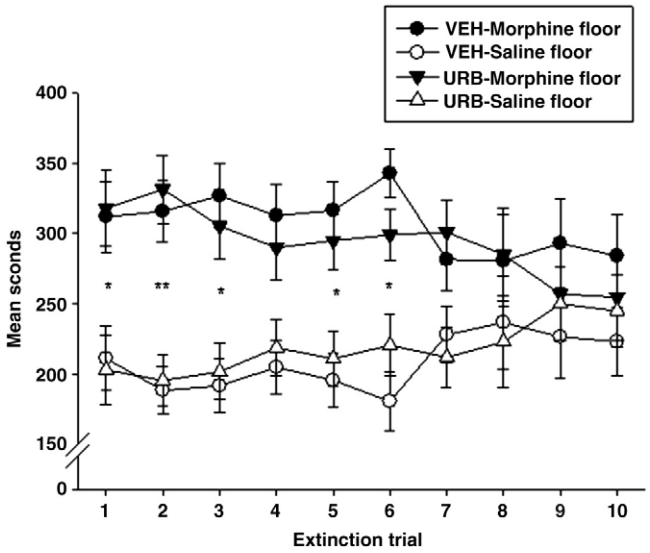

Fig. 5. Experiment 2d. Mean (+sem) seconds spent on the morphine-paired floor and on the saline-paired floor during each of 10 extinction test trials in Experiment 2d. Groups were treated with vehicle ($n=9$) or URB-597 ($n=8$) 2 hours prior to each extinction test trial. Asterisks indicate that rats spent more time on the morphine-paired floor than the saline-paired floor irrespective of pretreatment drug ($*=p<0.05$; $**=p<0.01$).

floor than the saline-paired floor on extinction trials 1–3 and 5–6, but not on trial 4 ($p<0.10$) or trials 7–10 (p's>0.10).

A morphine prime reinstated the extinguished morphine-induced conditioned preference, but prior treatment with URB-597 during extinction did not affect the strength of reinstatement, as can be seen in Fig. 6. The mixed factors ANOVA revealed only significant effects of conditioning floor, $F(1, 15)=16.4$; $p<0.001$, and a conditioning floor by trial interaction, $F(1, 15)=12.9$; $p<0.01$. On the morphine reinstatement test trial, but not on the saline reinstatement test trial, rats spent more time on the morphine-paired floor than on the saline-paired floor ($p<0.01$); however, prior treatment with URB-597 during extinction did not influence the strength of reinstatement.

3.3. Experiment 3: Effect of URB-597 and SR141716 on extinction of a naloxone-precipitated morphine withdrawal-induced conditioned aversion

Fig. 7 presents the extinction data depicted as 6 blocks of 4 trials each. Compared to the vehicle, it is clear that URB-597 facilitated extinction and SR141716 impaired extinction. There was a significant floor by block of trials interaction for each extinction group: Vehicle ($F(1, 24)=3.55$; $p<0.001$), URB-597 ($F(1, 24)=1.71$; $p=0.028$), and SR141716 ($F(1, 24)=2.75$; $p<0.001$). During Block 1 (extinction tests 1–4), all three groups showed a significant aversion to the withdrawal-paired floor in comparison to the saline-paired floor (all p's<0.01). During Blocks 2–4 (extinction tests 5–16) only the vehicle and SR141716 groups retained the aversion (p's<0.05). During Block 5 (extinction tests 17–20), only the SR141716 group retained the aversion ($p<0.01$). There was no significant floor effect for any group during Block 6 (extinction tests 21–24).

In the post-extinction, drug-free test 72 h after the last extinction trial, the mixed factor ANOVA revealed no significant effects, indicating that extinction was maintained in the absence of the treatment drugs. Previous treatment with either URB-597 or SR141716 during extinction training did not modify the strength of subsequent reinstatement of the naloxone-precipitated morphine withdrawal-induced conditioned aversion following extinction training, as can be seen in Fig. 8. Although the rats maintained extinction of the conditioned floor aversion following a saline prime, $F(1, 24)=0.5$, they did display a reactivated aversion following the naloxone-precipitated morphine withdrawal

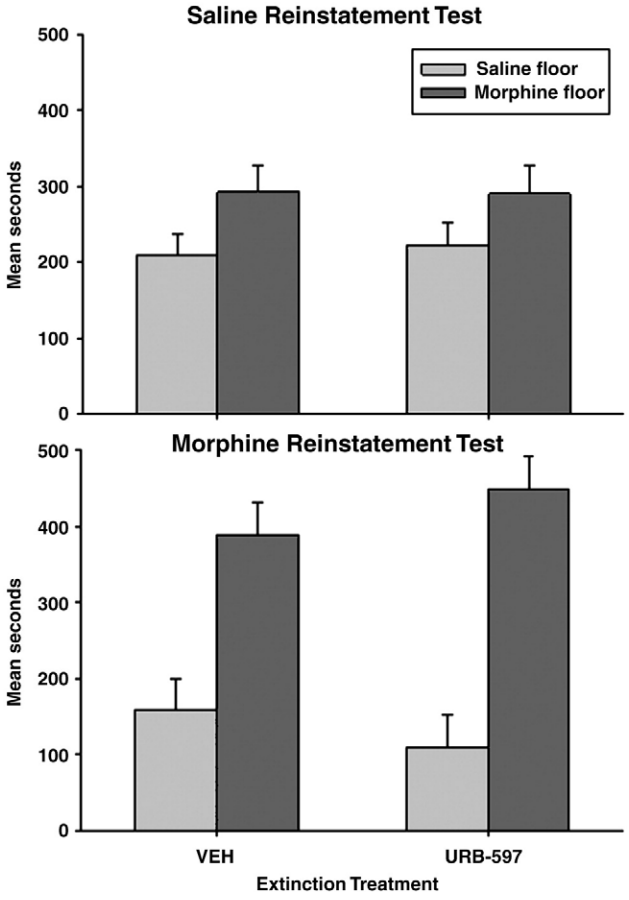

Fig. 6. Mean (+sem) seconds spent on the morphine-paired floor and on the saline-paired floor during the saline and morphine reinstatement tests for rats that had been treated with URB-597 ($n=8$) or VEH ($n=9$) during extinction in Experiment 2d.

prime, $F(1, 24)=4.3$, $p=0.05$. However, the prior extinction pretreatments did not modify the strength of the reinstated floor aversion as indicated by no significant interactions (Saline test, $F(2, 24)=1.1$; Withdrawal test, $F(1, 24)=0.3$).

4. Discussion

The data from Experiments 1–3 are consistent with an emerging consensus in the literature that manipulations of the eCB system selectively modify extinction of aversive, but not appetitive, learning (e.g. Lutz, 2007; Harloe et al., 2008, Hölter et al., 2005; Niyuhire et al., 2007). Administration of the FAAH inhibitor and indirect CB_1 agonist, URB-597, promoted the extinction of a naloxone-precipitated morphine withdrawal-induced conditioned aversion (Expt. 3), but did not affect extinction of a morphine-induced conditioned preference (Expts. 2a–d) nor sucrose-motivated operant responding (Expt. 1). Additionally, administration of the CB_1 inverse agonist/antagonist SR141716 interfered with the extinction of the conditioned aversion (Expt. 3), but did not interfere with extinction of responding motivated by sucrose reward, nor did AM-251 interfere with extinction of a morphine-induced place preference (Expt 2a). In fact, SR141716 suppressed operant responding during extinction, suggesting either a motivational or locomotor suppressant effect.

Four experiments evaluated the potential of CB_1 antagonist/inverse agonist, AM-251 (in Experiment 2a) or URB-597 (in Experiments 2b–d), to modify the rate of extinction of a morphine-induced conditioned floor preference. In Experiments 2a–c, manipulation of the eCB system occurred on the first extinction trial (as in Experiment 3), both during extinction by testing (Expts 2a, b) and during extinction by forced exposure (Expt 2c). Because the conditioned preference extinguished

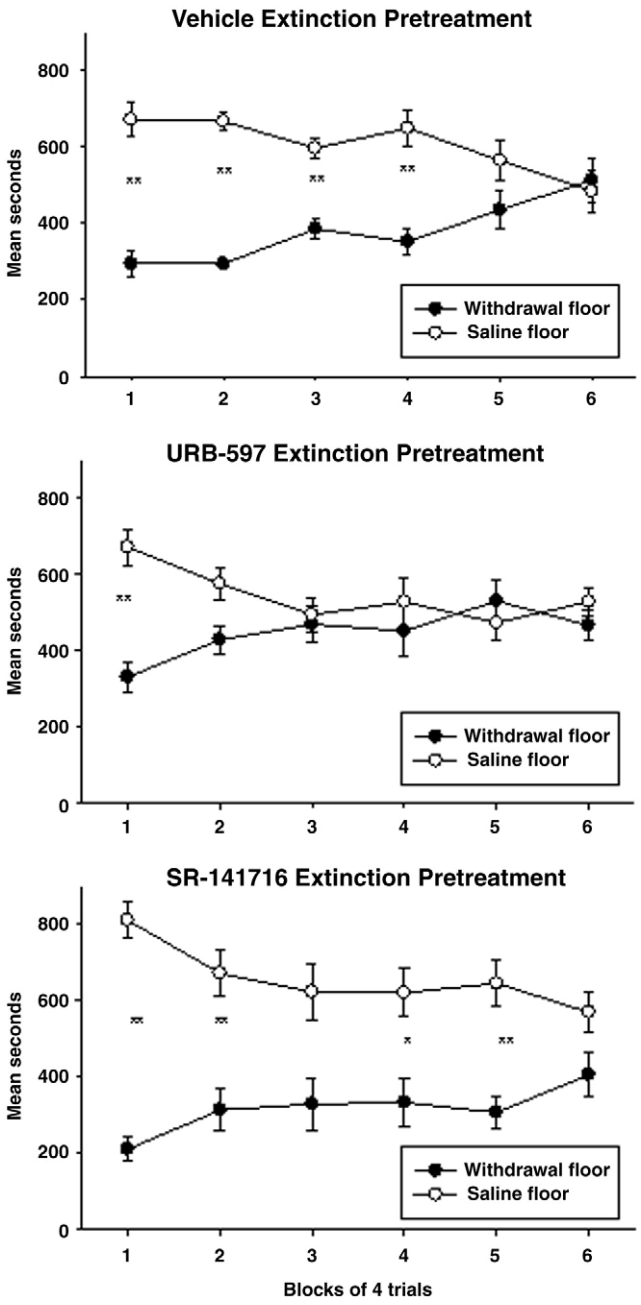

Fig. 7. Experiment 3. Mean (+sem) seconds spent on the naloxone-precipitated morphine withdrawal-paired floor and on the saline-paired floor during each block of 4 trials for rats pretreated with vehicle ($n=8$), URB-597 ($n=8$) or SR141716 ($n=8$) group. Asterisks indicate that rats spent less time on the withdrawal-paired floor than the saline-paired floor during block of trials ($*=p<0.05$; $**=p<0.01$).

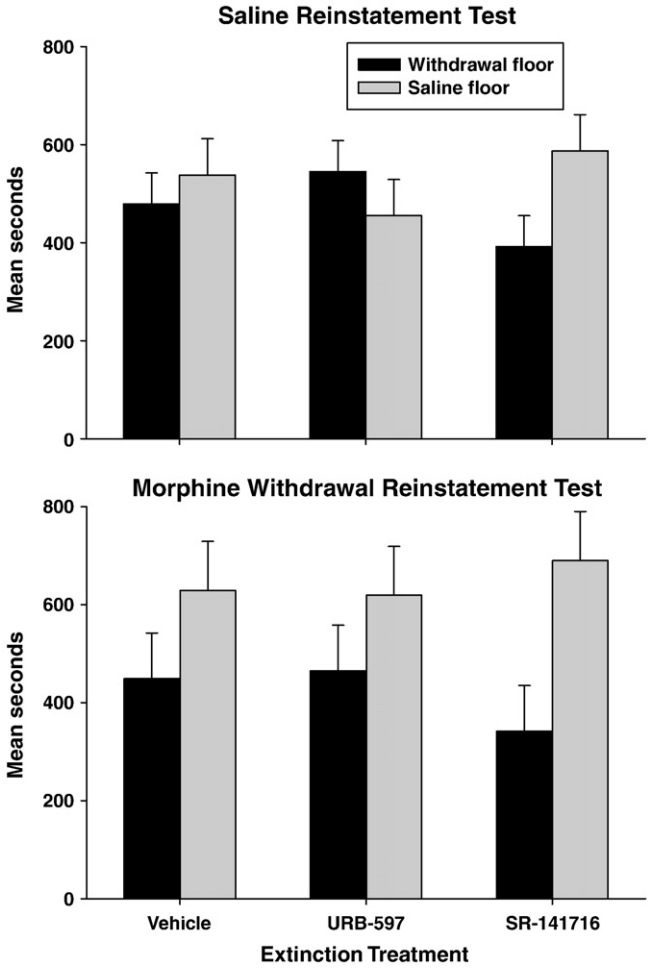

Fig. 8. Mean (+sem) seconds spent on the withdrawal-paired floor and on the saline-paired floor during the saline and naloxone-precipitated morphine withdrawal reinstatement tests for rats that had been treated with VEH ($n=8$), URB-597 ($n=8$) or SR141716 ($n=8$) during extinction in Experiment 3.

rapidly in Experiments 2a–c, Experiment 2d was designed to ensure more robust resistance to extinction of the conditioned preference. In Experiment 2d, the number of conditioning trials was increased to 8, the duration of the extinction/test trials was 10 min and they were spaced by 72–96 h (e.g.. Mueller et al., 2002; Paolone et al., 2009). Additionally, only rats that displayed a conditioned preference greater than 45 s in a probe test trial following conditioning were continued into extinction (e.g., Paolone et al. 2009). With these modifications, resistance to extinction was enhanced in Experiment 2d. Regardless of the method of extinction, manipulations of the eCB system had no effect on the speed of extinction of the conditioned floor preference.

Instead, it appeared that the failure of manipulations of the eCB system in Experiments 2a–d to affect extinction of a morphine-induced conditioned preference may have been a function of the appetitive nature of the task. This hypothesis was then tested by evaluating the potential of URB-597 to promote, and SR141716 to interfere with, the extinction of a conditioned floor aversion produced by an aversive stimulus, naloxone-precipitated morphine withdrawal (Experiment 3). In fact, URB-597 facilitated extinction of the conditioned aversion in comparison to vehicle alone, whereas SR141716 prolonged the aversion. These effects were not apparent during the first block of test trials, but emerged over repeated testing suggesting an effect on extinction, but not retrieval of the memory. These results are consistent with others (Harloe et al., 2008; Niyuhire et al., 2007) showing a selective effect of manipulation of the eCB system on learning motivated by an aversive stimulus, but not on learning motivated by a rewarding stimulus. Due to known interactions between the eCB and opioid systems (e.g., Navarro et al., 1998; Solinas and Goldberg, 2005), it is important to extend the generality of this finding to extinction of a conditioned floor aversion produced by other aversive drugs, such as lithium chloride.

It has been well established that extinction is not unlearning, but instead is new inhibitory learning that interferes with the originally learned response (e.g., Bouton, 2002). Indeed, simply presenting a priming dose of the unconditioned stimulus (US) drug prior to a test for a previously extinguished place preference (Parker and McDonald, 2000; Mueller et al., 2002) or a place aversion (Parker and McDonald, 2000) reinstates the learned response. Both a morphine prime

(Experiment 2d) and a naloxone-precipitated morphine withdrawal prime (Experiment 3) reinstated the conditioned floor preference and aversion respectively. However, prior treatment with neither URB-597 nor SR-141716 during extinction modified the strength of subsequent reinstatement by a drug prime.

The selective effects of manipulation of the eCB system on extinction of the conditioned floor aversion may be related to the known anxiolytic effects of URB-597 (e.g., Kathuria et al., 2003) and anxiogenic effects of SR141716 (e.g., Navarro et al., 1998; Mitchell and Morris, 2007). That is, maintenance of conditioned aversive associations may be promoted by conditioned fear and/or anxiety responses experienced at the time of testing. It is less likely that the hedonic effects of URB-597 or SR141716 influenced their ability to modify extinction of the conditioned aversion, because previous investigations have shown that URB-597 produces neither a conditioned place preference nor aversion (Gobbi et al., 2005) and SR141716 generally produces neither a conditioned place preference nor aversion (Chaperon et al., 1998; Mas-Nieto et al., 2001; Navarro et al., 2001; Li et al., 2008 but see Saňudo-Peňa et al., 1997).

Our data support and expand previous findings suggesting that the eCB system functions primarily in the extinction of aversively motivated behaviors and is mediated by an increase in available anandamide. Marsicano et al. (2002) reported that impaired extinction — but not acquisition and consolidation — of fear memory by the eCB system was mediated by elevated anandamide specifically in the basolateral amygdala (BLA). Although eCB levels were not measured in the present experiments, the facilitation of extinction of the conditioned aversion produced by URB-597 may also be mediated by elevated anandamide in the BLA, an area of the brain that has been implicated in the expression of conditioned place aversion learning (e.g., Zanoveli et al., 2007; Lucas et al., 2008). The finding that pretreatment with SR141716 interfered with the extinction of the conditioned aversion suggests that the effects are CB_1-mediated; however, future experiments that evaluate the potential of a CB_1 antagonist/inverse agonist to reverse the effects of URB-597 are warranted to ensure a CB_1 mechanism of action.

Recent evidence demonstrates that CB_1 receptor modulation of extinction requires memory reactivation/reconsolidation resulting from an initial presentation of the CS, an effect that appears to be mediated by CB_1 receptors in the amygdale (Lin et al., 2006). This suggests that the initial expression of a conditioned response may have a substantial eCB component that when manipulated either facilitates (CB_1 antagonists) or inhibits (CB_1 agonists) reconsolidation of recently activated memories. It is therefore conceivable that the effect of URB-597 and SR-141716 on the extinction of the conditioned floor aversion was mediated by their potential to modify reconsolidation rather than extinction per se.

The results reported here complement previous reports using different behavioural procedures (Chhatwal et al., 2005; Harloe et al., 2008; Hölter et al., 2005; Marsicano et al., 2002; Niyuhire et al., 2007; Pamplona et al., 2006; Varvel and Lichtman, 2002; Varvel et al., 2007), each suggesting that activation of the eCB system selectively facilitates extinction of aversively motivated behaviours. Converging lines of evidence suggest that manipulation of the eCB system may be a promising therapeutic target to promote extinction of aversive memories, such as those experienced by people suffering from post-traumatic stress disorder.

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