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Effect of Cannabinoid CB1 Receptor Antagonist SR141714A and CB1 Receptor Knockout on Cue-Induced Reinstatement of Ensure® and Corn-Oil Seeking in Mice

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The cannabinoid CB1 receptor antagonist SR141716A decreases cue-induced reinstatement of sucrose and drug seeking in rats. Reinstatement behavior is not well characterized in C57Bl/6 mice, including CB1 receptor knockout mice generated on a C57Bl/6 background. In the present study, male C57Bl/6, CB1 knockout (CB1 KO), and wild-type littermate (WT) mice were trained to respond for the sweet reinforcer Ensure® or corn oil. Responding was maintained on a fixed ratio 1 (FR1) schedule of reinforcement for 10 days, and then extinguished by the removal of the reinforcer and associated cues. Subsequently, the effect of either pretreatment with SR141716A or CB1 receptor knockout on cue-induced reinstatement of Ensure® or corn-oil seeking was assessed. Both 1.0 and 3.0 mg/kg SR141716A decreased reinstatement of Ensure® seeking in C57Bl/6 mice. A tenfold higher dose of SR141716A (10.0 mg/kg) was required to attenuate reinstatement behavior in C57Bl/6 mice responding for corn oil, suggesting that CB1 receptors may be selectively involved in the neurobiology underlying reinstatement of responding for some food reinforcers but not others. Whereas CB1 receptor antagonism selectively attenuated reinstatement of responding for Ensure®, genetic deletion of the CB1 receptor produced only a trend in decreasing reinstatement of Ensure® seeking, and did not attenuate reinstatement of corn-oil seeking. Baseline differences in levels of operant responding were also observed in WT *vs* CB1 KO mice maintained by Ensure® and corn oil. This and other possible reasons for the observed discrepancy between pharmacological blockade *vs* genetic invalidation of the CB1 receptor on reinstatement of Ensure® seeking are discussed.
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INTRODUCTION

In humans, the presence of cues associated with rewarding substances, such as palatable foods or drugs of abuse can contribute to relapse to uncontrollable eating or to drug use, respectively (Berthoud, 2004; Childress *et al*, 1988). Similarly, when laboratory animals are conditioned to associate cues with the availability of a food or drug reinforcer, these cues can subsequently reinstate responding after a period of extinction. This behavior is considered to be analogous to relapse in humans; therefore, cue-induced reinstatement paradigms have been used to examine the neurobiology underlying relapse across a wide range of reinforcers (Shaham *et al*, 2003; De Vries and Schoffelmeer, 2005). Nevertheless, little is currently known about the neurobiology underlying relapse to uncontrollable eating.

Preclinical as well as clinical evidence indicates that the cannabinoid CB1 receptor system is involved in relapse to food and drug taking. Results from recent clinical trials revealed that the CB1 receptor antagonist SR141716A (also known as rimonabant, Acomplia®) produced weight loss in obese human subjects during year 1 and decreased weight regain during year 2 (RIO-Lipid trial; Van Gaal *et al*, 2005). SR141716A also significantly attenuated relapse to cigarette smoking compared to placebo (STRATUS-US trial; Anthenelli and Despres, 2004). Furthermore, SR141716A is effective in reducing cue-induced reinstatement of cocaine (De Vries *et al*, 2001), heroin (De Vries *et al*, 2003), methamphetamine (Anggadiredja *et al*, 2004), nicotine (De Vries *et al*, 2005), ethanol (Economidou *et al*, 2006), and sucrose (De Vries *et al*, 2005; Economidou *et al*, 2006) seeking behavior in rats.

Whereas cue-induced reinstatement behavior has been extensively studied in rats, far fewer studies have assessed such behavior in mice (Highfield *et al*, 2002; Fuchs *et al*,

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2003; Yan *et al*, 2006; Mead *et al*, 2007). Therefore, the present study examined the effect of SR141716A on cue-induced reinstatement behavior in mice trained to respond for the sweet reinforcer Ensure®. Ensure® is a mixed macronutrient (94 total calories/tbsp; 2.25 g fat, 15 g carbohydrates, 3.38 g protein) that has been shown previously to maintain operant responding in mice (Caine *et al*, 1999; Barrett *et al*, 2004; Ward and Dykstra, 2005). Additionally, it is unknown whether the CB1 receptor system's involvement in relapse behavior extends to nonsweet palatable reinforcers. Recent experiments conducted in our laboratory show that several concentrations of corn oil also serve as a reinforcer in C57Bl/6 mice (Ward and Dykstra, 2005); therefore, we assessed whether SR141716A would attenuate cue-induced reinstatement in mice responding for 32% corn oil (120 total calories/tbsp; 14 g fat) as well.

Lastly, no studies to date have examined cue-induced reinstatement of food seeking in mutant mice, including mice lacking the CB1 receptor (CB1 KO mice). Other data from CB1 KO mice support the hypothesis that the CB1 receptor system can modulate intake of sweet palatable foods. It has been demonstrated, for example, that sucrose intake (Poncelet *et al*, 2003) and sucrose self-administration (Sanchis-Segura *et al*, 2004) are reduced in CB1 KO mice. Our laboratory recently reported that motivation for Ensure® is attenuated in CB1 KO mice, whereas motivation to consume corn oil is not attenuated in these mice (Ward and Dykstra, 2005). Therefore, we also assessed the effect of CB1 receptor knockout on cue-induced reinstatement of both Ensure® and corn-oil seeking.

In the present study, reinstatement of either Ensure®- or corn-oil seeking was assessed using a cue-induced reinstatement procedure adapted from a mouse model of relapse to cocaine seeking described by Fuchs *et al* (2003). In the present study, responding was maintained on an fixed ratio 1 (FR1) schedule of reinforcement of either 100% Ensure® or 32% corn-oil presentation for 10 days, and then extinguished by the removal of the reinforcer and cues previously associated with its delivery. The effect of either pretreatment with SR141716A (0.3–10.0 mg/kg) or CB1 KO was then assessed on cue-induced reinstatement of Ensure® or corn-oil seeking.

MATERIALS AND METHODS

Animals

Sixty-four C57Bl/6 male mice (The Jackson Laboratory), 12 male CB1-KO, and 12 male wild-type (WT) mice were used for these experiments. The CB1 knockout (CB1 KO) mice were originally generated on a full C57Bl/6 background by Zimmer and colleagues (1999) at the National Institutes of Health (NIH) by a targeted mutation of the large single coding sequence of the CB1 receptor gene. Nucleic acids that code for amino acids 32–448 were replaced with a phosphoglycerate kinase (PGK)-neo cassette through homologous recombination in embryonic stem cells (Zimmer, 1992). Heterozygous breeding pairs were obtained from a colony at Virginia Commonwealth University, and were bred and genotyped at the Julius L Chambers Biomedical/Biotechnology Research Institute at North Carolina Central University animal facilities to obtain WT and CB1 KO mice. Following their arrival at the animal facilities at UNC Chapel Hill or Temple University, mice were housed in groups of four, with *ad libitum* access to food and water throughout the duration of the operant studies. Lights were programmed on a 12 h light/dark cycle with lights off at 0700, so that all experimental testing occurred during the dark cycle of the animals' diurnal cycle. Mice weighed 20–25 g at the beginning of the experiments. Animal protocols were approved by the institutional animal care and use committees, and the methods were in accord with the *Guide for the Care and Use of Laboratory Animals*.

Drug

SR141716A (Research Triangle Institute, RTP, NC) was dissolved in a vehicle of 100% ETOH, Alkamuls EL-620 (Rhodia, Cranbury, NJ) and saline in a ratio of 1:1:18 and was injected intraperitoneally in a volume of 0.1 ml/10 g 15 min before behavioral testing.

Operant Procedure

Apparatus. Experiments were conducted in mouse operant conditioning chambers (21.6 × 17.8 × 12.7 cm, Model ENV-307W, Med Associates, Georgia, VT) located within ventilated sound attenuation chambers. The operant conditioning chambers were equipped with two nose-poke holes (1.2 cm diameter) equipped with internal amber stimulus lights. The operant chambers were also equipped with a motor-driven dipper for liquid food presentation. The receptacle opening for access to the food was located between the two nose-poke holes, and an amber stimulus light was located above the receptacle opening. The chambers were also equipped with a house light, ventilator fan, and tone generator.

Experiment 1: Ensure®-Maintained Responding

Acquisition. In Experiment 1, three separate groups of mice were trained to respond for undiluted Ensure®. Group 1 included 34 C57Bl/6 mice, and Group 2 and Group 3 included six WT and six CB1 KO mice, respectively. All mice were trained to nose poke for undiluted Ensure® under a FR1 schedule during 7 daily 30 min acquisition sessions (see Ward and Dykstra, 2005 for details).

Maintenance. Following acquisition, responding for Ensure® was maintained under an FR1 schedule during daily 1 h sessions for 10 days in Groups 1–3. During maintenance sessions, responses on the active, illuminated nose-poke hole resulted in the presentation of Ensure® for 8 s, paired with illumination of the stimulus light above the food receptacle and a 1 s tone. Responses on the inactive nose-poke hole and entry into the food receptacle were also recorded, but were without scheduled consequences. The house light and fan remained on throughout the 1 h test session. No time-out period was imposed following responding in the active nose-poke hole.

Extinction. Subsequently, responding for Ensure® was extinguished in Groups 1–3. During the extinction sessions, all cues associated with the delivery of Ensure® were

removed and responses had no scheduled consequences (ie the dipper arm did not enter the receptacle opening, the stimulus light above it was not activated, and there was no presentation of the tone). The active nose-poke hole remained illuminated and the house light and fan remained on throughout the session. Extinction sessions were conducted daily in Groups 1–3 until mice reached a criterion of $\leqslant$30% of the number of active nose-poke hole responses made on the last maintenance day. This criterion was chosen to engender a response rate on the final extinction session comparable to that reported for extinction of cocaine seeking in mice described by Fuchs *et al* (2003). Retaining this level of responding at the end of extinction helps guarantee that mice will respond in the active nose-poke hole at the start of the reinstatement session, which is required for the reintroduction of the previously associated cues (Fuchs personal communication).

Cue-induced reinstatement test. Mice in Group 1 were treated with either vehicle or SR141716A (0.3–3.0 mg/kg) 15-min before the reinstatement test to assess the effect of CB1 receptor antagonism on cue-induced reinstatement of Ensure® seeking. During the 1 h test session, responses on the active, illuminated nose-poke hole resulted in the presentation of the empty dipper cup paired with illumination of the stimulus light above the food receptacle and a 1 s tone in the absence of Ensure®. Responses in the inactive nose-poke hole had no programmed consequences. Groups 2 and 3 were tested for cue-induced reinstatement to assess the effect of CB1 KO on relapse to Ensure® seeking behavior as described above.

Experiment 2: Corn Oil-Maintained Responding

Acquisition. In Experiment 2, three separate groups of mice were trained to respond for a 32% concentration of corn oil (emulsified in 3% xanthan gum, Sigma Chemical Co., St Louis, MO) using methods identical to Ensure®-trained mice. Group 4 included 30 C57Bl/6 mice, and Group 5 and Group 6 included six WT and six CB1 KO mice, respectively.

Maintenance. Following acquisition, responding for 32% corn oil was maintained under conditions identical to those in the maintenance phase of Experiment 1.

Extinction. Subsequently, responding for corn oil was extinguished in Groups 4–6. The procedure for extinction of corn oil-maintained responding was identical to that described above for Ensure® extinction.

Cue-induced reinstatement test. Mice in Group 4 were treated with either vehicle or SR141716A (1.0–10.0 mg/kg) 15-min before the reinstatement test to assess the effect of CB1 receptor antagonism on cue-induced reinstatement of corn-oil seeking. Groups 5 and 6 were tested for cue-induced reinstatement to assess the effect of CB1 KO on relapse to corn-oil seeking behavior.

Statistical Analysis

The effect of SR141716A on reinstatement of Ensure® or corn-oil responding was analyzed using one-way analysis of variance (ANOVA). Two-way ANOVA was used to analyze the effect of CB1 KO on Ensure®- and corn oil-maintained responding, with genotype (WT *vs* KO) and session as factors of variation. Dependent variables included responses in both the active and inactive nose-poke holes and entries into the food receptacle during maintenance and extinction. The effect of CB1 KO during cue-induced reinstatement of responding for Ensure® or corn oil was analyzed using unpaired Student's *t*-tests.

RESULTS

Experiment 1: Ensure®-Maintained Responding

Effect of SR141716 in C57Bl/6 mice.

Acquisition of Ensure®-maintained responding: In Group 1, 29 out of 34 C57Bl/6 mice obtained the maximum number of reinforcers (10) on or before the last acquisition session (data not shown).

Active nose-poke hole responding: As shown in Figure 1a, Ensure®-maintained responding in the active nose-poke hole under an FR1 schedule of reinforcement in C57Bl/6 mice during the maintenance phase of Experiment 1. Figure 1a also shows that all mice met extinction criteria by the fifth extinction session. Responding for Ensure® was reinstated by the reintroduction of cues in mice pretreated with vehicle, and a one-way ANOVA revealed a significant effect of SR141716 on attenuation of cue-induced reinstatement ($F_{(3,32)} = 7.198$; $p < 0.01$). *Post hoc* analysis revealed a significant effect at the 1.0 and 3.0 mg/kg SR141716 doses as compared to vehicle.

Inactive nose-poke hole responding: Figure 1a also shows that C57Bl/6 mice exhibited very low levels of responding in the inactive nose-poke hole in relation to the active nose-poke hole during maintenance, extinction, and cue-induced reinstatement. Although one-way ANOVA revealed an effect of SR141716 on inactive hole responding during reinstatement ($F_{(3,32)} = 4.544$; $p < 0.05$), *post hoc* analysis revealed no significant difference between any dose of SR141716 and vehicle treatment.

Entries into the food receptacle: Figure 1b shows that head entries into the receptacle were made at least as many times as a reinforcer was earned in C57Bl/6 mice during maintenance. Figure 1b also illustrates an extinction burst, or initial increase in head entries on the first day of the extinction phase that was not seen for responses made into the active nose-poke hole. Lastly, during the cue-induced reinstatement test for Ensure®-responding, pretreatment with SR141716A significantly decreased entries into the food receptacle in C57Bl/6 mice ($F_{(3,32)} = 7.119$; $p < 0.01$). *Post hoc* analysis revealed a significant effect at the 1.0 and 3.0 mg/kg SR141716 doses compared to vehicle.

WT vs *CB1 KO.*

Acquisition of Ensure®-maintained responding: During acquisition of Ensure®-maintained responding, five out of six WT mice obtained the maximum number of reinforcers by the last training session, whereas three out of six CB1 KO

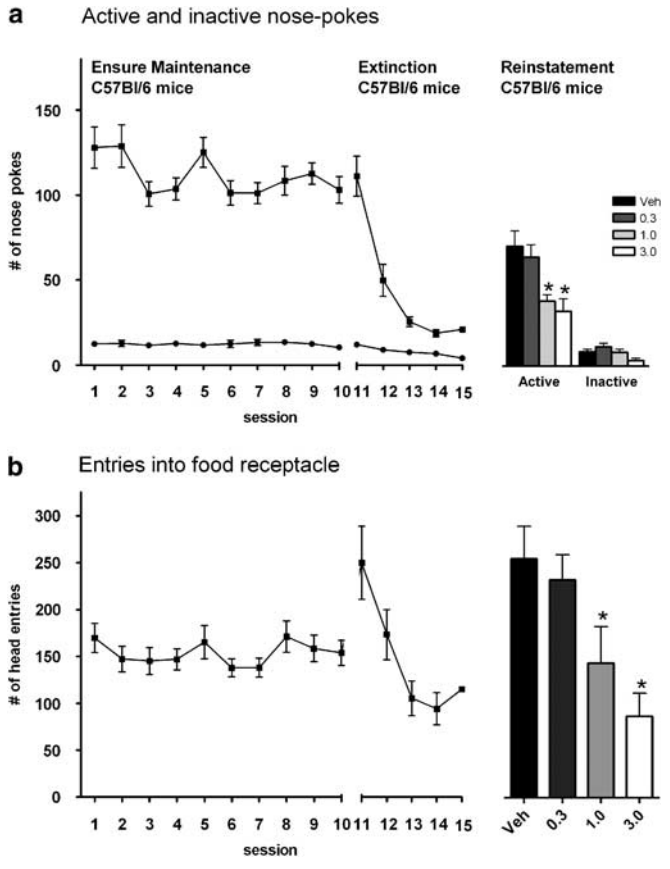

Figure 1 Responding for 100% Ensure® in C57Bl/6 mice during a cue-induced reinstatement procedure. (a) Ensure®-maintained responding in the active and inactive nose-poke holes during maintenance, extinction, and reinstatement in C57Bl/6 mice. Responses in the active nose-poke hole are represented by ■. Responses in the inactive nose-poke hole are represented by ●. The bar graph shows responses in both the active and inactive nose-poke holes during reinstatement in mice treated with vehicle or 0.3–3.0 SR141716A. (b) Head entries made into the food receptacle opening during maintenance, extinction, and cue-induced reinstatement in C57Bl/6 mice. The bar graph shows head entries during reinstatement in mice treated with vehicle or 0.3–3.0 SR141716A. Asterisks (*) indicate significant effect of SR141716A on responding during reinstatement as compared to vehicle ($p<0.05$).

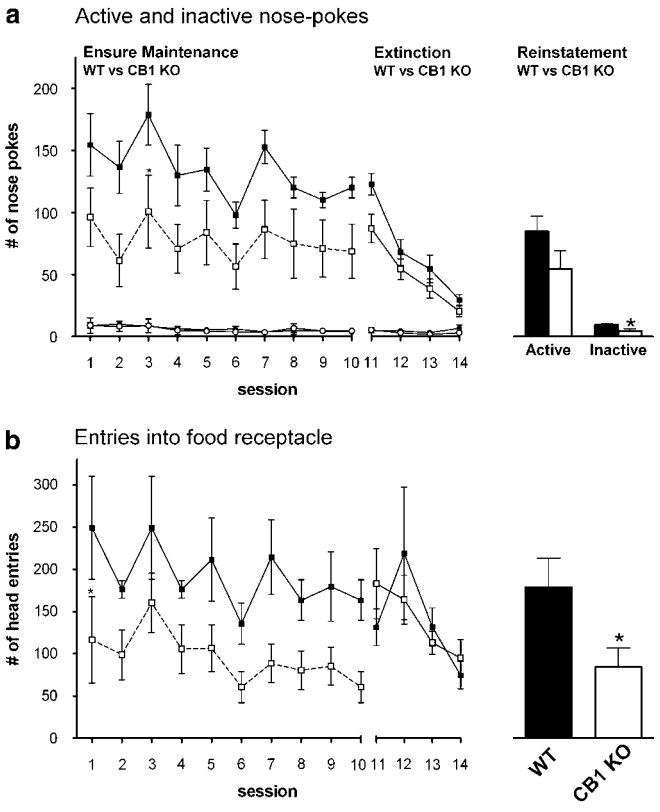

Figure 2 Responding for 100% Ensure® in CB1 KO and WT mice during a cue-induced reinstatement procedure. (a) The effect of CB1 KO on responding for Ensure® in the active and inactive nose-poke holes. Responses in the active nose-poke hole are represented by ■ for WT and □ for CB1 KO mice. Responses in the inactive nose-poke hole by WT (●) and CB1 KO (○) mice are also depicted. The bar graph shows responses in both the active and inactive nose-poke holes during reinstatement by WT (solid bars) and CB1 KO (open bars) mice. (b) Head entries made into the food receptacle opening during maintenance, extinction, and cue-induced reinstatement. Entries into the food receptacle are represented by ■ for WT and □ for CB1 KO mice. Significant effects of CB1 KO during either maintenance or extinction are indicated by a dashed connecting line (- -□- -; $p<0.05$). Asterisks (*) on the line graph indicate significant effect of genotype on select days revealed by *post hoc* analysis. The bar graph shows head entries during reinstatement by WT (solid bar) and CB1 KO (open bar) mice. Asterisks (*) on the bar graphs indicate significant effect of CB1 KO *vs* WT on responding during reinstatement ($p<0.05$).

mice obtained the maximum number of reinforcers by the last session (data not shown).

Active nose-poke hole responding: As shown in Figure 2a, Ensure®-maintained responding in the active nose-poke hole under an FR1 schedule of reinforcement in WT and CB1 KO mice during the maintenance phase of Experiment 1. For WT *vs* CB1 KO mice, two-way ANOVA showed a significant main effect of genotype ($F_{(1,10)}=8.477$; $p<0.05$) and of session ($F_{(9,10)}=4.442$; $p<0.05$), with no interaction ($F_{(9,100)}=0.46$; NS). Figure 2a also shows that WT and CB1 KO mice met extinction criteria by the fourth extinction session. Two-way ANOVA on the raw extinction data showed a significant main effect of session ($F_{(3,10)}=15.27$; $p<0.05$) but not genotype ($F_{(1,10)}=0.518$; NS), and no interaction ($F_{(3,100)}=0.922$; NS). Figure 2a also indicates that responding for Ensure® was reinstated by the reintroduction of cues in both WT and CB1 KO mice, and unpaired Student's *t*-test revealed no significant effect of CB1 KO.

Inactive nose-poke hole responding: Figure 2a also shows that WT and CB1 KO mice exhibited very low levels of responding in the inactive nose-poke hole in relation to the active nose-poke hole during the maintenance phase for Ensure® responding. Two-way ANOVA showed no main effect of genotype ($F_{(1,10)}=0.425$; NS) or session ($F_{(9,10)}=1.628$; NS), and no interaction ($F_{(9,100)}=0.164$; NS). Figure 2a also illustrates that responding in the inactive hole was low during extinction in both groups. For WTs *vs* CB1 KO, two-way ANOVA showed no main effect of genotype ($F_{(1,10)}=1.974$; NS) or session ($F_{(3,10)}=1.360$; NS), and no interaction ($F_{(3,100)}=0.754$; NS). Figure 2a does indicate, however, that responding in the inactive hole during reinstatement was significantly decreased in CB1 KOs as compared to WT mice ($p<0.05$).

Entries into the food receptacle: Figure 2b shows that head entries into the receptacle were made at least as many times as a reinforcer was earned in WT and CB1 KO mice during each session of the maintenance phase. Averaged across daily sessions, WT mice entered the food receptacle ~45% more often than they responded on the active hole, whereas CB1 KO mice entered the food receptacle ~25% more often than the active hole. Two-way ANOVA showed a significant main effect of genotype ($F_{(1,10)} = 6.893$; $p < 0.05$) and of session ($F_{(9,10)} = 5.452$; $p < 0.05$), with no interaction ($F_{(9,100)} = 0.588$; NS). Figure 2b also illustrates that entries into the food receptacle were extinguished in a similar manner in WT and CB1 KO mice. Two-way ANOVA showed a significant main effect of session ($F_{(3,10)} = 3.519$; $p < 0.05$) but not genotype ($F_{(1,10)} = 0.001$; NS), and no interaction ($F_{(3,100)} = 0.903$; NS). Figure 2b does indicate, however, that number of entries into the food receptacle during reinstatement was significantly decreased in CB1 KO mice as compared to WT mice ($p < 0.05$).

Extinction data expressed as percent baseline responding: Because CB1 KO produced a reduction of baseline responding for Ensure® under the FR1 schedule, the effect of CB1 KO during the extinction phase of the study was also analyzed by expressing these data as a percent of baseline (defined as the number of responses made during the last maintenance session). Analysis of the transformed data by two-way ANOVA showed no effect of genotype on extinction of active or inactive nose pokes, but did reveal that CB1 KO mice made significantly more entries into the food receptacle than WT controls during extinction sessions ($F_{(1,10)} = 7.815$; $p < 0.05$) (data not shown).

Experiment 2: Corn Oil-Maintained Responding

Effect of SR141716A in C57Bl/6 mice.

Acquisition of corn oil-maintained responding: In Group 4, 23 out of 30 C57Bl/6 mice obtained the maximum number of reinforcers (10) by the last acquisition session.

Active nose-poke hole responding: As shown in Figure 3a, corn oil-maintained responding in the active nose-poke hole under an FR1 schedule of reinforcement in C57Bl/6 mice during the maintenance phase of Experiment 2. Figure 3a also shows that all mice met extinction criteria by the sixth extinction session. Additionally, mice responding for corn oil exhibited a robust extinction burst on the first day of the extinction phase. Responding for corn oil was reinstated by the reintroduction of cues in mice pretreated with vehicle, and a one-way ANOVA revealed a significant effect of SR141716 on attenuation of cue-induced reinstatement ($F_{(3,29)} = 3.273$; $p < 0.05$). *Post hoc* analysis revealed a significant effect at the 10.0 mg/kg SR141716 dose as compared to vehicle.

Inactive nose-poke hole responding: Figure 3a also shows that C57Bl/6 mice exhibited very low levels of responding in the inactive nose-poke hole in relation to the active nose-poke hole during the maintenance, extinction, and cue-induced reinstatement. Pretreatment with SR141716 produced a significant effect on responding in the inactive hole during reinstatement ($F_{(3,29)} = 3.641$; $p < 0.05$). *Post hoc* analysis revealed significant difference between 3.0 mg/kg SR141716 and vehicle treatment.

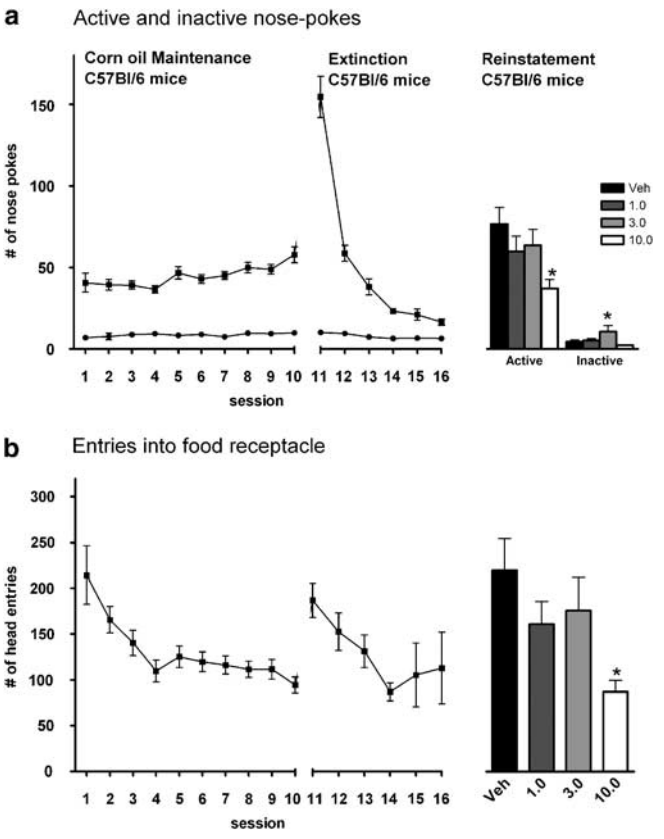

Figure 3 Responding for 32% corn oil in C57Bl/6 mice during a cue-induced reinstatement procedure. (a) Corn-oil maintained responding in the active and inactive nose-poke holes during maintenance, extinction, and reinstatement in C57Bl/6 mice. Responses in the active nose-poke hole are represented by ■. Responses in the inactive nose-poke hole are represented by ●. The bar graph shows responses in both the active and inactive nose-poke holes during reinstatement in mice treated with vehicle or 1.0–10.0 SR141716A. (b) Head entries made into the food receptacle opening during maintenance, extinction, and cue-induced reinstatement in C57Bl/6 mice. The bar graph shows head entries during reinstatement in mice treated with vehicle or 1.0–10.0 SR141716A. Asterisks (*) indicate significant effect of SR141716A on responding during reinstatement as compared to vehicle ($p < 0.05$).

Entries into the food receptacle: Figure 3b shows that head entries into the receptacle were made at least as many times as a reinforcer was earned in C57Bl/6 mice during maintenance. Figure 3b also illustrates extinction burst on the first 3 days of the extinction phase. Lastly, during the cue-induced reinstatement test for Ensure® responding, pretreatment with SR141716A significantly decreased entries into the food receptacle in C57Bl/6 mice ($F_{(3,29)} = 4.925$; $p < 0.01$). *Post hoc* analysis revealed a significant effect at the 10.0 mg/kg SR141716 dose as compared to vehicle.

WT vs *CB1 KO.*

Acquisition of corn oil-maintained responding: During acquisition of corn oil-maintained responding, all six WT mice obtained the maximum number of reinforcers by the last training session, and five out of six CB1 KO mice

obtained the maximum number of reinforcers by the last session.

Active nose-poke hole responding: As shown in Figure 4a, 32% corn oil-maintained responding in the active nose-poke hole under an FR1 schedule of reinforcement in WT and CB1 KO mice during the maintenance phase of Experiment 2. For WT *vs* CB1 KO mice, two-way ANOVA showed a significant main effect of genotype ($F_{(1,10)} = 10.04$; $p < 0.05$) and of session ($F_{(9,10)} = 2.097$; $p < 0.05$), with no interaction ($F_{(9,100)} = 0.707$; NS). Figure 4a also shows that WT and KO mice met extinction criteria by the fourth extinction session. WT mice exhibited an extinction burst on the first day of the extinction phase, and this burst in responding was seen in CB1 KO mice on the first and second extinction sessions as well. Two-way ANOVA showed a significant main effect of session ($F_{(3,10)} = 8.788$; $p < 0.05$) but not genotype ($F_{(1,10)} = 2.978$; NS), and no interaction ($F_{(3,100)} = 0.331$; NS) between WT and CB1 KO mice. Figure 4a also indicates that responding for corn oil was reinstated by the reintroduction of cues in both WT and CB1 KO mice, and unpaired Student's *t*-test revealed no significant effect of CB1 KO.

Inactive nose-poke hole responding: Figure 4a shows that WT and CB1 KO mice exhibited low levels of responding in the inactive nose-poke hole in relation to the active hole during the maintenance phase for corn-oil responding. For WT *vs* CB1 KO mice, two-way ANOVA showed a main effect of session ($F_{(9,10)} = 3.124$; $p < 0.05$) but not for genotype ($F_{(1,10)} = 0.144$; NS), and no interaction ($F_{(9,100)} = 2.610$; NS). Figure 4a also illustrates a tendency toward increased inappropriate responding in CB1 KO mice during extinction sessions compared to WT controls. Two-way ANOVA showed a trend toward a main effect of genotype ($F_{(1,10)} = 4.518$; $p = 0.059$), with no effect of session ($F_{(3,10)} = 1.231$; NS), and no interaction ($F_{(3,100)} = 0.367$; NS). CB1 KO did not significantly affect responding in the inactive hole during reinstatement.

Entries into the food receptacle: As shown in Figure 4b, head entries were made into the food receptacle at least as many times as a reinforcer was earned in WT and CB1 KO mice across the maintenance phase of Experiment 2. Averaged across daily sessions, WT mice entered the food receptacle ~98% more often than they responded on the active hole, whereas CB1 KO mice entered the food receptacle ~157% more often than the active hole. Two-way ANOVA showed no main effect of genotype ($F_{(1,10)} = 0.001$; NS) or session ($F_{(9,10)} = 0.775$; NS), but a significant interaction ($F_{(9,100)} = 2.207$; $p < 0.05$). Figure 4b also illustrates that entries into the food receptacle were extinguished in a similar manner in WT and CB1 KO mice. Two-way ANOVA showed a significant main effect of session ($F_{(3,10)} = 4.872$; $p < 0.05$) but not genotype ($F_{(1,10)} = 0.140$; NS), and no interaction ($F_{(3,100)} = 0.036$; NS). In addition, Figure 4b indicates that CB1 KO had no effect on entries into the food receptacle during reinstatement.

Extinction data expressed as percent baseline responding: Because baseline responding for corn oil under the FR1 schedule was reduced in CB1 KO mice, the effect of CB1 KO during the extinction phase of the study was also analyzed by expressing these data as a percent of baseline responding. Two-way ANOVA showed no significant main effect of genotype on active and inactive nose pokes or entries into the food receptacle during extinction (data not shown).

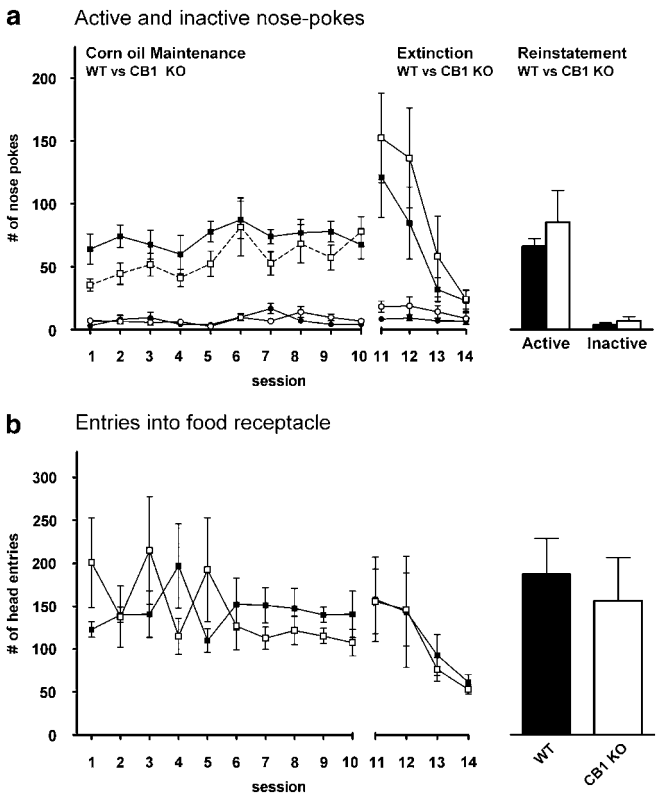

Figure 4 Responding for 32% corn oil in CB1 KO and WT mice during a cue-induced reinstatement procedure. (a) The effect of CB1 KO on responding for corn oil in the active and inactive nose-poke holes. Responses in the active nose-poke hole are represented by ■ for WT and □ for CB1 KO mice. Responses in the inactive nose-poke hole by WT (●) and CB1 KO (○) mice are also depicted. The bar graph shows responses in both the active and inactive nose-poke holes during reinstatement by WT (solid bars) and CB1 KO (open bars) mice. (b) Head entries made into the food receptacle opening during maintenance, extinction, and cue-induced reinstatement. Entries into the food receptacle are represented by ■ for WT and □ for CB1 KO mice. Significant effects of CB1 KO during either maintenance or extinction are indicated by a dashed connecting line (- -□- -; $p < 0.05$).

DISCUSSION

Our results demonstrate that C57Bl/6 mice will reinstate responding for both Ensure®- and corn oil-associated cues following several extinction sessions. Administration of the CB1 receptor antagonist SR141716A significantly and dose-dependently reduced Ensure®-seeking behavior with a minimal effective dose of 1.0 mg/kg. CB1 receptor KO also led to a decrease in active nose pokes, although this trend was not significant, and a significant decrease in head entries made into the receptacle during reinstatement of Ensure® seeking. Although SR141716A also attenuated cue-induced reinstatement of corn-oil seeking, a higher dose of 10 mg/kg SR141716A was required for this effect, and CB1

receptor KO had no effect on any measured behaviors during cue-induced reinstatement of corn-oil seeking. To the authors' knowledge, this is the first study to investigate the role of CB1 receptors in cue-induced reinstatement responding in C57Bl/6 and CB1 KO mice; importantly, the C57Bl/6 strain represents a common progenitor of several other knockout mouse models used to study addictive disorders (Crawley *et al*, 1997).

Several studies have demonstrated that SR141716A reliably and potently attenuates cue-induced reinstatement for several drugs of abuse in rats, for example heroin, nicotine (1.0 mg/kg; De Vries *et al*, 2003, 2005), ethanol, and cocaine (0.3 mg/kg; Economidou *et al*, 2006; De Vries *et al*, 2001). Our results support a key recent finding that blockade of the CB1 receptor system can also suppress conditioned seeking for palatable foods (De Vries *et al*, 2005; Economidou *et al*, 2006). In the present study, 1.0 mg/kg SR141716A was sufficient to attenuate cue-induced reinstatement of Ensure® seeking in mice, and De Vries *et al* (2005) demonstrated that this dose also decreased reinstatement of sucrose seeking in rats. In contrast, whereas corn oil also served as a palatable food reinforcer in the present study, a tenfold higher dose of 10 mg/kg SR141716A was required to attenuate relapse to corn-oil seeking. Taken together, these data demonstrate that although endocannabinoids may play a general role in conditioned seeking across drug and natural reinforcers, inactivation of CB1 receptors does not attenuate all palatable food seeking with the same potency, with SR141716A selectively affecting conditioned seeking of the sweet reinforcer Ensure®.

Although corn oil served as a palatable reinforcer in this and a previous report from our laboratory (Ward and Dykstra, 2005), 32% corn-oil intake was lower than 100% Ensure® intake during maintenance in all experimental groups. This may be indicative of a lower reinforcing strength of corn oil in the present assay; however, it is quite possible that the high caloric density of corn oil as a fat nutrient and its ability to elicit stronger satiety signals is a better explanation of the lower intake. The fact that C57Bl/6, WT, and CB1 KO mice trained to respond for corn oil exhibited strikingly robust bursts in responding during initial extinction sessions as compared with mice trained to respond for Ensure® actually suggests that corn oil served as a more efficacious reinforcer than Ensure® in these mice. Furthermore, reinstatement of corn-oil seeking behavior in vehicle-treated mice actually exceeded response levels during maintenance, whereas Ensure® seeking was lower in vehicle-treated mice during reinstatement as compared to maintenance. Indeed, it is possible that SR141716A attenuated Ensure® reinstatement at lower doses because the reinforcing strength of corn oil was more difficult to surmount pharmacologically. As mentioned previously, however, relatively low doses of SR141716A (<1.0 mg/kg) have been shown to reduce reinstatement of responding previously maintained by strong drug reinforcers, such as cocaine. Therefore, it is more likely that neural pathways mediating cue-induced reinstatement of corn-oil seeking do not completely overlap with those mediating conditioned seeking of other natural and drug reinforcers and are less dependent on CB1 activation. The principal neural substrates thought to play a key role in conditioned drug-seeking behavior include the prefrontal cortex, striatum, and amygdala, with high levels of CB1 receptors present in each region; however, the extent to which these neural pathways are involved in conditioned seeking of palatable foods, such as sucrose, Ensure®, and corn oil, is unknown. Further research should elucidate the neural substrates underlying cue-induced reinstatement of food seeking and reveal the extent to which they overlap across palatable food types and with those underlying drug-seeking behavior.

An assessment of response patterns in WT *vs* CB1 KO mice during maintenance and extinction of Ensure® and corn-oil responding provided further information regarding the role of CB1 receptors in food-directed behaviors. During the maintenance phase of Experiment 1, CB1 KO mice responded significantly less for Ensure® under an FR1 schedule of reinforcement. The effect of CB1 KO on Ensure®-maintained responding was robust and persistent, observed for active nose-poke hole responding and food entries, and complements other reports that CB1 KO mice responded significantly less than WT controls for sucrose under an FR1 schedule (Sanchis-Segura *et al*, 2004) and for sucrose and Ensure® under PR schedules of reinforcement (Sanchis-Segura *et al*, 2004; Ward and Dykstra, 2005). Conversely, this is the first report in the literature on the effect of CB1 KO on responding for a fat reinforcer under a fixed-ratio schedule. During the maintenance phase of Experiment 2, CB1 KO produced a significant decrease in responding for corn oil, but this effect was less robust than for Ensure® and only observed for active nose-poke hole responding. Interestingly, this potentially more potent effect of CB1 KO on Ensure® responding in this model is analogous to a previous finding in our laboratory showing that motivation for Ensure®, but not corn oil, was attenuated in CB1 KO mice responding under a progressive ratio schedule of reinforcement (Ward and Dykstra, 2005).

Assessment of extinction behavior in CB1 KO and WT mice trained to respond for Ensure® and corn oil revealed that both WT and CB1 KO mice showed a similar rapid extinction of Ensure®- and corn-oil seeking. This finding was somewhat surprising in the CB1 KO mice, in that this genotype displays a deficit in extinction learning under several experimental conditions, including spatial memory (Varvel *et al*, 2005) and conditioned fear learning (Marsicano *et al*, 2002). Our results indicated only a modest effect of CB1 KO on extinction behavior in mice trained to respond for Ensure®, in that they exhibited a significant increase in head entries into the food receptacle throughout the extinction sessions. This was the only indication in the present study that extinction of appetitive conditioning is impaired in CB1 KO mice. Hölter *et al* (2005) also reported that extinction of an appetitively motivated learning task was not impaired in CB1 KO mice, and we are continuing to assess the role of CB1 receptors in extinction of conditioned Ensure®- and corn-oil maintained behaviors with similar results. Taken together, these findings suggest that CB1 receptors may not be involved in the extinction of appetitively conditioned operant behaviors.

Although CB1 receptor blockade attenuated cue-induced reinstatement behavior in C57Bl/6 mice responding for Ensure® (and to a lesser extent corn oil), active nose-poke

hole responding was fully reinstated in both Ensure®- and corn-oil trained CB1 KO mice, in that their levels of responding returned to those observed during maintenance. This discrepancy in SR141716A-treated *vs* CB1 KO mice may be due to the observed baseline differences in operant responding in CB1 KO mice. It is possible, for example, that the initial blunted level of responding observed in the CB1 KO mice during maintenance reflects an overall decrease in a conditioned association between the cues and the reinforcer, so that a detection of further attenuation of conditioned responding during reinstatement is hampered in this model. Alternative parameters, which engender similar baseline levels of responding during maintenance in WT and CB1 KO mice (such as type and/or concentration of reinforcer used) may clarify whether baseline differences in responding masked potential differences in the two groups. This discrepancy may also be because of compensatory changes in feeding and/or reward pathways in CB1 KOs during development, which remains an issue in research using noninducible knockouts. Another possibility is that there is a qualitative difference in the pharmacological effect of receptor invalidation as opposed to putative inverse agonist activity at CB1 receptors produced by SR141716A, which may produce anticannabimimetic effects that are not necessarily indicative of endogenous cannabinoid activity (see Pertwee, 2005 for review). Lastly, the lack of effect in the CB1 KO mice responding for Ensure® may reflect an insufficient sample size to reach statistical significance. Seven to ten C57Bl/6 mice were tested at each SR141716A dose, whereas only six WT and six CB1 KO mice were available for use in the current study.

The present results support other findings from rat studies that the CB1 receptor system is involved in relapse to palatable food seeking by demonstrating that cue-induced reinstatement of Ensure®- and corn-oil seeking is attenuated in C57Bl/6 mice pretreated with SR141716A and in CB1 KO mice. However, inactivation of the CB1 receptor system by SR141716A or CB1 KO more potently suppressed cue-induced seeking of Ensure® *vs* corn oil, suggesting that either (1) the CB1 receptor system does not play an equivalent role in modulating conditioned seeking of Ensure® *vs* corn oil or (2) cue-induced reinstatement of corn-oil seeking behaviors was more difficult to surmount because corn oil served as a more robust reinforcer in the present study. Lastly, the use of CB1 KO mice in this procedure suggests that some behaviors related to palatable food seeking are attenuated in CB1 KO mice; however, further assessment of knockout mouse behavior in similar assays is warranted to reveal whether issues such as baseline behavioral differences or compensatory changes during development may limit the utility of noninducible knockout mice in reinstatement procedures.

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