Neuropsychopharmacology (2005) 30, 339–349

www.neuropsychopharmacology.org

CB_1 Receptor Knockout Mice Display Reduced Ethanol-Induced Conditioned Place Preference and Increased Striatal Dopamine D2 Receptors

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Cannabinoids and ethanol activate the same reward pathways, and recent advances in the understanding of the neurobiological basis of alcoholism suggest that the CB_1 receptor system may play a key role in the reinforcing effects of ethanol and in modulating ethanol intake. In the present study, male CB_1 receptors knockout mice generated on a CD1 background displayed decreased ethanol-induced conditioned place preference (CPP) compared to wild-type ($CB_1^{+/+}$) mice. Ethanol (0.5, 1.0, 1.5, and 2.0 g/kg) induced significant CPP in $CB_1^{+/+}$ mice at all doses tested, whereas it induced significant CPP only at the highest dose of ethanol (2.0 g/kg) in $CB_1^{-/-}$ mice. However, there was no genotypic difference in cocaine (20 mg/kg)-induced CPP. There was also no genotypic difference, neither in cocaine (10–50 mg/kg) nor in D-amphetamine (1.2–5 mg/kg)-induced locomotor effects. In addition, mutant and wild-type mice did not differ in sensitivity to the anxiolytic effects of ethanol (1.5 g/kg) when tested using the elevated plus maze. Interestingly, this decrease in ethanol efficacy to induce CPP in $CB_1^{-/-}$ mice was correlated with an increase in D2/D3 receptors, as determined by [^{3}H]raclopride binding, whereas there was no difference in D1-like receptors, as determined by [^{3}H]SCH23390 binding, measured in the striatum from drug-naïve mice. This increase in D2/D3 binding sites observed in CB_1 knockout mice was associated with an altered locomotor response to the D2/D3 agonist quinpirole (low doses 0.02–0.1 mg/kg) but not to an alteration of quinpirole (0.1–1.0 mg/kg)-induced CPP compared to wild-type mice. Altogether, the present results indicate that lifelong deletion of CB_1 receptors reduced ethanol-induced CPP and that these reduced rewarding effects of ethanol are correlated to an overexpression of striatal dopamine D2 receptors.
Neuropsychopharmacology (2005) **30,** 339–349, advance online publication, 22 September 2004; doi:10.1038/sj.npp.1300568

Keywords: cannabinoid; CB1 receptor; ethanol; reward; knockout; psychostimulants

INTRODUCTION

The endocannabinoid system has been implicated in a number of neurological and psychiatric disorders, including drug addiction (Van der Stelt and Di Marzo, 2003). Cannabinoids such as Δ9-tetrahydrocannabinol (Δ9-THC), the major psychoactive component of marijuana, produce their pharmacological effects by stimulating two types of G-protein-coupled cannabinoid receptors: the CB_1 receptor, mainly localized in the CNS and the CB_2 receptor primarily found in the immune system (Wilson and Nicoll, 2002).

Some clinical studies have suggested that genetic variants of the CNR1 gene might be associated with susceptibility to alcohol or drug dependence (Comings *et al*, 1997; Schmidt *et al*, 2002), although not all agree (Preuss *et al*, 2003). Several lines of evidence support the involvement of endocannabinoid system, and its CB_1 receptor, in the pharmacological and behavioral effects of ethanol (Hungund *et al*, 2002; Mechoulam and Parker, 2003). In this regard, chronic ethanol exposure leads to a selective increase in the levels of both endogenous cannabinoid agonists arachidonylethanolamide and 2-arachidonylglycerol in cultured SK-N-SH cells (Basavarajappa and Hungund, 1999a) or cerebellar granular neurons (Basavarajappa *et al*, 2000). Chronic ethanol exposure has also been shown to downregulate CB_1 receptor number and/or function in rodents and this observed downregulation may result from overstimulation of receptors via increased synthesis of endogenous CB_1 receptor agonists (Basavarajappa and Hungund, 1999b; Ortiz *et al*, 2004).

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Received 24 March 2004; revised 11 August 2004; accepted 12 August 2004
Online publication: 13 August 2004 at http://www.acnp.org/citations/Npp081304041139/default.pdf

Converging evidence suggests that the CB_1 receptor signaling system could play an important role in modulating alcohol-reinforcing effects and alcohol drinking behavior. Thus, studies have shown that the CB_1 receptor antagonist SR141716A reduces alcohol intake (Arnone *et al*, 1997; Colombo *et al*, 1998; Rodriguez de Fonseca *et al*, 1999) and the motivation to consume alcohol in a progressive ratio paradigm (Gallate and McGregor, 1999) in rats, while a CB_1 receptor agonist increased the motivation to consume alcohol alcohol in a progressive ratio paradigm (Gallate *et al*, 1999). In addition, ethanol (0.5–2.0 g/kg) has been shown to decrease operant responding to a greater extent in $CB_1^{-/-}$ mice than in wild-type mice, suggesting a possible role of CB_1 receptor in the rate disruptive effects of ethanol (Baskfield *et al*, 2004). Recently, we and others have shown that ethanol consumption and/or preference are decreased in $CB_1^{-/-}$ mice generated on a CD1 background (Naassila *et al*, 2004) or a C57BL/6J background (Poncelet *et al*, 2003). Two other studies have also shown that $CB_1^{-/-}$ mice generated on a C57BL/6J background consumed 70% less of a 12% ethanol solution compared to their wild-type counterparts (Hungund *et al*, 2003) and exhibited decreased ethanol preference when given a 20% ethanol solution (Wang *et al*, 2003). Furthermore, our previous study has shown that this decrease in voluntary ethanol intake and preference observed in $CB_1^{-/-}$ mice is associated with an increased ethanol sensitivity (hypothermia, sedation, locomotion) and ethanol withdrawal severity (Naassila *et al*, 2004).

Various studies in rats have suggested that the cannabinoid system may be involved in the rewarding effects of various types of reinforcers, such as drugs of abuse, food, or electrical brain stimulation (Chaperon *et al*, 1998; Deroche-Gamonet *et al*, 2001). Psychoactive cannabinoids increase the extracellular dopamine concentration (Tanda *et al*, 1997) and the activity (French, 1997) of dopaminergic neurons in the ventral tegmental area–mesolimbic pathway. These dopaminergic circuits are known to play a pivotal role in mediating the rewarding effects of ethanol (Di Chiara and Imperato, 1988; Weiss and Porrino, 2002). The relative contributions of different dopamine receptor subtypes in mediating rewarding effects of ethanol have been difficult to establish, in part, because of the paucity of pharmacological agents specific for each of the receptor subtypes within the two main families the D1-like (D1 and D5) and the D2-like receptors (D2, D3, and D4) (Cunningham *et al*, 2000). Mice lacking D1 or D2 receptors have been shown to display reduced ethanol-conditioned place preference (CPP) and/or ethanol self-administration (El-Ghundi *et al*, 1998; Phillips *et al*, 1998; Cunningham *et al*, 2000).

There is considerable evidence that endocannabinoids modulate the brain dopaminergic system and recently, functional interactions between endocannabinoid and dopaminergic systems have been demonstrated. Dopamine release in rat nucleus accumbens has been shown to increase after administration of exogenous cannabinoids (Tanda *et al*, 1997; Szabo *et al*, 1999). Activation of D2-like dopamine receptors, but not D1-like receptors, increased anandamide release in dorsal striatum (Giuffrida *et al*, 1999). Furthermore, chronic treatment with D2-like receptor antagonists upregulated CB_1 receptor expression in the rat striatum (Mailleux and Vanderhaeghen, 1993) and *in vitro* experiments have shown that a D2-like receptor antagonist attenuated the ethanol-induced formation of 2-arachidonylglycerol (Basavarajappa *et al*, 2000). In addition, pretreatment with the CB_1 antagonist SR141716A enhanced the hyperactivity elicited by administration of a D2-like receptor agonist, suggesting that endocannabinoid system may act as an inhibitory feedback mechanism on the hyperlocomotor effects induced by dopamine (Giuffrida *et al*, 1999). At the molecular level, a functional interaction between CB1 and D2 receptors has been recently demonstrated. In this regard, it has been shown that the D2 receptor may have a significant modulatory role in determining the G-protein coupling specificity of CB_1 receptor in HEK cells (Jarrahian *et al*, 2004).

Given the established importance of the cannabinoidergic system in modulating ethanol consumption and mediating ethanol effects, we used CB_1 receptor gene knockout mice (Ledent *et al*, 1999) in a CD1 background to investigate the rewarding effects of ethanol. The present study used a place conditioning task to determine whether CB_1 receptor deficiency produces an increase or a decrease in ethanol reward. Our goal was to establish whether the reduced ethanol consumption described in $CB_1^{-/-}$ mice actually results from an increase in ethanol reward (Cunningham *et al*, 2000). Since a functional interaction between CB_1 and D2 receptors has been demonstrated, sensitivity to the rewarding effects of the D2-like agonist quinpirole in $CB_1^{-/-}$ mice has also been investigated. The present study also determined whether the lifelong deletion of the CB_1 receptor could alter D1 or D2 receptor levels in striatum that may account for the differences in responding to ethanol.

MATERIALS AND METHODS

Animals

CB_1 null mutant mice were generated by homologous recombination as described (Ledent *et al*, 1999). Briefly, a PKG-Neo cassette was inserted between *Avr*II and *Sfi*I sites located 1073 bp apart, replacing the first 233 codons of the gene. Homologous recombination in R1 cells and aggregation with CD1 eight-cell-stage embryos were performed. A recombinant line was used to generate chimeras allowing germline transmission of the mutant gene. Heterozygous mice were bred for 15 generations on a CD1 background before generating the wild-type and CB_1 null littermates used in this study. The F14 generation of homozygous mice was genotyped and therefore used to produce the F15 generation that has been used for the experiments. Adult male wild-type and $CB_1^{-/-}$ mice (8–14 weeks old) weighing 20–30 g were used. All animals used in a given experiment were derived from the same breeding series, and were matched for age and weight. Mice were housed in groups of 10 in clear plastic cages and maintained in a temperature- ($\sim 22^\circ C$) and humidity-controlled room on a 12 h light/dark cycle. The number of animals was kept to a minimum and all efforts were made to avoid animal suffering. Experiments were carried out in strict accordance with both the Guide for the Care and Use of Laboratory Animals

(NIH) and the E.C. regulations for animal use in research (CEE No. 86/609).

Drugs

Cocaine hydrochloride, D-amphetamine, quinpirole, sulpiride, and SCH23390 were obtained from Sigma Chemicals (Paris, France). [^{3}H]raclopride (s.a. 80 Ci/mmol) and [^{3}H]SCH23390 (s.a. 85 Ci/mmol) were obtained from NEN (UK). Ethanol (95% (v/v)) was obtained from Carlo Erba réactifs (Val de Reuil, France). Ethanol was diluted to 20% (v/v) in physiological saline prior to the intraperitoneal (i.p.) injection. Cocaine and D-amphetamine injections were made in volumes of 1 ml/100 g and ethanol injections were made in volumes of 1.25 ml/100 g. Saline injections were made in volumes equal to that of the corresponding drug for each animal.

CPP Apparatus and Procedures

A two-chambered CPP apparatus was used (Bioseb, Chaville, France), which consisted of two $30 \times 20 \times 20\,cm^3$ compartments with distinct visual and tactile cues. One of the compartments had gray colored walls and a stainless-steel floor and the opposite compartment had black–white striped walls and a smooth floor. The two compartments were separated by a guillotine door. Distance and time spent in each compartments were measured by computer-interfaced infrared photobeams (16×16). Both compartments were illuminated by dim light with 40 lx brightness.

The procedure consisted of three different phases: preconditioning (day 1), conditioning (days 2–5), and postconditioning (day 6). To control possible innate preferences for one of the two conditioning compartments, mice underwent a single preconditioning session. Immediately after saline injection they were allowed free access to both conditioning compartments for 20 min. Initial place preference was determined by the side in which a mouse spent more than 600 s out of a 20-min trial. Place preference conditioning was conducted using an unbiased procedure (Cunningham *et al*, 2003). When a group of untrained mice showed a preference for one compartment (no more than 70% in one compartment), half of the animals received either ethanol or cocaine or quinpirole in the spontaneously preferred compartment and the other half in the nonpreferred compartment. Preconditioning showed no significant difference in the initial preference between mutant and wild-type mice (data not shown). We selected a counterbalanced protocol in order to reduce each mouse's initial preference, as discussed previously (Cunningham *et al*, 2003).

Animals were randomly assigned to undergo either drug conditioning in the morning and saline conditioning in the afternoon, or *vice versa*. Animals received a total of two injections per day. For drug conditioning, animals ($n = 7$–13/group) were randomly assigned to receive either saline or ethanol (0.5, 1.0, 1.5, and 2.0 g/kg i.p., prepared at 20% in saline) or cocaine (20 mg/kg i.p. prepared in saline) or quinpirole (0.1 and 1.0 mg/kg i.p.). Quinpirole, at the 0.1–1.0 mg/kg doses, has been demonstrated to induce CPP (Hoffman *et al*, 1988; Hoffman and Beninger, 1989). Immediately following administration, animals were confined to one of the two conditioning compartments for 20 min. The drug- and saline-paired conditioning compartments and the time of the day of the drug or saline conditioning session (morning or afternoon) were random and counterbalanced across all groups. Conditioning sessions were conducted twice daily for 4 days, with a minimum of 5 h between conditioning sessions. Previous studies have demonstrated that plasma levels of ethanol or cocaine in mice are $>80\%$ clear following this time period after a single i.p. injection (Benuck *et al*, 1987; Faulkner *et al*, 1990). On the day following the last conditioning session, animals were tested for CPP by placing them between the two compartments (guillotine door removed) and allowing free access to both conditioning compartments for 20 min. CPP was determined by comparing the time spent (in s) in the drug-paired compartment during the preconditioning session and the time spent in the drug-paired compartment during the test session.

Effects of Cocaine, D-Amphetamine, and Quinpirole on Locomotor Activity

Locomotor activity was assessed in LE 88811 IR motor activity monitor (BIOSEB, Chaville, France). Animals were confined to a $45\,cm^2$ clear acrylic plastic chamber, in which horizontal locomotion was measured from photocell beam interruptions. Photocell beams transected the chamber 2 cm above the floor at 16 sites along each side. Test chambers were shielded from external noise and light, but each test field was illuminated with a white fluorescent light and was fully ventilated. Mice ($n = 6$–15/group) were injected i.p. with saline or 2.5–50 mg/kg cocaine or 0.6–5.0 mg/kg D-amphetamine or 0.02–1.0 mg/kg quinpirole and placed immediately into activity monitors for a test duration of 20 min for cocaine or 15 min for D-amphetamine. Different control groups were used for the different drugs. For testing the effect of 0.02–0.1 mg/kg quinpirole on locomotor activity, quinpirole was injected i.p. 30 min before testing.

Anxiolytic Effects of Ethanol Measured in the Elevated Plus Maze

The elevated plus maze apparatus was a modification of that validated by Lister (1987) and consisted of two open ($30 \times 5 \times 0.25\,cm^3$) and two enclosed ($30 \times 5 \times 5\,cm^3$) arms that extended from a common central platform ($5 \times 5\,cm^2$). The apparatus was constructed from black Plexiglas and elevated 60 cm above the floor. In accordance with established procedures (Rodgers and Johnson, 1995), male $CB_1^{-/-}$ ($n = 13$) and wild-type ($n = 14$) mice were individually placed on the central platform of the maze facing an open arm immediately after an i.p. injection of either saline or 1.5 g/kg ethanol. A 5 min test duration was used, and the apparatus was thoroughly cleaned between test sessions. The conventional spatiotemporal measures (ie open arm time and entries) were scored. A mouse was considered to have entered an arm when all four of its paws were placed in the arm.

Radioligand Binding

Binding experiments were performed on mouse striatal membranes from drug-naïve animals as previously described (Asencio *et al*, 1999). Membranes were prepared from striatum homogenized in 10 volumes ice-cold 0.32 M sucrose at 1200 rpm for 10 strokes, then centrifuged at 1000*g* at 4°C for 10 min. The pellet was then homogenized and centrifuged as above. The resulting two supernatants were combined and centrifuged at 48 000*g* for 20 min at 4°C. The P2 pellet was washed three times using assay buffer and the membranes were then resuspended to approximately 1 mg/ml. The final pellet was frozen (−18°C) until use and a 10 μl aliquot was used for protein measurement by the method of Lowry *et al* (1951). The assay conditions for each of the ligands was as follows: (a) *D1 binding*: 50 μl of [^{3}H]SCH23390 (s.a. 85 Ci/mmol) (0.02–7.5 nM) was incubated in a final volume of 500 μl assay buffer (50 mM Tris, 4 mM $MgCl_2$, pH 7.4) with 100 μg membranes. SCH23390 (1 μM) was used to define nonspecific binding. Samples were incubated for 1 h at 30°C. (b) *D2 binding*: 50 μl of [^{3}H]raclopride (s.a. 80 Ci/mmol) (0.02–10 nM) was incubated in a final volume of 500 μl assay buffer (50 mM Tris, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM KCl, 120 mM NaCl, 0.1% ascorbate, pH 7.4) with 150 μg membranes. Sulpiride (10 μM) was used to define nonspecific binding. Samples were incubated for 1 h and 30 min at 25°C. After incubation, samples were filtered through Whatman GF/B (45 μm pore size) glass fiber filters presoaked in 0.5% polyethylenimine and washed with an additional 2 × 5 ml assay buffer. Radioactivity was determined using 5 ml of ACS scintillation fluid and counted in a Wallac 1414 Winspectral liquid scintillation counter (Perkin-Elmer, 60% efficiency for [^{3}H]). Binding parameters (K_d, B_{max}) were evaluated using MultiCalc Software (Perkin-Elmer).

Statistical Analysis

Statistical analyses were conducted using SigmaStat software (SPSS Inc., Erkrath, Deutschland). For the CPP experiments, data were analyzed using a repeated-measure two-way analysis of variance (RM-ANOVA) followed by a Tuckey's *post hoc* test (factors genotype × session). For the locomotor activity, the effect of genotype and drug was analyzed using two-way analysis of variance (two-way ANOVA) (genotype × dose) and Tuckey's *post hoc* test where appropriate. For the elevated plus maze test, the effect of genotype and treatment was analyzed using two-way ANOVA (genotype × treatment) and Tuckey's *post hoc* test where appropriate. Radioligand binding experiments were analyzed using Student's *t*-test. A significance level of 0.05 was used for all tests.

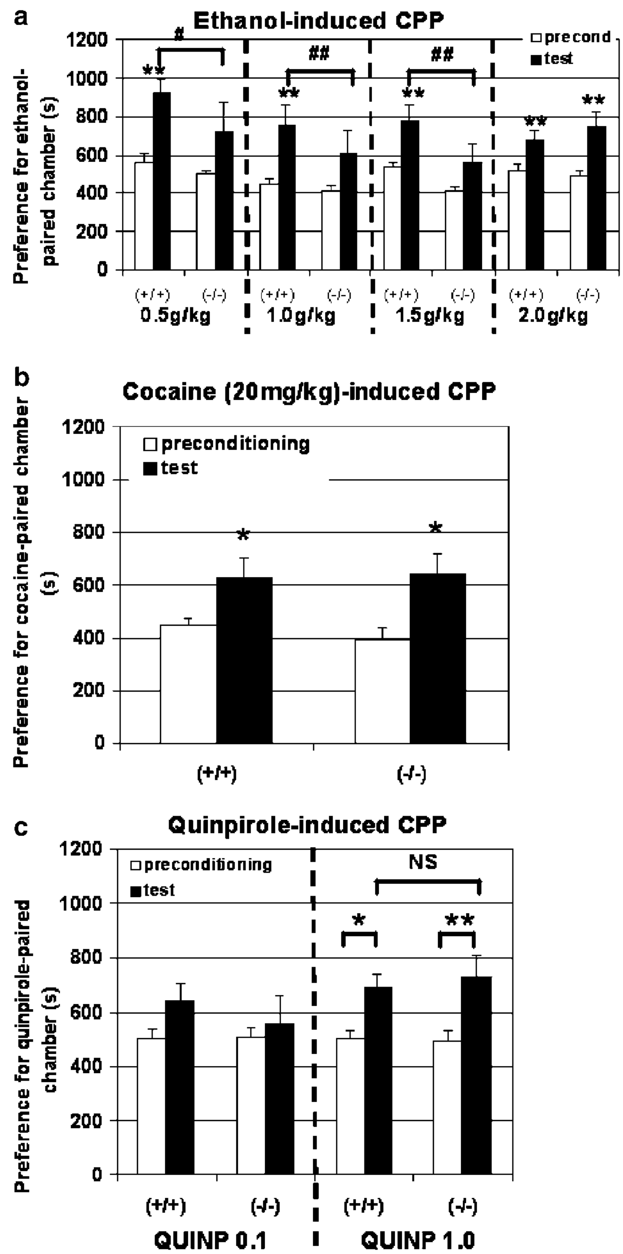

Figure 1 Rewarding effects of ethanol (0.5–2.0 g/kg, i.p.) (a), cocaine (20 mg/kg) (b), and quinpirole (0.1–1.0 mg/kg) (c) evaluated in the CPP paradigm. CB_1 wild-type mice ($n=7$–10/group for ethanol, $n=10$ for cocaine, and $n=10$/group for quinpirol) and mutant mice ($n=11$–13/group for ethanol, $n=8$ for cocaine, and $n=8$–10/group for quinpirole) were used in this experiment. Data are expressed as mean ± SEM time spent in the drug-paired compartment during preconditioning (□) and postconditioning (■) tests. *$p<0.05$, **$p<0.01$ compared to respective preconditioning session; $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to wild-type mice.

RESULTS

Ethanol-, Cocaine- and Quinpirole-Induced Conditioned Place Preference

Two-way RM-ANOVA (genotype × session) revealed a significant effect of genotype when ethanol 0.5–1.5 g/kg was used as the conditioning dose (Figure 1a; 0.5 g/kg: $F_{3,35}=4.04$, $p<0.05$; 1.0 g/kg: $F_{3,35}=8.53$, $p<0.01$; 1.5 g/kg: $F_{3,43}=9.52$, $p<0.01$) but not at the 2.0g/kg ethanol dose ($F_{3,37}=0.005$, NS). The repetitive administration of ethanol (0.5–1.0 g/kg) during 4 days resulted in the development of a place preference in wild-type mice (Tuckey's *post hoc* test, 0.5 g/kg: $p<0.01$; 1.0 g/kg: $p<0.005$; 1.5g/kg: $p<0.005$) but not in $CB_1^{-/-}$ mice. Tuckey's *post hoc* test revealed significant genotypic differences when test sessions were compared (0.5 g/kg: $p<0.05$; 1.0 g/kg: $p<0.001$; 1.5 g/kg:

$p < 0.001$), revealing that wild-type mice were more sensitive to the ethanol-induced place preference than mutant mice. After 4 days of conditioning with ethanol 2.0 g/kg, both genotypes developed a significant CPP ($+/+$: $p < 0.01$; $-/-$: $p < 0.001$) and no genotypic difference was observed ($F_{3,37} = 0.005$, NS). Ethanol at the dose of 0.5 g/kg produced maximal place conditioning effect in wild-type mice and two-way ANOVA revealed no main effect of dose in both wild-type mice ($F_{3,59} = 2.13$, NS) and mutant mice ($F_{3,86} = 2.18$, NS).

In addition, cocaine (20 mg/kg)-induced place preference did not differ between genotypes (Figure 1b). Two-way RM-ANOVA showed a significant session effect (preconditioning compared to test session) ($F_{1,35} = 13.12$, $p < 0.001$), revealing that cocaine induced CPP in both genotypes, but showed no significant main effect of genotype ($F_{1,35} = 0.13$, NS). The D2/D3 agonist quinpirole did not induce a significant place preference at the 0.1 mg/kg dose in both genotypes (Figure 1c; $F_{1,35} = 2.20$, NS). However, repeated administration of quinpirole (1.0 mg/kg) for 4 days resulted in significant development of CPP in both genotypes ($F_{1,41} = 16.10$, $p < 0.001$; $+/+$: $p < 0.05$; $-/-$: $p < 0.005$) and there was no genotypic difference ($F_{1,41} = 0.07$, NS).

Effects of Cocaine, D-Amphetamine, and Quinpirole on Locomotor Activity

The two-way ANOVA showed a main effect of cocaine dose ($F_{4,131} = 15.78$, $p < 0.001$) but no significant main effect of genotype ($F_{1,131} = 0.44$, NS) and no significant interaction ($F_{4,131} = 0.56$, NS) between the cocaine dose and genotype factors (Figure 2a). Similarly, the two-way ANOVA showed a main effect of D-amphetamine dose ($F_{3,73} = 3.77$, $p < 0.05$) but no significant main effect of genotype ($F_{1,73} = 0.21$, NS) and no significant interaction ($F_{3,73} = 0.93$, NS) between the D-amphetamine dose and genotype factors (Figure 2b). Significant main effects for dose ($F_{2,48} = 7.91$, $p < 0.001$) and genotype ($F_{1,48} = 18.63$, $p < 0.001$) were detected for the locomotor effects of quinpirole (Figure 2c). No significant interaction effect was detected ($F_{2,48} = 2.21$, NS).

Sensitivity to Ethanol-Induced Anxiolytic Effects

Basal levels of anxiety (ie anxiety levels in drug-naïve mice) were not statistically different between genotypes. Mutant and wild-type mice did not differ in sensitivity to the anxiolytic effects of ethanol when tested using the elevated plus maze (Figure 3). Ethanol increased the percentage time in open arms (main effect of treatment, $F_{1,48} = 4.73$, $p < 0.05$) and the number of open arms entries (main effect of treatment, $F_{1,48} = 13.48$, $p < 0.001$), but the genotypes did not differ in sensitivity to this effect (time, $F_{1,48} = 0.10$, NS; number, $F_{1,48} = 0.24$, NS). In addition, ethanol increased the number of total arm entries (main effect of treatment, $F_{1,48} = 12.63$, $p < 0.001$) in both genotypes and to the same extent ($F_{1,48} = 1.48$, NS).

Dopamine D1 and D2 Receptors in the Striatum

There was no significant genotypic difference in the maximum density of [^{3}H]SCH23390 binding sites (B_{max}): 932.25 ± 60.72 fmol/mg protein ($CB_1^{+/+}$) *vs* 1072.79 ± 75.52 fmol/mg protein ($CB_1^{-/-}$) (Figure 4a). The equilibrium dissociation constants (K_d) for the two genotypes were similar: 1.71 ± 0.23 nM ($CB_1^{+/+}$) *vs* 1.66 ± 0.17 nM ($CB_1^{-/-}$). However, there was a significant genotypic difference ($p < 0.05$) in the maximum density of [^{3}H]raclopride binding sites: 330 ± 41.26 fmol/mg protein ($CB_1^{+/+}$) *vs* 471 ± 39.92 fmol/mg protein ($CB_1^{-/-}$) (Figure 4b). No significant genotypic difference in the K_d values of

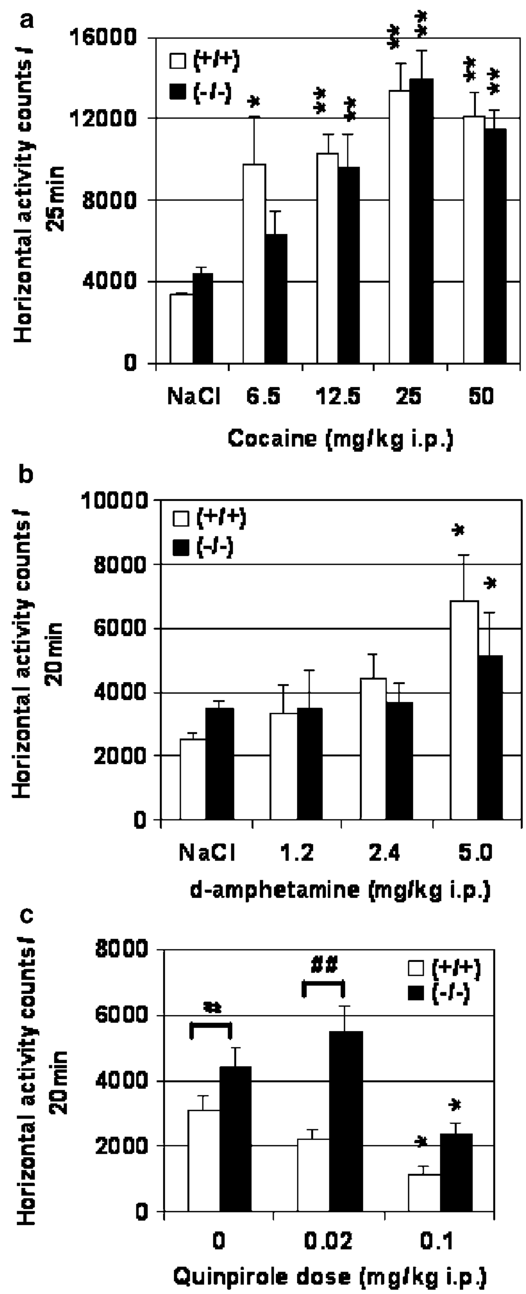

Figure 2 Locomotor effects of cocaine (a) ($n = 11–15$ mice per genotype), D-amphetamine (b) ($n = 8–17$ mice per genotype), and quinpirole (c) ($n = 6–11$ mice per genotype) in CB_1 wild-type (□) and mutant mice (■). There was no genotypic difference, neither in cocaine (10–50 mg/kg) nor in D-amphetamine (1.2–5 mg/kg). There was a genotypic difference in the D2/D3 agonist quinpirole (low doses 0.02–0.1 mg/kg)-induced locomotor effects. *$p < 0.05$, **$p < 0.01$ compared to respective NaCl group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to wild-type mice.

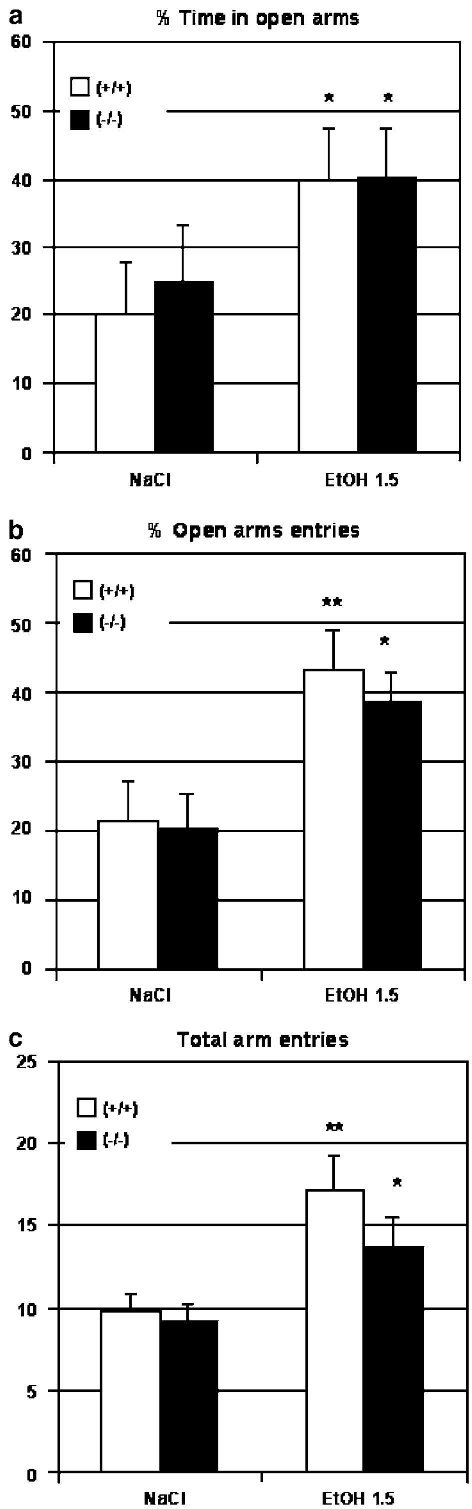

Figure 3 Anxiolytic-like behavior and the anxiolytic effects of ethanol in CB_1 wild-type (□) and mutant mice (■). Mice ($n = 13–14$ per genotype) received 1.5 g/kg ethanol or saline i.p. and were immediately tested on the elevated plus maze for 5 min. Values represent mean ± SEM. Ethanol produced an increase in the percentage time in open arms (a, $F_{1,48} = 4.73$, $p < 0.05$) and in the percentage open arm entries (b, $F_{1,48} = 13.48$, $p < 0.001$), and in the number of total arm entries (c, $F_{1,48} = 12.63$, $p < 0.001$). There was no significant genotypic difference in any parameters tested. *$p < 0.05$, **$p < 0.01$ compared to respective NaCl group.

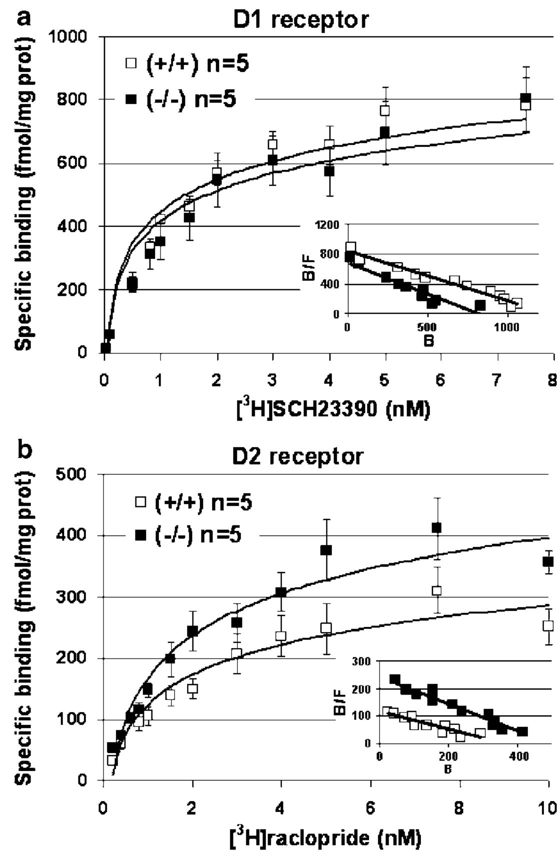

Figure 4 Representative saturation curves and showing the specific binding for [^{3}H]SCH23390 (a) and [^{3}H]raclopride (b) in the striatum of $CB_1^{+/+}$ (□) and $CB_1^{-/-}$ (■). Insets show representative Scatchard plots (B = bound and F = free). Five independent determinations per genotype. There was a significant genotypic difference ($p < 0.05$) in the maximum density of [^{3}H]raclopride binding sites: 330 ± 41.26 fmol/mg protein ($CB_1^{+/+}$) *vs* 471 ± 39.92 fmol/mg protein ($CB_1^{-/-}$). However, there was no genotypic difference, neither for the maximum density of [^{3}H]SCH23390 binding sites nor for the K_d values of both radioligands used.

[^{3}H]raclopride for D2 receptors was apparent between $CB_1^{+/+}$ and $CB_1^{-/-}$ mice: 2.38 ± 0.21 and 2.66 ± 0.42 nM, respectively.

DISCUSSION

In this study, we provide evidence suggesting that lifelong deletion of the CB_1 receptor reduces the rewarding effects of ethanol in a CPP paradigm. In this regard, $CB_1^{-/-}$ mice failed to display a CPP to an environment paired with a moderate (0.5–1.5 g/kg) but not a higher (2.0 g/kg) dose of ethanol (Figure 1a). Thus, it appears that the rewarding effects of ethanol are decreased in $CB_1^{-/-}$ mice and that higher doses of ethanol are needed in order to produce its motivational effects in these animals. These findings fit well with previously reported data showing that voluntary alcohol consumption and/or preference are decreased in $CB_1^{-/-}$ mice (Hungund *et al*, 2002, 2003; Poncelet *et al*, 2003; Wang *et al*, 2003; Naassila *et al*, 2004). There is a large body of experimental reports demonstrating reliable ethanol-induced CPP in inbred and outbred mice (Crabbe *et al*, 1992; Risinger and Oakes, 1996; Bormann and Cunningham, 1997). In the present study, ethanol at the dose of 0.5 g/kg produced maximal place conditioning effect in wild-type mice. This dose is somewhat lower than those previously reported to induce CPP in mice. Cunningham *et al* (1992a) reported a maximal conditioning effect with doses of 3 and 4 g/kg using DBA/2J mice, whereas Risinger *et al* (1996) demonstrated significant CPP in Swiss–Webster mice with ethanol doses of 1 and 2 g/kg. The higher potency of ethanol in our study compared with the previous reports might be explained by the shorter conditioning trials used in the present study (20 min) compared with 30 and 60 min in the cited study (Risinger and Oakes, 1996). Similarly, it has been recently shown in a study using shorter conditioning trials (20 min) that ethanol at the dose of 0.8 g/kg produced maximal place conditioning effect in mice (Kuzmin *et al*, 2003). The magnitude of the effects of CB_1 receptor deletion on reward forms a coherent picture of the role of these receptors in the rewarding effects of multiple classes of abused substances. Deletion of CB_1 receptors eliminates the rewarding effects of cannabinoids (Ledent *et al*, 1999), opiates (Ledent *et al*, 1999; Martin *et al*, 2000, Cossu *et al*, 2001; but see Rice *et al*, 2002), and nicotine (Castane *et al*, 2002), but leaves the rewarding effects of psychostimulants intact (Martin *et al*, 2000; Cossu *et al*, 2001). As previously described by Martin *et al* (2000), our present results also showed that CB_1 deletion did not influence cocaine (20 mg/kg)-induced CPP (Figure 1b). Surprisingly, ethanol (0.5–1.0 g/kg) elicited a more robust CPP than cocaine (20 mg/kg) in wild-type mice. Given that the affective properties of cocaine (both aversive and rewarding) have been reported to be dose dependent, it is possible to argue that the 20 mg/kg dose of cocaine used in the present study was not the optimal dose to induce CPP. For example, it has been shown that female rats developed CPP at cocaine doses of 5 and 10 mg/kg but not 20 mg/kg, while male rats required higher cocaine doses (20 mg/kg) (Russo *et al*, 2003). In addition, it has also been previously shown that ethanol (2.0 g/kg) and cocaine (15 mg/kg) induced the same degree of preference in the place preference paradigm in mice (McGeehan and Olive, 2003).

Furthermore, deletion of CB_1 receptors did not modify the locomotion elicited by psychostimulants (both cocaine and D-amphetamine) (Figure 2a and b), whereas ethanol-induced locomotor effects have been shown to be altered in $CB_1^{-/-}$ mice (Naassila *et al*, 2004). Thus, contrary to psychostimulants, both ethanol-induced CPP and ethanol-induced locomotor effects are altered in CB_1 knockout mice, suggesting that CB_1 receptors are essential for the expression of behavioral effects of ethanol. In addition, there was a genotypic difference in quinpirole-induced locomotor effects (Figure 2c). The enhanced sensitivity to locomotor effects of quinpirole observed in the present study may be related to the compensatory upregulation of D2 dopamine receptors in $CB_1^{-/-}$ mice (Figure 4b).

Psychoactive cannabinoids increase the extracellular dopamine concentration (Tanda *et al*, 1997) and the activity (French, 1997) of dopaminergic neurons in the ventral tegmental area–mesolimbic pathway. Since these dopaminergic circuits are known to play a pivotal role in mediating the rewarding effects of alcohol (Di Chiara and Imperato, 1988; Weiss and Porrino, 2002), the enhanced dopaminergic drive elicited by cannabinoids could affect ethanol reinforcing effects. Several lines of evidence have indicated that the positive reinforcing effects of ethanol result from activation of common biological mechanisms involving dopamine pathways. Low to moderate doses of ethanol have been extensively reported to increase the firing rate of ventral tegmental dopaminergic neurons (Gessa *et al*, 1985) and, in turn, dopamine release in the nucleus accumbens that has been implicated in stimulating spontaneous locomotor activity in rodents (Imperato and Di Chiara, 1986). An interesting possibility is that $CB_1^{-/-}$ mice have decreased sensitivity to the rewarding effects of ethanol because of the modulation of dopamine release by ethanol via CB1 receptors. Consistent with this hypothesis, it has been recently shown that $CB_1^{-/-}$ mice completely lacked acute alcohol-induced dopamine release in the nucleus accumbens (Hungund *et al*, 2003). The decreased rewarding effects of ethanol in $CB_1^{-/-}$ mice might therefore be related to alteration of ethanol-induced dopamine release via CB_1 receptors in the mesocorticolimbic reward pathway. Recent observations have shown that the endogenous cannabinoid system facilitates the perception or the effects of positive reinforcers such as electrical brain stimulation (Deroche-Gamonet *et al*, 2001) and drugs of abuse (Chaperon *et al*, 1998; Colombo *et al*, 1998). The lack of morphine self-administration in $CB1^{-/-}$ mice was also associated with the inability of morphine to stimulate dopamine release in the nucleus accumbens (Mascia *et al*, 1999), as observed for ethanol (Hungund *et al*, 2003). Previous studies have suggested that the rewarding properties of cannabinoids and opioids might be functionally linked (Tanda *et al*, 1997; Ledent *et al*, 1999; Navarro *et al*, 2001; Vacca *et al*, 2002) and many studies have also shown a complex interaction between ethanol and endogenous opioids (Gianoulakis, 2001). Acute alcohol consumption stimulates opioid peptide release in brain regions related to reward and reinforcement, whereas chronic alcohol consumption induces central opioid deficiency that may be perceived as opioid withdrawal, thereby promoting alcohol consumption via negative reinforcement mechanisms (Gianoulakis, 2001). Interestingly, a recent *in vitro* study suggested that D2 agonists or ethanol (ethanol acting through adenosine release and subsequent activation of A_{2A} adenosine receptors) could act synergistically with δ-opioid or CB_1 receptors to increase PKA signaling (Yao *et al*, 2003). This mechanism may account, in part, for drug-induced activation of medium spiny neurons in the nucleus accumbens and suggests that adenosine and inhibitory GTP-binding proteins are components of a postsynaptic molecular mechanism that hypersensitize dopaminergic signaling in the presence of cannabinoids and ethanol (Yao *et al*, 2003).

These results, taken together with the present results, suggest that the CB_1 null mutation specifically affects both ethanol and opioid self-administration and that this effect might be associated with the inability of these drugs of abuse to stimulate dopamine release in the nucleus accumbens. Heroin-induced CPP and operant heroin self-administration are drastically reduced in $CB_1^{-/-}$ mice (Ledent *et al*, 1999; Martin *et al*, 2000). It has been previously demonstrated that the CB_1 antagonist, SR141716A, reduces intravenous heroin self-administration but does not alter heroin-induced increases in extracellular dopamine levels in the nucleus accumbens shell, showing that CB_1 receptor antagonism reduces the reinforcing properties of heroin through a dopamine-independent mechanism (Caille and Parsons, 2003).

A notable finding in the present study is that the reduced alcohol self-administration (Naassila *et al*, 2004) and alcohol-induced CPP in mice lacking CB_1 receptors is correlated to a compensatory increase in striatal dopamine D2 receptors. The binding of [^{3}H]raclopride to D2 receptors was found to be increased in the striatum of $CB_1^{-/-}$ mice compared to wild-type mice and this difference in the B_{max} value was not associated with a difference in the K_d value (Figure 4b). There is considerable evidence that endogenous cannabinoids modulate the dopaminergic system. Within the striatum, CB_1 receptors have been shown to be localized on the same neurons as Gi-coupled dopamine D2 receptors and an interaction between D2 and CB_1 receptors has been established in primary striatal culture (Glass and Felder, 1997). Concurrent activation of D2 and CB_1 receptors results in an increase in cAMP accumulation in contrast to the inhibition of of cAMP accumulation normally observed with activation of either receptor alone (Glass and Felder, 1997). *In vivo* experiments suggested that chronic treatment with D2 receptor antagonists upregulate CB_1 receptor expression in the rat striatum (Mailleux and Vanderhaeghen, 1993). In addition, pretreatment with the CB_1 antagonist SR141716A enhanced the hyperactivity elicited by the administration of a D2-like receptor agonist, suggesting that the endocannabinoid system may act as an inhibitory feedback mechanism on the hyperlocomotor effects induced by dopamine (Giuffrida *et al*, 1999). Since this inhibitory feedback is lacking in $CB_1^{-/-}$ mice, this could explain the increase locomotor response to quinpirole observed in $CB_1^{-/-}$ mice and the previously reported hyperactivity observed in $CB_1^{-/-}$ mice (Naassila *et al*, 2004). Furthermore, in the present study, the observed alteration of dopamine receptor density was specific to the dopamine D2 receptor since no genotypic difference in the dopamine D1 receptor was observed (Figure 4a). Like D2 dopamine receptors, the CB_1 receptor is negatively coupled to adenylate cyclase via Gi/o protein. Therefore, the CB_1 receptors on the striatal dopamine neurons play a role in inhibiting the dopaminergic neuron activity. This may explain the compensatory upregulation of D2 dopamine receptor binding in mice lacking CB_1 receptors. Similarly, chronic treatment with D2-like receptor antagonists upregulated CB_1 receptor expression in the rat striatum (Mailleux and Vanderhaeghen, 1993). Interestingly, the current observations also reveal dissociations between acute quinpirole-induced locomotor effects and quinpirole-induced CPP. In this regard, $CB_1^{-/-}$ mice displayed different responses to the locomotor effect of low doses of the D2/D3 agonist quinpirole, but not a different sensitivity to its rewarding effects.

Overexpression of D2 receptors has been implicated in reduced self-administration of alcohol in rats (Thanos *et al*, 2001). Thus, it might actually be changes in both the D2 receptor and the CB_1 receptor that reduce alcohol rewarding effects and alcohol self-administration in the $CB_1^{-/-}$ mice. Similar results have been found in the μ-opioid receptor knockout mice that also exhibited increased D2 receptor expression and decreased ethanol-CPP (Roberts *et al*, 2000; Park *et al*, 2001; Tien *et al*, 2003). There is strong evidence of involvement of the D2 receptor in the behavioral effects of ethanol. For example, Cohen *et al* (1997, 1998) demonstrated that the D2 receptor is in involved in both the hyperlocomotor effects of ethanol and ethanol self-administration. With regard to the invovement of the D2 receptor in ethanol-CPP, the results are not clear. Ethanol (2.0g/kg)-CPP has recently been shown to be reduced in D2 receptor knockout mice (Cunningham *et al*, 2000) but not after the D2/D3 antagonist haloperidol treatment (Cunningham *et al*, 1992b; Risinger *et al*, 1992). Results of the present study are not consistent with previous studies that demonstrated lower ethanol intake (Phillips *et al*, 1998) or ethanol-CPP (Cunningham *et al*, 2000) in $D2^{-/-}$ mice but are consistent with previous studies that demonstrated reduced ethanol self-administration following overexpression of D2 receptors in nucleus accumbens (Thanos *et al*, 2001, 2004). It is important to note that CB_1 receptor is involved in ethanol-induced dopamine release in the nucleus accumbens (Hungund *et al*, 2003) and it is possible that this lack of ethanol-induced dopamine release in $CB_1^{-/-}$ mice has a more profound effect on the behavioral effects of ethanol than the 43% increase in the maximum density of D2 sites reported in the present study.

In the elevated plus maze test, mutant and wild-type mice showed equivalent basal level of anxiety (Figure 3) and these data are in line with previous study (Marsicano *et al*, 2002). Previous findings have shown that $CB_1^{-/-}$ mice display an increased sensitivity to the acute intoxicating effects of ethanol (Naassila *et al*, 2004); however, the present study showed that deletion of the CB_1 gene did not modify sensitivity to ethanol's anxiolytic effects, revealing that the ethanol phenotype of $CB_1^{-/-}$ mice is not simply due to a global, unidirectional change in acute sensitivity, but is behavior specific. Ethanol significantly increased the time spent in the open arms to the same extent in $CB_1^{-/-}$ mice compared to $CB_1^{+/+}$ mice. Both genotypes were equally sensitive to ethanol's low-dose locomotor stimulant effects as measured by counting total arm entries. In contrast to our previous study (Naassila *et al*, 2004) using a locomotor activity chamber, which showed ethanol-induced suppression of activity in $CB_1^{-/-}$ mice and ethanol-induced activation of activity in wild-type mice, the present study using the plus maze test showed ethanol-induced activation of activity in both genotypes. It is not immediately clear why the hyperlocomotor effects of 1.5 g/kg ethanol reported here are not consistent with the hypolocomotor effects of 1.5 g/kg ethanol reported previously in $CB_1^{-/-}$ mice (Naassila *et al*, 2004). However, there are number of notable differences between plus maze and locomotor activity chamber testing procedures, for example, the time and the environment of testing. Moreover, the number of total arm entries is not 'pure' measure of locomotor activity (Boerngen-Lacerda and Souza-Formigoni, 2000) and this parameter measured in the present study did not confirm the previously described hyperactivity in $CB_1^{-/-}$ mice (Naassila *et al*, 2004). Our goal was to establish that the anxiolytic properties of ethanol were not confounding our ability to assess ethanol's rewarding properties. The relationship between anxiety and ethanol has been a matter of considerable controversy. It has been demonstrated that a significant ethanol-CPP in rats previously selected to be anxious in the elevated plus maze, but not in the 'nonanxious rats' (Blatt and Takahashi, 1999). Spanagel *et al* (1995), showed a significantly higher intake and preference for ethanol in rats selected as anxious in the plus maze test, which led them to suggest that the degree of anxiety may underlie, at least in part, the initial motivation to drink alcohol. These results agree with the study of Stewart *et al* (1993), which indicated a higher degree of anxiety in ethanol-preferring than nonpreferring rats. In the present study, mutant and wild-type mice showed equivalent basal level of anxiety and equivalent sensitivity to ethanol's anxiolytic effects. Therefore, it is likely that the results of place preference conditioning were not confounded by either different levels of basal anxiety or the potential anxiolytic effect of ethanol.

Altogether, these previous data and the present study suggest that the decreased rewarding effects of ethanol observed in $CB_1^{-/-}$ mice might be related to both an absence of ethanol-induced dopamine release in the nucleus accumbens and an increase in number of dopamine D2 receptors in the striatum. The present results also demonstrated that the increased number of striatal D2 receptors in $CB_1^{-/-}$ mice is associated with a different response to the locomotor effect of low doses of the D2/D3 agonist quinpirole, but not to a different sensitivity to its rewarding effects. Finally, the present findings indicate that the compensatory upregulation of D2 dopamine receptors might be involved in the behavioral effects of ethanol in $CB_1^{-/-}$ mice and suggest that CB_1 receptors are essential for the expression of ethanol rewarding effects.

ACKNOWLEDGEMENTS

We thank D Desavoye and V Warnault for excellent technical assistance.

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