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SPECIAL ISSUE ARTICLE

ETHANOL INDUCES HIGHER BEC IN CB_1 CANNABINOID RECEPTOR KNOCKOUT MICE WHILE DECREASING ETHANOL PREFERENCE

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Abstract — Aims: Previous studies have shown that CB_1 cannabinoid receptors are involved in the behavioural effects induced by chronic ethanol administration in Wistar rats by using SR 141716, a CB_1 cannabinoid receptor antagonist. These studies have now been extended to investigate the effect of acute and chronic alcoholization on blood ethanol concentration (BEC) and ethanol preference in CB_1 knockout (−/−) mice. **Methods:** BEC was monitored for a period of 8 h in both $CB_1^{-/-}$ male mice and CB_1 male wild-type (+/+) mice, which had received an acute i.p. injection of ethanol in 1, 3 or 5 g/kg doses. Ethanol preference was assayed in both groups of male mice in non-forced ethanol administration and forced chronic pulmonary alcohol administration for 14 and 39 days, respectively. **Results:** After an acute intraperitoneal ethanol injection of 5 g/kg, $CB_1^{-/-}$ mice showed a significant higher BEC during the ethanol elimination stage than the $CB_1^{+/+}$ mice. However, those in the 1 and 3 g/kg groups showed no significant difference. A 2–3 fold increase in BEC was observed in $CB_1^{-/-}$ mice on days 10 and 11 after commencement of forced chronic pulmonary alcoholization in comparison with $CB_1^{+/+}$ mice, although comparable BEC values were assayed in both groups on day 12. In addition, these $CB_1^{-/-}$ mice showed a significantly lower preference for ethanol than $CB_1^{+/+}$ mice. **Conclusions:** The studies on $CB_1^{-/-}$ and $CB_1^{+/+}$ mice have clearly confirmed the involvement of CB_1 receptor on ethanol induced behavioural effects and also revealed that CB_1 receptors may be implicated in ethanol absorption/distribution, particularly after administration of high ethanol doses.

INTRODUCTION

The endocannabinoid system consists not only of G-protein-coupled cannabinoid receptors but also other components such as enzymes [i.e. fatty acid amidohydrolase (FAAH)] and endogenous ligands. There are two types of cannabinoid receptors, CB_1 and CB_2. The CB_1 cannabinoid (CB_1) receptor is predominantly expressed in brain at relatively high levels in hippocampus, cerebellum and spinal cord and thus, often referred to as the brain cannabinoid receptor. CB_1 receptors are also expressed at low levels in peripheral tissues including spleen, testis and leucocytes (Herkenham *et al*., 1991; Bouaboula *et al.*, 1993; Lévénès *et al*., 1998; Navarro *et al*., 1998). The CB_2 receptor is referred to as the peripheral cannabinoid receptor because it mainly shows peripheral expression in immune cells (Munro *et al*., 1993; Facci *et al*., 1995). To date, there are two endogenous ligands of CB_1 receptors, namely anandamide and 2-arachydonylglycerol, which mimic the pharmacological action of Δ^9-tetrahydrocannabinol, the active compound of marijuana and other synthetic agonists (Devane *et al*., 1992; Mechoulam and Fride, 1995; Mechoulam *et al*., 1995; Stella *et al*., 1997). There is growing evidence for the implication of endogenous cannabinoids in biological functions, including the control of appetite and food intake (Di Marzo *et al*., 2001), the modulation of some of the pharmacological effects of ethanol (Basavarajappa *et al*., 1998, 2000; Basavarajappa and Hungund, 1999a,b, 2002; Hungund and Basavarajappa, 2000a,b; Hungund *et al*., 2002) and drinking behaviour (Arnone *et al*., 1997; Colombo *et al*., 1998; Gallate *et al*., 1999; Gallate and McGregor, 1999; Rodríguez de Fonseca *et al*., 1999; Freedland *et al*., 2001; Colombo *et al*., 2002; Hungund *et al*., 2002).

Chronic ethanol administration has a dual effect on the cannabinoid receptor, increasing the level of both endogenous cannabinoid agonists, anandamide and 2-arachidonylglycerol, while downregulating the CB_1 receptor number and function, thereby suggesting a role for the endocannabinoid system in the neurobiological effects of ethanol (Basavarajappa *et al*., 1998b, 2000; Basavarajappa and Hungund, 1999a,b). Nonetheless, ethanol did not produce any effects on CB_1 receptor binding and mRNA levels in rats (Gonzalez *et al*., 2002). However, a recent study by Ortiz *et al*. (2004) showed that forced consumption of high quantity of ethanol for a long period significantly decreased the gene expression of the CB_1 receptors in the caudate-putamen, the ventromedial nucleus of hypothalamus and both CA1 and CA2 fields of the hippocampus. The last finding is in accordance with Basavarajappa *et al*. (1998) and Basavarajappa and Hungund (1999a). The result obtained by Gonzalez *et al*. (2002) could be due to differences in the quantity of ethanol and the duration of ethanol administration.

CP-55,940, a CB_1 receptor agonist, promoted alcohol craving in rats (Gallate *et al*., 1999), as well as voluntary ethanol intake in Sardinian alcohol-preferring (sP) rats (Colombo *et al*., 2002). WIN-55,212–2, another CB_1 receptor agonist, also promoted voluntary ethanol intake in sP rats (Colombo *et al*., 2002).

Numerous studies have shown that the CB_1 receptor antagonist SR 141716 reduces ethanol intake (Arnone *et al*., 1997; Colombo *et al*., 1998; Rodríguez de Fonseca *et al*., 1999; Freedland *et al*., 2001) and ethanol craving (Gallate and McGregor, 1999) in different rat strains. In addition,

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SR 141716 suppressed the ethanol deprivation effects (i.e. the temporary increase in ethanol intake after a period of ethanol withdrawal) in sP rats (Serra *et al*., 2002). All these results suggest that the blocking of CB_1 receptor decreases the consumption of ethanol. Nonetheless, it is also important to mention that in our previous study in Wistar rats, we showed that the cannabinoid receptor antagonist SR 141716 profoundly altered ethanol preference in chronically pulmonary alcoholised rats depending on the dose and time of administration. Doses of 3 or 10 mg/kg/day, administered during chronic pulmonary alcoholization enhanced ethanol preference whereas its administration during the ethanol withdrawal stage after alcoholization induced a decrease in ethanol preference (Lallemand and De Witte, 2001). We have also shown that the action of SR 141716 was dependent on a number of factors, including the duration of ethanol intoxication as well as the number of ethanol re-exposures and ethanol withdrawals (Lallemand *et al*., 2004).

All these previous studies used antagonists and agonists of CB_1 receptors. In certain circumstances, some antagonists have side-effects, which could alter/modify their actions. For example, SR-141716, a CB_1 receptor antagonist, can show agonist property (Shire *et al*., 1999).

An alternative to avoid these possible pharmacological side-effects is the use of null mutant mice. The development of transgenic CB_1 knockout mice has provided the opportunity to study the role of the CB_1 receptor system in the regulation of ethanol consumption (Ledent *et al*., 1999; Zimmer *et al*., 1999).

$CB_1^{-/-}$ mice with CD_1 background showed decreased ethanol intake and preference. These effects were associated with a dramatic sensitivity to the hypothermic and hypolocomotor effects in response to low doses of ethanol (Naassila *et al*., 2004). These mice also showed an increased intensity of ethanol withdrawal-induced convulsions. Female $CB_1^{+/+}$ mice consumed more ethanol than male $CB_1^{+/+}$ mice; in addition, this gender difference was observed in both genotypes — female $CB_1^{-/-}$ mice showed a decreased ethanol consumption compared with that of female $CB_1^{+/+}$ mice, but did consume the same quantity of ethanol as did male $CB_1^{+/+}$ mice. Hungund *et al*. (2003) observed similar results, although the gender difference in ethanol consumption observed between female and male $CB_1^{+/+}$ mice was abolished in $CB_1^{-/-}$ mice. These results were also observed in the study of Poncelet *et al*. (2003) using $CB_1^{-/-}$ mice with C57BL/6 × 129/Ola F2 background.

$CB_1^{-/-}$ with C57BL/6J background had a higher preference for ethanol but only for a few days (Racz *et al*., 2003). After the cessation of chronic ethanol administration, these mice did not exhibit withdrawal symptoms. After mild intermittent foot-shock stress, alcoholized $CB_1^{-/-}$ mice did not consume an increased amount of ethanol as did the $CB_1^{+/+}$ mice for the next 24 h. The activation of CB_1 receptors in wild-type mice will also contribute to the high ethanol preference exhibited by C57BL/6J mice (Wang *et al*., 2003) as SR 141716 is able to reduce ethanol drinking when administered to these mice and not in $CB_1^{-/-}$ mice. Young and old $CB_1^{-/-}$ mice with this genetic background displayed low ethanol preference. On the contrary, $CB_1^{+/+}$ mice presented an age-dependent decline in ethanol preference, suggesting that the decline in ethanol preference is related to a loss of cannabinoid signalling in the limbic forebrain.

It could be hypothesized that there was an interaction of gender and expression of phenotype associated with the CB_1 gene mutation. The total fluid intake was similar between the different genotypes, although differences were evident between males and females within the same genotype. $CB_1^{-/-}$ male mice did not show the acute ethanol-induced increase in dopamine levels in nucleus accumbens compared with $CB_1^{+/+}$ mice, which would indicate that activation of the limbic system was required for the reinforcing effects of ethanol (Hungund *et al*., 2003).

The purpose of our study was to investigate the effect of a low to high acute intraperitoneal ethanol injection on blood ethanol concentration (BEC), as well as the effects of non-forced ethanol administration and forced chronic pulmonary ethanol intoxication on ethanol preference by comparing $CB_1^{-/-}$ and $CB_1^{+/+}$ mice to ascertain the precise involvement of the cannabinoid system on ethanol-related behavioural effects.

MATERIALS AND METHODS

Homozygous $CB_1^{-/-}$ male mice were compared with homozygous $CB_1^{+/+}$ male wild-type mice. $CB_1^{+/+}$ and $CB_1^{-/-}$ mice were from a C57BL/6J × 129/Ola (Harlan) F2 genetic background and generated as described previously (Robbe *et al*., 2002; Ravinet-Trillou *et al*., 2003). No backcrosses were performed. These mice were provided by Sanofi-Recherche Synthélabo (Montpellier, France). The mice were housed in clear plastic cages with steel wire fitted tops and wood chip bedding under standard conditions (normal 12 h light–dark cycles, light on at 08:00 h, constant room temperature of 25 ± 1°C) with commercial lab chow diet and tap water available *ad libitum* during the entire experiments.

Acute ethanol experiments

BEC was assayed in $CB_1^{-/-}$ and $CB_1^{+/+}$, 30–32 g, 12-weeks-old, male mice, housed 5/cage, after an intraperitoneal injection of ethanol. The experiment was carried out in their home cages. Blood samples were collected from the retro-orbital sinus under slight ether anaesthesia where necessary, into haematocrit tubes at 20, 40 min and 1, 2, 4, 9 and 12 h, after either 1 or 3 g/kg ethanol doses (15% v/v), while an additional two samples at 14 and 16 h were also collected after the 5 g/kg dose. This procedure followed the schedule of blood drawing used by Bruguerolle and Dubus (1993), Bruguerolle *et al*. (1994) and Hettiarachchi *et al*. (2001). Blood from each haematocrit tube was transferred into microcentrifuge tubes containing sodium fluoride as an anticoagulant. The concentration of blood ethanol was assayed by an alcohol-dehydrogenase-based method (Aufrère *et al*., 1997).

Chronic ethanol experiments

Non-forced ethanol administration experiments. $CB_1^{-/-}$ and $CB_1^{+/+}$, 30–32 g, 12-weeks-old, male mice, were housed 2/cage. Fluid intake (water and 10% v/v ethanol when present) was recorded every 1 or 2 days, and body weight every week.

Free-choice period. Two drinking bottles were placed in each cage, one containing tap water and the other, 10% v/v ethanol solution. The mice had continuous access to the

drinking tips of both tubes. The position of the tubes was changed every day, in order to avoid possible bias due to place preference. The ratio of the 24 h intake from the ethanol bottle versus total fluid intake was used to define preference and the absolute amount (g/kg body weight/day) of ethanol consumed was also calculated.

Forced chronic pulmonary ethanol administration procedure. The motility of $CB_1^{-/-}$ and $CB_1^{+/+}$, 30–32 g, 12 weeks old, male mice, was recorded, after 3 weeks of acclimatization, for 18 h by the MacLab system, the recordings being combined for each hourly interval. The apparatus has been described in detail previously (Lallemand and De Witte, 2001).

Forced chronic alcoholization was induced in these mice, housed in pairs of two, within a plastic chamber ($120 \times 60 \times 60$ cm) by pulmonary inhalation of a mixture of ethanol and air. The mixture was pulsed into the chamber via a mixing system that allowed the quantity of ethanol to be increased every day, so that the average BEC continued to rise (Le Bourhis, 1975; Aufrère *et al*., 1997) during the experimental procedure. The animals remained for 12 days in the alcohol chamber. The chamber temperature ranged between 28–30°C (Terdal and Crabbe, 1994; Finn and Crabbe, 1999). BECs were determined regularly during the chronic alcoholization. Blood from each haematocrit tube was transferred into microcentrifuge tubes containing sodium fluoride as an anticoagulant. The concentration of blood ethanol was assayed by an alcohol-dehydrogenase-based method.

Withdrawal motility and free-choice period. At the end of the forced chronic pulmonary alcoholisation period the motility and ethanol preference was studied in these two groups of mice. For the measurement of ethanol preference, the mice from each strain underwent three successive steps (Le Bourhis, 1977) on cessation of the chronic ethanol intoxication. First, full beverage deprivations, i.e. the drinking bottles were removed during the last 6 h of the chronic alcoholization procedure and the following 18 h of the withdrawal period. The motility of each mouse was recorded during these 18 h using the same apparatus described above. Secondly, a 10% (v/v) ethanol solution was given as the sole drinking fluid during the following 24 h. Thirdly, a free-choice beverage situation [water vs 10% (v/v) ethanol solution] was presented for a period of 39 days. During this free-choice period, the fluid consumptions were recorded daily and ethanol consumption expressed as a percentage of total fluid intakes and as ethanol intake in g/kg of body weight. The positions of the drinking bottles were changed every day to avoid position preference. BECs were assayed at different time points during the free-choice period by the method described above. The weight of animals was recorded every 3 or 4 days.

In all experiments, the results are presented as mean ± standard error (SE) except where stated otherwise. In all experiments, groups were compared by two-way analysis of variance (ANOVA) (genotype; time) with repeated measures on time. Where appropriate, *post hoc* pair wise comparisons were analysed by the least-significant difference test of multiple comparisons (Fisher LSD protected *t*-test) (GB-STAT 5.3 for Windows, Dynamic Microsystems, Silver Spring, MD, USA). Criterion for significance was set at $P < 0.05$ for all tests.

The Belgian Governmental Agency under the authorized number LA 1220028 as well as the European Communities Council Directive concerning the Use of Laboratory Animals approved these experiments.

Products

Absolute ethanol, used in the free-choice paradigm and acute experiment, was obtained from Labotec (La Gleize, Belgium). Ethanol at 15% (w/v) was prepared for i.p. injection in 0.9% saline. Ethanol at 97% was obtained from Belgalco SA (Belgium). Sodium fluoride was from Sigma Aldrich, (Steinheim, Germany).

RESULTS

Acute ethanol experiments

The BECs were similar in both groups of mice after either 1 or 3 g ethanol/kg doses [$F(1,56) = 0.0005$, $P = 0.982$ and $F(1,56) = 0.1161$, $P = 0.7421$ respectively]. However, after an acute injection of 5 g/kg of ethanol, $CB_1^{-/-}$ mice showed significant differences compared to the $CB_1^{+/+}$ mice [$F(1,8) = 19.254$, $P = 0.0022$], with a significantly higher BEC than the $CB_1^{+/+}$ mice [$F(9,72) = 2.981$, $P = 0.0045$] (Fig. 1).

Chronic ethanol experiments

Non-forced ethanol administration experiments. After 1 week of measurements, $CB_1^{-/-}$ mice showed a significantly higher water consumption in comparison to $CB_1^{+/+}$ mice [$F(1,54) = 6.8364$, $P = 0.0176$] (data not shown). The mean water consumptions over the time of the experiment were 10.8 ± 1.24 and 13.8 ± 0.87 ml, respectively for $CB_1^{+/+}$ and $CB_1^{-/-}$ mice.

At the conclusion of the study, the $CB_1^{-/-}$ mice showed a significantly lower mean weight in comparison to controls [$F(1,38) = 7.3466$, $P = 0.01$]. The mean weights were 32.18 ± 0.62 and 30.16 ± 0.36 g, respectively, for $CB_1^{+/+}$ and $CB_1^{-/-}$ mice.

Free choice. $CB_1^{-/-}$ mice showed a significantly reduced ethanol preference (expressed as a percentage of total fluid intake) in comparison to control mice [$F(1,12) = 8.6787$; $P = 0.0122$] (Fig. 2A). There was also a significant interaction between genotype and time [$F(8,96) = 2.1965$, $P = 0.0342$]. Nonetheless, when ethanol preference is expressed as ethanol intake in g/kg of body weight, the genotype significance disappeared totally and only the interaction remained [$F(38,418) = 3.9539$; $P < 0.0001$] (Fig. 2B). The mean ethanol intake over the time of the experiment was 12.28 ± 0.48 and 13.12 ± 0.59 g/kg/day, respectively, for $CB_1^{-/-}$ and $CB_1^{+/+}$ mice.

When comparing liquid type consumptions, i.e. water and ethanol, ethanol volume consumed in mice of either genotype was not significantly different [$F(1,12) = 0.2861$, $P = 0.6025$]. On the contrary, $CB_1^{-/-}$ mice consumed significantly more water than $CB_1^{+/+}$ mice [$F(1,12) = 14.1872$, $P = 0.0027$].

During the free-choice period the total consumption (water + 10% v/v ethanol) of $CB_1^{-/-}$ mice was not significantly different in comparison to control mice [$F(1,26) = 3.3544$, $P = 0.0785$] (data not shown), but there was a significant interaction between genotype and time [$F(8,208) = 4.3224$, $P < 0.0001$]. Nonetheless, the total consumption of

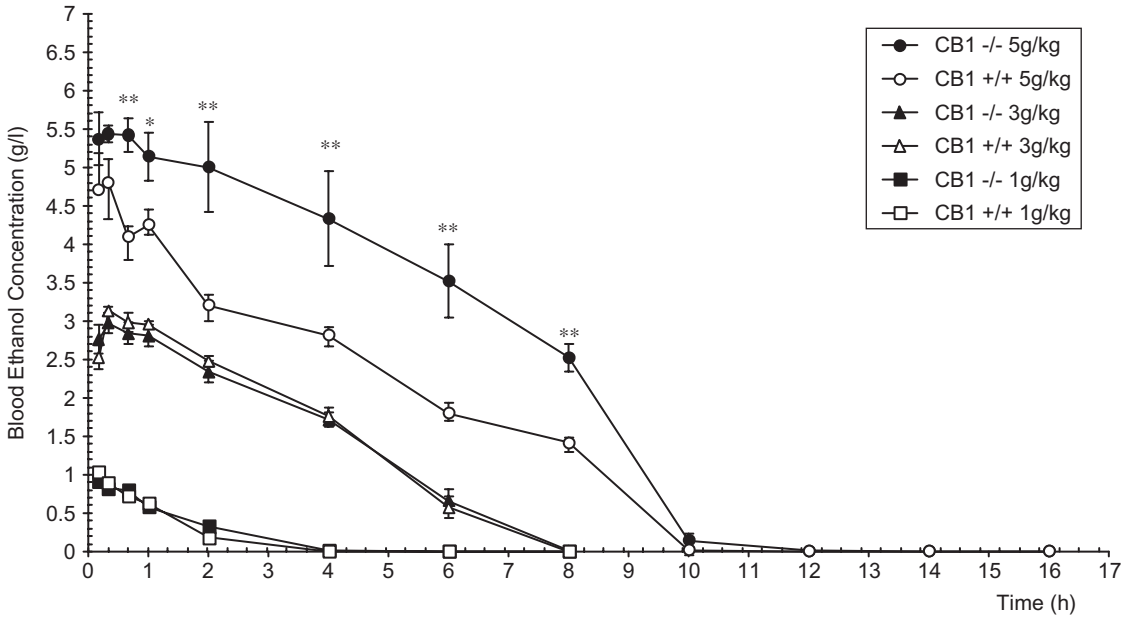

Fig. 1. Time course of BEC after an acute i.p. injection of 1, 3 or 5 g/kg ethanol in $CB_1^{-/-}$ and $CB_1^{+/+}$ mice. Open symbols represent the $CB_1^{+/+}$ mice and filled symbols represent $CB_1^{-/-}$ mice: circles for 5 g/kg ethanol, triangles for 3 g/kg ethanol and squares for 1 g/kg ethanol. Significant time points between $CB_1^{+/+}$ mice (control) and $CB_1^{-/-}$ mice are represented by $*P < 0.05$ and $**P < 0.01$, relative to their control. Results are presented as mean ± SE.

$CB_1^{-/-}$ mice was always above that of control mice. The mean consumptions over the time of the experiment were 9.67 ± 0.70 and 7.23 ± 0.33 ml/24 h, respectively, for $CB_1^{-/-}$ and $CB_1^{+/+}$ male mice.

Forced chronic ethanol pulmonary administration experiments. The motility of $CB_1^{-/-}$ mice, prior to forced chronic ethanol pulmonary administration, was not significantly different in comparison to $CB_1^{+/+}$ mice [$F(1,510) = 0.7872$, $P = 0.382$].

In the $CB_1^{-/-}$ mice the mean BEC assayed at different time points during the forced chronic alcoholization regime were significantly different than the mean levels in the $CB_1^{+/+}$ mice [$F(1,26) = 25.887$, $P < 0.0001$] characterized by a significant higher BEC level at both 10 and 11 days after the commencement of forced chronic pulmonary alcoholization. [$F(6,156) = 7.931$, $P < 0.0001$] (Fig. 3). At 10 days, the mean BEC was 3 fold higher in the $CB_1^{-/-}$ mice than in the $CB_1^{+/+}$ mice, whereas at 11 days, it showed a 2 fold increase. However, on Day 13 no significant difference in mean BEC was assayed.

During the forced chronic pulmonary alcoholization period, water consumption of $CB_1^{-/-}$ mice was not significantly different [$F(1,18) = 1.7514$, $P = 0.2023$] (data not shown). The mean water consumptions over the time of the experiment were 9.82 ± 0.5 and 8.76 ± 0.48 ml/24 h, respectively, for $CB_1^{-/-}$ and $CB_1^{+/+}$ mice.

$CB_1^{-/-}$ mice had a significantly lower body weight than $CB_1^{+/+}$ mice [$F(1,38) = 7.3466$, $P = 0.01$]. The mean weights over the time of the experiment were 30 ± 0.46 and 32 ± 0.62 g, respectively, for $CB_1^{-/-}$ and $CB_1^{+/+}$ mice.

Following forced chronic pulmonary alcoholization, similar motilities were assayed for both $CB_1^{-/-}$ and control mice [$F(1,340) = 0.8442$, $P = 0.3704$] as they were also similar prior to the chronic pulmonary alcoholization.

Free choice. During the first 24 h period after forced chronic pulmonary alcoholisation, there were no significant differences in ethanol consumption between $CB_1^{-/-}$ and $CB_1^{+/+}$ mice [$F(1,11) = 0.4936$, $P = 0.4969$]. The mean ethanol consumptions in the alcoholized $CB_1^{+/+}$ group and in the alcoholized $CB_1^{-/-}$ group were, 18.69 ± 1.69 ml and 16.75 ± 1.29 ml, respectively. During the free-choice period, ethanol preference, expressed as percentage of total fluid consumption, of $CB_1^{-/-}$ mice showed no significance at the genotype level when compared with $CB_1^{+/+}$ mice [$F(1,11) = 2.1819$, $P = 0.1677$] (Fig. 4A). There was also absence of significance when ethanol preference was expressed as ethanol intake/kg body weight [$F(1,11) = 1.6614$, $P = 0.2239$] (Fig. 4B). Nonetheless, in both representations of ethanol preference, there were always significant interactions between genotype and time [$F(38,418) = 2.345$, $P < 0.0001$ and $F(38,418) = 3.9539$, $P < 0.0001$, respectively, for percentage of total fluid consumption (Fig. 4A) and ethanol intake expressed in g/kg body weight (Fig. 4B)]. The mean ethanol intakes in all experiments were 17.96 ± 0.52 and 22.05 ± 0.69 g/kg, respectively, for $CB_1^{+/+}$ and $CB_1^{-/-}$ mice.

When the ethanol and water consumptions in each genotype were compared, no significant differences between the two liquids in both $CB_1^{+/+}$ [$F(1,12) = 0.0356$, $P = 0.8534$] and $CB_1^{-/-}$ mice [$F(1,10) = 3.5586$, $P = 0.0886$] were apparent. Nonetheless, there were always significant interactions between liquid type and time in both genotypes [$F(38,456) = 8.1675$, $P < 0.0001$ and $F(38,380) = 6.2574$, $P < 0.0001$, respectively, for $CB_1^{+/+}$ and $CB_1^{-/-}$]. In $CB_1^{+/+}$ mice, the consumptions of water

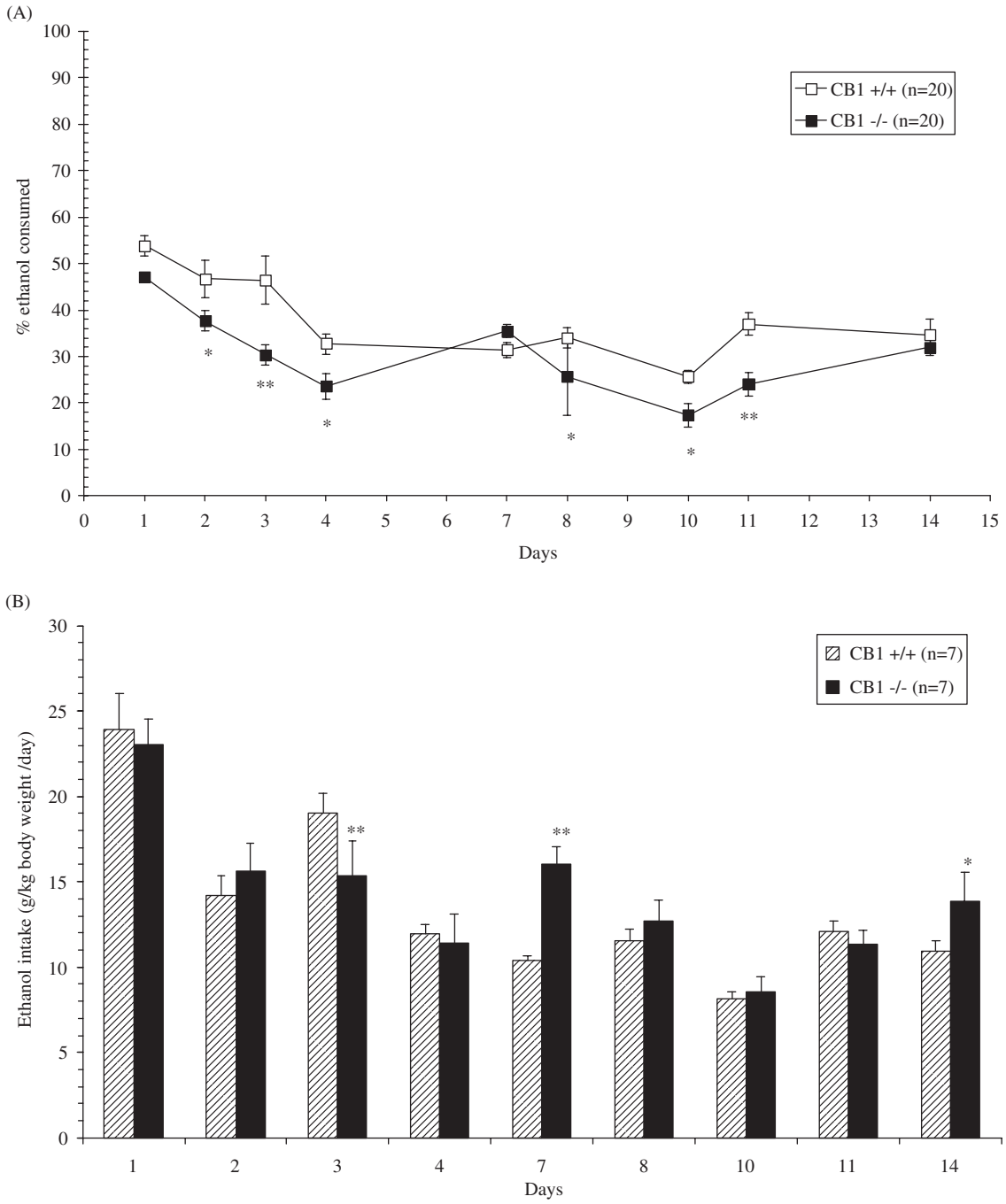

Fig. 2. (A) Time course of ethanol preference expressed as percentage of total fluid intake of $CB_1^{-/-}$ and $CB_1^{+/+}$ mice in non-forced ethanol intake free choice. Open squares represent the $CB_1^{+/+}$ mice and filled squares the $CB_1^{-/-}$ mice. Significant time points between $CB_1^{+/+}$ mice (control) and $CB_1^{-/-}$ mice are represented by $^*P < 0.05$ and $^{**}P < 0.01$. Results are presented as mean ± SE. (B) Time course of ethanol preference expressed as g/kg body weight/day ethanol intake of $CB_1^{-/-}$ and $CB_1^{+/+}$ mice in non-forced ethanol intake free choice. Hatched bars represent the $CB_1^{+/+}$ mice and filled bars the $CB_1^{-/-}$ mice. Significant time points between $CB_1^{+/+}$ mice (control) and $CB_1^{-/-}$ mice are represented by $^*P < 0.05$ and $^{**}P < 0.01$. Results are presented as mean ± SE.

and ethanol were very similar (8.42 ± 0.25 and 8.93 ± 0.23 ml, respectively, for ethanol and water). In contrast, in $CB_1^{-/-}$ mice, the ethanol consumption was always lower than the intake of water (8.81 ± 0.27 and 11.21 ± 0.21 ml, respectively, for ethanol and water). In addition, water intake of $CB_1^{-/-}$ mice was higher than in the $CB_1^{+/+}$ mice.

The total liquid consumption was not significantly different in $CB_1^{-/-}$ and $CB_1^{+/+}$ mice [$F(1,11) = 1.3981$, $P = 0.262$], although there was a significant interaction between genotype and time [$F(38,418) = 3.5209$, $P < 0.0001$]. The total consumption values for $CB_1^{-/-}$ mice were always greater or at the same level for those of the $CB_1^{+/+}$ mice.

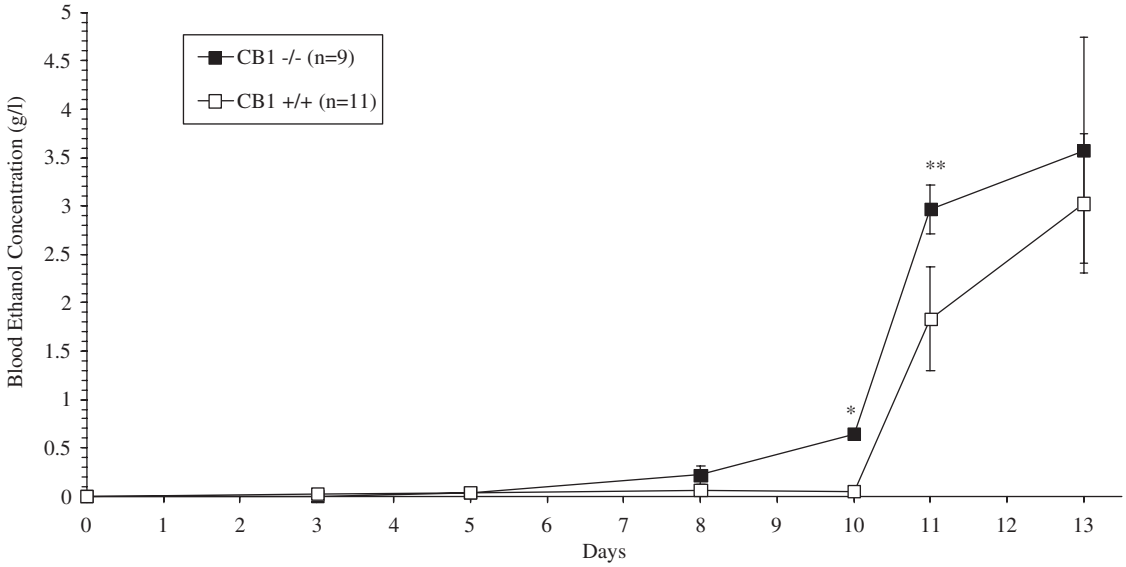

Fig. 3. Time course of BEC during forced chronic pulmonary alcoholization of $CB_1^{-/-}$ and $CB_1^{+/+}$ mice. Open squares represent the $CB_1^{+/+}$ mice and filled squares the $CB_1^{-/-}$ mice. Significant time points between $CB_1^{+/+}$ mice (control) and $CB_1^{-/-}$ mice are represented by $*P < 0.05$ and $**P < 0.01$. Results are presented as mean ± SE.

During the free-choice paradigm, there was no significant difference between the BEC values at the genotype level [$F(1,4) = 3.092$, $P = 0.1535$]; the values assayed being less than 0.02 g/l in both groups of mice (data not shown). However, there was a significant interaction between genotype and time [$F(5,20) = 2.849$, $P = 0.0422$], as well as for time [$F(5,20) = 20.192$, $P < 0.0001$].

During the free-choice period, $CB_1^{-/-}$ mice showed a significantly lower body weight than the $CB_1^{+/+}$ mice [$F(1,18) = 9.5004$, $P = 0.0064$] (data not shown). There was also a significant interaction between genotype and time [$F(10,180) = 6.851$, $P < 0.0001$]. The body weight of $CB_1^{-/-}$ mice at the beginning of the study was 31.2 ± 0.6 and 31.14 ± 0.67 g at its conclusion. The body weight of $CB_1^{+/+}$ mice was 32.36 ± 0.75 g at the beginning of the study and was 37.64 ± 1.39 g at the end.

DISCUSSION

Recently, mouse specific gene deletions have been used to investigate the role of the endocannabinoid system in alcohol research. In this study we assessed the effect of CB_1 receptor null mutation on ethanol preference in both non-alcoholized and chronically alcoholized mice as well as ethanol clearance after an acute ethanol i.p. injection. The acute ethanol injection in mice lacking the CB_1 receptor showed an unexpected result in that the ethanol peak concentration for the high ethanol dose, 5 g/kg, induced a significantly higher ethanol peak concentration in $CB_1^{-/-}$ mice. However, the ethanol elimination rates for the lower doses, 1 and 3 g/kg, were similar in both $CB_1^{-/-}$ and $CB_1^{+/+}$ mice. This has not been described previously in the literature for $CB_1^{-/-}$ mice. Nonetheless, the influence of the cannabinoid system on the metabolism of ethanol was reported in one study where the administration of cannabinoid receptor inhibitor SR 141716 induced no changes in ethanol metabolism in rats (Colombo *et al*., 1998). It is difficult to interpret these present results. As the 1 and 3 g/kg ethanol doses showed no significant change between $CB_1^{-/-}$ and $CB_1^{+/+}$, we hypothesized that, with respect to the high dose of ethanol used, the lack of CB_1 cannabinoid receptors in the enteric nervous system, particularly at the level of the gastrointestinal tract of $CB_1^{-/-}$ mice, might interfere with the absorption/distribution of ethanol (Batkai *et al*., 2001; Pertwee, 2001). However, this lack would intervene only with a high acute dose of ethanol.

In mice with non-forced ethanol administration, ethanol preference ratio was significantly reduced in $CB_1^{-/-}$ mice, but when ethanol preference was expressed as g/kg body weight per day, no significances appeared. These results are in agreement with those obtained by Wang *et al*. (2003) for ethanol preference ratio. Nonetheless, other studies by Hungund *et al*. (2003) and Poncelet *et al*. (2003) observed that ethanol intake expressed in g/kg body weight/day was significantly reduced in $CB_1^{-/-}$ mice as well. This discrepancy on the preference in ethanol intake was unclear. In our study, the absence of significance in preference as expressed in g/kg body weight/day is mainly the result of a higher, but not significant, total liquid intake of the $CB_1^{-/-}$ mice.

In chronically forced alcoholized mice, the BEC in $CB_1^{-/-}$ mice peaked faster than in $CB_1^{+/+}$ mice, although the maximum values obtained were not significantly different. This result has not been observed in previous studies, although Colombo *et al*. (1998b) showed that the antagonism at CB_1 cannabinoid receptors did not modify ethanol metabolism. In our study, the difference between $CB_1^{-/-}$ and $CB_1^{+/+}$ mice was noted only during the increase of BEC but not at the end of the chronic alcoholization period. Unlike other chronic alcoholization

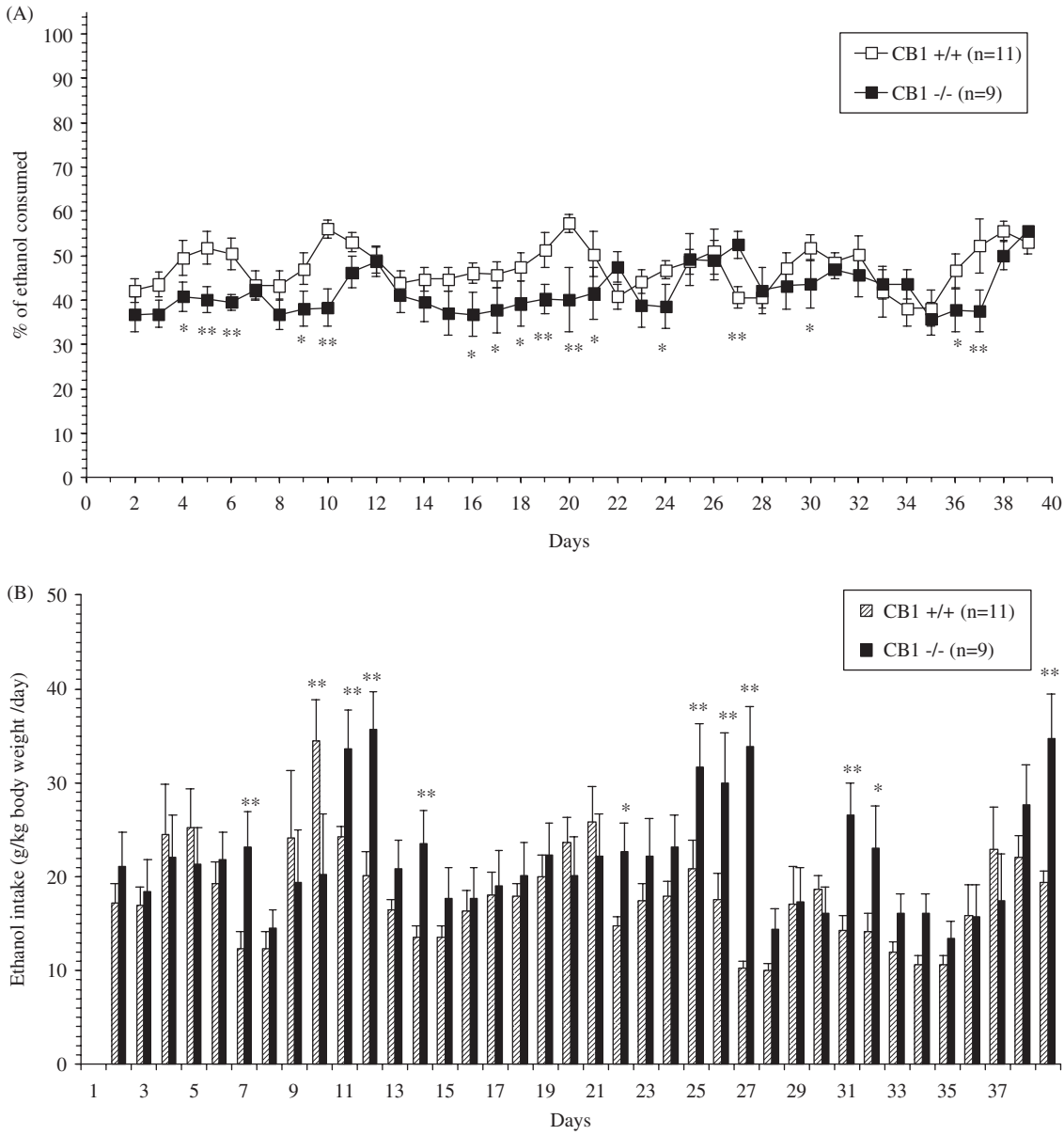

Fig. 4 (A) Time course of ethanol preference expressed as percentage of total fluid intake of $CB_1^{-/-}$ and $CB_1^{+/+}$ mice after forced chronic pulmonary alcoholization. Open squares represent the $CB_1^{+/+}$ mice and filled squares the $CB_1^{-/-}$ mice. Significant time points between $CB_1^{+/+}$ mice (control) and $CB_1^{-/-}$ mice are represented by $^*P < 0.05$ and $^{**}P < 0.01$. Results are presented as mean ± SE. (B) Time course of ethanol preference expressed as g/kg body weight/day ethanol intake of $CB_1^{-/-}$ and $CB_1^{+/+}$ mice after forced chronic pulmonary alcoholization. Hatched bars represent the $CB_1^{+/+}$ mice and filled bars the $CB_1^{-/-}$ mice. Significant time points between $CB_1^{+/+}$ mice (control) and $CB_1^{-/-}$ mice are represented by $^*P < 0.05$ and $^{**}P < 0.01$. Results are presented as mean ± SE.

procedures, our protocol of chronic alcoholization is a forced one, i.e. animals were unable to adjust the amount of ethanol ingested by themselves. Our procedure of chronic alcoholization induced other mechanisms involved in ethanol metabolism microsomal ethanol oxidizing system MeOS/ cytochrome P450IIE (Lieber, 1999) and alcohol dehydrogenase (Kishimoto *et al*., 1995), which have not been studied to date in these knockout animals.

After forced chronic pulmonary alcoholization, the ethanol consumption in $CB_1^{-/-}$ mice was similar to that of $CB_1^{+/+}$ when access to 10% (v/v) ethanol solution was given. In contrast, when $CB_1^{-/-}$ mice had access to both drinking bottles, i.e. free choice, their ethanol preference was significantly lower than $CB_1^{+/+}$ mice when expressed as percentage of total consumption. This result is in agreement with our previous study in Wistar rats of the action of the CB_1 cannabinoid receptor inhibitor SR 141716 (Lallemand and De Witte, 2001) and data reported recently by Hungund *et al*. (2003), Poncelet *et al*. (2003), Racz *et al*. (2003), Wang *et al*. (2003) and Naassila *et al*. (2004), which show that a CB_1 receptor

antagonist decreases ethanol consumption in rats and mice. Nevertheless, when ethanol preference is expressed as g/kg body weight/day, $CB_1^{-/-}$ mice presented significant ethanol intake time point higher than $CB_1^{+/+}$ mice.

In both non-forced alcoholized and chronically forced alcoholized experiment, the $CB_1^{-/-}$ mice showed a significantly lower weight than the $CB_1^{+/+}$ mice. This result was in contradiction to the results from a previous study (Wang *et al*., 2003) where no difference was observed when the animals had free access to the food. A weight difference between $CB_1^{-/-}$ and $CB_1^{+/+}$ mice has been described between gender (Hungund *et al*., 2003) when there is restricted food access. In another study, $CB_1^{-/-}$ mice gained less weight than $CB_1^{+/+}$ mice when fed with high fat diet (Ravinet-Trillou *et al*., 2003). Conversely, these data could be interpreted as a higher weight gain by $CB_1^{+/+}$ mice, which is in accordance with the results of Wang *et al*. (2003), although in our experiments the mice had full access to the food. This effect could be due to the length of the experiment and the presence of ethanol, which modulates endocannabinoid levels in neuronal cells (Gonzalez *et al*., 2002).

Both CB_1 genotypes showed no significant differences in their motility irrespective of whether they were chronically forced alcoholized or not. There was also no difference in motilities before and after chronic alcoholization. These results are in agreement with those observed in the study of Racz *et al*. (2003) where $CB_1^{-/-}$ mice showed no withdrawal symptoms when compared with $CB_1^{+/+}$ mice. In contrast, Naassila *et al*. (2004) reported an increased ethanol withdrawal severity in $CB_1^{-/-}$ mice. This discrepancy in the results obtained in those studies may be caused by the use of different measures for alcohol withdrawal symptoms.

In conclusion, these data showed: (1) a higher BEC in $CB_1^{-/-}$ mice after a high acute ethanol dose of 5 g/kg; (2) during forced chronic pulmonary alcoholization, higher BEC levels are reached at an earlier time point in $CB_1^{-/-}$ mice, and (3) $CB_1^{-/-}$ mice show a lower ethanol preference. These results strongly support an important role for the endocannabinoid–CB_1 receptor system in ethanol drinking behaviour as well as other actions of ethanol. Further studies of enzymes involved in the pharmacokinetics of ethanol are needed to explain the apparent differences in ethanol absorption/distribution observed in $CB_1^{-/-}$ mice after high doses of ethanol.

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