Genetic and Pharmacological Manipulations of the CB₁ Receptor Alter Ethanol Preference and Dependence in Ethanol Preferring and Nonpreferring Mice

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ABSTRACT Recent studies have indicated a role for the endocannabinoid system in ethanol-related behaviors. This study examined the effect of pharmacological activation, blockade, and genetic deletion of the CB₁ receptors on ethanol-drinking behavior in ethanol preferring C57BL/6J (B6) and ethanol nonpreferring DBA/2J (D2) mice. The deletion of CB₁ receptor significantly reduced the ethanol preference. Although the stimulation of the CB₁ receptor by CP-55,940 markedly increased the ethanol preference, this effect was found to be greater in B6 than in D2 mice. The antagonism of CB₁ receptor function by SR141716A led to a significant reduction in voluntary ethanol preference in B6 than D2 mice. A significant lower hypothermic and greater sedative response to acute ethanol administration was observed in both the strains of CB₁⁻/⁻ mice than wild-type mice. Interestingly, genetic deletion and pharmacological blockade of the CB₁ receptor produced a marked reduction in severity of handling-induced convulsion in both the strains. The radioligand binding studies revealed significantly higher levels of CB₁ receptor-stimulated G-protein activation in the striatum of B6 compared to D2 mice. Innate differences in the CB₁ receptor function might be one of the contributing factors for higher ethanol drinking behavior. The antagonists of the CB₁ receptor may have therapeutic potential in the treatment of ethanol dependence. Synapse 62:574–581, 2008.

INTRODUCTION

Chronic alcoholism is a serious mental disorder characterized by impaired control over drinking, leading to tolerance and physical dependence. Although, genetic and environmental factors have been shown to play important role in the development of ethanol dependence, the underlying mechanism is still unclear. Understanding the molecular mechanism of...
ethanol dependence is critical to the treatment of ethanol-related disorders, as withdrawal severity is a major risk factor for relapse.

In the recent years, a number of studies have suggested a close interaction between the endocannabinoid system and ethanol-related behaviors (Colombo et al., 2007; Lallemand et al., 2001; Vinod and Hungund, 2006). The endocannabinoid system, which consists of cannabinoid (CB) receptors, endocannabinoids, and proteins that are involved in the regulation and metabolism of endocannabinoids, has received much attention in understanding of its functional significance in drug abuse and other neuropsychiatric disorders. Two types of CB receptors, CB1 and CB2, have been identified till to date, which belong to the G-protein coupled receptor (GPCRs) family. The endocannabinoids, N-arachidonyl ethanolamide anandamide (AEA) is a partial agonist whereas 2-arachidonylglycerol is a full agonist for the CB receptors. Recent studies have also demonstrated that AEA act as a full agonist (endovanilloid) at the vanilloid (VR1) receptors (Sugiura et al., 2006).

The CB1 receptor is one of the most abundant neurenomodulatory GPCRs that is expressed in the cortex, striatum, hippocampus, and cerebellum (Howlett, 2005). Recent studies have also revealed the existence of CB2 receptors in the brain (Oana et al., 2006; Van Sickle et al., 2005). In the CNS, the endocannabinoids are synthesized in the postsynaptic neurons and act as retrograde messengers to activate presynaptically located G\(_i/o\)-coupled CB1 receptors that modulate adenyl cyclase, ion channels, and extracellular signal-regulated kinases (Howlett, 2005). Studies from our laboratory and others have suggested a role for both CB1 and CB2 receptors in alcohol addiction (Ishiguro et al., 2007; Vinod and Hungund, 2006).

Epidemiological studies have established that alcoholism is a familial disorder with significant genetic contribution (Heath et al., 2001). Several animal models have been developed to understand a genetic basis of alcoholism. In this regard, C57BL/6J (B6; ethanol preferring) and DBA/2J (D2; ethanol nonpreferring) mice have been shown to exhibit striking differences in ethanol related behavior. This study was designed to investigate a role of the CB1 receptor in ethanol-related behaviors using pharmacological and genetic manipulation of the CB1 receptor function. This study further examined whether the genetically predetermined differences in the brain CB1 receptor function could explain the changes in behavioral consequences of ethanol drinking behavior.

**MATERIALS AND METHODS**

**Animals**

The CB1 heterozygous (+/−) mice with mixed B6.129Sv(B6)-Cnr1^tmZim background were generated as described previously (Zimmer et al., 1999). These mice were further backcrossed for 10 generations with B6 background to reach genetic homogeneity. In addition, the heterozygous mice (CB1 +/-) with B6 background were interbred with D2 strain to generate CB1 +/- mice (F1) and were further backcrossed with D2 mice for 10 generations. A marker-assisted selection protocol was used to evaluate the amount of B6 and D2 background genes in each generation using microsatellite markers (Invitrogen, Carlsbad, CA) as previously described (Hospital et al., 1992). This technique is advantageous in that it significantly accelerates the pace of congenic strain development. Twelve to thirteen-week old male homozygous wild (+/+) and knockout (−/−) mice used in this study were derived from the intercrosses between the 10th generation heterozygous mice. Animal care and handling procedures were in accordance with Institutional and National Institutes of Health guidelines.

**Chemicals**

CP-55,940, HU-210 (Tocris Cookson, Ellisville, MO), and SR141716A (NIDA, Bethesda, MD) were dissolved in physiological saline with 1% Tween 80 and 1% DMSO. Ethanol (Pharmaco Products, Brookfield, CT) solution was prepared in physiological saline for ethanol tolerance and dependence studies. Ethanol (95%, David Sherman Corporation, St Louis, MO) solutions were prepared in tap water and used for drinking studies. Pyrazole (Sigma-Aldrich, St Louis, MO) was dissolved in saline and injected at a volume of 10 ml/kg (68 mg/kg).

**Ethanol drinking behavior**

Mice (−/− and +/+) were individually housed for at least 1 week prior to testing for ethanol preference using a “two-bottle” choice paradigm as previously described (Hungund et al., 2003; Vadasz et al., 2000). Briefly, the test consisted of six 3-day trials, in which mice were allowed to choose between ethanol and water. Animals were acclimatized to escalating concentrations of ethanol; a 3% solution for trial 1 (days 1–3) was increased to 6% in trial 2 (days 3–6), and further increased to 12% for trials 3 (days 6–9), 4 (days 9–12), 5 (days 12–15), and 6 (days 15–18). This arrangement provided multiple measures of ethanol preference at 12% concentration (v/v). The positions of water and ethanol tubes were alternated in each 3-day trial to avoid place preference. Data obtained from the trials 4, 5, and 6 were used to calculate the ethanol preference.

Acute effects of CP-55,940 (10, 30, and 50 μg/kg body wt, i.p.) and SR141716A (1, 3, and 5 mg/kg body wt, i.p.) on ethanol preference was examined using limited access protocol in B6 and D2 mice that were acclimatized to escalating concentrations of ethanol.
as previously described for unlimited access paradigm. One hour after the treatment with either drug or vehicle, mice were offered 12% ethanol and water for 6 h in the beginning of the dark cycle (reverse light/dark cycle). The consumption of ethanol and water was measured at the end of sixth hour. Data were calculated as grams of ethanol or water consumed/kg body wt/day and then the ratio of ethanol preference (ethanol intake/ethanol + water intake) was determined.

**Ethanol-induced hypothermia and sedation**

Sensitivity to ethanol-induced hypothermia and sedation was measured in ethanol-naive CB1 −/− and +/+ mice. Ethanol-induced hypothermia was assessed by monitoring rectal temperatures (Physiotemp Instruments, Clifton, NJ) after administration of a challenge dose of ethanol (2 g/kg body wt, i.p.). The rectal temperatures were recorded immediately prior to the ethanol challenge and 30 min thereafter. The difference in body temperature between pre- and post injection was used to determine the degree of hypothermia. Ethanol-induced sedation was measured by determining the time between loss of the righting reflex (LORR) and time to regain the righting reflex after the administration of a challenge dose of ethanol (3 g/kg body wt, i.p.) as previously described (Nowak et al., 2006).

**Handling-induced convulsions**

The ethanol inhalation paradigm was used to evaluate handling-induced convulsions (HIC). This procedure has been proven to be an effective and reliable method for producing functional dependence to ethanol (De Witte et al., 2003; Kliethermes et al., 2004; Nowak et al., 2006; Ripley et al., 2002). Briefly, mice were given a priming dose of ethanol (2 g/kg body wt, i.p.) in addition to pyrazole before being placed in the chamber. An alcohol dehydrogenase inhibitor, pyrazole (68 mg/kg body wt, i.p.), was given to stabilize the blood ethanol level (BEL). Animals were exposed to ethanol vapors for 72 h, with daily pyrazole injections. The BEL was determined every 24 h by an alcohol dehydrogenase inhibitor, pyrazole (68 mg/kg body wt, i.p.), was given to stabilize the blood ethanol level (BEL). Animals were exposed to ethanol vapors for 72 h, with daily pyrazole injections. The BEL was determined every 24 h by an

**Agonist-stimulated [35S]GTPγS binding Assay**

The CB1 receptor-stimulated [35S]GTPγS activation was measured in the crude synaptic membrane isolated from the cerebral cortex, hippocampus, striatum, and cerebellum of B6 and D2 mice (ethanol naive) as previously described with minor modifications (Vinod et al., 2006). Briefly, an aliquot of membrane (20–50 μg protein) was incubated in Tris-magnesium-ethylene diamine tetra acetic acid buffer containing 0.1% fatty acid-free BSA, 100 mM NaCl, 30 μM GDP, and 0.05 nM [35S]GTPγS in silicone-treated test tubes for 1 h at 37°C. The CB1 receptor agonist, CP-55,940 (1.5 μM) was used to examine the CB1 receptor-stimulated [35S]GTPγS binding to G-protein. Basal activity was measured in the absence of CP-55940. Nonspecific binding of the radioligand was determined in the presence of 10 μM GTPγS. Reaction mixture was rapidly filtered through nonpresoaked GF/B glass filters (Brandel, Gaithersburg, MD). Radioactivity was measured by liquid scintillation spectroscopy at an efficiency of 95% for [35S]. The CP-55940 stimulated [35S]GTPγS binding was presented as percentage over the basal value: ([stimulation − basal]/basal) × 100.

**Data analysis**

Statistical analysis was performed by independent-sample t test or two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test as appropriate (GraphPad software, San Diego, CA). Data are presented as mean ± SEM. Results were considered to be statistically significant at P < 0.05.
RESULTS
Effects of CB1 receptor deletion, SR141716A, and CP-55,940 on ethanol preference

The results of two-bottle choice between ethanol and water revealed that mice lacking CB1 receptor gene displayed a significantly lower preference for ethanol compared to their +/+ counterparts in both the strains (Fig. 1). There were main effects of drug (\(F_{1,26} = 38.32, P < 0.0001\)) and strain (\(F_{1,26} = 786.8, P < 0.0001\)) on ethanol preference with a significant drug \times strain interaction (\(F_{1,26} = 4.54, P < 0.05;\) Fig. 1). Two-way ANOVA analysis also revealed an effect of drug (\(F_{3,66} = 55.8, P < 0.0001\)) and strain (\(F_{3,66} = 301.0, P < 0.0001\)) on ethanol preference with significant drug \times strain interaction (\(F_{3,66} = 30.59, P < 0.0001\); Fig. 2A) when mice were administered with CB1 receptor antagonist, SR141716A. Interestingly, a lower dose of SR141716A (1 mg/kg) was able to significantly decrease the ethanol preference in B6 (\(P < 0.01\)) but not in D2 mice compared to vehicle-treated control groups (Fig. 2A). Pharmacological manipulation with the CB1 receptor agonist, CP-55,940, resulted in drug (\(F_{3,63} = 8.76, P < 0.0001\)) and strain (\(F_{1,63} = 119.8, P < 0.0001\)) dependent increase in ethanol preference with a significant drug \times strain interaction (\(F_{3,63} = 2.85, P < 0.05;\) Fig. 2B). Interestingly, a trend toward increase in ethanol preference with a higher dose (50 \(\mu\)g/kg) was observed only in B6 (\(P < 0.001\)) but not in D2 mice compared to their respective control groups.

Ethanol-induced hypothermic and sedative effects in CB1 \(\sim\sim\) mice

There was a main effect of ethanol-induced hypothermia on genotype (\(F_{1,35} = 35.0, P < 0.0001\)) and strain (\(F_{1,35} = 15.5, P < 0.0001\)) without a significant genotype \times strain interaction (\(F_{1,35} = 0.54, P = 0.466;\) Fig. 3A). Similarly, acute ethanol-induced a genotype (\(F_{1,37} = 19.11, P < 0.0001\)) and strain (\(F_{1,37} = 33.08, P < 0.0001\)) dependent increase in the duration of LORR without a significant interaction between genotype \times strain (\(F_{1,37} = 0.15, P = 0.69;\) Fig. 3B).
**Effect of genetic deletion and pharmacological blockade of the CB₁ receptor on HIC**

A marked effect of HIC on genotype \( F_{1,33} = 48.9, \ P < 0.0001 \) and strain \( F_{1,33} = 6.1, \ P < 0.01 \) with significant genotype x strain interaction \( F_{1,33} = 4.5, \ P < 0.05; \) Fig. 4A) was observed following 8 h after withdrawal from chronic ethanol exposure. The basal scores of HIC were not different between +/+ and −/− mice, which were exposed to circulating air without ethanol (data not shown). This finding was further supported by the pharmacological study in which cotreatment with SR141716A (3 mg/kg body wt) during chronic exposure led to drug \( F_{1,47} = 35.3, \ P < 0.0001 \) and strain \( F_{1,47} = 70.9, \ P < 0.0001 \) dependent reduction in severity of HIC with significant drug x strain interaction \( F_{1,47} = 4.2, \ P < 0.05; \) Fig. 4B). However, SR141716A alone did not show any effect on the HIC in control mice (data not shown). The strain-dependent differences in WIN 55,212-2 were greater in B6 than D2 mice. Although a reason for this differential effect is currently unknown, a higher efficiency of the striatal CB₁ receptor coupling could explain the greater effect of the striatal CB₁ receptor stimulation, which could explain the greater opposite effect of SR141716A.

**CB₁ receptor function in the brain of B6 and D2 mice**

The CP-55,940-stimulated \([35S]GTP_{\gamma}S\) activation was found to be significantly higher in the striatum of ethanol naïve B6 than D2 mice \( P < 0.05; \) Fig. 5). However, no differences in the G-protein activation were found in the cerebral cortex, hippocampus, and cerebellum between the groups.

**DISCUSSION**

This study examined whether stimulation and blockade of the CB₁ receptor regulate ethanol preference in mice with genetically predetermined differences in ethanol-related behaviors. Activation of the CB₁ receptor led to a marked increase in ethanol preference in both the strains with greater stimulation in B6 than D2 strains. The ethanol-reinforcing behavior via CB₁ receptor stimulation has been reported in ethanol-preferring rats (Colombo et al., 2002). Conversely, pharmacological blockade with SR141716A or genetic deletion of the CB₁ receptor significantly reduced ethanol preference in these mice. These results confirm earlier findings of decreased ethanol intake and conditioned place preference in the CB₁ −/− mice (Hungund et al., 2003; Lallemand et al., 2001; Naassila et al., 2004; Thanos et al., 2005; Wang et al., 2003). Interestingly, effects of CP-55,940 and SR141716A on ethanol preference were greater in B6 than D2 mice. Although a reason for this differential effect is currently unknown, a higher efficiency of the striatal CB₁ receptor coupling detected in B6 than D2 mice might be one of the contributing factors. This would certainly amplify responses to an agonist and may also unmask an endogenous tone, which could explain the greater opposite effect of SR141716A.

The reduction in ethanol preference particularly in D2 mice at higher dose of CP-55,940 (50 μg/kg) appears to be associated with catalepsy and decreased spontaneous activity by the CB₁ receptor stimulation (Chaperon and Thiebot, 1999; Wang et al., 2003). Similarly, no further increase in ethanol preference with this higher dose was also evident in B6 strain. The strain-dependent differences in WIN 55,212-2 (CB₁ receptor agonist) and SR141617A induced ethanol preference in B6 and D2 mice has been recently reported (Kelai et al., 2006). In addition, it is also of
importance to note a critical role of the CB$_2$ receptor in the neurobiology of alcohol addiction. For instance, enhancement of ethanol preference in mice treated with CB$_2$ receptor agonist under chronic stress model was prevented by CB$_2$ receptor antagonist (Ishiguro et al., 2007) suggesting a potential utility of the CB$_2$ receptor antagonist in the treatment of alcohol addiction.

Effects of acute ethanol challenge on hypothermia and sedation revealed genotype dependent changes. The CB$_1$ $\text{+/−}$ mice of both the strains showed a reduction in hypothermic effect. This finding is in agreement with the fact that the CBs elicit hypothermic effect via CB$_1$ receptor (Rawls et al., 2002; Zimmer et al., 1999) and therefore, absence of this receptor could have attributed to a lower hypothermic response. Although, this is not consistent with the previous study (Naassila et al., 2004), the possible explanation for the contrasting results could be due to differences in ethanol dosage employed. Conversely, ethanol-induced sedative effect was greater in CB$_1$ $\text{+/−}$ mice, which is similar to the observation made by Naassila et al. (2004).

To test whether genetically predetermined brain regional changes in the endocannabinoid system are associated with differences in ethanol preference in B6 and D2 mice, the status of CB$_1$ receptor function (G-protein activation) was examined. We observed a higher CB$_1$ receptor-mediated $[^{35}\text{S}]$GTP$_\gamma$S binding in the striatum of B6 mice. This is consistent with our previous finding that demonstrated a higher G-protein activation in the whole brain of B6 than D2 mice (Basavarajappa and Hungund, 2001). However, other brain regions that are associated with reward mechanism need to be also further examined. Although, the underlying mechanism(s) of varied ethanol-reinforcing behavior in these strains is yet to be determined, the higher CB$_1$ receptor function (CB$_1$ receptor-mediated G-protein signaling) in the striatum of B6 mice seems to play a critical role in the ethanol consummatory behavior. This hypothesis was partly supported by the existence of correlation between age-dependent decrease in ethanol preference and decline in the limbic CB$_1$ receptor signaling (Wang et al., 2003).

Ethanol intake has been shown to increase the limbic dopamine (DA) and AEA contents (Cheer et al., 2007; Gonzalez et al., 2002a; Hungund et al., 2003; Weiss et al., 1993). AEA also activates the mesolimbic dopaminergic system by increasing the DA content (Gessa et al., 1998; Solinas et al., 2006). A lack of ethanol-induced DA release has been shown in the NAc of CB$_1$ $\text{+/−}$ mice or wild-type mice treated with a CB$_1$ receptor antagonist (Cheer et al., 2007; Hungund et al., 2003). These functional interactions between endocannabinoid and DA systems might be partly associated with positive reinforcing effect of ethanol. The observed effect could also be due to a lower DA content in the mesostratial region in B6 than in D2 mice (George et al., 1995). The hypodopaminergic activity and hyperactivity of the CB$_1$ receptor function in limbic system might play a role in determining the susceptibility for a greater ethanol drinking behavior. Besides this difference, protein kinase A and CREB, which are downstream to the CB$_1$ receptor, have been found to be lower in the NAc of B6 than D2 mice (Misra and Pandey, 2003, 2006; Pandey et al., 2006).

A clinical report correlated the severity of ethanol withdrawal symptoms with the CB$_1$ gene polymorphism (Schmidt et al., 2002), lending indirect support for a role of the CB$_1$ receptor in withdrawal. To further assess the role of the endocannabinoid system in ethanol withdrawal symptoms, the effect of CB$_1$ receptor deletion was investigated. Severity of HIC was markedly decreased in the CB$_1$ $\text{+/−}$ mice. This observation was further ascertained by the CB$_1$ receptor antagonist, SR141716A. The concurrent administration of SR141716A (3 mg/kg) with ethanol exposure reduced severity of HIC. This anticonvulsant effect is not due to altered ethanol metabolism since SR141716A did not change the BEL. To our knowledge, this is the first pharmacological evidence linking the CB$_1$ receptor in ethanol withdrawal. In agreement with these findings, reduction of morphine withdrawal signs were also reported in CB$_1$ $\text{+/−}$ mice (Ledent et al., 1999) and mice cotreated with SR141716A (Mas-Nieto et al., 2001). We have previously demonstrated that mice treated with SR141716A during chronic exposure are more tolerant to challenge dose of ethanol (Nowak et al., 2006), suggesting a role for the CB$_1$ receptor in neuronal substrate mediating ethanol tolerance and dependence. However, the question of whether genetic differences in the endocannabinoid system associated with symptoms following ethanol withdrawal remains to be elucidated. Our findings are not consistent with a previous report that indicated increased ethanol-induced withdrawal signs in CB$_1$ $\text{+/−}$ mice in a liquid-diet paradigm (Naassila et al., 2004). On the contrary, lack of ethanol-induced withdrawal signs in these mice has been shown after voluntary ethanol consumption (Racz et al., 2003). This could be associated with the fact that CB$_1$ $\text{+/−}$ mice consume significantly less ethanol than $+/+$ mice. Besides this, genetic makeup of the mice, dosage, mode, and period of ethanol exposure and time at which the HIC were analyzed might also be important confounding factors for these contrasting results.

Chronic ethanol exposure increases the inhibitory and decreases the excitatory neurotransmissions. Subsequently, the physical signs of withdrawal have primarily been thought to result from reduced GABAergic and increased glutamatergic function (De Witte, 2003). Chronic ethanol exposure has also been shown to increase the endocannabinoid content and...
concomitant downregulation of CB1 receptors (Basavarajappa et al., 2000; Gonzalez et al., 2002b; Ortiz S et al., 2004; Vinod et al., 2006). Thus, SR141716A could block this effect and the endocannabinoid induced-desensitization of the CB1 receptors. Activation of the CB1 receptors that are located on the GABAergic and glutamatergic interneurons might also play a critical role in regulating the inhibitory and excitatory neurotransmission (Schlicker and Kathmann, 2001). An elevation in the AEA level following NMDA-induced excitotoxicity could be prevented by SR141716A (Hansen et al., 2002; Sommer et al., 2006). It is possible that the administration of SR14176A during ethanol exposure might counteract with functions of GABAergic and glutamatergic system. A recent study further revealed that deletion of CB1 receptors impairs neuroadaptations of both the NMDA and GABA receptors following chronic ethanol exposure suggesting a role for the endocannabinoid system in alcohol dependence (Warnau et al., 2007). Although, the classical tetrads effects of AEA are CB1 receptor-mediated (Wise et al., 2007), SR141716A has been shown to only partially attenuate the AEA-mediated antinociception, catalepsy, hypothermia, and suppression of spontaneous activity (Wiley et al., 2006). This raises the question whether AEA exerts its effect through non-CB1 receptors, for example, VR-1 receptor, DA, and Ca2+/K+ channels (Di Marzo et al., 2000; van der Stelt and Di Marzo, 2005). Because each of these systems may potentially influence the physiology and behavior, further systematic studies are essential in determining the underlying mechanism of ethanol withdrawal signs.

In conclusion, the present findings provide evidence for a critical role for the CB1 receptor in ethanol preference, sensitivity, and dependence. Genetically determined differences in the activity of the endocannabinoid system under basal conditions and in response to ethanol exposure may exist between ethanol-prefering and nonprefering strains. The CB1 receptor antagonist might be a potential therapeutic agent in the treatment for ethanol dependence.

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