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Upregulation of cannabinoid type 1 receptors in dopamine D2 receptor knockout mice is reversed by chronic forced ethanol consumption

Panayotis K. Thanos, PhD^{1,2}, Vanessa Gopez, BS², Foteini Delis, PhD², Michael Michaelides, MS^{2,3}, David K. Grandy, PhD⁴, Gene-Jack Wang, MD², George Kunos, MD, PhD⁵, and Nora D. Volkow, MD¹

¹ Laboratory of Neuroimaging, NIAAA, NIH, Department of Health and Human Services, Bethesda, MD

² Behavioral Neuropharmacology & Neuroimaging Lab, Department of Medicine, Brookhaven National Laboratory, Upton, NY

³ Department of Psychology, SUNY Stony Brook. Stony Brook, NY

⁴ Department of Physiology and Pharmacology, Oregon Health and Science University, Portland OR

⁵ Laboratory of Physiologic Studies, NIAAA, NIH, Department of Health and Human Services, Bethesda, MD

Abstract

Background—The anatomical proximity of the cannabinoid type 1 (CNR1/CB1R) and the dopamine D2 receptors (DRD2), their ability to form CB1R-DRD2 heteromers, their opposing roles in locomotion and their involvement in ethanol's reinforcing and addictive properties, prompted us to study the levels and distribution of CB1R after chronic ethanol intake, in the presence and absence of DRD2.

Methods—We monitored the drinking patterns and locomotor activity of *Drd2*^{+/+} and *Drd2*^{−/−} mice consuming either water or a 20% (v/v) ethanol solution (forced ethanol intake) for six months and used the selective CB1 receptor antagonist [³H]SR141716A to quantify CB1R levels in different brain regions with in-vitro receptor autoradiography.

Results—We found that the lack of DRD2 leads to a marked upregulation (approximately two-fold increase) of CB1R in the cerebral cortex, the caudate-putamen and the nucleus accumbens, which was reversed by chronic ethanol intake.

Conclusions—The results suggest that DRD2-mediated dopaminergic neurotransmission and chronic ethanol intake exert an inhibitory effect on cannabinoid receptor expression in cortical and striatal regions implicated in the reinforcing and addictive properties of ethanol.

Keywords

cannabinoid; CB1; dopamine; D2; knockout; ethanol; autoradiography

INTRODUCTION

Although the neurochemical mechanisms underlying alcohol addiction are not fully understood there is evidence that dopamine D2 receptors (DRD2) and cannabinoid type 1 receptors (CB1R) are involved in this behavior. A propensity to abuse natural and drug reinforcers, such as food, alcohol and cocaine, has been associated with low striatal DRD2 availability in humans (Stice et al., 2008; Volkow et al., 1996; Volkow et al., 2007; Wang et al., 2001; Wang et al., 2004) while post mortem studies show similarly diminished DRD2 levels in the caudate-putamen and the nucleus accumbens of alcoholic subjects (Tupala et al., 2004). Studies in rodents are in agreement with these observations, showing that alcohol preferring rats have lower DRD2 mRNA (Bice et al., 2008) and protein levels (Stefanini et al., 1992; Thanos et al., 2004). In addition, DRD2 over-expression in the nucleus accumbens of mice (Thanos et al., 2005c), Sprague-Dawley (Thanos et al., 2001), and alcohol preferring and non-preferring rats (Thanos et al., 2004) decreased their ethanol preference and intake. On the other hand, the complete absence of DRD2 in mice also leads to lower ethanol intake, preference, and conditioned place preference (Cunningham et al., 2000; Phillips et al., 1998; Risinger et al., 2000), and both DRD2 antagonists and agonists can attenuate ethanol's behavioral effects (Cohen et al., 1997) which suggest that the interactions between ethanol and DRD2 are complex.

The presence of CB1R is also critical for ethanol intake as CB1R antagonism or knockout leads to decreased ethanol intake and preference (Colombo et al., 1998; Lallemand et al., 2001; Wang et al., 2003) (Lallemand and de Witte, 2005; Naassila et al., 2004; Thanos et al., 2005a; Vinod et al., 2008), CB1R antagonism has been proposed as a potential therapeutic intervention for alcoholism (Basavarajappa, 2007) and CB1R downregulation after ethanol intake (Basavarajappa et al., 1998; Ortiz et al., 2004) may be a protective mechanism of the brain to prevent ethanol over-consumption. In contrast, ethanol intake is higher in rats treated with CB1R agonists (Colombo et al., 2002; Gallate et al., 1999) and in alcohol preferring C57BL/6 mice, which have higher affinity CB1R, compared to DBA/2, alcohol avoiding mice (Hungund and Basavarajappa, 2000).

Interactions between the CB1R and DRD2 have been reported at the systems, molecular, cellular, and behavioral levels. It is possible that the cannabinoid system facilitates the effects of positive reinforcers by modulating dopaminergic neurotransmission. Tetrahydrocannabinol, a cannabinoid receptor agonist, lowers the threshold for intracranial self-stimulation (Gardner et al., 1988) while CB1R antagonism has the opposite effect (Deroche-Gamonet et al., 2001). Tetrahydrocannabinol also increases the firing rate of DA neurons (French et al., 1997) and raises DA release in NAc (Tanda et al., 1997) while CB1 antagonism inhibits drug-induced phasic DA release, which suggests that the endocannabinoid tone facilitates the effects of drugs of abuse on DA release (Cheer et al., 2007).

The anatomical proximity of CB1R and DRD2 in the synapse (Pickel et al., 2006) enables the formation of CB1R-DRD2 heterodimers that influence cAMP levels in the cell (Glass and Felder, 1997; Mackie, 2005). These receptors influence each other's expression: (i) stimulation of either receptor increases the neurotransmitter levels of the other (Giuffrida et al., 1999; Tanda et al., 1997), (ii) the life-long absence of CB1R leads to DRD2 over-expression in the striatum (Houchi et al., 2005), and (iii) rodents and primates treated with antipsychotics (DRD2 antagonists), 6-OHDA or MPTP, as well as Parkinson's disease patients, show increased CB1 mRNA and receptor levels (Andersson et al., 2005; Lastres-Becker et al., 2001; Mailleux and Vanderhaeghen, 1993).

The close interaction between CB1R and DRD2 and their involvement in the rewarding and addictive effects of alcohol cued us to study the effects of chronic ethanol intake and DRD2 expression on CB1R levels and distribution. We studied CB1R levels in the brain of *Drd2* knockout mice that were exposed to chronic (6 months) ethanol consumption. Since CB1R levels increase after neuroleptic treatment (Andersson et al., 2005) and decrease after alcohol intake (Basavarajappa et al., 1998; Ortiz et al., 2004), we hypothesized that the absence of DRD2 would result in CB1R upregulation and that this effect would be attenuated by chronic alcohol intake.

2. MATERIALS AND METHODS

2.1 Animals

Twenty four male mice (mean weight: 20g; age: 8 weeks at the beginning of the experiment), bred in Brookhaven National Laboratory's Medical Department, were used for this study. Mice were housed on a reverse 12 hour dark/light cycle with lights off at 8 am and divided into four groups of six animals per group: i) *Drd2*^{+/+} mice drinking H₂O, ii) *Drd2*^{+/+} mice drinking 20% (v/v) ethanol, iii) *Drd2*^{-/-} mice drinking H₂O, and iv) *Drd2*^{-/-} mice drinking 20% (v/v) ethanol. The mice originated from breeding of *Drd2*^{+/-} mice obtained from Oregon Health and Science University, congenic (N5) on C57BL/6J strain (Kelly et al., 1998). For 24 weeks, all mice were given free access to food and fluids (water or ethanol solution). We chose a chronic, one bottle forced ethanol intake paradigm in order to model the condition of a heavy, long-term drinker and to achieve levels of ethanol intake as similar as possible between the animals. All experiments were conducted in conformity with the National Academy of Sciences Guide for the Care and Use of Laboratory Animals (NAS and NRC, 1996) and Brookhaven National Laboratory Institutional Animal Care and Use Committee protocols.

2.2 [³H] SR141716A *in-vitro* receptor autoradiography

The animals were sacrificed at the end of the 24th week of treatment. Water or ethanol solution was still available to the mice on the day of the sacrifice. All mice were anesthetized with a mixture of ketamine (100mg/kg) and xylazine (10mg/kg) and the brains were rapidly extracted, flash frozen in isopentane and stored at -80°C. Coronal sections, 12µm thick, were cut with a Leica cryostat and stored at -80°C until receptor binding was performed. One brain from the *Drd2*^{+/+} ETOH group was lost during the sectioning process. *In vitro* CB1R autoradiography was performed as previously reported (Thanos et al., 2008). The sections were pre-incubated for 10 min in assay buffer solution (50 mM Tris-HCl, 120mM NaCl, 5mM KCl, 2mM CaCl, 1mM MgCl, pH 7.4) at room temperature and then incubated for 90 min in assay buffer in the presence of 0.4 nM [³H] SR 141716A (Amersham) at room temperature. Non-specific binding was determined in the presence of 100µM HU-210 (Tocris Bioscience). Incubation was followed by 3×30 min washes in ice-cold assay buffer and a rapid rinse in ice-cold distilled H₂O. The sections were dried overnight in a desiccator, at room temperature and then placed into a glass slide cassette for image acquisition scanning using the β-Imager 2000 (Biospace, France). Using Betavision+ software (Biospace Measures, IN/US), regions of interest were drawn of the left and right caudate-putamen (CPu), nucleus accumbens (NAC), hippocampus (HP), thalamus (TH), hypothalamus (HYP), cerebellum (CB), and cingulate (CC), motor (MC), sensory (SC), and insular (IC) cortices of each brain slice. The data were expressed in counts per minute per millimeter squared and converted to µCi/g tissue using a brain paste standard of known radioactivity value and mass (Thanos et al., 2008).

2.3 Body weight and fluid intake

Body weight and intake of water or ethanol were measured three times a week throughout the study. Each mouse had access to one bottle of water or 20% (v/v) ethanol solution (i.e. one bottle, forced ethanol consumption) which was replenished when fluid intake was monitored. In order to estimate volume loss, an empty cage with a bottle of water and 20% (v/v) ethanol solution was placed on the same cage rack. Fluid intake is reported in g of ethanol or ml of water intake per kg of body weight (g/kg, ml/kg).

2.4 Open-field Locomotor Activity

Open-field locomotor activity was examined every two weeks for 24 weeks, during their dark cycle. The mice were transferred (cages covered with black, opaque cloth) from their home cage to an Optical Sensor Arena (Minimitter Corporation, Oregon, USA), which consisted of a cage (48 cm × 26 cm × 16 cm) and an optical sensor securely attached to the wire top. The number of beam breaks were collected every minute over a 90-minute session. Data collection was performed in the dark. All data were recorded using the Vitalview software (Minimitter Corporation, Oregon, USA).

2.5. Statistics

Statistically significant differences were examined with Analysis of Variance (ANOVA) followed by Tukey multiple comparisons. Repeated Measures ANOVA was applied for the analysis of all behavioral data, with Genotype and Treatment as between-subjects factors and Time as within-subjects factor.

3. RESULTS

3.1 [³H] SR141716A *in-vitro* receptor autoradiography

The effects of DRD2 knockout and chronic ethanol intake on specific binding of the CB1R antagonist/inverse agonist [³H] SR141716A are shown in Fig. 1 and Fig. 2. CB1R binding was significantly affected by *Drd2* gene knockout and by ethanol intake in areas of the cortex and the basal ganglia. CB1R binding was significantly higher in *Drd2* $-/-$ mice on water, compared to *Drd2* $+/+$ on water. In contrast, there were no differences in CB1R binding between the *Drd2* $+/+$ and *Drd2* $-/-$ mice on alcohol. These effects were observed in the caudate-putamen, the nucleus accumbens, and in motor, sensory and limbic regions of the cerebral cortex (caudate-putamen: Genotype × Treatment $F(1, 19) = 6.570$, $p = 0.020$; nucleus accumbens: Genotype × Treatment $F(1, 19) = 4.610$, $p = 0.045$; cingulate cortex: Genotype × Treatment $F(1, 19) = 5.611$, $p = 0.028$; motor cortex: Genotype × Treatment: $F(1, 19) = 5.234$, $p = 0.033$; parietal cortex: Genotype × Treatment: $F(1, 19) = 4.556$, $p = 0.040$; insular cortex: Genotype: $F(1, 19) = 4.600$, $p = 0.04$; Treatment $F(1, 19) = 9.808$, $p = 0.005$). In *Drd2* $+/+$ mice, ethanol intake produced a non significant decrease in CNR1 binding levels in all brain areas examined.

3.2 Body Weight

Body weights throughout the treatment are shown in Fig. 3. *Drd2* $+/+$ mice on water had the highest weight, followed by *Drd2* $+/+$ on alcohol and *Drd2* $-/-$ on water while *Drd2* $-/-$ on alcohol had the lowest weight. Body weight increased over time in mice drinking water. In *Drd2* $+/+$ mice on water, the weight during the last 12 weeks of treatment (weeks 12–24) was significantly higher compared to the first 8 weeks of treatment (weeks 1–8) ($p < 0.05$). Similarly, in *Drd2* $-/-$ mice on water, the weight during weeks 16–24 was significantly higher than during weeks 1–6 ($p < 0.05$). *Drd2* $+/+$ mice drinking ethanol showed minor statistically significant increases in body weight (weight on week 19 was higher than on weeks 1 and 6, $p < 0.05$). In contrast, *Drd2* $-/-$ mice on ethanol showed no significant weight

differences during the entire study. [Three-way Repeated Measures ANOVA: Genotype: $F(1, 20)=8.470$, $p=0.008$, Treatment: $F(1, 20)=4.654$, $p=0.04$, Time: $F(23, 460)=26.617$, $p<0.001$, Time $\times$ Genotype: $F(23, 460)=1.929$, $p=0.006$, Time $\times$ Treatment: $F(23, 460)=3.312$, $p<0.001$, Genotype $\times$ Treatment: $F(1, 20)=0.0002$, $p=0.396$, Time $\times$ Genotype $\times$ Treatment: $F(23, 460)=1.172$, $p=0.264$].

3.3 Fluid Intake

Fluid intake throughout the treatment is shown in Fig. 4. There were no significant differences in water or ethanol intake between *Drd2*^{+/+} and *Drd2*^{-/-} mice. In water treated animals, water intake decreased significantly during the first 5 weeks of treatment but remained stable thereafter [Two way repeated measures ANOVA: Genotype: $F(1, 10)=0.1774$, $p=0.682$; Time: $F(23, 230)=19.6537$, $p<0.0001$; Genotype $\times$ Time: $F(23, 230)=2.9727$, $p<0.0001$]. There were no differences in ethanol intake between *Drd2*^{+/+} and *Drd2*^{-/-} mice throughout the 24 weeks of ethanol exposure [Two-way repeated-measures ANOVA: Genotype: $F(1, 10)=0.011$, $p=0.919$; Time: $F(23, 230)=2.375$, $p=0.010$; Genotype $\times$ Time: $F(23, 230)=0.884$, $p=0.620$], and no differences in the amount of water they drank [Two-way repeated-measures ANOVA: Genotype: $F(1, 10)=0.007$, $p=0.733$; Time: $F(23, 230)=2.720$, $p<0.001$; Genotype $\times$ Time: $F(23, 230)=0.814$, $p=0.713$]. On average, mice that were exposed to the ethanol solution drank ~20% less water than mice on water treatment during the first four weeks of treatment [Three-way repeated-measures ANOVA: Genotype: $F(1, 20)=0.071$, $p=0.793$; Treatment: $F(1, 20)=20.172$, $p<0.001$; Genotype $\times$ Treatment: $F(1, 20)=0.142$, $p=0.701$; Time: $F(23, 460)=11.169$, $p<0.001$; Genotype $\times$ Time: $F(23, 460)=1.502$, $p=0.064$; Treatment $\times$ Time: $F(23, 460)=6.690$, $p<0.001$; Genotype $\times$ Treatment $\times$ Time: $F(23, 460)=1.709$, $p=0.022$].

3.4 Locomotor Activity

Locomotor activity throughout the treatment is shown in Fig. 5. Overall, locomotor activity was highest in *Drd2*^{+/+} mice on ethanol, followed by *Drd2*^{+/+} mice on water, and *Drd2*^{-/-} mice. Note that while *Drd2*^{+/+} mice on ethanol showed (at several time points during the first weeks of treatment) higher locomotor activity than *Drd2*^{+/+} mice on water, there were no differences between ethanol- and water-treated *Drd2*^{-/-} mice. Statistically significant differences were sporadic, observed between *Drd2*^{+/+} and *Drd2*^{-/-} mice drinking ethanol on weeks 6 and 14 and between *Drd2*^{+/+} and *Drd2*^{-/-} mice drinking water on week 22 [Three-way repeated-measures ANOVA: Genotype: $F(1, 20)=6.988$, $p=0.016$; Treatment: $F(1, 20)=24.812$, $p<0.001$; Genotype $\times$ Treatment: $F(1, 20)=0.100$, $p=0.920$; Time: $F(11, 220)=2.748$, $p=0.002$; Genotype $\times$ Time: $F(11, 220)=0.834$, $p=0.606$; Treatment $\times$ Time: $F(11, 220)=0.852$, $p<0.588$; Genotype $\times$ Treatment $\times$ Time: $F(11, 220)=1.441$, $p=0.156$].

4. DISCUSSION

DRD2 regulate CB1R levels

We show that in the lifelong absence of DRD2, CB1R undergo significant upregulation, which may reflect an increase in the number of receptors and/or their affinity. While gene knockout technology is an invaluable tool for the delineation of brain function, it does not come without limitations. In the current study, it is possible that our findings are not due to the lack of DRD2 per se but due to secondary changes and adaptations that the brain has undergone during development in order to compensate for the receptor deficiency. However, it is reasonable to suggest that the upregulation of CB1R is indeed a result of DRD2 deficiency, because previous studies in rodents have shown that antipsychotic treatment (i.e. DRD2 antagonism) and 6-OH-DA lesion of the dopaminergic terminals increased CB1R mRNA and protein levels in the striatum (Andersson et al., 2005; Mailleux and Vanderhaeghen, 1993). Similarly, MPTP lesion of the dopaminergic projections in non-

human primates also increased CB1R levels, a result that was reversed after DOPA administration, and Parkinson's disease patients also show higher CB1R levels compared to normal subjects (Lastres-Becker et al., 2001). Our result, together with the aforementioned studies, suggest that dopaminergic neurotransmission, via DRD2 signaling, exerts an inhibitory effect on CB1R levels. Antagonism or elimination of DRD2 increases gene expression of the *Cnr1* gene as well as membrane expression and function of the CB1R protein.

Our result contributes to a number of studies supporting the existence of an interaction between CB1R and DRD2 at the cellular, molecular, and behavioral level. CB1R and DRD2 are co-localized in neurons of the caudate-putamen (Martin et al., 2008), the nucleus accumbens (Pickel et al., 2006), the cerebral cortex, and the hippocampus (Khan et al., 1998; Moldrich and Wenger, 2000). There is evidence that these two receptors exist as monomers linked to $G_{i/o}$ proteins and as heterodimers linked to G_{α_s} proteins (Jarrahian et al., 2004; Navarro et al., 2008). CB1R agonists decrease the affinity of DA for DRD2 and this antagonistic interaction is believed to be mediated by the formation of CB1R-DRD2 heteromers (Marcellino et al., 2008). The two receptors have similar effects on neurotransmitter release. CB1R stimulation increases the firing rate of DA neurons (French et al., 1997) and DA release in nucleus accumbens (Tanda et al., 1997) while DRD2 stimulation increases the extracellular levels of anandamide, measured by microdialysis, in striatum (Giuffrida et al., 1999). The two receptors have opposite effects on locomotion. The stimulatory effects of quinpirol, a D2R agonist, are attenuated by endocannabinoids (Beltramo et al., 2000) and potentiated by the CB1R antagonist SR141716A (Giuffrida et al., 1999). On the other hand, the inhibitory effects of cannabinoid agonism on locomotion are attenuated by DRD2 agonism (Meschler et al., 2000). Finally, *Cnr1* $-/-$ mice show DRD2 upregulation in the striatum (Houchi et al., 2005), which is a result complementary to ours. It becomes apparent that CB1R and DRD2 are mutually regulated and that the expression of either receptor is under the negative control of the other.

Chronic ethanol intake downregulates CB1R in the absence of DRD2

We show that 24 weeks of forced chronic ethanol intake decreases CB1R binding in the absence, but not in the presence, of DRD2. The lower CB1R levels after chronic ethanol intake in the *Drd2* $-/-$ mice could underlie the lower reinforcing effects of ethanol in these mice, as shown in previous studies that used a choice paradigm.

Multiple studies provide evidence that CB1R are involved in ethanol's rewarding effects. Ethanol preference is higher in mice with higher affinity (Hungund and Basavarajappa, 2000) and increased coupling efficiency of CB1R (Basavarajappa and Hungund, 2001) and increases with CB1R agonist treatment (Colombo et al., 2002) while *Cnr1* gene knockout or CB1R antagonism decrease ethanol self-administration and preference (Arnone et al., 1997; Colombo et al., 1998; Gallate et al., 1999; Lallemand and de Witte, 2005; Lallemand et al., 2001; Thanos et al., 2005a; Thanos et al., 2005b; Wang et al., 2003). Ethanol intake increases endocannabinoid levels in cerebellar cell cultures (Basavarajappa et al., 2000) and the limbic forebrain but not in the midbrain (Gonzalez et al., 2002), and may (Basavarajappa et al., 1998; Ortiz et al., 2004) or may not (Gonzalez et al., 2002) lead to CB1 receptor downregulation. It is possible that, when occurring, ethanol-induced CB1R downregulation is an adaptation of the cannabinoid system to increased endocannabinoid levels that might have protective effects for the animal since it would prevent ethanol over-consumption.

In our experiment, 24 weeks of ethanol treatment had no effect on CB1R binding levels in *Drd2* $+/+$ mice. This may be due to the experimental paradigm (one bottle, forced ethanol consumption), the relatively high dose used, and the prolonged treatment period (6 months, as opposed to 4–50 days of previous studies) (Basavarajappa et al., 1998; Gonzalez et al.,

2002; Ortiz et al., 2004). It is possible that CB1R downregulation is a relatively short-term adaptation that is not sustained after prolonged intake of substantial ethanol amounts by the normal animal.

The age of the mice might be another critical factor. Wang et al. (Wang et al., 2003) showed that ethanol preference is linked to the presence of CB1R only in young animals. In their study, there were no differences in ethanol preference between wild type and *Cnr1* $-/-$ mice after the age of 4 months and CB1R coupling to the second-messenger cascade was reduced. Similarly, Ginsburg and Lamb (Ginsburg and Lamb, 2006) showed that SR141716A does not antagonize the effects of tetrahydrocannabinol on ethanol intake in aged rats (17 months old) that have been chronically exposed to ethanol. Finally, preliminary data from our lab (Piyis et al., 2007) show that 3 month old naive mice have almost twice as much CB1 binding, compared to the 8 month old control mice of the current study, which suggests that the endocannabinoid system undergoes significant changes as the animal ages and it may be differentially involved in various behaviors over time.

In *Drd2* $-/-$ mice, chronic forced ethanol intake leads to a significant decrease in [3 H]SR141716A binding, which may reflect a decrease in CB1R number and/or affinity. Given that CB1R most probably mediate ethanol intake, the observed downregulation may represent a protective adaptation of the central nervous system to the substantial levels of ethanol consumed. In our experimental model this downregulation occurs in the absence of DRD2, which suggests that the dopaminergic system affects the interaction between ethanol and the endocannabinoid system. Similarly, Alen et al. (2008) showed that pharmacological inactivation of DRD2 prevents cannabinoid-induced alcohol relapse. These results are in agreement with the hypothesis that DRD2-mediated neurotransmission is critical for ethanol-related neuronal signaling and suggest that a functional interaction between ethanol and the dopaminergic and endocannabinoid systems exists in the mammalian brain. They also imply that the lower alcohol preference in *Drd2* $-/-$ mice, as observed in two-bottle choice experiments, may be due, in part, to a downregulation of CB1R. If exposure to forced ethanol intake produces similar neurochemical changes in the brain compared to those induced by free access to similar amounts of ethanol, we would expect that chronic, two bottle choice alcohol intake would produce similar downregulation in CB1 levels in the knockout mice. This question remains to be addressed.

Finally, the absence of DRD2-mediated neurotransmission uncovers the inhibitory effect of ethanol on CB1R in DA-rich areas of the basal ganglia (caudate-putamen and nucleus accumbens) and in brain areas with intermediate levels of DA innervation (cerebral cortex), while brain regions with low levels of DA innervation (hippocampus, thalamus, cerebellum) were largely unaffected.

Behavioral measures

Fluid intake—Previous studies show that low DRD2 levels are associated with increased alcohol consumption (McBride et al., 1993; Stefanini et al., 1992) and that DRD2 over-expression reduces ethanol self-administration (Thanos et al., 2005c; Thanos et al., 2004; Thanos et al., 2001). However, multiple studies also agree that *Drd2* $-/-$ mice show reduced ethanol preference and intake, compared to wild-type, in the two-bottle choice experimental model (Palmer et al., 2003; Phillips et al., 1998; Risinger et al., 2000; Thanos et al., 2005c). If ethanol exerts its psycho-physiological effects in part via the dopaminergic system through DRD2 stimulation, then the complete absence of D2R would, indeed, lead to decreased ethanol intake and preference.

In this study, *Drd2* $-/-$ mice drank the same amount of ethanol as *Drd2* $+/+$ throughout the entire experiment. The two main differences between the current and the above-mentioned

studies are the following: (i) we used a one-bottle, forced ethanol consumption and (ii) the concentration of ethanol is rather high (20% v/v). Similar results have been reported for *Cnr1* $-/-$ mice: they show decreased ethanol preference and consumption in a two-bottle choice paradigm, compared to wild-type but they show no difference in ethanol intake when they are forced to drink ethanol in their diet as the sole source for fluids (Naassila et al., 2004). It is also known that *Drd2* $-/-$ mice may have reduced ethanol preference compared to wild-type but this difference decreases to non-significant levels if the animals have been pre-exposed to ethanol (Palmer et al., 2003). Therefore, the lack of a choice together with neurochemical adaptations after repeated exposure to relatively high levels of ethanol may account for the observed ethanol drinking pattern of the *Drd2* $-/-$ mice in our study.

There were no differences in the water drinking pattern of the two genotypes. Animals exposed to water drank the same amount over time, regardless of genotype, and the same was true for the water consumed by mice exposed to ethanol solution. These results suggest that the life-long absence of the DRD2 does not affect the motivation of the animal to drink water.

Locomotor activity—In this study, *Drd2* $+/+$ mice showed greater locomotor activity than *Drd2* $-/-$ mice and mice on forced ethanol intake showed greater locomotor activity than mice drinking water. Statistically significant differences were sporadic: *Drd2* $+/+$ mice on ethanol moved significantly more than *Drd2* $-/-$ on ethanol in the middle of the treatment period and *Drd2* $+/+$ on water significantly more than *Drd2* $-/-$ on water at the end of the treatment. Our results are in partial agreement with a previous study showing that *Drd2* $+/+$ mice, with or without prior exposure to ethanol, show no significant ethanol-induced locomotor stimulation or sensitization (Palmer et al., 2003). However, the same study showed that ethanol induces and sensitizes locomotion in *Drd2* $-/-$ mice, which we did not observe. *Drd2* $-/-$ mice showed no differences in locomotor activity between ethanol- and water-treated groups.

It is possible that the lack of significant ethanol effects is due to the high ethanol concentration in the continuous access, one-bottle, forced ethanol paradigm that was applied. At low doses ethanol has anxiolytic (Cohen et al., 1997) or stimulant effects (Imperato and Di Chiara, 1986) leading to behavioral disinhibition, which may be manifested as increased locomotor activity, while at higher doses, and, more so, in a condition where access to pure water is impossible, it has tranquilizing effects, which may be manifested as decreased locomotion and loss of muscle control (Cohen et al., 1997).

In addition, sensitization to ethanol, as well as to other drugs, is more likely to occur when the compound is administered intermittently as opposed to continuously (Palmer et al., 2003; Robinson and Becker, 1986). It is possible that the continuous access to ethanol in the current study does not permit the emergence of locomotor sensitization in *Drd2* $-/-$ mice, although the lack of information on the temporal patterns of alcohol intake and blood alcohol levels does not allow a conclusive statement.

Body weight—The lower body weight of water consuming *Drd2* $-/-$ mice, compared to wild-type, is in agreement with previously published findings showing that lack of DRD2 may result in increased energy expenditure (Yamaguchi et al., 1996) and decreased levels of pituitary and serum growth hormone (Diaz-Torga et al., 2002). They are also in agreement with studies of prenatal exposure to DRD2 antagonists, which would be a situation akin to that of *Drd2* gene knockout, that resulted in lower body weight of the offspring (Singh and Singh, 2002; Williams et al., 1992; Zuo et al., 2008). Mice drinking alcohol showed minor or no changes in body weight which is in agreement with previous studies showing that prolonged intake of substantial amounts of ethanol leads to weight loss or decreased weight

gain (Addolorato et al., 1997; Cascales et al., 1983; Levine et al., 2000; Preedy and Peters, 1988), possibly via increased lipid oxidation (Addolorato et al., 1997; Levine et al., 2000) or energy dissipation (Lieber, 2004).

5. Summary and limitations

We observed an upregulation of CB1R in *Drd2*^{-/-} mice that was reversed by chronic forced ethanol intake. Since fluid and alcohol intake, as well as locomotor activity, were similar between wild type and *Drd2*^{-/-} mice we may exclude the possibility that CB1R upregulation and its reversal by ethanol in the *Drd2*^{-/-} mice are due to different levels of ethanol intake or may reflect major differences in the motor capacity of the animals. Similar changes in CB1R levels after pharmacological antagonism of DRD2 in normal animals (Andersson et al., 2005) also allow us to suggest that the observed upregulation of the cannabinoid receptors is due to the lack of DRD2 *per se* and not to secondary adaptations to the life-long absence of DRD2. The downregulation of CB1 after alcohol intake in the *Drd2*^{-/-} animal could underlie the lower reinforcing effects of ethanol in these mice, which remains to be proven in a two-bottle choice paradigm. Finally, it should be noted that all the results of the current study have been obtained in male mice only. Furthermore, in contrast to males, female rodents show higher levels of ethanol intake (Basavarajappa et al., 2006; Malinen et al., 2009), lower levels of endogenous cannabinoids (Malinen et al., 2009) and have different mechanisms of CB1 regulation (Basavarajappa et al., 2006). In contrast to males, females have lower levels of dopamine release (Munro et al., 2006), that is more tightly regulated by the DRD2 autoreceptor and dopamine transporter interactions in the basal ganglia (Walker et al., 2006), and a higher proportion of dopamine projections to the frontal cortex (Kritzer and Creutz, 2008). These sex-dependent differences in the cannabinoid and the dopaminergic systems do not allow us to draw a general conclusion on the interactions between ethanol, CB1, and DRD2 for both sexes.

6. Conclusions

1. The membrane expression of CB1R is under the negative control of DRD2 in the basal ganglia (caudate-putamen and nucleus accumbens) and the cerebral cortex but not in the hippocampus, the thalamus, or the cerebellum.
2. Long-term ethanol intake, in the absence of DRD2, has inhibitory effects on CB1R levels, in areas of the cerebral cortex, the caudate-putamen and the nucleus accumbens. This receptor interaction may help explain previous reports on decreased reinforcing effects of ethanol in CB1R and DRD2 deficient mice.

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Figure 1. Effects of *Drd2* knockout and chronic ethanol intake on brain CB1 receptor autoradiographic localization

Specific binding (mean+s.e.m.) of the CB1 ligand [³H]SR141716 (0.4nM) in brain sections of *Drd2*^{+/+} and *Drd2*^{-/-} mice drinking water or 20% (v/v) ethanol solution for 24 weeks, **p*<0.05.

Figure 2. Autoradiographic distribution of [^3H]SR141716 in coronal sections of the mouse brain (A) *Drd2*^{+/+} and (B) *Drd2*^{-/-} mice drinking water; (C) *Drd2*^{+/+} and (D) *Drd2*^{-/-} mice drinking 20% ethanol solution. Ci; cingulate, Mo: motor, Sens: somatosensory, Ins: insular cortices, Str: striatum.

Figure 3. Effects of *Drd2* knockout and chronic ethanol intake on body weight

Average daily body weight (+ s.e.m.), in g, during the 24-week treatment period of *Drd2*^{+/+} and *Drd2*^{-/-} mice drinking water or 20% ethanol solution. **p*<0.05 in *Drd2*^{+/+} H₂O, #*p*<0.05 in *Drd2*^{-/-} H₂O.

Figure 4. Effects of *Drd2* knockout and chronic ethanol intake on fluid intake

Top: Average daily water intake (+ s.e.m.), in ml/kg body weight, during the 24-week treatment period of *Drd2*^{+/+} and *Drd2*^{-/-} mice drinking water, **p*<0.05. **Middle:** Average daily ethanol intake (+ s.e.m.), in g/kg body weight, during the 24-week treatment period of *Drd2*^{+/+} and *Drd2*^{-/-} mice drinking 20% ethanol solution. **Bottom:** Average daily water intake (+ s.e.m.), in ml/kg body weight, during the 24-week treatment period of *Drd2*^{+/+} and *Drd2*^{-/-} mice drinking 20% ethanol solution.

Figure 5. Effects of *Drd2* knockout and chronic ethanol intake on locomotor activity
 Beam breaks per 90min sessions (+ s.e.m.), during the 24-week treatment period of *Drd2*^{+/+} and *Drd2*^{-/-} mice drinking water or 20% ethanol solution. * $p < 0.05$ between ETOH drinking mice, # $p < 0.05$ between H₂O drinking mice.