

Involvement of A_{2A} receptors in anxiolytic, locomotor and motivational properties of ethanol in mice

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We have shown previously that mice lacking the adenosine A_{2A} receptor (A_{2A}R) generated on a CD1 background self-administer more ethanol and exhibit hyposensitivity to acute ethanol. We aimed to investigate if the increased propensity of A_{2A}^{-/-} mice to consume ethanol is associated with an altered sensitivity in the motivational properties of ethanol in the conditioned place preference (CPP) and conditioned taste aversion (CTA) paradigms and with an altered development of sensitization to the locomotor effects of ethanol. We also tested their sensitivity to the anxiolytic effects of ethanol. Our results show that A_{2A}^{-/-} mice produced on a CD1 background displayed a reduced ethanol-induced CPP and an increased sensitivity to the anxiolytic and locomotor-stimulant effects of ethanol, but they did not show alteration in ethanol-induced CTA and locomotor sensitization. Ethanol-induced CPP, ethanol consumption and the locomotor effects of ethanol were also tested in A_{2A}^{-/-} mice produced on a C57BL/6J background. Our results emphasized the importance of the genetic background because alteration in ethanol consumption and preference, ethanol-induced CPP and locomotor-stimulant effects were not found in knockout mice produced on the alcohol-preferring C57BL/6J genetic background. Finally, the A_{2A}R agonist, 2-p-(2-carboxyethyl)-phenylethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride (CGS 21680), reduced ethanol consumption and preference in C57BL/6J mice. In conclusion, A_{2A}R deficiency in mice generated on a CD1 background leads to high ethanol consumption that is associated with an increased sensitivity to the locomotor-stimulant/anxiolytic effects of ethanol and a decrease in ethanol-induced CPP.

Keywords: A_{2A} receptor, adenosine, anxiety, ethanol, knockout mice, reward, sensitization

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Alcoholism is a devastating illness with a profound public health impact and which is influenced by both genes and environment. The specific genes involved have not yet been identified and many genes, each with a small effect, are likely to predispose an individual to abuse alcohol. Some promising new targets are emerging by using rodent genetic animal models such as transgenics and null mutants (Crabbe *et al.* 2006). Thus, the generation of transgenic mice overexpressing or knockout mice deleted of a selected gene involved in neurotransmission has proven valuable for studying its implication in ethanol-mediated behaviors. Among the numerous targets that have been identified, we have previously shown in mice lacking the adenosine A_{2A} receptor (A_{2A}R) that this receptor mediates several behavioral effects of ethanol (Naassila *et al.* 2002). The A_{2A}R belongs to a family of four G-protein-coupled adenosine receptors that are strongly expressed in basal ganglia (Schiffmann *et al.* 2007). Numerous *in vitro* and *in vivo* studies showed that ethanol induces an increase in extracellular adenosine levels, with subsequent activation of adenosine receptors (Mailliard & Diamond 2004). In addition, A_{2A}R are highly expressed in the nucleus accumbens that has been shown to play a crucial role in drug-seeking behavior and drug reinforcement (Baldo *et al.* 1999), thus suggesting a role of these receptors in addiction.

Furthermore, there is emerging evidence that the adenosinergic system might be involved in alcohol addiction (Ferré *et al.* 2007; Mailliard & Diamond 2004). Preventing A_{2A} and dopamine D₂ receptor synergism through viral inhibition of βγ dimers necessary for this interaction significantly reduced ethanol preference in rats (Yao *et al.* 2002). We have previously shown that adenosine A_{2A}^{-/-} mice generated on a CD1 background drink more alcohol, are less sensitive to the intoxicating effects of alcohol (Naassila *et al.* 2002) and exhibit blunted ethanol withdrawal effects (El Yacoubi *et al.* 2001). Mice lacking the equilibrative nitrobenzylthioinosine-nucleoside transporter (ENT1), responsible for adenosine uptake, display elevated levels of ethanol consumption compared with wild-type mice (Choi *et al.* 2004). Choi *et al.* (2004) also showed that ENT1^{-/-} mice displayed a decrease in A₁ adenosine tone in nucleus accumbens and suggested that A_{2A}R activation could be decreased. This hypothesis of decreased A_{2A}R activation is in line with our previous results that showed an increase in ethanol consumption observed in A_{2A}^{-/-} mice (Naassila *et al.* 2002). It is noteworthy that

adenosine-transporter-binding sites have been shown to be decreased in the brain of $A_{2A}^{-/-}$ mice (Snell *et al.* 2000) and that reduced levels of $A_{2A}R$ expression have been observed in the brain of alcohol-preferring mice (Short *et al.* 2006). In addition, low dose of the $A_{2A}R$ antagonist 3,7-dimethyl-1-propargylxanthine (DMPX) enhanced operant self-administration of ethanol in rats, while high doses decreased ethanol self-administration (Arolfo *et al.* 2004).

Based on these studies, the purpose of the present study was to confirm and extend our previous data on the involvement of the $A_{2A}R$ in the behavioral effects of ethanol. We investigated if the increased propensity to consume alcohol in $A_{2A}^{-/-}$ mice generated on a CD1 background is associated with alteration in the sensitivity to the motivational properties [conditioned place preference (CPP) and conditioned taste aversion (CTA)] of ethanol. In addition, because a relationship has been found between heightened ethanol preference and susceptibility to ethanol-induced locomotor sensitization (Grahame *et al.* 2000; Lessov *et al.* 2001), anxiety (Spanagel *et al.* 1995) and locomotor activation (Wise & Bozarth 1987), we tested these behavioral responses to ethanol in mice produced on a CD1 background. As numerous studies have shown a critical influence of the genetic background, we also checked if the alteration in ethanol consumption, ethanol-induced CPP and ethanol-induced stimulant motor effects are found in mice generated on the alcohol-preferring genetic background C57BL/6J (Yoneyama *et al.* 2008) compared with knockout mice generated on an alcohol-avoiding (CD1) genetic background (Short *et al.* 2006). Besides the difference in ethanol consumption and preference, to our knowledge there is no study that compared the behavioral responses with ethanol between these two strains. As our previous data suggested that activating the $A_{2A}R$ may play a role in suppressing alcohol-drinking behavior, we measured the effect of the $A_{2A}R$ agonist 2-p-(2-carboxyethyl)-phenylethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride (CGS 21680) on ethanol consumption in the alcohol-preferring C57BL/6J strain.

Materials and methods

Animals

We used adult male wild-type and $A_{2A}^{-/-}$ mice (8–14 weeks of age) generated on a CD1 background as described previously (Ledent *et al.* 1997) and weighing 20–30 g. The first generation chimeric (129X1/SvJ \times 129S1) \times CD1 heterozygotes were bred for 15 generations on a CD1 outbred background (Charles River, St Germain sur l'Arbresle, France) to dilute the genetic background of the embryonic stem cells derived from the 129 mouse strain, with selection for the heterozygous mutant A_{2A} gene at each generation (Ledent *et al.* 1997). Fifteenth-generation heterozygotes were bred together to generate $A_{2A}R$ -deficient and control mice, and homozygotes from the F_{16} generation were used to produce all the experimental mice (F_{17} generation). In the present study, the number of breeders were 160 (85 wild-type and 75 knockout mice) and 172 (82 wild-type and 90 knockout mice), respectively, for female and for male mice. The breeders were randomly selected and have been used once or twice to generate the offspring. Therefore, at least 70 different breeding pairs that each contributed to one or two litters have been used and only one offspring per litter was used in one type of experiment. The generation of $A_{2A}^{-/-}$ mice and their genotyping by polymerase chain reaction amplification of tail DNA were described previously (Ledent *et al.* 1997). $A_{2A}^{-/-}$ and $A_{2A}^{+/+}$ mice were also generated on a C57BL/

6J background. The first generation chimeric (129X1/SvJ \times 129S1) \times C57BL/6J heterozygotes were backcrossed with C57BL/6J females for 10 generations before generating the $A_{2A}^{-/-}$ and $A_{2A}^{+/+}$ founders. All experimental $A_{2A}^{-/-}$ and $A_{2A}^{+/+}$ mice were of the F_{12} generation and were obtained after breeding homozygotes from the F_{11} generation. All experiments were performed under blind conditions. Knockout and wild-type control males (25–30 g) were housed 10 per cage (each genotype in a separate cage) in temperature-controlled ($21 \pm 1^\circ\text{C}$) and humidity-controlled ($55 \pm 10\%$) rooms with a 12-h light/12-h dark cycle (light between 0700 and 1900 h). Food and water were available *ad libitum* during all experiments. The number of animals was kept to a minimum. All efforts were made to avoid making the animals suffer, and animal procedures were conducted in accordance with the guidelines of the European Communities, Directive 86/609/European Economic Community regulating animal research and approved by the local ethical committee. All animals used in a given experiment originated from the same breeding series and were matched for age and weight. To test the effect of the $A_{2A}R$ agonist on ethanol consumption and preference, experiments were also performed on adult male C57BL/6J mice (Charles River, Saint Aubin les Elbeuf, France).

Drugs

Ethanol (95%, v/v) was obtained from Carlo Erba réactifs (Val de Reuil, France). Ethanol was diluted to 20% (v/v) in physiological saline before the intraperitoneal (i.p.) injection. Ethanol injections were made in volumes of 1.25 ml/100 g. Saline injections were made in volumes equal to that of the corresponding drug for each animal. A_{2A} -selective adenosine receptor agonist CGS 21680-HCl, was dissolved in saline and was obtained from Sigma Aldrich (Paris, France).

Alcohol intake test

Data for the alcohol intake study were collected from two experiments. Throughout the experiments, fluid intake and body weight were assessed everyday. C57BL/6J $A_{2A}^{-/-}$ mice (male, $n = 16$; female, $n = 13$) and $A_{2A}^{+/+}$ (male, $n = 13$; female, $n = 20$) were individually housed in plastic mouse cages with *ad libitum* access to standard rodent chow and habituated in their home cage to drinking from two bottles containing plain water for 1 week. Mice were then given 24-h access to two bottles, one containing water and the other containing ethanol in water. The ethanol concentration (v/v) was increased every 3 days; mice received 3%, 6%, 10%, 20% and finally 30% ethanol over the course of the experiment. The positions of the bottles were changed everyday to control for position preferences. Average ethanol consumption per day was obtained for each ethanol concentration. To obtain a measure of ethanol consumption that corrected for individual differences in mouse size, grams of ethanol consumed per kilogram of body weight per day were calculated for each mouse. As a measure of relative ethanol preference, an ethanol preference ratio was calculated by dividing total ethanol solution consumed by total fluid (ethanol plus water) consumption. To confirm our previous study on CD1 $A_{2A}^{-/-}$ mice, ethanol intake and preference were also measured in male $A_{2A}^{-/-}$ and $A_{2A}^{+/+}$ mice generated on a CD1 background ($A_{2A}^{+/+}$, $n = 10$; $A_{2A}^{-/-}$, $n = 10$).

CPP apparatus and procedures

Ethanol-induced CPP was measured in mice generated on a CD1 ($A_{2A}^{+/+}$, $n = 13$ and $A_{2A}^{-/-}$, $n = 13$ for each dose and each genotype) and a C57BL/6J ($A_{2A}^{+/+}$, $n = 10$ and $A_{2A}^{-/-}$, $n = 10$ for each dose and each genotype) background. A two-chambered CPP apparatus was used (Bioseb, Chaville, France), which consisted of two $30 \times 20 \times 20$ cm³ compartments with distinct visual and tactile cues (Houchi *et al.* 2005). One of the compartments had gray-colored walls and a stainless steel floor and the opposite compartment had black–white striped walls and a smooth floor. The two compartments were separated by a guillotine door. Distance and time spent in each compartment were measured by computer-interfaced infrared photo-beams (16×16). Both compartments were illuminated by dim light with 40 lx brightness. The procedure consisted of three different phases: preconditioning (day 1), conditioning (days 2–5) and

postconditioning (day 6). To control possible innate preferences for one of the two conditioning compartments, mice underwent a single preconditioning session. Immediately after saline injection, they were allowed free access to both conditioning compartments for 20 min. Initial place preference was determined by the side in which a mouse spent more than 600 seconds out of a 20-min trial. Place preference conditioning was conducted using an unbiased procedure (Cunningham *et al.* 2003). When a group of untrained mice showed a preference for one compartment (no more than 70% of time spent in one compartment), half of the animals received ethanol in the spontaneously preferred compartment and the other half in the nonpreferred compartment. We selected a counterbalanced protocol to reduce each mouse's initial preference, as discussed previously (Cunningham *et al.* 2003). Animals were randomly assigned to undergo either drug conditioning in the morning and saline conditioning in the afternoon, or vice versa. Animals received a total of two injections per day. For drug conditioning, animals were randomly assigned to receive either saline or ethanol (0.5, 1.0 and 2.0 g/kg i.p., prepared at 20% in saline). Immediately following administration, animals were confined to one of the two conditioning compartments for 20 min. The drug- and saline-paired conditioning compartments and the time of the day of the drug or saline conditioning session (morning or afternoon) were random and counterbalanced across all groups. Conditioning sessions were conducted twice daily for 4 days, with a minimum of 5 h between conditioning sessions. On the day following the last conditioning session, animals were tested for CPP by placing them between the two compartments (guillotine door removed) and allowing free access to both conditioning compartments for 20 min. CPP was determined by comparing the time spent (in seconds) in the drug-paired compartment during the preconditioning session and the time spent in the drug-paired compartment during the test session. The total number of entries in each compartment and the total distance traveled were measured during both preconditioning and test sessions.

Conditioned taste aversion

The aversive effect of ethanol was studied by the method of CTA at dose of 2.5 g/kg ethanol (i.p.) and only in mice produced on a CD1 background. The CTA procedure used in our study was according to the methods reported elsewhere (Palmer *et al.* 2004; Risinger & Cunningham 1998) and the 2.5 g/kg ethanol dose has been used in previous studies (Blednov *et al.* 2003; Diaz-Granados & Graham 2007). On the first day of the experiment, mice were individually housed for 3 days to adjust to single housing. Beginning on day 4, mice were placed on 2-h restricted water access from 0900 to 1100 h. This procedure lasted for 1 week. To evaluate the effect of the training procedure, the intake amount of water was recorded daily for each mouse. Mice were excluded from the test if the water intake amount was still not stable by the end of training procedure. On day 11, the mice were presented with a drinking tube containing 0.2 M NaCl in water instead of tap water that was used as the conditioned stimulus. The NaCl solution was available for 1 h (0900–1000 h). In the next 10 days, days 13, 15, 17, 19 and 21, all mice received 1 h (from 0900 to 1000 h) access to a solution of 0.2 M NaCl. The amount of NaCl solution consumed was recorded. Immediately after 1-h access period to the NaCl solution, mice received an injection of saline (control group) or ethanol (2.5 g/kg). To prevent possible dehydration, all mice (A_{2A}^{-/-}: NaCl, *n* = 11; ethanol, *n* = 13 and A_{2A}^{+/+}: NaCl, *n* = 7, ethanol, *n* = 11) were allowed access to water for 30 min, 5 h after the NaCl solution access period. On intervening days (on days 12, 14, 16, 18 and 20), mice were offered restricted access to tap water for 2 h (from 0900 to 1100 h), and the amount of water consumed was recorded. Mice were weighed daily just before NaCl solution or water presentation.

Effects of ethanol on locomotor activity

Locomotor activity was assessed in the LE 8811 IR motor activity monitor (Bioseb). Animals were confined to a 45-cm² clear acrylic plastic chamber, in which horizontal locomotion was measured from photocell beam interruptions. Photocell beams transected the cham-

ber 2 cm above the floor at 16 sites along each side. Test chambers were shielded from external noise and light, but each test field was illuminated with a white fluorescent light and was fully ventilated. Mice were injected i.p. with saline or 0.5–2.5 g/kg ethanol and placed immediately into activity monitors for 30 min. As no genotypic difference was observed during habituation period (data not shown), mice were not habituated to the activity chambers before testing ethanol response. Locomotor response was measured in wild-type and knockout mice generated on CD1 (*n* = 7 mice for each genotype and for each dose of ethanol) and C57BL/6J (*n* = 7–12 mice for each genotype and for each dose of ethanol) backgrounds. All animals were naive at the time of testing for each treatment.

Sensitization to the locomotor effects of ethanol

The general sensitization procedure was similar to that used by Boehm *et al.* (2008) and is described in Table 1. A_{2A}^{-/-} (NaCl, *n* = 12; EtOH, *n* = 12) and A_{2A}^{+/+} (NaCl, *n* = 12; EtOH, *n* = 12) mice generated on a CD1 background were subjected to a 14-day sensitization procedure in which they received daily ethanol (2.5 g/kg) or saline injections for 11 days starting on day 4 in their home cage (see protocol on Table 1). On the two first days, basal level of activity was measured after saline injection and on day 3 they were tested immediately after injection of 2.0 g/kg ethanol. On day 14, all mice received a challenge injection of ethanol (2.0 g/kg) and were immediately tested in the locomotor activity chambers. Locomotor activity was measured for 10 min on days 1, 2, 3 and 14. Blood ethanol concentrations were sampled immediately after testing and measured using an Analox Alcohol Analyser (Analox Instruments, Lille, France).

Anxiolytic effects of ethanol measured in the elevated plus-maze

The elevated plus-maze apparatus was a modification of that validated by Lister (1987) and consisted of two open (30 × 5 × 0.25 cm³) and two enclosed (30 × 5 × 5 cm³) arms that extended from a common central platform (5 × 5 cm²). The apparatus was constructed from black plexiglass and elevated 60 cm above the floor. In accordance with established procedures (Rodgers & Johnson 1995), male A_{2A}^{-/-} (NaCl, *n* = 10; EtOH, *n* = 10) and A_{2A}^{+/+} (NaCl, *n* = 10; EtOH, *n* = 10) mice generated on a CD1 background were individually placed on the central platform of the maze facing an open arm immediately after an i.p. injection of either saline or 1.5 g/kg ethanol and allowed to freely explore the apparatus for 5 min under 200 lx lighting. The apparatus was thoroughly cleaned between test sessions. The conventional spatiotemporal measures (i.e. open arm time and entries) were scored. A mouse was considered to have entered an arm when all four of its paws were placed in the arm. Behavior was scored by an experienced observer in the room.

Effect of CGS 21680 on ethanol intake in C57BL/6J mice

Male C57BL/6J mice (*n* = 12) (10 weeks old; Charles River Laboratories, L'Arbresle, France) were individually housed in plastic mouse cages with *ad libitum* access to standard rodent chow and were habituated in their home cage to drinking from two bottles containing plain water for 1 week. For the next 4 weeks, animals were allowed free choice between 10% ethanol in tap water or tap water. All

Table 1: Ethanol-induced Locomotor sensitization paradigm

	Test day 1–2, habituation	Test day 3, acute EtOH	No test days 4–13, daily treatments	Test day 14, sensitization expression
NaCl group	NaCl	NaCl	NaCl	2.0 g/kg
Repeated EtOH group	NaCl	2.0 g/kg	2.5 g/kg	2.0 g/kg

injections were made in saline each day 1 h before the dark period (lights off at 1900 h). During the first week saline was i.p. administered. The next week, mice were injected with 0.5 mg/kg CGS 21680 and they received 1.0 mg/kg CGS 21680 for an additional week. The last week, saline was injected to analyze the recovery from the CGS 21680 treatment. Ethanol consumption and preference were measured everyday and the positions of the bottles were changed everyday to control for position preferences.

Data analyses

Statistical analyses were conducted using STATVIEW 5.0 software. Ethanol consumption and preference were analyzed with a three-way (sex \times genotype \times concentration) repeated-measure analysis of variance (RM-ANOVA) for the C57BL/6J background and a two-way (genotype \times concentration) RM-ANOVA for the CD1 background, followed by a Tukey's *post hoc* test. For the CPP experiments, data were analyzed using a three-way (genotype \times dose \times pre-post conditioning) RM-ANOVA followed by a Tukey's *post hoc* test. For the CTA experiments, data were analyzed using a three-way (genotype \times day \times treatment) RM-ANOVA followed by a Tukey's *post hoc* test. For the locomotor activity, the effect of genotype and drug was analyzed using a two-way ANOVA (genotype \times dose) and Tukey's *post hoc* test. For the elevated plus-maze, the effect of genotype and treatment was analyzed using a two-way ANOVA (genotype \times treatment) and Tukey's *post hoc* test. The effects of CGS 21680 on ethanol consumption and preference were analyzed with a one-way RM-ANOVA, followed by a *post hoc* Tukey's test. A significance level of 0.05 was used for all tests.

Results

Alcohol intake test

In a two-bottle free-choice paradigm in which C57BL/6J wild-type and mutant mice could drink either water or an ascending series of ethanol concentrations (3%, 6%, 10%, 20% and 30%), three-way RM-ANOVA revealed that there were significant main effects of gender ($F_{1,928} = 122.75$; $P < 0.001$), ethanol concentration ($F_{4,928} = 144.22$; $P < 0.001$) and a significant gender \times ethanol concentration interaction ($F_{4,928} = 7.36$; $P < 0.001$), but no other interactions, for consumption of ethanol (Fig. 1a). There were significant main effects of gender ($F_{1,928} = 4.91$; $P < 0.05$), ethanol concentration ($F_{4,928} = 304.36$; $P < 0.001$) and a significant gender \times ethanol concentration interaction ($F_{4,928} = 4.1$; $P < 0.005$), but no other interactions, for ethanol preference (Fig. 1b). There was no difference in total fluid intake. In accordance with numerous studies (Naassila *et al.* 2002; Naassila *et al.* 2004), ethanol consumption and preference were increased in female mice compared with male mice generated on the C57BL/6J background.

Male null mice generated on a CD1 genetic background showed a strong increase of ethanol consumption and preference compared with wild-type mice (main effect of genotype, consumption: $F_{1,284} = 58.09$; $P < 0.001$ and preference: $F_{1,284} = 37.71$; $P < 0.001$; Fig. 1c,d). There were a main effect of ethanol concentration (consumption: $F_{4,284} = 14.43$; $P < 0.001$ and preference: $F_{4,284} = 48.95$; $P < 0.001$) and a genotype \times ethanol concentration interaction (consumption: $F_{4,284} = 2.57$; $P < 0.05$ and preference: $F_{4,284} = 4.01$; $P < 0.005$). There was no difference in total fluid intake.

Ethanol-induced CPP

Preconditioning showed no significant difference in the initial preference between mutant and wild-type mice in both

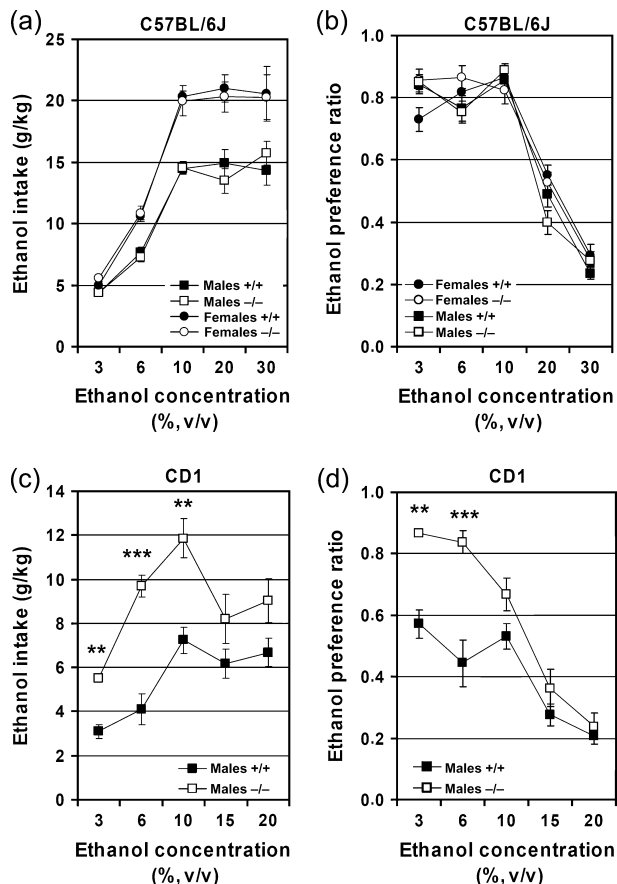


Figure 1: Ethanol consumption and preference. Voluntary ethanol consumption in mice generated on a C57BL/6J background (a, b) $A_{2A}^{-/-}$ mice (male, $n = 16$; female, $n = 13$) and $A_{2A}^{+/+}$ (male, $n = 13$; female, $n = 20$) and on a CD1 (c, d) genetic background ($A_{2A}^{+/+}$, $n = 10$; $A_{2A}^{-/-}$, $n = 10$). ** $P < 0.01$, *** $P < 0.001$, significant differences relative to wild-type mice for similar concentration of ethanol (Tukey's *post hoc* analyses).

genetic backgrounds (data not shown). However, there was a significant difference between the two genetic backgrounds. C57BL/6J mice spent more time (approximately 10%) in the compartment subsequently paired with ethanol compared with the CD1 mice [three-way (background \times genotype \times ethanol dose) ANOVA: main effect of background $F_{1,103} = 12.45$; $P < 0.001$].

In mice produced on a CD1 genetic background, three-way RM-ANOVA revealed a significant ethanol dose \times session \times genotype interaction ($F_{2,216} = 3.37$; $P < 0.05$). There were also significant session \times genotype ($F_{1,216} = 5.73$; $P < 0.05$) and ethanol dose \times session ($F_{2,216} = 5.51$; $P < 0.005$) interactions. Tukey's *post hoc* analyses indicated that the repetitive administration of ethanol (0.5–2.0 g/kg) during 4 days resulted in the development of a place preference in wild-type mice at both the 1.0 and 2.0 g/kg ethanol doses, whereas knockout mice displayed a significant place preference only at the highest dose (Fig. 2a), therefore revealing that knockout mice were less sensitive to the rewarding effects of ethanol.

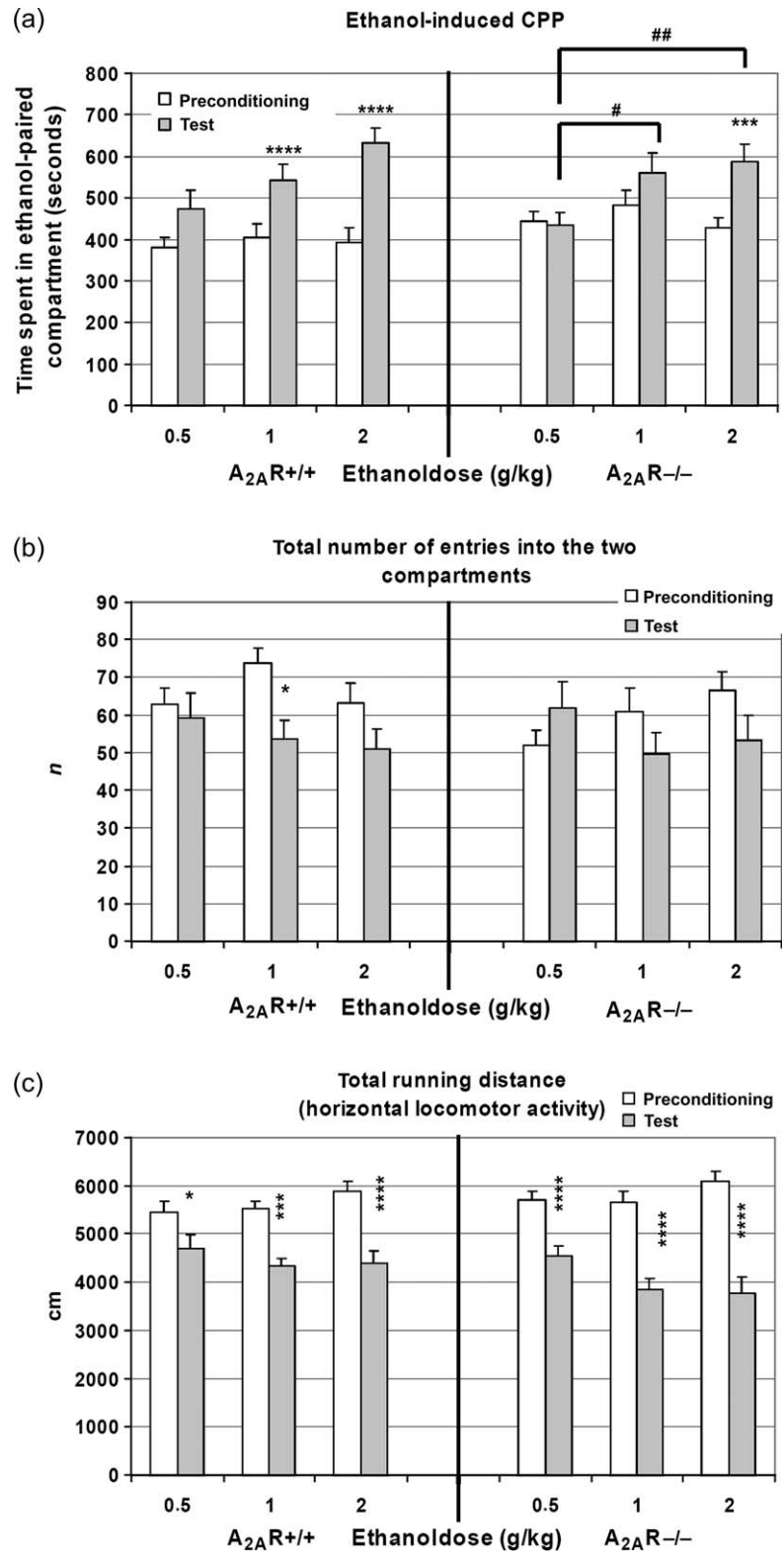


Figure 2: Ethanol-induced CPP in mice produced on the CD1 background. Effect of increasing doses of ethanol on the establishment of CPP in wild-type or knockout mice generated on a CD1 background (a). The bars represent the time spent on the ethanol-paired side from the preconditioning and the test session (mean ± SEM). Total number of entries in the two compartments (b) and total running distance (c). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, significant differences relative to respective preconditioning and ###*P* < 0.001 as indicated (Tukey's *post hoc* analyses). A_{2A}^{+/+}, *n* = 18 and A_{2A}^{-/-}, *n* = 18 for each dose and each genotype.

There was no significant ethanol dose \times session \times genotype interaction neither for the total number of entries ($F_{2,216} = 0.65$; $P > 0.05$) nor for the total running distance ($F_{2,216} = 0.79$; $P > 0.05$). For the total number of entries, there was a significant ethanol dose \times session interaction ($F_{2,216} = 3.09$; $P < 0.05$), and Tukey's *post hoc* analysis indicated that ethanol 1.0 g/kg induced a significant decrease of this parameter in $A_{2A}^{+/+}$ the day of the test (Fig. 2b). For the total running distance, there were significant session \times genotype ($F_{1,72} = 5.60$; $P < 0.05$) and ethanol dose \times session ($F_{2,72} = 4.45$; $P < 0.05$) interactions, and Tukey's *post hoc* analyses indicated that locomotor activity was significantly decreased during the test session similarly in both genotypes (Fig. 2c).

In contrast, in mice generated on a C57BL/6J background, three-way ANOVA revealed that ethanol (1.0–2.0 g/kg) did not induce CPP in both genotypes [no significant ethanol dose \times session \times genotype interaction ($F_{1,80} = 2.69$; $P > 0.05$) and no other interactions (Fig. 3a)]. There was no significant ethanol dose \times session \times genotype interaction neither for the total number of entries ($F_{1,80} = 0.22$; $P > 0.05$) nor for the total running distance ($F_{1,80} = 0.02$; $P > 0.05$). For the total number of entries, there were main effects of session ($F_{1,80} = 9.19$; $P < 0.005$), genotype ($F_{1,80} = 13.35$; $P < 0.0005$) and significant session \times genotype ($F_{1,80} = 5.75$; $P < 0.01$), dose \times genotype ($F_{1,80} = 6.37$; $P < 0.01$) and session \times ethanol dose ($F_{1,80} = 3.76$; $P < 0.05$) interactions. Tukey's *post hoc* analyses indicated that ethanol 1.0 g/kg induced a significant increase of this parameter in $A_{2A}^{+/+}$ the day of the test compared with the preconditioning day ($P < 0.001$) and with the $A_{2A}^{-/-}$ group ($P < 0.001$) (Fig. 3b). For the total running distance, there was a significant session \times genotype interaction ($F_{1,80} = 6.03$; $P < 0.05$) and Tukey's *post hoc* analyses indicated that locomotor activity was significantly decreased during the test session in $A_{2A}^{-/-}$ mice compared with the preconditioning day (1.0 and 2.0 g/kg: $P < 0.01$ and $P < 0.001$, respectively) and compared with wild-type mice (1.0 and 2.0 g/kg: $P < 0.01$ and $P < 0.05$, respectively) (Fig. 3c).

Conditioned taste aversion

Three-way ANOVA revealed that ethanol–NaCl pairings produced reductions in NaCl intake across trials similarly in both genotypes, and there were main effects of ethanol treatment ($F_{1,228} = 226.94$; $P < 0.001$), trials ($F_{5,228} = 4.06$; $P < 0.005$) and a significant ethanol treatment \times trials interaction ($F_{5,228} = 10.75$; $P < 0.001$), but no other interactions (Fig. 4).

Response to the locomotor effects of ethanol in both CD1 and C57BL/6J genetic backgrounds

In mice generated on a CD1 genetic background, two-way ANOVA revealed a main effect of ethanol dose ($F_{3,51} = 4.07$, $P < 0.05$) and a significant dose \times genotype interaction ($F_{3,51} = 4.025$, $P < 0.05$) (Fig. 5a). Tukey's *post hoc* analysis showed a significant increase of locomotion in $A_{2A}^{-/-}$ mice at the 1.5 g/kg dose ($P < 0.05$) with a significant genotypic difference ($P < 0.01$). In contrast, locomotion was unaltered by ethanol administration in $A_{2A}^{+/+}$ mice.

In mice generated on a C57BL/6J genetic background (Fig. 5b), the two-way ANOVA revealed no significant effect of the genotype ($F_{1,97} = 1.67$, $P > 0.05$), a main effect of ethanol dose ($F_{5,97} = 3.79$, $P < 0.005$) and a significant genotype \times ethanol dose interaction ($F_{5,97} = 4.025$, $P < 0.05$). Tukey's *post hoc* analysis indicated that locomotor activity was significantly decreased at the 2.5 g/kg ethanol dose in $A_{2A}^{+/+}$ mice ($P < 0.05$) and that locomotor activity was significantly reduced in $A_{2A}^{+/+}$ compared with $A_{2A}^{-/-}$ at higher doses (1.5 and 2.5 g/kg doses, $P < 0.05$).

Ethanol-induced locomotor sensitization

Repeated ethanol injection induced a significant locomotor sensitization similarly in both genotypes (Fig. 6). Three-way ANOVA revealed that there were main effects of day ($F_{1,179} = 13.52$; $P < 0.0005$), treatment ($F_{1,179} = 13.18$; $P < 0.0005$) and a significant day \times treatment interaction ($F_{1,179} = 3.73$; $P < 0.05$). Tukey's *post hoc* analysis indicated that repeated ethanol injection induced an enhancement of the locomotor response to ethanol on day 14 in both genotypes compared with mice that received repeated saline injection and compared with mice that received ethanol on day 3 ($P < 0.05$).

On day 14, blood ethanol concentrations did not differ between genotypes (in mg/dl, NaCl: $A_{2A}^{+/+}$ 154.0 ± 10.4 and $A_{2A}^{-/-}$ 145.0 ± 8.7 ; EtOH: $A_{2A}^{+/+}$ 149.9 ± 5.6 and $A_{2A}^{-/-}$ 144.2 ± 8.0).

Basal level of anxiety-like behavior and response to the anxiolytic effects of ethanol

Basal levels of anxiety were not statistically different between genotypes (% time open arm, $F_{1,74} = 2.47$, $P = 0.12$, Fig. 7a; % open arm entries, $F_{1,74} = 2.01$, $P = 0.16$, Fig. 7b). There was also no genotypic difference in the basal locomotor activity assessed by the total arm entries number ($F_{1,74} = 0.46$, $P = 0.5$; Fig. 7c).

Ethanol increased the percentage time in open arms (main effect of treatment, $F_{1,74} = 19.31$, $P < 0.001$) and the percentage open arm entries (main effect of treatment, $F_{1,74} = 41.22$, $P < 0.001$), revealing its anxiolytic effect in both genotypes. Two-way ANOVA revealed that $A_{2A}^{-/-}$ were more sensitive to the anxiolytic effect of ethanol (% open arm entries, $P < 0.05$ and % time open arm, $P < 0.01$). In addition, ethanol increased the number of total arm entries (main effect of treatment, $F_{1,74} = 14.84$, $P < 0.001$) in both genotypes and to the same extent.

Effect of the A_{2A} agonist CGS 21680 on ethanol intake and preference

A significant decrease was observed in ethanol consumption ($F_{3,318} = 29.89$, $P < 0.001$; Fig. 8a) and preference ($F_{3,318} = 38.62$, $P < 0.001$; Fig. 8b) after daily CGS 21680 treatment. *Post hoc* analyses revealed a significant effect of both the 0.5 and 1.0 mg/kg doses ($P < 0.05$) and a significant recovery when the treatment was discontinued ($P < 0.05$). At the 0.5 mg/kg dose of CGS 21680, both ethanol preference and consumption were significantly decreased the first

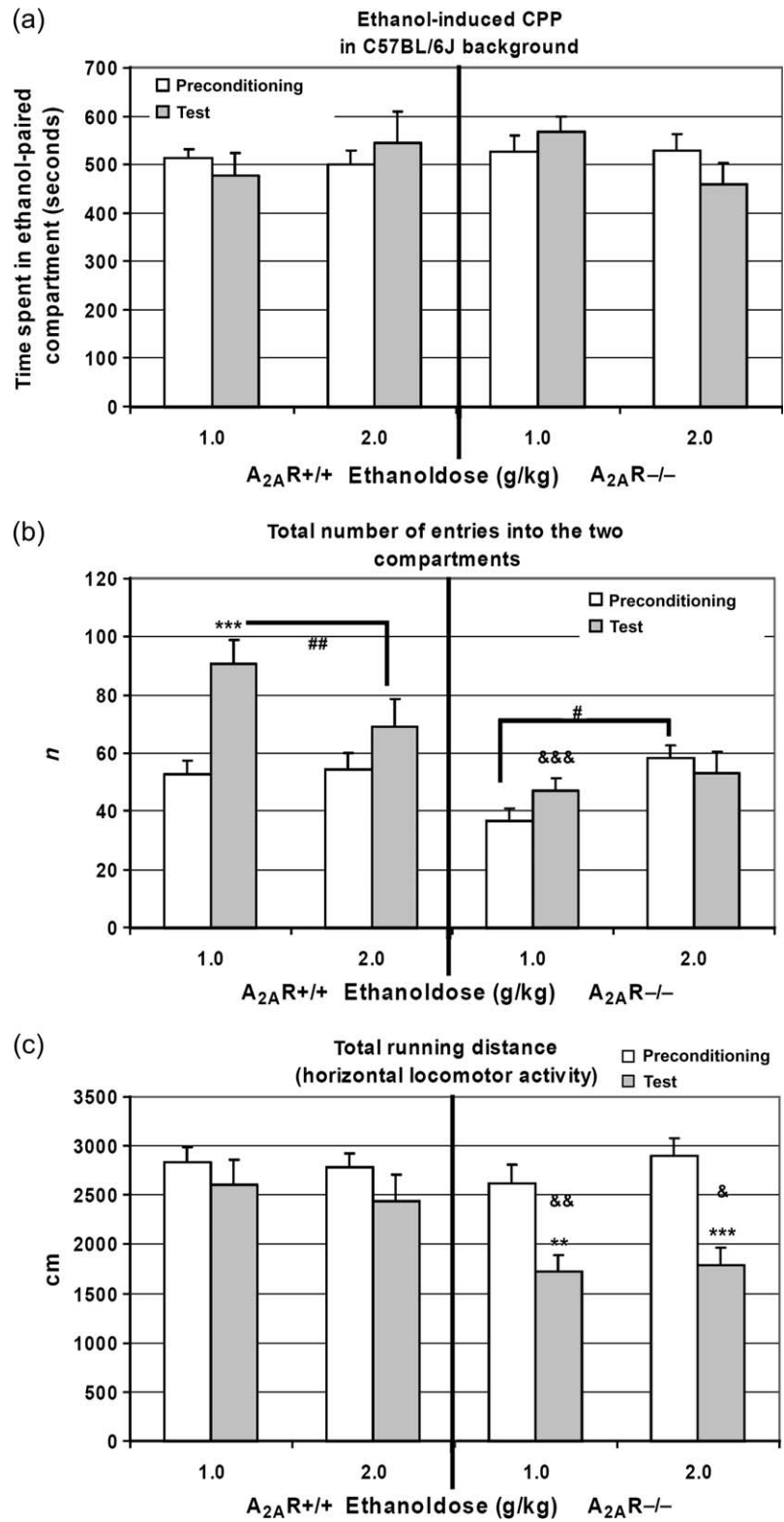


Figure 3: Ethanol-induced CPP in mice produced on the C57BL/6J background. Effect of ethanol (1.0 and 2.0 g/kg) on the establishment of CPP in wild-type or knockout mice generated on a C57BL/6J background (a). The bars represent the time spent on the ethanol-paired side from the preconditioning and the test session (mean ± SEM). Total number of entries in the two compartments (b) and total running distance (c). A_{2A}^{+/+}, n = 10 and A_{2A}^{-/-}, n = 10 for each dose and each genotype. **P < 0.01, ***P < 0.001 compared with respective preconditioning; #P < 0.05, ##P < 0.01 as indicated and &P < 0.05, &&P < 0.01, &&&P < 0.001 compared with respective A_{2A}^{+/+} group (Tukey's *post hoc* analyses).

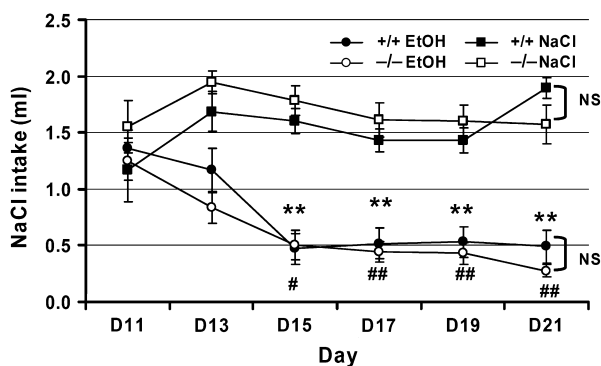


Figure 4: Effect of A_{2A} gene deletion on a CD1 background on conditioned taste aversion for ethanol ($n = 8-10$ per group). No difference was observed on basal NaCl solution consumption between the two genotypes. Ethanol (2.5 g/kg) induced CTA similarly in mice of both genotypes. $**P < 0.01$, significant differences relative to respective day 11 (D11) in wild-type mice group and $\#P < 0.05$, $\#\#P < 0.01$, compared with respective D11 in knockout mice group (Tukey's *post hoc* analyses). $A_{2A}^{-/-}$ mice (NaCl, $n = 11$; EtOH, $n = 13$) and $A_{2A}^{+/+}$ (NaCl, $n = 11$; EtOH, $n = 7$).

day of injection (with a maximum effect observed the first day) and the effect was diminished with repeated injections (data not shown). At the 1.0 mg/kg dose of CGS 21680, both ethanol preference and consumption were significantly decreased the first day of injection and the effect was enhanced with repeated injections (data not shown). The recovery to basal levels of both ethanol preference and consumption was achieved the first day after the end of treatment.

One-way RM-ANOVA indicated that CGS 21680 treatment did not alter total fluid consumption ($F_{3,318} = 1.64$, $P > 0.05$) (data not shown).

Discussion

The results of the present study can be summarized as follows: (1) deletion of the $A_{2A}R$ gene in mice generated on a CD1 background induced a decrease in ethanol-induced CPP, (2) the increased ethanol consumption is associated with an increased sensitivity to the motor stimulant and anxiolytic effects of ethanol in mice generated on a CD1 background, (3) A_{2A} agonist decreased both ethanol preference and consumption in C57BL/6J mice following 7 days of administration and (4) the genotypic difference in ethanol consumption, ethanol-induced CPP and motor-stimulant effects of ethanol observed in the CD1 background is not found in the C57BL/6J background.

We confirmed our previous study that has shown an increased ethanol preference and consumption in $A_{2A}^{-/-}$ mice generated on a CD1 background (Naassila *et al.* 2002). $A_{2A}^{-/-}$ mice displayed an increased ethanol consumption approximately twofold compared with wild-type mice, at the 3%, 6% and 10% ethanol concentrations. Ethanol consumption might be increased as a consequence of the lower sensitivity to the aversive effects of ethanol (Broadbent *et al.* 2002) or alteration of its rewarding properties. Our results

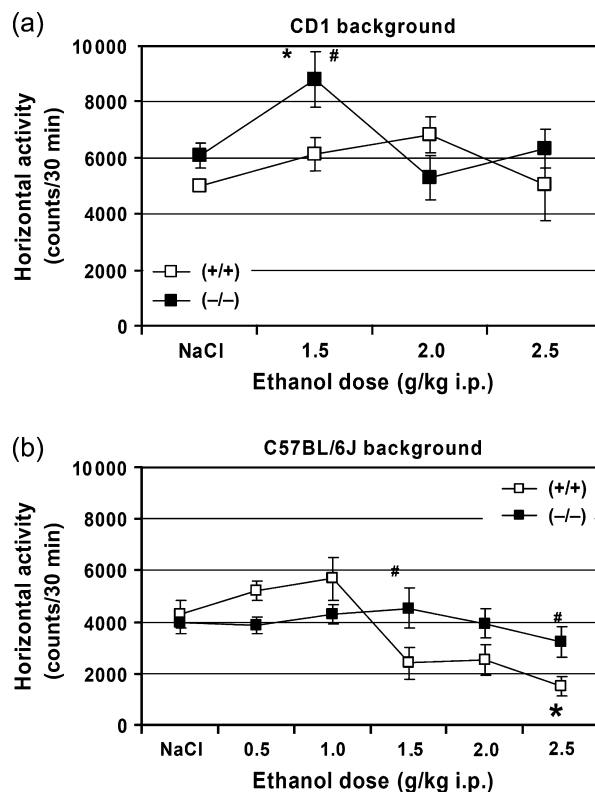


Figure 5: Effects of ethanol on locomotor activity. Locomotor effects of increasing doses of ethanol in wild-type and knockout mice generated on a CD1 (a) ($n = 7$ mice for each genotype and for each dose of ethanol) or on a C57BL/6J (b) ($n = 7-12$ mice for each genotype and for each dose of ethanol) background. Locomotor activity was measured for 30 min immediately after i.p. ethanol injection. $*P < 0.05$, significant differences relative to respective NaCl and $\#P < 0.05$, compared with wild-type mice for similar concentration of ethanol (Tukey's *post hoc* analyses).

indicate that the aversive properties of ethanol measured with the CTA procedure are not altered in knockout mice. However, this conclusion must be tempered by the fact that only one dose of ethanol was tested in the CTA procedure, as performed in other studies (Blednov *et al.* 2003; Diaz-Granados & Graham 2007). By contrast, the increase in ethanol consumption is associated with a decrease in ethanol-induced CPP in mice produced on a CD1 background. Previous study has shown that selective $A_{2A}R$ agonists attenuated the rewarding effects of brain stimulation, suggesting that adenosine, through $A_{2A}R$, may inhibit central reward processes (Baldo *et al.* 1999). In our experimental conditions for the CPP, it is noteworthy that we observed a significant difference in the initial time spent in the compartment subsequently paired with ethanol between the two genetic backgrounds, but it is unlikely that this difference in the initial time spent in the compartment subsequently paired with ethanol may affect the induction of the CPP. In this regard, we have previously shown using the same procedure in mice that the time spent in the compartment subsequently paired with ethanol

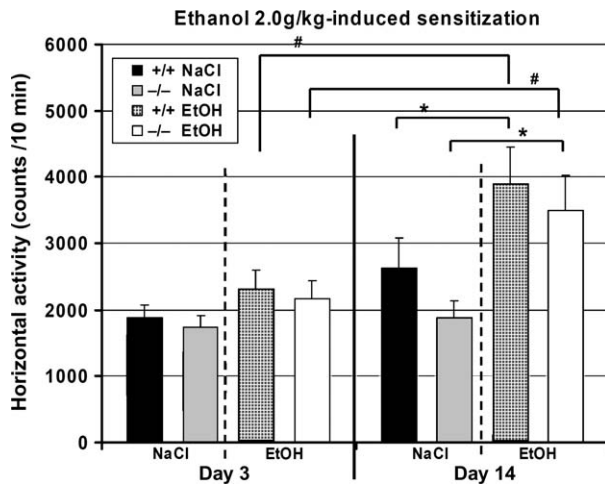


Figure 6: Ethanol 2.0 g/kg-induced behavioral sensitization. Mice generated on a CD1 background ($n = 12/\text{group}$) were subjected to a 14-day sensitization procedure in which they received daily ethanol (2.5 g/kg) or saline injections for 11 days starting on day 4 in their home cage. On day 3 and day 14, they were tested immediately after injection of 2.0 g/kg ethanol. $A_{2A}^{-/-}$ mice (NaCl, $n = 12$; EtOH, $n = 12$) and $A_{2A}^{+/+}$ (NaCl, $n = 12$; EtOH, $n = 12$). $*P < 0.05$ compared with respective group on day 14, $\#P < 0.05$, compared with respective group on day 3 (Tukey's *post hoc* analyses).

(400–600 seconds) did not influence the induction of CPP by ethanol (Houchi *et al.* 2005). In our paradigm, we also observed a decrease in the locomotor activity during the test session, it is unlikely that the decrease in locomotor activity observed during the test session may increase the induction of CPP by ethanol as suggested in a previous study (Gremel & Cunningham 2007). Our results show no correlation between the level of activity during the test session and the induction of CPP. Knockout mice generated on the C57BL/6J background displayed a decrease in locomotor activity during the test session as observed in the CD1 background. In addition, the C57BL/6J strain exhibited a decreased locomotor activity compared with the CD1 strain and did not develop significant CPP.

Our study also emphasized the importance of the genetic background. The inability to find a difference in alcohol consumption and preference in C57BL/6J knockout mice may reflect that C57BL/6J mice are an alcohol-preferring line and already exhibit exceedingly high alcohol preference, suggesting that there might be a ceiling effect. In other words, an increase in alcohol preference by a mutation in a mouse line that is already alcohol preferring is unlikely. Consistent with our data, a recent report indicated that CD1 mice displayed an alcohol-avoiding phenotype compared with C57BL/6J mice (Short *et al.* 2006). Short *et al.* (2006) also indicated that the basal level of $A_{2A}R$ expression (mRNA and receptor density) is decreased in the caudate putamen and nucleus accumbens of C57BL/6J mice compared with CD1 mice, suggesting that the alcohol-preferring phenotype is associated with lower expression of $A_{2A}R$ in the reward system. The present results also showed that the alterations

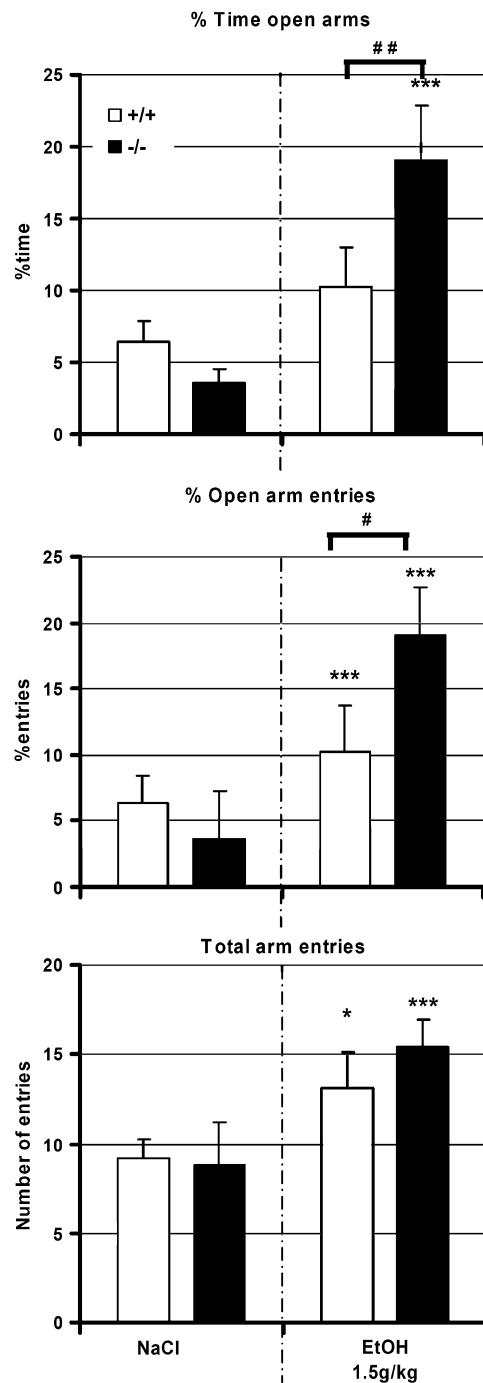


Figure 7: Effects of ethanol on anxiety-like behavior in the elevated plus-maze test. Ethanol (1.5 g/kg) effects on the percentage of time spent (a) and entries (b) in the open arms, and in the total number of entries (c) in mice submitted to the elevated plus-maze. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with respective NaCl and $\#P < 0.05$, $\#\#\#P < 0.01$ compared to respective wild-type mice (Tukey's *post hoc* analyses). $A_{2A}^{-/-}$ mice (NaCl, $n = 10$; EtOH, $n = 10$) and $A_{2A}^{+/+}$ (NaCl, $n = 10$; EtOH, $n = 10$) generated on a CD1 background.

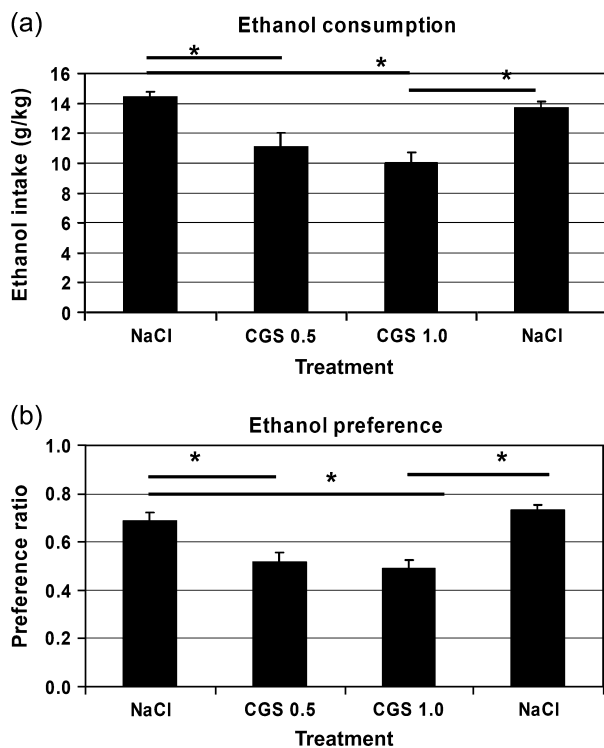


Figure 8: Effect of the A_{2A} receptor antagonist CGS 21680 on ethanol intake in C57BL/6J mice. Effect of CGS 21680 (0.5 and 1.0 mg/kg) on ethanol consumption (a) and preference (b) in C57BL/6J mice consuming a 10% ethanol solution or water. Each bar represents the mean \pm SEM of daily measurements for 1 week. * $P < 0.05$ as indicated (Tukey's *post hoc* analyses). A total of 12 mice was used for the experiment.

in the conditioned rewarding and motor-stimulant effects of ethanol were dependent on the genetic background because we showed that ethanol did not induce significant place conditioning and motor-stimulant effects in the C57BL/6J background. Previous reports have already shown that the behavioral responses to ethanol are highly influenced by the genetic background used to generate the knockouts (Bowers *et al.* 1999) and that the C57BL/6J background is resistant to the conditioned rewarding and locomotor-activating effects of ethanol (Cunningham *et al.* 1992).

Recent evidence suggests a relationship between heightened ethanol preference and susceptibility to ethanol-induced locomotor sensitization (Grahame *et al.* 2000; Lessov *et al.* 2001). Ethanol-stimulated activity has been hypothesized to reflect euphoria and possibly reward (Phillips & Shen 1996). Our procedure of repeated ethanol injection in the home cage produced the development of locomotor sensitization. A_{2A}R gene deletion in mice generated on a CD1 background did not alter the sensitized response, suggesting that A_{2A}R are not important for these ethanol-mediated responses.

In the elevated plus-maze test, mutant and wild-type mice produced on the CD1 background showed an equivalent basal level of anxiety and these data are inconsistent with a previous study (Ledent *et al.* 1997). This discrepancy may be explained

by the fact that in our experimental conditions the basal level of anxiety is high. Importantly our data show that A_{2A}^{-/-} mice generated on a CD1 background were more sensitive to the anxiolytic effects of ethanol (1.5 g/kg). The relationship between anxiety and ethanol has been a matter of considerable controversy. For example, the tension-reduction hypothesis, initially proposed by Conger (1956), predicts that certain individuals who are innately anxious and more sensitive to the anxiolytic effects of ethanol may show a higher predisposition for ethanol drinking and abuse (for a review see Greeley & Oei 1999). A significant ethanol CPP has been shown in rats previously selected to be anxious in the elevated plus-maze, but not in the 'non-anxious rats' (Blatt & Takahashi 1999). Spanagel *et al.* (1995) showed a significantly higher intake and preference for ethanol in rats selected as anxious in the plus-maze test, which led them to suggest that the degree of anxiety may underlie, at least in part, the initial motivation to drink alcohol. These results agree with the study of Stewart *et al.* (1993), which indicated a higher degree of anxiety in ethanol-preferring than nonpreferring rats. In the present study, mutant and wild-type mice showed equivalent basal level of anxiety, and mutant mice displayed an increase in sensitivity to ethanol's anxiolytic effects. Therefore, our results are in line with those suggesting a positive correlation between the level of ethanol intake and preference and the sensitivity to the anxiolytic effects of ethanol.

There is emerging evidence that adenosinergic mechanisms contribute to ethanol consumption. Mice lacking the adenosine transporter (ENT1^{-/-}), displayed a decreased A₁ adenosine tone in the nucleus accumbens and elevated levels of ethanol consumption compared with wild-type mice (Choi *et al.* 2004). In contrast, it has also been shown that ethanol operant self-administration is not altered by an A₁R antagonist while it is bimodally affected by an A_{2A}R antagonist (Arolfo *et al.* 2004). Choi *et al.* (2004) also suggested that ENT1^{-/-} mice may display a decrease in A_{2A}R activation that is consistent with our previous finding of increased alcohol consumption in A_{2A}^{-/-} mice (Naassila *et al.* 2002). However, Choi *et al.* (2004) reported that the decrease in A_{2A}R activation may not play a major role because the observed increase in ethanol consumption is corrected with an A₁R agonist. It is noteworthy that complex adaptations and compensations induced by lifelong gene deletion have been described in knockout mice. For example, adenosine transporter and A₁R binding sites were, respectively, decreased and increased in the brain of A_{2A}^{-/-} mice (Snell *et al.* 2000). As previous data suggested that activating the A_{2A}R may play a role in suppressing alcohol-drinking behavior (Naassila *et al.* 2002) and in reducing the rewarding effects of brain stimulation (Baldo *et al.* 1999), we tested the effect of the A_{2A} agonist CGS 21680 on ethanol consumption in the alcohol-preferring C57BL/6J strain. Our results confirmed this hypothesis because A_{2A}R activation by CGS 21680 (0.5–1.0 mg/kg) decreased both ethanol consumption and preference (~30% decrease). At the 1.0 mg/kg dose of CGS 21680, the drug was more effective in decreasing both ethanol preference and consumption with repeated administration. The present results are in line with a previous report that showed an increase in ethanol self-administration after A₂ antagonism with DMPX at low concentration (Arolfo *et al.* 2004). It is noteworthy that increasing the dose of DMPX has

been shown to decrease ethanol self-administration possibly through an action at distinct receptor population and/or a decrease in pharmacological selectivity (Arofo *et al.* 2004).

It should be noted that the breeding strategy used in the present study may have influenced the results as the experimental animals were produced from homozygote breeders. Therefore, there are potential background genetic effects, maternal effects and other environmental effects (e.g. each genotype has been housed separately) that could impact the results.

In summary, the lack of A_{2A}R gene expression changed an ethanol nonpreferring strain (CD1) into an ethanol-preferring strain at low ethanol doses and this phenotype was not maintained in mice produced on a C57BL/6J genetic background. The increased propensity to drink ethanol in A_{2A}^{-/-} mice generated on a CD1 background was associated with an increase in sensitivity to the motor stimulant and anxiolytic effects of ethanol and a decrease in ethanol-induced CPP. Drugs that activate A_{2A}R function might be beneficial in the treatment and prevention of high drinking, and our results shed light on the A_{2A}R agonism that may be considered as an innovative approach to reduce alcohol consumption.

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