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Anxiogenic and stress-inducing effects of peripherally administered acetaldehyde in mice: Similarities with the disulfiram–ethanol reaction

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ABSTRACT

Peripheral accumulation of acetaldehyde, the first metabolite of ethanol, produces autonomic responses in humans called "flushing". The aversive characteristics of flushing observed in some populations with an isoform of aldehyde dehydrogenase (ALDH2) less active, are the basis for treating alcoholics with disulfiram, an ALDH inhibitor. Although ethanol and centrally formed acetaldehyde have anxiolytic effects, peripheral accumulation of acetaldehyde may be aversive in part because it is anxiogenic.

Objectives: We investigated the effect of direct administration of acetaldehyde on behavioral measures of anxiety and on hormonal markers of stress in mice. The impact of disulfiram on the anxiolytic actions of ethanol was evaluated. Acetate (a metabolite of acetaldehyde) was also studied.

Methods: CD1 male mice received acetaldehyde (0, 25, 50, 75 or 100 mg/kg) at different time intervals and were assessed in the elevated plus maze and in the dark–light box. Corticosterone release after acetaldehyde administration was also assessed. Additional experiments evaluated the impact of disulfiram on the anxiolytic effect of ethanol (0 or 1 mg/kg), and the effect of acetate on the plus maze.

Results: Direct administration of acetaldehyde (100 mg/kg) had an anxiogenic effect at 1, 11 or 26 min after IP administration. Acetaldehyde was ten times more potent than ethanol at inducing corticosterone release. Disulfiram did not affect behavior on its own, but blocked the anxiolytic effect of ethanol at doses of 30 and 60 mg/kg, and had an anxiogenic effect at the highest dose (90 mg/kg) when co-administered with ethanol. Acetate did not affect any of the anxiety parameters.

Conclusions: Peripheral administration or accumulation of acetaldehyde produces anxiogenic effects and induces endocrine stress responses. This effect is not mediated by its metabolite acetate.

1. Introduction

The "alcohol flushing response" is a cutaneous vasodilation in response to moderate alcohol ingestion, which is accompanied by other autonomic symptoms (tachycardia, palpitation, dizziness, nausea, etc.; Chao, 1995; Eriksson, 2001; Von Wartburg, 1987). It occurs in individuals with at least one inactive aldehyde dehydrogenase (ALDH) allele (ALDH2*2) (Shibuya et al., 1989), which results in acetaldehyde, the first metabolite of ethanol, not being removed normally and accumulating in different tissues (Mizoi et al., 1983; Crabb et al., 1989; Isse et al., 2005). General acetaldehyde accumulation has been implicated as the major factor discouraging excessive drinking in this population (Harada et al., 1982; Luczak et al., 2002; Peng et al., 2007), and this factor is the basis for treating alcoholics with ALDH inhibitors like disulfiram or cyanamide (Kristenson, 1995; Valérdiz and Vázquez, 1989; Niederhofer et al., 2003). Disulfiram irreversibly inhibits ALDH (Marchner and Tottmar, 1978), and in individuals that consume alcohol it produces a reaction that is similar to the one observed in people with the genetic mutation, which acutely causes considerable distress (Kristenson, 1995). The autonomic symptoms produced by the interaction between ethanol and disulfiram (Johnsen et al., 1992; Peachey et al., 1983) resemble some of the vegetative responses observed after an acute anxiety episode.

It is well known that ethanol at moderate doses has anxiolytic effects in humans and rodents (Abrams et al., 2001; Boehm et al., 2002; Correa et al., 2008; Gallate et al., 2003; Tambour et al., 2005). In a previous study in mice, it was found that when catalase-mediated metabolism of ethanol into acetaldehyde is blocked, or acetaldehyde is inactivated by D-penicillamine, there is a suppressive effect of the anxiolytic actions of ethanol (Correa et al., 2008), suggesting that centrally formed acetaldehyde contributes to the anxiolytic effects of ethanol, since catalase has a minor role in peripheral ethanol metabolism (Hunt, 1996) but seems to have a critical role in brain ethanol metabolism (Aragon et al., 1992; Correa et al., 2009; Zimatkin and Deitrich, 1997; Zimatkin et al., 2006). Moreover, in rats, centrally administered acetaldehyde (intraventricular, ICV) is more efficacious at producing anxiolytic effects than centrally administered ethanol

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(Correa et al., 2003). In humans, the situation is more complicated because peripheral increases in acetaldehyde can lead also to elevated brain acetaldehyde levels. It has been postulated that the higher central nervous system (CNS) acetaldehyde levels in the flushers with the ALDH2*2 allele are responsible for the more behaviorally activating and, possibly, positive perceived effects of alcohol intoxication, and that peripheral sympathomimetic effects of acetaldehyde, known to be stronger than those induced by ethanol (Chao, 1995; Nishimura et al., 2002), may be responsible for the more negative aspects of flushing (Chao, 1995).

Acetate is the main metabolite of acetaldehyde (Zimatkin et al., 2006). It has been shown that when there is inhibition of the two main brain metabolizing enzymes, catalase and cytochrome P4502E1 (CYP2E1), it diminishes the production of acetaldehyde in rat brain homogenates and in the same proportion also reduces acetate accumulation (Zimatkin et al., 2006). In contrast, the ALDH inhibitor citral increased the accumulation of acetaldehyde and significantly reduced the formation of acetate (Zimatkin et al., 2006). Acetate has not shown anxiolytic effects when administered centrally (Correa et al., 2003), however it has never been determined if peripheral acetate administration can regulate anxiety.

The activation of the hypothalamic–pituitary–adrenal (HPA) axis leads to a neuroendocrine cascade that results in the secretion of glucocorticoids such as corticosterone from the adrenal gland (Rivier and Lee, 2001). This cascade represents a key element of the response of mammalian organisms to homeostatic threats (Rivier and Lee, 2001). Intense or repeated deviations from the normal pattern of HPA axis activation often results in pathologies caused by the dysregulated release of these hormones (Lee and Rivier, 2003). Drugs of abuse like ethanol, act as stressors in their stimulation of the HPA (Lee and Rivier, 2003; Pastor et al., 2008), and can lead to multiple adaptations of this stress regulatory system, including a compromised hormonal response and a sensitized brain stress response that together can contribute to dependence (Richardson et al., 2008). Previous studies have demonstrated that acute acetaldehyde accumulation, after ethanol administration and ALDH blockade with cyanamide, increases blood corticosterone levels in rats (Kinoshita et al., 2001). However, low doses of peripherally administered acetaldehyde did not increase corticosterone (Cao et al., 2007). The induction of corticosterone release usually requires strong stimuli or long-term exposure to them (Lee and Rivier, 2003; Pastor et al., 2008). For instance, ethanol doses (0.5 or 1.0 g/kg) that produce an anxiolytic response in mice (Correa et al., 2008) do not significantly affect corticosterone levels in the same strain of mice in which higher doses (2.0 g/kg) are required to induce plasma corticosterone levels (Pastor et al., 2004).

In the present study with CD1 mice, we investigated the impact of the direct administration of acetaldehyde at different times and doses using the elevated plus maze and the dark–light box. In addition, we studied the effect of peripherally accumulated acetaldehyde. Thus, anxiolytic doses of ethanol were evaluated in interaction with disulfiram (at doses that inhibit liver ALDH activity) on the elevated plus maze. We also assessed if acetate could mimic some of the effects of acetaldehyde in this behavioral paradigm. Finally, we determined if high doses of peripherally administered acetaldehyde increased blood corticosterone levels.

2. Material and methods

2.1. Subjects

CD1 male mice (30–40 g) were purchased from Harlan-Interfauna Iberica S.A. (Barcelona, Spain). Mice, 6–7 weeks old at the beginning of experiments, were housed in groups of three per cage, with standard laboratory rodent chow and tap water available *ad libitum*. They were maintained in the colony at 22 + 1 °C with lights on from 8:00 to 20:00 h. Mice were handled and habituated to the test room for one week before the anxiety tests were conducted or tissue samples were obtained. In the behavioral studies 10–12 animals were used per group, and in the biochemical studies n = 6–8 per group. All experimental procedures complied with the European Community Council directive (86/609/ECC) for the use of laboratory animal subjects and with the "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council 2003).

2.2. Drugs and selection of doses

All drugs were administered intraperitoneally (IP). Ethanol (96% v/v, Panreac Quimica S.A., Spain) was dissolved in physiological saline in a 20% v/v solution. Acetaldehyde (99% v/v, Panreac Quimica S.A., Spain) was dissolved in physiological saline in a 2% v/v solution. Disulfiram (Sigma-Aldrich Quimica S.A., Spain) was dissolved in peanut oil (Guinama SA, Spain). Sodium acetate (Sigma-Aldrich Quimica S.A., Spain) was dissolved in physiological saline, and hydrochloric acid (1 N, Panreac Quimica S.A., Spain) was used to bring the sodium acetate solution from pH 8.2 to pH 7.4. The selection of doses and times was based on pilot studies and on previous studies from our laboratory with this strain of mice (Correa et al., 2008).

2.3. Behavioral procedures

The plus maze is made of black polypropylene and is elevated 50 cm. It consists of two arms (65 cm L × 5 cm W) arranged in a plus configuration and intersecting in a central platform. The open arms have a 1 cm border around their perimeter and the closed arms have a 20 cm translucent wall. The dark–light box apparatus consisted of a polypropylene chamber divided in two compartments by a partition containing a small opening (5 cm H × 5 cm W). The light compartment (25 cm W × 25 cm H × 25 cm L) was open, painted in white and illuminated, while the dark compartment (25 cm W × 25 cm H × 18 cm L) was painted in black and enclosed by a removable ceiling. Elevated plus maze and dark–light box are behavioral paradigms used to evaluated anxiety responses and measure the avoidance that rodents show to high or bright open spaces respectively. Different experiments and treatment conditions were done in different groups of animals. Only for the acetaldehyde time course experiment, the same animal was first evaluated in the elevated plus maze for 5 min and then immediately placed and evaluated in the dark–light box. Every session was videotaped and several parameters were recorded during 5 min testing sessions for each behavioral paradigm. All parameters were manually registered afterwards by an observer unaware of the experimental condition. Animals were tested individually and both apparatus were cleaned with diluted isopropyl alcohol between animals. In the elevated plus maze the dependent variables were: latency to exit the first time to an open arm, time spent in the open arms, percentage of entries into the open arms compared to the total entries in the 4 arms, and total entries in all open and enclosed arms as an index of locomotion. An entry into an arm was recorded if the animal crossed the line that connected that arm with the central platform with all four legs. In the dark–light box we recorded the latency for the first entry into the bright compartment from the dark one, total entries into the bright compartment and time spent in the bright compartment.

After receiving the corresponding drug injection every mice was placed in a separate cage until testing started. In the elevated plus maze mice were placed in the central platform with their head pointing at one enclosed arm. In the dark–light box mice were initially placed in the dark compartment facing one corner.

For the acetaldehyde experiments different times after injection were assessed (elevated plus maze: 1, 11 or 21 min and dark–light box: 6, 16 or 26 min). These times were chosen based on previous behavioral reports indicating a very fast acting effect of peripherally

administered acetaldehyde (Quertemont et al., 2004). Disulfiram was administered 16 h before the ethanol injection. This time was chosen because ALDH inhibition induced by disulfiram develops between 8 and 12 h after administration but lasts for days (between 6 and 10 days) in humans and rodents (Yourick and Faiman, 1989). Behavior in the plus maze was assessed 10 min after ethanol administration. Acetate was injected 10 min before the plus maze test started.

2.4. Plasma corticosterone determination

Ethanol (0, 1, 2 or 3 g/kg) and acetaldehyde (0, 100, 200 or 300 mg/kg) were administered to naïve mice, and 60 min later trunk blood was collected. This time was chosen based on previous data from our laboratory and others (Pastor et al., 2004; Willey et al., in press) using different techniques, that show a reliable peak in blood corticosterone levels after 60 min. Animals were sacrificed by decapitation under anesthesia. Corticosterone measurements were based on previous published protocols (Pastor et al., 2004). Blood samples were collected in heparinized (15 units/ml of blood) Eppendorf tubes and centrifuged at 4000 rpm for 10 min. Supernatant was taken and stored at −20 °C until corticosterone determination. Plasma corticosterone levels were measured using a commercially available enzymatic immunoassay kit (Rodents Corticosterone Enzyme Immunoassay System, OCTEIA Corticosterone; Immunodiagnostic Systems LTD, Boldon, England). The ng/ml of blood corticosterone concentration was determined using a nonlinear (logarithmic) adjustment from the standard curve.

2.5. Determination of liver ALDH activity

In order to confirm that disulfiram was inhibiting liver low Km ALDH activity, naïve mice were used. Mice were pretreated with the different disulfiram doses (0, 30, 60 and 90 mg/kg) in the same time range as in the behavioral studies. Mice were anesthetized and perfused using 50 ml of heparinized (1000 IU/l) isotonic saline. One lobe of the liver was removed and frozen at −70 °C. On the assay day, the samples were homogenized in 0.25 M sucrose and 0.1 mM EDTA; the volume of this solution (ml) was equivalent to 10% of liver weight (expressed in grams). The liver homogenates were centrifuged at 800 g for 10 min at 4 °C in an AvantiTM 30 centrifuge (Beckman, USA). Supernatant aliquots (1.0 ml) were centrifuged at 10,000 g for 10 min at 4 °C. The precipitate was dissolved in 4.0 ml of a sucrose 0.25 M and triton ×100 1% v/v solution and was frozen at −80 °C, for 30 min, after which they were defrosted and centrifuged at 10,000 g for 10 min at 4 °C. Then 300 μl of supernatant aliquots were added to 500 μl of sodium pyrophosphate 50 mM (pH 8.8), adenine β-nicotinamide dinucleotide (NAD) 1.0 mM, rotenone 2.0 μM, 4-metilpirazol 0.2 mM and magnesic chloride 1.0 mM. ALDH activity was measured spectrophotometrically by following the production of NADH at 340 nm [$(\varepsilon)340 = 6.3\ l\,mmol^{-1}\,mm^{-1}$]. Following Gill et al. (1996), the assay mixture contained 50 mM of sodium pyrophosphate (pH 8.8), 1.0 mM of NAD, 2.0 μM of rotenone, 0.2 mM of 4-methylpyrazole, and 1.0 mM of magnesium chloride. The assay mixture was incubated with 0.1 ml of enzyme supernatant in a 25 °C water bath for 20 min. The reaction was started by the addition of 0.1 ml of the substrate acetaldehyde (50.0 μM for low Km). Each reaction was followed over a 10 min period. ALDH activity was expressed as nmol NADH produced/min/mg protein. Protein levels were determined from these supernatants (Bradford, 1976).

2.6. Statistical analysis

Results are reported as mean ± SEM. Data that follow a normal distribution were analyzed by means of factorial ANOVA. Planned comparisons were undertaken if a significant main effect or interaction was found at $p<0.05$. The non-parametric Kruskal–Wallis ANOVA by ranks test was performed when data did not follow a normal distribution. The non-parametric Mann–Whitney *U* test was used for paired comparisons when there was a significant Kruskal–Wallis ANOVA. A computerized statistical program (STATISTICA 4.1) was used in this study.

3. Results

3.1. Experiment 1: Effect of peripheral administration of different doses of acetaldehyde on the elevated plus maze

Fig. 1a–d shows the results of acetaldehyde treatment (administered 1 min before the behavioral test started) on different variables measured in the elevated maze. The ANOVA for the acetaldehyde dose (0, 25, 50, 75 or 100 mg/kg) was significant ($F(4,77) = 4.724$, $p<0.01$) for the dependent variable time spent in the open arms (Fig. 1a). Planned comparisons showed that the 0 mg/kg acetaldehyde group was significantly different from the 50 and 100 mg/kg acetaldehyde group ($p<0.05$). The ANOVA for the variable percentage of entries into the open arms was also significant ($F(4,77) = 3.352$, $p<0.01$) (Fig. 1b). Planned comparisons showed a significant difference between the 0 mg/kg acetaldehyde group and the 100 mg/kg acetaldehyde group ($p<0.05$). The Kruskal–Wallis ANOVA test indicated that there was a significant overall effect of acetaldehyde treatment ($p<0.01$) on the latency to enter an open arm (Fig. 1c). However, the non-parametric Mann–Whitney *U* test did not show any significant differences between any of the acetaldehyde doses and the control group. The total arm entries results (Fig. 1d) analyzed by means of one-way ANOVA revealed a significant effect of acetaldehyde dose ($F(4,77) = 3.695$, $p<0.01$). Planned comparisons showed significant differences between the 0 mg/kg and 50 mg/kg doses of acetaldehyde ($p<0.05$). The ANOVA for the entries in the enclosed arms was not significant, thus indicating that most of the activity was due to entries in the open arms.

3.2. Experiment 2: Effect of peripheral administration of acetaldehyde at different time intervals on the elevated plus maze

Fig. 2a–d shows the results from the time course study with the 100 mg/kg dose of acetaldehyde on the elevated plus maze. A pilot study did not reveal significant differences between the animals injected with saline at different times. Thus, in the experimental design we only included a group control (represented on the graphs by a line) that received saline at the intermediate time interval (11 min before the test). Data were analyzed using a one-way ANOVA (saline: 11 min, acetaldehyde 100 mg/kg: 1 min, 11 min or 21 min after administration). The ANOVA for the time spent in the open arms (Fig. 2a) revealed a significant effect ($F(3,76) = 3.624$, $p<0.01$) of time, and planned comparisons demonstrated significant differences between the group treated with saline and the groups that were treated with 100 mg/kg acetaldehyde and were introduced in the elevated plus maze 1 min ($p<0.01$) or 11 min ($p<0.05$) after this injection. The ANOVA for the percentage of entries in the open arms in relation to the total of entries (Fig. 2b) demonstrated a significant effect ($F(3,66) = 2.972$, $p<0.05$). As with the previous variable, planned comparisons revealed a significant difference between the saline group and the groups treated with acetaldehyde at 1 min and 11 min before the test ($p<0.01$ and $p<0.05$, respectively), but not for the group treated with acetaldehyde 21 min before the test. For the latency to enter into an open arm (Fig. 2c), the non-parametric Kruskal–Wallis ANOVA test indicated that there was not a significant overall effect. The ANOVA for the total arms entries (Fig. 2d) showed a significant effect ($F(3,66) = 4.113$, $p<0.01$). Planned comparisons showed significant differences between the saline group and all the other groups ($p<0.05$ for the 11 min group and $p<0.01$ for 1 and 21 min groups). The ANOVA for the entries in the enclosed arms

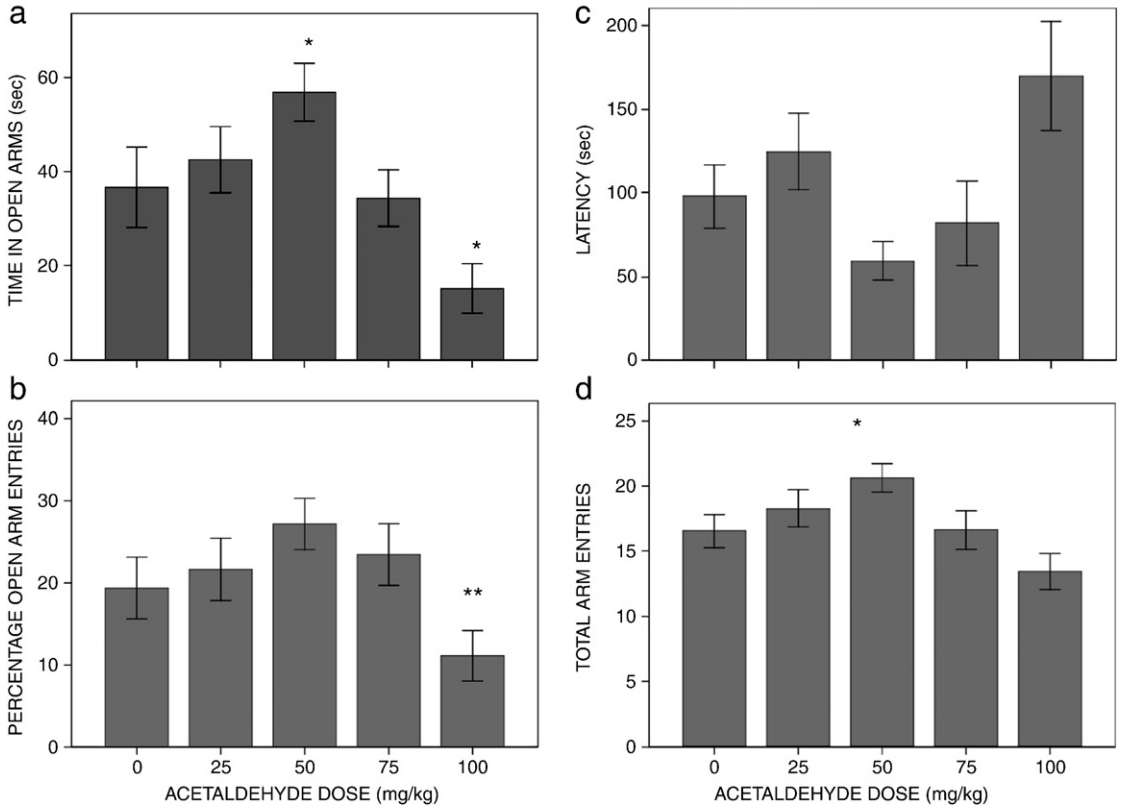

Fig. 1. Effect of different doses of acetaldehyde on the elevated plus maze. a) Time spent in the open arms. b) Ratio of entries in open arms relative to total number of entries in all four arms. c) Latency to enter an open arm for the first time. d) Total arm entries. Data are expressed as mean ± S.E.M. (*$p<0.05$, **$p<0.01$ compared to 0 mg/kg acetaldehyde group).

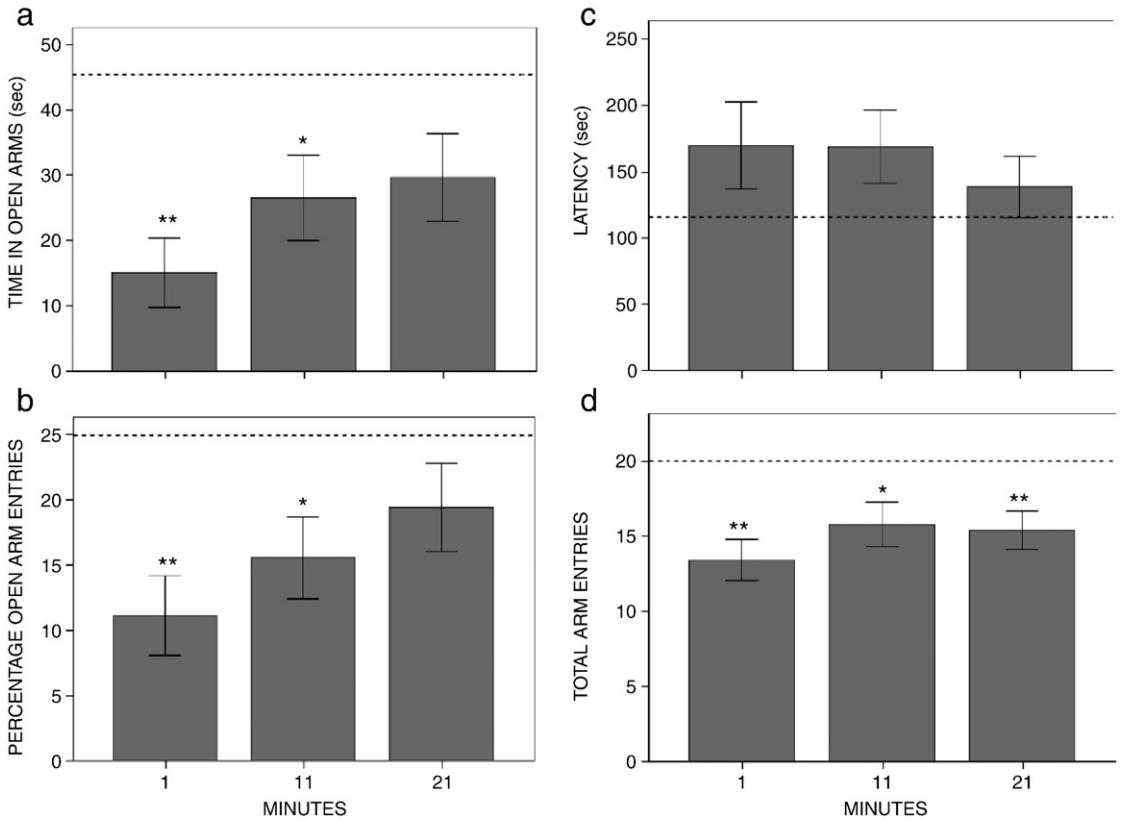

Fig. 2. Effect of acetaldehyde administered at different times (1, 11 or 21 min) before behavior was evaluated on the elevated plus maze. a) Time spent in the open arms. b) Ratio of entries in open arms relative to total number of entries in all four arms. c) Latency to enter an open arm for the first time. d) Total arm entries. Data are expressed as mean ± S.E.M. (*$p<0.05$, **$p<0.01$ compared to 0 mg/kg acetaldehyde group represented by the dotted line).

was not significant, thus indicating that any effect on activity was due to entries in the open arms.

3.3. Experiment 3: Effect of peripheral administration of acetaldehyde at different time intervals on the dark–light box

The effect of acetaldehyde (100 mg/kg) at different times after injection (6, 16 or 26 min) on the dark–light box compared with saline treated animals is shown in Fig. 3a–c. One-way ANOVA showed that there was a significant effect on the initial latency to enter the light compartment ($F(3,63)=2.972$, $p<0.05$) (Fig. 3a). Planned comparisons indicated a significant difference between the saline group and the group injected with acetaldehyde 100 mg/kg 6 min before being introduced in the dark–light box ($p<0.01$). The ANOVA for the frequency of crossings (Fig. 3b) showed that the treatment had a statistically significant effect ($F(3,63)=7.476$, $p<0.01$). Planned comparisons revealed a significant difference between the saline group and the groups administered with acetaldehyde 6 and 26 min before testing ($p<0.01$ and $p<0.05$ respectively). The ANOVA for the time spent in the illuminated compartment (Fig. 3c), revealed a significant effect ($F(3,63)=5.247$, $p<0.01$). Planned comparisons showed a significant difference between the saline group and the 6 and 26 min treatment groups ($p<0.01$ and $p<0.05$, respectively).

3.4. Experiment 4: Impact of disulfiram on the anxiolytic effects of ethanol in mice as measured in the elevated plus maze

Fig. 4a–d shows the effect of disulfiram on the anxiolytic effects of ethanol in the elevated plus maze. Several dependent variables, including time, percentage of entries in the open arms, and total arm entries, were analyzed independently by means of a two-way factorial ANOVA (dose of disulfiram (0, 30, 60 or 90 mg/kg) × dose of ethanol (0 or 1 g/kg)). The analysis of time spent in the open arms (Fig. 4a) showed a statistically significant effect for the disulfiram dose factor ($F(3,140)=3.31$, $p<0.05$), but no significant effect for the ethanol dose factor. However, the interaction between both factors was significant ($F(3,140)=3.63$, $p<0.01$). Planned comparisons revealed that ethanol had an anxiolytic effect, as indicated by the fact that the group that received 0 mg/kg disulfiram plus 1 g/kg ethanol showed a significant increase in the time spent in the open arms compared to the combined control group that received 0 mg/kg disulfiram plus ethanol 0 g/kg ($p<0.01$). Disulfiram did not have an effect on its own on the time spent in the open arms, since among the groups treated with ethanol 0 g/kg the different doses of disulfiram (30, 60 or 90 mg/kg) were not different from the dose 0 mg/kg of disulfiram. The groups treated with 60 or 90 mg/kg disulfiram plus 1 g/kg ethanol showed a significant reduction in time spent in the open arms compared to the 0 mg/kg disulfiram plus 1 g/kg ethanol group ($p<0.01$). Furthermore, among the groups treated with 90 mg/kg disulfiram, the 1 g/kg ethanol-treated group spent significantly less time in the open arms compared to the 0 g/kg ethanol group ($p<0.05$), indicating that disulfiram produced an anxiogenic effect when combined with ethanol. The ANOVA for the percentage of entries in the open arms (Fig. 4b) revealed a significant effect for the disulfiram factor ($F(3,140)=6.45$, $p<0.01$), but not for the ethanol factor. The interaction was statistically significant ($F(3,140)=2.67$, $p<0.05$). Planned comparisons revealed that, among the groups treated with ethanol 0 g/kg, the disulfiram groups were not

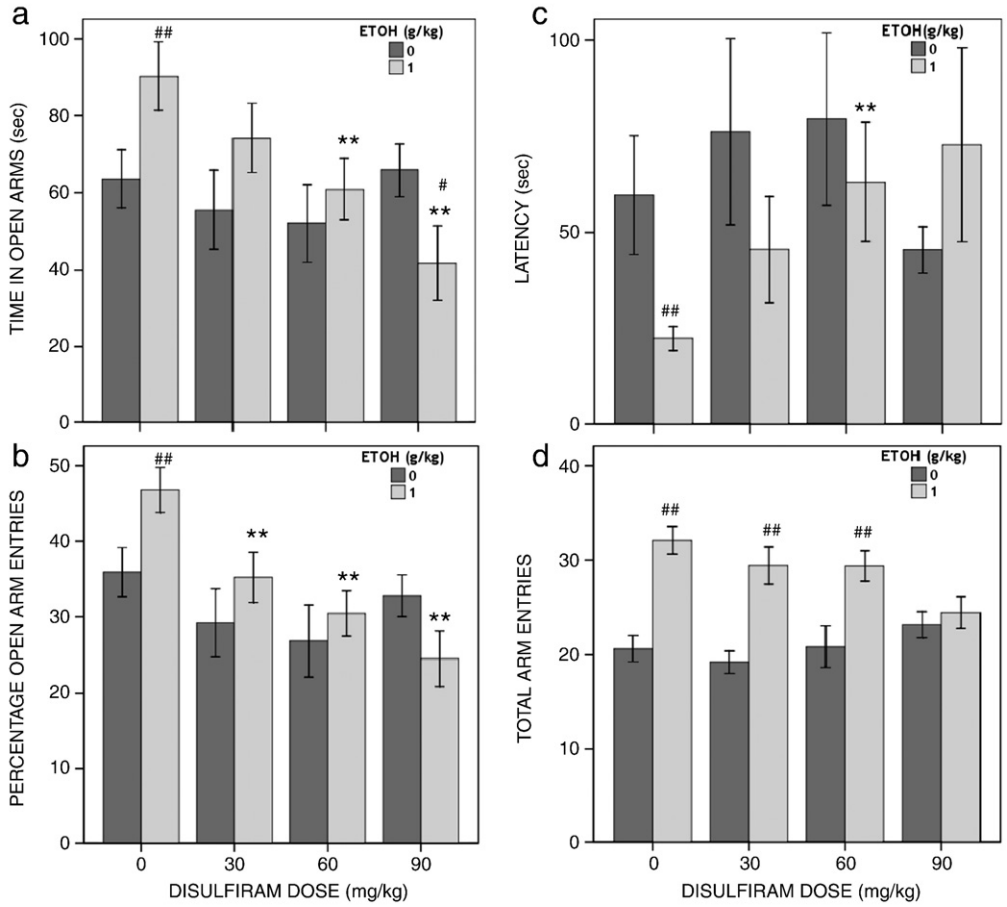

Fig. 3. Effect of acetaldehyde administered at different times (6, 16 or 26 min) before behavior was evaluated on the dark–light box. a) Latency to enter the lit compartment. b) Number of entries in the lit compartment. c) Time spent in the lit compartment. Data are expressed as mean ± S.E.M. (*$p<0.05$, **$p<0.01$ compared to 0 mg/kg acetaldehyde group represented by the dotted line).

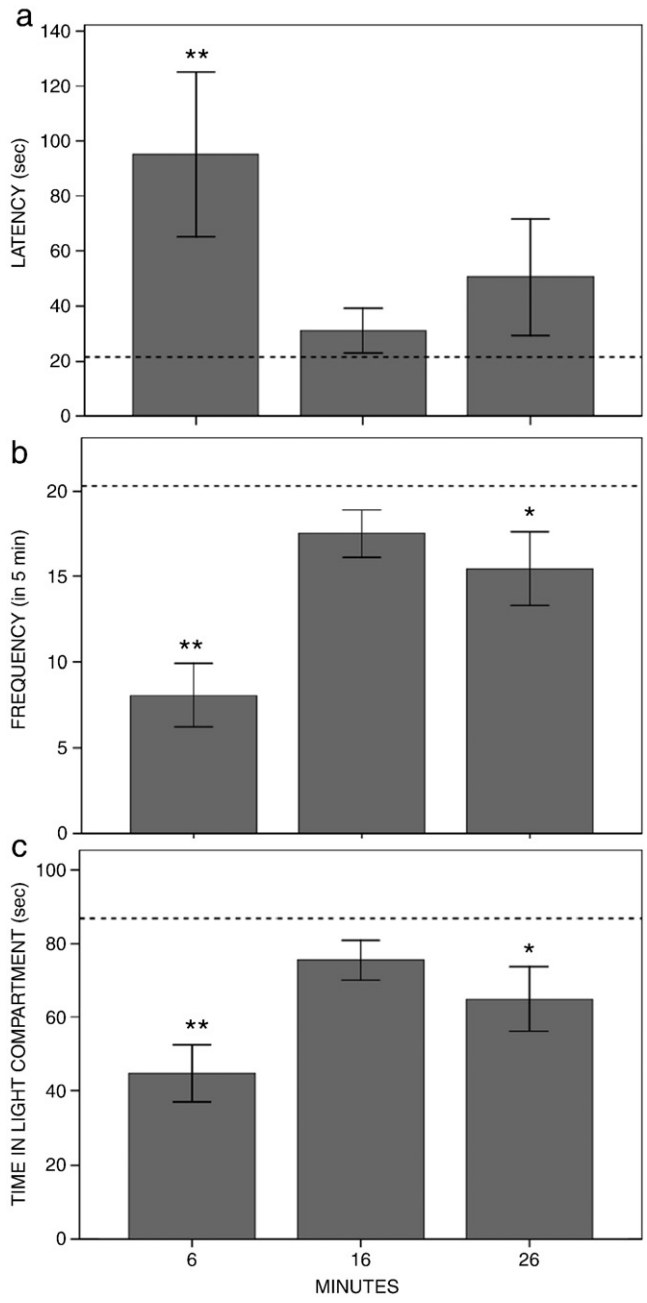

Fig. 4. Effect of disulfiram on ethanol-induced anxiolysis in the elevated plus maze. a) Time spent in the open arms. b) Ratio of entries in open arms relative to total number of entries in all four arms. c) Latency to enter an open arm for the first time. d) Total arm entries. Data are expressed as mean ± S.E.M. (**p<0.01 compared to 0 g/kg ethanol–0 mg/kg disulfiram group. #p<0.05, ##p<0.01 compared to 0 mg/kg ethanol in the respective disulfiram dose).

significantly different, indicating that there was no effect of disulfiram *per se*. Ethanol had an anxiolytic effect, since the group 0 mg/kg disulfiram plus 1 g/kg ethanol was significantly different from the group 0 mg/kg disulfiram plus 0 g/kg ethanol ($p<0.01$). The groups pretreated with 30, 60 or 90 mg/kg disulfiram plus 1 g/kg ethanol had a significant decrease in the percentage of entries in the open arms in relation to the group 0 mg/kg disulfiram plus 1 g/kg ethanol ($p<0.01$ for all groups). Latency for initial entry into an open arm (Fig. 4c) was analyzed by means of the non-parametric Kruskal–Wallis ANOVA by ranks test, because the data did not follow a normal distribution. This analysis demonstrated that there was a significant overall effect ($p<0.01$) on latency to enter into an open arm. The non-parametric Mann–Whitney *U* test demonstrated that among the 0 mg/kg disulfiram groups, 1 g/kg ethanol reduced the latency time compared to the saline treated group ($p<0.01$); however, none of the other ethanol treated groups were different from their respective saline treated groups, indicating that ethanol interacted with the different disulfiram doses to increase the latency to enter an open arm. Moreover, the group 1 g/kg ethanol plus 60 mg/kg disulfiram was different from the group 1 g/kg ethanol plus 0 mg/kg disulfiram ($p<0.01$). The two-way factorial ANOVA for the total number of arm entries (Fig. 4d) showed a significant effect for the ethanol factor ($F(1,140)=44.572$, $p<0.01$), and for the interaction between both factors ($F(3,140)=3.813$, $p<0.01$), but not for the disulfiram treatment factor. Planned comparisons revealed that there were no significant differences between the groups pretreated with the different doses of disulfiram plus 0 g/kg ethanol, demonstrating that the disulfiram doses used in the present experiments did not affect spontaneous locomotion. The 0 mg/kg disulfiram plus 1 g/kg ethanol group was significantly different from the 0 mg/kg disulfiram plus 0 g/kg ethanol group, indicating that this dose of ethanol had motor stimulating properties ($p<0.01$). This induction of locomotion was also observed among the groups pretreated with 30 or 60 mg/kg disulfiram and 1 g/kg ethanol in relation to their own control groups (0 g/kg ethanol; $p<0.01$). However, the group 90 mg/kg disulfiram plus 1 g/kg ethanol was not significantly different from the group 90 mg/kg disulfiram plus 0 g/kg ethanol, indicating that disulfiram was only able to block the motor stimulant properties of 1 g/kg of ethanol at the highest dose. The ANOVA for the entries in the enclosed arms only showed a significant effect of the ethanol main effect ($F(1,140)=36.84$, $p<0.01$), indicating that ethanol increased activity both in the open and also in the enclosed arms independently of the disulfiram dose.

3.5. Experiment 5: Effect of different doses of disulfiram on hepatic ALDH activity

Hepatic ALDH activity after disulfiram administration (Table 1) was analyzed by means of one-way ANOVA for the disulfiram factor, which was significant ($F(3,28)=6.381$, $p<0.01$). Planned comparisons revealed significant differences between the group treated with 0 mg/kg disulfiram and the groups treated with 60 and 90 mg/kg disulfiram ($p<0.01$).

3.6. Experiment 6: Effect of peripheral administration of acetate on the elevated plus maze

Table 2 lists the data from the elevated plus maze after acetate treatment. A one-way ANOVA for the acetate dose (0, 50, 100 or 200 mg/kg) was performed for several dependent variables. The results of independent ANOVAs did not show any significant effects of acetate treatment on the time spent in the open arms, percentage of entrances in the open arms, total arm entries or enclosed arm entries.

3.7. Experiment 7: Effect of peripheral administration of ethanol or acetaldehyde on plasma corticosterone levels

Table 3 shows plasma corticosterone levels after different doses of ethanol (0, 1, 2 or 3 g/kg). A one-way ANOVA revealed a statistically

Table 1
Effect of different doses of disulfiram on liver low Km ALDH activity.

Disulfiram dose (mg/kg)	Hepatic ALDH activity (nmol NADH/min/mg protein)
0	0.0687 ± 0.0059
30	0.0619 ± 0.0029
60	0.0536 ± 0.0034**
90	0.0456 ± 0.0028**

Disulfiram was administered IP, 16 h before determination. Data are expressed as mean ± S.E.M. (n = 6 per group).
** p<0.01, compared to disulfiram 0 mg/kg.

Table 2
Effect of different doses of acetate on the elevated plus maze.

	Acetate (mg/kg)			
	0	50	100	200
Time in open arm	43.8 ± 36.5	36.5 ± 23.6	59.0 ± 29.4	36.8 ± 29.9
Percentage entries	24.5 ± 5.4	24.5 ± 4.2	32.8 ± 4.3	23.8 ± 3.8
Latency	133.5 ± 112.6	88.1 ± 74.5	83.6 ± 78.6	78.5 ± 79.6
Total entries	20.1 ± 5.9	17.9 ± 4.6	19.0 ± 5.4	18.5 ± 5.8

Acetate was administered IP, 10 min before test. Data are expressed as mean ± S.E.M. (n = 12 per group).

significant effect of ethanol treatment ($F(3,21) = 7.37$, $p<0.01$). Planned comparisons showed that groups treated with 2 or 3 g/kg of ethanol had significant increases in plasma corticosterone levels compared with the 0 mg/kg ethanol group ($p<0.05$ and $p<0.01$, respectively). The results of acetaldehyde on plasma corticosterone levels are also shown in Table 1. The one-way ANOVA for the acetaldehyde dose (0, 100, 200 or 300 mg/kg) demonstrated a significant effect on plasma corticosterone levels ($F(3,26) = 11.86$, $p<0.01$). Planned comparisons showed a significant increase in the groups treated with 200 and 300 mg/kg acetaldehyde compared to the group treated with 0 mg/kg ($p<0.01$).

4. Discussion

In the present set of experiments, it was shown that peripheral acetaldehyde produced anxiogenic effects in two animal models of anxiety, and also increased an endocrine marker of stress. First of all, acetaldehyde was administered at several doses, and behavior was observed 1 min after administration. This time was chosen because acetaldehyde administered IP to mice has demonstrated to produced peak blood levels between 1 and 3 min after administration (Isse et al., 2005). The results seen in the elevated plus maze demonstrated that acetaldehyde at the highest dose (100 mg/kg) reduced percentage of entries and time spent in the open arms, with no significant effect on total entries, indicating that it has anxiogenic actions independent of locomotion effects. The lack of effects of 100 mg/kg acetaldehyde administered peripherally on locomotion in outbred mice has been previously observed (Font et al., 2005). On the other hand, it was also found an increase in the percentage of open arm entries with 50 mg/kg acetaldehyde. This effect can be explained by the significant increase in total activity found at this dose (seen in the total arm entries), more so when considering that this increase was not accompanied by an increase in the time spent in the open arms. The stimulating effect of this dose of acetaldehyde on locomotion is consistent with previous studies in high ethanol drinking rats UChB (Tampier and Quintanilla, 2002).

The duration of the anxiogenic effect of peripherally administered acetaldehyde was evaluated by extending the time interval between acetaldehyde administration and the beginning of the behavioral observation. The effective dose of acetaldehyde at 1 min (100 mg/kg) was chosen and, in addition to the plus maze, a second behavioral test for anxiety, the dark–light box, was also used. The results demonstrated that acetaldehyde had anxiogenic properties at least during a period of 11 min after injection in the elevated plus maze, and for an even longer period (26 min) when anxiety was evaluated in the dark–light box.

Table 3
Plasma corticosterone levels (ng/ml).

Ethanol (g/kg)		Acetaldehyde (mg/kg)	
0	21.4 ± 3.5	0	21.3 ± 1.6
1	25.0 ± 5.8	100	26.6 ± 4.2
2	40.1 ± 6.1*	200	40.8 ± 2.6**
3	53.7 ± 6.6**	300	41.9 ± 2.3**

Effect of different doses of ethanol and acetaldehyde (administered IP, 60 min before determination) on plasma corticosterone levels. Data are expressed as mean ± S.E.M. (n = 6–8 per group), (**p<0.01, *p<0.05 compared to their respective control group).

In the subsequent experiment we evaluated if the accumulation of acetaldehyde in the periphery after ethanol administration at anxiolytic doses in animals that received an ALDH inhibitor, reversed the anxiolytic effects of ethanol. A pharmacological treatment that in humans has extensively been used to discourage alcohol intake (*i.e.*, disulfiram), produced a blockade of the anxiolytic effects of alcohol in the plus maze, and even induced anxiety at doses that inhibit hepatic low Km ALDH activity.

Since ALDH inhibition can produce peripheral as well as central acetaldehyde accumulation, and it has been demonstrated that administration of 100 mg/kg of acetaldehyde can lead to detectable levels in the brain (Quertemont et al., 2004), the possible actions produced by acetaldehyde reaching the brain cannot be discounted as a factor in the total anxiogenic response outcome observed in the present set of results. Nevertheless, it has been previously shown that centrally formed acetaldehyde (via the actions of catalase) is responsible for the anxiolytic effects of ethanol in mice (Correa et al., 2008). Moreover, in rats it also was demonstrated that acetaldehyde administered in the cerebral ventricles had anxiolytic properties in an open field (Correa et al., 2003). In the present experiments, if there were any central effects of acetaldehyde, they would be to reduce the strength of the anxiogenic response. Thus, we hypothesize that it is peripheral, and not central acetaldehyde accumulation, that is responsible for the anxiogenic response. In previous reports (Quertemont et al., 2004; Tambour et al., 2005), peripheral administration of acetaldehyde at doses similar to those used in the present work did not affect anxiety responses in mice in an elevated plus maze, although they impaired motor performance, an effect that was not found in the present results. The authors showed that blood and brain acetaldehyde levels detected after peripheral administration were similar in both tissues (Quertemont et al., 2004). Thus, it is possible that the actions of these levels of acetaldehyde in the periphery and in the brain could be counteracting each other in terms of the overall outcome of the anxiety response generated.

Acetate has demonstrated to be a behaviorally active metabolite of ethanol (Carmichael et al., 1991; Israel et al., 1994; Arizzi et al., 2003; Correa et al., 2003). It has been detected in plasma after ethanol administration, as the portion of acetate that has not been metabolized hepatically is released into the blood (Lundquist et al., 1962). Acetate is then redistributed throughout the body, and is rapidly taken up into the brain by a carrier-mediated process (Oldendorf, 1973). An alternative central source of acetate is ethanol brain metabolism (Zimatkin et al., 2006). Since in the present work disulfiram, when combined with ethanol, could be not only increasing acetaldehyde levels, but also reducing acetate levels, and on the other hand, is also possible that acetate levels would rise as a result of peripheral administration of acetaldehyde, the possible contribution of acetate to the present effects was assessed. Acetate administered at injection times and doses similar to acetaldehyde did not modify any of the behavioral parameters of anxiety. In summary, although acetate has been involved in the motor suppressing effects of ethanol in mice (Israel et al., 1994) and rats (Correa et al., 2003), it does not seem to mediate its anxiolytic actions (Correa et al., 2003), nor does it seem to be involved in the anxiogenic response produced by a bolus injection of acetaldehyde in the periphery.

The present data also showed that high doses of peripherally administered acetaldehyde produced an endocrine response considered a physiological marker of stress. Peripherally injected acetaldehyde was 10 times more potent than peripherally injected ethanol at increasing corticosterone release. Decades ago it was demonstrated that acetaldehyde, rather than ethanol *per se*, is responsible for direct adrenal stimulation (Cobb et al., 1981; Cobb and Van Thiel, 1982). In humans with the ALDH2*2 isoform, ethanol increases plasmatic levels

of cortisol (Wall et al., 1994; Nishimura et al., 2002). In rats, it has been shown that ethanol administration after enzymatic blockade of ALDH produced acetaldehyde accumulation in blood as well as the activation of the HPA axis leading to increases in corticotrophin-releasing factor (CRF) and in plasma corticosterone concentrations (Kinoshita et al., 2001). On the other hand, it has been demonstrated that centrally formed acetaldehyde correlates inversely with corticosterone release. When catalasemic metabolism of ethanol is blocked (thus mainly reducing central rather than peripheral acetaldehyde formation), corticosterone release increases (Pastor et al., 2004). This suggests that central accumulation of low doses of acetaldehyde does not seem to produce an acute stress reaction, as marked by corticosterone levels.

In several human populations the ALDH2*2 allele, which encodes for an inactive ALDH form, appears to protect against alcoholism (Luo et al., 2006; Mulligan et al., 2003; Peng et al., 2007), and it has been demonstrated in a number of studies that this mutation produces a rise in acetaldehyde levels (for a review see Eriksson, 2001). In animals, Aldh2 KO mice accumulate higher levels of acetaldehyde in several organs and show a reduction in their preference for ethanol relative to wild type mice (Isse et al., 2002, 2005). UChA (low drinkers) and UChB (high drinkers) rats have ALDH2 polymorphisms (Sapag et al., 2003) that relate to their ethanol consumption (Quintanilla et al., 2005). In addition, alcohol-avoiding (ANA) and low alcohol-preference (LAP) rats show higher blood acetaldehyde levels than alcohol-preferring (AA) and high alcohol-preference (HAP) rats (Eriksson, 1973; Koivisto et al., 1993; Nishiguchi et al., 2002). Thus, acetaldehyde levels seem to have a direct impact on alcohol consumption; they are inversely related to the quantity of alcohol voluntarily consumed in humans and rodents (Eriksson, 2001; Quintanilla et al., 2005, 2007; Rivera-Meza et al., 2010). In this sense, ALDH inhibitors such as disulfiram (Antabuse) and calcium carbimide (Abstem, Temposil) have been used with mixed success to stop consumption and to prevent relapse in alcoholics. Support for the general use of disulfiram is equivocal, mostly being found in the form of reduced quantity of alcohol consumed and a reduced number of drinking days. Evidence for an effect on increasing the proportion of patients who achieve abstinence is not clear (Hughes and Cook, 1997). For example, in a special group of patients selected for been highly motivated to stop drinking, the success in being abstinent after antabuse was only 40% (Sereny et al., 1986). In animal studies it has been demonstrated that its main effect of reducing ethanol consumption occurs when the rats were inexperienced in consuming alcohol, but its therapeutic properties are not manifested if the animal has been consuming ethanol voluntarily for a long time (Tampier et al., 2008; Garver et al., 2000). Thus, the aversive properties of peripheral acetaldehyde accumulation in some cases can deter ethanol consumption, especially in individuals with little experience with alcohol in which an increase in anxiety is probably a very salient effect that competes with any other effects of alcohol. This seems to be also the case for individuals that show alcohol dependence and post-traumatic stress disorder in which disulfiram seems to be a good therapeutic agent (Barth and Malcolm, 2010; Kozarić-Kovacić, 2009). In other subjects the aversive effects can be partly responsible for the lack of compliance with the treatment. In general, disulfiram has a reputation as an effective medication when given in a way that minimizes the risk of a disulfiram–ethanol reaction (Wright and Moore, 1989).

5. Conclusions

In view of the present results, it could be argued that, among other well known symptoms produced by a burst in blood acetaldehyde levels (Eriksson, 2001), anxiogenic effects can be added as one more of the aversive effects of peripheral acetaldehyde accumulation. This anxiogenic response has been studied in ethanol naive animals. Thus, our results suggest that the aversive anxiogenic response would be effective in naive subjects during their first encounters with alcohol consumption. Additionally, the elevation of corticosterone levels induced by peripherally administered acetaldehyde could potentiate neural changes that can modulate future responses to alcohol (Weiss et al., 2001).

Disclosure statement

All authors declare no financial or personal conflict of interest.

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