



Differential effects of early exposure to alcohol on alcohol preference and blood alcohol levels in low- and high-anxious rats

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Received: 10 September 2019 / Accepted: 20 July 2020 / Published online: 27 September 2020
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Abstract

Emotional disturbances emerge following alcohol withdrawal. The anxiolytic effect of alcohol may be one important motivation for its consumption in conditions where alcohol intake is anxiety reducing. Besides, early alcohol experience will predict future alcohol-related problems at some point in their lives. Rats classified according to their anxiety-like behavior phenotype show a higher preference for alcohol. Yet, despite preclinical studies have identified the behavioral and physiological effects of alcohol withdrawal, little has been shown on relapse to alcohol after a period of abstinence following intermittent long-term alcohol consumption in low-(LA) and high-anxiety (HA) phenotype rats. This study attempts to assess the role of emotional reactivity of early-aged LA and HA rats on later alcohol preference, through an operant response procedure. For this, a sweetened liquid fading procedure associated with a long-term and intermittent voluntary alcohol drinking was used, with the animals being tested on different withdrawal periods. Alcohol withdrawal symptoms were recorded, and blood alcohol levels were assessed at two intervals to examine the influence of anxiety phenotype on blood alcohol clearance. Data from HA control and HA withdrawn rats show that emotionality selectively influences alcohol preference. Blood alcohol decay was faster in HA than in LA alcohol pretreated rats. HA rats showed an ascending curve of alcohol intake, exhibiting a high level of alcohol drinking on withdrawal and withdrawal symptoms. Moreover, HA alcohol experienced rats show a high frequency of lever-pressing response during extinction, associated with a facilitation of bar-pressing recovery trials, an indication of alcohol-seeking behavior.

Keywords Alcohol withdrawal · Anxiety-like behavior · Rats · Operant response · Extinction · Recovery

Introduction

Alcoholism, like other addictive diseases, is defined as a chronic relapsing disorder that develops in a very organized and gradual manner and encompasses three main steps: an

occasional intermittent acute drug intake, a chronic but controlled drug consumption, and a compulsive, out of control alcohol drinking. Withdrawal at this stage follows intense and protracted and often leading to relapse (Le et al. 2001; Heilig et al. 2010; Jesse et al. 2017). Currently, alcoholism is a continuing problem faced by many societies. Several studies have pointed out the contribution of genetic and environmental factors on alcohol abuse development (Mayfield et al. 2008; Kendler et al. 2011). Also, the period of the initiation of alcohol use seems to be crucial to predicting alcohol-related problems later in life (Bates and Labouvie 1997; Brown and Tapert 2004; Schramm-Sapyta et al. 2008; Marshall 2014; Dyer et al. 2019). Alcohol disturbs the normal growth of the adolescent brain, making it more vulnerable to the effects of alcohol itself in adulthood. These changes occur in many levels, including neural, cognitive, and behavioral domains (Welch et al. 2013). Adolescents who have contact with alcohol at this early stage of life will often display in the future regular and higher levels of alcohol intake in adulthood and likely meeting the criteria

Communicated by Thomas Deller.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00221-020-05932-6>) contains supplementary material, which is available to authorized users.

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for alcohol dependence at some point in their lives (Grant and Dawson 1997; Hingson and Zha 2009; McCambridge et al. 2011; Marshall 2014).

Alcohol use and abuse can be facilitated in individuals prone to alcohol dependence such as anxious subjects. For example, some studies have reported a high level of comorbidity between anxiety disorders and alcohol problems (Kushner et al. 1990; Kessler et al. 1997). There is also evidence that anxiety may increase the probability of relapse and negatively influence the treatment prognosis of alcohol use disorders (Fox et al. 2007; Sinha and Li 2007). Besides, as reported by Easey et al. (2019), child and adolescent anxiety was positively correlated with later alcohol use. Thus, the anxiolytic effect of alcohol may be one important motivation for alcohol drinking. In fact, in situations where alcohol drinking is anxiety reducing, this effect reinforces drinking and may promote future alcohol intake (Conger 1956). Therefore, it is expected that anxious individuals should benefit more from the anxiolytic effect of alcohol and consume more alcohol than less anxious individuals, especially if the initial contact with alcohol occurs at the early stages of the development. In preclinical studies, rats previously classified as anxious have a significantly higher intake and preference for alcohol during the initiation phase of short voluntary alcohol drinking (Spanagel et al. 1995). Also, alcohol-preferring rats have exhibited higher anxiety than non-preferring counterparts (Hwang et al. 2004). In these animals, the rate of blood-alcohol elimination has shown a faster decline than that of LA rats (Ezequiel Leite and Nobre 2012).

Despite epidemiological data highlight the age of alcohol drinking onset as a crucial factor for later alcohol use and abuse patterns little has been shown regarding future alcohol preference of early-aged LA and HA rats exposed to voluntary long-term alcohol intake. In the present study, we go one step further on this issue to test the hypothesis that peri-adolescent HA alcohol-withdrawn rats are more sensitive to the reinforcing effects of alcohol itself and show increased alcohol preference and faster blood alcohol elimination than their LA equivalents. To achieve this goal, alcohol drinking was strengthened through an intermittent voluntary alcohol intake procedure. It has been shown that this procedure leads to the escalation of alcohol intake and preference in both mice and rats (Melendez 2011; Carnicella et al. 2014). This approach attempts to reproduce what is commonly observed in humans who consume more alcohol when its availability obeys a periodic pattern (Holloway et al. 1984).

Materials and methods

Animals

Two-hundred and eighty-eight male Wistar rats (21 days, ± 45 –50 g) from the campus of Ribeirão Preto,

University of São Paulo, were used in this study. Following arrival, the animals remained undisturbed by additionally 48 h, to be acclimatized to the living conditions in the Laboratory of Neuropsychopharmacology. Rats were housed in groups of 4 in Plexiglas cages (45 × 35 × 15 cm), lined with wood shavings that were changed every 3 days. They were maintained in a 12/12-h light/dark cycle (lights on at 07:00 a.m.) at 24 ± 1 °C with free access to food and water during the homecage treatments.

Ethical statements

We declare that this study was performed under the formal endorsement (process 08.1.1547.53.3) from the Committee on Animal Research and Ethics (CEUA) of the University of São Paulo and following the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. The number of animals used was the minimum required to ensure the reliability of the results, and every effort was made to minimize animal suffering.

Experimental procedure

We started our experiments using the elevated zero-maze (EZM) to separate rats into LA or HA considering the time spent in the open arms. Three days after, the animals underwent 3 days of operant training to modeling the lever-pressing response using sweetened tap water as the reinforcing stimulus. After that, the intermittent access to increased concentrations of alcohol in a two-bottle-choice drinking paradigm (Wise 1973; Simms et al. 2008) was initiated and lasted for 68 days. Later, the animals were tested at 24 h (day 70), 48 h (day 71), 72 h (day 72), and 96 h (day 73) following the interruption of chronic alcohol intake (see Fig. 1—experimental timeline).

Selection of rats with low (LA) or high anxiety-like (HA) behavior

Rats were allocated to LA or HA groups according to their natural avoidance of the open arms of the EZM. The EZM is an elevated annular platform (50 cm from the floor, ed: 100 cm; id: 80 cm; width: 10 cm) constructed from dark plywood, with two opposite enclosed quadrants and two opposite open quadrants, located inside a room with constant background noise (50 dB). Luminosity at the level of the center of the EZM was 60 lx. Behaviors were recorded using a camera (Everfocus, Duarte, CA, USA) linked to a monitor located outside the experimental room. Experimental sessions were conducted between 09:00 a.m. and 05:00 p.m. The maze was cleaned with 20% alcohol followed by deionized water before each test. The use of the EZM as opposing to the traditional elevated plus-maze as the main predictor of

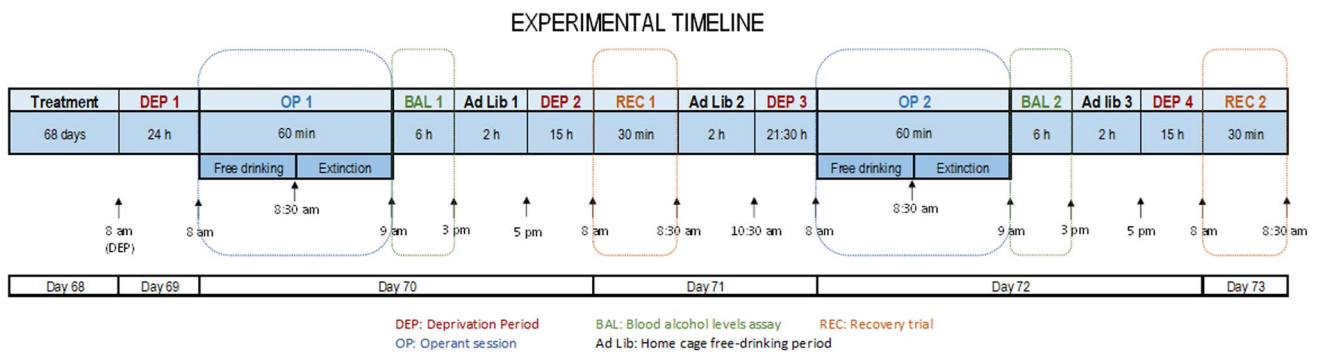


Fig. 1 The figure shows the experimental timeline used in our study. As informed, the animals were tested on 24, 48, 72, and 96 h from the interruption of the intermittent voluntary intake of alcohol

anxiety-like behavior in rats has been proposed in previous studies (Shepherd et al. 1994; Kulkarni et al. 2007; Braun et al. 2011). The dependent variable recorded was the percentage of the time spent in the open arms, relative to the total time of the test. The animals belonging to the distal quarters above (less time in the open arms) or below the median (more time spent in the open arms) were allocated to the HA or LA groups, respectively (144 rats). The remaining animals whose data were located in the two quarters near either side of the mean were removed and used in other experiments. The animals were placed individually in one of the closed arms of the maze and allowed 5 min of free exploration. Like the elevated plus-maze, an arm entry or exit was defined as all four paws entering or exiting an arm, respectively.

Experimental groups

Four groups were formed based on the data obtained from the EZM ($n = 36$ for each group). Later, eleven animals were discarded from the study since they did not achieve basal operant rates of lever-pressing behavior throughout the three training sessions. Thus, after the operant training was finished, the groups were composed as follows: water LA ($n = 30$), water HA ($n = 34$), alcohol LA ($n = 33$), and alcohol HA ($n = 36$).

Operant training device

The experiments were conducted in a rat-operant chamber (35 × 35 × 50 cm) housed in a sound-attenuating shell (70 × 80 × 80 cm) with a stainless-steel grid floor, a ventilation fan, and controlled luminosity (20 lx, incandescent white-bulb). A liquid receptacle was installed in the middle of the rear wall, 5 cm above the grid floor with two stainless-steel response levers located on each side, 10 cm apart. Two 200 ml pipettes located behind the wall, outside the chamber, and linked to each one of the two levers, allowed

the experimenter to control the total volume of solutions delivered during each session. The free operant response procedure used herein aimed to allow the deprived animals to drink water or alcohol solutions as much as they wish. The operant chamber was interfaced with a PC, and all data were automatically recorded (Insight, São Paulo, Brazil).

Operant training design

At this stage, the animals have a mean bodyweight of 100 g (27 days’ age). Three 2-h daily training sessions were performed. These instrumental sessions occurred from Wednesdays to Fridays when the animals could drink a solution of sweetened tap water as much as they wish (saccharin 0.2%, Synth, São Paulo, Brazil). The operant training used here has been described, with minor changes, previously (Ezequiel Leite and Nobre 2012; Nobre 2016). It consisted of the animals learn to press one of the two levers randomly active during trials to receive reinforcement. The active lever was signaled by one of the two white LED bulbs positioned 10 cm above each lever. The fluid was supplied by a dipper fluid system adjusted to deliver 100 µl of fluid after each lever press (FR1) following by 10 s of delay; the period in which pressing the lever did not result in reinforcing. A grille allowed the unconsumed liquid to drain away. This was later subtracted from the total volume of the solution offered, to obtain the actual consumption. Finished these 3 days of the operant training period, we start the third phase of the experiment, involving the chronic and intermittent voluntary intake of water or alcohol solutions.

Intermittent access to 20% alcohol with saccharin fading

Alcohol was prepared by diluting a stock solution of pure alcohol (99.5%, Synth, São Paulo, Brazil) with tap water (Nobre 2016). To induce robust alcohol intake, we used an association of intermittent voluntary alcohol drinking with a fading initiation procedure, where decreased amounts of

the non-caloric sweetener saccharin were added to increasing concentrations of alcohol. The sweetener was removed from the study at the end of the sixth week of treatment to compensate for the possible influence of saccharin on alcohol intake (Simms et al. 2008). Despite criticism on the use of saccharin to induce alcohol drinking, several studies have pointed out the efficacy of sweeteners, including “super-sac” to speed up and strengthen this behavior (Tolliver et al. 1988; Melendez 2011; Broadwater et al. 2013; Carnicella et al. 2014). Also, joining a sweetener to alcohol was important taking into account that rats do not appreciate alcohol taste and considering that, in our study, we offered high alcohol dosages to the animals, up to 20% v/v.

Procedure for alcohol exposure in the home-cage

Each cage was equipped with two graduated glass bottles (500 ml each) with stainless-steel drinking spouts inserted through the stopper. An internal stainless-steel sphere in the spout avoided liquid drip during handling. The positions of the bottles were changed every time the box was cleaned, when solutions were changed, to prevent animals from becoming habituated to the same locations. Exposure to control and alcohol solutions lasted for 68 days, followed by one day of fluids deprivation. Fluid changes and box cleaning occur at 08:00 a.m. on Mondays, Wednesdays, and Fridays. To control rats were offered two bottles of water or one bottle of water and one bottle of water + saccharin. Experimental groups had access to two bottles of water or one bottle of water and one bottle of alcohol solution + saccharin. Therefore, water was available to the animals 24 h a day. Following 30 intermittent days of alcohol exposure and 1 day of deprivation/abstinence period, the animals were submitted to four instrumental sessions and two BAL assessments, with an interval of 24 h between them (Fig. 1).

First operant session (alcohol drinking and extinction)

The mean body weight of rats was 400 g (± 50 g). Sessions were 60 min in duration. Operant drinking was allowed during the first 30 min. Control and alcohol groups were reinforced with 100 μ l of tap water or alcohol 10%, contingent on pressing the right (water signaling) or the left lever (alcohol signaling) of the operant chamber, respectively. Consequently, both control and experimental groups have access to alcohol at this stage. In our study, the concentration of 10% of alcohol solution proved to be the best choice for the evaluation of voluntary alcohol intake since the disruptive effects of high levels of alcohol, including alteration of motor skills, confusion or sedation, can cause a general decrease in operant performance (Carnicella et al. 2011). The dependent variable recorded was the total alcohol intake (g/kg) and the frequency of lever-pressing

behavior. After 30 min from the beginning of the session, alcohol delivery was blocked to record the frequency of lever-pressing on the alcohol lever (30-min extinction). The persistence of operant response maintained by previously alcohol-paired environmental cues has been commonly used to measure endurance to extinction (Shahan and Burke 2004; Jimenez-Gomez and Shahan 2007). Since previous studies have shown that BAL rates of different doses of alcohol decay to undetectable levels within 3–6 h post-injection (Schulteis and Liu, 2006) BAL measurements were assessed over the next 6 h, a period when withdrawal symptoms begin to occur (Zhang et al. 2007).

Determination of blood-alcohol levels Blood alcohol assessment was conducted using the headspace gas chromatography technique, a useful method that reduces problems related to contamination and sample carry-over. Tail-vein blood samples were obtained 15 min and 60 min after finished extinction, and on 120, 240, and 360 min subsequently. For this, a 1 cm cut was made in the final third part of the tail and the cut was reopened at each sampling assessment. Five animals of each group were randomly chosen for BAL analysis, and five samples were taken from each animal. The second BAL test was carried out on five other animals, other than those used in the previous analysis. Each blood sample (50 μ l) was collected from the rat-tail vein through capillary tubes into heparinized rounded borosilicate glass vials (20 ml headspace vials, Sun-Sri, Rockwood, TN, USA). Half a gram of NaCl and 0.1 g NaF was added, with butanol serving as the internal standard. This solution was placed in the vials, which were sealed with silicone septa and aluminum caps, mixed, and placed in a preheated oven (60 °C) for 15 min, to allow the headspace to reach equilibrium (Wilkinson et al. 1975; Tangerman, 1997). A 100- μ l gas aliquot was withdrawn through the silicon septa using a 250- μ l gas-tight syringe (Hamilton, USA) and injected directly into the gas chromatograph. The syringe was previously maintained at 60 °C to prevent internal condensation on the walls. An additional group (no treatment) was included in the study to control the dissemination of alcohol vapor in the air of the experimental room. The gas chromatography (GC) analysis was carried out using a G8000 gas chromatograph (G-CROM, São Paulo, Brazil) equipped with a flame ionization detector (FID) and a Carbowax capillary column ($l=30$ m, $ID=0.25$ mm, $dF=0.25$ μ m). The GC conditions were as follows: column temperature, 65 °C; injection temperature, 200 °C; detector temperature, 250 °C; column pressure, 70 kPa; and nitrogen carrier gas flow rate, 12 mL/min. Chromatograms were recorded and processed with Peak Simple software (version 3.93). The main variable in the study was the height of the peak, calculated using an external calibration curve.

Withdrawal symptoms Withdrawal symptoms were recorded twice. Once after the last BAL measurement at the end of the first extinction session, and then again at the end of the second extinction session. Since groups performed similarly (no statistical difference between both records) data were collapsed in a single value. Evaluation of withdrawal intensity was conducted considering the studies of Hunter et al. (1975) and Schulteis et al. (1995). Symptoms recorded were tail rigidity, hypoactivity, irritability, vocalization, muscle rigidity, and tremors. These symptoms were classified according to their appearance as light incidence (1) or heavy incidence (2).

Second operant session (first lever-pressing recovery)

On the day after the first operant test, the animals returned to the experimental chamber and the motivation for alcohol drinking was evaluated as measured by the recovery of lever-pressing response following extinction. The session remained for 30 min. The dependent variable recorded was the frequency of lever presses. Finished these sessions the animals were returned to the animal house with free access to food and water for the next two hours. After, they were again fluid deprived for an additional 21 h and 30 min.

Third operant session (alcohol drinking and extinction)

This session was performed just like the 1st operant trial (2.3.7.). Fluid intake, withdrawal symptoms, and BAL analysis were recorded similarly.

Fourth operant session (second lever-pressing recovery)

Sessions were performed in the same way as the 2nd operant trial (2.3.8.).

Euthanasia

At the end of the experiments, the animals were deeply anesthetized with an overdose of sodium pentobarbital (60 mg/kg). Ten minutes later, death was determined by loss of the righting reflex, spontaneous respiration, and lack of response to tail pinch. They were then placed in a holding freezer.

Statistical analysis

EZM and homecage drinking

The Student's *t* test was applied to examine differences between EZM variables. The variables recorded during the homecage drinking period were the total volume of fluid intake (ml), the total volume of water and alcohol intake (ml), the amount of saccharin and alcohol intake (g/kg), and

alcohol preference (%). In the 38 days where water was the only fluid available water intake showed to be quite similar between groups. Therefore, we considered for statistical comparisons only the data recorded in the 30 days were water, saccharin, and alcohol were the fluids available to both groups. For this, we use a repeated-measures (RM) ANOVA with the anxiety-like phenotype as the independent variable and the 30 days of drug exposure as the within-dependent variable. The *t* test was used to determine the main differences between the absolute averaged values of LA and HA groups.

First and second operant sessions (alcohol drinking, extinction, and lever-pressing recovery)

The amount of alcohol intake (g/kg) was analyzed using a two-way ANOVA with drug effects and anxiety phenotype as the main factors. The concurrent frequency of lever-pressing behavior was examined with a two-way ANOVA (treatments \times anxiety levels) with repeated measures (reinforced \times non-reinforced responses). The frequency of bar-pressing during extinction was examined with a simple two-way ANOVA. A repeated-measures ANOVA was also performed to test the main differences between LA and HA rats on BAL at each time point. When necessary, the Tukey HSD test was used for post hoc comparisons. The level of *p* was set at ≤ 0.05 .

Withdrawal symptoms

Statistical examination of the withdrawal scores was performed through the Kruskal–Wallis Multiple–Comparison *Z* value Test considering each one of the variables in the analysis.

Results

Elevated zero maze (EZM)

The Student's *t* test confirmed that the groups selected as LA ($n = 72$) or HA responders ($n = 72$) differed between them regarding anxiety-like behavior as determined by the percentage of the open arms time (water: $t_{64} = -23.31$, $p < 0.001$; alcohol: $t_{69} = -22.58$, $p < 0.001$) (Table 1).

Home cage drinking

Total volume drinking (water group)

The repeated measures ANOVA revealed main differences in anxiety levels ($F_{1,62} = 90.04$; $p < 0.0001$) and days of treatment ($F_{29,1798} = 41.42$; $p < 0.0001$), and main interaction

between factors as well ($F_{29,1798} = 3.13$; $p < 0.0001$). Post hoc comparisons indicate that this difference was related to the increased volume of fluid drinking of HA animals ($n = 30$) comparing with the LA ones ($n = 34$) along with the treatment, particularly after saccharin removal

Table 1 Data recorded from the elevated zero-maze (EZM) were divided into quartiles (1–4) according to the percentage of the time spent in the open arms (total $n = 288$)

Groups	% Open arms	T test	p
Water			
LA	25.40	−23.31	<0.001
HA	5.50		
Alcohol			
LA	27.40	−22.58	<0.001
HA	6.00		

Animals belonging to the quartile 1 (less time in open arms) were assigned to the high-anxiety (HA) group. Animals belonging to quartile 4 (more time in the open arms) were allocated to the low-anxiety (LA) group. Animals whose means were around the median (25% below or above) were discarded and used in other experiments ($n = 144$). Therefore, at this stage, four groups were composed ($n = 36$ /group). To determine the statistical significance between groups, the Student t-test was used. The level of p was set at 5%

(Fig. 2a). Student's t test highlights this statistical difference ($t_{62} = -4.76$, $p < 0.0001$) (Fig. 2a, inset).

Total volume drinking (alcohol group)

The RM ANOVA detected the volume drinking is similar between LA and HA rats during treatment ($F_{1,67} = 0.16$; $p > 0.05$), but significant differences were found on the factor days of treatment ($F_{29,1943} = 37.29$; $p < 0.0001$) (Fig. 2b). The Tukey–Kramer test determines that this difference was related to the increased volume of alcohol drinking of both groups along with the treatment. Overall, average fluid intake was identical between LA and HA groups as showed by the t test ($t_{67} = -0.29$, $p = 0.77$) (Fig. 2b, inset).

Water intake (water group)

ANOVA revealed significant differences on factors anxiety ($F_{1,62} = 124.72$; $p < 0.0001$), and days of treatment ($F_{29,1798} = 99.48$; $p < 0.0001$). Significant interaction was found ($F_{29,1798} = 12.82$; $p < 0.0001$). Post hoc analysis revealed that HA rats increase water consumption immediately following saccharin removal (Fig. 3a). The overall increase is evidenced by the student's t test when applied

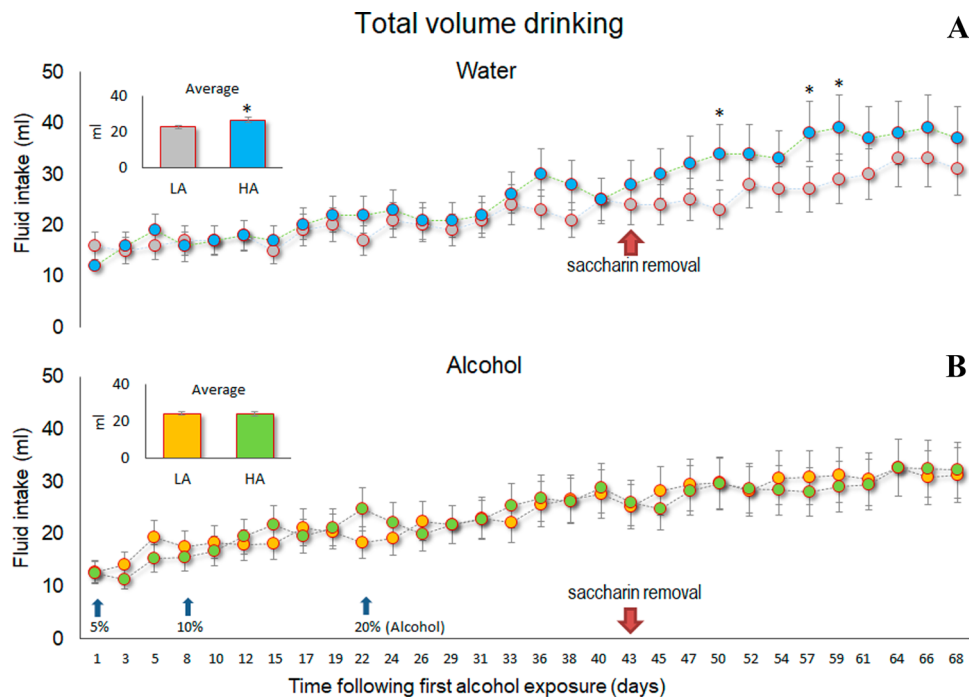


Fig. 2 Mean daily volume drinking of LA and HA rats of water (a) and alcohol (b) groups during the 30 days of homecage drinking where water and saccharin/water solution (control group), or water and saccharin/alcohol solution (experimental group) were available to the animals. The 38 days where water alone was offered to both groups are not shown. Saccharin was removed from treatments on day 43. Treatment was initiated with alcohol 5%. Alcohol 10%

was offered from day 8 to day 19, from which the animals received alcohol 20% until the end of the treatment. Groups were as follows: water LA ($n = 34$), water HA ($n = 30$), alcohol LA ($n = 36$), alcohol HA ($n = 33$). Statistical analysis was performed with a two-way RM ANOVA. Insets show the mean overall volume of drinking. When appropriate, the t -test was used to determine the main differences between LA and HA groups. Probability was set at 5%

to average water intake ($t_{62} = -11.17, p < 0.0001$) (Fig. 3a, inset).

Water intake (alcohol group)

The amount of water intake in LA and HA rats was similar ($F_{1,67} = 3.75; p > 0.05$) and showed to be increased along with treatment ($F_{29,1943} = 46.92; p < 0.0001$) (Fig. 3b). Overall groups mean showed to be identical ($t_{67} = 1.93, p \geq 0.05$) (Fig. 3b, inset).

Saccharin intake (water group)

The repeated-measures ANOVA pointed out significant differences on factors anxiety ($F_{1,62} = 8.68; p < 0.005$), and days of treatment ($F_{17,1054} = 34.43; p < 0.0001$), and also a significant interaction between factors ($F_{17,1054} = 4.86; p < 0.0001$). These differences were due to the increased amount of saccharin intake by LA animals (Fig. 4a) as determined by the *t* test ($t_{62} = 2.94, p \leq 0.005$) (Fig. 4a, inset). Tukey post hoc showed that this occurs mainly at the initial days of the saccharin exposure.

Saccharin intake (alcohol group)

Significant differences were found for all factors (anxiety levels: $F_{1,67} = 189.42; p < 0.0001$; days of treatment: $F_{17,1139} = 14.57; p < 0.0001$). Interaction between factors also achieves statistical significance ($F_{17,1139} = 3.37; p < 0.0005$) (Fig. 4b). This difference was due to the increased saccharin

amounts consumed by HA rats along the first 3 weeks of saccharin exposure. Student’s *t* test highlights this statistical difference ($t_{67} = -13.76, p < 0.0001$) (Fig. 4b, inset).

Alcohol intake

The volume of alcohol drinking was greatly influenced by anxiety levels ($F_{1,67} = 243.97; p < 0.0001$) and the period of treatment ($F_{29,1943} = 37.46; p < 0.0001$). These factors interact significantly ($F_{29,1943} = 5.89; p < 0.0001$) (Fig. 5a). The same was found regarding the amount of alcohol consumed (anxiety factor: $F_{1,67} = 194.94; p < 0.0001$, days of treatment: $F_{29,1943} = 92.47; p < 0.0001$, interaction: $F_{29,1943} = 6.70; p < 0.0001$) (Fig. 5b). Tukey test revealed that HA rats have increased alcohol intake, comparing with LA ones. This increase tends to remain stable during the treatment. On the other hand, LA rats increase fluid drinking following saccharin removal in such a way that LA and HA groups perform identically at the end of the period of homecage drinking.

Alcohol preference

The same above was found for alcohol preference (anxiety levels: $F_{1,67} = 228.98; p < 0.0001$, days of treatment: $F_{29,1943} = 2.83; p < 0.0001$, interaction: $F_{29,1943} = 4.92; p < 0.0001$) (Fig. 5c). Tukey post hoc showed that HA rats have increased preference for alcohol and this preference stabilizes throughout the 30 days of alcohol exposure. LA rats increase alcohol drinking following saccharin removal. Insets in Fig. 5 show the main difference between LA and

Fig. 3 Mean daily water intake of LA and HA rats of water (a) and alcohol group (b) during the 30 days of homecage drinking where water and saccharin/water solution control group, or water and saccharin/alcohol solution were available to the animals. The 38 days where water alone was offered to both groups are not shown. Two-way RM ANOVA with *p* set as 5%. Insets show the mean total volume of drinking. When appropriate, the *t* test was used to determine the main differences between LA and HA groups (5%)

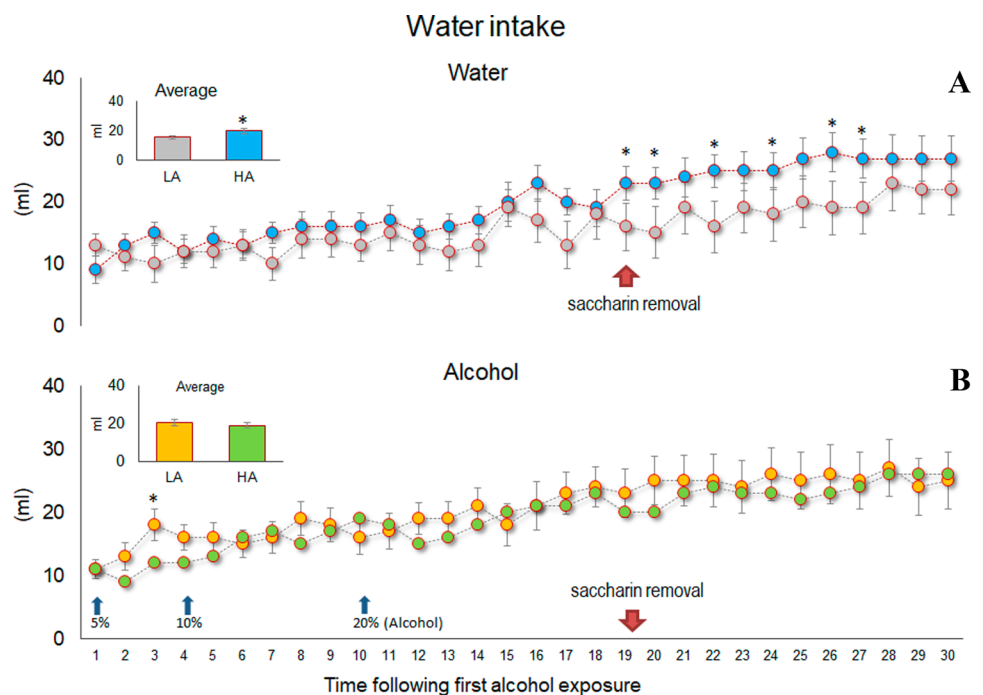


Fig. 4 The average consumption of saccharin (a and b) and saccharin fluid intake (c and d) of LA and HA rats of water and alcohol groups during the 30 days of homecage drinking where water and saccharin/ water solution (control group), or water and saccharin/alcohol solution were available to the animals. The 38 days where water alone was offered to both groups are not shown. Two-way RM ANOVA with *p* set as 5%. Insets show the mean total volume of drinking. When appropriate, the *t*-test was used to determine the main differences between LA and HA groups (5%)

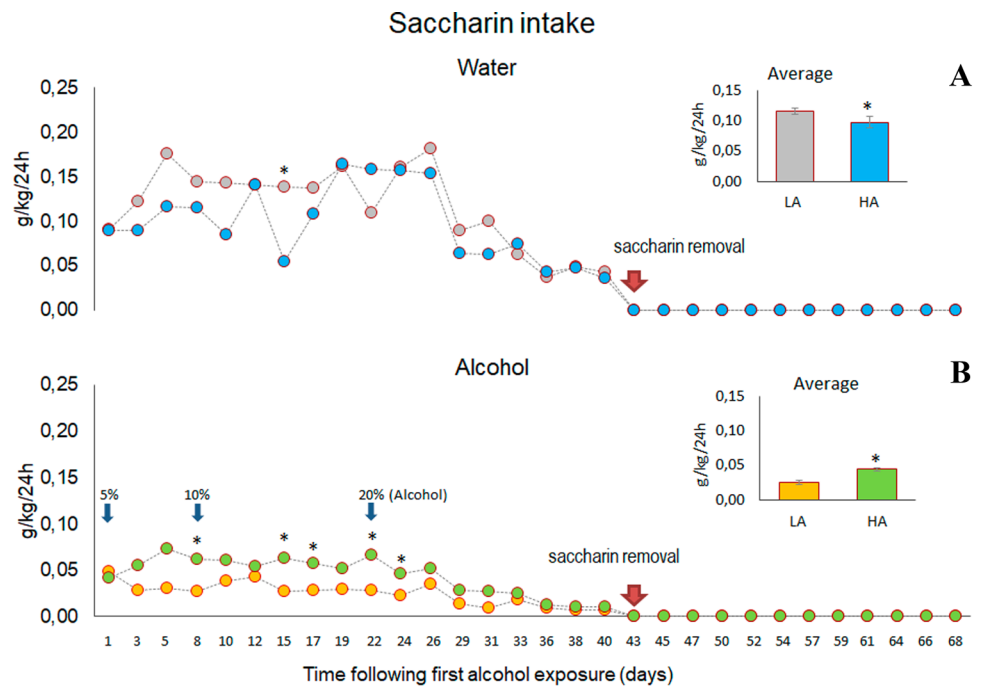
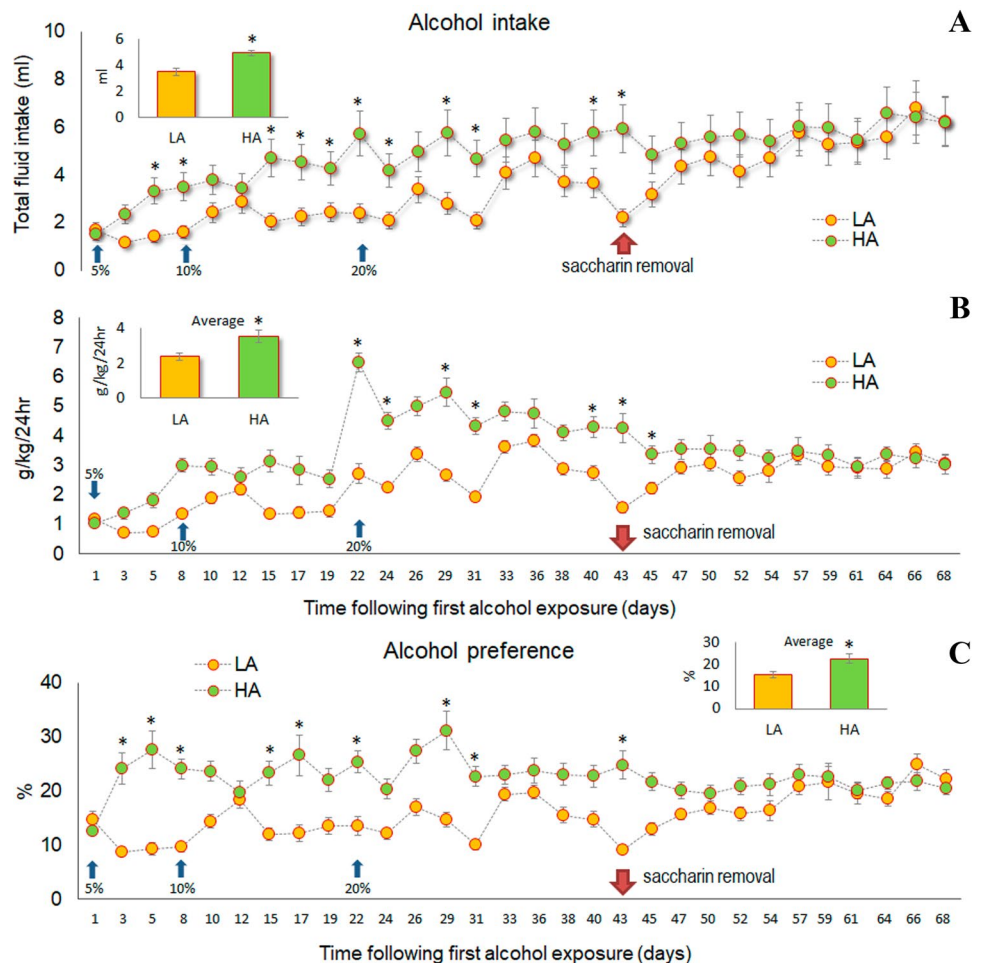


Fig. 5 Average alcohol intake (a and b) and alcohol preference (c) of LA and HA rats of water and alcohol groups during the 30 days of homecage drinking where water and saccharin/ water solution (control group), or water and saccharin/alcohol solution were available to the animals. The 38 days where water alone was offered to both groups are not shown. Two-way RM ANOVA with *p* set as 5%. Insets show the mean total volume of drinking. The student's *t* test was used to determine the main differences between LA and HA groups (5%)



HA regarding the volume of alcohol intake ($t_{33} = -15.62$, $p < 0.0001$), the amount of alcohol consumed ($t_{33} = -13.96$, $p < 0.0001$), and alcohol preference ($t_{33} = -15.13$, $p < 0.0001$), respectively (insets in Fig. 5).

First operant session (alcohol drinking, extinction, and lever-pressing recovery)

The two-way RM ANOVA applied to the data obtained in the first operant session highlighted the main differences between water and alcohol groups ($F_{1,129} = 45.04$; $p < 0.0001$) (Fig. 6a). Besides, LA and HA rats also differed between them considering the amount of alcohol consumed ($F_{1,129} = 15.89$; $p < 0.0001$). Significant interaction between factors was also found ($F_{1,129} = 45.04$; $p < 0.0001$). Post hoc comparisons showed that these differences were due to the increased alcohol intake of HA alcohol pre-treated rats as revealed by the increased rate of lever-pressing behavior ($F_{1,129} = 20.18$; $p < 0.0001$) (Fig. 6, inset). Regarding this variable, the two-way RM ANOVA also detected the main difference in the factor lever (reinforced \times non-reinforced bar-pressing) ($F_{1,129} = 23.47$; $p < 0.0005$). Tukey post hoc indicated that the HA alcohol group increases de response on the alcohol-release lever, comparing with HA controls rats. However, they also emit a similar rate of non-reinforced responses. Regarding the frequency of lever-pressing during extinction (Fig. 6b). Main differences were found on treatment ($F_{1,129} = 47.06$; $p < 0.001$) and anxiety factors

($F_{1,129} = 10.76$; $p < 0.005$), with these factors interacting between them ($F_{1,129} = 40.29$; $p < 0.005$). Post hoc test showed that this was due to increases in the lever-pressing response behavior of HA alcohol rats. Bar-pressing recovery was also affected by treatments (Fig. 6d) ($F_{1,129} = 36.81$; $p < 0.001$), and significantly influenced by anxiety levels ($F_{1,129} = 29.00$; $p < 0.05$). Post hoc analysis revealed that HA rats increase the amount of alcohol intake during this phase of the experiments as shown by the elevated rates of lever-presses in both alcohol-signaling and non-signaling lever.

Second operant session (alcohol drinking, extinction, and lever-pressing recovery)

Groups showed to diverge significantly from each other regarding treatment ($F_{1,129} = 109.80$; $p < 0.0001$) and anxiety levels ($F_{1,129} = 32.93$; $p < 0.0001$). Main interaction between factors was absent ($F_{1,129} = 32.93$; $p > 0.02$). Tukey post hoc indicated that high anxiety levels and previous alcohol experience increase alcohol intake, mainly in HA alcohol pre-treated rats. As a matter of fact, except for the LA controls, all the other groups showed increased rates of alcohol intake when compared with the data collected from the first operant trial (Fig. 7E). In parallel, lever pressing response was greatly affected by treatments ($F_{1,129} = 97.43$; $p < 0.0005$), anxiety levels ($F_{1,129} = 43.18$; $p < 0.0001$), and the choice of the lever (reinforced \times non-reinforced) ($F_{1,129} = 97.62$; $p < 0.005$). Post hoc comparisons showed that the rate of

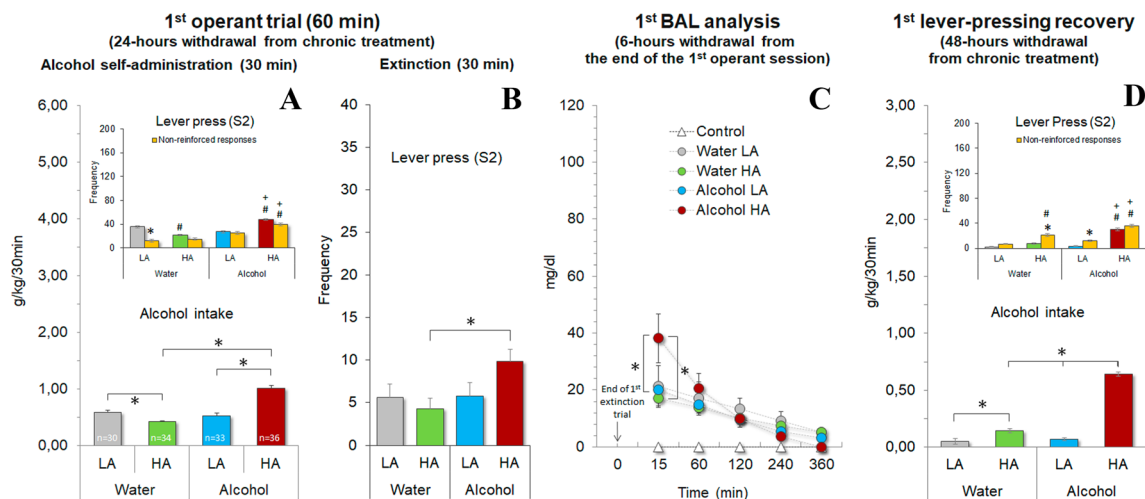


Fig. 6 Histograms (mean \pm SEM) depict the total alcohol intake (a) and the frequency of reinforced and not reinforced lever presses (inset). In b is represented the frequency of alcohol-reinforced lever pressing (S2) following 30 min of extinction of the operant response. The blood alcohol concentration recorded during the 6-h withdrawal from the end of the 1st operant trial is shown in c. Finally, in d are depicted the total alcohol intake (g/kg/30 min) and lever-presses recovery following 48-h withdrawal from the chronic treatment (inset). Operant trials were initiated 24-h after withdrawal

from chronic, voluntary, and intermittent alcohol exposure (30 min of free alcohol drinking followed by 30 min of extinction of operant bar-pressing). First BAL analysis was conducted along with the 6-h following extinction. On the day after, the 1st operant bar-pressing recovery trial was performed (48-h withdrawal from chronic alcohol exposure). Groups were as follows: water LA ($n = 34$), water HA ($n = 30$), alcohol LA ($n = 36$), alcohol HA ($n = 33$). Data were analyzed by Two-way ANOVA followed by Tukey’s post hoc for unequal N . The value of p set at ≤ 0.05

reinforced and non-reinforced responses is quite similar in HA rats, but it is significantly reduced in the other groups (Fig. 7e, inset). Considering the data recorded from the second extinction trial, two-way ANOVA pointed out the effect of treatments ($F_{1,129} = 143.81$; $p < 0.0001$), anxiety levels ($F_{1,129} = 247.34$; $p < 0.00001$), and main interaction between factors ($F_{1,129} = 120.31$; $p < 0.0001$) (Fig. 7f). A similar result was found during the 2nd lever-pressing recovery session. Two-way RM ANOVA applied to the amount of alcohol intake revealed main effects of treatment ($F_{1,129} = 102.80$; $p < 0.0001$) an anxiety levels ($F_{1,129} = 83.17$; $p < 0.001$) and significant interaction between factors ($F_{1,129} = 46.08$; $p < 0.001$) (Fig. 7h). Post hoc comparisons highlighted that HA alcohol rats enhanced substantially alcohol intake comparing with HA controls or the LA alcohol counterpart. In the same way, two-way RM ANOVA showed that lever-pressing behavior was significantly increased in this group ($F_{1,129} = 102.27$; $p < 0.0001$) (Fig. 7h, inset).

Determination of blood-alcohol levels (BAL) in LA and HA rats tested on 24 and 72 h of withdrawal from chronic treatment.

BAL time courses were compared with a repeated-measures ANOVA to analyze BAL with treatments (water \times alcohol) and anxiety levels (LA \times HA) as the main between factors and the five moments of blood

assay (15, 60, 120, 240, and 360 min) as the within repeated measure. There was a significant effect of time course of blood assay ($F_{4,64} = 288.8$; $p < 0.0001$), and a drug \times time ($F_{4,64} = 45.77$; $p < 0.0001$), anxiety \times time ($F_{4,64} = 5.55$; $p < 0.0007$), and drug \times anxiety \times time interaction ($F_{4,64} = 13.64$; $p < 0.0001$). Tukey test revealed that these differences were mainly due to the elevated levels of alcohol on blood plasma of HA alcohol-withdrawn rats throughout the first 15 min of blood sampling (Fig. 6c). This difference tends to disappear along with the six hours of BAL analysis.

The BAL recorded after the second extinction trial showed a similar pattern but with additional main effects on treatment and anxiety. Previous alcohol experience increases the blood alcohol content of LA and HA alcohol pre-treated rats ($F_{1,16} = 167.38$; $p < 0.0001$). Anxiety levels were also a predictor for alcohol intoxication in LA controls ($F_{1,16} = 25.10$; $p < 0.0001$). Moreover, the interaction was found for the time course of blood assay \times drug ($F_{4,64} = 138.24$; $p < 0.0001$), and time \times anxiety levels ($F_{4,64} = 77.62$; $p < 0.0001$). Post hoc comparisons revealed that HA anxious rats showed to be highly intoxicated, with mean blood content achieving up to 80 mg/dl. LA alcohol rats and HA controls also differed significantly from LA controls showing that previous alcohol experience and anxiety phenotype interact to facilitate voluntary alcohol intoxication (Fig. 7g).

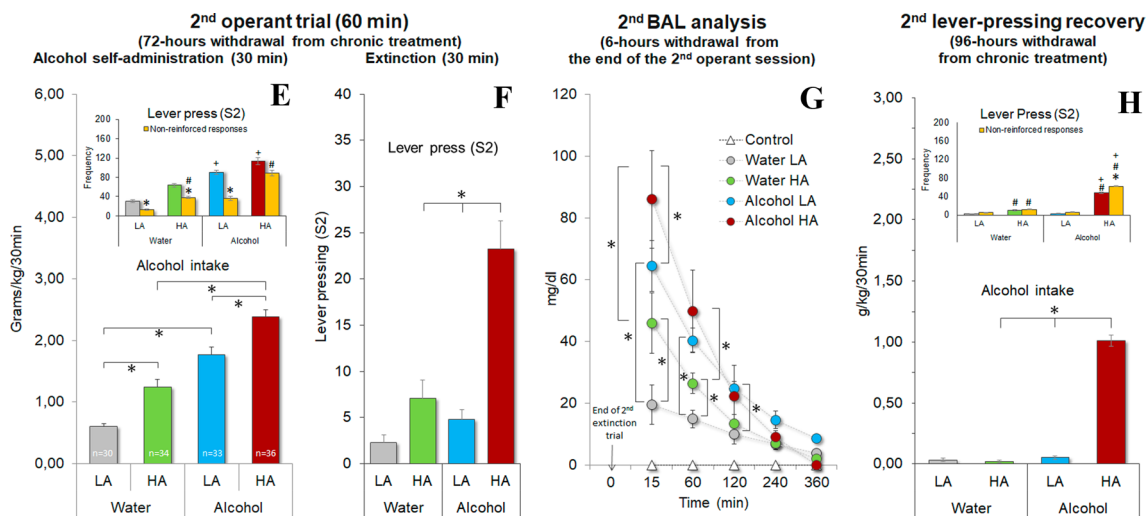


Fig. 7 In **e** are depicted the total alcohol intake and the frequency of reinforced and not reinforced lever presses during the 2nd operant trial (inset). **f** represents the frequency of alcohol-reinforced lever pressing (S2) following 30 min of extinction of the 2nd operant response test. The blood alcohol concentration recorded during the 6-h withdrawal from the end of the 2nd operant trial is shown in **g**. In **h** are depicted the total alcohol intake (g/kg/30 min) and lever-presses recovery (inset). The 2nd operant trial was initiated 72-h after with-

drawal from chronic, voluntary, and intermittent alcohol exposure (30 min of free alcohol drinking followed by 30 min of extinction of operant bar-pressing). A second BAL analysis was conducted during the 6-h after ending extinction. On the day after, the operant bar-pressing recovery trial was performed (96-h withdrawal from chronic alcohol exposure). Two-way ANOVA followed by Tukey's post hoc for unequal N with p set at ≤ 0.05

Withdrawal symptoms

The non-parametric Kruskal–Wallis was performed to compare the mean among the four groups considering each one of the variables in the analysis. Hypoactivity, irritability, and vocalization were the main variables affected ($p < 0.05$). Dunn’s test showed that this effect was dependent on previous alcohol experience and was positively correlated with anxiety levels, with the HA rats exhibiting heavy signals of alcohol withdrawal (Table 2).

Discussion

In our study, 144 animals, previously separated into LA or HA responders were submitted to sixty-eight days of intermittent exposure to water and an alcohol solution up to 20% v/v. A procedure of saccharin fading was used to facilitate alcohol drinking. Later, they were tested in a voluntary operant response to analyzing the frequency of lever-pressing response and alcohol intake, the frequency of lever-pressing during extinction, and lever-pressing recovery. These measurements were recorded following two consecutive periods of alcohol withdrawal where BAL was also assessed. Our data showed that two factors contribute to enhancing alcohol consumption during the operant tests, the anxiety phenotype, and the previous experience with alcohol.

The main results of this study were as follows. Considering the measures recorded during the home cage period, the total volume of fluid intake was similar between LA and HA groups until the saccharin was removed from fluid drinking. From that point on, and unlike HA groups, LA animals start to increase water intake. It is worth noting that LA control rats have increased the amount of saccharin, twice as much as the HA animals. Our data is supported by the previous

report of Dess and Minor (1996) where rats selectively bred for high saccharin ingestion proved to be less emotional than the low-saccharin rats. It has been sustained that ingestion and emotionality interact significantly (Dess 1991). These data can also indicate that LA rats tend to compensate for the absence of saccharin increasing the volume of water, following saccharin removal from fluid drinking. Considering this hypothesis, the change in fluid intake of LA control rats can indicate that an affective response of these animals to a sweet taste can be raised by long-term saccharin experience.

At large, during the home cage period, HA alcohol-exposed rats showed to develop a great affinity for alcohol. At the end of alcohol exposure, however, the volume and amount of alcohol intake and alcohol preference was quite similar between LA and HA groups. This is particularly due to the increase in alcohol intake of LA rats following the clearance of saccharin from the fluid. Conversely, the alcohol intake of HA rats remains stable. Despite several divergences concerning the positive correlation between anxiety states and alcohol intake have been found (Henniger et al. 2002; Da Silva et al. 2004; Langen and Fink 2004), our result is supported by the recent translational study of Jadhav et al. (2017) where a preclinical model was adapted to implement the operational definitions used in the DSM criteria. They found that anxiety trait was positively correlated with the propensity to develop addiction-like traits in rats.

The early contact with alcohol effects is a marker of future alcohol dependence in humans (DeWit et al. 2000). In rodents, it has been shown that alcohol exposure increases alcohol preference in adult life (Hayashi and Tadokoro 1985). In another study, the age of drinking onset was not related to later alcohol preference (Siegmund et al. 2005). However, despite these mixed results, the age of drinking increases the susceptibility of stress-induced alcohol intake. In the present report, the influence of alcohol drinking over

Table 2 The table shows the incidence of alcohol withdrawal symptoms in low- (LA) and high-anxiety (HA) rats

Groups	Incidence	Trail rigidity	Hypoactivity	Irritability	Vocalization	Muscle rigidity	Tremors
Water							
LA (n = 34)	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
HA (n = 30)	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
Alcohol							
LA (n = 36)	1	0	5%	19%	3%	0	0
	2	0	0	14%	6%	0	0
HA (n = 33)	1	0	49%	15%	32%	5%	0
	2	0	26%	43%	29%	7%	0

Data on columns describe the percentage of animals affected by the variable in the study considering the total number of animals in each group. Withdrawal symptoms were recorded twice but data were collapsed in a single value. Withdrawal scores were analyzed through the non-parametric Kruskal–Wallis Multiple–Comparison Z value test

time in periadolescent aged and adult rats on later relapse-like behavior and stress-induced alcohol drinking was investigated with three different independent variables being considered: (i) periadolescent rats were early segregated into LA or HA phenotypes, (ii) we used an intermittent procedure for alcohol delivery, (iii) a sweetened solution fading procedure was used to facilitate alcohol drinking. With the use of this methodology, we found that later alcohol drinking can be facilitated in high- but not low-anxious control subjects. This effect is increased in alcohol experienced rats. These data fit with the well-known “tension reduction hypothesis” (Conger 1956) which predicts that the negative-reinforcing effect of alcohol facilitates future alcohol intake. Also, our results endorse previous findings from our (Ezequiel Leite and Nobre 2012) and other laboratories (Stewart et al. 1993; Spanagel et al. 1995; Möller et al. 1997) (Jadhav et al. 2017) showing a positive correlation between anxiety and the reinforcing effects of alcohol in rats. Anyway, others have not found any evidence of this relationship (Overstreet et al. 1997; Henniger et al. 2002). Contrary to was expected, when examining the relation of anxiety and alcohol intake Langen and Fink (2004) found that rats prone to anxiety-like behavior consume less alcohol than LA rats no matter exposed to a two-bottle choice procedure or a progressive-ratio operant paradigm. A similar phenomenon is described in the study of Henniger et al. (2002). The differences found among these studies can be attributed to several methodological distinctions. As discussed above, different from much of these reports, in our experiments, it was used an intermittent and long-term voluntary access to alcohol in a two-bottle choice drinking paradigm, associated with a sweetened solution fading procedure. This method allows the animals to consume significant levels of alcohol, similar to those drinking by selectively inbred alcohol-preferring rats (Dyr and Kostowski 2000; Bell et al. 2006), an effect not attributed to the influence of saccharin on alcohol taste, as already discussed.

The rate of acquisition of operant response is positively correlated with the saliency of a reinforcer (Donjam 1982). On the other hand, the rate of extinction and bar-pressing recovery is associated with the strength of conditioning to environmental cues (Katner et al. 1999). In the present study, to analyze the motivation for alcohol intake, control, and alcohol pre-treated rats were submitted to a 30 min session of extinction in the same the operant trial in which both water and alcohol were presented as a reinforcer. Our results showed that in the first operant session HA rats from the alcohol group increase the rate of lever pressing on the alcohol-signaling lever, with a consequent increase in the amount of alcohol intake. They also emitted similar rates of non-reinforced responses during the null period, an effect probably due to the increased motivation for alcohol intake associated with low control of impulsive behavior resulting from the long-term alcohol experience. This reinforcing

effect of previous alcohol exposure is visible during extinction when lever-pressing behavior is enhanced in the absence of the reinforcer. This robust response on the alcohol-signaling lever indicated that alcohol was effective as a behavioral reinforcer mainly for HA alcohol rats. We argue that the animals also learn to respond to the context in which the drug was delivered and increase their rates of lever pressing in the absence of the reinforcing stimuli. Therefore, results from the first extinction phase reveal an increase in alcohol-seeking behavior in HA alcohol pre-treated animals tested during withdrawal. Thus, for HA individuals, the consequence of alcohol intake on behavior is quite dependent on their previous drinking history in such a way that, during a long-term alcohol experience, the drinker seems to learn that alcohol alleviates withdrawal symptoms in a negative reinforcing-like profile. This is especially important in the context of our experiments since we used an intermittent procedure for alcohol delivery where several withdrawal sessions were introduced over the length of alcohol treatment. So, it is speculated that the aversive nature of alcohol withdrawal could have the ability to reinforce future alcohol drinking to mitigate the negative effects of alcohol withdrawal, among them, increased anxiety-like symptoms (Koob 2013). Therefore, it is supposed that alcohol intake following 6 h of alcohol withdrawal is driven mainly by the emotional component, but it does not rule out that physical withdrawal does not affect alcohol consumption either since, at this point, overt withdrawal symptoms were found. Other studies have reported the appearance of more severe physical withdrawal symptoms at several time points including 6 h after alcohol interruption (Macey et al. 1996; Saglam et al. 2006; Kayir and Uzbay 2008). However, different from ours, these studies used the method of forced alcohol consumption where the drug is administered in a nutritionally complete liquid food. This technique is well-known to induce higher rates of alcohol intake; more than any other approaches (Lieber et al. 1989), and consequently induces higher levels of alcohol dependence and withdrawal symptoms but lacks the common voluntary approach to alcohol drinking, as seen in humans. Importantly, our results endorse the previous assumption that individuals that go through subsequent alcohol withdrawal experiences will display more severe withdrawal symptoms than those who undergo a single withdrawal episode and this is an indicator that the previous experience of alcohol withdrawal can alter in the future the response of the animal to the drug (Maier and Pohorecki 1989).

Data collected from gas chromatography revealed that blood-alcohol elimination in HA animals was faster than those observed in LA rats. This discrepancy is even greater when blood alcohol levels were assessed following the second operant session of alcohol drinking that was conducted following three days after the interruption of the home cage

drinking. In a previous report, it was demonstrated that alcohol withdrawal symptoms can be detected after acute alcohol treatment, when blood-alcohol levels are no longer detectable (Schulteis and Liu 2006). In our report, HA alcohol pre-treated rats showed increased blood-alcohol clearance, since alcohol intoxication showed to be higher during the first minute of blood-alcohol assessment but equivalent to the other groups, six hours after. To our knowledge, a specific correlation between anxiety and alcohol metabolism is lacking. Nevertheless, in humans (Willinger et al. 2002) and rodents (Spanagel et al. 1995), a positive correlation between anxiety and alcohol preference has been found. Moreover, high alcohol-preferring mice, which achieve blood alcohol levels like alcohol-dependent humans when submitted to a free choice alcohol drinking, metabolize alcohol faster than alcohol naïve mice. It is supposed that this rapid alcohol clearance results from metabolic tolerance as revealed by the induction of the hepatic enzyme Cytochrome P450 (Matson et al. 2013). In this context, Chester and Barrenha (2007) showed the existence of an acceptable correlation between startle response and alcohol preference in alcohol-preferring (HAP1/HAP2) and non-preferring (LAP1/LAP2) mice with male and female HAP lines exhibiting high startle amplitude and a significant decrease in their blood-alcohol content 6 h after an alcohol injection. Thus, it seems that the factor anxiety by itself also impacts somehow blood alcohol clearance. Given that increases in startle have been used as an index of anxiety-related behavior, we can suppose the existence of genetic mechanisms underlying the reinforcing effects of alcohol in highly anxious subjects.

In our study, BAL rates recorded in HA rats were shown to be elevated. These levels remained higher even after a secondary analysis of the BAL data samples and corrections of minor errors detected. However, it has been shown that the amount of alcohol consumed, and the resulting BAL levels, are strongly influenced by energy level and meal composition in both rats (Dilley et al. 2018), and humans (Lin et al. 1976; Pikaar et al. 1988). Importantly, alcohol concentration also affects alcohol absorption since concentrations of alcohol above 30% led to reduced gastric motility and slower alcohol absorption (Bujanda 2000). Besides, alcohol by itself increases stomach acidification which also helps to slow alcohol absorption (Aures et al. 1982) and changes in gastric emptying strongly impact alcohol absorption (Holt 1981). Also, and this is important for our study, a high BAL can be more related to reduced alcohol dilution than high alcohol intake (Dilley et al. 2018). All this information, and some particularities of our study, may be responsible for the discrepancy between our and other reports. We use a long-term and intermittent alcohol exposure procedure. With this experimental scheme, the animals were maintained up to 24 h of liquid deprivation before each one of the tests (15 h was the minor time of deprivation). This is important

to note since daily food intake has been found to reduce progressively during water deprivation (Armstrong et al. 1980). Therefore, our results on BAL levels can be powerfully influenced by the positive impact of water deprivation on alcohol absorption, and by the influence of the negative reinforcing profile of alcohol withdrawal on the volume of alcohol ingested by HA rats.

One of the main drawbacks of our study is that conclusions about age-specific effects cannot be justified since we do not include the appropriate comparator groups (i.e., a young adult group exposed to the identical procedure). Also, although the reduction in the open-arms exploration in EZM is related to anxiety-like behavior in rodents (Shepherd et al. 1994; Kulkarni et al. 2007; Braun et al. 2011), selection studies, like ours, often select for traits other than those originally intended. For example, it is widely recognized that rats find novel, exposed environments relatively aversive due to the potential risk of predation (Litvin et al. 2008). Considering the age of the animals, previous reports have stressed lower levels of exploration by adolescent rats in the open-field (Candland and Campbell 1962) and lower levels of open arm activity in the conventional elevated plus-maze, compared to adults (Doremus et al. 2003). Opposed results were found by Doremus-Fitzwater (2009) with open-arms exploration being greatly facilitated in adolescent than adult rats. In mice, marked differences were found in alcohol-related behaviors across adolescent development, with early adolescents showing great sensitivity to the stimulatory effects of alcohol on motor behavior paralleled by the alcohol-induced anxiolytic-like response, compared with older mice (Hefner and Holmes 2007). Besides, changes in open-arms activity, which can also be attributed to general activity, neophobia, and risk-taking, are all alternative interpretations of the phenotype of reduced open arm exploration, each of which is critical determinants of high alcohol consumption. In this way, it is expected that alcohol intake acquires strong reinforcing-like properties in young than adult rats. This gap may limit some statements of the current study where young rats were early selected as LA or HA responders. Therefore, our conclusions on this matter must be interpreted with due care.

Conclusions

Chronic alcohol exposure can increase future alcohol intake and this effect correlates with anxiety levels. In our study, we sought to determine whether premature alcohol experience of LA and HA rats could facilitate future preference for alcohol drinking. Despite in the present study the emphasis on motivation is not justified (it will only with progressive schedules of reinforcement), increases on lever-pressing frequency may indicate that alcohol drinking and possibly

the motivation to consuming alcohol is differentially driven in LA and HA rats. Anxious subjects tend to increase alcohol intake over time with the amount of alcohol drinking being related to the incidence of withdrawal symptoms. Therefore, different from LA animals, HA rats submitted to an early and long-term intermittent alcohol exposure later exhibit a high level of alcohol drinking on withdrawal, and withdrawal symptoms, associated with a high frequency of lever-pressing during extinction and recovery of bar pressing, an indicative of alcohol-seeking behavior. The changes observed in alcohol metabolism of HA rats is a matter for future studies, but it may be due to enzymatic tolerance resulting from long-term alcohol drinking.

Acknowledgements This study was supported by FAPESP (Proc. No. 2010/15157-4). M.J. Nobre is the recipient of a Productivity Research Grant from CNPq (303144/2015-7). R.F. Sgobbi holds a post-doctoral fellowship from FAPESP (2014/09685-9). We declare that the sponsors have not been involved in or influenced the design, collection, analysis or interpretation of study data, nor the writing of the report or the decision to submit it for publication.

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