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Binge drinking in alcohol-preferring sP rats at the end of the nocturnal period

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Abstract

Sardinian alcohol-preferring (sP) rats have been selectively bred for high alcohol preference and consumption using the standard 2-bottle “alcohol (10%, v/v) vs. water” choice regimen with unlimited access; under this regimen, sP rats daily consume 6–7 g/kg alcohol. The present study assessed a new paradigm of alcohol intake in which sP rats were exposed to the 4-bottle “alcohol (10%, 20%, and 30%, v/v) vs. water” choice regimen during one of the 12 h of the dark phase of the daily light/dark cycle; the time of alcohol exposure was changed daily in a semi-random order and was unpredictable to rats. Alcohol intake was highly positively correlated with the time of the drinking session and averaged approximately 2 g/kg when the drinking session occurred during the 12th hour of the dark phase. Alcohol drinking during the 12th hour of the dark phase resulted in (a) blood alcohol levels averaging approximately 100 mg% and (b) severe signs of alcohol intoxication (e.g., impaired performance at a Rota-Rod task). The results of a series of additional experiments indicate that (a) both singular aspects of this paradigm (i.e., unpredictability of alcohol exposure and concurrent availability of multiple alcohol concentrations) contributed to this high alcohol intake, (b) alcohol intake followed a circadian rhythm, as it decreased progressively over the first 3 h of the light phase and then maintained constant levels until the beginning of the dark phase, and (c) sensitivity to time schedule was specific to alcohol, as it did not generalize to a highly palatable chocolate-flavored beverage. These results demonstrate that unpredictable, limited access to multiple alcohol concentrations may result in exceptionally high intakes of alcohol in sP rats, modeling – to some extent – human binge drinking. A progressively increasing emotional “distress” associated to rats’ expectation of alcohol might be the neurobehavioral basis of this drinking behavior.

Keywords

Time schedule of alcohol drinking; Limited and unpredictable access to alcohol; Experimental model of binge drinking; Sardinian alcohol-preferring (sP) rats

Introduction

In an attempt to develop proper rodent models of excessive intake of alcohol (possibly up to intoxicating levels), multiple procedures involving temporally limited accesses to alcohol have been tested. For instance, repeated daily exposures to 10% (v/v) alcohol, for limited time periods of 1 h, during the light phase of the daily light/dark cycle, and with fixed time of access on each day, resulted in relatively high intakes of alcohol (0.8–1.0 g/kg) in selectively bred, alcohol-preferring Alko Alcohol (AA) rats (Sinclair, Hyytiä, & Nurmi, 1992). The repeated, concurrent availability of multiple alcohol concentrations (0%, 15%, and 30%, v/v) in two daily 1-h drinking sessions, 3 h apart during the dark phase, resulted in mean alcohol intakes exceeding 2.5 g/kg/session in selectively bred, Indiana alcohol-preferring (P) rats (Bell, Rodd, Lumeng, Murphy, & McBride, 2006); these alcohol intakes, together with the corresponding blood alcohol levels (BALs; averaging approximately 120 mg%), met the proposed criteria for binge-like drinking (Bell et al., 2006). Notably, the amounts of alcohol consumed by AA and P rats under the above limited-access paradigms were much higher than those recorded in single bouts of alcohol drinking when AA and P rats were continuously (24 h/day) exposed to alcohol (Bell et al., 2006; Sinclair et al., 1992). Comparable data have also been collected in mice: exposure to a single alcohol bottle (10%, 20%, or 30%, v/v) in daily 2- or 4-h drinking sessions, occurring early in the dark phase (leading to this procedure being named with the sharp appellation of “Drinking-in-the-Dark”), resulted in elevated intakes of alcohol and pharmacologically significant BALs in alcohol-preferring C57BL/6J mice (Rhodes, Best, Belknap, Finn, & Crabbe, 2005).

Sardinian alcohol-preferring (sP) rats constitute one of the few rat lines selectively bred for high alcohol preference and consumption (see Bell et al., 2012; Colombo, Lobina, Carai, & Gessa, 2006). When exposed to the standard, home cage 2-bottle “alcohol (10%, v/v) vs. water” choice regimen, with unlimited access for 24 h/day, sP rats daily consume 6–7 g/kg alcohol. Under this regimen, alcohol intake in sP rats (a) is stable over long periods of time, (b) occurs mainly during the dark phase of the daily light/dark cycle, (c) is fractioned in 3–4 daily bouts, (d) gives rise to BALs in the 50–70 mg% range at each bout, and (e) produces measurable psychopharmacological effects, including amelioration of genetically determined, anxiety-related behaviors (see Colombo et al., 2006). Nevertheless, sP rats exposed to the 2-bottle “alcohol (10%, v/v) vs. water” choice regimen and unlimited access rarely – if ever – lose control over alcohol and become intoxicated (see Colombo et al., 2006), suggesting that the basal procedure of alcohol drinking under which sP rats have been selectively bred can lead to high alcohol drinking but not to development of alcohol dependence. To date, escalations in alcohol drinking up to intoxicating levels and development of “uncontrolled” and “compulsive”-like alcohol-taking behaviors in sP rats have only been observed when rats were exposed to the intermittent (once every other day) access to 20% (v/v) alcohol: under this regimen, mean daily alcohol intake rose to 9–10 g/kg and alcohol drinking occurred despite (a) aversive consequences (adulteration of alcohol solutions) and (b) presence of a competing reinforcer (a saccharin solution) (both indexes of “behavioral” dependence on alcohol) (Loi et al., 2010, 2014).

The present study was designed as a further attempt to identify experimental procedures capable of producing remarkably high intakes of alcohol in sP rats. To this end, the present

study assessed the efficacy of a procedure that combined different aspects of the previously mentioned paradigms known to successfully induce escalations in alcohol drinking in alcohol-preferring AA and P rats and C57BL/6J mice. Specifically, in the present experiment, sP rats were exposed to daily drinking sessions (a) of limited duration [1 h; according to the pioneering study by Sinclair et al. (1992) with AA rats], (b) during the dark phase of the light/dark cycle [according to the propensity of sP rats for bout-like alcohol drinking during the dark phase (see Colombo et al., 2006) and taking in to account the outcomes of the “Drinking-in-the-Dark” model (Rhodes et al., 2005)], and (c) with concurrent access to multiple concentrations of alcohol [10%, 20%, and 30%, v/v; reproducing, to some extent, the experimental design of the study by Bell et al. (2006) with P rats]. The procedure tested in the present study also incorporated an additional aspect: the unpredictability of time of alcohol availability; specifically, time of access to alcohol was changed every day in a semi-random order to ensure that, over 12 consecutive days, rats experienced all 12-h time periods of the dark phase as periods of access to alcohol.

Materials and methods

All experimental procedures employed in the present study were in accordance with the Italian law on the “Protection of animals used for experimental and other scientific reasons.”

Animals

Male sP rats from the 83rd and 84th generation were used. Rats were approximately 60 days old at the start of each experiment (see below). In all experiments, rats were singly housed in standard plastic cages with wood chip bedding; single housing started at the age of approximately 45 days. The animal facility was under an inverted 12:12 h light/dark cycle (lights on at 8:00 PM) at a constant temperature of 22 ± 2 °C and relative humidity of approximately 60%. Food pellets (2018 Diet; Harlan, San Pietro al Natisone, UD, Italy) and water were always available in the home cage. Independent groups of rats were used in each experiment.

Experimental procedure

Experiment 1 – characterization of alcohol drinking behavior; assessment of BALs and alcohol intoxication

Aims: This first experiment was designed to assess alcohol intake in rats concurrently exposed to multiple alcohol concentrations in daily drinking sessions of 1 h; the time of each drinking session was changed daily in a semi-random order, so that (a) each hour of the 12 h of the dark phase of the daily light/dark cycle was tested and (b) time of the drinking session was unpredictable to rats. This experiment also addressed the research question whether alcohol drinking during the 1st and 12th hour of the dark phase was associated to signs of intoxication (measured by changes in the rat performance at a Rota-Rod task). BALs deriving from alcohol drinking during the 1st and 12th hour of the dark phase were also assessed.

Procedure: Schematic representation of Experiment 1 is given in Fig. 1, panel A. Starting from the day of single-cage housing, rats ($n = 36$) were exposed to daily training sessions at

the Rota-Rod [accelerating Rota-Rod Treadmill for rats (Ugo Basile, Comerio, VA, Italy)]. A total of 28 daily training sessions were conducted; 11 occurred before the start of Phase 1 (see below), 8 occurred during Phase 1, and 9 occurred during Phase 2 (see below). In each training session, rats were removed from the home cage and exposed to the Rota-Rod for 15 min; training sessions were conducted during the 12th hour of the light phase of the light/dark cycle. Rotation speed was kept constant (4 rpm) for 5 min, accelerated (from 4 to 14 rpm) over the following 5-min period and finally held at 14 rpm for the final 5-min period. The time each rat managed to remain on the revolving drum was recorded. Time recording was initiated at the beginning of the acceleration phase. The 24 rats displaying the best Rota-Rod performance over the initial 11 training sessions were selected for the study; the remaining rats ($n = 12$) were excluded.

At the age of approximately 60 days, the selected rats were exposed to the home-cage, 4-bottle choice regimen between water and 10%, 20%, and 30% (v/v) alcohol with unlimited access (24 h/day) for 12 consecutive days (Phase 1). A 24-h period of alcohol withdrawal, with water as the sole fluid available, was interposed between Phases 1 and 2.

Rats were then exposed to the above 4-bottle choice regimen, with access limited to 1 h/day, for 13 consecutive days (Phase 2). The initial drinking session of Phase 2 ("acclimatization" session) differed from all subsequent sessions as rats were not yet accustomed to the limited-access regimen; it was predicted that their alcohol drinking could be influenced by this novel condition (namely, abrupt and unexpected removal of the alcohol bottles). Therefore, data from this initial drinking session were evaluated separately. The time of access to alcohol was established semi-randomly so that, over 12 consecutive days, all 12 h of the dark phase were tested (sequence used: 4, 9, 1, 8, 5, 12, 3, 7, 10, 2, 11, and 6; as specified above, the "acclimatization" session was not included in data analysis and was replicated on the 8th day of the sequence). Water was always available.

In both Phases 1 and 2, on a daily basis, bottles were refilled with fresh solution and their positions changed randomly to avoid development of position preference. Alcohol and water intake was expressed in g/kg of pure alcohol and mL/kg water, respectively, and monitored by weighing the bottles with a 0.01-g accuracy (a) every day immediately before the start of the dark phase in Phase 1, and (b) immediately before and immediately after each daily drinking session in Phase 2. Possible fluid spillage was calculated by using multiple bottles filled with the different alcohol concentrations and positioned in empty cages interspersed in the cage racks; mean spilled volumes were subtracted before data analysis.

On the day after the last drinking session of Phase 2, the Rota-Rod test was conducted. Specifically, rats were divided into two groups of $n = 12$, matched for alcohol intake over the entire Phase 2, and exposed to an additional drinking session occurring during the 1st or 12th hour of the dark phase ("1st Hour" and "12th Hour" rat groups, respectively). The Rota-Rod test was conducted as previously described by Hoffman and Tabakoff (1984) and consisted of two trials on the Rota-Rod: the first trial ("pre-alcohol" performance) was identical to the training sessions [15-min duration; rotation speed kept constant (4 rpm) for 5 min, accelerated (from 4 to 14 rpm) over 5 min, and finally held at 14 rpm]. Recording of the time each rat managed to remain on the revolving drum was initiated at the beginning of

the acceleration phase. This first trial occurred approximately 1 h before the start of the drinking session. The second trial (“post-alcohol” performance) occurred immediately after the end of the drinking session and lasted 11 min; the drum rotated at 4 rpm for 1 min, then acceleration began (from 4 to 14 rpm, in 5 min); for the last 5 min, the speed was maintained at 14 rpm. Once again, the time spent by each rat on the drum from the beginning of the acceleration phase was recorded.

Immediately after the end of Rota-Rod performance, blood samples (50 μ L) were collected from the tip of the tail of each rat for BAL determination. Blood samples were analyzed by means of an enzymatic system [GL5 Analyzer (Analox Instruments, London, UK)] based on measurement of oxygen consumption in the alcohol-acetaldehyde reaction.

Statistical analysis: Data on alcohol intake over the 12 drinking sessions of Phase 2 were (a) analyzed by 1-way ANOVA with repeated measures and (b) exposed to regression analysis (mean alcohol intake vs. time of the drinking session over the dark phase) and calculation of the Pearson correlation coefficient. Data on (a) alcohol intake on the day devoted to the Rota-Rod test, (b) BALs, and (c) Rota-Rod performance in “1st Hour” and “12th Hour” rat groups were analyzed by the 2-tailed Student *t* or Mann–Whitney test (depending on their parametric distribution) and exposed to regression analysis and calculation of the Pearson correlation coefficient.

Experiment 2 – evaluation of the contribution of (a) multiple alcohol concentrations and (b) unpredictability

Aims: This experiment was designed to address two different research questions: (a) the contribution of the availability of multiple alcohol concentrations to alcohol intake, and (b) the contribution of the unpredictability factor to alcohol intake. The first question was addressed comparing alcohol intake in two rat groups exposed to either 4 (0%, 10%, 20%, and 30% alcohol) or 2 (0% and 10% alcohol) bottles in daily drinking sessions of 1 h with an unpredictable time schedule over the dark phase of the daily light/dark cycle. Notably, the 2-bottle “alcohol (10%) vs. water” choice regimen – although with unlimited access for 24 h/day – is the standard procedure under which sP rats have been selectively bred (see Colombo et al., 2006). The second research question was addressed comparing alcohol intake between one rat group given alcohol in daily drinking sessions of 1 h with an unpredictable time schedule over the dark phase of the daily light/dark cycle and two different rat groups given alcohol regularly at the 1st and 12th hour, respectively, of the dark phase; all these three rat groups had alcohol under the 4-bottle (0%, 10%, 20%, and 30%) choice regimen.

Procedure: Schematic representation of Experiment 2 is given in Fig. 1, panel B. This experiment employed $n = 32$ rats. For 24 rats, Phase 1 was identical to that of Experiment 1 [12 consecutive days with unlimited access to water and 10%, 20%, and 30% (v/v) alcohol]. For the remaining 8 rats, Phase 1 was made up of 12 consecutive days with unlimited access to water and 10% (v/v) alcohol under the 2-bottle choice regimen. In Phase 2, the $n = 24$ rats previously exposed to the 4-bottle choice regimen were divided into three groups of $n = 8$, matched for alcohol intake over Phase 1. One rat group, serving as control (“4-Bottle/

Unpredictable”), was exposed to 13 daily 1-h drinking sessions with access to water and 10%, 20%, and 30% (v/v) alcohol. The other two rat groups were exposed to 13 daily 1-h drinking sessions, with access to water and 10%, 20%, and 30% (v/v) alcohol, taking place constantly at the 1st (“1st-Hour/Fixed”) and 12th (“12th-Hour/Fixed”) hour of the dark phase, respectively. The rat group previously exposed to the 2-bottle choice regimen was exposed to 13 daily 1-h drinking sessions with access to water and 10% (v/v) alcohol (“2-Bottle/Unpredictable”). For both “4-Bottle/Unpredictable” and “2-Bottle/Unpredictable” rat groups, (a) the drinking sessions occurred over the dark phase of the daily light/dark cycle and (b) the time of exposure to alcohol was established semi-randomly. The sequence used was the following: 9, 3, 7, 11, 2, 6, 10, 1, 4, 12, 8, and 5.

Comparable to Experiment 1, the initial drinking session (“acclimatization” session) was not included in data analysis. (In the “4-Bottle/Unpredictable” and “2-Bottle/Unpredictable” rat groups, it was replicated on the 2nd day of the sequence.) Water was always available. Bottles were handled as described above; data on alcohol and water intake were collected as described above.

Statistical analysis: Data on alcohol intake from “1st-Hour/Fixed” and “12th-Hour/Fixed” rat groups over the 12 drinking sessions of Phase 2 were analyzed by 2-way (rat group, day) ANOVA, with repeated measures on the factor day, followed by the LSD test for *post hoc* multiple comparisons. Data from each single rat group over the 12 drinking sessions of Phase 2 were also analyzed separately by 1-way ANOVA with repeated measures.

Data on alcohol intake from “4-Bottle/Unpredictable” and “2-Bottle/Unpredictable” rat groups over the 12 drinking sessions of Phase 2 were (a) analyzed by 2-way (rat group, hour) ANOVA, with repeated measures on the factor day, followed by the LSD test for *post hoc* multiple comparisons, and (b) exposed to independent regression analyses (mean alcohol intake vs. time of the drinking session over the dark phase) and calculation of the Pearson correlation coefficient.

Data on alcohol intake from “1st-Hour/Fixed” and “4-Bottle/Unpredictable” rat groups over the 12 drinking sessions of Phase 2 were compared by 2-way (rat group, access time) ANOVA, with repeated measures on the factor access time, followed by the LSD test for *post hoc* multiple comparisons; the *post hoc* test was limited to the day on which both rat groups concurrently had access to alcohol during the 1st hour of the dark phase (8th day of the sequence).

Data on alcohol intake from “12th-Hour/Fixed” and “4-Bottle/Unpredictable” rat groups over the 12 drinking sessions of Phase 2 were compared by 2-way (rat group, access time) ANOVA, with repeated measures on the factor access time, followed by the LSD test for *post hoc* multiple comparisons; the *post hoc* test was limited to the day on which both rat groups concurrently had access to alcohol during the 12th hour of the dark phase (10th day of the sequence).

Experiment 3 – comparison of alcohol drinking between the dark and light phase

Aims: This experiment was designed to compare alcohol intake in rats unpredictably exposed to the 4-bottle choice regimen in 1-h drinking sessions occurring during the dark or light phase of the daily light/dark cycle.

Procedure: Schematic representation of Experiment 3 is given in Fig. 1, panel C. This experiment employed 24 rats. Phase 1 was identical to that of Experiment 1 [12 consecutive days with unlimited access to water and 10%, 20%, and 30% (v/v) alcohol]. In Phase 2, rats were divided into two groups of $n = 12$, matched for alcohol intake over Phase 1, and exposed to 8 daily drinking sessions of 1 h and access to water and 10%, 20%, and 30% (v/v) alcohol over the dark (“Dark” rat group) or light (“Light” rat group) phase. For both rat groups, time of exposure to alcohol was established semi-randomly; the sequence used was the following: 5, 1, 7, 12, 2, 10, and 3. In the same way as in Experiments 1 and 2, the initial drinking session (“acclimatization” session) was not included in data analysis and was replicated on the 6th day of the sequence. Water was always available.

Bottles were handled as described above; data on alcohol and water intake were collected as described above.

Statistical analysis: Data on alcohol intake from “Dark” and “Light” rat groups over the 7 drinking sessions of Phase 2 were (a) analyzed by 2-way (phase, hour) ANOVA with repeated measures on the factor hour and (b) exposed to separate regression analyses (mean alcohol intake vs. time of the drinking session) and calculation of the Pearson correlation coefficient.

Experiment 4 – extension of schedule sensitivity to chocolate drinking

Aims: This experiment was conducted with the intent of assessing whether sensitivity of alcohol drinking to time schedule, observed in Experiments 1–3, was specific for alcohol or extendable to an alternative, non-drug reinforcer such as a highly palatable chocolate-flavored beverage.

Procedure: Schematic representation of Experiment 4 is given in Fig. 1, panel D. Alcohol-naïve rats were divided into two groups ($n = 12$), matched for body weight, and exposed to the 2-bottle choice regimen between water and either 1% (“1% Chocolate” rat group) or 5% (“5% Chocolate” rat group) (w/v) Nesquik® (Nestlé Italiana, Milan, MI, Italy) in water, with limited access for 1 h/day (the first hour of the dark phase) and 12 consecutive days (Phase 1). Water was always available. A 24-h period of withdrawal from the chocolate-flavored beverage, with water as the sole fluid available, was interposed between Phases 1 and 2.

In Phase 2, rats of both groups were exposed to the 2-bottle choice regimen (same concentrations of the chocolate-flavored beverage as in Phase 1), with limited access for 1 h/day and 10 consecutive days. All drinking sessions occurred during the dark phase of the daily light/dark cycle. In all drinking sessions, time of access to the chocolate-flavored beverage was established semi-randomly [sequence used: 5, 12, 11, 3, 8, 1, 6, 9, and 2; as

for the “alcohol” experiments (see above), the initial drinking session (“acclimatization” session) was not included in data analysis; it was replicated on the 7th day of the sequence]. Water was always available.

Chocolate-flavored beverages were prepared daily. To prevent any deposit of the chocolate powder, bottles were shaken immediately before the start of each drinking session. Intake of the chocolate-flavored beverage and water was expressed in mL/kg and monitored by weighing the bottles with a 0.01-g accuracy immediately before and immediately after each daily drinking session.

Statistical analysis: Data on intake of the chocolate-flavored beverage from “1% chocolate” and “5% chocolate” rat groups over the 9 drinking sessions of Phase 2 were (a) analyzed by 2-way (chocolate concentration, hour) ANOVA with repeated measures on the factor hour and (b) exposed to separate regression analyses (mean intake of the chocolate-flavored beverage vs. time of the drinking session) and calculation of the Pearson correlation coefficient.

Results

Experiment 1 – characterization of alcohol drinking behavior; assessment of BALs and alcohol intoxication

During Phase 1 (unlimited access), all rats rapidly acquired alcohol drinking behavior, as indicated by a mean daily intake of alcohol as high as approximately 4.5 g/kg on the initial day of exposure to the 4-bottle choice regimen; mean daily alcohol intake rose progressively and then stabilized, over the last 5 days, around 6.4 g/kg. Mean daily water intake decreased progressively, compensating for the increase in mean daily alcohol intake (data not shown).

In the “acclimatization” session of Phase 2, rats were exposed to alcohol during the 7th hour of the dark phase and their alcohol intake averaged 1.13 ± 0.07 g/kg. ANOVA revealed highly significant differences in alcohol intake during the subsequent 12 drinking sessions of Phase 2 [$F(1,11) = 29.65, p < 0.0001$]. Specifically, mean alcohol intake varied largely, from a minimum value of 0.69 ± 0.05 g/kg, when the drinking session occurred during the 1st hour of the dark phase, to a maximum value of 1.96 ± 0.07 g/kg, when the drinking session occurred during the 12th hour of the dark phase. The association between mean alcohol intake and time of access approached 1.00 ($r = 0.984$, slope = 0.107, intercept = 0.665, $p < 0.0001$, $n = 24$) (Fig. 2). In terms of intake from the three different alcohol bottles, 10% concentration was found to be the preferred concentration (approximately 50% of total alcohol intake over entire Phase 2), followed by 20% (approximately 30% of alcohol intake), and 30% (approximately 20% of alcohol intake) with modest differences among the 12 h of the dark phase (data not shown). Mean water intake was negligible (<0.5 mL/kg) in each drinking session.

The Rota-Rod experiment was conducted the day after completion of Phase 2 and employed two rat groups matched for equal alcohol intake and schedule sensitivity over the entire Phase 2 (“1st Hour”: $r = 0.983$, slope = 0.105, intercept = 0.692, $p < 0.0001$, $n = 12$; “12th Hour”: $r = 0.970$, slope = 0.109, intercept = 0.649, $p < 0.0001$, $n = 12$). Rats accessing

alcohol at the 12th hour of the dark phase consumed a mean alcohol intake (2.00 ± 0.07 g/kg) that was approximately 2.3 times higher than that consumed by rats accessing alcohol in the 1st hour of the dark phase (0.86 ± 0.06 g/kg) ($p < 0.0001$, Student *t* test) (Fig. 3, panel A). Alcohol intake in the “12th Hour” rat group was associated with marked levels of motor incoordination, as indicated by an approximately 60% impairment at the Rota-Rod task; conversely, motor coordination ability was minimally affected by alcohol drinking in the “1st Hour” rat group, as indicated by an approximately 6% impairment at the Rota-Rod task ($p < 0.005$, Mann–Whitney test) (Fig. 3, panel C). Large differences were also recorded in BALs (measured immediately after completion of the Rota-Rod task), as they averaged 101.1 ± 8.1 and 26.9 ± 6.8 mg% in the “12th Hour” and “1st Hour” rat groups, respectively ($p < 0.0001$, Mann–Whitney test) (Fig. 3, panel B).

Regression analyses, conducted combining the data from both rat groups, indicated that BALs were positively correlated to alcohol intake ($r = 0.907$, slope = 0.012, intercept = 0.641, $p < 0.0001$, $n = 24$) (Fig. 3, panel D); impairment at the Rota-Rod task was positively correlated to alcohol intake ($r = 0.753$, slope = 0.013, intercept = 1.015, $p < 0.0001$, $n = 24$) (Fig. 3, panel E) and BALs ($r = 0.730$, slope = 0.920, intercept = 34.335, $p < 0.0001$, $n = 24$) (Fig. 3, panel F).

Experiment 2 – evaluation of the contribution of (a) multiple alcohol concentrations and (b) unpredictability

In Phase 1, data from all rats exposed to the 4-bottle choice regimen were highly similar to those collected in Experiment 1. Specifically, all rats rapidly acquired alcohol-drinking behavior, as indicated by a mean daily alcohol intake as high as 5 g/kg already on the first day and then progressing to approximately 6.5 g/kg. In the rat group exposed to the 2-bottle choice regimen, mean daily alcohol intake was highly similar to that recorded in the rat group exposed to the 4-bottle choice regimen, starting from approximately 5 g/kg and increasing to approximately 6.7 g/kg. In both rat groups, mean daily water intake decreased progressively, compensating for the increase in mean daily alcohol intake (data not shown).

In the “acclimatization” session of Phase 2, rats of the “4-Bottle/Unpredictable” and “2-Bottle/Unpredictable” groups were exposed to alcohol during the 3rd hour of the dark phase and their alcohol intake averaged 1.07 ± 0.13 and 0.94 ± 0.12 g/kg, respectively; alcohol intake in the “1st-Hour/Fixed” and “12th-Hour/Fixed” rat groups averaged 1.01 ± 0.09 and 1.84 ± 0.20 g/kg, respectively.

Comparison between the rat groups with fixed time of access to alcohol

2-way ANOVA revealed a significant effect of rat group [$F(1,14) = 12.46$, $p < 0.005$], but not of day [$F(11,154) = 1.08$, $p > 0.05$], and a significant interaction [$F(11,154) = 2.04$, $p < 0.05$] on alcohol intake over Phase 2. Mean alcohol intake in the “1st-Hour/Fixed” rat group tended to increase on continuing exposure, from a minimum value of 0.78 ± 0.14 g/kg on Day 1 to a maximum value of 1.22 ± 0.12 g/kg on Day 11 [1-way ANOVA: $F(11,77) = 1.94$, $p < 0.05$] (Fig. 4, panel A). Mean alcohol intake in the rat group constantly exposed to drinking sessions during the 12th hour of the dark phase (“12th-Hour/Fixed”) was relatively steady over the 12-day period, with a minimum value of 1.14 ± 0.23 g/kg on Day 7 and a

maximum value of 1.68 ± 0.25 g/kg on Day 1 [1-way ANOVA: $F(11,77) = 1.40$, $p > 0.05$] (Fig. 4, panel A); however, it was significantly higher ($p < 0.05$, LSD test), than that recorded in the “1st-Hour/Fixed” rat group on Days 1–6, 8–10, and 12. Mean water intake was negligible (<1.0 mL/kg in both rat groups) in each drinking session (data not shown).

Comparison between the rat groups with 2- or 4-bottle choice

2-way ANOVA revealed a relevant statistical trend of rat group [$F(1,14) = 4.26$, $p = 0.058$], a highly significant effect of hour [$F(11,154) = 15.32$, $p < 0.0001$], and no significant interaction [$F(11,154) = 0.74$, $p > 0.05$] on alcohol intake over Phase 2. In both “4-Bottle/Unpredictable” and “2-Bottle/Unpredictable” rat groups, mean alcohol intake was highly positively correlated with time of access to alcohol (“4-Bottle/Unpredictable” rat group: $r = 0.970$, slope = 0.098, intercept = 0.784, $p < 0.0001$, $n = 8$; “2-Bottle/Unpredictable” rat group: $r = 0.925$, slope = 0.066, intercept = 0.800, $p < 0.0001$, $n = 8$). In the “4-Bottle/Unpredictable” rat group, alcohol intake varied from a minimum value of 0.83 ± 0.12 g/kg, when the drinking session occurred during the 1st hour of the dark phase, to a maximum value of 2.03 ± 0.10 g/kg, when the drinking session occurred during the 12th hour of the dark phase (Fig. 4, panel B). In the “2-Bottle/Unpredictable” rat group, alcohol intake varied from a minimum value of 0.87 ± 0.09 g/kg, when the drinking session occurred during the 1st hour of the dark phase, to a maximum value of 1.66 ± 0.08 g/kg, when the drinking session occurred during the 11th hour of the dark phase (Fig. 4, panel B). Mean alcohol intake was relatively similar in the two rat groups when the drinking session occurred over the first hours of the dark phase, while it tended to differ, with higher intakes in the “4-Bottle/Unpredictable” rat group, when the drinking session occurred over the last hours of the dark phase (Fig. 4, panel B); accordingly, differences at the *post hoc* analysis were observed when the drinking session occurred at the 9th and 12th hour ($p < 0.05$, LSD test) and a tendency toward differences was observed when the drinking session occurred at the 10th and 11th hour. The largest difference was recorded when the drinking session occurred during the 12th hour. In that specific drinking session, alcohol intake in the “4-Bottle/Unpredictable” rat group was approximately 30% higher than in the “2-Bottle/Unpredictable” rat group (Fig. 4, panel B). Mean water intake was negligible (<0.8 and 1.1 mL/kg in the “4-Bottle/Unpredictable” and “2-Bottle/Unpredictable” rat groups, respectively) in each drinking session (data not shown).

Comparison between the rat groups with fixed or unpredictable access to alcohol

“1st-Hour/Fixed” vs. “4-Bottle/Unpredictable”—2-way ANOVA revealed a significant effect of rat group [$F(1,14) = 17.34$, $p < 0.001$] and access time [$F(11,154) = 6.81$, $p < 0.0001$], as well as a significant interaction [$F(11,154) = 8.20$, $p < 0.0001$] on alcohol intake over Phase 2. *Post hoc* analysis comparing alcohol intake in the “4-Bottle/Unpredictable” rat group when the drinking session occurred during the 1st hour (0.83 ± 0.12 g/kg) with alcohol intake in the “1st-Hour/Fixed” rat group recorded on the same day (8th day of the sequence) (0.88 ± 0.12 g/kg) revealed no difference ($p > 0.05$, LSD test) (see arrows in Fig. 4, panel C).

“12th-Hour/Fixed” vs. “4-Bottle/Unpredictable”—2-way ANOVA revealed a significant effect of access time [$F(11,154) = 6.19$, $p < 0.0001$] but not of rat group [$F(1,14)$

= 0.30, $p > 0.05$], and a significant interaction [$F(11,154) = 3.93$, $p < 0.0001$] on alcohol intake over Phase 2. Alcohol intake in the “4-Bottle/Unpredictable” rat group when the drinking session occurred during the 12th hour (2.03 ± 0.10 g/kg) was significantly higher ($p < 0.05$, LSD test), by approximately 40%, than that in the “12th-Hour/Fixed” rat group recorded on the same day (10th day of the sequence) (1.45 ± 0.21 g/kg) (see arrows in Fig. 4, panel D).

Experiment 3 – comparison of alcohol drinking between the dark and light phase

Data from Phase 1 were highly similar to those collected in Experiments 1 and 2. Specifically, all rats rapidly acquired alcohol-drinking behavior, as indicated by a mean daily alcohol intake exceeding 4.0 g/kg already on the first day and then progressing to approximately 6.5 g/kg. Mean daily water intake decreased progressively, compensating for the increase in mean daily alcohol intake (data not shown).

In the “acclimatization” session of Phase 2, “Dark” and “Light” rats were exposed to alcohol during the 10th hour of the dark and light phase, respectively; their alcohol intake averaged 1.85 ± 0.12 and 0.75 ± 0.07 g/kg, respectively. ANOVA revealed a highly significant effect of phase [$F(1,22) = 17.37$, $p < 0.0005$] and hour [$F(6,132) = 10.37$, $p < 0.0001$], as well as a significant interaction [$F(6,132) = 57.73$, $p < 0.0001$], on alcohol intake during the subsequent 7 drinking sessions. Specifically, mean alcohol intake in the “Dark” rat group varied largely, from a minimum value of 0.65 ± 0.07 g/kg, when the drinking session occurred during the 1st hour, to a maximum value of 2.17 ± 0.11 g/kg, when the drinking session occurred during the 12th hour; mean alcohol intake was found to be highly positively correlated ($r = 0.959$, slope = 0.134, intercept = 0.649, $p < 0.001$, $n = 12$) with time of access to alcohol (Fig. 5). In the “Light” rat group, mean alcohol intake was relatively high (1.58 ± 0.12 g/kg) when the drinking session occurred during the 1st hour, and decreased progressively when the drinking session occurred during the 2nd and 3rd hour, remaining relatively stable (0.70–0.85 g/kg) over the subsequent times of access; regression analysis revealed a mild negative correlation between mean alcohol intake and time of access to alcohol ($r = -0.778$, slope = -0.057 , intercept = 1.317, $p < 0.05$, $n = 12$) (Fig. 5). Mean water intake was negligible (< 0.2 mL/kg in both rat groups) in each drinking session (data not shown).

Experiment 4 – extension of schedule sensitivity to chocolate drinking

In Phase 1, temporally fixed availability of the chocolate-flavored beverage during the 1st hour of the dark phase resulted in an intense and relatively stable consummatory behavior in all rats, with intakes averaging approximately 15 and 60 mL/kg per session in the rat groups given 1% (“1% Chocolate”) and 5% (“5% Chocolate”) chocolate powder, respectively. Mean water intake was negligible (< 1 mL/kg in both rat groups) in each drinking session (data not shown).

In the “acclimatization” session of Phase 2, rats were exposed to the chocolate-flavored beverage during the 6th hour of the dark phase; intake of the chocolate-flavored beverage averaged 18.38 ± 3.01 and 85.33 ± 4.42 mL/kg in the “1% Chocolate” and “5% Chocolate” rat groups, respectively. ANOVA revealed a highly significant effect of chocolate

concentration [$F(1,22) = 192.65, p < 0.0001$] and hour [$F(8,176) = 6.93, p < 0.0001$], but not of significant interaction [$F(8,176) = 1.30, p > 0.05$] on intake of the chocolate-flavored beverage over the subsequent 9 drinking sessions; mean intake of the chocolate-flavored beverage was more than 2 times higher in the “5% Chocolate” than “1% Chocolate” rat group (Fig. 6). In both rat groups, intake of the chocolate-flavored beverage resulted in relatively flat curves over time (“1% Chocolate”: $r = 0.317$, slope = 0.749, intercept = 29.593, $p > 0.05$, $n = 12$; “5% Chocolate”: $r = 0.589$, slope = 1.467, intercept = 77.613, $p > 0.05$, $n = 12$) (Fig. 6).

Discussion

Overall, the results of the present study indicate that exposure of selectively bred, alcohol-preferring sP rats to concurrent limited access (1 h/day) to multiple alcohol concentrations (0%, 10%, 20%, and 30%, v/v), with an unpredictable time schedule and over the dark phase of the daily light/dark cycle, resulted in exceptionally high and intoxicating intakes of alcohol. As seen in each “alcohol” experiment (Experiments 1–3), alcohol drinking in sP rats was highly sensitive to the time of alcohol access: alcohol intake increased progressively as the drinking session moved from the earliest hours to the latest hours of the dark phase. When alcohol was made available during one of the latest hours of the dark phase (11th and 12th hour), alcohol intake was more than 2 times higher than that recorded over the first hours (1st and 2nd hour) of the dark phase, with intermediate – yet progressively increasing – values over the in-between hours. In each experiment, the positive correlation between alcohol intake and time of alcohol exposure over the dark phase was remarkably high and statistically significant.

Alcohol intakes as high as those recorded when the drinking session occurred during the latest hours of the dark phase (averaging approximately 2 g/kg) (a) are 3–4 times higher than those recorded at each hourly drinking episode in sP rats given unlimited access to alcohol (Agabio et al., 1996) and (b) had rarely been observed in sP rats exposed to drinking sessions of limited duration. Alcohol drinking at the 12th hour of the dark phase resulted in BALs that apparently meet the criterion posed for binge drinking in humans [BALs higher than 80 mg% after an episode of heavy use of alcohol (NIAAA National Advisory Council, 2004)], underlining the relevance of these results in depicting a new experimental model of binge drinking. Additionally, alcohol intake during the latest hours of the dark phase resulted in clear signs of intoxication, as demonstrated by the severe impairment displayed by rats when exposed to the Rota-Rod task immediately after completion of the 12th-hour drinking session. With the sole exception of a recent study in which sP rats were exposed to intermittent (once every other day) access to the 2-bottle choice regimen with water and 20% (v/v) alcohol (Loi et al., 2010), alcohol drinking up to intoxicating levels had never previously been observed in sP rats.

Visual observation of rat behavior suggested that the majority of alcohol drinking occurred over the first 10–15 min of the drinking session. Unfortunately, the lack of automated equipment for detailed recording of alcohol intake hindered evaluation of the drinking pattern over the session (the frequent removal and weighing of the four bottles was inadvisable, as it would have likely disrupted the rat behavior). However, the observation

that most of the alcohol drinking occurred over the first part of the session is in agreement with multiple observations on the pattern of alcohol taking in sP rats exposed to brief (30–60 min) sessions of operant, oral alcohol self-administration (e.g., Maccioni et al., 2009); additionally, these observations suggest that BALs and the degree of alcohol intoxication might have been even higher if recordings had been undertaken at earlier time points during the drinking session.

Alcohol intake and BALs recorded when the drinking session occurred during the 12th hour of the dark phase were comparable to those observed in alcohol-preferring P rats exposed to another model of binge drinking, comprising 2–3 daily, 1-hour drinking sessions, interspersed at fixed hours over the dark phase of the daily light/dark cycle, and with concurrent access to 0%, 15%, and 30% (v/v) alcohol (named “Drinking-in-the-Dark *plus* Multiple Schedule Access”) (Bell et al., 2006). Additionally, the drinking outcomes recorded in the present study when the drinking session took place during the latest hours of the dark phase meet most of the criteria recently proposed to define excessive alcohol drinking in rodents (Bell et al., 2006); accordingly, alcohol intake (a) occurred under a free-choice condition (water was always available as an alternative fluid), (b) resulted in BALs in the range of 100 mg% or higher, and (c) produced signs of intoxication. Further experiments will be set up to test two additional criteria: development of tolerance to alcohol-induced effects and development of dependence upon alcohol.

Experiment 2 was aimed at assessing the contribution of each singular aspect of the present experimental design (i.e., unpredictability of alcohol exposure and concurrent availability of multiple alcohol concentrations) to the high levels of alcohol intake recorded. In order to run all proper comparisons, Experiment 2 included four different rat groups exposed to: (a) 4 bottles (0%, 10%, 20%, and 30% alcohol) in daily drinking sessions of 1 h occurring regularly at the 1st hour of the dark phase; (b) 4 bottles in daily drinking sessions of 1 h occurring regularly at the 12th hour of the dark phase; (c) 4 bottles in daily drinking sessions of 1 h with an unpredictable time schedule and during the dark phase; (d) 2 bottles (0% and 10% alcohol) in daily drinking sessions of 1 h with an unpredictable time schedule and during the dark phase.

Firstly, comparison between the two “fixed” rat groups, depicted in Fig. 4, panel A, indicates that availability of alcohol at the end of the dark phase (“12th-Hour/Fixed” rat group) resulted in higher alcohol intake than when alcohol was made available at the beginning of the dark phase (“1st-Hour/Fixed” rat group). These differences tended to be greater at the beginning of the 12-day period, subsequently declining toward the end, as the likely consequence of an adaptation process of alcohol drinking to the schedule of access. Irrespective of this progressive decrement in the difference in alcohol intake between the two rat groups, the higher intakes recorded in the “12th-Hour/Fixed” rat group suggest that exposure to alcohol over the final portion of the dark phase was capable of increasing alcohol intake regardless of predictability/unpredictability of the time of alcohol access.

Comparison between the “4-Bottle/Unpredictable” and “2-Bottle/Unpredictable” rat groups, depicted in Fig. 4, panel B, demonstrated that schedule sensitivity of alcohol drinking was not limited to the regimen with multiple alcohol concentrations, but also developed in the rat

group exposed to the single alcohol concentration. However, the multiple-alcohol-concentration condition appeared to be more sensitive to time schedule: alcohol intake was virtually identical or minimally different when the drinking session occurred during the first hours of the dark phase, while it tended to be higher – in the “4-Bottle/Unpredictable” than “2-Bottle/Unpredictable” rat groups – when the drinking session occurred over the last hours of the dark phase. Specifically, (a) the two regression lines intersected at the 1st-hour time point and then diverged progressively, and (b) when the drinking session occurred during the 12th hour, alcohol intake in the “4-Bottle/Unpredictable” rat group was approximately 30% higher than in the “2-Bottle/Unpredictable” rat group (2.03 ± 0.10 and 1.59 ± 0.07 g/kg, respectively). These data suggest that, when demand for alcohol was at its height (i.e., latest hours of the dark phase), sP rats apparently took advantage of the availability of multiple alcohol concentrations to increase their alcohol consumption.

Comparison between the “4-Bottle/Unpredictable” and “1st-Hour/Fixed” rat groups provided clues regarding the effect of unpredictability on alcohol intake at the beginning of the dark phase. We considered that the most appropriate comparison was that conducted on the day when both rat groups were concurrently exposed to alcohol during the 1st hour of the dark phase (this concurrence occurred on the 8th day of the sequence; see arrows in Fig. 4, panel C). On that specific day, alcohol intake was virtually identical between the two rat groups (0.83 ± 0.12 and 0.88 ± 0.12 g/kg in “4-Bottle/Unpredictable” and “1st-Hour/Fixed” rat groups, respectively), suggesting that contribution of unpredictability was absent when the drinking session occurred over the initial part of the dark phase. Conversely, comparison between the “4-Bottle/Unpredictable” and “12th-Hour/Fixed” rat groups provided clues on the effect of unpredictability on alcohol intake at the end of the dark phase. The most appropriate comparison was that performed on the 10th day of the sequence, when both rat groups had access to alcohol during the 12th hour of the dark phase (see arrows in Fig. 4, panel D). On that specific day, alcohol intake was approximately 40% higher in “4-Bottle/Unpredictable” (2.03 ± 0.10 g/kg) than “12th-Hour/Fixed” (1.45 ± 0.21 g/kg) rat groups. All together, these results suggest that unpredictability of time of alcohol access had an influence on alcohol drinking; however, this influence was only evident once several hours of the nocturnal period had elapsed.

The positive correlation between alcohol intake and the time of alcohol access appears to be a property limited to the nocturnal period (i.e., the time period of maximal activity in rats). Indeed, when drinking sessions occurred over the light phase of the daily light/dark cycle (Experiment 3), the drinking behavior of sP rats changed remarkably: alcohol intake (a) was relatively high (1.58 ± 0.12 g/kg) when the drinking session occurred during the 1st hour, (b) decreased rapidly and progressively over the subsequent 2 h (0.98 ± 0.11 g/kg when the drinking session occurred during the 3rd hour), and (c) was relatively low and stable in each drinking session occurring during later hours. Overall, a slightly negative correlation between alcohol intake and time of exposure to alcohol was observed over the light phase. Together, these results depict a circadian rhythm of alcohol demand and intake, as they suggest that alcohol drinking – after having increased progressively over the dark phase – took some time (the first 2–3 h of the light phase) to return to levels that were then maintained throughout the majority of the light phase and the initial part of the dark phase.

The “chocolate” experiment (Experiment 4) was conducted to assess the specificity of alcohol intake to the effect of time schedule of availability. To this end, an independent set of alcohol-naïve sP rats was exposed to a highly palatable, chocolate-flavored beverage. With the intent of closely following the experimental design of “alcohol” experiments (Experiments 1–3), access to the chocolate-flavored beverage was limited to daily drinking sessions lasting 1 h and occurring over the dark phase of the daily light/dark cycle with an unpredictable time schedule. This experiment employed two independent groups of rats, exposed to two different concentrations of the chocolate powder (1% and 5%) (and therefore to different levels of palatability and intake of the chocolate-flavored beverages). These different concentrations were used to rule out possible “ceiling” intakes of the chocolate-flavored beverage that would have masked the effect of schedule. As expected (e.g., Maccioni, Pes, Carai, Gessa, & Colombo, 2008), intake of the chocolate-flavored beverage was higher in the rat group given the 5%-powder concentration. However, in both rat groups, intake of the chocolate-flavored beverage was completely insensitive to time of the drinking session, suggesting the specificity of alcohol intake to the schedule effect.

Additional studies will be undertaken to investigate the neurobiological correlate(s) underlying the sensitivity of alcohol drinking of sP rats to the time schedule of alcohol access. However, at present, several hypotheses may be put forward. Rats of the sP line have repeatedly been found to display a unique “emotional” profile comprising (a) high levels of anxiety-related behaviors at the elevated plus maze (Colombo et al., 1995; Leggio et al., 2003; Richter, Zorilla, Basso, Koob, & Weiss, 2000; Roman & Colombo, 2009; Roman et al., 2012) and elevated zero maze (Cagiano et al., 2002), (b) low levels of spontaneous locomotor activity and high levels of thigmotaxis when exposed to an open-field arena (Agabio et al., 2001) and to the multivariate concentric square field (Roman & Colombo, 2009; Roman et al., 2012), and (c) low exploratory drive, high risk assessment, and low risk-taking behavior in the multivariate concentric square field (Roman & Colombo, 2009; Roman et al., 2012). Additionally, voluntary alcohol drinking markedly reduced the anxiety-related behaviors of sP rats exposed to the elevated plus maze (Colombo et al., 1995) and Social Interaction test (Lobina, Gessa, & Colombo, 2013), suggesting that anxiolysis is likely one of the alcohol effects that drive sP rats to alcohol drinking. Therefore, it may be hypothesized that the unpredictable schedule of alcohol access used in the present study, together with the expectation of alcohol availability over the nocturnal period, may have generated a progressively increasing – as time elapsed – “emotional distress,” leading sP rats to progressively increase (up to intoxicating levels) their intakes of alcohol. Accordingly, additional experiments have been planned to assess possible changes in blood levels of the stress-related hormones, corticosterone and adreno-corticotrop hormone, with the intent of providing an initial outlook on the neuroendocrine profile of this drinking response as well as of the likely stressful, negative affective state associated with expectation of alcohol availability. Further experiments should also investigate (a) possible alterations in the brain levels of the neurotransmitters GABA, glutamate, and dopamine, whose extracellular concentrations have been found to display a circadian rhythm (see Albrecht, 2011), and (b) genes making up the molecular clock that regulate sensitivity to drugs of abuse and to their rewarding properties (see McClung, 2007).

In summary, the results of the present study provide evidence for an alcohol drinking behavior in alcohol-preferring sP rats that meets the criteria to warrant classification as an experimental model of binge drinking. Singular features of this procedure are (a) daily, brief drinking sessions, (b) concurrent availability to multiple alcohol concentrations, and (c) unpredictability of the time schedule of alcohol access. When the drinking session occurred over the last part of the dark phase of the daily light/dark cycle, alcohol intake peaked up to approximately 2 g/kg in 1 h, resulted in BALs averaging approximately 100 mg%, and produced severe signs of intoxication.

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**A Experiment 1 - (a) Characterization of alcohol drinking behavior
(b) Assessment of BALs and alcohol intoxication**

**B Experiment 2 - Evaluation of the contribution of:
(a) multiple alcohol concentrations
(b) unpredictability**

C Experiment 3 - Comparison of alcohol drinking between the dark and light phase

D Experiment 4 - Extension of schedule sensitivity to chocolate drinking

Fig. 1. Schematic representation of the experimental design of the four experiments conducted in the present study.

Fig. 2.

Alcohol intake in Sardinian alcohol-preferring (sP) rats exposed to 12 consecutive daily 1-h drinking sessions under the 4-bottle “alcohol (10%, 20%, and 30%, v/v) vs. water” choice regimen. Drinking sessions occurred during one of the 12 h of the dark phase of the daily light/dark cycle; time of the drinking session was changed daily in a semi-random order and was unpredictable to rats. Alcohol intake is expressed in grams of pure alcohol per kg body weight. Each point is the mean $\pm$ SEM of $n = 24$ rats.

Fig. 3.

Alcohol intake (panel A), blood alcohol levels (BALs; panel B), and impairment at the Rota-Rod task (panel C) in Sardinian alcohol-preferring (sP) rats exposed to a 1-h drinking session under the 4-bottle “alcohol (10%, 20%, and 30%, v/v) vs. water” choice regimen occurring during the 1st (“1st-Hour”) or 12th (“12th-Hour”) hour of the dark phase of the daily light/dark cycle; time of the drinking session was unpredictable to rats. Alcohol intake is expressed in grams of pure alcohol per kg body weight. The Rota-Rod task was performed immediately after the end of the 1-h drinking session; data are expressed as % impairment in comparison to a pre-test session (see text for details). BALs were measured immediately after completion of the Rota-Rod task and are expressed in mg%. In panels A–C, each bar is the mean $\pm$ SEM of $n = 12$ rats. [#] $p < 0.0001$ with respect to “1st-Hour” rat group (Student t test); ^{*} $p < 0.005$ and ^{**} $p < 0.0001$ with respect to “1st-Hour” rat group (Mann–Whitney test). Panels D–F illustrate the correlation between these three variables; in these panels, each point is the value collected in each single rat of the two groups.

Fig. 4.

Alcohol intake in Sardinian alcohol-preferring (sP) rats exposed to 12 consecutive daily 1-h drinking sessions under four different experimental conditions: (a) 4-bottle “alcohol (10%, 20%, and 30%, v/v) vs. water” choice regimen with drinking sessions occurring always at the 1st hour of the dark phase of the daily light/dark cycle (“1st-Hour/Fixed”) (panels A and C); (b) 4-bottle “alcohol (10%, 20%, and 30%, v/v) vs. water” choice regimen with drinking sessions occurring always at the 12th hour of the dark phase (“12th-hour/Fixed”) (panels A and D); (c) 4-bottle “alcohol (10%, 20%, and 30%, v/v) vs. water” choice regimen with drinking session occurring during one of the 12 h of the dark phase, changed daily in a semi-random order and unpredictable to rats (“4-Bottle/Unpredictable”) (panels B–D); (d) 2-bottle “alcohol (10%, v/v) vs. water” choice regimen with drinking session occurring during one of the 12 h of the dark phase, changed daily in a semi-random order and unpredictable to rats (“2-Bottle/Unpredictable”) (panel B). Alcohol intake is expressed in grams of pure alcohol per kg body weight. Arrows indicate values collected on the day when both rat groups were concurrently exposed to alcohol during the 1st (panel C) and 12th (panel D) hour of the dark phase. Each point is the mean $\pm$ SEM of $n = 8$ rats.

Fig. 5.

Alcohol intake in Sardinian alcohol-preferring (sP) rats exposed to 7 consecutive daily 1-h drinking sessions under the 4-bottle “alcohol (10%, 20%, and 30%, v/v) vs. water” choice regimen. Drinking sessions occurred during one of the 12 h of the dark (“Dark”) or light (“Light”) phase of the daily light/dark cycle; time of the drinking session was changed daily in a semi-random order and was unpredictable to rats. Alcohol intake is expressed in grams of pure alcohol per kg body weight. Each point is the mean $\pm$ SEM of $n = 12$ rats.

Fig. 6.

Intake of a chocolate-flavored beverage in Sardinian alcohol-preferring (sP) rats exposed to 9 consecutive daily 1-h drinking sessions under the 2-bottle choice regimen with water and 1% chocolate powder in water ("1% Chocolate"), or water and 5% chocolate powder in water ("5% Chocolate"). Drinking sessions occurred during one of the 12 h of the dark phase of the daily light/dark cycle; time of the drinking session was changed daily in a semi-random order and was unpredictable to rats. Intake of the chocolate-flavored beverage is expressed in mL fluid per kg body weight. Each point is the mean $\pm$ SEM of $n = 12$ rats.