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Research report

Restraint stress enhances alcohol intake in adolescent female rats but reduces alcohol intake in adolescent male and adult female rats

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ABSTRACT

Adolescents may be more sensitive to stress-induced alcohol drinking than adults, which would explain the higher prevalence of alcohol abuse and dependence in late adolescence than in adulthood. The present study analyzed the impact of restraint stress on the initiation of alcohol intake across 2 weeks of intermittent, two-bottle choice intake in male and female adolescent rats and adult female rats. Restraint stress significantly increased alcohol intake and preference in female adolescent rats but decreased alcohol intake and preference in male adolescent and female adult rats. The effects of restraint stress on alcohol intake were mitigated in adolescent females following administration of the κ opioid receptor antagonist norbinaltorphimine. Adolescent but not adult female rats that were subjected to restraint stress spent more time on the open arms of the elevated plus maze. Female adolescents exposed to stress also exhibited greater risk-taking behaviors in a concentric square field test compared with non-stressed controls. These results indicate age- and sex-related differences in the sensitivity to alcohol-stress interactions that may facilitate the initiation of alcohol use in female adolescents. The facilitatory effect of stress on alcohol intake was related to greater exploratory and risk-taking behaviors in young females after stress exposure.

1. Introduction

The age at which alcohol is first consumed is a drinking milestone that distinguishes individuals who will subsequently display controlled drinking from those who will exhibit a greater prevalence of alcohol use [1] and alcohol use disorders (AUDs; [2]). Early alcohol exposure may induce changes in brain systems that are responsible for processing stressful stimuli [3]. Animal research has revealed greater stress-induced alcohol-drinking in male [4] and female [5] rats that were exposed to alcohol during adolescence compared with rats that were exposed to alcohol in adulthood. Conversely, early drinking may be facilitated by a history of stress exposure. In an epidemiological study [6], alcohol ingestion in adulthood was significantly greater in individuals who had both an early onset of alcohol drinking and a history of stressful life events. This interaction was expanded on by Buchmann et al. [7], who found that early alcohol initiation promoted subsequent drinking in an attempt to reduce or control negative emotional states. These studies suggest that subjects who begin drinking at an early age learn to manage negative mood states associated with adolescence through the anxiolytic effects of alcohol.

The aforementioned studies indicate the relevance of analyzing facilitatory effects of stress on the first drinking experiences during adolescence and potential treatments to ameliorate stress-induced drinking. The main aim of the present study was to evaluate whether restraint stress during adolescence promotes the initiation of alcohol intake during adolescence and whether these effects are different in females and males. According to official guidelines [8], we included both males and female animals and focused on alcohol drinking in females, which has been traditionally neglected in basic research [9].

In Experiment 1, restraint stress reliably facilitated the initiation of alcohol intake in female but not male adolescent rats. In Experiment 2, we replicated the facilitatory effect of stress on the initiation of alcohol intake in female adolescent rats and sought to inhibit such stress-induced alcohol intake by administering the κ opioid receptor (KOR) antagonist norbinaltorphimine (nor-BNI) prior to restraint stress exposure. There is considerable interest in the use of KOR antagonists to

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alter alcohol intake. Norbinaltorphimine reduces the stress-induced enhancement of alcohol's rewarding effects [10] and generally blocks the effects of acute stressors [11].

In Experiment 3 we assessed behavioral effects of restraint stress that likely underlie its ability to promote alcohol intake in female adolescent rats. That experiment evaluated the potential mechanisms that may have led to greater alcohol intake in adolescent female rats in Experiments 1 and 2. Specifically, Experiment 3 assessed anxiety-like behavior in the elevated plus maze (EPM) and basal and alcohol-induced exploration in a modified version of the concentric square field (CSF) test. The EPM provides measures of the avoidance of open and bright spaces [12], whereas the CSF [13] combines the layout of the light-dark box test and the open field test, and adds the possibility of exploring an elevated and brightly illuminated sector, and a sector which is only accessible via jumping. Therefore, the CSF provides measures of risk taking and shelter seeking. Developmental studies should strive to determine whether the effects of a given treatment persist when applied to older subjects. This was achieved by testing stress-induced alcohol drinking and behavior in the EPM in adult female rats in Experiments 4a and b.

2. Methods

2.1. Subjects and housing conditions

One-hundred and fifty-one outbred Wistar rats were used. Specifically, Experiment 1 employed 14 adolescent males and 14 females, whereas Experiments 2–3 employed 30 and 45 females, respectively. Experiments 4a and 4b employed 24 adult female rats each. In each experiment, the animals were naive to any experimental manipulation. The animals were obtained from the vivarium of Instituto de investigaciones Médicas M. y M. Ferreyra (INIMEC-CONICET-UNC), a producer of specific pathogen-free animals.

The vivarium, the maintenance rooms and the rooms used for behavioral testing or for the assessment of voluntary alcohol intake were maintained under a 12 h/12 h light/dark cycle (lights on at 8:00 AM), at 22–24 °C and 45–55% humidity. Corn cob was used as bedding. The procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 2011), as adopted and promulgated by the NIH and the EU, and were certified by the Institutional Animal Care and Use Committee at INIMEC-CONICET-UNC. The experiments also complied with the Declaration of Helsinki. Across experiments, only one rat per litter was assigned to a given group. This was done to avoid litter effects.

The pregnant rats came from the regular stock of the vivarium. To provide subjects for the study, they were mated with a single male. Each couple was housed in standard maternity cages (60 cm length × 40 cm width × 25 cm height) with *ad libitum* access to water and food. Births were checked each day at 1000. Parturition day was considered PD0. The offspring were weaned on PD21 when they were transferred to the animal maintenance room of our laboratory. They were kept in standard maternity cages in same-sex groups of four each. Following recommendations from our institutional animal care committee the rats were pair-housed (cage size: 46 cm length × 30 cm width × 20 cm height, subsequent described as "maintenance cages") in same-sex couples on PD35. This considers the relationship between size of the cage and weight of the animal. Alcohol intake tests were conducted individually, in half of a maintenance cage, as described in Section 2.3.2.

2.2. Experimental designs

Experiment 1 measured alcohol intake after termination of a phase of chronic restraint stress in adolescent rats using a 2 (Sex) × 2 (Stress: 120 min of restraint per day for 5 days or non-stressed) factorial design. Each of the four groups was composed by 7 subjects.

Experiment 2 assessed the effects of nor-BNI on restraint stress-induced alcohol drinking in female rats using a 2 (nor-BNI treatment before stress exposure: 0.0 [vehicle control] or 10 mg/kg) × 2 (Stress: 120 min of restraint stress per day for 5 days or non-stressed) factorial design. Each group was composed of eight rats, with the exception of the basal control group (i.e., vehicle-treated non-stressed group; $n = 6$).

Experiment 3 assessed, in a different set of female adolescent rats, behavior in the EPM and basal and alcohol-induced exploration of the CSF, using the same factorial design as in Experiment 2. Each group was composed of eleven rats, with the exception of the basal control group (i.e., vehicle-treated non-stressed group; $n = 11$).

Experiment 4a measured alcohol intake in female adult rats after termination of a phase of chronic restraint stress. The rats were distributed into two groups: 12 stressed and 12 non-stressed. Experiment 4b evaluated behavior in the EPM in a separate group of 24 female adult rats (12 stressed and 12 non-stressed).

2.3. Apparatus and procedures

2.3.1. Restraint stress procedures

Restraint stress was applied in Experiments 1–4 using procedures that are routinely used in our laboratory and, with modifications, in other laboratories [14–16]. On postnatal day 30–34 (PD30-34; adolescents) or PD70-74 (adults), the animals were confined to polyvinylchloride restraint tubes for 2 h. Control rats were weighed and returned to their home cage. Three tube sizes were used to accommodate differences in the size of the animals.

2.3.2. Alcohol intake tests

These tests were used in Experiments 1, 2, and 4a as previously described [17]. Between tests, the rats were housed in a standard maintenance cage with a same-sex conspecific, and had *ad libitum* access to food and water. At the beginning of each intake session, the maintenance cage was divided into two halves by a divider made of Plexiglas. The animals were then weighed and individually housed in half (section size: 23 cm length × 30 cm width × 20 cm height) of their home cage. Each section was equipped with two 100 ml bottles and *ad libitum* food. One bottle contained an alcohol solution (4% v/v during the first week and 5% v/v during the second week; vehicle: tap water), and the other bottle contained tap water only. The rationale for using these, relatively low, concentrations is that they are similar to those of alcoholic beverages (e.g., beer) preferred by adolescents. Studies scrutinizing beverage choice found that more than half of the volume of alcohol consumed by adolescents corresponded to beer [18], and beer was also the beverage of choice for most (67%) late adolescents (18–20 years old) who incurred in binge drinking in the U.S [19]. Moreover, we assessed the effects of stress on the first alcohol drinking experience (i.e., initiation), in which the use of relatively low alcohol concentrations is expected.

The bottles were weighed before and after each session to provide measures of fluid intake. Absolute alcohol intake (g/kg), the percentage of alcohol preference ([alcohol intake/overall fluid intake] × 100), and overall fluid intake (milliliters of fluid per 100 g of body weight [ml/100 g]) were calculated. Leakage of the fluid was accounted for by conducting pre- and post-session readings of two bottles that were placed in an empty cage. In Experiments 1 and 4, the alcohol intake protocol began on PD37 (adolescence) or 77 (adulthood), respectively, and lasted 2 weeks. A total of six sessions were conducted within these two weeks. The sessions began at 3:00 PM on Monday, Wednesday, and Friday and lasted until 9:00 AM the next day. This is, the voluntary drinking sessions took place during the last section of the light phase and throughout the dark phase. Experiment 1 revealed that the facilitatory effect of restraint stress on alcohol intake dissipated after session 4 in adolescent females. Experiment 2 analyzed the effects of KOR blockade on stress-induced drinking and consisted of only four intake

sessions, the first on PD37.

2.3.3. Norbinaltorphimine and alcohol treatment

In Experiments 2 and 3, the rats were administered nor-BNI intraperitoneally, 24 h before the first session of restraint stress, at a dose of 10.0 mg/kg (vehicle: 0.9% saline; injection volume: 0.01 ml/g). Norbinaltorphimine is a long-lasting KOR antagonist with significant pharmacological actions that last up to 14 days after a single administration [20]. The 10.0 mg/kg dose was chosen based on prior work [10,21].

Alcohol was administered at the 10 min time point in the CSF test in Experiment 3 at a dose (0.5 g/kg, volume of stock solution: 8.4%) that induces anxiolytic responses in adolescent Wistar rats tested in the elevated plus maze test [22].

2.3.4. Test of anxiety-like behavior

The EPM test was conducted on PD37 (Experiment 3) or PD77 (Experiment 4b), between 800 AM and noon. The standard EPM apparatus was elevated 50–60 cm above the floor. A full description of the apparatus and procedure is described in [23]. The 5-min test was video recorded to measure the latency to the first entry into the closed arms, time spent on the open arms and closed arms, and number of entries into the open arms and closed arms. Each open arm was virtually divided into three equally sized sections. The time spent in the whole arm or only in the two most distal sections (including the section that featured a "cliff" on the outer border) was measured. We also calculated the duration per visit in each of the EPM sections.

2.3.5. Test of risk-taking and shelter-seeking behavior

The adolescent female rats of Experiment 3 were tested for risk-taking and shelter-seeking behavior on PD40, between 800 AM and noon. The CSF (48 cm × 48 cm × 48 cm; full description in [24]) consisted of a central open field (OF) that connected three corridors. One of the corridors led to a sheltered (SHEL) sector that had a rubber ceiling and was not illuminated. The other two corridors led to the challenge (CHA) sector via a hole in the doorway, elevated 10 cm above the floor. Access to the CHA was not possible via regular horizontal locomotion, nor was possible to preview its interior via stretching or nose poking. The animals had to perform a risk taking behavior (i.e., jump through the hole) to reach the CHA sector. One of the corridors allowed access to a ramp (R) and a section that featured an elevated structure (i.e., the BRIDGE) that was made of metallic mesh that the rats could climb and explore. Illumination of the apparatus was the following: SHEL (0 lx), corridors and CHA sector (20–30 lx), and R and BRIDGE (600–650 lx).

The 20-min CSF test began by placing the animal in the OF. At the 10 min time point, the rats were removed from the apparatus and administered 0.5 g/kg alcohol. The rationale for administering alcohol at the 10 min time point was to obtain a baseline of spontaneous behavior prior to assessing alcohol's effects. The time spent and the frequency of entries in the OF, SHEL sector, CHA sector, R, and BRIDGE were recorded by a trained experimenter using JWatcher 0.9. The time spent and frequency of entries in the SHEL, OF and CHA sections were considered measures of shelter seeking, exploratory behavior and risk taking, respectively. The time spent and frequency of entries in the R and BRIDGE reflected risk taking. The duration per visit in each of the sections was also calculated.

It should be noted that in Experiment 3, the animals were repeatedly tested: they were tested in the EPM on PD37 and three days later (i.e., PD40) they were assessed for risk-taking and shelter-seeking in the CSF. There was no counterbalancing (i.e., all the animals were tested first in the EPM and three days later in the CSF). The rationale was that animals received an alcohol dose during the CSF test. Had we counterbalanced, some animals would have been tested in the EPM after an alcohol exposure. Within each test, the order of testing was alternated between stressed and non-stressed animals; and the observers who recorded and rated the tests were blind to the experimental condition of each subject.

2.4. Data analysis

Body weight (across experiments, recorded prior to each restraint stress session or prior to the test sessions), absolute alcohol intake (g/kg), percent alcohol preference, and overall fluid intake during the intake sessions in Experiments 1, 2, and 4a were analyzed by repeated-measures analysis of variance (ANOVA). Depending on the experiment, Stress, nor-BNI, or Sex served as between-subjects factors. Session (Experiments 1 and 4a: sessions 1–6; Experiment 2: sessions 1–4) was the within-subjects factor.

Each behavior measured in the EPM was analyzed using independent factorial ANOVAs (Experiment 3, with Stress and nor-BNI as between-subjects factors) or by independent *t*-tests (grouping factors: stress condition; Experiment 4b). Time spent and frequency of entries into each section of the CSF were independently analyzed with an ANOVA with Stress and nor-BNI as the between-subjects factors and interval of assessment (1 or 2) as the repeated measure. Similar ANOVAs analyzed mean duration (s) of visit to the OF, BRIDGE, SHELL and R sections of the CSF apparatus. Mean duration (s) of visit to the CHA could not be analyzed because some animals spent zero time at this section during either the first or second section of the test. This resulted in missing data and low sample size. Tukey's post hoc test ($\alpha = 0.05$) was used to analyze significant main effects and interactions between factors, and Cohen's partial eta squared (η^2p) was used to describe effect sizes. Planned comparisons were used to analyze significant between factor × within factor interactions. The rationale for this distinction is that there is a lack of appropriate post hoc tests to analyze interactions that involve both between- and within-subjects factors [25].

3. Results

3.1. Experiment 1

Body weight (Table 1) significantly increased across days ($F_{10,240} = 1214.17$, $p = 0.000$, $\eta^2p = 0.98$) and was significantly greater in males than in females ($F_{1,24} = 20.16$, $p = 0.0001$, $\eta^2p = 0.46$), yet similar across stressed and non-stressed animals.

As shown in Fig. 1, alcohol intake (g/kg) was affected by restraint stress, with differential effects in male and female adolescents. The ANOVA yielded significant main effects of Sex and Session ($F_{1,24} = 4.91$, $p = 0.04$, $\eta^2p = 0.17$, and $F_{5120} = 6.91$, $p = 0.001$, $\eta^2p = 0.22$, respectively), with significant Sex × Stress and Stress × Session interactions ($F_{1,24} = 16.90$, $p = 0.001$, $\eta^2p = 0.41$, and $F_{5120} = 8.62$, $p = 0.001$, $\eta^2p = 0.26$, respectively). The three-way Sex × Stress × Session interaction achieved significance ($F_{5120} = 2.31$, $p = 0.04$, $\eta^2p = 0.09$).

To further analyze the significant three-way interaction, follow-up Stress × Session repeated-measures ANOVAs were conducted for each sex. The ANOVA for males yielded significant main effects of Stress and Session ($F_{1,12} = 9.30$, $p = 0.01$, $\eta^2p = 0.44$, and $F_{5,60} = 6.59$, $p = 0.001$, $\eta^2p = 0.35$, respectively) and a significant Stress × Session interaction ($F_{5,60} = 8.07$, $p = 0.001$, $\eta^2p = 0.40$). The pair-wise comparisons indicated significantly less alcohol intake (g/kg) in stressed than in non-stressed males in all sessions, with the exception of session 4. The ANOVA for females revealed significant main effects of Stress and Session ($F_{1,12} = 7.61$, $p = 0.01$, $\eta^2p = 0.39$, and $F_{5,60} = 2.51$, $p = 0.04$, $\eta^2p = 0.17$, respectively) and a significant Stress × Session interaction ($F_{5,60} = 4.26$, $p = 0.002$, $\eta^2p = 0.26$). Stressed females drank significantly more alcohol than non-stressed females during the second, third, and fourth sessions.

The analysis of percent alcohol preference (Fig. 1, lower panel) yielded significant main effects of Sex and Session ($F_{1,24} = 4.58$,

Table 1
Body weight (g) in adolescent rats during stress days on postnatal days 30–34 and during the subsequent, two-bottle intakes sessions (Exp. 1 and 2). In Exp. 2 the animals were given a single dose of 0.0 (vehicle) or 10.0 mg/kg norbinaltorphimine (nor-BNI) 24 h before the commencement of stress treatment. The data are presented as mean ± SEM.

		Experiment 1				Experiment 2			
		Females		Males		0.0 mg/kg nor-BNI		10.0 mg/kg nor-BNI	
		Stressed (n = 7)	Non-Stressed (n = 7)	Stressed (n = 7)	Non-Stressed (n = 7)	Stressed (n = 8)	Non-Stressed (n = 6)	Stressed (n = 8)	Non-Stressed (n = 8)
Restraint Stress	Session 1	86.93 ± 6.58	92.50 ± 5.29	106.79 ± 5.09	104.36 ± 7.33	101.38 ± 2.60	95.33 ± 2.01	97.63 ± 2.20	92.13 ± 2.79
	Session 2	90.57 ± 7.15	97.79 ± 5.37	112.71 ± 5.66	112.21 ± 8.21	106.31 ± 2.50	103.42 ± 1.90	101.19 ± 2.21	100.75 ± 3.00
	Session 3	94.79 ± 7.43	105.14 ± 5.64	120.00 ± 5.82	122,79 ± 9.24	110.19 ± 2.80	109.33 ± 2.25	107.06 ± 2.54	107.25 ± 2.64
	Session 4	100.14 ± 7.54	112.00 ± 5.72	127.00 ± 6.54	129.50 ± 10.04	116.81 ± 2.67	116.50 ± 2.29	112.44 ± 2.34	116.13 ± 3.13
	Session 5	104.86 ± 7.62	117.21 ± 5.85	133.21 ± 6.39	137.36 ± 10.48	121.56 ± 2.85	123.67 ± 2.28	118.31 ± 2.48	122.31 ± 3.63
Intake Session	Session 1	117.00 ± 7.79	132.93 ± 5.70	155.64 ± 7.46	161.79 ± 12.03	127.56 ± 4.17	137.42 ± 2.78	132.25 ± 2.80	136.63 ± 2.87
	Session 2	125.71 ± 8.32	142.71 ± 6.07	171.07 ± 8.23	177.57 ± 13.73	143.13 ± 3.08	146.67 ± 2.72	141.38 ± 2.71	144.69 ± 3.41
	Session 3	134.79 ± 7.83	151.14 ± 5.50	186.86 ± 8.43	192.93 ± 14.53	151.81 ± 3.15	153.67 ± 3.39	151.38 ± 3.40	155.50 ± 3.36
	Session 4	149.50 ± 7.65	164.14 ± 5.56	206.50 ± 9.29	219.07 ± 15.17	165.00 ± 2.41	166.58 ± 3.18	162.44 ± 3.34	166.44 ± 3.85
	Session 5	155.21 ± 7.71	170.64 ± 5.59	228.71 ± 9.86	227.29 ± 15.87	174.00 ± 3.69	175.08 ± 3.39	172.19 ± 3.86	173.69 ± 4.40
	Session 6	162.29 ± 7.67	174.21 ± 5.47	245.79 ± 10.25	244.71 ± 17.56	170.94 ± 4.10	178.67 ± 4.33	176.94 ± 4.10	180.06 ± 4.56

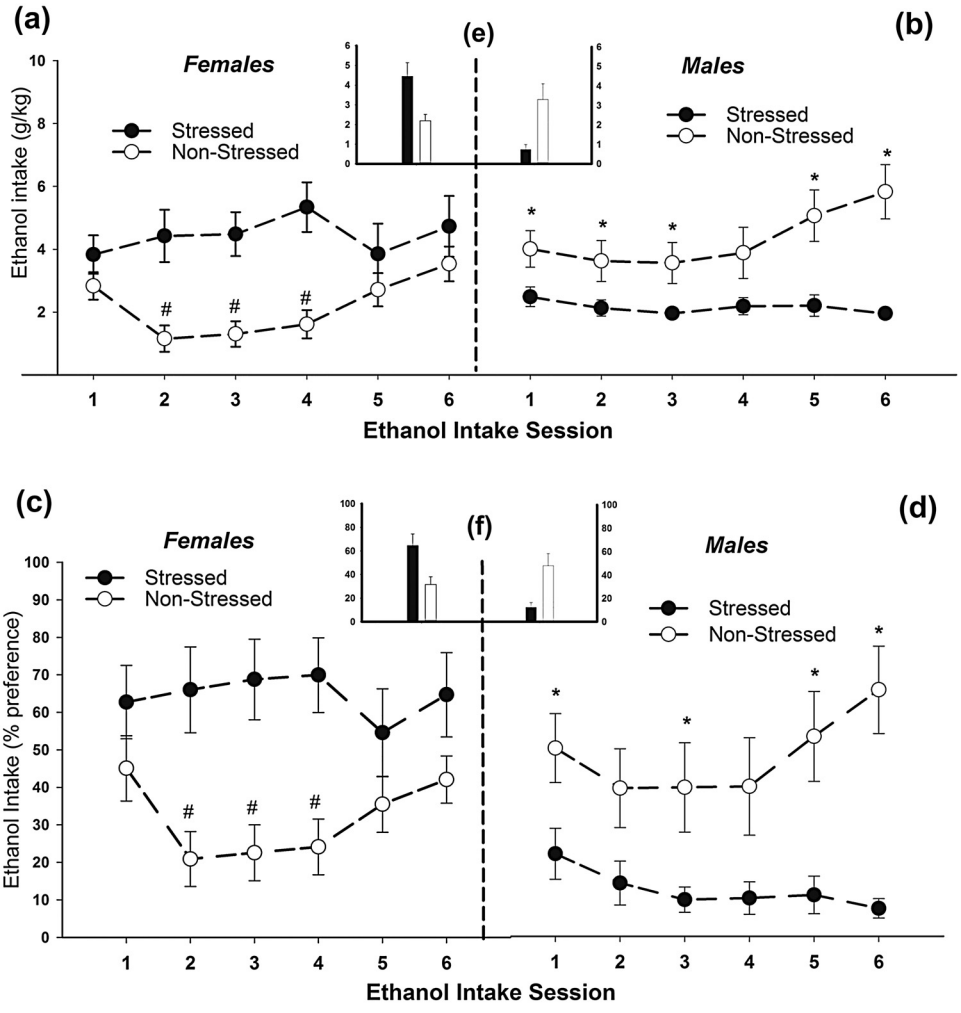

Fig. 1. (A, B) Absolute alcohol intake (g/kg) and (C, D) percent alcohol preference in male and female adolescent Wistar rats as a function of day of assessment and stress treatment (5 days of restraint stress or non-stressed). The asterisk and pound signs indicate, for males and females, respectively, a significant difference in alcohol intake between stressed and non-stressed rats in a given alcohol intake session. Refer to the text for a full description of significant differences yielded by the corresponding ANOVAs. (E, F) Absolute alcohol intake (g/kg) and percent alcohol preference collapsed across days of assessment. Vertical lines indicate SEM.

Table 2
Overall liquid intake (ml/100 g) in adolescent rats during stress days on postnatal days 30–34 and during the subsequent, two-bottle intakes sessions, in Experiments 1 and 2 (upper and lower sections, respectively). The data are presented as mean ± SEM.

	Female Group		Male Group	
	Stress	Non-Stress	Stress	Non-Stress
Session 1	19.33 ± 0.54	19.95 ± 2.61	16.17 ± 0.53	17.72 ± 1.31
Session 2	20.68 ± 0.79	17.56 ± 1.28	15.74 ± 0.74	18.15 ± 1.26
Session 3	20.26 ± 1.21	18.76 ± 0.96	16.31 ± 0.53	18.00 ± 1.01
Session 4	19.26 ± 0.98	18.17 ± 1.05	19.05 ± 1.25	17.66 ± 1.06
Session 5	18.05 ± 1.50	20.29 ± 1.19	16.38 ± 0.95	19.91 ± 1.50
Session 6	19.42 ± 1.97	21.73 ± 1.56	16.99 ± 0.65	19.27 ± 1.29
	Stressed Group		Non-Stressed Group	
	Vehicle	nor-BNI	Vehicle	nor-BNI
Session 1	26.97 ± 2.52	23.07 ± 1.75	20.43 ± 2.03	20.83 ± 1.28
Session 2	21.98 ± 1.43	21.04 ± 1.77	17.76 ± 0.82	19.36 ± 1.32
Session 3	21.96 ± 1.16	22.51 ± 1.84	17.85 ± 1.20	19.44 ± 1.15
Session 4	22.02 ± 1.55	21.84 ± 1.39	16.35 ± 1.87	19.76 ± 1.51

$p = 0.04$, $\eta^2p = 0.16$, and $F_{5120} = 4.33$, $p = 0.001$, $\eta^2p = 0.15$, respectively), significant Sex × Stress and Stress × Session interactions ($F_{1,24} = 16.97$, $p < 0.000$, $\eta^2p = 0.41$, and $F_{5120} = 7.32$, $p = 0.001$, $\eta^2p = 0.23$, respectively), and a significant three-way Sex × Stress × Session interaction ($F_{5120} = 2.35$, $p = 0.05$, $\eta^2p = 0.09$). The follow-up Stress × Session repeated-measures ANOVA for male rats indicated significant main effects of Stress and Session ($F_{1,12} = 10.14$, $p = 0.007$, $\eta^2p = 0.46$, and $F_{5,60} = 3.26$, $p = 0.011$, $\eta^2p = 0.21$, respectively) and a significant Stress x Session interaction ($F_{5,60} = 3.96$, $p = 0.003$, $\eta^2p = 0.25$). Planned comparisons indicated that male rats that were exposed to restraint stress drank less alcohol than non-stressed control rats in sessions 1, 3, 5, and 6. The repeated-measures ANOVA for females revealed a significant main effect of Stress ($F_{1,12} = 7.04$, $p = 0.002$, $\eta^2p = 0.37$) and a significant Stress × Session interaction ($F_{5,60} = 5.57$, $p = 0.000$, $\eta^2p = 0.32$). Stress increased the percentage of alcohol preference in females during self-administration sessions 2–4.

The ANOVA of overall fluid intake (descriptive data on Table 2) only revealed a significant Stress × Session interaction ($F_{5130} = 4.09$, $p = 0.001$, $\eta^2p = 0.14$). Stressed rats consumed slightly but significantly less fluid in session 5 than non-stressed rats.

3.2. Experiment 2

Experiment 2 evaluated whether the stress-induced increases in alcohol intake and preference in female rats could be inhibited by pre-treatment with nor-BNI. Body weight across sessions (see Table 1) was similar across groups. The ANOVA of alcohol intake (in g/kg; Fig. 2) yielded a significant main effect of Session ($F_{3,78} = 5.36$, $p = 0.002$, $\eta^2p = 0.17$), with greater alcohol intake in sessions 1 and 4 than in sessions 2 and 3 across groups. The Stress × nor-BNI interaction was significant ($F_{1,26} = 6.60$, $p < 0.016$, $\eta^2p = 0.20$). The *post hoc* tests indicated significantly greater alcohol intake in vehicle-treated stressed female rats compared with vehicle-treated non-stressed females and nor-BNI-treated stressed females. Alcohol intake in nor-BNI-treated stressed females was similar to nor-BNI-treated non-stressed females. These results indicate that nor-BNI inhibited the facilitatory effect of restraint stress on alcohol intake.

The ANOVA of the percentage of alcohol preference indicated a significant main effect of Session ($F_{3,78} = 3.13$, $p = 0.030$, $\eta^2p = 0.11$) and a significant Stress × nor-BNI interaction ($F_{1,26} = 5.09$, $p = 0.032$, $\eta^2p = 0.16$). The *post hoc* tests revealed a greater percentage of alcohol preference in the vehicle-treated stressed group than in the vehicle-treated non-stressed group. The percentage of alcohol preference in the vehicle-treated non-stressed group was similar to the nor-BNI-treated stressed group, indicating that nor-BNI reduced the preference for alcohol in stressed females (Fig. 2).

Overall fluid intake was greater in stressed rats than in non-stressed rats ($F_{1,26} = 6.05$, $p = 0.020$, $\eta^2p = 0.19$; descriptive data in lower section of Table 2) and unaffected by nor-BNI treatment.

3.3. Experiment 3

Experiment 3 evaluated the behavioral effects of restraint stress (i.e., anxiety-like behavior in the EPM and exploratory behavior in the CSF) that may be associated with its ability to affect alcohol intake, in adolescent females. The ANOVA of body weight (Table 3) indicated significant main effects of Stress and Day ($F_{1,38} = 6.86$, $p = 0.01$, $\eta^2p = 0.15$, and $F_{5190} = 1155.18$, $p = 0.00$, $\eta^2p = 0.97$, respectively) and a significant Stress × Day interaction ($F_{5190} = 26.92$, $p = 0.00$, $\eta^2p = 0.41$). Stressed females weighed significantly less than non-stressed controls on days 3–5 of the restraint stress protocol, but not thereafter.

The ANOVA of the time spent on the open arms (all sections) and the ANOVA of the time spent in the distal section of the open arms of the EPM yielded a significant effect of Stress ($F_{1,39} = 4.90$, $p = 0.03$, $\eta^2p = 0.11$, and $F_{1,39} = 5.43$, $p = 0.03$, $\eta^2p = 0.12$, respectively). As

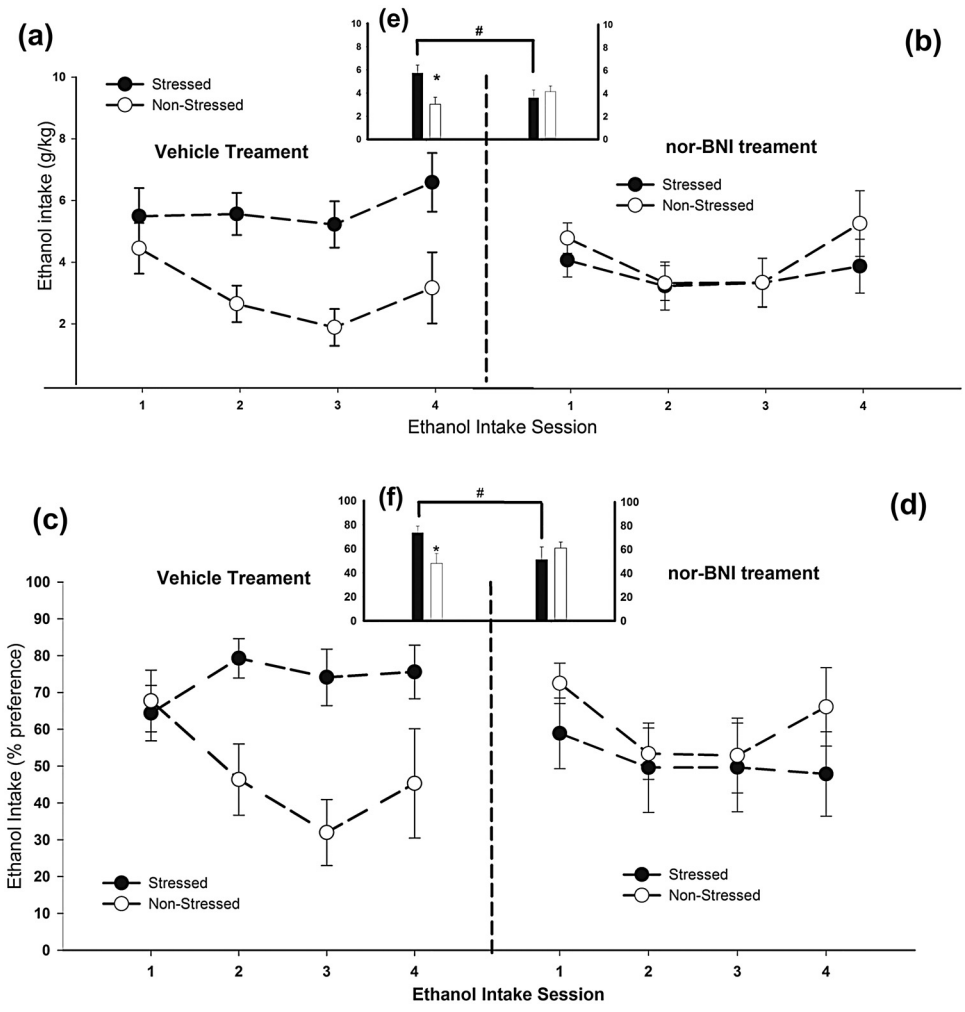

Fig. 2. (A, B) Absolute alcohol intake (g/kg) and (C, D) percent alcohol preference in female adolescent Wistar rats as a function of day of assessment, stress treatment (5 days of restraint stress or non-stressed), and pharmacological treatment (a single dose of 0.0 [vehicle] or 10.0 mg/kg norbinaltorphimine [nor-BNI]) given 24 h before stress exposure. (E, F) Absolute alcohol intake (g/kg) and percent alcohol preference collapsed across days of assessment. The statistical analyses indicated significantly greater alcohol intake in vehicle-treated stressed females compared with vehicle-treated non-stressed females and nor-BNI-treated stressed females. Significant effects are indicated by the asterisk and pound signs, respectively. Vertical lines indicate SEM.

Table 3
Body weight (g) in adolescent rats during stress days on postnatal days 30–34, in Exp. 3. The animals were given a single dose of 0.0 (vehicle) or 10.0 mg/kg norbinaltorphimine (nor-BNI) 24 h before the commencement of stress treatment. The data are presented as mean ± SEM.

		Experiment 3			
		0.0 mg/kg nor-BNI		10.0 mg/kg nor-BNI	
		Stressed	Non-Stressed	Stressed	Non-Stressed
Restraint Stress	Session 1	96.82 ± 1.96	96.58 ± 1.34	92.82 ± 1.72	96.09 ± 1.31
	Session 2	101.73 ± 1.89	102.67 ± 1.47	96.64 ± 1.79	100.45 ± 1.51
	Session 3	105.82 ± 2.04	109.92 ± 1.31	101.18 ± 1.93	107.91 ± 1.25
	Session 4	109.73 ± 2.27	116.58 ± 1.37	105.64 ± 2.25	114.55 ± 1,38
	Session 5	115.45 ± 2.30	124.42 ± 1.65	110.82 ± 2.35	118.09 ± 1.93

shown in Fig. 3, stressed animals spent more time than non-stressed controls in both of these open, fear-inducing sections of the maze, a result that was similar in both nor-BNI- and vehicle-treated rats. Latency to the first entry into the closed arms and overall locomotor activity (i.e., total number of transfers between arms) were similar across groups (vehicle-treated non-stressed: 7.67 ± 2.77 and 18.67 ± 2.85; vehicle-treated stressed: 11.36 ± 3.96 and 22.45 ± 2.17; nor-BNI-treated non-stressed: 16.11 ± 5.32 and 22.44 ± 2.53; nor-BNI-treated stressed: 18.27 ± 6.15 and 20.64 ± 2.29; for latency and transfers, respectively). The ANOVA of the mean duration (s) of visit to the open arms and the ANOVA of the mean duration (s) of visit to the closed arms did not reveal significant main effects or significant interactions. Mean duration (s) of visit to the open and closed arms was 7.86 ± 1.30 and 34.33 ± 7.01 (group Non Stressed − Vehicle administration), 8.20 ± 1.43 and 21.13 ± 2.84 (group Stressed − Vehicle administration), 5.94 ± 0.85 and 23.38 ± 3.46 (group Non Stressed − norBNI administration) and 8.76 ± 1.75 and 24.03 ± 4.46 (group Stressed − norBNI administration).

Adolescent rats were tested for basal and alcohol-induced (first and second 10-min intervals of the test, respectively) exploration of the CSF. The ANOVA for total number of section entries (i.e., overall level of activity) indicated a significant effect of Interval of evaluation,

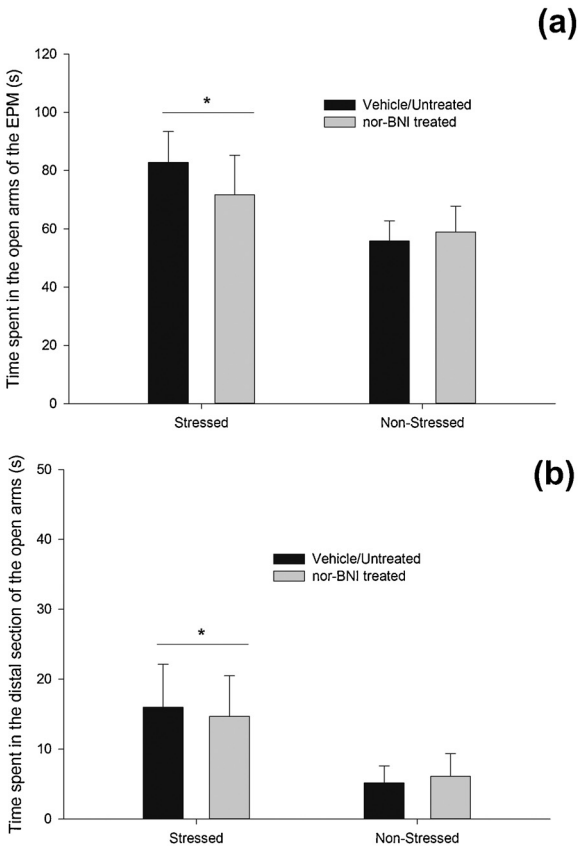

Fig. 3. Time spent (in s) in (A) all sections of the open arms or (B) only the two most distal sections of the open arms (upper and lower panels, respectively) of the elevated plus maze in female adolescent Wistar rats as a function of stress exposure during postnatal days 30–34 (5 days of restraint stress or non-stressed) and pharmacological treatment (a single dose of 0.0 [vehicle] or 10.0 mg/kg norbinaltorphimine [nor-BNI]). The asterisk indicates that stressed rats spent more time than non-stressed controls in the open, fear-inducing section of the maze. Vertical lines indicate SEM.

$F_{1,39} = 10.18$, $p = 0.001$, $\eta^2p = 0.21$. Mean and SEM number of section entries during the first and second half of the test were as follows: Non Stressed − Vehicle administration 69.00 ± 4.79 and 50.83 ± 6.56, Stressed − Vehicle administration 72.90 ± 5.01 and 61.82 ± 5.16, Non Stressed − norBNI administration 76.33 ± 6.12 and 55.44 ± 12.42, and Stressed − norBNI administration 64.00 ± 5.79 and 60.36 ± 7.93.

The ANOVAs of time spent or frequency of entries in the SHEL, OF, R and BRIDGE sector did not reveal significant main effects or interactions. The time spent in the CHA sector, but not the number of entries in that sector, was significantly greater in stressed rats than in non-stressed rats during the second 10-min interval of the test (significant Stress × Interval: $F_{1,39} = 4.10$, $p < 0.05$, $\eta^2p = 0.10$). Fig. 4 depicts time spent in each sector the CSF. Data for number of entries is shown in Table 4. The ANOVAs of the mean duration (s) of visit to the OF, SHELL and R sections of the CSF during the first and second section of the test (descriptive statistics at Table 4) revealed a lack of significant main effects or significant interactions.

3.4. Experiment 4

This experiment assessed stress-induced alcohol drinking (Exp. 4a) and behavior in the EPM in adult female rats (Exp. 4b). In both experiments Body weight was unaffected by stress (see Table 5).

The ANOVA of fluid intake in Experiment 4a revealed greater overall intake in the first session than in the subsequent sessions ($F_{5110} = 3.31$, $p = 0.008$, $\eta^2p = 0.13$; descriptive data on Table 5). Stress exposure significantly decreased alcohol intake in adult females (Fig. 5). The analysis of absolute (g/kg) and percent alcohol intake revealed significant main effects of Day ($F_{5110} = 5.11$, $p = 0.000$, $\eta^2p = 0.18$, and $F_{1,22} = 18.48$, $p = 0.001$, $\eta^2p = 0.46$, respectively) and Stress ($F_{5110} = 3.41$, $p = 0.006$, $\eta^2p = 0.13$, and $F_{1,22} = 14.22$, $p = 0.001$, $\eta^2p = 0.39$). Pair-wise comparisons indicated that alcohol intake (g/kg) was significantly greater on the first test day than on the subsequent test days and significantly lower in stressed females compared with non-stressed females. The percentage of alcohol preference was significantly greater on the first test day than on the last two test days and significantly lower across days in stressed rats than in non-stressed rats.

The *t*-tests indicated that restraint stress did not time spent in the open arms of the EPM, number of transfers between arms, and latency to enter the closed arms (the first of these variables is shown in Fig. 6, others not shown). The *t*-tests also indicated that restraint stress did not significantly alter the mean duration (s) of visit to the open arms or the mean duration (s) of visit to the closed arms. Mean duration (s) of visit to the open and closed arms was 34.11 ± 5.78 and 48.20 ± 20.21 (group Non Stressed) and 65.70 ± 30.99 and 48.81 ± 14.35 (group Stressed).

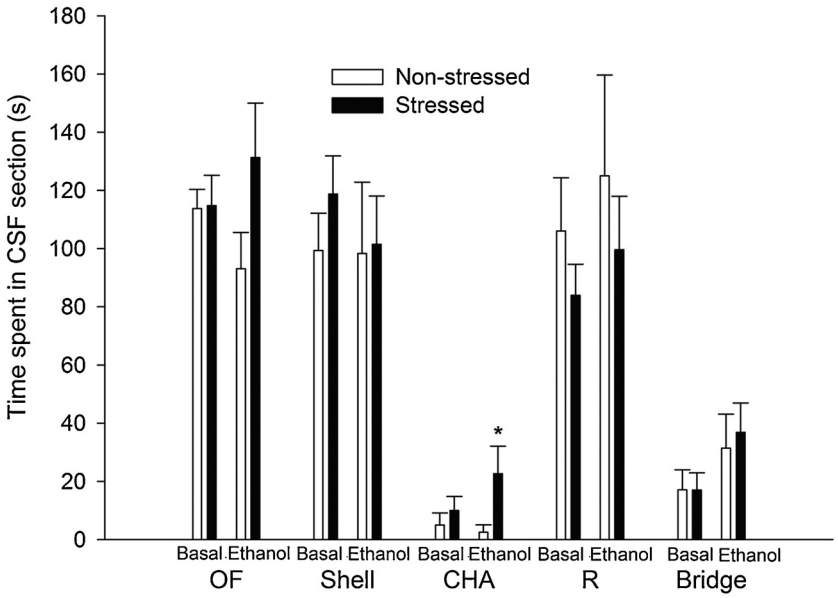

Fig. 4. Time spent in the different sections of the concentric square field (CSF) in female adolescent Wistar rats as a function of stress exposure during postnatal days 30–34 (5 days of restraint stress or non-stressed) pharmacological treatment (a single dose of 0.0 [vehicle] or 10.0 mg/kg norbinaltorphimine [nor-BNI) given 24 h before stress exposure for each interval of evaluation. The adolescents were tested for basal (first 10-min interval of the test) and alcohol-induced (second 10-min interval of the test, following administration of 0.5 g/kg alcohol) exploration of the CSF. The data are expressed as mean ± SEM. The data were collapsed for nor-BNI treatment. Norbinaltorphimine treatment did not exert a significant main effect and did not significantly interact with the other factors. The asterisk indicates that stressed animals spent a significantly longer time in the challenge sector compared with their control counterparts.

Table 4
Number of entries into (upper numbers of each cell) and mean duration of visit to (s, lower numbers of each cell) the different sections of the concentric square field (CSF) in female adolescent Wistar rats as a function of stress exposure. The adolescents were tested for basal (first 10-min interval of the test) and alcohol-induced (second 10-min interval of the test, following administration of 0.5 g/kg alcohol) exploration of the CSF. The data, which are expressed as mean ± SEM, has been collapsed for nor-BNI treatment.

		Experiment 4			
		Stressed		Non-Stressed	
		1 st interval	2nd interval	1 st interval	2nd interval
Area of The CSF apparatus	SHEL	7.31 ± 0.53 15.55 ± 1.04	5.86 ± 0.57 19.48 ± 2.86	7.29 ± 0.59 13.46 ± 1.67	4.86 ± 0.73 23.75 ± 7.23
	OF	22.09 ± 1.30 5.17 ± 0.39	18.82 ± 1.28 7.69 ± 1.6	22.95 ± 1.22 5.06 ± 0.27	16.80 ± 2.09 5.61 ± 0.40
	R	4.95 ± 0.53 16.84 ± 1.23	6.14 ± 0.81 16.71 ± 2.34	5.90 ± 0.78 19.34 ± 2.75	5.05 ± 0.98 35.87 ± 13.60
	BRIDGE	0.64 ± 0.20 26.75 ± 6.00	1.32 ± 0.30 27.17 ± 5.75	1.00 ± 0.32 13.66 ± 2.68	1.29 ± 0.43 24.61 ± 4.98
	CHA	0.45 ± 0.19 28.15 ± 2.74	0.77 ± 0.33 48.90 ± 14.04	0.24 ± 0.19 20.57 ± 0.78	0.10 ± 0.10 26.46 ± 0.78

Table 5
Upper section. Body weight (g) in adult rats during stress days (Experiments 4a and 4b) and during the subsequent, two-bottle intakes sessions (Exp. 4a only). **Lower section.** Overall liquid intake (ml/100 g) in stressed and non-stressed rats during the two-bottle intakes sessions (Exp. 4a only). The data are presented as mean ± SEM.

		Experiment 5a		Experiment 5b	
		Stressed	Non-Stressed	Stressed	Non-Stressed
		(n = 12)	(n = 12)	(n = 12)	(n = 12)
Restraint Stress	Session 1	239.42 ± 4.22	248.42 ± 3.37	267.17 ± 4.71	257.92 ± 4.97
	Session 2	246.92 ± 3.80	251.75 ± 3.59	265.25 ± 5.10	259.92 ± 5.13
	Session 3	246.42 ± 3.96	251.17 ± 3.81	265.33 ± 5.33	260.42 ± 4.36
	Session 4	247.33 ± 3.96	253.08 ± 3.99	265.67 ± 5.05	260.92 ± 5.31
	Session 5	248.83 ± 3.59	256.5 ± 3.87	265.17 ± 5.24	260.83 ± 5.10
Intake Sessions	Session 1	250.67 ± 3.95	258.83 ± 4.41	20.01 ± 1.60	23.45 ± 2.17
	Session 2	253.5 ± 3.96	258.42 ± 4.23	16.4 ± 1.69	19.82 ± 1.37
	Session 3	254.5 ± 4.19	260.83 ± 4.63	17.73 ± 1.58	20.99 ± 1.47
	Session 4	256.17 ± 4.22	262.33 ± 4.81	16.49 ± 1.74	18.6 ± 2.08
	Session 5	258.00 ± 4.25	262.00 ± 4.56	15.69 ± 1.30	18.95 ± 2.18
	Session 6	260.92 ± 4.65	264.42 ± 5.29	15.7 ± 1.70	17.69 ± 1.121

4. Discussion

Restraint stress induced sexually dimorphic, opposite effects in adolescent rats. Female adolescent rats exhibited, after exposure to stress, significant enhancement of alcohol intake in Experiment 1, which was replicated in Experiment 2. Stress-exposed male adolescent rats exhibited a significant reduction of alcohol intake and preference. The facilitatory effect of restraint stress on alcohol intake was age-dependent, in which reductions of alcohol intake and preference were observed in stress-exposed adult females (Exp. 4a).

In humans, men usually consume more alcohol than women, measured in terms of frequency and overall quantity, and present a greater incidence of AUD [26]. This gap between sexes has been shrinking [27,28], particularly among adolescents. Among college students who drink, females have a higher risk of AUD than their male counterparts [29], and women tend to experience greater impulsivity after acute alcohol consumption [30,31]. The present results further elucidate sex- and age-related vulnerability to AUD. Female adolescents, but not male adolescents or female adults, may have a particularly high risk of initiating alcohol intake after early stress. It has been shown that pubertal status is a significant modulator of alcohol intake [32]. An important point is that puberty occurs earlier in female (~PD33) than in male (~PD41) Wistar rats [33]. Therefore, and of relevance to interpret the sex differences here reported, female animals in the present study had already completed puberty when alcohol intake tests started while males were just shortly before puberty onset. In other words, it is likely that the repeated stressor and the alcohol intake sessions impacted the two sexes at different times during their pubertal maturation, perhaps contributing to the observed sex differences.

The differential effect of stress in adolescent vs. adult female rats is perhaps the most relevant result of this study. The discussion of this age-dependent phenomenon could benefit from a description of the development of the hypothalamic-pituitary-adrenal (HPA) axis. This axis controls, by a complex cascade of signals, the release of stress-related hormones (e.g, corticosterone, CORT) [34]. Excessive activation of this system can result in a variety of negative outcomes, including neurotoxicity [35]. Notably, it has been shown that restraint stress exposure for 30 min induces a more prolonged CORT release in adolescent than in adult female rats [36]. This effect was independent of the levels of ovarian hormones and seemed to be the result of a greater sensitivity to the adrenocorticotropic hormone at the level of the adrenal cortex. Other studies also showed greater HPA axis activity in adolescent than in adult rats or mice, after exposure to hypoxia or other stressors (for review and references, see [34]) It is thus possible that the age differences in stress-reactive alcohol drinking, reported in the present study, ultimately obeyed to age differences in the response of the HPA axis to restraint stress. Therefore, a caveat is that CORT levels were not measured in the present work. It is important to note that, in a prior study [37], we exposed adolescent and adult Wistar rats (albeit only males) to five sessions of restraint stress, one each day for five days. Three days later we measured baseline and stress-induced (exposure to a brightly lit chamber) CORT levels. The acute stressor

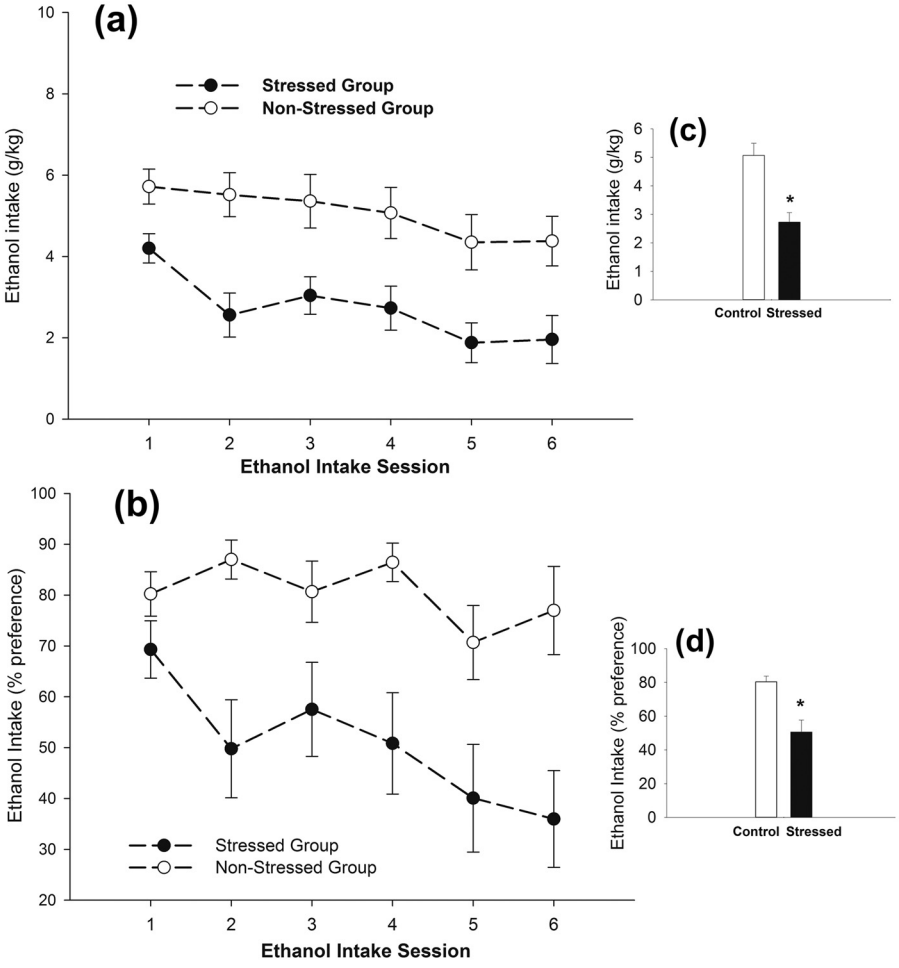

Fig. 5. (A) Absolute alcohol intake (g/kg) and (B) percent alcohol preference in female adult Wistar rats as a function of day of assessment (intake test sessions 1–6) and stress (5 days of restraint stress or non-stressed). The small inset graphs C and D depict g/kg and percent alcohol preference collapsed across days of assessment. The asterisk indicates a significant difference in alcohol intake between stressed and non-stressed rats. Vertical lines indicate SEM.

enhanced CORT response, yet this increase was similar in adolescents and adults, and in animals exposed or not to restraint stress [37].

The facilitatory effect of restraint stress on alcohol consumption was inhibited by nor-BNI. This is consistent with previous studies that reported that KORs mediate stress-induced alcohol intake. The pharmacological blockade of KORs inhibited the facilitatory effect of stress on alcohol-induced CPP [10]. The influential studies by Walker and Koob [38] and Walker, Zorrilla and Koob [39] found that nor-BNI disrupted alcohol self-administration in alcohol-dependent rats. Some reports, however, have indicated that KORs also mediate some of the aversive, post-ingestive effects of alcohol intoxication [40,41]. In the present study, nor-BNI was devoid of nonspecific effects on alcohol intake. This is, nor-BNI administration blocked the promoting effect of stress upon alcohol intake but did not exert significant effects upon alcohol intake patterns of non-stressed animals.

A factor that likely underlies the facilitatory effect of restraint stress on alcohol intake is the anxiogenic-like effect of restraint stress [42]. However, in female rats, behavior in the EPM and CSF indicated no increases in anxiety- or fear-related behavior. Stressed females exhibited significantly greater exploration of the open arms. These females also spent significantly more time in the CHA sector of the CSF [i.e., a risk-taking area; as shown by [13]] compared with non-stressed females. This effect was only observed during the second 10-min interval of the test (i.e., after administration of a low dose of alcohol [0.5 g/kg]). This raises the possibility that stressed females but not non-stressed females exhibit alcohol-induced enhancement of risk-taking behavior. Two studies from our laboratory observed greater alcohol intake in male rats after treatments that either enhanced impulsivity or impaired risk assessment behaviors. In one of these studies, male rats that were subjected to restraint stress spent more time in the white area of a light-dark maze and exhibited greater alcohol intake than their non-stressed counterparts [37]. These results suggest that restraint stress-induced cognitive alterations may promote alcohol intake. Similar to the present study, a subsequent study exposed adolescent male Wistar rats to chronic environmental enrichment and found increases in exploration of the CHA sector of the CSF and alcohol intake [24]. Restraint stress appears to increase alcohol intake in females by facilitating the exploration of novel stimuli and increasing risk-taking behaviors.

The present study has several limitations. Stress exposure affected alcohol intake in the adult females yet did not alter exploration patterns in the EPM. Blood alcohol levels were also not measured, although in a previous study we found similar alcohol metabolism in adolescent or adult Wistar rats, exposed or not to restraint stress [37]. Also, in the present study we reported a restraint stress-induced decrease in alcohol intake in adolescent males, whereas in [37] adolescent males exhibited a transient increase in alcohol intake after the same stress protocol. These seemingly disparate findings may be explained by the presence of additional sources of stress during, and before, the test conducted by Fernández et al. Specifically, the latter study measured alcohol intake in 2 h sessions preceded by significant dehydration-induced stress (i.e., 22 h of water deprivation). Also, the rats in Fernández et al. in [37] were isolated during the test and confined to small hanging cages. It should be noted that, in the present study, the stress induced by the individual housing during alcohol intake sessions was minimized by housing animals in side-by-side compartments (i.e., during the intake tests they were separated by a Plexiglass divider that allowed perception of the odor cues of the counterpart) and by reuniting the animals in-between sessions.

The lack of measurement of the estrous cycle in our female rats can

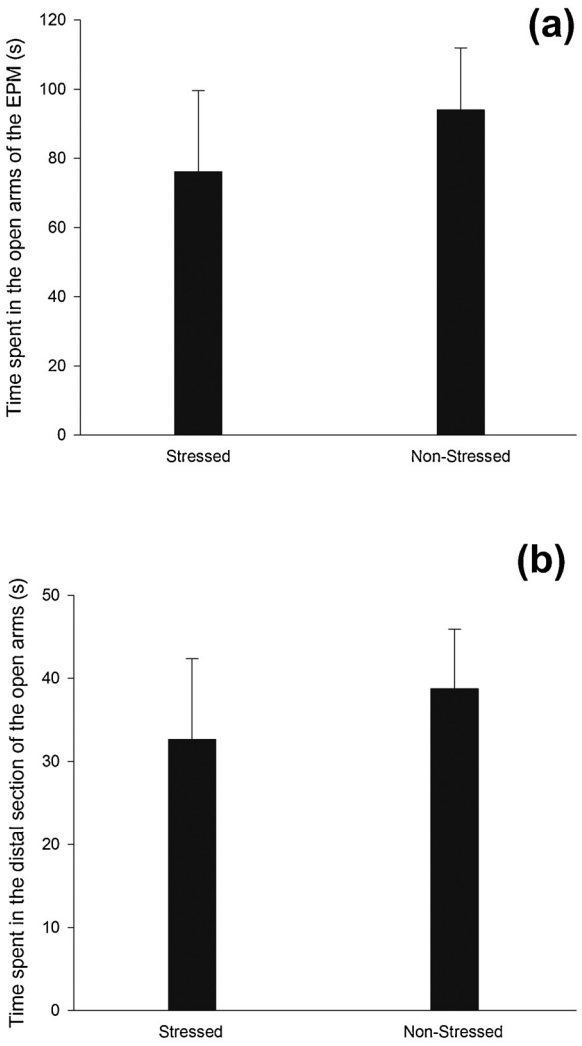

Fig. 6. Time spent (in seconds) in (A) all sections of the open arms or (B) only the two most distal sections of the open arms (upper and lower panels, respectively) of the elevated plus maze in female adult Wistar rats as a function of stress exposure during postnatal days 70–74 (5 days of restraint stress or non-stressed). Vertical lines indicate SEM.

also be thought of as a limitation of the study. Recent studies, however, have severely questioned the notion that female cyclicity adds significant variability to neurobiological studies [43], even to those interested in stress responses. This that does not imply, of course, that levels of sex hormones are not important for a variety of phenomena. It seems, however, that the variability of the estrous cycle within females, regardless they are housed individually or in group, cannot explain most of the sex differences found in the mice [44] or rat [45] literature. Many studies that measured anxiety, stress and locomotor activity using validated tests (e.g., EPM, LDB, open-field tests, forced swim test) have reported a lack of significant effects when the phase of estrous cycle was used as a grouping variable [43,46–48].

Some data inconsistencies in body weight and overall fluid intake were observed. Specifically, overall fluid intake in Experiment 1 was slightly but significantly reduced in stressed rats. This effect, however, was only observed at session 5 and was similar in male and female rats. As described, these sexes exhibited significantly different patterns of stress-induced alcohol drinking. On the contrary, in Experiment 2 overall fluid intake was actually greater in stressed than in non-stressed, female adolescent, rats. Still, this effect of stress was independent of the reduction of stress-induced drinking, observed after nor-BNI treatment. Body weight in adolescents was mostly unaffected by stress, although in Experiment 3 stressed females weighed significantly less than controls in the last days of the stress protocol. These differences, however, were transient and disappeared by the time the animals were submitted to the alcohol intake protocol. In sum, it seems that none of these effects were associated with the main results reported. If anything, these results are consistent with previous work indicating that adolescent rats may be more sensitive to the effects of stress upon body weight and food intake, than adult rats [49]. Neither overall fluid intake nor body weight were significantly affected by stress in adults.

Compared with adults, adolescents are significantly less sensitive to the sedative [37,50] and aversive [51] effects of alcohol, but they are more sensitive to the appetitive [52], motor-activating [53], and social-facilitating [54,55] effects of the drug. This idiosyncratic pattern of responses to alcohol may convey a higher risk for alcohol initiation or escalation in adolescents [56,57]. The sensitivity to stress-induced drinking that was observed in adolescents, albeit only in females, but not in adults in the present study may represent yet another age-related difference in the sensitivity to alcohol.

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The funding source had no involvement in the study design; in the collection, analysis, and interpretation of data; in writing the report; or in the decision to submit the article for publication.

Declaration of interest

The authors declare no competing interest or conflict of interest related to the data presented in the manuscript.

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