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ANXIETY RESPONSE AND RESTRAINT-INDUCED STRESS DIFFERENTIALLY AFFECT ETHANOL INTAKE IN FEMALE ADOLESCENT RATS

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Abstract—Anxiety disorders are more likely to occur in women than in men, usually emerge during adolescence and exhibit high comorbidity with alcohol use disorders (AUD). Adolescents with high levels of anxiety or heightened reactivity to stress may be at-risk for developing AUD. An approach to analyze if high levels of inborn anxiety predict greater ethanol drinking is to assess the latter variable in subjects classified as high- or low-anxiety responders. The present study assessed ethanol drinking in adolescent, female Wistar, rats classified as high-, low- or average-anxiety responders and exposed or not to restraint stress (RS, Exp. 1). Classification was made through a multivariate index derived from testing anxiety responses in an elevated plus maze and a light–dark box tests. RS was applied after animals had been initiated to ethanol drinking. Intake of sweetened ethanol was unaffected by level of anxiety response. Adolescents with high levels of inborn anxiety exhibited significantly higher intake of unsweetened ethanol than counterparts with standard levels of anxiety, yet this effect was inhibited by RS exposure. Experiment 2 assessed FOS immunoreactivity after RS. Stress induced a significant increase in FOS immunoreactivity at the paraventricular nucleus, yet this effect was unaffected by level of anxiety response. Female adolescents with high levels of basal anxiety may be at-risk for exhibiting increased predisposition for ethanol intake and preference. The study also indicates that stress may exert differential effects on adolescent ethanol intake as a function of the level of anxiety response.

Key words: adolescents, ethanol intake, stress, anxiety, Fos immunoreactivity.

INTRODUCTION

Anxiety disorders, which are more likely to occur in women than in men (Pisu et al., 2016), usually emerge during adolescence (Cunningham et al., 2002) and exhibit high comorbidity with alcohol use and alcohol use disorders (AUD) (Hobbs et al., 2011). A recent study indicated that social anxiety disorder significantly predicted AUD in both African American and European American adolescents (Sartor et al., 2016). This is consistent with the postulate that individuals with high levels of anxiety may be more sensitive to the negative reinforcing effects of alcohol (hereinafter referred to as ethanol) and thus may be at-risk for developing AUDs (Kushner et al., 1994).

Ethanol intake has been measured in animals selectively bred to exhibit high- or low-inborn anxiety (Henniger et al., 2002) and vice versa, anxiety responses have been analyzed in animals selectively bred for high- and low-ethanol intake. Rats selected for high anxiety-response drank more ethanol than rats selected for their predisposition to explore dangerous environments (Izidio and Ramos, 2007) and the ethanol-preferring P rats exhibit significantly greater sensitivity to exteroceptive nociceptive stimulation and spend less time in the open arms of an elevated plus maze than their ethanol-nonpreferring (NP) counterparts (Stewart et al., 1993). An anxious phenotype has also been described in the genetically selected Marchigian Sardinian alcohol-preferring rats (Ciccocioppo et al., 2006). These animals exhibited reduced propensity to explore the open arms of the elevated plus maze and the central zone of the open field (Roman et al., 2012). Similarly, the Warsaw alcohol high-preferring (WHP) rats exhibited enhanced acoustic startle response than their low-preferring WLP counterparts (Acewicz et al., 2012). Other studies, however, have failed to replicate these results (Da Silva et al., 2004) or yielded a negative association between anxiety and ethanol intake (Henniger et al., 2002). For instance, the WHP rats exhibited less anxiety (i.e., more

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Abbreviations: AA, average-anxiety responder; ANOVAs, analyses of variance; Arc, arcuate nucleus; AUD, alcohol use disorders; BLA, basolateral amygdala nucleus; CEA, central amygdala nucleus; EPM, elevated plus maze; Fos-ir, FOS immunoreactivity; HA, high-anxiety responder; LA, low-anxiety responder; LDB, light–dark box; mPFC, medial prefrontal cortex; PD, postnatal day; PVC, polyvinyl chloride; PVN, paraventricular nucleus; RS, restraint stress; WHP, Warsaw alcohol high-preferring.

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time spent in the central section of an open field) than the WLP rats (Acewicz et al., 2014).

Another approach to analyze if high levels of inborn anxiety predict greater ethanol drinking is to submit subjects to a validated animal model of anxiety [e.g., elevated plus maze or light–dark box, EPM and LDB, respectively] (Kumar et al., 2013). The animals are classified as high- or low-anxiety responders as a function of performance on the test and then ethanol drinking is assessed. This approach has yielded evidence for an association between inborn anxiety and ethanol intake at adulthood (Spanagel et al., 1995; Bahi, 2013). For instance, Primeaux et al. (2006) found greater preference for 4% ethanol and 6% ethanol in rats classified as anxious as a function of performance on the elevated plus maze, compared with non-anxious rats.

High-anxiety responders may be more sensitive to aversive and stressful stimulation (Muigg et al., 2008), and adolescents have been found to be more sensitive to stress, and to stress–ethanol interactions, than adults. Five days of restraint stress (RS) significantly enhanced ethanol intake and reduced ethanol-induced sleep time in adolescent but not in adults (Fernandez et al., 2016). A single, 90-min session of RS increases anxiety, as shown by reduced social investigation, in both adults and adolescent rats. This effect of stress is reversed by ethanol in adolescent, but not in adult, rats (Varlinskaya and Spear, 2012). Another relevant interaction between adolescence, stress and ethanol is that male (Siegmund et al., 2005) or female (Fullgrabe et al., 2007) rats that started to drink ethanol during adolescence, but not during adulthood, were sensitive to foot-shock induced facilitation of ethanol drinking. This could result from an ethanol-induced alteration in the brain circuits involved in the stress response. Rats given vapor ethanol exposure during postnatal days (PD) 28–42, for instance, exhibited reduced expression of corticotropin releasing factor mRNA in the paraventricular nucleus (PVN), after a challenge with ethanol in early adulthood (Allen et al., 2011). These results highlight the importance of assessing stress-induced drinking early in development. Stress during adolescence may facilitate onset and escalation of drinking, likely to a greater extent than stress during adulthood, and sub-populations characterized by high levels of inborn anxiety may be particularly vulnerable to ethanol drinking and stress-induced drinking.

The present study assessed ethanol drinking throughout the duration of adolescence, in female rats classified as high-, low- or AAs and exposed or not to RS (Exp. 1). Classification was made through a multivariate index of anxiety, and RS was applied after animals had been initiated to ethanol drinking. It has been indicated that animals need several intake sessions to learn about ethanol's anti-anxiety effects (Samson et al., 1998). Experiment 2 assessed neural activation (FOS immunoreactivity, Fos-ir) after RS in the three anxiety groups, in the basolateral and central amygdala (BLA and CEA, respectively), and in the PVN and arcuate nucleus (Arc).

These brain areas are involved in the stress response and regulate baseline levels of anxiety response. Briski and Gillen (2001) and later Keshavarzy et al. (2015) observed significant Fos-ir in PVN and Arc after a 2-h or a 1-h RS session, respectively [also see Kwon et al. (2006)]. Rats classified as HAs, but not those classified as LAs, exhibited decreased expression of central corticotropin-releasing factor in the PVN, after chronic RS (5 weeks, 3 h/day; Wisowska-Stanek et al., 2016). The role of the amygdala in mediating anxiety responses and in the acquisition of conditioned fear has been studied at length (Maren and Quirk, 2004). Rats bilaterally lesioned in CEA exhibited a blunted response to experimentally induced anxiety and a significant reduction in ethanol intake (Moller et al., 1997). The CEA also endures plastic changes (for instance, after chronic drug treatment) that result in greater anxiety and sensitivity to stress (Koob, 2009). The BLA, in turn, projects to several other areas, including CEA, ventral hippocampus and medial prefrontal cortex (mPFC). A recent study found an increase in anxiety-like behavior after the optogenetic activation of the BLA-mPFC pathway, whereas the inhibition of this circuit was associated with decreased anxiety-like behavior (Felix-Ortiz et al., 2016). Alterations in anxiety were also observed after manipulating the projections between the BLA and the CEA (Tye et al., 2011) or between the BLA and the ventral hippocampus (Felix-Ortiz et al., 2013). Moreover, several studies have reported a reduction of experimental anxiety after microinjection of benzodiazepines into the BLA or CEA, underscoring a casual role of these structures in the expression of anxiety-like behaviors (Menard and Treit, 1999; Engin and Trait, 2008).

Our hypotheses were that HAs would exhibit greater ethanol intake and that this would be exacerbated by RS. We expected these behavioral differences to translate into greater neural response to RS; i.e., greater RS-induced Fos-ir, with likely regional differences, in high-anxiety than in low-anxiety or average-anxiety females. We focused on females due to the greater prevalence of anxiety disorders in women and because, among college students who drink, women are more likely than men to develop AUD (Perkins, 2002). Female rats also consume more ethanol than males (Lancaster et al., 1996; Doremus et al., 2005).

EXPERIMENTAL PROCEDURES

Experimental designs

A 3 (level of baseline anxiety response: high-anxiety, average-anxiety and low-anxiety; HA, AA and LA, respectively) × 2 (stress exposure: stressed or non-stressed; S and NS, respectively) factorial design was employed in both Experiments 1 and 2. Number of animals in each group was as follows: HA-S = 12, HA-NS = 14; AA-S = 9, AA-NS = 18; LA-S = 9, LA-NS = 17 (Experiment 1), and HA-S = 5, HA-NS = 5; AA-S = 5, AA-NS = 5; LA-S = 5, LA-NS = 5 (Experiment 2). The uneven number of subjects in the groups of Exp. 1 was because group assignment as a function of anxiety response occurred after completion of experimental procedures. A separate group of rats

was left untreated (UT, $n = 5$) in Experiment 2. These animals were pair-housed up to the perfusion, to control for any unspecific effects of housing that could affect Fos-ir.

Subjects

A total of 220 Wistar adolescent female rats, representative of 43 litters were employed (Experiment 1, 132 animals, 25 litters; Experiment 2, 88 animals, 18 litters). Rats were born and reared at the vivarium of the Instituto de Investigaciones Médicas M. y M. Ferreyra (INIMEC–CONICET-Universidad Nacional de Córdoba, Argentina). The vivarium had controlled temperature (22 ± 1 °C) and humidity (45%) and a 12 h/12 h light/dark cycle (lights on at 8:00 AM). Three adult females undergoing proestrum, as indicated by inspection of vaginal smears, were housed with a single male for three days, approximately. The day of birth was considered postnatal day 0 (PD0) and on PD1 the litters were culled to 10 animals (five males and five females, whenever possible). Animals were housed with their dams until weaning on PD 21. Females were then transferred to standard cages in groups of four and had *ad libitum* access to water and lab chow (ACA Nutricion, Buenos Aires, Argentina). The experiments were endorsed by the Ministry of Animal Care of INIMEC–CONICET-UNC and were in agreement with the NIH Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

Behavioral testing for anxiety (Exps. 1 and 2)

A summary of the tests conducted to assess inborn anxiety response is in the upper section of Fig. 1. On PD30 [i.e., two days after the normative beginning of adolescence in rats, see (Spear, 2000)] animals were withdrawn from their homecage and transferred to an adjacent room equipped with a light–dark box (LDB) apparatus and an EPM. A video camera was fixed in a metal rail hanging from the ceiling and moved to appropriately capture the behavior of the animal in each apparatus. Videos were scored through Etho-Log software (Ottoni, 2000). The apparatuses were made of black Plexiglas and cleaned after each animal with sodium peroxide (6%).

The animals were gently placed in the central section of the EPM, facing the open arm. They remained in the EPM apparatus for five minutes and then were immediately transferred to the LDB for another 5 min. The EPM was located 50 cm above the floor and was supported by four rounded metal bars. It had two open (45 cm × 5 cm) and two enclosed arms (45 cm × 5 cm × 45 cm; length, width and height, respectively), which converged in a central platform (5 cm × 5 cm). A warm fluorescent lamp (75 W) provided light, resulting in an illumination level of ≈100 lux. The main dependent variable was the percent number of entries into the open arms. Entries into an open or closed arm were counted when the four paws crossed the arm.

The LDB featured a large, bright section (25 l × 25 w cm × 30 h) illuminated by a 75-W white bulb adjusted to generate an illumination level of 400 lux; and a smaller section (18 l × 25 w × 30 h) without illumination (i.e., 0 lux). The sections were connected by a gate (6.5 × 6.5 cm) located at floor level. Testing began by gently placing the rat in the center of the bright compartment facing the communicating gate. The main dependent variable was the latency to exit the bright compartment. Time spent in the bright compartment was also measured.

Both anxiety assays were conducted on the same day to better capture the anxiety trait of the animal, which can exhibit day-to-day fluctuations due to non-specific factors (e.g., prandial state, recent changes in bedding). Same-day testing makes both tests affected by the same non-specific states, thus increasing the reliability of the assessment (Ramos et al., 2008). Also, previous studies from our lab indicated that the relationship between EPM scores and ethanol intake (albeit only measured in a single, 24-h session; Acevedo et al., 2014) was not affected by whether the EMP was conducted before or after another behavioral testing. Based on this information, we did not counterbalance test presentation in the present study.

Group assignment as a function of anxiety response

Adolescent animals were classified as a function of anxiety response as low-anxiety, average-anxiety and high-anxiety response (LA, AA and HA groups, respectively), as a function of a composite, general index of anxiety response. This multivariate index (further referred as "overall anxiety score") comprised percent number of entries into the open arms of the EPM and latency to exit the bright area of the LDB test (defined as placing all four paws into the smaller compartment). Scores corresponding to these dependent variables were standardized (z-scores, relative to the entire sample of subjects) and then added up and divided by two, to obtain a single anxiety score for each animal. The resulting score represented an overall anxiety score encompassing positive and negative values. Higher (and more positive) values indicated a relatively low anxiety response; and lower (and more negative) values, derived from relatively low number of entries into the open arms of the EPM and shorter latency to escape from the bright side of the LDB, indicated a relatively high anxiety response. Similar multivariate aversive indexes have been previously employed to successfully reveal expression of conditioned disgust reactions in adult (Parker, 1995) and preweanling (Pautassi et al., 2008) rats. The middle section of Fig. 1 succinctly describes the equation to calculate overall anxiety scores and provides examples obtained from animals classified as high- or LAs.

LA and HA subjects corresponded to those exhibiting the 20% higher and lower overall anxiety scores, respectively. Specifically, LA subjects fell between percentiles 99–80 of the distribution of this variable, whereas HA subjects fell within percentiles 1–20. Please note that a high overall anxiety score actually reflects a relatively low anxiety response. AA subjects were those that fell in between percentiles 40 and 60 of the distribution of overall anxiety scores. Therefore, using the

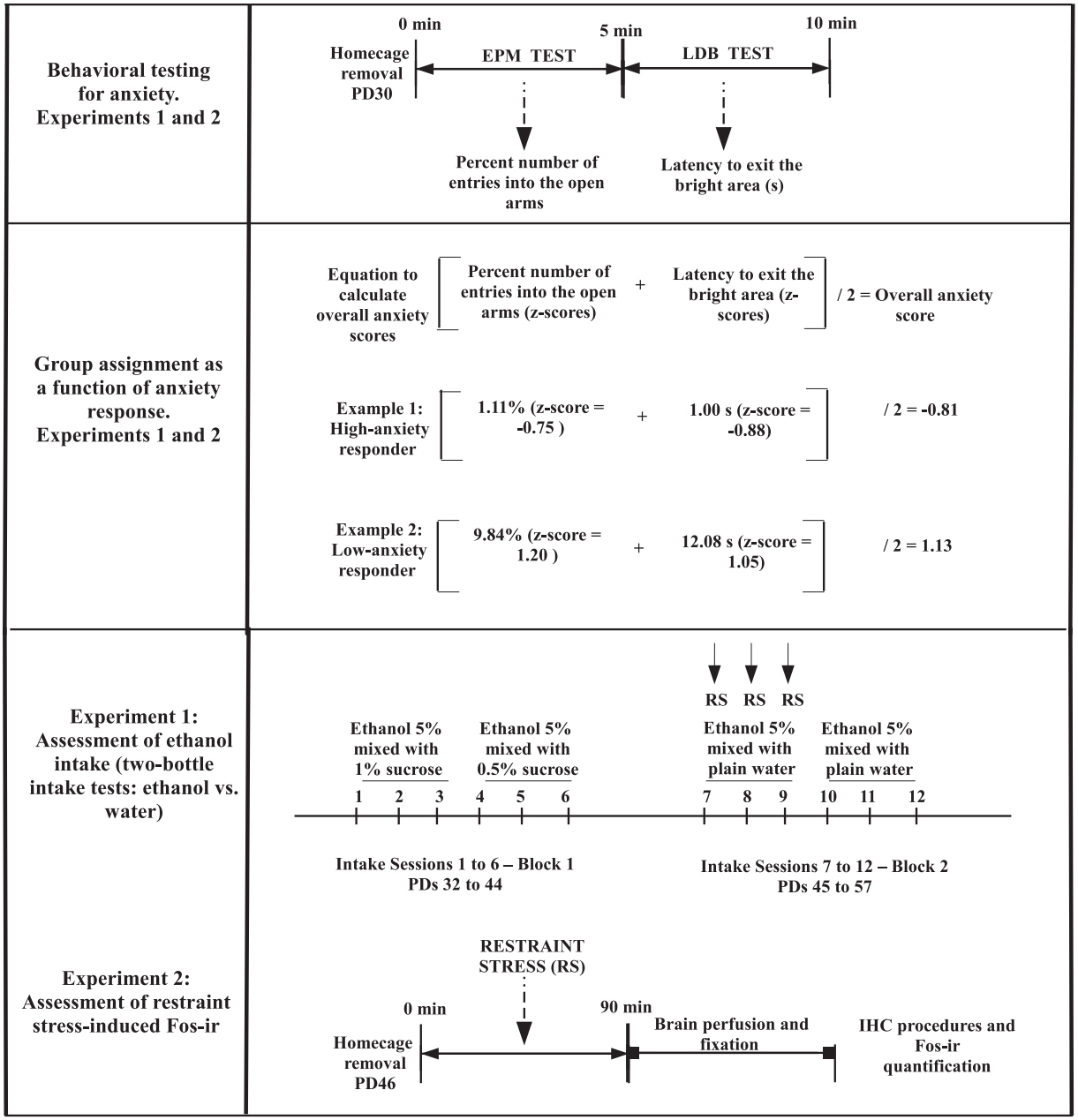

Fig. 1. Methods for the analysis of the relationship between inborn anxiety response and ethanol intake in adolescent female rats. Upper section: On postnatal day (PD) 30, the rats were removed from the homecage and underwent behavioral screening. Behavioral testing began by gently placing the rat in the central platform of the elevated plus maze (EPM). Five minutes later they were transferred to a light–dark box (LDB). The animals remained in LDB for 5 min. Middle section: The percent number of entries into the open arms of the EPM and the latency (s) to exit the bright area of the LDB were employed to calculate a multivariate, overall anxiety score. Specifically, these scores were standardized (*z*-scores), added up and divided by two. This equation is exemplified using the scores registered in two animals classified as high- or low-anxiety responders (Examples 1 and 2, respectively). Lower section: Animals were assessed for ethanol intake (Experiment 1) during four weeks, from PD 32 to PD 57. During each week animals were given three every other day, two-bottle choice tests, followed by two rest days. Ethanol was mixed with 1% (week 1, intake sessions 1–3) or 0.5% (week 2, intake sessions 4–6) w/v sucrose, or with plain water (weeks 3 and 4, intake sessions 7–12). Experiment 2 was conducted at PD46. The female rats were exposed for 90 min to restraint stress and then submitted to the perfusion and immunohistochemical (IHC) procedure. Fos-ir was quantified at several, stress-sensitive, brain areas.

HA-AA-LA split criteria, animal were selected on the basis of their performance in two different and validated tests for anxiety response, EPM and LDB. Splitting through upper and lower percentiles has been employed in adult (Klebaur and Bardo, 1999; Nadal et al., 2005), adolescent (Acevedo et al., 2010), an infant (Arias et al., 2009) rats to characterize subpopulations with differential susceptibility to ethanol intake or drug or ethanol reactivity.

Ethanol Intake procedures (Exp. 1)

As indicated in the lower section of Fig. 1, Ethanol intake was assessed during four weeks, from PD 32 (i.e., two

days after the anxiety screening) to PD 57, following procedures similar to those described in Fabio et al. (2015). This length of testing was selected to expose the youth to alcohol throughout the adolescent stage of development. During each week animals were given three every other day, two-bottle choice tests, followed by two rest days. Intake sessions began at 1500 and ended at 0900 of the following day. Animals were weighed to the nearest gram and individually housed in cages lined with pine shavings. Each cage had two bottles equipped with ball-point tubes. One bottle was filled with tap water and the other bottle was filled with a 5% v/v ethanol solution. Ethanol was mixed with 1% (week 1, intake sessions 1–3) or 0.5% (week 2, intake sessions 4–6) w/v sucrose, or with plain water (weeks 3 and 4, intake sessions 7–12).

In summary, ethanol intake was divided into two blocks of 6 sessions. Animals began responding for a mildly sweetened ethanol solution (block 1, weeks 1 and 2). The sweetener was then faded out and during block 2 (weeks 3 and 4) animals self-administered unsweetened 5% ethanol. Tap water served as vehicle for all solutions. The position of the water and ethanol bottles was randomly switched across sessions to prevent place-preference effects. Water and ethanol bottles were put in an empty cage and used to correct intake scores due to accidental leaking (i.e., a spillage control). Between tests, animals were housed in groups of 4–5 with *ad libitum* access to water and chow.

The selection of a sucrose-fade procedure with a relatively low concentration of ethanol was based on previous studies indicating that adolescent, uninitiated Wistar rats generally drink low amounts of ethanol concentrations >5–6%, unless mixed with sucrose (Fabio et al., 2014), or unless animals are subjected to substantial water deprivation (Ponce et al., 2008) or a lengthy initiation protocol. We preferred using the sucrose-fading procedure to rapidly stabilize daily intake and to avoid the stress associated with dehydration. Our intermittent access procedure also avoided continuous exposure to isolation-induced stress, as animals were immediately reunited with a same-sex counterpart after termination of each ethanol intake session. It has been indicated that intermittent access to highly concentrated ethanol (e.g., 20%, Simms et al., 2008) can promote escalation of ethanol intake in adult Wistar rats, without the need for a sucrose fade. We, however, conducted pilot experiments in preparation for the present study and found very little, if any, intake of 20% ethanol, in female adolescent Wistar rats exposed to the drug every other day.

Restraint-Induced Stress (Exps. 1 and 2)

In Experiment 1, animals were given 90 min of stress or remained untreated in their home cage, immediately before commencement of intake sessions 7, 8 and 9 (i.e., third week of intake test, see lower section of Fig. 1). Restraint was conducted on those days to allow animals experience the pharmacological effects of ethanol prior to stress exposure. Moreover, a previous study from our lab, that used the two-bottle overnight test of the present study, indicated that ethanol intake and preference in adolescents significantly escalated during these sessions, in which animals first experience unsweetened ethanol access (Fabio et al., 2015). There was no stressor prior to intake sessions 10, 11 and 12, nor in any of the sessions of Block 1.

In Experiment 2, animals were given a single, 90-min exposure to RS, on PD 46. The rationale for selecting this PD was that, in Experiment 1, the first exposure to RS and the first session of access to unsweetened ethanol occurred on PD46. Immediately after termination of the stress session they were submitted to the perfusion (see Fig. 1, lower section). In both experiments the animals from the stress groups were withdrawn from their home cage, transferred to a separate room, and confined in tubes made of white polyvinyl chloride (PVC) and closed through PVC lids with small holes to allow a proper respiration. Three tube sizes were available to accommodate differences in the size of animals: 15×4.2 cm, 15×3.3 cm and 20×5.8 cm; length and maximal internal diameter, respectively.

Brain tissue preparation, immunohistochemistry procedures and Fos-ir quantification (Exp. 2)

In Experiment 2, animals were anesthetized with i.p. injections of chloral hydrate (dose: 0.001 ml/g of a 30 heparinized saline (10 U/ml) and 4% paraformaldehyde (Sigma–Aldrich, USA) in 0.1 M phosphate buffer (PB; pH 7.4), immediately after termination of RS procedures. Brains were removed and transferred to a 30% sucrose-PB solution at 4 °C for at least 72 h.

Free-floating 40-μm coronal sections were cut and placed in 0.01 M PB, using a freezing microtome. Three series of sections were kept at 20 °C with a conservative solution. A fourth series was used for immunohistochemistry. A peroxidase blockade was carried out by placing the brain cuts in a solution composed of 10% hydrogen peroxide, 10% methanol and 0.01 M PB for 1 h. Sections were rinsed three times in 0.01 M PB, and in order to block non-specific binding sites were incubated in 5% normal horse serum (NHS, Invitrogen, New Zealand) for 1 h. Next, the free-floating sections were incubated overnight at room temperature under continuous agitation, with a rabbit monoclonal antibody against the c-Fos protein (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:2000 in 0.01 M PB containing 0.3% Triton X-100 and 1% NHS. Brains were washed three times in 0.01 M PB, incubated with biotinylated donkey anti-rabbit secondary antibody (Jackson Laboratories, West Grove, PA, USA) diluted 1:500 in 1% NHS, and washed three times more in 0.01 M PB for 2 h. Afterward, the sections were incubated 2 h with the avidin–biotin–peroxidase complex (ABC Elite Kit; Vector Labs, Burlingame, CA, USA) diluted 1:50 in 1% NHS. Next, sections were incubated for 5 min with 0.05% 3–3′-diaminobenzidine tetra hydrochloride (DAB, Sigma–Aldrich, St. Louis, MO, USA) and 0.03% hydrogen peroxidase in 0.1 M PB, to give rise to a brown reaction product (c-Fos mark). Finally, immunostained sections were mounted onto gelatin-coated glass slides (with Albrecht's gelatine, 1.5% gelatine-80% ethanol), dehydrated and

coverslipped with DPX, a slide mounting medium (Sigma–Aldrich, USA).

Cells exhibiting c-Fos staining were localized by examining tissue sections under a light microscope (Primo Star iLed), equipped with an AxioCam ERc 5-s camera (Zeiss, Jena, Germany). FIJI Is Just Image J software, 1.49 K version was used to digitize images and to determine the number of immune positive neurons. All cell counts were performed by an observer blind to the experimental treatments. An area of the same size and shape (200μm^2) was considered to count cells of each brain structure. Following the rat brain atlas of Paxinos and Watson (2007), slices for PVN and Arc were taken from bregmas −1.72, −1.80 and −1.92 mm (i.e., plates 47–49, respectively), and the slices for CEA and BLA were taken from bregmas −2.04, −2.16 and −2.28 mm (i.e., plates 50–52; respectively). Fig. 2 depicts the coordinates for each brain region under analysis.

For each animal, at least three brain sections from the labeled structures were counted bilaterally. Preliminary analysis indicated that Fos-ir patterns across brain sections counted were similar. Analyses of variance (ANOVAs) in which section were considered as repeated measures, did not yield significant main effect of section or significant interactions between this factor and the remaining factors: anxiety-response (low-, average-, or HA) or stress exposure (stressed or no-stressed) across all structures. Therefore, data from the brain sections of each hemisphere were averaged. These counts were in turn averaged for the subsequent statistical analysis.

Statistical analysis

Unless specified, results are expressed as mean ± SEM. The effectiveness of the split procedure was assessed in each Experiment through a one-way ANOVA (between factor: anxiety response group, dependent variable: overall anxiety response). Body weight at the beginning and termination of ethanol intake sessions in Experiment 1 (PD 32 and PD 57, respectively) was separately analyzed through a two-way factorial ANOVA (stress exposure × anxiety response).

In Experiment 1, ethanol intake (g/kg and percent preference) during the first and second block of intake sessions (block 1: sessions 1–6; block 2: sessions 7–12) were separately analyzed through ANOVAs. Each included anxiety response group and stress exposure as between group factors, and intake session as the repeated measure. A similar set of ANOVAs was employed to analyze overall intake scores (ml/100 g of body weight). Ethanol intake (g/kg and % preference) and overall liquid intake (ml/100 g) were also analyzed in adolescents that fell between high- and average-anxiety responders, and between average- and low-anxiety responders. These intake variables were separately analyzed for intake block 1 and 2 through two-way mixed ANOVAs.

We also examined ethanol drinking (g/kg and % preference) as a function anxiety-like behavior on each test. The split procedure and the analyses were those just described, yet animals were classified as high-, average- or low-anxiety responders as a function of their behavior in only of the tests. The corresponding ANOVAs indicated that neither of the classification criteria significantly affected ethanol intake (all $ps > 0.05$).

In Experiment 2, one-way ANOVAs – one for each brain structure analyzed – were conducted to confirm that the triage/screening procedures did not result in unspecific Fos-ir induction. These analyses included the non-stressed groups (HA-NS, AA-NS and LA-NS) and the group of naïve animals (UT group, $n = 5$) that had not been tested for anxiety response and remained untreated in the homecage until the perfusion. Stress-induced Fos-ir at CEA, BLA, Arc and PVN (Experiment 2) was analyzed via separate factorial ANOVAs [between factors: level of baseline anxiety response: (low anxiety, average anxiety and high-anxiety) and stress exposure (stressed or non-stressed)]. The dependent variable was the number of positive cells.

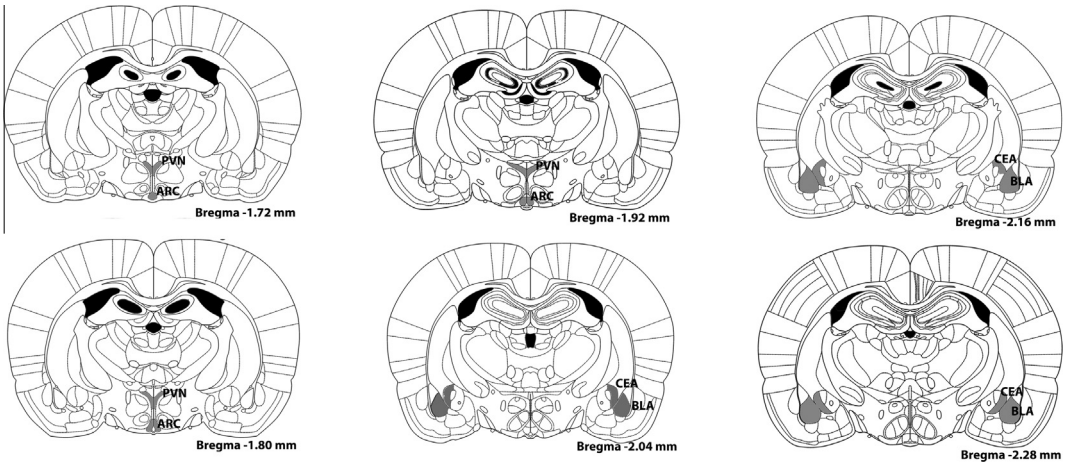

Fig. 2. Schematic diagram of brain sections analyzed in this study, based on Paxinos and Watson (2007). Figures represent antero-posterior levels to bregma of selected areas. Localization of the considered section of paraventricular nucleus (PVN), arcuate nucleus (Arc), central amygdala (CEA) and basolateral amygdala (BLA) is indicated with the corresponding legend, and highlighted anatomically in gray when necessary.

Table 1. Latency to exit from and time spent in the bright side of the light–dark box (LBD), total number of arm entries, total time spent and % number of entries into the open arms of the elevated plus maze (EPM) in female adolescent rats classified as low- average- or high-anxiety (LA, AA or HA) as a function of their baseline anxiety response

	Baseline anxiety response		
Dependent variable	Low anxiety (LA)	Average anxiety (AA)	High anxiety (HA)
Latency to exit the bright side of the LDB	11.34 ± 1.75	5.62 ± 0.70	1.76 ± 0.21
Time spent in the bright side of the LDB	23.08 ± 3.21	11.37 ± 1.86	6.73 ± 1.21
Percent number of entries into the open arms of the EPM	26.47 ± 3.54	9.43 ± 1.62	1.81 ± 0.42
Total number of arm entries in the EPM	62.15 ± 4.74	50.52 ± 2.12	51.15 ± 3.82
Total time (s) spent in open arms in the EPM	43.04 ± 5.94	14.17 ± 2.15	2.73 ± 0.77

This resulted from averaging the count obtained in each hemisphere, which was the average of three slides.

Follow-up ANOVAs and post hoc tests were used to find the loci of significant main effects or significant interactions. Specifically, follow-up ANOVAs and Tukey's tests were employed for analysis of simple main effects or interactions comprising "between" factors. Interactions involving repeated measures were scrutinized by orthogonal planned comparisons. There is a lack of appropriate post hoc tests to analyze interactions that involve between × within factors (Winer et al., 1991). Therefore, to better control for the numerous comparisons made, significant between × within factor interactions were analyzed via planned comparisons and only when supported by our *a priori* hypothesis. The partial Eta squared (η^2p) was employed to estimate effect sizes. *Alpha* level was $\leqslant$0.05 across analyses.

RESULTS

Experiment 1

Overall anxiety scores in HA, AA and LA animals were −0.72 ± 0.02, −0.10 ± 0.02 and 1.03 ± 0.09, respectively. Standardized scores in EPM and LDB tests were as follows: −0.69 ± 0.03 and −0.75 ± 0.04, −0.13 ± 0.12 and −0.08 ± 0.12 and 1.14 ± 0.26 and 0.92 ± 0.31; for HA, AA and LA animals, respectively. A one-way ANOVA confirmed that the groups exhibited significant differences in overall anxiety response, $F_{(2,76)} = 291.36$, $p < 0.001$, $\eta^2p = 0.88$. Post-hoc tests indicated that each group significantly differed from the others (all *ps* < 0.001).

The analysis of time spent in the bright side of the LDB revealed a significant main effect of anxiety response, $F_{(2,76)} = 13.93$, $p < 0.001$, $\eta^2p = 0.27$. Post-hoc tests revealed a significant, twofold increase in time spent in the bright area in low-anxiety subjects spent, when compared to average- or high-anxiety counterparts. There were no differences between the latter groups, which could be the result of them exhibiting a functional floor effect. The ANOVA for time (s) spent in the open arms of the EPM revealed a significant main of anxiety response [$F_{(2,76)} = 32.11$, $p < 0.001$, $\eta^2p = 0.46$], with HA adolescent spending significantly less time in the open arms than LA or AA counterparts. There was also a trend ($p = 0.07$) for LA rats to spend more time in the open arms than AA rats. The analysis for total number of arm entries in the EPM indicated that locomotor activity was fairly similar between HA, AA and LA adolescents (both *ps* > 0.05). Table 1 presents descriptive data (mean ± SEM) for each of these variables and for the raw, untransformed scores of latency to exit the bright side of the LDB and the % of entries into the open arms of the EPM, across groups.

Body weights were similar across groups defined by stress exposure and anxiety response, at both the beginning and termination of ethanol intake sessions. The ANOVAs indicated the lack of significant main effects or significant interactions. Body weights (g) at PD32 and PD57 were as follows: 106.08 ± 2.68 and 215.67 ± 5.27 for HA-S; 100.57 ± 2.21 and 214.36 ± 4.22 for HA-NS; 105.33 ± 4.46 and 209.89 ± 2.99 for AA-S; 102.44 ± 1.76 and 208.94 ± 3.51 for AA-NS; 106.33 ± 4.27 and 221.22 ± 5.98 for LA-S; 105.82 ± 2.97 and 207.82 ± 3.77 for LA-NS animals.

As depicted in Fig. 3, ethanol acceptance during the first block of intake sessions (sessions 1–6; in which ethanol was sweetened with sucrose) was very similar across groups. The ANOVAs for ethanol intake during the first block of sessions revealed a significant main effect of session, $F_{(5,365)} = 15.51$, $\eta^2p = 0.18$ and $F_{(5,365)} = 10.59$, $\eta^2p = 0.13$, both $p < 0.001$; for g/kg and percent preference, respectively. Planned comparisons indicated that absolute and percent ethanol intake was greater during the first session than during the remaining sessions. Planned comparisons also indicated that, after the significant drop in session 2, absolute ethanol intake remained stable across sessions, whereas percent preference exhibited a significant increment in session 4 relative to sessions 2 and 3.

Patterns of ethanol intake during block 2 (i.e., sessions 7–12), varied substantially as a function of stress exposure and anxiety response. During these sessions the animals ingested unsweetened ethanol. It seems that animals with high baseline levels of anxiety response consumed more ethanol than AAs, but only when not exposed to stress. Stress exposure appeared to induce a reduction in ethanol acceptance in HAs; and these patterns seemed to be similar for gram per kilogram of ethanol ingested and for percent preference for ethanol. Fig. 4 depicts the data in separate graphs, one for each level of anxiety response; whereas Fig. 5 depicts the same data separated by stress condition, so as to facilitate comparison of (stressed or non-stressed) HA, AA and LA rats on the same graph.

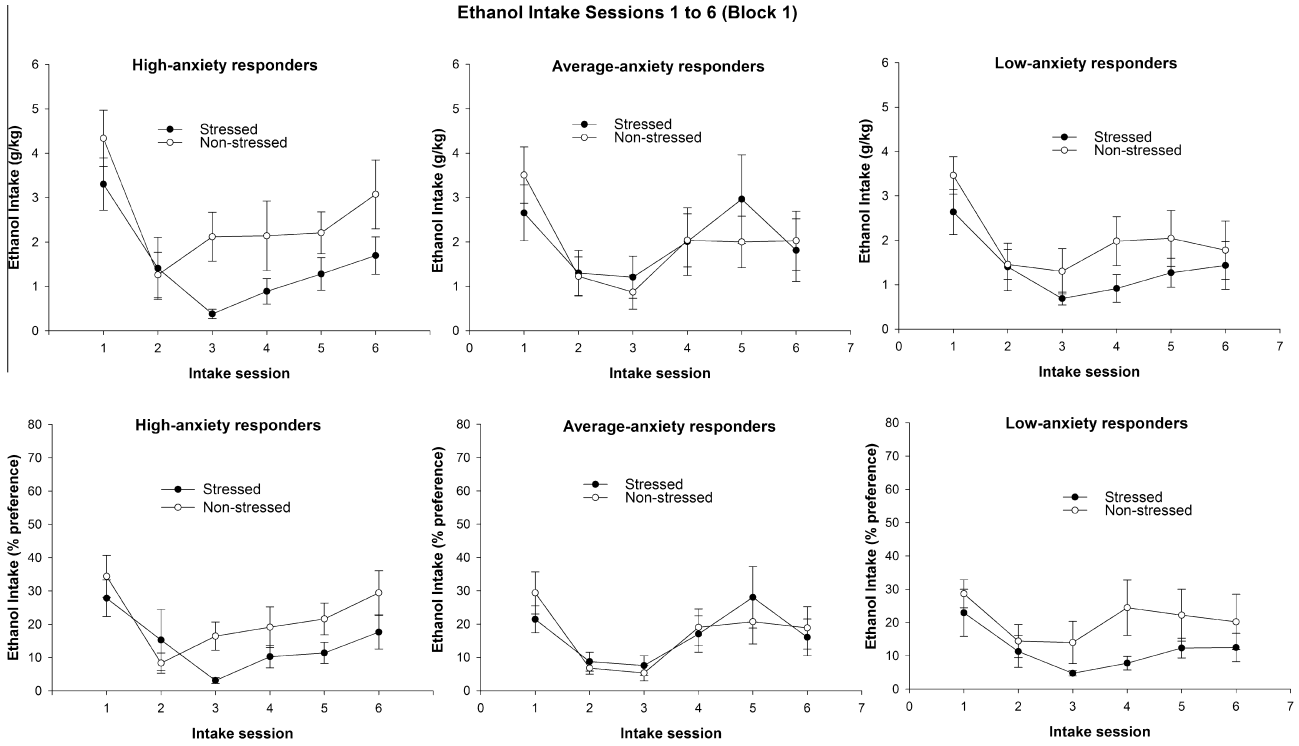

Fig. 3. Ethanol intake (g/kg and percent preference) (upper and lower panels, respectively) in female adolescent rats as a function of anxiety response (high-, average- or low-anxiety response), stress exposure (stressed or non-stressed) and ethanol intake session (1 to 6, Block 1). During intake sessions 1–3 and 4–6 ethanol (5% v/v) was mixed with 1% or 0.5% sucrose, respectively. Please note that no stress exposure occurred during Block 1. The ANOVAs indicated that, across groups, absolute and percent ethanol intake was greater during the first session than during the remaining sessions. Please refer to the text for a full account of the significant differences observed. Percent preference exhibited a significant increment in session 4 relative to sessions 2 and 3. The data are expressed as mean ± SEM.

The ANOVAs revealed significant main effects of Session [$F_{(5,365)} = 17.45$, $\eta^2p = 0.19$ and $F_{(5,365)} = 14.26$, $\eta^2p = 0.16$; for g/kg and percent preference, respectively, $ps < 0.001$)] and Stress [$F_{(1,73)} = 3.89$, $\eta^2p = 0.05$ and $F_{(1,73)} = 5.16$; for g/kg and percent preference, respectively, $ps < 0.001$)]. The three-way interaction between anxiety response, stress exposure and session also achieved significance, $F_{(10,365)} = 1.87$, $\eta^2p = 0.05$ and $F_{(10,365)} = 2.04$, $\eta^2p = 0.05$, for g/kg and percent preference, respectively, $ps < 0.05$.

To further understand the loci of the three-way interaction, two sets of follow-up ANOVAs were conducted. In the first set, ANOVAs [between factor: anxiety response, within factor: intake session 7–12] were separately conducted for stressed and for non-stressed animals. The aim was to assess the effect of anxiety response on ethanol acceptance. The ANOVAs for the stressed animals revealed only a significant main effect of session, $F_{(5,135)} = 14.42$, $\eta^2p = 0.35$ and $F_{(5,135)} = 13.48$, $\eta^2p = 0.33$, for g/kg and percent preference, respectively, $ps < 0.001$. Planned comparisons indicated that ethanol intake in stressed animals gradually increased from sessions 7–8 to sessions 9–10 and then peaked at sessions 11–12. Group assignment as function of anxiety response and the interaction between the latter factor and session did not achieve significance, for neither of these variables.

For non-stressed animals, the ANOVA for g/kg ethanol consumed revealed a significant main effect of session and a significant session × level of anxiety response interaction, $F_{(5,230)} = 6.97$, $\eta^2p = 0.13$, $p < 0.001$, and $F_{(10,235)} = 2.16$, $\eta^2p = 0.09$, $p < 0.05$. The ANOVA for percent ethanol preference, in turn, indicated significant main effects of Level of anxiety response and Session [$F_{(2,46)} = 3.25$, $\eta^2p = 0.12$ and $F_{(5,230)} = 6.13$, $\eta^2p = 0.12$, both $p < 0.05$], as well as a significant anxiety response × session interaction, $F_{(10,230)} = 2.67$, $\eta^2p = 0.10$, $p < 0.005$. Planned comparisons indicated that in sessions 9, 10, 11 and 12 percent ethanol intake was significantly greater in subjects with high level of anxiety (HA) than in adolescents with average level of anxiety (AA). Similarly, planned comparisons also indicated that g/kg of ethanol consumed was significantly greater in HA, when compared to AA counterparts, in sessions 10, 11 and 12. LAs exhibited an intermediate level of ethanol intake and preference, not differing at any session from HA or AA subjects.

The second set of follow-up ANOVAs were conducted for each level of anxiety response, and included stress exposure (i.e., stressed or non-stressed) and session as between and within factors, respectively. The aim of these analyses was to understand the locus of the significant simple and interactive effects of stress on ethanol intake, as found in the general ANOVA.

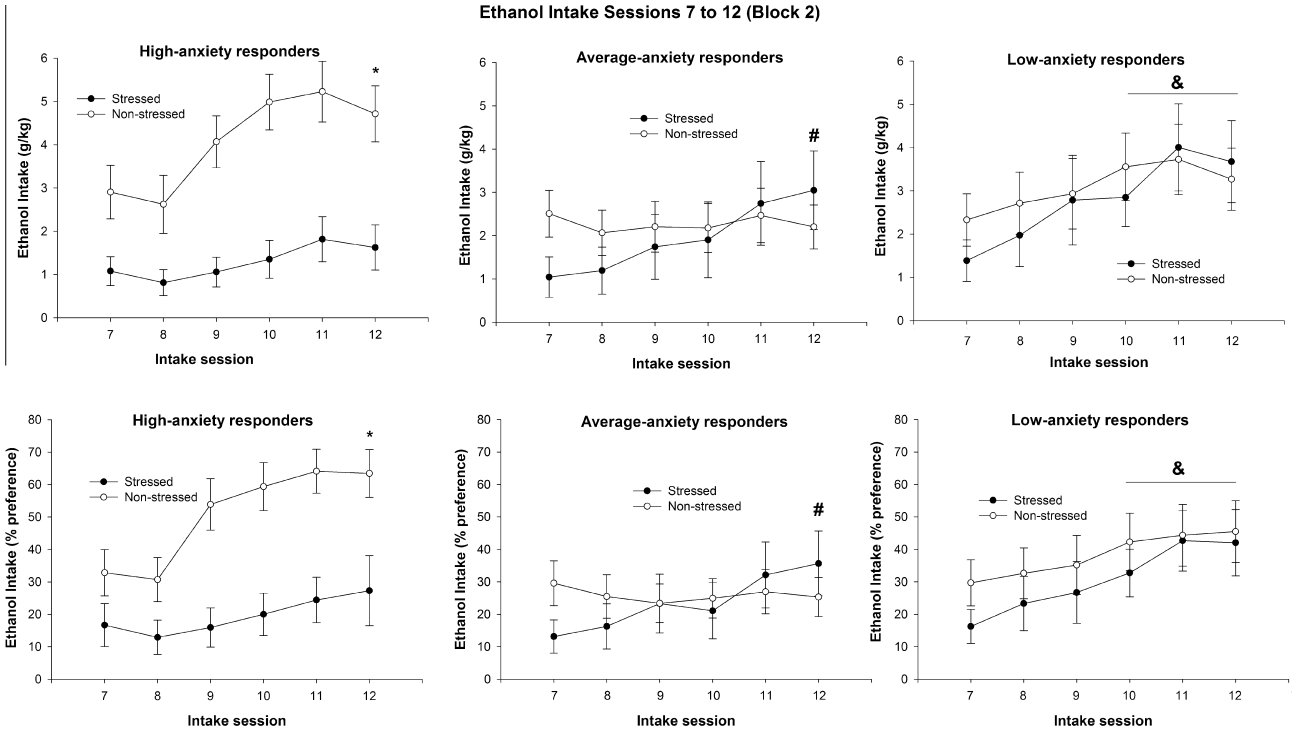

Fig. 4. Ethanol intake (g/kg and percent preference) (upper and lower panels, respectively) in female adolescent rats as a function of anxiety response (high-, average- or low-anxiety response), stress exposure and ethanol intake session (7–12, Block 2). Throughout these sessions animals self-administered unsweetened 5% ethanol. Stress was applied only during the first week of Block 2 (i.e., immediately before sessions 7, 8 and 9). The asterisks indicate that ethanol preference and intake significantly increased from session 7 to 12 ($p < 0.05$ and $p < 0.005$, respectively) in high-anxiety, non-stressed, animals. The pound signs indicate that ethanol intake (g/kg and %) in stressed AA adolescents was significantly greater in the last than in the first session ($p < 0.05$). The ampersand signs indicate that among low-anxiety responders, both stressed or non-stressed, ethanol consumption and preference was significantly greater in the sessions 10–12 than in the sessions 7–9 ($p \leqslant 0.05$). The data are expressed as mean ± SEM.

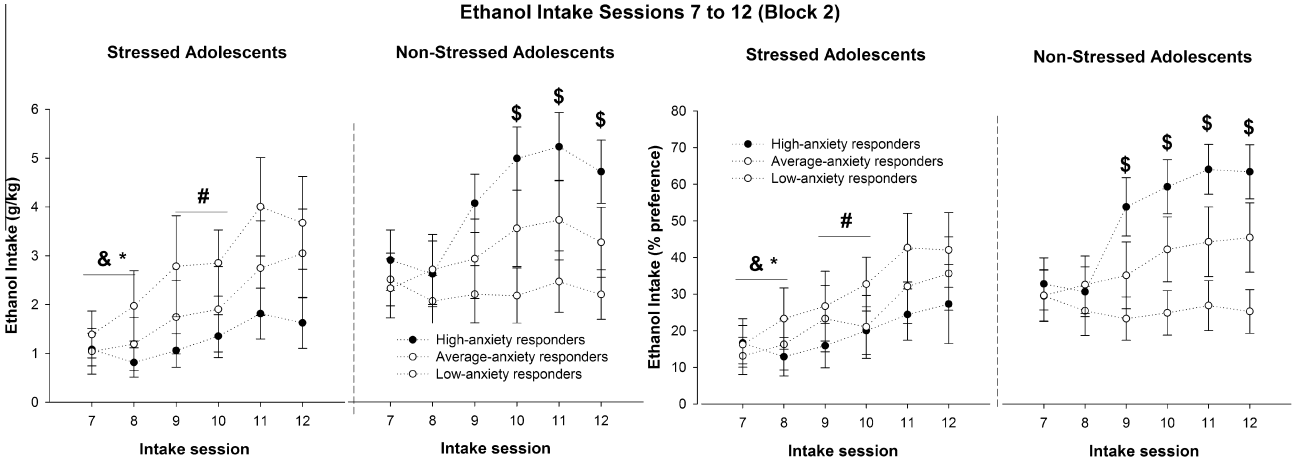

Fig. 5. This figure depicts the same data as Fig. 4 (please refer to its legend for a full account of the groups and variables depicted), yet separated by stress condition, so as to facilitate comparison of ethanol intake in (stressed or non-stressed) high-, average-and low-anxiety rats on the same graph. The ampersand and the asterisk signs indicate that stressed adolescents, regardless the level of anxiety response, exhibited significantly greater ethanol intake and preference in sessions 9–10 ($p < 0.05$) or in sessions 11–12 ($p < 0.001$), respectively, than in sessions 7–8. The pound sign indicates that stressed adolescents, regardless the level of anxiety response, exhibited significantly greater ethanol intake and preference in sessions 11–12 than in sessions 9–10 ($p < 0.05$). The $ sign indicates a significant difference ($p < 0.05$) in ethanol intake or preference, for a given testing session, between adolescents with high level of anxiety and adolescents with average level of anxiety. The data are expressed as mean ± SEM.

The ANOVAs for the HA group indicated a significant main effect of Session and Stress [$F_{(5,120)} = 9.47$, $\eta^2p = 0.28$, $p < 0.001$; $F_{(1,24)} = 10.54$, $\eta^2p = 0.30$, $p < 0.05$ and $F_{(5,120)} = 10.86$, $\eta^2p = 0.31$, $p < 0.001$; $F_{(1,24)} = 10.51$, $\eta^2p = 0.30$, $p < 0.001$; for absolute (g/kg) and percent ethanol intake, respectively] and a

Table 2. Overall Fluid Intake (ml/100 g) in female adolescent rats classified as low-, average- or high-anxiety (LA, AA or HA) as a function of their baseline anxiety response during block 1 and 2 of the intake protocol

	BLOCK 1					
Baseline anxiety response	Session 1 (PD32)	Session 2 (PD34)	Session 3 (PD36)	Session 4 (PD39)	Session 5 (PD41)	Session 6 (PD43)
LA	34.48 ± 2.55	35.18 ± 2.62	34.53 ± 2.51	27.85 ± 1.64	27.60 ± 1.74	27.89 ± 1.72
AA	34.93 ± 2.62	38.88 ± 3.14	40.70 ± 2.90	28.79 ± 1.94	27.52 ± 1.76	27.44 ± 2.04
HA	34.71 ± 2.18	36.97 ± 2.66	35.16 ± 2.58	25.88 ± 1.63	27.39 ± 2.01	26.93 ± 2.25
	BLOCK 2					
Baseline anxiety response	Session 7 (PD46)	Session 8 (PD48)	Session 9 (PD50)	Session 10 (PD53)	Session 11 (PD55)	Session 12 (PD57)
LA	21.10 ± 1.37	20.82 ± 1.38	20.79 ± 0.99	21.51 ± 1.00	21.30 ± 0.73	19.62 ± 0.87
AA	21.37 ± 1.54	19.59 ± 0.98	20.45 ± 1.02	20.40 ± 1.00	20.84 ± 1.10	20.62 ± 1.08
HA	23.08 ± 1.67	20.36 ± 1.36	20.69 ± 1.35	21.84 ± 2.01	21.66 ± 1.42	19.80 ± 1.31

significant interaction between stress and session for percent ethanol intake, $F_{(5,120)} = 2.41$, $\eta^2p = 0.09$, $p < 0.05$. Stressed HA subjects, drank significantly less (g/kg and % preference) than unstressed HA counterparts. Planned comparisons indicated that ethanol preference and intake significantly increased from session 7–12 in HA unstressed animals, but remained stable in HA, stressed animals.

The ANOVAs for adolescents with average-anxiety patterns (AA group) indicated a main effect of session [$F_{(5,125)} = 3.33$, $\eta^2p = 0.12$, $p < 0.01$] for g/kg ethanol ingested and a significant interaction between stress and session, [$F_{(5,125)} = 2.99$, $\eta^2p = 0.11$ and $F_{(5,125)} = 2.46$, $\eta^2p = 0.09$, $p < 0.05$; for absolute (g/kg) and percent ethanol intake, respectively]. Planned comparisons revealed that ethanol intake (g/kg and %) in stressed AA adolescents was significantly greater in the last than in the first session. Ethanol intake in non-stressed, AA subjects remained stable across sessions.

The ANOVA for animals classified as LAs (LA Group) only yielded a significant main effect of session [$F_{(5,120)} = 6.66$, $\eta^2p = 0.22$ and $F_{(5,120)} = 5.20$, $\eta^2p = 0.20$, both $p < 0.005$; for absolute (g/kg) and percent ethanol intake]. Planned comparisons indicated greater ethanol consumption and preference in the sessions 10–12 than in the sessions 7–9.

Overall fluid intake was similar across groups. The ANOVA for overall fluid intake (ml/100 g) scores during the first block of sessions (when animals where given a bottle of 5% ethanol mixed in 1% or 0.5% sucrose and a bottle of tap water) revealed only a significant main effect of session, $F_{(5,365)} = 37.86$, $\eta^2p = 0.34$, $p < 0.001$. Post-hoc tests indicated greater liquid intake during the first three than during the last three sessions. The ANOVA for the second block of sessions (when animals where given a bottle of 5% ethanol mixed in tap water and a bottle of tap water) revealed the lack of significant main effects or significant interactions between the factors under analysis. Descriptive data for overall fluid intake (mean and SEM) across groups can be found in Table 2.

Ethanol intake scores were also analyzed in subjects that fell between high- and AAs, and between average- and LAs (i.e., unclassified subjects). The ANOVAs for scores during block 1 yielded a significant main effect of Session, $F_{(5,240)} = 9.25$, $\eta^2p = 0.22$, $F_{(5,235)} = 6.20$, $\eta^2p = 0.11$, both $p < 0.001$, for absolute and percent ethanol preference, respectively. Ethanol intake was greater in the first intake sessions than in the subsequent sessions. The ANOVA for g/kg ethanol ingested during block 2 revealed significant main effects of stress exposure [$F_{(1,50)} = 7.21$, $p < 0.01$, $\eta^2p = 0.12$] and session, $F_{(5,250)} = 7.77$, $\eta^2p = 0.13$, $p < 0.001$. Planned comparisons revealed greater absolute and percent ethanol consumption in sessions 10, 11 and 12 than in session 1; as well as less ethanol intake in session 2 than the last session. Perhaps more important, planned comparisons also indicated that average absolute ethanol intake was significantly lower in stressed (1.60 ± 0.31 g/kg) than in control, unstressed subjects (3.19 ± 0.39 g/kg). Stressed subjects also exhibited less percent ethanol preference than unstressed controls (24.63 ± 5.10 vs 39.47 ± 4.47), [significant main effect of Stress, $F_{(1,50)} = 4.42$, $p < 0.05$, $\eta^2p = 0.08$].

Experiment 2

Overall anxiety scores in HA, AA and LA animals were −0.79 ± 0.033, −0.06 ± 0.02 and 0.92 ± 0.08, respectively. Standardized scores in EPM and LDB tests were as follows: −0.82 ± 0.04 and −0.77 ± 0.05, −0.17 ± 0.14 and 0.52 ± 0.15, 0.81 ± 0.83 and 1.03 ± 0.45 for HA, MA and LA animals, respectively. Significant differences in overall anxiety response between the groups were confirmed by a one-way ANOVA, $F_{(2,39)} = 286.03$, $\eta^2p = 0.94$, $p < 0.001$. Post-hoc tests indicated that each group was different from the others.

The one-way ANOVAs that included the untreated group (UT) revealed similar level of Fos-ir between the non-stressed group (NS) and the UT group of animals (all *p*s > 05). This confirmed that the test for anxiety response did not induce unspecific neural activation. The ANOVAs for number of positive Fos-ir at CEA, BLA and Arc did not yield significant main effects of stress

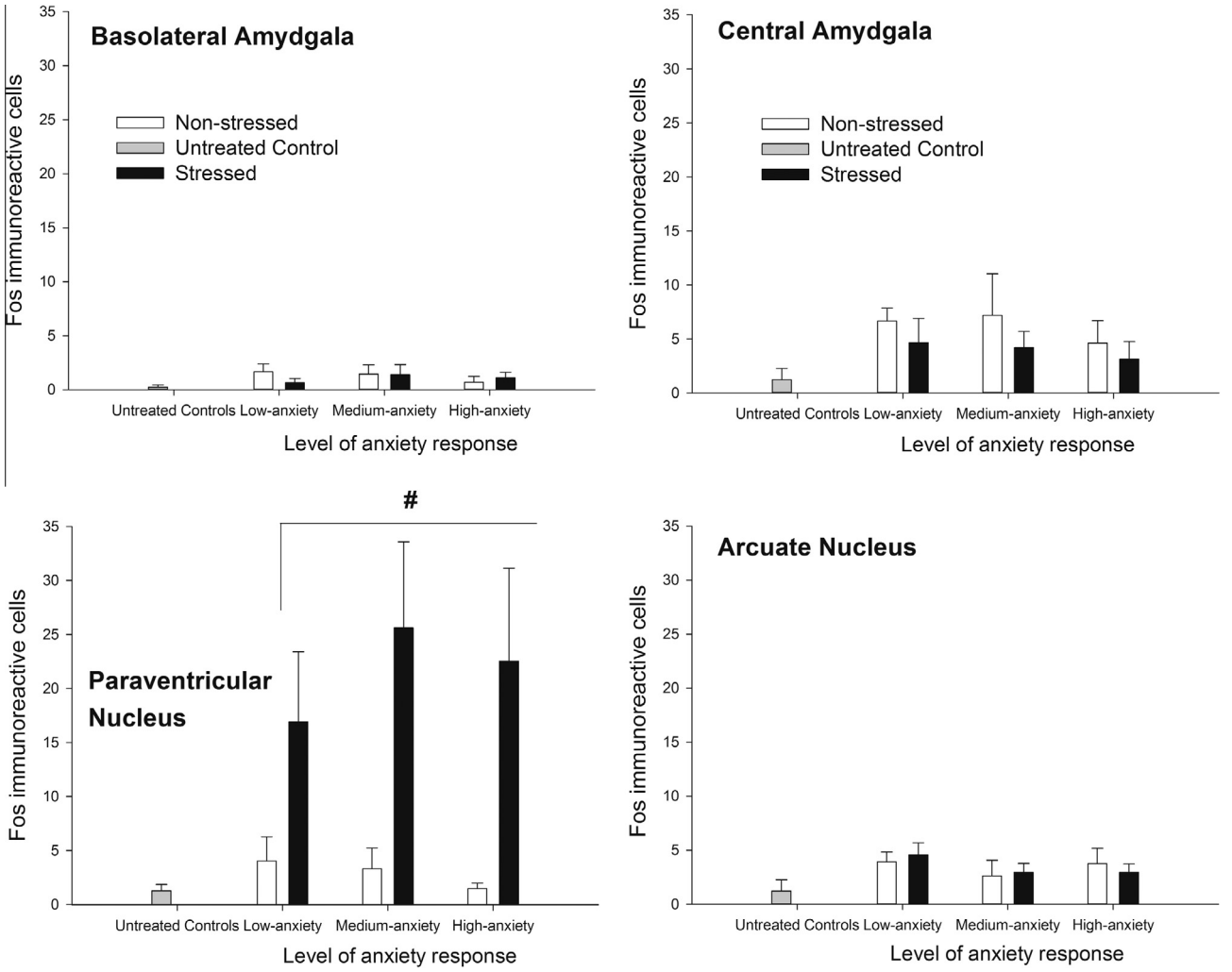

Fig. 6. Fos immunoreactivity in basolateral and central amygdala, paraventricular nucleus and arcuate nucleus of female adolescent rats that had been exposed or not to acute restraint stress (Stressed and Non-Stressed groups, respectively), as a function of anxiety response (low-, average- or high-anxiety, response). The level of anxiety response was assessed by the elevated plus maze and light–dark box tests. A separate group of rats was left untreated during this screening. These animals were pair-housed up to the perfusion, to control for any unspecific effects of housing that could affect Fos-ir. The pound sign indicates a significant main effect of stress treatment ($p < 0.001$) upon Fos-immunoreactivity in paraventricular nucleus. Vertical bars indicate the standard error of the means.

exposure or level of anxiety response. The interaction between these factors was also not significant. The ANOVA for Fos-ir at PVN, in turn, yielded a significant effect of stress exposure [$F_{(1,24)} = 16.76$, $\eta^2 p = 0.41$, $p < 0.001$]. RS induced a significant, fourfold increase in Fos-ir at PVN, which was similar in HA, AA and LA animals. These results can be observed in Fig. 6. Fig. 7 illustrates the pattern found at PVN via representative photomicrographs.

DISCUSSION

The main finding of the present study was that those female youth with high levels of inborn anxiety exhibited significantly higher ethanol intake than counterparts with normal levels of anxiety. High-anxiety responders exhibited a twofold increase in absolute ethanol intake and a threefold increase in percent ethanol intake, in comparison with counterparts that exhibited normal or average-anxiety response. Notably, the facilitating influence of elevated inborn anxiety on ethanol intake was only observed in animals devoid of explicit stress exposure. Differences as a function of baseline anxiety were observed only in sessions in which ethanol was mixed with tap water. No differences across groups were found when ethanol was mixed with slightly sweetened sucrose, nor were differences observed in terms of overall intake across tests.

These results are consistent with some, but not all, studies that analyzed anxiety-driven ethanol in adulthood. An important caveat in these comparisons is that most of these previous studies employed only male subjects. Spanagel et al. (1995) found significantly greater ethanol intake in ‘‘anxious” than in ‘‘non-anxious” adult males, throughout an 8-day procedure in which Wistar rats were given 2% or 4% ethanol solutions.

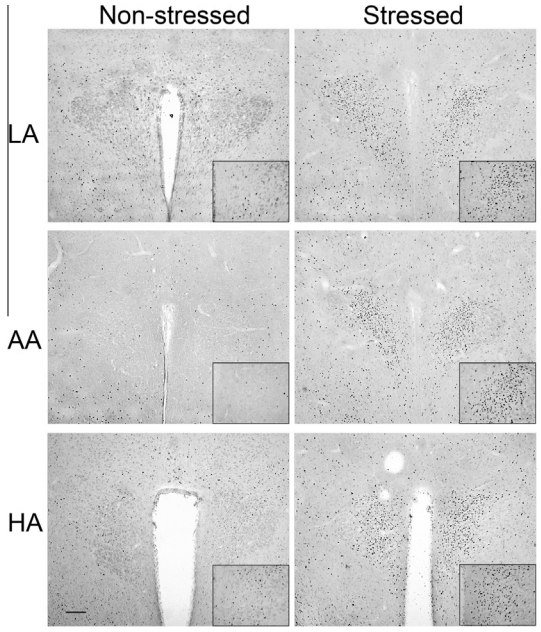

Fig. 7. Microphotographs illustrating neurons exhibiting Fos-immunoreactivity in the paraventricular nucleus of adolescent female rats that had been exposed or not to acute restraint stress (Stressed and Non-Stressed groups, respectively), as a function of anxiety response (low- average- or high-anxiety response). The level of anxiety response was assessed at PD30 by the elevated plus maze and light–dark box tests. Horizontal line represents 200 μm.

Spanagel et al. (1995) observed that blood ethanol concentrations achieved by anxious rats were similar to those inducing anxiolytic effects in rats tested in an elevated plus maze; and other researchers (Blatt and Takahashi, 1999) found that male Wistar rats classified as "anxious" after an EPM test, exhibit conditioned place preference by ethanol at doses that those rats classified as "normal" or "non-anxious" did not.

Results obtained in rats selectively bred for high-anxiety response or for high ethanol intake have been, however, contradictory. Ethanol-preferring (P) male rats spent less time in the open arms of an elevated plus maze and exhibit greater conditioned fear than ethanol-non preferring (NP) rats (Stewart et al., 1993). Female Floripa L rats, selected for their low scores of locomotion in the central section of an open field (which is considered an index of anxiety) exhibited greater percent predilection for ethanol than male rats and than Floripa H rats, selected for high scores of locomotion in the central section of the open field (Izidio and Ramos, 2007). Moreover, male rats selectively bred for high reactivity to stress (i.e., reduced swim activity after a shock) consume high quantities of ethanol (West et al., 2015). Although these studies seem to favor the hypothesis of a positive association between anxiety response and ethanol predilection, other studies with male and female Floripa rats found no differences in ethanol intake between the lines (Da Silva et al., 2004); and Henniger et al. (2002) found an inverse relationship, with LAB rats (selectively bred for low-anxiety behaviors) drinking more than HAB counterparts selected for exhibiting high-anxiety behaviors, an effect that was fairly similar in males and females.

An interesting feature of the present study is that variables extracted from two different tests were used to differentiate populations of subjects exhibiting differential anxiety responses. The use of a composite, multivariate index [similar to those employed in Parker (1995) and Pautassi et al. (2008)] favored the detection of a significant effect of level of inborn anxiety response on ethanol intake. The analyses examining the relationship between anxiety-like behavior on each test alone and subsequent ethanol drinking indicated that neither test had, by itself, a significant relationship with ethanol drinking.

The anxiety-screening strategy of the present study is in line with the suggestion of Ramos (2008). This author proposed that, since different tests of anxiety reflect different aspects of emotionality, a more reliable assessment of anxiety response requires the integration of measures derived from more than one test. Ramos (2008) proposed the so-called "triple test", a physical integration of EPM, open field and LDB in a single apparatus (Ramos et al., 2008). The alternative strategy employed in the present paper consisted of collapsing information on anxiety response derived from sequential tests. It has been similarly observed (Nielsen et al., 1999) that turning and locomotor activity together (but not activity alone) predicted voluntary ethanol consumption in Long-Evans rats. Locomotor activity in an inescapable open-field has been widely used to predict ethanol-induced reinforcement and ethanol intake. Animals exhibiting enhanced locomotor activity or lack of habituation exhibited greater ethanol intake (Bisaga and Kostowski, 1993; Nadal et al., 2002). A study by Acevedo et al. (2010) suggests that sensitivity to ethanol-induced motor activity may also predict ethanol intake in adolescent female rats.

Previous studies indicated that stress can exacerbate ethanol drinking and that male (Siegmund et al., 2005) and female (Fullgrabe et al., 2007) adolescents may be more sensitive than adults to stress-reactive drinking. Thus, our expectation was that stress would further increase ethanol consumption in HA responders. This was not corroborated. RS exerted a suppressive effect upon ethanol consumption in animals with high-anxiety response. High-anxiety female rats exposed to stress not only consumed significantly less ethanol than unstressed, high-responders, but also failed to exhibit a significant increase in ethanol consumption across the second block of intake sessions. This is, the suppressing effect of RS in HA rats persisted after cessation of stress exposure (i.e., intake stayed low rather than recovering after stress). A suppressive effect of stress was also observed in the animals that remained in-between groups (i.e., unclassified subjects), but not in those exhibiting average overall anxiety scores. Average-anxiety animals exposed to stress, but not those that were non-stressed, exhibited a significant increase in ethanol from session 7 to session 12.

This pattern of results suggests that stress exerted differential effects on ethanol intake as a function of the level of inborn anxiety response. Stress effects on ethanol intake have been often complex and contradictory. Studies assessing foot-shock effects on ethanol preference have revealed increased, decreased

or unaltered ethanol ingestion and preference, depending on parameters such as intensity and frequency of nociceptive stimulation (Pautassi et al., 2010). The differential effects of stress as a function of anxiety response may be explained by the Yerkes-Dodson law [reviewed in (Miczek et al., 2008)], which predicts enhanced and reduced drug intake following moderate and intense stress, respectively. In other words, the relationship between stress and drug intake fits an inverted U-shaped curve and, therefore, greater intake following stress would be observed if subjects are in the "sweet point" of the curve. Under this reasoning, HAs in this study may have perceived the restraint-induced stress as more intense than average or low-anxiety counterparts. It is also possible to attribute the stress-induced suppression of ethanol intake in rats with high level of anxiety to depression-like behavior in response to stress. A study from our lab (Fernandez et al., 2016) observed changes in exploratory behavior after exposure to RS during adolescence, albeit the RS was given to males only and in a more extended fashion than in the present study.

It may result surprising that subjects in most groups tended to drink more ethanol after the sucrose fade, which contrast with the drop reported by other studies. The rats in Samson et al. (1999) drank ≈1.4 g/kg/session when 10% ethanol was mixed with 10% sucrose, but only drank 0.4 g/kg/session when 10% ethanol was presented unadultered. It should be noted, however, that the usual sucrose fading procedure involves extensive initiation. Rats undergo ≈20–22 sessions in which levels of ethanol are increased (from 0% to 10%) and levels of sucrose are decreased (from 10% to 0%), before they are exposed for the first time to unadultered 10% ethanol. In the present study, the rats were exposed to mild concentrations of sucrose (1.0% or 0.5%) for only six sessions and ethanol concentration was kept at 5% throughout testing. These procedural differences likely explain the lack of a drop in ethanol intake after removal of the sweetener. The very mild concentration of sucrose employed during sessions 1–6 may also explain why no group differences in ethanol or vehicle intake were observed during these sessions. Decreased preference for low concentrations of sucrose may reflect anhedonia (Gross and Pinhasov, 2016).

The present study, based upon the notion that HAs may drink more ethanol because they seek the negative reinforcing of this drug (Kushner et al., 1994), has several limitations. The results confirmed the association between anxiety and predisposition to drink ethanol (Experiment 1), yet provided little information toward clarifying the mechanisms underlying this association. Experiment 2 was meant to add mechanistic information, by assessing if high-responders were more sensitive, at the neural level, to aversive and stressful stimulation (Muigg et al., 2008). Such a result would have explained the suppression of increased drinking in rats with high level of anxiety. Yet the behavioral differences in anxiety response and ethanol intake, as observed in Experiment 1, did not translate into neural differences in response to acute RS. In agreement with studies conducted in adult male mice [e.g., (Kwon et al. (2006))], RS induced significant Fos-ir in PVN, yet this effect was independent of the level of anxiety response. It could be argued that this may be due to the length of the treatment, with chronic vs. acute stress being applied in ethanol intake and Fos-ir assays, respectively. Yet the suppressive effect of RS upon the ethanol intake of HA rats was observed immediately after the first stress exposure. It may be still unclear why we measured Fos-ir after only one RS exposure, when the behavioral differences were most evident after three exposures. The rationale was to avoid the potential confound of habituation to repeated stress exposure. Several studies have indicated that frequent presentation of a stressor is often associated with habituation of the neural or hormonal response to that same stressor (Weinberg et al., 2009).

The lack of significant, RS-induced Fos-ir at Arc and BLA was somehow surprising. Arc mediates stress reactivity via communication with PVN (Dallman et al., 1995), and exhibits robust RS-induced Fos-ir (Kwon et al., 2006). The integrity of the BLA, in turn, is needed for the emission of acute RS-induced neuroendocrine responses (Bhatnagar et al., 2004). Stress-induced Fos-ir at CEA was also not different from that of UT or non-stressed controls. An important limitation, however, is that we treated the BLA and the CEA as homogeneous structures. Recent work in male mice, however, have indicated that these structures feature sub-regions whose activation induces differential, even opposite, effects on anxiety measures (Haubensak et al., 2010). Specifically, the CEA can be divided into at least three sections: centrolateral (CEL), centromedial (CEM) and capsular central amygdala. The CEM is the primary output region of the amygdala, and its activation results in autonomic and behavioral responses indicative of anxiety (Tye et al., 2011). The CEL, in turn, can exert a feed-forward inhibition of CEM output. Activation of BLA somata as a whole produces anxiogenic effects, yet stimulation of the BLA-CEL projection induces acute anxiolytic effects, likely the result of this projection activating the feed-forward inhibition of CEM. Given the heterogeneous structure of CEA, it is possible that our examination of Fos-ir across the entire structure failed to reveal a clear relationship with stress exposure or with anxiety levels.

This pattern of results suggests that, under the present experimental circumstances, the overall level of neural response to the acute stressor was low. This may be because our study was conducted in adolescent instead of adult animals. Although most of the studies [e.g., (Song et al., 2007)] indicate that adolescents may be more sensitive to stress than adults, the reverse pattern occurs in certain variables (e.g., modulation of ethanol-induced sleep time; Fernandez et al., 2016) and a recent meta-analytic study (Noori et al., 2014) suggested that adolescents may be less sensitive than adults to RS. Most of these studies, however, have only focused on males. Despite this discussion, the marked sensitivity of PVN to RS – as observed in the present study -- further cements the notion that PVN is the key structure in the integration of stress information and initiation of stress responses (Jankord and Herman, 2008). In addition to featuring neurons that produce corticotrophin releasing hormone, the PVN exhibits extensive dopaminergic

afferents and projects to periaqueductal gray matter, parabrachial nucleus, nucleus of the tractus solitarius and other structures involved in the activation of the hypothalamic–pituitary–adrenal axis (Ulrich-Lai and Ryan, 2014). Other limitations were the use of females and acute stress exposure only. It has been observed that RS-induced Fos-ir desensitizes after repeated exposure in adult male mice (Kwon et al., 2006). Perhaps Fos-ir differences between high- and LAs would have been observed after repeated RS exposure. Also, we did not measure the initial (i.e., first 30–60 min) levels of ethanol intake in each session, when meaningful blood ethanol levels are typically observed. These levels might have had a significant correlation with the anxiety response of the subjects.

CONCLUSION

This study suggests that certain female adolescents, those with high levels of basal anxiety, exhibit increased predisposition for ethanol intake and preference. It also indicates that stress may exert differential effects on ethanol intake as a function of the level of pre-existing anxiety. This animal model holds promise as a benchmark for detecting subjects at-risk for AUD, assessing neural mechanisms involved and, ultimately, discovering novel therapeutics to reduce these harmful interactions between ethanol availability and inborn anxiety patterns.

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REFERENCES

Acevedo MB, Molina JC, Nizhnikov ME, Spear NE, Pautassi RM (2010) High ethanol dose during early adolescence induces locomotor activation and increases subsequent ethanol intake during late adolescence. Dev Psychobiol 52:424–440.

Acevedo MB, Nizhnikov ME, Molina JC, Pautassi RM (2014) Relationship between ethanol-induced activity and anxiolysis in the open field, elevated plus maze, light-dark box, and ethanol intake in adolescent rats. Behav Brain Res 265:203–215.

Acewicz A, Mierzejewski P, Jastrzebska A, Kolaczkowski M, Wesolowska A, Korkosz I, Samochowiec J, Bienkowski P (2012) Acoustic startle responses and prepulse inhibition of acoustic startle responses in Warsaw alcohol high-preferring (WHP) and Warsaw alcohol low-preferring (WLP) rats. Alcohol Alcohol 47:386–389.

Acewicz A, Mierzejewski P, Jastrzebska A, Korkosz I, Karas K, Sienkiewicz-Jarosz H, Samochowiec J, Kolaczkowski M, Bienkowski P (2014) Anxiety- and depressive-like traits in Warsaw alcohol high-preferring (WHP) and Warsaw alcohol low-preferring (WLP) rats. Pharmacol Biochem Behav 22:261–265.

Allen CD, Rivier CL, Lee SY (2011) Adolescent alcohol exposure alters the central brain circuits known to regulate the stress response. Neuroscience 182:162–168.

Arias C, Mlewski EC, Miller S, Molina JC, Spear NE (2009) Novelty modulates the stimulating motor effects of ethanol in preweanling rats. Pharm Biochem Behav 92:448–456.

Bahi A (2013) Individual differences in elevated plus-maze exploration predicted higher ethanol consumption and preference in outbred mice. Pharmacol Biochem Behav 105:83–88.

Bhatnagar S, Vining C, Denski K (2004) Regulation of chronic stress-induced changes in hypothalamic-pituitary-adrenal activity by the basolateral amygdala. Ann New York Acad Sci 1032:315–319.

Bisaga A, Kostowski W (1993) Individual behavioral differences and ethanol consumption in Wistar rats. Physiol Behav 54:1125–1131.

Blatt SL, Takahashi RN (1999) Experimental anxiety and the reinforcing effects of ethanol in rats. Braz J Med Biol Res 32:457–461.

Briski K, Gillen E (2001) Differential distribution of Fos expression within the male rat preoptic area and hypothalamus in response to physical vs. psychological stress. Brain Res Bull 55:401–408.

Ciccocioppo R, Economidou D, Cippitelli A, Cucculelli M, Ubaldi M, Soverchia L, et al. (2006) Genetically selected Marchigian Sardinian alcohol-preferring (msP) rats: an animal model to study the neurobiology of alcoholism. Addict Biol 11:339–355.

Cunningham MG, Bhattacharyya S, Benes FM (2002) Amygdalo-cortical sprouting continues into early adulthood: implications for the development of normal and abnormal function during adolescence. J Comp Neurol 453:116–130.

Da Silva GE, Ramos A, Takahashi RN (2004) Comparison of voluntary ethanol intake by two pairs of rat lines used as genetic models of anxiety. Braz J Med Biol 37. 1511-117.

Dallman MF, Akana SF, Strack AM, Hanson ES, Sebastian RJ (1995) The neural network that regulates energy balance is responsive to glucocorticoids and insulin and also regulates HPA axis responsivity at a site proximal to CRF neurons. Ann N Y Acad Sci 771:730–742.

Doremus TL, Brunell SC, Rajendran P, Spear LP (2005) Factors influencing elevated ethanol consumption in adolescent relative to adult rats. Alcohol Clin Exp Res 29:1796–1808.

Engin E, Trait D (2008) The effects of intra-cerebral drug infusions on animals' unconditioned fear reactions: A systematic review. Prog Neuropsychopharmacol Biol Psychiatry 32:1399–1419.

Fabio MC, Macchione AF, Nizhnikov ME, Pautassi RM (2015) Prenatal ethanol increases ethanol intake throughout adolescence, alters ethanol-mediated aversive learning, and affects mu but not delta or kappa opioid receptor mRNA expression. Eur J Neurosci 41:1569–1579.

Fabio MC, Nizhnikov ME, Spear NE, Pautassi RM (2014) Binge ethanol intoxication heightens subsequent ethanol intake in adolescent, but not adult, rats. Dev Psychobiol 56:574–583.

Felix-Ortiz AC, Beyeler A, Seo C, Leppla CA, Wildes CP, Tye KM (2013) BLA to vHPC inputs modulate anxiety-related behaviors. Neuron 79:658–664.

Felix-Ortiz AC, Burgos-Robles A, Bhagat ND, Leppla CA, Tye KM (2016) Bidirectional modulation of anxiety-related and social behaviors by amygdala projections to the medial prefrontal cortex. Neuroscience 321:197–209.

Fernandez MS, Fabio MC, Miranda-Morales RS, Virgolini MB, De Giovanni LN, Hansen C, et al. (2016) Age-related effects of chronic restraint stress on ethanol drinking, ethanol-induced sedation, and on basal and stress-induced anxiety response. Alcohol 51:89–100.

Fullgrabe MW, Vengeliene V, Spanagel R (2007) Influence of age at drinking onset on the alcohol deprivation effect and stress-induced drinking in female rats. Pharm Biochem Behav 86:320–326.

Gross M, Pinhasov A (2016) Chronic mild stress in submissive mice: Marked polydipsia and social avoidance without hedonic deficit in the sucrose preference test. Behav Brain Res 298:25–34.

Haubensak W, Kunwar PS, Cai H, Ciocchi S, Wall NR, Ponnusamy R, Biag J, Dong HW, Deisseroth K, Callaway EM, Fanselow MS, Lüthi A, Anderson DJ (2010) Genetic dissection of an amygdala microcircuit that gates conditioned fear. Nature 468:270–276.

Henniger MS, Spanagel R, Wigger A, Landgraf R, Holter SM (2002) Alcohol self-administration in two rat lines selectively bred for extremes in anxiety-related behavior. Neuropsychopharmacology 26:729–736.

Hobbs JD, Kushner MG, Lee SS, Reardon SM, Maurer EW (2011) Meta-analysis of supplemental treatment for depressive and anxiety disorders in patients being treated for alcohol dependence. Am J Addict 20:319–329.

Izidio GS, Ramos A (2007) Positive association between ethanol consumption and anxiety-related behaviors in two selected rat lines. Alcohol 41:517–524.
Jankord R, Herman JP (2008) Limbic regulation of hypothalamo–pituitary–adrenocortical function during acute and chronic stress. Ann N Y Acad Sci 1148:64–73.
Keshavarzy F, Bonnet C, Behzadi G, Cespuglio R (2015) Expression patterns of c-Fos early gene and phosphorylated ERK in the rat brain following 1-h immobilization stress: concomitant changes induced in association with stress-related sleep rebound. Brain Struct Funct 220:1793–1804.
Klebaur JE, Bardo MT (1999) Individual differences in novelty seeking on the playground maze predict amphetamine conditioned place preference. Pharm Biochem Behav 63:131–136.
Koob GF (2009) Brain stress systems in the amygdala and addiction. Brain Res 1293:61–75.
Kumar V, Bhat ZA, Kumar D (2013) Animal models of anxiety: a comprehensive review. J Pharmacol Toxicol Methods 68:175–183.
Kushner MG, Sher KJ, Wood MD, Wood PK (1994) Anxiety and drinking behavior: moderating effects of tension-reduction alcohol outcome expectancies. Alcohol Clin Exp Res 18:852–860.
Kwon MS, Seo YJ, Shim EJ, Choi SS, Lee JY, Suh HW (2006) The effect of single or repeated restraint stress on several signal molecules in paraventricular nucleus, arcuate nucleus and locus coeruleus. Neuroscience 142:1281–1292.
Lancaster FE, Brown TD, Coker KL, Elliott JA, Wren SB (1996) Sex differences in alcohol preference and drinking patterns emerge during the early postpubertal period. Alcohol Clin Exp Res 20:1043–1049.
Maren S, Quirk GJ (2004) Neuronal signalling of fear memory. Nat Rev Neurosci 5:844–852.
Menard J, Treit D (1999) Effects of centrally administered anxiolytic compounds in animal models of anxiety. Neurosci Biobehav Rev 23:591–613.
Miczek KA, Yap JJ, Covington 3rd HE (2008) Social stress, therapeutics and drug abuse: preclinical models of escalated and depressed intake. Pharmacol Ther 120:102–128.
Moller C, Wiklund L, Sommer W, Thorsell A, Heilig M (1997) Decreased experimental anxiety and voluntary ethanol consumption in rats following central but not basolateral amygdala lesions. Brain Res 760:94–101.
Muigg P, Hetzenauer A, Hauer G, Hauschild M, Gaburro S, Frank E, et al. (2008) Impaired extinction of learned fear in rats selectively bred for high anxiety–evidence of altered neuronal processing in prefrontal-amygdala pathways. Eur J Neurosci 28:2299–3309.
Nadal R, Armario A, Janak PH (2002) Positive relationship between activity in a novel environment and operant ethanol self-administration in rats. Psychopharmacology 162:333–338.
Nadal R, Rotllant D, Marquez C, Armario A (2005) Perseverance of exploration in novel environments predicts morphine place conditioning in rats. Behav Brain Res 165:72–79.
National Research Council (1996) Guide for the care and use of laboratory animals. Washington, D.C.: National Academy Press.
Nielsen DM, Crosley KJ, Keller Jr RW, Glick SD, Carlson JN (1999) Ethanol induced differences in medial prefrontal cortex dopamine asymmetry and in nucleus accumbens dopamine metabolism in left- and right-turning rats. Brain Res 823:207–212.
Noori HR, Helinski S, Spanagel R (2014) Cluster and meta-analyses on factors influencing stress-induced alcohol drinking and relapse in rodents. Addict Biol 19:225–232.
Ottoni EB (2000) EthoLog 2.2: a tool for the transcription and timing of behavior observation sessions. Behav Res Methods Instrum Comput 32:446–449.
Parker LA (1995) Rewarding drugs produce taste avoidance, but not taste-aversion. Neurosci Biobehav Rev 19:143–151.
Pautassi RM, Arias C, Molina JC, Spear NE (2008) Domperidone interferes with conditioned disgust reactions but not taste avoidance evoked by a LiCl-paired taste in infant rats. Dev Psychobiol 50:343–352.
Pautassi RM, Camarini R, Quadros IM, Miczek KA, Israel Y (2010) Genetic and environmental influences on ethanol consumption: perspectives from preclinical research. Alcohol Clin Exp Res 34:976–987.
Paxinos G, Watson C (2007) The rat brain in stereotaxic coordinates. 6th ed. Elsevier Academic Press: San Diego.
Perkins HW (2002) Surveying the damage: a review of research on consequences of alcohol misuse in college populations. J Stud Alcohol Suppl 2002:91–100.
Pisu MG, Garau A, Boero G, Biggio F, Pibiri V, Dore R, et al. (2016) Sex differences in the outcome of juvenile social isolation on HPA axis function in rats. Neuroscience 320:172–182.
Ponce LF, Pautassi RM, Spear NE, Molina JC (2008) Ethanol-mediated operant learning in the infant rat leads to increased ethanol intake during adolescence. Pharmacol Biochem Behav 90:640–650.
Primeaux SD, Wilson SP, Bray GA, York DA, Wilson MA (2006) Overexpression of neuropeptide Y in the central nucleus of the amygdala decreases ethanol self-administration in ‘‘anxious” rats. Alcohol Clin Exp Res 30(5):791–801.
Ramos A (2008) Animal models of anxiety: do I need multiple tests? Trends Pharmacol Sci 29:493–498.
Ramos A, Pereira E, Martins GC, Wehrmeister TD, Izidio GS (2008) Integrating the open field, elevated plus maze and light/dark box to assess different types of emotional behaviors in one single trial. Behav Brain Res 193:277–288.
Roman E, Stewart RB, Bertholomey ML, Jensen ML, Colombo G, Hyytiä P, et al. (2012) Behavioral profiling of multiple pairs of rats selectively bred for high and low alcohol intake using the MCSF test. Addict Biol 17:33–46.
Samson HH, Slawecki CJ, Sharpe AL, Chappell A (1998) Appetitive and consummatory behaviors in the control of ethanol consumption: a measure of ethanol seeking behavior. Alcohol Clin Exp Res 22:1783–1787.
Samson HH, Files FJ, Denning C (1999) Chronic ethanol self-administration in a continuous-access operant situation: the use of a sucrose/ethanol solution to increase daily ethanol intake. Alcohol 19:151–155.
Sartor CE, Jackson KM, McCutcheon VV, Duncan AE, Grant JD, Werner KB, Bucholz KK (2016) Progression from First Drink, First Intoxication, and Regular Drinking to Alcohol Use Disorder: A Comparison of African American and European American Youth. Alcohol Clin Exp Res 40:1515–1523.
Siegmund S, Vengeliene V, Singer MV, Spanagel R (2005) Influence of age at drinking onset on long-term ethanol self-administration with deprivation and stress phases. Alcohol Clin Exp Res 29:1139–1145.
Simms JA, Steensland P, Medina B, Abernathy KE, Chandler LJ, Wise R, Bartlett SE (2008) Intermittent access to 20% ethanol induces high ethanol consumption in Long-Evans and Wistar rats. Alcohol Clin Exp Res 32:1816–1823.
Song M, Wang X-Y, Zhao M, Wang X-Y, Zhai H-F, Lu L (2007) Role of stress in acquisition of alcohol-conditioned place preference in adolescent and adult mice. Alcohol Clin Exp Res 31:2001–2005.
Spanagel R, Montkowski A, Allingham K, Stohr T, Shoaib M, Holsboer F, et al. (1995) Anxiety: a potential predictor of vulnerability to the initiation of ethanol self-administration in rats. Psychopharmacology 122:369–373.
Spear LP (2000) The adolescent brain and age-related behavioral manifestations. Neurosci Biobehav Rev 24:417–463.
Stewart RB, Gatto GJ, Lumeng L, Li TK, Murphy JM (1993) Comparison of alcohol-preferring (P) and nonpreferring (Np) rats on tests of anxiety and for the anxiolytic effects of ethanol. Alcohol 10:1–10.
Tye KM, Prakash R, Kim SY, Fenno LE, Grosenick L, Zarabi H, Thompson KR, Gradinaru V, Ramakrishnan C, Deisseroth K (2011) Amygdala circuitry mediating reversible and bidirectional control of anxiety. Nature 471:358–362.
Ulrich-Lai YM, Ryan KK (2014) Neuroendocrine circuits governing energy balance and stress regulation: functional overlap and therapeutic implications. Cell Metab 19:910–925.

Varlinskaya EI, Spear LP (2012) Increases in anxiety-like behavior induced by acute stress are reversed by ethanol in adolescent but not adult rats. Pharmacol Biochem Behav 100:440–450.

Weinberg MS, Bhatt AP, Girotti M, Masini CV, Day HE, Campeau S, Spencer RL (2009) Repeated ferret odor exposure induces different temporal patterns of same-stressor habituation and novel-stressor sensitization in both hypothalamic-pituitary-adrenal axis activity and forebrain c-fos expression in the rat. Endocrinology 150:749–761.

West CH, Boss-Williams KA, Ritchie JC, Weiss JM (2015) Locus coeruleus neuronal activity determines proclivity to consume alcohol in a selectively-bred line of rats that readily consumes alcohol. Alcohol 49:691–705.

Winer B, Brown DR, Michels KM (1991) Statistical principles in experimental design. 3rd ed. New York: McGraw-Hill.

Wisowska-Stanek A, Lehner M, Skórzewska A, Krząścik P, Paźnik A (2016) Behavioral effects and CRF expression in brain structures of high- and low-anxiety rats after chronic restraint stress. Behav Brain Res 310:26–35.

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