Pharmacology, Biochemistry and Behavior 176 (2019) 6–15

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

The offspring of rats selected for high or low ethanol intake at adolescence exhibit differential ethanol-induced Fos immunoreactivity in the central amygdala and in nucleus accumbens core

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ARTICLE INFO

Keywords:
Short-term selective breeding
Adolescents
Ethanol-induced Fos
Maternal care

ABSTRACT

Adolescents exhibit, when compared to adults, altered responsivity to the unconditional effects of ethanol. It is unclear if this has a role in the excessive ethanol intake of adolescents. Wistar rats from the third filial generation (F_3) of a short-term breeding program which were selected for high (STDRHI) vs. low (STDRLO) ethanol intake during adolescence, were assessed for ethanol-induced (0.0, 1.25 or 2.5 g/kg) Fos immunoreactivity (Fos-ir) in the central (Ce), basolateral (BLA) and medial (Me) amygdaloid nuclei; nucleus accumbens core and shell (AcbC, AcbSh), ventral tegmental area (VTA), as well as prelimbic and infralimbic (PrL, IL) prefrontal cortices. Following i.p. administration of saline, and across the structures measured, Fos-ir was significantly greater in STDRHI than in STDRLO rats. Across both lines, baseline Fos-ir was significantly lower in BLA than in any other structure, whereas PrL, IL and Shell did not differ between each other and exhibited significantly greater level of baseline neural activation than Ce, Me, AcbC and VTA. STDRLO, but not STDRHI, rats exhibited ethanol-induced Fos-ir in Ce. STRDHI, but not STDRLO, rats exhibited an ethanol-induced Fos-ir depression in AcbC. Key maternal care behaviors (i.e., grooming of the pups, latency to retrieve the pups, time spent in the nest and time adopting a kiphotic posture) were fairly similar across lines. There were significant intergenerational variations in the amount self-licking behaviors in STDRHI dams as well as an increased amount of exploration of the cage in these animals, when compared to STDRLO counterparts. These results indicate that short term selection for differential alcohol intake during adolescence yields heightened neural activity at baseline (i.e., after vehicle) in STRDHI vs. STDRLO adolescent rats, and differential sensitivity to ethanol-induced Fos immunoreactivity in Ce and in AcbC. It is unlikely that rearing patterns explained the neural differences reported, between STDRHI and STDRLO rats.

1. Introduction

Animal models of genetic risk for alcohol use disorders (AUDs) include rat lines selected for high or low-ethanol consumption during adulthood (McBride and Li, 1998). These are valuable models [e.g., alcohol preferring and non-preferring (P, NP), University of Chile abstinent and bibulous (UChA, UChB), Alko-alcohol (AA) and Alko-non-alcohol (ANA)] generated by bi-directionally selection, across several generations, of males and females that during adulthood exhibited low or high preference for ethanol.

In humans, however, ethanol intake normatively begins, and often escalates, during adolescence (Pilatti et al., 2017). The greatest percentage of individuals diagnosed with AUD are aged between 17 and 25 years (Windle and Zucker, 2010); and, in mice and rats studies, greater ethanol intake and preference in adolescents vs. adults is a common outcome (Vetter et al., 2007). The reasons underlying the apparent greater vulnerability to AUD in adolescents are not completely understood (Doremus-Fitzwater and Spear, 2016). The postnatal days 21 to 27 [PD 21–27] in the rat have been identified as analogous to the juvenile period in humans, while PD 28–42 and PD 46–59 parallel early/mid-adolescence and late adolescence, respectively (Doremus-Fitzwater and Spear, 2016)(Burke and Miczek, 2014; Karanikas et al., 2013; Spear, 2000).

We performed a short-term bi-directional selective breeding program for low and high levels of ethanol drinking (Fernandez et al., 2017), with the innovation that the selection was initiated during adolescence. In a progenitor (F_0) generation we tested ethanol intake at PD 32–57 followed by bi-directional selective mating for animals with

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https://doi.org/10.1016/j.pbb.2018.11.004
Received 11 July 2018; Received in revised form 18 October 2018; Accepted 8 November 2018
Available online 09 November 2018
0091-3057/

high and low ethanol intake. The selection process yielded lines with high and low ethanol intake (hereinafter referred to as STDRHI and STDRLO, respectively) and was repeated in filial generations 1 and 2 (F_1 and F_2, respectively). Heritability scores peaked at 0.61 for F_1 males and significant differences in ethanol drinking between STDRHI and STRDLO rats were observed across the procedure. STDRHI, alcohol naïve, rats exhibited, when compared to STDRLO counterparts, greater taste neophobia and significantly less time spent in the bright area of a light-dark chamber. This was accompanied by significantly reduced ethanol-induced taste aversion and significantly greater ethanol-induced motor stimulation (Fernandez et al., 2017). These published results suggest that the genes responsible for modulating these behavioral traits may be responsible for modulating alcohol consumption during adolescence. The neural underpinnings of these phenomena are largely unknown.

An underlying assumption of the short-term bi-directional selection program conducted in our lab is that the mating of animals that, as adolescents, exhibited high- or low- ethanol intake yields lines of rats with innate differences in sensitivity to the motivational effects of ethanol. It is possible, however, that the idiosyncratic pattern of response to ethanol in STDRHI vs. STDRLO is the consequence of other differences between the selected lines, for example, differences in maternal care (Ponce et al., 2011).

The main objective of the present study was to describe baseline (0.0 g/kg; i.e., after administration of vehicle) and ethanol-induced (1.25 or 2.5 g/kg) levels of neural activation via quantification of an inducible transcription factor (Fos immunoreactivity, Fos-ir), in STDRHI and STDRLO, male and female, rats of the third filial generation (i.e., F3). We also analyzed maternal care behaviors (i.e., time spent in a breastfeeding stance, grooming of the pups, etc.) in STDRHI and STDRLO F1 and F2 litters, on postnatal days (PDs) 7 and 12.

These F3 rats employed in the present study were derived from mating high and low ethanol-drinking F_2 parents that, as adolescents, drank weekly averages of approximately 4.0 or 1.0 g/kg ethanol/24 h, respectively [see Fig. 2 in (Fernandez et al., 2017)]. The immunoreactivity assays were conducted in brain regions that play a key role in the integration of anxiety responses or that are involved in the interactions between ethanol and stress or anxiety (i.e., basolateral, central and medial amygdala: BLA, Ce and Me, respectively) (Pucci et al., 2018), as well as brain areas involved in the processing of ethanol's motivational effects (ventral tegmental area, nucleus accumbens core and shell, and prelimbic and infralimbic medial prefrontal cortex: VTA, AcbC, AcbSh, PrL and IL, respectively) (Adriani et al., 2018; Koob and Simon, 2009).

The use of Fos-ir as a marker of ethanol-induced activation has provided important information about the mechanisms underlying ethanol's effects. Seminal studies (Chang et al., 1995; Ryabinin et al., 1997) indicated that acute administration of the drug (0.75–3.0 g/kg, i.p.) activates a wide range of brain areas, including Ce. Subsequent studies confirmed that ethanol administration (2.0–2.5 g/kg) induces Fos-ir in Ce and AcbSh and identified that, at least part of, these neurons are GABAergic [Ce: Morales et al., 1998; AcbSh: Leriche et al., 2008] or cholinergic GABAergic [AcbSh: Herring et al., 2004]. Also relevant are studies that employed measures of neuronal activity other than Fos-ir. These studies found greater basal local cerebral glucose utilization (LCGU) in P than in NP rats (Strother et al., 2008; Strother et al., 2005). This effect was found at several brain areas (e.g., Ce, BLA, AcbSh) and was more pronounced at adolescence than at adulthood.

Our previous study (Fernandez et al., 2017) revealed increased anxiety and greater taste neophobia in STDRHI vs STDRLO rats. Hence, our hypotheses were that F_3 STDRHI adolescents would exhibit, when compared to F_3 STDRLO counterparts, greater baseline neural activity in brain areas related to the integration and processing of fear and anxiety responses, namely BLA, Ce and Me. This pattern could be exacerbated by differences in maternal care between the lines. The dam-pup dyad interaction regulates the expression of glucocorticoid receptors, which are densely present in amygdala. Our previous study also revealed a blunted response to ethanol-induced aversion and greater sensitivity to ethanol-induced behavioral stimulation. We expected these behavioral differences to translate into greater ethanol-induced Fos-ir in VTA, accumbens and prefrontal areas.

2. Materials and methods

2.1. Experimental design

2.1.1. Experiment 1

Maternal care behaviors were assessed in 20 STDRHI (8 F_1, 12 F_2) and 20 STDRLO (11 F_1, 9 F_2) dams. The unit of analysis in this 2 (Line: STDRHI vs. STDRLO) × 2 (Filial generation: F_1 vs. F_2) factorial design was each litter. The litters used in this experiment were derived from the second parturition provided by these F_1 and F_2 dams.

2.1.2. Experiment 2

A total of 49 (25 male, 24 female), 37-day old, Wistar rats were employed. These F_3 animals were derived from 10 F_2 dams (5 STDRHI, 5 STDRLO). A 2 (Sex) x 2 (Line: STDRHI vs. STDRLO) × 3 (Ethanol treatment: 0.0, 1.25 or 2.5 g/kg) factorial design (n = 4 per group but for group STDRLO males 2.5 g/kg in which n = 5). The unit of analysis in this design was the pup, and each group had no more than one male and one female from each litter. These litters, different from those employed in Experiment 1, were derived from the first parturition provided by the F_2 dams.

2.2. Subjects

The rats were born and reared in the production vivarium of the Instituto de Investigaciones Médicas M.y M. Ferreyra (INIMEC-CONICET-UNC, Córdoba, Argentina), a producer of specific pathogen-free animals. The vivarium was kept under a controlled light/dark cycle (12 h/12 h, lights on at 8:00 AM) and temperature ranged between 22 and 24 °C.

The short-term selection and production of our high and low ethanol consumption lines of rats has been described in detail elsewhere (Fernandez et al., 2017). Briefly, ethanol intake during adolescence (PD32–57) was tested in a foundational nucleus (i.e., F_0) of 120 (60 male and 60 female) genetically heterogeneous Wistar rats. Animals were tested in intermittent-access intake protocol (three sessions per week, 24 h sessions, ethanol concentration was 4% and 5% in the first and second testing weeks, and 6% in the last two weeks). The 12 males and 12 females that exhibited the highest absolute ethanol intake and average percentage of ethanol preference (during the three sessions of week 3 and the three sessions of week 4) were mated together, and the same mating was done between those exhibiting the lowest ethanol intake and preference scores. Litter mates were not mated together to prevent endogamy.

These F_0 couples generated 120 F_1 STDRHI rats and 120 F_1 STDRLO rats. Ethanol intake testing and mating as a function of high or low ethanol intake preference was conducted within these F_1 STDRHI and F_1 STDRLO rats, as described for the foundational nucleus. These 24 litters made from high and low F_1 rats (i.e., 12 STDRHI and 12 STDRLO) produced 120 F_2 STDRHI rats and 120 F_2 STDRLO rats. Assessment of maternal care behaviors for the present study was made in 19 (8 STDRHI, 11 STDRLO) of these F_1 couples. The ethanol intake and mating selection process was repeated in F_2 STDRHI rats and in F_2 STDRLO rats, to generate the F_3 offspring that were used in the present study to assess basal and ethanol-induced neural activity. Maternal behavior was assessed, for the present study, in 21 (12 STDRHI, 9 STDRLO) of these F_2 litters. The F_3 rats, male or females, were not tested for ethanol intake. The neural assays reported in Experiment 2 of the present study employed a sub-set of these F_3 rats.

2.2.1. Ethical statement

Rearing and experimental procedures were reviewed and certified by the Institutional Animal Care and Use Committee at INIMEC-CONICET-UNC and were also reviewed by the Biological Studies study section of the National Agency for the Promotion of Science and Technology. The procedures complied with the Declaration of Helsinki, the ARRIVE guidelines, and the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) as promulgated by the NIH and the EU. The animals did not undergo unnecessary suffering and extensive measures were taken to prevent pain and distress. Sample sizes are similar to those published in our initial breeding experiment.

2.3. Procedures

2.3.1. Assessment of maternal care behavioral repertoire

The dams were kept undisturbed throughout gestation and lactation, aside from the cage/bedding changes of the standard operating procedures of our vivarium. On testing days (i.e., PD7 and PD 12), the dams and their pups were placed in new, clean cages for 60 min (the pups were scattered to different sections of the home cage prior to placement of the dam) and their interactions were video recorded (Flip Video Ultra, Cisco, Irvine, CA, USA) for 60 min. The video files were later decoded, using JWatcher 1.0, by a trained researcher in 5 min on, 15 min off, fractions. Following prior studies from our lab (Ponce et al., 2011), we assessed the time spent in the nest (s), time (s) spent grooming the pups and time (s) adopting a breastfeeding stance (i.e., kyphotic or arched-back nursing position, a posture that promotes nipple attachment by the pup). Two non-maternal behaviors [time spent (s) self-licking and time spent exploring the cage] were also evaluated.

2.3.2. Ethanol administration and assessment of FOS-ir

At PD37, a developmental period in which we had found significant differences in responsivity to ethanol between the selected lines (Fernandez et al., 2017), the offspring of the F_2 STDRHI and F_2 STDRLO dams were administered vehicle [i.e., 0.0 g/kg; 0.9% v/v sodium chloride (Sigma Aldrich, St. Louis)] or ethanol (1.25 or 2.5 g/kg), intraperitoneally (i.p.). The doses were generated by injecting 0.01 ml/g of a 7 or 14% v/v ethanol solution, between the diaphragm and the genitalia, approximately. Ethanol doses and post-administration sampling time were selected based on a study (Fabio et al., 2015) that measured ethanol-induced For-ir in PD37 adolescents derived from dams exposed or not to ethanol in-utero. The rationale for not using a third ethanol dose was that in this previous study (Fabio et al., 2015) the pattern of FOS-ir induced by 3.25 g/kg ethanol was similar to that induced by 2.5 g/kg. Moreover, doses lower than 2.0 g/kg usually fail to induce significant FOS-ir.

Ninety-minutes after the ethanol or vehicle administration, the rats were anesthetized with Chloral hydrate (dose: 0.001 ml/g of a 30% v/v solution) and perfused with a solution made of 0.9% heparinized saline and 4% paraformaldehyde (PFA, Sigma Aldrich, St. Louis, MO, EEUU), in 0.1-M phosphate buffer (PB; pH 7.4). Brains were left overnight in the skull, and then they were removed and stored in 30% sucrose at 4 °C until processing. Once the brains sank, four series of 40um thick sections were obtained with a freezing microtome, of which two were stored for other studies. The remaining two series were processed for FOS-ir, as described in Fabio et al. (2015). Briefly, deactivation of endogenous peroxidases of sections by incubating in peroxidase block solution of 1% H_2O_2, 10% methanol and 0.01 M PBS for 1 h., rinsed three times in PBS and incubated for 1 h. in 5% normal horse serum (NHS, Invitrogen, New Zealand). Afterwards sections were directly incubated in rabbit polyclonal antibody against c-Fos protein (sc-253; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000 in 0.1 M PBS with 1% NHS and 0.3% Triton X-100 overnight at room temperature. Later, sections were rinsed three times in 0.01 PBS and incubated for 1 h. with biotinylated donkey anti- rabbit secondary antibody (Jackson Laboratories, West Grove, PA) diluted 1:500 in 1% NHS. After three rinses the sections were then incubated for 1 h. with the avidin–biotin–peroxidase complex (ABC Elite Kit; Vector Labs, Burlingame, CA) diluted in 1% NHS. Sections were subsequently incubated for 5 min with a solution containing 0.05% 3–3-diamino-benzidine tetra hydrochloride (DAB, Sigma Aldrich, St. Louis, MO, USA) and 0.01% hydrogen peroxidase. The sections were then mounted on gelatinized slides, dehydrated and covered with DPX.

Three sections per animal were selected from each of the brain regions under analysis, following the descriptions of Paxinos G.(Paxinos and W., 2007). Specifically, for the BLA, Ce and Me, three sections were taken at bregmas −3.34, −3.00 and −2.76 mm. IL and PrL were taken at bregmas 3.34, 3.00 and 2.76 mm; whereas AcbC and AcbSh were taken from bregmas 1.68, 1.44 and 1.08 mm. VTA was taken at bregmas −6.72 and −6.84 mm. The photographs were acquired with a Primo Star iLed microscope, equipped with an AxicamERc 5 s Microscope camera (Zeiss, Jena, Germany). The counting tool of Photoshop (Adobe Systems, USA) was used to count the number of cells with activated nuclei in each structure. Data from the three sections were averaged for the subsequent statistical analysis.

2.4. Statistical analyses

Each of the maternal or non-maternal behaviors were analyzed via repeated measures ANOVAs that considered Line: (STDRHI, STDRLO) and Filial generation (F1, F2) as independent factors, and testing day (i.e., PD7 and PD12) as the within-subject measure. Data for 4 F2 STDRLO dams could not be calculated on PD7 because of corruption of video files. For similar reasons we lost the PD12 maternal data for one STDRHI F2 litter. These data were not replaced.

Potential baseline differences between STDRHI and STDRLO rats, in Fos-ir activation across the structures measured were analyzed via a three-way RM ANOVA (comparative factors between groups: Line and Sex; the within-subject factor was composed by the different brain structures under analysis: BLA, Ce, Me, AcbC, AcbSh, IL, Prl), conducted only in animals from the postnatal control groups (i.e., administered 0.0 g/kg ethanol). As shown in the results section, the analysis indicated significant baseline differences in the mean number of immunoreactive cells in STDRHI vs. STDRLO rats, and significant baseline differences in the level of baseline neural activation across the areas. Therefore, subsequent analyses of ethanol-induced Fos-ir were conducted separately for each line and structure. Specifically, separate two-way ANOVAs (comparative factors: Dose and Sex) were conducted for each brain structure under analysis, for each line. To reduce the type I error associated with these ANOVAs, we reduced the threshold of the p level to ≤ 0.006 (i.e., 0.05/8 structures analyzed in each line), using a Bonferroni-like correction.

The significant main effects and significant interactions were analyzed via *Tukey* post-hoc tests. The partial eta-squared (η^2p) was used to describe effect sizes, which were interpreted according to the guidelines described in Lakens (2013) [small ($\eta^2p = 0.01–0.05$), medium ($\eta^2p = 0.06–0.13$), and large ($\eta^2p = \geq 0.14$)].

2.4.1. Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

3. Results

The ANOVAs conducted on time spent in the nest or adopting a breastfeeding stance did not yield significant main effects or significant interactions. Frequency of grooming of the pups was higher in F_1 than in F_2 litters (significant main effect of filial generation), $F_{1, 26} = 20.97$, $p < 0.001$, $\eta^2p = 0.45$, yet similar across STDRHI and STDRLO litters

Table 1
Maternal care and non-maternal behaviors in STDRHI and STDRLO dams.

	STDRLO		STDRHI	
Variable	DP 7	DP 12	DP 7	DP 12
F_2 Generation				
Time spent in the nest	117 ± 49	180 ± 71	48 ± 36	27 ± 14
Time spent in breastfeeding stance	240 ± 82	368 ± 61	255 ± 118	418 ± 97
Grooming of the pups	180 ± 38	176 ± 37	86 ± 11	165 ± 37
Time spent exploring	463 ± 58	325 ± 46	729 ± 117	394 ± 89
Self-licking	63 ± 15	54 ± 24	44 ± 11	34 ± 12
F_3 Generation				
Time spent in the nest	110 ± 40	99 ± 39	154 ± 58	109 ± 61
Time spent in breastfeeding stance	452 ± 80	375 ± 60	350 ± 84	350 ± 56
Grooming of the pups	58 ± 13	83 ± 10	71 ± 12	55 ± 14
Time spent exploring	409 ± 39	446 ± 50	378 ± 21	368 ± 28
Self-licking	56 ± 13	59 ± 6	64 ± 11	94 ± 24

`Time spent in the nest (s), time (s) spent grooming the pups, time (s) adopting a breastfeeding stance, time spent (s) self-licking and time spent exploring the cage by dams belonging to lines selected for high or low ethanol drinking during adolescence (STDRHI and STDRLO, respectively). The measurements were conducted on postnatal days 7 and 12 of their offspring, in 20 STDRHI (8 F_1, 12 F_2) and 20 STDRLO (11 F_1, 9 F_2) dams. The data are expressed as mean ± SEM and shown as a function of filial generation (2 or 3, F_2 and F_3, respectively). Time exploring the cage was significantly greater in STDRHI F_1 dams than in any of the other groups ($F_{1,\ 26} = 6.42$, $p < 0.05$, $\eta^2p = 0.20$). No differences in self-licking, grooming of the pups, time spent in the nest or adopting a breastfeeding stance were found between STDRHI and SRDLO dams of either generation (ps > 0.05).

and across testing days. The ANOVA for self-licking behaviors yielded a significant Generation x Line interaction ($F_{1,\ 26} = 4.32$, $p < 0.05$, $\eta^2p = 0.14$). The post-hoc tests indicated significantly greater emission of self-licking in F_2 STDRHI vs. F_1 STDRHI dams; whereas the levels of this behavior were similar in STDRLO F_2 or F_3 dams. No differences in self-licking, however, were found between STDRHI and SRDLO dams of either generation. The ANOVA for time exploring the cage indicated a significant Line × Generation interaction, ($F_{1,\ 26} = 6.42$, $p < 0.05$, $\eta^2p = 0.20$), with the *post-hoc* tests indicating significantly greater exploration in STDRHI F_1 dams than in any of the other groups. Table 1 presents mean ± SEM for each behavior.

As shown in Fig. 1, baseline level of neural activity varied across structure and line. The ANOVA conducted in control animals (i.e., those receiving 0.0 g/kg ethanol) revealed significant main effects of Line and Structure ($F_{1,9} = 6.01$, $p = 0.04$, $\eta^2p = 0.40$, and $F_{7,63} = 45.80$, $p = 0.001$, $\eta^2p = 0.84$, respectively), but no significant main effect of Sex ($F_{1,9} = 4.02$, $p = 0.08$, $\eta^2p = 0.31$). The interactions between Line and Sex ($F_{1,9} = 0.00$, $p = 0.99$, $\eta^2p = 0.00$), Line and Structure ($F_{7,63} = 0.84$, $p = 0.55$, $\eta^2p = 0.09$), Sex and Structure ($F_{7,63} = 1.60$, $p = 0.15$, $\eta^2p = 0.15$) and the three-way interaction (i.e., Line × Sex × Structure: $F_{7,63} = 0.98$, $p = 0.46$, $\eta^2p = 0.09$) were not significant. The overall (i.e., across structures) level of Fos-ir activation was significantly greater in STDRHI than in STDRLO rats, with this effect surpassing the threshold for a big effect size. The *post-hoc* tests also revealed that – across both Lines – Fos-ir was significantly lower in BLA than in any other structure. PrL, IL and Shell did not differ between each other and exhibited significantly greater level of neural activation than Ce, Me, AcbC and VTA. Sex did not exert a significant main effect nor significantly interacted with the remaining variables. Microphotographs illustrating these results at Ce, Me and AcbC can be observed in the upper panels of Figs. 2, 3 and 4, respectively.

Fig. 5 shows Fos-ir positive cells as a function of ethanol dose, in STDRLO and STDRHI adolescents. The ANOVAs conducted in STDRLO rats revealed no significant main effects or significant interactions in BLA, Me, AcbC, AcbSh, VTA and IL. There was a significant main effect

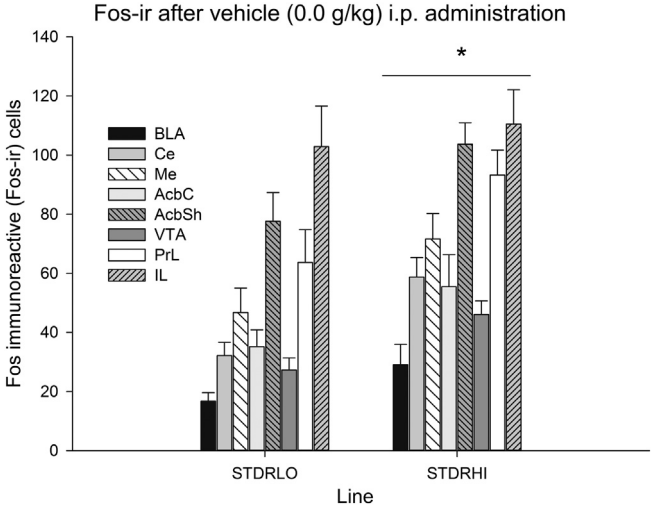

Fig. 1. Baseline (i.e., 90 min after vehicle i.p. administration) Fos immunoreactivity (Fos-ir) in different brain areas [central, basolateral and medial amygdaloid nucleus (BLA, Ce and Me, respectively), nucleus accumbens core and shell (AcbC, AcbSh), ventral tegmental area (VTA) and prelimbic and infralimbic (PrL, IL) prefrontal cortex] of male and female adolescent Wistar rats of short-term lines selected for high and low ethanol consumption (STDRHI and STDRLO, respectively). Each group was composed by 4 rats/sex, except group STDRLO males 2.5 g/kg in which n = 5. The statistical analysis (ANOVA) included sex as a factor yet revealed that sex did not exert a significant main effect upon baseline Fos-ir nor significantly interacted with the remaining factors. Therefore, the data (mean ± SEM number of immunoreactive cells) is depicted collapsed across sex. The ANOVA yielded a significant main effect of Line, with the STDRHI rats exhibiting significantly greater Fos-ir across all structures compared with STDRLO rats. This significant main effect is indicated by the asterisk. The ANOVA also revealed a significant main effect of structure. Please refer to the text for a full account of the statistical results yielded by the ANOVA. Vertical bars indicate the standard error of the means.

of Dose in Ce ($F_{2,19} = 11.09$, $p = 0.001$, $\eta^2p = 0.54$). The Sex × Dose interaction did not reach significance ($F_{2,19} = 0.80$, $p = 0.46$, $\eta^2p = 0.08$). The post hoc conducted to understand the significant effect of Dose indicated significantly greater ethanol-induced Fos-ir in animals treated with 1.25 or 2.5 g/kg ethanol than in control, vehicle-treated, counterparts. The microphotographs located in the left column of Figs. 2, 3 and 4 illustrate the ethanol-induced Fos-ir found in SDTDRLO rats at Ce, Me and AcbC, respectively.

The ANOVAs conducted in STDRHI rats revealed a trend for greater Fos-ir at Me in rats given 2.5 g/kg ethanol, when compared to 0.0 g/kg controls, which did not survive correction for multiple comparisons (main effect of dose: $p = 0.04$). The ANOVA for Fos-ir at AcbC revealed significant main effects of Dose and Sex ($F_{2,18} = 6.84$, $p = 0.006$, $\eta^2p = 0.43$, and $F_{1,18} = 9.55$, $p = 0.006$, $\eta^2p = 0.34$, respectively). The post hoc tests indicated that STDRHI rats treated with ethanol (either 1.25 or 2.5 g/kg) had significantly lower neural activation at AcbC than those treated with vehicle. In regards with the significant main effect of Sex, the overall level of neural activity within this area (i.e., the average values in male or female STDRHI rats at this structure, regardless ethanol dose received) was significantly greater in females STDRHI (49 ± 8) than in males STDRHI (30 ± 2). The Fos-ir patterns exhibited by STDRHI rats at Ce, Me and AcbC can be appraised in the right section of Figs. 2, 3 and 4, respectively.

4. Discussion

The present study assessed baseline and ethanol-induced differences in neural activity in a unique animal model for the assessment of risk of adolescent ethanol use, namely adolescent F_3 rats from lines selected for high (STDRHI) or low (STDRLO) ethanol drinking during

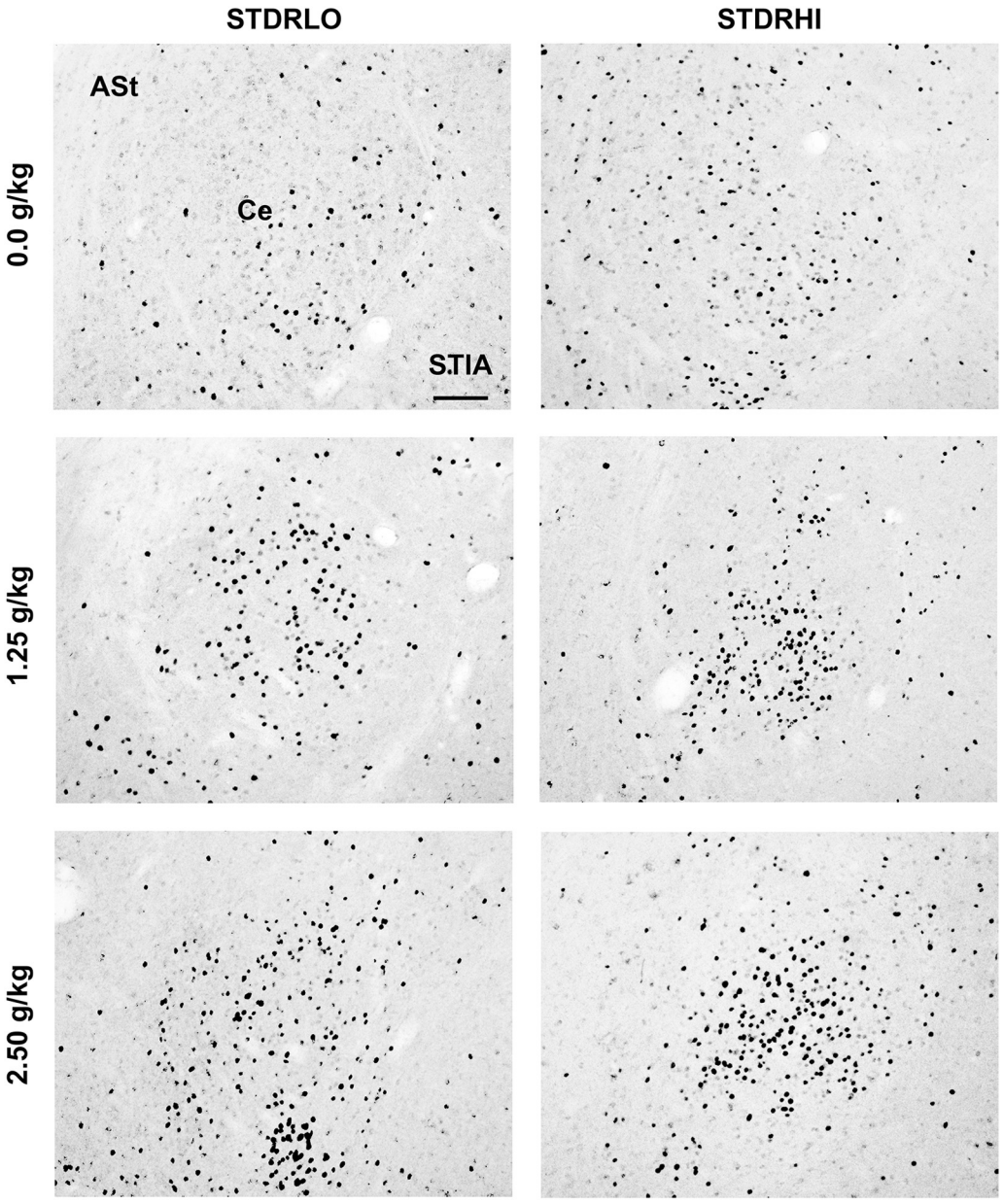

Fig. 2. Representative microphotographs illustrating Fos immunoreactivity in central amygdaloid nucleus (Ce) of adolescent Wistar rats derived from short-term lines selected for high and low ethanol consumption (STDRHI and STDRLO, respectively). On postnatal day 37 the animals were challenged with 0.0, 1.25 or 2.5 g/kg of ethanol (i.p.), 90 min before brain extraction. Microphotographs taken at 10× resolution are shown. STIA = Bed Nucleus Stria Terminalis; ASt = Amygdalotriatal Transition. The horizontal size bar represents100 um.

adolescence. We measured Fos-ir in the brain 90 min after i.p. administration of vehicle or after the intoxication induced by a moderate (1.25 g/kg) or a relatively high (2.5 g/kg) ethanol dose. These measurements were conducted in brain areas involved in the processing of fear and rewarding stimuli (Koob and Simon, 2009; Sharp, 2017; Silbermann et al., 2008).

One of the main new pieces of information derived from this study is that STDRHI, ethanol-naïve, adolescent, rats, exhibit significantly greater neural activity than STDRLO peers. The relative greater levels of baseline neural activity (i.e., after administration of 0.0 g/kg ethanol) in STDRHI vs. STDRLO rats were statistically similar across the brain areas measured, although particularly conspicuous at the amygdala nuclei. The amygdala has a key role in the processing of aversive stimuli and in the acquisition and expression of aversive conditioning (Kolber et al., 2008). Fear-inducing stimuli entering from the cortex and sensory areas are seemingly integrated by the BLA, whose glutamatergic neurons project to Ce and are also connected to the medial prefrontal cortex (Sharp, 2017). It should be noted, however, that the observed higher expression of Fos-ir in STDRHI vs. STDRLO rats may be due to innate line differences alone, differential stress effects associated with i.p. vehicle injection, or a combination of innate line differences x differential stress response.

A caveat of the present study is that we did not scrutinize the functional identity of the neurons activated by ethanol. Yet it has been shown that acute ethanol can potentiate GABAergic inhibition at Ce (Morales et al., 1998), AcbSh (Leriche et al., 2008) and BLA (Silberman et al., 2008), an effect associated with its anti-anxiety effects. Previous studies also indicated that most of the Ce neurons that showed Fos-ir after ethanol expressed pro-enkephalin (Criado and Morales, 2000), although a minor fraction expressed corticotrophin releasing factor. These neurons may have a role in the maintenance of ethanol dependence, as the administration of muscimol, a GABA agonist, into the Ce

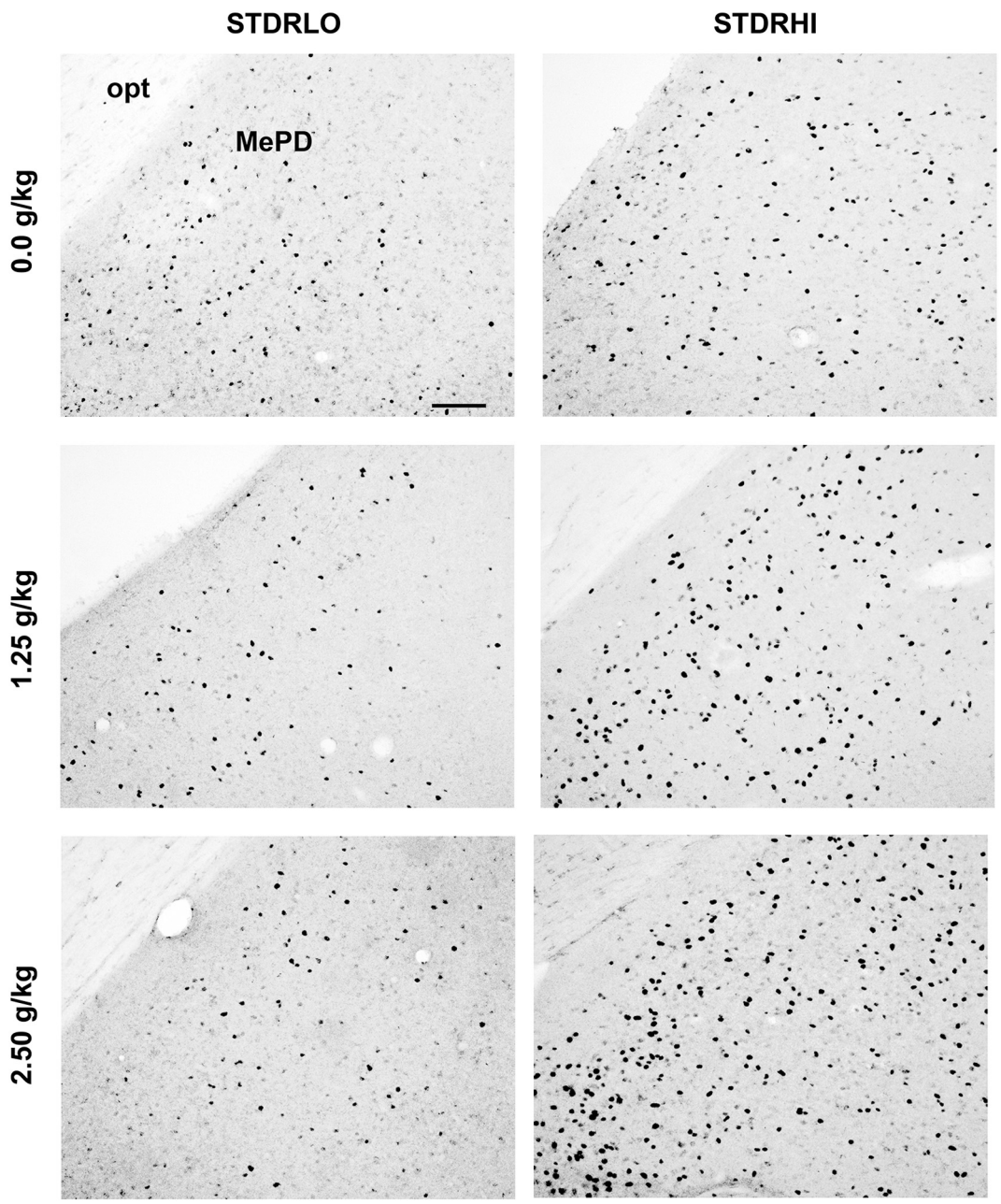

Fig. 3. Representative microphotographs illustrating Fos immunoreactivity in medial amygdaloid nucleus (Me) of adolescent Wistar rats derived from short-term lines selected for high and low ethanol consumption (STDRHI and STDRLO, respectively). On postnatal day 37 the animals were challenged with 0.0, 1.25 or 2.5 g/kg of ethanol (i.p.),90 min before brain extraction. Microphotographs taken at 10× resolution are shown. Opt = optic tract; MePD = medial amygdala postero dorsal. The horizontal size bar represents 100 um.

reduces ethanol drinking in dependent rats (Roberts et al., 1996), whereas administration of a GABAA antagonist decreased ethanol drinking in non-dependent rats (Hyytia and Koob, 1995). Physiological activation of the Ce and Me, in turn, activates behavioral (e.g., freezing) and hormonal responses via modulation of brain stem and hypothalamic structures (Kolber et al., 2008; Lin et al., 2011), although there is also output control from these areas of aminergic tracts and prefrontal areas.

In our previous study (Fernandez et al., 2017) we found several behaviors reflecting enhanced anxiety in the STDRHI line: reduced exploration of an open-field, greater time spent in the closed sections of a light-dark box and greater taste neophobia than the STDRLO counterpart. In the present study no measurement of anxiety was conducted, yet it is tempting to suggest that the greater baseline Fos-ir reflects an anxiety-prone phenotype in STDRHI animals. This explanation is congruent with the possibility that the baseline measurement conducted in our study actually depicts FOS-ir as a function of a mild stressor (i.e., the injection of vehicle). Also, previous work have reported an association between hyperexcitability of Ce neurons and greater anxiety responsiveness (Jiang et al., 2014), and have suggested a key role of Ce in regulating ethanol drinking (McBride, 2002). Consistent with this, rats lesioned in Ce exhibit a reduced anxiety response and a reduction of ethanol intake (Moller et al., 1997).

Fos-ir expression after the mild stressor of vehicle i.p. has been assessed in lines of rats selected for high- or low ethanol consumption during adulthood [the P and NP lines and the AA and ANA lines, see (Thiele et al., 1997)]. Unlike our results, FOS-ir after vehicle was fairly similar at AcbC, AcbSh and Ce, between these preferring and non-

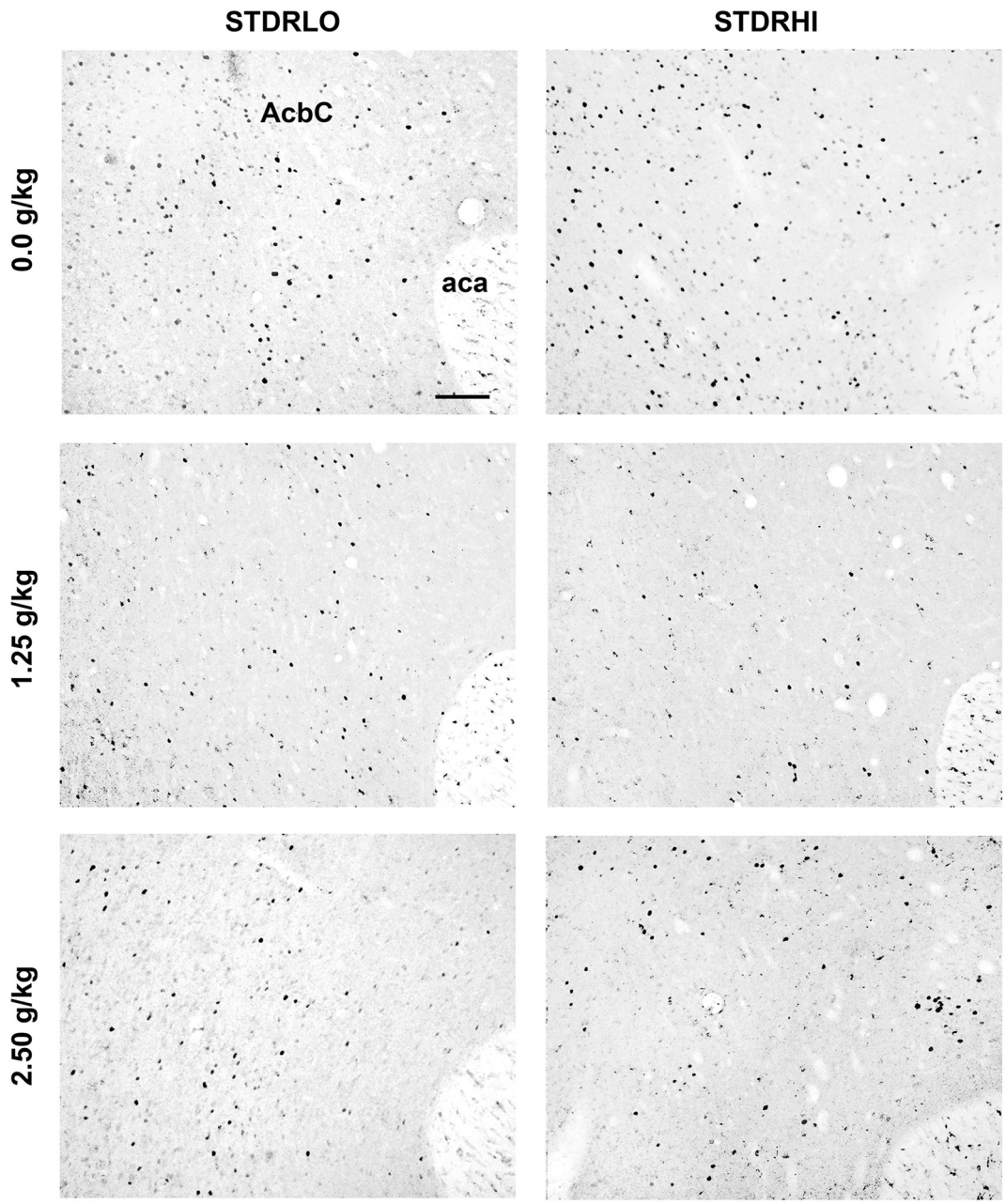

Fig. 4. Representative microphotographs illustrating Fos immunoreactivity in nucleus accumbens core (AcbC) of adolescent Wistar rats derived from short-term lines selected for high and low ethanol consumption (STDRHI and STDRLO, respectively). On postnatal day 37 the animals were challenged with 0.0, 1.25 or 2.5 g/kg of ethanol (i.p.),90 min before brain extraction. Microphotographs taken at 10× resolution are shown aca = anterior commissure. The horizontal size bar represents100 um.

preferring rats of both lines. Also in contrast with the results of the present study, Moller et al. (1997) found insensitivity to punishment and reduced innate anxiety in the AA rat line. This suggests that the anxiety reactivity pattern Fernandez et al. (2017) found in the STDRHI line, selected for high ethanol consumption during adolescence, is qualitatively different from that found after using a line in which the selection was conducted at adulthood. Yet the greater baseline Fos-ir in STDRHI vs. STDRLO rats is reminiscent of studies of functional neuronal activity, conducted in P and NP rats using LCGU. These studies observed greater basal LCGU in P than in NP rats (Strother et al., 2005), a difference which was more pronounced in adolescent P rats, which exhibited greater LDGU than their NP counterparts in several of the areas measured in the present study, including frontal cortex, Ce, BLA and AcbSh (Strother et al., 2008). Also relevant is a study (Herman et al., 2013) that revealed heightened GABAergic transmission in the Ce of genetically selected Marchigian Sardinian rats.

Ethanol's motivational, appetitive and aversive, effects are closely linked to its impact on the mesocorticolimbic pathway, which originates in the VTA, projects to the nucleus accumbens and terminates in the prefrontal cortex (Camarini and Pautassi, 2016). The nucleus accumbens shell receives major afferent input (deCampo and Fudge, 2013; Shirayama and Chaki, 2006) from the so-called extended amygdala (i.e., Ce, basal nucleus of the stria terminalis and sublenticular region) and the prefrontal cortex, in turn, contains the PrL and IL regions, which seemingly exert opposing actions upon drug-seeking behaviors (Millan et al., 2011). Phenotypes at-risk for AUDs exhibit differential ethanol-induced neural activity in this pathway. For instance, adolescent rats given prenatal ethanol exposure drink more ethanol

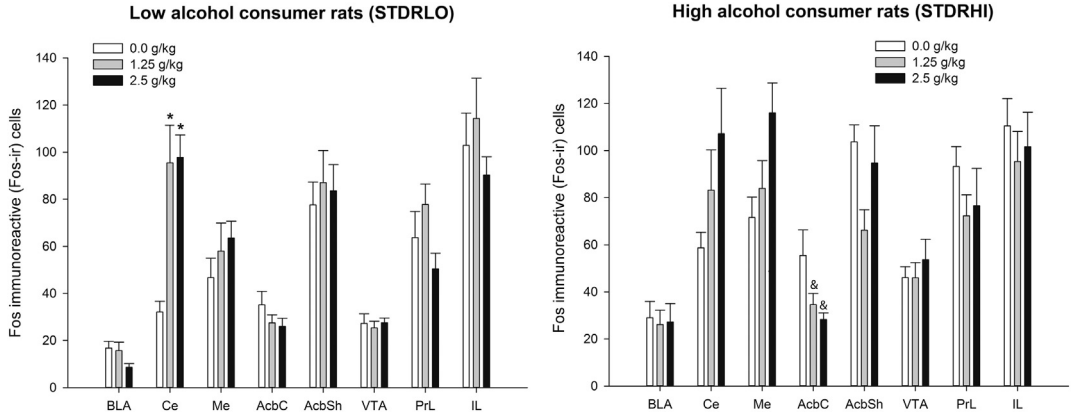

Fig. 5. Ethanol-induced Fos immunoreactivity (Fos-ir) in different brain areas [central, basolateral and medial amygdaloid nucleus (BLA, Ce and Me, respectively), nucleus accumbens core and shell (AcbC, AcbSh), ventral tegmental area (VTA) and prelimbic and infralimbic (PrL, IL) prefrontal cortex] of male and female adolescent Wistar rats of short-term lines selected for high and low ethanol consumption (STDRHI and STDRLO, respectively). On postnatal day 37 the animals were treated with ethanol (1.25 or 2.5 g/kg) or vehicle (i.e., 0.0 g/kg) 90 min before brains extraction. Each group was composed by 4 rats, except group STDRLO males 2.5 g/kg in which n = 5. The statistical analyses (separate ANOVAs for each line) included sex as a factor yet revealed that, for the most part, sex did not exert a significant main effect upon ethanol-induced Fos-ir nor significantly interacted with the remaining factors. Therefore, the data (mean ± SEM number of immunoreactive cells) is depicted collapsed across sex. The ANOVAs conducted in STDRLO rats revealed no significant main effects or significant interactions in BLA, Me, AcbC, AcbSh, VTA and IL. There was a significant main effect of Dose in Ce. The ANOVAs conducted in STDRHI rats revealed no significant main effects or significant interactions in Me, whereas the ANOVA for Fos-ir at AcbC revealed significant main effects of Dose and Sex. Fos-ir at AcbC was significantly greater in females STDRHI (49 ± 8) than in males STDRHI (30 ± 2). The post hoc tests indicated (a) significantly greater Fos-ir at Ce in STDRLO rats given 1.25 or 2.5 g/kg ethanol than in STDRLO peers given vehicle and (b) significantly reduced Fos-ir at AcbC in STDRHI rats given 1.25 or 2.5 g/kg ethanol than in STDRHI peers given vehicle. These effects are indicated by the asterisks and the ampersand signs, respectively. Vertical bars indicate ± SEM.

than control counterparts and exhibit greater neural ethanol-induced activity in the VTA and AcbC, yet reduced baseline neural activation at Il (Fabio et al., 2015). A recent work indicated that chronic adolescent ethanol exposure disrupts dopamine signaling in VTA neurons, an effect associated with heightened risk-taking and maladaptive decision-making (Schindler et al., 2016).The second important novel information derived from the present study is the differential (i.e., STDRHI vs. STDRLO) pattern of activation induced by the ethanol administration, which comprised several of these structures.

When challenged with ethanol the STDRHI, but not the STDRLO, rats exhibited ethanol-induced depression at nucleus accumbens core. The pattern observed at nucleus accumbens core was not consistent with our original hypothesis of finding greater ethanol-induced FOS-ir in the accumbens of STDRHI vs. STDRLO animals. It is possible, however, that this result implies greater ethanol-induced GABAergic activity which in turns results in reduced functional neural response. Future studies should try to scrutinize this possibility.

STDRLO but not STDRHI rats exhibited ethanol-induced activation at central amygdala. Studies with heterogeneous, non-selected, adult Sprague-Dawley rats have indicated heightened FOS-ir at Ce after 1.5 or 3.0 g/kg ethanol (Chang et al., 1995), thus similar to the pattern observed here in STDRLO rats. A blunted response to ethanol-induced Fos-ir at Ce, similar to the pattern exhibited by the STDRHI line, is more commonly observed after protracted ethanol exposure (Chang et al., 1995) and is usually considered a neural index of the development of tolerance to ethanol. It is possible that this low-level of Ce response the pharmacological effects of ethanol, in STDRHI subjects, serves to drive engagement in ethanol self-administration, as a mean to achieve to a desired level of intoxication. However, the lack of significant ethanol-induced Fos-ir at Ce in STDRHI rats may also relate to the fact the STDRHI line exhibited significantly greater Fos-ir after vehicle alone. This could have resulted in a ceiling effect that prevented observing further up-regulation of Fos-ir after ethanol.

It could be postulated, however, that the differences in ethanol-induced Fos-ir are due to differences in the circulating levels of ethanol. In a previous study, however, we found no significant differences between STDRHI and STDRLO in the peak level of blood ethanol concentration achieved after i.p. administration of 1.25 or 2.5 g/kg ethanol. Differential metabolic processing of ethanol is, however, just one of the multiple differences between STDRHI and STDRLO that could explain their differential level of ethanol intake. Experiment 1 assessed maternal and non-maternal behaviors emitted by dams of either line. The quality and quantity of maternal care are powerful modulators of drug-seeking behaviors (Francis and Kuhar, 2008), probably due to perinatal programming of the HPA axis. Pre-clinical studies have shown that pharmacological [maternal intoxication with ethanol (Ponce et al., 2011)] or non-pharmacological [e.g., prolonged pup-dam separation occurring daily from postnatal days 1 to 21(Roman and Nylander, 2005)] interventions that disrupt maternal care result in greater stress response at adulthood and enhanced ethanol intake (Cruz et al., 2008).

It is very unlikely, however, that rearing patterns explained the neural differences reported in this work, or the behavioral differences reported in previous work, between STDRHI and STDRLO rats. There were no significant line differences in key maternal behaviors; i.e., grooming of the pups, latency to retrieve the pups, time spent in the nest and time adopting a kiphotic posture that allow pups access to the nipples. There were some subtle, yet significant intergenerational variations in the amount self-licking behaviors in STDRHI dams as well as an increased amount of exploration of the cage in these animals, when compared to STDRLO counterparts. These effects could be yet other indices of heightened anxiety in STDRHI rats, yet it is unlikely that could have a direct effect in the pup and certainly did not affect the emission of the other maternal behaviors measured.

A caveat of the present study is that we discuss results found between different generations. In any selection program, the selection trajectory for the phenotype is not linear and of a fixed slope. Thus, comparing different dependent variables across different generations of selection can introduce unwanted confounding factors. Also, we have not measured if the histochemical alterations found in adolescents STDRHI rats last until adulthood. Furthermore, an inherent limitation of this selection program is that it introduces the confound of adolescent ethanol exposure having epigenetic effects (Pucci et al., 2018) that are passed down to subsequent generations. Additionally, in F_1 and F_2 generations, the interactions between the individual's now altered epigenetics and additional ethanol exposure further complicate the

interpretations of the final generation being tested in the present study.

Moreover, we report results obtained with i.p. administrations of high doses of ethanol, which may have little relationship to mechanisms involved in regulating ethanol drinking. Previous studies, however, have shown significant Fos-ir after ethanol self-administration, in many of the areas reported in the present study. For instance, (Wscieklica et al., 2016) reported that extended self-administration of ethanol by male Wistar rats significantly modulated Fos-ir in, among other areas, Ce, Me and AcbSh. In an intriguing study, Long-Evans rats that drank ethanol, in a limited access (2 h/day for four days) paradigm, were tested for anxiety behavior in an elevated plus maze immediately after the fourth self-administration session and sacrificed for measurement of Fos-ir 120 min later (Sharko et al., 2013). Rats that drank relatively high ethanol levels exhibited reduced anxiety and heightened Fos-ir in Ce, when compared to rats that drank relatively low levels of ethanol or that drank only water.

Female rats, both adolescents and adults, usually drink more ethanol than male rats (Doremus et al., 2005) and the prevalence of anxiety disorders is greater in women than in men (McLean et al., 2011). Moreover, in our previous study (Fernandez et al., 2017) we observed that some of behavioral differences between STDRHI and STDRLO, notably ethanol-induced locomotion, were sex-dependent. It was thus also important to assess if the expected pattern of basal and ethanol-induced Fos-ir differed across sex. This hypothesis was not corroborated: the patterns found were fairly similar across sex (although Fos-ir at AcbC was significantly greater in females STDRHI than in males STDRHI). This lack is not completely surprising, as other studies –some from our lab – also reported a lack of sex-related differences in ethanol-induced neural activity, although measured at the level of the Delta-Fos-B protein (Wille-Bille et al., 2017). A limitation was the relatively low sample size used (i.e., 4–5 per group), which could have precluded the detection of differences in ethanol-induced Fos-ir, particularly sex-related differences. These n-values, however, are similar to those reported in previous assessments of ethanol-induced Fos-ir [e.g, (Fabio et al., 2015; Fabio et al., 2013) n = 5; (Thiele et al., 1997) n = 4; (Morales et al., 1998) n = 6; (Varela et al., 2014) n = 6].

It is tempting to conclude that the data pinpoint to Ce, and AcbC, as areas involved in the differential intake of STDRHI vs. STDRLO rats. Yet, not only we did not explicitly measure ethanol intake in the present study, but the results were obtained with i.p. administrations of high doses of ethanol, which may have little relationship to mechanisms involved in regulating alcohol drinking. It is worth noting, however, that previous studies have found that voluntary ethanol drinking in conditions of limited access increased Fos-ir in similar areas as of those of the present study (Burnham and Thiele, 2017)(Bachtell et al., 2002).

Overall, these results indicate that short term selection for differential alcohol intake during adolescence yields heightened neural activity at baseline (i.e., after vehicle) in STRDHI vs. STDRLO adolescent rats, and differential sensitivity to ethanol-induced Fos-ir in Ce and in AcbC. All of the main effects reported surpassed the threshold for a big effect size. It is unlikely that rearing patterns explained the neural differences reported in this work, between STDRHI and STDRLO rats.

Declaration of interest

We declare having no competing interest nor conflict of interest related to our MS or its results.

Acknowledgments and funding sources

This work and was supported by PICT 2015-0325 to RMP. The authors would like to thank Paul Meyer (State University of New York at Buffalo, USA) for his suggestions in a preliminary version of the MS. The funders had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Competing financial interests statement

The authors declare having no competing interest nor conflict of interest related to our MS or its results.

Authorship

Fernandez and Pautassi designed the study. Fernandez, Pautassi. De Olmos and Ferreyra run the neurochemical and behavioral tests, and Pautassi, Ferreyra and Fernandez run the statistical analysis. Pautassi, De Olmos and Fernandez wrote the first draft of the manuscript. All authors contributed and approved the final manuscript.

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