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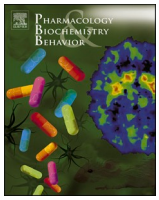

Adolescent alcohol exposure modifies adult anxiety-like behavior and amygdala sensitivity to alcohol in rats: Increased c-Fos activity and sex-dependent microRNA-182 expression

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ABSTRACT

Adolescent binge alcohol drinking is a serious health concern contributing to adult alcohol abuse often associated with anxiety disorders. We have used adolescent intermittent ethanol (AIE) administration as a model of binge drinking in rats in order to explore its long-term effect on the basolateral amygdala (BLA) responsiveness to alcohol and anxiety-like behavior. AIE increased the number of BLA c-Fos positive cells in adult Wistar rats and anxiety-like behavior assessed by the open field test (OFT). Additionally, in adult female rats receiving AIE BLA over expression of miR-182 was found. Therefore, our results indicate that alcohol consumption during adolescence can lead to enduring changes in anxiety-like behavior and BLA susceptibility to alcohol that may be mediated by sex-dependent epigenetic changes. These results contribute to understanding the mechanisms involved in the development of alcohol use disorders (AUD) and anxiety-related disorders.

1. Introduction

Alcohol abuse is a serious health problem often associated with anxiety disorders. Comorbidity between alcohol use disorders (AUD) and neuropsychiatric disorders related to anxiety has been described (Stoychev et al., 2021). Amongst various factors, alcohol exposure during adolescence contributes to the emergence of these disorders, serving as a significant factor in promoting the later-life comorbidity of anxiety and AUD (Pandey et al., 2017).

Adolescence is a challenging phase of life characterized by the transition from childhood dependence to adult independence (Spear, 2000). Elevated anxiety levels during adolescence have been linked to alcohol consumption (Pandey et al., 2017; Spear, 2018). In general, the consumption of alcohol amongst adolescents is characterized by episodes of heavy drinking followed by periods of abstinence. In rodents, the adolescent intermittent ethanol (AIE) model is used to explore the consequences of ethanol exposure similar to that induced by adolescent binge-drinking. It involves administering ethanol intermittently via different routes including either voluntary drinking or forced administration to mimic the binge pattern. Previous research has demonstrated that this model yields long-lasting effects (Crews et al., 2019, 2016). Using this model, it has been found that exposure to alcohol during adolescence leads to increased alcohol consumption (Gilpin et al., 2012; Maldonado-Devincci et al., 2010; Spear, 2013; Strong et al., 2010) and anxiety-like behavior in adulthood (Coleman et al., 2014; Crews et al., 2019; Healey et al., 2022).

The impact of adolescent alcohol on brain development underlies these behavioral changes that are evident long after adolescence. During this late developmental period, the brain circuits critical for processing rewards and affective stimuli undergo significant changes, making them highly vulnerable to alcohol. Hence, alcohol produces enduring functional modifications in the brain areas responsive to alcohol and stressful stimuli (Spear, 2018). The basolateral amygdala (BLA) is a major candidate, as it is highly vulnerable to alcohol (Ernst and Fudge, 2009; Wassum and Izquierdo, 2015) and has been proposed as a key region in the ontogeny of both alcoholism and stress/anxiety disorders (Agoglia and Herman, 2018). These disorders exhibit sex-specific vulnerabilities that can arise from the differential organizing effects of the

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sex hormones acting in BLA during sensitive developmental periods (Price and McCool, 2022).

Basolateral amygdala has been extensively studied for its involvement in fear conditioning (McDannald and Galarce, 2011) and other anxiety and stress responses (Rau et al., 2015). Although its role in alcohol intake is not well-understood, the BLA seems to be involved in the acquisition of motivational properties (Millan et al., 2015) as well as in the seeking of alcohol (Butler et al., 2014). The BLA is sensitive to prolonged alcohol exposure, which leads to synaptic remodeling and alterations in neurotransmission (Christian et al., 2012; Diaz et al., 2011; Kyzar et al., 2019). This impact is even more pronounced as studies on ΔFosB expression have found that alcohol induces higher BLA activity during this period than in adulthood (Wille-Bille et al., 2017). Alcohol exposure during adolescence also induces persistent synaptic alterations in adulthood. An overpopulation of wide spines in the BLA following AIE has been reported (Jury et al., 2017). The fact that functional modifications in the adolescent BLA may be responsible for increased adult alcohol consumption is supported by lesion studies. Moaddab et al. (2017) found that BLA lesions reduce alcohol consumption in adults with a history of adolescent alcohol use. Hence, these authors proposed that BLA hyperactivity is comorbid with anxiety and alcohol use disorders. Nevertheless, the nature of the changes and the processes involved in the long-term effects of alcohol binge drinking on the BLA during adolescence remain largely unknown.

Various epigenetic processes have been proposed to play a role in the reprogramming of the amygdala by alcohol exposure during adolescence, making adults more vulnerable to alcohol and anxiety (Kyzar and Pandey, 2015; Pandey et al., 2017; Spear, 2013). It has been demonstrated that AIE results in chromatin alterations, including histone acetylation and DNA methylation in the amygdala. These changes have been associated with anxiety-related behavior and alcohol consumption in adulthood (Kokare et al., 2017; Kyzar et al., 2017; Pandey et al., 2015; Sakharkar et al., 2014, 2019). Additionally, another emerging epigenetic mechanism of interest is microRNAs which are small non-coding RNAs of 21 to 33 nucleotides in length that regulate mRNA translation (Bartel, 2009). Whilst several studies have investigated the effects of adolescent alcohol exposure on miRNA in other brain areas, such as the hippocampus (Vázquez-Ágredos et al., 2022, for a review), research specifically focused on the BLA is scarce. The only published study observed increased miR-137 levels in the amygdala of adult rats exposed to AIE. It is worth noting that the authors concentrated on tissue encompassing the central and medial amygdala, whilst only a small portion of the BLA was included (Kyzar et al., 2019). Regarding other miRNAs, miR-182 down regulation has been linked to promote long-term fear memory formation in the amygdala (Griggs et al., 2013). Changes in this miRNA expression have also been related with ethanol intoxication in humans (Ibáñez et al., 2020) as well as stress and depression-like behaviors (Li et al., 2016).

Therefore, our objective was to investigate the impact of alcohol administration during adolescence on both the adult BLA responsiveness to alcohol and the BLA miRNA expression, as well as its effects on anxiety-like behavior. To achieve this, we used AIE in rats as a model of binge drinking (Spear and Swartzwelder, 2014). Once the rats reached adulthood, we performed immunohistochemistry to assess c-Fos expression as an indicator of BLA activity, anticipating hyper-activation in response to acute alcohol administration. As a control for assessing potential AIE-induced cell loss Cresyl violet staining was used. We evaluated miRNA expression through an epigenome-wide analysis using tissue primarily composed of the BLA, although small portions of other amygdala nuclei could not be ruled out. We expected changes in miRNA expression that have previously been associated with alcohol consumption or stress in other brain areas (Vázquez-Ágredos et al., 2022). Finally, we also obtained behavioral measures of anxiety using an open field test (OFT) in adult rats in order to confirm the effectiveness of AIE.

2. Materials and method

2.1. Animals

Six timed-pregnant Wistar rats were obtained from Charles River Laboratories (Wilmington, MA, USA) and housed individually. A total of 59 pups from the six litters (30 males; 29 females) were weaned on postnatal day (PND) 21 and group-housed with males and females in separate cages. The rats were maintained on a 12/12 h light/dark cycle with ad libitum access to food and tap water. They were randomly assigned to either the adolescent intermittent ethanol (AIE) or the adolescent intermittent saline (AIS) group. Both sex and litter origin was counterbalanced so that the litter effect was avoided. They were exposed to either adolescence intermittent ethanol injections (2 g/kg, i.p.; AIE, $N = 29$) or volume-matched saline (AIS, $N = 30$) on a 2-day on/off schedule from PND28 to PND41, resulting in a total of eight injections (Fig. 1). The ethanol dose applied is widely used (Kyzar et al., 2019; Robinson et al., 2020; Sakharkar et al., 2019) to achieve blood ethanol concentration levels exceeding 0.10 g/dL (Bloom et al., 1982; Sakharkar et al., 2014) similar to those defining binge drinking in humans (Crews et al., 2019). All injections were administered in the rats' home cages. The animals were humanely euthanized using sodium pentobarbital (100 mg/kg, i.p.) when they reached adulthood at PND98-102.

A sub-set of both AIS ($n = 20$) and AIE ($n = 19$) adult rats were randomly selected to undergo c-Fos immunohistochemistry in adulthood whilst miRNA expression was evaluated in the rest of the animals (AIS, $n = 10$; AIE, n = 10). Those assigned to c-Fos immunohistochemistry were previously tested in the OFT as shown in Fig. 1. The OFT was assessed in an open box made of black painted wood (52 × 52 × 40 cm). Overhead lighting illuminated the testing area reducing room context information. Each animal was allowed to freely explore the empty open-field arena for 5 min after previous handling and room acclimatization. The dimensions of the center of the box were set at 25 × 25 cm. Sessions were recorded with an overhead video camera. One of the AIE videos was lost leading to a reduced number of samples ($n = 18$). The Smart Video Tracking System 3.0 software (Panlab, Harvard) was used to automatically calculate the following parameters: number of entries into the center, time spent in the center, speed, distance traveled and rearing behavior.

Then, all the animals belonging to AIE and AIS groups were exposed to an acute injection of 2 g/kg, i.p. ethanol (PND101–PND102) prior to sacrifice, which occurred 90 min after injection for c-Fos immunohistochemistry.

The procedures were approved by the University of Granada Ethics Committee for Animal Research and by the Regional Ministry of Agriculture, Fisheries, and Rural Development of Andalusia (1/06/2022/078).

2.2. Tissue collection

For c-Fos immunohistochemistry and Cresyl violet staining, animals belonging to AIE and AIS groups were euthanized 90 min after the acute injection of ethanol. They were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.). The brains were removed and placed in a 4 % paraformaldehyde solution for 72 h at 4 °C before being transferred to a 30 % sucrose solution until they sank for cryoprotection. Coronal sections of 20 μm were cut at −3.36 relative to Bregma using a cryostat (Leica CM1900).

For microRNA analysis, the brains of rats belonging to AIE ($n = 11$) and AIS ($n = 10$) groups were immediately extracted, and the amygdala was freshly dissected at −3.36 relative to Bregma, according to The Rat Brain in Stereotaxic Coordinates, Fourth Edition Atlas (Paxinos, 1998). The tissue included BLA, although small portions of other amygdala nuclei could not be excluded. The tissue was flash frozen in liquid nitrogen and stored at −80 °C until processed.

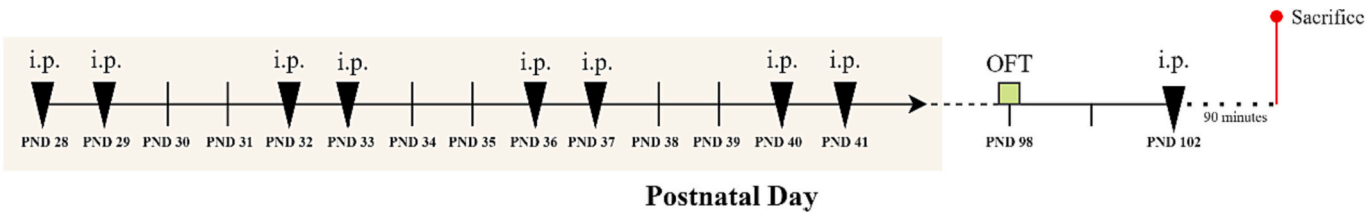

Fig. 1. Timeline of the adolescent intermittent ethanol administration. Intraperitoneal (i.p.) injections of ethanol (AIE group) or volume-matched saline (AIS group) were applied following a 2-days-on, 2-days-off schedule from the postnatal day (PND) 28 to 41. The open-field test (OFT) was applied at PND98 in those animals selected to undergo c-Fos immunohistochemistry and Cresyl violet staining. The challenge ethanol injection was applied at PND102 both to the subjects belonging to the c-Fos immunohistochemistry and the mi-RNA studies.

2.3. C-Fos immunohistochemistry

The tissue sections, floating freely, were washed with phosphate-buffered saline (PBS 0.01 M, pH 7.4), treated with 3 % hydrogen peroxide for 15 min, washed again, and then incubated in a solution containing 3 % normal goat serum and 0.4 % Triton X-100 in PBS for 30 min. The sections were then exposed overnight at 4 °C to a primary antibody against c-Fos (1:5000; Anti-c-Fos ABE-457, Millipore). After rinsing with PBS, the sections were incubated with a secondary antibody (Goat Anti-Rabbit IgG Antibody, 1:500; Millipore) for 120 min at room temperature. The primary and secondary antibody solutions were combined with a mixture of 2 % normal goat serum, 0.4 % Triton X-100, and PBS. Subsequently, the sections were rinsed, subjected to the ABC kit (Vector Laboratories, Burlingame, CA), and the reaction was visualized using the peroxidase substrate kit DAB (Vector Laboratories, Burlingame, CA). Finally, the sections were rinsed, mounted on gelatin-coated slides, dehydrated with ethanol and xylenes and covered with a cover slip.

2.4. Cresyl violet staining

The mounted sections underwent a series of ethanol solutions with decreasing concentrations, including 100 %, 96 %, 70 %, and ultimately 50 %, to ensure proper hydration. Following the ethanol treatment, the sections were rinsed thoroughly with distilled water to remove any residual ethanol. The tissue sections were immersed in a Cresyl violet solution (0.2 g in 200 mL of distilled water) for 10 min to allow for optimal staining. Post-staining, the sections were once again rinsed in distilled water and briefly exposed to a solution of acetic acid (20 mL in 200 mL of distilled water) ensuring that excess Cresyl violet was removed. To complete the procedure, the sections were dehydrated using ethanol and treated with xylene and then covered with a cover slip for preservation.

2.5. Transcriptome sequencing

RNA was extracted from BLA samples through homogenization using the RNeasy Lipid Tissue Mini Kit (Qiagen). Total cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, USA). For reverse transcription, 200 ng of total RNA was used from each sample. A solution-phase assay was carried out (Applied Biosystems). The extraction of small RNA was performed using the MiRNAe Micro Kit (Qiagen), and the purity and integrity of the RNA were checked using Nanodrop (Thermo Fisher) and Bioanalyzer (Agilent), respectively. The preparation of the small RNA library involved several enzymatic steps to include only the small RNA fragments in the final library. Sequencing of multiplexed libraries was performed using the NextSeq 500 equipment (Illumina). FastQs were obtained using Illumina's bcl2fastq software. The quality of the sequences was assessed using fastQC and multiQC (Ewels et al., 2016). First, the raw sequence adapters were removed using the cutadapt software (Martin, 2011). Then, 4 bps and low-quality bps (<20 in Phred scale) at each end were trimmed. The expression of miRNA was obtained using the miARma-Seq pipeline (Andrés-León et al., 2016), and the sequences were aligned using bowtie2 (Langmead and Salzberg, 2012). The miRNAs were annotated using the miRbase database (Kozomara et al., 2019).

2.6. Data acquisition

After excluding damaged tissue, sections containing the basolateral amygdala (BLA) (AIE $n = 11$; AIS $n = 12$) were identified using an optical microscope (Olympus B × 41) and the Stereo Investigator Software (mbf Bioscience) from a coronal section approximately at −3.36 mm relative to Bregma, according to Paxinos and Watson (Paxinos, 1998). Within each section, six microphotographs at 40× magnification were captured for the BLA, following a dorso-ventral-medio-lateral axis to cover the entire extent. The microphotographs were labeled with a sequential number (ranging from 1 to 6) to indicate their position (Fig. 2 (A)). As a control area, we selected the perirhinal cortex (PRh) located in the same section as BLA. To analyze this area, we captured 2 microphotographs at 40× magnification for each section whilst following a dorso-ventral-medio-lateral axis to cover the entire extent (Fig. 2 (A)).

The number of cells positive for c-Fos was determined using Image J Software (National Institute of Mental Health). In each microphotograph, objects meeting specific criteria (black circular dots on a white background), including a defined size range (35–150 μm^2) and circularity values (0.50–1.00), were automatically identified by the software as c-Fos positive cells. To standardize the microphotographs and minimize background noise, they were converted to 8-bit images, and the background was brightened by 50.0 pixels. Representative microphotographs from different experimental groups are shown in Fig. 2 (B). As the immunohistochemical mounting procedure on slides did not allow determination of the hemisphere to which brain slices belonged, the mean number of c-Fos positive cells was calculated for both hemispheres in each section, adopting a randomized approach to mitigate potential confounding effects. For a general morphological study, some of the brain slices from each age group (AIE $n = 3$, AIS $n = 4$) were randomly selected to be stained with Cresyl violet. The acquisition and processing of the microphotographs was performed in the same manner as the c-Fos images. Representative microphotographs stained with Cresyl violet are displayed in Fig. 2 (B).

We used miRNA target gene prediction software miRTarBase and DianaTarBase to identify experimentally validated miRNA target genes and miRDB to identify predicted miRNA target genes. We combined the results of this database and we specifically excluded gene targets with a target prediction score lower than 80. Additionally, we conducted Gene Ontology (GO) enrichment analysis using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) software to elucidate the functions of these target genes. The analysis encompassed three distinct categories: biological process (BP), cellular component (CC), and molecular function (MF). Furthermore, we used the same set of target genes to perform a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, also using the DAVID software. The purpose of the KEGG pathway analysis was to predict the metabolic pathways

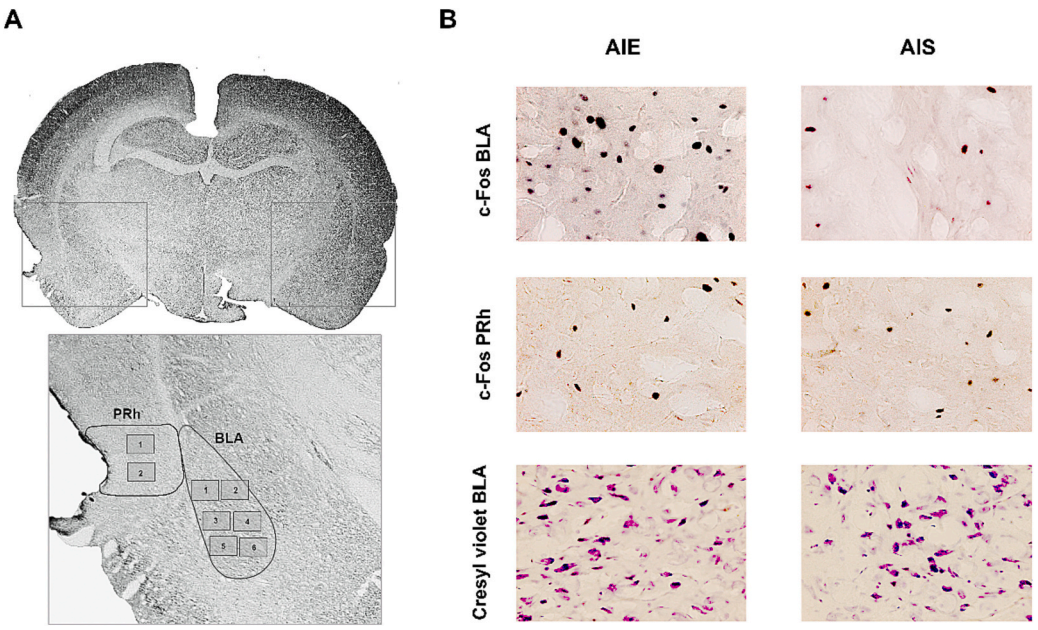

Fig. 2. (A) Schematics of the microphotographs (40×) of the basolateral amygdala and the perirhinal cortex. The images were numbered following a dorso-ventral and medio-lateral axis. (B) Representative microphotographs obtained at 40× magnification of c-Fos immunohistochemistry of the basolateral amygdala and the perirhinal cortex in the different groups. Representative microphotographs obtained at 40× magnification of Cresyl violet staining of the basolateral amygdala in the different groups. (*AIE: adolescent intermittent ethanol*; *AIS: adolescent intermittent saline*).

associated with the gene products by mapping the target genes to relevant biological pathways.

2.7. Statistical analysis

Data corresponding to c-Fos immunohistochemistry and Cresyl violet staining as well as the OFT were analyzed using the JAMOVI software (*The Jamovi Project*, 2022). A two-factor analysis of variance (ANOVA) (sex × group) or *t*-test was used, or a Kruskal-Wallis test was used if the normality or homoscedasticity criterions were not met. Statistical significance was set at $p < 0.05$.

Regarding the analysis of differentially significant miRNAs, the expression was normalized with NOISeq (Tarazona et al., 2011) following the approach of Trimmed Mean of M (Gu et al., 2016). Principal component analysis (PCA) was performed by visually representing the variance of the data and evaluating quality issues such as contaminated samples, processing errors, or anomalous measurements. Differential expression analysis was performed following the default recommendations of DESeq2 (Love et al., 2014). DESeq2 performs internal normalization for each gene, fits negative binomial generalized linear models for each gene and uses the Wald test for significance testing (Love et al., 2014). All the plots were generated using ggplot2 (Wickham, 2016). A miRNA was considered differentially expressed when the log2FC was >±1.5, and the adjusted *p*-value was <0.05. Enrichment analysis in GO and KEGG was conducted using the DAVID software, and we used the Benjamini-Hochberg p-value < 0.05 in order to correct for multiple comparisons.

3. Results

3.1. AIE increases c-Fos activity in the adult basolateral amygdala

The normality of the data was assessed using the Shapiro-Wilk showing a normal distribution ($W = 0.932$ $p = 0.124$) and the homogeneity of variance was also assessed using Levene's test, which yielded non-significant results ($F(3,19) = 0.523$, $p = 0.672$). Then, we conducted a 2 × 2 two-way ANOVA (Group × Sex) to investigate differences in c-Fos positive cell counts within the adult BLA between the AIE and AIS groups. The results of the ANOVA unveiled a significant main effect of group ($F(1,19) = 21.869$; $p < 0.001$; $\eta^2p = 0.535$) (Fig. 3 (A)), indicating that animals exposed to AIE exhibited a notably higher number of active c-Fos-positive cells in the BLA compared to the AIS group. However, no statistically significant effects were observed for sex, nor did we find any significant interaction between group and sex.

To identify whether this hyperactivation is specific to the BLA or to a general activation, we analyzed c-Fos in the PRh as a control area. Once the assumptions of normality (Shapiro-Wilk = 0.947, $p = 0.251$) and homogeneity of variance (Levene's test yielded ($F(3,19) = 2.76$, $p = 0.070$)) had been verified, we conducted a 2 × 2 two-way ANOVA (group × sex) to investigate differences in c-Fos positive cell counts within the adult PRh between the AIE and AIS groups. The result of this analysis revealed no significant effect associated with either factor (sex or group), nor did it indicate any interactions between these factors (all *p*-values > 0.1) (Fig. 3B), indicating that the activation of this area had not been affected by the alcohol procedure.

3.2. Cresyl violet staining indicates no effect of AIE on cell number

A semi-random selection process was used to choose a sub-set of brains for cell quantification, resulting in the inclusion of 7 Cresyl violet stained brains (4 from the AIE group and 3 from the AIS group). We conducted an independent-means *t*-test to evaluate potential disparities in the number of Cresyl violet stained cells within the BLA between AIS and AIE rats. The normality of the data was confirmed using the Shapiro-Wilk ($W = 0.956$ $p = 0.781$). Levene's Test to analyze the homogeneity of variance, which also yielded non-significant results ($F(1,5) = 0.978$; $p = 0.368$).

Remarkably, the outcome of this analysis did not reveal any significant effect associated with ethanol exposure ($t(5) = -0.148$, $p = 0.888$) (Fig. 4). This absence of statistical significance implies that there were no discernible differences in the total number of stained cells within the BLA between the AIS and AIE groups. Consequently, this finding permits us to confidently exclude the possibility that the discrepancies observed in the number of c-Fos positive cells between the two groups were attributable to variations in tissue quality.

The normality of the data collected from the OFT was confirmed

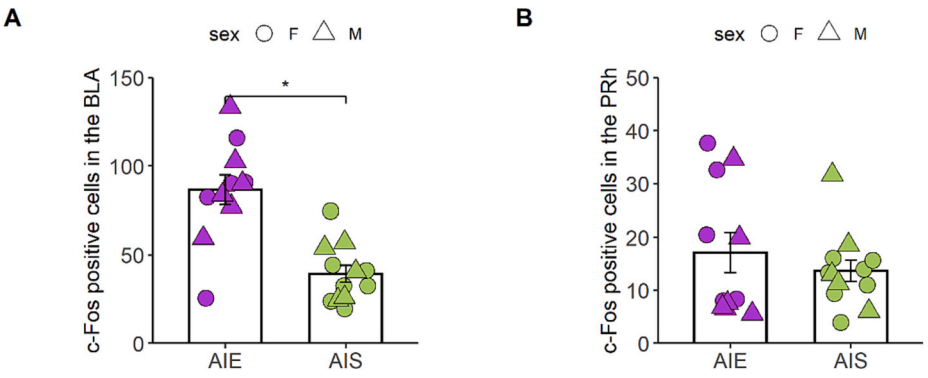

Fig. 3. (A) Mean (± SEM) of c-Fos positive cells in the basolateral amygdala for both groups adolescence intermittent ethanol administration (AIE) and adolescence intermittent saline (AIS). (B) Mean (± SEM) of c-Fos positive cells in the perirhinal cortex for AIE and AIS group. *AIE: adolescent intermittent ethanol*; *AIS: adolescent intermittent saline*. * = $p < 0.05$.

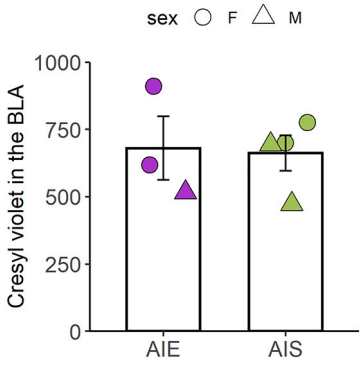

Fig. 4. Mean (± SEM) of Cresyl violet stained cells in the basolateral amygdala for both groups, adolescence intermittent ethanol administration (AIE) and adolescence intermittent saline (AIS).

using the Shapiro-Wilk test for the following variables: entries into the center, center time, total distance traveled and average speed.

We also performed a Levene's Test to analyze the homogeneity of variance which yielded non-significant results. Entries into the center ($F(3,34) = 0.764$; $p = 0.522$); total distance traveled ($F(3,34) = 0.090$; $p = 0.965$) and average speed ($F(3,34) = 0.091$; $p = 0.964$). We conducted a two-way ANOVA (Group × Sex). The results of this analysis revealed significant group effects for entries into the center ($F(1,34) = 4.902$; $p = 0.034$; $\eta^2p = 0.126$), total distance traveled ($F(1,34) = 4.7506$; $p = 0.036$; $\eta^2p = 0.123$), and average speed ($F(1,34) = 4.7455$; $p = 0.036$; $\eta^2p = 0.122$), indicating group-related differences (Fig. 5 (A), (B), (C)). These findings show that the AIE group entered the center fewer times, traveled a shorter total distance and had a lower average speed than the AIS group, suggesting higher levels of anxiety-like behavior.

Additionally, sex had a significant effect on total distance traveled ($F(1,34) = 22.1286$; $p < 0.001$; $\eta^2p = 0.394$) and Average speed ($F(1,34) = 22.1884$; $p < 0.001$; $\eta^2p = 0.395$), but no interactions were observed (Fig. 5 (B), (C)). These results indicate that females traveled longer distances and at a higher speed than the males. For center time and rearing, the Kruskal-Wallis test was used, revealing no significant differences in center time but significant differences in rearing concerning sex ($H = 6.17$, $p = 0.013$) (Fig. 5 (D), (E)). The females exhibited a greater number of rearing behaviors than the males. These findings offer valuable insights into the influence of AIE on OFT behavioral measures, along with discerning sex-related distinctions.

3.3. Principal component analysis (PCA) of the expressed miRNA

Five amygdala samples were taken from each group, one per animal. Principal component analysis (PCA) is shown in Fig. 6. PCA is a statistical valuable for data visualization, noise reduction, feature selection, and detecting relationships within the data. The results of our PCA analysis highlight the presence of distinct patterns in miRNA expression within our dataset. PC1 (46.85 %) and PC2 (29.04 %) collectively explain a substantial proportion of the variance (75.89 %), suggesting that these components are biologically meaningful and might reflect underlying regulatory mechanisms of cellular responses.

3.4. AIE induce miR-182 overexpression in adult amygdala of female rats

Given the pivotal role of miRNAs in gene regulation, this study delves into miRNA expression to uncover potential molecular mechanisms underlying the observed behavioral and functional differences induced by the AIE procedure. Regarding sex-dependent effects, we found no differentially expressed miRNAs between the AIE and AIS groups in adult males. However, a noteworthy distinction emerged in adult female rats subjected to the AIE procedure. Specifically, miR-182 exhibited significant differential expression in the amygdala when compared to their AIS counterparts, with a marked overexpression (log2FC = 2.15, padj = 0.03) (Fig. 7).

To elucidate the potential downstream effects of miR-182, we performed miRNA target gene prediction using specialized software and subsequently performed comprehensive GO and KEGG pathway analyses on these target genes. As depicted in Fig. 8 (A), the analysis revealed the top 10 significant GO terms across biological processes, cellular components, and molecular functions ($p < 0.05$). Furthermore, our KEGG pathway analysis identified the top 15 significant pathways, as illustrated in Fig. 8 (B).

4. Discussion

In this study, we have used the AIE administration model to investigate the long-term changes induced by adolescent alcohol exposure, which alter the adult BLA responsiveness to alcohol and cause anxiety-like behavior. Several findings can be highlighted.

Firstly, AIE leads to significant BLA hyperactivation in response to acute alcohol injection, observed in both male and female adult rats, as indicated by c-Fos expression. To the best of our knowledge, this is the first report of increased BLA Fos reactivity to alcohol in adult rats exposed to AIE administration during adolescence. Previous research found that adolescent rats exhibited a greater number of ethanol-induced ΔFosB-positive cells in the BLA compared to adults in response to voluntary drinking during two-bottle intake sessions (Wille-Bille et al., 2017). However, long-lasting AIE impact on adult responses to alcohol was not explored. Moreover, that study identified differences between adolescent and adult rats in alcohol consumption, with a progressive escalation observed in adolescents. This aligns with previous

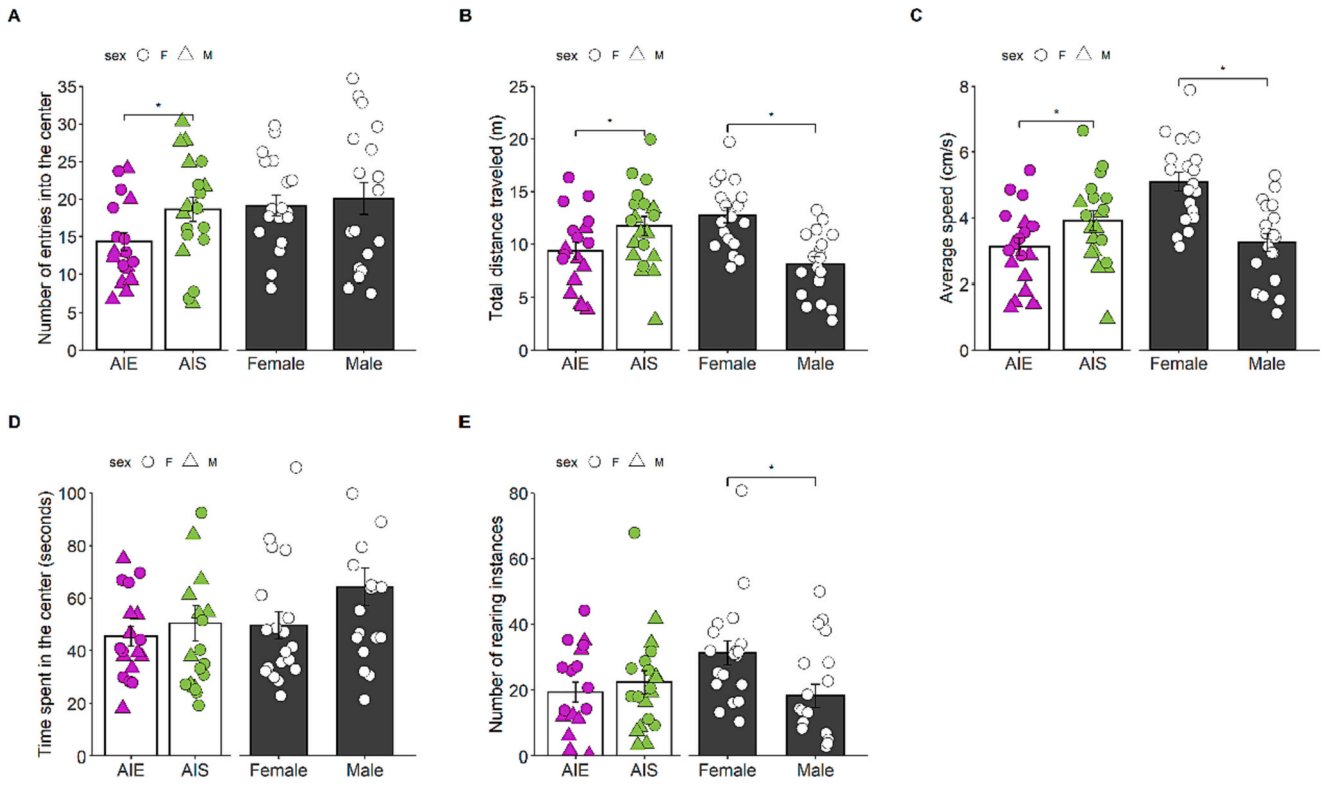

Fig. 5. Effect of adolescent intermittent ethanol (white columns) and sex (black columns) on adult anxiety-like behavior measured in the OFT. (A) Mean ± SEM number of entries in the center. (B) Mean ± SEM distance traveled (cm). (C) Mean ± SEM speed (cm/s) (D) Mean ± SEM time spent in the center (s) (E) Mean ± SEM rearing instances.
AIE: adolescent intermittent ethanol; *AIS: adolescent intermittent saline*. * = $p < 0.05$.

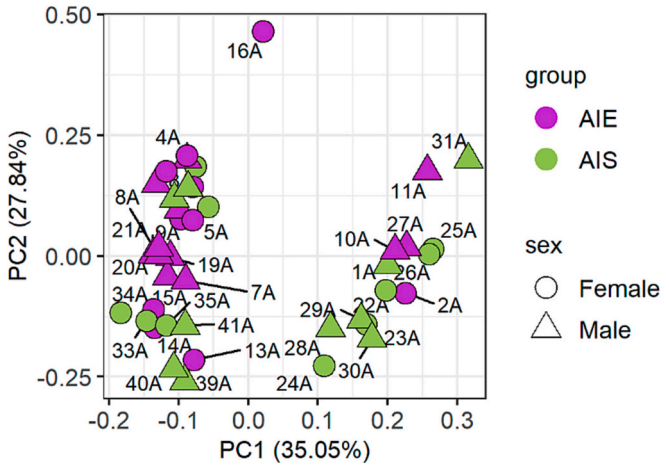

Fig. 6. Principal component analysis (PCA) of the expressed miRNA in adult rats. The results highlight the presence of distinct patterns in miRNA expression within our dataset. The first principal component (PC1), accounted for a substantial portion of the variance in miRNA expression, explaining approximately 46.85 % of the total variance. The second principal component (PC2), contributed significantly to the overall variance in miRNA expression, explaining approximately 29.04 % of the variance. PC1 and PC2 collectively explain a substantial proportion of the variance, suggesting that these components are biologically meaningful.

results in our lab, which demonstrated higher voluntary alcohol intake in adolescent compared to adult rats using a four-bottle procedure (García-Burgos et al., 2009). In this study the forced i.p. administration method allowed a precise control over adolescent alcohol exposure, thereby mitigating the potential influence of variability in voluntary consumption. The administration route which is well documented and widely used also allowed us to apply a lower dose than alternative routes such as vapor inhalation or intra gastric administration. The increased number of c-Fos positive cells in response to ethanol injection that we have found in the adult BLA of rats exposed to alcohol during adolescence suggests an enhanced sensitivity and reactivity of the BLA to alcohol following early alcohol exposure in a binge-like regime. In fact, the impact of intermittent adolescent alcohol exposure overshadows the potential novelty effect that could increase BLA activity. Thus, the absence of a control group exposed to a saline adult injection, which could be considered a limitation in order to control the novelty effect, does not prevent the conclusion that increased adult BLA sensitivity to alcohol occurs after intermittent adolescent exposure. Neither can we exclude that AIE has increased BLA response to any aversive event such as the i.p. injection. However, this does not decrease the relevance of the results reported. Even if the long-term effect of AIE on BLA hyperactivation is non-specific affecting any adult challenge, it is clearly evident in response to alcohol. Considering that the BLA integrates sensory cues associated with alcohol and subsequently modulates reward-seeking behavior as well as emotional responses (Chaudhri et al., 2013; Tavares et al., 2023; Wassum and Izquierdo, 2015), its hyperactivation may contribute to the reinforcing properties of alcohol. Moreover, regarding the anatomical organization of the BLA, it has been reported that specific cellular groups respond to stimuli of opposing valence (Kim et al., 2016). Specifically, the anterior BLA has been involved in processing negative emotional experiences, whilst the posterior BLA appears to be associated with responses to positive valence stimuli. Accordingly, we found increased activation of the posterior BLA in adulthood after AIE. This supports the long-lasting effects of early intermittent alcohol exposure on the positive emotional and rewarding effects of alcohol. Our results do not allow us to draw conclusions on the specific cell type responsible for the increased BLA activity reported in

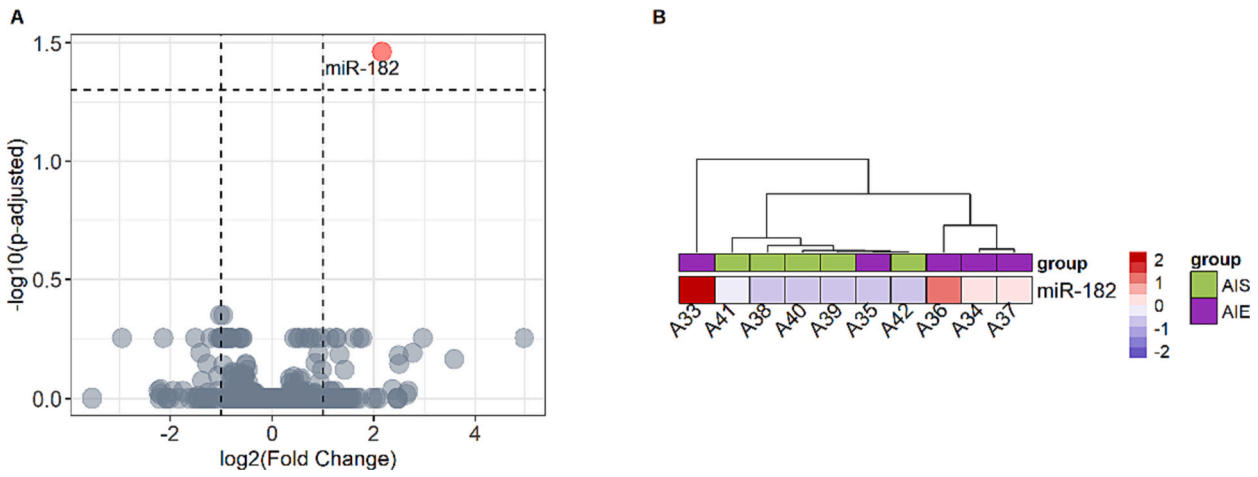

Fig. 7. (A) Volcano plot of the expressed miRNA in adult female rats. The results show that miR-182 was overexpressed in the BLA of adult female rats (log2FC = 2.15, padj = 0.03). (B) Clustered heat map of miR-182, differentially expressed in AIS female rats compared with AIE female rats. The heat map shows that there is an overexpression of this miRNA in the samples of AIE female rats.

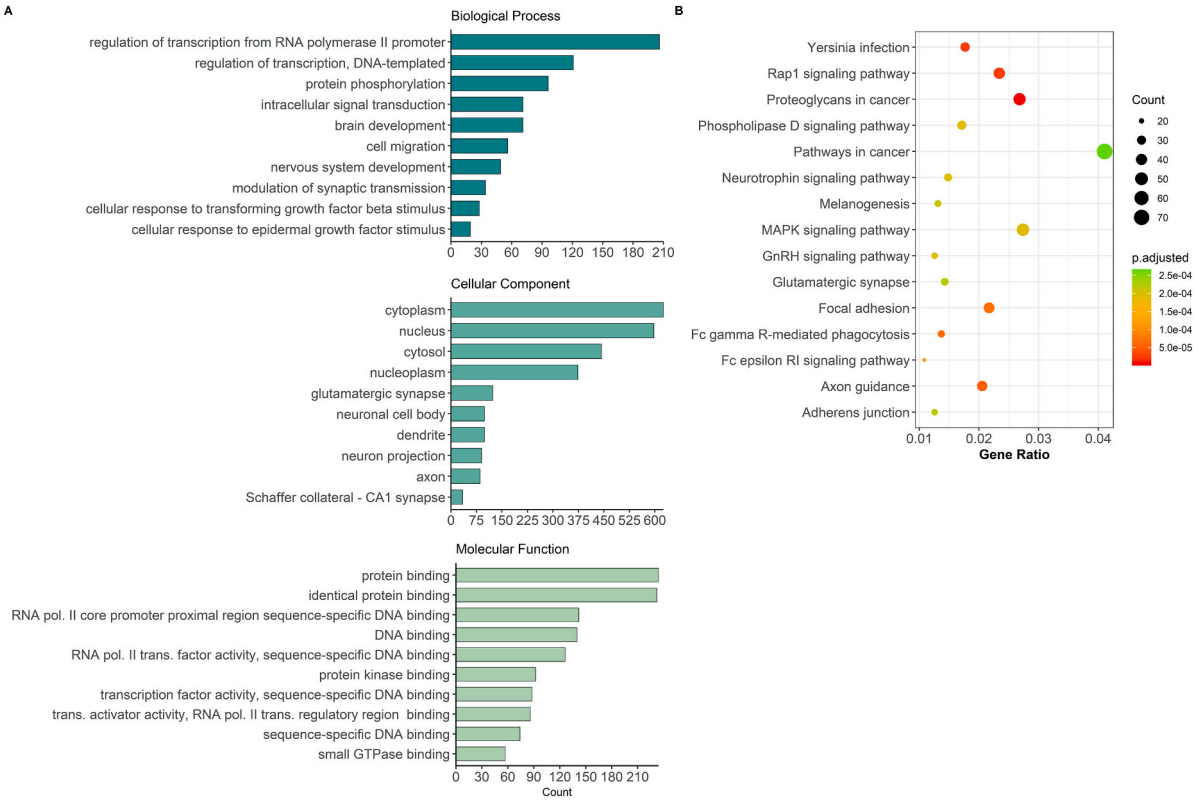

Fig. 8. (A) Top 10 significant gene ontology (GO) terms for miR-182 target genes. The results reveal that miR-182 target genes are involved in crucial biological processes, such as protein phosphorylation, intracellular signal transduction, brain development, cell migration, and nervous system development, amongst others. In the cellular component category, notable locations include cytoplasm, nucleus, cytosol, glutamatergic synapse, neuronal cell body, dendrite, and more. Regarding molecular function, they are involved in protein binding, DNA binding, protein kinase binding, and so forth. (B) Top 15 significant KEGG pathways for miR-182 target genes. The results indicate that these target genes actively participate in various pathways, including Yersinia infection, Rap1 signaling pathway, phospholipase D signaling pathway, neurotrophin signaling pathway, MAPK signaling pathway, glutamatergic synapse, etc.

the AIE group. However, it is feasible that activation of excitatory glutamatergic pyramidal cells would play a critical role since they represent the majority of the total BLA cell population. It has been reported that chronic ethanol and withdrawal alters the balance between glutamatergic and GABAergic local circuits regulating BLA pyramidal excitability (Diaz et al., 2011). Acute alcohol decreases pyramidal cells activity as a result of GABAergic activation (Perra et al., 2008). This has been associated with its anxiolytic effect. If the increased BLA activity in this study is attributable to pyramidal cell activity, our results would indicate a long-lasting facilitating effect of AIE on pyramidal cell excitability. A similar increase in BLA pyramidal cell excitability has been found after adolescent chronic stress induced by social isolation (Rau et al., 2015). Consequently, a heightened rewarding effect of alcohol, combined with increased anxiety responses following AIE

exposure, may promote alcohol consumption in adulthood. This finding is consistent with previous results showing that neurotoxic BLA lesions disrupt the voluntary alcohol drinking in adult rats that had access to alcohol during adolescence (Moaddab et al., 2017). Hence, it can be proposed that alcohol-induced BLA plasticity during development may underlie an elevated risk of adult alcohol consumption. Further research using voluntary consumption models is needed to confirm the influence of the BLA activity changes induced by adolescent intermittent alcohol exposure on adult behavior.

Secondly, AIE results in increased anxiety-related behavior in adulthood, as assessed in OFT. Although decreased distance traveled and speed can reflect the effect of early alcohol on adult locomotion activity, the fact that AIE also reduced the number of center entries also supports enhanced anxiety. This finding is consistent with previous reports using various behavioral tests (Coleman et al., 2014; Crews et al., 2019; Healey et al., 2022; Van Skike et al., 2015). It is plausible to suggest that the enhanced anxiety observed in adulthood is attributable to the AIE-induced hyperactivation of the BLA. In accordance with this, the BLA has been implicated in the regulation of anxiety and fear responses, and its hyperactivation may contribute to the development of anxiety disorders, which frequently co-occur with alcohol use disorders (Agoglia and Herman, 2018; Silberman et al., 2009; Tye et al., 2011). In fact, biological and hormonal factors may interact with alcohol exposure during critical BLA developmental periods to shape individual vulnerabilities and excitability (Price and McCool, 2022). This explains the sex differences that we found in OFT performance. Females exhibited lower levels of anxiety, as evidenced by measures such as total distance, average speed, and the number of rearing instances in the OFT. Nevertheless, these sex-related differences did not show any significant interaction with AIE exposure. Some studies have reported sex-dependent behavioral effects of AIE exposure (Matthews et al., 2022). Regarding anxiety-like behavior, however, the scarce evidence indicates that the sex differences in adult behavior resulted from early alcohol exposure are consistently observed. These differences appear to depend on the task involved, being more evident in those tasks that induce higher anxiety, such as restrain-induced stress (Healey et al., 2023) and elevated plus maze (Healey et al., 2022). We can hypothesize that OFT performance is sensitive to sex differences in exploratory behavior but may not detect sex-dependent effects of AIE due to its relatively safe testing environment. This would be in accordance with the sex-related differences in OFT behavioral parameters irrespective of alcohol adolescent exposure. We found sex differences only in total distance, average speed, and number of rearing instances that might indicate motion/arousal differences but not in center time or entries that reflect anxiety. This is a common finding in most of the previous studies although there are some reporting sex differences in all the OFT parameters (Knight et al., 2021). These authors propose the size of the groups as the explanation for this discrepancy. They used a larger group size (47 animals per group) whilst we have included 5 animals per group as is usual in most of the studies. Therefore, in our study we cannot exclude the presence of anxiety-like behavior sex differences.

Finally, we found sex-dependent changes in adult BLA miRNA expression as a result of AIE exposure. The dysregulation observed in the BLA following AIE may be associated with alterations in its neurotransmission. Previous studies have reported changes in various neurotransmitter systems, including glutamate and gamma-aminobutyric acid (GABA), within the BLA in response to alcohol exposure (Gass et al., 2011; Marron Fernandez De Velasco et al., 2023; Nimitvilai-Roberts et al., 2023). Amongst the molecular and cellular alterations induced by alcohol, epigenetic dysregulation during the adolescent critical developmental period plays a crucial role in modulating amygdala synaptic plasticity (Kyzar and Pandey, 2015; Pandey et al., 2017). In fact, the reprogramming of the amygdaloid circuitry through epigenetic interventions has been shown to restore the long-term effects of AIE (Teague and Nestler, 2022). It is interesting to note that most studies were centered on the central and medial amygdala. Some of these epigenetic processes are regulated by changes in miRNA expression. Accordingly, AIE increased miR-137 expression in adult amygdala. This was related to AIE-induced altered alcohol drinking and anxiety-like behavior that were restored after infusion of miR-137 antagomir in the central nucleus of the amygdala (Kyzar et al., 2019). Although to the best of our knowledge there are no previous reports of selective AIE effects on adult BLA, our results indicate that modulation of miRNA expression may be involved in the epigenetic remodeling of this amygdala region.

In our study, adult females with a history of AIE exhibited and overexpression of miR-182 in the amygdala. We did not find, however, any changes in microRNA expression in adult males. To the best of our knowledge, this research represents the first attempt to conduct an epigenome-wide analysis of miRNAs in the context of AIE. We attribute our results primarily to the BLA, as the extracted tissue contained mainly this region. Although the technique used does not allow us to entirely exclude the presence of small portions of other amygdala nuclei, their potential contribution appears to be negligible given the larger size of the BLA in comparison. The identification of miR-182 as significantly overexpressed in the BLA of adult females with AIE provides novel insights into the molecular pathways associated in AIE-related changes. Previous reports linked miR-182 overexpression with long-term fear memory formation in the amygdala (Griggs et al., 2013) and depression-like behavior which can be prevented by miR-182 inhibition (Li et al., 2016). Interestingly, our findings are consistent with a previous report demonstrating a sex-dependent miR-182 overexpression in the hippocampus of AIE-exposed mice (Pascual et al., 2021). Regarding the sex effect, however, it is noteworthy that the impact of adolescent alcohol exposure seems to be opposite in the hippocampus and BLA. Whilst the effect was evident in the hippocampus of males (Pascual et al., 2021), we observed a significant effect in the BLA of females. This points to a sex-dependent vulnerability of the hippocampus and amygdala possibly related to different developmental trajectories. Also sex differences in miR-182 expression throughout development that might modify the influence of alcohol exposure cannot be ruled out. Although data on sex-dependent miR-182 expression during adolescence are scarce, lower levels in human plasma have been reported.

The fact that AIE increased BLA hyperactivation both in male and female adult rats whilst only females exhibited BLA miR-182 overexpression suggests that they are independent effects. However, the possibility that they are linked cannot be ruled out, at least in females, through processes not detected by c-Fos immunohistochemistry. It is well known that c-fos expression is a marker of neuronal activation whilst inhibition might be the process regulated by miR-182. Regarding OFT, the effect of AIE was not sex-dependent, but there were sex differences in anxiety-like behavior. Females exhibited lower levels of anxiety. Interestingly, miR-182 has been implicated not only in alcohol-related processes but also in stress responses in various brain regions (Kamens et al., 2021; McCreary et al., 2016; Pascual et al., 2021). The overexpression of miR-182 in adult female rat could be related with the behavioral differences in OFT cannot be ruled out. In fact, its multifaceted role suggests that miR-182 may serve as a critical regulator of the interaction between alcohol consumption and stress reactivity, potentially contributing to the development of AUDs. Moreover, exploring the GO terms associated with miR-182 targets revealed intriguing patterns related to developmental processes, including brain development, cell migration and cerebral cortex neuron differentiation. This finding suggests that the dysregulation of miR-182 in the BLA during AIE may disrupt normal developmental trajectories, ultimately leading to long-term consequences on brain function and behavior. Our findings complement the results reported by Kyzar et al. (2019), who observed increased miR-137 expression in the adult amygdala following AIE exposure.

To sum up, our study underscores the enduring impact of intermittent alcohol consumption during adolescence on the BLA, potentially heightening susceptibility to alcohol consumption and the development

of AUD in adulthood. This connection may also be linked to the comorbidity between AUD and anxiety disorders. Gaining a deeper understanding of the relationship between BLA hyperactivity and anxiety-related behavior is crucial for unraveling the complex interplay amongst alcohol exposure, amygdalar function and the development of comorbid psychiatric disorders. Future research in this area would greatly benefit from a focus on investigating the specific neurochemical alterations that underlie the hyperactivation observed in our study. This would provide a more comprehensive understanding of the neurobiological mechanisms that drive the long-term consequences of AIE exposure. Furthermore, our results contribute to the growing body of research on the effects of AIE and highlight the significance of studying epigenetic mechanisms, particularly the involvement of miR-182 in mediating alcohol-induced brain alterations. This finding enhances our comprehension of the neurobiological foundations of alcohol-related behavior and suggests that epigenetic dysregulation in the BLA may play a crucial role in the initiation and persistence of AUDs. Further research in this field is essential for advancing our knowledge and addressing the complex interactions between alcohol, behavior, epigenetics and brain function.

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CRediT authorship contribution statement

Ana Vázquez-Ágredos: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Visualization. **Marta Valero:** Conceptualization, Data curation, Investigation, Methodology. **Teresa Aparicio-Mescua:** Data curation, Formal analysis. **Raquel García-Rodríguez:** Data curation, Formal analysis. **Fernando Gámiz:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing. **Milagros Gallo:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

The datasets generated for this study can be found in “Open Science Framework” at doi:10.17605/OSF.IO/HWRS8. This experiment is part of a pre-registered project that can be found at doi:10.17605/OSF.IO/6EQKZ.

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References

Agoglia, A., Herman, M., 2018. The Center of the Emotional Universe: alcohol, stress, and CRF1 amygdala circuitry. Alcohol 72, 61–73. https://doi.org/10.1016/j.alcohol.2018.03.009.

Andrés-León, E., Núñez-Torres, R., Rojas, A.M., 2016. miARma-Seq: a comprehensive tool for miRNA, mRNA and circRNA analysis. Sci. Rep. 6, 25749. https://doi.org/10.1038/srep25749.

Bartel, D.P., 2009. MicroRNA target recognition and regulatory functions. Cell 136, 215–233. https://doi.org/10.1016/j.cell.2009.01.002.

Bloom, F., Lad, P., Pittman, Q., Rogers, J., 1982. Blood alcohol levels in rats: non-uniform yields from intraperitoneal doses based on body weight. Br. J. Pharmacol. 75, 251–254. https://doi.org/10.1111/j.1476-5381.1982.tb08780.x.

Butler, T.R., Chappell, A.M., Weiner, J.L., 2014. Effect of β3 adrenoceptor activation in the basolateral amygdala on ethanol seeking behaviors. Psychopharmacology (Berl) 231, 293–303. https://doi.org/10.1007/s00213-013-3238-y.

Chaudhri, N., Woods, C.A., Sahuque, L.L., Gill, T.M., Janak, P.H., 2013. Unilateral inactivation of the basolateral amygdala attenuates context-induced renewal of Pavlovian-conditioned alcohol-seeking. Eur. J. Neurosci. 38, 2751–2761. https://doi.org/10.1111/ejn.12278.

Christian, D.T., Alexander, N.J., Diaz, M.R., Robinson, S., McCool, B.A., 2012. Chronic intermittent ethanol and withdrawal differentially modulate basolateral amygdala AMPA-type glutamate receptor function and trafficking. Neuropharmacology 62, 2430–2439. https://doi.org/10.1016/j.neuropharm.2012.02.017.

Coleman, L.G., Liu, W., Oguz, I., Styner, M., Crews, F.T., 2014. Adolescent binge ethanol treatment alters adult brain regional volumes, cortical extracellular matrix protein and behavioral flexibility. Pharmacol. Biochem. Behav. 116, 142–151. https://doi.org/10.1016/j.pbb.2013.11.021.

Crews, F.T., Vetreno, R.P., Broadwater, M.A., Robinson, D.L., 2016. Adolescent alcohol exposure persistently impacts adult neurobiology and behavior. Pharmacol. Rev. 68, 1074–1109. https://doi.org/10.1124/pr.115.012138.

Crews, F.T., Robinson, D.L., Chandler, L.J., Ehlers, C.L., Mulholland, P.J., Pandey, S.C., Rodd, Z.A., Spear, L.P., Swartzwelder, H.S., Vetreno, R.P., 2019. Mechanisms of persistent neurobiological changes following adolescent alcohol exposure: NADIA consortium findings. Alcohol. Clin. Exp. Res. 43, 1806–1822. https://doi.org/10.1111/acer.14154.

Diaz, M.R., Christian, D.T., Anderson, N.J., McCool, B.A., 2011. Chronic ethanol and withdrawal differentially modulate lateral/basolateral amygdala paracapsular and local GABAergic synapses. J. Pharmacol. Exp. Ther. 337, 162–170. https://doi.org/10.1124/jpet.110.177121.

Ernst, M., Fudge, J., 2009. A developmental neurobiological model of motivated behavior: anatomy, connectivity and ontogeny of the triadic nodes. Neurosci. Biobehav. Rev. 33, 367–382. https://doi.org/10.1016/j.neubiorev.2008.10.009.

Ewels, P., Magnusson, M., Lundin, S., Käller, M., 2016. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics 32, 3047–3048. https://doi.org/10.1093/bioinformatics/btw354.

García-Burgos, D., González, F., Manrique, T., Gallo, M., 2009. Patterns of ethanol intake in preadolescent, adolescent, and adult Wistar rats under acquisition, maintenance, and relapse-like conditions. Alcohol. Clin. Exp. Res. 33, 722–728. https://doi.org/10.1111/j.1530-0277.2008.00889.x.

Gass, J.T., Sinclair, C.M., Cleva, R.M., Widholm, J.J., Olive, M.F., 2011. Alcohol-seeking behavior is associated with increased glutamate transmission in basolateral amygdala and nucleus accumbens as measured by glutamate-oxidase coated biosensors. Addict. Biol. 16, 215–228. https://doi.org/10.1111/j.1369-1600.2010.00262.x.

Gilpin, N.W., Karanikas, C.A., Richardson, H.N., 2012. Adolescent binge drinking leads to changes in alcohol drinking, anxiety, and amygdalar corticotropin releasing factor cells in adulthood in male rats. PloS One 7, e31466. https://doi.org/10.1371/journal.pone.0031466.

Griggs, E.M., Young, E.J., Rumbaugh, G., Miller, C.A., 2013. MicroRNA-182 regulates amygdala-dependent memory formation. J. Neurosci. 33, 1734–1740. https://doi.org/10.1523/JNEUROSCI.2873-12.2013.

Gu, Z., Eils, R., Schlesner, M., 2016. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. Bioinformatics 32, 2847–2849. https://doi.org/10.1093/bioinformatics/btw313.

Healey, K.L., Kibble, S.A., Bell, A., Kramer, G., Maldonado-Devincci, A., Swartzwelder, H. S., 2022. Sex differences in the effects of adolescent intermittent ethanol exposure on exploratory and anxiety-like behavior in adult rats. Alcohol 98, 43–50. https://doi.org/10.1016/j.alcohol.2021.11.002.

Healey, K.L., Kibble, S., Dubester, K., Bell, A., Swartzwelder, H.S., 2023. Adolescent intermittent ethanol exposure enhances adult stress effects in male rats. Pharmacol. Biochem. Behav. 223, 173513 https://doi.org/10.1016/j.pbb.2022.173513.

Ibáñez, F., Ureña-Peralta, J.R., Costa-Alba, P., Torres, J.-L., Laso, F.-J., Marcos, M., Guerri, C., Pascual, M., 2020. Circulating microRNAs in extracellular vesicles as potential biomarkers of alcohol-induced neuroinflammation in adolescence: gender differences. IJMS 21, 6730. https://doi.org/10.3390/ijms21186730.

Jury, N.J., Pollack, G.A., Ward, M.J., Bezek, J.L., Ng, A.J., Pinard, C.R., Bergstrom, H.C., Holmes, A., 2017. Chronic ethanol during adolescence impacts corticolimbic dendritic spines and behavior. Alcohol. Clin. Exp. Res. 41, 1298–1308. https://doi.org/10.1111/acer.13422.

Kamens, H.M., Miller, C.N., Caulfield, J.I., Zeid, D., Horton, W.J., Silva, C.P., Sebastian, A., Albert, I., Gould, T.J., Fishbein, D., Grigson, P.S., Cavigelli, S.A., 2021. Adolescent stress reduces adult morphine-induced behavioral sensitization in C57BL/6J mice. Front. Behav. Neurosci. 15, 678102 https://doi.org/10.3389/fnbeh.2021.678102.

Kim, J., Pignatelli, M., Xu, S., Itohara, S., Tonegawa, S., 2016. Antagonistic negative and positive neurons of the basolateral amygdala. Nat. Neurosci. 19, 1636–1646. https://doi.org/10.1038/nn.4414.

Knight, P., Chellian, R., Wilson, R., Behnood-Rod, A., Panunzio, S., Bruijnzeel, A.W., 2021. Sex differences in the elevated plus-maze test and large open field test in adult Wistar rats. Pharmacol. Biochem. Behav. 204, 173168 https://doi.org/10.1016/j.pbb.2021.173168.

Kokare, D.M., Kyzar, E.J., Zhang, H., Sakharkar, A.J., Pandey, S.C., 2017. Adolescent alcohol exposure-induced changes in alpha-melanocyte stimulating hormone and neuropeptide Y pathways via histone acetylation in the brain during adulthood. Int. J. Neuropsychopharmacol. 20, 758–768. https://doi.org/10.1093/ijnp/pyx041.

Kozomara, A., Birgaoanu, M., Griffiths-Jones, S., 2019. miRBase: from microRNA sequences to function. Nucleic Acids Res. 47, D155–D162. https://doi.org/10.1093/nar/gky1141.

Kyzar, E.J., Pandey, S.C., 2015. Molecular mechanisms of synaptic remodeling in alcoholism. Neurosci. Lett. 601, 11–19. https://doi.org/10.1016/j.neulet.2015.01.051.

Kyzar, E.J., Zhang, H., Sakharkar, A.J., Pandey, S.C., 2017. Adolescent alcohol exposure alters lysine demethylase 1 (LSD1) expression and histone methylation in the amygdala during adulthood. Addict. Biol. 22, 1191–1204. https://doi.org/10.1111/adb.12404.

Kyzar, E.J., Bohnsack, J.P., Zhang, H., Pandey, S.C., 2019. MicroRNA-137 drives epigenetic reprogramming in the adult amygdala and behavioral changes after adolescent alcohol exposure. eNeuro 6. https://doi.org/10.1523/ENEURO.0401-19.2019. ENEURO.0401-19.2019.

Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359. https://doi.org/10.1038/nmeth.1923.

Li, Y., Li, S., Yan, J., Wang, D., Yin, R., Zhao, L., Zhu, Y., Zhu, X., 2016. miR-182 (microRNA-182) suppression in the hippocampus evokes antidepressant-like effects in rats. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 65, 96–103. https://doi.org/10.1016/j.pnpbp.2015.09.004.

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550. https://doi.org/10.1186/s13059-014-0550-8.

Maldonado-Devincci, A.M., Alipour, K.K., Michael, L.A., Kirstein, C.L., 2010. Repeated binge ethanol administration during adolescence enhances voluntary sweetened ethanol intake in young adulthood in male and female rats. Pharmacol. Biochem. Behav. 96, 476–487. https://doi.org/10.1016/j.pbb.2010.07.008.

Marron Fernandez De Velasco, E., Tipps, M.E., Haider, B., Souders, A., Aguado, C., Rose, T.R., Vo, B.N., DeBaker, M.C., Luján, R., Wickman, K., 2023. Ethanol-induced suppression of G protein–gated inwardly rectifying K+–dependent signaling in the basal amygdala. Biol. Psychiatry. https://doi.org/10.1016/j.biopsych.2023.04.006. S0006322323012027.

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17, 10–12. https://doi.org/10.14806/ej.17.1.200.

Matthews, D.B., Scaletty, S., Trapp, S., Kastner, A., Schneider, A.M., Schreiber, A., Rossmann, G., 2022. Chronic intermittent ethanol administration during adolescence produces sex dependent impairments in behavioral flexibility and survivability. Brain Sci. 12, 606. https://doi.org/10.3390/brainsci12050606.

McCreary, J.K., Erickson, Z.T., Hao, Y., Ilnytskyy, Y., Kovalchuk, I., Metz, G.A.S., 2016. Environmental intervention as a therapy for adverse programming by ancestral stress. Sci. Rep. 6, 37814. https://doi.org/10.1038/srep37814.

McDannald, M.A., Galarce, E.M., 2011. Measuring Pavlovian fear with conditioned freezing and conditioned suppression reveals different roles for the basolateral amygdala. Brain Res. 1374, 82–89. https://doi.org/10.1016/j.brainres.2010.12.050.

Millan, E.Z., Reese, R.M., Grossman, C.D., Chaudhri, N., Janak, P.H., 2015. Nucleus accumbens and posterior amygdala mediate cue-triggered alcohol seeking and suppress behavior during the omission of alcohol-predictive cues. Neuropsychopharmacology 40, 2555–2565. https://doi.org/10.1038/npp.2015.102.

Moaddab, M., Mangone, E., Ray, M., McDannald, M., 2017. Adolescent alcohol drinking renders adult drinking BLA-dependent: BLA hyper-activity as contributor to comorbid alcohol use disorder and anxiety disorders. Brain Sci. 7, 151. https://doi.org/10.3390/brainsci7110151.

Nimitvilai-Roberts, S., Gioia, D., Lopez, M.F., Glaser, C.M., Woodward, J.J., 2023. Chronic intermittent ethanol exposure differentially alters the excitability of neurons in the orbitofrontal cortex and basolateral amygdala that project to the dorsal striatum. Neuropharmacology 228, 109463. https://doi.org/10.1016/j.neuropharm.2023.109463.

Pandey, S.C., Sakharkar, A.J., Tang, L., Zhang, H., 2015. Potential role of adolescent alcohol exposure-induced amygdaloid histone modifications in anxiety and alcohol intake during adulthood. Neurobiol. Dis. 82, 607–619. https://doi.org/10.1016/j.nbd.2015.03.019.

Pandey, S.C., Kyzar, E.J., Zhang, H., 2017. Epigenetic basis of the dark side of alcohol addiction. Neuropharmacology 122, 74–84. https://doi.org/10.1016/j.neuropharm.2017.02.002.

Pascual, M., López-Hidalgo, R., Montagud-Romero, S., Ureña-Peralta, J.R., Rodríguez-Arias, M., Guerri, C., 2021. Role of mTOR-regulated autophagy in spine pruning defects and memory impairments induced by binge-like ethanol treatment in adolescent mice. Brain Pathol. 31, 174–188. https://doi.org/10.1111/bpa.12896.

Paxinos, G., 1998. The Rat Brain in Stereotaxic Coordinates. Academic Press.

Perra, S., Pillolla, G., Luchicchi, A., Pistis, M., 2008. Alcohol inhibits spontaneous activity of basolateral amygdala projection neurons in the rat: involvement of the endocannabinoid system. Alcohol. Clin. Exp. Res. 32, 443–449. https://doi.org/10.1111/j.1530-0277.2007.00588.x.

Price, M.E., McCool, B.A., 2022. Chronic alcohol dysregulates glutamatergic function in the basolateral amygdala in a projection-and sex-specific manner. Front. Cell. Neurosci. 16, 857550 https://doi.org/10.3389/fncel.2022.857550.

Rau, A.R., Chappell, A.M., Butler, T.R., Ariwodola, O.J., Weiner, J.L., 2015. Increased basolateral amygdala pyramidal cell excitability may contribute to the anxiogenic phenotype induced by chronic early-life stress. J. Neurosci. 35, 9730–9740. https://doi.org/10.1523/JNEUROSCI.0384-15.2015.

Robinson, S.L., Dornellas, A.P.S., Burnham, N.W., Houck, C.A., Luhn, K.L., Bendrath, S.C., Companion, M.A., Brewton, H.W., Thomas, R.D., Navarro, M., Thiele, T.E., 2020. Distinct and overlapping patterns of acute ethanol-induced C-Fos activation in two inbred replicate lines of mice selected for drinking to high blood ethanol concentrations. Brain Sci. 10, 988. https://doi.org/10.3390/brainsci10120988.

Sakharkar, A.J., Tang, L., Zhang, H., Chen, Y., Grayson, D.R., Pandey, S.C., 2014. Effects of acute ethanol exposure on anxiety measures and epigenetic modifiers in the extended amygdala of adolescent rats. Int. J. Neuropsychopharmacol. 17, 2057–2067. https://doi.org/10.1017/S1461145714001047.

Sakharkar, A.J., Kyzar, E.J., Gavin, D.P., Zhang, H., Chen, Y., Krishnan, H.R., Grayson, D.R., Pandey, S.C., 2019. Altered amygdala DNA methylation mechanisms after adolescent alcohol exposure contribute to adult anxiety and alcohol drinking. Neuropharmacology 157, 107679. https://doi.org/10.1016/j.neuropharm.2019.107679.

Silberman, Y., Bajo, M., Chappell, A.M., Christian, D.T., Cruz, M., Diaz, M.R., Kash, T., Lack, A.K., Messing, R.O., Siggins, G.R., Winder, D., Roberto, M., McCool, B.A., Weiner, J.L., 2009. Neurobiological mechanisms contributing to alcohol-stress-anxiety interactions. Alcohol 43, 509. https://doi.org/10.1016/j.alcohol.2009.01.002.

Spear, L.P., 2000. The adolescent brain and age-related behavioral manifestations. Neurosci. Biobehav. Rev. 24, 417–463. https://doi.org/10.1016/S0149-7634(00)00014-2.

Spear, L.P., 2013. Adolescent neurodevelopment. J. Adolesc. Health 52, S7–13. https://doi.org/10.1016/j.jadohealth.2012.05.006.

Spear, L.P., 2018. Effects of adolescent alcohol consumption on the brain and behaviour. Nat. Rev. Neurosci. 19, 197–214. https://doi.org/10.1038/nrn.2018.10.

Spear, L.P., Swartzwelder, H.S., 2014. Adolescent alcohol exposure and persistence of adolescent-typical phenotypes into adulthood: a mini-review. Neurosci. Biobehav. Rev. 0, 1–8. https://doi.org/10.1016/j.neubiorev.2014.04.012.

Stoychev, K., Dilkov, D., Naghavi, E., Kamburova, Z., 2021. Genetic basis of dual diagnosis: a review of genome-wide association studies (GWAS) focusing on patients with mood or anxiety disorders and co-occurring alcohol-use disorders. Diagnostics (Basel) 11, 1055. https://doi.org/10.3390/diagnostics11061055.

Strong, M.N., Yoneyama, N., Fretwell, A.M., Snelling, C., Tanchuck, M.A., Finn, D.A., 2010. "Binge" drinking experience in adolescent mice shows sex differences and elevated ethanol intake in adulthood. Horm. Behav. 58, 82–90. https://doi.org/10.1016/j.yhbeh.2009.10.008.

Tarazona, S., García-Alcalde, F., Dopazo, J., Ferrer, A., Conesa, A., 2011. Differential expression in RNA-seq: a matter of depth. Genome Res. 21, 2213–2223. https://doi.org/10.1101/gr.124321.111.

Tavares, G.E.B., Bianchi, P.C., Yokoyama, T.S., Palombo, P., Cruz, F.C., 2023. Involvement of cortical projections to basolateral amygdala in context-induced reinstatement of ethanol-seeking in rats. Behav. Brain Res. 448, 114435 https://doi.org/10.1016/j.bbr.2023.114435.

Teague, C.D., Nestler, E.J., 2022. Teenage drinking and adult neuropsychiatric disorders: an epigenetic connection. Sci. Adv. 8, eabq5934. https://doi.org/10.1126/sciadv.abq5934.

The Jamovi Project, 2022.

Tye, K.M., Prakash, R., Kim, S.-Y., Fenno, L.E., Grosenick, L., Zarabi, H., Thompson, K.R., Gradinaru, V., Ramakrishnan, C., Deisseroth, K., 2011. Amygdala circuitry mediating reversible and bidirectional control of anxiety. Nature 471, 358–362. https://doi.org/10.1038/nature09820.

Van Skike, C.E., Diaz-Granados, J.L., Matthews, D.B., 2015. Chronic intermittent ethanol exposure produces persistent anxiety in adolescent and adult rats. Alcohol. Clin. Exp. Res. 39, 262–271. https://doi.org/10.1111/acer.12617.

Vázquez-Ágredos, A., Gámiz, F., Gallo, M., 2022. MicroRNA regulation of the environmental impact on adolescent neurobehavioral development: a systematic review. Front. Cell. Neurosci. 16, 956609 https://doi.org/10.3389/fncel.2022.956609.

Wassum, K.M., Izquierdo, A., 2015. The basolateral amygdala in reward learning and addiction. Neurosci. Biobehav. Rev. 57, 271–283. https://doi.org/10.1016/j.neubiorev.2015.08.017.

Wickham, H., 2016. ggplot2, Use R! Springer International Publishing, Cham. https://doi.org/10.1007/978-3-319-24277-4.

Wille-Bille, A., De Olmos, S., Marengo, L., Chiner, F., Pautassi, R.M., 2017. Long-term ethanol self-administration induces ΔFosB in male and female adolescent, but not in adult, Wistar rats. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 74, 15–30. https://doi.org/10.1016/j.pnpbp.2016.11.008.