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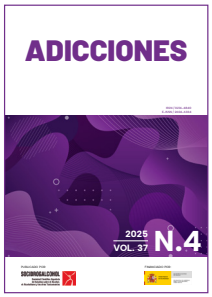

ORIGINAL

Sex differences in emotional and cognitive tasks in rats exposed to alcohol binges and controls during early adulthood

Diferencias sexuales en tareas emocionales y cognitivas en ratas expuestas a atracones de alcohol y en controles durante la adultez temprana

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Abstract

A sex perspective in the behavioural effects induced by alcohol binge exposure during youth or early adulthood remain limited. This study aimed to evaluate the effects of high doses of ethanol on emotional behaviour and cognition in 8-weeks old rats, from a sex perspective. Male and female animals were exposed to ethanol binges (3 g/kg, i.g.; 3 times/day x 4 days) in a 2days on-2days off paradigm and assessed in the elevated plus maze (EPM), forced swimming test (FST), saccharin preference test (SPT), Morris water maze (MWM) and novel object recognition (NOR) test.

Baseline differences between control male and females were observed in emotional, motivational and cognitive tasks. Additionally, Intensive Alcohol Exposure (IAE) exerted sex-specific effects in: a) EPM: males showed anxiety and no effect in females; b) FST: depressive-like symptoms in both sexes but more pronounce immobility time in females; c) NOR test: impairment in a short-term memory in both sexes but females displayed improved performance in a long-term versus their controls. No IAE-related effects were found in the SPT or MWM.

These results suggest inherent sex-differences in rodent performance in behavioural tests assessing emotional, motivational and cognitive behaviours. Additionally, IAE may impact male and females differently during abstinence in early adulthood. These findings underscore the importance of considering sex as a critical variable for preclinical studies.

Key words: Alcohol, youth, behaviour, binge drinking, sex differences

Resumen

Integrar la perspectiva de sexo en estudios preclínicos sobre la exposición excesiva a alcohol durante la juventud o la adultez temprana sigue siendo limitada. Este estudio tuvo como objetivo evaluar los efectos de altas dosis de etanol en el comportamiento emocional y la cognición en ratas de 8 semanas de edad, considerando diferencias de sexo. Machos y hembras fueron expuestos a atracones de etanol (3 g/kg, vía oral; 3 veces al día durante 4 días) en un paradigma de dos días alternos, y evaluados en el laberinto elevado en cruz (EPM), la prueba de natación forzada (FST), la prueba de preferencia de sacarina (SPT), el laberinto acuático de Morris (MWM) y la prueba de reconocimiento de objetos nuevos (NOR).

Se observaron diferencias basales entre machos y hembras controles en tareas emocionales, motivacionales y cognitivas. Además, la Exposición Intensiva al Alcohol (EIA) ejerció efectos específicos por sexo en: a) EPM: los machos mostraron ansiedad y ningún efecto en las hembras; b) FST: síntomas similares a la depresión en ambos sexos, pero un tiempo de inmovilidad más pronunciado en las hembras; c) Prueba NOR: Se observó deterioro de la memoria a corto plazo en ambos sexos, pero las hembras mostraron un mejor rendimiento a largo plazo en comparación con sus controles. No se observaron efectos relacionados con la EIA en las pruebas de conducta SPT ni en MWM.

Estos resultados sugieren diferencias inherentes entre sexos en el rendimiento de los roedores en pruebas conductuales que evalúan las conductas emocionales, motivacionales y cognitivas. Además, la EIA podría afectar de forma diferente a machos y hembras durante la abstinencia en adultez temprana. Estos hallazgos subrayan la importancia de considerar el sexo como una variable crítica en los estudios preclínicos.

Palabras clave: Alcohol, juventud, comportamiento, atracones, diferencias sexuales

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Traditionally, most of the preclinical studies in animal models have been performed in males and, therefore, there is a significant lack of information and bias by sex in a great deal of biomedical and psychobiological results. Over the last decade, it has been a dizzying turn of the scientific community on this issue, urging the incorporation of the sex variable in all preclinical investigations and also the gender perspective in humans' studies. Indeed, policy changes such as the 2016 mandate by the National Institutes of Health (NIH), emphasize the inclusion of sex as a critical biological variable in preclinical and clinical research (Campbell et al., 2024; Costa-Valle et al., 2022; Kaluve et al., 2022). This emerging urge in understanding sex differences in preclinical investigation also affects studies related with alcohol abuse. Although the efforts to address these methodological biases have been intensified over the last years, exploring sex-based differences in the context of alcohol abuse is still scarce, specifically the impact of alcohol abuse in behavioural responses, including emotional and cognitive subdomains.

Several lines of research studying the impact of alcohol binge drinking (ABD) on youth or early adulthood, including our own experience, have led to important biological and behavioural consequences of such a dangerous pattern of alcohol consumption without specific focus in the sex variable (Antón et al., 2017, 2018) and only more recently we addressed this important issue (López-Valencia et al., 2024; Orio et al., 2018).

ABD consists of an intensive alcohol exposure (IAE). It is defined as the consumption of 4 or 5 standard drink units (SDU) in a short period of time. It is accepted that the acute rise in blood ethanol levels (BELs) achieved during this pattern of alcohol consumption (≥ 80 g/dL) contribute to the neurotoxic effects and alterations in brain plasticity (Patrick et al., 2021; Waszkiewicz et al., 2018) and it is associated with neuroinflammation and emotional and cognitive alterations (Antón et al., 2017; Crews et al., 2016; Orio et al., 2018; Pascual et al., 2007, 2014). Despite its adverse outcomes, ABD is particularly prevalent among adolescents and youths, who are often drawn to this pattern of consumption due to its association with behavioural disinhibition. The WHO and other global monitoring systems have reported the prevalence of heavy episodic drinking across different age groups, emphasizing its impact on public health (World Health Organization, 2024). Young people are more vulnerable to the reinforcing effects of drugs, especially alcohol, which is often easily accessible in many environments. Indeed, a combination of biological vulnerability and a predisposition for seeking new experiences increase the risk of substance use and abuse during adolescence (Chung et al., 2018; Maldonado-Devincci et al., 2022; Sicher et al., 2022; Spear, 2018).

This study aims to investigate the effects of IAE comparatively on young males and females' rats during early abstinence, using a validated animal model and focusing on cognitive and emotional behaviours. Searching for possible sex-specific differences in the performance of several tests will potentially help us to better understand basal differences in normal behaviour and the underlying mechanism of alcohol abuse for each sex in a near future.

Material and methods

Animals

Fifty-seven Wistar rats (Envigo©, Barcelona, Spain) aged seven weeks at arrival were used across all experiments. Females and males were housed in different isolated rooms in groups of 3-4 per cage. They were maintained at constant conditions of temperature (21 ± 1°C) and humidity (59 ± 10%) under a 12 h dark-light inversed cycle (lights on at 8:00 p.m.) with free access to food and water. Animals were habituated to these conditions for one week before the experiments and then they were handled gently to acclimate to the experimenters and gavage procedure.

All procedures were approved and adhered to the guidelines of the Animal Welfare Committee of the Complutense University of Madrid (Ethical approval reference: PROEX 312/19; PROEX 122.7/23) following European legislation (2010/63/EU).

Experimental design

Rats were randomly assigned to control and ethanol groups: male control group (n=9), male ethanol group (n=10), female control group (n=18), and female ethanol group (n=20). Rats received intragastric (i.g.) intermittent doses of ethanol or water three times per day for four days using specific cannulae (16-G needle, Fisher Scientific, Waltham, MA, USA), in a 2-day on/2-day off protocol (Fig. 1A). This is a modified protocol (from Antón et al., 2017; Obernier et al., 2002; Rodríguez-González et al., 2021) previously used by our group (Lopez-Valencia et al., 2024), which introduces a period of abstinence, a relevant factor involved in the harmful effects of binge drinking consumption (Pascual et al., 2007). The experiments in females were conducted in duplicate to increase the internal validity of the results, which are presented as a pool of data from two identical experiments.

Behavioural Assessment

The behavioural assessment was scheduled by sufficient time interval to avoid interferences between tests, and they were performed during the dark phase (see Fig. 1B for experimental timeline). Rats were evaluated in traditional tests used to assess emotional and cognitive alterations, following an alternation of experimental groups in all tests.

The analyses were performed by a double-blind protocol to ensure the truthfulness of the results.

Anxiety-like behaviour: Elevated Plus Maze

To check anxiety-like behaviour, we performed the Elevated Plus Maze (EPM) test 12 h after the last ethanol binge. EPM is based on a balance of fear and curiosity towards novelty, and it is designed to test general anxiety-related behaviours in rodents (Cosquer et al., 2005; Pellow et al., 1985).

The EPM was performed on two open black and grey plastic arms (50 x 10 cm) and two perpendicular enclosed arms of the same size but with opaque walls 50 cm high. The junction of the four arms formed a central square area (10 cm^2). The apparatus was elevated 65 cm above the floor. The light intensity was set up at 20 lux. On the test day, each rat was placed on the central platform facing one closed arm and opposite to the experimenter position. Then, the animal was allowed to freely explore de maze for 5 min. Between animals, the maze was carefully cleaned with ethanol 5% to remove possible odour. One rat fell from the EPM initially and it was excluded from the analysis. The number of entries and the time spent in all the arms were measured by a computer-controlled system (Mazesoft-4) recording the interruptions of infrared photo beams located along each arm. The percentage of each was calculated upon total entries into any arms and upon the total time spent in both arms, respectively. It was considered a visit whenever an animal entered an arm with all four limbs. Anxiety-like behaviour was defined as a decrease in the number of entries and time spent in the opened arms related to the total entries and total time, respectively.

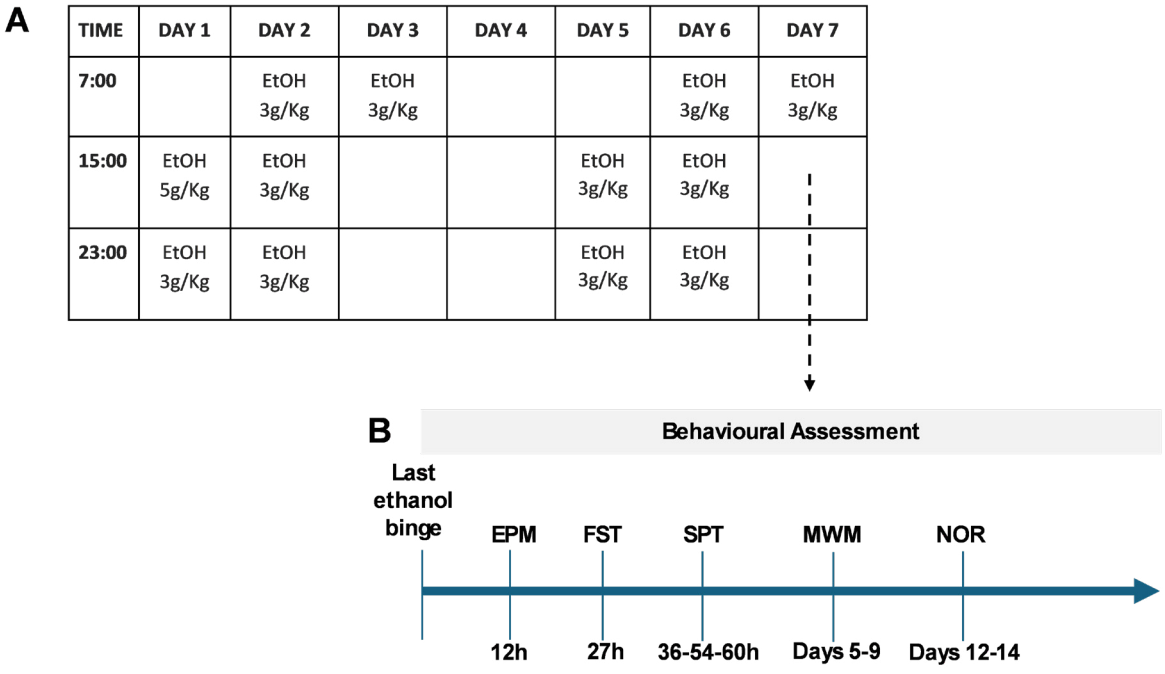
Depressive-like behaviour: Forced Swimming Test

The Forced Swimming Test (FST) is based on the method described by Porsolt (Porsolt et al., 1977) and it is one of the most common assays for the study of depressive-like behaviour in rodents (Slattery & Cryan, 2012; Yankelevitch-Yahav et al., 2015), although there are contradictory opinions (Armario, 2021).

Animals were tested 27 h after the last ethanol administration (see Fig 1 for experimental design). They were placed individually into transparent cylinders (47 x 35 cm) filled with water (25 ± 1ºC) for 5 min. Escape-directed behaviours were analysed, such as swimming, horizontal movements throughout the water tank; climbing, vertical movements of the forepaws; immobility and latency to immobility. All the assays were performed under red light conditions and recorded for subsequent analysis. Either an increase in the immobility or a decrease in the swimming, climbing and latency times were considered depressive-like behaviour, reflecting a failure of persistence in escape (Cryan et al., 2002; Detke et al., 1995).

Figure 1

Schematic representation of the experimental design



Note. A) Alcohol binge drinking treatment; binges were administered intragastrically at 3g/kg, except for the initial loading dose of 5 g/kg every 8h in a 2days on-2days off- 2days on paradigm; B) Timeline of behavioural testing. EtOH: ethanol; ***EPM***: elevated plus maze; ***FST:*** forced swimming test; ***SPT:*** saccharin preference test; ***MWM***: Morris water maze test; ***NOR***: novel object recognition test.

Motivational behaviour: Saccharin Preference Test

The Saccharin Preference Test (SPT) is used to measure the sensitivity to reward in rodents (Scheggi et al., 2018). Once animals were finishing the FST, around 27h after last binge, they were dried with a towel and housed individually, provided with food *ad libitum*. In each individual cage, rats were offered a free choice between 2 bottles, one with 0.1% saccharin (w/v) and another with tap water. Bottles were weighed to determine the liquid consumption and placed into the cage with an alternated position of the water vs. saccharin to avoid place preference. Liquid consumption was measured at specific abstinence times from last binge (36h, 54h, 60h) and accumulated drinking was calculated. The saccharin preference was shown as the percentage of consumed saccharin over the total amount of liquid intake. A decrease in the preference for the sweet solution (natural reward) is considered a reflection of anhedonia, a core symptom of a depressive-like behaviour (Scheggi et al., 2018; Slattery et al., 2007).

Spatial Memory: Morris Water Maze

The Morris Water Maze (MWM) test is used to measure spatial learning and memory in rodents (Rendeiro et al., 2009). This test was carried out during 5 consecutive days in a circular pool (diameter 122 cm). The water in the tank was made opaque with white nontoxic tempera paint (temperature 24 ± 1ºC). The pool was in a room with visible external cues and light intensity controlled. The experimenter worked also as a cue. A platform (diameter 10 cm) was submerged 1-2 cm below the water surface in one of four equal imaginary quadrants. During 4 consecutive days, animals were trained to find the submerged platform in a fixed location of the MWM. Each day consisted of 4 trials in which animals were released facing the wall from different points. Each trial had a maximum latency of 60 s, where animals freely explored the swimming pool to reach the platform. All rats were allowed to stay on the platform for 10 s more before being removed from the water. Latencies to find the platform were recorded in each trial and the average was calculated for each day and animal. After each trial, mice were dried and returned to their home cages.

The fifth day, the test was carried out without the platform for 60 s with a new start position in the pool to ensure that the mice remember the goal location rather than a specific swim path. Here, we measured the latency to reach the previous platform location, the number of platform-site crossovers and the time spent within an imaginary ring (diameter 30 cm) around where the platform had been. All the assays were recorded by a video camera located above the pool for further analysis (Vorhees & Williams, 2006).

Recognition Memory: Novel Object Recognition Test

The Novel Object Recognition (NOR) test was performed to study possible memory impairments based on the tendency of rodents to interact more with a novel object than a familiar one (Bevins & Besheer, 2006).

The test was performed in a square arena (80x80x42cm) with black matte-painted walls and floor. The arena was subdivided into 4 equal sections, allowing the evaluation of 4 rats simultaneously. The NOR was carried out in accordance with previous studies (Marco et al., 2013; Moya et al., 2022) under low-light conditions (20 lux). The test was organized in three phases: habituation (time = 0), a training phase (pre-test) and two test sessions 4h and 24h after the training phase. During the habituation, animals were allowed to freely explore the arena during 5 minutes without objects. In the training phase, 2 identical objects (glass bottles) were in opposite corners of the arena, and animals were allowed to freely explore them for 3 min. During the test session 4h after the training phase, one of the familiar objects (F, glass bottle) was substituted by a novel object (N1, green ashtray), and the rats were allowed to explore both objects for 5 min. In the 24h session, the novel N1 was replaced by another novel object (N2, money box) and the object positions in the arena were alternated in order to avoid possible place preferences. Both the training and test sessions were video recorded (Sony DCRDVD310E, Spain). Exploration of an object was considered whenever animals pointed their nose toward an object at a distance 1 cm, while turning around, climbing or biting the objects was not considered exploration. The latency to first explore the novel object in the test sessions was registered, and the discrimination index (DI) calculated as the difference between the time spent exploring the novel object and the familiar one in relation to the total time spent exploring the objects.

Statistical Analysis

All data are expressed as the mean ± S.E.M and were analysed using a two-way analysis of variance (2-way ANOVA), comparing the factors [alcohol/water] versus sex [male/female], when normality was verified; otherwise, a Kruskal-Wallis test was used. The saccharin preference test was analysed using a three-way ANOVA with time (36h, 54h, 60h), ABD treatment (water versus alcohol) and sex (males vs. females) as independent factors. *Post hoc* comparisons (Bonferroni or Dunn's) were performed in case of significant interaction between factors. Homoscedasticity was checked by Barlett's test, and data were transformed (sqrt, $\log_{10}$) when appropriate. The outliers were excluded using Grubbs' test. A p value <0.05 was set as the threshold for statistical significance in all statistical analyses. All data were analysed using GraphPad Prism version 8.01 (GraphPad Software, Inc., La Jolla, CA, USA).

Ethical aspects

All protocols have been approved by the Animal Welfare Committee of Complutense University of Madrid (reference: PROEX 312/19) following European legislation (2010/63/EU).

Results

Effects on anxiety

In the EPM, we observed an apparent opposite effect of alcohol in males and females versus their controls at 12h of abstinence, regarding the percentage of entries in the open arms (Fig. 2A). The Kruskal-Wallis test was significant (Kruskal-Wallis = 10.66, p=0.0137), and the Dunn's *post hoc* test suggested an anxiogenic behaviour in alcohol-treated males compared to their controls (trend, very close of significance (p=0.055), that was not observed in females. Indeed, data in females appear to follow the opposite pattern and, despite no significant difference between control and ethanol groups in females, the ethanol-treated female rats entered more in the open arms compared to ethanol-treated males (Fig. 2A; p=0.0168).

Regarding the percentage of time spent in the arms (Fig. 2B), the 2-way ANOVA reported a significant interaction between ethanol and sex ($F_{(1,49)}$=14.01, p=0.0005) and an ethanol main effect ($F_{(1,49)}$=7.290, p=0.0095). Bonferroni *post hoc* comparisons revealed a difference between male and female controls, with females spending less time in the open arms (p<0.05). IAE induced a clear anxiogenic effect in males (p<0.01) but not females (p>0.05, n.s.) compared with their respective controls (Fig. 2B), as suggested also in the mentioned data from Fig. A.

Effects on the depressive-like behaviour

In the FST, ethanol binge treatment induced reductions in the latency to first immobility (Fig. 3A; $F_{(1,53)}$=0.01111, p=0.0090), with no sex differences, indicative of a depressive-like behaviour during early alcohol abstinence (~ 27h).

The interaction between factors (treatment and sex) was in the limit of significance for immobility (Fig. 3B; $F_{(1,53)}$=3.964, p=0.0517) and significant for the swimming time (Fig. 3C; $F_{(1,52)}$=4.622, p=0.0362), with Bonferroni *post hoc* tests indicating that ethanol-treated females may have higher immobility versus their female controls (p<0.05). A sex main effect for immobility and swimming ($F_{(1,53)}$=146.1, p<0.0001 and $F_{(1,52)}$=126.5, p<0.0001, respectively) was indicative than females showed higher immobility duration and lower swimming time, an effect also showed after Bonferroni *post hoc* test (Fig. 3B, p<0.0001; Fig, 3C, p<0.0001, respectively).

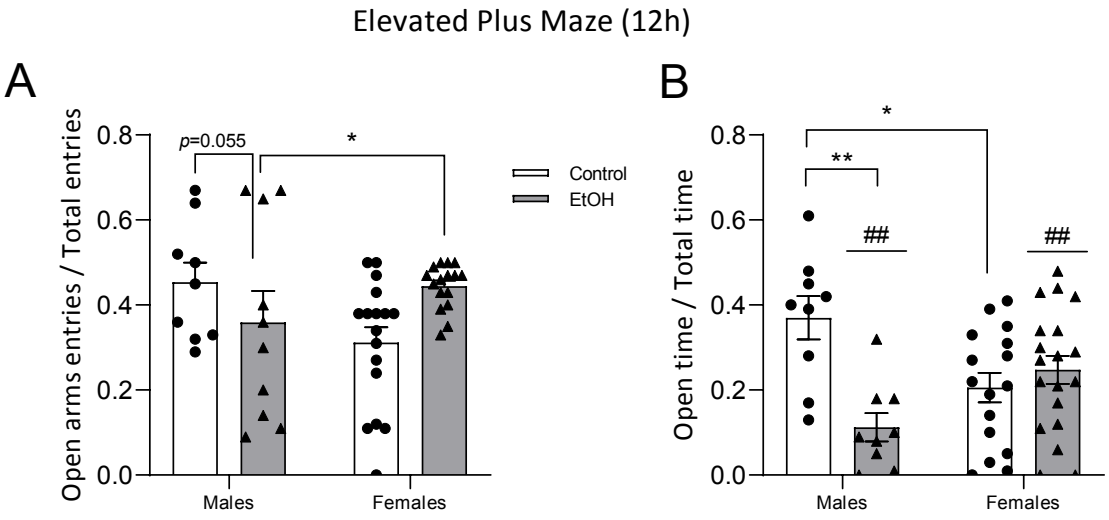
No differences were observed in the climbing times between experimental groups (Fig. 3D; EtOH main effect: $F_{(1,52)}$=1.241, p=0.2705; Sex main effect: $F_{(1,52)}$=0.2063, p=0.6516; Interaction between factors: $F_{(1,52)}$=0.8724, p=0.3546).

Effects on negative motivational state or anhedonia

The SPT started 27h after last binge exposure and we recorded accumulative drinking over the 36h, 54h and 60h of alcohol abstinence (Fig. 4). Repeated-measures 3-way ANOVA, comparing treatment, sex and time, found main effects of abstinence time ($F_{(1.768,85.73)}$=4.034, p=0.0255) and sex ($F_{(1,52)}$=55.90, p<0.0001), being females the sex showing higher saccharin preference over time. Since the

Figure 2

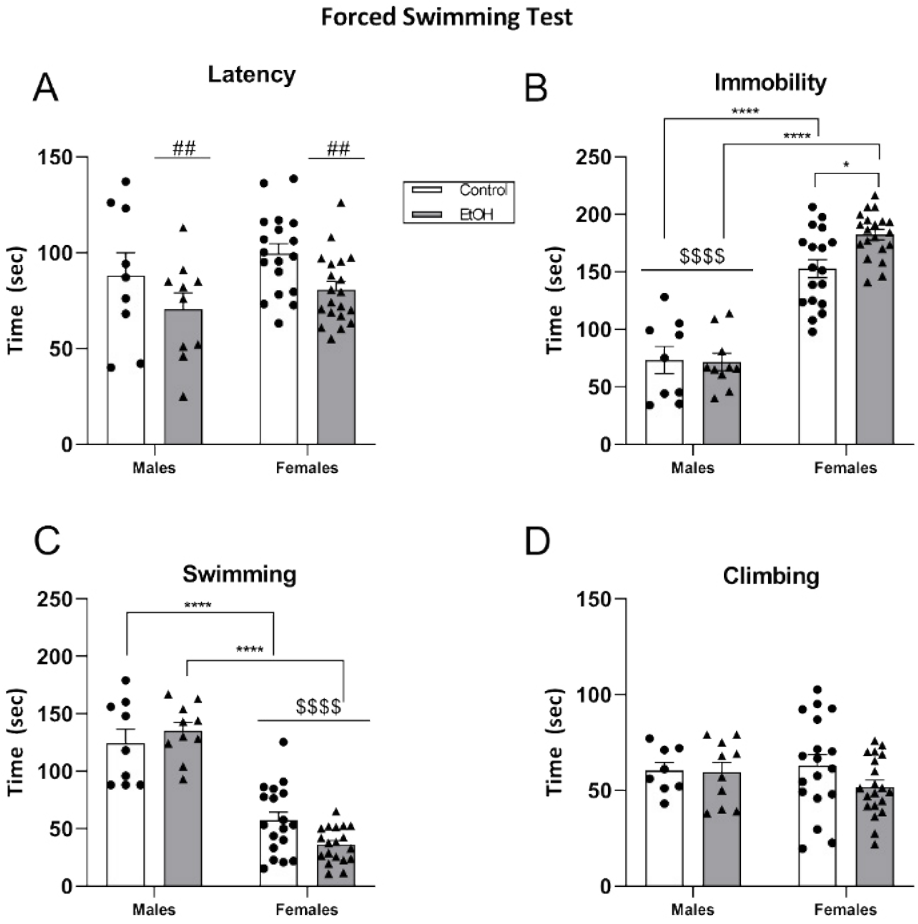
Elevated Plus Maze to assess anxiety-like behaviour in male and females



Note. A) Ratio of entries in the open arms over the total entries; B) Ratio of time spent in the open arms over the total time spent in both arms. Animals were assessed in the test 12h after the last alcohol binge administration. Results represent the mean ± S.E.M. (n=9-10 in males and n=18-20 in females: pool of two experiments). Non-parametric Kruskal-Wallis followed by Dunn's ***post hoc*** test (A): $^{*}p < 0.05$. Two-way ANOVA (B): Interaction followed by Bonferroni ***post hoc*** test (Fig.2A): $^{*}p < 0.05$, $^{**}p < 0.001$. Ethanol (EtOH) main effect: $^{\#\#}p < 0.001$.

Figure 3

Forced Swimming test to assess depressive-like behaviour



Note. A) Latency to immobility; B) Immobility time; C) Swimming time; D) Climbing time. Animals were assessed in the test around 27h after the last ethanol binge. Results represent the mean ± S.E.M. (n=9-10 in males and n=18-20 in females= pool of two identical experiments). Two-way ANOVA: EtOH main effect $^{\#\#}p < 0.001$; sex main effect $^{\$\$\$\$}p < 0.0001$; interaction followed by Bonferroni ***post hoc*** test: ethanol-treated females differ from control females $^{*}p < 0.05$; female groups differ from male groups $^{*}p < 0.0001$.

3-way ANOVA indicated an interaction between time and sex ($F_{(2,97)}$=3.191, p=0.0455) disregarding of ethanol treatment, we performed a 2-way ANOVA (time x sex) and Bonferroni *post hoc* test confirmed that females showed higher preferences at 36h (p<0.0001), 54h (p<0.001), and 60h (p<0.0001) (Figure 3).

Despite of these differences, at the times of evaluation in this experiment the possible effects of IAE on anhedonic behaviour were not evident, since we found no alcohol effects in males or females versus their controls at any time tested ($p > 0.05$, n.s.).

Effects on spatial memory

To study if alcohol consumption affected the spatial learning and memory differentially in males and females, we conducted the MWM test. During the learning curve, there were not significant differences any day between groups (Fig. 5A) and the time needed to find the platform decreased as the sessions progressed for all subjects.

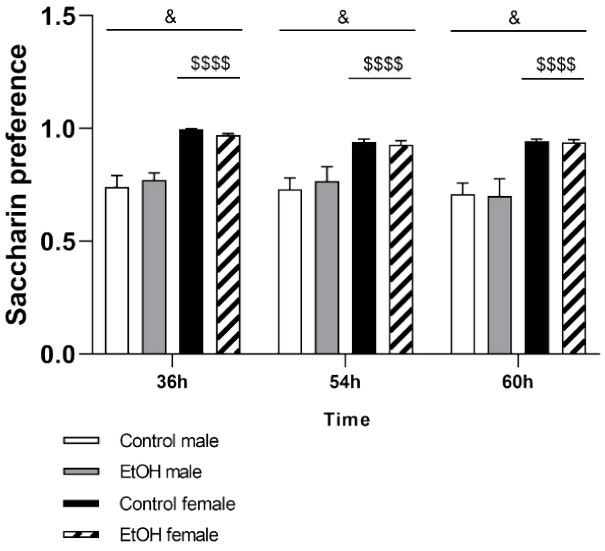
In the probe trial, we did not observe significant effects of alcohol binges in the latency, crossing or quadrant exploration variables of the test (Fig. 5B,C,D; ($F_{(1,51)}$=3.051, p=0.0867; $F_{(1,52)}$=2.535, p=0.1174; $F_{(1.53)}$=0.009591, p=0.9224, respectively), although a trend for increases latency can be observed in ethanol-treated females versus their controls (Fig. 5B, p=0.0867, n.s.). However, it was a main effect of sex on platform crosses and swimming time around the quadrant of platform location (Fig 5C,D; $F_{(1,52)}$=7.144, p=0.0100; $F_{(1,53)}$=14.76, p=0.0003, respectively), with higher scores in females than males.

Effects on recognition memory

In the NOR, we observed a main effect of alcohol in the latency to first approach to the novel object 4h after

Figure 4

Saccharin preference test to assess anhedonia



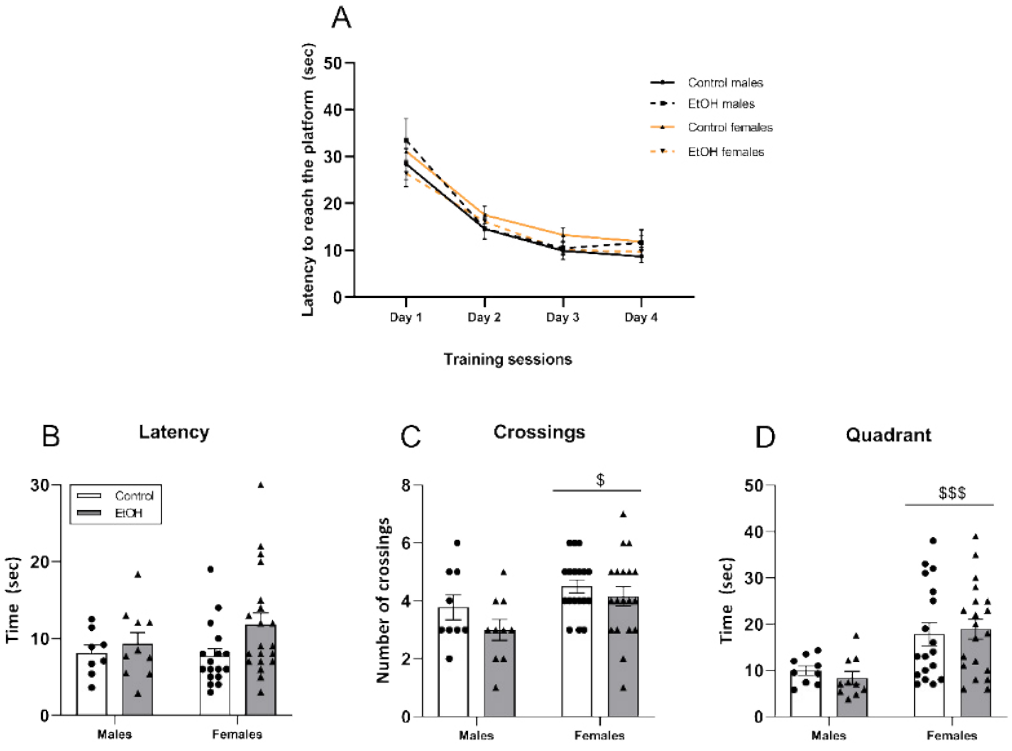
Note. The saccharin preference was calculated as the percentage of consumed saccharin over the total amount of liquid intake. Animals were tested for a cumulative response, starting around 27h after last binge and with measurements at 36h, 54h or 60h of alcohol abstinence. Data represent the mean ± S.E.M. (n=9-10 in males and n=18-20 in females: pool of two experiments). Repeated-measures 3-way ANOVA (sex x treatment x time): sex main effect: effect $^{\$\$\$\$}p < 0.0001$; time main effect $^{\&}p < 0.05$; interaction (time x sex) (not represented in the figure).

the training phase (Fig 6A, $F_{(1,46)}$=4.491, p=0.0395), as both male and female ethanol-treated animals displayed higher latencies than controls, indicative of alterations in the short-term memory. The interaction between factors (sex x treatment) was near of significance ($F_{(1,46)}$=3.551, p=0.065), data suggesting that the effect of alcohol may be stronger for males (Fig. 6A). There was no main effect of sex ($F_{(1,46)}$=0.8515, p=0.3609). Regarding the DI, the interaction was again near of significance (Fig. 6C; $F_{(1,49)}$=3.399, p=0.0713, n.s.) and no alcohol of sex main effects were found ($F_{(1,49)}$=0.1159, p=0.7350, $F_{(1,49)}$=0.5682, p=0.4546, respectively).

In a long-term, 24h after the training phase, the 2-way ANOVA revealed ethanol and sex main effects (Fig 6B $F_{(1,44)}$=5.153, p=0.0282; $F_{(1,44)}$=10.09, p=0.0027, respectively) with an interaction between factors ($F_{(1,44)}$=7.816, p=0.0076). *Post hoc* comparisons revealed than female control animals had higher latencies at 24h to explore the novel object than male controls (p<0.01). In the long-term test, only female ethanol-treated rats showed reduced latency versus their female controls (p<0.001) (Fig. 6B). Regarding the DI, 2-way ANOVA did not show interaction between factors (Fig. 6D; $F_{(1,51)}$=0.04861, p=0.8264), ethanol or sex main effects 24h after the training phase (Fig. 6D; $F_{(1,51)}$=1.037, p=0.3132, $F_{(1,51)}$=1.330, p=0.2541, respectively).

Figure 5

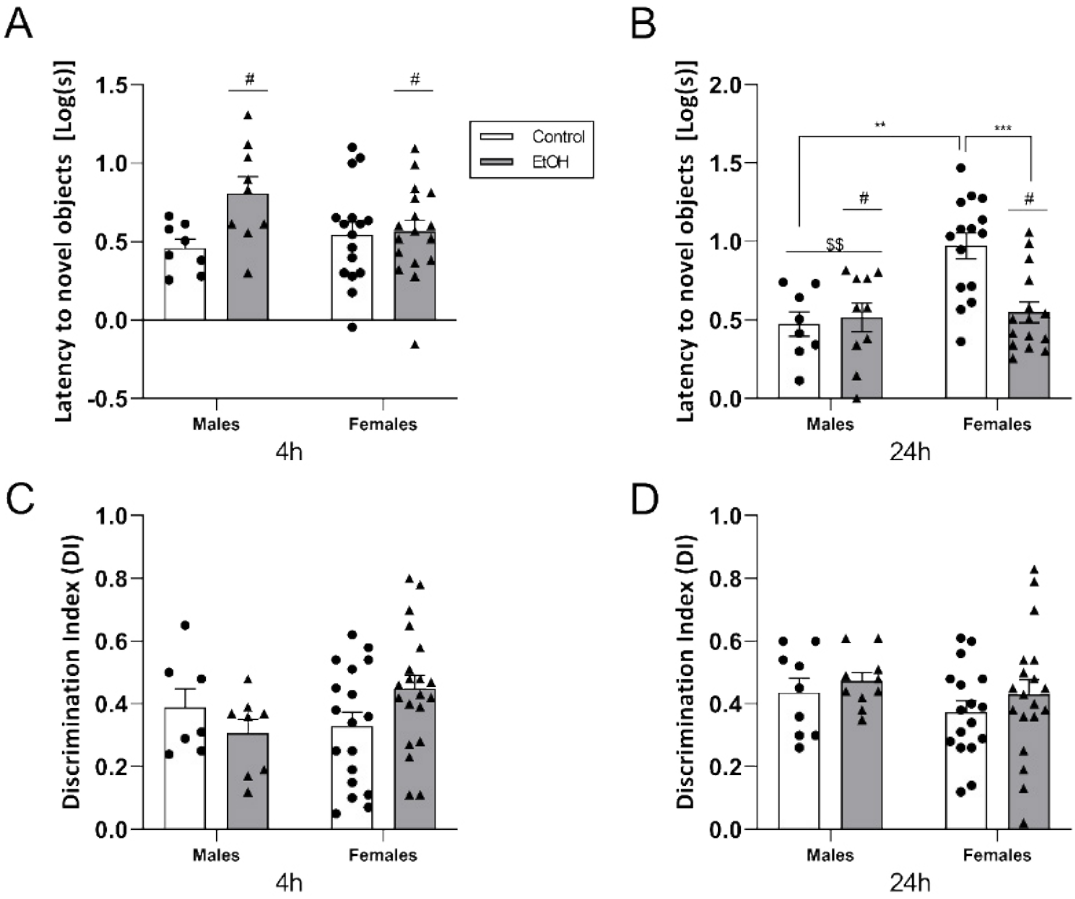
Morris Water Maze test to study spatial memory



Note. A) Learning curve including the average of each experimental group during the four trials. B) Latency to reach the platform location. C) Number of platform-site crossovers. D) Time spent around the platform location. Data is expressed as the mean ± S.E.M. Two-way ANOVA: sex main effect $^{\$}p < 0.05$, $^{\$\$\$}p < 0.0001$.

Figure 6

Novel Object Recognition test to study recognition memory



Note. A) Latency to novel object at 4h session. B) Latency to novel object at 24h session. C) Discrimination index at 4h session. D) Discrimination index at 24h session. Results represent the mean ± S.E.M. Two-way ANOVA: EtOH main effect $^{\#}p < 0.05$; sex main effect $^{\$\$}p < 0.001$; interaction followed by Bonferroni *post hoc* test: $^{**}p < 0.001$, $^{***}p < 0.0001$.

Altogether, the analysis of latency in the NOR reveals a different pattern of behaviour in males and females in the short- and long-term tests. IAE appears to negatively affect the short-term memory in both sexes (Fig. 6A), whereas in the long-term, IAE induce the opposite effect (decrease in latency) in females only (Fig. 6B).

Discussion

This study explores the differential effects of alcohol binge exposure in male and female rats during early adulthood. By focusing on both cognitive and emotional behaviours, it aims to uncover sex-specific differences that shape animal behaviour under physiological (control) conditions in several tests widely used in preclinical studies, and the impact of IAE during early adulthood with this sex perspective.

Our results suggest some sex differences both in control and ethanol conditions depending on the test, highlighting the strong need of including both sexes for any preclinical study evaluating emotional and cognitive behaviour in animals. The implications of these differences are discussed below.

Sex differences in behaviour in physiological (control) conditions

In terms of emotional state, sex-differences were observed in anxiety and depressive-like behaviours under control conditions, as showed by animal performance in the EPM and FST. The EPM is a test widely used to evaluate anxiety-like behaviour in rodents. These tasks exploit the conflict between rodents' exploratory drive and their tendency to avoid open spaces (Campbell et al., 2024; Chen et al., 2024; Donner & Lowry, 2013). Our results indicate that females spend significantly less time in the open arms of the EPM compared to males under normal conditions. While the literature generally establishes that females tend to exhibit less anxious behaviour, showing more interest in open areas than males (Campbell et al., 2024), many authors propose that sexual differences in this behavioural pattern may reflect alternative coping strategies or environmental exploration strategies, rather than true anxiety-related behaviours (Donner & Lowry, 2013).

The FST and the SPT are standard methods for assessing antidepressant effects in rodents (Scheggi et al.,

2018; Slattery et al., 2007), although the usefulness of FST for this purpose has been questioned recently (Armario, 2021). In the FST, swimming and climbing behaviours represent the animals' adaptative response to an adverse situation, suggesting that the absence of these behaviours or an increased tendency for immobility are indicators of behavioural despair or depressive-like behaviour (Dalla et al., 2010). On the other hand, the SPT evaluates motivational behaviour. It assumes that animals will consume more saccharin due to its hedonic properties, and the decrease in the preference for it (a natural reward) represents anhedonia, a core symptom of depression, which is induced by different stressors (Campbell et al., 2024; Sayd et al., 2015). It is common to find sex differences related to basal emotional state in the literature, indicating that females show more depressive-like behaviours compared to males in tasks such as the FST (Dalla et al., 2010), aligning with our results. Nevertheless, the results in tasks related to depressive-like behaviour are inconsistent; as Kokras et al. (2015) point out, in the FST, one-third of studies show greater immobility in females, as we showed here, one-third in males (Pavlova et al., 2020; Xia et al., 2023) and one-third find no sex differences. In contrast, in the SPT, females seem to show a greater preference for saccharin, both in our study and in previous works, exhibiting a different impact of it in the reward system, which calls into question the use of these tests as a measure of depressive behaviour (Dalla et al., 2010; Kokras et al., 2015). This underlines the need to validate representative tasks that correctly describe behavioural differences between male and female animals, in order to translate the results to human behaviour.

Regarding cognitive performance, the MWM provides insights about learning ability and spatial memory through measurements such as the time it takes for the animal to find the platform or the time it spends swimming around it (Zorzo et al., 2024). While most previous work comparing males and females on spatial navigation has found superiority in male performance (Bowman et al., 2022; Gutiérrez-Menéndez et al., 2023; Zorzo et al., 2024), in our study, no sex differences were found during the learning phase, in line with previous research (Qi et al., 2016), although on the day of the test, females showed more crosses and swimming time around the platform than males. Neurobiological studies related to this ability have described different strategies used by the two groups; males tend to rely on global special strategies, while females seem to favour more by spatial cues from the environment (Shansky, 2018; Zorzo et al., 2024). The high number of visual cues arranged in our experimental setup may have favoured the female's performance.

Another cognitive function related to memory is the ability to recognise new objects. The NOR test allows to evaluate this ability obtaining measures on the approach latency and the novel object discrimination index, in a short-term (4h after the training phase) or in a long-term (24h after the training phase) (Marco et al., 2013; Moya et al., 2022). Our results suggest that there are no differences in the performance of control males and females in this test in a short-term, although females perform worst in a long-term. The absence of sexual differences in the recognition memory agrees with most of the studies (Campbell et al., 2024; Van Hees et al., 2022) and only a few studies described a slight superiority of females, in different phases of the estrous cycle (Koszałka et al., 2023; van Goethem et al., 2012).

Table 1

Summary of sex differences in behaviour in physiological (control) conditions

Behavioural test	Sex differences in control animals
EPM	Females spend less time in the open arms than males
FST	Females spend less time swimming and more time immobile than males
SPT	Females showed greater preference for saccharin than males
MWM	No differences during learning phase; females showed more crosses and swimming time during test day than males
NOR	No differences at 4h; worst performance in females at 24h

Sex differences in the effects of intensive alcohol exposure

Most of the studies exploring sex differences on alcohol consumption were performed in C57BL/65 mice, with intermittent and voluntary access paradigms -such as the paradigm of choosing two bottles, or drinking in the dark (DID)-, in which the effects of long-term consumption are studied (Ardinger et al., 2024; Leonardo Jimenez Chavez et al., 2020; Magee et al., 2024; Maldonado-Devincci et al., 2022; Rath et al., 2020; Rivera-Irizarry et al., 2023). In rats, there are studies of voluntary alcohol consumption using the two-bottle paradigm (Abderrahim et al., 2022; Albrechet-Souza et al., 2020; Buján et al., 2024). Only few studies with sex perspective employed intragastric administration of ethanol, usually for long periods, and the results remain mixed (Healey et al., 2023; Jia et al., 2024; Penta et al., 2024; Varlinskaya et al., 2020). To the best of our knowledge, no prior research has examined sex differences in IAE with this specific administration protocol, using high doses of ethanol administration in a short period of time. Our results suggest that forced alcohol binge exposure impact differently in male and female rats only in some tests, which are discussed below.

Our results showed that the effect of IAE on anxiety in the EPM was clear for males, in agreement with our

Table 2

Summary of sex differences in behaviour after IAE

Behavioural test	Sex differences in ethanol-treated animals
EPM	Ethanol-treated males entered the open arms less and spent less time there than their control group whereas this effect was not shown in females.
FST	Ethanol-treated females exhibited more time immobile than female controls and this effect was not observed in males.
NOR	In a long-term phase, ethanol improves performance in females versus their controls and this effect it not observed in males.

previous studies with this animal model (Antón et al., 2017) but this effect is not present in ethanol-treated females, that showed no differences with their female controls. Note that ethanol-treated females showed a tendency to the opposite direction (anxiolytic effect), and this possible sex difference has been highlighted also by other authors using other paradigms of ethanol administration (Albrechet-Souza et al., 2020; Buján et al., 2024; Costa-Valle et al., 2022).

Besides that, our study provide evidence that binge exposure has depressive effects in both sexes, but predominantly in females, who display longer immobility times in the FST, in agreement with other studies. Alcohol-induced depressive-like behaviour during abstinence in males is in agreement with our previous data using this ABD model (Antón et al., 2017) and studies using ethanol vapor exposure (Bach et al., 2021; Walker et al., 2010).

Reductions in saccharin preference in the SPT would be related to this depressive effect. However, in our study, we did not find effects of IAE on this test. We have previously observed that an inflammatory state induced by lipopolysaccharide administration, which is known to be increased after IAE (Antón et al, 2017, 2018), induces an anhedonic state in males (Sayd et al., 2015), but we have never tested the animals under this IAE model before. It is possible that the abstinence period at the start of the test was too late to observe this effect, since the decrease in saccharin preference is highly dependent on the inflammatory status of the animal (Abderrahim et al., 2022; Sayd et al., 2015).

In terms of the cognitive function assessed in the MWM, in our study we did not observe significant effects of alcohol on spatial memory, although a trend (non-significant effect) can be observed in ethanol-treated females, which showed an apparent higher latency in the time taken to locate the platform. Other studies described negative effects of alcohol on spatial memory in males following a protocol of intermittent and voluntary alcohol access (Sanz-Martos et al. 2023), although no ethanol effects were observed in males in our study. In the NOR, we found that the ethanol-treated groups show different behaviours in the two test sessions 4h and 24h after the training phase. At 4h, alcohol shows significant effects on both sexes, increasing the latency of approaching the novel object, with a possible stronger effect in males (trend; interaction non-significant p=0.06). However, at 24h, males treated with alcohol experience an increase in latency as they did in the short-term, but females exhibited lower latency compared to their respective female control group. This effect was surprising, and it could be favoured by the high latency times observed in female controls. Other authors have shown that different paradigms of ethanol administration have negative effects on the ability to recognize the object measured by this task (Lamont et al., 2020; Moya et al., 2022), but no sex differences have been reported (Van Hees et al., 2022). These results in females would need to be confirmed in future studies, and the possible role of IAE in memory consolidation in a long-term.

Taken together, these results suggest that many behavioural tests that have been widely used to date to assess emotional and cognitive tasks in preclinical animal models may be performed differently by male and females in physiological conditions, which has implications for a proper extrapolation of results. Males and females biologically differ in several aspects, including the hormone profile. Numerous studies have demonstrated the critical influence of hormones in regulating behaviour (Hamson et al., 2016; Shirazi et al., 2021), so this could be a contributing factor to the observed variations between male and female outcomes. It is important to note that adolescence and early adulthood are crucial periods for maturation, during which hormone levels fluctuate significantly over time and vary by sex, playing a key role in the neurobiological development (Vijayakumar et al., 2018). Sex hormones such as estradiol (predominant in females) and testosterone (predominant in males) exerts distinct effects on adolescent brain development. Recent evidence suggests that estradiol enhances neurogenesis and plasticity in regions such as the hippocampus, which could be related to the superior performance of females in the MWM. In contrast, elevated testosterone levels appear to be associated with increased reinforcement sensitivity and a greater propensity for risk-taking behaviours, potentially explaining the observed sex differences in saccharin preference and exploratory behaviour in the EPM (Erol & Karpyak, 2015; He et al., 2024).

On the other hand, the effects of IAE may induce differential effects in male and females, as for example, alcohol binges induced a clear anxiogenic effect in males in the EPM and worsen recognition ability in the NOR, consistent with previous literature, whereas IAE appears to have a more disinhibitory effect in the EPM performance at the same time of abstinence and an enhancing of the recognition memory in the NOR, which is intriguing and deserve further investigation. Some authors suggest that hormonal fluctuations across the phases of the estrous

cycle influence both animal behaviour and the effects of alcohol exposure (Corbett et al., 2024; Klein Marcondes et al., 2001; Paiva-Santos et al., 2022; Scholl et al., 2019; Sircar, 2019). Scientific evidence also suggests that alcohol consumption may disrupt circulating levels of sex hormones. For instance, acute alcohol intoxication, as seen in IAE, is linked with a decrease in testosterone levels. Since testosterone has been associated with a propensity for risk-taking behaviours, the ethanol-induced reductions in entries and time in open arms in the EPM could be partially explained by this effect. In our present study we did not explore the estro cycle of females or the hormone levels, so this hypothesis remains speculative and needs further experimentation.

Additionally, some authors suggest that sex-related hormonal differences may interfere with alcohol metabolism, affecting its absorption and distribution throughout the body, ultimately leading to distinct behavioural effects (Erol & Karpyak, 2015). In this regard, some authors suggested that females have a faster metabolism of alcohol than males (Baraona et al., 2001; Desroches et al., 1995; Kishimoto et al., 2002; Thomasson, 2002). The faster elimination of alcohol by females could be due to a higher activity of hepatic enzymes (Kishimoto et al., 2002; Elena Quintanilla et al., 2007; Simon et al., 2002), although other authors found no differences (Livy et al., 2003; Lopez et al., 2003). These sexual differences in alcohol metabolism could explain some of the behavioral discrepancies that IAE induced in male and females in our study. In those experiments, we did not measure BELs or hepatic enzymes, so we cannot confirm this hypothesis, and this is a limitation of the study.

Recently, it has been shown that the pattern of IAE used in this study affects the intestinal microbiota, the integrity of the intestinal barrier, causing the translocation of pathogens into the systemic circulation and triggering a peripheral inflammatory response and disruption of the blood-brain barrier that contributes to alcohol-induced neuroinflammation, which is related to emotional and cognitive alterations (Antón et al., 2018; Orio et al., 2019; Orio, 2020; Rodriguez-Gonzalez & Orio, 2020; Rodriguez-Gonzalez et al., 2021; Rodriguez-Gonzalez et al., 2023). Specific research on the microbiota has shown that sex hormones and diet affect the proliferation of different microbial communities, which in turn impact the immune system, and these differences being more evident during adolescence (Org et al., 2016). Most of the mentioned studies have been done in males and only recently we have some data about the differential impact of this IAE protocol in the gut-brain axis of male and females (López-Valencia et al., 2024). Anyhow, ABD seems to affect cognitive performance differently in young men and women (Lees et al., 2019) and this could be related to inflammatory components (Orio et al., 2018).

Despite providing interesting data about possible sex differences in behaviour, we recognize several limitations of the current study. First, although the behavioural tests used in this study are widely described in the literature and they were administered sequentially according to a design to avoid between-test interferences (Moya et al., 2022, Moya et al., 2021; Marco et al., 2017), it should be noted that most of them were validated in males, so it is possible that their interpretation for females is biased and we cannot discard interferences in female animals. Secondly, this is a study that exclusively focus on behaviour; the absence of biochemical measures such as BELs, hormones or hepatic enzymes limits the interpretation of results. Finally, an intrinsic limitation of using this IAE model is that it does not allow to identify significant differences in the pattern of binge drinking between males and females (higher in females), which compromises the translational character of the study.

Our findings highlight the importance of a rigorous characterization of the test widely used to assess behaviour in preclinical animal models considering the differential performance of females in some of those tests, to enhance the translational relevance of the investigation. In terms of translational value, making sex-specific differences in the effects of IAE during early adulthood visible is essential to questioning the assumption that alcohol exerts identical effects across sexes, breaking the homogeneous perspective that persists regarding the effects of alcohol across sexes in its mechanism of action, toxicity, prevention and treatment. Thus, incorporating a sex-based perspective into future research is vital for the early detection of alcohol-related problems in both sexes, as well as for guiding the design of more effective interventions.

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Conflict of interest

The authors declare no conflict of interest.

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