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Unraveling the molecular mechanisms involved in alcohol intake and withdrawal in adolescent mice exposed to alcohol during early life stages.

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ABSTRACT

Alcohol interferes with foetal development and prenatal alcohol exposure can lead to adverse effects known as foetal alcohol spectrum disorders. We aimed to assess the underlying neurobiological mechanisms involved in alcohol intake and withdrawal in adolescent mice exposed to alcohol during early life stages, in discrete brain areas. Pregnant C57BL/6 female mice were exposed to binge alcohol drinking from gestation to weaning. Subsequently, alcohol seeking and taking behaviour were evaluated in male adolescent offspring, as assessed in the two-bottle choice and oral self-administration paradigms. Brain area samples were analysed to quantify AMPAR subunits GluR1/2 and pCREB/CREB expression following alcohol selfadministration. We measured the expression of mu and kappa opioid receptors both during acute alcohol withdrawal (assessing anxiety alterations by the EPM test) and following reinstatement in the two-bottle choice paradigm. In addition, alcohol metabolism was analyzed by measuring blood alcohol concentrations under an acute dose of 3 g/kg alcohol. Our findings demonstrate that developmental alcohol exposure enhances alcohol intake during adolescence, which is associated with a decrease in the pCREB/CREB ratio in the hippocampus, prefrontal cortex and striatum, while the GluR1/GluR2 ratio showed a decrease in the hippocampus. Moreover, PLAE mice showed behavioural alterations, such as increased anxiety-like responses during acute alcohol withdrawal, and higher BAC levels. No significant changes were identified for mu and kappa opioid receptors mRNA expression. The current study highlights that early alcohol exposed mice increased alcohol consumption during late adolescence. Furthermore, a diminished CREB signalling and glutamatergic neuroplasticity are proposed as underpinning neurobiological mechanisms involved in the sensitivity to alcohol reinforcing properties.

Keywords: alcohol exposure, prenatal and lactational periods, reward, neuroplasticity.

ABBREVIATIONS

2BC: two bottle choice

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AMY: amygdala

BAC: blood alcohol concentration

CNS: central nervous system

CREB: cyclic adenosine monophosphate response element binding protein

DID: drinking in the dark

EPM: elevated plus maze

FAS: foetal alcohol syndrome

FASD: foetal alcohol spectrum disorders

FR: fixed ratio

GD: gestational day

HPC: hippocampus

KOR: kappa opioid receptor

MOR: mu opioid receptor

NAc: nucleus accumbens

PAE: prenatal alcohol exposure

PD: postnatal day

PFC: prefrontal cortex

PLAE: prenatal and lactational alcohol exposure

PR: progressive ratio

STR: striatum

VTA: ventral tegmental area

1. Introduction

Alcohol can interfere with foetal development (Goodlett and Horn, 2001). Thus, prenatal alcohol exposure (PAE) may lead to adverse effects on the foetus (Bandoli et al., 2019). Such effects are globally known as foetal alcohol spectrum disorders (FASD), including the most severe type, the so-called foetal alcohol syndrome (FAS) (Stratton et al., 1996). FAS is characterized by facial abnormalities, growth retardation, central nervous system (CNS) dysfunction and neurobehavioural disabilities (Stratton et al., 1996). The prevalence of FASD, in the United States and Western Countries, might number as high as 1 to 5 per 100 school children, although it could be completely prevented by abolishing alcohol consumption during pregnancy (May et al., 2018). The percentage of women who drink alcohol during pregnancy has increased in the past years (Denny et al., 2017), and specially, the number of pregnant women reporting binge alcohol drinking (NIAAA, 2016). Binge drinking, an excessive but episodic alcohol consumption pattern, constitutes a Public Health problem (EMCDDA, 2018). It has become a regular practice during adolescence and youth, which is of special concern, since these periods of life are characterized by a heightened vulnerability to the neurotoxic effects of alcohol and addictive substances (Jones et al., 2018).

Human studies have reported a link between gestational alcohol exposure and the increased probability of alcohol abuse and illicit drugs during adolescence (Alati et al., 2006; Baer et al., 2003; Pfinder et al., 2014) and adulthood (Goldschmidt et al., 2019; Pfinder et al., 2014). Preclinical studies in rodent models reported PAE to raise alcohol palatability in adolescent animals by diminishing the aversion to its smell, taste and oral irritation (Glendinning et al., 2012). Numerous studies in animal models have also hypothesized that alterations in the pharmacological reinforcing properties of alcohol following PAE might be underlying the increased postnatal alcohol acceptance (Pautassi et al., 2012). However, the exact mechanisms by which early alcohol exposure predisposes an individual to alcohol dependence later in life are still not well understood.

Exposure to drugs of abuse, including alcohol, induces aberrant plasticity leading to the development of addictive processes (Koob and Volkow, 2016). The glutamatergic system plays a crucial role in learning processes and neuroplasticity, constituting one of the neurobiological substrates altered by drugs of abuse (Hopf, 2017). In particular, the ionotropic glutamatergic N-methyl-D-aspartate receptor is clearly involved in the acute and chronic effects of alcohol. Nevertheless, the role of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) in mediating the effects of alcohol is still unclear. AMPARs composed of GluR1–4 subunits are one of the three types of ionotropic receptors, whose main function is related to

the mediation of excitatory glutamatergic signalling at the hyperpolarized physiological resting potential in neurons (Hopf, 2017). Alcohol exposure or intake leads to the expression of calcium-permeable AMPARs in different brain nuclei, which have been linked to heavy alcohol use (Koob and Volkow, 2016; Vranjkovic et al., 2017). The calcium-permeable AMPARs were reported to increase in the nucleus accumbens (NAc) of rodents when subjected to a number of behavioural conditions such as alcohol or cocaine exposure, stress or novelty (Hopf and Mangieri, 2018). Thus, alcohol-related neuroadaptations in AMPA glutamate receptors are of great interest since their role in alcohol abuse is not fully understood.

Previous studies demonstrated that opioid neurotransmission modulates alcohol intake. Indeed, the activation of the opioid system constitutes the initial mechanism responsible for the rewarding effects of alcohol. Specifically, both mu- (MOR) and delta-opioid receptors have been involved in the reinforcing effects of alcohol (Becker et al., 2002; Di Chiara et al., 1996; Koob, 2003; van Rijn et al., 2012) by mediating alcohol-induced dopamine release in the ventral tegmental area (VTA) (Herz, 1997). Fabio et al. (2015) showed that alcohol exposure during the early developmental period enhanced relative MOR mRNA expression in the VTA, leading to increased alcohol-induced dopaminergic activity. Furthermore, the blockade of MOR inhibited the facilitating effect of PAE on alcohol preference and intake (Chotro and Arias, 2003; Díaz-Cenzano and Chotro, 2010). The dynorphin/kappa opioid receptor (KOR) system has also been reported to mediate the motivational and dysphoric effects of alcohol (Walker and Koob, 2008). Moreover, KOR plays a key regulatory function in alcohol-related stress (Anderson et al., 2016). Indeed, Nizhnikov et al. (2014) revealed a down-regulation of synaptosomal KOR expression following antenatal alcohol exposure in brain areas involved in the motivational effects of the substance, such as the NAc, the amygdala (AMY) and the hippocampus (HPC). In addition, KOR antagonism partially reversed the effects induced by PAE on postnatal alcohol acceptance (Díaz-Cenzano et al., 2014).

The intracellular pathways activated by opioid and glutamatergic signalling converge in the modulation of the cyclic adenosine monophosphate response element binding protein (CREB), which is a transcriptional factor implicated in many aspects of CNS functions, including longterm memory formation, synaptic plasticity and drug consumption (Alberini, 2009; Barrett and Wood, 2008; Carlezon et al., 2005). Alterations in the regulation of activated/phosphorylated CREB (pCREB) have been detected in several brain areas during alcohol exposure and withdrawal (Moonat et al., 2010). However, the regulation of these proteins by alcohol differs depending on the brain region and experimental conditions. An increase in pCREB expression in the AMY was observed after acute alcohol exposure in adult rats, while withdrawal after chronic alcohol

treatment decreased CREB expression (Pandey et al., 2008). Nevertheless, hippocampal expression of CREB as well as pCREB was significantly reduced by developmental alcohol exposure in animal models (Dong et al., 2014; Krahe et al., 2009; Subbanna et al., 2014).

The aim of the present study is focused on the evaluation of the molecular mechanisms underlying the increased likelihood to alcohol dependence in adolescent male mice exposed to the drug during early-life developmental stages. Binge-like alcohol exposure took place from the beginning of the gestation period until the end of the lactation period, since the last trimester of the human pregnancy occurs during the first postnatal days (PD) in mice (Patten et al., 2014), thus covering the whole human-equivalent prenatal development. Then, alcohol intake and preference were evaluated using the self-administration (SA) and the two-bottle choice (2BC) paradigms in prenatal and lactation alcohol exposed (PLAE) mice and its counterparts during adolescence. To assess the possible mechanisms by which developmental alcohol exposure could be affecting the future responsiveness towards alcohol in offspring mice, the expression of AMPAR subunits GluR1 and GluR2 and molecular factors related to neuroplasticity (pCREB/CREB) were evaluated in the prefrontal cortex (PFC), HPC and striatum (STR), key mesolimbic brain structures known to be involved in the regulation of alcohol intake and reinforcement, in addition to their role in the formation of contextual memories encompassing drug experiences (Gilpin and Koob, 2008; Luo et al., 2011). Furthermore, the mRNA expression of MOR and KOR was analysed during alcohol withdrawal stage and after reinstatement in the 2BC paradigm in the PFC and the STR, but also in the AMY, due to its important role in the regulation of negative emotional and stress-related states, particularly associated with the suppression of alcohol consumption (Gilpin et al., 2015). In addition, the opioid transmission within the AMY has been described as an important mediator of contextinduced alcohol seeking behaviour (Marinelli et al., 2010). Possible alterations of alcohol metabolism that could have an impact on alcohol's effects in our mouse model of FASD were also assessed.

2. Material and methods

2.1. Animals

10 to 12 week-old male and female C57BL/6 inbred mice, an alcohol-preferring strain (Fuller, 1964) (Charles River, Barcelona, Spain) arrived at our animal facility (UBIOMEX, PRBB) to be used as breeders. The mice were housed in couples in standard cages and in controlled laboratory conditions, with a temperature of 21 ± 1 °C and 55 ± 10% humidity. Mice were allowed to acclimatize to the new environmental conditions for at least 1 week prior to the experiments. All tests took place during the first few hours of the dark phase of a reversed light/dark cycle (lights off at 08:00 h and on at 20:00 h). After successful mating, pregnant females were observed

daily for parturition. For each litter, the date of birth was designated as PD-0. Pups remained with their mothers for 21 days and were then weaned (PD-21). After weaning, male offspring were housed in groups of four. Female offspring were used for other experiments. Food and water were available *ad libitum*, except when water was substituted by alcohol according to the drinking in the dark (DID) procedure and during the behavioural testing of the offspring. Investigators were blinded to the experimental conditions of the subjects in each experiment. All procedures were conducted in compliance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and were approved by the local ethics committee (CEEA-PRBB).

2.2. Drugs

Alcohol (Ethyl alcohol, EtOH) was purchased from Merck Chemicals (Darmstadt, Germany) and diluted in tap water to obtain a 20% (v/v) alcohol solution for the DID test, and 10% (v/v), 15% (v/v) and 20% (v/v) alcohol solutions for the two-bottle choice paradigm. For the oral selfadministration procedure, absolute alcohol (Merck, Madrid, Spain) was dissolved in water using a w/v percentage, i.e. a 6% (w/v) alcohol solution equivalent to a 7.6% (v/v) alcohol solution. Saccharin sodium salt (Sigma, Madrid, Spain) was diluted in water.

2.3. Drinking in the dark (DID) test

This procedure was conducted as previously reported, with some minor modifications (Cantacorps et al., 2017; 2018), commencing two days after mating. Pregnant females were randomly assigned to two groups: alcohol- and water-exposed (control). Briefly, food was removed, and the water bottles were replaced with 10-ml graduated cylinders fitted with sipper tubes containing either 20% (v/v) alcohol in tap water or only tap water 3 h after the lighting was deactivated. Following a 2 h-access period, individual intakes were recorded, and food and water bottles were returned to the home cage. During this period, mice were individually housed, and each corresponding male breeding pair was removed from the home cage. The procedure was repeated on days 1 to 3, and fresh fluids were provided each day (from Tuesday to Thursday). On day 4 (Friday), alcohol or water cylinders were left for 4 h and fluid intakes were recorded. Fluid intakes (g/kg body weight) were calculated from average 2day body weight values as dams were weighed at 2-day intervals. The procedure was maintained throughout the 3-week gestation and/or 3-week lactation periods (gestation day (GD)-1 to PD-21). As previously reported (Cantacorps et al., 2017), blood alcohol concentration (BAC) for dams reached levels of ~80 mg/dL after the last binge drinking session (4-hr access) of the gestation and lactation periods following the same procedure. Thus, we assume that similar levels were reached by

females in the current study. In addition, no alterations in litter size or pup's body weight have been reported using this model (Cantacorps et al., 2019). Male offspring of each litter were randomly distributed to the different experimental conditions to avoid litter effects. The effects of developmental alcohol exposure on the following alcoholrelated behaviours were assessed in different groups of mice, as outlined in Figure 1.

2.4. Oral alcohol self-administration (SA)

Our procedure was based on the model used by Navarrete et al. (2014) with some modifications. Oral ethanol SA was carried out in 7 modular operant chambers (Med Associates, Inc.) and Med-PC IV software controlled stimuli and fluid delivery and recorded operant responses. The chambers had two small holes with adjacent photocells to detect nose-poke responses. The active nose-poke delivered 37 µl of fluid combined with a 0.5s stimulus light and a 0.5s buzzer beep, which was followed by a 6s time-out period, whilst inactive nose-pokes had no consequences.

To evaluate the effects of alcohol exposure on alcohol SA, animals underwent a 3-phase experiment: training, saccharin substitution and 6% alcohol consumption.

Training phase (8 days)

Two days prior to the experiment, access to the standard diet was restricted to 1 h per day. Before the first training session, water was withdrawn for 24 h, and food allotment was provided 1 h prior to the 1-h eating session to increase lever-pressing motivation. On the following 3 days, water was provided *ad libitum*, except during the 1-h food-access period prior to the start of each session, in which the water bottle was removed from the cages (postprandial). On the following four days, and for the rest of the experiment, food access was provided for 1 h after each daily session and water was available ad libitum to avoid alcohol consumption due to thirst (pre-prandial). The food restriction schedule produced a weight loss in the mice of around 15% of their free-feeding weight. The mice were trained to press on the active lever to receive 37 µl of 0.2% (w/v) saccharin reinforcement.

Saccharin substitution (9 days)

The saccharin concentration was gradually decreased as the alcohol concentration was gradually increased (Samson, 1986; Roberts et al., 1998). Both solutions were administered for three consecutive days with the following concentrations (0.15% Saccharin − 2% EtOH; 0.10% Saccharin − 4% EtOH; 0.05% Saccharin − 6% EtOH).

Alcohol consumption (6% w/v) (6 days)

The aim of the last phase was to evaluate the number of responses on the active lever, the 6% alcohol (w/v) intake and the motivation to drink. To achieve this goal, during the last phase, the number of effective responses and alcohol consumption (ml) were measured under fixed ratio 1 (FR1) for 5 daily consecutive sessions. The learning task criteria for FR1 were: (1) reaching ≥ 70% of preference for the active hole; (2) ≥ 10 reinforced trials by session, and (3) ≤ 30% deviation in the number of reinforced trials, all during three consecutive days. Animals that achieve these criteria underwent to a progressive ratio (PR) schedule of response. The response requirement to achieve reinforcements escalated in accordance with the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. To evaluate motivation for alcohol consumption, the breaking point for each animal was calculated as the maximum number of consecutive responses to achieve one reinforcement, on the basis of the previous scale. Each session lasted 1 h, except for the PR schedule of reinforcement session that lasted 2 h.

2.5. Two-bottle choice (2BC) paradigm

This procedure, adapted from Portero-Tresserra et al., (2018), involved escalating alcohol concentrations. Animals were initially habituated to the procedure by allowing them free access to two drinking bottles filled with water for three days. After habituation, mice were allowed to choose between water and alcohol solutions, the concentration (v/v) of which was progressively increased every three days in three phases (10%, 15% and 20%). After the final alcohol-20% session, the animals were alcohol-deprived for three days (abstinence period), and were then provided with 10%-alcohol, corresponding to the reinstatement stage. Water and alcohol consumptions were measured every 24h, and the locations of drinking bottles were switched in order to control for possible position preference. The average alcohol intake (g/kg/day) and alcohol preference ratio, representing alcohol consumption (ml/day) as a percentage of total fluid consumption, were calculated every day.

2.6. Elevated plus maze (EPM)

EPM was performed in the offspring during the abstinence period, 24h after the final session of the 2BC (Gracia-Rubio et al., 2016). The apparatus (Panlab s.l.u., Barcelona, Spain) consisted of a black maze with four arms (16 × 5 cm) set in the form of a cross from a neutral central square (5 × 5 cm). Two of the arms were enclosed by high walls (closed arms), while the other two perpendicular arms were open and exposed (open arms). The maze was situated 30 cm above the floor in dim lighting conditions (30 lux). At the outset of the 5-min observation session, each mouse was placed in the central area, facing an open arm. The total number of entries (placing

all four paws into the arm) and the time spent in the open and closed arms (as a percentage of the total test time) were recorded by automated tracking software (Smart, Panlab s.l.u., Barcelona, Spain).

2.7. Alcohol metabolism evaluation

The alcohol metabolism was measured using a standard protocol (Ehringer et al., 2009). Mice were moved to the testing room and allowed to acclimate for at least 30 min prior to receiving an alcohol injection (3 g/kg; i.p.). Tail blood was collected at 30, 60 or 120 minutes after the injection in heparin coated tubes kept in ice and centrifuged at 13,000 rpm for 10 min at 4ºC. Supernatant (cell-free plasma) was stored at -80ºC until the quantification analysis was performed. BAC was measured using the EnzyChrom™ Ethanol Assay Kit (Bioassay Systems, USA), following the manufacturer's instructions.

2.8. Preparation of tissue extract

Three different groups of mice (n=4-6 per group) were sacrificed by cervical dislocation for the tissue extraction at three different time points. The first cohort of animals was sacrificed immediately after the PR session in the SA procedure and the PFC, HPC and STR were dissected. The second cohort of animals was separated in two sets of mice. One of them was sacrificed 24h after the last 2BC session (alcohol 20%), during the alcohol withdrawal stage, while the other set of mice was sacrificed 24 h after being exposed to alcohol 10% (reinstatement) in the 2BC (Figure 1). The PFC, STR and AMY structures were dissected from the second cohort of animals. After extraction, brains were placed in the trough of the brain slicer matrix for the dissection of discrete brain regions. During use, the cutting block was kept cold on crushed ice. Blades (kept also on ice) were carefully inserted through the cutting channels slicing the brain at right angles to the sagittal axis (channel used adds 1 mm to the thickness of the slice). The initial razor blade sliced through the coronal plane of the brain at the level of the body of the anterior commissure. The position of the initial razor blade served as a reference point from which brain sections were obtained (Heffner et al., 1980). Individual brain areas were dissected from the slices formed. All nuclei were dissected following this procedure and then stored in dry ice at -80ºC on an ice-cold plate. A diagram showing the stereotaxic coordinates at which brain areas were dissected is included in Figure 1.

2.9. Sample preparation and western blot analyses

Brain tissues of the PFC, HPC and STR were first homogenized in cold lysis buffer (0.05M Tris- HCl pH7.4, 0.15M NaCl, Triton X-100 1%, Glycerol 10% and EDTA 0.001M). Homogenates were kept

on ice for 30 min and centrifuged at 15,000 rpm for 15 min, and the supernatant was collected to determine the protein concentration. The lysate protein concentration was detected by means of the DC TM Protein Assay (Bio-Rad; Barcelona, Spain). Membranes were incubated overnight at 4 ºC with primary antibodies: Anti-β-Tubulin (anti-mouse, 1:5000, BD Biosciences), anti-phospho CREB (anti-rabbit, 1: 1000, Merck Milipore, Spain), anti-CREB (anti- rabbit, 1: 500, Merck Milipore, Spain), anti-GluR1 and anti-Glur2 (anti-rabbit, 1:1000, Merck Milipore, Spain). After washing with TBS/T, blots were incubated with their respective fluorescent secondary antibodies: anti-mouse (1:2500, Rockland) and anti-rabbit (1:2500, Rockland). Blots were quantified using a LI-COR Odyssey scanner and software (LI-COR Biosciences, Lincoln, USA). For comparative purposes, control values were normalized to 100%, and the respective protein expression values were adjusted according to the normalization factor.

2.10. Gene expression analyses. Real time PCR

Total RNA was extracted from brain tissue nuclei (PFC, HPC and AMY) and purified using the RNeasy Lipid Tissue Mini Kit (Qiagen). 1 µg of total RNA was reverse-transcribed using the Applied Biosystems High-Capacity cDNA Reverse Transcription kit. Quantitative RT-PCR analysis was performed on an Applied Biosystems Prism 7900 System using Power SYBR Green PCR Master Mix (Applied Biosystem). Transcripts were amplified by means of the following forward and reverse primers: GAC CGC TAC ATT GCT GTG TG and GCA AGG AGC ATT CAA TGA CA for kappa opioid receptor; GTG TAG TGG GCC TCT TTG GA and CCA CGT TCC CAT CAG GTA GT for mu opioid receptor; and AGC ATA CAG GTC CTG GCA TC and TTC ACC TTC CCA AAG ACC AC for cyclophilin A. Cyclophiline A was used as the endogenous normalizer. Amplifications were carried out in triplicate and the data for each target gene were normalized to the endogenous reference gene, and the fold-change in target gene mRNA abundance was determined using the 2(-ΔΔCt) method (Livak and Schmittgen, 2001).

2.11. Statistics

Two-way ANOVA with repeated measures was calculated to analyse alcohol intake during the DID test with Group (Water-Alcohol) as between-subject factors and Day as a within-subject factor. One-way ANOVA with repeated measures was used to analyse alcohol intake in the alcohol-drinking group of dams during the DID test. Data obtained from the SA (FR1) tests were evaluated using a repeated measures two-way ANOVA with Group (Water-Alcohol) as between-subject factors and Day as a within-subject factor. For the 2BC paradigm, a three-way ANOVA with repeated measures, with Group (Control-PLAE) as a between variable and Day (1, 2 and 3) and Alcohol concentration (10%, 15% and 20%) as within variables. The BAC data were analysed

by means of a two-way ANOVA with Group (Water-Alcohol) as a between subject factor and Time (30 min- 60 min- 120 min) as a within variable. Subsequent post-hoc comparisons were calculated using the Bonferroni test when required. The unpaired twotailed Student's t-test was performed for the PR (breaking point and 6% alcohol consumption) in the SA paradigm, the 10% alcohol preference in the 2BC reinstatement phase, the biochemical analyses and the transcript expression. Pearson's correlation analysis was used to explore correlations between the variables. Results are expressed as the mean ± SEM, and statistical significance was set at $p<0.05$. Statistical analyses were performed using SPSS Statistics v23.

3. Results

3.1. DID test in dams

As shown in Figure 2, dams were exposed to alcohol under the DID test procedure from the gestational period GD-1 to PD-21 in order to mimic voluntary binge alcohol drinking. Two-way ANOVA analysis of water and alcohol consumption (ml) during DID testing showed a significant effect of Day [$F(23,391) = 16.555$; $p<0.001$], Group [$F(1,17) = 50.188$; $p<0.001$] and betweenfactor interaction [$F(23,391) = 8.543$; $p < 0.001$]. Bonferroni post-hoc comparisons revealed a significant increase in fluid consumption from days 16 to 24, corresponding to the lactational period when compared to the other days (1 to 15) ($p<0.05$). In this period, all mice consumed more water than alcohol, from days 17 to 24 of the test ($p<0.05$, for all cases) (Figure 2a).

One-way ANOVA with repeated measures analysis of alcohol intake (gEtOH/kg) (Figure 2b) showed a significant effect of Day [$F(23,230)= 4.048$; $p<0.001$]. Post-hoc comparisons revealed significant differences between the 4-h drinking sessions compared with the 2-h sessions on days 1 to 3, in the first ($p<0.01$), second, fourth and sixth week ($p<0.05$, in all cases).

3.2. Effects of PLAE on oral alcohol SA

The two-way ANOVA for the number of responses (nose-pokes) (Figure 3a) in the FR1 for alcohol-induced SA revealed a significant effect of Group [$F(1,42)=5.333$; $p<0.05$] and Days [$F(4,168)=2.913$; $p<0.05$] with no interaction between the two factors. PLAE mice responded to a higher extent when compared to the control group ($p<0.05$). The statistical analysis for alcohol consumption in the FR1 (Figure 3b) revealed a significant effect for the variable Group [$F(1,42)=5.103$; $p<0.05$] and Days [$F(4,168)=2.898$; $p<0.05$] with no between-factor interaction (Figure 3b). The PLAE-group mice showed higher alcohol consumption compared to the control group ($p<0.05$). (Figure 3a and 3b).

The PR evaluated motivation for alcohol consumption, the breaking point for each animal was calculated as the maximum number of consecutive responses to achieve one reinforcement following the scale presented in the methodology section. The unpaired Student's t-test showed that the animals exposed to alcohol during the developmental stage presented higher breaking point values and higher 6%-alcohol intake than their water-exposed counterparts (t_{42}=2.104, $p<0.05$ and t_{42}=2.402, $p<0.05$, respectively) (Figures 3c and 3d).

3.3. Maternal binge-like alcohol drinking increased alcohol intake in the 2BC paradigm.

We evaluated alcohol consumption using the 2BC paradigm to determine the preference for alcohol intake compared to water in the offspring mice (Figure 4a). Three-way ANOVA showed an effect of Group [$F(1,23) = 39.865$; $p<0.01$], Alcohol concentration [$F(2,46) = 8.116$; $p<0.01$] and the interaction Alcohol concentration x Day [$F(4,92) = 4.051$; $p<0.01$]. PLAE mice consumed more alcohol than their control-group counterparts ($p<0.01$). The highest percentage of alcohol concentration consumption corresponded to the 10% ($p<0.01$). Furthermore, in the 10% of alcohol concentration, animals drank more liquid in the first day when compared with the third one ($p<0.05$); however, in the 15% of alcohol concentration the second day mice drank less when compared with the first and the third days ($p<0.01$ in both cases).

Following the 2BC procedure, the animals were subjected to three days of abstinence, before being exposed once more to 10%-alcohol (reinstatement phase). The unpaired Student's t-test calculated for alcohol consumption during the reinstatement phase indicated a higher alcohol preference for the PLAE mice when compared to the control group (t_{21}= -2.267, $p<0.05$) (Figure 4b).

3.4. Alcohol-exposed offspring mice presented increased anxiety-like behaviour

Offspring mice were assessed for anxiety-like behaviour in the EPM after 24h of alcohol withdrawal in the 2BC (Figure 5). The Student's t-test revealed significant differences for the percentage of time spent in the open arms (t_{15}= 2.856, $p<0.05$), showing that the PLAE mice spent less time there compared to the control group (Figure 5a). No changes in the number of total entries were found after PLAE (Figure 5b).

3.5. PLAE mice showed higher BAC than control mice after 3 g/kg alcohol administration

The BAC of the offspring mice was measured at 30, 60 and 120 min after alcohol (3g/kg) administration. Two-way ANOVA with repeated measures showed an effect of Group [$F(1,9) = 27.60$; $p<0.001$] with no effect of Time or between-factor interaction. Thus, mice exposed to

PLAE presented higher levels of BAC than the control animals at each experimental time point (Figure 6).

3.6. GluR1/GluR2 expression was altered in PLAE mice

The GluR1/GluR2 expression was assessed in the PFC (Figure 7a), HPC (Figure 7b) and STR (Figure 7c) of the offspring mice. When the PFC was analysed, the Student's t-test did not show any significant differences for GluR1, GluR2 or the GluR1/GluR2 ratio. The Student's t-test showed an effect in the STR for GluR2 (t_{10}= 2.134, p=0.05), decreasing the levels of GluR2 in PLAE animals when compared to the control group, and a tendency in the GluR1/GluR2 ratio (t_{10}= -1.989, p=0.07). Furthermore, a significant effect for the GluR1/GluR2 ratio in the HPC (t_8= 2.218, p=0.05) was found, showing that mice exposed to PLAE exhibited a lower GluR1/GluR2 expression compared to the control group.

In addition, Pearson's correlation analyses were performed between data obtained from the biochemical studies of offspring and each corresponding maternal alcohol intake (mean of binge sessions), as shown in Figure 8. The expression of GluR1 (p=0.06) and GluR1/GluR2 ratio in the HPC positively correlated with the maternal alcohol intake.

3.7. PLAE mice showed diminished pCREB/CREB protein ratio expression

pCREB/CREB protein expression was assessed in the PFC, HPC and STR of the offspring mice (Figure 7; lower panel). When the PFC was analysed, the unpaired Student's t-test revealed an effect for CREB (t_{10}=- 2.638, p<0.05), increasing its levels in PLAE animals when compared with the control group. In addition, when the pCREB/CREB protein ratio expression was measured in the same structure, the PLAE mice showed a lower pCREB/CREB protein expression in comparison with the control group (t_{10}= 2.861, p<0.05). Furthermore, decreased levels of pCREB and a reduction in the pCREB/CREB ratio protein expression in PLAE animals were found in the STR (t_9= 2.126, p=0.05 and t_8= 2.478, p<0.05), respectively. The Student's t-test showed an effect in the HPC for pCREB (t_{10}= 4.317, p<0.01) and the pCREB/CREB protein expression (t_9= 2.418, p<0.05), and the PLAE mice showed a protein decrease when compared to the control group.

A significant correlation between CREB and pCREB/CREB expression in the PFC with the maternal alcohol intake was found. pCREB expression in the HPC negatively correlated with the maternal alcohol intake (Figure 8). Correlations among the other factors analysed were not statistically significant.

3.8. Mu and Kappa opioid receptor mRNA expression was not altered in PLAE mice

The effects of PLAE on MOR (Figure 9; upper panel) and KOR (Figure 9; lower panel) mRNA expression were evaluated in the PFC, STR and AMY structures at two different time points: 24 h after the last 2BC session (access to 20% alcohol) during the acute withdrawal period and 24 h after the reinstatement phase (access to 10% alcohol). The unpaired Student t-test showed no significant differences for the mRNA expression of these receptors in any of the brain structures analysed at either of the time points.

4. Discussion

The CNS function is highly vulnerable to the effects of alcohol exposure during prenatal and early postnatal developmental stages (Caputo et al., 2016). In this context, our findings demonstrate that exposure to alcohol during pregnancy and lactation periods, heightens alcohol intake during adolescence as assessed by different paradigms including the oral alcohol SA, the 2BC and following abstinence (after intensive alcohol exposure in the 2BC). Moreover, PLAE mice showed behavioural alterations, such as increased anxiety-like responses during acute alcohol withdrawal. The molecular analysis of discrete brain areas after alcohol consumption in the operant SA paradigm showed a decrease in the GluR1/GluR2 ratio of AMPARs in the HPC, followed by a reduction in the pCREB/CREB ratio in the PFC, STR and HPC. Finally, no significant changes were identified in the mRNA expression of MOR and KOR in the PFC, STR and AMY during alcohol withdrawal and after reinstatement.

In our study, a voluntary mouse model of binge-like alcohol drinking during pregnancy and lactation was used (Cantacorps et al., 2017; 2018). Using this procedure, mice can reach BAC levels of intoxication (~80 mg/dl) (NIAAA, 2016), like previously reported (Cantacorps et al., 2017). We have previously demonstrated that maternal binge-like alcohol drinking induces long-lasting cognitive, emotional and motivational impairments in offspring mice, increases pro-inflammatory factors and decreases the expression of myelin proteins in different brain areas (Cantacorps et al., 2017; 2018; 2020). According with previous studies (Cantacorps et al., 2017; Montagud-Romero et al., 2019), we observed an escalation in the consumption of water during the lactation period when compared to the prenatal period in water-exposed dams during the DID test, which has been linked to an increased fluid demand to support milk production.

In utero alcohol exposure produces persistent alterations in brain areas controlling motivational and reward circuits. Furthermore, alcohol exposure during the prenatal developmental period seems to be predictive of adolescent alcohol use and misuse (Baer et al., 2003; Popova et al., 2016). Premature exposure to alcohol in rodents may induce a heightened affinity for the drug

in later stages of life (Spear and Molina, 2005). In addition, PAE has been reported to increase alcohol tolerance and sensitization to ulterior alcohol intake (Barbier et al., 2008; 2009), while decreasing the sensitivity to the aversive effects of alcohol, such as hypothermia and sedation, in young adult animals (Abel et al., 1981; Barbier et al., 2009; Gore-Langton and Spear, 2019). Although there is a vast body of preclinical, clinical and epidemiological evidence supporting the link between early alcohol exposure and later alcohol use from adolescence to adulthood, the mechanisms underlying such deleterious effects call for further understanding (Gaztañaga et al., 2020; Popova et al., 2016). Recently, Cantacorps and colleagues (2018) have shown that the degree by which alcohol can induce approaching behaviours towards a conditioned context is reduced in PLAE mice, as assessed using the conditioned place preference paradigm. Such results may indicate a shift to the right in the dose-response curve for alcohol-induced conditioned place preference in PLAE mice, suggesting a development of tolerance following developmental alcohol exposure. Similarly, Pautassi et al. (2012) showed that PAE makes subjects more tolerant to the motivational effects of alcohol. These data are consistent with our results, showing an increase in alcohol consumption in the 2BC and SA models that would seem to indicate a lower sensitivity to alcohol's reinforcing effects. In other words, PLAE mice would need to consume higher amounts of alcohol to elicit the same rewarding effect than in control group. Accordingly, PLAE mice responded to a higher extent at nose-poking behaviour when compared to the control group during the FR1 schedule of reinforcement, showing higher alcohol consumption. When the schedule of reinforcement in the alcohol SA changed to the PR, in which the effort required to obtain a single dose of alcohol is progressively increased, we also obtained a significant difference between PLAE and control group. PLAE mice showed a higher breaking point and consumed more alcohol during the PR session than their counterparts, indicating an increased motivation or '*wanting*' to obtain the drug. Similarly, March et al. (2009) reported an enhanced acquisition of response-stimulus associations when alcohol is given as an intraoral reinforcer in animals prenatally exposed to alcohol. Preclinical findings also showed that a single binge-like exposure to alcohol in mice, during a period equivalent to the third trimester of neural development, would seem to be enough to exacerbate dependence-like alcohol consumption in mice (Bosse et al., 2018). Hence, there is a large body of evidence supporting the link between maternal alcohol drinking and increased alcohol intake later in life, together with the appearance of alcohol-related problems.

In addition, we analysed the BAC (30, 60 and 120 min) after administering a 3 g/kg alcohol treatment to assess the impact of maternal alcohol drinking on offspring's alcohol metabolism. The acute dose of 3 g/kg alcohol yielded higher BAC levels in PLAE than control mice, suggesting alterations in the mechanisms involved in alcohol's metabolism and clearance as a result of

developmental alcohol exposure. Likewise, Popoola et al. (2017) reported higher BACs in rats prenatally exposed to alcohol following alcohol i.p. injections (3.5-4.5 g/kg) at PD-42, together with a reduced sensitivity to alcohol's hypnotic effects. Also, pups derived from alcohol-treated dams had slower elimination rates of alcohol from the blood than control groups when treated with identical doses of alcohol (Nizhnikov et al., 2006). Notwithstanding, other preclinical studies have shown a lack of effect of PAE on alcohol pharmacokinetics (Becker et al., 1993; Arias et al., 2008) or even higher metabolic rates in alcohol-exposed offspring (Perez et al., 1983; Sze et al. 1976). These discrepancies might be due to differences on alcohol dosage, developmental timing of alcohol exposure or time of assessment. Thus, the higher BAC levels observed in PLAE mice after a challenging dose of alcohol could be related with a reduction in their sensitivity to alcohol's sedative effects, which may account for differences observed in operant paradigms of alcohol intake. Indeed, low sensitivity to alcoholinduced hypnosis has been strongly associated with high-risk alcohol consumption patterns (Naassila et al., 2002; Spear, 2014; Towner and Varlinskaya, 2020).

A history of binge alcohol-drinking, in both humans and laboratory animals, enlarges symptoms of negative affect and dysphoria during periods of abstinence (Lee et al., 2017). PAE has been associated with alterations of social behaviour and anxiety disorders. Furthermore, PAE may result in altered behavioural responsiveness to stressful and anxiety-provoking test situations, including the EPM in rodents (Díaz et al., 2016). Previous findings have shown increased anxiety levels in PLAE mice (Cantacorps et al., 2018; Montagud-Romero et al., 2019). Nevertheless, experimental findings are rather inconsistent and show both increased and decreased anxiety-like behaviour in the EPM after chronic gestational alcohol exposure (Kleiber et al., 2011; Liang et al., 2014). Our results obtained in the EPM 24 h after the last day of alcohol (20%) consumption in the 2BC paradigm, that is during the withdrawal period, show a reduction in the percentage time spent in the open arms in PLAE group compared with their counterparts. Therefore, we could assume that PLAE mice showed an increase in anxiety-like behaviour. However, we could not rule out that the effect observed during the acute withdrawal phase (24h after 2BC) was not a consequence of early alcohol exposure, as PLAE mice has been reported to already show increased basal levels of anxiety-like behaviour. Therefore, the effects observed in the present work could be attributed to a combination of both, PLAE and withdrawal-induced effects. Exposure to alcohol during prenatal and lactation stages produces changes in the neurobiological substrates that modulate emotional responses, which at the same time could influence the rewarding effects to ethanol, as the neurobiological systems controlling motivation and emotional responses are interconnected, providing a higher vulnerability to drug abuse (Koob and Schulkin, 2018).

We have also analysed molecular parameters that may underlie some of the behavioural effects observed. Glutamatergic neurotransmission has been involved in neuroplasticity, learning and memory (Bell et al., 2016). Ionotropic glutamate receptors (such as the AMPARs) are ligand-gated ion channels involved in fast excitatory transmission in the CNS and they also play a role in conditioned alcohol-seeking responses (Backstrom and Hyytia, 2004; SanchísSegura et al., 2006). Our results reveal a decreased GluR1/GluR2 ratio expression in PLAE mice when compared to the control group in the HPC. This reduction of GluR1/GluR2 expression in the HPC may indicate a reduction in the excitatory neurotransmission, possibly affecting the processes involved in learning and memory. Compelling evidence in rodent models has shown hippocampal cognitive defects produced by alcohol consumption during pregnancy in which a role of glutamatergic synaptic transmission has been described (Mira et al., 2020). In addition, it has been found that alcohol consumption increases synaptic levels of GluR1 and GluR2 in AMPAR currents in the dorsal-medial STR (Wang et al., 2012). Furthermore, acute alcohol drinking increased the synaptic expression of GluR1 and led to a long-lasting enhancement in the function of GluR2-lacking AMPARs in the NAc (Beckley et al., 2016). In accordance, our study shows a tendency ($p=0.07$) to an increased GluR1/GluR2 in the STR of PLAE mice, which may also contribute to the enhancement of the neuroplasticity routes attributed to alcoholseeking behaviour. Indeed, it is thought that long-term brain plasticity adaptations lead to aberrant engagement of normal learning processes that over-consolidate the transition from goal-directed behaviours to habits related with drug addiction (Robbins and Everitt, 1999).

Our results also demonstrate that PLAE mice show a decreased ratio of pCREB/CREB proteins expression when compared to the control group in the three brain structures analysed (PFC, STR and HPC). The diminished expression of the pCREB/CREB protein ratio expression in these areas could mediate the increase of alcohol consumption observed in PLAE mice by enhancing their desire to seek and take the drug. CREB is a molecular key factor for synaptic plasticity and neuronal functions addressed to control gene transcription underlying long-term potentiation and memory formation, storage and retrieval (Bartolotti and Lazarov, 2019). The phosphorylation of CREB (pCREB) plays a major role in memory acquisition and consolidation (Bartolotti and Lazarov, 2019) and it is inhibited by alcohol exposure (Pandey et al., 2001). Decreased pCREB and reduced expression of CREB-related genes (such as brain-derived neurotrophic factor and corticotrophin-releasing factor) have been observed in cortical structures of alcohol-withdrawn/deprived rodents (Moonat et al., 2010; Pandey et al., 2001). Moreover, Dominguez and colleagues (2014) found that alcohol exposure and withdrawal reduce dorsal CA1 region pCREB expression, suggesting that the hippocampal CREB function may contribute to the behavioural deficits commonly observed during alcohol exposure and

abstinence. In addition, a large body of evidence associates CREB with the acquisition and continued dependence on drugs of abuse (Carlezon et al., 2005; Nestler, 2014). CREB activation in the NAc has been linked to a variety of emotional responses (Barrot et al., 2002; Carlezon et al., 2005). Moreover, drugs of abuse other than alcohol induced the reduction of CREB activity in specific brain structures related to the rewarding system, a mechanism that may be involved in the hypohedonic state associated with protracted abstinence (Misra et al., 2001). Additionally, it has been previously demonstrated that CREB function modulates NMDAR transmission and perturbs levels of AMPA subunits (Huang et al., 2008; Marie et al., 2005; Middei et al., 2013). The increased adolescent alcohol consumption of PLAE mice could be a confounding factor in the results obtained in the biochemistry studies, as it makes it difficult to discern whether changes observed are due to mere *in utero* alcohol exposure, or are due to a combination of both prenatal and adolescent alcohol exposure. However, the significant correlations between the expression of the different factors and each corresponding maternal alcohol intake supports the notion that PLAE induces long-lasting alterations in GluR1 and GluR2 expression, as well as pCREB and CREB expression.

Alcohol reinforcement mechanisms involve, at least partially, the activation of the endogenous opioid system. Alcohol modifies opioid transmission at different levels, including the binding of endogenous ligands to opioid receptors (Mendez and Morales-Mulia, 2008). Opioid activity is modulated during alcohol withdrawal and previous studies have shown changes in endogenous opioid peptides (Anderson et al., 2017). For instance, alcohol consumption was significantly reduced in the absence of β-endorphins in mice (Racz et al., 2008), and mice lacking pre-prodynorphin showed a decrease in alcohol consumption (Blednov et al., 2006). In our experimental conditions, no changes were identified in mRNA expression of the opioid receptors during alcohol withdrawal and after reinstatement in the 2BC paradigm, while other studies have shown an activation of the endogenous opioid system following moderate foetal alcohol exposure and the involvement of opioid activity in the reinforcing effects of alcohol (Gaztañaga et al., 2015; Miranda-Morales et al., 2020). Wille-Bille et al. (2018, 2020) reported increased mRNA levels of KOR in the VTA, the PFC and AMY of adolescent offspring prenatally exposed to alcohol, as well as increased levels of prodynorphin. Notwithstanding, effects upon mRNA expression do not necessary reflect the same changes on protein. For instance, a reduced expression of MOR and KOR proteins was found in the NAc of PAE offspring rats, while no changes in mRNA expression were described (Bordner and Deak, 2015). The opioid receptors can be regulated through desensitization mechanisms mediated by β-arrestin proteins, which in turn, have been shown to modulate alcohol consumption (Björk et al., 2008; Lamberts and Traynor, 2013). Hence, even though our results suggest that PLAE does not have an impact on

the expression MOR and KOR transcripts, we cannot discard that developmental alcohol exposure may have an impact on functional changes of these receptors by modulating their protein redistribution on the cell surface. In addition, it should be noted that epigenetic mechanisms owing to developmental alcohol exposure, which have been previously described in our mouse model (Cantacorps et al., 2019), might be playing a role here.

5. Conclusion

The present study highlights the involvement of glutamatergic neuroplasticity and CREB signalling adaptations in key brain areas for the regulation of drug's reinforcing effects as putative mechanisms underlying the increased likelihood for ulterior alcohol intake and preference in adolescent offspring mice exposed to binge alcohol during early ontogeny. The enhanced appetitive reinforcing properties of alcohol, together with the development of tolerance and reduced sensitivity to the aversive effects might be underpinning the heightened drug-seeking behaviour and motivation for alcohol consumption in PLAE mice. Even though no changes in opioid receptors transcripts were found, protein trafficking of MOR and KOR could be playing a role in the future responsiveness towards alcohol in animals prenatally exposed to the substance. Understanding the mechanisms by which foetal alcohol exposure favours the later alcohol consumption will let the development of strategies to mitigate the behavioural effects resulting from early-life exposure to the drug.

Conflict of interest

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Experimental timeline. Schematic representation of the experimental timeline and stereotaxic coordinates for the brain areas dissected.

Figure 2. **Maternal DID (drinking in the dark) test.** Data are expressed as (mean ± SEM) (a) Volume of water and alcohol consumed (ml) during DID procedure during prenatal and lactation periods (n=15 per group). *p<0.05 Day effect; #p<0.05 Group effect, (b) Alcohol intake (gEtOH/kg) during DID test (n=15). *p<0.05, **p<0.01 compared with the 2h session during the week, Bonferroni post-hoc test.

Figure 3. **Effects of prenatal and postnatal alcohol exposure in alcohol self-administration (SA).** Data are expressed as (mean ± SEM) (n=20-22 per group). (a) The number of effective responses (nose-pokes) during FR1 (fixed ratio 1) (b) The volume of 6% EtOH consumption during FR1 (c) Breaking point achieved during PR (progressive ratio) schedule of responses (d) 6% alcohol consumption during PR (progressive ratio) schedule. Student's t-test *p < 0.05 in relation to the control group.

Figure 4. Effects of early alcohol exposure on the two-bottle choice (2BC) procedure. Data are expressed as (mean ± SEM) (n=11-15 per group). (a) Percentage of alcohol preference daily, across the three alcohol solutions. Bonferroni post-hoc test **p<0.01 in relation to 15% and 20% alcohol. (b) Alcohol (10% v/v) intake following three days of abstinence, Student's t-test # p<0.05 compared to the control group.

Figure 5. **Effects of prenatal and postnatal alcohol exposure on the elevate-d plus maze (EPM) after 24 h of alcohol abstinence.** Data are expressed as (mean ± SEM) (n=8-9 per group). (a) Percentage of time spent in the open arms of the maze was assessed in the offspring. *p<0.05 compared to the control mice (Student's t-test). (b) Total number of entries.

Figure 6. The blood alcohol concentration (BAC) levels at 30 min, 60 min and 120 min after the injection of alcohol (3 mg/kg). Data are expressed as (mean ± SEM) (n=8-11 per group). **p<0.01 compared to the control mice (Group main effect).

Figure 7. GluR1,GluR2, CREB and pCREB levels in the prefrontal cortex (PFC), striatum (STR) and hippocampus (HPC) of the gestational and lactational alcohol- or water-exposed mice after SA. Data are expressed as (mean ± SEM) (n=4-6). (a) GluR1, GluR2 levels and GluR1/GluR2 ratio in the PFC, (b) STR and, (c) HPC (upper panel); (d) pCREB, CREB levels and pCREB/CREB ratio in the PFC, (e) STR and, (f) HPC (lower panel). *p≤0.05, **p<0.01, compared to the control group (Student t-test).

Figure 8. Pearson's correlation analyses with maternal alcohol intake. Correlations between GluR1, GluR2, GluR1/GluR2, pCREB, CREB and pCREB/CREB expression in the PFC, STR and HPC and each corresponding mean of maternal alcohol intake during binge sessions. Colour of each cell represents the degree of each corresponding r value, as shown on the right panel, from -1 (blue) to 1 (red). Significant correlations are indicated by *p<0.05; **p<0.01.

Figure 9. Mu opioid receptor (MOR) and kappa opioid receptor (KOR) levels in the prefrontal cortex (PFC), striatum (STR) and amygdala (AMY) of the gestational and lactational alcohol/water exposed mice in the 2BC paradigm. Data are expressed as (mean ± SEM) (n=46). (a) Evaluation of mu opioid receptor levels 24h after the last exposure to 20% alcohol in the 2BC, during the acute withdrawal phase. (b) Mu opioid receptor levels assessed during the reinstatement phase (10% alcohol) in the 2BC paradigm. (c) Kappa opioid receptor levels 24h after the last exposure to the 20% of alcohol in 2BC paradigm. (d) Kappa opioid receptor levels during the alcohol (10%) reinstatement phase.

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Figure 1

Schematic representation of experimental timeline.

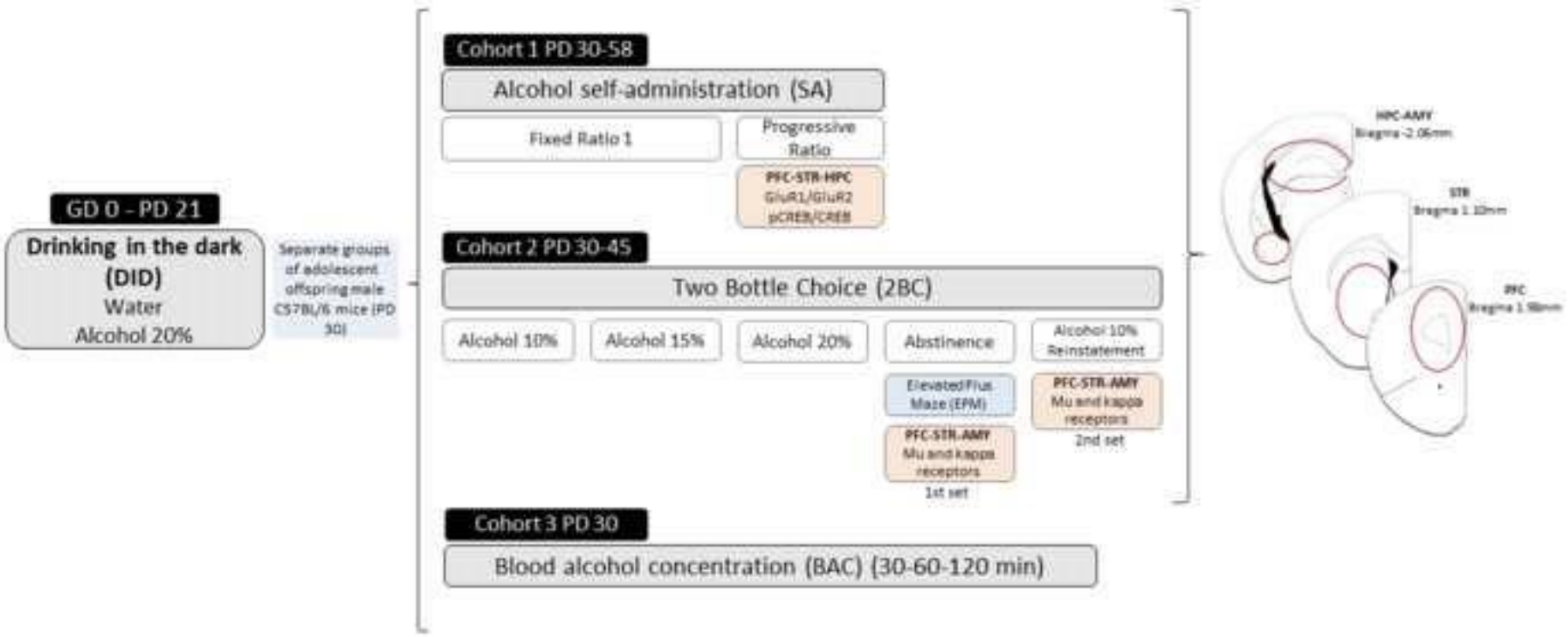

Figure 2

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Figure 3

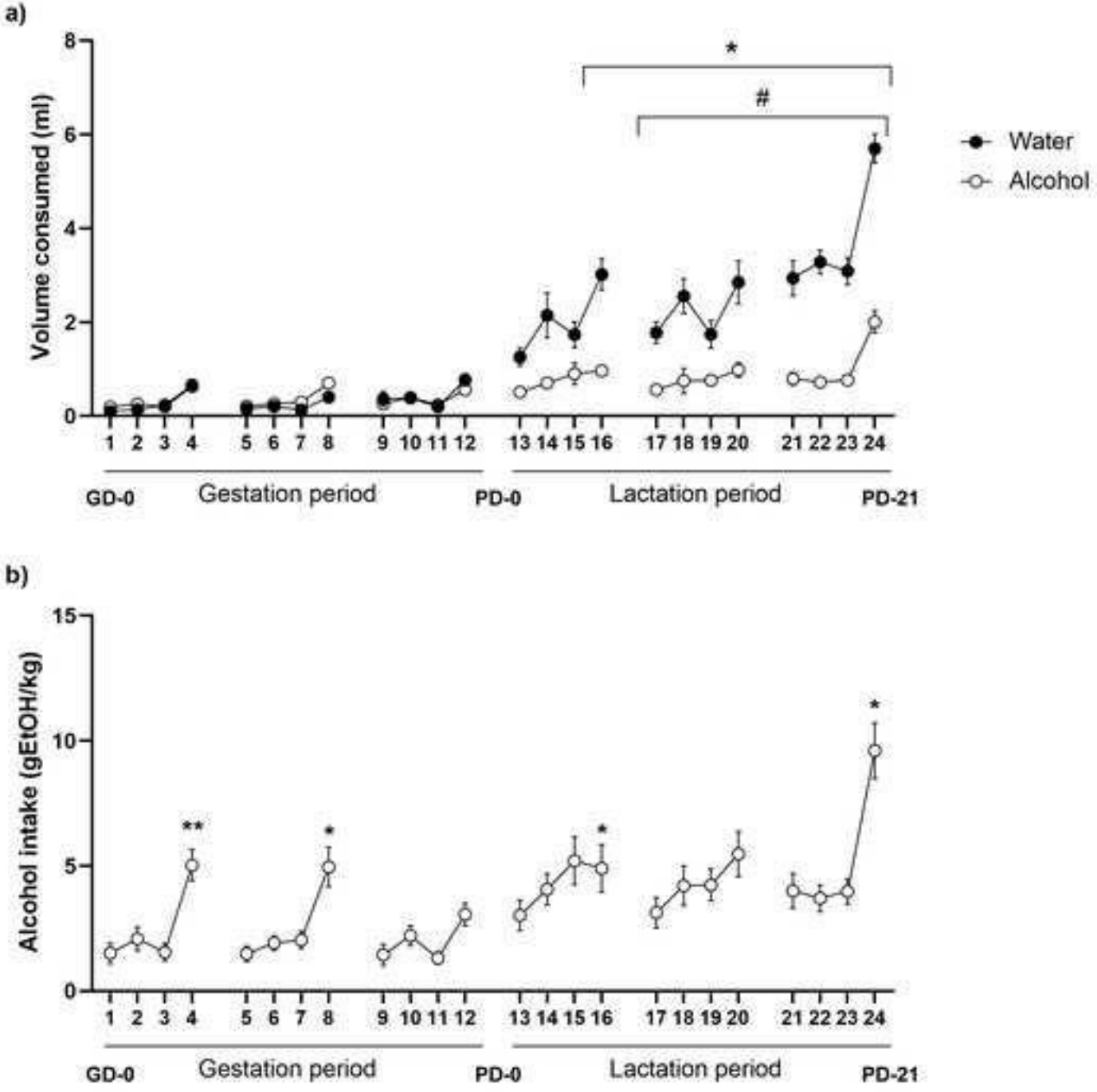
a)
Volume consumed (ml)
0
2
4
6
8
*
#
Water
Alcohol
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
GD-0
Gestation period
PD-0
Lactation period
PD-21
b)
Alcohol intake (gEtOH/kg)
0
5
10
15
**
*
*
*
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
GD-0
Gestation period
PD-0
Lactation period
PD-21

Figure 4

Click here to download Figure Figure3-R1.tif

Figure 5

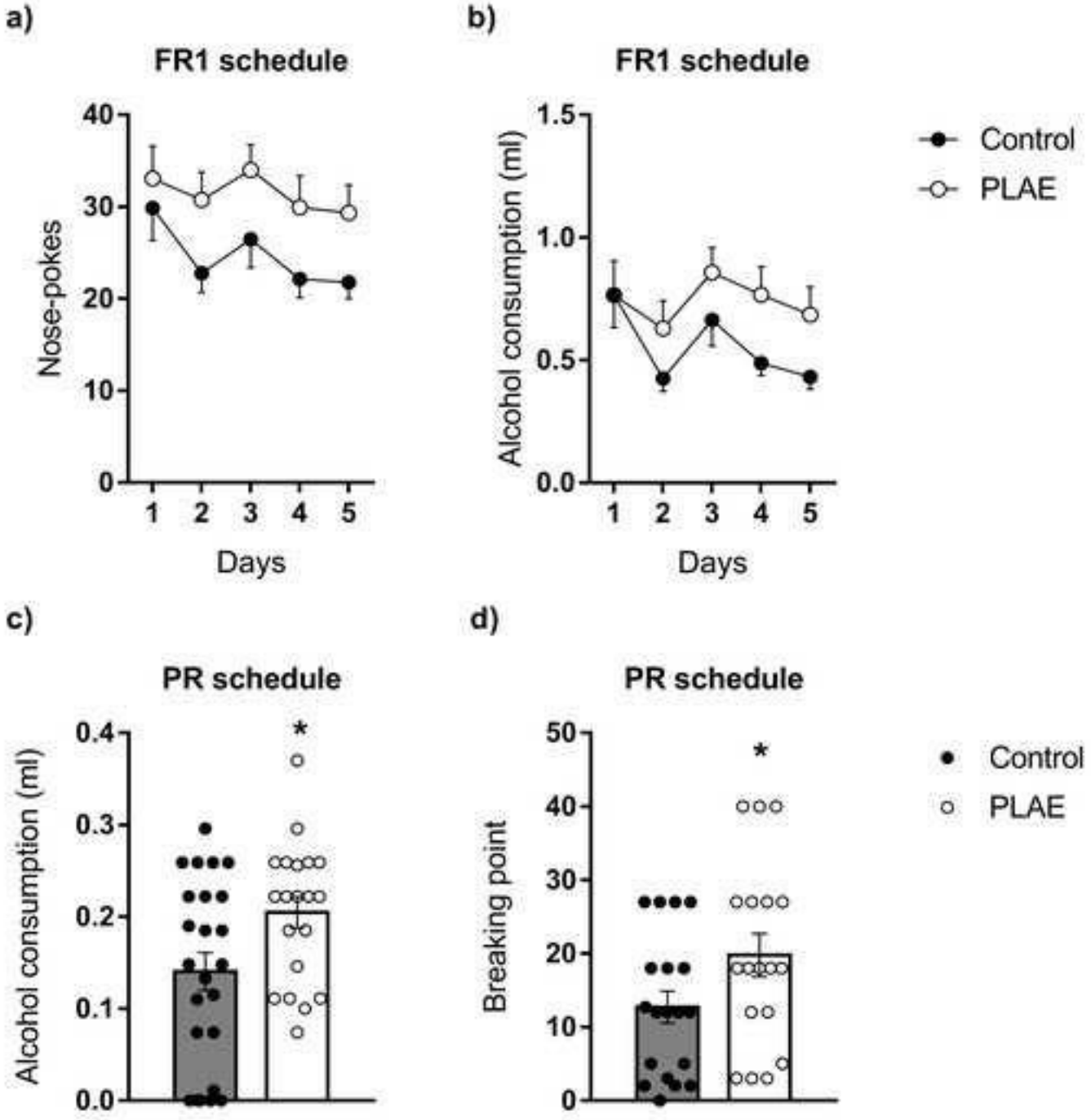
a)
FR1 schedule
Nose-pokes
40
30
20
10
0
1
2
3
4
5
Days
b)
FR1 schedule
Alcohol consumption (ml)
1.5
1.0
0.5
0.0
1
2
3
4
5
Days
Control
PLAE
c)
PR schedule
Alcohol consumption (ml)
0.4
0.3
0.2
0.1
0.0
*
d)
PR schedule
Breaking point
50
40
30
20
10
0
*
Control
PLAE

Figure 6

Click here to download Figure Figure4-R1.tif

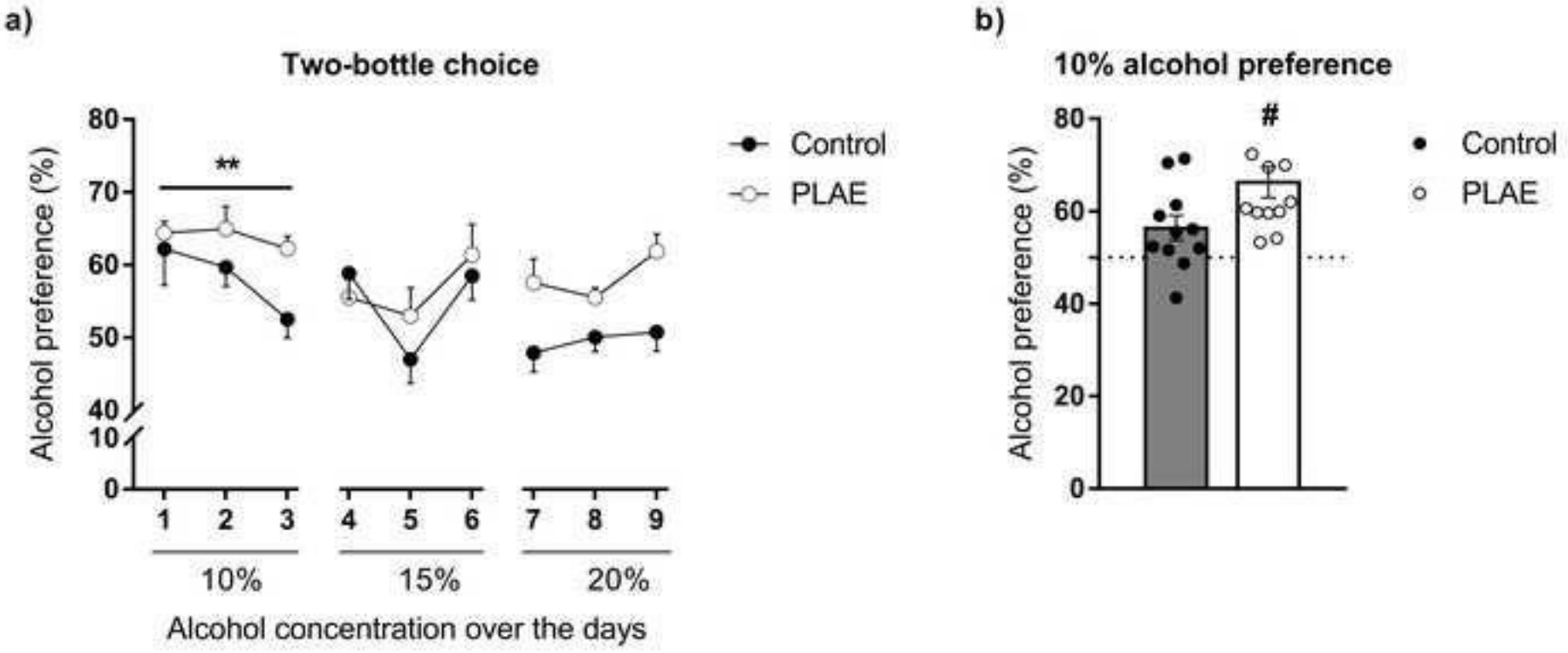

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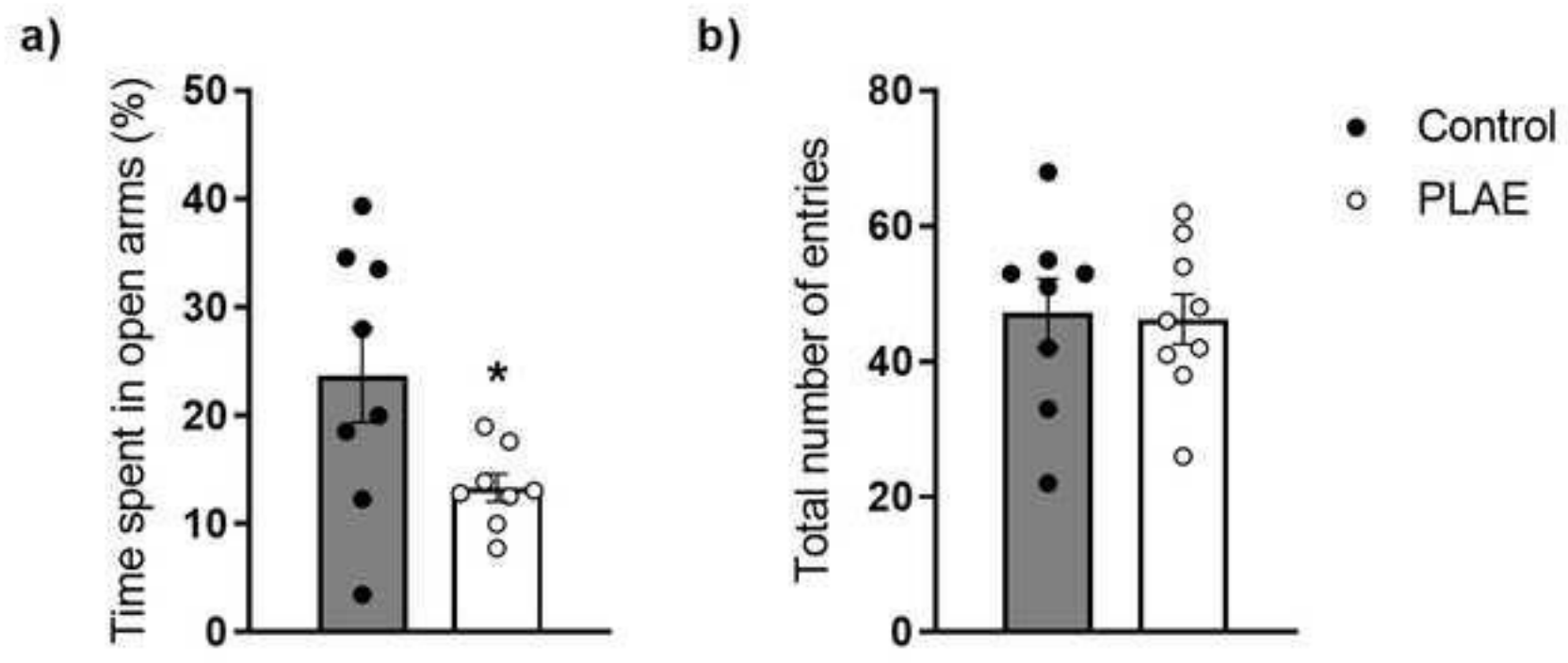
Figure 7



Click here to download Figure Figure6-R1.tif

Figure 8


BAC determination
Blood alcohol levels (mg/dl)
800
600
400
200
0
***
Control
PLAE
30
60
120
30
60
120
Minutes after alcohol injection

Figure 9

Click here to download Figure Figure7-R1.tif

Figure 10

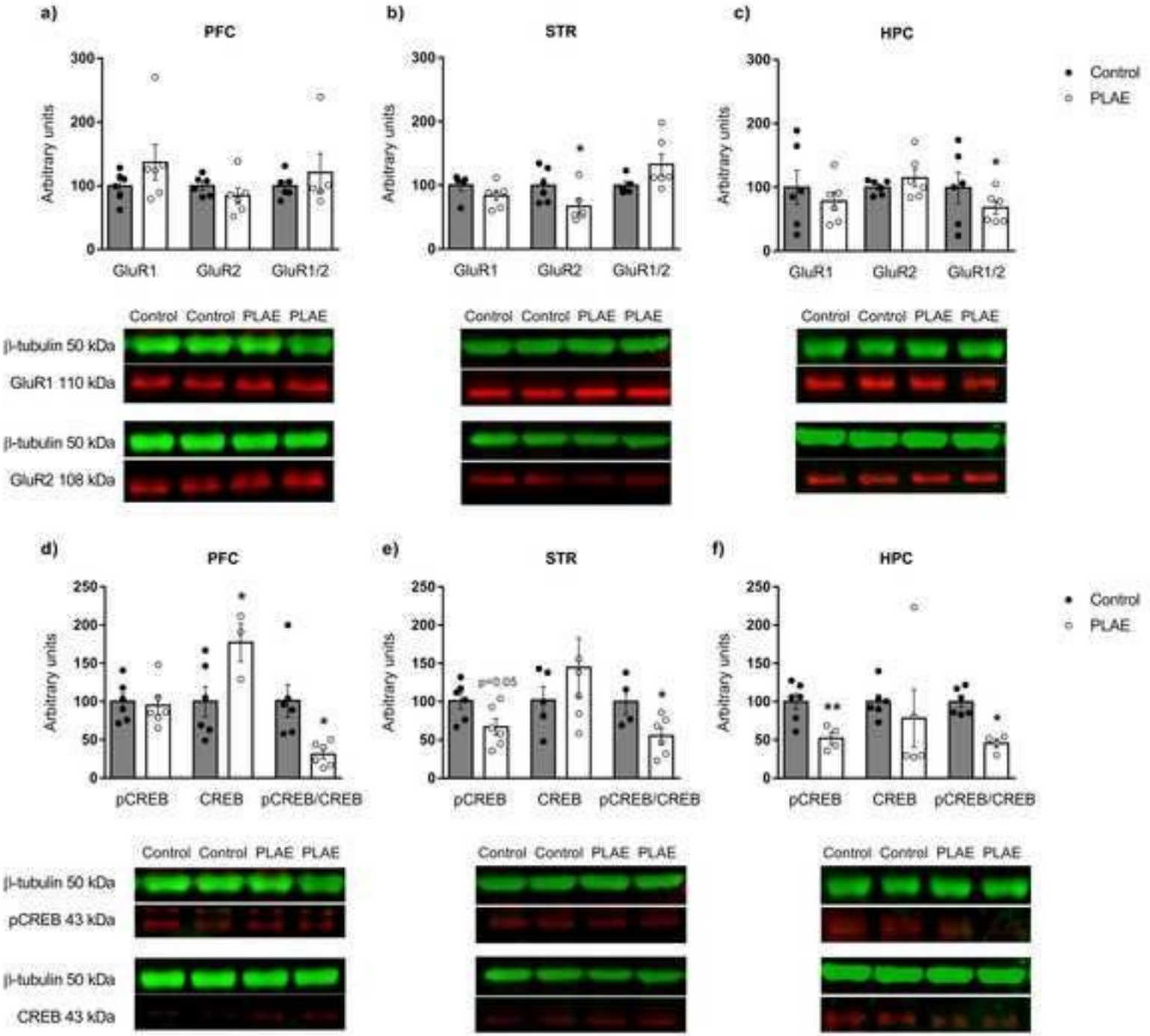

a)
PFC
b)
STR
c)
HPC
Control
PLAE
Arbitrary units
300
200
100
0
GluR1
GluR2
GluR1/2
Control Control PLAE PLAE
β-tubulin 50 kDa
GluR1 110 kDa
GluR2 108 kDa
d)
PFC
e)
STR
f)
HPC
250
150
50
pCREB
CREB
pCREB/CREB
p=0.05
β-tubulin 50 kDa
pCREB 43 kDa
CREB 43 kDa

Figure 11

Click here to download Figure Figure8-R1.tif

Figure 12

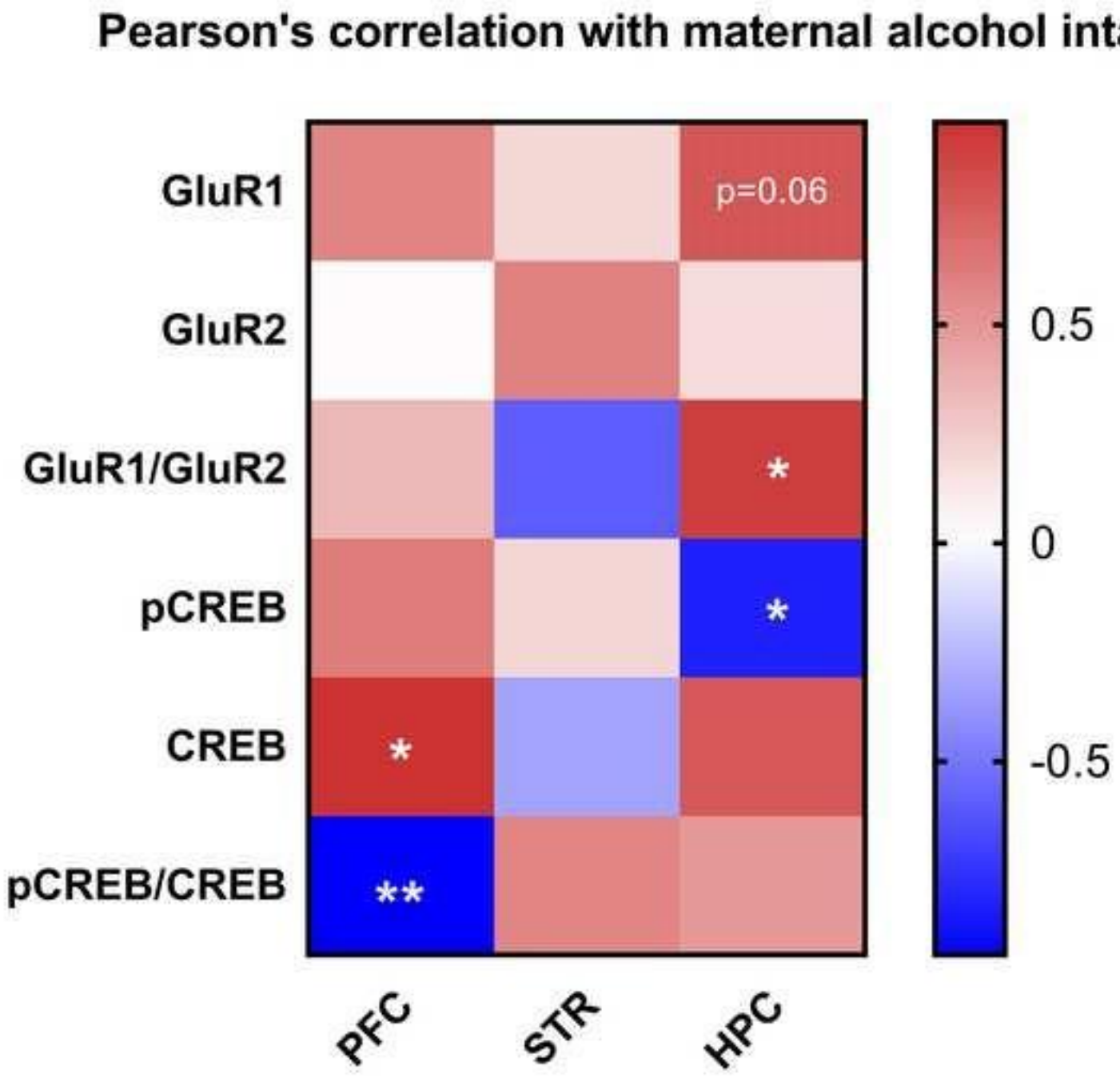
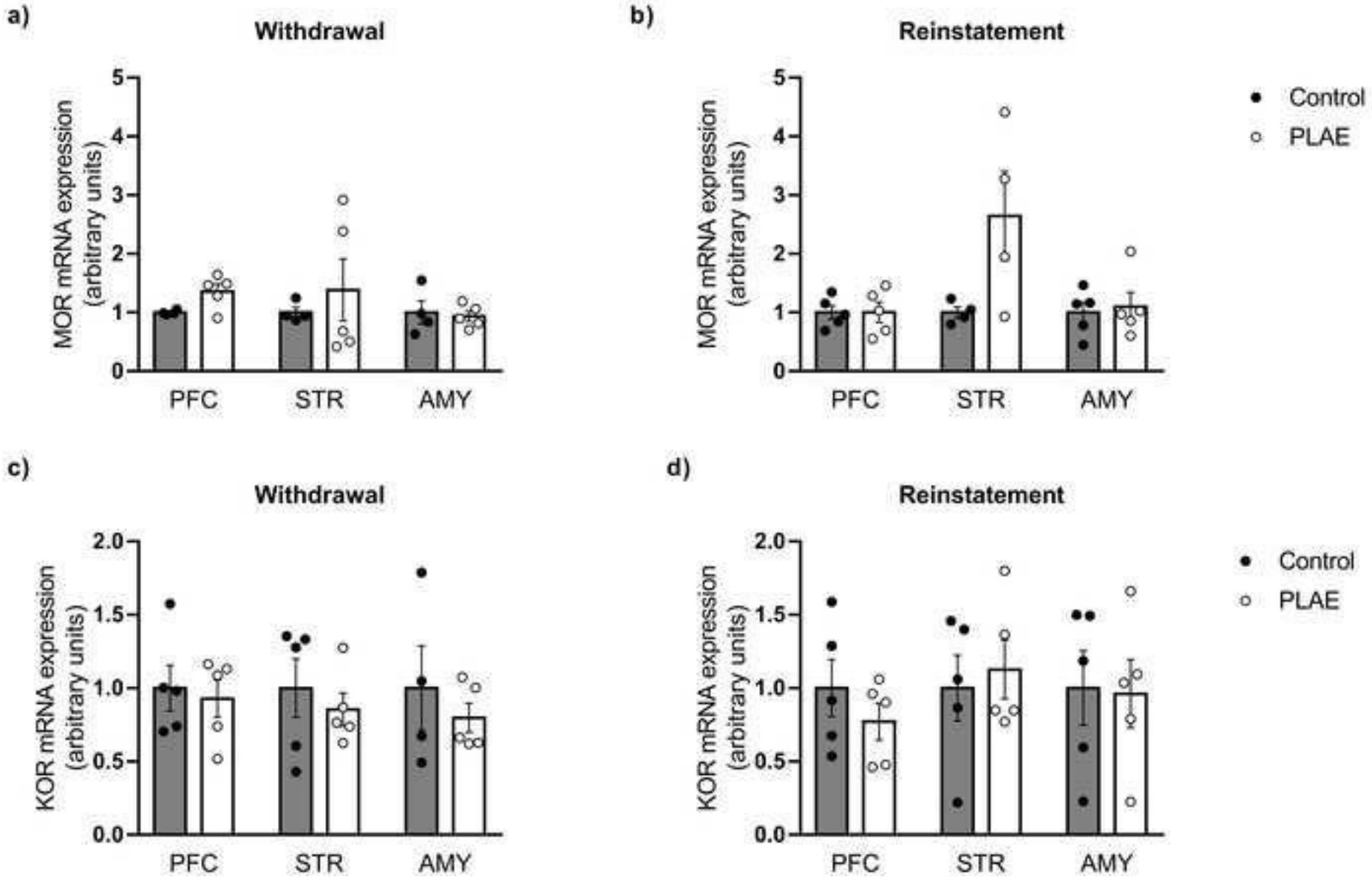

Pearson's correlation with maternal alcohol intake
GluR1
GluR2
GluR1/GluR2
pCREB
CREB
pCREB/CREB
p=0.06
*
*
*
**
PFC
STR
HPC
0.5
0
-0.5

Figure 13

Click here to download Figure Figure9-R1.tif

Figure 14


a)
Withdrawal
MOR mRNA expression (arbitrary units)
0
1
2
3
4
5
PFC
STR
AMY
b)
Reinstatement
MOR mRNA expression (arbitrary units)
0
1
2
3
4
5
PFC
STR
AMY
Control
PLAE
c)
Withdrawal
KOR mRNA expression (arbitrary units)
0.0
0.5
1.0
1.5
2.0
PFC
STR
AMY
d)
Reinstatement
KOR mRNA expression (arbitrary units)
0.0
0.5
1.0
1.5
2.0
PFC
STR
AMY
Control
PLAE

Declaration of competing interest

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Ethical Statement

All procedures were conducted in compliance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and were approved by the local ethics committee (CEEA-PRBB).

Author contributions

SM-R, LC, JM, MR-A, MVM and OV were responsible for the study concept and design. SMR, JFF-G, LC, and CN carried out the experimental studies. SM-R, LC and OV drafted the manuscript and SM-R, JFF-G, LC, JM, MR-A, MVM and OV participated in the

interpretation of findings. All authors critically reviewed the content and approved the final version for publication.