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Altered brain functional connectivity and behaviour in a mouse model of maternal alcohol binge-drinking.

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Abstract

Prenatal and perinatal alcohol exposure caused by maternal alcohol intake during gestation and lactation periods can have long-lasting detrimental effects on the brain development and behaviour of offspring. Children diagnosed with Foetal Alcohol Spectrum Disorders (FASD) display a wide range of cognitive, emotional and motor deficits, together with characteristic morphological abnormalities. Maternal alcohol binge drinking is particularly harmful for foetal and early postnatal brain development, as it involves exposure to high levels of alcohol over short periods of time. However, little is known about the long-term effects of maternal alcohol binge drinking on brain function and behaviour. To address this issue, we used pregnant C57BL/6 female mice with time-limited access to a 20% v/v alcohol solution as a procedure to model alcohol binge drinking during gestation and lactational periods. Male offspring were behaviourally tested during adolescence (30 days) and adulthood (60 days), and baseline neural metabolic capacity of brain regions sensitive to alcohol effects were also evaluated in adult animals from both groups. Our results show that prenatal and postnatal alcohol exposure caused age-dependent changes in spontaneous locomotor activity, increased anxiety-like behaviour and attenuated alcohol-induced conditioned place preference in adults. Also, significant changes in neural metabolic capacity using cytochrome c oxidase (CCO) quantitative histochemistry were found in the hippocampal dentate gyrus, the mammillary bodies, the ventral tegmental area, the lateral habenula and the central lobules of the cerebellum in adult mice with prenatal and postnatal alcohol exposure. In addition, the analysis of interregional CCO activity correlations in alcohol-exposed adult mice showed disrupted functional brain connectivity involving the limbic, brainstem, and cerebellar regions. Finally, increased neurogenesis was found in the dentate gyrus of the hippocampus of alcohol-exposed offspring, suggesting neuroadaptive effects due to early alcohol exposure. Our results demonstrate that maternal binge-like alcohol drinking causes long-lasting effects on motor and emotional-related behaviours associated with impaired neuronal metabolic capacity and altered functional brain connectivity.

Keywords: Anxiety-like behaviour, Alcohol preference, Maternal alcohol binge drinking, Functional connectivity, Cytochrome oxidase, Hippocampal neurogenesis

1. Introduction

The central nervous system (CNS) is particularly vulnerable to the adverse effects of alcohol, especially during critical periods of brain development. Foetal alcohol spectrum disorders (FASD) comprise a wide range of morphological and neurocognitive anomalies as a result of *in utero* alcohol exposure in offspring, including learning and memory deficits, poorer motor function and executive functioning impairments. Furthermore, psychological deficits and secondary disabilities, such as hyperactivity, attention deficits, social difficulties and psychiatric problems, are often associated with Foetal Alcohol Syndrome (FAS), which is the most severe consequence of maternal alcohol drinking during pregnancy (Gupta et al., 2016). In pregnant women, 1 of 33 reported binge drinking during the previous 30 days (CDC, 2016) and the estimated global prevalence of FAS is 14.6 per 10,000 people (Popova et al., 2017). FASD, therefore represents a major public health concern and is the leading known preventable cause of mental retardation in western countries.

In our recent study (Cantacorps et al., 2017), we reported spatial memory deficits and motor coordination impairments in a mouse model of FASD. Furthermore, with a view to elucidate the underlying molecular mechanisms in neurobehavioural phenotypes, we showed that maternal binge-like alcohol drinking during gestation and lactation periods produces an upregulation of proinflammatory signalling and myelination defects in the prefrontal cortex and hippocampus of adult offspring mice. Binge alcohol drinking during pregnancy has also been identified as a risk factor for the development of mental disorders later in adulthood, such as depression, anxiety and substance abuse (Barr et al., 2006; Hellemans et al., 2010). Indeed, higher rates of anxiety have been found among the FASD population (Hellemans et al., 2008). Also, attention deficit hyperactivity disorder (ADHD) is often diagnosed as a comorbid condition in FASD-affected humans (Jacobson and Jacobson, 2002; Popova et al., 2016). Similarly, previous animal studies reported increases in anxiety-like behaviour following prenatal alcohol exposure (Cullen et al., 2013; Dursun et al., 2006; Kleiber et al., 2011; Liang et al., 2014). Moreover, alterations in locomotor activity have been described in rodent offspring prenatally exposed to alcohol (Abate et al., 2017; Dursun et al., 2006; Kim et al., 2013; Muñoz-Villegas et al., 2017; Sanchez Vega et al., 2013; Schambra et al., 2016).

There is scarce research in the available literature as to the association between prenatal alcohol exposure and subsequent drug abuse in exposed offspring (Behnke and Smith, 2013). Clinical data support an increased risk of alcohol abuse later in life following prenatal exposure (Alati et al., 2006; Parolin et al., 2016; Yates et al., 1998). However, the neurobiological mechanisms underlying such susceptibility are unknown. We hypothesized that developmental alcohol exposure may heighten the risk of drug abuse later in life, altering reward-related brain functional networks.

Changes in the metabolic capacity of the brain could be sensitive to alcohol effects (González-Reimers et al., 2014). In this line, cytochrome c oxidase (CCO) is the mitochondrial enzyme responsible for cellular oxygen consumption in aerobic energy metabolism and it is also an intracellular measure of oxidative metabolic capacity as CCO activity is critically linked to ATP generation within the mitochondria of brain cells (Wong-Riley, 1989). For this reason, CCO activity may be used as a metabolic marker for neuronal activity as it is mainly required for neuronal membrane repolarization associated with neuronal activation (Wong-Riley, 1989). Therefore, quantitative CCO histochemistry is regarded as a method for measuring long-term changes in neuronal oxidative capacity related with the energy metabolism (protein-synthesis-dependent enzyme induction over hours) of brain regions (Gonzalez-Lima and Cada, 1998; Riha et al., 2011).

Neurotoxicity associated with ethanol exposure during gestation and lactation may be caused by the induction of oxidative stress and mitochondrial dysfunction leading to apoptotic brain cell death (Ramachandran et al., 2001; Reddy et al., 2013). CCO activity would be particularly impaired by oxidative stress as a consequence of alcohol exposure in neurons, a mechanism also reported for the most common neurodegenerative diseases such as Alzheimer's or Parkinson's and aging (Lin and Beal, 2006; Onyango et al., 2016). In this regard, the effects of alcohol exposure on brain CCO activity are still not well known. Only one study reported chronic intermittent or binge-like alcohol exposure in young and aged rats to decrease CCO activity in cortical and cerebellar cortices together with the locus coeruleus (Jaatinen et al., 2003). However, several studies showed inconsistent results as to the effects of alcohol on brain CCO activity, with some studies reporting no changes in rats' whole-brain CCO activity following foetal alcohol exposure (Marin-Garcia et al., 1996) or chronic alcohol exposure (Thayer and Rottenberg, 1992), decreased whole-brain CCO activity (Marin-Garcia et al., 1995)

and decreased hippocampal CCO mRNA expression after chronic alcohol exposure (Hyun Kim et al., 2001). Such discrepancies between studies could be related to the different methods and protocols used to measure CCO activity in brain homogenates or brain regions and the different protocols used to evaluate alcohol exposure. To our knowledge, this is the first study to evaluate changes in regional brain CCO in the male offspring of mice exposed to alcohol during gestation and lactation periods. In addition, functional connectivity among brain regions in the offspring was also calculated using cross-correlations of CCO activity.

In the present study, our aim was to assess the effects of prenatal and lactational alcohol exposure (PLAE) on anxiety-related behaviour, locomotor activity and alcohol-related motivational properties. Furthermore, we evaluated the cumulative changes in the baseline metabolic capacity of brain regions sensitive to maternal alcohol consumption using cytochrome c oxidase (CCO) histochemistry. Changes in brain CCO activity after prenatal and lactational alcohol exposure could be caused by altered neurogenesis during postnatal development or changes in neuronal oxidative metabolism leading to abnormal synaptic activity. In order to discriminate between the possible causes of changes in CCO activity, neurogenesis in the dentate gyrus (DG) of the hippocampus was assessed to explore the possible mechanisms underlying changes observed in brain metabolic capacity.

2. Materials and Methods

2.1 Animals

Male (n=24) and female (n=24) C57BL/6 inbred mice were purchased from Charles River (Barcelona, Spain) and transported to our animal facility (UBIOMEX, PRBB) to be used as breeders. Animals were 12 weeks old when breeding commenced and were individually housed in standard cages in a temperature- ($21^{\circ} \pm 1^{\circ}\text{C}$), humidity- ($55\% \pm 10\%$), and light-cycle-controlled room. Lighting was maintained on a 12-hour reverse light-dark cycle with lights turned off between 08:00 and 20:00. Mice were allowed to acclimatize to the new environmental conditions for at least one week prior to experimentation, which took place during the dark phase under a dim red light. After successful mating, pregnant females were observed for parturition on a daily basis. For each litter, the date of birth was designated as postnatal day (PD) 0. Pups remained with their mothers for 21 days and were then weaned (PD21). After weaning, male offspring

were housed in groups of 4. Food and water were available *ad libitum* except when water was substituted for alcohol according to DID procedure and during behavioural testing of the offspring. Different cohorts of mice were used for behavioural training at PD30 and PD60 ($n=7-13$ per group), as well as, separate groups of mice were used for biochemical analyses ($n=5-8$ per group). Every effort was made to minimize the number of animals used and their suffering. All animal care and experimental procedures were conducted in accordance with the European Communities Directive 86/609/EEC regulating animal research and were approved by the local ethics committee (CEEA-PRBB).

2.2 Drugs

Ethyl alcohol was purchased from Merck Chemicals (Darmstadt, Germany) and diluted in tap water to obtain a 20% (v/v) alcohol solution.

2.3 Drinking-in-the-dark (DID) test

The procedure was conducted as previously reported (Cantacors et al., 2017). Two days after mating, pregnant females were randomly assigned to one of two groups: alcohol and water-exposed (control). 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 3h after the lighting was turned off. Following a 2h-access period, individual intake was recorded and food and water bottles were returned to the home cage. During this time, the mice were individually housed and each corresponding male breeding pair was removed from the home cage for the DID procedure. The procedure was repeated on days 2 and 3 and fresh fluids were provided each day (from Monday to Wednesday). On day 4 (Thursday), alcohol or water cylinders were left for 4h and fluid intakes were recorded. Two empty control cages (water and alcohol) were placed in the rack to measure general liquid loss (leakage/evaporation) and drip values were subtracted from the drinking values. Fluid intakes (g/kg body weight) were calculated on the basis of average 2-day body weight values, as dams were weighed at 2-day intervals (Mondays and Wednesdays). This procedure was initiated during pregnancy and maintained throughout lactation until offspring were weaned.

2.4 Spontaneous locomotor activity

Offspring mice were evaluated for their basal locomotor activity during adolescence (PD30) and adulthood (PD60). Animals were placed in locomotor activity boxes (24x24x24 cm) (LE8811 IR, Panlab s.l.u., Barcelona, Spain) and horizontal (deambulations) and vertical (rearings) movements were automatically recorded for 45 min as previously described (Gracia-Rubio et al., 2016).

2.5 Elevated plus maze (EPM)

EPM was performed to evaluate anxiety-like behaviour in the offspring at PD30 and PD60 as previously reported (Gracia-Rubio et al., 2016; Simonin et al., 1998). The apparatus (Panlab s.l.u., Barcelona, Spain) consisted of a black maze with four arms (16x5 cm) set in the form of a cross from a neutral central square (5x5 cm). Two arms were closed up by vertical walls (closed arms) while the other two perpendicular arms had open edges (open arms). The maze was elevated 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).

2.6 Alcohol-induced conditioned place preference (CPP)

The rewarding effects of alcohol were assessed in adult mice (PD60) using an unbiased CPP procedure, adapted from Roger-Sánchez et al., (2012). The apparatus consisted of two conditioning compartments that differed in visual and tactile cues (30x29x35 cm) connected by a grey-coloured tunnel (14x29x35 cm) (Cibertec S.A., Madrid, Spain). One compartment had white-painted walls with prismatic textured flooring and the other had black walls with a smooth floor. All the compartments were equipped with infrared emitter/detector pairs, 2.2 cm above the floor and 1 cm apart. This procedure was conducted in three different phases. In the preconditioning phase (day 1), mice were placed in the central compartment and were allowed to explore both compartments of the apparatus for 20 min. Mice showing strong unconditioned aversion (< 33% of the session time) or preference (> 67%) for either compartment were not included in the study. In the conditioning phase (days 2-9), half the animals from each group received the drug in one compartment and the other half in the other compartment through four pairings. Thus, mice received an i.p injection of 1 or 2 g/kg alcohol immediately prior to

being confined to the drug-paired compartment for 5 min on days 2, 4, 6 and 8. On the alternate days (3, 5, 7 and 9), mice received physiological saline before being confined to the vehicle-paired compartment for 5 min. Control animals were administered saline prior to confinement in one of the two compartments every day. The central area was blocked with guillotine doors during conditioning. The test (day 10) was conducted in the same conditions applied in the preconditioning phase. The time spent in each compartment during preconditioning and testing phases was recorded. The CPP score was calculated as the difference of time spent in the drug-paired compartment during the test and time spent in the pre-conditioning phase for each subject.

2.7 Cytochrome c oxidase histochemistry

Adult mice (PD60) were sacrificed and their brains were quickly removed, frozen in isopentane at -70°C (Sigma–Aldrich, Madrid, Spain) and stored at -40°C to preserve brain tissue and enzyme activity. Next, 30 μm -thick coronal brain sections were obtained using a cryostat microtome (Microm International GmbH, model HM 505-E, Heidelberg, Germany). The sections were mounted on slides and stored at -40°C prior to processing with quantitative CCO histochemistry.

A modified version of the quantitative CCO histochemical method developed by Gonzalez-Lima and Jones (1994) was used. Staining variability across different staining baths was controlled by including sets of tissue standards. These standards were obtained from sections of brain homogenates of previously known CCO activity determined spectrophotometrically. Following the previously described protocol by Conejo et al. (2013), sets of standards cut at different thicknesses (10, 30, 50 and 70 μm) were included with each batch of slides. Each set of slides was fixed for 5 min with a 0.5% glutaraldehyde solution, rinsed three times in phosphate buffer (PB) and preincubated during 5 min in a solution containing 0.05 M Tris buffer at pH 7.6 with 275 mg/l cobalt chloride, 10% (w/v) sucrose, and 5 ml dimethylsulfoxide. The sections were then rinsed in PB (pH 7.6; 0.1 M) and were incubated at 37°C for 1 h, in the dark and with continuous stirring, in a solution containing 50 mg 3,3'-diaminobenzidine, 15 mg cytochrome c (Sigma, St. Louis, MO, USA) and 4 g sucrose per 100 ml PB (pH 7.4; 0.1 M). The reaction was stopped by fixing the tissue in buffered formalin (10% w/v sucrose and 4% formaline) for 30 min at room temperature. The slides were then

dehydrated, cleared with xylene and coverslipped with Entellan (Merck, Darmstadt, Germany).

2.8 Evaluation of regional brain cytochrome c oxidase activity

CCO histochemical staining intensity was measured by densitometric analysis using a computer-assisted image analysis workstation (MCID, InterFocus Imaging Ltd., Linton, England), which includes specific image analysis software. Four measurements of relative optical density in three consecutive sections were obtained for each region, making up a total of twelve measures per region. To establish comparisons and consider possible staining variations across brain sections from different staining baths, measurements were also obtained from CCO-stained brain homogenate standards. Regression curves between section thickness (10, 30, 50, 70 micrometer-thick sections) and CCO activity, previously measured by spectrophotometric assay in each set of standards were calculated. Finally, average relative optical density measured in each brain region was converted into CCO activity units (1 unit: 1 μmol of cytochrome c oxidized/min/g tissue wet weight at 23°C) using the previously calculated regression curve in each homogenate standard. The average measure per region was carried out for each section and animal. These included cingulate (CG), prelimbic (PL), and infralimbic areas (IL) of the medial prefrontal cortex and primary motor cortex (M1). In addition, the following regions were also analysed: striatum (STR), nucleus accumbens (ACB), lateral septal nucleus (LS), bed nucleus of the stria terminalis (BST), ventral pallidum (substantia innominata, PALv), anterodorsal (AD) and anteroventral (AV) thalamic nuclei; hippocampal subfields including CA1, CA3 and DG of the dorsal (CA1d, CA3d, DGd) and ventral hippocampus (CA1v, CA3v, DGv); lateral (LHb) and medial (MHb) habenula; medial (MeAb), basal (BaA), lateral (LaA) and central (CeA) amygdaloid nuclei; medial (MM), medial lateral part (LMM) and lateral (LM) nuclei of the mammillary bodies; pars reticularis (SNr) and pars compacta (SNc) of the substantia nigra and ventral tegmental area. Likewise, granular and molecular layers of the central (lobules II, III), culmen (lobules IV-V) and uvula (lobules IX) of the cerebellum were taken into account. Figure 6 shows representative microphotographs of cytochrome c oxidase (CO) stained coronal sections of the dorsal hippocampus in a control mouse brain and in a PLAE mice brain. The selected brain regions were defined according to an interactive online atlas, the Allen Mouse Brain Atlas (version 2, 2011; <http://atlas.brain-map.org/>).

2.9 BrdU immunofluorescence staining

On PD60, neurogenesis was evaluated in the DG. Animals were injected three times at 2h intervals with 5'-bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich, Madrid, Spain) (100 mg/kg, i.p.) as previously described with minor modifications (Chen et al., 2012; Johansson et al., 2015). Brain tissue was collected 3 or 30 days after the last BrdU injection to determine neural cell proliferation and survival, respectively. Mice were anesthetized and transcardially perfused with 0.1M PB followed by ice-cold 4% paraformaldehyde PB solution. Brains were removed, postfixed in 4% paraformaldehyde PB solution overnight at 4 °C and stored in 30% v/v sucrose in 0.1M PB at 4 °C. After freezing in dry ice, 30 µm-thick coronal tissue sections were obtained using a microtome. Subsequently, floating brain sections were washed three times in 0.1M phosphate saline buffer (PBS), treated with 2N HCl for 30min at 37°C and neutralized in tap water, followed by immunofluorescent staining. After extensive washes in PBS, sections were blocked with a PBS solution containing 0.25% Tween 20 and 5% normal goat serum for 1h at room temperature. The following primary antibodies were then incubated in the same solution for 48h at 4°C: rat anti-BrdU (1:300; Abcam, Cambridge, UK), mouse anti-neuronal specific nuclear protein (NeuN) (1:1000; Merck Millipore, Darmstadt, Germany). After three washes in PBS, floating sections were incubated with fluorescent secondary antibodies for 2h at room temperature without light: goat anti-rat IgG Alexa Fluor 488 (1:500; Invitrogen, Barcelona, Spain), goat anti-mouse IgG Alexa Fluor 555 (1:500; Invitrogen). Finally, sections were mounted on slides using Fluoroshield (Sigma-Aldrich) and coverslipped for microscopy.

2.10 Image analysis

Six brain sections containing the hippocampus of each subject were analysed bilaterally. Samples were visualized and digitized under a 20x objective using a laser scanning confocal microscope (Leica TCS SP5; Mannheim, Germany). Z-plane image stacks (1,024x1,024 pixels) were taken at 2 µm. BrdU positive cells with NeuN colabelling in the DG of the hippocampus were quantified using ImageJ software (NIH, Bethesda, MD, USA) and the average of each sample was calculated.

2.11 Statistical analysis

Data obtained from the DID test (volumes of water and alcohol consumption) were analysed using two-way analysis of variance (ANOVA) with group as a between-subject factor and day as a within-subject factor, followed by Bonferroni post-hoc comparisons when required. One-way ANOVA with repeated measures was used to analyse alcohol intake in the alcohol-drinking group of dams during the DID test. Locomotor activity results were analysed using two-way ANOVA with group and time as factors of variation or by unpaired two-tailed Student's *t*-test to compare total deambulations and rearings between groups. Data from EPM were analysed using unpaired or paired two-tailed Student's *t*-test. Data from CPP were evaluated using two-way ANOVA with group and treatment as factors of variation. Immunofluorescence counting data were analysed using unpaired two-tailed Student's *t*-test. Group differences in mean brain CCO activity measured in each brain region were analysed by Student's *t*-tests. Results are expressed as mean $\pm$ SEM. Differences were considered statistically significant when $p < 0.05$. Analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA).

In order to evaluate possible changes in functional relationship between regional brain activity caused by the DID test, regional CCO activity data were analysed in terms of pair-wise correlations within each experimental group (Puga et al., 2007). Pearson product-moment correlations were calculated for interregional correlation analysis, including pair-wise comparisons of each region showing a mean between-group difference as revealed by Student's *t*-tests. CCO activity values were normalized by dividing the measured activity of each brain region by the average CCO activity value of the hippocampal areas measured for each animal. This was done in order to reduce possible differences in the intensity of CCO staining not resulting from experimental manipulations. To ensure the reliability of correlations, a 'jackknife' procedure (Shao and Tu, 1995) was performed based on the calculation of all possible pair-wise correlations resulting from the removal of one subject each time and only considering the correlations that remained significant ($p < 0.01$) across all possible combinations. This is a conservative method, sensitive to outliers, which avoids magnified type-1 errors caused by the large number of interregional correlations computed in relation to the sample sizes. Statistical analysis was performed using the statistics module featured in SigmaPlot 12 software (Systat Software Inc., Germany).

3. Results

1.1. Maternal alcohol consumption

As shown in Figure 1, dams were exposed, under the DID test procedure throughout the gestation and lactation periods, in order to mimic voluntary binge alcohol drinking. Two-way ANOVA analysis of water and alcohol volumes of consumption during DID testing (Fig. 1A) showed a significant effect of day [$F(23,437)=2.135$; $p<0.01$], an insignificant effect of group [$F(1,19)=2.724$; n.s.] and a significant interaction between factors [$F(23,437)=2.135$; $p<0.01$]. Bonferroni post-hoc comparisons revealed a significant increase in water consumption compared to alcohol consumption on day 24 of the test ($p<0.001$).

One-way ANOVA with repeated measures analysis of alcohol intake showed a significant effect of day [$F(5.72,57.2)=10.8$; $p<0.001$]. Furthermore, post-hoc comparisons revealed significant differences between day 5 ($p<0.05$) and day 6 ($p<0.01$) (2h drinking sessions) compared to day 8 (4h drinking session) (Fig. 1B).

Maternal body weight was measured at 2-day intervals throughout the DID procedure (Fig. 1C). Two-way ANOVA analysis revealed a significant effect of day [$F(11,209)=192.8$; $p<0.001$], but no significant effect of group [$F(1,19)=0.9391$; n.s.] and no significant interaction between factors [$F(11,209)=1.485$; n.s.].

1.2. Prenatal and postnatal alcohol exposure induces alterations in spontaneous locomotor activity

The effects of prenatal and postnatal alcohol exposure on spontaneous locomotor activity were assessed in adolescent (PD30) and adult (PD60) offspring. Two-way ANOVA analysis of horizontal movements on PD30 (Fig. 2A) showed an effect of time [$F(8,72)=14.8$; $p<0.001$], but no significant effect of group [$F(1,9)=3.407$; n.s.] or interaction between factors [$F(8,72)=0.323$; n.s.]. Unpaired two-tailed Student's t-test of total deambulations (Fig. 2B) showed a statistically significant difference ($t_{17}=2.436$; $p<0.05$). Two-way ANOVA analysis of vertical movements (Fig. 2C) showed an effect of time [$F(8,80)=4.653$; $p<0.001$], group [$F(1,10)=6.05$; $p<0.05$] but no significant interaction between factors [$F(8,80)=1.45$; n.s.]. Student's t-test of total rearings (Fig. 2D) showed a significant difference between groups ($t_{18}=2.881$; $p<0.01$).

Two-way ANOVA analysis of horizontal movements on PD60 (Fig. 3A) showed an effect of time [$F(8,168)=32.52$; $p<0.001$], group [$F(1,21)=5.713$; $p<0.05$] but no

significant interaction between factors [$F(8,168)=0.7715$; n.s.]. Student's *t*-test of total deambulations (Fig. 3B) showed a statistically significant difference ($t_{21}=2.39$; $p<0.05$). Two-way ANOVA analysis of vertical movements (Fig. 3C) showed an effect of time [$F(8,168)=10.7$; $p<0.001$], group [$F(1,21)=5.678$; $p<0.05$] but no significant interaction between factors [$F(8,168)=1.242$; n.s.]. Student's *t*-test of total rearings (Fig. 3D) showed a significant difference between groups ($t_{21}=2.383$; $p<0.05$).

1.3. Alcohol-exposed offspring mice exhibit an anxiogenic-like response

Mice were assessed for their anxiety-like behaviour on the EPM at PD30 and 60 (Fig. 4). Student's *t*-test analysis revealed a statistically significant difference in the percentage of time spent in the open arms ($t_{18}=2.602$; $p<0.01$) (Fig. 4A) but no between-group differences were found in the percentage of entries into open arms ($t_{18}=0.8776$; n.s.) (Fig. 4B) or in the total number of entries ($t_{18}=0.1902$; n.s.) at PD30 (Fig. 4C). Similarly, we observed a significant decrease in the percentage of time spent in the open arms ($t_{21}=1.846$; $p<0.05$) (Fig. 4D), whereas no differences were found in the percentage of entries into open arms ($t_{21}=0.2885$; n.s.) (Fig. 4E) or the total number of entries ($t_{21}=0.3611$; n.s.) at PD60 (Fig. 4F).

1.4. Maternal binge-like alcohol consumption attenuates alcohol-induced rewarding effects in the offspring

As illustrated in Figure 5, PLAE mice showed an attenuation of alcohol-induced CPP. Two-way ANOVA analysis showed an effect of group [$F(1,54)=12.97$; $p<0.001$], treatment [$F(2,54)=15.48$; $p<0.001$] and an interaction between factors [$F(2,54)=3.538$; $p<0.05$]. Bonferroni post-hoc comparisons revealed statistically significant differences in the CPP score between saline and 1g/kg EtOH-treated control mice ($p<0.01$), and between saline and 2g/kg EtOH-treated control mice ($p<0.001$). Furthermore, alcohol-exposed mice treated with 2g/kg EtOH showed a significant CPP score reduction when compared with their counterparts ($p<0.01$).

1.5. Quantitative CCO histochemistry

Mean CCO values

Statistical analysis revealed significant increases in CCO activity in the DG ($t_{14}=2.51$; $p=0.02$) of the dorsal hippocampus, the CeA ($t_{14}=2.63$; $p=0.02$) of the amygdaloidal

complex, the medial lateral mammillary nucleus ($t_{14}= 2.25$; $p<0.05$) and the molecular ($t_{14}= 3.83$; $p<0.01$) and granular ($t_{14}= 3.97$; $p=0.001$) layers of the lobule II of the cerebellar cortex in PLAE mice when compared to the control group. In contrast, significantly lower CCO activity was detected for the PLAE group in the lateral habenula ($t_{14}= 2.68$; $p=0.018$) and ventral tegmental area ($t_{14}= 2.18$; $p<0.05$). Table 1 shows the mean CCO activity values measured in the 36 regions of interest of both the experimental groups.

Interregional within-group correlations

The analysis of pairwise cross-correlations in CCO activity showed a widely distributed network of significant correlations including cerebral and cerebellar regions in the control group (Fig. 7A). However, the PLAE group (Fig. 7B) showed three more closed and discrete networks within the brain regions analysed (amygdala complex, cerebellum and ventral hippocampus with brainstem regions).

1.6. Effects of early alcohol exposure on neurogenesis

As illustrated in Figure 8, neurogenesis in the DG of the hippocampus was assessed using BrdU assay. Student's t-test showed a significant increase in BrdU-labeled cells co-localizing with NeuN-labelled cells following 3-day sacrifice (neuron proliferation study) in PLAE mice ($t_6=3.31$; $p<0.05$) (Fig. 8A) and 30-day sacrifice (evaluation of new-born neuron survival) ($t_7=6.422$; $p<0.001$) (Fig. 8B).

2. Discussion

The results of the present study demonstrate that binge-like alcohol exposure during foetal and early postnatal development induces persistent neurobiological effects involved in anxiety-related behaviour, locomotor behaviour and alcohol-rewarding properties. Furthermore, we found that alterations in functional brain circuitry may indeed underlie such neurobehavioural consequences.

We reported different alterations in spontaneous locomotor activity depending on age, as adolescent mice were hyperactive in response to early alcohol exposure, while adult mice showed diminished locomotor activity. It is noteworthy that such differences were only significant when data were analysed by collapsing the whole 45-min period. As indicated by some studies, it is during adolescence that hyperactivity is more often diagnosed in FASD-affected humans and the alteration may become less pronounced

during adulthood (Kleiber et al., 2011). In accordance with our findings, previous studies have reported locomotor activity to be increased by alcohol dosage and reduced with age in prenatal alcohol exposed rats (Kim et al., 2013; Muñoz-Villegas et al., 2017). Similarly, hyperactivity in juvenile but hypoactivity in adult rats as a consequence of early exposure to alcohol has been previously reported (Marche et al., 2011). The differences obtained between pre- and post-pubertal stages are probably due to the major brain maturation processes that occur during adolescence (Fuhrmann et al., 2015; Sisk and Foster, 2004). Also, diminished locomotor activity was appreciated when assessed at adulthood in other rat studies (Dursun et al., 2006; Ohta et al., 2010). Hypoactivity at adulthood may be a result of damage to the developing frontal cortices, as we previously reported (Cantacorps et al., 2017), affecting the frontostriatal circuitry, like others suggested (Alberry and Singh, 2016; Schambra et al., 2016). However, other studies using a lowest dose of alcohol and shorter periods of exposure reported no differences in basal locomotor activity after developmental alcohol exposure in mice (Alberry and Singh, 2016; Brady et al., 2012), suggesting that a higher dose of alcohol and longer periods of exposure are required to induce changes in spontaneous locomotor activity.

Also, an increase in anxiety-related behaviour was found in the offspring of mothers exposed to binge alcohol during gestation and lactation. This anxiogenic effect assessed in the EPM was observed at adolescence and maintained throughout adulthood. Consistently, recent studies have reported an increase in anxiety-related behaviour following foetal alcohol exposure in mice (Abbott et al., 2016; El Shawa et al., 2013; Kleiber et al., 2011; Schambra et al., 2016) and rats (Baculis et al., 2015; Cullen et al., 2013; Dursun et al., 2006). However, other studies showed no effect on anxiety-like responses in mice (Alberry and Singh, 2016; Boehm et al., 2008; Sanchez Vega et al., 2013) and rats (Barbier et al., 2008; Muñoz-Villegas et al., 2017) or even an anxiolytic effect in rats (Ohta et al., 2010). Such differences may be due to the pattern of consumption used (binge versus moderate or continuous), the period of exposure, the route of alcohol administration (intragastric intubation versus voluntary oral consumption), the species and the strain used.

Few studies have actually addressed the effects of prenatal alcohol exposure on the rewarding properties of alcohol using the CPP paradigm. In our study, an attenuation of the conditioned response to the drug-associated compartment was found in alcohol-

exposed offspring mice. This attenuation was more pronounced in the highest dose of alcohol administered (2 g/kg), although both doses used in the present study are effective doses for control mice. Such results lead us to hypothesize that alcohol-exposed mice are less sensitive to the rewarding effects of alcohol and may need higher doses of alcohol to obtain the same reinforcement as control mice. Barbier et al. (2008) reported no differences in the conditioned response to 1g/kg alcohol in the CPP paradigm in 2-month old prenatally alcohol-exposed rats, although the study did use a different procedure of pre- and postnatal alcohol exposure in a different species. Moreover, unlike in our study, the control group did not show any conditioned preference for the compartment paired with 2g/kg alcohol.

Cumulative changes in the baseline metabolic capacity of different brain regions were reported in the present study. Increased CCO activity was observed in the DG of the hippocampus, the CeA, the medial lateral nucleus of the mammillary bodies and the granular and molecular layers of the central lobules of the cerebellum, while a reduction in CCO activity was found in the VTA and the LH of PLAE mice consistent with the observed attenuation of the rewarding effects induced by alcohol in these mice. In fact, some of these areas have been associated with emotional and reward processing. For instance, the CeA processes emotionally relevant sensory information and its dysfunction has been linked with both anxiety and substance abuse disorders, and, specifically, with alcohol use disorders (Gilpin et al., 2015). In agreement with our findings, prenatal alcohol exposure has been reported to attenuate GABAergic inhibition in the basolateral amygdala (BLA), which projects to the CeA and other brain regions, leading to neuronal hyperexcitability and anxiety-like behaviour in rats (Zhou et al., 2010). This result agrees well with the anxiety-like responses observed in PLAE mice at the two different ages evaluated (PD30 and PD60). Moreover, the CeA is a critical region for alcohol dependence, since ethanol would also seem to regulate glutamate neurotransmission in this region by increasing neuronal excitability involved in anxiety-like behaviour (Silberman and Winder, 2015).

In addition, the mammillary bodies have been related to anxiety-like behaviour as a brain region critical for the effects of anxiolytic drugs (González-Pardo et al., 2006; Kataoka et al., 1982). In particular, increased CCO activity in the medial lateral nucleus of the mammillary bodies has been reported following chronic alcohol exposure in rats (Rubio et al., 1996). In agreement with our results, increased brain glucose utilization in

the mammillary bodies, together with the central amygdala and the hippocampus, has also been reported in rats after a history of alcohol consumption (Williams-Hemby et al., 1996). Therefore, increased CCO in these limbic brain regions may be linked to increased emotional-and anxiety-like behaviour in PLAE mice.

Although the LH is associated with negative emotions, receiving inputs from the limbic system and basal ganglia, it also controls dopamine levels in the striatum and plays a role in reward processes. Dysfunction of the habenula has been implicated in depression and the effects of drugs of abuse (Velasquez et al., 2014). In addition, the LH is particularly involved in alcohol use and withdrawal according to recent studies (Shah et al., 2017). In particular, inhibition of the LH by high-frequency electrical stimulation has been reported to reduce alcohol intake in rats (Li et al., 2016). Our results show decreased CCO activity in the LH of PLAE mice, which may be related to the attenuated conditioned response to alcohol in the CPP paradigm found in PLAE mice.

The VTA comprises part of the mesocorticolimbic system, which plays a central role in reward processing, with dopaminergic projections to limbic structures, such as the nucleus accumbens, the amygdala and the prefrontal cortex. The VTA is one of the targets of alcohol, as it increases the firing of dopaminergic neurons projecting to the NAcc. Therefore, the activation of dopamine neurons in the VTA is involved in alcohol-reward processing (Engel and Jerlhag, 2014). In the present study, lower neuronal activity in the VTA was found in PLAE mice. We hypothesize that such an effect may account for the reduced sensitivity to the rewarding effects of alcohol observed in PLAE mice during adulthood. Indeed, it has been previously reported that prenatal alcohol exposure induces alterations in the mesocorticolimbic dopaminergic system in rodents, including the reduced electrical activity of dopaminergic neurons located in the substantia nigra and the VTA (Choong and Shen, 2004; Shen and Choong, 2006), thus contributing to a decreased dopaminergic function. Conversely, Hausknecht et al. (2015) reported that prenatal alcohol exposure persistently increased excitatory synaptic strength in VTA dopaminergic neurons by enhancing the calcium-permeable AMPA receptor function. Fortunately, this finding does not clash with previous studies, as the decreased activity in the VTA after prenatal alcohol exposure is thought to be due to a decrease in the number of spontaneously active VTA dopaminergic neurons (Hausknecht et al., 2015). Thus, the decrease in VTA dopaminergic neuron activity is caused by over-excitation blocking depolarization in a proportion of VTA dopaminergic

neurons (Shen and Choong, 2006). It is worth noting that such a mechanism is also observable following repeated exposure to drugs of abuse following prolonged withdrawal, as a transient increase first occurs in neuronal activity, which is linked to enhanced addiction risk (Shen and Choong, 2006). Also, it has previously been reported that, in an alcohol-preferring line of rats, 2 week-long alcohol deprivation decreased brain glucose utilization in the VTA, as compared to alcohol-preferring rats with scheduled access to alcohol (Smith et al., 2001). Accordingly, our CCO results support the hypothesis to the effect that the mesocorticolimbic system involved in reward processing is functionally impaired in PLAE mice, a result that would underlie alcohol relapse and drug craving following prenatal and early postnatal alcohol exposure.

The cerebellum is involved in motor learning and coordination, and its dysfunction may produce alterations in movement coordination. Thus, we hypothesize that increased cerebellar activity may account for the impairment in the rotarod performance previously reported in our model (Cantacors et al., 2017). However, recent insights into the role of the cerebellum in emotive and cognitive processing (Adamaszek et al., 2017; Shakiba, 2014; Strata, 2015) lead us to postulate the involvement of cerebellar dysfunction in emotional disturbances observed in alcohol-exposed mice.

The DG of the dorsal hippocampus mediates the encoding of multiple sensory inputs and memory-based spatial pattern separation (Kesner, 2017). Therefore, disruptions in the activity of the DG may account for memory impairments. However, we hypothesise that the increase observed in CCO hippocampal activity may be due to an increase in neurogenesis, as the subgranular zone of the DG is one of the main brain regions in which new neurons are continually generated (Chambers et al., 2015). An increase in BrdU+ cells was found in PLAE mice, suggesting an increase in neurogenic responses at adulthood. Previous studies, however, showed no effect of prenatal and/or early postnatal alcohol exposure on cell proliferation and survival in the DG (Choi et al., 2005; Hamilton et al., 2016; Helfer et al., 2009; Uban et al., 2010; Wozniak et al., 2004) or even an increase in immature neuron differentiation in young adult animals following prenatal and early postnatal binge-like alcohol exposure (Boehme et al., 2011; Gil-Mohapel et al., 2011). However, reduced hippocampal neurogenesis later in adulthood has been also reported (Ieraci and Herrera, 2007; Klintsova et al., 2007; Redila et al., 2006), suggesting that the capacity for neurogenesis is highly conserved in early life and that deficits would tend to appear later in life (Gil-Mohapel et al., 2014). In contrast,

other studies reported increased hippocampal neurogenesis in mice after acute or chronic alcohol consumption associated with enhanced brain-derived neurotrophic factor (BDNF) expression in this region together with impaired learning and memory (Stragier et al., 2015). In this regard, our results may be interpreted in terms of an epigenetic response to alcohol exposure due to the neuroadaptive mechanisms caused by early alcohol exposure (Palmisano and Pandey, 2017).

The analysis of interregional within-group correlations in CCO activity showed alterations in functional brain networks activated in PLAE mice when compared to control mice. Three discrete and parallel closed reciprocal functional brain networks were detected involving the amygdala complex, the cerebellum and the ventral hippocampus together with brainstem regions. Conversely, a more complex single brain network was detected in the control group, with numerous cross-correlations between anatomically-related brain regions comprising the prelimbic cortex, the dorsal and ventral hippocampal regions, the amygdala complex, the mammillary body, brainstem regions (SNR, VTA) and cerebellar regions. In addition, the only negative cross-correlation was found only between the prelimbic region (PL) and the lateral septum (LS) in PLAE mice. Activation of the LS has been directly related with dopamine-related motivational and reinforcing effects of ethanol exposure (Jonsson et al., 2017). Since the PL indirectly projects to the LS via the IL (Vertes, 2004), the negative correlation found in CCO activity could be related with abnormal modulation of the LS by the PL and/or other anatomically-related brain regions involved in motivation or anxiety-related behaviour. Such results indicate that PLAE mice clearly display disrupted functional brain network connectivity as compared with control mice. Altered fronto-cerebellar and cortico-limbic connectivity has been described in neuroimaging studies in individuals with alcohol use disorders (Herting et al., 2011; Rogers et al., 2012) and in rats exposed intermittently to alcohol during adolescence (Broadwater et al., 2017). Accordingly, disrupted functional brain connectivity has been reported in humans (Donald et al., 2015) and rats (Rodriguez et al., 2016) with prenatal alcohol exposure using magnetic resonance imaging techniques. Other studies also have reported alterations in intraneocortical circuitry (El Shawa et al., 2013) and gene networks involved in developmental processes (Kleiber et al., 2012) in the offspring of mice exposed to alcohol during gestation. Altered brain functional connectivity in PLAE mice would therefore be related to possible cognitive, emotional, motivational

and motor deficits, as demonstrated in this and in previous studies (Cantacorps et al., 2017) and in studies for human foetal alcohol spectrum disorders (Moore et al., 2014).

In conclusion, we observed that early alcohol exposure impaired motor and emotional-related behaviours and attenuated alcohol rewarding effects. Furthermore, for the very first time, we have reported long-lasting effects on neural oxidative metabolism in some brain areas, in addition to alterations in functional brain networks due to maternal binge-like alcohol drinking.

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Ethical Statement

Mice were allowed to acclimatize to the new environmental conditions for at least one week before starting the experiments. Every effort was made to minimize animal suffering and reduce the number of animals used. All procedures were conducted in accordance with national (BOE-2013-1337) and EU (Directive 2010-63EU) guidelines regulating animal research, and were approved by the local ethics committee (CEEA-PRBB).

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Figure legends

Figure 1. Maternal DID test. A) Volume of water and alcohol consumed during DID procedure throughout prenatal and lactation periods. Alcohol was available 2h/day on days 1 to 3 and 4h on day 4 of the series. Two-way ANOVA and Bonferroni post-hoc test. *** $p < 0.001$ Water vs. Alcohol. B) Alcohol intake (g EtOH/kg) during DID test. RM one-way ANOVA and Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$ compared with day 8. C) Maternal body weight measured at 2-day intervals of DID test. Two-way ANOVA and Bonferroni post-hoc test (n.s.). Data are expressed as [mean $\pm$ SEM] (n=10-11 per group).

Figure 2. Effects of prenatal and postnatal alcohol exposure on spontaneous locomotor activity on PD30. Horizontal activity (deambulations) (A and B) and vertical activity (rearings) (C and D) were measured in male adolescent offspring (PD30). Data are expressed as [mean $\pm$ SEM] (n=9-11 per group). Student's t-test * $p < 0.05$, ** $p < 0.01$ Control vs. PLAE.

Figure 3. Effects of prenatal and postnatal alcohol exposure on spontaneous locomotor activity on PD60. Horizontal activity (deambulations) (A and B) and vertical activity (rearings) (C and D) were measured in male adult offspring (PD60). Data are expressed as [mean $\pm$ SEM] (n=11-12 per group). Student's t-test * $p < 0.05$, ** $p < 0.01$ Control vs. PLAE.

Figure 4. Effects of prenatal and postnatal alcohol exposure in the elevated plus maze. The percentage of time spent in open arms (A and D), percentage of entries into open arms (B and E) and the number of total entries (C and F) were assessed in male offspring on PD30 and 60. Data are expressed as [mean $\pm$ SEM] (n=10-13 per group). Student's t-test * $p < 0.05$, ** $p < 0.01$ Control vs. PLAE.

Figure 5. Effects of maternal binge-like alcohol consumption on alcohol-induced rewarding effects in the CPP paradigm. CPP score results are expressed as [mean $\pm$ SEM] (n=8-12 per group). Bonferroni post-hoc test ** $p < 0.01$, *** $p < 0.001$ within-group comparisons; ### $p < 0.001$ between-group comparisons.

Figure 6. Representative microphotographs of cytochrome c oxidase (CO) stained coronal sections of the dorsal hippocampus showing decreased CO staining

intensity in a control mouse brain (A) as compared with a PLAE brain (B). The effects reported in this paper are based on reproducible, statistically reliable differences determined by direct densitometric analysis of the sections themselves. DG, dentate gyrus. Scale bar: 500 μ m.

Figure 7. Pair-wise interregional activity correlations by group (PD60). Schematic diagram showing significant correlations in CCO activity in control (A) and PLAE (B) groups. Solid arrows indicate significant positive correlations in metabolic activity between two regions; dashed arrows indicate significant negative correlations ($p \leq 0.001$). The pattern of functional CCO cross-correlations found in the PLAE group involves a widespread distributed network between the measured cerebral and cerebellar regions. Abbreviations are the same as in Table 1.

Figure 8. Effects of prenatal and postnatal alcohol exposure on neuronal progenitor proliferation and survival at PD60. Data are presented as [mean of BrdU+ cells $\pm$ SEM] ($n=4-5$ per group) in proliferation (A) and survival (B) studies. Student's t-test * $p < 0.05$, *** $p < 0.001$ Control vs. PLAE.

Table 1. Regional brain cytochrome c oxidase activity. Mean $\pm$ SEM values are represented for each experimental group. *($p < 0.05$) and ⁺($p \leq 0.001$) Control vs. PLAE.

	n	CONTROL	n	PLAE
CORTEX				
Prelimbic region (PL)	8	27.03 $\pm$ 0.78	8	25.41 $\pm$ 0.89
Infralimbic region (IL)	8	25.30 $\pm$ 0.87	8	24.75 $\pm$ 1.03
Cingulate region (CG)	8	26.41 $\pm$ 0.82	8	26.57 $\pm$ 1.43
Motor (M1)	8	28.60 $\pm$ 0.99	8	29.54 $\pm$ 1.44
HIPPOCAMPUS				
Dorsal CA1 area (CA1d)	8	25.37 $\pm$ 0.92	8	26.75 $\pm$ 0.70
Dorsal CA3 area (CA3d)	8	25.57 $\pm$ 0.85	8	25.86 $\pm$ 0.80
Dorsal DG (DGd)	8	24.25 $\pm$ 0.95	8	31.32 $\pm$ 0.68*
Ventral CA1 area (CA1v)	8	28.99 $\pm$ 0.76	8	29.93 $\pm$ 1.20
Ventral CA3 area (CA3v)	8	30.34 $\pm$ 0.84	8	31.42 $\pm$ 1.01
Ventral GD (DGv)	8	26.52 $\pm$ 0.75	8	25.74 $\pm$ 1.47
DIENCEPHALON				
Anterodorsal Thalamic Nucleus (AD)	8	40.01 $\pm$ 1.14	8	46.41 $\pm$ 1.26
Anteroventral Thalamic Nucleus (AV)	8	35.32 $\pm$ 1.36	8	38.14 $\pm$ 0.73
Medial Medial Mammillary Nuclei (MM)	8	29.26 $\pm$ 1.19	8	29.24 $\pm$ 1.07
Medial Lateral Mammillary Nuclei (LMM)	8	28.87 $\pm$ 0.67	8	31.49 $\pm$ 0.95*
Lateral Mammillary Nuclei (LM)	8	35.44 $\pm$ 0.97	8	36.24 $\pm$ 1.37
Medial Habenula (MHb)	8	25.83 $\pm$ 0.86	8	26.22 $\pm$ 0.89
Lateral Habenula (LHb)	8	41.75 $\pm$ 0.59	8	37.63 $\pm$ 1.41*
AMYGDALA COMPLEX				
Basolateral Nucleus (BaA)	8	28.59 $\pm$ 0.85	8	28.90 $\pm$ 0.90
Medial Nucleus (MeA)	8	21.42 $\pm$ 0.67	8	21.04 $\pm$ 1.05
Central Nucleus (CeA)	8	26.32 $\pm$ 0.63	8	28.87 $\pm$ 0.73*
Lateral Nucleus (LaA)	8	25.15 $\pm$ 1.42	8	23.61 $\pm$ 0.97
STRIATUM AND BASAL FOREBRAIN				
Dorsal Striatum (STR)	8	25.57 $\pm$ 1.40	8	24.53 $\pm$ 1.35
Nucleus Accumbens (ACB)	8	28.27 $\pm$ 1.26	8	30.31 $\pm$ 0.57
Lateral Septum (LS)	8	27.46 $\pm$ 0.72	8	29.11 $\pm$ 1.71
Ventral Pallidum (PALv)	8	28.16 $\pm$ 0.88	8	27.01 $\pm$ 0.90
Bed Nucleus of Stria Terminalis (BST)	8	24.54 $\pm$ 1.21	8	25.64 $\pm$ 0.79
MIDBRAIN				
Substantia nigra, pars reticularis (SNr)	8	23.76 $\pm$ 1.13	8	23.31 $\pm$ 0.83
Substantia nigra, pars compacta (SNc)	8	24.18 $\pm$ 0.91	8	24.36 $\pm$ 0.76
Ventral Tegmental Area (VTA)	8	23.50 $\pm$ 0.66	8	21.12 $\pm$ 0.86*
CEREBELLUM				
Lobule II, granular layer (L2gr)	8	35.14 $\pm$ 1.29	8	39.33 $\pm$ 0.68 ⁺
Lobule II, molecular layer (L2ml)	8	34.08 $\pm$ 0.61	8	37.74 $\pm$ 0.93 ⁺
Lobule III, granular layer (L3gr)	8	33.08 $\pm$ 0.94	8	34.98 $\pm$ 0.95
Lobule III, molecular layer (L3ml)	8	33.79 $\pm$ 0.66	8	35.27 $\pm$ 0.90
Lobules IV/V, granular layer (L4/5gr)	8	31.45 $\pm$ 0.73	8	32.90 $\pm$ 0.44
Lobules IV/V, molecular layer (L4/5ml)	8	32.08 $\pm$ 0.93	8	32.63 $\pm$ 0.95
Lobule IX, granular layer (L9gr)	8	33.56 $\pm$ 0.64	8	34.55 $\pm$ 2.18

Fig. 1 Maternal DID test

Fig. 2 Spontaneous locomotor activity (PD30)

Fig. 3 Spontaneous locomotor activity (PD60)

Fig. 4 Elevated plus maze

Fig. 5 Alcohol-induced CPP (PD60)

Fig. 6

Control group (B)

PLAE group (B)

Fig. 7

Fig. 8

Highlights

- Maternal alcohol binge drinking disrupts neural functional connectivity in offspring
- Prenatal and lactational alcohol exposure alters adult brain oxidative metabolism
- Maternal alcohol exposure causes long-term changes in anxiety and alcohol preference