Maternal separation increases alcohol-seeking behaviour and reduces endocannabinoid and monoamine levels in the mouse striatum.

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Abstract

Childhood adversity is associated with an increased risk of mood, anxiety and substance use disorders. Maternal separation is a reliable rodent model of early life adversity that leads to depression-like symptoms, which may increase the vulnerability to alcohol consumption during adolescence. However, the specific alterations in the pattern of alcohol consumption induced by maternal separation, and the underlying molecular mechanisms are still unclear. The purpose of this study was to evaluate the long-term effects of maternal separation with early weaning (MSEW) on emotional and social behaviour, alcohol reward properties, and alcohol consumption, abstinence and relapse in adolescent male C57BL/6 mice. In addition, endocannabinoid and monoamine levels were analysed in discrete brain areas after maternal separation. Results showed that MSEW mice presented emotional alterations related to depressive-like behaviour and modified endocannabinoid levels in the striatum and the prefrontal cortex. MSEW mice also showed impairments in alcohol-induced conditioned place preference and higher alcohol intake in a model of binge drinking. Moreover, MSEW animals displayed a higher propensity to relapse in the two-bottle choice paradigm following a period of alcohol abstinence associated with reduced monoamine levels in the striatum. Such results indicate that exposure to early life stress increased the vulnerability to alcohol binge-drinking during adolescence, which may be partially explained by decreased sensitivity to alcohol rewarding properties and the ability to potentiate alcohol drug-seeking behaviour following a period of abstinence.

Keywords: Alcohol, maternal separation, adolescence, endocannabinoids, monoamines, striatum.

Running title: Maternal separation and alcohol seeking behaviour.

Abbreviations:

- **2-AG:** 2-arachidonoylglycerol
- **2-LG:** 2-linoleoylglycerol
- **2-OG:** 2-oleoylglycerol
- **5-HIAA:** 5-hydroxyindoleacetic acid
- **5-HT:** Serotonin
- **AEA:** N-arachidonoylethanolamine
- **AEA-d4:** N-arachidonoyl ethanolamide-d4
- **AUD:** Alcohol Use Disorders
- **BAC:** Blood Alcohol Concentration
- **CPP:** Conditioned Place Preference
- **DA:** Dopamine
- **DEA:** N-docosatetraenoylethanolamine
- **DHEA:** N-docosahexaenoylethanolamine
- **DHEA-d4:** N-docosahexaenoylethanolamine-d4
- **DID:** Drinking in the Dark
- **DOC:** Deoxycorticosterone
- **EC:** Endocannabinoid
- **EPM:** Elevated Plus Maze
- **EtOH:** Alcohol
- **FID:** Flame Ionization Detector
- **HPA:** Hypothalamic-pituitary-adrenal
- **HPLC:** High Performance Liquid Chromatography
- **ISTD:** Internal Standard Solution
- **LC-MS/MS:** Liquid Chromatography–Tandem Mass Spectrometry
- **LEA:** N-linoleoylethanolamine
- **LEA-d4:** N-linoleoylethanolamine-d4

- **MSEW:** Maternal Separation with Early Weaning
- **NAE:** N-acylethanolamide
- **OEA:** N-oleoyl ethanolamide
- **OEA-d4:** N-oleoylethanolamine-d4
- **PEA:** N-palmitoylethanolamine
- **POEA:** N-palmitoleoylethanolamine
- **PD:** Postnatal Day
- **PFC:** Prefrontal Cortex
- **SEA:** N-stearoylethanolamine
- **SN:** Standard Nesting
- **SRM:** Selective Reaction Monitoring
- **Trp:** Tryptophan
- **TST:** Tail Suspension Test
- **Tyr:** Tyrosine

Highlights:

- MSEW increases despair-like behaviour and diminishes social novel interaction.
- Early-life stress reduces sensitivity to alcohol-rewarding effects.
- MSEW reduces endocannabinoid levels in the striatum and prefrontal cortex.
- MSEW increases alcohol-seeking behaviour.
- MSEW reduces corticosteroid and monoamine levels similarly to alcohol abstinence.

1. Introduction

Childhood adversity is associated with an increased risk of anxiety, depression and substance use disorders (Norman et al., 2012; Teicher and Samson, 2016). Specifically, early postnatal life is considered to be a period of high vulnerability in which exposure to extreme and prolonged stressors may lead to long-lasting deleterious effects on brain neurodevelopment and function, as is suggested by both clinical (Lupien et al., 2009; Teicher et al., 2003; Teicher and Samson, 2013) and preclinical (Kaufman et al., 2000; Ladd et al., 2000; Sarro et al., 2014) studies. These previous findings suggest that early life events exert a sustained influence over neural systems mediating stress reactivity.

Maternal separation is a validated rodent model of early life stress frequently used to replicate early adversities, entailing early separation of pups from dams for long periods during the first two weeks after birth (Tractenberg et al., 2016). Previous studies have shown that maternal separation affects the formation of neuronal networks and exerts long-lasting effects on neural function (Nishi et al., 2014). Moreover, maternal separation leads to high levels of anxiety-like behaviour and high stress hormone responsiveness (Caldji et al., 2000; Plotsky and Meaney, 1993), depression-like behaviour assessed as anhedonia, despair behaviour and a decrease in behavioural responses to novelty (George et al., 2010; Gracia-Rubio et al., 2016a; Lukkes et al., 2017; Matthews et al., 1996; Matthews and Robbins, 2003; Rüedi-Bettschen et al., 2004). In addition, maternal separation produces neurochemical alterations such as an imbalance in the regulation of hypothalamic-pituitary-adrenal (HPA) axis responses (Nishi et al., 2014), decreased neurogenesis (Lajud et al., 2012) or BDNF levels in the hippocampus (Martini and Valverde, 2012). Reduced 5-HT1A receptor levels are also found in maternally separated mice, indicating a monoaminergic dysfunction in these animals (Bravo et al., 2014; Gracia-Rubio et al., 2016b; Leventopoulos et al., 2009), in addition to higher pro-inflammatory cytokines levels in serum (Réus et al., 2015) associated with increased neuroinflammatory responses (Gracia-Rubio et al., 2016a; Roque et al., 2016).

Adolescence is a critical period for brain development and maturation, and is a sensitive period to develop psychiatric illness, including anxiety, mood and substance abuse disorders (Paus et al., 2008). The earlier drug use is initiated, the more likely it is for addiction to progress (Degenhardt et al., 2008) and adolescents exhibit increased risk taking and novelty seeking behaviour. On a worldwide basis, the most commonly abused drug is alcohol (Johnston et al., 2009). Alcohol intoxication during adolescence has been reported to induce brain damage (Lisdahl et al., 2013; Pascual et al., 2014),

and is considered to be one of the main risk factors for emerging neuropsychiatric disorders (Johansson et al., 2015; Ros-Simó et al., 2013; Skogen et al., 2014), including alcohol use disorder (AUD) during adulthood (Dawson et al., 2008; Kyzar et al., 2016).

Additionally, the individual risk of developing AUD is affected by early life stress (Sinha, 2008), suggesting the involvement of common neurobiological substrates of susceptibility to addictive and affective disorders (Polter and Kauer, 2014). The dysregulation of the brain reward function induced by adverse early-life experiences may be related to the development of drug addiction (Cheetham et al., 2010), presumably through the modulation of the dopaminergic (Nestler and Carlezon, 2006) and endocannabinoid (EC) systems (Agrawal et al., 2012; Parsons and Hurd, 2015), which are involved in reward, mood and stress processing.

Accordingly, previous reports have shown that maternally separated animals present a lower density of dopamine transporter sites in the striatum, greatly reduced D2 (Gracia-Rubio et al., 2016b) and D3 dopamine receptor binding (Brake et al., 2004), and a dysregulation in the transcriptional factors Nur1 and Pitx3 related to dopaminergic activity in the ventral tegmental area, indicating a dysfunction of the dopaminergic system (Gracia-Rubio et al., 2016b). Moreover, regarding the EC system, a decreased CB1 receptor expression has been described in the hippocampus and prefrontal cortex (PFC) induced by maternal separation, while, in the striatum, an increase has been reported (López-Gallardo et al., 2012; Romano-López et al., 2012; Suárez et al., 2009), which may contribute to a proclivity to alcohol ingestion (Parsons and Hurd, 2015). Several studies have concluded that animals exposed to maternal separation exhibited high patterns of alcohol consumption (Daoura et al., 2011; García-Gutiérrez et al., 2016; Gondré-Lewis et al., 2016; Gustafsson and Nylander, 2006; Ploj et al., 2003; Roman and Nylander, 2005) during adulthood. Nevertheless, only a few studies have evaluated the effects of maternal separation on alcohol consumption and underlying neurobiological mechanisms during adolescence (García-Gutiérrez et al., 2016) and previous literature has not investigated the effects of MSEW during alcohol abstinence and relapse.

It is, therefore, of interest to understand the underlying neurobiological mechanisms by which early life stress may contribute to the vulnerability to AUD during adolescence. Thus, the purpose of our study is to evaluate the effects of maternal separation on despair-like and social behaviour, alcohol rewarding properties, voluntary alcohol intake and relapse following abstinence in adolescent male C57BL/6 mice. Monoaminergic precursors and metabolites, such as tyrosine (Tyr), tryptophan (Trp)

and 5-hydroxyindoleacetic acid (5-HIAA), and EC compounds, such as 2-arachidonoylglycerol (2-AG) and anandamide (AEA), were assessed in the striatum and PFC of maternally separated mice as related key targets in the abuse liability of alcohol.

2. Methods

2.1 Animals

Outbred C57BL/6 mice male and female adult mice purchased from Charles River (France) were used as breeders. On arrival, breeding pairs were formed and housed in 45×25×15 cm plastic boxes. After approximately 2 weeks, the male was removed, and pregnant females were housed individually. A final group of 108 offspring male mice were used to conduct the different experiments. Animals were housed in an acclimatized room (temperature 21±1°C, humidity 55±10%, lights on from 08:00 to 20:00 h), except during the alcohol consumption procedure, when the room was lit from 20:00 to 8:00 h. Water and pellet food were available *ad libitum*, except during the drinking in the dark (DID) test, as explained in section 2.7. 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).

2.2 Drugs

Alcohol (Ethyl alcohol, ethanol, 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 5% (v/v), 10% (v/v), 15% (v/v) and 20% (v/v) for the two-bottle choice paradigm. For the conditioned place preference (CPP) procedure, alcohol (1 and 2 mg/kg) was dissolved in sterile physiological saline (0.9% NaCl) and injected intraperitoneally (i.p) in a volume of 0.1 ml/10g.

2.3 Behavioural procedures

2.3.1 Rearing conditions

Pregnant female mice were checked daily prior to parturition. For each litter, the date of birth was designated as postnatal day 0 (PD0). Mice were randomly assigned to one of two different experimental groups: Standard nest (SN) and Maternal separation with early weaning (MSEW). In the MSEW group, offspring were separated from their mothers for 4 h per day on PD2-5 (9:30–13:30) and 8 h per day on PD6-16 (9:30–17:30 h). For separation, mothers were moved to another cage, while the offspring remained in their home cages with a heating blanket (32–34 °C) for thermoregulation. Once the mothers had been removed, the offspring were taken to another room to prevent the

progenitors from being stressed by their vocalizations (George et al., 2010; Gracia-Rubio et al., 2016a, 2016b). Pups were weaned at PD17, and to facilitate their access to food and avoid possible dehydration, wet regular chow and hydrogel (Bio-Services, Uden, The Netherlands) were provided in their home cages until PD21. In the SN group, the offspring remained with their mothers for 21 days and were then weaned (PD21). After weaning, the male offspring were housed in groups of three or four. Different cohorts of male mice were used for each experimental procedure commencing on PD28 (Fig. 1).

2.3.2 Tail suspension test (TST)

Mice were individually suspended by the tail from a horizontal ring-star bar (50 cm above the floor) using adhesive tape (2 cm from the tip of tail) for a 6-min period as described by Steru et al. (1985). The time of immobility during this period was duly recorded, with higher immobility scores corresponding to higher levels of despair-like behaviour.

2.3.3 Three-chamber social test

The social approach apparatus was an open-topped acrylic box (63 cm L × 42 cm W × 23 cm H) divided into three chambers with two walls. Dividing walls had retractable doorways allowing access into each chamber. The wire cup used to enclose the novel mice was made of cylindrical chrome bars at 1 cm intervals (10 cm H; bottom diameter: 10 cm). The test was conducted as reported (Segura-Puimedon et al., 2014) with some minor modifications. Test mice were confined in the centre chamber at the beginning of each phase. To initiate each 10-minute phase, the doorways to the side chambers were opened and the mice were allowed to explore freely. During the habituation phase, each of the two side chambers contained an empty inverted wire cup. During the sociability phase, an unfamiliar mouse (novel mouse 1) was enclosed in one of the wire cups in a side chamber. The location of novel mouse 1 alternated between the two side chambers across test mice. During the social novelty phase, a new unfamiliar mouse (novel mouse 2), from a different cage, was enclosed in the wire cup which had remained empty during the sociability phase. The exploration of an enclosed mouse or wire cup was defined as when a test mouse climbed onto a cup or when the distance between nose and cup was less than 1 cm. The time spent in each chamber and the time spent exploring enclosed novel mice or empty cups (novel objects) throughout

the different phases were recorded with an overhead camera and analysed by an automated tracking program (Smart, Panlab).

2.3.4 Elevated plus maze (EPM)

The EPM was a black plastic structure with four arms (16×5 cm) forming a cross from a neutral central square (5×5 cm). Two arms were protected by vertical walls (closed arms), while the other two perpendicular arms had unprotected edges (open arms). The maze was raised 30 cm above the floor and illuminated from above (100 lx). The experiments were conducted as previously reported (Simonin et al., 1998). At the beginning of the 5-min observation session, each mouse was placed in the central neutral area, facing one of the open arms. The total number of visits to the open arms and the cumulative time spent in the open arms were then observed on a monitor through a video camera system (ViewPoint, Lyon, France) and analysed by an automated tracking program (Smart, Panlab). The time spent in the open arms was recorded when the mouse moved its head and two forepaws into the arm. The percentage of time spent in the open arms of the EPM was considered as a measure of anxiety-like behaviour.

2.3.5 Alcohol-induced conditioned place preference (CPP)

The procedure, unbiased in terms of initial spontaneous preference, was divided into three phases: preconditioning phase, conditioning phase and test phase, adapted from Roger-Sánchez et al. (2012). The apparatus consisted of two main conditioning compartments (30×29×35 cm) connected to a smaller, central compartment (14×29×35 cm) (Cibertec S.A., Madrid, Spain). The conditioning compartments were endowed with different visual and tactile cues. One compartment had white walls with prismatic textured flooring while the other had black walls with flat, smooth flooring. The central compartment consisted of grey-coloured walls with neutral flooring. All the compartments were equipped with infrared emitter/detector pairs, 2.2 cm above the floor and 1 cm apart, covering the whole perimeter of the box. During the preconditioning phase (day 1), mice were placed in the central compartment and were allowed to access both compartments of the structure for 20 min. Animals showing strong unconditioned aversion (< 33% of the session time) or preference (> 67%) for any particular compartment were discarded from the study. In the conditioning phase (days 2-9), half of the animals from each group received the drug in one compartment

and the remaining half in the other through four pairings. Thus, mice received an i.p injection of 1 or 2 g/kg alcohol immediately prior to confinement in the drug-paired compartment for 5 min on days 2, 4, 6 and 8. On alternate days (3, 5, 7 and 9), the mice received physiological saline prior to confinement in the vehicle-paired compartment for 5 min. Control animals received saline prior to confinement in one of the two compartments. The central area was blocked with guillotine doors and was always inaccessible during conditioning. Alcohol dosage and trial duration were selected on the basis of previous experiments in our laboratory. The test (day 10) was conducted under the same conditions applied in the preconditioning phase. The time spent in each compartment during the preconditioning and test phases was duly recorded. The CPP score was calculated for each subject as the difference in seconds between the time spent in the drug-paired compartment during the test and time spent in the preconditioning phase, as a measure of the degree of conditioning induced by the drug.

2.3.6 Drinking in the dark (DID) test

This procedure was conducted as previously reported (Esteve-Arenys et al., 2017; Rhodes et al., 2005; Ros-Simó et al., 2012; 2013) with some minor modifications. Mice were individualized and acclimatized to the room and light cycle for 1 week before experimentation began. Each week, on days 1 to 3, the water bottle and food were removed from the animals' cage and a 10-ml graduated pipette containing 20% (v/v) alcohol solution in tap water, or only tap water, was added for two hours (11am-13pm). Individual alcohol intake was recorded and food and water bottles were replaced. On day 4, the same procedure was followed, but the pipette was made available for 4 hours (11am- 15pm) in order to induce binge drinking. This process was repeated for two weeks. Immediately after the final alcohol intake period (day 4, week 2), blood samples were collected from the dorsal tail vein in order to assess blood alcohol concentrations (BAC) (detailed in 2.10).

2.3.7 Two bottle free-choice paradigm

The procedure involved escalating alcohol concentrations adapted from Vadasz et al., (2007). Animals were firstly habituated to the procedure by allowing them free

access to two drinking bottles filled with water for three days. They were secured to the cage grid with metal strings. Liquid loss due to spillage was measured by using separate empty cages and revealed negligible loss compared to average oral consumption. 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 four phases (5%, 10%, 15% and 20%), and, finally, alcohol (10%) was presented to mice for three days following three days of alcohol deprivation. Each 24h, the water and alcohol consumption was measured and the bottles were refreshed and their location 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 for each 24h period.

2.4 Biochemical analysis

2.4.1 Quantification of plasma alcohol

Blood samples were placed on ice for slow coagulation and were centrifuged at 10,000 g for 40 min at 4ºC to obtain cell-free plasma, which was then stored at -80ºC until alcohol quantification analysis was carried out. Frozen plasma samples were allowed to reach room temperature before processing. 5 µl aliquots of each sample were transferred to sample microvials, combined with 5 µl of internal standard solution (0.2 g/l isopropanol) and placed in a water bath at 50ºC for 5 min. Then, 1 ml of the headspace gas was injected manually with a Hamilton syringe into the gas chromatograph (Agilent 7890A GC-system) equipped with a flame ionization detector (FID). The alcohol and internal standards were separated in less than 6 min on a capillary column (Supelcowax-10®, 30m x 250µm x 0.25µm) using helium as a carrier gas at a constant flow rate of 2.0ml/min (split ratio 20:1). The column oven temperature was isothermal at 55ºC and the injector and the FID system were selected at 150 and 200ºC, respectively. Blood alcohol concentrations (BAC) were quantified from linear standard curves (0.1 – 2 g/l alcohol) using the peak area ratios of alcohol to the internal standard using Agilent Chemstation software.

2.4.2 Quantification of endocannabinoids and related compounds by liquid chromatography–tandem mass spectrometry (LC-MS/MS)

The determination of ECs and related compounds: N-acylethanolamide (NAE) compounds was based on methodology previously described in plasma (Pastor et al.,

2014), and adapted for the extraction of ECs from brain tissue (Busquets-Garcia et al., 2011). The following 2-acyl glycerols and N-acylethanolamines were quantified: 2-arachidonoylglycerol (2-AG), 2-linoleoylglycerl (2-LG), 2-oleoylglycerol, N-arachidonoylethanolamine or anandamide (AEA), N-docosatetraenoylethanolamine (DEA), N-docosahexaenoylethanolamine (DHEA), N-linoleoylethanolamine (LEA), N-oleoylethanolamine (OEA), N-palmitoylethanolamine (PEA), N-palmitoleoylethanolamine (POEA), and N-stearoylethanolamine (SEA). Mice were sacrificed by decapitation, the brain was rapidly removed, and the striatum and the PFC were dissected using a brain tissue blocker. Striatum and PFC tissue of mice (mean weights: 13.9 ± 0.56 mg; 13.2 ± 0.8 mg respectively) were kept at -80ºC until homogenization. For homogenization, tissues were placed in a 1 mL Wheaton glass homogenizer and spiked with deuterated internal standards. Tissues were homogenized on ice with a mixture of 50 mM Tris-HCl buffer (pH 7.4): methanol (1:1). The homogenization process took less than five minutes per sample and homogenates were kept on ice until organic extraction to minimize the ex-vivo generation of ECs began. Homogenates were extracted with chloroform. The organic phase was transferred to clean tubes, evaporated under a stream of nitrogen, and extracts were reconstituted in 100 μL of a mixture water:acetonitrile (10:90, v/v) with 0.1% formic acid (v/v) and transferred to High performance liquid chromatography (HPLC) vials with microvials. 20 μL were injected into the LC-MS/MS systems. For LC-MS/MS analysis, an Agilent 6410 triple quadrupole (Agilent Technologies, Wilmington, DE), equipped with a 1200 series binary pump, a column oven and a cooled autosampler (4ºC), was used. Chromatographic separation was carried out with a Waters C18-CSH column (3.1 x 100 mm, 1.8 μm particle size) maintained at 40ºC with a mobile phase flow rate of 0.4 mL/min. The composition of the mobile phase was: A: 0.1% (v/v) formic acid in water; B: 0.1% (v/v) formic acid in acetonitrile. ECs and related compounds were separated by gradient chromatography. The ion source was operated in the positive electrospray mode. The selected reaction monitoring (SRM) mode was used for the analysis. Quantification was carried out by means of isotope dilution with the response of the internal standards. Solvents and reagents were acquired from Merck (Darmstadt, Germany) and internal standards from Cayman Chemical (Ann Harbor, MI, USA).

2.4.3 Quantification of monoamine metabolites and corticosteroids by LC-MS/MS

Mice were sacrificed by decapitation, the brain was rapidly removed, and the striatum was dissected using a brain tissue blocker. All samples were immediately frozen in dry ice and stored at −80 °C. Monoamine neurotransmitters and corticosteroids were determined by LC-MS/MS using an Acquity UPLC coupled to a Quattro Premier triple quadrupole mass spectrometer (Waters Associates, Milford, MA, USA). The nitrogen desolvation temperature was set to 450ºC and the source temperature to 120ºC. Collision gas was argon at a flow of 0.21 ml/min. The LC separations were performed at 55 ºC using an Acquity BEH C18 column (100 mm, 2.1 mm i.d., 1.7 µm) (Waters Associates) at a flow rate of 300 µL min-1. Water and methanol, both with formic acid (0.01% v/v) and ammonium formate (1mM), were selected as mobile phase solvents. Analytes were determined by SRM methods, acquiring two transitions for each analyte. Quantification was performed after area peak integration of the analytes and the Internal Standard solution (ISTD) and comparison with a solvent calibration curve injected both before and after the batch. MassLynx software was used for data management. Neurotransmitters were quantified using a previously reported method (Marcos et al., 2016). Briefly, for sample processing, 500 µl of ice-cold buffer (0.5 mN sodium metabisulfate, 0.2 N perchloric acid and 0.5 mM EDTA) (Biskup et al., 2012) and 30 µl of the ISTD (containing serotonin-d4, dopamine-d4, 5-hydroxyindoleacetic acid-d5 and 3-methoxytyramine-d4) were added to the tissue, which was then homogenized using a sonicator. Samples were centrifuged for 10 min (10.000 g at 4 °C) and the supernatant was kept on ice until the analysis was conducted. 500 µL of the extract were evaporated under nitrogen at 20 ºC. The dry residue was dissolved in 100 µL of water and 10 µL were injected into the LC-MS/MS system. Corticosteroids were determined based on a previously described method (Marcos et al., 2014). Briefly, after adding 50 µL of the ISTD (containing cortisol-d4, testosterone-d3 and cortisone-d8), 2 mL of a saturated NaCl solution and 250 µL of a 25% (w/v) K2CO3 solution, the sample was extracted with 6 mL of ethyl acetate after being shaken in a rocking mixer at 40 oscillations per minute for 20 min. Following centrifugation (3000 g, 5 min), the organic layers were separated and evaporated to dryness under a stream of nitrogen in a water bath at 40 ºC. The extracts were reconstituted with 100 µL of a mixture of deionized water: acetonitrile (9:1, v/v) and 10 µL were injected into the LC-MS/MS system.

2.5 Statistical analysis

Statistical analysis was performed using one-way ANOVA in order to compare between Group differences in the TST, 3-chamber social test, DID test, two-bottle choice test, ECs analysis (groups: SN vs MSEW) and, in the EPM, corticosteroid and monoaminergic analysis (groups: SN-water, MSEW-water, SN-EtOH, MSEW-EtOH). The effects of MSEW in the CPP were evaluated using two-way ANOVA, with rearing groups (SN and MSEW) and treatment (saline, 1g/kg and 2g/kg) factors. The Bonferroni test was used for all the post-hoc comparisons. Data are presented as mean ± SEM. P-value < 0.05 was considered statistically significant. SPSS v21 package was used for all statistical analyses.

3. Results

3.1 Effects of maternal separation on despair-like behaviour and social behaviour: TST and 3-chamber social test

During the TST, mice exposed to MSEW exhibited a significant increase in despair-like behaviour. One-way ANOVA revealed a significant increase of immobility time in MSEW mice compared with the SN group [$F_{(1, 29)}$=12.706; p<0.001] (Fig. 2A). In the 3-chamber social test, MSEW group showed a decrease in the percentage of novel interaction time [$F_{(1, 19)}$=7.677; p<0.05], while no statistically significant differences were found in the percentage of sociability time [$F_{(1, 19)}$=2.939; n.s.]. No bias or initial preference for one of the two compartments was detected as no statistically significant differences were found during the habituation phase [Right: $F_{(1, 19)}$=0.051; n.s.; Left: $F_{(1, 19)}$=0.58; n.s.] (Fig. 2B).

3.2 Effects of maternal separation on alcohol consumption during adolescence: CPP, DID test and two-bottle choice procedures

As shown in Fig. 3, expression of alcohol-induced CPP was prevented in maternally separated animals. Two-way ANOVA analysis showed statistically significant differences in Group [$F_{(1, 57)}$=16.727; p<0.001], Treatment [$F_{(2, 57)}$=12.423; p<0.001] and interaction Group x Treatment [$F_{(2, 57)}$=6.055; p<0.01] factors. Post-hoc testing revealed differences in SN mice between saline group and alcohol-treated groups in both 1g/kg and 2g/kg (p<0.001), but no differences were found in MSEW mice between saline and alcohol groups in either of the two doses (1g/kg: p=0.08 and 2g/kg: p< 0,05). Statistically significant differences between SN and MSEW in 1g/kg (p<0.05) and in 2g/kg (p<0.001) were also found.

In the DID test, one-way ANOVA showed increased alcohol intake in the MSEW group compared to the SN group on day 7 [$F_{(1, 19)}$=5.486; p<0.05] and 8 [$F_{(1, 19)}$=5.826; p=0.05] (Fig. 4A). Water intake was recorded in both groups of mice throughout the procedure and no significant differences were found between groups on any of the testing days. BAC analysis also showed an increase in the alcohol levels of the MSEW group compared to the SN group [$F_{(1, 19)}$=4.294; p=0.05] (Fig. 4B), indicating that only MSEW animals achieve binge drinking. Pearson correlation

analyses showed significant positive correlation between the levels of BAC and alcohol intake on day 8 ($r = 0.465$, $p<0.05$) (Fig. 4C).

As for the two-bottle choice, all mice consumed similar amounts of alcohol in the different concentrations supplied, as no statistically significant group effects were observed in terms of alcohol intake (g/kg/24h) (all $F>0.1$, n.s.). However, following the three days of abstinence, MSEW mice did show a higher alcohol intake (10% v/v) [$F_{(1,27)}= 4.581$; $p<0.05$] (Fig. 5A). Specifically, one-way ANOVA of daily alcohol intake revealed that MSEW animals consumed more alcohol on day 16 [$F_{(1,27)}= 6.705$; $p<0.05$] and day 17 [$F(1,27)=4.973$; $p=0.05$] (Fig. 5B).

3.3 Effects of maternal separation on the endocannabinoid system

The analysis of the effects of MSEW on the EC system in the striatum of adolescent mice revealed a decrease in AEA [$F_{(1,17)}= 5.283$; $p<0.05$], DEA [$F_{(1,19)}= 4.610$; $p<0.05$] and DHEA [$F_{(1,18)}=4.877$; $p<0.05$] in the MSEW group (Fig. 6B). While no significant differences were appreciated for LEA and OEA, the MSEW group did tend to show decreased levels [$F_{(1,18)}= 3.803$; $p=0.06$; $F_{(1,18)}= 3.724$; $p=0.07$, respectively] (Fig. 6B). No statistically significant between-group values were observed after the analysis of 2-AG, 2-LG and 2-OG [$F_{(1,19)}= 0.327$; n.s; $F_{(1,19)}=1.418$; n.s; $F_{(1,19)}=2.75$; n.s.], respectively (Fig. 6A).

One-way ANOVA analysis of EC compounds in the PFC of adolescent mice revealed a decrease in the MSEW group for 2-AG, 2-LG and 2-OG levels [$F_{(1,16)}=4.473$; $p=0.05$; $F_{(1,16)}=4.738$; $p<0.05$; $F_{(1,16)}=5.193$; $p<0.05$, respectively] (Fig. 7A). No statistically significant differences were found for AEA, DEA, DHEA, LEA, OEA, PEA, POEA or SEA [$F_{(1,16)}= 0.320$; n.s.; $F_{(1,16)}= 2.652$; n.s., $F_{(1,16)}=0.388$; n.s.; $F_{(1,16)}=1.018$; n.s.; $F_{(1,16)}=3.082$; n.s.; $F_{(1,16)}=1.652$; n.s; $F_{(1,16)}=0.089$; n.s.; $F_{(1,16)}=1.789$; n.s., respectively] (Fig. 7B).

3.4 Effects of maternal separation during alcohol abstinence: EPM, corticosteroids and monoamine metabolites

On the third day of alcohol abstinence, EPM was carried out to assess anxiety-like behaviour. One-way ANOVA showed a significant effect of Group [$F_{(3,33)}= 5.932$; $p<0.01$]. Post-hoc testing revealed that both groups of mice, which had consumed alcohol during the two-bottle choice procedure (SN and MSEW), registered a decrease

in the percentage of time spent in the open arms compared to SN-water animals ($p<0.05$; $p<0.01$, respectively). Nevertheless, the MSEW mice, which had consumed water also presented differences compared to the SN-water mice ($p<0.01$) (Fig. 8A). Thus, MSEW animals exhibited similar levels of anxiety-like behaviour to SN mice during alcohol abstinence. To evaluate the animals' stress during alcohol abstinence, corticosteroid blood levels were assessed after the EPM. One-way ANOVA showed between-group statistical differences [$F_{(3,21)}= 12.709$; $p<0.001$]. Post-hoc analysis revealed that all groups displayed lower levels of deoxycorticosterone (DOC) compared to SN-water mice ($p<0.0001$ vs SN-EtOH and MSEW-EtOH and $p<0.05$ vs MSEW-water) (Fig. 8B). Moreover, in order to study the monoaminergic system in striatum during abstinence, one-way ANOVA analyses revealed between-group statistical differences in Tyr [$F_{(3,29)}= 6.420$; $p<0.01$], 5-HIAA [$F_{(3,29)}= 4.722$; $p<0.01$] and Trp [$F_{(3,29)}= 3.842$; $p<0.05$] compounds. Specifically, both adolescent MSEW and SN mice presented a significant decrease of the three selected monoaminergic compounds in the striatum during alcohol abstinence compared to SN-water mice (all $p<0.05$). However, MSEW-water group also showed a basal decrease in these compounds compared to SN-water mice (all $p<0.05$) (Fig. 9A-C).

4. Discussion

The present study demonstrates that adolescent mice exposed to MSEW showed behavioural alterations, such as increased despair-like behaviour, deficits in social behaviour and decreased EC levels in the striatum and PFC, all of which are changes related to depression-like symptoms. Such alterations were also associated with a higher vulnerability to alcohol seeking behaviour and alcohol binge drinking due to a decrease in alcohol-induced rewarding effects. Moreover, MSEW adolescent mice showed a facilitation of alcohol seeking following a period of abstinence and, subsequently, higher alcohol consumption during relapse. In addition, a reduction in the levels of several monoaminergic system compounds and higher anxiety-like behaviour during alcohol abstinence were observed in MSEW adolescent mice.

Previous studies have shown maternal separation to produce persistent alterations in emotional reactions, including despair-like behaviour during adolescence (García-Gutiérrez et al., 2016; Gracia-Rubio et al., 2016a; Lukkes et al., 2017; Martini and Valverde, 2012). In accordance with such studies, our results confirm that MSEW mice exhibited despair-like behaviour during adolescence, as assessed in the TST. Moreover, the present results show that adolescent MSEW mice presented impaired social novelty behaviour, suggesting heightened levels of social anxiety. Such findings are in agreement with previous reports showing that adult maternally separated mice presented a reduced interest in social novelty (Tsuda and Ogawa, 2012), but contrast with other studies showing that social behaviour was not altered in adult mice exposed to a maternal separation procedure (Harrison et al., 2014). Nevertheless, although there is no previous data regarding social behaviour during adolescence in MSEW mice, there is evidence to the effect that social defeat stress seriously affects social behaviour and induces significant social avoidance in adolescent mice (Iñiguez et al., 2014) and that maternal separation leads to reduced behavioural responsiveness to novelty (Matthews et al., 1996). Such data support the view that maternal separation is a well-established animal model of early-life stress to develop depression-like behaviour, as despair-behaviour and social anxiety are two core symptoms of depression (Lukkes et al., 2017; Millstein and Holmes, 2007; Tractenberg et al., 2016).

Furthermore, MSEW mice showed reduced EC levels in the striatum and the PFC compared to SN mice, thus suggesting a disruption of stress and mood-related processing. Specifically, MSEW mice showed a decrease in AEA, and other N-

acylethanolamines (DEA and DHEA) in the striatum and decreased 2-AG and other 2-acylglycerols (2-LG and 2-OG) in the PFC, suggesting that one of the mechanisms through which MSEW may induce depressive-like behaviour could be related to the modulation of the endogenous cannabinoid system. Indeed, several data demonstrate that early life stress affects the EC signalling leading to alterations in emotional processing, stress responses and dopaminergic dysfunction (Karhson et al., 2016; Morena et al., 2016; Viveros et al., 2012). Previous studies have also shown a decline in AEA content in several brain areas such as the striatum, amygdala, hippocampus and PFC in animal models of depression (Hill et al., 2008). Moreover, it has been demonstrated that both acute and chronic exposure to stress produces a bidirectional regulation of both types of EC compounds, resulting in a reduction of AEA and increased 2-AG respectively, particularly within the amygdala, hippocampus and PFC (Morena et al., 2016). The downregulation of AEA signalling appears to contribute to manifestations of anxiety and HPA axis activation, and may also have a role in the development of anhedonia and hyperalgesia. However, the behavioural influences of 2-AG are less characterized (Morena et al., 2016). In contrast, although increased CB1 cannabinoid receptor levels have been found in the striatum of maternally separated rats, lower values have been observed in the PFC and hippocampus (López-Gallardo et al., 2012; Romano-López et al., 2012; Suárez et al., 2009). Therefore, such results lead us to hypothesize that changes in striatal CB1 receptors may be a compensatory mechanism due to the alterations induced by chronic stress on EC levels, as shown by reduced AEA and other NAE compounds in this brain structure. However, the EC reduction found in the PFC, which mostly affected 2-acylglycerol compounds (2-AG, 2-LG and 2-OG), does not seem to modulate the expression of CB1 cannabinoid receptors in the same way as was observed in the striatum and, thus, other mechanisms would seem to be involved. In addition, the dysregulation of EC signalling has also been detected in the pathophysiology of social functioning deficits observed in major depressive disorder (Karhson et al., 2016). Thus, there are various indications as to the EC system's inhibitory role in stress response, as its disruption may be associated with mood changes occurring in depression (Morena et al., 2016; Vinod and Hungund, 2006). Indeed, EC hydrolytic enzyme inhibitors, such as fatty acid amide hydrolase, have become potential therapeutic targets for major depressive disorder (Ogawa and Kunugi, 2015).

Traumatic early life events may increase the risk of developing AUD, and maternal separation is a risk factor for alcohol consumption during adolescence and adulthood (Delavari et al., 2016; Holgate and Bartlett, 2015). The present study shows, for the first time, that adolescent mice exposed to MSEW exhibited a reduction in alcohol-induced rewarding effects compared to SN mice as assessed in the CPP paradigm. Such results agree with previous studies reporting that maternal separation attenuates alcohol second-order conditioning in adult rats (Pautassi et al., 2012), decreases the behavioural responses to conditioned reward stimuli (Matthews and Robbins, 2003) and produces anhedonia as assessed in the saccharin test (Gracia-Rubio et al., 2016a). Indeed, anhedonia displayed by maternal separation models may blunt the capacity to experience pleasure, one of the core clinical features of major depressive disorder. Therefore, the present results support a lack of alcohol-induced rewarding effects or a shift to the right in the dose-response curve in mice exposed to chronic stress produced by maternal separation. Nevertheless, some discrepancies are found in the relationship between the exposure to stress and the response to reward. Thus, a decrease in amphetamine or morphine-induced rewarding effects was reported in rodents exposed to chronic mild stress (Papp et al., 1991; Valverde et al., 1997), and a decrease in the cocaine-induced CPP was also found in a neonatal model of stress (Hays et al., 2012), in adolescent MSEW mice (Gracia-Rubio et al., 2016b), and in adult maternally separated rats exposed to the cocaine self-administration paradigm (Martini and Valverde, 2012; Matthews et al., 1999). However, enhanced rewarding effects of cocaine were found in adolescent mice exposed to social defeat (Rodríguez-Arias et al., 2017) and in adult mice exposed to chronic stress (McLaughlin et al., 2003), and enhanced alcohol-induced reward in the CPP was exhibited in mice after exposure to stress (Moreira-Silva et al., 2014). Hence, the characteristics of stressful situations, the age at which the animals' stress situation took place or was evaluated, and the particular paradigm used to evaluate rewarding effects may well be considered important factors in understanding divergences in the results.

Considering that a decrease in alcohol-rewarding properties may modify the vulnerability to alcohol consumption, adolescent MSEW mice were assessed for their alcohol intake using two different models: the DID test and the two-bottle choice procedure. In the current study, maternal separation increased alcohol intake (20% v/v) on day 7 and binge-day (day 8) in the DID test and during the relapse phase in the two-bottle choice paradigm (10% v/v). Our data suggest that MSEW mice were more

vulnerable to alcohol consumption using the intermittent alcohol access paradigm in agreement with previous studies showing that maternal separation increases alcohol intake in intermittent patterns of drinking during adulthood (Cruz et al., 2008; Daoura et al., 2011; Nylander and Roman, 2013). In contrast, only few studies have focused on adolescent rodents and some discrepancies with our findings were found. While Daoura and coworkers (2011) found that maternal separation did not affect alcohol consumption, Garcia-Gutierrez et al. (2016) showed that adolescent maternally separated mice presented higher alcohol consumption in alcohol-induced self-administration procedures In our study, although no differences were found in the alcohol intake during the two-bottle choice prior to the abstinence period, the MSEW mice did, afterwards, show increased alcohol consumption.

It has been previously shown that the EC system is involved in the motivational effects caused by several types of drug abuse, such as alcohol (Parsons and Hurd, 2015; Serrano and Parsons, 2011; Solinas et al., 2008). Therefore, the reduction in the EC levels found in MSEW animals may be related to the lack of alcohol-rewarding properties. One of the underlying mechanisms is explained by the release of AEA, which increases extracellular dopamine (DA) levels in the nucleus accumbens in a CB1 receptor-dependent manner (Solinas et al., 2006). Therefore, CB1 receptor knockout mice exhibited lower alcohol-induced CPP (Houchi et al., 2005; Thanos et al., 2005), but also an increased sensitivity to alcohol intoxication and severe withdrawal (Naassila et al., 2004). The effects of CB1 receptor antagonism on alcohol reward result, in part, from a diminished ability of the drug to augment nucleus accumbens DA release (Cheer et al., 2007). In accordance with the present results, previous studies have shown that, following maternal separation, adult rats present increased CB1 receptor expression in the ventral striatum (Romano-López et al., 2012) and reduced EC degrading enzymes in the nucleus accumbens (Romano-López et al., 2016), which may contribute to inducing the proclivity to ingest alcohol. The stress and reward networks are highly interactive, and ECs may modulate such interactions. ECs influence the response of the reward circuitry to stress by modulating the stress-induced changes in sensitivity to natural rewards that may result in anhedonia following chronic stress exposure. ECs also enhance the incentive salience of strong rewards which, through chronic stress, may increase the vulnerability to drug use later in life (Volkow et al., 2017).

The present study supports the view that maternal separation increases alcohol consumption following stressful events (García-Gutiérrez et al., 2016) as acute

withdrawal during abstinence from chronic alcohol exposure is associated with increased anxiety-like behaviour (Eisenhardt et al., 2015). Hence, early life stress may increase the vulnerability to later-life stress, enhancing stress reactivity and therefore alcohol-seeking behaviour. Indeed, an association between alcohol abuse and depression has been suggested in humans (Boden and Fergusson, 2011; Heinz et al., 2001). In the present study, anxiety-like behaviour assessed during the period of abstinence revealed that adolescent mice previously exposed to alcohol exhibited a lower percentage of time in the open arms when compared to SN-water group of mice, independently of rearing conditions. Thus, both groups of animals presented withdrawal-induced anxiety effects during the cessation of alcohol consumption. Our results agree with previous studies showing alcohol-induced withdrawal signs during abstinence in mice (Kliethermes, 2005; Perez and De Biasi, 2015). A reduction of plasma DOC was also found in both alcohol abstinent mice and MSEW-water mice (non-abstinent mice). Interestingly, previous results show that plasma DOC levels were elevated following acute alcohol administration (Porcu et al., 2011) and decreased during chronic alcohol exposure (Khisti et al., 2005) in agreement with our results for alcohol abstinent mice. Furthermore, DOC and its metabolites are also linked to affective disorders (Eser et al., 2007; Pisu and Serra, 2004). Indeed, previous results revealed that the administration of tetrahydrodeoxycorticosterone, which is a metabolite of DOC, attenuated the behavioural and neuroendocrine consequences of maternal separation (Patchev et al., 1997), probably due to its ability to modulate HPA axis activity through its interaction with $GABA_A$ receptors (Brunton, 2016). Altogether, our data show that MSEW-water (non-abstinent) mice exhibited a higher anxious phenotype and, consequently, showed reduced DOC levels, as previously found after chronic stress (Gunn et al., 2011) and maternal separation (Nishi et al., 2014).

During alcohol abstinence, the monoamine levels in the striatum were analysed by Tyr quantification, as a DA precursor, Trp and 5-HIAA, as a precursor and metabolite of serotonin (5-HT), respectively. Our results showed a decrease in these compounds for both groups of mice during alcohol abstinence. Alterations in both DA and 5-HT activity have been associated with alcohol withdrawal and relapse. For instance, decreased reward circuitry activation may lead to higher alcohol consumption to enhance DA release (Leyton and Vezina, 2014). Deficits of 5-HT in limbic areas and other brain structures are also known to lead to higher alcohol consumption (Müller and Homberg, 2015). Nevertheless, low monoamine metabolite levels were also found in

MSEW-water mice. Previous results propose that one of the pathophysiological mechanisms of prolonged adverse effects induced by neonatal maternal separation is related to monoaminergic system alterations (Burke and Miczek, 2014). Indeed, a reduction of monoamine levels in the mesolimbic reward circuit is associated with the appearance of depressive-like symptoms, such as anhedonia and reduced motivation (Nestler and Carlezon, 2006). Subsequently, maternal separation led to a decreased D2 receptor expression in nucleus accumbens (Gracia-Rubio et al., 2016b) and altered the densities of DA and 5-HT in the hippocampus (Lee et al., 2007) and PFC (Braun et al., 2000). Hence, this result may contribute to supporting the attenuation of alcohol-induced reward and the depressive and anxious phenotypes in MSEW mice. Since ECs are capable of modulating reward-seeking behaviour through the regulation of DA content in the ventral tegmental area, our results prompt us to hypothesize that monoaminergic dysfunction in the striatum could also be mediated by early-life stress-induced EC downregulation.

Our study demonstrates that exposure to MSEW led to a decreased functioning of EC and monoaminergic systems in brain areas controlling reward mechanisms, which may contribute to reduced alcohol rewarding effects, increased alcohol-seeking behaviour following abstinence and a persistent depressive phenotype. Our findings emphasize the relevance of early periods of life in the development of some psychiatric disorders such as mood disorders and substance use disorders.

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Figure Legends

Figure 1. Schematic representation showing the schedule of all the behavioural, biochemical and alcohol intake procedures. All the procedures were performed in adolescent male C57BL/6J mice, commencing on PD28 (see Methods for details). Postnatal day (PD); maternal separation with early weaning (MSEW); standard nest (SN); tail suspension test (TST); conditioned place preference (CPP); drinking in the dark (DID); blood alcohol concentration (BAC); elevated plus maze (EPM); deoxycorticosterone (DOC); tyrosine (Tyr); 5-hydroxyindoleacetic acid (5-HIAA); tryptophan (Trp).

Figure 2. Effects of maternal separation in despair-like behaviour and social interaction. (A) Mean ±SEM immobility time (s). Effect of MSEW procedure in the TST. The MSEW group showed an increase in despair-like behaviour (** $p<0.01$). (B) Percentage of time in the indicated chamber, expressed as the mean percentage ± SEM of the total time spent (15 min). MSEW showed a decrease in novel interaction (* $p<0.05$). N=10 mice per group.

Figure 3. Effects of maternal separation on the rewarding effects of alcohol (EtOH) in the CPP. Bars represent the difference between the time in Post- vs. Pre-C tests by mean ±SEM. *** $p<0.001$ Saline vs SN 1g/kg and SN 2g/kg. # $p<0.05$ SN 1g/kg vs MSEW 1g/kg. ## $p<0.001$ SN 2g/kg vs MSEW 2g/kg. N=9-11 mice per group.

Figure 4. Effects of maternal separation on alcohol binge drinking. (A) Consumption of alcohol (EtOH) (gEtOH/kg) in the DID test for two consecutive weeks. The alcohol concentration used was 20% v/v. Data are presented as mean ± SEM. (B) BAC values (mg/dL) calculated from blood samples collected on the last day of alcohol intake. (C) Correlation between gEtOH/kg in the DID test and BAC. The MSEW group showed higher alcohol intake on days 7 and 8, which correlated with BAC * $p<0.05$. N=10 mice per group.

Figure 5. Effects of maternal separation in the two-bottle choice procedure. (A) Mean ± SEM of daily alcohol (EtOH) consumption expressed as g/kg of body weight. (B) Daily alcohol intake expressed as g/kg across the five alcohol solutions. MSEW animals presented increased alcohol intake following abstinence (10% v/v), specifically on days 16 and 17. * $p<0.05$. N=15 for SN and N=13 for MSEW groups.

Figure 6. Effects of maternal separation on EC levels in the striatum. (A) Values for 2-AG, 2-LG and 2-OG expressed as mean ±SEM (nmol/g of tissue); (B) AEA, DEA, DHEA, LEA, PEA, POEA and SEA expressed as mean ± SEM (pmol/g of tissue). All compounds were analysed by LC-MS/MS. *p<0.05. N=9-10 per group.

Figure 7. Effects of maternal separation on EC levels in the PFC. (A) Values for 2-AG, 2-LG and 2-OG expressed as mean ± SEM (nmol/g of tissue); (B) AEA, DEA, DHEA, LEA, PEA, POEA and SEA expressed as mean ± SEM (pmol/g of tissue). All compounds were analysed by LC-MS/MS. *p≤0.05. N=9 per group.

Figure 8. Effects of maternal separation during alcohol abstinence in (A) the EPM presented as mean ± SEM of percentage of time in the open arms, and in (B) DOC plasma analysis from the four experimental groups during abstinence represented by mean ±SEM of ng/g. *p<0.05; ** p<0.01; ***p<0.001. N=7-8 per group.

Figure 9. Effects of maternal separation during alcohol abstinence in the levels of analytes from the monoaminergic system. Striatum samples were analysed using LC–MS/MS chromatography. Histograms represent the average of striatum samples from the four experimental groups during abstinence represented (mean ± SEM) of ng/mL of (A) Tyrosine, (B) 5-HIAA and (C) Tryptophan metabolites. Posthoc vs SN-water: *p<0.05; ** p<0.01; ***p<0.001. N=7-8 per group.