Maternal separation increases alcohol-drinking behaviour and reduces endocannabinoid levels in the mouse striatum and prefrontal cortex.

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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 is to evaluate the long-term effects of maternal separation with early weaning (MSEW) on emotional and social behaviour, alcohol rewarding 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. 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 intake following a period of abstinence.

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

Running title: Maternal separation and alcohol drinking behaviour.

Abbreviations:

- **2-AG:** 2-arachidonoylglycerol
- **2-LG:** 2-linoleoylglycerol
- **2-OG:** 2-oleoylglycerol
- **5-HIAA:** 5-hydroxyindoleacetic acid
- **AEA:** N-arachidonoyl ethanolamine
- **BAC:** Blood Alcohol Concentration
- **CPP:** Conditioned Place Preference
- **DEA:** N-docosatetraenoyl ethanolamine
- **DHEA:** N-docosahexaenoyl ethanolamine
- **DID:** Drinking in the Dark
- **DOC:** Deoxycorticosterone
- **EC:** Endocannabinoid
- **EPM:** Elevated Plus Maze
- **EtOH:** Alcohol
- **LC-MS/MS:** Liquid Chromatography–Tandem Mass Spectrometry
- **LEA:** N-linoleoyl ethanolamine
- **MSEW:** Maternal Separation with Early Weaning
- **NAE:** N-acyl ethanolamide
- **OEA:** N-oleoyl ethanolamide
- **PEA:** N-palmitoyl ethanolamine
- **POEA:** N-palmitoleoyl ethanolamine
- **PD:** Postnatal Day
- **PFC:** Prefrontal Cortex
- **SEA:** N-stearoyl ethanolamine
- **SN:** Standard Nesting
- **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 binge-like drinking behaviour.
- MSEW reduces corticosteroid and monoamine levels similarly to alcohol abstinence.

1. Introduction

Early postnatal life is a period of high vulnerability in which prolonged exposure stressors may lead to long-lasting deleterious effects on brain neurodevelopment and function. Therefore, preclinical (Kaufman et al., 2000; Sarro et al., 2014) and clinical studies (Lupien et al., 2009; Teicher and Samson, 2013) 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 the early separation of pups from dams for long periods 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, 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 and Robbins, 2003; Rüedi-Bettschen et al., 2004). In addition, maternal separation decreases neurogenesis (Lajud et al., 2012), reduces 5-HT1A receptor levels (Bravo et al., 2014; Gracia-Rubio et al., 2016b), and increases pro-inflammatory cytokines levels in serum (Réus et al., 2015) associated with increased neuroinflammatory responses (Gracia-Rubio et al., 2016a). Behavior and molecular alterations induced by maternal separation appear during adolescence and persist until adulthood (Gracia-Rubio et al., 2016a).

In this sense, adolescence is a critical period for brain development and maturation and is a sensitive period to develop psychiatric illnesses, 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 at the same time, risk taking and novelty seeking are hallmarks of typical adolescent behaviour (Wolf et al., 2013). Alcohol is the most commonly abused drug during adolescence and alcohol intoxication was reported to induce brain damage (Pascual et al., 2014). Therefore, alcohol intake during adolescence is considered one of the main risk factors contributing to the development of neuropsychiatric disorders later in life (Skogen et al., 2014), including alcohol use disorder (Kyzar et al., 2016). Additionally, the individual risk of developing alcohol use disorder is affected by early life stress (Sinha, 2008), suggesting that the dysregulation of the brain reward function induced by early life

adverse experiences may be related to the development of drug addiction (Cheetham et al., 2010), presumably through the modulation of the dopaminergic and endocannabinoid (EC) systems (Parsons and Hurd, 2015), which are involved in reward, mood and stress processing. Accordingly, previous reports showed 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). Moreover, regarding the EC system, a decreased CB1 receptor expression was described in the hippocampus and prefrontal cortex (PFC) due to maternal separation, while in the striatum, an increase was reported (López-Gallardo et al., 2012; Romano-López et al., 2012), which may contribute to a proclivity to alcohol ingestion (Parsons and Hurd, 2015). Several studies have concluded that animals exposed to maternal separation exhibit high patterns of alcohol consumption (García-Gutiérrez et al., 2016; Gondré-Lewis et al., 2016; Roman and Nylander, 2005) during adulthood. Nevertheless, only a few studies have evaluated the effects of maternal separation on alcohol consumption during adolescence (García-Gutiérrez et al., 2016), and previous literature has not investigated the effects of maternal separation 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 develop alcohol use disorders during adolescence, specially the contribution of the EC and the monoaminergic systems as related key targets in the abuse liability of alcohol. In this context, the aim of the present study was first to examine whether maternal separation may increase the propensity to alcohol consumption, and second to determine if it modifies the EC and the monoaminergic systems in the PFC and striatum in adolescent male C57BL/6 mice. For this purpose, to verify the effects of maternal separation on despair-like and social behaviour, the tail suspension test (TST) and the three-chamber social test were assessed respectively. Moreover, to evaluate the effects of maternal separation on alcohol rewarding properties, voluntary alcohol intake, binge drinking and relapse following abstinence were analysed. Finally, monoaminergic precursors and metabolites, such as tyrosine (Tyr), tryptophan (Trp) and 5-hydroxyindoleacetic acid (5-HIAA), and EC compounds were assessed in the striatum and PFC of maternally separated mice.

2. Experimental procedures

2.1 Animals

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 plastic boxes. After 2 weeks, the male was removed, and pregnant females were housed individually. A final group of 150 offspring male mice were used to conduct the different experiments. Offspring female mice were dismissed for the experiment and were used for other experiments. Animals were housed in an acclimatized room (temperature $21\pm1^{\circ}\text{C}$, humidity $55\pm10\%$). 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 food were available *ad libitum*, except during the drinking in the dark (DID) test. 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 (CEEAA-PRBB).

2.2 Drugs

Alcohol (Ethyl alcohol, EtOH) was purchased from Merck Chemicals (Darmstadt, Germany) and diluted in tap water to obtain a 20% (v/v) alcohol solution for the DID test and 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 (100 and 200 mg/ml) 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

Additional information on behavioural procedures is available in Supplementary Material

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), as previously reported (Gracia-Rubio et al., 2016a). In order to control the possible litter effect on the behavioural tests performed, one-way

ANOVA was calculated for the different experiments in which more than one pup from the same litter were included in the same experimental group (Table 1).

2.3.2. Experimental schedule

Different cohorts of male mice were used for each experimental procedure commencing on PD28 (Fig 1). Three different set of animals were used in order to perform the whole experiment: 1) 30 mice MSEW and 30 mice SN were used to evaluate CPP. Previously, a set of 15 animals per group was used to analyse the TST and 10 animals per group in the 3-chamber social test. After the CPP animals from the saline group were sacrificed in order to perform the EC analysis (day 41). 2) 20 mice MSEW and 20 mice SN were used for the DID Test and the BAC analysis. 3) 50 mice were used for the two bottle choices; during the third abstinence day a set of 30 mice were tested in the EPM and sacrificed to carry out the blood analysis of DOC and the striatum monoamines.

2.3.3. Tail Suspension Test

Mice were individually suspended by the tail from a horizontal bar for a 6-min period as described by Steru et al. (1985).

2.3.4. 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 and two wire cups. The test was conducted in three different phases, as reported (Segura-Puimedon et al., 2014). Briefly, the first 10-minute were the *Habituation Phase*, in which mice were allowed to explore freely the three chambers. Next, during the *Sociability Phase*, an unfamiliar mouse was enclosed in one of the wire cups in a side chamber whereas the second wire cup remained empty. Finally, during the *Social Novelty Phase*, a new unfamiliar mouse was enclosed in the wire cup, which had remained empty during the Sociability Phase (Figure 2C). The time spent in each chamber and the time spent exploring enclosed novel mice was recorded. The exploration of an enclosed mouse or wire cup was considered when the mouse approached its nose to the cup and the distance between the nose and cup was less than 1 cm or climbed onto a cup.

2.3.5. Elevated plus maze

The elevated plus maze (EPM) experiments were conducted as previously reported (Simonin et al., 1998).

2.3.6. Alcohol-induced conditioned place preference

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 connected to a smaller, central compartment (Cibertec S.A., Madrid, Spain). 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.

2.3.7. Drinking in the dark test

This procedure was conducted as previously reported (Esteve-Arenys et al., 2017; Rhodes et al., 2005) with some minor modifications.

2.3.8. 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. 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

Immediately after the last alcohol intake of the DID test (day 4, week 2), blood samples were collected and placed on ice for slow coagulation and were centrifuged at 10,000 g for 40 min at 4°C to obtain cell-free plasma, which was then stored at -80°C 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 50°C 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. BACs 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 ECs 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-linoleoylglycerol (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 -80°C until homogenization. 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 (4°C), was used. 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, brain areas were dissected and were immediately frozen in dry ice and stored at -80°C . Monoamine compounds 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). Analytes were determined by selected reaction monitoring methods, acquiring two transitions for each analyte. Quantification was performed after area peak integration of the analytes and the Internal Standard solution and comparison with a solvent calibration curve injected both before and after the batch. MassLynx software was used for data management. Monoamine compounds were quantified using a previously reported method (Marcos et al., 2016). Corticosteroids were determined based on a previously described method (Marcos et al., 2014).

2.5. Statistical analysis

Statistical analysis was performed to study the possible effect of the litter on the different behaviour and biochemical studies. Therefore, one-way ANOVA was calculated between different experimental groups. Once, the litter effect was discarded (Table 1), we calculated Student's t-test to compare between Group differences in the TST, 3-chamber social test, DID test, two-bottle choice test, ECs analysis (groups: SN vs MSEW). The effects of maternal separation in the CPP, EPM, corticosteroid and monoaminergic compounds analysis were evaluated using two-way ANOVA, with rearing groups and treatment factors. The Bonferroni test was used for all the post-hoc comparisons. Data are presented as mean $\pm$ 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

MSEW mice exposed to the TST exhibited a significant increase in despair-like behaviour. There was a significant increase of immobility time in MSEW mice compared with the SN group [$t_{(28)}=-3.565$; $p=0.001$] (Fig. 2A). In the 3-chamber social test, MSEW group showed a decrease in the percentage of novel interaction time [$t_{(18)}=2.771$; $p<0.05$], without differences in the percentage of sociability time [$t_{(18)}=-1.714$; 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: $t_{(18)}=0.761$; n.s.; Left: $F_{(18)}=-0.226$; n.s.] (Fig. 2B).

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

As depicted 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 100 mg/ml and 200 mg/ml ($p<0.001$), without differences in MSEW mice between saline and alcohol groups in either of the two doses (100 mg/ml: $p=0.08$ and 200 mg/ml, n.s.). Statistically significant differences between SN and MSEW in 100 mg/ml ($p<0.05$) and in 200 mg/ml ($p<0.001$) were also found.

In the DID test, MSEW group showed an increase in the alcohol intake compared to the SN group on day 3 [$t_{(18)}=-2.342$; $p<0.05$] and 4 [$t_{(18)}=-2.414$; $p=0.05$] during the second week (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 [$t_{(18)}=-2.172$; $p=0.05$] (Fig. 4B). Pearson correlation analyses showed significant positive correlation between the BACs and alcohol intake on the 4th day of the second week ($r = 0.465$, $p<0.05$) (Fig. 4C).

Therefore, the higher the individual BAC level the higher the alcohol intake during the binge session.

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) [$t_{(26)} = -2.140$; $p < 0.05$] (Fig. 5A). Specifically, daily alcohol intake revealed that MSEW animals consumed more alcohol on day 16 [$t_{(26)} = -2.589$; $p < 0.05$] and day 17 [$t_{(26)} = -2.230$; $p < 0.05$] (Fig. 5B). Likewise, difference in the alcohol preference ratio was also found following the three days of abstinence. Two-way ANOVA showed a significant effect of the alcohol concentration [$F_{(4,104)} = 19,693$; $p < 0.001$], no group effect [$F_{(1,26)} = 1,401$; n.s.], and interaction between these two factors [$F_{(4,104)} = 2,471$; $p < 0.05$]. Subsequent post-hoc comparisons indicated a higher alcohol preference for MSEW mice ($66,63\% \pm 1,3$ ml) comparing with SN mice ($58,72\% \pm 2,2$ ml) during the alcohol consumption days after abstinence ($p < 0.01$) (Data not shown).

3.3. Effects of maternal separation on the EC system

The analysis of the effects of MSEW on the EC system in the striatum of adolescent mice revealed no statistically significant between-group values after the analysis of 2-AG, 2-LG and 2-OG (Fig. 6A). However, a decrease in AEA [$t_{(16)} = 2.298$; $p < 0.05$], DEA [$t_{(18)} = 2.147$; $p < 0.05$] and DHEA [$t_{(17)} = 2.208$; $p < 0.05$] was appreciated in the MSEW group (Fig. 6B). No statistically significant between-group values were observed after the analysis of 2-AG, 2-LG and 2-OG, whereas for LEA and OEA, the MSEW group did tend to show decreased levels [$t_{(17)} = 1.950$; $p = 0.06$; $t_{(17)} = 1.93$; $p = 0.07$, respectively] (Fig. 6B).

The 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 [$t_{(16)} = 2.115$; $p = 0.05$; $t_{(16)} = 2.177$; $p < 0.05$; $t_{(16)} = 2.279$; $p < 0.05$, respectively] (Fig. 7A). No statistically significant differences were found for AEA, DEA, DHEA, LEA, OEA, PEA, POEA or SEA (Fig. 7B).

3.4. Effects of maternal separation during alcohol abstinence: anxiety-like behaviour, corticosteroids blood levels and striatum monoamine metabolites

On the third day of alcohol abstinence, EPM was carried out to assess anxiety-like behaviour. Two-way ANOVA showed a significant effect of Group [$F_{(1,33)} = 11.310$; $p < 0.01$] and Treatment [$F_{(1,33)} = 4.627$; $p < 0.05$] and interaction between Group x Treatment [$F_{(1,33)} = 4.857$; $p < 0.05$]. Post-hoc testing revealed that both groups of mice, which had consumed alcohol during the two-bottle choice procedure (SN-EtOH and MSEW-EtOH), 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 presented differences compared to the SN-water mice ($p < 0.01$) (Fig. 8A). Thus, MSEW receiving water animals exhibited similar levels of anxiety-like behaviour to SN and MSEW mice during alcohol abstinence. To evaluate the animals' stress during alcohol abstinence, corticosteroid blood levels were assessed after the EPM. Two-way ANOVA showed statistical differences in Group [$F_{(1,21)} = 8.329$; $p < 0.01$], Treatment [$F_{(1,21)} = 30.494$; $p < 0.0001$], and interaction between Group and Treatment [$F_{(1,21)} = 5.639$; $p < 0.05$]. 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 respectively, and $p < 0.05$ vs MSEW-water) (Fig. 8B). Moreover, in order to study monoaminergic compounds in striatum during abstinence, two-way ANOVA analysis showed statistically significant differences in Treatment for Tyr [$F_{(1,29)} = 13.068$; $p < 0.001$], 5-HIAA [$F_{(1,29)} = 8.712$; $p < 0.01$] and Trp [$F_{(1,29)} = 7.098$; $p < 0.01$], interaction Group x Treatment for Tyr [$F_{(1,29)} = 4.456$; $p < 0.05$], 5-HIAA [$F_{(1,29)} = 4.114$; $p < 0.05$], but not for Trp [$F_{(1,29)} = 2.499$; n.s.] and no differences in Group (all $F < 2.9$, n.s.) factors. Post-hoc analysis showed that adolescent MSEW and SN mice presented a significant decrease of the Tyr and 5HIAA compounds in the striatum during alcohol abstinence compared to SN-water mice (all $p < 0.05$). Moreover, MSEW-water group also showed a basal decrease in Tyr and 5-HIAA compared to SN-water mice (all $p < 0.05$). No significant changes were observed for the Trp levels (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. Such alterations were also associated with a higher vulnerability to develop alcohol drinking behaviour due to a decreased sensitivity to alcohol-induced rewarding effects. Moreover, MSEW adolescent mice showed a facilitation of alcohol drinking following a period of abstinence and, subsequently, showed higher alcohol consumption during relapse. In addition, a reduction in the levels of several monoaminergic system compounds and higher anxiety-like behaviour were observed in MSEW adolescent mice compared to control animals.

4.1 Maternal separation produced persistent alterations in emotional reactions and reduced levels of EC in the striatum and PFC

In accordance with previous studies (García-Gutiérrez et al., 2016; Gracia-Rubio et al., 2016a; Lukkes et al., 2017), our results confirm that MSEW mice exhibited despair-like behaviour during adolescence, as assessed in the TST. Moreover, the present results also show that adolescent MSEW mice presented impaired social novelty behaviour, supporting 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 are in 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 showing that social defeat stress seriously affects social behaviour and induces significant social avoidance in adolescent mice (Iñiguez et al., 2014). 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).

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

(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). 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 hypothalamic-pituitary-adrenal 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). 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 we hypothesized in the striatum and, thus, other mechanisms would 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 inhibitors, have become potential therapeutic targets for major depressive disorder (Ogawa and Kunugi, 2015).

4.2 Maternal separation produced higher vulnerability to alcohol drinking behaviour and modified levels of monoamine compounds in the mouse striatum

Traumatic early life events may increase the risk of developing alcohol use disorders, and maternal separation is a risk factor for alcohol consumption during adolescence and adulthood (Delavari et al., 2016). 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 are in agreement 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 the 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 (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 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 paradigm used to evaluate reward may 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 3 and binge-day (day 4) in the second week of 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 (Daoura et al., 2011; Nylander and Roman, 2013). In contrast, scarce studies have focused on adolescent rodents and some discrepancies with our findings were found. While Daoura et al. (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; 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 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), but also an increased sensitivity to alcohol intoxication and severe withdrawal (Naassila et al., 2004). The effects of CB1 receptor antagonism on alcohol reward may be due to, in part, a diminished ability of the drug to augment nucleus accumbens dopamine 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 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-drinking behaviour. In the present study, both groups of animals presented withdrawal-induced anxiety effects during the cessation of alcohol consumption independently of rearing conditions. Moreover, 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, 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 hypothalamic-pituitary-adrenal 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 maternal separation (Nishi et al., 2014).

During alcohol abstinence, monoamine compounds levels in the striatum were analysed by Tyr quantification, as a dopamine precursor, Trp and 5-HIAA, as a precursor and metabolite of serotonin, respectively. Our results showed a decrease in these compounds for both groups of mice during alcohol abstinence. Alterations in both dopamine and serotonin activity have been associated with alcohol withdrawal and relapse (Leyton and Vezina, 2014; Müller and Homberg, 2015). Nevertheless, low monoamine metabolite levels were also found in MSEW-water mice. In agreement, 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). Subsequently, maternal separation led to

a decreased D2 receptor expression in nucleus accumbens (Gracia-Rubio et al., 2016b) and altered the densities of dopamine and serotonin in the hippocampus (Lee et al., 2007) and PFC (Braun et al., 2000). Hence, this finding may contribute to the attenuation of alcohol-induced reward and to the appearance of depressive and anxious phenotypes in MSEW mice. Since ECs are capable of modulating reward-seeking behaviour through the regulation of dopamine 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.

In conclusion, 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-drinking 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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Table 1. Effects of the litter in each behavioural test.

TST	Three Chamber test	EPM
$F_{(19,29)} = 1.423$; $p > 0.05$	Sociability and Social Novelty: Both $F_{(16,19)} > 0.575$; $p > 0.05$	$F_{(11,32)} = 2.118$; $p > 0.05$
DID Test	2-Bottle Choice	Monoamines
All the 8 drinking days: $F_{(11,39)} > 4.25$; all $p > 0.05$	All the ethanol intake per day: $F_{(15,27)} > 1.85$; all $p > 0.05$	Tyr, 5-HIAA and Trp: All $F_{(11,30)} > 0.662$; $p > 0.05$

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/6 mice, commencing on PD28 (see Methods for details).

Figure 2. Effects of maternal separation in the tail suspension and three-chamber tests. (A) Mean $\pm$ 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 $\pm$ SEM of the total time spent (15 min). MSEW showed a decrease in novel interaction (* $p < 0.05$). N=10 mice per group. (C) Scheme of the procedure used in the three-chamber social test.

Figure 3. Effects of maternal separation on the rewarding effects of EtOH in the CPP. Bars represent the difference between the time in Post- vs. Pre-C tests by mean $\pm$ SEM. *** $p < 0.001$ Saline vs SN 100 mg/ml and SN 200 mg/ml. # $p < 0.05$ SN 100 mg/ml vs MSEW 100 mg/ml. ## $p < 0.001$ SN 200 mg/ml vs MSEW 200 mg/ml. N=9-11 mice per group.

Figure 4. Effects of maternal separation on alcohol binge drinking. (A) Consumption of EtOH (gEtOH/kg) in the DID test for two consecutive weeks. The alcohol concentration used was 20% v/v. Data are presented as mean $\pm$ 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 $\pm$ SEM of daily 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 $\pm$ SEM (nmol/g of tissue); (B) AEA, DEA,

DHEA, LEA, PEA, POEA and SEA expressed as mean $\pm$ 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 $\pm$ SEM (nmol/g of tissue); (B) AEA, DEA, DHEA, LEA, PEA, POEA and SEA expressed as mean $\pm$ SEM (pmol/g of tissue). All compounds were analysed by LC-MS/MS. * $p \leq 0.05$. N=9 per group.

Figure 8. Effects of maternal separation during alcohol abstinence in (A) the EPM presented as mean $\pm$ 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 $\pm$ 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 $\pm$ SEM) of ng/mL of (A) Tyr, (B) 5-HIAA and (C) Trp metabolites. Posthoc vs SN-water: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. N=7-8 per group.