Maternal separation induces neuroinflammation and long-lasting emotional alterations in mice

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Abbreviations: Cornu Ammonis (CA); Dentate gyrus (DG); Hippocampus (HC); Interleukin-1 beta (IL-1 β); Interleukin-6 (IL-6); Internal Standard Solution (ISTD); Ionized calcium-binding adapter molecule 1 (Iba1); Kynurenine (KYN); Liquid Chromatography Mass Spectrometry (LC-MS/MS); Maternal separation with early weaning (MSEW); *N*-methyl-D-aspartate (NMDA); Non-significant (NS); Phosphate buffer (PB); Phosphate buffer saline (PBS); Postnatal day (PD); Prefrontal cortex (PFC); Selected Reaction Monitoring (SRM); Serotonin (5-HT); Standard nesting (SN); Tryptophan (TRP); Tumor necrosis factor alpha (TNF-α).

Abstract

Early life experiences play a key role in brain function and behaviour. Adverse events during childhood are therefore a risk factor for psychiatric disease during adulthood, such as mood disorders. Maternal separation is a validated mouse model for maternal neglect, producing negative early life experiences that result in subsequent emotional alteration. Mood disorders have been found to be associated with neurochemical changes and neurotransmitter deficits such as reduced availability of monoamines in discrete brain areas. Emotional alterations like depression result in reduced serotonin availability and enhanced kynurenine metabolism through the action of indoleamine 2, 3-dioxygenase in response to neuroinflammatory factors. This mechanism involves regulation of the neurotransmitter system by neuroinflammatory agents, linking mood regulation to neuroinmunological reactions. In this context, the aim of this study was to investigate the effects of maternal separation with early weaning on emotional behaviour in mice. We investigated neuroinflammatory responses and the state of the tryptophan-kynurenine metabolic pathway in discrete brain areas following maternal separation. We show that adverse events during early life increase risk of long-lasting emotional alterations during adolescence and adulthood. These emotional alterations are particularly severe in females. Behavioural impairments were associated with microglia activation and disturbed tryptophan-kynurenine metabolism in brain areas related to emotional control. This finding supports the preeminent role of neuroinflammation in emotional disorders.

Key words: Early life experiences, maternal neglect, despair behaviour, neuroinflammation, tryptophan-kynurenine metabolism.

1. Introduction

Emotional disorders, including major depression, are the most prevalent psychiatric disorders worldwide, importantly contributing to the global burden of diseases (Murray and Lopez, 2013). The World Health Organization predicts that depressive disorders will be the greatest contributor to the global burden of disease by 2030 (Mathers *et al.*, 2005; Stuart and Baune, 2014). Major depression is thought to comprise a heterogeneous group of diseases caused by genetic, epigenetic and environmental factors (Nestler, 2014). More than a quarter of depressed patients fail to achieve remission despite trying multiple treatments (Felger and Lotrich, 2013), and a high percentage of patients relapse (Raedler, 2011), highlighting the need for more effective therapies.

Early life experiences are thought to play a key role in brain function and behaviour (Lupien *et al.*, 2009). In humans, detrimental early life events, such as maternal neglect or abuse during childhood, are associated with increased risk of emotional disorders that may persist into adulthood (Heim and Nemeroff, 2001; Gross and Hen, 2004; Heim and Binder, 2012). Experimental and clinical studies have shown that the immaturity and plasticity of the central nervous system during childhood make it particularly sensitive to stress at a young age, which may cause significant changes in brain structure and function (Lupien *et al.*, 2009). In recent years, various rodent behavioural models of early life stress, such as maternal separation, have been used to study the neurobiological basis of emotional and motivational disorders *(*Pryce *et al.*, 2001; Martini and Valverde, 2012; Fuentes *et al.*, 2014). Maternal separation with early weaning (MSEW) (George *et al.*, 2010) attempts to reduce any potential compensatory maternal care after maternal deprivation.

Recent clinical and experimental data suggest that the pathophysiology of several neuropsychiatric disorders, including depressive syndromes, involves activation of the immune system in response to inflammatory agents. These studies report that depressed patients present elevated plasma levels of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin -1β (IL-1β) and the tumour necrosis factor (TNF-α) (Miller *et al.*, 2009; Dantzer *et al.*, 2011; Anderson *et al.*, 2014). The production of these pro-inflammatory mediators has been related to the pathophysiological effects of stress and depressive states (Myint *et al.*, 2007; Miller *et al.*, 2009). Indeed inflammation also appears to be an immunological consequence due to early life neglect (Danese *et al.*, 2007). Thus, the production of cytokines contributes to a deregulation of the hypothalamic-pituitary-adrenal

axis and promotes abnormalities in the neural plasticity, including a decrease of the neurotrophic support and an impaired neurogenesis. Further evidence suggests that pro-inflammatory cytokines alter tryptophan (TRP) metabolism, affecting the activity of serotonin (5-HT) neurotransmitter system (Miller *et al.,* 2009; Christmas *et al.,* 2011). Therefore, the metabolic tryptophan route becomes imbalanced during depression, increasing kynurenine (KYN) synthesis and enhancing the alternative TRP metabolic pathway by activating indoleamine 2,3-dioxygenase, (Christmas *et al.,* 2011) (see Figure S1), decreasing the availability of TRP to be metabolized in 5HT. Interestingly, metabolites of the TRP-KYN pathway may regulate the brain homeostasis as well as modulate other different neurotransmitter systems including glutamate and dopamine (Miller *et al.,* 2009; Myint, 2012). In fact, kynurenic acid, an intermediate metabolic product of the TRP-KYN pathway behaves as a NMDA antagonist, displaying neuroprotective actions in the brain and inhibiting the release of the excitatory neurotransmitter glutamate and inhibiting the release of dopamine in discrete brain areas (Borland and Michael, 2004; Sas *et al.,* 2007; Klein *et al.,* 2013). On the other hand, quinolinic acid, one of the pathway's final products, seems to act as an NMDA agonist, promoting glutamate release and contributing to excitotoxicity and oxidative stress in the brain (Muller and Schwarz, 2007; McNally *et al.,* 2008). Similarly, 3-hydroxykynurenine also displays neurotoxic effects by promoting the formation of oxygen species and causing neuronal apoptosis (Okuda *et al.,* 1998; Stone, 2001). Taken together, the imbalance of this metabolic tryptophan route induces a detrimental 5-HT synthesis that has been directly associated to the development of depressive symptoms in humans and in experimental animal models (Laugeray *et al.,* 2010; Gabbay *et al.,* 2010; Steiner *et al.,* 2011). Considering these facts, it seems of relevance the evaluation of the possible imbalance of the metabolic tryptophan route under our experimental conditions in which long-lasting emotional alterations are observed.

In this study, we investigated behavioural alterations induced by early life adversity in male and female CD1 mice, and explored the interplay between depressive manifestations in behavioural models, neuroinflammation, and alterations in the TRP-KYN pathway. Using MSEW in CD1 mice, we aimed to elucidate behavioural, neuroimmunological and neurochemical changes induced by maternal separation. We used CD1 mice to evaluate the effects of two experimental rearing paradigms on emotional behaviour during adolescence and into adulthood, MSEW and a Standard Nest (SN). We performed a range of tests for anxiety- and emotional-related behaviours, and evaluated neuroinflammatory responses in specific brain areas of mice reared under each paradigm. We evaluated microglia activation in

the prefrontal cortex (PFC) and hippocampus, and analysed metabolites of the TRP-KYN pathway to explore the link between depressive disorders and inflammatory reactions.

2. Materials and methods

2.1. Animals

We used 12 male and 12 female outbred CD1 mice as breeders for this study (provided by Charles River, Barcelona, Spain), and shipped to our animal facility, UBIOMEX, PRBB. Animals were 10 weeks old at the start of breeding and were housed individually in standard cages in a temperature- (21°± 1°C), humidity- (55% ± 10%), and light-cycle-controlled room. The room was lit between 8:00h and 20:00h, and experiments were conducted during the light phase (8:30h to 15:00h), except for the evaluation of maternal behaviour, as indicated. Food and water were available *ad libitum* except during behavioural testing of the offspring. Mice were allowed to acclimatize to the new environmental conditions for at least one week before starting the experiments. Every effort was made to minimize animal suffering and reduce the number of animals used. All procedures were conducted in accordance with national (BOE-2013-1337) and EU (Directive 2010-63EU) guidelines regulating animal research, and were approved by the local ethics committee (CEEA-PRBB).

2.2. Rearing conditions

Mice were randomly assigned to one of two different experimental groups, SN and MSEW. For each group, breeding pairs (one male, and one female) were housed in Plexiglas cages (369 x 156 x 132 mm), and the males were removed when the females were about 10 days pregnant. Pregnant females were observed daily at 9 and 17h for parturition. For each litter, the date of birth was designated postnatal day (PD) 0. In the MSEW group, offspring were separated from their mothers for 4h per day on PD2-5 (9:30-13:30) and 8h per day on PD6-16 (9:30-17:30h). For separation, mothers were moved to another cage, while the offspring remained in their home cages with a heating blanket (32-34ºC) for thermoregulation. After removing the mothers, offspring were taken to another room to avoid their mothers to become stressed from hearing their vocalizations (George *et al.,* 2010). Pups were weaned at PD17, and to facilitate their access to food and avoid a possible dehydration, wet regular chow and hydrodrogel (Bio-Services, Uden, The Netherlands) were provided in their home cages until PD21. In the SN group, offspring remained with their mothers for 21 days and were then weaned (PD21). Cages remained untouched until PD10, when they were cleaned. After

weaning, offspring were housed in groups of 4 to 5 animals of the same sex. For the experiments, 5 and 4 females were assigned to the MSEW and SN groups, respectively. We observed no significant difference between groups in the total number of offspring, or the number of males or females. Average litter size was 13 (53% male). A different group of mice was used to evaluate adult behavioural parameters. In this case, 5 females were assigned to each group, and we observed no differences between groups in the number or sex of the offspring (average litter size, 12; 56% males).

2.3. Maternal care

We recorded the biological mother's spontaneous maternal behaviour 3 times per day (8:15, 17:30 and 20:15) from PD1 to PD16 according to an adapted version of a previously described protocol (Dimitsantos *et al.,* 2007; Fodor *et al.,* 2012). The long break between the morning and afternoon maternal care evaluation session was consistent with the 8 h maternal separation period performed during PD6-16 (9:30-17:30 h). Behaviour was recorded on-line by an observer who remained silent in the room. Observations of the maternal care behaviour were performed at three periods of the day, at 8.15 h, 17.30 h and 20.15 h. Within each observation period, the behaviour of each mother was scored 25 times spaced 3 min each one (25 observations x 3 periods per day x 16 days = 1200 observations/mother).The following behaviours were scored as present or absent and quantified in a check list: 1) mother licking and grooming any offspring (body + anogenital region); 2) mother nursing offspring in an arched-back posture with rigid limbs ("high kyphosis"); 3) mother nursing in a "blanket" posture, i.e. lying on the offspring or her limbs are rigid but she has a low dorsal arch posture ("low/partial kyphosis"); 4) mother nursing in a "passive" posture ("supine nursing"), i.e. lying on her back or side while the offspring nurse; and 5) mother "off" offspring (no maternal contact).

2.4. Behavioural procedures

The offspring's body weight was recorded at PD10, 17, 30, 62 and 83 to evaluate their nutritional status. The mice were observed to evaluate spontaneous behaviour during adolescence (starting at PD30) with a maximum discrepancy of three days due to date of birth. For some experiments, the behaviour of adult mice (starting at PD90) was also evaluated (spontaneous locomotor activity, elevated plus maze, tail suspension test, saccharin preference test and passive avoidance test). All tests were carried out between 8:30 and

15:00h. Animals were transferred to the experimental room ≥30 min before the test to acclimatize them to the test environment (Fig. 1). Distinct groups of mice were used to evaluate adolescent and adult behaviour for all the experiments performed. The spontaneous locomotor activity, the elevated plus maze test and the tail suspension test were performed in the same group of mice. Distinct groups of animals were used for the experiments related to pain threshold, passive avoidance model and the saccharin test, respectively.

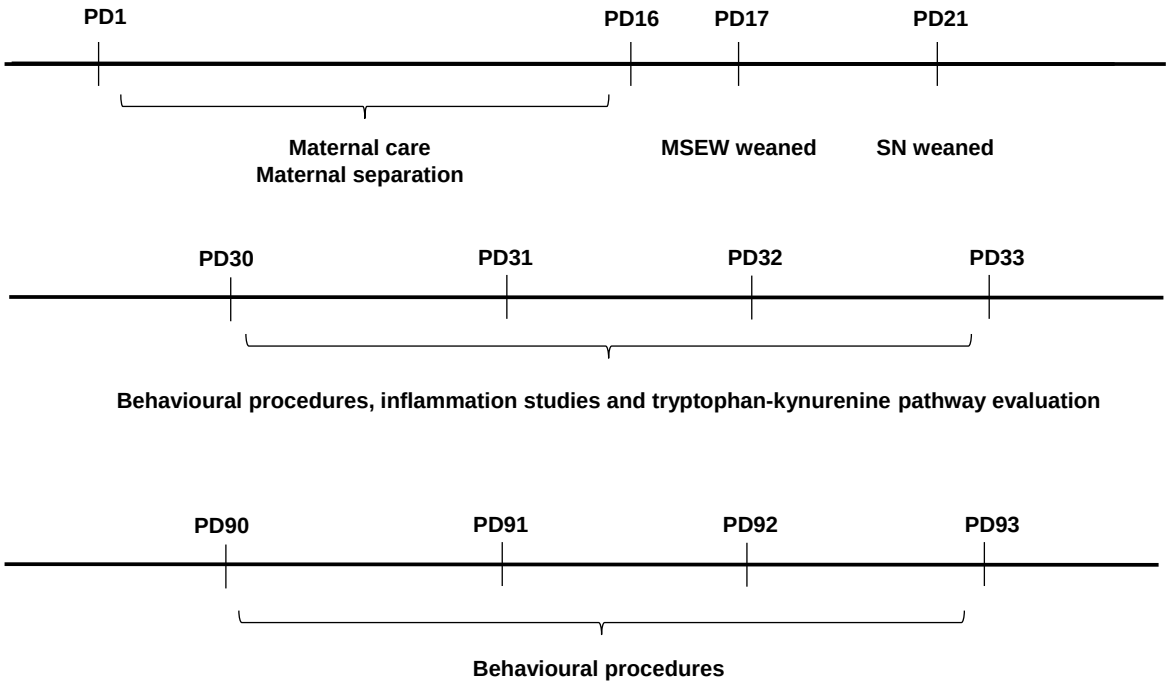

Figure 1. Experimental schedule. See Material and Methods for details. Postnatal day (PD); maternal separation with early weaning (MSEW); standard nest (SN).

2.4.1. Basal Locomotor Activity

Animals were evaluated for spontaneous activity, as previously described (Ros-Simó *et al.*, 2012): horizontal (deambulations) and vertical (rearings) movements were recorded automatically for 20 min on PD31-33 and PD91-93 respectively using locomotor activity boxes (24 x 24 x 24 cm) (LE8811 IR, Panlab S.L., Barcelona, Spain) in a low luminosity room (20 lux) with white noise.

2.4.2. Elevated plus maze

Elevated plus maze (EPM) was performed on PD32-35 and PD92-95 respectively (Panlab S.L., Barcelona, Spain) using a similar procedure to that reported previously (Pellow *et al.*, 1985; Simonin *et al.*, 1998). Briefly, a black maze was elevated 30 cm above the ground and illuminated from the top (100 lux). Each mouse was placed in the centre of the maze for 5 min. The percentage time spent in the open arms and percentage of entries into the open arms were recorded. The total number of entries (placing all four paws in the arm) was also quantified.

2.4.3. Tail suspension test

Mice underwent the tail suspension test on PD33-36 and PD93-96, as described previously *(*Steru *et al.*, 1985; Aso *et al.*, 2008). Briefly, each mouse was suspended individually (using adhesive tape attached 1 cm from the tip of the tail) 50 cm above a bench top for 6 minutes. The percentage time the animal was immobile during this interval was recorded.

2.4.4. Saccharin test

Saccharin test was performed on PD 34-40 and PD94-100 in a different group of mice. Mice were individually housed and exposed to a saccharin solution (0.33% w/v) (Sigma-Aldrich, Madrid, Spain) and tap water during 72 h, according to Lu *et al.*, (2005) and Disse *et al.*, (2010) with minor modifications. The position of the bottles was switched each 24 h, in order to prevent a possible effects of side preference in drinking behaviour. No previous food or water deprivation was applied to the mice before the test. The consumption of water and saccharin solution was evaluated simultaneously to all experimental groups by weighing the bottles every 24 h. Mice were also weighed every day. A control cage without animals was placed to control the amount of liquid spontaneously loss from the bottles. The saccharin preferences were calculated at the time points 24 h, 48 h and 72 h after the exposure to saccharine solutions, according to the following ratio: saccharin intake (g)/[saccharin intake (g) + water intake (g)] × 100.

2.4.5. Electrical nociceptive threshold

A separate group of adolescent mice (PD30) were used to assess the pain threshold to electrical stimulus in accordance to a previously procedure (Martin *et al.*, 2002; Tsuji *et al.*, 2003) with minor modifications. We used the dark compartment of the passive avoidance device (see 2.4.5 Passive avoidance section). Mice were allowed 5 min to habituate to the

compartment before a range of inescapable shocks exposure. During this period, locomotor activity was evaluated by measuring the number of the rearings and the squared crossed (4.6-4.8 cm). The electric foot shock delivery consisted in 10 shocks spaced 30 s at 0.5 mA and 3 s of duration. The number of jumps (all paws off the grid floor) and number of vocalizations during the trial were recorded. The mice used to evaluate the electrical nociceptive threshold were not used for other behavioural procedure.

2.4.6. Passive avoidance test

Two separate groups of animals underwent the passive avoidance test, in adolescence (PD30-33) and adulthood (PD90-93). This test was conducted as previously described (Saavedra *et al.*, 2013) with minor modifications. The mice involved in this test did not undergo any other behavioural procedure. The experiment was conducted in a device divided into a weakly and brightly lit compartment (2-5 and 160 lux, respectively; dimensions, 19 x 19 x 27 cm) (Panlab S.L., Barcelona, Spain). The dark chamber had a stainless steel grid floor for shock delivery. On the acquisition day, each mouse was placed into the bright compartment. A sliding door between the compartments was opened after 30 s, and the latency to enter the dark compartment was recorded for up to 90 s. Upon entering the dark compartment, the door was closed and mice received a foot shock (0.5 mA, 3 s), and were immediately removed from the apparatus. The mice were returned to the brightly lit compartment 24 h later, and the procedure was repeated but omitting the foot shock (retention trial). The latency to enter the dark compartment was recorded for up to 300 s.

2.5. Inflammation studies

2.5.1. Evaluation of neuroinflammatory responses

On PD30, we evaluated microglia activation in naïve animals to study neuroinflammatory responses in the PFC and three hippocampal regions, namely the cornu ammonis 1 (CA1) and 3 (CA3), and the dentate gyrus (DG). We evaluated the presence of ionized calcium-binding adapter molecule 1 (Iba1) in microglia by immunofluorescence using rabbit polyclonal anti-Iba1 staining (1:300; Wako Pure Chemical Industries, Ltd., Japan), as previously reported (Tourino *et al.*, 2010; Ros-Simo *et al.*, 2012). Mice were anesthetized with a ketamine/xylazine mixture (100 and 20 mg/kg, respectively), and perfused transcardially with 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde. The brain was removed and postfixed in the same solution for 4h and cryoprotected in 30% v/v sucrose in 0.1 M in PB (pH 7.4; 24h at 4°C). After freezing in dry ice, the brain was sliced into 35 μm coronal

sections. A mouse brain atlas (Paxinos and Franklin, 2004) was used to identify the anatomical location of the PFC, CA1, CA3 and DG (3 samples per area per mouse, evaluated bilaterally). Floating brain sections were washed three times in 0.1 M phosphate buffer saline (PBS) and incubated in 3% v/v normal donkey serum (Jackson ImmunoResearch, Laboratories. Inc, West Grove, PA, USA) and 0.3% triton X-100 (Sigma-Aldrich, Madrid, Spain) for 2h at room temperature. Sections were incubated at 4ºC overnight with the previous primary antibody. They were then washed three times for 10 min in 0.1 M PBS, and incubated at room temperature for 2h with a fluorescent secondary antibody, namely donkey anti-rabbit IgG Alexa Fluor 488 (1:500; RD systems, Barcelona, Spain). Finally, sections were mounted on slides with a fluorescence mounting medium composed of Mowiol 40–88 (Sigma-Aldrich), 87% glycerol, water, and 2,5%1,4-diazabicyclo-[2.2.2]octane, and coverslipped for microscopy and photography.

2.5.2. Image analysis

Three images were taken of each brain structure bilaterally. Sample areas were visualized under a 20X or 40X objective in a Leica DMR microscope, and digitized using a Leica DFC 300 FX digital camera (Vashaw Scientific Inc, Atlanta, USA). Microglia cells were quantified using ImageJ and samples stained with Iba1 were selected. The background was subtracted by adjusting the detection threshold density, and we only considered the signal density above the threshold. The investigator was blind to the groups analysed. The number and percentage of stained pixels per area was measured automatically, and the average of each sample was calculated.

2.6. Tryptophan-kynurenine pathway analysis

2.6.1. Chemicals and reagents

TRP, 5-HT, 5-hydroxyindoleacetic acid, KYN, kynurenic acid, 3-hydroxykynurenine, xanthurenic acid, and ammonium formate (HPLC grade) were obtained from Sigma-Aldrich (St Louis, MO, USA), ritalinic acid from Steraloids Inc. (Newport, RI, USA), and formic acid (liquid chromatography-tandem mass spectrometry (LC/MS) grade) and methanol (LC gradient grade) from Merck (Darmstadt, Germany). Ultrapure water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

2.6.2. Quantification of metabolites by Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

At PD30, naïve mice were sacrificed by decapitation, the brain was rapidly removed, and the PFC was dissected using a brain tissue blocker. All samples were immediately frozen in dry ice and stored at -80ºC. For tissue 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 internal standard solution (ISTD) (ritalinic acid 1 µg/mL in methanol:water (1:1) with 20 mM ascorbic acid) were added to the tissue, which was then homogenized using a sonicator. Samples were centrifuged for 10 min (10.000 g at 4ºC) and the supernatant was kept on ice until analysis. 75 µL of extract were diluted with 75 µL of water for quantification of TRP, 5-HT, 5-hydroxyindoleacetic acid and KYN. 500 µL of extract were evaporated under nitrogen at 20ºC for quantification of kynurenic acid, 3-hydroxykynurenine and xanthurenic acid. The dry residue was dissolved in 75 µL of 0.5 M acetic acid. 20 µL of extract were injected into the LC-MS/MS system, which consisted of a triple quadrupole (Xevo) mass spectrometer (Waters Associates, Milford, MA, USA) coupled to an Acquity UPLC system (Waters Associates) for chromatographic separation. LC separation was performed using an Acquity BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 µm) (Waters Associates), at a flow rate of 300 µL min^{-1}. The mobile phase solvents were water and methanol, each containing 0.01% v/v formic acid and 1 mM ammonium formate. The gradient and Selected Reaction Monitoring (SRM) method are detailed in supplementary information. Analyte quantification was based on the integral of the analyte and ISTF peaks, and a calibration curve constructed before and after the batch.

2.7. Statistical analysis

We compared the effects of maternal manipulation on maternal care using two-way ANOVA (group and day), followed the Bonferroni post-hoc test. We evaluated the effects of rearing conditions on offspring body weight, behavioural performance, neuroinflammatory response, and tryptophan-kynurenine pathway activity using a two-way ANOVA (group and sex*)*, followed the Bonferroni post-hoc test. Data are represented as mean ± SEM. A p-value < 0.05 was considered statistically significant. Data were analyzed using SPSS v19.

3. Results

3.1. Maternal separation alters spontaneous maternal care

To study the effect of rearing conditions on maternal behaviour, spontaneous maternal care was recorded 3 times every day from PD1 to 16 (Fig. 2). Two-way ANOVA of ***arched-back nursing, blanket posture*** and ***off-nest behaviour*** showed group and day effects, and an interaction between these factors (except in the number of times mother was out of the nest) (Table 1). Bonferroni post-hoc analysis calculated for ***arched-back nursing*** (rearing conditions) showed significant differences between the SN and MSEW groups from PD3 to PD8 ($p<0.05$), indicating that MSEW mothers exhibited the arched-back posture more often than SN mothers (Fig. 2A). Post-hoc analysis calculated for ***blanket posture*** (rearing groups) showed significant differences between SN and MSEW groups at PD9 and PD 14 ($p<0.01$) and from PD10 to PD15 ($p<0.05$), indicating that MSEW mothers exhibited the blanket posture more often than SN mothers (Fig. 2B). Post-hoc analysis for ***off-nest behaviour*** (rearing groups) showed significant differences between SN and MSEW groups at PD3 and from PD9 to PD 14 ($p<0.05$), and from PD4 to PD8 ($p<0.01$). These results show that MSEW mothers spent less time out of the nest than SN mothers (Fig. 2C). We observed no significant differences for other parameters related to maternal care.

Table 1. Two-way ANOVA for maternal care evaluated from PD1 to PD16.

	Maternal care					
	Arched-back nursing		**Blanket posture**		**Off-nest**	
	F	**P <**	**F**	**P <**	**F**	**P <**
Rearing Group	F(1,8)=21.160	0.01	F(1,8)=19.054	0.01	F(1,8)=24.689	0.01
Day	F(15,105)=102.230	0.001	F(15,105)=100.616	0.001	F(15,105)=54.173	0.001
RGxD	F(15,105)=7.917	0.05	F(15,105)=9.794	0.01	F(15,105)=0.81	NS

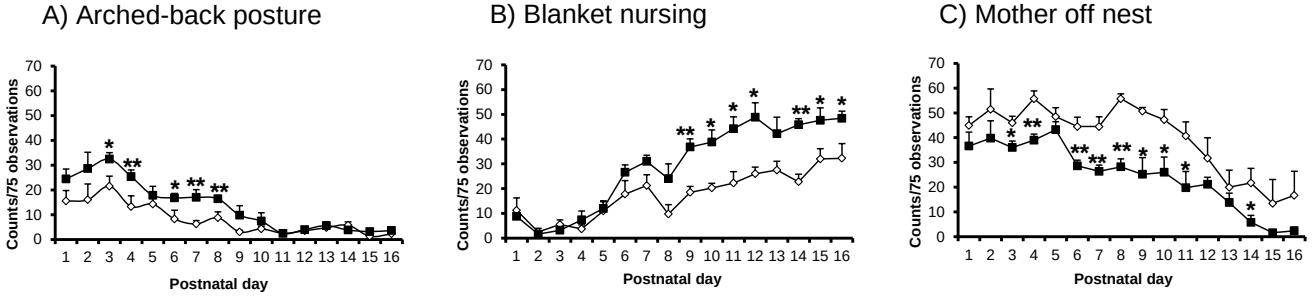

Figure 2. Effect of maternal separation on maternal behaviour. Maternal behaviours were evaluated three times per day from PD1 to 16 (8:15h, 17:30h and 20:15h): arched-back posture (Panel A), blanket nursing (Panel B) and off-nest behaviour (Panel C). White symbols represent SN group of mice; Black symbols represent MSEW group of mice. Data are expressed as the mean daily (± SEM) count (75 observations/day) of each behaviour, N=4-5 mothers per group. * $p < 0.05$; ** $p < 0.01$ rearing groups comparisons.

3.2. Maternal separation does not affect body weight

Body weight was measured in a group of mice at PD10, 17, 30, 62 and 83. Significant differences were observed between rearing groups at PD10 and PD17. Two-way ANOVA calculated for body weight at PD10 and PD17 showed a rearing group effect ($F(1,59)=27.183$; $p<0.001$) and ($F(1,59)=17.211$; $p<0.001$), respectively, sex effect ($F(1,59)=20.898$; $p<0.001$) and ($F(1,59)=8.7791$; $p<0.001$), respectively, without interaction between both factors. Bonferroni post-hoc analysis showed that mice from MSEW group had lower body weight than mice from SN in male and female ($p<0.001$). Moreover, male mice exhibited higher body weight than female in SN group ($p<0.001$) and MSEW group ($p<0.05$) (data not shown).

3.3. Maternal separation induces changes in locomotor activity among offspring

Spontaneous locomotor activity was evaluated in adolescent mice (PD31-33; Fig. 3A and B) and in adulthood (PD91-93, Fig 3C and D). Two-way ANOVA for ***horizontal activity*** (deambulations) showed a rearing group effect ($F(1,71)=9.560$; $p<0.01$), but no sex effect, and no interaction between these two factors. Bonferroni post-hoc test calculated for horizontal activity showed that male mice from the MSEW group exhibited lower locomotor activity than those from the SN group ($p<0.05$; Fig. 3A). Two-way ANOVA of ***vertical activity*** (rearing) also showed a significant rearing group effect ($F(1,71)=5.416$; $p<0.05$), but no sex effect and no interaction between these two factors. Post-hoc analysis indicated that male mice from the MSEW group exhibited a lower vertical activity than those from the SN group ($p<0.05$) (Fig. 3B). We observed no significant differences in locomotor activity in adult mice (PD91-93) (Fig. 3C and D).

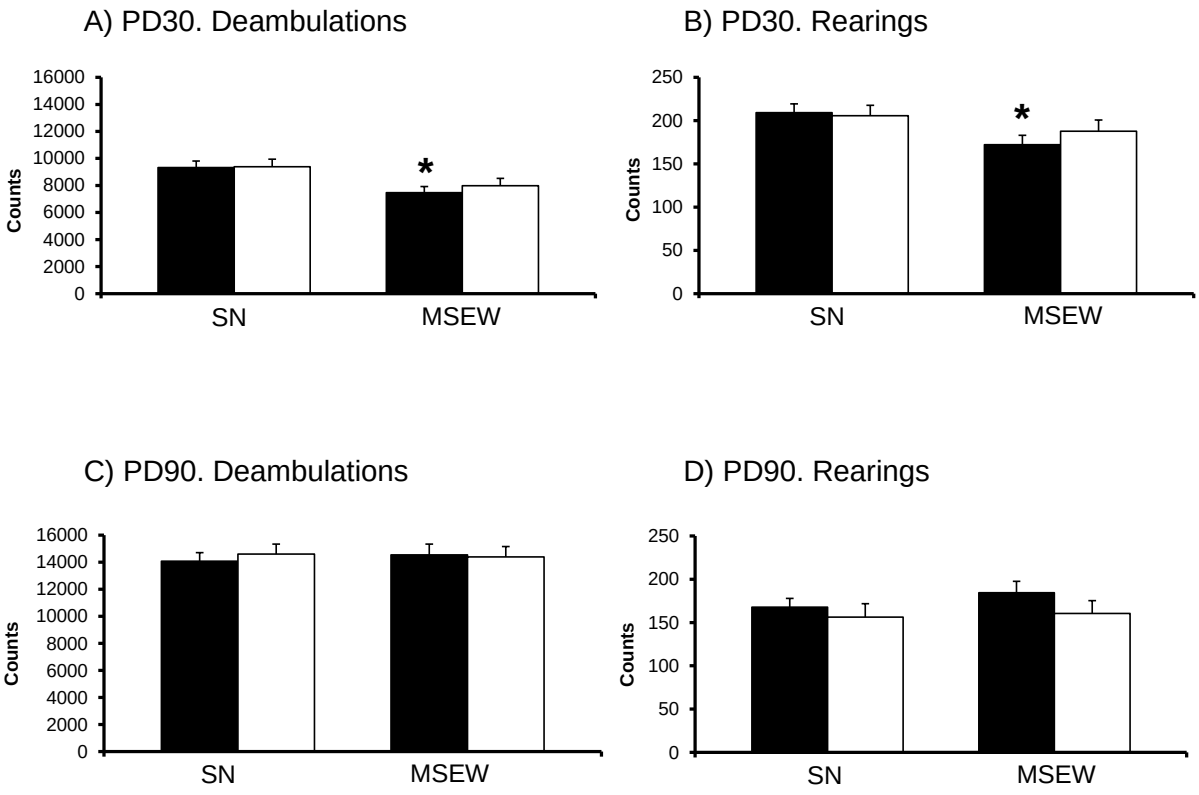

Figure 3. Effects of maternal separation on spontaneous locomotor activity among offspring. Horizontal activity (deambulations) (A and C) and vertical activity (rearings) (B and D) were evaluated in male and female adolescent offspring on PD31-33 and PD91-93. Black bars represent male groups of mice and white bars represent female groups of mice. Data are expressed as the mean (± SEM) of photocell counts during a 20 min period. N=14-23 mice per group at PD31-33 and N=16-18 at PD91-93. * $p < 0.05$ rearing groups comparisons.

3.4. Effect of maternal separation on elevated plus maze

Mice were exposed to the EPM during adolescence, at PD32-35, and in adulthood, at PD92-95 (separate groups of mice for PD32-35 and PD92-95 experiments).

For adolescent mice (PD32-35), two-way ANOVA calculated for ***the percentage time spent in open arms*** showed a rearing group effect ($F(1,63)=12.609$; $p<0.001$), but no sex effect, and no interaction between these two factors (Fig. 4A). Bonferroni post-hoc test showed lower percentage of time spent in the open arm in the MSEW group than in the SN group for both males ($p<0.05$) and females ($p<0.01$) mice (Fig. 4A). Two-way ANOVA calculated for the ***percentage of entries into the open arms*** of the maze showed a rearing group effect ($F(1,63)=21.609$, $p< 0.001$), but no sex effect, and no interaction between these two factors (Fig. 4B). Post-hoc analysis showed that mice from the MSEW group made significantly less entries into the open arms of the maze than mice from the SN group, in both males and

females ($p<0.01$) (Fig. 4B). We observed no differences in the ***total number of entries into the maze***, indicating that there was no significant difference in general activity between the two experimental groups in this model (Fig. 4C).

We evaluated the response in the EPM during adulthood (PD92-95). Our results indicate that females were more sensitive to the deleterious effects of maternal separation in this model, and that these effects persisted into adulthood. Two-way ANOVA for the ***percentage time spent in open arms*** showed a significant rearing group effect ($F(1,58)=6.875$; $p<0.05$), but no sex effect and no interaction between these two factors (Fig. 4D). Bonferroni post-hoc analysis showed that female adult mice from the MSEW group exhibited a higher anxiety-like responses that those from the SN group ($p<0.05$). Two-way ANOVA for the ***percentage of entries into the open arms*** of the maze showed a rearing group ($F(1,58)=15.909$, $p< 0.001$) effect and sex effect ($F(1,58)=4.120$, $p< 0.05$), but no interaction between these factors. MSEW female mice showed less number of entries into open arms than SN mice ($p<0.001$) (Fig. 4E). We also found a sex effect in the SN group. Post-hoc analysis showed than females performed a higher number of entries in the open arms than male mice ($p<0.05$). No difference in the ***total number of entries into the maze*** (Fig. 4F).

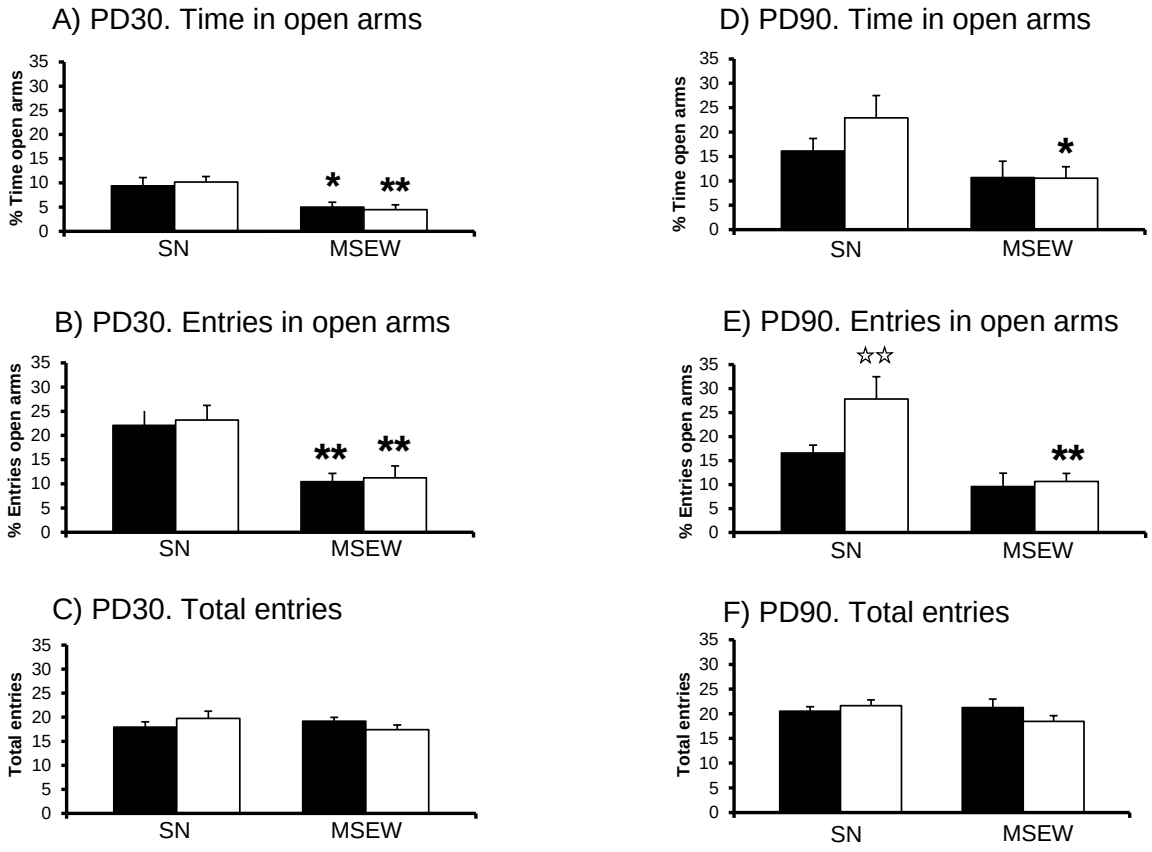

Figure 4. Effects of maternal separation in elevated plus maze. The percentage time spent in open arms (Panels A and D), the percentage of entries into open arms (Panels B and E) and the number of total entries (Panels C and F) were assessed in male and female offspring on PD32-35 and PD92-95. Black bars represent male groups of mice and white bars represent female groups of mice. Data are expressed as the mean ± SEM, N=14–19 mice per group at PD32-35, and N=13-16 mice per group at PD92-95. * $p < 0.05$, ** $p < 0.01$; rearing groups comparisons.

3.5. Effect of maternal separation on tail suspension test

The effects observed in the tail suspension test were evaluated in female and male adolescent mice (PD33-36) and at adulthood (PD93-96). Two-way ANOVA of the ***time spent immobile*** showed a significant rearing group ($F(1,75)=37.510$; $p<0.001$) and sex effect ($F(1,77)=15.270$; $p<0.001$), but no interaction between these two factors. Bonferroni post-hoc test showed that MSEW mice spent more time immobile than SN mice, in both males ($p<0.01$) and females ($p< 0.01$) (Fig. 5A). We also observed a significant sex effect. Post-hoc analysis showed a significant difference in time spent immobile between males and females from the MSEW group ($p<0.01$) (Fig. 5A). Interestingly, the greater despair behaviour observed in the MSEW group persisted into adulthood (PD93-96) in both males and females. Consequently, two-way ANOVA revealed a significant rearing group effect ($F(1,65)=18.011$; $p<0.001$), but no sex effect, and no interaction between these factors. Subsequent post-hoc analysis showed that mice from the MSEW group spent more time immobile than those from the SN group, in both males ($p<0.01$) and females ($p<0.05$) (Fig. 5B).

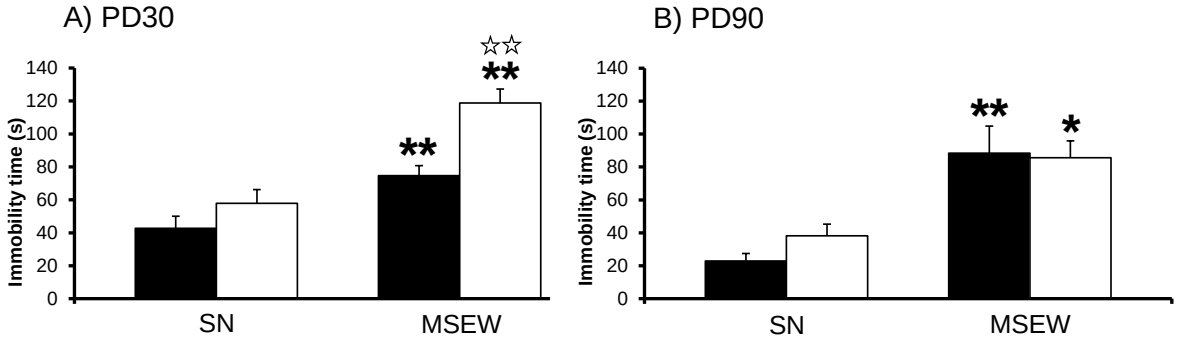

Figure 5. Effects of maternal separation on tail suspension test. Time spent immobile was evaluated in male and female offspring at PD 33-36 (A) and PD 93-96 (B). Black bars represent male groups of mice and white bars represent female groups of mice. Data are expressed as the mean ± SEM of time spent immobile (s), N =18-20 mice per group at PD33-36, and N = 14-18 mice per group at PD93-96). * $p < 0.05$, ** $p < 0.01$; rearing groups comparisons. Two white stars $p < 0.01$ sex comparisons.

3.6. Effect of maternal separation on saccharin preference test

Saccharin test was performed in adolescent mice (PD34-40) (females and males) to evaluate the anhedonia-like effects in mice. We evaluated saccharin preference over water and saccharin intake. Two-way ANOVA for ***preference of saccharin at 24h, 48h*** and ***72h*** revealed group effect (except at 48h) and sex effect (except 24h) without interaction between both factors (Table 2). Bonferroni post-hoc of ***saccharin preference at 24h*** of group factor showed reduced saccharin preference in MSEW mice when compared with SN mice, in both males ($p<0.01$) and females ($p<0.05$) (Fig. 6). Post-hoc analysis of ***saccharin preference at 48h*** indicated that males showed decreased saccharin preference when compared with females, in SN group ($p<0.01$) and MSEW group ($p<0.05$) (Fig. 6). Bonferroni post-hoc analysis of ***saccharin preference at 72h*** revealed that males showed reduced saccharin preference when compared with females, in both SN group ($p<0.05$) and MSEW group ($p<0.01$) (Fig.6).

At adulthood (PD93-96), two-way ANOVA for ***preference of saccharin at 24h, 48h*** and ***72h*** revealed no significant differences between rearing groups, no sex effect and no interaction between both factors (Table 2).

Table 2. Two-way ANOVA calculated for saccharin test.

	% Saccharin preference					
	24h		**48h**		**72h**	
PD30	**F**	**P**	**F**	**P**	**F**	**P**
Rearing group	F(1,32)=14.744	<0.001	F(1,32)=1.031	NS	F(1,32)=5.983	0.05
Sex	F(1,32)=3.744	NS	F(1,32)=15.360	<0.001	F(1,32)=14.214	<0.001
GxS	F(1,32)=0.085	NS	F(1,32)=0.245	NS	F(1,32)=0.024	NS
	24h		**48h**		**72h**	
PD90	**F**	**P**	**F**	**P**	**F**	**P**
Rearing group	F(1,57)=3.441	NS	F(1,57)=0.767	NS	F(1,57)=1.907	NS
Sex	F(1,57)=1.095	NS	F(1,57)=4.294	NS	F(1,57)=0.177	NS
GxS	F(1,57)=0.238	NS	F(1,57)=0.002	NS	F(1,57)=0.001	NS

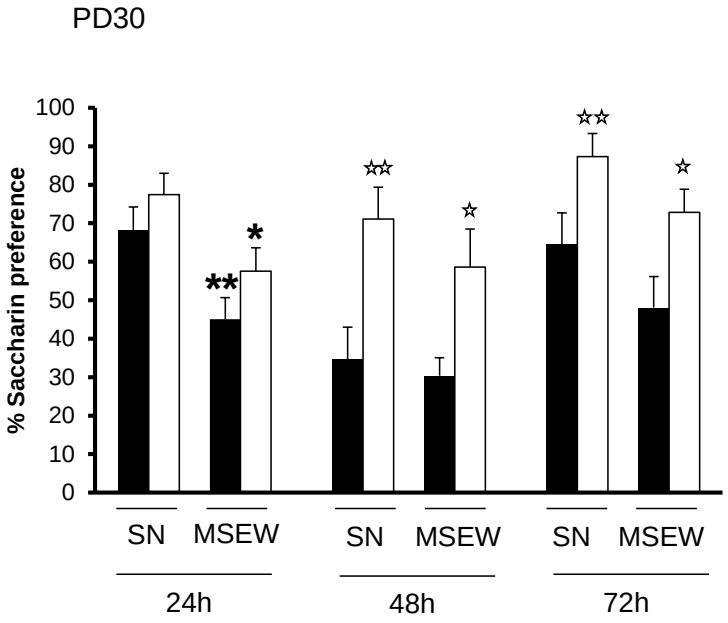

Figure 6. Effects of maternal separation on the saccharin preference test at adolescence. Saccharin test was evaluated at PD34-40 in males and females mice. Saccharin intake was calculated as saccharin preference. Black bars represent male groups of mice and white bars represent female groups of mice. Data are expressed as mean ± SEM, N=7-8 mice per group. * $p < 0.05$, ** $p < 0.01$; rearing groups comparisons. One white star $p < 0.05$; two white stars $p < 0.01$ sex comparisons.

3.7. Nociceptive threshold

We evaluated the nociceptive threshold in mice by measuring the initial behavioural reactivity to electric foot-shock exposure in mice at PD30 (female and male) (Table 3). No significant differences were found between groups in the foot-shock reactivity (number of jumps and vocalisations), indicating a similar basal response. Two-way ANOVA for the number of jumps showed no rearing group effect ($F(1,39)=0.382$; NS), no sex effect ($F(1,39)=0,266$; NS), and no interaction between both factors ($F(1,39)=0,005$; NS) (Table 3). Moreover, no significant differences between rearing groups were found in the locomotor activity (number of rearings and squares crossed) during the habituation period. Two-way ANOVA for the number of rearing and squares crossed showed no rearing group effect ($F(1,39)=0.51$; NS) and ($F(1,39)=4.088$; NS), respectively, no sex effect ($F(1,39)=0.110$; NS) and ($F(1,39)=0.985$; NS), respectively, and no interaction between both factors ($F(1,39)=2.082$; NS) and ($F(1,39)=1.105$; NS), respectively) (Table 3).

Table 3. Effects of maternal separation on electric nociceptive threshold.

	Locomotor activity during habituation		Electric foot-shock reactivity	
	Rearings	**Squares crossed**	**Jumping**	**Vocalisation**
SN Male	25.7±4.36	80.3±8.34	30.2±2.94	10±0
SN Female	30.3±5.35	101.2±4.45	28.2±2.94	10±0
MSEW Male	29.9±2.51	94.2±5.64	28.5±3.41	10±0
MSEW Female	26.9±1.71	100.8±7.97	26.9±2.23	10±0

Table 3. Electric foot-shock reactivity was evaluated in male and female offspring on PD30. Data are expressed as mean ± SEM of the number of behavioural parameters observed, N=10 mice per group. No significant differences were observed between groups in any behavioural parameters recorded. (Two-way ANOVA).

3.8. Effect of maternal separation on passive avoidance

We conducted a passive avoidance test to evaluate differences in emotional memory (associative learning) associated with maternal separation. In adolescent mice (PD30-33) (females and males), we did not find differences between rearing groups for ***latency in acquisition trials.*** Two-way ANOVA of ***latency in retention trial*** showed a significant effect of rearing group ($F(1,43)=5.402$; $p<0.05$) and sex ($F(1,43)=6.604$; $p<0.01$), but no interaction between these two factors. Bonferroni post-hoc analysis showed that female MSEW mice exhibited shorter latency in enter the dark compartment than SN mice ($p<0.05$). No differences were observed between rearing groups in males (Fig. 7A). Therefore, there was significant difference between males and females in the response to retention trials ($p<0.01$) (Fig 7A). At adulthood (PD90-93) we observed no differences between rearing groups for ***latency in acquisition trials*** for any of the factors investigated. In adult mice (PD90-93), two-way ANOVA of ***latency in retention trials*** showed a significant rearing effect ($F(1,66) = 13.908$; $p<0.01$), no sex effect, and interaction between these factors ($F(1, 66)=4.340$; $p<0.05$). Bonferroni post-hoc test showed significant differences in retention trials between female MSEW and SN mice ($p<0.01$). These results confirmed that the impaired passive avoidance responses related to maternal separation persists into adulthood in female mice (Fig 7B).

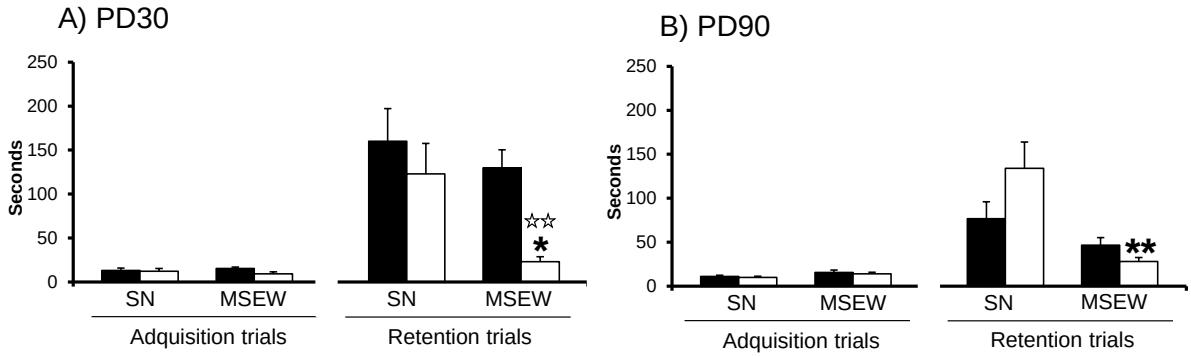

Figure 7. Effects of maternal separation on the passive avoidance test. Latency time in entering the dark compartment in acquisition and retention trials in male and female offspring at PD30-33 (A) and PD90-93 (B) were evaluated. Black bars represent male groups of mice and white bars represent female groups of mice. Data are expressed as mean ± SEM of the latency time, N=10-12 mice per group at PD30-33, and N=14-17 mice per group at PD90-93. * $p < 0.05$, ** $p < 0.01$; rearing groups comparisons. Two white stars $p < 0.01$ sex comparisons.

3.9. Maternal separation induces neuroinflammation in different brain areas

Microglia activation was evaluated using Iba1 staining in the PFC, and the CA1, CA3 and DG regions of the hippocampus. In adolescent mice (PD30), two-way ANOVA of ***the percentage of stained area in the PFC*** showed a rearing group effect ($F(1,15)=8.397$; $p<0.05$), and sex effect ($F(1,15)=1.862$; $p<0.05$), without interaction between these factors. Bonferroni post-hoc analysis showed that female MSEW mice had greater stained area than female SN mice ($p<0.01$) (Fig. 8A). We found no such difference between rearing groups in male mice (Fig. 8A). For the ***hippocampal CA1 region***, two-way ANOVA showed a rearing group ($F(1,15)=22.438$; $p<0.001$) and sex effect ($F(1,15)=6.805$; $p<0.05$), and interaction between these factors ($F(1,15)=8.351$; $p<0.05$). Subsequent post-hoc analysis showed that female MSEW mice had a higher percentage of stained area than SN mice ($p<0.01$; Fig. 8B). We also observed a significant difference between male and female MSEW group of mice ($p<0.01$), indicating that female mice had greater microglia activation than male mice (Fig. 8B). For the ***hippocampal CA3 region,*** two-way ANOVA showed a significant rearing group effect ($F(1,15)=5.721$; $p<0.05$), but no sex effect, and no interaction between these factors. Post-hoc analysis showed that female MSEW mice had higher microglia activation than female SN mice ($p<0.05$) (Fig. 8C). We observed no such differences in males. Finally, for the ***hippocampal DG region,*** two-way ANOVA of microglia activation showed a significant rearing group ($F(1,15)=4.842$; $p<0.05$) and sex effect ($F(1,15)=8.143$; $p<0.05$), but no

interaction between these factors. Bonferroni post-hoc analysis showed that female MSEW mice presented a higher percentage of stained area than SN female mice ($p<0.05$), and that female MSEW mice had higher microglia activation than male MSEW mice ($p<0.05$) (Fig. 8D).

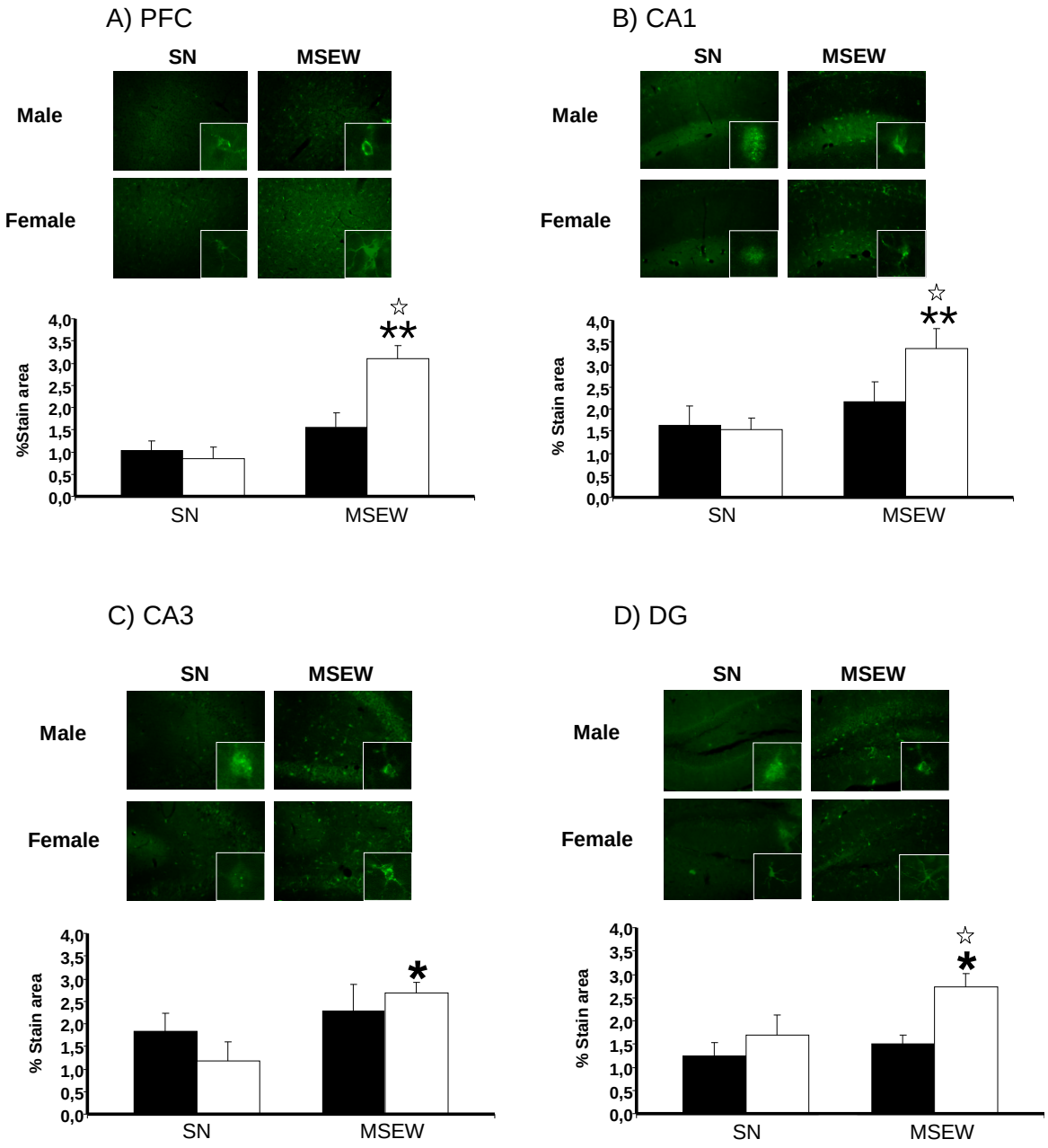

Figure 8.Effects of maternal separation on Iba1 staining. Microglia activation was evaluated using Iba1 in the PFC (A), CA1 (B), CA3 (C), and DG (D) regions of the hippocampus in MSEW and SN mice at PD30. Black bars represent male groups of mice and white bars represent female groups of mice. Data on microglia staining quantification are expressed as mean the percentage of stain area ± SEM, N =4 mice per group. * $p < 0.05$, ** $p < 0.01$; rearing groups comparisons. One white star $p < 0.05$, sex comparisons.

3.10. Maternal separation produces alterations in the tryptophan-kynurenine metabolic pathway.

To assess whether TRP-KYN metabolism was altered in the PFC, a representative brain area that receives dense 5-HT innervation and plays an important role in behavioural and emotional responses (Palazidou, 2012), we evaluated the tryptophan-kynurenine pathway in a distinct group of animals from those used for the behavioural experiments. We quantified the following products of this metabolic pathway in PFC samples from adolescent mice: TRP, 5-HT, 5-hydroxyindolacetic acid, KYN, kynurenic acid, 3-hydroxykynurenine and xanthurenic acid. To evaluate the activity of the main enzymes involved, we also calculated the ratios between precursors and metabolic products of both the TRP-5HT and TRP-KYN pathways. Analysis of the ***5-hydroxyindolacetic acid/5-HT*** ratio showed a rearing group ($F(1,30)=11.138$; $p<0.01$) and sex effect ($F(1,30)=19.917$; $p<0.001$), and an interaction between these factors ($F(1,30)=7.668$; $p<0.01$). Bonferroni post-hoc analysis showed that female MSEW mice have a higher ***5-hydroxyindolacetic acid/5-HT*** ratio than female SN mice ($p<0.01$). We observed no difference in male mice (Table 4). Analysis of ***kynurenic acid*** levels showed a significant rearing group effect ($F(2,28)=14.854$; $p<0.001$), but no sex effect, and no interaction between these two factors. Bonferroni post-hoc test showed that male MSEW mice had lower kynurenic acid levels than male SN mice ($p<0.01$) (Table 4). Two-way ANOVA of the ***kynurenic acid/KYN*** ratio showed a significant rearing group effect ($F(1,28)=15.226$; $p<0.001$), but no sex effect, and no interaction between these factors. Post-hoc analysis showed that MSEW mice had a higher kynurenic acid/KYN ratio than SN male ($p<0.01$) and females ($p<0.01$) mice (Table 4). We observed no differences between rearing groups in ***3-hydroxykynurenine*** levels and the ***3-hydroxykynurenine/KYN* ratio**. Analysis of the ***3-hydroxykynurenine/kynurenic acid ratio*** showed a significant rearing group ($F(1,27)=19.379$; $p<0.001$) and sex effect ($F(1,27)=15.067$; $p<0.001$), but no interaction between these two factors. Post-hoc test showed that MSEW mice had a higher 3-hydroxykynurenine/kynurenic acid ratio than SN mice, in both males ($p<0.01$) and females ($p<0.05$) groups of mice (Table 4). These results reflected an imbalance in the TRP-KYN pathway in MSEW mice, suggesting that this pathway is involved in behavioural alterations induced by early life experiences.

Table 4. Effects of maternal separation on metabolites of the tryptophan-kynurenine pathway in the PFC.

	SN		MSEW		
	Male	**Female**	**Male**	**Female**	
TRP	438,6±28,6	472,2±36,6	431,2±17,2	590,4±119,4	
5-HT	61,61±6,15	51,32±6,05	75,84±4,28	65,13±8,10	
5-HT/TRP	2,70±0,30	2,14±0,15	3,53±0,18	2,43±0,32	
5-HIAA	15,34±1,49	29,33±3,24	17,44±1,13	25,84±2,88	
5-HIAA/5-HT	5,25±0,59	12,22±1,33	4,70±0,44	6,32±1,20	**
KYN	6,03±0,60	4,71±0,69	5,13±0,59	6,94±0,88	
KYN/TRP	0,28±0,03	0,20±0,02	0,23±0,02	0,26±0,04	
KA	0,45±0,05	0,41±0,06	0,18±0,03	** 0,32±0,04	
KA/KYN	1,47±0,12	1,87±0,34	0,71±0,09	** 1,01±0,08	**
3-HK	0,80±0,11	0,46±0,09	0,71±0,10	0,60±0,03	
3-HK/KYN	2,75±0,40	1,90±0,11	2,78±0,31	1,72±0,12	
3-HK/KA	37,40±5,97	25,62±4,63	73,32±8,44	** 40,40±4,07	*
XA	nd	1,20±0,25	nd	0,86±0,22	
XA/KYN	nd	3,32±0,55	nd	3,58±0,62	

Table 4. Metabolites of the tryptophan-kynurenine pathway were evaluated in the PFC of male and female offspring on PD30. Data are expressed as mean ± SEM of the pg/mg, N=6-8 mice per group. Tryptophan (TRP), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), kynurenine (KYN), kynurenic acid (KA), 3-hydroxykynurenine (3-HK), xanthurenic acid (XA), not detected (nd). * $p < 0.05$, ** $p < 0.01$; rearing groups comparisons.

4. Discussion

In this study, we have evaluated for the first time the behavioural and neurochemical effects of a model of maternal separation reflecting early life neglect on emotional disorders and neuroinflammation. Animals raised under the MSEW model showed enhanced spontaneous maternal care, probably to compensate for maternal separation. However, increased maternal care failed to recover MSEW-related impairment of neurobiological functions and behaviours during adolescence, like locomotion deficits, responses to stress and the manifestation of anhedonia related to a lack of preference for saccharine. We found that several of these alterations were more striking in females, reflecting their greater vulnerability to stress, as previous reported (Becker *et al.*, 2007; Fuentes *et al.*, 2014).

Most strikingly, behavioural changes that appeared during adolescence generally persisted into adulthood. The behavioural alterations here identified are unlikely to be due to nutritional changes because the differences in body weigh observed during lactation (PD10 and PD17) disappeared several days after weaning (PD30) and did not re-emerge during the adolescence or adulthood. Previous studies using similar procedures revealed apparently conflicting results regarding the impact of maternal separation procedure in the offspring's body weight. Thus, whereas George *et al.*, (2010) did not observe differences in the body weight of mice, other studies (Fabricius *et al.*, 2008; McIntosh *et al.*, 1999) described a decrease in the offspring´s body weight after maternal separation that was later recovered at PD65 (Fabricius et al., 2008) or that remained until adulthood (Mclntosh *et al.*, 1999). In these protocols no special chow were availed in mice´s home cage. Changes in nutritional state are relevant since previous studies using MSEW have associated this model with the incidence of anatomical and functional alterations in the central nervous system, including smaller brain size and developmental deficits, although they did not find differences in the body weight (Carlyle et al., 2012; Duque et al., 2012). Therefore, early life stress experiences and the lack of maternal care represent vulnerability factors that could influence on normal brain development, particularly during early postnatal stages (Greenough *et al.*, 1987; Finlay and Darlington, 1995; Johnson, 2001; Kolb and Gibb, 2011; Cai *et al.*, 2015), spoiling normal development of emotional and cognitive behaviours in adult mammals (Kaffman and Meaney, 2007).

Interestingly, the maternal behaviour observed in mice from MSEW group was enhanced during the interval between post-maternal separation periods, and was attenuated just before maternal separation, when mothers and offspring had been together for some time (Pryce *et al.*, 2001b; Llorente-Berzal *et al.*, 2011). In addition, MSEW mothers progressively increased

care during the experiment, suggesting that they development a sensitized behaviour associated with repeated periods of separation from their offspring (Own and Patel, 2013). Previous studies proposed that enhanced maternal care after periods of stress during early life could counteract the deleterious effects of neglect on behaviour (Macri *et al.,* 2008). In fact, the consequences in adulthood of early exposure to stress depend on the severity of neonatal stress; exposure to moderate stress during the neonatal period is associated with reduced stress reactivity in adulthood (Macri *et al.,* 2011), producing greater ability to cope with stressful situations. Previous studies showed that early weaning alone without maternal separation also induces behavioural and neurochemical alterations in rodents (Kikusi *et al.,* 2009; Kikusi and Mori, 2009). In these previous studies, mice were weaned at PD14, and showed at adulthood higher levels of anxiety and aggressive behaviour. In addition, neurochemical deficits were also detected in these mice such as decreased neurogenesis and hippocampal BDNF and increased corticosterone plasma levels. These changes have been also observed in other models that exposed the mice only to maternal separation (Macri et al., 2008; Martini and Valverde, 2012). Nevertheless, our findings suggest that early weaning due to maternal separation has a stronger effect than moderate stress, inducing long-lasting effects.

MSEW mice show disrupted emotional reactivity, and associated neurochemical alterations as a consequence of maternal separation (increased inflammatory responses and alterations in the TRP-KYN metabolic pathway). Maternal separation is associated with enhanced anxiety-like behaviour in the offspring (van Oers *et al.,* 1997; Huot *et al.,* 2001; Martini and Valverde, 2012). Consistent with previous literature, our results demonstrate that both adolescent (male and female) and female adult MSEW mice display greater anxiety, and that this anxiety persists into adulthood in females. These results emphasize the long-lasting emotional effects of maternal separation and female mice's propensity to become anxious in response to stress (Rhodes and Rubin, 1999).

Our study also suggests that male and female MSEW mice exhibit emotional alterations related to an anhedonic-like state and a despair behaviour that persisted into adulthood. Moreover, adolescent male MSEW mice exhibited a hypolocomotor phenotype that is likely related to apathetic behaviour (Sobin and Sackeim, 1997; Lavretsky *et al.,* 1999). This particular hypolocomotor phenotype was not manifested during adulthood. This fact supports that the emotional alterations observed in mice exposed to MSEW was not due to the impairment in the locomotor activity or a deficient performance in the behavioural models due to locomotor impairments. Thus, the hypolocomotor phenotype was only observed in

adolescent male mice when investigated the spontaneous locomotion (Figure 3), but not in other paradigms that indirectly provide data from locomotion (EPM, habituation for the evaluation of the nociceptive threshold). Additionally, our results showed that adolescent MSEW group of mice reduced the consumption of saccharin, suggesting that early life neglect induced responses related to anhedonia, a core symptom of depressive states that could be observed in rodents after the exposure to a chronic stress (D´Aquila *et al.,* 1997; Harkin *et al.,* 2002; Strekalova *et al.,* 2004), like the MSEW procedure. Our results agree well with previous data showing a reduced in the saccharin consumption in two accepted model to induce a depressive-like state in rodents as chronic mild stress (Harkin *et al.,* 2002; Pijlman *et al.,* 2003; Schweizer *et al.,* 2009), and social defeat (Furay *et al.,* 2011; Shimamoto *et al.,* 2015).

As expected, we also observed a significant difference between sexes in various tests, in which female MSEW mice were more sensitive to adverse emotional conditions, in agreement with experimental (Palanza, 2001; Palanza *et al.,* 2001b; Renoir *et al.,* 2011) and clinical (Kessler, 2003; Altemus, 2006) studies showing that depressive symptoms are more common in females than in males (Kudielka and Kirschbaum, 2005; Sherin and Nemeroff, 2011). Regarding this point, gonadal hormones display profound effects in the central nervous system. Both, estradiol and progesterone influence the expression of anxiety-like behaviour in rodents by acting on estrogenous receptors (Lund *et al.,* 2005; Osterlund *et al.,* 2005; Spiteri *et al.,* 2010 and Tomihara *et al.,* 2010). In this sense, experimental data has shown that estradiol and progesterone play an anxiolytic effect in different procedure to evaluate anxiety in rodents (Zimmerberg and Benton, 1993; Fernandez-Guasti and Picazo, 1997; Mora *et al.,* 1997; Palanza *et al.,* 2001b). In addition, we have also demonstrated that low progesterone and estrogenous levels such as seen in diestrus and in male mice can increase susceptibility to anxiogenic effects induced by cocaine (Martini *et al.,* 2014). Several studies have shown the effects of estrous cycle in the responsiveness of stress (Zimmerberg and Benton, 1993; Fernandez-Guasti and Picazo, 1997; Mora *et al.,* 1997; Palanza *et al.,* 2001b). These studies have shown a reduction in behavioural levels of anxiety during proestrus phase, when levels of ovarian steroids hormones are higher (Butcher *et al.,* 1974). In the present study, the estrous cycle was not considered due to the complexity of the experimental schedule and the different manipulations performed in the mice. Therefore, we cannot discard the participation of hormonal milieu in the emotional alterations observed in female mice after the exposure to MSEW. In this sense, a recent study has shown that maternal separation impaired the influence of estrous cycle on feeding behaviour in rats (Iwasaki and Inoue, 2015).

Clinical studies note that a substantial proportion of depressed patients also manifest cognitive deficit and retardation that affects learning and memory (Eriksson *et al.*, 2012), including declarative memory, executive functions and emotional processing; these deficiencies have been reported to be associated with altered monoaminergic neurotransmission (Tsuji *et al*., 2003; Clark *et al.*, 2009). In fact, acute TRP depletion modulates 5-HT availability in the brain, which plays a crucial role in resistance to distracting negative information (Roiser *et al.*, 2008). In agreement with these findings, female MSEW mice exhibited an impaired performance in passive avoidance task, a learning task that has been associated to emotional learning (Erikssen *et al.*, 2012; Saavedra *et al.*, 2013). In our experimental conditions, the compromised response observed in the group of female MSEW mice could not be attributed to changes in locomotion or in the electric nociceptive threshold. In fact, whereas previous studies have identified a reduction in thermal pain sensitivity after maternal separation (Weaver *et al.*, 2007), our experiments revealed no modifications in the electric nociceptive threshold neither for rearing conditions nor for sex (Table 3). Changes in the performance in passive avoidance test could be attributed to neuroinflammatory reactions induced by the chronic exposure to MSEW (Lykhmus *et al.*, 2015; Elmore *et al.*, 2015).

Therefore, to explore the proposed involvement of the immunological system in the pathophysiology of emotional alterations (Motivala *et al.*, 2005; Miller *et al.*, 2009; Felger and Lotrich, 2013), we explored central inflammatory responses under each rearing paradigm. Our findings show that neuroinflammation occurs in discrete brain areas such as the PFC and hippocampus (CA1, CA3 and DG), which are involved in cognitive functions (Vertes, 2006). Interestingly, we observed enhanced microglial activation in female, but not male, MSEW mice. Microglial cells are a primary source of pro-inflammatory cytokines in the brain. Under our experimental conditions, the highest levels of activated microglia in female mice were observed in the medial PFC and DG, CA1 and CA3 areas of the hippocampus, brain regions that are involved in the control of emotional and cognitive functions (Palazidou, 2012). Interestingly, the hippocampus regulates the hypothalamic-pituitary-adrenal axis, which is known to become deregulated in patients with depression (Nestler, 2014; Cai et al., 2015). In fact, activation of microglial cells was enhanced in the CA1 and CA3 regions in female MSEW mice; these regions are susceptible to damage after the chronic impact of corticoid signalling in the brain due to excessive stress-induced activation of the hypothalamic-pituitary-adrenal axis (Fanselow, 2000). Our findings agree with previous studies showing greater activation of microglia in depressed mice under chronic mild unpredictable stress, an accepted model for inducing depression-like states in rodents (Farooq *et al.*, 2012).

Alteration of the TRP-KYN pathway has previously been associated with impaired functionality of various neurotransmitter systems, including those related to mood (Miller *et al.,* 2009). In microglial cells, KYN is preferentially converted to quinolenic acid, which is neurotoxic; in contrast, kynurenic acid has a neuroprotective role (Muller and Schwarz, 2007; McNally *et al.,* 2008). The impact of MSEW on the activation of microglial cells seems to be important for the development of depressive states (Miller *et al.,* 2009), and could be attributed to an imbalance in TRP-KYN metabolism (Miller *et al.,* 2009; Myint, 2012). Our findings emphasize the deleterious effects of inflammatory reactions on neuronal repair and survival. This is consistent with our previous work showing that a single episode of maternal separation results in decreased brain derived neurotrophic factor in discrete brain areas, including the hippocampus and amygdala (Martini and Valverde, 2012), which shows that negative early life experiences are associated with impaired neuroplasticity. Accordingly, our female MSEW mice showed decreased 5-HT metabolism in the PFC (lower 5-hydroxyindoleacetic acid/5-HT ratio), which leads us to propose that MSEW produces a functional reduction in 5-HT neurotransmission that may contribute to the development of a depressive-like state in MSEW animals. We can speculate that these functional changes in 5-HT neurotransmission are not only due to 5-HT availability. In line with this statement, reduced expression of 5-HT1A receptors is thought to explain anxiety-like behaviour in rats exposed to prenatal stress (White and Birkle, 2001) and maternal separation (Leventopulos *et al.,* 2009; Bravo *et al.,* 2014). Additionally, the PFC is one of the main brain areas in where post-synaptic 5-HT1A receptors are located (Palacios *et al.,* 1990). Thus, the reduction of 5-HT transporter sites and 5-HT1A receptors levels have been observed in the PFC of post-mortem patients of major depression (Lopez-Figueroa *et al.,* 2004), indicating that cortical 5-HT neurotransmission is altered (Le François *et al.,* 2015), in agreement with our present findings. Analysis of the TRP-KYN pathway in MSEW mice also shows a decrease in the kynurenic acid/ KYN ratio, indicating a decrease in the synthesis of kynurenic acid, a metabolite with neuroprotective effects (Sas *et al.,* 2007; Klein *et al.,* 2013). Interestingly, our results regarding the ratio between 3-hydroxykynurenine (neurotoxic) and kynurenic acid (neuroprotective) indicate a significant increase in levels of the former compound in MSEW mice. Therefore, we propose that the KYN metabolic pathway becomes unbalanced in favour of the production of neurotoxic metabolites such as 3-hydroxykynurenine and quinolenic acid. Since we observed no change in 3-hydroxykynurenine levels, we explored the possibility that KYN is metabolized to quinolenic acid through anthranilic acid, an alternative metabolic pathway. However, the concentrations of quinolenic acid from our selected brain areas were

too small to be accurately quantified. Clinical studies have reported an imbalance between neuroprotective and neurotoxic metabolites of the KYN metabolic pathway in serum samples from depressive patients, who had a lower kynurenic acid/KYN ratio than healthy controls, indicating a decrease in the neuroprotective metabolic route (Myint *et al.,* 2007). Additional clinical studies support our hypothesis, and show that quinolenic acid was increased post-mortem in the brains (e.g. the PFC) of patients with major depression and bipolar disorder (Steiner *et al.,* 2011). Furthermore, an increase in KYN and the 3-hydroxyanthranilic/KYN ratio in serum was also observed in adolescents with melancholic depression (Gabbay *et al.,* 2010), which is consistent with our proposal. Taken together, our results demonstrate for the first time that MSEW mice have imbalanced KYN metabolism, which could explain the impaired emotional behaviour observed in these animals. Based on these results, we propose the involvement of KYN metabolic pathways in the pathophysiology of emotional alterations states possibly due to an impaired TRP metabolic route that leads to an insufficient 5-HT production in discrete brain areas.

In conclusion, our results lead us to propose that detrimental early life events such as maternal neglect reproduces most of the behavioural alterations associated with emotional alterations in mice. These alterations persist into adulthood. We also found that females were more sensitive to adverse conditions than males. Our study also supports the notion that imbalanced TRP-KYN metabolism and associated neuroinflammatory reactions underlie these emotional impairments.

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Highlights:

1. MSEW produces long-term emotional alterations in mice that persist into adulthood.
2. MSEW induces neuroinflammation and an imbalanced in tryptophan metabolism.
3. Female mice were more sensitive to emotional perturbations.

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SUPPLEMENTARY INFORMATION

LC Gradient

Mobile phases solvents: (A) Water with formic acid (0.01% v/v) and ammonium formate (1 mM); (B) methanol with formic acid (0.01% v/v) and ammonium formate (1 mM). Analytes were separated using a linear gradient of the percentage of solvent B, as follows: 0 min, 1%; 0.5 min, 1%; 7 min, 40%; 8.5 min, 90%; 9 min, 90%; 9.5 min, 1%; 12 min, 1%. The total run time of the analysis was 12 min.

SRM method

Analytes were determined using an SRM method by acquiring two transitions for each compound (**Table S1**). The most specific transition was selected for quantitative purposes. Data were managed using the MassLynx software.

Analyte	MW	Ionization	Precursor m/z	Cone voltage (V)	Collision energy (eV)	Product m/z
TRP	204	ESI+	205	15	10	188*
5-HT	176	ESI+	177	10	5	160*
5-HIAA	191	ESI+	192	25	20	146*
KYN	208	ESI+	209	15	10	192*
3-HK	224	ESI+	225	15	10	208*
KA	189	ESI+	190	20	20	144*
XA	205	ESI+	206	20	20	160*

Table S1. Molecular weight (MW) and SRM conditions for the analytes studied (* transition used for quantification). Tryptophan (TRP), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), kynurenine (KYN), kynurenic acid (KA), 3-hydroxykynurenine (3-HK), xanthurenic acid (XA).

Figure S1. Schematic representation of the Tryptophan-kynurenine (TRP-KYN) metabolic pathway (adapted from Christmas *et al.*, 2011).