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Limited effects of early life manipulations on sex-specific gene expression and behavior in adulthood

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ABSTRACT

Exposure to childhood adversity is associated with increased vulnerability to stress-related disorders in adulthood which has been replicated in rodent stress models, whereas environmental enrichment has been suggested to have beneficial effects. However, the exact neurobiological mechanisms underlying these environment influences on adult brain and behavior are not well understood. Therefore, we investigated the long-term effects of maternal separation (MS) or environmental enrichment (EE) in male and female CD1 mice. We found clear sex-specific effects, but limited influence of environmental manipulations, on adult behavior, fecal corticosterone metabolite (FCM) levels and stress- and plasticity related gene expression in discrete brain regions. In detail, adult females displayed higher locomotor activity and FCM levels compared to males and EE resulted in attenuation in both measures, but only in females. There were no sex- or postnatal manipulation-dependent differences in anxiety-related behaviors in either sex. Gene expression analyses revealed that adult males showed higher *Fkbp*5 mRNA levels in hippocampus, hypothalamus and raphe nuclei, and higher hippocampal *Nos1* levels. Interestingly, MS elevated *Nos1* levels in hippocampus but reduced *Fkbp*5 expression in hypothalamus of males. Finally, we also found higher *Maoa* expression in the hypothalamus of adult females, however no differences were observed in the expression levels of *Bdnf*, *Crhr1*, *Nr3c1* and *Htr1a*. Our findings further contribute to sex-dependent differences in behavior, corticosterone and gene expression and reveal that the effects of postnatal manipulations on these parameters in outbred CD1 mice are limited.

1. Introduction

Stress vulnerability or resilience depends on the complex interplay between genetic, epigenetic and environmental influences, and each individual show different responses to stress. In keeping, pre-natal period, early childhood and adolescence have been shown to be crucial regulatory windows that impact an individual's stress-sensitivity and resilience [1]. Most human and even animal studies have focused on early life, which is accepted as the most vulnerable stage to stress due to its high impact on numerous neurodevelopmental processes [1,2]. However, these processes represent adaptive physiological and psychological responses of the organism to cope with environmental demands [2]. In other words, exposure to early-life stress or early-life enrichment can have both beneficial and adverse outcomes depending on the subsequent life experience of the organism [3,4]. Early-life stress (ELS) or enrichment (EE) have been shown to impact similar centrally-regulated systems and brain regions such as frontal cortex, striatum and nucleus accumbens, amygdala, hippocampus, hypothalamus and raphe nuclei [5–8], albeit in differing fashions; thus, additional studies assessing the impact of both in parallel are warranted.

Numerous studies have shown that ELS and EE can alter the expression and function of components of the hypothalamic-pituitary-adrenal (HPA) axis, which is involved in the pathogenesis of psychiatric disorders such as anxiety disorders [9], bipolar disorder [10], major depression [11] and ADHD [12]. For example, genetic variance in the corticotrophin-releasing hormone (CRH) receptor (*Crhr1*) gene has been associated with impaired HPA axis reactivity, but only in individuals reporting early life trauma [13,14]. Rodent studies have

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recapitulated these findings as functional polymorphisms in *Crhr1* gene in male CD1 mice act as long-term regulators of HPA axis and stress vulnerability [15]. Another level of the HPA axis that is affected by ELS and EE is the glucocorticoid receptor (GR, encoded by *Nr3c1*) and its important regulator FKBP5, a co-chaperone of heat shock protein 90, encoded by *Fkbp5* [16–22]. Studies have revealed altered GR expression in the dorsolateral prefrontal cortex and hippocampus in patients with mood disorders, [16–19,23]. Human studies have also shown a clear link between *Fkbp5* genetic variance, early life stress and adult psychiatric symptoms [20,21,24], as well as elevated cortisol response in patients with major depression [25]. In keeping, different rodent studies linked EE to reduced *Nr3c1* and *Fkbp5* mRNA levels [22,26]. Such alterations in GR signaling caused by ELS or EE have been associated with increased hypothalamic CRH and brain-derived neurotrophic factor (*Bdnf*) expression, which itself is associated with psychiatric disorders and in antidepressant (AD) actions [27–30] downstream of their effects on monoamine systems [31,32]. Moreover, stress also has an impact on this system, for example, a polymorphism in the monoamine oxidase A (*Maoa*) gene has been shown to mediate the relationship between early childhood maltreatment and adult antisocial behavior and violence [33–35]. Further, a major hypothesis for the delay in the onset of action of ADs is related to their effect at 5-HT1 A autoreceptors (*Htr1a*) [36–38] and recent studies revealed that environmental enrichment in adult rats resulted in elevated levels of *Htr1a* mRNA in hippocampus and increased neural plasticity [39]. In contrast, chronic unpredictable stress lead to downregulation of hippocampal *Htr1a* and impaired corticosterone (main type of glucocorticoid in rodents) responses [40]. In addition, the sexual dimorphism in *Htr1a* expression, and its effect on stress regulation, has been shown by many animal and human studies as females have different raphe-hippocampus feedback control than males under different environmental conditions [37]. Although not a key component of HPA axis, neuronal nitric oxide synthase (*Nos1*) has been implicated in stress-related disorders such as depression, anxiety and schizophrenia [41–44] and been shown to regulate serotonergic neurotransmission [45] and glucocorticoid signaling [45–48]. Inhibition or complete deletion of *Nos1* gene has also been linked to anti-depressive like behavior in both mice and rats [46,49,50].

Sex-specific differences in sensitivity to early life manipulations, such a higher prevalence for psychiatric disorders in females, are continually noted [51,52] but unfortunately overlooked. To date, only limited number of rodent studies investigated both sexes. While several rodent studies have shown different activity levels, anxiety- or depressive-like behavior, stress or drug (e.g. ADs) response in various strains of female mice and rats compared to their males, there are still some inconsistencies in literature [53–57]. ELS and EE have been also shown to differentially influence regulation of some genes and proteins in distinct brain regions, which could trigger neurochemical, behavioral mechanisms and sex-specific regulation of neurotransmitters [58–62]. To date, sex-specific changes have mostly been explained by differences in estrogen and androgen systems that have been also indicated to influence HPA axis regulation [58,59,71]. However, the mechanisms underlying such sex-specific differences are not fully understood and require further investigation.

In a previous study, we showed that sex- and early-life experiences have a strong influence on DNA methylation of the aforementioned genes [63]. Specifically, female offspring were more susceptible to programming by postnatal environmental manipulations. However, whether these epigenetic modifications lead to alterations in gene expression and/or behavior was not assessed. Therefore, the aim of the present study was to determine the sex-dependent effects of early life adversity and environmental enrichment on adult behavior, corticosterone and mRNA expression levels. In both studies, we employed CD1 mice, an outbred stock that has advantages as they resemble the variation in the human population to a better extent than inbred mice.

2. Material and methods

2.1. Animals

Six- to eight-week-old male and female CD1 mice purchased from Charles River Laboratories (Sulzfeld, Germany) (n = 30 females, n = 15 males) were used for breeding. Each male was housed with two females for one week for the mating period and then removed from the cage. Pregnant mice were weighed daily, and the day of birth was considered the postnatal day zero (P0). All mice were housed in Makrolon Type III (840 cm^2) rat cages with their dams (n = 2 per cage, total n = 10 dams per group) until the pups were weaned. Two (male or female) pups per litter, were pseudo-randomly selected for experiments. After weaning, offspring were housed in Makrolon Type II (375 cm^2) mouse cages under a 12 h: 12 h light-dark (lights on at 07:00) cycle at ambient temperature of 21 ± 2 °C and humidity of 55 ± 2%. Food and water were available ad libitum. In each experiment, mice were tested in a randomized order between 10:00 - 16:00 to avoid any daytime-dependent activity differences at ambient temperature of 21 ± 2 °C. Test equipment were cleaned with Aerodesin 2000 (Lysoform, Berlin, Germany) before and after each mouse to avoid olfactory cues. All animal protocols have been reviewed and approved by the review board of the Government of Lower Franconia and the University of Würzburg. Animal experiments were conducted at the Center of Experimental Molecular Medicine (University of Würzburg) according to the Directive of the European Communities Council of 24 November 1986 (86/609/EEC).

2.2. Rearing conditions and experimental design

Mouse pups were kept with their dams until the day of weaning in Makrolon Type III (840 cm^2) rat cages. For the control group (Control), pups were kept under standard housing conditions in their cages with their dams until P21. For environmental enrichment (EE) group, pups were kept in their cages with their dams until P21. In this case, cages were equipped either with one large (wheels, wooden tubes, wooden rockers, wooden roofs) or two small (wooden ladders, mirrors with bells, wooden cylinders) toys which were exchanged weekly to maintain novelty. For maternal separation (MS) group, litters were separated from their dams daily for 3 h (11:00 − 14:00) between P2 and P20. During maternal separation, pups were kept under controlled temperature and humidity conditions. In order to compensate for the lack of body heat of the dam, the litter were irradiated with an infrared lamp (˜30 °C). In order to ensure a sufficient humidity of 55%, the cages were also covered with damp cloths. Temperature and humidity were continuously measured electronically. The pups were weaned on P21. Same-sex and cagemate pups were housed under standard conditions (n = 2 per cage). Offspring were randomly separated into two groups each consisting of three experimental sub-groups (Control, MS, EE). EE continued throughout the experiments. Half of the offspring (n = 10 per group and sex) were used for the behavioral testing starting at the age of 11 weeks. Other half of the offspring (n = 13 per group and sex) were used for gene expression analysis and sacrificed at the age of 6–8 weeks.

2.3. Behavioral testing

2.3.1. Open field (OF)

The open field apparatus was used to assess general locomotor activity and exploratory behavior. The OF apparatus is a square chamber (51 cm x 51 cm) surrounded with black Perspex walls (H: 40 cm). Mice were placed into the OF next to one of the walls facing the arena and allowed to explore the chamber (˜120 lx) for 30 min. Total distance travelled and time spent in the center of the arena (25 cm x 25 cm) were recorded using the VideoMot2 video tracking system (TSE Systems GmbH, Germany).

2.3.2. Light dark box (LDB)

The LDB apparatus consists of two compartments, a dark enclosed compartment (20 × 40 cm, walls: black infraredpermeable plexiglass) and a light compartment (40 × 40 cm, walls: transparent plexiglass) connected via a central floor opening (5 × 6 cm). Each mouse was placed in the dark compartment and allowed to explore both compartments for 5 min. Time spent in the light (˜120 lx) and dark compartments (˜0-5 lx), transitions between compartments, and latency to enter the light compartment (with all four paws) were recorded using the VideoMot2 video tracking system (TSE Systems GmbH, Germany).

2.3.3. Elevated plus-maze (EPM)

The Elevated Plus-Maze (EPM) is a cross-shaped elevated platform (62 cm above floor) consisting of central 5 × 5 cm square with two opposite enclosed arms (L: 30 cm, W: 5 cm, H: 15 cm; inner walls: black infrared-permeable plexiglass) and two opposite open arms (L: 30 cm, W: 5 cm, H: 2 mm). Mice were placed in the central square facing one of the open arms and allowed to explore the apparatus for 5 min. Illumination was ˜110 lx in the open arms, ˜25 lx in the closed arms, and ˜75 lx in the center area. Behavior of the mice such as distance travelled in the apparatus and time spent in the open arms were recorded using the VideoMot2 video tracking system (TSE Systems GmbH, Germany).

2.4. Corticosterone metabolites

Fecal samples were collected from the pair-housed cages at the age of 8 weeks, 24 h after weekly maintenance of the cages and kept at −20 °C for fecal corticosterone metabolites (FCM) analysis. Preparation of fecal samples for the CM measurement was performed as previously described [64]. Fecal samples kept at -20 °C were thawed and incubated at 80 °C for 2 h. Afterwards, they were homogenized with mortar and pestle one by one and 0.05 *g* of each sample was incubated for 30 min with 80% methanol on a multi-vortex at room temperature. After centrifugation for 10 min at 5200 rpm, 0.5 ml aliquot for each sample was stored at -20 °C until analysis. FCM were measured with a 5α-pregnane-3β,11β,21-triol-20-one EIA (enzyme immunoassay) as previously described [65].

2.5. Relative gene expression analysis

2.5.1. Tissue preparation

A separate set of experimentally naive CD1 mice that were reared under the same postnatal environmental paradigms (Control, MS and EE) were anaesthetized with isoflurane anesthesia and then decapitated at the age of 6–8 weeks. Brains were rapidly removed and snap-frozen in isopentane on a block of dry ice and kept at 80 °C until dissection. Six brain regions (frontal and motor cortex, striatum with nucleus accumbens, hippocampus, hypothalamus, amygdala and raphe nuclei) were dissected according to mouse brain atlas [3] on a refrigerated plate (+4 °C) using an Olympus SZX7 stereomicroscope (Olympus GmbH, Germany). Collected tissue was placed immediately in nuclease-free tubes on dry ice and stored at −80 °C until RNA isolation.

2.5.2. RNA isolation and quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using the RNeasy Mini Kit and RNase-Free DNase Set (Qiagen, Germany), PeqGold RNAPure (PeqLab, Germany) and chloroform (Carl-Roth, Germany) according to the manufacturer instructions, suspended in nuclease-free water and stored at −80 °C. RNA quality and yield were quantified using NanoDrop ND100 (PeqLab, Germany) and Experion™ automated electrophoresis system (Bio-Rad Laboratories, Inc., Germany). Only RNA samples with 260/280 ratio between 1.8–2.0, the 260/230 ratio between 2.0–2.2 and the RQI between 8.1–10 were used in the study. cDNA was synthesized from isolated total RNA using iScript™ cDNA Synthesis Kit (BioRad, Germany) according to the manufacturer instructions. The resulting cDNA was diluted at 1:5 ratio with nuclease-free water and stored at −20 °C. 1 µl of each cDNA was used for qRT-PCR in 10 µl reaction mixes per sample consisting of 200 nM forward and reverse primers (Table 1) and 1x SYBR Green Master Mix (Life Technologies, Germany). All samples, no-reverse transcriptase controls and negative controls were tested in duplicates in 384-well plates using the CFX384 Touch™ Real-Time PCR Detection System (BioRad, Germany). Amplification was performed using pre-incubation at 95 °C for 2 min followed by 40 cycles of amplification as denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, terminal denaturation at 95 °C for 10 s followed by a melting curve step from 65 °C to 95 °C with increments for 5 s. *B2m* and *Hprt*, the expression of which did not differ across the groups, were used as reference genes to normalize expression levels of genes of interest. Primer efficiencies were tested with linear regression software LinReg [66]. Relative gene expression analysis was performed with the Bio-Rad CFX Manager 3.0 software (Bio-Rad, Germany) using the comparative cycle threshold method [67].

Table 1
The sequences of oligonucleotide primers (5′→ 3′) used for PCR and qRT-PCR (Mm: Mus musculus).

Gene	Forward Sequence	Reverse Sequence
Mm_*Crhr1*	GCCTTTTTCTACGGTGTCCG	CGTTGAGAATCTCCTGGCACT
Mm_*Maoa*	TCGGGAGAATTTTACCCAAACCA	AACTCTATCCCGGGCTTCCA
Mm_*Htr-1a*	AACAAGACTGGAAAGGGGGC	GCGGCATGTTGCACTTAGTT
Mm_*Nos1*	CATCGCTCCCTTCCGAAGTT	ATTGTCGACACCCGAAGACC
Mm_*Bdnf*	ACTGGGTTAACTTTGGGAAATGC	GTCATCACTCTTCTCACCTGG
Mm_*Fkbp5*	GGCGAGGGATACTCAAACCC	ACACCACATCTCGGCAATCA
Mm_*Nr3c1*	CAAGGGTCTGGAGAGGACAAC	GCTGGACGGAGGAGAACTCA
Mm_*B2M*	ACTGACCGGCCTGTATGCTA	CAATGTGAGGCGGGTGGAA
Mm_*Hprt*	TGCTGACCTGCTGGATTACA	TTTATGTCCCCCGTTGACTGA

2.6. Statistical analysis

Behavioral, FCM and log2 transformed relative gene expression data was analyzed using twoway ANOVA with sex and environmental manipulation as independent variables. Where appropriate, repeated measures ANOVA was used. In cases of strong main effects for sex, data from males and females was analyzed separately. Pairwise comparisons with Bonferroni corrections were performed to explore main effects. Outliers were detected using box-plot using 1.5 × IQR (interquartile range). Normality and homogeneity of variances were tested using ShapiroWilk ($p > 0.05$) test and Levene's test ($p > 0.05$). In case these tests failed, nonparametric tests (Kruskal-Wallis and MannWhitney U) were used to detect statistical differences. All data are presented as mean ± SEM. All analyses were performed using IBM SPSS Statistics 22.0 Software (IBM, United States) and graphs were prepared using GraphPad Prism 5 (GraphPad Software, United States).

3. Results

3.1. Corticosterone metabolite levels

In this study, basal FCM levels were measured from samples which were collected at the age of 8 weeks (Fig. 1A). Fecal samples of pair-housed mice were pooled, yielding one data point per each cage. Overall, females had higher FCM levels compared to males (sex effect: $\chi^2(1) = 19.286$, $p \leq 0.001$), while postnatal manipulation had no effect on FCM ($\chi^2(2) = 2.286$, $p = 0.319$). Due to the main effect of sex, FCM data from males and females was analyzed separately. In females, postnatal EE resulted in lower corticosterone levels in adulthood ($\chi^2(2) = 8.077$, $p = 0.018$; $U = 0$, $p = 0.025$), while MS did not significantly alter FCM levels ($U = 6$, $p > 0.05$). While we did not observe a significant postnatal manipulation effect on FCM levels in males ($\chi^2(2) = 5.540$, $p = 0.063$), pairwise comparisons revealed a trend for

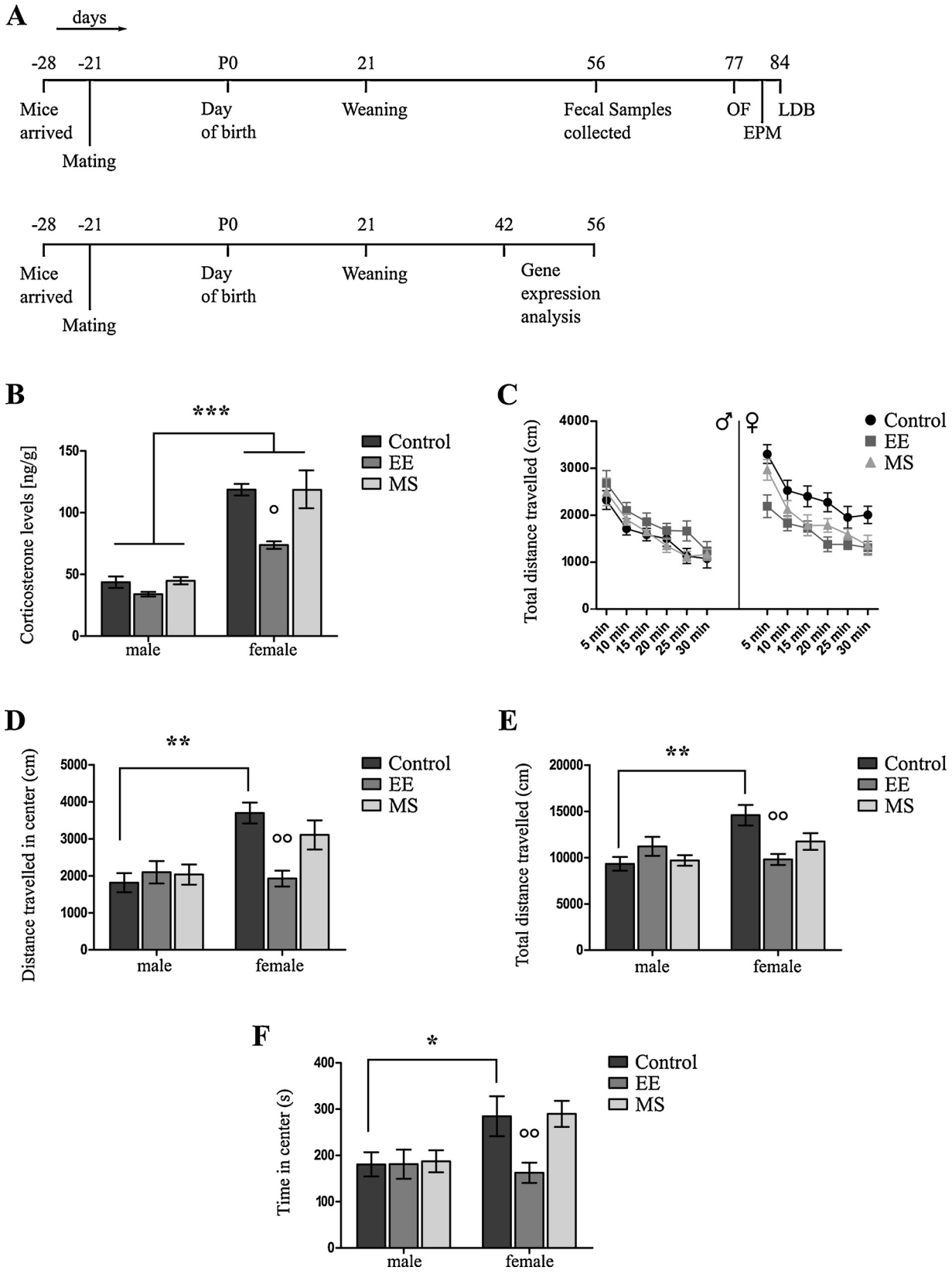

Fig. 1. Females show higher FCM levels and activity in open field test. **A)** Time schedule for experimental protocols. **B)** FCM levels are higher in female CD1 mice, however EE significantly reduces adult FCM levels in females (males: n = 5 per group, females: n = 3 for controls, n = 5 for EE and n = 4 for MS; n accounts for the pooled sample due to pair-housing.). **C–E)** Control and MS females show higher locomotor activity in the open field arena whereas EE attenuates this behavior. **F)** There was a significant increase in time spent in the center of the open field compared to males in control and MS females, whereas EE resulted a reduction in time spent in the center. Data is shown as Mean ± S.E.M. Open field test: n = 9 for Control, n = 9 for EE and n = 10 for MS in males; n = 9 for Control, n = 7 for EE, and n = 8 for MS in females. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 male vs female mice, °p ≤ 0.05, °°p ≤ 0.01 environmental manipulation vs respective control. OF: open field, EPM: elevated plus maze, LDB: light dark box, FCM: fecal corticosterone metabolites.

EE to decrease adult FCM levels (Control vs EE: U = 4, p = 0.076; Control vs MS: U = 11, p = 0.754).

3.2. Open field

General locomotor activity and exploratory behavior were assessed using the open field test. A significant interaction of sex and postnatal manipulation was found on time spent in the center of the open field ($F(2,46) = 3.439$, $p = 0.041$), distance travelled in the center ($F(2,46) = 7.206$, $p = 0.002$), and total distance travelled ($F(2,46) = 8.859$, $p = 0.001$) (Fig. 1B–E). Females were more active in terms of the total distance travelled ($p = 0.027$) and distance travelled in the center ($p = 0.002$), however, this effect was mainly due to the difference between control groups ($p = 0.001$ for both measures). On the other hand, EE reduced locomotor activity levels only in females ($p = 0.005$ and $p = 0.002$, respectively). Time spent in the center was also higher in female controls compared to male controls ($p = 0.013$). Nevertheless, this effect might be due to increased activity in female mice, as EE also reduced time spent in center compared to female controls ($p = 0.018$). When analyzed in 5-min intervals, all groups displayed habituation to the open field across time ($F(3.558,184.996) = 8.859$, $p < 0.001$). There was also a significant interaction effect for sex and postnatal manipulation ($F(2,52) = 7.442$, $p = 0.001$): EE females showed lower activity whereas EE males' activity was similar compared to their respective controls.

3.3. Light dark box

Anxiety-like behavior towards novelty and brightly illuminated areas was tested in LDB (Fig. 2). There were no main effects for either postnatal manipulation or sex and no interaction effect of these factors in terms of transitions between light and dark compartment ($F(2,46) = 0.114$, $p = 0.892$; $F(1,46) = 0.645$, $p = 0.426$; $F(2,46) = 1.805$, $p = 0.176$ respectively), time spent in light compartment ($F(2,46) = 1.152$, $p = 0.325$; $F(1,46) = 0.247$, $p = 0.621$; $F(2,46) = 0.167$, $p = 0.847$ respectively) and latency to enter to light compartment ($F(2,46) = 1.261$, $p = 0.293$; $F(1,46) = 0.512$, $p = 0.478$; $F(2,46) = 0.643$, $p = 0.531$ respectively).

3.4. Elevated plus maze

EPM was also used to assess anxiety-related behaviors in terms of inert aversion to elevated open areas. There was a significant interaction of sex and postnatal manipulation for total distance travelled ($F(2,46) = 4.897$, $p = 0.012$) but not for percentage of time spent in open arms ($F(2,46) = 2.218$, $p = 0.120$) or number of entries to open arms ($F(2,46) = 3.081$, $p = 0.055$) in EPM apparatus (Fig. 3). There was only a main effect of sex for total distance travelled ($F(2,46) = 5.247$, $p = 0.027$) but this seems to be due to interaction effect as only EE males showed higher locomotor activity (male EE vs male Control: $p = 0.012$; male EE vs female EE: $p = 0.012$) but no significant difference in other measures (all other p-values higher than 0.05).

3.5. Relative gene expression

As specified above, the chosen genes and the examined brain areas have been shown to be sensitive to gene x environment regulation and involved in susceptibility to psychiatric disorders. ***Fkbp5***: Male mice had higher expression levels in the hippocampus ($F(1,65) = 4.797$, $p = 0.032$) compared to female mice regardless of the postnatal manipulation (Table 2). Nonparametric tests also revealed sex-dependent differences in *Fkbp5* expression in hypothalamus and raphe nuclei. In hypothalamus, males had higher expression compared to females ($U = 378.000$, $p = 0.041$; Table 2). Kruskal-Wallis H-test revealed differences in males due to postnatal manipulation ($\chi^2(2) = 6.234$, $p = 0.044$): maternal separation reduced hypothalamic *Fkbp5* expression in adulthood (Control vs MS: $U = 28$, $p = 0.011$; Control vs EE: $U = 71$, $p = 0.703$). In females, no significant difference in *Fkbp5* expression in the hypothalamus was detected ($\chi^2(2) = 0.611$, $p = 0.737$). Male mice also showed higher *Fkbp5* expression in raphe nuclei according to Mann-Whitney U test ($U = 453.500$, $p = 0.019$). EE or MS did not cause any significant difference in the *Fkbp5* expression in this region (males: $\chi^2(2) = 3.585$, $p = 0.167$; females: $\chi^2(2) = 6.234$, $p = 0.790$). No significant differences were detected in other regions ($p > 0.05$). ***Nos1***: There was a significant main effect for sex ($F(1,68) = 4.520$, $p = 0.037$) as males had higher expression levels, and postnatal manipulation ($F(2,68) = 4.009$, $p = 0.023$) but no interaction effect ($F(2,68) = 0.219$, $p = 0.804$) in hippocampus. Post-hoc

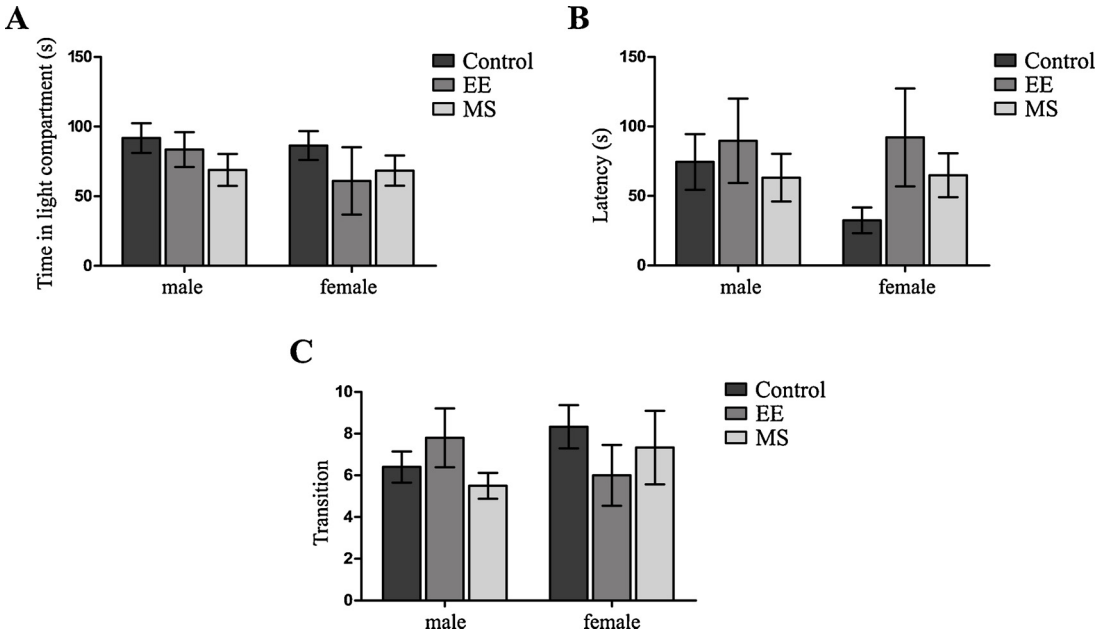

Fig. 2. No significant differences were detected in the light dark box. Data is shown as Mean ± S.E.M. n = 9 for Control, n = 9 for EE and n = 10 for MS in males; n = 9 for Control, n = 7 for EE, and n = 8 for MS in females.

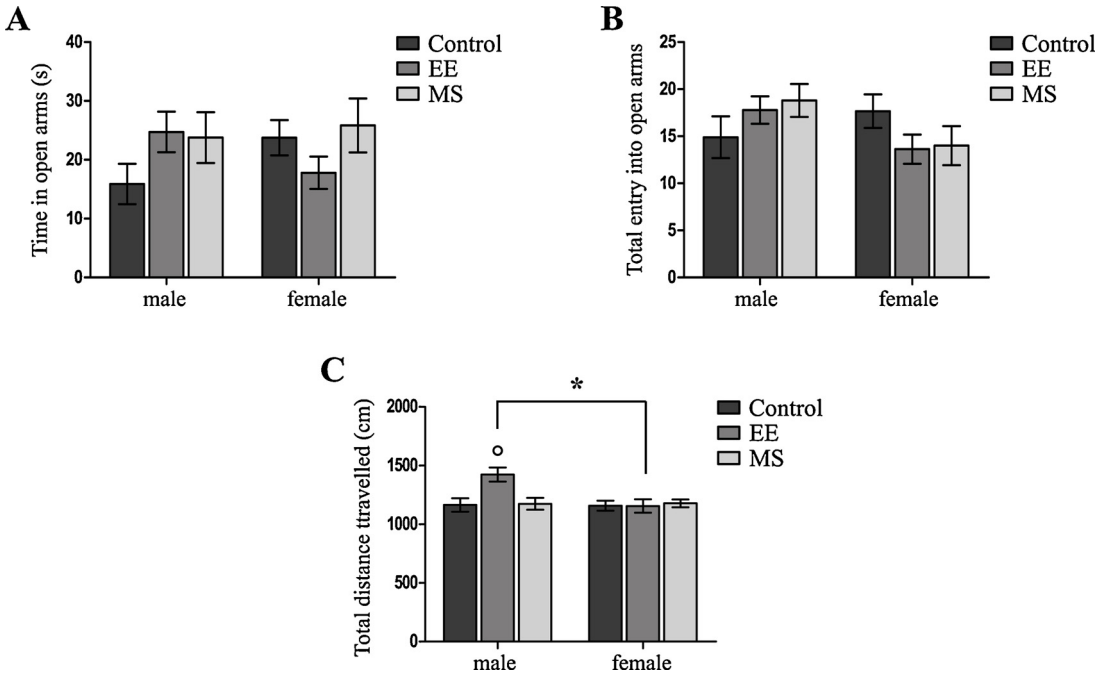

Fig. 3. Results from elevated plus maze revealed no significant differences in anxiety-related behavior **(A–B)**, however EE males showed higher locomotor activity compared to their female counterparts **(C)**. Data is shown as Mean ± S.E.M. *p ≤ 0.05 male vs female mice, °p ≤ 0.05 environmental manipulation vs respective control, n = 9 for Control, n = 9 for EE and n = 10 for MS in males; n = 9 for Control, n = 7 for EE, and n = 8 for MS in females.

Bonferroni test showed that mice which underwent MS had marginally higher *Nos1* expression (p = 0.061) in the hippocampus regardless of sex. ***Maoa***: Females had higher *Maoa* expression levels in hypothalamus regardless of the postnatal manipulation ($F(1,62) = 4.646$, $p = 0.035$). There was neither main effect for postnatal manipulation ($F(2,62) = 1.011$, $p = 0.370$) nor interaction effect of sex and postnatal manipulation ($F(2,62) = 0.810$, $p = 0.450$) in this region. We could not detect any other significant differences in other regions ($p > 0.05$). In addition, we measured the expression levels of *Bdnf*, *Crhr1*, *Nr3c1* and *Htr1a* in six brain regions (frontal cortex, striatum with nucleus accumbens, hippocampus, hypothalamus, amygdala and raphe nuclei), but could not detect significant between-group differences in the expression levels of any of these genes in any of the brain regions (all p values higher than 0.05; Table 2).

4. Discussion

In this study, we demonstrated a strong influence of sex on adult gene expression pattern and locomotor activity, but limited impact of ELS and EE on these measures. Female mice displayed higher FCM levels and locomotor activity in the open field test, whereas both effects were reduced by EE. We also showed that specific genes such as *Nos1*, *Fkbp5* and *Maoa* were differentially expressed in male and female mice. However, there was only a mild effect of environmental manipulations on the mRNA levels of these genes with the main effect observed being increased *Nos1* expression following MS.

4.1. Corticosterone response

Glucocorticoids play a major role in the regulation of stress mechanisms, cognitive processes, and growth. Their metabolites can be measured non-invasively in the feces to limit the stress exposure of the animals and were found to reflect adrenocortical activity well [68,69]. As previously found [58,70], CM levels in fecal samples from female mice had overall higher levels compared to male mice (Fig. 1), regardless of postnatal manipulation.

Contrary to previous studies suggesting that MS resulted in an increase in peripheral corticosterone levels [60,71], we did not find a significant difference in FCM levels between MS and Control mice. However, this may be explained by differences in experimental design between studies such as strains used in the studies (CD1 vs BALB/c or C57BL/6 J), duration of MS (15 min to 24 h), timing of MS (P1 to P14 or P21), model of MS (isolation of dams vs pups individually) and the measurement methods (fecal samples vs blood) [72,73]. Timing of maternal deprivation has been shown to be an important factor for regulation of HPA axis reactivity in CD1 mice [74]. In a previous study, 24-h MS during early postnatal days (i.e. P3) was shown to induce long-term elevated corticosterone response, however MS on P8 or repetitive MS on P3 and P8 did not reproduce this effect [74]. Moreover, the mice in the present study were housed in groups of two per cage to avoid the stressful outcomes of single housing [61]. Therefore, the time between weaning (at the age of 3 weeks) and collection of fecal samples (at the age of 8 weeks) in social housing might have diminished the effects of MS paradigm. Similar to our findings, a previous study on male Balb/c mice also show that neither early-life stress alone, nor combination of early life stress and adult chronic stress, induced any change in corticosterone levels [75]. However, in line with literature, we documented that EE caused a decrease in FCM levels [22,76]. Taken together, this underscores that enrichment plays a significant role in decreasing HPA axis reactivity, especially in females.

4.2. Sex-specific differences in adult behavior

Numerous studies, in both rodents and humans, have documented that enriched or stressful environments during critical periods of brain development can trigger long-lasting changes in the regulation of the HPA axis and, consequently, behavior. In this study, we showed that female mice display higher locomotor activity in open field compared with males (Fig. 1). The elevated activity in females was reduced by EE, which also resulted in decreased time spent in the center of the open field. Although this is an accepted measure of anxiety-like behavior, no differences were observed in other anxiety-sensitive tests such as LDB and EPM (Table 2). This suggests that hypolocomotion may explain the shorter time spent in center of open field. In our study, we could not

Table 2
Relative gene expression levels. Expression levels were analyzed according to a sex x postnatal treatment design.

Region	Gene	Males			Females		
		Control	EE	MS	Control	EE	MS
FC-MC	BDNF	4.70 ± 0.05, n = 11	4.71 ± 0.09, n = 13	4.69 ± 0.07, n = 13	4.85 ± 0.14, n = 13	4.75 ± 0.06, n = 12	4.89 ± 0.07, n = 12
	CRHR1	3.99 ± 0.07, n = 10	3.84 ± 0.07, n = 11	4.06 ± 0.1, n = 13	3.87 ± 0.13, n = 13	3.97 ± 0.11, n = 12	4.05 ± 0.10, n = 11
	NR3C1	1.56 ± 0.07, n = 12	1.56 ± 0.04, n = 12	1.65 ± 0.08, n = 12	1.62 ± 0.12, n = 13	1.56 ± 0.09, n = 12	1.51 ± 0.08, n = 12
	FKBP5	1.95 ± 0.08, n = 13	1.83 ± 0.05, n = 12	1.91 ± 0.07, n = 13	1.77 ± 0.14, n = 13	1.83 ± 0.11, n = 12	1.92 ± 0.08, n = 12
	MAOA	0.73 ± 0.10, n = 13	0.82 ± 0.04, n = 12	0.78 ± 0.08, n = 13	0.63 ± 0.09, n = 12	0.70 ± 0.05, n = 12	0.73 ± 0.07, n = 12
	HTR1 A	2.86 ± 0.09, n = 13	2.87 ± 0.07, n = 13	2.84 ± 0.05, n = 9	3.00 ± 0.04, n = 13	2.79 ± 0.08, n = 12	3.01 ± 0.07, n = 12
	NOS1	1.89 ± 0.15, n = 13	1.92 ± 0.09, n = 13	1.98 ± 0.04, n = 12	1.71 ± 0.12, n = 13	1.93 ± 0.08, n = 12	1.96 ± 0.16, n = 12
Striatum	BDNF	2.79 ± 0.23, n = 13	2.47 ± 0.36, n = 12	2.14 ± 0.26, n = 12	2.59 ± 0.30, n = 13	2.40 ± 0.42, n = 10	2.37 ± 0.30, n = 13
	CRHR1	2.77 ± 0.13, n = 13	2.58 ± 0.11, n = 13	2.56 ± 0.16, n = 13	2.45 ± 0.13, n = 13	2.51 ± 0.09, n = 12	2.53 ± 0.10, n = 13
	NR3C1	1.87 ± 0.12, n = 11	2.03 ± 0.08, n = 13	1.95 ± 0.09, n = 13	1.91 ± 0.10, n = 13	2.01 ± 0.07, n = 12	1.93 ± 0.05, n = 13
	FKBP5	3.00 ± 0.11, n = 12	2.86 ± 0.07, n = 12	2.85 ± 0.09, n = 13	2.81 ± 0.09, n = 12	2.90 ± 0.08, n = 12	2.90 ± 0.07, n = 12
	MAOA	1.05 ± 0.10, n = 12	1.20 ± 0.12, n = 13	1.01 ± 0.05, n = 12	1.07 ± 0.07, n = 12	1.14 ± 0.06, n = 12	1.21 ± 0.07, n = 12
	HTR1 A	1.53 ± 0.15, n = 13	1.51 ± 0.19, n = 13	1.33 ± 0.16, n = 13	1.55 ± 0.18, n = 13	1.40 ± 0.14, n = 12	1.51 ± 0.11, n = 13
	NOS1	2.46 ± 0.06, n = 9	2.55 ± 0.08, n = 10	2.48 ± 0.09, n = 12	2.34 ± 0.09, n = 13	2.64 ± 0.10, n = 12	2.58 ± 0.08, n = 13
Hippocampus	BDNF	4.91 ± 0.08, n = 12	4.84 ± 0.09, n = 13	4.80 ± 0.08, n = 11	4.88 ± 0.06, n = 12	4.79 ± 0.13, n = 11	4.92 ± 0.05, n = 13
	CRHR1	2.99 ± 0.14, n = 12	2.73 ± 0.14, n = 13	2.99 ± 0.12, n = 12	2.73 ± 0.14, n = 13	2.74 ± 0.13, n = 11	2.80 ± 0.14, n = 13
	NR3C1	1.74 ± 0.14, n = 12	1.69 ± 0.07, n = 13	1.71 ± 0.07, n = 12	1.64 ± 0.09, n = 13	1.66 ± 0.10, n = 12	1.64 ± 0.10, n = 13
	FKBP5	3.04 ± 0.11, n = 12	3.03 ± 0.07, n = 10	3.18 ± 0.08, n = 12	**2.89 ± 0.13*, n = 13**	**2.83 ± 0.10*, n = 12**	**3.00 ± 0.08*, n = 12**
	MAOA	0.90 ± 0.07, n = 11	1.03 ± 0.05, n = 12	1.07 ± 0.07, n = 12	0.92 ± 0.08, n = 13	0.90 ± 0.04, n = 10	0.97 ± 0.09, n = 13
	HTR1 A	3.68 ± 0.06, n = 12	3.89 ± 0.08, n = 13	3.70 ± 0.08, n = 11	3.67 ± 0.09, n = 12	3.66 ± 0.09, n = 12	3.88 ± 0.05, n = 12
	NOS1	3.06 ± 0.15, n = 11	3.07 ± 0.10, n = 13	**3.29 ± 0.09#, n = 12**	**2.86 ± 0.11*, n = 13**	**2.80 ± 0.10*, n = 12**	**3.17 ± 0.14*#, n = 13**
Hypothalamus	BDNF	4.56 ± 0.08, n = 11	4.82 ± 0.10, n = 13	4.75 ± 0.13, n = 12	4.87 ± 0.16, n = 12	4.94 ± 0.11, n = 12	4.84 ± 0.07, n = 11
	CRHR1	2.29 ± 0.09, n = 10	2.22 ± 0.13, n = 12	2.14 ± 0.11, n = 12	2.17 ± 0.09, n = 11	2.13 ± 0.16, n = 12	2.16 ± 0.13, n = 13
	NR3C1	1.34 ± 0.10, n = 11	1.20 ± 0.07, n = 12	1.20 ± 0.06, n = 12	1.37 ± 0.04, n = 9	1.42 ± 0.12, n = 12	1.24 ± 0.06, n = 13
	FKBP5	1.86 ± 0.08, n = 12	1.80 ± 0.11, n = 13	**1.56 ± 0.09#, n = 12**	**1.55 ± 0.02*, n = 8**	**1.55 ± 0.16*, n = 11**	**1.54 ± 0.07*, n = 10**
	MAOA	1.54 ± 0.04, n = 9	1.72 ± 0.06, n = 12	1.67 ± 0.06, n = 12	**1.75 ± 0.06*, n = 10**	**1.76 ± 0.08*, n = 12**	**1.77 ± 0.08*, n = 13**
	HTR1 A	2.34 ± 0.10, n = 11	2.44 ± 0.06, n = 12	2.39 ± 0.08, n = 10	2.46 ± 0.13, n = 12	2.24 ± 0.09, n = 12	2.27 ± 0.08, n = 12
	NOS1	2.64 ± 0.06, n = 12	2.60 ± 0.11, n = 13	2.53 ± 0.06, n = 8	2.65 ± 0.14, n = 11	2.53 ± 0.12, n = 12	2.58 ± 0.12, n = 13
Amygdala	BDNF	4.77 ± 0.05, n = 10	4.89 ± 0.05, n = 11	4.75 ± 0.05, n = 12	4.85 ± 0.05, n = 12	4.77 ± 0.11, n = 12	4.87 ± 0.08, n = 12
	CRHR1	3.40 ± 0.15, n = 13	3.41 ± 0.06, n = 11	3.49 ± 0.14, n = 13	3.34 ± 0.15, n = 12	3.31 ± 0.14, n = 12	3.38 ± 0.12, n = 12
	NR3C1	1.65 ± 0.11, n = 13	1.56 ± 0.11, n = 13	1.60 ± 0.09, n = 13	1.56 ± 0.11, n = 13	1.62 ± 0.09, n = 12	1.64 ± 0.07, n = 10
	FKBP5	2.61 ± 0.12, n = 13	2.76 ± 0.06, n = 11	2.57 ± 0.12, n = 12	2.56 ± 0.12, n = 12	2.53 ± 0.09, n = 12	2.44 ± 0.11, n = 13
	MAOA	1.20 ± 0.08, n = 13	1.18 ± 0.09, n = 13	1.16 ± 0.08, n = 13	1.06 ± 0.05, n = 11	1.08 ± 0.10, n = 12	1.09 ± 0.06, n = 10
	HTR1 A	2.99 ± 0.08, n = 13	3.12 ± 0.06, n = 13	3.12 ± 0.08, n = 13	3.06 ± 0.08, n = 13	3.07 ± 0.05, n = 12	2.96 ± 0.09, n = 13
	NOS1	3.32 ± 0.07, n = 12	3.47 ± 0.09, n = 12	3.28 ± 0.07, n = 11	3.14 ± 0.11, n = 13	3.38 ± 0.12, n = 11	3.19 ± 0.18, n = 13
Raphe nuclei	BDNF	4.88 ± 0.06, n = 12	4.86 ± 0.08, n = 13	4.92 ± 0.09, n = 13	4.90 ± 0.11, n = 12	4.89 ± 0.09, n = 12	5.00 ± 0.06, n = 11
	CRHR1	3.23 ± 0.12, n = 12	3.35 ± 0.13, n = 13	3.22 ± 0.15, n = 13	3.33 ± 0.12, n = 13	3.34 ± 0.12, n = 12	3.28 ± 0.09, n = 11
	NR3C1	1.26 ± 0.08, n = 13	1.31 ± 0.07, n = 11	1.18 ± 0.10, n = 13	1.27 ± 0.08, n = 13	1.28 ± 0.10, n = 12	1.23 ± 0.08, n = 12
	FKBP5	1.69 ± 0.08, n = 13	1.79 ± 0.04, n = 11	1.59 ± 0.07, n = 13	**1.59 ± 0.09*, n = 13**	**1.56 ± 0.08*, n = 12**	**1.50 ± 0.05*, n = 11**
	MAOA	1.85 ± 0.08, n = 13	1.94 ± 0.05, n = 12	1.88 ± 0.07, n = 13	1.94 ± 0.04, n = 11	1.82 ± 0.03, n = 10	2.01 ± 0.08, n = 11
	HTR1 A	2.75 ± 0.05, n = 13	2.83 ± 0.08, n = 13	2.62 ± 0.06, n = 13	2.71 ± 0.07, n = 13	2.76 ± 0.05, n = 11	2.73 ± 0.08, n = 12
	NOS1	2.71 ± 0.10, n = 13	2.76 ± 0.10, n = 12	2.63 ± 0.09, n = 12	2.81 ± 0.07, n = 12	2.73 ± 0.10, n = 12	2.57 ± 0.13, n = 11

Data is represented as Mean ± S.E.M. *p ≤ 0.05 for main effect of sex. #p ≤ 0.05 for main effect of postnatal treatment (compared to respective controls).

detect any significant behavioral differences between MS and control mice in either sex. Previous studies also show inconsistent effects of ELS and EE and thus, the stress protocol, sex, age and testing paradigms should be considered all when performing such experiments [77–79]. For example, early life stress protocols are highly heterogeneous with MS protocols varying from 15 min to 3 h or between P1-to either P14 or P21, as well as some requiring 24 h complete deprivation from mothers or individual isolation of pups. Moreover, other ELS/EE protocols are centered on impoverished environment such as limited nesting material or food as well as additive stress as restriction [72–75,80,81], which differs from our EE group that had an enriched environment during development. Most importantly, there are several studies showing that strain-specific difference is a very important factor to consider while testing long-term effects of stress or enrichment [82,83]. In a recent study, maternal separation (3 h a day, postnatal days 0–13 i.e. one-week shorter than the current study) was tested in eight different strains of mice for anxiety- and depressive- related traits in adulthood. In accordance with our study, they also did not show any behavioral differences between MS and their respective controls, with the behavioral differences a consequence of sex, strain, or both [82]. In another study, C57BL/6 mice were subjected to a 3-h MS paradigm during the first 2 postnatal weeks and adult male MS group displayed higher anxiety-like behavior, whereas adult female MS group displayed reduced anxiety compared to their controls [84]. On the other hand, CD1 mice subjected to early social enrichment between postnatal days 1–25 also displayed sex-dependent behavioral differences in adulthood in terms of elevated anxiety in enriched adult males, but not in females [85]. EE in the present study did not affect anxiety-related behavior in either sex; although this may be due to the maintenance of EE throughout the entire experiment.

4.3. Sex-specific differences in adult gene expression

In a previous study, we demonstrated that both ELS and EE lead to sex-specific alterations in DNA methylation in a number of genes implicated in stress coping and/or anxiety-related behavior [63]. Here we expand on those findings and could show that both the hippocampus and the hypothalamus show sex-specific differences in gene expression levels (Table 2). This study also revealed that there were no significant differences in the expression levels of *Bdnf*, *Crhr1*, *Nr3c1* and *Htr1a* in six brain regions (frontal cortex, striatum with nucleus accumbens, hippocampus, hypothalamus, amygdala and raphe nuclei) in MS or EE groups compared to Controls regardless of sex. EE after weaning has been shown to reduce *Crhr1* gene expression in basolateral amygdala [22] and to reduce hippocampal and amygdalar *Nr3c1* but induce hippocampal and striatal *Bdnf* gene expression in mice [86], as well as

Bdnf levels in the hippocampus and amygdala [86–88]. Whereas these were decreased in frontal cortex [89]. However, experimental design differences between our and their studies should be considered, as well as the use of outbred CD1 mice in the current study. This is an important caveat as the aforementioned studies were performed using inbred mouse lines, different timing and type of the stress and enrichment procedures. Despite the differences in adult FCM levels documented in Section 3.1, females showed no influence of postnatal manipulations on the *Bdnf*, *Crhr1*, *Nr3c1* and *Htr1a* expression levels. However, corticosterone levels are under strong influence of many effectors, which may be affected by EE, but this requires further investigation.

FKBP5, encoded by *Fkbp5,* is an important regulator of glucocorticoid receptor. Despite the fact that we could not detect any difference in *Nr3c1* levels, here we show higher levels of *Fkbp5* expression in the hippocampus, hypothalamus and raphe nuclei in male CD1 mice compared to females (Table 2). There is a relative dearth of information regarding how stress regulates the expression pattern of *Fkbp5* gene, but several studies revealed that glucocorticoid treatment and different stress stimuli influence the expression levels of *Fkbp5* mRNA [90,91]. This was not observed in our study.

Low *Maoa* activity, due to a receptor polymorphism, in combination with adverse childhood environment has been shown to increase the risk for adult antisocial and criminal behavior [33,92–94]. On the contrary, our study did not reveal a significant main effect of environment on gene expression levels of *Maoa*. However, we showed a significant sex effect that females had lower *Maoa* levels in hypothalamus (Table 2), which is in keeping with it being a X-linked gene and might be one of the underlying factors of stress vulnerability in females. We have recently showed a sex-specific effect on epigenetic regulation regardless of environmental manipulation as females showing higher DNA methylation on *Maoa* gene in many brain regions [63], supporting our findings in this study.

The major effects of early-life stress were observed in relation with the *Nos1* gene. Prior studies performed on adult male B6129SF2 mice have shown that exposure to 3 weeks of chronic mild stress induced long term increases in *Nos1* mRNA and protein levels in hippocampus as well as its enzymatic activity [46,49]. Interestingly, acute restraint stress on adult Wistar rats has been shown to induce an increase in *Nos1* mRNA levels in medial prefrontal cortex but down-regulation in hippocampal *Nos1* expression [95,96]. In addition, EE has been shown to reduce *Nos1* mRNA levels in hippocampus of aged mice [97]. Here we further show that early life adversity also causes elevation of *Nos1* mRNA expression hippocampus, independent of sex (Table 2). Interestingly, we also show that male CD1 mice had higher hippocampal *Nos1* expression compared to females. Sex hormones have been previously shown to affect *Nos1* expression in brain [98–100], therefore effects of environmental manipulations on sex-specific *Nos1* expression still require further investigation.

Finally, it should be discussed that mice in our study were cohoused and it has been repeatedly demonstrated that social context during early life is an important contributor to neurodevelopmental deficits and anxiety-related phenotypes in adulthood [73,102–105]. Thus, group-housing may have compensated for the effects of ELS.

Limitations: The small sample size may limit our power to find main effects. However, this seems unlikely as postnatal manipulation did not lead to any anxiety-related phenotype in CD1 adult mice when we consider the overall results from the behavioral test battery. The temporal dissociation between the behavioral and gene expression findings also limit correlation between the observations. However, both data sets were obtained in adult mice (e.g. older than 6–8 weeks of age) and are thus likely to reflect the consequences of MS and/or EE. Finally, while the gene expression findings are revealing, they need to be verified at the protein level.

5. Conclusion

In conclusion, our study adds to the literature showing that early life experiences have different influences on the stress-regulatory mechanisms in males and females. Females showed higher levels of locomotor activity, as well as higher FCM levels, compared with males. While EE led to a reduction in FCM levels in females, neither ELS nor EE caused alterations in the expression profile of several genes those involved in plasticity and stress regulation (*Bdnf*, *Crhr1*, *Nr3c1* and *Htr1a*). Moreover, sex differences in *Nos1*, *Fkbp5* and *Maoa* were observed, especially within the hippocampus and hypothalamus, and MS caused a sex-independent increase in hippocampal *Nos1* expression. Last but not least, we used CD1 outbred mice in our study due to their genetic variability as mentioned previously. As a result, we could demonstrate the effects of environmental manipulations on above-mentioned phenotypes by acknowledging the inter-individual differences observed in human population. As each individual (i.e. each mouse in this study) would have a slightly different genetic background, early-life environmental manipulations might also affect their response to these manipulations differentially [101]. This could also explain the phenotypic variances that we see in our data.

Taken together, our findings further contribute to sex-dependent differences in behavior, corticosterone and gene expression and reveal that the effects of postnatal manipulations on these parameters in outbred CD1 mice are limited.

Disclosure

Authors in this study declare no conflict of interest.

Author contributions

AR and AP designed the study. USD and AP performed the behavioral experiments. RP performed FCM analysis. EC performed gene expression analysis, statistical analysis and wrote the manuscript. DAS and AOL revised the data and the manuscript.

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