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Postnatal impoverished housing impairs adolescent risk-assessment and increases risk-taking: A sex-specific effect associated with histone epigenetic regulation of *Crfr1* in the medial prefrontal cortex

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ABSTRACT

While increasing evidence posits poor decision-making as a central feature of mental disorders, very few studies investigated the effects of early-life stress (ELS) on specific components of reward-related choice behaviors. Risk-taking (RT) involves the exposure to some danger, or negative consequences, in order to achieve a goal-directed behavior. Such behaviors are likely to be preceded by risk-assessment (RA), which is a dynamic cognitive process involving the acquisition of information in potentially dangerous situations. Here, we investigated the effects of being raised in impoverished housing conditions during early life (P2–P9) on RT, RA and dopaminergic and corticotrophinergic gene expression of adolescent male and female mice. Phenotypes were assessed by two protocols: the elevated plus-maze (EPM) and the predator-odor risk-taking (PORT). We found decreased RA in mice exposed to impoverished housing in the absence of a reward (EPM), with a more pronounced effect among females. Moreover, when exposed to a predatory olfactory cue, increased RT was observed in these females in a reward-related task (PORT), as well as decreased HPA axis responsivity. This sex-specific behavioral effect was associated with increased *Crfr1* mRNA expression in the medial prefrontal cortex (mPFC) and higher levels of the histone mark H3R2^{me2s}, a histone modification known to be involved in transcriptional activation, within the promoter of the *Crfr1* gene. These findings revealed that ELS exposure can impair the acquisition of environmental information in dangerous situations and increase RT in reward-related scenarios among females, with an important role regarding epigenetic regulation of the *Crfr1* gene.

1. Introduction

There is considerable evidence showing that early-life stress (ELS) exposure can negatively affect brain development, producing an array of clinically relevant behavioral and cognitive alterations (Blair and Raver, 2016; Harrison and Baune, 2014). These consequences may prime such vulnerable individuals toward the development of neuropsychiatric illnesses during adolescence as well as young adulthood (Grassi-Oliveira et al., 2008). While increasing evidence posits poor decision-making as a central feature of mental disorders (Kluwe-Schiavon et al., 2016b; Steward et al., 2016), very few studies investigated the effects of ELS on specific cognitive components of choice behaviors. Of particular interest, risk-assessment (RA) is a dynamic cognitive process that involves the acquisition of environmental information in potentially dangerous situations (Reis et al., 2012). Impairments in RA are often associated with risky choices and increased risk-taking (RT) particularly in reward-related situations, such as impulsive behavioral patterns towards reinforcing stimuli despite the negative consequences associated with such actions (Kusev et al., 2017; Reske et al., 2015). However, how ELS could potentially affect RA and RT later in life is presently unknown.

Substantial evidence suggests that the medial prefrontal cortex (mPFC) is a highly sensitive brain region to the effects of ELS (Chocyk et al., 2013), while the mPFC has a key contribution for behavioral

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control, risk perception and reward processing (Crowley et al., 2017; Schall, 2001). In particular, dopaminergic neurotransmission through D1 and D2 receptors modulate neuronal inputs between the mPFC and the ventral striatum during reward processing (Jenni et al., 2017). Evidence indicates that pharmacological blockade of D1 or D2 receptors in the mPFC and striatum could hinder RA processing through alterations in risk or reward sensitivity (Jenni et al., 2017; Sonntag et al., 2014). In addition to dopaminergic neurotransmission, corticotrophin releasing factor (CRF) is also involved in RA and RT (Guillaume et al., 2013), particularly with respect to the influence of stress on reward sensitivity (Viola et al., 2016), as well as risk-seeking approach or avoidance (Georgiou et al., 2018). For example, CRF receptor type 1 (CRFR1) gene expression in the mPFC correlates with risk-avoidance behaviors following predator odor exposure in rodents (Schreiber et al., 2017), while the blockade of this receptor reverses stress-induced cognitive and executive impairments (Uribe-Marino et al., 2016). In this sense, stress can dynamically modulate the mesocorticolimbic dopamine system (*e.g.* mPFC and striatum) via actions of the neuropeptide CRF on its receptors (Holly et al., 2015).

Dopaminergic and corticotrophinergic signaling therefore represent good candidates for an investigation into the effects of ELS on RA and RT. Furthermore, emerging evidence supports the idea that the transcription of dopaminergic and corticotrophinergic genes is regulated through the activity of epigenetic mechanisms that can act as a rheostat, serving to turn up or turn down levels of gene expression in response to rapidly changing environmental demands (Baker-Andresen et al., 2013; Takase et al., 2013). More recently, it has been shown that epigenetic mechanisms can also prime genes for responsivity to future events (Stroud et al., 2017), and that the early postnatal environment has a crucial role on the corresponding changes in DNA or histones that is accompanied by gene expression and enduring behavioral phenotypes (Vialou et al., 2013). Therefore, the study of the epigenetic landscape is also of particular interest since it has been implicated in the neurobiology of a variety of cognitive and behavioral processes (Marshall and Bredy, 2016), but little is known regarding its role on gene expression regulation implicated on RA and RT processing.

On these bases, using a mice model of impoverished housing during early infancy, we investigated the effects of early life adversity on adolescent RA and RT behavior. We utilized the elevated plus-maze (EPM) for the investigation of anxiety and RA when a reward is not presented, and the predator-odor risk-taking (PORT) task for the investigation of RT behavior associated with a reward stimulus (Dent et al., 2014). The PORT task explores a conflict between two biologically relevant stimuli for rodents: the motivation to consume a sweet and highly palatable solution while being threat by predatory olfactory cues. Since previous studies highlighted sex differences in decision-making and RT behavior (Georgiou et al., 2018), as well as regarding vulnerability to the effects of ELS (Walker et al., 2017), we aimed to extend these findings with an investigation of the performance of both adolescent male and female mice in these behavioral tasks. In order to determine to what extent the dopaminergic and corticotrophinergic signaling may account for the effects of stress and sex in RA and RT processing, we measured gene expression levels of *Drd1*, *Drd2*, *Crf* and *Crfr1* in the mPFC and in the striatum.

Finally, histone acetylation/deacetylation and methylation of specific lysine residues on nucleosomal histone proteins (*i.e.*, H3-K9) within promoter regions are ways that chromatin remodeling can influence gene transcription. In particular, modifications of histone H3 in the mPFC have been associated with the effects of stress and fear on cognition (Bredy et al., 2007). Therefore, we also investigated the levels of $H3K9^{me3}$, a histone mark associated with transcriptional repression, and the levels of $H3R2^{me2s}$, a histone mark associated with gene expression, within the promoter region of behaviorally relevant candidate genes.

2. Methods

2.1. Animals

This study was performed with male and female C57BL/6 mice obtained from the colony of the Center for Experimental Biological Models (CeMBE), Pontifical Catholic University, Porto Alegre, RS, Brazil. The CeMBE is a facility devoted for the breeding of rodents in accordance with the sanitary standard SPF (Specific Pathogen Free). This building consists of breeding rooms, quarantine rooms, expedition services, material disinfection and sterilization, a warehouse, cold chambers, a diagnostic laboratory and operating rooms. In order to avoid any stress associated with animal transportation between different facilities, as well as to generate concomitant births, mice were bred in-house at the CeMBE, with technical support for the management of animals by a veterinarian staff team, duly registered at the Regional Veterinary Medicine Board. Two adult female C57BL/6 mice were bred with an adult male for 72 h. After this period, the male animal was moved to a new cage, while females remained together. Following two weeks and at the end of gestational period, females were separated in individual cages. Then, single-housed pregnant females were visually checked daily for the presence of pups. Following birth, 30 different mice litters were used, in which 15 were randomly assigned to standard reared group, and 15 were randomly assigned to impoverished housing condition (Rice et al., 2008). Each litter contributed with one to three pups per sex for each experimental group.

All animals were housed under a 12 h/12 h light–dark cycle in ventilated Plexiglas cages with temperature maintained at 21 ± 1 °C. Food and water were available *ad libitum*, with the exception when animals underwent water restriction for PORT behavioral training and testing. The experiments were conducted in accordance with the NIH laboratory animal care guidelines and approved by the Ethical Committee on the Use of Animals of the Pontifical Catholic University of Rio Grande do Sul. The experimental design and timeline of experiments are depicted in Fig. 1. All behavioral testing were conducted in the light phase of the light-dark cycle, with luminosity at the level of 75 lx. Mice were weighted at the postnatal day (P) 9, P30 and P40.

2.2. Model of impoverished housing during early infancy

We used the limited bedding and nesting (LBN) protocol (Rice et al.,

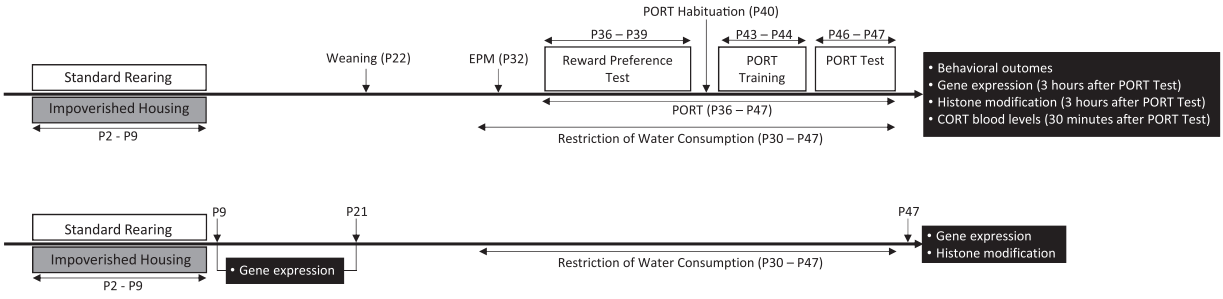

Fig. 1. Experimental design and study outcomes; EPM, elevated plus-maze; PORT, predator-odor risk-taking task.

2008). From P2 to P9, standard rearing dams and litters were placed in cages with standard amounts of wood shavings and 4 g of cotton as nesting material. Therefore, dams shredded the cotton to create an ample nest area for control pups. Dams assigned to impoverished housing conditions had reduced amount of nesting material (1 g of cotton) for the period of 8 days (P2–P9). Moreover, the cage floor was covered with an aluminum mesh platform, and wood shavings were placed underneath it. Thus, while mouse droppings could fall below the platform without trapping the pups, impoverished housing dams could not reach the wood shavings. Following P9, impoverished housing dams and litters were allocated into new cages prepared according with standard rearing control conditions. Both groups were left completely undisturbed from P2 to P22, except for regular cage changes (fresh wood shavings and nesting material) at P9, P16 and P21. All pups were weaned at P22 and remained together with their same-sex littermates (2 or 4 animals per cage), under standard housing conditions.

2.3. Maternal behavior analysis

Previous evidence showed that limited nesting material following birth is associated with altered maternal behaviors of the mouse dam toward pups (Rice et al., 2008). Therefore, observations of maternal behavior (n = 10 dams per group) were conducted from P2 to P9. The observations were conducted seven times over a period of 18 min (0, 3rd, 6th, 9th, 12th, 15th and 18th min) and were performed during three shifts of the day – 9 a.m., 3:30 p.m., and 7:15 p.m. The frequency of behaviors evaluated were nursing (N), licking pups (L), contact with pups (C), no interaction with pups (X) and eating/drinking (E). The behaviors N, L, B and C were categorized as maternal care related behaviors. Thus, the percentage of maternal care was computed by dividing the sum of all maternal care related behaviors by the number of all observations. Additionally, the frequency of exits of the dam from the nest was recorded during all observations. Maternal behavior was recorded by two independent observers, which were blind to postnatal rearing conditions.

2.4. PORT task

One hundred animals were used for behavioral training (n = 25 offspring per group). The PORT is a behavioral protocol that allows the investigation of RT under reward-seeking conditions (Dent et al., 2014). The rewarding stimulus used was a solution of condensed milk (10%) mixed with water (90%). Water restriction was required for 15 h daily, beginning at P30 and lasting until the final day of testing (P47). Water bottles were withdrawn at 6 pm, and animals only had access to water again in the morning of the next day, or after the completion of behavioral tasks. PORT behavioral procedures were performed between 8 a.m. and 11:30 am.

Habituation to the rewarding solution was performed when mice were at P33. A Falcon tube cap containing the sweet solution (2 mL) was placed inside animal's home cages for the period of 48 h. From P36 to P39, animals were daily tested for their preference regarding the consumption of the sweet solution over water. These 15 min sessions were performed in Plexiglas open field boxes (30 cm × 30 cm × 30 cm) containing two Falcon tube caps, one containing the sweet solution (2 mL) and the other containing only water (2 mL). To prevent side preference, the position of the caps was switched between sessions. Sweet solution and water consumption was measured by weighing the caps before and after test. The percentage of reward preference was computed using this equation [volume of sweet solution consumption/ volume of sweet solution consumption + volume of water consumption]. Mice that did not present at least 60% of reward preference in the last testing session, were excluded from the analysis.

Habituation to the PORT apparatus was performed when animals were at P40, for 15 min. The PORT apparatus is a Plexiglas box divided into three equal compartments (30 cm × 30 cm × 30 cm each chamber). Compartments are connected by doors (5 cm × 5 cm), allowing animals to fully explore the apparatus. In all PORT sessions the floor of the central compartment was covered with wood shaving. From P42 to P43, mice performed training sessions to learn where to collect the sweet solution (40 uL) inside the PORT apparatus. Each animal performed 4 trials per day (total of 8 trials). At each trial, the animal was placed in one of the lateral compartments and allowed to explore the three compartments. The sweet solution (40 uL) was positioned in the center of the opposed lateral compartment. Therefore, animals had to cross the apparatus to collect the sweet solution. Some mice moved from left to right and others right to left, but the direction was fixed for each individual subject throughout trials. Following reward consumption animals were placed back in their home cages and waited for the next trial (approximately 5 min of interval). The time spent to collect the reward was recorded for each trial.

Tests were performed similarly to training trials. Animals were placed in the initial compartment and allowed to explore the PORT apparatus until reward consumption. In the neutral condition test (P46), the floor of the central compartment was covered with wood shavings just as a training trial. However, in the final test (P47), wood shavings were mixed with coyote urine (2 mL) to generate a potentially dangerous environment within the PORT central compartment (Wang et al., 2013).

The time spent in the initial or in the central compartment, before the animal reached the reward-containing area, and the time spent to collect the reward were recorded. In addition, when the animal hesitated and stretched/sniffed with front paws before entering the central compartment, the frequency of this specific behavior was recorded as a measure of RA in the PORT task. PORT data was video recorded and analyzed by two independent observers, which were blind to postnatal rearing conditions. Any discordance during this process was discussed with another author to reach a final agreement.

Importantly, of the 100 animals used for behavioral training, 30% did not show at least 60% of reward preference in the last preference test (two-way analysis of variance (ANOVA) - no significant group effect [$F(3,96) = 0.43$, $p = 0.512$; Fig. A1 in Supplementary material], and no significant sex effect [$F(3,96) = 0.40$, $p = 0.527$]), and therefore were excluded from the study (no group differences in exclusion, $x^2 = 0.95$; $p = 0.810$). Thus, the final sample size for behavioral analysis (*e.g.* EPM and PORT) was: males exposed to impoverished housing (n = 19), males with standard rearing (n = 16), females exposed to impoverished housing (n = 17), and females with standard rearing (n = 18).

Regarding estrous cycle effects on behavioral outcomes, the prospective vaginal swab is the most accurate method to identify the phase of the estrous cycle, but this method could serve as a selective stressor that could impact performance and interpretation of the data. So, we used the visual inspection method (Byers et al., 2012) to identify the estrous cycle in the last days of testing, which provided us a quick and stress-free method of evaluation. We found that all female mice were in diestrus stage of the estrous cycle during PORT tests (P46 and P47).

2.5. EPM test

Given that anxiety traits could fundamentally affect the perception of risk (Reis et al., 2012), the EPM was used for the study of anxiety and also RA. The EPM apparatus was constructed with black Plexiglas and consisted of two open arms (30 cm × 5 cm) and two closed arms (30 cm × 5 cm × 15 cm) connected via a central platform (5 cm × 5 cm). The apparatus was raised to a height of 50 cm above the ground. Before PORT behavioral training and specifically at P32, mice were transported to the experimental room and left undisturbed for 15 min prior to testing. They were placed individually in the center of the maze facing a closed arm and allowed 5 min of free exploration. The time spent in the open or closed arms, as well as in the center of the maze was recorded.

In addition, the frequency of the following RA behaviors (Reis et al., 2012) was recorded: 1) when mice dipped their heads below the level of the maze floor (head dipping); 2) when stretching the head/shoulders from the center of the maze towards open arms (peeping out); and 3) when the animal stretches to its full length with the forepaws keeping the hind paws in the same place and turns back to the anterior position while exploring the center of the maze (stretched-attend posture). The sum of these behaviors was computed as RA behaviors in the EPM. In addition, EPM data was video recorded and analyzed by two independent observers, which were blind to postnatal rearing conditions. Any discordance during this process was discussed with another author to reach a final agreement.

2.6. Brain tissue collection

Three hours after the final test of the PORT task mice were euthanized. We selected this time point since previous evidence indicated enhanced gene expression of neurotransmitter receptors after 3 h of a single exposure to predatory olfactory cues (Liu et al., 2010). Additionally, cohort of younger animals (P9 and P21) and animals (P47) that did not perform behavioral training (but were exposed to water restriction) were generated to verify changes in gene expression that could not be related to the effects of the PORT task. The brains were removed immediately after cervical dislocation by decapitation, and the mPFC and the striatum (ventral and dorsal subregions as a single tissue) were dissected. Tissues were then frozen on dry ice and stored at −80 °C until used for molecular analysis.

2.7. Gene expression analysis

Total RNA was isolated from 6 samples per group, randomly selected, using QIAzol (Qiagen; Hilden, Germany) and chloroform standard protocols. Such samples were randomly chosen in order to avoid any selection biases. RNA concentration was measured using the NanoDrop spectrophotometer. Total of 500 ng of RNA from each sample was reverse transcribed using the miScript II RT Kit (Qiagen). The following Quantitect primers (Qiagen) were used: *Crf* (QT0029389) and *Crfr1*; and the following IDT primers were used: *Drd1* (Forward): ATGGCTCCTAACACTTCTACCA; *Drd1* (Reverse): GGGTATTCCCTAAG AGAGTGGAC; *Drd2* (Forward): ACCTGTCCTGGTACGATGATG; *Drd2* (Reverse): GCATGGCATAGTAGTTGTAGTGG; *Pgk* (Forward): TGCACG CTTCAAAAGCGCACG; *Pgk* (Reverse): AAGTCCACCCTCATCACGACCC. Each SYBR Green PCR reaction was run in duplicate for each sample using a Rotor Gene Real-Time PCR machine (Qiagen). The fold change relative expression was calculated using the ΔΔCt method with the male standard reared group as a reference. *Pgk* ct values were used as endogenous control for mRNA analysis. To verify primer specificities, melting curve analyses and agarose gels were performed.

2.8. Histone modification analysis

Chromatin immunoprecipitation (ChIP) was performed following modification of the Invitrogen ChIP kit protocol. Tissues from two mice were randomly selected from each group, pooled and counted as one sample (each group had five samples). Samples were fixed in 1% formaldehyde and cross-linked cell lysates were sheared by sonication (5 repeats of 15 s 90% pulses, with 30 s of interval) in 1% SDS lysis buffer to generate chromatin fragments with an average length of 300bp. The chromatin was then immunoprecipitated overnight at 4 °C using the specific Abcam antibodies to each target: H3R2 symmetric dimethylation – $H3R2^{me2s}$ (AB194684 – 2 ug per sample) and H3K9 trimethylation – $H3K9^{me3}$ (AB8898 – 4 ug per sample). Protein-DNA-antibody complexes were precipitated with protein G-magnetic beads for 1 h at 4 °C, followed by three washes in low salt buffer, and three washes in high salt buffer. The precipitated protein-DNA complexes were eluted from the antibody with 1% SDS and 0.1 M NaHCO3, then incubated overnight at 60 °C in 200 mM NaCl to reverse formaldehyde cross-link. Following proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation, samples were subjected to qPCR using primers specific for 200bp segments corresponding to the promoter region of the *Crfr1* gene: Upstream transcription start site (TSS): Forward – GGTGAACCTCCTGGATGGC; Reverse – GCAGGCAGCCTTTCTTCTCT; Downstream TSS: Forward – GGCGCCTGGGAGCAG; Reverse – CTTCA CGAGCCGGAGCTG. Additionally, 200bp primers were designed for the upstream region of the TSS of the *Gapdh* gene: Forward – TCAGCACG TCCCTGGATGG; Reverse – GGCTCAGAGGCCTCCTATAGTATC. Samples with no immunoprecipation (input) were used to normalize qPCR data from immunoprecipitated samples. The ΔΔCt method was used for ChIP analysis with the female standard reared group as a reference.

2.9. Corticosterone levels

As a measure of the hypothalamic-pituitary-adrenal (HPA) axis functioning, trunk blood was collected and corticosterone (CORT) levels were measured from 8 animals per group 30 min after the final PORT test. A total of 5 u L of plasma were used for analyses performed with the Corticosterone Enzyme Immunoassay (Arbor Assays) ELISA kit, according to the manufacturer's instructions. The optical density was analyzed at 450 nm wavelength in an ELISA plate reader, and the data was subsequently transformed into concentration (pg/mL) using standard curve parameters.

2.10. Statistical analysis

Experimental differences were assessed by Student's *t*-test for maternal care behavior, body weight, gene expression analysis throughout development (P9, P21 and P47 without behavioral test), and for histone modification data. Two-way ANOVA was used for behavioral (EPM), HPA axis, and gene expression data analysis, with a sex factor (male x female) and a group factor (impoverished housing x standard rearing). The PORT data was analyzed by means of repeated measures ANOVAs, with a condition factor (no odor x coyote urine) and a group factor (impoverished housing x standard rearing), for each sex individually. To identify specific effects in pairwise comparison, ANOVAs were followed by Tukey-Kramer *post-hoc* tests for multiple comparisons between all 4 conditions (standard reared males, standard reared females, impoverished housing males, impoverished housing females). Linear regression analysis was used to evaluate the association between behavioral and gene expression data. Statistical significance was defined as $p < 0.05$ and results are expressed as the mean ± SEM.

3. Results

3.1. Alterations in maternal behavior and body weight during infancy due to impoverished housing conditions

Exposure to impoverished housing led to a fragmentation in maternal care, manifested by an increased frequency of exits of the dam from the nest/pups in the impoverished housing group compared with standard reared group [$t(18) = 3.07$, $p = 0.044$; Fig. 2A]. No significant differences were detected regarding the percentage of maternal care behaviors between groups. Additionally, impoverished housing exposure reduced the body weight in infant animals (P9), as compared to standard-reared animals [$t(38) = 3.20$, $p = 0.003$; Fig. 2C], although no differences were detected at P30 and P40.

3.2. Females exposed to impoverished housing showed decreased RA behaviors in the EPM

No significant group, or group x sex interaction effects were detected in a 2-way ANOVA regarding the exploration time of the open

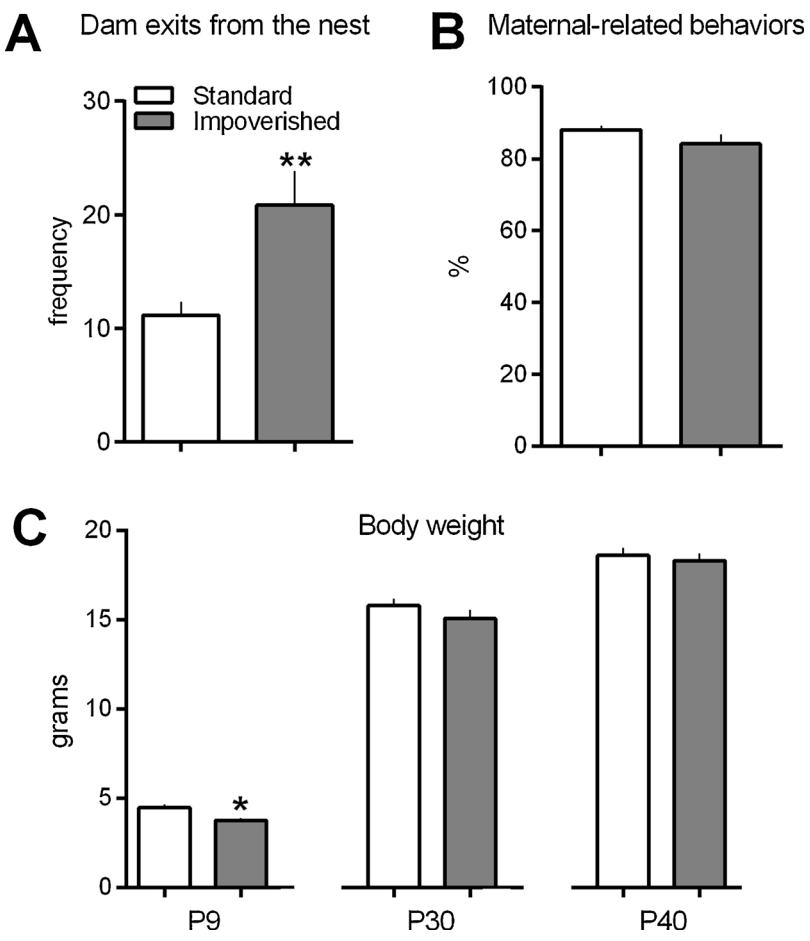

Fig. 2. Maternal behavior and body weight analyses; (A) frequency of the exits of the dam from the nest; (B) percentage of maternal-related behaviors (licking pups, nursing pups and contact with pups); (C) body weight at P9, P30 and at P40 in grams; *, $p < 0.05$ (*t*-test); **, $p < 0.01$ (*t*-test); results are expressed as the mean ± SEM.

(Fig. 3A) and closed arms (Fig. 3B), and regarding the time spent at the center of the EPM (Fig. 3C). Regarding RA behaviors, a significant group effect was observed showing that impoverished housing exposure was associated with less RA behaviors compared with standard rearing condition [$F(3,64) = 9.16$, $p = 0.004$; Fig. 3D], while no significant group x sex interaction was detected. *Post-hoc* analysis revealed that this effect was specific to impoverished housing female mice, given that these animals had less RA behaviors compared with standard reared females ($p = 0.048$).

3.3. Females exposed to impoverished housing showed increased RT under reward-seeking conditions and decreased HPA axis response to coyote urine

First, we analyzed the PORT data (Fig. 4A) exploring potential effects of group and sex on the main PORT dependent variable, the latency to collect the reward in the last test (following coyote urine odor exposure) by means of a two-way ANOVA. We found a significant group x sex interaction [$F(1,66) = 5.20$, $p = 0.028$]. Taking into account this interaction effect, we analyzed all the remaining PORT data for each sex separately, in order to further explore the PORT condition (no odor x coyote urine) as a main factor in repeated measures ANOVAs.

Male mice presented a significant reduction in the latency to collect the reward among training trials [condition effect: $F(7,252) = 9.57$, $p < 0.001$; Fig. 4B], while no group differences were detected in this learning process. In the test sessions, coyote urine exposure induced an increase in the latency to collect the reward compared with the neutral condition [condition effect: $F(1,33) = 4.82$, $p = 0.035$], but similarly no group differences were detected.

Female mice also presented a significant reduction in the latency to collect the reward among training trials [condition effect: $F(7,231) = 16.06$, $p < 0.001$; Fig. 4C], while no group differences were detected during PORT learning. Similarly, coyote urine exposure induced an increase in the latency to collect the reward compared with the neutral condition in the testing sessions [condition effect: $F(1,33) = 7.67$, $p = 0.009$]. However, a reduction in the latency to collect the reward was observed in females exposed to impoverished housing in comparison with standard reared females [group effect: $F(1,33) = 4.37$, $p = 0.044$], and such differences were specific to the coyote urine testing trial (*Post-hoc* analysis, $p = 0.036$). Furthermore, this effect could not be attributed to changes in reward preference (Fig. A2 in Supplementary material), as both groups of males [$F(3,96) = 20.82$, $p < 0.001$] and females [$F(3,90) = 19.62$, $p < 0.001$] increased their preference for the consumption of the sweet solution over water, while no effects of impoverished housing exposure were observed regarding the acquisition of reward preference.

Since the predatory olfactory cues were introduced in the central compartment of the PORT apparatus, we examined potential differences between groups regarding the time spent in this area. Male mice presented a significant increase in the exploration time of the central compartment when exposed to coyote urine compared with the neutral condition [condition effect: $F(1,33) = 4.95$, $p = 0.033$, Fig. 4D], but no group differences were detected. Female mice also presented a significant increase in the exploration time of the central compartment when exposed to coyote urine compared with the neutral condition [condition effect: $F(1,33) = 30.91$, $p < 0.001$, Fig. 4E]. However, this significant increase was specific to the standard reared females [group x condition interaction effect: [$F(1,33) = 5.99$, $p < 0.020$, *post-hoc* analysis (no odor x coyote urine), $p < 0.001$, *post-hoc* analysis (standard reared x impoverished housing in the coyote urine trial), $p = 0.030$], given no differences were observed in females exposed to impoverished housing.

This specific effect among standard reared females prompted us to

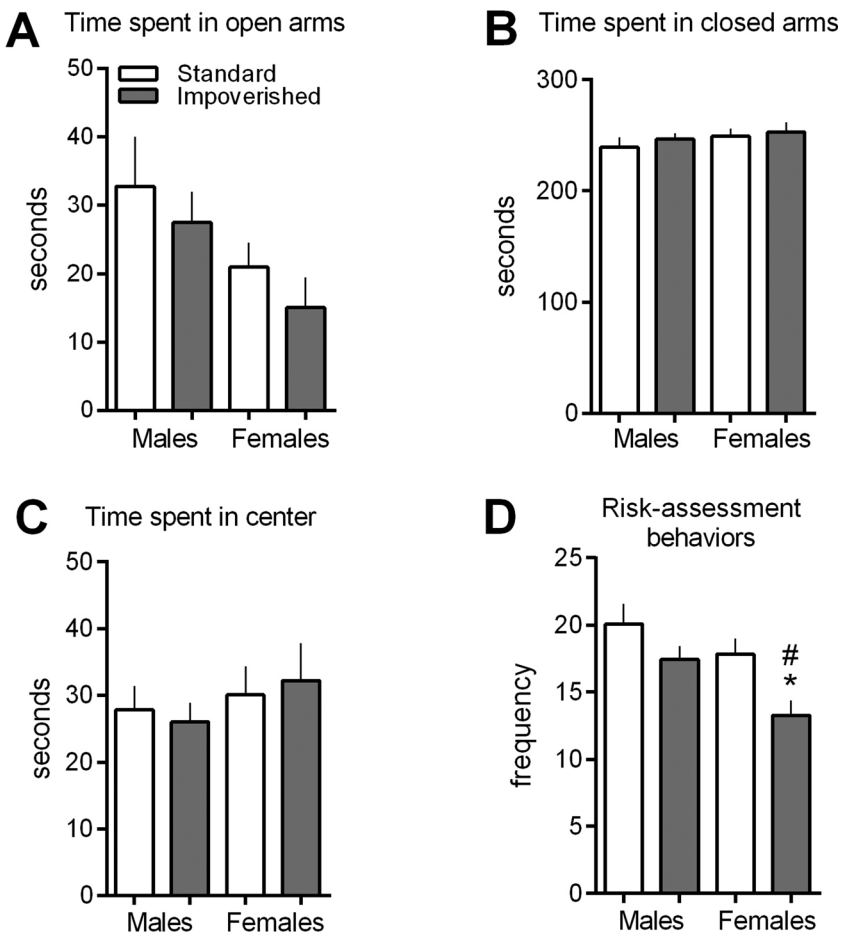

Fig. 3. Analyses of the parameters of the EPM; (A) time spent in open arms; (B) time spent in closed arms; (C) time spent in center area; (D) frequency of RA behaviors (head dipping, peeping out, stretched-attend posture); #, significant group-effect in the 2-way ANOVA; *, $p < 0.05$ (Tukey *post-hoc* test); results are expressed as the mean ± SEM.

investigate if the increase in the time spent in central compartment was specific to the coyote urine odor or, given to novelty-seeking effects, of distinct odors mixed with wood shavings (Fig A5 in Supplementary material). Therefore, in an independent cohort of control animals we observed a significant increase [$F(2,42) = 8.73$, $p > 0.001$] in the exploration time of the central compartment when females were exposed to the coyote urine in the PORT test ($p = 0.012$), but not when they were exposed to citronella ($p = 0.450$), compared with the no odor condition.

Regarding the time spent in the initial compartment of the PORT apparatus, coyote urine exposure induced an increase in the latency to enter central compartment compared with the neutral condition in the testing sessions in females [condition effect: $F(1,33) = 10.44$, $p = 0.003$, Fig A3 in Supplementary material], but not in males. Moreover, no group differences were observed in both females and males. Regarding the frequency of hesitations and stretching/sniffing with front paws before entering the central compartment, coyote urine exposure induced an increase in the frequency of such behavior compared with the neutral condition in the testing sessions in females [condition effect: $F(1,33) = 4.34$, $p = 0.045$, Fig A4 in Supplementary material], but similarly not in males. No group differences were observed in both females and males.

Then, we explored HPA axis functioning 30 min after the PORT test with coyote urine, and no group differences were observed. However, a significant group x sex interaction effect was detected ($F(3,33) = 48.48$, $p < 0.001$; Fig. 4F), showing that females exposed to impoverished housing had lower CORT levels when compared to standard reared females ($p < 0.001$).

3.4. Association between female higher RT induced by impoverished housing conditions and Crfr1 gene expression in the mPFC

After the PORT test with coyote urine, no significant sex or group effects were detected in the mRNA levels of *Drd1* (Fig. 5A), *Drd2* (Fig. 5B) and *Crfr1* (Fig. 5D) in the striatum. A significant group effect was detected in the mRNA levels of *Crf* ($F(3,21) = 4.40$, $p = 0.048$; Fig. 5C), showing higher *Crf* expression in both males and females exposed to impoverished housing compared with standard reared mice.

Similarly, no significant sex or group effects were detected in the mRNA levels of *Drd1* (Fig. 5E), *Drd2* (Fig. 5F) and *Crf* (Fig. 5G) in the mPFC. Regarding *Crfr1* mRNA levels, no group effect was observed, but a significant group x sex interaction effect was detected ($F(3,21) = 16.06$, $p > 0.001$; Fig. 5H). This interaction effect revealed that only females exposed to impoverished housing had significant higher mRNA levels of *Crfr1* in the mPFC compared with standard reared females ($p = 0.012$). Furthermore, linear regression analysis revealed a significant negative association between *Crfr1* gene expression in the mPFC and the latency to collect the reward in the PORT test with coyote urine among females ($R = 0.59$; $R^2 = 0.39$; $p = 0.029$; Fig. 5I), but not among male mice.

Considering this interaction and sex-specific effect, we investigated whether the changes in *Crfr1* expression induced by impoverished housing exposure could be identified at earlier stages of female mPFC development (Fig. 5J). Significant lower mRNA levels of *Crfr1* were detected at the P9 [$t(10) = 3.49$, $p < 0.004$] in mice exposed to impoverished housing, as compared to the standard-reared group, but not at P21. Moreover, in the P47, a trend to reduction was observed in animals exposed to impoverished housing that did not perform behavioral training [$t(10) = 2.21$, $p = 0.07$]. These results demonstrated that impoverished housing exposure is associated with changes in the

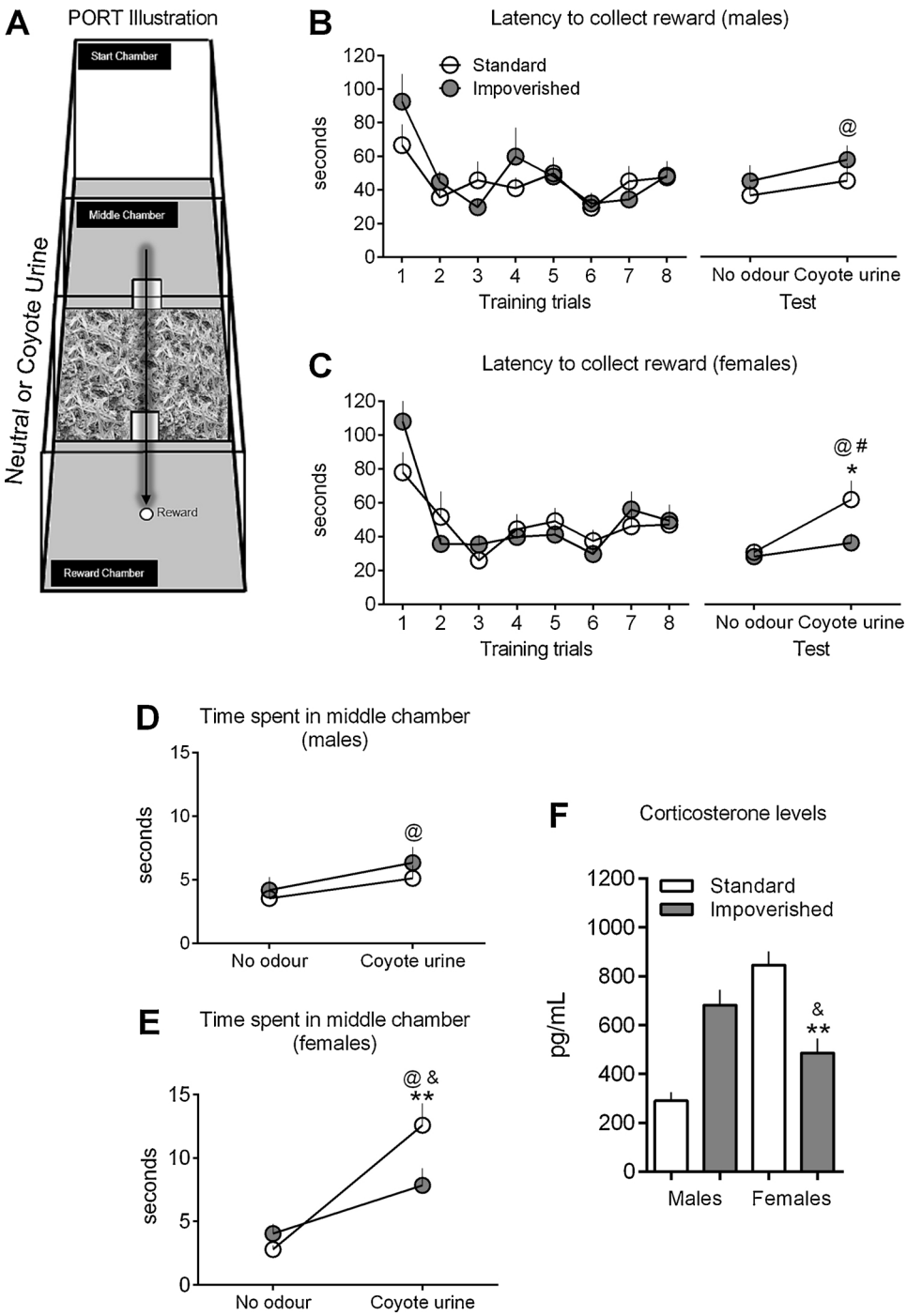

Fig. 4. Analyses of the parameters of the PORT; (A) illustration of the PORT apparatus; (B) latency to collect the reward among male mice; (C) latency to collect the reward among female mice; (D) time spent in the middle chamber of the PORT among male mice; (E) time spent in the middle chamber of the PORT among female mice; (F) blood levels of CORT 30 min following the PORT test with coyote urine (coefficients of variation, standard reared males = 0.38; impoverished housing males = 0.27; standard reared females = 0.18; impoverished housing females = 0.38); @, condition-effect (no odor X coyote urine) in the repeated measures ANOVA; #, significant group-effect in the repeated measures ANOVA; &, significant group x condition interaction-effect in the repeated measures ANOVA; *, $p < 0.05$ (Tukey *post-hoc* test); **, $p < 0.01$ (Tukey *post-hoc* test); results are expressed as the mean ± SEM.

expression of *Crfr1* in the mPFC throughout early postnatal life and adolescence, specifically in female mice, manifested as a decrease in the expression throughout development and an increase following the PORT test with coyote urine.

3.5. Higher levels of the histone mark H3R2^{me2s} in the mPFC of female mice exposed to impoverished housing conditions

This notable dynamic change of *Crfr1* gene expression, prompted us to investigate the chromatin status surrounding the promoter region of the *Crfr1* gene following the PORT test with coyote urine, specifically in the mPFC. The level of histone H3 methylation (H3R2^{me2s} and H3K9^{me3}) was investigated by ChIP analysis, particularly 200bp upstream the TSS, and 200bp downstream the TSS. No significant sex or group effects were observed regarding the levels of H3K9^{me3} (Fig. 6A), a histone mark associated with transcriptional repression, upstream or downstream the TSS. Similarly no significant sex or group effects were observed in the levels of H3R2^{me2s} (Fig. 6B), a histone mark associated with gene expression, upstream or downstream the TSS. However, a significant interaction effect (sex x group) was detected ($F(3,17) = 4.56$, $p = 0.047$), revealing that females exposed to impoverished housing had significant higher H3R2^{me2s} levels upstream the TSS of the promoter region of the *Crfr1* gene in the mPFC, compared with standard reared females ($p < 0.045$). Taking into account such differences between female mice groups, we investigated both H3R2^{me2s} and H3K9^{me3} levels at 200bp upstream of the TSS of the constitutively expressed *Gapdh* gene, but no group differences were observed on both histone marks (Fig. A6 in Supplementary material).

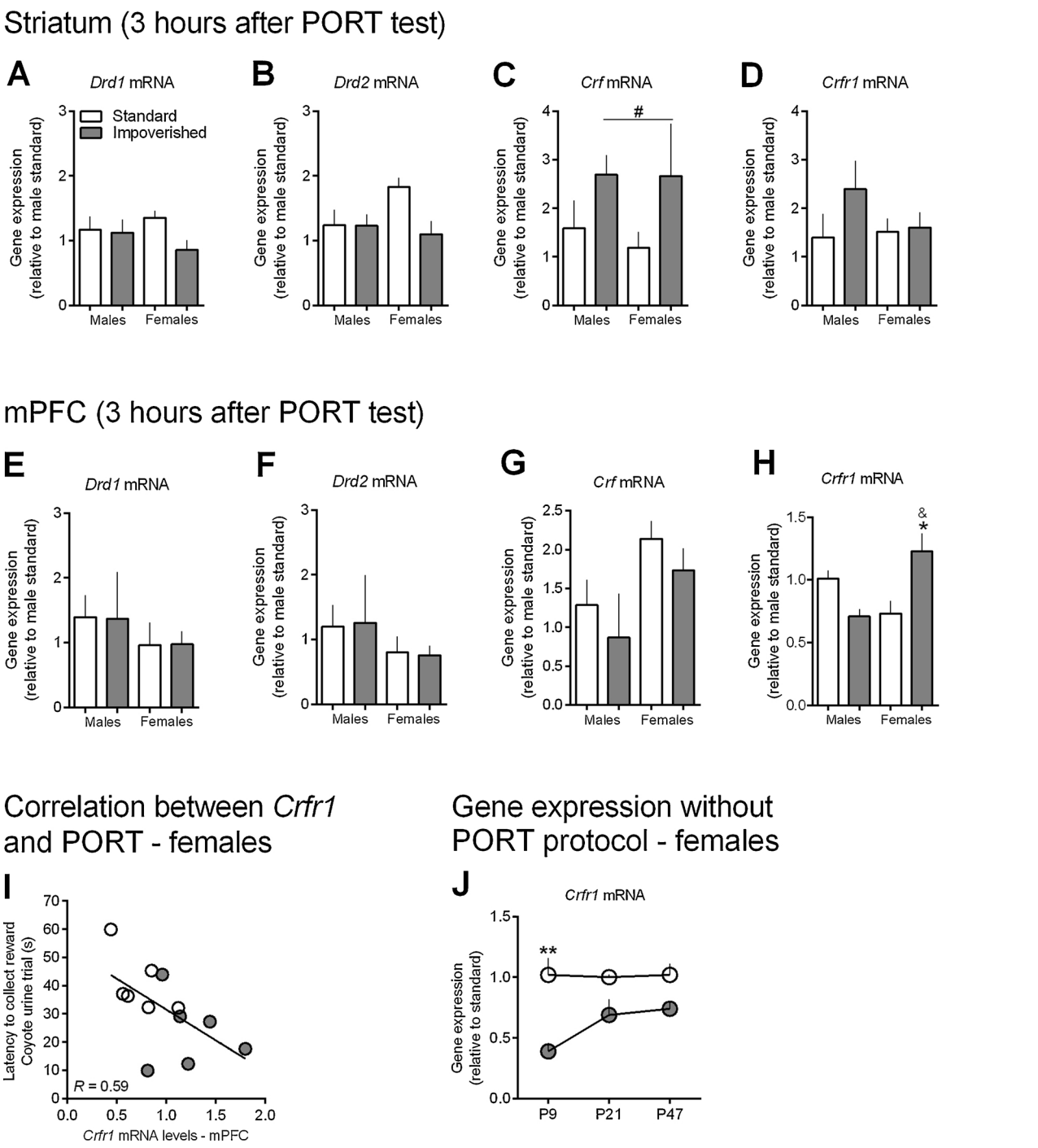

Fig. 5. Gene expression analyses in the striatum 3 h following the PORT test with coyote urine; (A) *Drd1* mRNA levels; (B) *Drd2* mRNA levels; (C) *Crf* mRNA levels; (D) *Crfr1* mRNA levels; Gene expression analyses in the mPFC 3 h following the PORT test with coyote urine; (E) *Drd1* mRNA levels; (F) *Drd2* mRNA levels; (G) *Crf* mRNA levels; (H) *Crfr1* mRNA levels; (I) linear regression analysis showing a negative association between *Crfr1* gene expression in the mPFC and the latency to collect the reward in the PORT test with coyote urine among females; (J) *Crfr1* mRNA levels at P9, P21 and at P47 in female animals that did not perform behavioral training/test; #, significant group-effect in the 2-way ANOVA; &, interaction-effect in the 2-way ANOVA; *, $p < 0.05$ (Tukey *post-hoc* test); **, $p < 0.01$ (*t*-test); results are expressed as the mean ± SEM.

In order to verify if these histone changes occurred in the female mPFC only following the PORT test, we investigated both histone marks at the *Crfr1* promoter region of adolescent female mice (P47) that did not perform behavioral training. We did not detect any significant group differences between impoverished housing or standard rearing conditions in the levels of H3K9^{me3} (Fig. 6C) and H3R2^{me2s} (Fig. 6D), suggesting that higher levels of the histone mark H3R2^{me2} in the mPFC of females exposed to impoverished housing is dynamic, particularly occurring after the last test of the PORT behavioral protocol.

4. Discussion

In this study, we investigated the effects of ELS on RA and RT processes by using a mouse model of impoverished housing exposure during early infancy. Consistent with previous studies of early life adversity (Rice et al., 2008), impoverished housing led to reduced body

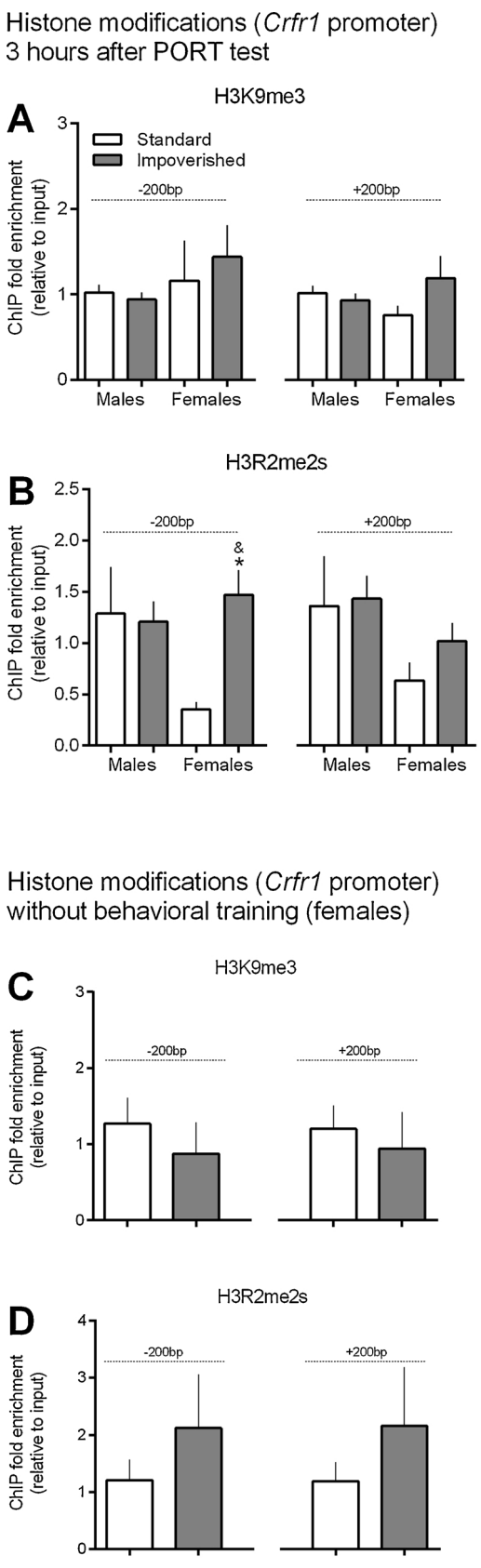

Fig. 6. Histone modification analyses at the promoter region of the *Crfr1* gene upstream (200bp) and downstream (200bp) the transcription start site–TSS in the mPFC; (A) H3K9me3 levels 3 h following the PORT test with coyote urine; (B) H3R2me2s levels 3 h following the PORT test with coyote urine; (C) H3K9me3 levels in animals that did not perform behavioral training/test; (D) H3R2me2s levels in animals that did not perform behavioral training/test; &, interaction-effect in the 2-way ANOVA; results are expressed as the

weight of pups and altered maternal care across the first 9 days of the postnatal period. Furthermore, being raised in an impoverished environment resulted in altered behavioral and molecular phenotypes during adolescence, with distinguishable effects between sexes. Specifically, decreased RA processing was observed in female mice exposed to impoverished housing in the absence of a reward (EPM). Moreover, when exposed to a predatory olfactory cue, increased RT was observed in these females in a reward-related task (PORT), as well as decreased HPA axis responsivity. These patterns of RT behavioral alterations were associated with higher *Crfr1* gene expression in the mPFC, which correlated with an euchromatin state at the proximal promoter region of the *Crfr1* gene, exhibited as higher levels of the histone mark H3R2^{me2s}. Together, these results revealed that ELS-related phenotypes could be extended to impaired acquisition of environmental information in potentially dangerous situations and increased RT in reward-related scenarios, and that the *Crfr1* gene has an important role on the relationship between ELS consequences later in life in females.

Human studies simulating real-life risky decisions have showed that neuropsychiatric patients present patterns of impulsive behaviors due to the difficulties in delaying gratification, and impaired perception of risks and benefits (Kluwe-Schiavon et al., 2016a). RT involves the exposure to some danger, or negative consequences, in order to achieve a goal-directed behavior (Furby, 1992). In our set of experiments using the PORT task, mice had the motivation to consume a sweet and highly palatable solution while being threat by predatory olfactory cues. Standard reared females presented a typical pattern of approach/avoidance conflict when exposed to predatory olfactory cue, since both the latency to collect the reward and the time spent in the central compartment of the PORT, a particular risky area in this environment, were significantly higher compared to the neutral condition. Such behaviors are associated with less RT.

In contrast, risk-prone animals would present less latency to get the rewarding stimulus in such high-risk scenario, disregarding that a predator may be, or has recently been, nearby. In female mice exposed to impoverished housing during early life, we observed such behavioral pattern, coincident with less exploration time of the central compartment of the PORT. In males, however, although we observed a significant effect of the predatory olfactory cue on the latency to collect the reward, postnatal rearing conditions did not alter RT behavior. Thus, these data suggest that early adversity contributes to higher levels of impulsivity and RT behavior in females during adolescence, particularly in reward-seeking conditions, as well as reduced emotional reactivity, which was supported by the findings of lower HPA axis responsivity facing this high-risk scenario. Human studies corroborate this hypothesis since disadvantageous patterns of decision-making was observed in the subjects with the lowest cortisol levels (van Honk et al., 2003), while a history of childhood adversity has been shown to negatively affect decision-making processes through an interaction with key HPA axis genes (Guillaume et al., 2013).

Moreover, males and females naturally differ in how they cope with risk, and this disparity might be an important element of our sex-specific effect produced by early impoverished housing exposure. Females are more risk-averse, more influenced by available contingencies and more responsive to changes in their current environment than males, which are more risk-prone (van den Bos et al., 2012). For instance, a study that exposed rodents to a predator odor found that females only started to display high levels of exploratory behavior after repeated exposures to this adverse olfactory stimulus, when the risk had relatively diminished (Jolles et al., 2015). Thus, the female-specific impoverished housing effect in the PORT task highlights that ELS only affected the more responsive sex to risky situations. In this sense, our results corroborated with the idea that sex may play a significant role in determining the effects of early life adversity on behavioral outcomes (Manzano-Nieves et al., 2018), and that such differences could be attributed to sex-differences in the neurobiological basis underlying RT processing.

RT is likely to be preceded, at least at some level, by the cognitive process of gathering and assessing information about the nature of the danger during exposure to risk-related circumstances (Meyerson et al., 2006). In the PORT task, such RA-related behaviors were investigated by the frequency of hesitations and stretching/sniffing with front paws before entering the central compartment. We observed a significant increase in such behaviors when animals were exposed to coyote urine compared with the neutral condition, but there were no significant group differences in this outcome in both sexes. Given that both RT and RA are highly influenced by the expected benefit and motivational processes (Meyerson et al., 2006), it seems that in a reward-seeking condition, rather than reduced RA, ELS exposure in females led to increased RT behavior during adolescence. However, because of the short duration of the PORT test (usually less than 60 s), it is possible that a floor effect influenced these results, in which usually mice performed less than 3 RA behaviors during testing sessions.

In the EPM mice were exposed to a novel situation that created a conflict between the motivation to explore the novel environment (*e.g.* open arms of the maze) and the fear of novelty (Roy and Chapillon, 2004). Lower frequency of RA-related behaviors (*e.g.* head dipping, peeping-out, stretched-attend posture) was observed in mice exposed to impoverished housing as indicated by a significant group effect in the 2-way ANOVA. In males, however, such differences did not reach statistical significance in *post-hoc* tests, revealing a more significant role of being raised in an impoverished environment on reduced baseline RA processing among females. This is in agreement with previous findings showing that poor RA is often associated with subsequent risky choices and increased RT (Roman and Colombo, 2009), as observed in the PORT task. However, the selective reduction of RA in the EPM among impoverished housing females led us to hypothesize that in a situation were no reward-seeking behavior is implicated, this is the more notable phenotype associated with the effects of ELS during adolescence, rather than higher RT behavior.

Although this phenomenon requires further study, the opposite effects of impoverished housing in RA and RT may be partially related to differences in *Crfr1* gene expression in the mPFC. The primary role of the CRF system is to activate the HPA axis. In parallel, CRF neurons are found in extrahypothalamic areas, including the mPFC, where they may play a significant role in cognitive processes (George et al., 2012). While extensive evidence demonstrated a critical role for CRF-CRFR1 system on behavioral adaptation to stress (Albrechet-Souza et al., 2017), few studies explored the role of CRFR1 on cognitive function, including decision-making processes (Georgiou et al., 2018). Our molecular analyses revealed that females exposed to impoverished housing had significantly higher mRNA levels of *Crfr1* in the mPFC than standard reared females and male animals following the PORT test. Furthermore, a negative association between *Crfr1* mRNA levels and RT in the PORT test was observed among female, but not male mice. Although we did not detect group differences in *Crf* mRNA levels in the mPFC, we observed increased *Crf* in the striatum of mice exposed to impoverished housing, and together with published data (Uribe-Marino et al., 2016), these results suggest that behavioral changes induced by ELS are at least partly dependent on mesocorticolimbic CRF-CRFR1 signaling.

Our results also demonstrated that impoverished housing in female mice was associated with decreased *Crfr1* mRNA levels in the mPFC throughout development, and only following the PORT test enhanced gene expression was observed. It is therefore possible that reduced baseline mPFC *Crfr1* mRNA is associated with less RA (as shown in the EPM), while the presentation of a reward and predatory olfactory cues, when a cost-analysis is required (as in PORT), enhanced *Crfr1* gene expression is a mechanism associated with high levels of RT. For instance, in rodents with high CRF-CRFR1 signaling in the cortex, CRFR1 antagonism in the mPFC could produce risk-prone behavioral phenotypes (Schreiber et al., 2017). Considering that the PORT test is also a mild stressor (*e.g.* coyote urine exposure), previous data already revealed that acute stress increases *Crfr1* mRNA levels in the mPFC, together with higher levels of CREB phosphorylation, and such effects can mediate a variety of alterations in behavior and cognitive process (Uribe-Mariño et al., 2016).

This dynamic change in gene transcription could be potentially associated with epigenetic mechanisms that can regulate gene transcription, particularly regarding how chromatin and histone-DNA complex was re-organized in the mPFC after the PORT test. We showed that female mice exposed to impoverished housing had higher levels of the histone mark H3R2^{me2s}, particularly in the region upstream the TSS of the *Crfr1* gene, compared with standard reared female mice, while no group differences were observed among males, as indicated by a significant interaction effect in the 2-way ANOVA. The deposition of the histone modification H3R2^{me2s} leads to a poised euchromatin structure that is essentially associated transcriptional activation (Migliori et al., 2012), which corroborates our finding of higher mRNA levels in the mPFC following the PORT test. Moreover, it has been recently reported that the H3R2^{me2s} mark is mostly detected on the –1 nucleosome relative to the TSS, where it has an important role in preventing other nucleosomes from assembling in the surrounding promoter region (Migliori et al., 2012).

Given that no group differences were detected in the accumulation of the H3R2^{me2s} mark at the *Crfr1* promoter region in the mPFC of animals that were not exposed to the PORT behavioral test, we assume that this epigenetic mechanism controls dynamic changes in *Crfr1* gene expression associated with higher levels of RT behavior, specifically detected in females exposed to early adversity. It should be noted, however, that we did not observe significant lower levels of H3R2^{me2s} mark in animals not exposed to PORT, although such mice had decreased *Crfr1* mRNA levels in the mPFC compared with standard reared females. Considering the histone code hypothesis, it is proposed that a combination of several histone modifications may regulate the outcome of gene expression. Given that we did not detect any significant effect in the levels of H3K9^{me3}, it is possible that other repressive histone marks, or even DNA methylation (Jabbar et al., 2016), were acting in order to attenuate *Crfr1* gene expression in early-life stressed females throughout development and before PORT testing. These findings suggest that both protocols (*e.g.* impoverished housing and PORT) do not appear to affect histone H3 in general, but rather specific combinations of histone marks associated with specific promoters (*e.g.* H3R2^{me2s}). This is in agreement with findings showing specific histone modifications around the promoters of stress-sensitive genes, such as *Bdnf* (Bredy et al., 2007). Despite that, to draw a more direct link between these, it seems that pharmacological or genetic manipulation of this methyl histone mark should be performed, to generate causal evidence of its role in driving changes in *Crfr1* expression that mediate distinct behavioral phenotypes.

Although accumulating evidence implicates the dopaminergic system in decision-making (Georgiou et al., 2018), and the performance of rodents in the PORT task (Blomeley et al., 2018), we did not find any effects of impoverished housing on *Drd1* or *Drd2* expression in the mPFC. Nevertheless, since we did not investigate protein levels of dopamine receptors as well as their signaling mechanisms, we cannot rule out that the cortical dopaminergic system may contribute to the relationship between impoverished housing and altered RT and RA processing.

5. Conclusion

These findings indicate a potent effect of impoverished housing exposure early in life on RA and RT behavior in adolescent female mice, which could be detrimental for cognitive performance in potentially dangerous situations, and suggest that histone epigenetic regulation of the *Crfr1* gene may represent an important factor in mediating the relationship between ELS and altered decision-making processes later in life in females. Furthermore, our observations supports previous

evidence suggesting that *Crfr1* is critically involved in the regulation of behavioral and neuroendocrine stress-related phenotypes by epigenetic mechanisms (Sotnikov et al., 2014), but also offers new evidence regarding the role of the histone mark H3R2^{me2s} on the regulation of euchromatin states specifically at the promoter region of the *Crfr1* gene. Given that the mPFC has a delayed development, which continues throughout adolescence (Andersen and Teicher, 2009), our results highlight that this brain region and its associated cognitive and behavioral functions are highly vulnerable to the effects of early life adversity, and that females may to be more sensitive to the consequences of being raised in an impoverished environment.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2018.08.032.

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