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ORIGINAL ARTICLE

Early-Life Stress Reprograms Stress-Coping Abilities in Male and Female Juvenile Rats

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Abstract

Prenatal stress (PS) is a major risk factor for the development of emotional disorders in adulthood that may be mediated by an altered hypothalamic–pituitary–adrenal axis response to stress. Although the early onset of stress-related disorders is recognized as a major public health problem, to date, there are relatively few studies that have examined the incidence of early-life stressors in younger individuals. In this study, we assessed PS impact on the stress-coping response of juvenile offspring in behavioral tests and in the induced molecular changes in the hippocampus. Furthermore, we assessed if pregnancy stress could be driving changes in patterns of maternal behavior during early lactation. We found that PS modified stress-coping abilities of both sex offspring. In the hippocampus, PS increased the expression of *bdnf*-IV and *crfr1* and induced sex difference changes on glucocorticoids and BDNF mRNA receptor levels. PS changed the hippocampal epigenetic landscape mainly in male offspring. Stress during pregnancy enhanced pup-directed behavior of stressed dams. Our study indicates that exposure to PS, in addition to enhanced maternal behavior, induces dynamic neurobehavioral variations at juvenile ages of the offspring that should be considered adaptive or maladaptive, depending on the characteristics of the confronting environment. Our present results highlight the importance to further explore risk factors that appear early in life that will be important to allow timely prevention strategies to later vulnerability to stress-related disorders.

Keywords Prenatal stress · Maternal behavior · Juvenile offspring · Sex differences · Stress-coping response · Hippocampus

Abbreviations

5-hmC	5-Hydroxymethylcytosine
5-mC	5-Methylcytosine
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
C	Control group
CRF	Corticotrophin-releasing factor
CRFR1	Corticotrophin-releasing factor receptor 1
DNMT	DNA methyl transferase
EPM	Elevated plus maze behavioral test
FI	Fragmentation index for maternal behavior assessment
FST	Forced swimming behavioral test
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal axis
LDB	Light-dark box behavioral test
MR	Mineralocorticoid receptor
p75-NTR	P75 neurotrophin receptor
PND	Postnatal day
PS	Prenatal stress group
qPCR	Polymerase chain reaction
RA	Risk assessment
SHRP	Stress hypo-responsive period
SEM	Standard error of the mean
TET	Ten-eleven translocation proteins
TrkB	Neurotrophic receptor tyrosine kinase 2

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Introduction

Stress is an essential adaptive response to cope with experiences that threaten or are perceived to be threatening self-safeness, by evoking the classical fight or flight response [1]. Although some stressors arise from situations that are life-threatening such as accidents or natural disasters, the most frequent stressors are common daily life experiences as could be time pressures, economic uncertainty, and interpersonal conflicts at work or home. If the individual is unable to cope with environmental demands or daily hassles, chronic stress can become an important risk factor for the development of emotional disorders, including depression and anxiety [2]. Vulnerability to stress-related disorders depends on the ability of the hypothalamic–pituitary–adrenal (HPA) axis to react to stressors. The nature, intensity, duration, and age of exposure to stressors can dictate the magnitude of the HPA response [1, 3, 4]. However, not all individuals respond to stress in the same way or develop emotional disorders when exposed to chronic stress. In animals ranging from rodent to non-human primates, as well as in humans, the perinatal life is a stage of development where the brain has increased plasticity and, thereby, is particularly vulnerable to modulating environmental influences such as stress [5–7]. Different studies in animals and humans agree in highlighting that exposure of a pregnant female to either stress or synthetic stress hormones induces an early activation of the fetal HPA axis response that enhances individual susceptibility to toxic stress and increases the vulnerability to develop emotional disorders in later life [8–10]. These findings indicate that regardless of the genetic background of the individual, exposure to stressors in early life can program the HPA function determining the future physiological and behavioral traits of an individual response throughout the lifespan [1, 3, 8, 11].

In our laboratory, we have a long-standing interest in the effects of prenatal stress (PS) on neurodevelopment in animal models. We apply a restraint stress protocol to pregnant rats during the last week of gestation that was reported to increase depressive-like behavior in dams [12, 13]. We have previously demonstrated that PS induces changes in neuronal pathways of the adult male offspring brain, in areas related to mood regulation and response to stress (for reviews, see [14, 15]). In particular, the hippocampus was one of the brain areas most affected by the effects of PS. Among the most significant changes for the development of this study, we can highlight that PS reduced the expression of MAP2 protein, suggesting that dendrite arborization in this structure was decreased [16, 17]. In addition, we found that PS alters mRNA levels of different neural plasticity-related genes [18–20]. Such changes may be leading to a variety of alterations in offspring behavior ranging from enhanced conditioned place preference induced by cocaine [21] and increased anxiety-like behavior [22].

The prevailing mechanistic transducers of maternal stress to the developing brain during pregnancy are the glucocorticoids hormones (corticosterone in rodents). Glucocorticoids rise upon maternal HPA activation and reach the developing fetuses across the placenta [1, 3, 4, 8, 10]. Once in the fetal brain, glucocorticoids affect different aspects of neuronal structure and circuits in brain structures with high levels of glucocorticoid receptors such as in the hippocampus [3]. In particular, the hippocampus has a major role in the regulation of the HPA axis response to stress and it is highly sensitive to the effects of stress in early life since its development continues until early childhood [3, 5, 6].

It was reported that PS alters the expression and function of different components and mediators of the HPA by affecting hippocampal negative feedback regulation, modifying glucocorticoids response to stress [23–26]. For example, variations in one of the major regulator system of the stress response in the hippocampus, the corticotrophin-releasing factor (CRF) and its related receptor 1 (CRFR1), are linked with the etiology of anxiety-like behaviors by impaired HPA reactivity, as was reported by studying rodents with *crfr1* gene polymorphisms [27] or by exposure to postnatal stress [28, 29]. PS also modifies the availability of glucocorticoid receptors and their modulators by affecting their sensitivity, translocation to the nucleus, DNA binding, and transcriptional effects on target genes, leading to a variety of emotional disorders [30, 31]. Variance in glucocorticoid receptors could also modify the expression of many other hippocampal genes related to neuronal plasticity and the modulation of learning and memory processing of stressful events, such as the brain-derived neurotrophic factors (BDNF) [18] and various neurotransmitter receptors and synaptic proteins in this area [14, 17, 18, 20]. Changes in glucocorticoids and glucocorticoid receptors levels can also induce modifications on the epigenome of the developing hippocampus [1, 5, 8, 11, 26]. Several authors reported that in addition to the programming effects of glucocorticoids, stress during pregnancy might also modify maternal mood during lactation affecting her caregiving faculties [32]. In turn, variations in maternal pup-directed behaviors can also influence the epigenome and the maturation and functioning of the hippocampus [33–35].

Remarkably, most of the abovementioned phenotypic alterations induced by stress in early life should be thoroughly interpreted in relation to the environmental context, sex, and age in which such changes are evaluated. In this regard, although there is an extensive literature regarding how stress in early life is associated with increased vulnerability to stress-related disorders in adulthood both in

animal models and in humans [1, 3, 8, 9, 26], little is known about the consequences of such stressors in younger individuals. In fact, even when children can experience symptoms of depression and anxiety in much the same way as adults do, they nevertheless display and react to those symptoms in a different way than adults [36]. Childhood in humans (2–13 years) and juvenility or pre-puberty in rats (21–32 days) represent similar stages of a "brain in transition." At this stage, juvenile rats as much as children complete several neurodevelopmental goals related to their relationship with their parents and their interaction with the surrounding environment. Therefore, juvenile rats have been validated as models of human childhood in recent years [7, 37].

Based on the abovementioned background, in the present work, we sought to deeply explore the consequences of PS on juvenile offspring, with the aim to better characterize stress-related coping abilities in PS male and female younger rats and its implication to early onset of stress-related disorders.

Materials and Methods

Animals and Gestational Stress Paradigm

Eighteen virgin adult female Wistar rats (250–300 g) were obtained from outbred rats from the animal facility at the "Universidad de Buenos Aires." Rats were kept under standard laboratory conditions in a 12–12-h light/dark cycle (lights off at 19:00 h), controlled temperature of 25 °C, and humidity of 55%, with ad libitum access to water and standard diet rat chow (Asociación de Cooperativas Argentinas-Buenos Aires, Argentina). All experiments agreed with the standards for the care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals (NIG Publications No. 8023, revised 1978) and were approved by the Institutional Animal Care and Use Committee (CICUAL Res (CD) N° 2235/2016, School of Medicine, Universidad de Buenos Aires). Care was taken to minimize the number of animals used and their suffering.

Females were individually mated with a sexually experienced male in a mating cage. The day on which vaginal plugs were found was designated as the first gestational day. Pregnant rats were individually housed in maternal cages (dimensions 470 × 290 × 190 mm) and randomly assigned to either control (C) or prenatal stress (PS) group. Control rats ($n = 9$) were left undisturbed in the home-cage until delivery, while PS dams ($n = 9$) were individually subjected to a restraint stress procedure that took place three times daily in transparent plastic cylinders under bright light for 45 min (9:00, 12:00, and 16:00 h) from gestational day 14 until the end of pregnancy [21]. Pregnant female body weight growth, food intake, and water consumption were daily measured in both stressed and control groups (see Supplementary Material 1). Emotional behavioral reactivity in the dams was assessed at weaning (Supplementary Material 2).

The day of parturition was considered postnatal day (PND) 0. Litters were culled to 10 pups each on PND 1, maintaining a similar number of males and females when possible [38]. Dams remained with their pups until weaning at PND 21. Male and female offspring of the same experimental condition were then housed in separate cages with no more than 5 rats per cage, under standard conditions. To avoid litter effects, a maximum of two pups per sex and per experimental group were tested for the behavioral set of experiments. The behavioral tests were carried out between 8:00 and 12:00 h in the same room in which the rats were housed.

Male and female offspring were tested from PND 25 to PND 28 since juvenile rats (PND 21–32) represent similar stages of brain development as in human children (2–13 years) [7]. Behavioral testing order in offspring was as follows: (1) elevated plus maze (EPM), (2) light–dark box (LDB), and (3) forced swimming test (FST). All animals underwent the testing batteries in the same order of tests to minimize possible carryover effects of the different behavioral tests. The sequence of tests was arranged from the least to the most stressful, with a gap of the 24-h resting period between each test. The apparatuses were cleaned with 50% ethanol and completely dried between each animal testing. All experiments were videotaped, and the videos were analyzed using Solomon Coder Software (RRID:SCR_016041) by a researcher blind to experimental groups.

Assessment of corticosterone response to an acute stress and brain dissection for experiments of genes expression profile analyses were conducted at PND 28, in a separate set of offspring from those that underwent behavioral testing (to avoid interference from behavioral tests on gene expression [39]). The timeline in Fig. 1 illustrates the experimental design.

Maternal Behavior Observations

On postpartum days 4, 7, and 10, between 9:00 and 11:00 h, and immediately after the cage cleaning routine, we monitored the patterns of maternal behavior in all dams at different time points during early lactation [40]. The change of the whole litter to a clean cage with fresh bedding was rapidly performed (i.e., in less than 2 min) and the litter was placed in the same location as it was in the dirty cage. Maternal behaviors were then video recorded for 30 min to assess both self- and pup-directed behaviors, as well as the overall activity. Data were aggregated across days and the total of time spent licking/grooming pups, nursing pups, and nest building was recorded as measures of "pup-directed behaviors."

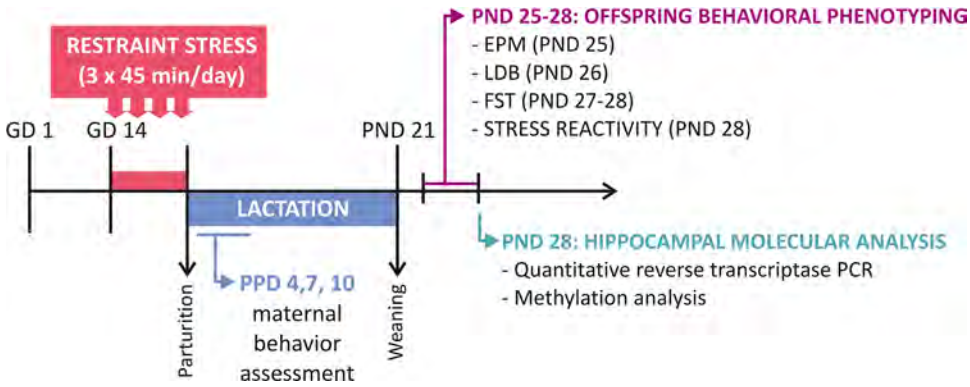

Fig. 1 Experimental design and study protocols. *GD*: gestational day; *PPD*, postpartum day; *PND*, postnatal day; *EPM*, elevated plus maze; *LDB*, light/dark box test; *FST*, forced swimming test

Among "pup non-directed behaviors," we measured "time off nest," as well as other behavior not implying directed care towards the pups (hover over and mouthing) [13, 41].

To determine whether the consistency of maternal care was affected by the treatment, an observation was extracted from maternal behavior recordings every 2 min within each 30-min observation period. Then, a "fragmentation index" (FI) was calculated [42]: if behaviors changed from one observation to the following, we assigned a "1." If behaviors remained consistent, lasting for more than 2 min, we graded the second observation a "0." Thus, for 16 consecutive observations of activity, the maximal FI achievable if behavior changed at every observation was 15. We then divided this score by 15 (total possible number of behavior changes) obtaining a score between 0 and 1. The higher the ratio, the more erratic the dam's behavior; contrariwise, the lower this ratio, the more consistent the behavioral pattern.

Analysis of Juvenile Offspring Behavior

Elevated Plus Maze Test

The EPM test was used to assess anxiety-like behavior and also risk assessment (RA) on PND 25 rats, following a protocol previously implemented in our lab [21, 22]. The test is based on a conflict between the rodent's preferences for protected areas and its motivation to explore novel environments. Increased avoidance of the open arms by an animal is considered to be an anxiety-like behavior [43]. The EPM apparatus consisted of two open (45-cm length × 10-cm width) and two closed-black arms (45-cm length × 10-cm width × 50-cm height) connected by a central platform (10 × 10 cm). The EPM was elevated 65 cm above the ground. Each rat was placed at the intersection of the 4 arms, facing an open arm, and allowed to freely explore the maze for 5 min. An entry was recorded when the rat entered with all four paws into an arm [21]. We quantified time spent in the central platform, in open and closed arms. Total arms entries were calculated as the total number of entries into any arm of the maze to distinguish between impaired exploratory behaviors, exploration limited to closed arms (avoidance), and free exploration. "Time ratio in open arms" was calculated as the percentage time spent in the open arms of the EPM divided by the total time spent in all arms of the maze. "Ratio of open arms entries" was calculated as the percentage number of entries into the open arms of the EPM divided by the total number of entries into all arms. Higher values for both ratios were considered lower anxiety in the rats [21]. We further recorded the frequency of the following RA behaviors: (1) rats dipped their heads below the level of the maze floor (head dipping); (2) stretching the head/shoulders from the center of the maze towards open arms (peeping out); and (3) when the rat 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 according to Viola et al. [44].

Light/Dark Box Test

One day after the EPM test (PND 26), the same sets of rats were tested in the LDB test for further assessment of anxiety-like behavior. The LDB test is based on an approach-avoidance conflict between exploration of novel environments and avoidance of brightly lit open space, and although it is a similar conflict as in the EPM, LDB does not produce identical behaviors [45]. The LDB apparatus consisted of the following: 1 white box (31 × 30 × 30 cm, 400 lx) and one black box (15 × 30 × 30 cm) with dim light (< 15 lx). Both boxes were connected via a small opening of 12 × 8 cm at floor level that enables transitions between boxes. The test started when each rat was individually placed in the center of the white area, facing away from the black area, and it was allowed to explore both compartments during 5 min. The time spent in each compartment was measured. "Time ratio in light chamber" was calculated as the percentage time spent in the light chamber divided by the total time spent in chambers. A higher ratio was considered lower anxiety in the rats [46].

Porsolt's Forced Swimming Test

The test was performed on PND 27 and 28. On both days, rats were individually forced to swim in a glass cylinder (60-cm high, 20-cm diameter) filled to a depth of 30 cm with tap water at 23–25 °C preventing the rats from touching the bottom of the cylinder. On the first day (PND 27), each rat underwent a 15-min pretest swim. On the following morning (i.e., 24 h later, PND 28), each rat was individually tested in the same cylinder for 5 min and video recorded for subsequent behavioral analysis. Time spent *swimming* (i.e., large forepaw movements that displaced water and moved the body around the cylinder), *climbing* (i.e., vigorous movements of the forepaws in and out of the water, usually directed against the wall of the tank), and *immobility* (i.e., the absence of motion of the entire body and only small movements necessary to keep the animal's head above the water is an indicator of despair) were recorded. Both after the pretest or test session, rats were immediately removed from the cylinder and dried with a paper towel. They were then returned to their home-cage. The water was changed after each rat to avoid the influence of urinary of fecal material and to maintain the same temperature for all individuals [12, 47]. We used Porsolt's Forced Swimming Test to assess the shift from active (swimming and climbing) to passive (immobility) coping strategies that occurs over time whenever rodents are exposed to an inescapable swim situation [47, 48]. The immobile behavior is supposed to reflect either a failure to persist in escape-directed behaviors after stress ("behavioral despair") or the development of passive stress-coping behavior [47, 48].

Corticosterone Response to Acute Restraint Stress

To test the responsiveness of the HPA axis, rats were individually exposed to a single 45 min restraint stress, starting at 9.00 h, in a transparent cylinder under bright light as described above. Afterwards, blood samples were collected from the rat trunk after rapid decapitation (STRESS, $N=7$ per sex/experimental group). Other sets of rats were returned to its home-cage and housing facility after acute restraint and left undisturbed for 2 h until collection of blood samples after decapitation at 12.00 h (RECOVERY, $N=7$ per sex/experimental group).

In addition, BASAL levels of corticosterone ($N=7$ per sex/experimental group) were analyzed from animals that did not undergo acute restraint. Rats were euthanized by decapitation at 9.00 h and truncal blood was collected. The hippocampus from this group was dissected and immediately homogenized in TRIzol Reagent (Life Technologies, Rockville, New York, USA). Homogenates were frozen on dry ice and stored at – 80 °C until further molecular analyses.

To improve serum separation from whole blood, samples were allowed to clot at room temperature for 30 min before being centrifuged at 1000 *g* for 10 min. Serum was collected and stored at – 20 °C until analysis. All samples were run in triplicate to determine serum corticosterone levels by specific radioimmunoassay previously validated and described by our collaborator [49]. Corticosterone standard curve was 0.05–50 µg/dL. CVs intra- and inter-assay were 4–6% and 8–10% respectively.

Molecular Analyses

Total RNA and genomic DNA were isolated from hippocampus homogenates with DirectZol RNA Miniprep (Zymo Research, Irvine, CA, USA) following manufacturer instructions.

RNA Extraction, cDNA Synthesis, and Real-Time Polymerase Chain Reaction (qPCR).

Complementary DNA was synthesized by retro transcription using oligo-dT and SuperScript® II Reverse Transcriptase enzyme (Life Technologies) according to manufacturer instructions. Non-transcribed samples were used to control for possible contamination of gDNA. All qPCRs' reactions were carried out in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, California, USA) in triplicate, using Power SYBR Green mix (Thermo Fisher Scientific). Specific forward and reverse primers were used at a final concentration of 0.3 or 0.5 µM. Table 1 shows the primers used in this study, which were designed by using sequence information from GeneBank at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and Ensembl (useast.ensembl.org, RRID:SCR_008356). Primer's specificity was checked using a melt curve. Differences in expression levels were assessed with LinReg PCR by comparing the initial quantities of the template by a linear regression [50]. Then, we carried out normalization with references genes [51, 52]. For datum normalization, we measured mRNA levels of two reference genes: *cyclo-a* and *ywhaz*. Normalization with both reference genes resulted in almost identical data [18, 53]. After normalization, gene expression was compared between treatments, C vs. PS.

Dot Blot Assay

Genomic DNA (100 ng) was denatured by heat and manually spotted on nitrocellulose membranes. As a loading control, membranes were stained with 4% methylene blue solution. The membranes were then incubated with antibody anti-5-methylcytosine (5-mC) or antibody anti-5-hydroxymethylcytosine (5-hmC) (both Zymo Research, Irvine, CA, USA) and with HRP-conjugated anti-rabbit secondary antibody

Table 1 Sequences of primers used in RT-qPCR reactions. Stress-related genes: *nr3c1*, glucocorticoid receptor; *nr3c2*, mineralocorticoid receptor; *fkbp4*, FKBP52, immunophilin protein class (FK506) binding protein of 52 kDa (increases GR sensitivity); *fkbp5*, FKBP51, FK506 binding protein 51 kDa (decreases GR sensitivity); *ppid*, co-chaperone peptidylprolyl isomerase D (positive modulator of GR); *bag-1*, co-chaperone BCL-2-associated athanogene (negative modulator of GR); *crf*, corticotrophin-related factor; *crfr1*, crf receptor type 1; *crfbp*, crf binding protein. Plasticity-related genes: *bdnf-IXa*, brain-derived neurotrophic factor (BDNF) exón 9 (total *bdnf*); *bdnf-IV*, BDNF transcript variant 4; *bdnf-VI*, BDNF transcript variant 6; *ntrk2*, neurotrophic receptor tyrosine kinase 2; *ntrk2-truncated*, ntrk2, transcript truncated T1 isoform; *ngfr*, nerve growth factor receptor p75-NTR. Chromatin methylation factors genes: *dnmt1*, DNA methyl transferase isoform 1; *dnmt3a*, DNA methyl transferase 3 alpha; *mecp2*, methyl CpG binding protein 2; *tet 1/2*, ten-eleven translocation family proteins 1 or 2. Reference genes: *cyclo-a*, cyclophilin-a; *ywhaz*, tyrosine 3-monooxygenase/tryptophan5-monooxygenase activation protein, zeta polypeptide

Stress system regulation-related genes			
Gene	ID	Forward sequence (5'–3')	Reverse sequence (5'–3')
nr3c1	NM_012576.2	AACTGGAATAGGTGCCAAGG	GAGGAGAACTCACATCTGGT
nr3c2	NM_013131.1	CAGCTCACCTCCATTACGCAT	CTTCACGACCTGGCTCATCTG
fkbp4	NM_001191863.1	CTTTTCTTCCCCGTTATACATG	CAAACAGAGGGAGCAAAATAAA
fkbp5	NM_001012174.1	AAGGACCACCGCACTCAAGA	GTGGTAAGGTCCTGACGTGATG
bag1	NM_001256084.1	AGCAGGCTAAAAAGGAAGAATTTG	TGCTCCACTGTATCACACTCTGCTA
ppid	NM_001004279.1	GGCTGTTATTGAGAAAGCAGATGTATC	CAAGCACCAATATTCAGCACACA
crfr1	NM_030999.4	CATCACCTACATGTTGTTCTTC	GTAGAACACAGACACAAAGAAG
crf	NM_031019.1	CGCCCATCTCTCTGGATCTC	GCCCTGGCCATTTCCAA
crhbp	NM_139183.2	GGAGCTGCTGGGAGGAACT	AACACAGGTCCACTAAGAGCATCA
Plasticity-related genes			
Gene	ID	Forward sequence (5'–3')	Reverse sequence (5'–3')
bdnf-IXa	NM_001285422.1	TAAATGAAGTTTATACAGTACAGTGGTTCTACA	AGTTGTGCGCAAATGACTGTTT
bdnf-IV	NM_001270632.1	GAGAGAGAGTCAGATTTTGGAG	CAAGAGTCTATTCCAGCCTAC
bdnf-VI	NM_001270634.1	TTGTCACTGGGACCTGAAA	AAGTCAAAACTTTCACTTCCTC
ntrk2	NM_012731.2	GTTTTAGCCTGTGTATGAGAAG	TATGGTAAAGCTTCTTTCCCTT
ntrk2-truncated	NM_001163168.2	CATGTCTTCTGGACTCTTTAGA	TACTAAGGACACCATGAAGATG
ngfr	NM_012610.2	ATTGGTCTATTCTGATGGAGTC	ACTAACAGATTCATCTCTCCAC
Methylation-related factors genes			
Gene	ID	Forward sequence (5'–3')	Reverse sequence (5'–3')
dnmt1	NM_053354.3	CATGGTGCTGAAGCTCACACTG	AACAGAGGCAGCTTCTCTCCAG
dnmt3a	NM_001003957.1	TCCAGACTCGCGTGCAATAA	ATGTGGCTGACAGATTCAAAATCA
mecp2	NM_001011924.1	GAGACACCTCCTTGGACCCTAAT	GTGGTTTCTGTTCTCTCCTGGAA
tet1	XM_003751959.1	GAACGGCATTCGGAAACAGA	TCGCCACGCCACCAA
tet2	XM_227694.5	CCCCACTCATGGGTCAATTC	CGATTGGGCTTGTTTACTTTGG
References genes			
Gene	ID	Forward sequence (5'–3')	Reverse sequence (5'–3')
cyclo-a	NM_022536.2	AAGCATACAGGTCCTGGCATCT	CATTCAGTCTTGGCAGTGGCAG
ywhaz	NM_013011.3	GATGAAGCCATTGCTGAACTTG	GTCTCCTTGGGTATCCGATGTC

(Sigma). Antigen–antibody complexes were detected according to Enhanced Chemiluminescence (ECL) Western blotting protocol SuperSignal West Pico chemiluminescent substrate (Thermo Scientific Pierce, Waltham, MA USA) [53].

Statistical Analyses

All molecular analyses results were analyzed by *t*-Student's test to evaluate the effects of the "stress" factor. Data for juvenile offspring behavioral testing were analyzed by two-way analysis of variance (ANOVA) to evaluate the effects due to "stress," "sex," and possible "stress × sex" interactions between both factors. When interactions were found, simple effects ANOVA analyses were done. Data from offspring body weight growth and corticosterone levels in serum were analyzed by three-way ANOVA to evaluate effects due to "stress," "sex," and "age" or "stress," "sex," and "acute stress" factors, respectively, as well as interactions between factors. For all ANOVA analyses, Tukey's post hoc test was performed to test differences between more than two groups. Statistical analyses of maternal behavior during lactation and FI were done using linear general models by using the "nlme package version 3.1–142" to perform a generalized

least-squares analysis of the relationship between each behavior and prenatal treatment. To account for the within-group correlation of data, each mother was entered into a correlation structure. The best statistical model for each behavior was later selected following the Akaike information criterion. The significance of fixed effects was obtained by the ANOVA test. For the analysis of RA behaviors, we used *MASS package version 7.3–51-4* to fit a negative binomial generalized linear model. The significance of fixed effects was obtained by the Wald test.

Shapiro–Wilk's and Levene's tests were applied to verify data normal distribution and homogeneity of variances, respectively. Response variables were transformed for the analyses if normal distributions of residuals were not achieved. Visual inspection of histograms, qq plots, and random distributions of fitted values was checked. Non-parametric analyses were achieved for "time in immobility" in the FST and for differences in transcript levels of several genes since homogeneity of variances or normal distribution could not be achieved respectively. In those cases, Kruskal–Wallis and Mann–Whitney *U* tests were run. Partial eta-square (η^2) was performed to calculated effect sizes for ANOVA results with $\eta^2 = 0.01$ defined as small, $\eta^2 = 0.06$ medium, and $\eta^2 > 0.14$ defined as large effect sizes [54]. Cohen's effect sizes (d) were calculated for all statistically different pair-wise comparisons with $d = 0.2$ defined as small, $d = 0.5$ medium, and $d > 0.8$ large effect size. The results are presented as mean ± standard error of the mean (SEM). The observed differences were considered to be statistically significant when $p < 0.05$. *N* values reported in figures represent the number of litters. Analyses of data were performed using SPSS 15.0 version (RRID:SCR_019096), Infostat 2018 (RRID:SCR_014310), or R Statistical Software 3.6.2 (RRID:SCR_002394). Graphical artworks were created with GraphPad Prism (RRID:SCR_002798) and ImageJ (RRID:SCR_003070) software. Final figures construction was created by CorelDRAW (RRID:SCR_014235).

Results

Gestational Stress Enhances Maternal Care Behavior on Lactating Dams

Maternal care behavior on lactating dams, assessed at postpartum days 4, 7, and 10, is shown in Fig. 2. The qualitative analysis of pup-directed or pup non-directed behaviors in both experimental groups demonstrated a main effect of stress treatment, with stressed dams spending significantly more time in licking/grooming behavior than non-stressed ones (Fig. 2a, 2b—*linear general model analysis*: $F_{(4.05)} = 10.26$; $p = 0.002$; $\eta^2_p = 0.182$). We did not find any significant differences between groups in other maternal behavior measures or time off nest. To determine whether the consistency of maternal care was affected by treatment, we calculated the FI by evaluating changes in the dam's behavior during consecutive 2-min epochs within each 30-min observation period. Thus, for 16 epochs, the maximal FI score achievable if behavior changed at every epoch was 15. We then divided this score by 15 (i.e., total possible number of behavior changes) obtaining a FI between 0 and 1. The higher the ratio, the more erratic the dam's behavior [42]. Although differences between treatments were not evident (Fig. 2c—*ANOVA*: $F_{(4.16)} = 0.04$, $p = 0.85$, $\eta^2_p = 0.001$), we did find a tendency for "postpartum day factor" when comparing the FI at PND 4 and 7 (*Tukey's* post hoc: $t_{(2.23)} = 2.53$, $p = 0.07$, $d = 0.408$), probably due to the fact that mothers take greater care of their offspring during first postpartum days, while their maternal behavior consolidates after a week.

Prenatal Stress Increases Offspring Body Weight Growth at Weaning

Offspring body weight was monitored weekly from birth to weaning (Table 2). Three-way ANOVA statistical analysis showed a significant interaction between "age × stress" factors ($F_{(3,111)} = 56.84$, $p = 0.0014$, $\eta^2_p = 0.1285$), but no effect due to sex and no interaction between "age × sex" or "stress × sex" factors were found. Therefore, data from male and female offspring were grouped and simple effects analysis due to PS was evaluated in each age. We found that PS body weight at PND 21 was significantly higher than C rats ($F_{(3,28)} = 14.59$, $p = 0.0007$, $\eta^2_p = 0.3425$ for stress factor).

Prenatal Stress Decreases Offspring Anxiety-Like Measures

Anxiety-like behavior was first measured in the EPM (Fig. 3a to 3c). We found a clear-cut PS main effect in both sexes' offspring performance in the EPM: PS rats had an enhanced ratio of open arm entries and spend more time exploring the open arm (i.e., reduced anxiety-like behavior) than C rats (Fig. 3a, 3b; *two-way ANOVA*: $F_{(1,28)} = 10.94$, $p = 0.0028$, $\eta^2_p = 0.28095$ for stress factor in open arm entries ratio; $F_{(1,28)} = 13.63$, $p = 0.0011$, $\eta^2_p = 0.327$ for stress factor in time ratio in open arms). No statistical differences were found for sex or stress or interaction between both factors when analyzing the total number of arm entries pointing out that the changes found were not due to an enhanced locomotion in the PS group (Fig. 3c; $F_{(1,28)} = 3.3 \times 10^{-4}$, $\eta^2_p = 1.18 \times 10^{-6}$). Frequency in RA was increased in both sex PS offspring (PS males: 18 ± 2.89 vs. C males: 13.71 ± 2.45; PS females: 14 ± 2.34 vs. C females: 8.71 ± 1.7), but not statistical differences were found (*negative binomial generalized linear model*: $z_{(1,65)} = 1.84$; $p = 0.0646$).

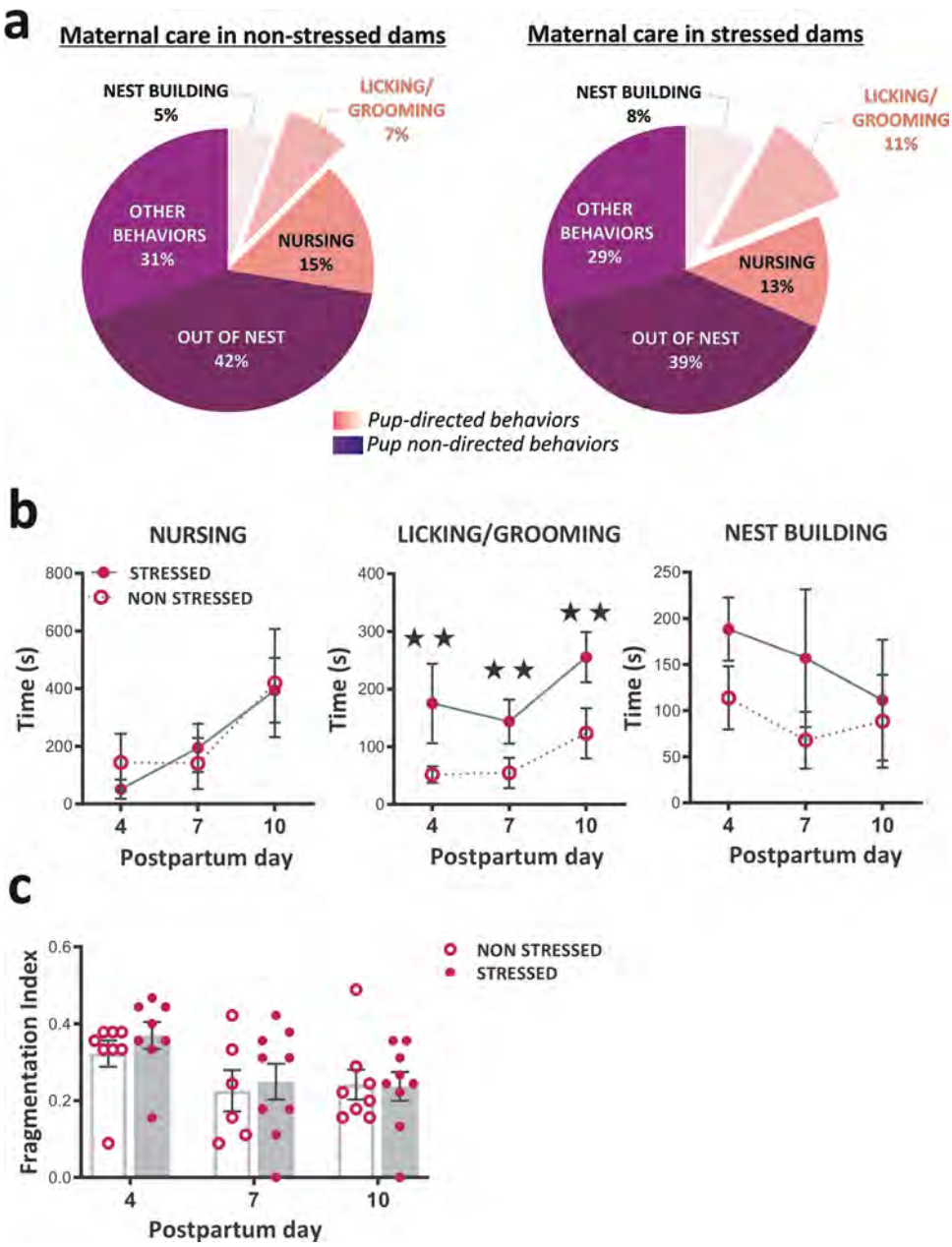

Fig. 2 Effects of gestational stress on maternal care behaviors during the early lactation period. **a** Qualitative assessment of the effects of stress during pregnancy on the percent occurrence of *pup-directed* and *pup non-directed* maternal behaviors during early lactation in exposed dams vs. non-stressed dams. **b** Values represent mean ± SEM of time in pup-directed behaviors (i.e., "nursing," "licking/grooming," and "nest building") for dams belonging to both experimental groups at postpartum days 4, 7 and 10. Groups with no letters in common for mean time in licking/grooming behavior are significantly different with $p < 0.001$ (main effect due to "stress" factor but not "time" linear general model analysis). **c** The graphic representation illustrates the maternal behavior consistency of both group dams within each observation period. Values represent mean fragmentation index (FI) ± SEM of stressed and non-stressed dams. FI = #behavior changes/#total possible number of behavior changes per 2-min epochs within the 30-min observation period. The higher the ratio, the more erratic the dam's behavior; the lower this ratio, the more consistent the behavioral pattern. In all cases, $N = 8–9$ dams per group

LDB testing was conducted after EPM to further assess PS consequences on offspring anxiety-like behavior (Fig. 3d). No statistical changes were found between experimental groups on the time ratio in the light chamber (*two-way ANOVA:* $F_{(1,27)} = 3.715$, $p = 0.0645$, $\eta^2_p = 0.10299$ for stress factor).

Prenatal Stress Modifies Offspring Stress-Coping Behavior in an Inescapable Situation in a Sex-Specific Manner

FST was used to assess if PS changes stress-coping behavior in an inescapable swim in juvenile offspring. Only PS male rats demonstrated significantly decreased time in immobility in the FST than other groups (Fig. 3e; Kruskal–Wallis test: $H = 5.59$, $p = 0.0175$, $d = 1.546326$ for stress factor in male). When analyzing time in mobility behaviors in the FST, we observed that both male and female PS offspring spent most time in swimming behavior (Fig. 3e, 3f; *two-way ANOVA*: $F_{(1,26)} = 6.65$, $p = 0.0159$, $\eta^2_p = 0.2036$ main effect for stress factor) than climbing when compared with C counterparts (*two-way ANOVA*: $F_{(1,26)} = 4.15$, $p = 0.0520$, $\eta^2_p = 0.1376$ stress factor).

Table 2 Effects of prenatal stress on litter body weight gain during lactation. *C*, control; *PS*, prenatal stress; *PND*, postnatal day. Values represent mean body weight ± SEM. Plus symbols demonstrate statistical differences due to the "age" factor for body weight gain with $p < 0.01$ (three-way ANOVA, main effects analysis for age factor). Stars in PS male and PS female demonstrate the presence of statistical differences between C and PS individuals in PND 21 offspring with $p < 0.001$ (three-way ANOVA, simple effect analysis for "stress" factor effect at PND 21). $N = 7$–9 pups per group

Groups	Weight of pups (g)			
	PND 1	PND 7	PND 14	PND 21
C male	7.0 ± 0.3	$15.1 \pm 0.4^{++}$	$26.9 \pm 1.5^{++}$	$41.3 \pm 1.6^{++}$
PS male	7.3 ± 0.2	$15.7 \pm 0.6^{++}$	$30.0 \pm 1^{++}$	$46.6 \pm 1.4^{++***}$
C female	7.0 ± 0.3	$15.3 \pm 0.6^{++}$	$25.8 \pm 1.9^{++}$	$38.6 \pm 1.5^{++}$
PS female	7.3 ± 0.2	$15.3 \pm 0.6^{++}$	$28.2 \pm 1.5^{++}$	$45.6 \pm 1.4^{++***}$

Prenatal Stress Affects Corticosterone Secretion to an Acute Stress in a Sex-Specific Way

HPA axis response to an acute restraint stress was measured in serum samples belonging to PND 28 offspring that were not tested in the behavioral battery. In both sexes and prenatal experimental groups, serum corticosterone levels raised after acute stress and return to basal levels after 2 h of recovery in the home-cage for all rats (Fig. 4; *three-way ANOVA*: $F_{(2,80)} = 110.67$, $p < 0.001$, $\eta^2_p = 0.7598$ postnatal stress factor). Three-way ANOVA statistical analysis revealed a significant interaction between "sex × postnatal stress" factors ($F_{(2,80)} = 29.97$, $p < 0.001$, $\eta^2_p = 0.4613$). Therefore, data from males and females was analyzed separately. In males, we did not find statistical differences between C and PS groups in serum corticosterone levels for any of the measured conditions. However, we observed a decrease in serum corticosterone levels in PS offspring after acute stress (Fig. 4a, $F_{(1,70)} = 1.226$, $p = 0.07$, $\eta^2_p = 0.0176$). In females, we observed a significant PS effect on corticosterone levels in serum compared with C females: corticosterone levels were reduced after an acute stress (Fig. 4b; three-way ANOVA simple components analysis: $F_{(1,70)} = 4.57$, $p < 0.05$, $\eta^2_p = 0.0612$) and also after recovery (Fig. 4b; three-way ANOVA simple components analysis: $F_{(1,70)} = 4.875$, $p < 0.05$, $\eta^2_p = 0.065$).

Prenatal Stress Changes Relative Genes Expression in Offspring Hippocampus

Prenatal Stress Increases the Expression of Important Genes Related to the Hippocampal Stress System Modulation

To study the modulation of the hippocampal HPA system, we explored basal levels of glucocorticoids gene expression: *nr3c1* (that codes for glucocorticoid receptor 1) and *nr3c2* (that codes for mineralocorticoid receptor). In addition, we explore the expression levels of co-chaperon (*fkbp4*, *fkbp5*) and modulator factors (*ppid*, *bag1*), which influence the glucocorticoids receptors function by modulating receptor sensitivity, translocation to the nucleus, DNA binding, and transcriptional effects on glucocorticoid's receptors target genes [55, 56]. We also evaluated CRF/CRFR1 system by assessing the expression of hippocampal *crf*, CRF binding protein (*crfbp*), and CRFR1 (*crfr1*). In males, we did not found differences between PS and C rats in the expression of glucocorticoids receptors and neither of their modulators (Fig. 5a). The analysis of genes related with CRF pathway showed an increase in *crf1* expression levels in PS male rats (Fig. 5b; *t-Student's test*: $t_{(10)} = 2.802$, $p = 0.0187$, $d = 1.6198$). In females, PS increased the levels of hippocampal *nr3c2* expression (Fig. 5c; *t-Student's test*: $t_{(12)} = 2.915$, $p = 0.013$, $d = 0.1558$). In addition, and similarly to what we observed for PS males, the expression of *crfr1* was increased in PS females (Fig. 5d; *t-Student's test*: $t_{(11)} = 3.023$, $p = 0.0116$, $d = 3{,}804$) in comparison to C counterparts.

Prenatal Stress Alters Genes Related with BDNF Signaling in the Hippocampus

Bdnf gene expression content is regulated by different isoforms depending on brain region, cell type, sex, and developmental stage (as reviewed by Foltran and Diaz [57]). Among all isoforms, *bdnf*-exon IV and VI were shown to be the most affected by early-life stress [13, 58, 59]. Under our experimental conditions, *bdnf*-exon VI mRNA yielded such low amplification rates that it was not possible to carry out a reliable quantification. In both sexes, PS increased the levels of *bdnf*-exon IV (Fig. 6; *t-Student's test*: $t_{(10)} = 2.756$, $p = 0.0203$, $d = 0.9196$ for males Fig. 6d; *t-Student's test*: $t_{(9)} = 2.624$, $p = 0.0276$, $d = 0.2802$ for females Fig. 6g) without changing the transcript levels of total *bdnf* (i.e., exon IX).

There are two distinct pathways for BDNF signaling that induce opposite effects on the cell depending on the right balance between pro-BDNF and mature BDNF: pro-BDNF has a high affinity for the p75 neurotrophin receptor (p75-NTR), and after binding, it initiates dendritic atrophy and mediates synaptic depression and cellular apoptosis, whereas mature BDNF binds to tropomyosin receptor kinase B (TrkB) and contributes to synaptic potentiation and promotes cell survival [57]. Furthermore, the T1 truncated-TrkB isoform lacks the intracellular catalytic portion and can act as a dominant negative inhibitor of BDNF signaling, becoming a further point of modulation on BDNF signaling [57]. In males, we found that PS reduced the mRNA levels of BDNF-specific receptor (*ntrk2*) in comparison to C rats (Fig. 6b;

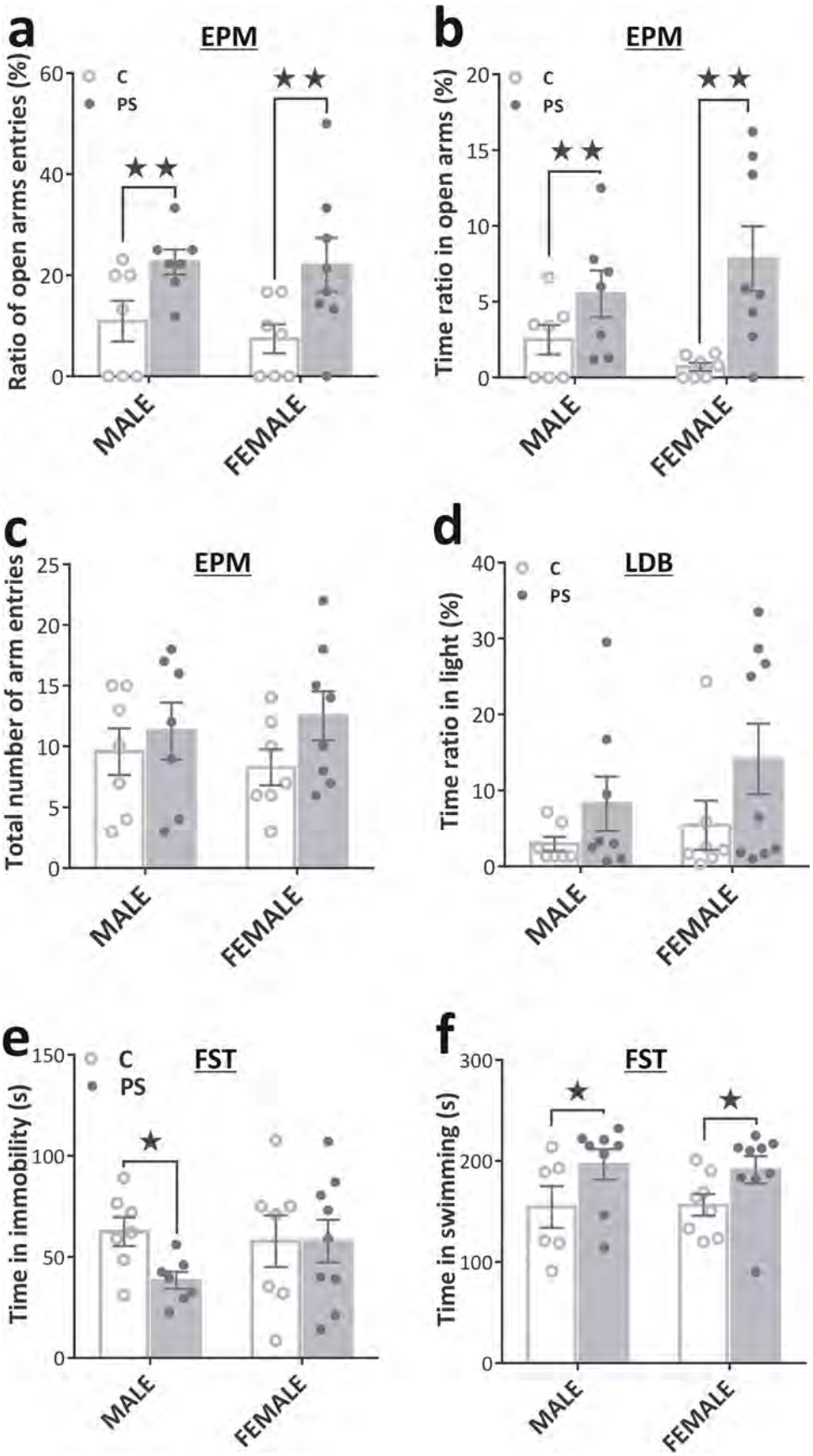

Fig. 3 Stress-coping behavioral analysis in male and female juvenile offspring. C, control; PS, prenatal stress; EPM, elevated plus maze; LDB, light–dark box test; FST, forced swimming test. **a**, **b**, and **c** EPM measures; **d** LDB test; **e** and **f** FST measures in male and female offspring, respectively. Bars represent the mean ± SEM of each parameter. For "ratio in open arms entries" and for "time ratio in open arms," stars denote statistical differences among C and PS groups with $p < 0.01$ (two-way ANOVA—main effect due to "stress" factor). For "time in immobility" and for "time in swimming" data, star marks statistical differences between C and PS groups with $p < 0.05$ (Kruskal–Wallis and two-way ANOVA respectively, "stress factor" effects PS vs. C). $N = 7–9$ pups per group

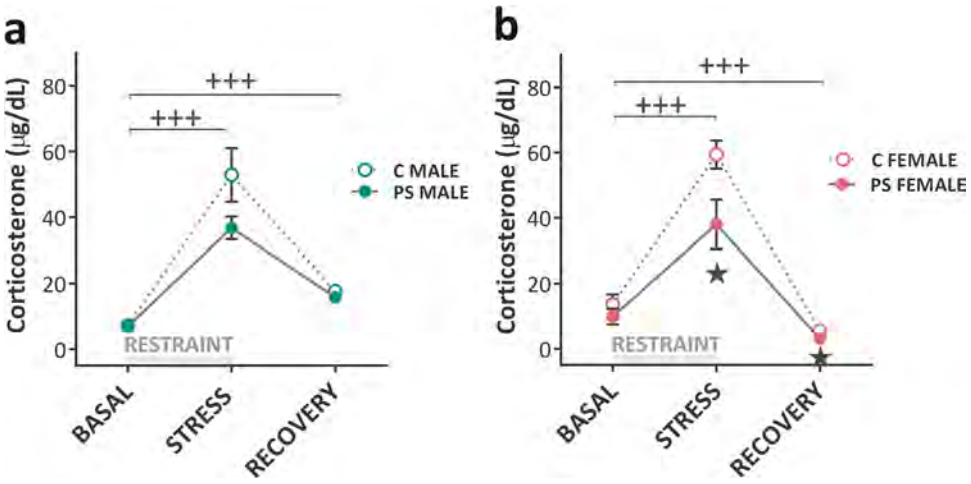

Fig. 4 Effects of prenatal stress on male (a) and female (b) offspring corticosterone levels after an acute stress. C, control; PS, prenatal stress. Values represent the mean ± SEM serum corticosterone at basal, after an acute restraint stress and after 2 h of recovery in the home-cage. "Plus symbol" denotes statistical differences between "basal" and "stress" or "recovery" conditions with $+++p<0.001$. Stars demonstrate the presence of statistical difference between C and PS group for corticosterone after "acute stress" and "recovery" conditions in females with $*p<0.05$. Analysis was performed by three-way ANOVA, followed by simple components analyses. $N=6$ pups per experimental condition

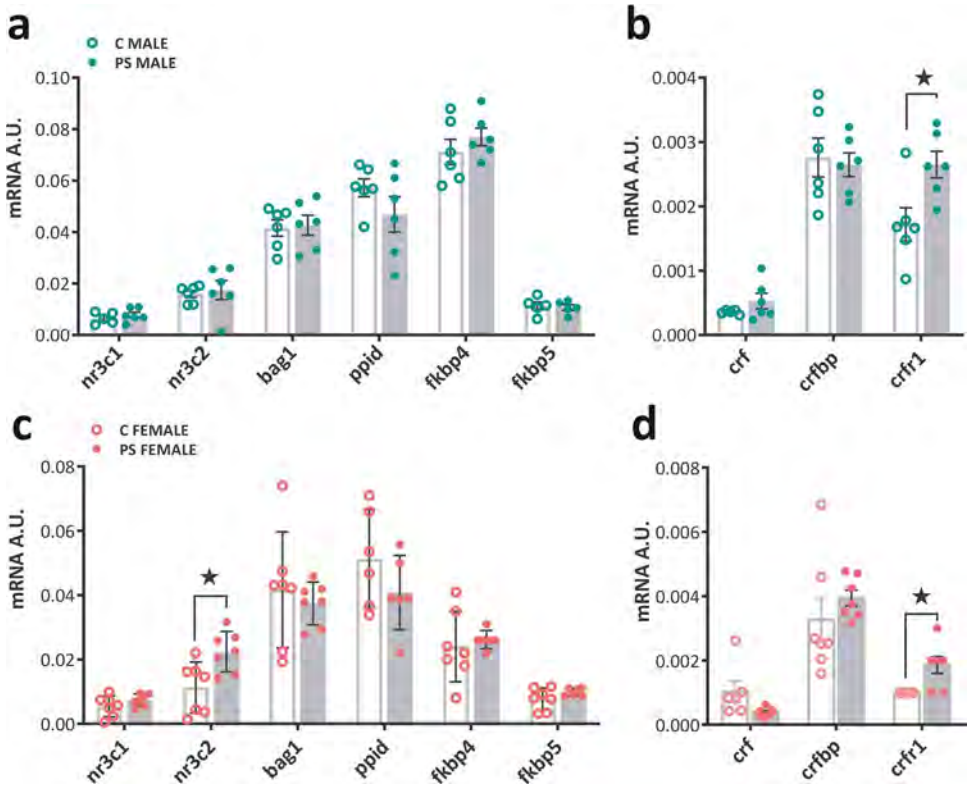

Fig. 5 Effects of prenatal stress on the hippocampal expression of stress-related genes in male (**a**, **b**) and female (**c**, **d**) offspring. C, control; PS, prenatal stress. Bars represent the mean ± SEM of real-time reverse transcriptase PCR data for genes encoding for glucocorticoid receptors and modulators (**a**, **c**) and *crf/crfr1* genes (**b**, **d**). Transcript levels in the hippocampus are expressed relative to the housekeeping genes *ciclo* and *ywaz*. Stars demonstrate the presence of statistical differences between C and PS groups for each sex ($*p<0.05$, *t-student test*). $N=6$–9 samples per group

t-Student's test: $t_{(10)}=4.79$, $p=0.0007$, $d=0.2766$), but did not influence the transcript levels of truncated-TRKB (*ntrk2-trnk*) or p75-NTR (*ngfr*) BDNF receptors. In females, the magnitude and direction of BDNF receptor expression differed from those changes observed in PS males: PS significantly increased the transcript levels of *ntrk2-trunc* (Fig. 6f; $t_{(10)}=4.431$, $p=0.0013$, $d=1.4314$) and *ngfr* (Fig. 6e; *t-Student's test*: $t_{(10)}=3.135$, $p=0.0106$, $d=1.9704$) without changing the transcript levels of *ntrk2* in comparison to C female rats.

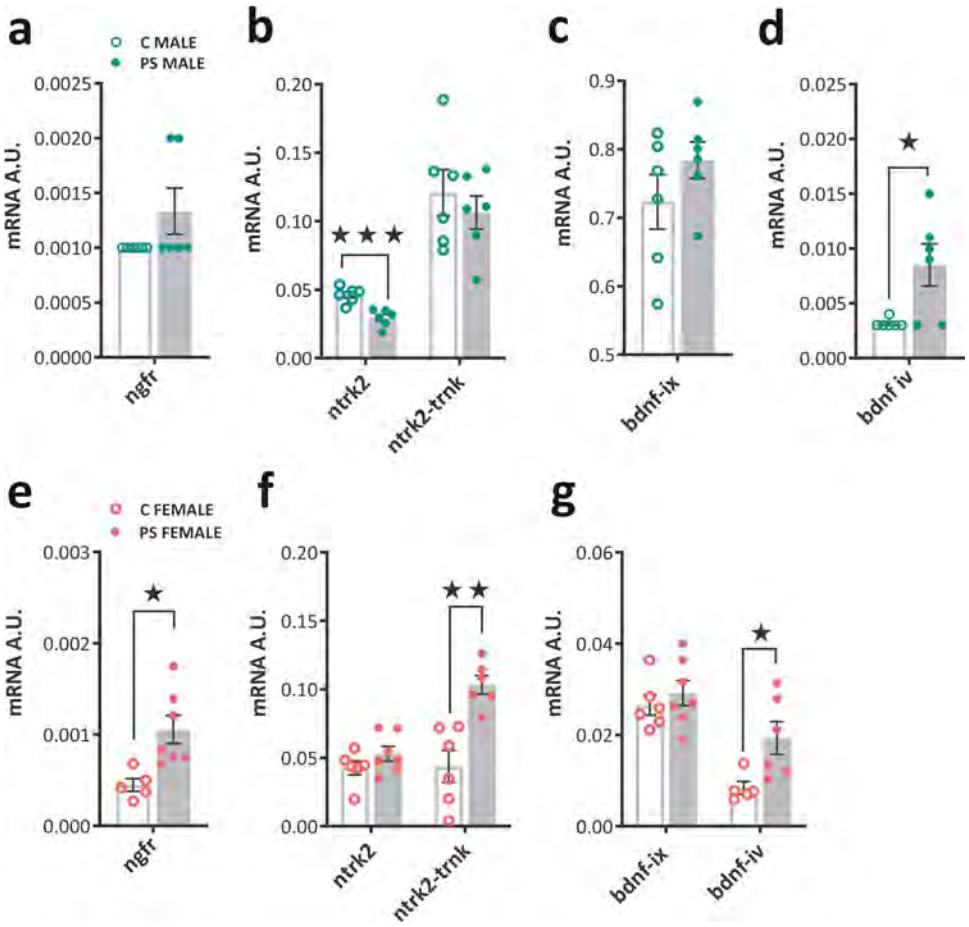

Fig. 6 Effects of prenatal stress on the expression of plasticity-related genes in the hippocampus of male (**a** to **d**) and female offspring (**e** to **g**). C, control; PS, prenatal stress. Bars represent the mean ± SEM of real-time reverse transcriptase PCR data for genes encoding for BDNF receptors *ngfr1* (**a**, **e**), *ntrk2* and *ntrk2-truncated* (**b**, **f**), and BDNF transcripts (**c**, **d**, **g**). All measured transcript levels in the hippocampus are expressed relative to the housekeeping genes *ciclo* and *ywaz*. Stars demonstrate the presence of statistical differences between C and PS groups for each sex (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, t-Student's test). $N = 7$–9 samples per group

Prenatal Stress Affects Hippocampus Chromatin Remodeling in a Sex-Specific Way

Methylation changes in chromatin are introduced by different proteins like DNA methyl transferases (DNMT1 and DNMT3A) and interpreted by methylated DNA binding proteins (such as MECP2). Thereafter, the DNA demethylation process is initiated by the oxidation of 5-mC into 5-hmC by methylcytosine dioxygenases (ten-eleven translocation proteins): TET1 and TET2. Thus, we first analyzed if PS altered the transcript levels for those proteins. Next, we evaluated possible differences in global levels of 5-mC and 5-hmC in the hippocampus to further assess PS consequences on methylation/hydroxymethylation dynamics [53].

In our experimental conditions, we were unable to amplify *dnmt1* gene levels. In males, PS increased the mRNA levels of *dnmt3a* (Fig. 7a; Mann–Whitney *U*-test: $U = 15.51$, $p = 0.0043$, $d = 1.5987$), *mecp2* (*t-Student's test:* $t_{(10)} = 3.025$, $p = 0.0128$, $d = 1.3751$) and *tet1* (*t-Student's test:* $t_{(10)} = 4.644$, $p = 0.0009$, $d = 2.6817$) in comparison to C rats. When analyzing total content of 5-mC and 5-hmC global DNA contents, we observed that PS male rats show decreased levels of 5-mC than C rats (Fig. 7b—right; *t-Student's test*: $t_{(11)} = 4.419$, $p = 0.01$, $d = 0.27737$) with no changes in 5-hmC global DNA content between experimental groups (Fig. 7c).

In females, we only found a significant increase on the levels of *mecp2* in PS offspring (Fig. 7d; *t-Student's test*: $t_{(11)} = 2.690$, $p = 0.0210$, $d = 0.9421$). We did not find statistical differences in the mRNA levels of the other measured genes, neither in 5-mC/5-hmC content (Fig. 7e, 7f).

Discussion

Although there is an extensive literature regarding how early-life stress is associated with increased vulnerability to stress-related disorders in adulthood, little is known about the consequences of early-life stressors in younger individuals. In the present study, we show that although several differences were found between sexes, exposure to stress during gestation influences the stress-coping abilities of juvenile offspring by inducing changes in the performance of PS offspring in anxiety-related and stress-coping behavioral tasks, on the pattern of hippocampal gene

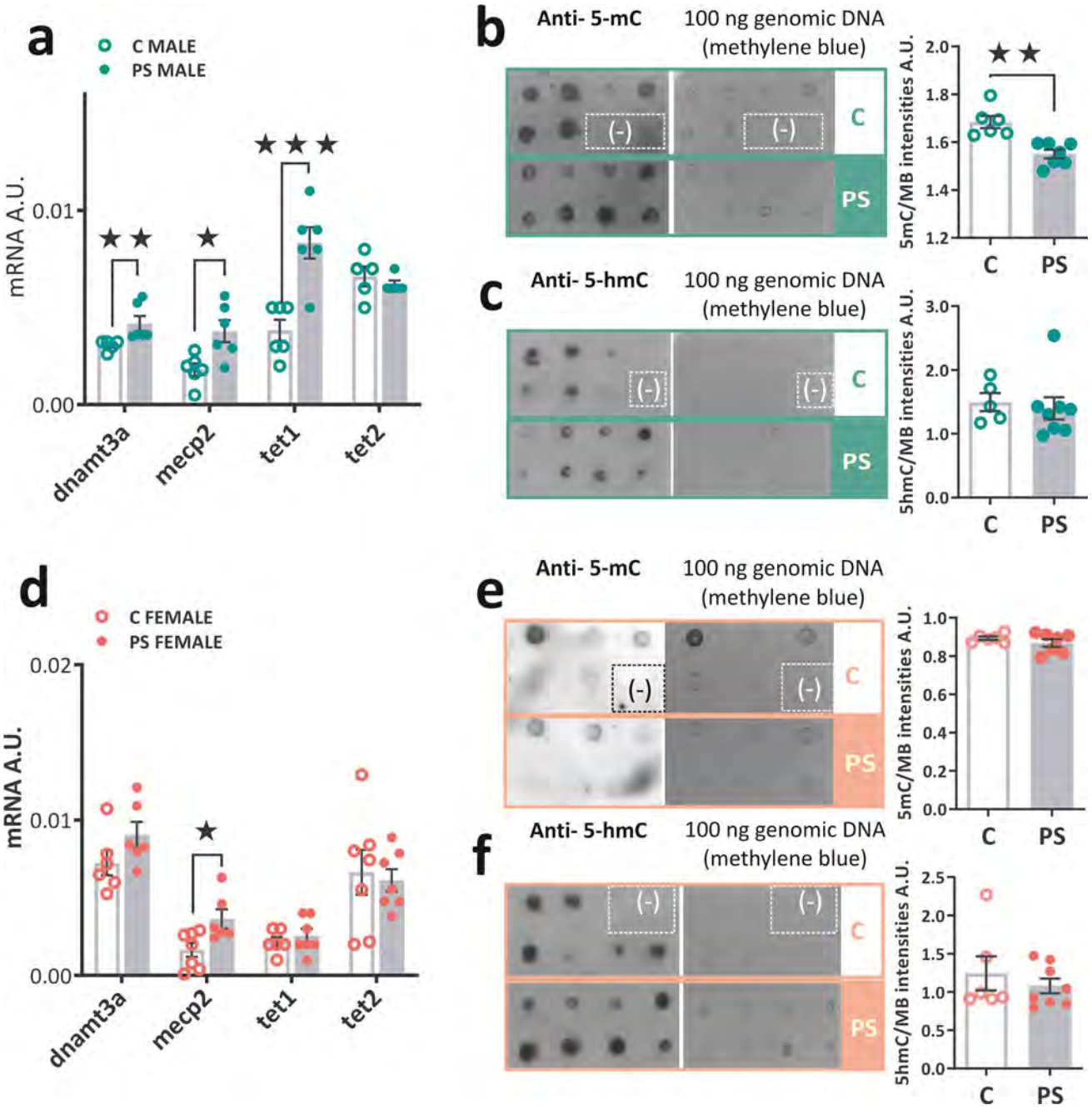

Fig. 7 Effects of prenatal stress on chromatin remodeler factors in male (**a** to **c**) and female (**d** to **f**) offspring hippocampus. **a**, **d** Bars represent the mean ± SEM of real-time reverse transcriptase PCR data for chromatin methylation-related factors: *dnamt3a*, *mecp2*, *tet1*, and *tet2*. All transcript levels are expressed relative to the housekeeping genes *ciclo* and *ywaz*. Stars demonstrate the presence of statistical differences between C and PS groups (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, *t*-Student's test or Mann–Whitney *U*-test). Dot blot assay with anti-5-mC (**b**, **e**) or anti-5-hmC (**c**, **f**) antibodies showing representative samples of genomic DNA (100 ng/dot) spotted on a nitrocellulose membrane. Both in **b**, **e**, **c**, **f**: Left, methylene blue staining showing loading control. For negative controls, we spotted water onto the membrane. Right panel, dot blot semi-quantification (**$p = 0.01$, *t*-Student's test). $N = 6$–8 samples per group

expression and on chromatin methylation. In addition, stress experience during pregnancy slightly enhances pup-directed maternal behavior of the dams after giving birth. In summary, differential outcomes in juvenile offspring are a result of the interaction between early exposure to stress and sex, but also to maternal care during early lactation being an additional mediator on those interactions (Table 3).

Table 3 Summary of hypothesized adaptive/maladaptive significance of prenatal stress (PS) consequences on stress-coping abilities in both sex juvenile offspring. We hypothesized as *maladaptive* the observed behavioral/molecular outcomes that might decrease chances of survival in a threatening environment or that contribute to long-lasting damaging changes in the hippocampus

	PS males	PS females	Possible significance (adaptive/maladaptive)
Body weight gain	⇑	⇑	*Adaptive*
Behavioral stress-coping abilities			
Anxiety-like behavior (EPM)	⇓	⇓	*Adaptive/maladaptive depending on the characteristics of the confronting environment*
Anxiety-like behavior (LDB)	⇓	⇓	
Depressive-like behavior (FST)	⇓	ns	
Time in swimming (FST)	⇑	⇑	
Stress response to acute stress	ns	⇓	
Molecular changes in the hippocampus			
nr3c1, *fkbp5*, *fkbp4*, *bag1*, *ppid*	ns	ns	*Possible adaptive changes for hippocampal stress response in females*
nr3c2	ns	⇑	
crf	ns	ns	*Possible maladaptive changes for hippocampal stress- and plasticity-related pathways on offspring of both sexes*
crfr1	⇑	⇑	
bdnf-IV	⇑	⇑	
bdnf IX (total *bdnf*)	ns	ns	
ntrk2	⇓	ns	
truncated-ntrk2	ns	⇑	
ngfr	ns	⇑	
dnmt3a	⇑	ns	*Enhanced epigenetic footprint mainly in male offspring*
mecp2	⇑	⇑	
tet1	⇑	ns	
5-mC DNA content	⇓	ns	
5-hmC DNA content	ns	ns	

Maternal Behavior in Dams Exposed to Stress During Pregnancy

Epidemiological studies in humans suggest that mothers who experience chronic psychological stress during pregnancy are more likely to neglect their children more often than mothers who were not stressed [60, 61]. Although many studies in rodents have explored the consequences of PS on maternal behavior, the findings diverge since several authors reported a reduction in pup-directed maternal behavior following PS [62–64] and others described the opposite behavior [32, 65]. In light of these contradictory results and since variations in maternal pup licking/grooming strongly regulate the offspring HPA response to stress [33, 34], we explored the maternal behavior in our rodent model. In rats, the period extended from PND 4 to 14 is known as the "stress hypo-responsive period" (SHRP) and it is very important for the maturation of the offspring HPA axis. The SHRP is characterized by low basal corticosterone levels, reduced sensitivity to CRF, and lack of a stress response to a variety of usual stressors that takes place during this period. Active maternal behavior during SHRP leads to sensory inputs to the brain that reduce HPA axis tone, protects the brain against the deleterious effects of enhanced corticosterone levels, and hence contributes to a proper constitution of the pup's stress response. Indeed, several brain areas that regulate HPA axis response (e.g., the hippocampus) also complete to maturate their structure and connectivity during this period [6, 66]. Therefore, any variation in maternal care received during the SHRP could affect HPA response. We carefully assessed maternal care in the home-cage to reduce the influence of any other variations in maternal behavior that could interfere with the main effect of stress during pregnancy (e.g., maternal separation, deprivation, or handling). During postpartum days 4, 7, and 10, we observed that the consistency of the maternal behavior was similar in both stressed and non-stressed dams (i.e., similar FI): maternal behavior tended to be more erratic during first lactation days, but it was progressively consolidated as the lactation period advances. However, the assessment of the qualitative distribution of maternal behavior in stressed and non-stressed dams revealed that stressed dams spend more time licking/grooming their pups than their control counterparts. This observation seems to be in line with Schmidt et al.'s (2018) speculation indicating that protective maternal behavior might be stimulated during pregnancy in response to stress resulting in an enhanced maternal behavior towards the pups during lactation [32]. In addition, our findings of

enhanced body weight gain found in PS offspring at weaning strongly support this hypothesis.

To further assess the impact of pregnancy stress on dams, we corroborated previous findings from Darnaudéry et al. (2004) showing that gestational restrain stress reduces dam's body weight gain at the end of pregnancy and induces long-term effects on dam's emotional reactivity (Supplementary material 1 and 2) [12]. Furthermore, we assessed the relationship between maternal care and juvenile offspring behavioral outcomes. In our hands, we observe that maternal care quality could be predictive for later offspring behavioral performance only in female's anxiety-related tasks. However, that relationship is lost by PS (Supplementary material 3). Although we are conducting further experiments to unravel these outcomes, these results imply that, at least in female offspring, the overall outcome on stress-coping behavior could be the result of the prenatal insult received during gestation plus the effect of the changed maternal care during lactation.

Prenatal Stress and Stress-Related Behavioral Outcomes in Juvenile Offspring

Behavioral data show that PS produces similar effects in both male and female juvenile offspring but differ on what we have previously described for adult offspring [22]. These differences might be explained due to age-dependent variations in stress response and stress-related behaviors [3, 4]. Although the behavioral performances of age-matched control rats remain relatively stable and without changes between sexes, we found that PS juvenile offspring displayed an enhanced activity and more time spent in the open arms of the EPM and an increased time (although not significant) in the light chamber of the LDB test. In PS males, we also found a reduced time in immobility in the FST and an enhanced time in swimming for both sexes. We speculated that these different action patterns between C and PS offspring could be due to enhanced stress response to novelty that could be driving different coping strategies in novel environments (e.g., increased swimming behavior in the FST). However, we found that PS rats were less responsive to an acute stress and such effect was more pronounced in female PS rats. It is important to pinpoint that corticosterone response to stress was assessed in a separate set of rats to differentiate if the stress reactivity was attributable to a brief emotional arousal after the behavioral test exposure or due to early stress.

Several studies reported similar results when assessing the effects of early adversity consequences on anxious- and depressive-like behavior in young offspring. However, the same results allow opposite interpretations [32, 67, 68]. On the one hand, the performance in a battery of the behavioral test in PS offspring could be positively interpreted (i.e., *resilience*): enhanced active coping behaviors when exposed to novelty (e.g., EPM results) or into an inescapable stress (e.g., reduced passive coping behavior in the FST in males and reduced stress response in females) [32, 67]. Similarly, reduced stress reactivity and anxiolytic-like behavior were observed in offspring with a high level of maternal behavior [33, 34]. On the other hand, the differences in emotional behaviors in PS rats might be negatively interpreted (i.e. *vulnerability*): PS rats take unnecessary risks (spent more time in the unsafe compartments of the EPM and LDB), are hyperactive (more time in swimming behavior), and have reduced stress response to novelty [32, 68].

We consider that beyond the significance of our results in terms of *resilience* or *vulnerability*, our findings need to be thoroughly interpreted in relation to the age in which those behaviors were evaluated and in terms of their *adaptive* or *maladaptive* contribution for survival when confronted with a potentially challenging environment during the juvenile age. Taking into account that PS brains seem to be programmed by early stressors to live in a harsh and unpredictable environment [10, 69], such behavioral outcomes observed might decrease chances of survival in a threatening environment (i.e. *maladaptive*) but might have positive consequences in an environment without that particular threat (i.e. *adaptive*). On the other hand, it is important to note that enhanced risk taking, hyperactivity, and reduced stress to novelty also resemble pathological patterns (e.g., attentional deficit hyperactivity disorder) [9, 68]. In a previous study from our group, we found that individual differences in cocaine-induced conditioned place preference in PS male offspring during adulthood were related to pubertal anxiety levels, in such a way that individuals with lower anxiety levels at puberty developed higher cocaine-induced place preference when reaching adulthood [21]. Therefore, those results together with the present study support the notion that the effects of PS on the behavioral response profile of PS rats are dynamic implying that behavioral outcomes change throughout the rat lifespan and symptomatology of stress-related disorders differ between ages. Hence, we highlight the importance of further exploring characterizations of behavioral consequences of early life adversities in younger individuals.

Prenatal Stress and Differences in Hippocampal Stress- and Plasticity-Related Gene Expression in Juvenile Offspring

Stress in early life, as well as a reduction in pup licking/grooming behaviors by dams during lactation, was shown to dysregulate glucocorticoid receptors MR (mineralocorticoid receptor) and GR (glucocorticoid receptor) mRNA expression, affecting different aspects of basal and stress-induced response regulation [1, 23, 24, 26, 31]. In this

study, we explored transcript levels of glucocorticoid receptors MR (*nr3c2*) and GR (*nr3c1*) and also evaluated the expression of genes encoding for positive (*fkbp4*, *ppid*) and negative (*fkbp5*, *bag-1*) regulators of glucocorticoid receptors function to explore if mediators of the receptors response could be intrinsically affected by PS and might be involved in the different outcomes we found in PS offspring [24, 31, 55]. From all the assessed genes, we only found differences due to PS in females, where PS upregulated the levels of *nr3c2* in coincidence with the observation that PS females had reduced response to an acute stress. Rozebom et al. (2007) achieved a similar finding while inducing chronic elevations of forebrain MR in a transgenic mice model: increased levels of *nr3c2* resulted in a moderate suppression of the stress response in female mice. Their results suggest that, at least in females, MR could contribute to negative feedback information during a stressful event beyond its well-known role in maintenance of basal HPA tone due to its higher affinity for glucocorticoids [70]. Indeed, MR overexpression decreased anxiety-like behavior in both transgenic mice sexes pointing out that in addition to glucocorticoids receptor's role in HPA axis regulation, GR and MR could have a potential role in anxiety modulation [70]. Ter Heegde et al. (2015) demonstrated an interesting role of MR as an important contributor of adaptive stress response and reduced risk for psychiatric disorders, by reviewing clinical and preclinical studies on increased MR functionality and gene expression [71]. These authors also explained that females should be more benefited by MR effects due to the enhanced affinity of the female steroid hormones for that glucocorticoid receptor [71].

In addition to MR and GR, the hippocampal CRF/CRFR1 system also plays a major role in stress response and emotional regulation by facilitating plasticity-related changes of adaptive responses to stress-coping (e.g., learning and memory of the stressful event) [72, 73]. Dysregulation of hippocampal CRF/CRFR1 system by early-life stress or prenatal administration of synthetic glucocorticoids was shown to trigger stress-related mood disorders [74, 75] and to induce morphological changes in hippocampal neurons dendrites [72]. However, mice lacking CRFR1 (but with a functional glucocorticoid system) were resistant to the detrimental effects of early-life stress on hippocampal function suggesting an important role of CRF/CRFR1 in mediating PS effects on offspring hippocampus [28]. In this study, we extended those findings by reporting that PS increased CRFR1 mRNA expression in the hippocampus of juvenile offspring of both sexes, suggesting that the hippocampal CRF/CRFR1 system might be altered in PS rats. Extended research is necessary to fully characterize the functional relevance of the observed changes and to link its impact on the behavioral outcomes we found on juvenile PS offspring.

The neurotrophin BDNF and TrkB receptor are also downstream targets of the glucocorticoid signaling pathway, being their protein and mRNA expression levels altered when HPA function is dysregulated [76]. In addition to glucocorticoids, hippocampal activation of CRF/CRFR1 signaling also regulates BDNF gene expression and later BDNF signaling by modulating the proteolytic conversion of pro-BDNF to mature BDNF levels and TrkB gene expression [76]. Dysregulation on such CRFR1/BDNF signaling activation has also been identified as pathophysiological factors contributing to mood disorders [76, 77]. Hence, BDNF has been well studied as a prime modulator of neuronal signaling in different models of early life adversities [13, 18, 58, 59]. From all possible *bdnf*-exon isoforms, PS showed to affect mainly those containing exons IV and VI by inducing differential epigenetic modulation at their promotor levels [13, 57, 58]. In addition to changes in *bdnf* gene expression, BDNF signaling in the hippocampus was also show to be altered by early-life stress [13, 25, 58]. In previous studies, we found that PS diminished total hippocampal *bdnf* (i.e., exon IX) gene expression levels in adult PS male offspring [18]. In this study, we extended such analysis by further assessing the detailed contribution of exons IV and VI to total *bdnf* mRNA levels and by exploring BDNF main receptors gene expression to assess BDNF signaling. We found no differences between experimental groups or sex on the levels of hippocampal *bdnf*-exon IX transcripts, despite an upregulation on *bdnf*-exon IV in both sexes' PS offspring. These findings suggest that differential exon usage in PS offspring hippocampus might be contributing to highly regulate transcriptional control of *bdnf* gene in response to early stress. Interestingly, the exon IV was reported to be predominant and supposed to contribute mostly to overall *bdnf* expression at the same offspring age as in our study [25, 58]. However, a big limitation of our research is that we were not able to amplify *bdnf*-exon VI and could not measure the contribution of other exons on overall *bdnf* expression, leaving this hypothesis speculative. In an attempt to further study BNDF signaling, we found marked sex differences in the expression of BDNF receptor genes between PS offspring: in males, PS downregulated *ntrk2* gene (i.e., reduced TrkB receptor), while in females, PS upregulated *ngfr1* and *truncated-ntrk2* gene expression (i.e., increased p75-NTR and increased truncated-TrkB). Given that we did not found differences in the total transcript levels of *bdnf* gene in neither male or female PS rats, the findings on BDNF receptors gene expression pointed out that despite the sex differences, the direction of the findings seems to be the same: early-life stress might increase *bdnf*-exon IV transcription probably by activating CRFR1/BDNF signaling to compensate total *bdnf* transcript levels, but disrupt BDNF signaling in

offspring hippocampus by differential pathways in male or female rats. New studies are currently being conducted to unravel the mechanistic understanding of these results.

Prenatal Stress Epigenetic Signature on Juvenile Offspring Hippocampus

As shown, PS exerted the most remarkable impact on hippocampal BDNF, CRF, and CRFR1 mRNA expression profile. Changes in the DNA methylation status of the promoter of those genes have been attributed to both PS [13, 25, 58, 74] and variations in maternal care [28, 75]. Hence, we explored whether PS might introduce an epigenetic signature on the hippocampus of juvenile offspring. In adult male offspring, we have already demonstrated that PS modified the expression of chromatin remodeler genes with enhanced expression of *dnmt3a* and *mecp2* and reduced levels of *tet1*. Those changes were correlated with a global decrease in hippocampal 5-hmC DNA levels [18, 53]. Accordingly, our results in this study strongly support the fact that early life modifies the epigenetic landscape of offspring hippocampus but in an age- and sex-dependent way: we found stronger changes in the measured parameters in PS male rats. Surprisingly, the directions of changes in juvenile rats slightly differ from those described for adult ones. Since 5-mC is a stable and long-lasting covalent modification to DNA with a major role in experience-dependent plasticity [78], the increased expression of chromatin methylation factors could be in line with the reduction in 5-mC content to restitute 5-mC global DNA levels to those found in control ones. On the other hand, despite the increase in *tet1* transcript levels, we did not find the reduction in 5-hmC content that we reported for adult PS rats [53]. It was reported that among TET isoforms, TET1 specifically regulates 5-mC in the central nervous system with decreased levels of *tet1* expression correlated with a global decrease in 5-hmC levels [79]. We speculate that the increase in *tet1* transcription might be responsible for a later decrease reported for aged PS male rats. In the mammalian brain, 5-hmC is enriched in genes related to synaptic function [79]. Thus, variations in 5-hmC have been linked to alterations in synaptic plasticity leading to vulnerability to mood-related disorders [78]. Thus, the absence of differences in 5-hmC content between PS and C rats might be underlying positive synaptic plasticity changes on individuals exposed to early stress in young individuals, but the reduced expression of *tet1* and a reduced 5-hmC content in adult rats may explain, in part, the behavioral deficits induced by prenatal stress in adult life [1, 3, 5, 8, 26].

Final Conclusions and Implications

Herein, we provide novel insights about the consequences of gestational stress programming on young offspring at the behavioral and molecular levels. Our results reveal that PS drives a "changed performance" in the behavioral tasks tested, while induces several sex-specific differences in the expression of stress- and plasticity-related genes that in the overall denotes an altered neuroplasticity milieu and epigenetic landscape in the PS offspring hippocampus. Although the extent of our findings needs to be further explored, most of our findings in juvenile rats are different—and sometimes opposite—from what we previously reported for adult offspring. Furthermore, we highlight that the caregiving quality of the mother during early lactation could be an additive factor that strongly accounts for such programming at least in female offspring.

Our results demonstrate that PS induces dynamical and transient changes in the individuals providing a potential adaptive advantage in a non-stressful environment at younger stages, but it may be deleterious and increase the risk to pathology across the lifespan. Hence, identifying risk factors that appear early in life is primal for detecting vulnerable individuals and thus allowing timely prevention strategies that will avoid risk to stress-related disorders in later life.

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Author Contributions M.E.P. designed, performed, analyzed all the experiments, and wrote the manuscript. M.C.M. helped with the development of real-time qPCR and chromatin remodeling experiments. V.P. helped with the development of prenatal stress protocol. J.G.B. analyzed behavioral data from maternal behavior. A.A. performed RIA analyses to detect corticosterone levels in serum. M.A.B. supervised the design, development, and analyses of molecular experiments. M.C.A. supervised the whole study design, development, and data analyses. M.C.A. also contributed to the final version of the paper. All authors made manuscript revisions and approved the submitted version.

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Data Availability The data support the findings of this research are available from the corresponding author (mpallares@fmed.uba.ar) upon reasonable request. The authors will take the responsibility for maintaining availability.

Declarations

Ethics approval This research was performed in accordance with the standards for the care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals (NIG Publications No. 8023, revised 1978). All protocols were approved by the Institutional Animal Care and Use Committee (CICUAL), Facultad de Medicina (School of Medicine), Universidad de Buenos Aires.

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Conflict of interest The authors declare no competing interests.

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