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Research report

Prenatal and postnatal experiences associated with epigenetic changes in the adult mouse brain

Felix Mattern[a], Antonia Post[b], Franziska Solger[a], Aet O'Leary[b], David A. Slattery[b], Andreas Reif[b], Thomas Haaf[a,*]

[a] Institute of Human Genetics, Julius-Maximilians-University, Würzburg, Germany
[b] Department of Psychiatry, Psychosomatic Medicine and Psychotherapy, University Hospital Frankfurt, Frankfurt am Main, Germany

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ABSTRACT

To analyze the influences of early-life history on the brain epigenome, the offspring of mouse dams kept in an enriched or standard environment were exposed postnatally to enriched, standard, or adverse conditions. The methylation patterns of 7 candidate genes (9 loci) involved in developmental programming of stress vulnerability/resilience and psychiatric disease were analyzed in 6 brain regions of adult male and female mice. Exposure to an enriched prenatal environment was associated with widespread epigenetic changes (all of small effect size), affecting 29 of 324 (9%) gene/region-specific methylation patterns. The effects of either adverse or enriched postnatal conditions were tested separately in the two prenatal cohorts. Significant changes were observed in 2 of 324 (0.6%) loci in offspring of dams in a standard environment and 6 of 324 (1.9%) loci in animals that were exposed prenatally to an enriched environment. Prenatal life experiences appear to have a bigger effect on the adult brain epigenome than postnatal experiences. Positive prenatal life experiences may increase epigenetic plasticity of the brain later in life. All observed between-group differences were sex-specific, consistent with largely different developmental trajectories of the male and female brain. Multiple changes of small effect size are consistent with a multifactorial model of developmental programming of adult behavior and disease susceptibility.

1. Introduction

Since psychiatric disorders are amongst the most common causes of global disease burden [1], a better understanding of the molecular mechanisms underlying an individual's resilience to successfully adapt to and recover from severe life adversity is urgently needed. Accumulating evidence in both rodent models and humans suggests that early-life experiences can have a persistent impact on the brain, modulating behavioural and psychiatric disease risk [2–5]. Consistent with the high brain plasticity during prenatal and early postnatal development, these may be the most sensitive time periods for programming of stress-related adult diseases. The most likely mechanism for translating the effects of early-life experiences into disease susceptibility later in life is epigenetic dysregulation of signaling pathways that are involved in the regulation of stress response [3–8].

Epigenetic mechanisms control the temporally and spatially highly coordinated gene expression in the brain without altering the DNA sequence. The most stable epigenetic modification is DNA methylation, more precisely methylation of cytosine carbon 5 at CpG dinucleotides. Promoter methylation during development, differentiation, or due to environmental exposures leads to an inactive chromatin structure and gene silencing, whereas gene body methylation is usually associated with active genes [9]. Epigenetic modifications reflecting gene-environment interactions during sensitive time periods are primary candidates for mediating the persistent effects of early-life experiences on the stress vulnerability and resilience in later life [3,7,10].

Compared to numerous studies on the long-term effects of adverse experiences, there is limited knowledge on the impact of positive experiences in early life. In mammals, the bond to the mother is probably the strongest factor in early postnatal environment. High levels of maternal care can have long-lasting effects on the epigenome, brain structure/function and behavior of the offspring [11–13]. In rats, environmental enrichment (EE) after weaning influences hippocampal gene expression and can protect from the negative effects of stressors [14,15]. In this study, we focused on the long-term effects of varying prenatal and postnatal life experiences on the brain epigenome. Pregnant mouse dams were kept under EE or standard conditions. Their offspring were reared under adverse (maternal separation, MS), control,

* Corresponding author at: Institute of Human Genetics, Julius-Maximilians-Universität Würzburg, Biozentrum, Am Hubland, 97074 Würzburg, Germany.
E-mail address: thomas.haaf@uni-wuerzburg.de (T. Haaf).

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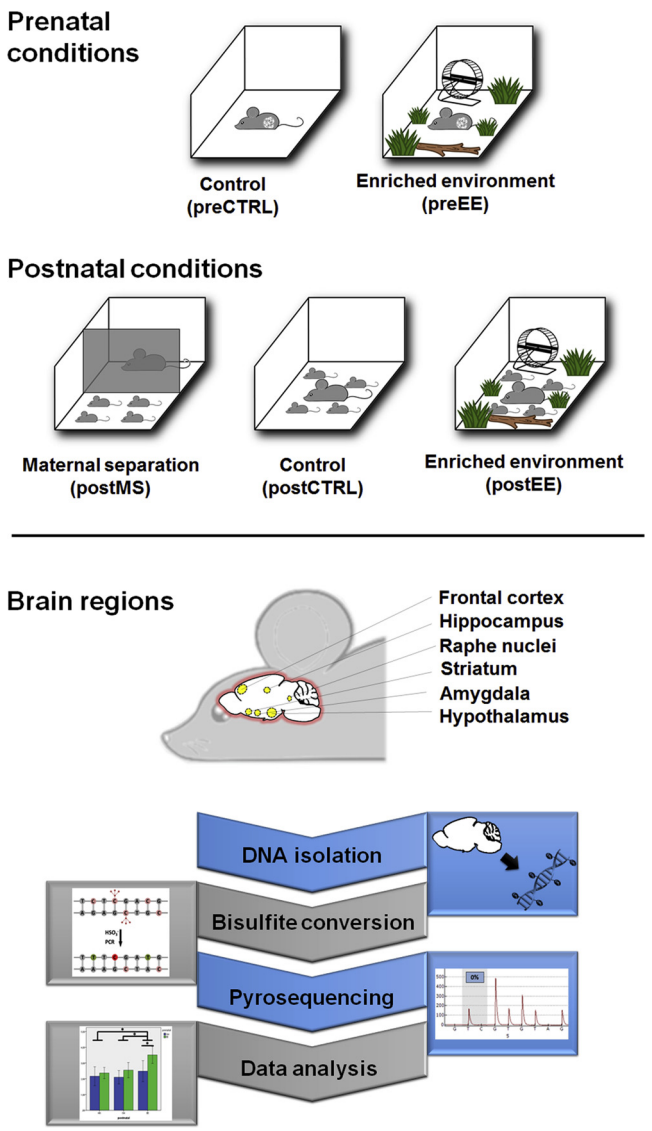

Fig. 1. Study design. Mouse dams were kept under standard housing conditions (preCTRL) or under enriched environmental conditions (preEE). For each prenatal group, the resulting offspring was randomized in three postnatal groups: stress in the form of maternal separation (postMS), control (postCTRL), and continued positive environment (postEE). Six different brain regions (amygdala, frontal cortex, hippocampus, hypothalamus, raphe nuclei, and striatum) were dissected from adult brains. After DNA isolation and bisulfite conversion, DNA methylation of 7 candidate genes (*Bdnf*, *Crhr1*, *Fkbp5*, *Htr1a*, *Maoa*, *Nos1*, and *Nr3c1*) was analyzed by pyrosequencing. For each gene, brain region and sex, the data was analyzed in the 6 offspring groups (2 prenatal x 3 postnatal conditions).

or enriched (EE) conditions. The methylation patterns of candidate genes, previously shown to be implicated in gene-environment interactions and psychiatric diseases, were studied in adult brains of mice with the resulting 6 different life histories. Given that previous studies suggested a sex effect on epigenetic programming of the stress response [16–18], we assessed both male and female offspring separately.

2. Methods

2.1. Animal treatment and DNA sample preparation

Experiments were performed in accordance with the EU directive for animal experiments. One group of primiparous pregnant dams of CD1 wild-type mice (preCTRL) were kept under standard laboratory conditions and their offspring was randomized into 3 groups (Fig. 1). The first group was raised under standard laboratory conditions (postCTRL), the second group encountered postnatal maternal separation (postMS) stress, and the third group was raised in postnatal environmental enrichment (postEE) conditions. A separate group of primiparous pregnant dams was exposed prenatally to an enriched environment (preEE). Their offspring was randomized into the same three groups as the control animals. Two to three pups per litter were used for each group. Maternal separation and environmental enrichment have been shown to affect maternal care, which can subsequently affect methylation [4]. However, as it would not be possible to disentangle the environmental manipulation from any alteration in maternal care, we did not assess it in this study.

For standard condition (preCTRL and postCTRL), dams and their pups were kept in standard Makrolon rat cages (surface area 900 cm^2) with nesting material available. For maternal separation (postMS), dams and their litters were also housed in standard Makrolon rat cages with nesting material available, but the pups were removed from the dams for 3 h daily from postnatal day 2 up to weaning at postnatal day 21. For both pre- and postnatal environmental enrichment (preEE and postEE), the standard Makrolon rat cages were enriched with either two small toys (wooden ladder, mirror with a bell, wooden cylinder) or one larger toy (running wheel, wooden tubs, wooden swing, or wooden shelter). The toys were changed weekly throughout the experiment. All animals were kept at a light- and temperature-controlled animal room (12/12 h light-dark cycle, lights on at 7:00 a.m.; 21 ± 2 °C) with food and water available ad libitum.

Brains of 13 adult (6–8 weeks) animals from each sex were prepared from the 6 resulting offspring groups; Cohort 1: preCTRL/postMS, preCTRL/postCTRL, and preCTRL/postEE, and Cohort 2: preEE/postMS, preEE/postCTRL, and preEE/postEE. Each cohort was raised and analyzed separately. Frontal cortex, amygdala, hippocampus, hypothalamus, striatum, and raphe nuclei were dissected, resulting in a total of 936 samples (6 regions x 13 animals x 2 sexes x 3 groups x 2 cohorts) for further analysis. Genomic DNA from 5 to 40 µg tissue was isolated with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Bisulfite conversion was performed with 500 ng of genomic DNA, using the EpiTect Fast 96 Bisulfite Conversion Kit (Qiagen).

2.2. DNA methylation analysis

We focused on 7 different genes that have been implicated in psychiatric disorders, namely *Bdnf*, *Crhr1*, *Fkbp5*, *Htr1a*, *Maoa*, *Nos1*, and *Nr3c1*, all of which have all been repeatedly implicated, from our group as well as others, to show either expression (mRNA or protein), methylation and/or functional alterations in response to stress exposure and/or environmental enrichment [2,19–23]. DNA methylation was analyzed by bisulfite pyrosequencing. Since *Bdnf* is endowed with multiple transcripts, two assays were designed covering the promoter regions of 5 (*Bdnf*-201, -206, -207, -208, and 210; termed *Bdnf-1*) and 4 transcripts (*Bdnf*-203, -204, -205, and -211; termed *Bdnf-2*), respectively. For the glucocorticoid receptor NR3C1, we designed an assay for the promoter region (of transcripts *Nr3c1*-201, -203, -206, and -208) and another one for the nerve growth factor protein A (NGFI-A)-binding site (in intron 1 of *Nr3c1*-203, -205, and -207). PCR and sequencing primers (Supplementary Table S1) were designed using the PyroMark Assay Design 2.0 software (Qiagen). Assays were established using the EpiTect PCR Control DNA set (Qiagen) with 0%, 25%, 50%, 75%, and 100% methylation.

The PCR mastermix for one 96-well plate consisted of 125 µl 10x PCR Reaction Buffer (with 20 mM $MgCl_2$), 25 µl PCR Grade Nucleotide Mix, 50 µl of each primer (10 mM), 10 µl (50 U) FastStartTaq DNA Polymerase (Roche Diagnostics, Mannheim, Germany), and 940 µl of RNAse-free water. The PCR reactions for one gene were performed in 96 well plates, each reaction containing 24 µl mastermix and 1 µl (approximately 100 ng) bisulfite converted template DNA. Amplification

Table 1
Effects of postnatal environmental manipulations on adult brain methylation patterns of prenatal cohort 1 (standard environment) in a sex-, region-, and gene-specific manner.

Brain region	Gene	Males			Females		
		Control	MS	EE	Control	MS	EE
Amygdala	*Bdnf-1*	3.19 ± 0.32	2.65 ± 0.25	3.94 ± 0.40	2.62 ± 0.31	3.17 ± 0.58	3.69 ± 0.41
	Bdnf-2	2.60 ± 0.25	2.28 ± 0.18	2.6 ± 0.17	3.22 ± 0.28	2.43 ± 0.22	2.66 ± 0.24
	Crhr1	2.33 ± 0.19	2.14 ± 0.19	1.91 ± 0.20	2.46 ± 0.27	2.72 ± 0.36	**4.2 ± 0.48** [b,c]
	Fkbp5	3.91 ± 0.33	3.55 ± 0.26	4.28 ± 0.43	3.38 ± 0.36	4.33 ± 0.37	3.26 ± 0.43
	Htr1a	1.71 ± 0.25	1.94 ± 0.31	1.48 ± 0.33	2.13 ± 0.21	2.31 ± 0.24	2.43 ± 0.21
	Maoa	1.66 ± 0.17	1.26 ± 0.12	1.42 ± 0.24	**33.65 ± 1.27** [c]	**33.57 ± 1.13** [c]	**33.62 ± 0.72** [c]
	Nos1	2.43 ± 0.20	2.93 ± 0.19	2.50 ± 0.24	2.37 ± 0.24	2.25 ± 0.29	1.70 ± 0.16
	Nr3c1	1.35 ± 0.29	0.94 ± 0.11	0.80 ± 0.09	1.83 ± 0.21	1.60 ± 0.16	**2.21 ± 0.27** [c]
	NGFI-A	2.19 ± 0.21	2.78 ± 0.15	2.29 ± 0.25	2.71 ± 0.41	2.41 ± 0.12	2.27 ± 0.15
Frontal cortex	*Bdnf-1*	3.42 ± 0.24	4.02 ± 0.36	3.26 ± 0.31	2.71 ± 0.21	2.69 ± 0.39	4.16 ± 0.64
	Bdnf-2	3.06 ± 0.48	2.87 ± 0.48	2.93 ± 0.36	**1.61 ± 0.14** [c]	**1.73 ± 0.24** [c]	**2.19 ± 0.23** [c]
	Crhr1	3.13 ± 0.10	3.50 ± 0.34	3.35 ± 0.55	3.37 ± 0.26	3.01 ± 0.18	3.14 ± 0.21
	Fkbp5	4.48 ± 0.48	4.51 ± 0.54	4.03 ± 0.33	4.82 ± 0.41	3.80 ± 0.23	4.91 ± 0.66
	Htr1a	2.67 ± 0.24	1.93 ± 0.44	2.06 ± 0.31	2.10 ± 0.26	1.97 ± 0.14	2.27 ± 0.23
	Maoa	1.53 ± 0.30	1.69 ± 0.14	1.73 ± 0.32	**32.42 ± 1.03** [c]	**33.34 ± 1.49** [c]	**33.55 ± 0.96** [c]
	Nos1	2.10 ± 0.19	2.29 ± 0.30	2.50 ± 0.31	1.94 ± 0.19	2.16 ± 0.14	2.16 ± 0.17
	Nr3c1	1.46 ± 0.21	1.14 ± 0.16	1.38 ± 0.14	2.06 ± 0.92	1.16 ± 0.33	0.68 ± 0.16
	NGFI-A	1.94 ± 0.19	1.77 ± 0.20	1.88 ± 0.18	**1.46 ± 0.12** [c]	**1.30 ± 0.08** [c]	**1.21 ± 0.08** [c]
Hippocampus	*Bdnf-1*	3.88 ± 0.45	3.03 ± 0.22	3.29 ± 0.16	2.80 ± 0.29	2.87 ± 0.17	3.29 ± 0.16
	Bdnf-2	1.81 ± 0.15	2.17 ± 0.14	2.09 ± 0.20	2.13 ± 0.15	2.05 ± 0.17	1.64 ± 0.22
	Crhr1	3.19 ± 0.26	3.00 ± 0.22	2.73 ± 0.29	4.22 ± 0.20	3.81 ± 0.24	**4.04 ± 0.41** [c]
	Fkbp5	3.87 ± 0.38	3.74 ± 0.39	3.27 ± 0.32	3.25 ± 0.62	3.06 ± 0.50	2.63 ± 0.43
	Htr1a	1.89 ± 0.29	1.80 ± 0.22	1.71 ± 0.22	1.87 ± 0.23	1.80 ± 0.22	1.27 ± 0.25
	Maoa	1.61 ± 0.59	1.99 ± 0.80	1.44 ± 0.26	**35.11 ± 1.26** [c]	**33.80 ± 1.32** [c]	**31.25 ± 0.79** [c]
	Nos1	2.11 ± 0.33	2.33 ± 0.21	2.21 ± 0.18	2.24 ± 0.22	2.54 ± 0.27	2.27 ± 0.28
	Nr3c1	0.74 ± 0.14	0.70 ± 0.10	1.18 ± 0.15	1.11 ± 0.15	0.93 ± 0.10	0.99 ± 0.27
	NGFI-A	2.51 ± 0.46	2.37 ± 0.15	1.83 ± 0.14	1.94 ± 0.33	2.32 ± 0.28	1.98 ± 0.17
Hypothalamus	*Bdnf-1*	2.41 ± 0.35	2.64 ± 0.19	2.95 ± 0.90	3.36 ± 0.44	3.00 ± 1.06	4.06 ± 0.43
	Bdnf-2	2.01 ± 0.26	2.29 ± 0.17	2.28 ± 0.20	2.06 ± 0.20	2.44 ± 0.16	1.98 ± 0.23
	Crhr1	1.50 ± 0.29	1.69 ± 0.14	1.90 ± 0.41	2.95 ± 0.56	3.47 ± 0.71	3.20 ± 0.48
	Fkbp5	3.84 ± 0.13	3.72 ± 0.21	4.27 ± 0.25	3.84 ± 0.34	4.78 ± 0.51	4.57 ± 0.32
	Htr1a	2.22 ± 0.24	2.47 ± 0.23	2.06 ± 0.40	2.00 ± 0.28	2.91 ± 0.68	2.04 ± 0.25
	Maoa	2.07 ± 0.23	2.39 ± 0.70	2.26 ± 0.19	**34.07 ± 0.84** [c]	**35.58 ± 2.56** [c]	**35.79 ± 1.32** [c]
	Nos1	4.30 ± 0.69	3.33 ± 0.28	3.40 ± 0.48	2.97 ± 0.18	**n/a**	3.49 ± 0.17
	Nr3c1	0.74 ± 0.11	0.91 ± 0.14	1.09 ± 0.17	0.98 ± 0.13	1.34 ± 0.28	0.85 ± 0.17
	NGFI-A	2.69 ± 0.26	2.20 ± 0.15	3.07 ± 0.51	2.83 ± 0.40	5.44 ± 2.64	2.84 ± 0.53
Raphe nuclei	*Bdnf-1*	9.29 ± 1.75	4.09 ± 1.39	5.04 ± 1.38	2.36 ± 0.29	3.27 ± 0.36	3.52 ± 0.55
	Bdnf-2	1.75 ± 0.16	2.09 ± 0.18	2.19 ± 0.21	1.91 ± 0.23	1.95 ± 0.19	1.98 ± 0.3
	Crhr1	3.77 ± 0.57	3.95 ± 0.90	3.62 ± 0.41	3.38 ± 0.40	3.26 ± 0.30	4.37 ± 0.74
	Fkbp5	3.58 ± 0.51	3.64 ± 0.42	3.51 ± 0.43	4.37 ± 0.45	4.06 ± 0.68	3.00 ± 0.22
	Htr1a	2.96 ± 0.75	2.31 ± 0.21	2.23 ± 0.38	2.45 ± 0.27	2.39 ± 0.45	2.40 ± 0.51
	Maoa	7.65 ± 1.1	7.01 ± 2.18	6.45 ± 1.82	**33.51 ± 0.95** [c]	**35.28 ± 1.08** [c]	**33.39 ± 1.33** [c]
	Nos1	4.58 ± 0.54	5.15 ± 0.79	3.66 ± 0.67	4.19 ± 0.48	4.10 ± 0.38	4.87 ± 0.35
	Nr3c1	1.37 ± 0.20	1.54 ± 0.37	1.78 ± 0.50	1.01 ± 0.20	1.05 ± 0.33	1.58 ± 0.27
	NGFI-A	3.12 ± 0.42	2.75 ± 0.33	3.12 ± 0.36	2.88 ± 0.46	2.74 ± 0.40	2.49 ± 0.56
Striatum	*Bdnf-1*	2.62 ± 0.44	2.81 ± 0.28	2.62 ± 0.18	2.55 ± 0.37	2.19 ± 0.29	**3.47 ± 0.30**[c]
	Bdnf-2	2.91 ± 0.30	3.34 ± 0.37	2.42 ± 0.26	2.63 ± 0.28	2.17 ± 0.20	**3.12 ± 0.29** [b]
	Crhr1	2.93 ± 0.21	2.65 ± 0.27	3.05 ± 0.44	3.01 ± 0.31	2.80 ± 0.28	3.95 ± 0.69
	Fkbp5	3.54 ± 0.73	3.74 ± 0.34	4.21 ± 0.50	4.95 ± 0.32	4.38 ± 0.71	4.52 ± 0.56
	Htr1a	2.24 ± 0.35	1.64 ± 0.22	1.54 ± 0.18	3.25 ± 0.92	2.37 ± 0.34	3.83 ± 0.9
	Maoa	1.37 ± 0.11	1.21 ± 0.12	1.28 ± 0.19	**30.66 ± 2.39** [c]	**31.87 ± 1.13** [c]	**30.03 ± 2.04** [c]
	Nos1	3.15 ± 0.32	4.14 ± 0.32	3.75 ± 0.54	3.03 ± 0.25	3.49 ± 0.49	2.88 ± 0.56
	Nr3c1	0.97 ± 0.10	1.31 ± 0.34	0.93 ± 0.27	1.70 ± 0.66	1.40 ± 0.39	1.71 ± 0.92
	NGFI-A	1.67 ± 0.20	2.30 ± 0.32	1.47 ± 0.22	2.21 ± 0.31	2.10 ± 0.17	2.23 ± 0.29

Data represent mean ± SEM (n = 11–13).
a = $p < 0.05$ vs. respective control group; b = $p < 0.05$ vs. respective MS group; c = $p < 0.05$ vs. respective male group.

was performed with an initial denaturation step at 95 °C for 5 min, 40 cycles of 95 °C for 30 s, primer-specific annealing temperature (52 °C for *Crhr1*, *Nr3c1* promoter and NGFI-A binding site, 56 °C for *Bdnf*-2, *Fkbp5*, *Htr1a*, and *Maoa*, 60 °C for *Bdnf*-1 and *Nos1*) for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. Aliquots (10 µl) of the generated PCR products were immobilized on Streptavidin Sepharose HP beads (GE Healthcare Life Sciences, Freiburg, Germany). Pyrosequencing was performed on a PyroMark Q96 MD system with PyroMark Gold Q96 CDT reagents (Qiagen). Methylation values were quantified using the Pyro Q-CpG software (Qiagen). For a given gene and sample, the measured methylation values of all (4–9) targeted CpG sites were averaged. In our experience, the average methylation difference between technical replicates (including bisulfite conversion, PCR and pyrosequencing) is in the order of one percentage point.

2.3. Statistical analyses

Both descriptive and inferential statistical analyses were performed with IBM SPSS version 23 (http://www.spss.com). For within-cohort comparisons, depending on the data distribution either nonparametric Mann-Whitney U or parametric two-way ANOVA followed by Bonferroni post-hoc tests were performed. A p-value < 0.05 was

considered as significant.

3. Results

Using bisulfite pyrosequencing, we determined the DNA methylation level of 7 genes (9 loci), *Bdnf*-1 and -2, *Crhr1*, *Fkbp5*, *Htr1a*, *Maoa*, *Nos1*, and *Nr3c1* promoter and NGFI-A binding site in 6 different brain regions (amygdala, frontal cortex, hippocampus. hypothalamus, raphe nuclei, and striatum) of mice which were subjected to different prenatal (CTRL, EE) and postnatal (MS, CTRL, EE) conditions (Fig. 1). Ideally, 13 samples were analyzed for each gene, brain region and sex, however sometimes a few measurements had to be excluded because they did not meet the quality standards for pyrosequencing. Supplementary Figs. 1 and 2 demonstrate 54 bar diagrams (9 loci x 6 brain regions) each with the mean methylation values in the 6 offspring groups, preCTRL/postMS, preEE/postMS, preCTRL/postCTRL, preEE/postCTRL, preCTRL/postEE, and preEE/postEE, in males and females, respectively.

3.1. Epigenetic effects of enriched prenatal environment

To estimate the epigenetic effects of prenatal environmental enrichment, we compared animals which were exposed prenatally to an enriched vs. a standard environment within each of the three postnatal groups: preCTRL/postMS vs. preEE/postMS, preCRTL/postCTRL vs. preEE/postCTRL, and preCTRL/postEE vs. preEE/postEE, resulting in 324 (3 postnatal conditions x 9 loci x 6 brain regions x 2 sexes) between-group comparisons. Altogether we detected 29 (9%) significant differences between the two prenatal groups (indicated by vertical lines in Supplementary Figs. 1 and 2). In 11 cases preEE was associated with an hypermethylation and in 18 cases with an hypomethylation. Significant changes were observed in all genes, in both sexes, and in all three postnatal groups, and with the notable exception of hippocampus in all brain regions.

As one example for a gene that may be susceptible to an enriched intrauterine environment, the last bar diagram in Supplementary Fig. 2 shows methylation of *Nr3c1* NGFI-A in female striatum. In all three postnatal treatment groups (MS, CTRL, and EE), methylation was higher in mice which had been exposed prenatally to an enriched environment. However, only the effect in the postnatal EE group (preCTRL/postEE vs. preEE/postEE) was significant.

3.2. Epigenetic effects of postnatal conditions

Effects of postnatal treatment (postMS vs. postCTRL, postEE vs. postCTRL, and postMS vs. postEE) were analyzed in the two prenatal cohorts separately, resulting in 324 (3 postnatal contrasts x 9 loci x 6 brain regions x 2 sexes) between-group comparisons for each cohort. Only two (0.6%) significant differences between postEE and postMS were observed in the prenatal control cohort (Table 1; Supplementary Table S2), namely *Crhr1* methylation in female amygdala (4.2% preCTRL/postEE vs. 2.7% preCTRL/postMS) and *Bdnf*-2 methylation in female striatum (3.1% in preCTRL/postEE vs. 2.2% preCTRL/postMS). In contrast, 6 (1.9%) significant differences due to postnatal environment were detected in the prenatal EE cohort (Table 2; Supplementary Table S3): *Crhr1* methylation in female amygdala (3.0% preEE/postEE vs. 5.2% preEE/postCTRL), *Htr1a* in female amygdala (3.2% preEE/postMS vs. 1.8% preEE/postCTRL), *Nos1* in male frontal cortex (3.4% preEE/postEE vs. 2.5% preEE/postMS), *Nr3c1* promoter in female hypothalamus (1.3% preEE/postEE vs. 0.5% preEE/postCTRL), *Bdnf*-2 in male raphe nuclei (1.7% preEE/postEE vs. 2.9% preEE/postCTRL), and *Nr3c1* NGFI-A in female striatum (3.8% preEE/postEE vs. 2.5% preEE/postMS). Changes due to postnatal conditions were three times more frequent in the preEE than in the preCTRL cohort. Six of 8 significant changes occurred in female animals, compared to only two in males.

Consistent with an X-linked gene which is subject to X-inactivation, *Maoa* methylation differed dramatically (in the order of 30–35 percentage points) between males and females in all analyzed brain regions. The remaining 8 autosomal loci showed 10 sex-specific methylation differences in the preCTRL and 20 in the preEE cohort, all of small effect size. Notably in all 6 offspring groups (preCTRL/postCTRL, preCTRL/postMS, preCTRL/postEE, pre EE/postCTRL, preEE/postMS, and preEE/postEE) *Nr3C1* NGFI-A methylation was significantly lower (by 0.4–1.2 percentage points) in female than in male frontal cortex (Tables 1 and 2).

4. Discussion

Our aim was to study the long-term epigenetic effects of a prenatal environmental enrichment (preEE vs. preCTRL) in combination with three different postnatal (postMS, postCTRL, and postEE) conditions. Quantitative methylation analyses of 7 candidate genes that have been associated with HPA axis activity, anxiety-like and social behavior were performed on adult brain regions of mice with 6 different life histories. The serotonergic system which plays a key role in many central nervous system processes originates in the raphe nuclei and extends throughout the corticolimbic system. The serotonin-1A receptor (*Htr1a*) and monoamine oxidase A (*Maoa*) can modulate emotional behavior. HTR1A binding in the hippocampus is susceptible to programming by prenatal stress [24,25]. Peripuberty adverse experiences can trigger prefrontal *Maoa* expression and aggressive behavior [24,26]. The corticotropin-releasing hormone receptor 1 (*Crhr1*) is involved in activation of the HPA axis following stress [22]. Variants in *Crhr1* and the FK506 binding protein 5 (*Fkpb5*) have been associated with the cortisol response to stress [21]. Alterations of the glucocorticoid receptor *Nr3c1* and glucocorticoid levels can modulate the HPA axis and prenatal stress vulnerability [27,28]. Early life experiences have been associated with changes in hippocampal/hypothalamic expression of the brain-derived neurotrophic factor (*Bdnf*) and social behavior in later life [23]. Hippocampal nitric oxide synthetase 1 (*Nos1*) is involved in regulating the behavioural effects of glucocorticoids as well as many synaptic and neuronal processes [29].

Altogether we observed widespread DNA methylation changes due to prenatal enrichment, affecting all studied genes, 5 of 6 brain regions, and both sexes. One limitation of our study is that due to space constraints in our mouse facility the prenatal control cohort (preCTRL/postCTRL, preCTRL/postMS, and preCTRL/postEE) and the prenatal EE cohort (preEE/postCTRL, preEE/postMS, and preEE/postEE) were raised and analyzed in sequential years. Although conditions were highly standardized, we cannot exclude the formal possibility of batch effects, mimicking differences between prenatal cohorts. In this light, it is reassuring that identical changes were observed with different assays and across different cohorts, i.e. *Bdnf* hypomethylation (promoter region 1 and 2) in preEE/postMS vs. preCTRL/postMS male amygdala and *Nr3c1* hypermethylation (promoter and NGFI-A binding site) in the preEE/postMS vs. preCTRL/postMS male striatum (Supplementary Fig. S1). None of the 29 (9%) observed between-group differences was of large effects size (> 5 percentage points). Both exposed and control animals varied within the normal range of methylation variation. Consistent with a multifactorial model, epigenetic programming of stress vulnerability/resilience by life history may be the result of multiple (both stochastic and/or environmentally induced) subtle changes rather than of a large effect on a single or a few genes in specific brain regions. This may also reflect the relative dearth of alterations in the hippocampus; a region previously shown to be sensitive to early-life manipulations [30]. However, we assessed alterations across the whole hippocampus and it has become apparent that the dorsal and ventral hippocampus display differential methylation patterns in response to environmental enrichment [10]. Moreover, a recent study demonstrated that *Hdac1* overexpression, linked with DNA methylation, in the medial prefrontal cortex, but not the dorsal or ventral hippocampus could mimic the behavioural effect of early-life stress [31]. Therefore,

Table 2
Effects of postnatal environmental manipulations on adult brain methylation patterns of prenatal cohort 2 (enriched environment) in a sex-, region-, and gene-specific manner.

Brain region	Gene	Males			Females		
		Control	MS	EE	Control	MS	EE
Amygdala	*Bdnf-1*	3.30 ± 0.53	4.51 ± 0.54	3.40 ± 0.5	3.63 ± 0.67	3.21 ± 0.40	3.27 ± 0.22
	Bdnf-2	2.48 ± 0.15	2.93 ± 0.14	2.39 ± 0.13	2.19 ± 0.16	2.47 ± 0.21	2.52 ± 0.24
	Crhr1	2.13 ± 0.29	1.68 ± 0.12	1.70 ± 0.20	**5.23 ± 1.01** [c]	3.49 ± 0.42	**3.02 ± 0.26** [a]
	Fkbp5	3.56 ± 0.33	4.03 ± 0.40	4.25 ± 0.41	3.51 ± 0.44	3.96 ± 0.43	3.61 ± 0.29
	Htr1a	1.81 ± 0.36	1.04 ± 0.25	1.44 ± 0.28	1.78 ± 0.24	**3.24 ± 0.43** [a,c]	2.10 ± 0.15
	Maoa	1.31 ± 0.19	1.46 ± 0.17	1.58 ± 0.36	**32.91 ± 2.60** [c]	**33.95 ± 1.32** [c]	**35.42 ± 1.71** [c]
	Nos1	2.45 ± 0.29	2.33 ± 0.25	2.58 ± 0.26	2.01 ± 0.22	2.40 ± 0.15	2.29 ± 0.30
	Nr3c1	0.95 ± 0.21	1.10 ± 0.21	0.81 ± 0.12	**1.87 ± 0.22** [c]	**2.39 ± 0.25** [c]	**1.78 ± 0.18** [c]
	NGFI-A	1.85 ± 0.21	2.58 ± 0.23	3.92 ± 1.51	2.24 ± 0.13	2.46 ± 0.13	2.59 ± 0.24
Frontal cortex	*Bdnf-1*	3.18 ± 0.37	3.27 ± 0.28	3.79 ± 0.33	2.57 ± 0.33	2.88 ± 0.36	2.57 ± 0.54
	Bdnf-2	2.48 ± 0.31	2.75 ± 0.22	2.36 ± 0.40	1.87 ± 0.21	2.34 ± 0.56	1.91 ± 0.24
	Crhr1	2.65 ± 0.13	3.68 ± 0.41	2.97 ± 0.32	2.81 ± 0.35	2.53 ± 0.23	2.89 ± 0.22
	Fkbp5	3.53 ± 0.37	3.81 ± 0.41	3.49 ± 0.36	4.62 ± 0.39	4.29 ± 0.34	4.03 ± 0.36
	Htr1a	2.70 ± 0.49	2.22 ± 0.28	1.95 ± 0.33	2.24 ± 0.20	2.44 ± 0.22	1.79 ± 0.17
	Maoa	2.56 ± 0.50	1.22 ± 0.26	2.28 ± 0.36	**33.62 ± 0.69** [c]	**31.64 ± 1.25** [c]	**32.00 ± 0.83** [c]
	Nos1	2.68 ± 0.22	2.46 ± 0.14	**3.36 ± 0.18** [b]	2.32 ± 0.22	**1.63 ± 0.15** [c]	**2.18 ± 0.23** [c]
	Nr3c1	1.32 ± 0.21	0.99 ± 0.14	1.27 ± 0.15	0.67 ± 0.18	1.17 ± 0.21	0.82 ± 0.18
	NGFI-A	1.86 ± 0.14	2.18 ± 0.14	2.69 ± 0.42	**1.48 ± 0.11** [c]	**1.44 ± 0.13** [c]	**1.48 ± 0.25** [c]
Hippocampus	*Bdnf-1*	2.80 ± 0.17	2.84 ± 0.29	3.17 ± 0.19	3.17 ± 0.30	2.40 ± 0.22	3.14 ± 0.31
	Bdnf-2	2.08 ± 0.19	2.21 ± 0.34	2.12 ± 0.21	1.89 ± 0.17	1.99 ± 0.12	1.68 ± 0.20
	Crhr1	2.64 ± 0.27	2.59 ± 0.27	3.54 ± 0.44	3.65 ± 0.22	**4.25 ± 0.29** [c]	3.80 ± 0.47
	Fkbp5	3.09 ± 0.26	2.96 ± 0.35	4.07 ± 0.44	2.18 ± 0.24	2.36 ± 0.44	**2.00 ± 0.39** [c]
	Htr1a	1.75 ± 0.13	2.19 ± 0.50	1.51 ± 0.21	1.43 ± 0.16	1.39 ± 0.19	1.91 ± 0.20
	Maoa	2.06 ± 0.46	1.96 ± 0.27	1.93 ± 0.35	**33.42 ± 1.33** [c]	**35.20 ± 0.64** [c]	**33.27 ± 1.67** [c]
	Nos1	1.89 ± 0.14	2.46 ± 0.32	2.16 ± 0.13	2.05 ± 0.26	2.46 ± 0.32	2.13 ± 0.15
	Nr3c1	0.69 ± 0.13	0.83 ± 0.13	0.90 ± 0.12	0.85 ± 0.15	0.76 ± 0.12	1.08 ± 0.15
	NGFI-A	1.69 ± 0.12	2.03 ± 0.17	1.73 ± 0.24	2.00 ± 0.10	1.94 ± 0.27	2.20 ± 0.18
Hypothalamus	*Bdnf-1*	1.91 ± 0.42	1.93 ± 0.36	2.78 ± 0.34	**3.77 ± 0.34** [c]	**3.58 ± 0.46** [c]	3.05 ± 0.20
	Bdnf-2	2.04 ± 0.22	2.19 ± 0.21	2.26 ± 0.11	1.94 ± 0.13	1.84 ± 0.15	2.18 ± 0.15
	Crhr1	1.88 ± 0.13	1.76 ± 0.12	1.87 ± 0.22	2.39 ± 0.23	**2.76 ± 0.34** [c]	2.61 ± 0.14
	Fkbp5	3.29 ± 0.21	3.83 ± 0.22	3.58 ± 0.29	3.71 ± 0.33	4.21 ± 0.26	3.76 ± 0.24
	Htr1a	2.15 ± 0.67	2.09 ± 0.31	1.82 ± 0.16	1.72 ± 0.23	2.00 ± 0.12	2.13 ± 0.14
	Maoa	2.58 ± 0.35	2.87 ± 0.42	2.60 ± 0.36	**35.75 ± 0.84** [c]	**36.12 ± 0.89** [c]	**34.26 ± 0.75** [c]
	Nos1	2.51 ± 0.21	2.77 ± 0.34	2.68 ± 0.23	3.42 ± 1.19	2.27 ± 0.19	2.81 ± 0.09
	Nr3c1	1.07 ± 0.13	0.95 ± 0.12	1.38 ± 0.39	0.51 ± 0.13	0.76 ± 0.10	**1.30 ± 0.18** [a]
	NGFI-A	2.37 ± 0.12	2.21 ± 0.18	2.91 ± 0.29	2.42 ± 0.23	2.72 ± 0.24	2.19 ± 0.18
Raphe nuclei	*Bdnf-1*	5.48 ± 1.66	6.53 ± 1.83	1.76 ± 0.40	2.91 ± 0.40	**1.96 ± 0.19** [c]	2.42 ± 0.43
	Bdnf-2	2.92 ± 0.52	1.79 ± 0.19	**1.69 ± 0.17** [a]	1.89 ± 0.19	2.15 ± 0.22	1.46 ± 0.10
	Crhr1	3.53 ± 0.26	3.43 ± 0.45	3.25 ± 0.33	3.51 ± 1.08	3.62 ± 0.32	5.00 ± 1.28
	Fkbp5	3.66 ± 1.17	4.42 ± 0.86	3.30 ± 0.37	4.20 ± 0.64	4.40 ± 0.55	5.96 ± 1.53
	Htr1a	2.15 ± 0.27	2.22 ± 0.35	2.15 ± 0.28	1.34 ± 0.31	3.06 ± 0.86	1.72 ± 0.53
	Maoa	10.6 ± 2.56	10.4 ± 2.67	9.21 ± 2.07	**30.9 ± 1.09** [c]	**35.46 ± 1.27** [c]	**31.19 ± 0.82** [c]
	Nos1	2.72 ± 0.64	3.05 ± 0.89	3.13 ± 0.83	4.99 ± 0.44	5.15 ± 0.34	4.08 ± 0.54
	Nr3c1	1.18 ± 0.34	2.16 ± 0.69	0.75 ± 0.13	1.79 ± 0.65	2.63 ± 0.89	2.24 ± 0.27
	NGFI-A	4.58 ± 0.87	2.57 ± 0.26	3.79 ± 0.62	2.32 ± 0.36	3.61 ± 0.98	2.91 ± 0.33
Striatum	*Bdnf-1*	2.53 ± 0.45	2.91 ± 0.32	2.91 ± 0.29	3.15 ± 0.41	3.48 ± 0.30	2.29 ± 0.53
	Bdnf-2	2.71 ± 0.23	2.75 ± 0.43	3.12 ± 0.48	3.11 ± 0.25	2.96 ± 0.28	2.26 ± 0.58
	Crhr1	2.95 ± 0.41	2.95 ± 0.32	2.45 ± 0.28	4.90 ± 1.57	4.08 ± 1.60	2.50 ± 0.21
	Fkbp5	3.75 ± 0.23	3.58 ± 0.20	2.79 ± 0.24	4.03 ± 0.53	3.60 ± 0.39	3.70 ± 0.35
	Htr1a	1.70 ± 0.21	1.51 ± 0.21	1.59 ± 0.27	**3.39 ± 0.66** [c]	2.36 ± 0.31	3.27 ± 0.57
	Maoa	1.55 ± 0.35	4.96 ± 2.94	2.33 ± 0.84	**30.18 ± 1.90** [c]	**36.03 ± 2.13** [c]	**31.25 ± 1.21** [c]
	Nos1	2.95 ± 0.30	3.98 ± 1.54	3.94 ± 0.30	3.51 ± 0.62	2.98 ± 0.61	3.33 ± 0.29
	Nr3c1	1.22 ± 0.22	0.64 ± 0.13	1.13 ± 0.24	2.52 ± 0.68	1.98 ± 0.76	2.27 ± 0.59
	NGFI-A	1.85 ± 0.34	1.19 ± 0.23	1.91 ± 0.27	**3.14 ± 0.43** [c]	**2.51 ± 0.22** [c]	**3.84 ± 0.53** [b,c]

Data represent mean ± SEM (n = 11–13).
a = $p < 0.05$ vs. respective control group; b = $p < 0.05$ vs. respective MS group; c = $p < 0.05$ vs. respective male group.

future studies comparing maternal separation and environmental enrichment should assess more discrete brain nuclei. When using adult brain methylation patterns as end point, prenatal enrichment had a larger impact than postnatal conditions. However, this needs to be tested in future experiments in which pre- and post-natal enrichment can be directly compared. Our results support the conclusion from nutritional studies that the environmental sensitivity of the epigenome is the highest in early stages of development and is decreasing during later life [32].

Since we cannot rule out possible batch effects, the epigenetic signatures of adverse and enriched postnatal conditions were analyzed in each prenatal cohort separately. Of the 8 significant methylation changes due to postnatal treatment, 6 were found in the preEE and only two in the preCTRL group. This promotes the idea that prenatal enrichment establishes a plastic epigenetic state [33], which increases the sensitivity to adverse or enriched postnatal conditions. Overall, female offspring appeared to be more susceptible to programming by postnatal environment, compared to males. This is explained by sexual dimorphism of the brain circuits underlying fetal programming of behavior.

5. Conclusions

The developing brain in the fetal and early postnatal period is particularly susceptible to epigenetic reprogramming by environmental exposures. An enriched environment of mouse dams appears associated with multiple DNA methylation changes in different genes and brain regions of the offspring. Moreover, prenatal enrichment may increase the sensitivity to enriched or adverse postnatal experiences given that we observed more alterations after prenatal manipulations. Widespread epigenetic changes of small effect size may mediate the long-lasting effects of early life experiences on stress vulnerability/resilience in later life. Essentially all observed epigenetic effects were sex-specific highlighting the role of gender in developmental programming of the brain.

Author contributions

AR designed the study. FM and FS performed DNA methylation analyses. AP performed the mouse work. AOL and DAS performed statistical analyses and revised the manuscript. FM and TH prepared the manuscript.

Competing interests

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bbr.2018.10.037.

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