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Original Article

Prenatal Dexamethasone Exposure in Male Rats Alters Gene Expression Patterns of Epigenetic Enzymes in Hippocampus and Cortex

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ABSTRACT

Objective: This study aimed to examine the effects of prenatal stress (PS) induced by dexamethasone exposure on gene expression levels of epigenetic enzymes in hippocampus and cerebral cortex of male rats through relative mRNA levels of histone acetyltransferases (activating transcription factor 2, P300), histone deacetylases (HDAC1, HDAC2), and DNA methyltransferases (DNMT1, DNMT3a, DNMT3b).

Methods: Pregnant rats were daily injected subcutaneously with dexamethasone (0.2 mg/kg) or saline during the third week of gestation. After birth, male rats were killed at 90 days of age (n = 5 for control and dexamethasone groups). Hippocampal and cortical tissues were used for gene expression analyses. The effects of dexamethasone on epigenetic mechanisms were investigated by real-time polymerase chain reaction through relative mRNA levels of DNMT1, DNMT3a, DNMT3b, activating transcription factor 2, P300, HDAC1, and HDAC2. Statistical comparisons were performed with Student's t-test.

Results: Prenatal dexamethasone exposure (PDE) caused increased DNMT1, DNMT3a, DNMT3b, activating transcription factor 2 and decreased P300 mRNA levels in hippocampus while increased DNMT3a, DNMT3b, activating transcription factor 2, P300, HDAC1, and HDAC2 mRNA levels were achieved in cortex. Furthermore, no significant differences were obtained in cortical DNMT1 and hippocampal HDAC1 and HDAC2 gene expression levels between control and prenatally stressed rats.

Conclusion: Our results emphasize the effect of prenatal dexamethasone exposure on gene expression levels of epigenetic enzymes involved in histone acetylation/deacetylation and DNA methylation in male rats and suggest that prenatal stress may lead to epigenetic dysregulation through alterations in hippocampal and cortical gene expression patterns of DNMT1, DNMT3a, DNMT3b, activating transcription factor 2, P300, HDAC1, and HDAC2.

Keywords: Dexamethasone, epigenetic enzymes, prenatal stress, rat

INTRODUCTION

The brain is vulnerable to stress factors during the perinatal life due to its high neuroplasticity. Prenatal exposure to environmental factors is assumed to change gene expression profiles throughout life and has been shown to have various negative effects on both health and cognition in animals and humans. The alterations in gene expression profiles can be caused by epigenetic mechanisms through histone or DNA modifications.[1,2] Moreover, prenatal stress has been proposed to affect the risk of developing mental or metabolic disorders such as anxiety disorder, depression, hypertension, and type 2 diabetes mellitus.[3-5]

Epigenetic mechanisms, which include histone modifications, non-coding RNAs, DNA methylation, and hydroxymethylation, are involved in the regulation of gene activity without modifying the DNA sequence.[6] The addition of acetyl groups to histones is catalyzed by histone acetyltransferases (HATs) while histone deacetylases (HDACs) catalyze the removal of acetyl groups.[7] Activating transcription factor 2 (ATF2) functions as HAT and associates with p300, which also works as HAT and transcriptional co-activator.[8] HDAC1 and 2, class I HDACs, are localized in hippocampus and cortex.[9] DNA methylation represses transcription and is catalyzed by DNA methyltransferases (DNMTs) including DNMT1, 2, and 3.[10]

The hypothalamic-pituitary-adrenal (HPA) axis plays an emerging role in stress adaptation. The hippocampus and the frontal cortex are involved in regulating the functioning of the HPA axis and are connected highly with hypothalamus.[11] Laboratory animals have been employed as prenatal glucocorticoid exposure (prenatal stress) models by administering corticosterone or its synthetic

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analogs throughout pregnancy. Dexamethasone (Dex), a synthetic glucocorticoid that is fat soluble, can pass through the placenta, and has been used to induce prenatal stress in rodents.[12] It has been demonstrated that the glucocorticoid receptor (GR) plays a critical role in modulating cellular responses to stress and circulating glucocorticoids. To regulate transcription and modify the structure of chromatin, GR works in conjunction with the epigenetic enzymes such as methyltransferases (DNMT1, DNMT3a, and DNMT3b), HATs (ATF2, p300), and HDACs (HDAC1, HDAC2).[13]

The present study examined for the first time how prenatal Dex exposure at a dose of 0.2 mg/kg during the last week of gestation affected the gene expression profiles of DNMTs (DNMT1, DNMT3a, and DNMT3b), HATs (ATF2, p300), and HDACs (HDAC1, HDAC2) concurrently.

METHODS

Animals and Treatment

The present study was approved by Ethics Committee on Animal Experiments of Ege University (May 24, 2017; Reference No. 2017-023). All efforts were made to minimize animal suffering. Six virgin female Sprague-Dawley rats, 210 ± 10 g, were housed randomly under a 12-hour light-dark cycle in plastic cages at 19-24°C and allowed continuous access to rat chow and water. Two female rats in proestrus were coupled with 1 male rat overnight. Pregnancies were confirmed by the vaginal smears and pregnant rats were divided into 2 groups as control or prenatal stress.[14] Rats were injected subcutaneously with Dex (Cat. No. D1159, Sigma-Aldrich, St. Louis, MO, USA) at a dose of 0.2 mg/kg or saline of equal volume during the third week of gestation. At end of treatments, pregnant rats delivered normally. All offspring were weaned on postnatal day 21 (PN21). Prenatal Dex exposure at a dose of 0.2 mg/kg resulted in lethality in the offspring. For this reason, we had to limit the number of male rats in the experimental groups to 5. At PN90, male rats were killed by decapitation. Hippocampus and cortex were dissected immediately, frozen, and stored at −80°C.[12]

Total RNA Extraction, Reverse Transcription, and Real-Time Quantitative Polymerase Chain Reaction

The cortical and hippocampal samples were used for total RNA extraction with MasterPure Complete DNA and RNA Purification Kit (Cat. No. MC85200, LGC Biosearch Technologies, Wis, USA). The concentration and purity of RNA samples were measured using the CLARIOstar Plus microplate reader with the LVIS plate (BMG LABTECH, Ortenberg, Germany). The absorbance$_{260\ nm}$/absorbance$_{280\ nm}$ ratio of RNA samples was determined, and a ratio of 1.95-2.00 was accepted as pure for RNA samples. One microgram of total RNA was reverse transcribed using cDNA synthesis kit containing RevertAid Reverse Transcriptase, 5X Reaction Buffer, Random Hexamer Primer, dNTP Mix, RiboLock RNase Inhibitor, and nuclease-free water (Cat. No. K1622, Thermo Fisher Scientific, Mass, USA). The amplification protocol for reverse transcription consisted of 1 cycle of 25°C for 5 minutes, 42°C for 1 hour, and 70°C for 5 minutes.

Real-time quantitative polymerase chain reaction (PCR) experiments were performed with SYBR Green and AriaMx RT-PCR System (Agilent Technologies, Calif, USA). cDNA (1 µL), 250 nM forward and reverse primers (1 µL), and DNase- and RNase-free water (7 µL) were amplified with Master Mix (10 µL). Primer-BLAST was used for primer designs, and the sequences of primers were confirmed with in silico PCR amplification.[15,16] The primers used in this experiment were given in Table 1. The amplification protocol consisted of 1 cycle of 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds. The dissociation curve analyses were performed at end of the amplification to control desired PCR products. The real-time PCR experiments were repeated 3 times. We determined the relative levels of mRNAs by comparative C_T method ($2^{-\Delta\Delta CT}$) and normalized to reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with Agilent software.[17]

Statistical Analysis

Data were analyzed with Statistical Package for the Social Sciences for Windows (SPSS 25.0, IBM SPSS Corp., NY, USA) using Student's *t*-test. Data were representative of 3 independent experiments and given as mean ± standard error. $P < .05$ was considered statistically significant.

RESULTS

Effects of PDE on Relative mRNA Levels of DNMTs

To examine the effects of PDE on gene expression levels of DNMTs, we measured relative mRNA levels of DNMT1, 3a, and 3b in hippocampal and cortical tissues of PDE group and control (Figure 1). The Tm values of DNMT1, 3a, and 3b were found to be 80.5°C, 89.0°C, and 83.5°C, respectively. In hippocampus of control and PDE groups, relative mRNA levels of DNMT1 were 1.000 ± 0.023 and 1.221 ± 0.035, respectively. In cortical samples, relative mRNA levels of DNMT1 in control and PDE groups were 0.997 ± 0.012 and 0.992 ± 0.024, respectively. No significant differences were found in cortical DNMT1 mRNA levels between the groups but PDE increased hippocampal DNMT1 mRNA levels significantly when compared to control. We also found significant differences in DNMT1 gene expression levels between hippocampal and cortical tissues of PDE group (Figure 1, $^{a}P < .001$ vs. control in hippocampus and vs. Dex in cortex). The relative mRNA levels of DNMT3a in hippocampal samples of control and PDE groups were 0.999 ± 0.026 and 1.319 ± 0.028, respectively. In cortical tissues of control and PDE groups, relative mRNA levels of DNMT3a were found as 1.003 ± 0.018 and 1.316 ± 0.036, respectively. When compared to control group, DNMT3a mRNA levels of PDE group in hippocampus and cortex

Main Points

- Prenatal stress was shown to change mRNA levels of epigenetic enzymes involved in histone acetylation/deacetylation and DNA methylation.
- Prenatal dexamethasone (Dex) exposure altered the gene expression patterns of DNMT1, DNMT3a, DNMT3b, activating transcription factor 2 (ATF2), and P300 in hippocampus.
- Prenatal Dex exposure altered the gene expression patterns of DNMT3a, DNMT3b, ATF2, P300, HDAC1, and HDAC2 in cortex.

Table 1. The Sequences for Forward and Reverse Primers Used in RT–qPCR

Primer	NCBI Ref. No	Primer Sequence	Position	Length
DNMT1	NM_053354.3	F: 5'–GAGGCACTGTCCGTCTTTGA–3' R: 5'–AAGTGACCGCGACTGCAATA–3'	1247	107 bp
DNMT3a	NM_001003958.1	F: 5'–ACGATAATACCTTCTCTGAAGCCC–3' R: 5'–CTTCCTTTCGATCATCCTCCCG–3'	88	150 bp
DNMT3b	NM_001396349.1	F: 5'–GATGAGGAGAGCCGAGAACG–3' R: 5'–CAGAGCCCACCCTCAAAGAG–3'	1488	128 bp
ATF2	NM_031018.2	F: 5'–GGATTGGTTAGGGCCCAGTC–3' R: 5'–CTCTTCTTCGACGGCCACTT–3'	1184	136 bp
P300	AB066220.1	F: 5'–AAGGTCTGGTAGTTCCCCCA–3' R: 5'–TGTGCCATTGGGCTTTTGAC–3'	254	129 bp
HDAC1	NM_001025409.1	F: 5'–CTCCATCTTCTCTCCAAGTCCC–3' R: 5'–GAGTTCTCCCAGTACCACTGC–3'	1480	150 bp
HDAC2	NM_053447.1	F: 5'–GGCCTCAGGATTCTGCTACG–3' R: 5'–CGGTCATCACGCGATCTGTT–3'	640	149 bp
GAPDH	NM_017008.4	F: 5'–AGTGCCAGCCTCGTCTCATA–3' R: 5'–AACTTGCCGTGGGTAGAGTC–3'	49	187 bp

GAPDH, glyceraldehyde–3–phosphate dehydrogenase; ATF2, activating transcription factor 2; HDAC, histone deacetylase; DNMT, DNA methyltransferase; RT–qPCR, reverse transcriptase quantitative polymerase chain reaction.

elevated significantly (Figure 1, $^{b}P < .001$ vs. control in hippocampus; $^{c}P < .001$ vs. control in cortex). The relative mRNA levels of DNMT3b in hippocampal samples were found as 0.998 ± 0.026 and 1.299 ± 0.031 in control and PDE groups, respectively. In cortical samples of control and PDE groups, relative mRNA levels of DNMT3b were 1.002 ± 0.029 and 1.250 ± 0.036, respectively. It was found that PDE led to significant enhancement of DNMT3b mRNA levels of PDE group in hippocampus and cortex when compared to control (Figure 1, $^{d}P < .001$ vs. control in hippocampus; $^{e}P < .001$ vs. control in cortex).

Effects of PDE on Relative mRNA Levels of Histone Acetyltransferases

To examine the effects of PDE on gene expression levels of HATs, we measured relative mRNA levels of ATF2 and P300 in hippocampal and cortical samples of PDE group and control (Figure 2). The Tm values of ATF2 and P300 were found to be 89.0°C and 83.0°C, respectively. The relative ATF2 mRNA levels in hippocampus and cortex of control were found as 1.002 ± 0.020 and 1.000 ± 0.022, respectively. In the PDE group, relative ATF2 mRNA levels in hippocampus and cortex were 1.602 ± 0.013 and 1.229 ± 0.028, respectively. When compared to control, the relative ATF2 mRNA levels were found elevated significantly in hippocampus and cortex of PDE group. We also found significant differences in ATF2 gene expression levels between hippocampus and cortex in the PDE group (Figure 2, $^{a}P < .001$ vs. control in hippocampus and vs. Dex in cortex; $^{b}P < .001$ vs. control in cortex). In hippocampal samples of control and PDE groups, relative mRNA levels of P300 were 0.999 ± 0.011 and 0.879 ± 0.008, respectively. The relative mRNA levels of P300 in cortical samples of control and PDE groups were found as 1.002 ± 0.016 and 1.350 ± 0.044, respectively. P300 gene expression levels of PDE group showed significant differences between hippocampus and cortex. We showed that P300 mRNA levels of PDE group significantly decreased in hippocampus, but were elevated in cortex compared to control (Figure 2, $^{c}P < .001$ vs. control in hippocampus and vs. Dex in cortex; $^{d}P < .001$ vs. control in cortex).

Effects of PDE on Relative mRNA Levels of Histone Deacetylases

To examine the effects of PDE on gene expression levels of HDACs, we measured relative mRNA levels of HDAC1 and 2 in hippocampal and cortical samples of PDE group and control (Figure 3). The Tm values of HDAC1 and HDAC2 were found to be 79.5°C and 81.0°C, respectively. In hippocampus and cortex of control group, relative mRNA levels of HDAC1 were found as 1.017 ± 0.040 and 1.014 ± 0.039, respectively. In PDE group, relative mRNA levels of HDAC1 in hippocampus and cortex were 1.131 ± 0.022 and 2.198 ± 0.083, respectively. We showed that HDAC1 mRNA levels of PDE group were significantly elevated in cortex, but no differences were found in hippocampus when compared to control. In PDE group, HDAC1 mRNA levels significantly differed between hippocampus and cortex (Figure 3, $^{a}P < .001$ vs. control in cortex and vs. Dex in hippocampus). The relative mRNA levels of HDAC2 were found as 1.000 ± 0.022 and 0.947 ± 0.024 in hippocampus of control and PDE groups, respectively. In cortical samples of control and PDE groups, the relative mRNA levels of HDAC2 were 0.999 ± 0.025 and 1.216 ± 0.011, respectively. We found that levels of HDAC2 mRNA were elevated significantly in cortex of PDE group compared to control. There were also significant differences in HDAC2 expression levels between hippocampus and cortex in the PDE group (Figure 3, $^{b}P < .001$ vs. control in cortex and vs. Dex in hippocampus).

DISCUSSION

The administration of dexamethasone, which is a fat-soluble synthetic glucocorticoid, during pregnancy has been used as a prenatal stress model in laboratory animals.[12] Through increased

Figure 1. Effects of prenatal Dex exposure on mRNA expression levels of DNMTs in hippocampus and cortex. The relative mRNA levels of DNMTs were determined with $2^{-\Delta\Delta CT}$ method and normalized to GAPDH. Data were expressed as mean ± standard error. $^{a}P < .001$ vs. control in hippocampus and vs. Dex in cortex; $^{b,d}P < .001$ vs. control in hippocampus; $^{c,e}P < .001$ vs. control in cortex (n = 5 for each group). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Dex, dexamethasone; DNMTs, DNA methyltransferases.

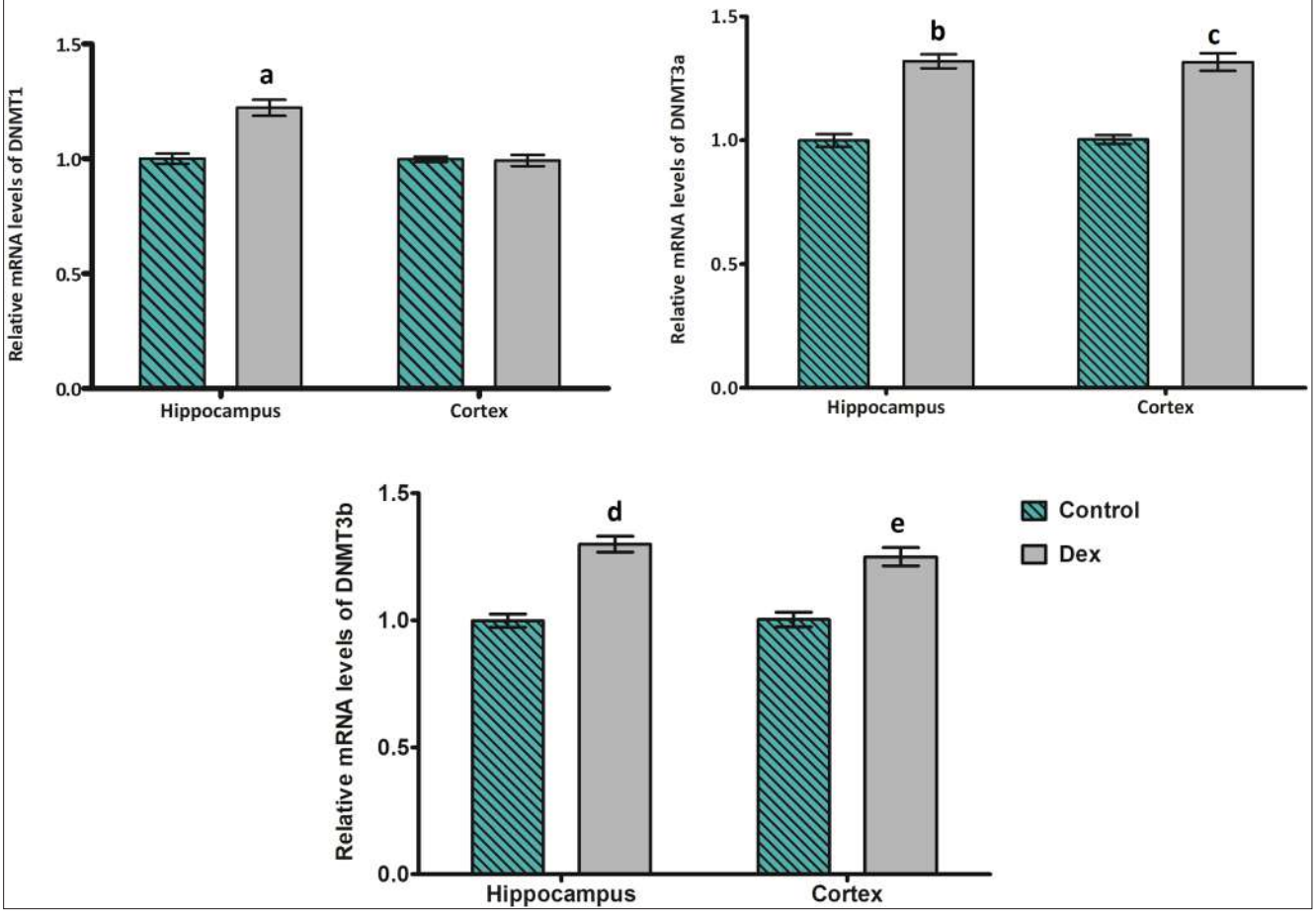


Figure 2. Effects of prenatal Dex exposure on mRNA expression levels of HATs in hippocampus and cortex. The relative mRNA levels of HATs were determined with $2^{-\Delta\Delta CT}$ method and normalized to GAPDH. Data were expressed as mean ± standard error. $^{a,c}P < .001$ vs. control in hippocampus and vs. Dex in cortex; $^{b,d}P < .001$ vs. control in cortex (n = 5 for each group). HATs, histone acetyltransferases; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Dex, dexamethasone.

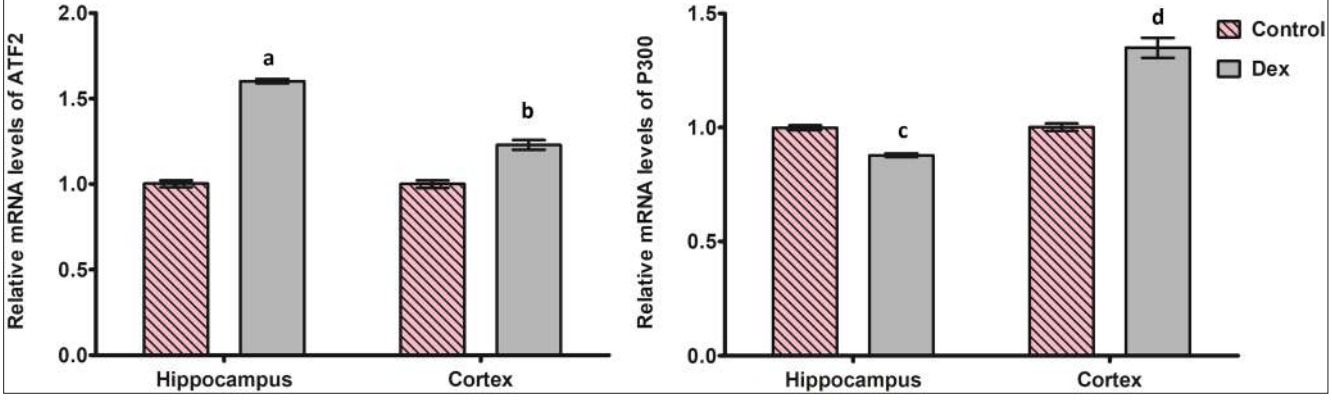
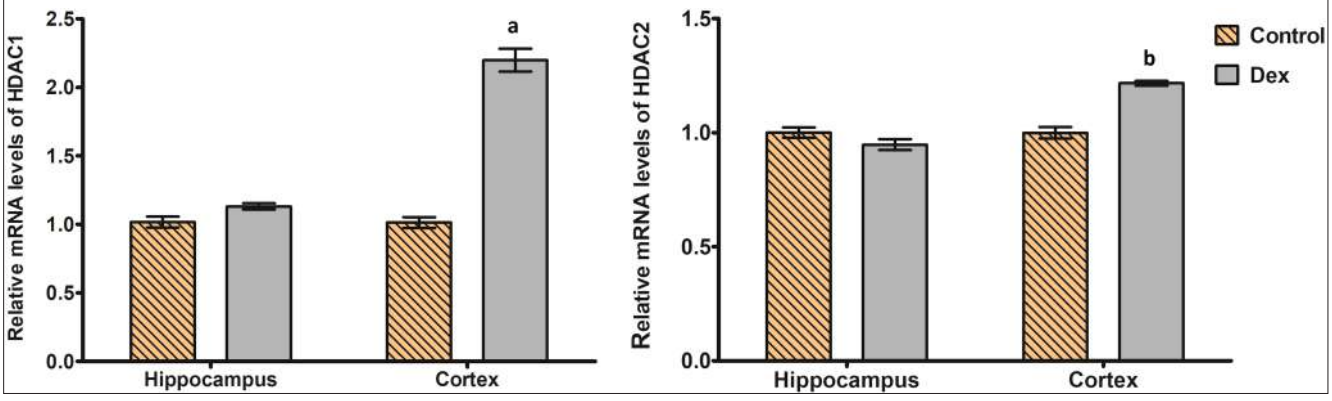


Figure 3. Effects of prenatal Dex exposure on mRNA expression levels of HDACs in hippocampus and cortex. The relative mRNA levels of HDACs were determined with $2^{-\Delta\Delta CT}$ method and normalized to GAPDH. Data were expressed as mean ± standard error. $^{a,b}P < .001$ vs. control in cortex and vs. Dex in hippocampus (n = 5 for each group). HDACs, histone deacetylases; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Dex, dexamethasone.



levels of adrenal corticotropin-releasing hormone and corticosterone, upregulation of corticotropin-releasing hormone in the hypothalamus, downregulation of mineralocorticoid receptors, and upregulation of 11-beta-hydroxysteroid dehydrogenase-1 in the hippocampus in adult male rats, prenatal Dex exposure has been shown to alter hippocampal drive on HPA axis activity.[18] Prenatal stress has been proposed to interact with genetics as well as epigenetics to alter the risk for various mental or metabolic disorders.[3-5]

The epigenetic regulation of gene activity, which includes DNA and histone modifications, is involved in various stages of neuronal function and are reported to have lifelong effects on mature neurons, neuroplasticity, and cognition.[6] The epigenetic processes causing changes in gene expression profiles during pre- and postnatal period may be responsible for development of the longer-lasting effects of prenatal stress.[19] Moreover, epigenetic mechanisms have attracted great attention as therapeutic strategy in diagnosis and treatment of neurodevelopmental disorder and neurodegeneration.[6,20,21] In our study, prenatal Dex exposure resulted in increased DNMT1, DNMT3a, DNMT3b, ATF2 and decreased P300 mRNA levels in hippocampus while increased DNMT3a, DNMT3b, ATF2, P300, HDAC1, and HDAC2 mRNA levels were found in cortex. Furthermore, there were no significant differences in cortical DNMT1, hippocampal HDAC1, and HDAC2 gene expression levels between control and prenatally stressed rats.

Activating transcription factor 2 regulates the expression of specific genes involved in inflammation, proliferation, apoptosis, transformation, and repair.[8] In the rat models of depression induced by lipopolysaccharide or chronic unpredicted mild stress, significant increases of ATF2 activation were obtained in hippocampus.[22] Neuroinflammation that occurs in particular brain regions in response to external stress stimuli is usually linked to depression. Chronic unpredicted mild stress-induced ATF2 activation in the hippocampus may be due to its role in inflammatory processes. Kucharczyk et al[11] showed activation of ATF2 in frontal cortex of male rats which were prenatally exposed to immobilization stress. In contrast to the frontal cortex, ATF2 activation has not been seen in the hippocampus.[11] In our study, PDE resulted in enhanced ATF2 mRNA levels both in hippocampus and cortex. The reason why we found increased expression of ATF2 in the hippocampus may be because we used a different prenatal stress protocol than Kucharczyk and colleagues.

P300 plays important roles during the neurodevelopmental stages and may involve in neuroplasticity of mature neurons.[23] The nuclear receptor interaction domain of the P300 interacts with the GR. P300 is required for transcriptional coactivation of target genes with glucocorticoid response elements.[13] In the study by Hu et al[24], pregnant rats were injected with Dex (0.2 mg/kg) from GD9 to 20 and PDE led to significant increase in P300 mRNA levels of fetal rats. P300 mRNA levels were found to be decreased significantly in hippocampus but increased in cortex of PDE group in our study. The differences in P300 expression pattern may be due to the fact that Hu et al used fetal hippocampal tissues in their study and we used hippocampal tissues from 3-month-old rats.

DNA methylation causes inhibition of transcription and is involved in regulation of gene expression and development.[10] Glucocorticoid receptor function can be regulated by DNMTs, and vice versa, GR can control the expression and activity of DNMTs.[13] Lui et al[25] induce PS using Dex at a different dose (0.1 mg/kg) from our study and demonstrated elevated DNMT1 mRNA levels in hippocampus of male rats. Boersma et al[2] demonstrated increased DNMT1 and 3a mRNA levels in hippocampus and amygdala of male rats exposed to different stressors including swim, social, and restraint stress during prenatal period.[2] In the study by Lei et al. PS induced by restraint stress caused enhancement in hippocampal protein levels of DNMT1 and 3a in female rats, but not in male offspring.[19] In the study by Grégoire et al.[26] prenatal restraint stress in male mice caused significant decrease in hippocampal DNMT3b and cortical HDAC1 mRNA levels whereas increase in hippocampal DNMT1 expression was

observed. In the present study, PDE caused enhanced mRNA levels of DNMT3a and DMNT3b both in hippocampus and cortex but DNMT1 mRNA levels increased only in hippocampus. Our findings supported previously reported studies describing increased DNMT1, DNMT3a, and DNMT3b expressions in the hippocampus as a result of prenatal stress.[2,25,26] The inhibition of HDAC activity plays important roles in cellular and molecular processes that are involved in neuroplasticity, oxidative stress, apoptosis, transcription, and neuroprotection through modification of histone acetylation levels.[27] Zheng et al[28] demonstrated that prenatal restraint stress increased DNMT1, HDAC1, and HDAC2 gene expressions in the hippocampal samples of male mice. Wei et al[29] discovered that GR occupancy was enhanced at the HDAC2 promoter glucocorticoid response element and enhances the transcription of HDAC2 in repeatedly stressed rats. We found that HDAC1 and HDAC2 mRNA levels elevated significantly in cortex but not altered in hippocampus of prenatally stressed rats. Using a different prenatal stress model and animal type from Zheng et al. may explain the difference in HDAC expressions.

There are some limitations in our study. We could obtain more effective results in gene expression analyses by increasing the number of animals in the experimental groups. We did not have the opportunity to analyze the protein levels of HATs, HDACs, and DNMTs. Detecting gene expression levels may not always give a precise result about the amount of a protein. We also did not separate subregions of cortical and hippocampal tissues. Therefore, protein quantification and microdissection techniques are required for a more detailed analysis.

CONCLUSION

Further studies detecting mRNA and protein levels of HATs, HDACs, and DNMTs throughout neurodevelopment and in later life are needed to determine the changes in epigenetic mechanisms. To the best of our knowledge, this study shows for the first time that prenatal Dex exposure at a dose of 0.2 mg/kg during the final week of gestation changed the gene expression profiles of DNMTs (DNMT1, DNMT3a, DNMT3b), HATs (ATF2, P300), and HDACs (HDAC1, HDAC2) concurrently in the cortex and hippocampus of male rats. Conclusively, our results will serve as an experimental contribution to understanding the neurodevelopmental effects of prenatal stress on epigenetic programming and suggest that prenatal stress may cause disruption of epigenetic regulation through changes in hippocampal and cortical gene expression patterns of DNMT1, DNMT3a, DNMT3b, ATF2, P300, HDAC1, and HDAC2 in male rats.

Ethics Committee Approval: Ethical committee approval was received from Ethics Committee on Animal Experiments of Ege University (Date: May 24, 2017, Decision no: 2017-023).

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Declaration of Interests: The authors declare that they have no competing interest.

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