Erzincan Üniversitesi
Fen Bilimleri Enstitüsü Dergisi
2022, 15(2), 609-621
ISSN: 1307-9085, e-ISSN: 2149-4584
Araştırma Makalesi

Erzincan University
Journal of Science and Technology
2022, 15(2), 609-621
DOI: 10.18185/erzifbed.1126806
Research Article

The Effects of Prenatal Stress on Cortical and Hippocampal Gene Expression Profiles of DNA Methyltransferases and Histone Deacetylases in Female Rats

Ezgi TURUNÇ[1*], Yiğit UYANIKGİL[2], Ayfer YALÇIN[3], Tijen KAYA-TEMİZ[4]

1 Izmir Katip Celebi University, Faculty of Pharmacy, Department of Biochemistry, İzmir, Turkey
2 Ege University, Faculty of Medicine, Department of Histology and Embryology, İzmir, Turkey
3 Ege University, Faculty of Pharmacy, Department of Biochemistry, İzmir, Turkey
4 Izmir Katip Celebi University, Faculty of Medicine, Department of Pharmacology, İzmir, Turkey

Geliş / Received: 06/06/2022, Kabul / Accepted: 10/08/2022

Abstract

The aim of this study was to investigate the effects of prenatal stress (PS) on mRNA levels of DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) in cerebral cortex and hippocampus of female rats. PS was induced in rats with dexamethasone (Dex). From gestation day 14 to 21, pregnant rats were injected daily with Dex (100 µg/kg) or saline. After birth, at 3 months of age, female rats were decapitated (n=5). For the first time, effects of Dex (100 µg/kg) on epigenetic enzymes were searched by real-time PCR through mRNA levels of DNMT1, DNMT3a, DNMT3b, HDAC1 and HDAC2 in female rats. Statistical significant differences were determined with one-way analysis of variance. Prenatal Dex exposure caused significant increases in DNMT3a, HDAC1 and HDAC2 mRNA levels in cortex and hippocampus. We further found that DNMT3b mRNA levels significantly increased in hippocampus but decreased in cortex of Dex group. No significant differences were found in DNMT1 mRNA levels. It was concluded that PS may trigger dysregulation of epigenetic mechanisms in cortex and hippocampus of female rats through alterations in gene expression profiles of DNMT3a, DNMT3b, HDAC1 and HDAC2.

Keywords: Prenatal stress, dexamethasone, DNA methyltransferase, histone deacetylase, female rat

Dişi Sıçanlarda Prenatal Stresin DNA Metiltransferazların ve Histon Deasetilazların Kortikal ve Hipokampal Gen Ekspresyon Profilleri Üzerindeki Etkileri

Öz

Çalışmanın amacı prenatal stresin (PS) dişi sıçanların serebral korteks ve hipokampüsünde DNA metiltransferazlar (DNMT) ve histon deasetilazların (HDAC) mRNA düzeylerine etkilerini araştırmaktır. PS sıçanlarda deksametazonla (Dex) indüklendi. Gebeliğin 14. gününden 21. gününe, gebe sıçanlara Dex (100 µg/kg) veya salin enjekte edildi. Doğumdan sonra 3 aylıkken dişi sıçanlar (n=5) dekapite edildi. İlk kez dişi sıçanlarda Dex'in (100 µg/kg) epigenetik enzimler üzerindeki etkileri gerçek zamanlı PCR ve DNMT1, DNMT3a, DNMT3b, HDAC1, HDAC2 mRNA düzeyleriyle araştırıldı. İstatistiksel farklılıklar tek yönlü varyans analiziyle yapıldı. Prenatal Dex maruziyeti korteks ve hipokampüste DNMT3a, HDAC1 ve HDAC2 mRNA düzeylerinde anlamlı artışa neden oldu. Ayrıca Dex grubunda DNMT3b mRNA düzeylerinin hipokampüste anlamlı şekilde artarken kortekste azaldığı bulundu. DNMT1 mRNA düzeylerinde anlamlı bir farklılık bulunmadı. PS'in dişi sıçanların korteks ve hipokampüsünde; DNMT3a, DNMT3b, HDAC1 ve HDAC2 genlerinin ekspresyon profillerindeki değişiklikler yoluyla epigenetik mekanizmaların düzensizliğini tetikleyebileceği sonucuna varıldı.

Anahtar Kelimeler: Prenatal stres, deksametazon, DNA metiltransferaz, histon deasetilaz, dişi sıçan

***Corresponding Author:** ezgi.turunc@ikcu.edu.tr
Ezgi TURUNÇ, https://orcid.org/0000-0002-7587-7443
Yiğit UYANIKGİL, https://orcid.org/0000-0002-4016-0522
Ayfer YALÇIN, https://orcid.org/0000-0003-0407-3218
Tijen KAYA-TEMİZ, https://orcid.org/0000-0002-0069-6576

1. Introduction

Prenatal stress has been shown to affect health and cognitive function negatively in animals and humans [1]. The exposure to stressors during early life may have negative effects on behaviors and physiological functions, including metabolism, growth, and inflammatory response [2-4]. Stress related outcomes may be affected by age, gender, species, type, and duration of stress [5]. Prenatal stress has been proposed to interact with genetics as well as epigenetics to alter the risk for various diseases such as hypertension, type 2 diabetes mellitus, cardiovascular problems, depression, and anxiety disorder [6-9].

During the perinatal period, the brain is very sensitive to stress factors as its plasticity is high. The prenatal environment is hypothesized to alter the expression of genes during neurodevelopment and throughout life. The differences in gene expression are thought to be caused by changes in the epigenome through DNA methylation or histone modifications [10]. The long-term effects of early life stress may be related to epigenetic mechanisms that lead to alterations in gene expression patterns during the embryonic period and later life [5]. Inheritable modifications in the function and expression of the genes without changes in the DNA sequence are included in the research field of epigenetics. Non-coding RNAs, histone and DNA modifications are some of the epigenetic mechanisms which exhibit reversible and dynamic patterns [11]. Post-translational modifications including acetylation and methylation can alter histone proteins [1]. Histone acetyltransferases (HATs) are responsible for the addition of acetyl groups, whereas histone deacetylases (HDACs) remove acetyl groups [12]. DNA methylation represses transcription and has an emerging role in gene regulation and development. DNA methyltransferase enzymes (DNMT1, 2, 3a, 3b and 3l) catalyze the addition of methyl groups to DNA. DNMT1 is a maintenance methyltransferase, while DNMT3 proteins are *de novo* methyltransferases [13]. Increasing evidence suggests that epigenetic regulation during early life can have intense effects on neurodevelopment and brain function. However, the relationship between prenatal stress and its lifelong effects has not been fully elucidated. The long-term effects of prenatal stress may be mediated by epigenetic mechanisms, which are also powerful therapeutic strategies for various neurodevelopmental and neurodegenerative disorders [14-16]. Epigenetic alterations may be passed from one generation to the next through behavioral intervention or fetal programming, which can be influenced by stress [17,18]. Clarifying the epigenetic mechanisms concerning stress response regulation may help to understand the fetal and developmental programming of the hypothalamic–pituitary–adrenal (HPA) axis.

The hypothalamus is highly interconnected with the frontal cortex and hippocampus, which regulate the functioning of the HPA axis actively [19]. Fetal glucocorticoid exposure has been widely used as a prenatal stress model in animals by the administration of glucocorticoids or synthetic corticosteroid analogues such as dexamethasone (Dex) to pregnant animals [20-22]. The neural circuits in the developing brain may be affected by glucocorticoids, which have long-term effects on brain architecture and neuroplasticity, and these effects may differ depending on gender [5,23]. The female offspring have been shown to be more sensitive to early life stressors, which can be caused by increased adrenal sensitivity in females. At the same time, females are found to be more likely than males to suffer from neuropsychiatric problems

and stress [23-25]. Thus, we used female rats for our prenatal stress model. For the first time, the impacts of prenatal Dex exposure at a dose of 100 μg/kg during the last week of gestation on epigenetic enzymes were investigated in the brains of female offspring. The present study examined the effects of Dex induced prenatal stress on epigenetic mechanisms in the cerebral cortex and hippocampus of female rats through alterations in gene expression profiles of histone deacetylases (HDAC1, HDAC2) and DNA methyltransferases (DNMT1, DNMT3a, DNMT3b).

2. Materials and Methods

2.1. Prenatal Stress Model:

In vivo experiments were carried out at Ege University Laboratory Animals Application and Research Center. Six time-mated nulliparous female Sprague-Dawley rats, weighing 200-220 g, were randomly housed in plastic cages under standard 12h light and dark cycle in a room at 22 ± 3°C and were given continuous access to food and water. Vaginal smears were taken daily for the detection of the estrus cycle. Female rats in proestrus were coupled with males. Pregnancy was confirmed by the sperm presence in the vaginal smear [26].

Prenatal stress was induced in rats with Dex (synthetic glucocorticoid). From gestation day (GD) 14 to GD21, pregnant rats were daily injected with Dex at a dose of 100 μg/kg s.c. (Dex group) or saline (control group). After the termination of exposures, the rats were housed individually. Litters were left with their dam throughout rearing and they were not culled. All offspring were weaned on postnatal day (PD) 21 and put in social groups of the same treatment and sex. At 3 months of age (PD90), female rats were decapitated (n=5). Cerebral cortical and hippocampal tissues were dissected immediately on ice [20]. Tissue samples were placed in a storage tank with liquid nitrogen and transferred to Izmir Katip Celebi University Faculty of Pharmacy and stored at −80°C.

2.2. Total RNA Isolation, Reverse Transcription and Real-time PCR

Gene expression studies were conducted at Izmir Katip Celebi University Faculty of Pharmacy Research Laboratory-1. Total RNA was isolated from cortical and hippocampal samples with the MasterPure Purification Kit (Epicentre Bioctechnologies, Cat. No. MC85200) according to the manufacturer's protocol. 1 μg of total RNA was used for synthesis of cDNA with the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Cat. No. K1622).

Real-time PCR amplifications were carried out with SYBR Green I reagent and the AriaMx Real-time PCR System (Agilent Technologies, California, USA). 1 μL of cDNA, 1 μL of 250 nM primers, 7 μL of nuclease-free water were amplified with 10 μL of Master Mix containing SYBR Green I. Primer-BLAST was used for primer design [27]. The specific forward and reverse primers for DNMT1, DNMT3a, DNMT3b, HDAC1, HDAC2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were listed in Table 1.

The amplification protocol included an initial melting step for 3 min at 95°C; 40 cycles of melting steps for 5 s at 95°C; annealing and elongation steps for 10 s at 60°C. At the end of the

amplification, dissociation curve analysis was performed to determine the purity of PCR products. We assessed the relative mRNA levels using Agilent software with the comparative C_T ($2^{-\Delta\Delta CT}$) method and normalized to GAPDH [28].

Table 1. Specific primers of DNMT1, DNMT3a, DNMT3b, HDAC1, HDAC2 and GAPDH for real-time PCR.

Primer:	GenBank No:	Primer Sequence:
DNMT1	NM_053354.3	F: 5'-GAGGCACTGTCCGTCTTTGA-3' R: 5'-AAGTGACCGCGACTGCAATA-3'
DNMT3A	NM_001003958.1	F: 5'-ACGATAATACCTTCTCTGAAGCCC-3' R: 5'-CTTCCTTTCGATCATCCTCCCG-3'
DNMT3B	NM_001003959.1	F: 5'-GATGAGGAGAGCCGAGAACG-3' R: 5'-CAGAGCCCACCCTCAAAGAG-3'
HDAC1	NM_001025409.1	F: 5'-CTCCATCTTCTCTCCAAGTCCC-3' R: 5'-GAGTTCTCCCAGTACCACTGC-3'
HDAC2	NM_053447.1	F: 5'-GGCCTCAGGATTCTGCTACG-3' R: 5'-CGGTCATCACGCGATCTGTT-3'
GAPDH	NM_017008.4	F: 5'-AGTGCCAGCCTCGTCTCATA-3' R: 5'-AACTTGCCGTGGGTAGAGTC-3'

2.3. Statistical Analysis

Statistical Package for the Social Sciences for Windows (SPSS version 25.0) was used for statistical analysis. The statistical significant differences were determined with one-way analysis of variance. Tukey's test was used for post-hoc analysis. Data were representative of three independent experiments, and values were given as mean ± standard error. Statistically significiant p level was determined as <0.05.

3. Results and Discussion

3.1. Effects of Prenatal Stress on mRNA Expression Levels of DNA Methyltransferases

To investigate the effects of prenatal stress on mRNA expression levels of DNA methyltransferases, we measured relative DNMT1, DNMT3a, and DNMT3b mRNA levels in cortical and hippocampal samples of control and Dex groups (Fig. 1, 3, 5). Figure 1 displays the relative expression of DNMT1 in cortical and hippocampal samples of experimental groups.

Relative DNMT1 mRNA levels in cortical samples of the control and Dex groups were 1.002 ± 0.030 and 1.126 ± 0.072, respectively. In hippocampal samples, relative DNMT1 mRNA levels were found to be 1.009 ± 0.077 and 1.100 ± 0.014 in control and Dex groups, respectively. There were no significant differences in DNMT1 mRNA levels between the experimental groups (Fig. 1). The Tm value for DNMT1 was found to be 80.5°C (Fig. 2).

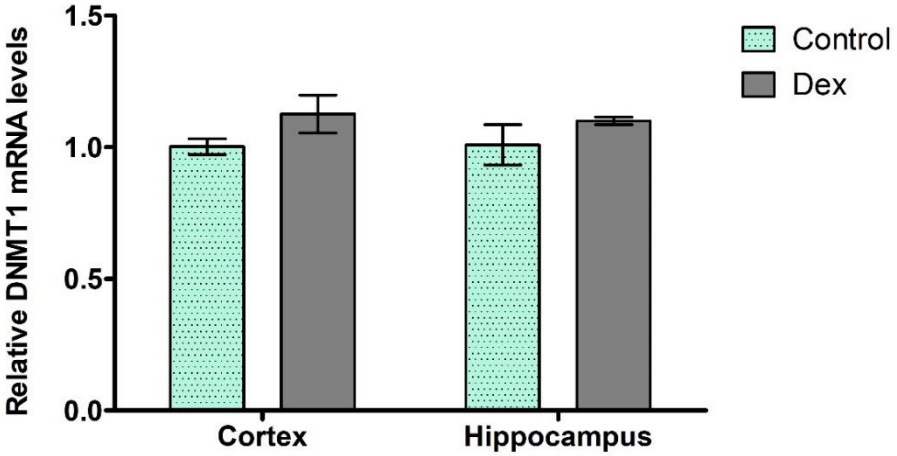

Figure 1. Effects of dexamethasone treatment on DNMT1 mRNA expressions in cortex and hippocampus of rats. The relative DNMT1 mRNA levels were assessed with comparative C_T method ($2^{-\Delta\Delta CT}$) and normalized to GAPDH. The results were given mean ± S.E.; n=5 in each group.

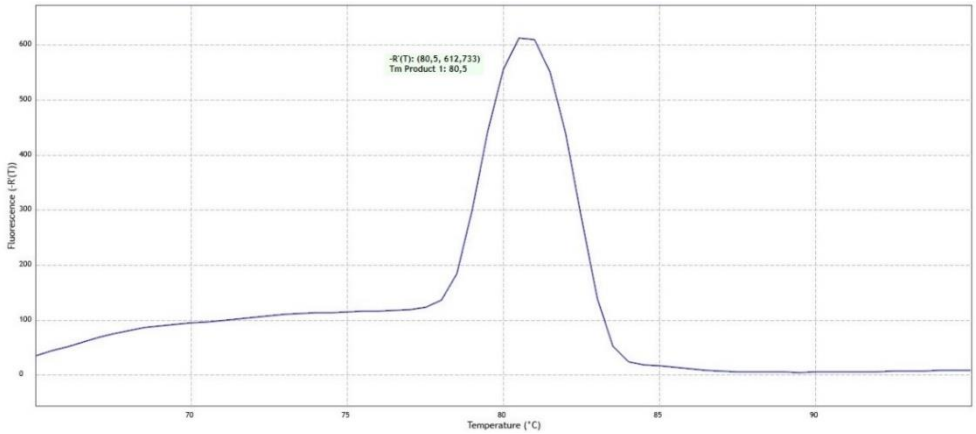

Figure 2. Melting curve analysis of DNMT1 real-time PCR product. The Tm value of DNMT1 was found to be 80.5°C.

Figure 3 displays the relative expression of DNMT3a in cortical and hippocampal samples of experimental groups. Relative mRNA levels of DNMT3a in cortical samples of the control and Dex groups were 1.000 ± 0.030 and 1.669 ± 0.076, respectively. In hippocampal samples, relative DNMT3a mRNA levels were found to be 0.999 ± 0.026 and 1.662 ± 0.104 in control and Dex groups, respectively. In the Dex group, DNMT3a mRNA levels in both cortex and hippocampus were found to be significantly increased when compared to control (Fig. 3, $^{a,b}p<0.001$). The Tm value for DNMT3a was found to be 89.0°C (Fig. 4).

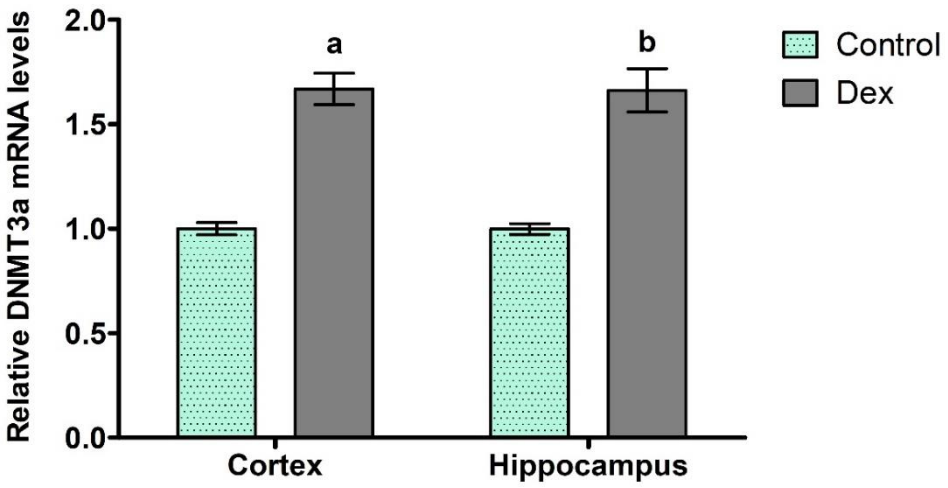

Figure 3. Effects of dexamethasone treatment on DNMT3a mRNA expressions in cortex and hippocampus of rats. The relative DNMT3a mRNA levels were assessed with comparative C_T method ($2^{-\Delta\Delta CT}$) and normalized to GAPDH. The results were given mean ± S.E.; n=5 in each group. $^{a}p<0.001$ versus control group in cortex, $^{b}p<0.001$ vs control group in hippocampus.

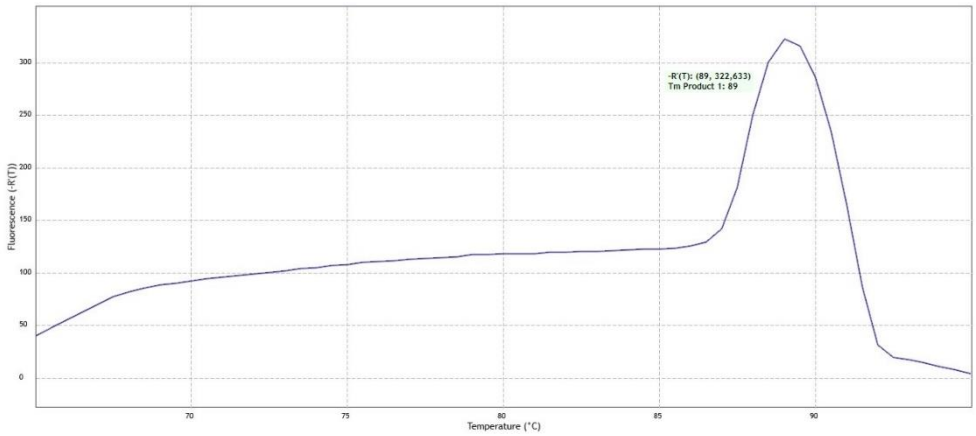

Figure 4. Melting curve analysis of DNMT3a real-time PCR product. The Tm value of DNMT3a was found to be 89.0°C.

Figure 5 displays the relative expression of DNMT3b in cortical and hippocampal samples of experimental groups. Relative mRNA levels of DNMT3b in cortical and hippocampal samples of control group were 1.003 ± 0.027 and 1.000 ± 0.009, respectively. In the Dex group, relative DNMT3b levels in cortical and hippocampal samples were 0.795 ± 0.024 and 1.685 ± 0.084, respectively (Fig. 5). It was found that DNMT3b levels of the Dex group decreased significantly in the cortex ($^{a}p<0.03$) but increased in the hippocampus compared to control ($^{b}p<0.001$). DNMT3b expression levels differed significantly between cortex and hippocampus in the Dex group ($^{c}p<0.001$). The Tm value for DNMT3b was found to be 83.5°C (Fig. 6).

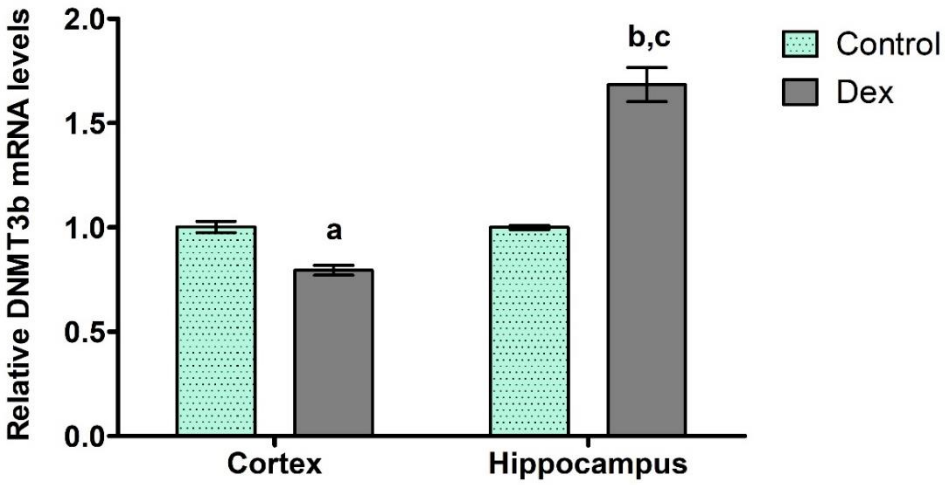

Figure 5. Effects of dexamethasone treatment on DNMT3b mRNA expressions in cortex and hippocampus of rats. The relative DNMT3b mRNA levels were assessed with comparative C_T method ($2^{-\Delta\Delta CT}$) and normalized to GAPDH. The results were given mean ± S.E.; n=5 in each group. $^{a}p<0.03$ vs control group in cortex, $^{b}p<0.001$ DNMT3b vs control group in hippocampus, $^{c}p<0.001$ vs Dex group in cortex.

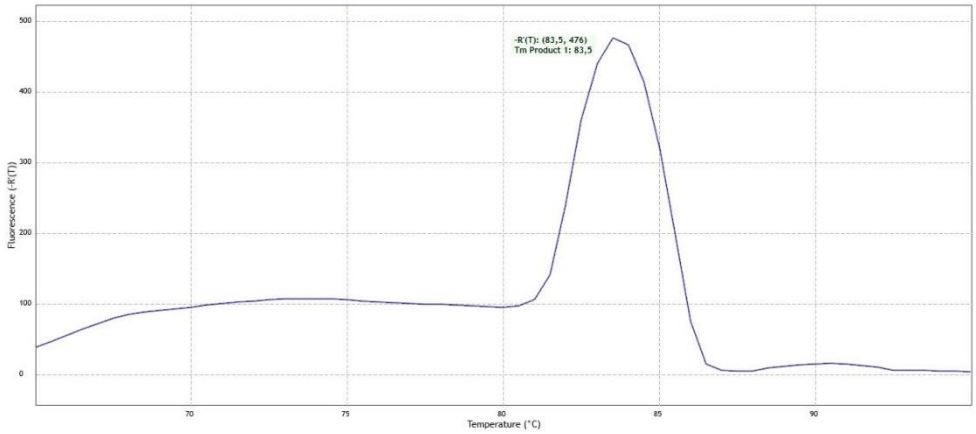

Figure 6. Melting curve analysis of DNMT3b real-time PCR product. The Tm value of DNMT3b was found to be 83.5°C.

3.2. Effects of Prenatal Stress on mRNA Expression Levels of Histone Deacetylases

To investigate the effects of prenatal stress on mRNA expression levels of histone deacetylases, we measured relative HDAC1 and HDAC2 mRNA levels in cortical and hippocampal samples of control and Dex groups (Fig. 7, 9). Figure 7 displays the relative expression of HDAC1 in cortical and hippocampal samples of experimental groups. Relative mRNA levels of HDAC1 in cortical and hippocampal samples of the control group were 1.000 ± 0.015 and 1.002 ± 0.034, respectively. In the Dex group, relative HDAC1 levels in cortical and hippocampal samples were 1.466 ± 0.063 and 1.308 ± 0.045, respectively. Prenatal Dex exposure caused significant increases in both cortical and hippocampal HDAC1 mRNA levels when compared to control (Fig. 7, $^{a}p\leq0.001$, $^{b}p<0.001$). The Tm value for HDAC1 was found to be 79.5°C (Fig. 8).

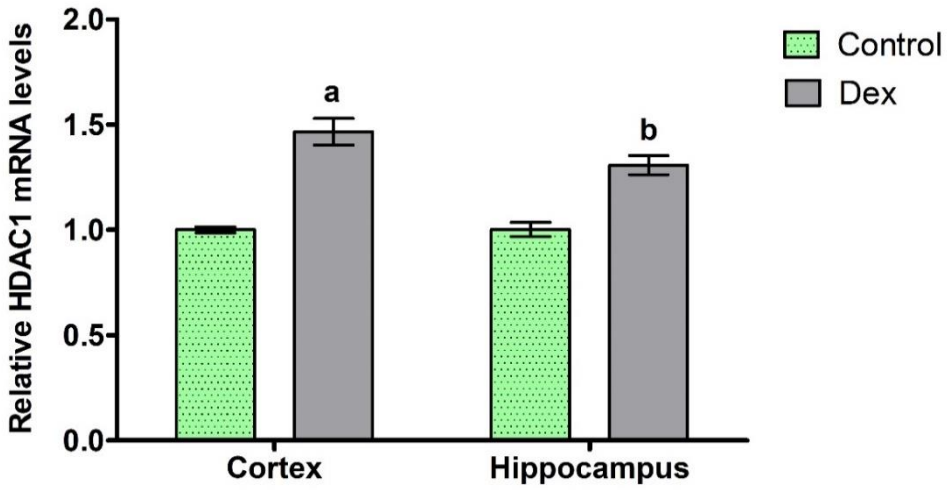

Figure 7. Effects of dexamethasone treatment on HDAC1 mRNA expression in cortex and hippocampus of rats. The relative HDAC1 mRNA levels were assessed with comparative C_T method ($2^{-\Delta\Delta CT}$) and normalized to GAPDH. The results were given mean ± S.E.; n=5 in each group. $^{a}p<0.001$ versus control group in cortex, $^{b}p<0.001$ vs control group in hippocampus.

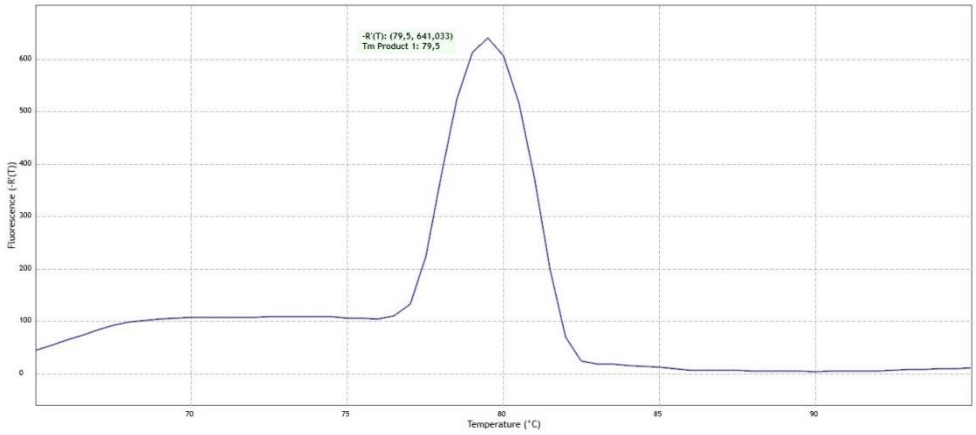

Figure 8. **Melting curve analysis of HDAC1 real-time PCR product.** The Tm value of HDAC1 was found to be 79.5°C.

Figure 9 displays the relative expression of HDAC2 in cortical and hippocampal samples of experimental groups. Relative mRNA levels of HDAC2 in cortical samples of the control and Dex groups were 1.001 ± 0.030 and 1.412 ± 0.052, respectively. In hippocampal samples, relative HDAC2 mRNA levels were found to be 1.003 ± 0.043 and 1.268 ± 0.059 in control and Dex groups, respectively. HDAC2 mRNA levels were significantly elevated in cortical and hippocampal samples of the Dex group when compared to control (Fig. 9, $^{a}p<0.001$ and $^{b}p<0.01$). The Tm value for HDAC2 was found to be 81.0°C (Fig.10).

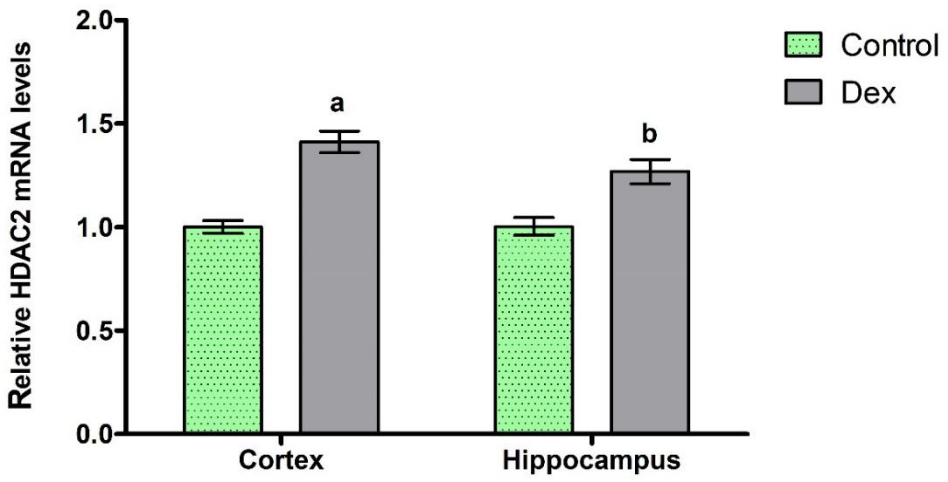

Figure 9. Effects of dexamethasone treatment on HDAC2 mRNA expression in cortex and hippocampus of rats. The relative HDAC2 mRNA levels were assessed with comparative C_T method ($2^{-\Delta\Delta CT}$) and normalized to GAPDH. The results were given mean ± S.E.; n=5 in each group. $^{a}p<0.001$ versus control group in cortex, $^{b}p<0.001$ vs control group in hippocampus.

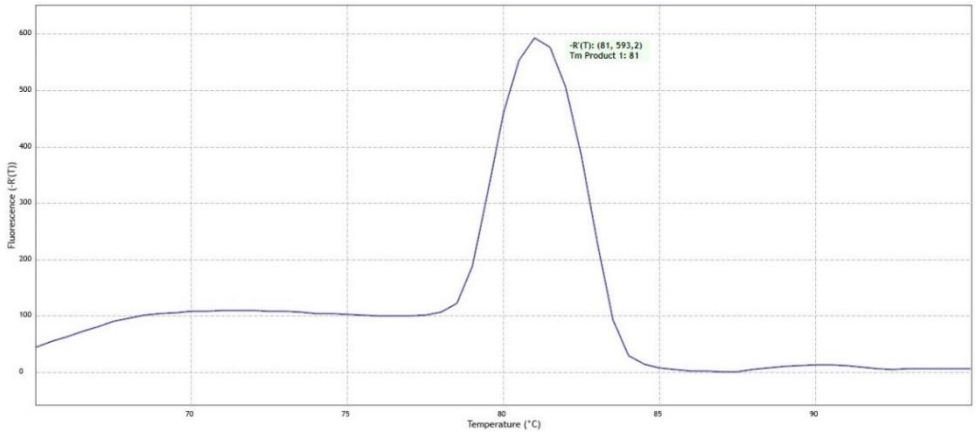

Figure 10. Melting curve analysis of HDAC2 real-time PCR product. The Tm value of HDAC2 was found to be 81°C.

The fact that we did not separate neurons and glia cells and the low number of rats in the groups may have reduced the effectiveness of our study in detecting significant differences in gene expression profiles between control and Dex groups. Additionally, we were not able to analyze protein levels of DNMTs and HDACs. Understanding whether PS-induced epigenetic changes occur in either neurons or glia or in both would increase our knowledge about the effects of PS related to epigenetic factors. Further studies, including protein quantification and microdissection techniques, are required for a more refined analysis. In our study, PS resulted in increased DNMT3a, HDAC1 and HDAC2 mRNA levels in the cortex and hippocampus of female rats. Furthermore, prenatal Dex exposure exhibited increased hippocampal DNMT3b mRNA levels, whereas cortical gene expression levels of DNMT3b were found to be decreased. There were no significant differences in DNMT1 gene expression levels in both cortex and hippocampus between the control and Dex groups.

The study by Benoit et al. indicated that chronic unpredictable stress induced PS impaired spatial memory in male and female mice, as well as prenatally stressed females had higher corticosterone and DNMT1 levels and less histone H3 acetylation than males. As a result, it can be concluded that female brain may be more sensitive to the effects of PS [1]. In the study by Boersma et al., PS was induced by a variable stress paradigm (cold exposure, social, restraint and and swim stress) during the third week of gestation and the expression levels of DNMT1 and 3a were found to be increased in amygdala and hippocampus of prenatally stressed male rats [10]. Lei and colleagues showed increased hippocampal DNMT1 and DNMT3a protein levels in female rats prenatally exposed to restraint stress, whereas no differences were found in the protein levels of DNMT1 and DNMT3a in prenatally stressed male rats compared to control. They also reported that prenatal stress induced neurodevelopmental abnormalities in female rats are DNA methylation-dependent [5]. Similar to ours, Lui and and colleagues induced PS with Dex at dose of 100 μg/kg from GD14 to GD21, whereas male offspring were used in their study. They reported increased hippocampal DNMT1 mRNA levels in Dex group [22]. In contrast, we did not find any significant differences in cortical and hippocampal DNMT1 mRNA levels of Dex group. Grégoire and colleagues showed that prenatal restraint stress caused sex-specific alterations in mRNA expression of epigenetic- and stress-related genes in cortex and hippocampus. In their study, male mice exposed to prenatal restraint stress showed increased hippocampal DNMT1 but decreased hippocampal DNMT3b and cortical HDAC1 expression. They also reported no differences were found in expression of DNMTs and HDACs between untreated and prenatally stressed female mice [29]. In the study by Zheng et al., PS was induced with combination of 24-hour light disturbance and restraint stress to pregnant mice during gestation. Prenatally stressed dams showed depressive- and anxiety-like behaviors. Male mice exposed to PS exhibited attenuated expression of brain-derived neurotrophic factor (BDNF), elevated expressions of DNMT1, HDAC1, and HDAC2 in the hippocampus as compared to control group [9]. Our findings support previously reported study describing increased HDAC2 expression in the hippocampus as a result of Dex exposure. In the study by Huang et al., female rats received prenatal Dex exhibited increased hippocampal glucocorticoid receptors (GR) and HDAC2 expressions, whereas expression of BDNF was found to be decreased. They further observed that 0.5 μM Dex treatment to the fetal hippocampal neuron cells (H19-7) caused alterations in the GR-HDAC2-BDNF pathway and administration of RU486 (GR antagonist) reversed the increase in HDAC2 [23]. Growing evidence suggests that stress-induced long-term effects are mediated by epigenetic factors. Changes in histone modifications and DNA methylation have been detected in response to prenatal stress. Moreover, epigenetic mechanisms have become important therapeutic targets in the diagnosis and treatment in various neurodevelopmental and neurodegenerative diseases [14-16].

4. Conclusion

Detailed investigation for mRNA and protein levels of DNMTs and HDACs throughout development in control and prenatally stressed rats is necessary to determine epigenetic changes. Nevertheless, our data suggest that prenatal Dex exposure induced alterations in mRNA expression levels of DNMT3a, DNMT3b, HDAC1 and HDAC2 in the cortex and

hippocampus of female rats. As a conclusion, our findings highlight the impact of prenatal stress on epigenetic enzymes involved in DNA methylation and histone deacetylation in female rats and suggest that prenatal Dex exposure may trigger dysregulation of epigenetic processes through alterations in cortical and hippocampal gene expression profiles of DNMT3a, DNMT3b, HDAC1 and HDAC2. Overall, to our knowledge, this study shows for the first time that prenatal Dex (100 μg/kg) exposure during the last week of gestation caused changes in gene expression profiles of DNA methyltransferases and histone deacetylases in cortex and hippocampus of female rats and our results will provide experimental contributions for elucidating neurodevelopmental effects of prenatal stress on epigenetic programing.

Ethics in Publishing

This study was approved by the Utilization Committee of Ege University (Ref. No:2017-023).

Author Contributions

ET: contributed to the design, sample and data collection, performed the prenatal stress model and gene expression analyses and edited the manuscript; YU: performed the decapitations and dissections, contributed to sample collection; TT and AY: reviewed the manuscript.

Acknowledgements

This study was supported by a grant of İzmir Katip Çelebi University (2018-ONAP-ECZF-0001).

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