Mol Neurobiol
DOI 10.1007/s12035-016-0143-5

Effect of Maternal Stress Prior to Conception on Hippocampal BDNF Signaling in Rat Offspring

Somayeh Niknazar[1,2] · Arezo Nahavandi[2] · Ali Asghar Peyvandi[1] · Hassan Peyvandi[1] · Fatemeh Zare Mehrjerdi[3] · Mohsen Karimi[4]

Received: 15 June 2016 / Accepted: 19 September 2016

Abstract Environmental factors, especially stress, can remain pervasive effects across the lifespan. Traumatic experiences are risk factors for the behavioral and emotional disorders. Since brain-derived neurotrophic factor (BDNF) is the important regulator of neural survival, development, and its genetic and epigenetic alterations which have been linked with several neuropsychiatric disorders, the present study investigated the effect of maternal adulthood stress on molecular changes of BDNF and tyrosine kinase-coupled receptor (TrkB) in the hippocampus of 30-day-old offspring. To induce stress, we employed a repeated forced swimming model for female rats across 21 days. Then, they were divided into two parental breeding groups: stressed mother (SM) and non-stressed mother (NSM) or control group. Anxiety-like behavior was tested in adult female rats and 30-day-old pups by using the elevated plus maze (EPM). The level of serum corticosterone was also measured by ELISA. BDNF and TrkB gene methylation and protein expression in the hippocampus were detected using real-time PCR and Western blotting in all groups. Thirty-day-old male and female pups from SM groups had a significantly more serum corticosterone concentration, DNA methylation levels of BDNF and TrKB, and lower expression of these genes compared to pups from the control groups. Also, male pups from stressed mother exhibited significant anxiety-like behavior compared to male pups from the control mothers. These findings suggest that molecular changes formed by maternal stress experience even before conception persist to the next generation and will negatively influence on phenotypes of offspring.

Keywords Forced swim stress · Maternal stress · Offspring · Anxiety · Corticosterone · DNA methylation · BDNF and TrKB

Introduction

Stress is one of the most common words of modern life, used widely to describe different types of situations, events, and emotions [1]. Stress susceptibility can be transmitted to the next generation. The maternal stress effect on next generation was regarded. Several studies have been shown that maternal stress during pregnancy and early life period influences offspring in the behavioral, genetic, and epigenetic aspects in human and animal models [2–5]. In this study, we investigated DNA methylation of brain-derived neurotrophic factor (BDNF) and tyrosine kinase-coupled receptor (TrkB) genes in pups' hippocampus following maternal stress induction prior to conception. DNA methylation causes long-term changes in gene expression that tends to silence genes [6]. In exposure to a stressor, high levels of glucocorticoids can reduce the expression of BDNF [7, 8]. In animal studies, maternal stress during pregnancy leads to weight loss as well as increased hypothalamic-pituitary-adrenal (HPA) axis activity, anxiety, sleep disorders and mortality, learning and memory dysfunction, and depressive-like behaviors in offspring [9–11]. Stress in pregnant mice in the last week of gestation was disrupted

✉ Arezo Nahavandi
niknazar.somayeh@gmail.com; arezo_nahavandi@yahoo.com

[1] Hearing Disorders Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[2] Neuroscience Research Center, Department of Physiology, Iran University of Medical Sciences, Tehran, Iran

[3] Neurobiomedical Research Center, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

[4] Biotechnology Research center, Department of Molecular Medicine, Pasteur Institute of Iran, Tehran, Iran

Published online: 10 October 2016

memory and spatial learning in children that is associated with inhibition of neurogenesis in the hippocampus [12]. In addition, inappropriate behavior of mothers with offspring in the early life period leads to permanent changes in DNA methylation of the BDNF in the prefrontal cortex in adulthood [13]. BDNF expression in response to acute stress in infancy creates a persistent functional impairment. Exclusion of mice pups from the mother for 24 h on the ninth day of birth leads to decreased levels of BDNF in the hippocampus in adulthood. [14] In human, exposure of pregnant women with physical and mental stress can affect children's learning and mood disorders [15, 16]. It is well known that most of the BDNF effects are mediated through its high-affinity TrkB receptor [17]. It has been reported that removal of TrkB gene in neurons of mice pups increased anxiety-like behavior in adulthood [18], while mice with over-expression of this receptor show weak anxiety-like behavior [19]. Based on the abovementioned data, it is required to investigate whether maternal stressful adulthood life before conception can cause molecular changes of BDNF and TrkB in the hippocampus of their offspring.

Materials and Methods

Animals

Male and female Wistar rats with 3 months of age and average weight of 200–250 g were obtained from the Animal Experiment Center of Iran Medical University. Animals were housed under a steady temperature (21 ± 1 °C) and 12:12-h light/dark cycle. In this study, female rats were randomly divided into two groups: the repeated swim stress (RSS) group (n = 20) and the control group (n = 20). Male rats (n = 10) received standard no-stress treatment.

Stress Procedure

Animals were placed in 25 °C water in a plexiglas cylindrical container (70 × 40 × 80 cm) and 35 cm height of water and forced for 10 min daily at 08:30 a.m. for 21 consecutive days. In this study, unstressed animals which were habituated by daily handling were decapitated at the same time [20].

Elevated plus Maze

Elevated plus maze (EPM) is used for behavioral assay for rodents, and it is validated for assessment of anxiety-related behavior. The EPM apparatus includes four elevated arms above the floor, organized in two contrary closed arms, two contrary open arms, and a center area. Rats were located in the central section of the four arms of the EPM, and their behaviors were taped for 5 min. To measure anxiety-like behavior, all stressed animals were tested in the EPM on days 0 (before) and 21 of the repeated swimming test. Open arm entries (100 × open / total entries) and time spent (100 × open / (open + enclosed)) percentage were scored to measure anxiety-like behavior.

Corticosterone Assay

Twenty-four hours after the final swim stress, blood samples were obtained from adult rats (stressed and unstressed) to assess the corticosterone level. Samples were collected by intracardiac puncture between 10:00 and 11:00 a.m. and were centrifuged at 4 °C (at 3000 rpm for 15 min) to separate the serum. One male and one female pup from each mother (six male and six female pups from each breeding group) were subjected to blood sampling for corticosterone measurement on postnatal days 30. Serum samples were stored at −20 °C. Corticosterone levels were measured by ELISA kit (ALPCO Diagnostics, USA).

Tissue Preparation

Half of female rats were decapitated 24 h after the end of the phase of stress and the brains immediately removed. The entire hippocampus was dissected in a cold Petri dish, frozen in liquid nitrogen, and stored at −80 °C until used for the real-time PCR and Western blotting tests.

Breeding and Experimental Design

The rest of the animals formed breeding groups. Each breeding group of one male and two female rats were organized as a group of stressed females (n = 10) and non-stressed males (n = 5) (stressed mother group, SM) and a group in which both males (n = 5) and females (n = 10) received standard no-stress treatment (non-stressed mother group, NSM, or control group). After mating (10–15 consecutive days), males were removed. Each female rat was housed individually near the parturition time. For litter-size standardization, dams with approximately equal number of pups were selected. In each group, dams with 8–10 pups formed the highest percentage of dams with equal number of pups. Final sample size for each of the breeding groups was six dams. Pups were weaned from the dams after 4 weeks. Fathers never had any contact with the offspring.

DNA Methylation Analysis Using Methylation Sensitive Restriction Enzymes

Restriction endonucleases diagnose specific target sequences and cleave the DNA in the specified site. The cutting activity of many restriction enzymes relates to modifications of the DNA in their recognition sequence. If an enzyme is sensitive to the methylation of a cytosine residue in a CpG dinucleotide in its target sequence, it can be used for methylation analysis [21]. We used the isoschizomers HpaII (methylation-sensitive) and MspI (methylation-insensitive), which recognize the same sequences (the restriction site CCGG), with different sensitivity to methylation of the recognition site [22].

DNA Isolation

Frozen hippocampus samples were homogenized, and genomic DNA was isolated according to the manufacturer's instructions (E0008 KogeneBiotech, Korean). Purity of DNA was assessed by the ratio of optical density 260/280 nm. Genomic DNA was digested by HpaII and MspI restriction enzymes in separate reactions. Briefly, 1 μg of genomic DNA was diluted in 50 μl NEBuffer1 (New England Biolabs, MA, USA). Diluted DNA was divided into three aliquots, which were digested by HpaII (New England Biolabs, MA, USA) or MspI (New England Biolabs, MA, USA) and undigested (to serve as the background control). Digestion mixtures were incubated at 37 °C for 2 h and then stored at −20 °C [23].

Real-Time PCR

The real-time PCR reactions were performed using a StepOne™ Plus system (PE, Applied Biosystems, CA, USA). Quantitative real-time PCR was used to detect the DNA methylation status of the BDNF and TrkB promoter. Detection of methylated DNA in the BDNF exon was performed using the following primers: forward CpG island (5′_AAG ACT GCA GTG GAC ATG TCC _3′) and reverse (5′_CCT TCG TGT AAC CCA TGG GAT_3′). Methylation of DNA in the TrkB promoter was detected by the following primers: forward (5′ _TTC AGC TGC TGT TGC TGC TTC_3′) and reverse (5′_AGC AAC TGC GGT AGC AGG AC_3′). PCR parameters were 40 cycles at 94 °C for 15 s, 30 s at 63 °C for BDNF and 60 °C for TrkB, and 30 s at 72 °C. SYBR Green (PR081A, TaKaRa) PCR assays for each sample for each gene were performed in duplicate in 96-well optical plates. ΔΔCT for each sample was calculated using the following formula: ΔΔCT = ΔCT (MSPI-UD) − ΔCT (HpaII-UD) [23].

Protein Extraction

The hippocampal tissues were homogenized in a RIPA lysis buffer system (Santa Cruz Biotechnology, sc-24948, USA). The homogenates were centrifuged, supernatants were collected, and the total protein concentration of the supernatants was determined via Bradford's assay protocol [24], using bovine serum albumin as protein concentration reference.

Western Blotting

BDNF and TrkB protein levels were evaluated by Western blot. After electrophoresis (on 10 % polyacrylamide SDS gel), the separated proteins were blotted onto a nitrocellulose membrane. Non-specific binding sites were blocked in Tris-buffered saline (TBS) overnight at 4 °C, containing 0.1 % Tween-20 and anti-BDNF (AB, 6201, 1-1000; Abcam, Cambridge, UK) or anti-TrkB (AB, 33655, 1-1000; Abcam, Cambridge, UK), washed with 0.1 % Tween-20 in TBS (TBST) three times for 10 min, and probed with anti-goat IgG secondary antibodies (AB, 6721, 1-2000; Abcam, Cambridge, UK) for 2 h at room temperature. After rinsing with buffer, the immune complexes were visualized using chemiluminescence system ECL kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) according to the manufacturer's instructions and exposed to X-ray film. The film signals were scanned and then analyzed using TotalLab software (Nonlinear Dynamics Ltd., USA). Actin was used as an internal control.

Statistical Analysis

Data are presented as mean ± S.E., and statistical significance was analyzed by the Student t test. P values less than 0.05 were considered significant.

Results

Effect of Stress on Adult Female Rats in the EPM

EPM test was done 2 h after the final swim. In the EPM, the percentage of entries and time spent in the open arms was considered as anxiety-like behavior. For adult female rats, EPM test on the 21st day of the stress phase was compared to self-control groups (day 0). t Test analysis showed that after 21 days of the swimming phase, stressed female rats (n = 20) had lower entries ($P < 0.001$) and time spent ($P < 0.001$) in the open arms compared to the self-control groups (Fig. 1a, b).

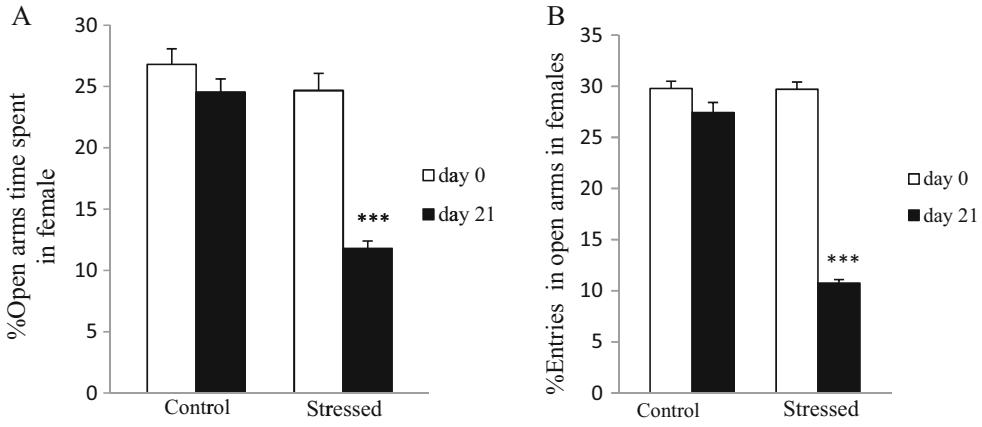

Fig. 1 Effect of chronic stress on behavioral parameters of adult female rats in the EPM (mean ± SEM). ***$P < 0.001$ compared to the self-control groups (day 0), (Student's *t* test)

Effect of Stress on the Corticosterone Levels in Adult Female Rats

After 21 days of repeated swim stress, serum corticosterone concentration was measured by ELISA in female rats. According to *t* test analysis, an increase in the level of serum corticosterone was detected in the adult stressed female ($n = 20$, $P < 0.001$) compared to the control females ($n = 20$), (Fig. 2).

DNA Methylation Analysis of BDNF and TrkB Gene in the Hippocampus of Adult Female Rats

Methylation analysis by *t* test showed that repeated swim stress significantly increases methylation in the targeted exon IV of BDNF ($n = 5$, $P < 0.001$) and TrkB ($n = 5$, $P < 0.01$) (Fig. 3) genes in adult female rats compared to non-stressed female rats ($n = 5$).

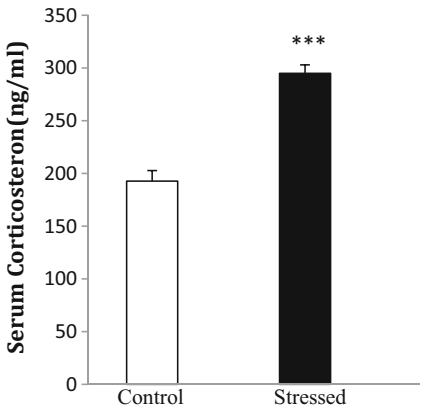

Fig. 2 Effect of chronic stress on serum corticosterone level in adult female rats (mean ± SEM). ***$P < 0.001$ compared to the control group (Student's *t* test)

Western Blot Analysis of BDNF and TrkB Protein Levels in the Hippocampus of Adult Female Rats

Protein samples were used from the hippocampus to assay changes in BDNF and TrkB expressions. Antibodies against BDNF recognized a single band on the blot at about 28 kDa. Antibodies against TrkB were recognized at 140 kDa. Results of densitometry showed that after the chronic stress induction phase, BDNF and TrkB protein expressions (***$P < 0.001$) in the hippocampus of female rats ($n = 5$) were decreased in comparison with the control group in adult female rats ($n = 5$) (Fig. 4).

Dam and Pup Characteristics

There were no significant differences in body weight between stressed (243.6 ± 1.47) and control (246.5 ± 0.67) dams. No significant differences were observed in litter size or pups' weight between stressed and control breeding groups.

Effect of the Maternal Stress on the 30-Day-Old Pups in the EPM

The EPM test was applied for the 30-day-old pups from each group. Our findings revealed that the 30-day-old male pups but not female pups of the SM ($n = 6$, $P < 0.05$) groups had lower percent of time spent in the open arms compared to the 30-day-old pups of the NSM ($n = 6$) group (Fig. 5a). No statically significant difference was detected in the percentage of entries into the open arms (Fig. 5b).

Effect of the Maternal Stress on the Pups' Serum Corticosterone

Using an ELISA assay, serum corticosterone was analyzed in 30-day-old pups ($n = 6$) from each group. According to *t* test

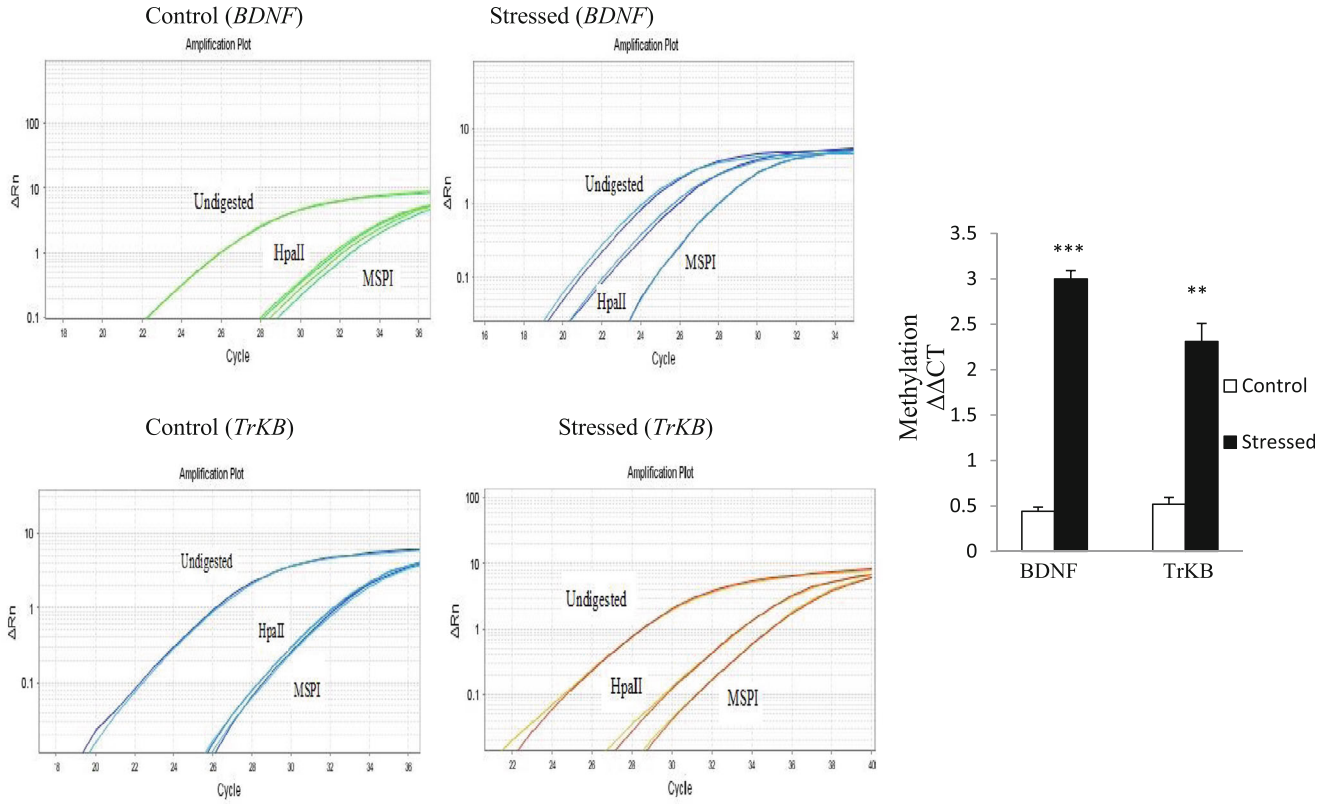

Fig. 3 Effect of chronic stress on BDNF and TrkB methylation in adult female rats (mean ± SEM). $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the control group (Student's *t* test)

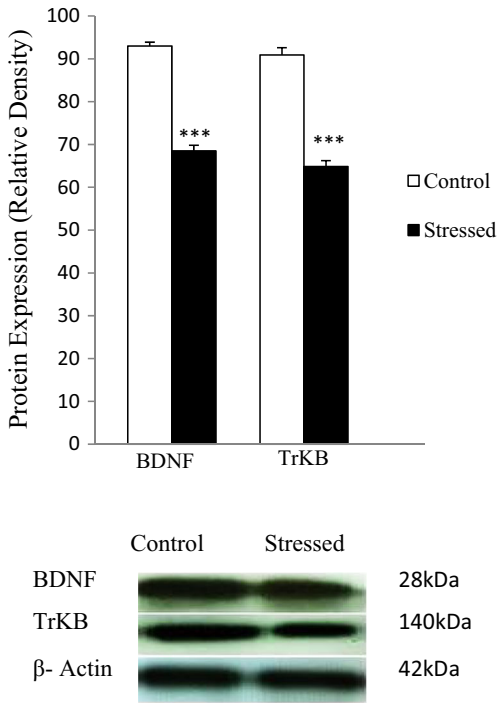

Fig. 4 Effects of chronic stress on BDNF and TrkB protein expression in adult female rats. $^{***}P < 0.001$ compared to the control group (Student's *t* test). β actin expression does not change in control and stressed rats (Student's *t* test)

analysis, serum corticosterone levels in 30-day-old male ($P < 0.001$) and female ($P < 0.001$) pups from the SM group were higher than in pups of the NSM group (Fig. 6).

Effect of the Maternal Stress on the Pups' Hippocampal BDNF and TrkB DNA Methylation

Figure 7 displays the percentage of DNA methylation of the BDNF exon IV region. Methylation of BDNF exon IV was different in control and stressed offspring in the hippocampus. According to *t* test analysis, methylation of BDNF and TrkB level in male ($P < 0.001$, $P < 0.01$) and female ($P < 0.01$, $P < 0.05$) pups from the SM group was higher than in pups of the NSM group, respectively (Fig. 7a, b).

Effect of Maternal Stress on the Pups' BDNF and TrkB Protein Expression in the Hippocampus

Results of densitometry showed that BDNF ($^{***}P < 0.001$) and TrkB ($^{***}P < 0.001$) protein expressions in the pups' hippocampus of stressed mother were decreased in comparison with the control group (Fig. 8a, b).

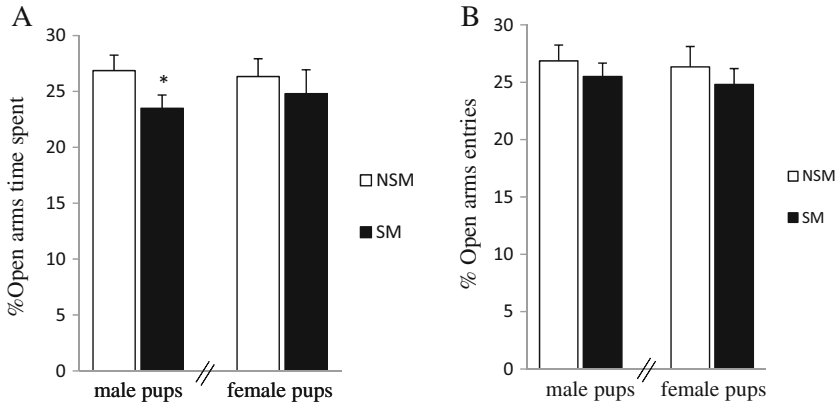

Fig. 5 Effect of maternal stress on the percent of entries and time spent (mean ± SEM) in open arms. *NSM* non-stressed mother; *SM* stressed mother. **a** Maternal stress decreased open arms time spent in the 30-day-old pups. $^{*}P < 0.05$ compared to the non-stressed mother group. **b** No significant stress effect was detected on the percent of entries between pups (Student's *t* test)

Discussion

In this study, as expected, repeated exposure of adult female rats to forced swimming for 10 min/day across 21 days increased serum corticosterone levels, which was associated with behavioral disorders. The HPA system is a one of the known endocrinological coping mechanisms against various stressful stimuli [25]. The HPA axis plays a main role in the response to stress in the neural pathway. Corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and glucocorticoid hormones are important factors in the HPA axis. Many studies have shown that stress has a main effect on the HPA axis function, is closely associated with cognitive and behavioral responses, and stimulates the synthesis and release of CRH from the hypothalamus [26–28]. Our results are consistent with previous studies which have shown that plasma and serum corticosterone levels in rats significantly increase after 14 and 21 days of repeated swimming in comparison with the control group [29, 30]. It has shown that chronic stress increases expression of CRH in the paraventricular nucleus and decreases glucocorticoid receptor expression in the hippocampus [26, 31]. The current study also showed that animal behavior was affected by the stressor in EPM and their presence was reduced in the open arms. It is reported that acute and repeated forced swim stress can induce anxiety-like behavior in the open field test and EPM [32].

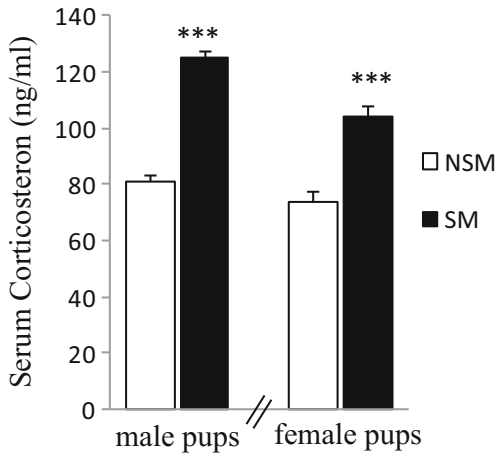

Fig. 6 Effect of maternal stress on serum corticosterone level (mean ± SEM). *NSM* non-stressed mother; *SM* stressed mother. $^{***}P < 0.001$ compared to the non-stressed mother group (Student's *t* test)

In addition, results of this study revealed that chronic stress causes an increase in BDNF and TrkB gene methylation which is followed by a significant reduction in protein expression of these genes in the hippocampus of adult female rats, consistent with our previous work [23]. The role of BDNF in stressed and emotional disorders was studied over 10 years. Studies have shown that 3 h of incubation with glucocorticoid decreases activity-dependent BDNF messenger RNA (mRNA) expression in hippocampal neurons [7, 8]. In contrast to these reports, Marmigère et al. demonstrated that plasma corticosterone level increases after 15, 60, and 180 min of exposure to stressful factors that is accompanied by increased expression of BDNF [33, 34]. It is demonstrated that BDNF and glucocorticoids adjust CRF homeostasis in the hypothalamus [35]. Heightened glucocorticoids are related to decreased BDNF levels that have been associated with depressive behavior [36, 37]. Inversely, increased glucocorticoid levels of the depressed person can be normalized by antidepressant administration, which depends on BDNF signaling function [38, 39]. BDNF and its receptor TrkB in the hippocampus regulate the HPA axis and therefore have an important role in stress response [40]. HPA axis dysfunction influences BDNF function negatively, which leads to psychiatric disorders [25]. High level of glucocorticoids induced by chronic stress exposure modifies BDNF signaling and increases anxiety behavior [41]. Exogenous glucocorticoid administration also decreases BDNF mRNA expression in the hippocampus [42]. In addition, increased hippocampal BDNF mRNA expression was observed after adrenalectomy [43]. Kikusui et al. have also found that

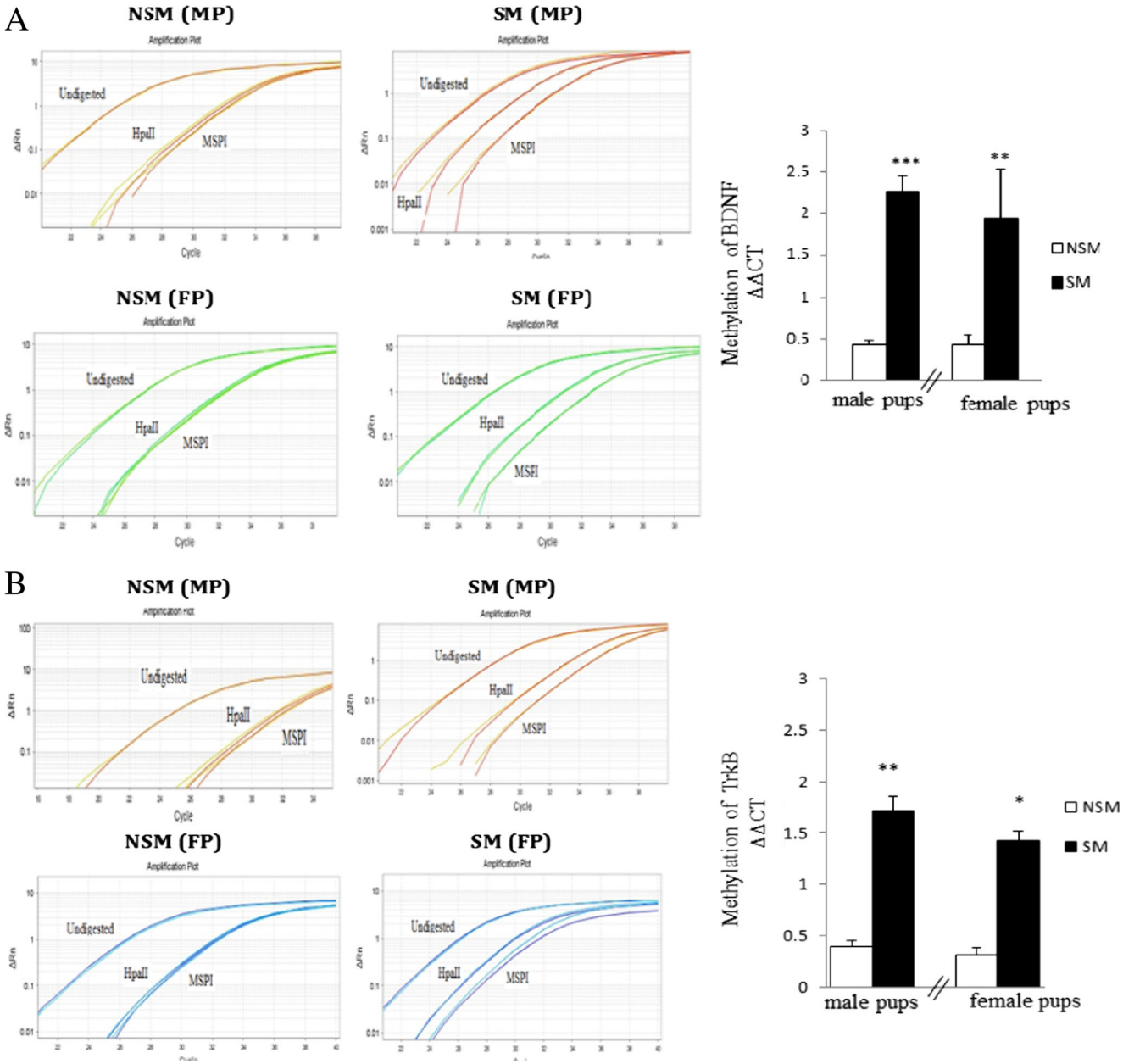

Fig. 7 Effect of maternal stress on BDNF (**a**) and TrkB (**b**) DNA methylation (mean ± SEM). *NSM* non-stressed mother; *SM* stressed mother; *MP* male pup; *FP* female pup. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to the non-stressed mother group (Student's *t* test)

a higher glucocorticoid level in early weaned mice reduces BDNF and neurogenesis in the hippocampus. Neuronal function has mainly been linked to BDNF and glucocorticoid interplay, and changes in neuronal function can induce psychiatric disorder [44]. Post-traumatic stress disorder (PTSD) rats have key changes in methylation of exon IV that increase DNA methylation in the BDNF gene associated with reduced expression of the BDNF gene [45]. Corticosterone markedly leads to downregulation of the BDNF gene resulting from exon IV BDNF mRNA reduction in the dentate gyrus [46]. However, the underlying mechanism of this interaction has not been fully understood and future researches are required to explore how BDNF and glucocorticoid signaling changes plasticity of the HPA axis on behavioral development and pathological situations. The impact of stress on TrkB DNA methylation has been investigated in a limited study and shows that hypermethylation and downregulation of TrkB in the Wernicke area do not relate to depression and suicidal behavior [47]. However, Ernst et al. showed that the TrkB promoter hypermethylation CpG2 site in frontal cortex is accompanied by suicidal behavior and leads to decreased expression of TrkB mRNA [48].

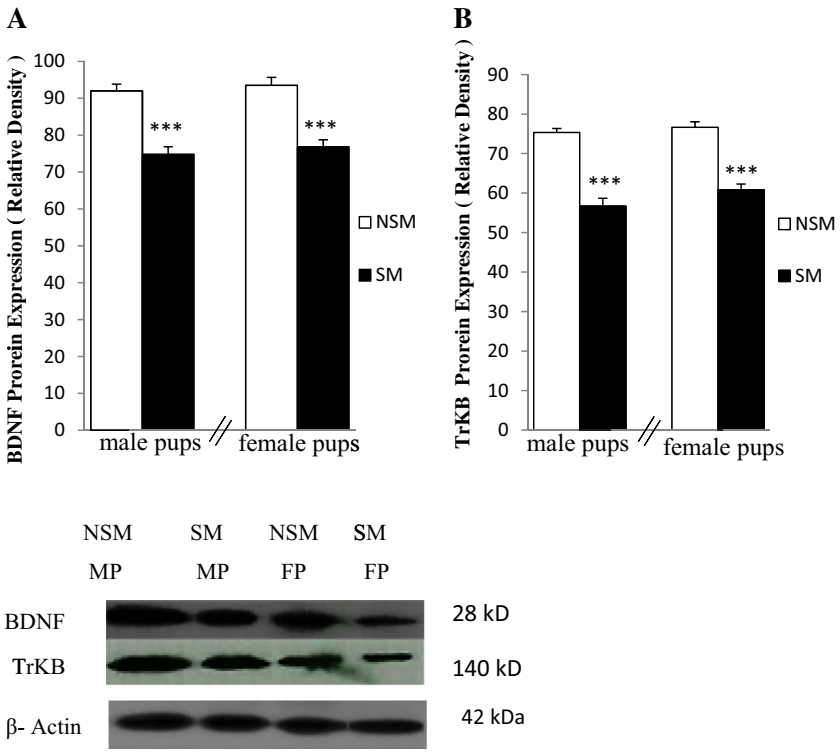

Fig. 8 Effect of maternal stress on BDNF (**a**) and TrkB (**b**) protein expressions (mean ± SEM). *NSM* non-stressed mother; *SM* stressed mother; *MP* male pup; *FP* female pup. ***$P < 0.001$ compared to the non-stressed mother group (Student's *t* test)

In addition, other findings of this study showed that maternal stress during adolescence and before pregnancy has significant effects on the incidence of behavioral, hormonal, and molecular changes in their male and female offspring. Serum corticosterone levels in 30-day-old male and female pups of the SM group were significantly higher than those in the control group. In addition, male pups of the SM group spent less time in the open arms compared to the control group. Also, more epigenetic alterations in hippocampal BDNF and TrkB genes were observed in offspring of female that were affected by stress before pregnancy than in pups of the control group. Maternal stress impacts glucocorticoid receptor (GR) expression and increases GR methylation in their pups' hippocampus [49]. Decrease of the hippocampal GR expression in offspring of stressed mother suggests that a higher level of glucocorticoid hormone is needed to stimulate this brain area. In fact, offspring of stressed mother show more hormonal response to stress exposure [50]. In addition, in the current study, decreased hippocampal BDNF and TrkB expression and increased methylation of these genes were observed in pups born of mothers that had experienced a preconception stressful situation compared to the control pups. Caporal et al. have proved maternal environmental enrichment (EE) experiences exclusively in the pre-reproductive period but not in gestational and postpartum periods, which can have an influence on the phenotype of offspring [51]. Several mechanisms such as germ cell, somatic transmission, fetus development, and maternal nutrition could be involved in transgenerational inheritance of environmentally induced alterations [52–55]. Studies have shown that environmentally induced changes in the gestational period can have an influence on the fetus directly through environmental signals like sounds, smell, and physical stimuli or indirectly via mother's behavioral and physiological (HPA axis and nutrition) modifications [56–58]. Also, maternal epigenetic changes could be passed to the next generation through germ cells or the placenta and milk (hormones, antioxidants, and antibodies) [54]. Maternal experiences have been transmitted through altered maternal care. Maternal care has lasting epigenetic impression on offspring's important plastic-related molecules such as BDNF and NMDA receptors [52, 53]. Cutuli et al. reported that maternal pre-reproductive EE improves maternal care quality which enhances hippocampal BDNF level in their offspring that is associated with cognitive development [59]. Also, we previously demonstrated that preconception maternal negative experiences reduce frequency of licking/grooming (LG) behavior in dams that lead to behavioral and hormonal alterations in pups. Indeed, improper maternal care changes HPA reactivity that processes a signal for activation of the endocrine and behavioral stress responses in offspring [30]. In current research, behavioral, biochemical, and molecular alterations in the offspring indicate that mothers' negative experiences before pregnancy were passed on to their offspring to prepare their progeny to cope with that situation.

Conclusion

In summary, this study revealed that repeated stress exposure prior to conception in female parent impacts offspring's genotype and phenotype measures. These findings suggest that molecular changes formed by maternal stress experience even before conception persist to the next generation and will negatively influence phenotypes of offspring.

Acknowledgment This work was supported by the Department of Physiology of Iran and the Hearing Disorder Research Center of Shahid Beheshti University of Medical Sciences.

Compliance with Ethical Standards

This study was confirmed by the Ethical Committee of Iran University of Medical Sciences based on National Institutes of Health Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985).

Conflict of Interest The authors declare that they have no conflict of interest.

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