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Experimental Research Article

Exploring expression alterations in *Mef2a*, *Tcf3*, and *Bdnf* genes following maternal separation in the rat brain: implications for neural plasticity

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ABSTRACT

Introduction: Mother-infant interaction is critical for neural development and maturation, with long-term molecular and behavioral impacts on offspring. Rats' neonatal maternal separation (MS) has been introduced as a model of early life stress, which may be the basis of mental health condition in adulthood. In the present study, the gene expression of *Mef2a*, *Tcf3*, and *Bdnf* was studied in various brain regions of rat offspring that experienced MS to provide further insight into the molecular mechanism of MS.

Methods: In this experimental study, MS was applied in rat litters separated from their mothers for four hours/day from PND 2 to PND 14. *Mef2a*, *Tcf3*, and *Bdnf* gene expression in various brain areas of adult offspring was measured by qPCR, and results were compared with the control group by t-test statistics.

Results: Quantitative analyses indicated that *Mef2a* has been downregulated in the amygdala of offspring that experienced MS. *Tcf3* gene expression was increased in the insula and striatum, while its level was decreased in the PFC of MS offspring. *Bdnf* was also upregulated in the insula but downregulated in the MS group's PFC.

Conclusion: Neonatal MS-induced gene expression changes the molecular drivers of neural plasticity in the offspring's central nervous system, which may be the basis of behavioral changes in adulthood. Further investigation of signaling pathways and behavioral modifications of rats that experienced MS may uncover the underlying mechanism of MS.

Keywords:
Maternal separation
Mef2a
Tcf3
Bdnf
Brain

Introduction

It has been well established that early life experiences have significant impacts on brain development and its function. Neurite arborization and synaptogenesis rapidly occur during this period, leading to neural network formation (Gilmore et al., 2018). Given that these pro-

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cesses are arranged in a highly tuned manner, early life stress, even a transient one, can interrupt normal neural development, producing persistent effects on the brain structure and function (Chen and Baram 2016). Brain development impairment alters the expression pattern of molecular factors of neurogenesis and plasticity across the brain regions, leading to long-lasting changes in brain function and a higher susceptibility to mental disorders (Schiavone et al., 2015).

Mother-infant interaction is vital for brain development and maturation, leaving long-lasting molecular and behavioral impacts on offspring (Čater and Majdič 2022). It was shown that maternal care in infancy modulates empathy (Asadi et al., 2021), neurogenesis, memory formation, and the mesolimbic dopamine (DA) system, as well as the hypothalamic-pituitary-adrenal axis (Čater and Majdič 2022). The maternal separation (MS) model in rats represents a useful model that mimics early life stress, leading to disturbances in brain development, behavior, and stress responses, which may be persistent throughout adulthood (Récamier-Carballo et al., 2017). This model involves four hours/daily removal of pups from their mother for about two weeks during their early lives. Previous research provided various lines of evidence about the impact of MS on the function and structure of the CNS (Nishi 2020), as well as the risk of psychiatric disorders and stress responsiveness (Schiavone et al., 2015). However, many have remained about the molecular players and signaling pathways affected by MS (Ohta et al., 2017).

Myocyte-specific enhancer factor 2A (*Mef2a*) is expressed widely across the nervous system, which regulates gene transcription (Liu et al., 2023). Previous studies showed that during brain development and throughout adulthood, MEF2 proteins are tuned by synaptic activity, leading to the recruitment of various epigenetic effectors affecting the structure of chromatin and the expression of genes (Assali et al., 2019). Consequently, they transform sensory input into modifications in the structure and function of neural networks (Assali et al., 2019). MEF2a has an important impact on the activity-dependent differentiation and plasticity of postsynaptic dendrites, a process that links environmental stimuli to developmental changes in the brain. MEF2a also restricts the number of excitatory synapses and contributes to long-term synaptic depression (Andzelm et al., 2019).

Transcription factor 3 (*Tcf3*) is another gene transcription regulatory protein and a final factor of the Wnt pathway that inhibits target genes from functioning in the absence of a Wnt signal. The Wnt pathway is crucial for regulating cell polarization, division, and the determination of cell fate during different developmental processes (de Jaime-Soguero et al., 2018). It has been reported that *Tcf3* is expressed highly in the hippocampus, contributing to the fear memory (Pai et al., 2018).

Brain-derived neurotrophic factor (*Bdnf*) is known to have an important role in the survival and differentiation of neurons as well as in the formation of synapses in the mammalian CNS (Rivera-Olvera et al., 2016). According to several lines of evidence, *Bdnf* seems to have contributed to long-term plasticity, modulating the morphology and function of the synapses (Kowiański et al., 2018), and is a critical regulator of long-term potentiation (LTP) in the hippocampus and other regions of the brain (Wang et al., 2022).

Maternal separation has been extensively studied, and numerous reports have highlighted its impact on the behaviors of offspring. In 2000, Lehmann et al. examined the long-term biobehavioral consequences of maternal separation in rats (Lehmann and Feldon 2000). Additionally, Mansouri et al. identified behaviors resembling autism in rats subjected to maternal separation (Mansouri et al., 2020), and Wang et al. investigated anxiety-related behaviors in these rats (Wang et al., 2020). Thus, there is substantial evidence regarding the impact of maternal separation on offspring behaviors. This study was designed to investigate the impact of MS during infancy on the expression of several genes related to neural plasticity, including *Mef2a*, *Tcf3*, and *Bdnf* gene expression in several brain regions, including the amygdala, hippocampus, insula, PFC, and striatum in rats.

Materials and Methods

Animals

In this experimental study, Wistar rats obtained from Pasture Institute (Tehran, Iran) were kept in a controlled environment with a temperature of 22 ºC ± 2, humidity at 60%, and a 12h light/dark cycle (lights on at 7:00 am) throughout the investigation. They were housed in a group of four consisting of one male and three random females. To determine their pregnancy status, the female rats were taken out of the cages after four days and placed in separate housing. Every animal received

TABLE 1: The sequences of *Mef2a*, *Tcf3*, *Bdnf*, and *β-Actin* primers and the size of their PCR products.

Gene Name	Forward primer	Reverse primer	Product size (bp)
Mef2a	AGAGGAACCGACAGGTGAC	CCGAGTTCGTCCTGCTTTC	207
Tcf3	GGTCTCCATGGCCTGAGTAA	TGGCATGGTTATGCAAAAGA	235
Bdnf	TCCCTGGCTGACACTTTTGA	GAAGTGTACAAGTCCGCGTC	104
β-Actin	TCTATCCTGGCCTCACTGTC	AACGCAGCTCAGTAACAGTCC	122

ad libitum access to regular rat feed and water.

Maternal separation

On the first postnatal day (PND 1), pups were assigned randomly to the MS or control group. In the MS group, litters were separated from their mothers 4 hours/day (between 9:00 h and 13:00 h) for 13 consecutive days (from PND 2 to PND 14). During the separation time, they were kept in an incubator at the same temperature as the body temperature of their mothers, without any physical contact among them (Rincel and Darnaudéry 2020). Litters were separated from their mothers on PND 21 after being weaned. Male subjects were kept in groups of four until PND 56 for molecular analysis. Three to six pups from different mothers were assigned to the control and MS groups.

Gene expression evaluation

Using a guillotine, rats were decapitated after being sedated by breathing in CO_2. After brain extraction, sterile instruments were used to dissect various sections including the amygdala, hippocampus, insula, PFC, and striatum, which were then snap-frozen in liquid nitrogen and preserved at -80 ºC. Dissection was performed according to the standard protocols (Aboghazleh et al., 2024) by the expert investigator preventing any cross-contamination between regions.

YTzol Pure RNA kit (Yektatajhiz azma, Tehran, Iran) was used for the extraction of total RNA from brain tissues, and the quality of the extracted RNA was assessed by an ND-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Reverse transcription was performed using Prime Script First Strand cDNA Synthesis Kit (Yektatajhiz azma, Tehran, Iran).

The *Mef2a*, *Tcf3*, and *Bdnf* primers were designed to measure the expression of these genes (Table 1) and Real Q-PCR Master Mix Kit for qPCR (Amplicon, Herlev, Denmark) was used for performing real-time PCR reactions with the following protocol: 15 minutes at 95ºC, repeating three steps (15-seconds denaturation at 95 ºC, 30-seconds annealing at 60 ºC for all primers, and 30-seconds extension at 72 ºC) for 40 cycles. Melting curves were checked before data analysis to ensure the specificity of amplification. The *β-Actin* gene was used for normalizing the expression of the studied genes.

Statistical Analysis

GraphPad Prism 9 was used to conduct statistical analysis. Expression levels of *Mef2a*, *Tcf3*, and *Bdnf* were calculated using the ΔΔCT method and were compared between the control and MS groups. Normality assumption was checked for each group of data, and then differences between groups were evaluated using t-test statistics. The P value less than 0.05 was considered significant and the data were presented as mean±SEM.

Results

Mef2a

Results from the real-time PCR measurements indicated that MS reduced the expression of *Mef2a* significantly in the amygdala (Fig. 1A; P=0.022). Its expression in the hippocampus (P=0.740), insula (P=0.499), PFC (P=0.450), and striatum (P=0.953) didn't change significantly (Fig. 1B-1E).

Tcf3

Tcf3 gene expression upregulated in insula (Fig. 2C, P=0.022) and striatum (Fig. 2E, P=0.007) of rats experienced MS but its expression downregulated in the PFC of MS rats (Fig.2D, P=0.018). The control and MS groups weren't different in *Tcf3* expression levels in the amygdala (Fig. 2A, P=0.516) as well as the hippocampus (Fig. 2B, P=0.414).

Bdnf

The qPCR results revealed a significant increase in the expression of *Bdnf* gene in the insula of MS animals (Fig. 3C, P=0.003), whereas *Bdnf* gene expression was

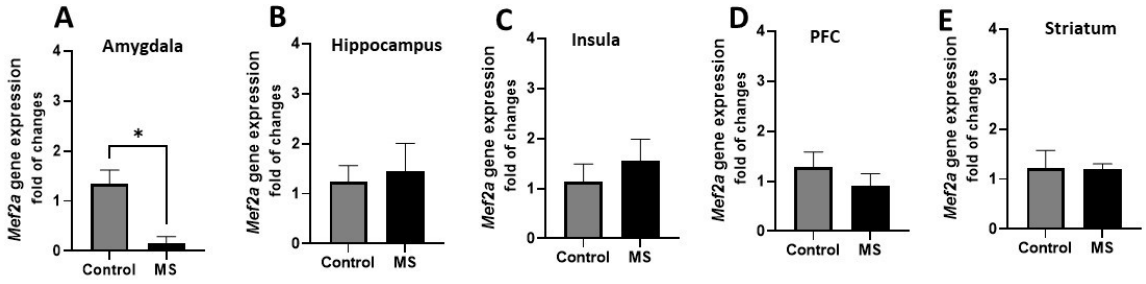

FIGURE 1. Relative expression level of *Mef2a* mRNA. A. amygdala; B. hippocampus; C. insula; D. prefrontal cortex; E. striatum. Data is presented as mean ± SEM ($^{*}P < 0.05$).

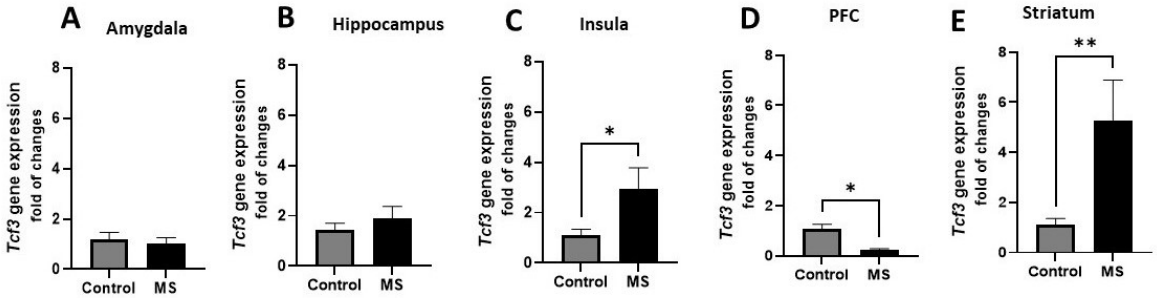

FIGURE 2. *Tcf3* expression level in various brain regions. A. amygdala; B. hippocampus; C. insula; D. prefrontal cortex; E. striatum. Data is presented as mean ± SEM ($^{*}P < 0.05$).

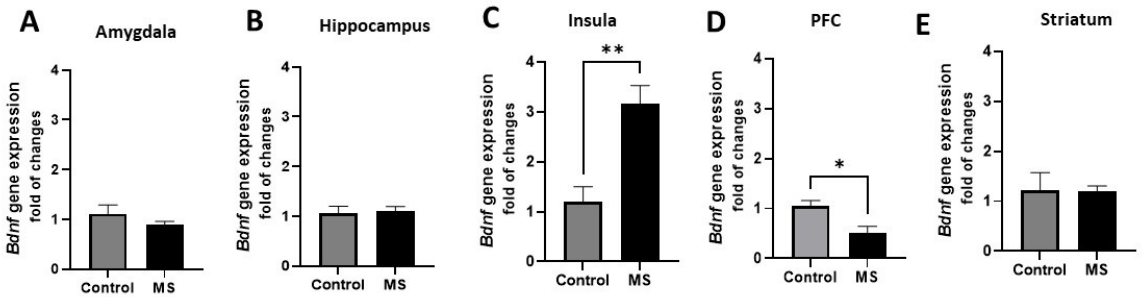

FIGURE 3. The mRNA expression level of *Bdnf* in the brain. A. amygdala; B. hippocampus; C. insula; D. prefrontal cortex; E. striatum. Data is presented shown as mean ± SEM ($^{*}P < 0.05$).

downregulated in PFC of the MS group in comparison with the control rats (Fig. 3D, P=0.016). In other brain regions, no significant alterations were detected (amygdala: Fig. 3A, P=0.453; hippocampus: Fig. 3B, P=0.874; striatum: Fig. 3E, P=0.953).

Discussion

Due to the findings of this study, neonatal MS modulated the expression of molecular players of neural plasticity, such as *Mef2a*, *Tcf3*, and *Bdnf*, in some brain regions. These modifications can last till adulthood. Downregulation of *Mef2a* has been detected in the amygdala of offspring experienced MS. *Tcf3* gene expression was increased in the insula and striatum, while it was decreased in the PFC of MS offspring. *Bdnf* was also upregulated in the insula but was downregulated in the PFC.

Parental care has a significant impact on the cognitive and emotional development of offspring (Morris et al., 2017). Previous studies have revealed that the postnatal

mother's tactile stimulation is connected with long-term behavioral changes; hence, MS can be connected with consistent alterations in the structure and functionality of the brain (Teicher and Samson 2016). Early life stress can interfere with neural differentiation and brain maturation, which may account for the behavioral deficits that are observed in animal models of psychiatric disorders like depression (LeMoult et al., 2020), post-traumatic stress disorder (Lee et al., 2018), early life neglect (Kundakovic and Champagne 2015), and autism (Khaledi et al., 2023).

Mef2s are key regulators of activity-dependent neural epigenetics, so they are considered as one of the mediators connecting environmental conditions to the internal situation of the brain, leading to better coping. Based on the associated co-factors, they can either repress or activate the expression of the genes (Pon and Marra 2016). During brain development, the role of MEF2a becomes more prominent, and it supports postsynaptic dendrite plasticity and enhances activity-dependent differentiation (Liu et al., 2023). The effect of MS on *Mef2a* gene expression was not investigated before. We detected its downregulation in the amygdala of offspring of MS rats, suggesting the role of *Mef2a* in this brain area in transcriptional regulation. It was reported that reducing the function of MEF2 in the amygdala and dentate gyrus promotes fear formation and spatial memory, respectively (Wang et al., 2018); thus, lower levels of MEF2a in the amygdala of rats that experienced MS may induce fear memory in these animals, which needs further validation. Akhtar et al. observed that MEF2a has only a subtle role in regulating hippocampal synaptic function (Akhtar et al., 2012). We've also not detected significant alteration in its hippocampus expression level compared to controls.

Comparison of *Tcf3* gene expression between control and MS groups in the present study revealed *Tcf3* upregulation in the insula and striatum as well as its downregulation in the PFC of the MS group. We have not found any studies evaluating the impact of early life stress or MS on the expression of *Tcf3*. TCF3 is the terminal factor of the Wnt/βCatenin pathway in embryonic stem cells. In the absence of a Wnt signal, TCF3 inhibits target sequences connecting developmental signals to the regulatory circuitry of ES cells to fine-tune the balance between differentiation and pluripotency (Koelman et al., 2022). There are limited reports regarding dysregulation of the β-catenin/TCF signaling in the experimental models of mood disorders (Mulligan and Cheyette 2017). The presented results suggested a connection between dysregulated *Tcf3* expression and mental disturbances induced by MS.

BDNF is one of the fundamental regulators of neural maturation, development, and synaptic plasticity in CNS (Récamier-Carballo et al., 2017). Numerous researches have looked at the influence of MS on *Bdnf* gene expression; however, the results have been conflicting. Récamier-Carballo et al. reported increased levels of *BDNF* in the amygdala and hippocampus but decreased expression in the frontal cortex of MS offspring (Récamier-Carballo et al., 2017). There are also reports detecting *Bdnf* downregulation in the hippocampus of the rats experienced MS (Zaletel et al., 2017), whereas other studies have found notable increases in BDNF protein concentration in the hippocampus (Wang et al., 2015). In the current study, significant increase in *Bdnf* expression was detected in the insula but a reduced expression of *Bdnf* in mPFC of MS animals was observed. It seems that observed discrepancies between the reported *Bdnf* expression level in MS animals relate to its sensitivity to the various factors including animals' handling (Récamier-Carballo et al., 2017), technical variations, and the age of experimental animals. Greisen et al. reported that differences in postweaning experiences of animals that experienced the same MS protocol resulted in different BDNF concentrations (Greisen et al., 2005). It was also observed that *Bdnf* expression was decreased with age in both the control and MS rats (Wang et al., 2015), so it is suggested that these effects may have age-related manifestations, leading to the conflicting results (Li et al., 2008).

Results of the present study detected upregulation of *Tcf3* and *Bdnf* in the insula and striatum as well as their downregulation in the PFC. Insula regulates social cognition, decision-making, empathy, responsiveness to emotional cues, arousal, and processing of somatic pain (Pavuluri and May 2015). Dysfunctional connectivity of the insula has been identified in ASD, and the pattern of functional connectivity of the insula can be used to discriminate individuals with ASD from typically developing children (Guo et al., 2019). Increased insula reactivity to specific kinds of emotional stimuli, such as fear and anger has been reported in patients with anxiety disorders (Stein et al., 2007; Uddin et al., 2017). We've also

detected *Bdnf* as well as *Tcf3* expression alteration in the insula of offspring experienced MS, which may result in dysfunctional connectivity in this region. Meanwhile, PFC is involved in various cognitive and emotional functions, and it was shown that stress impairs its structure and function (Woo et al., 2021). Reduced functional connectivity of the PFC was reported in individuals with depression (Murrough et al., 2016) and those with higher susceptibility to MDD (Pizzagalli and Roberts 2022). Decreased expression of *Bdnf* in the mPFC of MS animals was also previously reported (Wang et al., 2015). Moreover, a reduced number of inhibitory neurons and synapses in the mPFC and social deficit were also identified as a consequence of repeated MS (Tenkumo et al., 2020) which may be associated with decreased *Bdnf* expression.

It should be noted that to gain a comprehensive understanding of the changes that resulted from maternal separation, we have to consider the entire timeline to gain a comprehensive understanding of the changes resulted from maternal separation. If we do not find any changes in the expression of the genes in a particular region, such as the hippocampus, it does not imply that no changes occurred throughout the rat' development in this region. Moreover, the studied genes which are typically hub genes regulating various molecular pathways. We've not measured the expression of downstream genes which would allow us to determine the precise effects of their expression changes on brain function and the plasticity of neural circuits. Results of the current study have just provided clues that expression changes of genes contributing to the neural plasticity in particular brain regions may be the basis of behavioral alterations resulted from MS leading to susceptibility to mood disorders.

Conclusion

In conclusion, our observations showed that neonatal MS induced gene expression changes in some molecular drivers of neural plasticity in the brain of rat offspring which may be the basis of behavioral changes. It should be noted that interactions between the pups and their mothers are complicated and age, sex, environmental condition, as well as the level of applied early life stress may act as confounders that influence the expression of genes. Accordingly, the examination of other molecular factors in various developmental stages can further reveal the mechanisms by which maternal care influences the offspring's neurodevelopment and subsequent molecular and behavioral changes in adulthood. More investigation is essential for better understanding the underlying process of MS, which is currently poorly known.

Acknowledgments

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Conflict of interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval

The current investigation was approved by the local ethical committee, Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.PHNS.REC.1396.124).

Author's contribution

K.R., E.A.; Methodology, Investigation, Formal analysis, Writing - Original Draft; F.K.; Conceptualization, Resources, Writing - Review & Editing, Project administration; MA. M; Validation, Formal analysis, Writing - Review & Editing; S.A.; Conceptualization, Formal analysis, Writing - Original Draft, Review & Editing, Project administration, Funding acquisition. All authors read and approved the final version of the study.

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