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Effect of prenatal stress on density of NMDA receptors in rat brain

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ABSTRACT

N-methyl-D-aspartate (NMDA) receptors are important excitatory receptors which contribute to many brain functions. Altered NMDA receptor levels cause maldevelopment of corticostriatal and corticolimbic pathways, which is a neurobiological predisposing factor for development of epilepsy, schizophrenia and other idiopathic psychotic disorders. It was hypothesized that prenatal stress could play a role in pathophysiology of these disorders by affecting expression of the receptors through releasing corticosterone. Sixty-eight virgin female Wistar rats were selected and mated with male rats with the same genotype. Then, the pregnant rats were subjected to restraint or predator stress on 15th, 16th and 17th gestation days. Prenatal stress consisted of restraint or predator stresses of the dams under normal room conditions. After parturition, the pups were studied in terms of density of NMDA receptors in brain at different time points. Meanwhile, blood sample was obtained and corticosterone blood level (CBL) was measured. The pups were then compared with the pups born to unstressed dams. Stress induced significant rise in CBL and NMDA receptors in brain of the offspring. CBL was significantly higher among the stressed rats compared to the control ones; there was significant difference between the two stresses and between the two sexes. The male pups were affected more severely. Stressful events during gestation had important effects on NMDA receptors of the offspring. It can be concluded that stress-induced elevation of NMDA receptors and corticosterone might mediate altered susceptibility to epilepsy and decrease ability of learning and memory and other stress-induced neurologic disorders.

1. Introduction

Stress is a condition which is experienced by everyone and includes not only major life events but also hassles of daily life. Reactions to a stressful incident are highly variable, which reflects experience of an individual and genetic background of stress (McEwen, 2000, 2002). Exposure to stress during gestation affects brain development and results in permanent alterations that may predispose the offspring to subsequent cognitive or neuropsychiatric disorders (Fumagalli et al., 2009). There is a causative relationship between prenatal stress (PS) and abnormal cognitive, behavioral and psychosocial outcomes in both animals and humans. PS effects on the offspring's brain have been demonstrated in animal studies (Charil et al., 2010). PS may cause some of these outcomes through alternating expression of glutamate receptors. Corticosterone in rodents is a glucocorticoid and the primary end product of the hypothalamic–pituitary–adrenal (HPA) axis; it is an important component of stress system in rodents; so, it is known as a stress hormone (Charil et al., 2010).

NMDA receptor is one of the subclasses of ionotropic glutamate receptors which has a major role in memory, learning, motor coordination and neurodegeneration; stress can alter these conditions by changing expression of this receptor (Dingledine et al., 1999; Hawkins et al., 1999). The NMDA receptor is a heterotetramer composed of a family of related subunits. The GluN2B subunit of the receptor appears to be essential for some forms of memory and is particularly vulnerable to change with age in both the hippocampus and cerebral cortex. An increased association of GluN2B-containing

Abbreviations: NMDA, N-methyl-D-aspartate; CBL, corticosterone blood level; COS, corticosterone; PS, prenatal stress; HPA, hypothalamic–pituitary–adrenal; GD, gestation day; EDTA, ethylenediaminetetraacetic acid; RIA, radioimmunoassay; IHC, immunohistochemistry; TBS, tris-buffered saline; 11β-HSD2, 11-beta hydroxyl steroid dehydrogenase; GluN2B, glutamate [NMDA] receptor subunit 2B.

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NMDA receptors with synaptic scaffolding proteins in aged animals may have contributed to the age-related memory declines (Zamzow et al., 2013). GluN2B subunit is expressed from early embryonic stages in brain development, and GluN2B-containing NMDARs confer high Mg^{2+} sensitivity, large channel conductance, and potentiation by protein kinase C (Akashi et al., 2009). These properties have led to the proposal that this subunit is critically involved in activity-dependent brain functions, such as synapse refinement, synaptic plasticity, and cognitive function (Mori and Mishina, 1995). Changes in glutamate receptor subtype levels in different forebrain regions of adult rats suggest that development and formation of corticostriatal and corticolimbic pathways may be permanently altered as a result of prenatal stress suffering. Maldevelopment of these pathways may provide a neurobiological substrate for development of schizophrenia and other idiopathic psychotic disorders (Berger et al., 2002). Corticosterone alters expression of mRNA for some subunits of NMDA receptor in brain regions after birth, which is associated with stress response (Lee et al., 2003). It has been noted that glucocorticoids release after stress down-regulates hippocampal NMDA receptors in adult rats (Harvey et al., 2004). Son et al. showed that PS for 7 days impaired some NMDA receptor-dependent synaptic plasticity in CA1 area of hippocampal slices (Son et al., 2006). Long term stress causes increased NMDA receptors in medial prefrontal cortex, dorsal frontal cortex, hippocampal CA1, medial caudate-putamen as well as shell and core regions of nucleus accumbens (Berger et al., 2002). However, no study has investigated influence of maternal stress exposure during gestation on density of glutamate receptors including NMDA in the offspring's brain. Therefore, this study was designed to investigate effect of prenatal stress on density of NMDA receptors in brain of rats. In this study, two kinds of stressors as restraint stress and predator stress were used.

2. Materials and methods

Male and female Wistar rats (200–250 g) were obtained from the animal facility at University of Urmia, Iran. They were 10 weeks old on delivery. The rats were housed in groups of four per cage in the animal facility and kept under standard conditions as follows: 12-h light/dark cycle, 22 ± 2 °C and food and water ad libitum. All the experimental protocols and procedures complied with the guidelines of 1975 Declaration of Helsinki as reflected in the guidelines of Medical Ethics Committee, Ministry of Health, Iran. In addition, this study was approved by Ethics Committee, Urmia University of Medical Sciences. All the 12-week-old female rats were mated with a sexually experienced male from the same genotype. Each female was paired with one male at 9:00 am and checked for vaginal plug at 3:00 pm, which were immediately housed 4 rats per cage for the entire gestation. If a plug was not observed, the animal was returned to her home cage until the next morning for a new mating procedure. Pregnant rats were divided evenly to three control, restraint stressed and predator stressed groups ($n = 7$).

2.1. Restraint stress procedure

This procedure has been previously described (Sadaghiani and Saboory, 2010). Briefly, restraint stress group was stressed on 15th, 16th and 17th gestation days (GD15, 16 and 17, respectively). Stress involved transport of the home cage to the experimental room and placement of the pregnant female in a restraint chamber under normal room conditions. The animals were restrained for 60 min twice a day (between 800 and 900 h and between 18:00 and 19:00 h). This procedure has been previously shown to cause alterations in regulation of the HPA axis in the offspring (Ahmadzadeh et al., 2011; Sadaghiani and Saboory, 2010).

2.2. Predator stress procedure

This procedure has been previously described (Ahmadzadeh et al., 2011; Sadaghiani and Saboory, 2010). In short, stress involved transport of the home cage to the experimental room and placement of the pregnant female at 30 cm distance from the cat's cage under normal room conditions. The animals were kept in this position for 60 min between 800 and 900 h every day. This group was stressed on GD15, 16 and 17. The control group, consisting of seven pregnant females, was transported to the experimental room on GD15, 16 and 17 and handled similar to other groups but without any stress. After parturition, male and female pups from each litter were sorted to three uneven groups which were called P2, P6 and P15. To reduce possible litter effects, the maximum of four infant pups (two males and two females) from any litter were used as the subjects of this experiment. P2, P6 and P15 groups were decapitated on 2nd, 6th and 15th days after birth, respectively, under ether anesthesia at 08:00 h to collect trunk blood into 1.5 mL of EDTA-coated micro-centrifuge tubes. The samples were kept on ice and later centrifuged for 15 min at 9000 rpm at 4 °C. Plasma was transferred to clean 1.5-mL micro-centrifuge tubes and the plasma samples were stored frozen at −20 °C until determining COS levels. Plasma COS was measured using radioimmunoassay (RIA) commercial kit (Isotope, Budapest, Hungry) and the values were expressed in nanogram per milliliter (ng/mL). When the animals were decapitated, their brains were removed and divided into two parts of right and left hemispheres. Their left hemisphere was stored at −80 °C and kept for another study. Their right hemisphere was stored in 10% buffer formalin at least for three days and processed for histopathology for immunohistochemistry (IHC).

2.3. Immunohistochemistry

The rat brains were fixed by 4% formaldehyde and 4 μm of frontal sections were cut. These sections were treated with xylole, ethanol (100%), ethanol (96%) and water twice for 5 min and were then incubated in Tris–EDTA buffer (pHof9.0) and placed in a microwave with 100% power until reaching the boiling point. Afterward, the power was reduced to 40% and, after 15 min, it was cooled down, washed out with water and treated with TBS buffer for 5 min. Then, they were transferred to a dark and wet place; the blocker solution was used for about 10 min and it was washed with TBS buffer for 5 min. The sections were incubated for 30 min with NMDAR2B antibody (1.0 mg/mL, Cat No. NB100-74476, Novus Biological, Littleton, USA) as primary antibodies and washed with TBS solution for 5 min again. They were then treated with the polymer labeled peroxidase for 30 min in a wet place and washed for 5 min with TBS buffer. Then, they were treated with chromogen and substrate for 10 min and washed with TBS buffer and water for 5 min, respectively. They were then stained with hematoxylin, stocked in lithium carbonate for 5 min, washed with water and finally dehydrated with increasing concentrations of ethanol and finally xylole. The applied lams were covered with entellan and lamel. The samples were examined by a light microscope (Olympus BH2, Japan; X40) and observations were recorded. This study visualized and identified NMDAR2B subunit containing cells in the right hemisphere of rats' brain. The receptor density was counted in hippocampus, fornix and frontal cortex.

2.4. Statistical analyses

The results were expressed as mean ± SEM. SPSS v.15 software (SPSS, Chicago, IL, USA) was used for data analysis. The data related to CBL and receptor density were normally distributed. Therefore, they were analyzed using parametric techniques. Two-group comparisons were made using *t*-test whereas multiple-group comparisons were performed using one-way analysis of variance (ANOVA). When appropriate, post hoc analyses, such as Tukey's test, were done. Effects of restraint and predator stress on density of NMDA receptors were analyzed using two-way ANOVA for two factors. All the tests considered significance level of $p < .05$.

3. Results

3.1. Effects of restraint or predator prenatal stress on CBL in infant rats

Effects of gestational restraint or predator stress on CBL were determined in two male and two female pups (maximum) from each litter on P2, P6 and P15. CBL was significantly different between control and stressed rats. Also, there was significant difference between the two stresses (Tables 1 and 2).

Moreover, both stresses affected the pups sex dependently (Fig. 1).

3.2. Effects of restraint and predator stress on density of NMDA receptors

In order to analyze effects of restraint and predator stress during gestation on density of NMDA receptors, two-way ANOVA was used for two factors of stress and time after birth. There was significant interaction between stress and the day after birth (stress × day, $p < 0.001$). Therefore, subgroup analysis was performed. In this respect, mean of NMDA receptors was separately compared on P2, P6 and P15. On P2, there was no significant difference between the groups ($p = 0.131$). On P6, there was significant difference between the groups ($p = 0.018$). Post hoc analysis by Tukey's test represented that only restraint stressed group had larger value of receptor

Table 1
Effect of gestational restraint or predator stress during the third week of pregnancy on CBL in male pup rats (ng/mL).

Group	P2 ($n=8$)	P6 ($n=8$)	P15 ($n=8$)
Control	0.94 ± 0.04	0.32 ± 0.03	0.21 ± 0.02
Restraint	$3.5 \pm 0.2^*$	$3.03 \pm 0.17^*$	$2.54 \pm 0.22^*$
Predator	$5.59 \pm 0.23^*$	$4.34 \pm 0.13^*$	$3.22 \pm 0.14^*$
ANOVA	$F(2)=79.58$, $p<0.001$	$F(2)=123.13$, $p<0.001$	$F(2)=51.1$, $p<0.001$
	Tukey, $p<0.001$	Tukey, $p<0.001$	Tukey, $p<0.009$

Note: There were significant differences between control group and all stressed animals on P2, P6 and P15. Moreover, there was significant difference between restraint and predator stressed pups on P2, P6 and P15.

Table 2
Effect of gestational restraint or predator stress during the third week of pregnancy on CBL in female pup rats (ng/mL).

Group	P2 ($n=8$)	P6 ($n=8$)	P15 ($n=8$)
Control	0.92 ± 0.07	0.37 ± 0.08	0.26 ± 0.06
Restraint	$2.7 \pm 0.18^*$	$2.25 \pm 0.21^*$	$1.62 \pm 0.13^*$
Predator	$3.74 \pm 0.32^*$	$3.2 \pm 0.19^*$	$2.32 \pm 0.18^*$
ANOVA	$F(2)=44.47$, $p<0.001$	$F(2)=70.52$, $p<0.001$	$F(2)=62.36$, $p<0.001$
	Tukey, $p<0.005$	Tukey, $p<0.002$	Tukey, $p<0.003$

Note: There were significant differences between control group and all stressed animals on P2, P6 and P15. Moreover, there was significant difference between restraint and predator stressed pups on P2, P6 and P15.

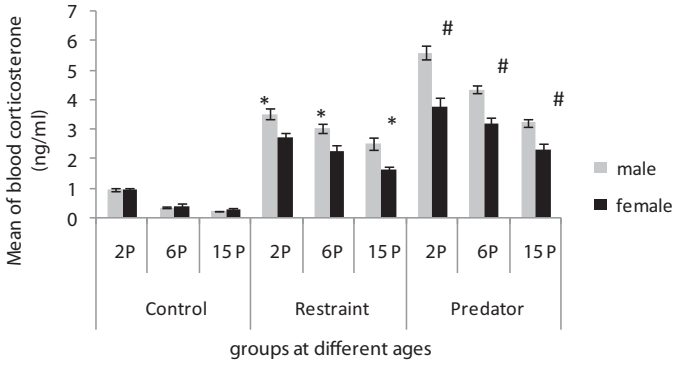

Fig. 1. Effect of prenatal stress on CBL in male and female rat pups. The dams were exposed to restraint or predator stress and CBL was determined in their pups on P2, P6 and P15. Each bar depicts mean ± SEM and $n=8$. There was significant difference between male and female pups in stressed animals. $^*p<0.009$, t test, $t=2.89$ on P2, $t=2.86$ on P6 and $t=3.6$ on P15. $^\#p<0.001$, t test, $t=4.7$ at P2, $t=4.9$ on P6 and $t=4.07$ on P15.

Table 3
Effect of gestational restraint or predator stress during the third week of pregnancy on levels of NMDA receptors in all pup rats.

Group	P2 ($n=12$)	P6 ($n=12$)	P15 ($n=12$)
Control	250.04 ± 58.658	125.25 ± 20.076	104.46 ± 9.278
Restraint	274.33 ± 23.115	146.96 ± 39.619*	123.00 ± 15.676*
Predator	247.04 ± 61.126	134.00 ± 6.115	148.37 ± 41.165*

density than the control group. On P15, there was significant difference between any pair of groups ($p<0.01$). In this respect, mean of NMDA receptors in predator stressed group was significantly larger than those in restraint stressed and control groups. Similarly, in restraint stressed group, it was larger than in the control group (Table 3).

Stress induced a sex specific alteration in NMDA receptor expression with a robust effect on male pups. At P2, density of NMDA receptors significantly increased in stressed male (but not female) pups compared to the control ones. There was a remarkable difference between male and female pups. At P6, stress induced a non-significant increase in density of NMDA receptors in both sexes compared to the control pups. At P15, predator stress by itself induced significant elevation in NMDA receptor expression of both sexes (Fig. 2).

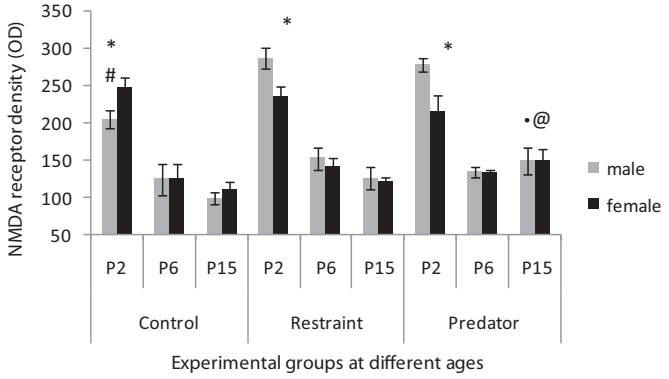

Fig. 2. Effect of prenatal stress on NMDA receptor level in male and female rat pups (each bar, $n=6$). The dams were exposed to restraint or predator stress and NMDA receptor level was determined in their pups on P2, P6 and P15. Each bar graph depicts mean ± SEM of NMDA receptors OD (optical density). There was significant difference between male and female pups in all groups on P2. $^*p<0.05$, male vs. female in each group on P2. Stresses induced a significant increase in density of NMDA receptor among male (but not female) pups at P2; $^\#p<0.01$ for control male pups vs. stressed male pups at P2. $^\wedge p=0.003$ for control male vs. predator male at P15; $^@p<0.001$ for control female vs. predator female at P15.

Images of NMDA receptor immunoreactivity are presented in Fig. 3. The images represent staining of the receptor in frontal cortex of infant rats at P2, P6, and P15.

4. Discussion

Effect of prenatal restraint or predator stress on density of NMDA receptors as functional proteins was assessed using immunohistochemistry method. Restraint stress significantly increased expression of these receptors on P6 and both restraint and predator stresses up-regulated expression of these receptors on P15 (Table 3). Stressful events in early life seem to have significant effects on HPA function. Previous studies have shown increased and prolonged COS secretion in response to stress (Carter et al., 2006; Viltart et al., 2006). Other studies have indicated that PS can reprogram the HPA axis and induce either basal or stress-related secretion of glucocorticoid hormones (Figueiredo et al., 2003; Owen et al., 2005; Weinstock, 2005). Only extreme challenges such as severe stressors can compromise role of placenta as a structural and chemical barrier and expose fetal brain

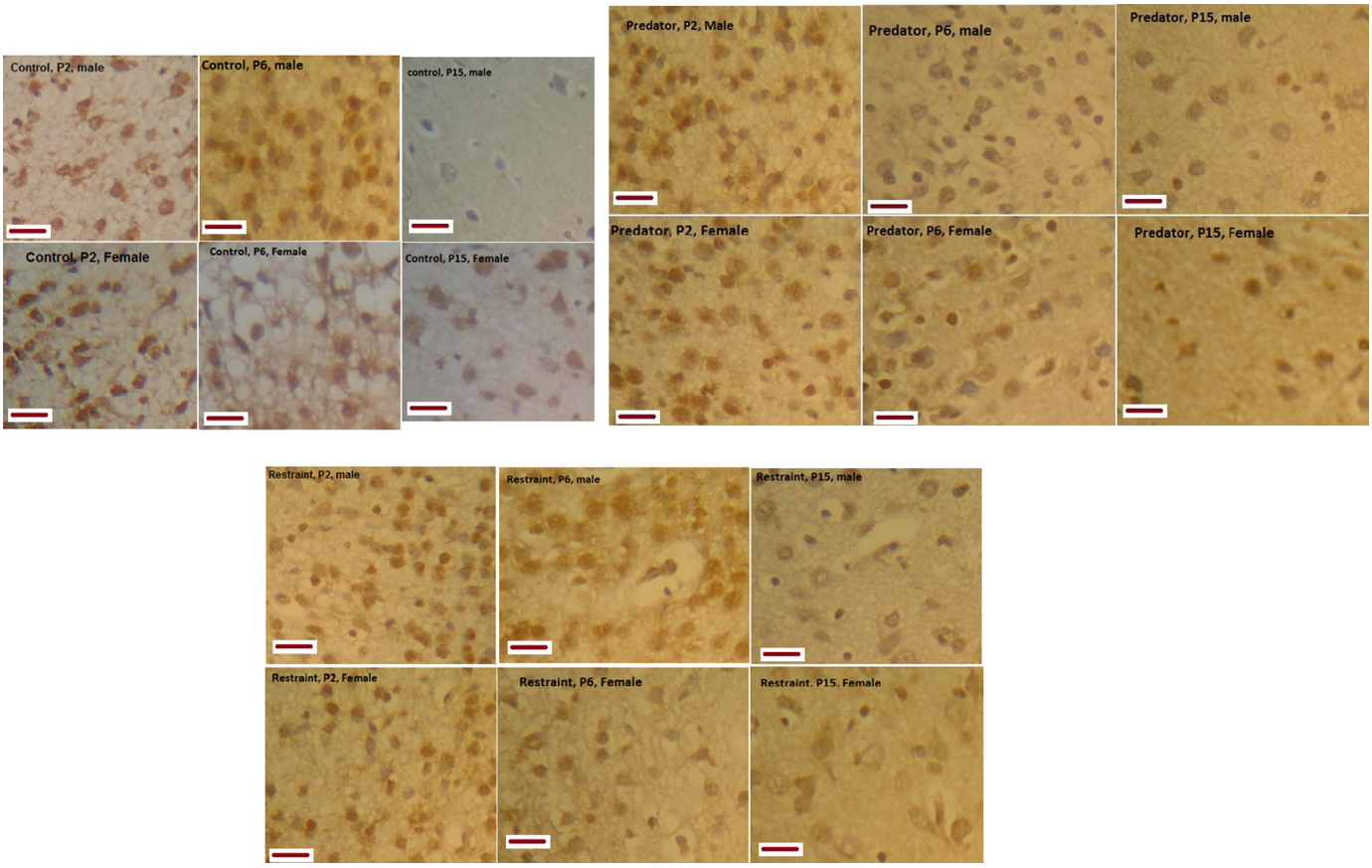

Fig. 3. Representative photomicrographs (40×) of NMDA receptor subunit (NMDAR2B) immunoreactivity in frontal gray matter for each of the groups at P2, P6 and P15; scale bar = 50 μm.

to glucocorticoids (Mueller and Bale, 2008; Seckl, 2004; Welberg et al., 2000). A validated model of early stress in rats is Prenatal Restraint Stress (PRS) (Seckl, 2004; Welberg et al., 2000). The offspring which their mothers have suffered from PRS have impaired HPA axis function (Darnaudery and Maccari, 2008; Maccari and Morley-Fletcher, 2007). PRS also increases age-related HPA axis dysfunction (Maccari and Morley-Fletcher, 2007). Predator stimuli are stressful for rats and mice. Exposure to natural predators and their odors elevate monoaminergic and stress hormone release in rats (Adamec et al., 1998; Saboory et al., 2011) and mice (Belzung et al., 2001). Therefore, to obtain more accurate results, both types of stress were applied in this study. According to Murmu et al., level of response to stress depended on type of stressors in pregnant dams. They showed that restraint increased COS level more effectively than forced swimming and crowding. They related this difference to degree of control that animals had over stressors (Murmu et al., 2006). PRS procedure's efficacy was previously proved by Sadaghiani and Saboory (2010). To confirm that the animals were really stressed, CBL was measured in this study. The two stressed groups showed more CBL than the control on all three days (p2, p6 and p15), which marked explicit correlation between CBL and NMDA receptor expression in brain. This issue might be due to reduction in protective activity of 11-beta hydroxyl steroid dehydrogenase (11β-HSD2) in placenta and subsequent fetal exposure to maternal glucocorticoids (Welberg et al., 2000). Mother's glucocorticoids reach the fetus and thus increase activity of HPA axis and change fetal brain development (Barbazanges et al., 1996). Long term PS on rats results in a small (10%) but significant increase of NMDA receptors in hippocampal CA1 region, but not in CA3 region (Berger et al., 2002). Principal above-mentioned findings indicated that restraint stress applied to rat mothers during the last week of their pregnancy produced functional alterations in corticostriatal glutamatergic systems of their offspring that persisted into adulthood (Berger et al., 2002). Uno et al. suggested hippocampus as the first target affected by glucocorticoid steroids secreted after pre- and also post-natal stressful events in primates (Uno et al., 1994). Hippocampus is one of the major regions which regulates HPA axis and its development is affected by PS (Uno et al., 1994).

NMDA receptors have notable effects on many brain functions. Researchers have shown that NMDA receptors may play a key role in pathophysiology of several neurological diseases including epilepsy (Ghasemi and Schachter, 2011; Novelli and Tasker, 2007). In various studies, activity and expression of NMDA receptors can be altered in association with epilepsy which is one of the most common neurologic disorders (Scher, 2003), particularly in some specific seizure types (Ghasemi and Schachter, 2011). It is well-established that NMDA receptors play a key role in etiology of epilepsy (Novelli and Tasker, 2007). So, events affecting NMDA receptors during prenatal life can cause epileptical conditions (Scher, 2003). Also, recent data suggests that the glutamatergic system is involved in the pathophysiology and treatment of major depressive disorder, and the NMDA receptor is a potential target for antidepressant drugs. Some preclinical and clinical evidence suggests that magnesium may be useful in the treatment of depression. Magnesium treatment in stressed animals increases the level of GluN2B in the prefrontal cortex (Pochwat et al., 2013). The essential role of NMDA receptors in synaptic plasticity is well-documented (Scher, 2003). Long term synaptic plasticity in rodent hippocampus which is necessary for spatial learning and memory depends on molecular events that are underlined by these

receptors (Stamatakis et al., 2009). NMDA receptors are also essential elements in pathways of neural death in some pathological conditions (Watanabe et al., 1994). Lee et al. simulated stress conditions by giving high dose COS to adrenalectomized rats after birth and showed an increase in expression of mRNA of some NMDA receptor subunits (Lee et al., 2003). When studying only male offspring, Berger et al. showed that deficiency of neurotransmission caused compensation over expression and perhaps up-regulation of NMDA receptors in different forebrain regions. This phenomenon was proposed to play a key role in pathophysiology of schizophrenia and other psychotic disorders (Berger et al., 2002). They exposed mothers to stress as much as three times per day for 7 days. In the present study, it was evaluated whether stress for fewer time and days (twice a day for three days) could alter NMDA receptors or not. The results were in line with those in the literature and indicated that PS significantly increased density of NMDA receptors in the right hemisphere of brain. Berger et al. investigated effect of PRS on expression of dopamine and glutamate receptors as late as ninety days after birth (Berger et al., 2002). In contrast to their study, here, level of NMDA receptors was separately assessed in infant rats on P2, P6 and P15 in male and female pups. The present work was consistent with their study as it showed that up-regulation of NMDA receptors was significant at least up to P15. Glutamatergic transmission might play a role in excitotoxicity, which is an important factor in a variety of neurological disorders such as Alzheimer, cerebral trauma, ischemia and epileptic disease (Ayala and Tapia, 2003). We showed that exposure to PS increased NMDA receptor level on 6th and 15th days of infancy which in turn might affect brain development and increase susceptibility to epilepsy and other neuropathologic diseases later in life through changing this glutamatergic transmission. The present data indicated no difference between the experimental and control groups on P2; but, there was significant difference in terms of P6 and P15 as a result of data analysis for both sexes (mixed data). However, analyzing the data separately for each sex represented significant difference between control and stressed rats on P2 (both stresses) and P15 (predator by itself), but not on P6. The controversy over days in this study might be due to differentially affected male and female pups. As illustrated in Fig. 2, density of NMDA receptors was higher in female pups than male ones on P2 in control group while it was the opposite in stressed pups where the value was significantly higher among male pups. These data indicated that male pups were affected more severely than females when their mothers were exposed to stress in late pregnancy. The present finding was consistent with results of previous investigations. In parallel to these data, it has been reported that, in the case of PS, male pups showed higher CBL, severe pilocarpine- and PTZ-induced seizure and higher mortality rate than females (Ahmadzadeh et al., 2011; Sadaghiani and Saboory, 2010). Mueller and Bale suggested that male fetuses were more sensitive to PS effects than female ones (Mueller and Bale, 2008), which could be due to activity and sensitivity of placental 11β-HSD2 that was sex-related and made male fetuses more exposed to stress hormones. Male fetuses expressed more glutamate receptors than females on the second day after partum; hippocampal glutamate receptors of the unstressed females were more than those of males on the same day (Burton and Waddell, 1994; Kerzner et al., 2002). In the present study, no significant difference was found between male and female pups in control and stressed groups on days 6 and 15 post-partum while there was a decreasing trend of NMDA receptor density with age from P2 to P15 in all the groups. Wenk et al. demonstrated reduced NMDA receptors in CA region of hippocampus with the age ranging from 3 to 31 months old (Wenk and Barnes, 2000). The present study was consistent with these investigations and showed reduced NMDA receptors in all three groups at a shorter time period.

5. Conclusion

Stressful events during gestation have important effects on excitatory NMDA glutamate receptors of the offspring which can affect many adulthood brain disorders such as epilepsy, schizophrenia and other psychotic disorders. Effects of stress on NMDA receptors were sex- and age-dependent, which affected male pups more severely than females at the very beginning of life after birth. Differential changes in CBL and density of NMDA receptors between male and female pups and their age-dependent trend might explain some of sex-specific behavioral responses of males and females later in life during the adulthood.

Conflicts of interest

The authors have no conflicts of interest to declare regarding the study described in this article and its preparation.

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