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INVOLVEMENT OF PGLUR1, EAAT2 AND EAAT3 IN OFFSPRING DEPRESSION INDUCED BY PRENATAL STRESS

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Abstract—It is widely known that prenatal stress (PS) exposure causes depression-like behaviour to offspring, as well as maladaptive responses including neurobiological and physiological changes. However, the underlying mechanism of PS induced juvenile-onset depression remains largely unravelled. The inadequacies of monoamine deficiency hypothesis, the emerging evidence of altered glutamate neurotransmission in mood disorders, as well as our previous studies inspired us to assess the potential role of glutamatergic system in the pathogenesis of juvenile depression. In this research, we examined the expression of phosphorylated GluR1 subunit of ionotropic receptor alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), the Na+-dependent glutamate transporters excitatory amino acid transporter 2 (EAAT2) and EAAT3 in the hippocampus, striatum and frontal cortex of 1-month-old rat offspring after mid and late PS exposure. Prenatally stressed offspring rats showed significantly prolonged duration of immobility and shortened immobility latency in tail suspension test. We also detected that PS significantly altered the expression of glutamate receptor and glutamate transporters of these depressed rats. In brief, the changes of phosphorylated GluR1 subunit of AMPAR protein level in the hippocampus and frontal cortex, as well as markedly decreased EAAT2 mRNA expression in the hippocampus, striatum and frontal cortex and EAAT3 mRNA expression in the hippocampus of stressed rats were both observed. These results underpinned that glutamate receptors and glutamate transporters might be involved in the progress of depression-like behaviour in juvenile rat offspring induced by PS.

Key words: prenatal stress, depression, pGluR1, EAAT2, EAAT3.

INTRODUCTION

Compelling evidence from epidemiological studies demonstrate that exposure to stressful events during pregnancy could raise the risk of psychiatric disorders, such as schizophrenia, attention deficit hyperactivity disorder (ADHD), depression and anxiety in the offspring (Bogoch et al., 2007; Field and Diego, 2008; Rice et al., 2010). Numerous animal studies have also reported that offspring of gestational stressed rodents showed increases in affective-related behaviour (Abe et al., 2007; Maccari and Morley-Fletcher, 2007; Weinstock, 2008). However, the underlying mechanism of how prenatal stress (PS) relates to the offspring depression remains largely unknown. The pathogenesis of depression is associated with a series of genetic, neurotransmitter disturbance, hormone dysregulation and diverse psychosocial factors (Nestler et al., 2002; Kalia, 2005; Krishnan and Nestler, 2008; Schroeder et al., 2011), among which monoamine deficiency is one of the broadly acknowledged hypothesis (Hammen et al., 2010; Uher and McGuffin, 2010; Carr and Lucki, 2011; Snyder, 2011). However, it hardly accounts for the delayed effect of the antidepressants like selective serotonin reuptake inhibitors (SSRIs) and serotonin norepinephrine reuptake inhibitors (SNRIs) (Cowen, 2008) as well as the unfavorable fact that less than 35% of patients reach full remission (Trivedi et al., 2008).

Recently a wealth of data has suggested that glutamatergic system played a pivotal role in major depression (MD) and bipolar disorder (BPD). L-Glutamate is the major excitatory acid neurotransmitter in the mammalian CNS, and in the meanwhile an important neurotoxin. It can cause deleterious effects including neuronal cell death when accumulated in the extracellular fluid at high concentration. Increased glutamate levels have been found in the hippocampus and amygdala in preclinical stress models (Paul and Skolnick, 2003; Reznikov et al., 2007) and in the frontal cortex from patients with BPD and MD (Hashimoto et al., 2007). Other research has reinforced this

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Abbreviations: AMPAR, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BPD, bipolar disorder; CON, control group; EAAT, excitatory amino acid transporter; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LPS, late prenatal stress group; MD, major depression; MPS, mid prenatal stress group; PBST, phosphate-buffered saline with 0.1% Tween-20; pGluR1, phosphorylated GluR1 subunit of AMPAR; PS, prenatal stress; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TST, tail suspension test.

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hypothesis by stating that there is also a positive correlation between plasma glutamate levels and the severity of depressive symptoms (Mitani et al., 2006). Our previous work also revealed that PS markedly elevated the glutamate level in the hippocampus and decreased the protein level of the NR1 subunit of *N*-methyl-D-aspartate receptor (NMDAR) of the one-month-old rat offspring (Jia et al., 2010). These findings drove us to focus on glutamatergic system to verify the mechanism of offspring depression-like behaviour induced by PS.

It is of note that glutamate can be regulated at different levels, such as the assembly into the synaptic vesicles, the release into synaptic cleft, and the uptake by the glial cells and neurons (Ontko et al., 2005). When released from presynaptic terminals, glutamate can activate postsynaptic glutamate receptors to mediate excitatory signalling (Weller et al., 2008), or be taken in via high affinity Na^+-dependent glutamate transporters—excitatory amino acid transporters (EAATs) which are located on the plasma membrane of the neuron and surrounding glia.

Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) is one of the major ionotropic receptor, and there are already a few indications that AMPA receptors may be critically involved in mood disorders (Alt et al., 2006; Bleakman et al., 2007). AMPAR has four different subunits, which are denominated as GluR1-4 or GluRA-D. Several lines of evidence have indicated the decreased level of the GluR1 subunit of AMPAR in the prefrontal cortex and striatum of subjects with MD and BPD (Meador-Woodruff et al., 2001; Beneyto et al., 2007). This is also in accordance with the transgenic animals study that GluR1 knock-out rats exhibited increased depressive-like behaviours (Chourbaji et al., 2008). Ser-845 phosphorylation was prime to AMPAR activity-dependent synaptic trafficking (Derkach et al., 2007). Study has convinced that PKA phosphorylation of GluR1 at Ser-845 site increases AMPA receptor cell surface expression as the result of a combination of increased receptor insertion and decreased internalization (Man et al., 2007). However, little is done about the phosphorylation state of AMPAR in prenatally stressed juvenile offspring rats.

EAATs play the predominant role in the reuptake of glutamate in the brain and maintain the proper concentration of glutamate level in the synaptic cleft. Five different glutamate transporters have been cloned in rodents to date: GLAST, GLT-1, EAAC1, EAAT4 and EAAT5. Their human homologs are: EAAT1, EAAT2, EAAT3, EAAT4 and EAAT5, respectively. All five transporters are localized in different ways in various brain regions. EAAT2 is the most abundant among the five, and locates on the membrane of the glia in the forebrain, hippocampus, cerebral cortex striatum, etc. While EAAT3 is widely distributed in the cerebellum and brain regions containing EAAT2, however, it is mainly expressed in postsynaptic neurons, especially the dendrite and the dendritic spines. Human post-mortem studies have revealed decreased EAAT1 and EAAT2 expression in the forebrain of patients with MD (Choudary et al., 2005), and decreased EAAT3, EAAT4 mRNA levels in the striatum (McCullumsmith and Meador-Woodruff, 2002). Functional genomics study of the depression patients also showed that the irregular expression of SLCA2 and SLCA3, the genes encoding EAAT2 and EAAT3, had close relationship with psychiatric disorders (Altar et al., 2008). Besides, chronic administration with Ceftriaxone, a β-Lactam antibiotic, has antidepressant-like effect to C57BL/6J mice, and this may occur through the up-regulation of the EAAT2 gene, which can then enhance the uptake of glutamate from synaptic cleft (Mineur et al., 2007). All these evidence have implied the potential regulating effects of EAATs in depression-like behaviour induced by PS.

Giving the close relationship between glutamate receptor, glutamate transporters and depressive disorder, we examined the changes of the main glial transporter EAAT2, the main neuronal glutamate transporter EAAT3 and the phosphorylation state of GluR1 subunit of AMPAR in the hippocampus, frontal cortex and striatum of offspring rats, thus to provide evidence that EAAT2, EAAT3 and phosphorylated GluR1 subunit of AMPAR (pGluR1) might be involved in offspring depression-like behaviour induced by PS. Since most individuals experience their first depression sometime during adolescence (Hankin, 2006), we tested the depression-like behaviour of 1-month-old rat offspring. The mid and late period during pregnancy is when stress can result in postpartum depressive-like behaviour (Smith et al., 2004; O'Mahony et al., 2006) in the dam and a period of stress that affects offspring outcomes (Darnaudéry and Maccari, 2008; Weinstock, 2008). To determine whether there was a critical time window of PS on behavioural responses and glutamatergic system, rats were exposed to restraint stress during the second or third gestational week.

EXPERIMENTAL PROCEDURES

Animals and procedures

All procedures were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animals Care and Use Committee at Xi'an Jiaotong University. All efforts were made to minimize the number of animals used and their suffering. Female Sprague–Dawley rats weighting 230–250 g and male Sprague–Dawley rats weighting 280–350 g were used. Animals were provided by the experimental animal centre of Xi'an Jiaotong University Medical College. The rats were housed in an animal room with controlled temperature (22–28 °C) and humidity (60%) on a 12-h-light/dark cycle (light on from 08:00 to 20:00) with free access to food and water. Virgin female rats were placed overnight with adult male rats (3:1) for mating. Vaginal smear was examined on the following morning (before 8:00). The day on which the smear was sperm positive was determined as embryonic day 0. Each pregnant rat was then housed separately. The pregnant rats were randomly assigned to mid prenatal stress

group (MPS, $n = 6$), late prenatal stress group (LPS, $n = 6$) and control group (CON, $n = 6$).

The pregnant rats of MPS and LPS group were separately exposed to restraint stress on days 7–13, 14–20 of pregnancy three times daily for 45 min (Zhu et al., 2004; Viltart et al., 2006). To prevent habituation of animals to the daily procedure, restraint periods were randomly shifted within certain time periods (08:00–10:00, 11:00–13:00, and 15:00–17:00). The restraint device was a transparent plastic tube (6.8 cm in diameter) with air holes for breathing and closed end. The length could be adjusted to accommodate the size of the animals. The pregnant rats of the CON were left undisturbed. On postnatal day 21, after all offspring were weaned, male and female pups were separated and housed four in each cage, respectively until testing at 1 month of age. At the end of postnatal day 30, two male and two female offspring rats from the same biological mother were selected with a random choice in three experiments.

Tail suspension test (TST)

Ten male and 10 female rats from each group were used for TST. The TST was performed according to the procedure described by Steru with modifications (Steru et al., 1985; Chermat et al., 1986; Izumi et al., 1997). One-month-old SD rat was hung for 6 min by the tail (approximately 2 cm from the tip of the tail) using a clamp covered with sponge which is bound to a metal rod. The rod was fixed 65 cm above the floor in a sound-isolated room. The head of the rat was about 20 cm beyond the floor and the rats were placed in a space surrounded by a V-shaped smooth slope on which the rats place their forepaws in order to alleviate their suffering. All the movements of the rat were recorded by a video camera in front of the rat and the total duration of immobility and the latency of immobility were calculated by a trained observer blinded to the groups. Rats were considered immobile only when they were totally motionless. All the tests were conducted between 10:00 and 16:00.

Western-blot

Brain sample preparation. At 1 month age, six male and six female rats which did not experience TST from MPS, LPS and CON group were all decapitated. The hippocampus, striatum and frontal cortex were rapidly dissected on ice and then frozen in liquid nitrogen for 30 min and then conserved in −80 °C refrigerator.

Brain tissue was homogenized in 1000 μL ice-cold Tris–HCl buffer (10 mM, pH 7.4), containing 1% sodium dodecyl sulfate (SDS), 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma, Santa Clara, CA, USA), 1× phosphatase inhibitor cocktail Tablets (Fermentas, Canada) (B1). Subcellular fraction was performed according to pervious description (Yaka et al., 2003). The tissue homogenates were centrifuged at 1000*g* for 10 min at 4 °C to remove nuclei and large debris (P1). The supernatant (S1) was then transferred to another tube and centrifuged again at 18,000*g* for 30 min at 4 °C to obtain a clarified fraction of cytosolic and light membrane fraction (S2) and a pellet corresponding to the crude synaptosomal fraction (P2), which was then resuspended in the same buffer and centrifuged at 25,000*g* to obtain a synaptosomal membrane fraction (LP1). Finally, the pellet was resuspended in 100 μL the same buffer (B1) added with 320 mM sucrose. Protein concentrations were determined using a Nano Drop ND-1000 spectrophotometer (Nano Drop, USA). The protein was conserved in a sterile 1.5 mL Eppendorf tube in −80 °C refrigerator. Before loading the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel, 25 μL 5× loading buffer was added into each sample protein and then denaturized in 60 °C water-bath for 1 h.

Western-blot. One hundred and seventy five micrograms of each sample protein was separated by SDS–PAGE electrophoresis using a 10% running gel and then transferred to 0.45 μm polyvinylidene difluoride (PVDF) membrane (Millipore, Boston, MA, USA). The membranes were then incubated for 2 h at room temperature in 5% free-fat dry milk in phosphate-buffered saline with 0.1% Tween-20 (PBST). Immunoblotting was carried out overnight at 4 °C with the primary antibodies against phospho Ser845-GluR1 (1:1000, ab3901, Abcam, England) in PBST buffer. The membranes were stripped three times with PBS-Tween-20 0.1% and incubated with appropriate HRP-conjugated goat anti-rabbit secondary antibody (1:8000, Pioneer, China). After three times of rinsing in PBST buffer, immunoreactivity was detected with an ECL Western Blotting Detection Kit (Millipore, Boston, MA, USA). Results were standardized to β-actin control protein, which was detected by evaluating the band density at 43 kDa after probing with a mouse anti-rat polyclonal antibody (1:10,000, Pioneer, China). The lanes were calculated by the Quantity One software.

RNA isolation and RT-PCR

Preparation of the hippocampus, striatum and frontal cortex. At 1 month age, six male and six female rats that were not participated in TST from three groups were anesthetized with urethane and were decapitated. The hippocampus, striatum and frontal cortex were separated from the brain quickly and were frozen at liquid nitrogen for 30 min and then transferred into −80 °C refrigerator.

RNA isolation. The tissues were homogenized in the 0.9% RNAase-free saline and the total RNA was extracted by the RNAfast 200 RNA kit (XianfengBiotech, China) in line with the manufacturer's protocol. To verify the quality of RNA, parallel samples were loaded onto the agarose gel stained with ethidium bromide. Only samples with clearly defined 18S and 28S RNA peaks were used in the study.

RT-PCR. Reverse transcription. One thousand nanograms of RNA from each sample was reverse transcribed, and the reverse transcription reaction was performed using RevertAid First Strand cDNA Synthesis Kit (#K1621, #K1622, Fermentas, Canada). Briefly, the reaction was performed in the GeneAmp PCR System 9700 (Bio-Rad, Hercules, CA, USA) at 30 °C 10 min, 42 °C 45 min, 95 °C 5 min, and then cooled down to 4 °C.

PCR and gel electrophoresis. The PCR was performed in the GeneAmp PCR System 9700 (Bio-Rad, USA) and reaction mixes included 2–3 μL cDNA, 12.5 μL 2× Master Mix, 1.0 μL forward and reverse primer, and total reaction volume is supplemented by ddH_2O to 25 μL. The thermal cycling programme consisted of 5 min at 94 °C, followed by 20–25 cycles of 30 s at 95 °C, 45 s at 57 °C (for EAAT2) or 56 °C (for EAAT3) or 58 °C (for glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) and 1 min at 72 °C, and then 6 min at 72 °C and cooled down to 4 °C. The sequences for PCR primers are shown in Table 1.

To quantitate the levels of mRNAs for the two transporters, the RT-PCR products were loaded onto an ethidium bromide-stained 2.0% agarose gel and the bands corresponding to EAAT2, EAAT3 and GAPDH were quantified by densitometry. The lanes were calculated by the Quantity One software.

Statistics

All values reported were represented as mean ± SEM. Offspring data analyses were explored by a two-way analysis of variance (ANOVA) (stress × sex) using the software SPSS 18.0. The main effects of the PS (MPS, LPS and CON,) and sex (female and male), as well as the interaction between PS and sex were analysed. When appropriate, post hoc least significant difference (LSD) tests were used to analyse the multiple comparisons. A difference was considered significant at $P < 0.05$ level.

RESULTS

PS induced behavioural despair in TST to 1–month-old rat offspring

There were no differences in the litter size or sex distribution of the litters among stress and control group. The effects of late and mid PS on depression-like behaviour in TST rats are shown in Fig. 1. The immobility time was significantly affected by PS ($F(2,57) = 10.053$; $P < 0.0001$), but not by sex ($F(1,58) = 0.291$; $P > 0.05$) and the interaction between sex and PS treatment ($F(2,54) = 0.291$; $P > 0.05$). Post hoc test revealed that both male and female juvenile rat offspring of LPS and MPS groups spent significantly more time immobile compared to the CON group ($P < 0.05$, $P < 0.05$) (Fig. 1a). Besides, a significant main effect of PS ($F(2,57) = 4.484$; $P < 0.01$) was also found on the latency of immobility in the TST, and while no significant effects of sex ($F(1,58) = 7.887$; $P > 0.05$) and the interaction between sex and PS treatment ($F(2,54) = 0.291$; $P > 0.05$) (Fig. 1b). This was due to the significantly reduced latency of immobility of latency in MPS group ($P < 0.01$). These results showed that both MPS and LPS can induce behaviour despair in TST in juvenile rat offspring.

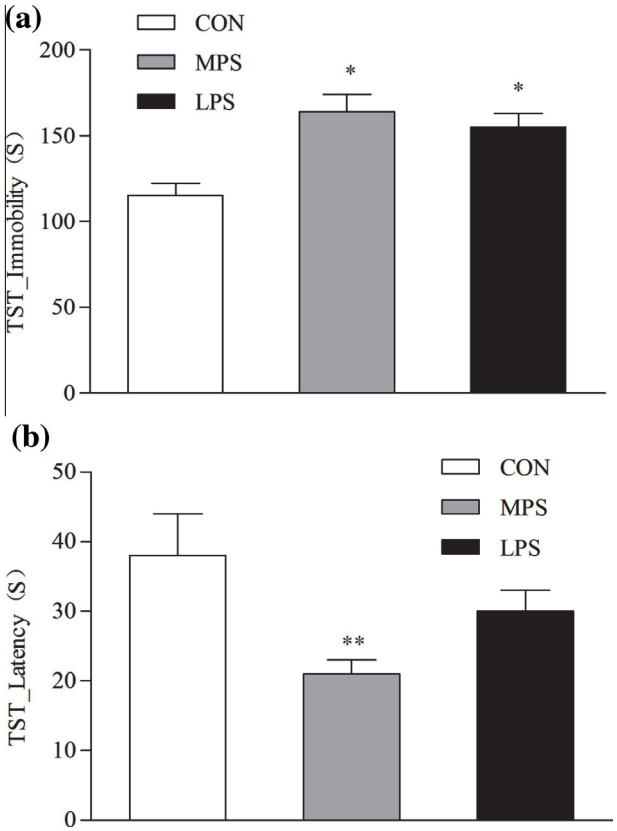

Fig. 1. Effects of prenatal stress in the tail suspension test of 1-month-old rat offspring. Immobility time and latency of immobility, shown by control (CON) and MPS and LPS offspring rats at 1 month of age ($n = 20$, 10 males and 10 females). Data are expressed as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. CON.

PS altered pGluR1 expression in both the hippocampus and frontal cortex of juvenile rat offspring

In order to investigate the influence of PS on glutamate transmission in juvenile offspring rats, we first analysed

Table 1. The forward and reverse primers of EAAT2, EAAT3 and GAPDH

	Forward primer (from 5′ to 3′)	Reversed primer (from 5′ to 3′)	Sequence length (bp)
EAAT2	TTTGCCTGTCACCTTCCG	GACTGCGTCTTGGTCATT	462
EAAT3	GCAATCCACTCCCTCGTA	ACCTGCTCCAGCTCCTTC	539
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG	496

the phosphorylation state of GluR1 subunit of AMPAR, which is critical for AMPAR trafficking. In the hippocampus, pGluR1 (Ser845) expression was significantly affected by PS ($F(2,33) = 6.895$; $P < 0.05$), but not by sex ($F(1,34) = 2.373$; $P > 0.05$) and the interaction between stress and sex ($F(2,30) = 2.969$; $P > 0.05$). As shown in Fig. 2a, PS significantly attenuated pGluR1 (Ser845) level of MPS group ($P < 0.05$). The reduction of pGluR1 might inhibit the trafficking of AMPAR to the postsynaptic membrane, thereby affecting the availability of AMPAR. While in the striatum, pGluR1 (Ser845) levels were not altered either by prenatal manipulation ($F(2,30) = 0.241$; $P > 0.05$) or by sex ($F(1,34) = 0.338$; $P > 0.05$) (Fig. 2b). Besides, pGluR1 levels in the frontal cortex manifested a significant main effect of sex ($F(1,34) = 5.608$; $P < 0.05$), whereas no effects of PS ($F(2,33) = 0.246$; $P > 0.05$) and the interaction between stress and sex ($F(2,30) = 0.047$; $P > 0.05$). This was due to the significant difference between female and male in both LPS and MPS groups ($P < 0.05$).

PS reduced EAAT2 and EAAT3 mRNA expression in the hippocampus, striatum and frontal cortex of juvenile rat offspring

To further explore the alteration of glutamate transporters in prenatally stressed rats, we examined EAAT2 and EAAT3 mRNA expression in the hippocampus, striatum and frontal cortex. The results showed significant reductions of EAAT2 and EAAT3 mRNA expression, which implied the anomaly of glutamate reuptake.

Firstly, the main glial glutamate transporter EAAT2 mRNA expression in the hippocampus revealed a significant main effect of PS ($F(2,33) = 7.848$; $P < 0.05$), whereas no significant effects of sex ($F(1,34) = 1.185$; $P > 0.05$) and the interaction between stress and sex ($F(2,30) = 0.022$; $P > 0.05$). As shown in Fig. 3a, mid and late prenatal manipulation significantly decreased EAAT2 expression of both male and female juvenile rat offspring ($P < 0.01$, $P < 0.01$). Besides, EAAT2 mRNA expression in the striatum was significantly affected by PS ($F(2,33) = 3.328$; $P < 0.05$), but not by sex ($F(1,34) = 1.960$; $P > 0.05$) and the interaction between stress and sex ($F(2,30) = 2.523$; $P > 0.05$). Fig. 3b indicates EAAT2 level in the striatum of both female and male juvenile rat offspring in the LPS group was significantly reduced compared to CON ($P < 0.05$) group and MPS ($P < 0.05$) group. Furthermore, a significant main effect of PS ($F(2,33) = 8.238$; $P = 0.001$) on EAAT2 mRNA expression was also tested in the frontal cortex, but not sex ($F(1,34) = 3.544$; $P > 0.05$) and the interaction between stress and sex ($F(2,30) = 0.024$; $P > 0.05$). This was due to the significant reduction in the LPS group compared to the CON group ($P < 0.01$) (Fig. 3c).

The evaluation of the neuronal glutamate transporter EAAT3 mRNA expression in the hippocampus revealed a significant main effect of PS ($F(2,33) = 3.386$; $P < 0.05$), but not sex ($F(1,34) = 0.765$; $P > 0.05$) and the interaction between stress and sex ($F(2,30) = 0.250$; $P > 0.05$). Specifically, this was due

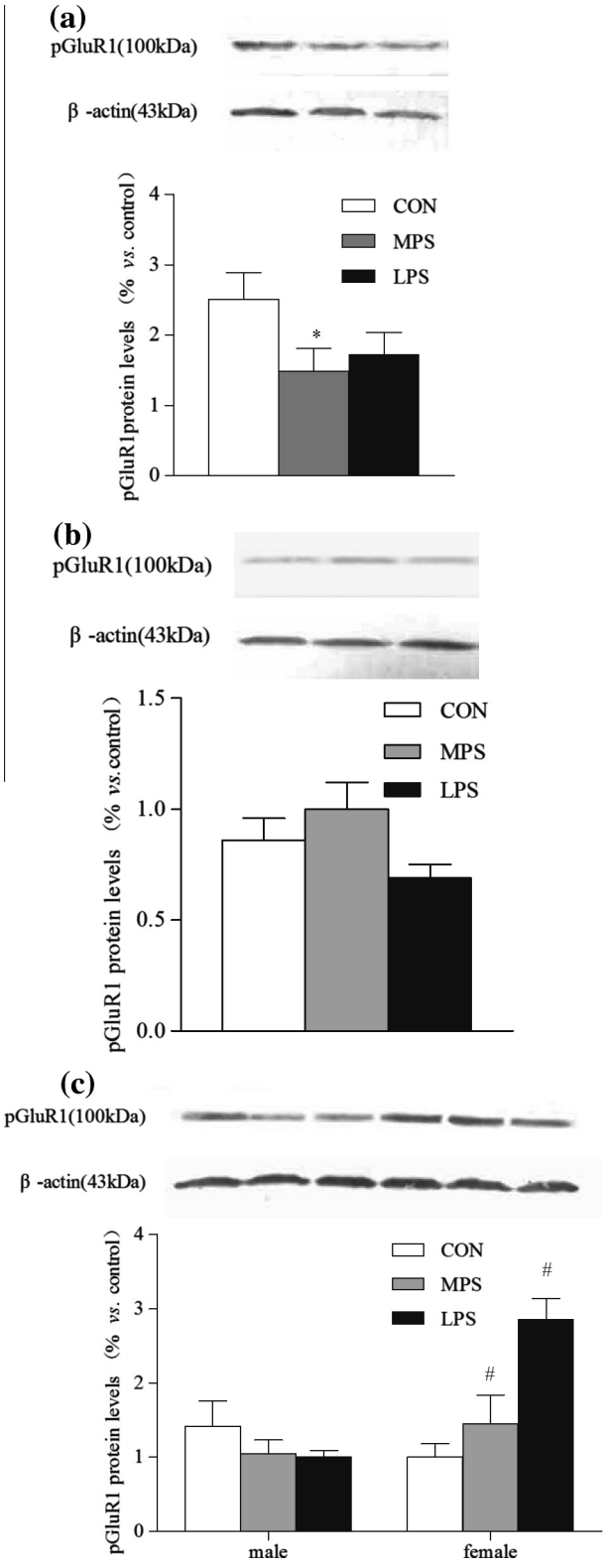

Fig. 2. Effects of prenatal stress on the phosphorylation of ionotropic glutamate receptor AMPAR GluR1(Ser-845) subunit, assessed in the hippocampus (a), striatum (b) and frontal cortex (c) of male (left panel) and female (right panel) rats at 1 month of age. The results, expressed as % of control/unstressed rats, represent the mean ± SEM of 6 (for frontal cortex) or 12 (for hippocampus and striatum, six males and six females) independent determinations. Data are expressed as mean ± SEM. $^{*}P < 0.05$ vs. CON, $^{\#}P < 0.05$ vs. male.

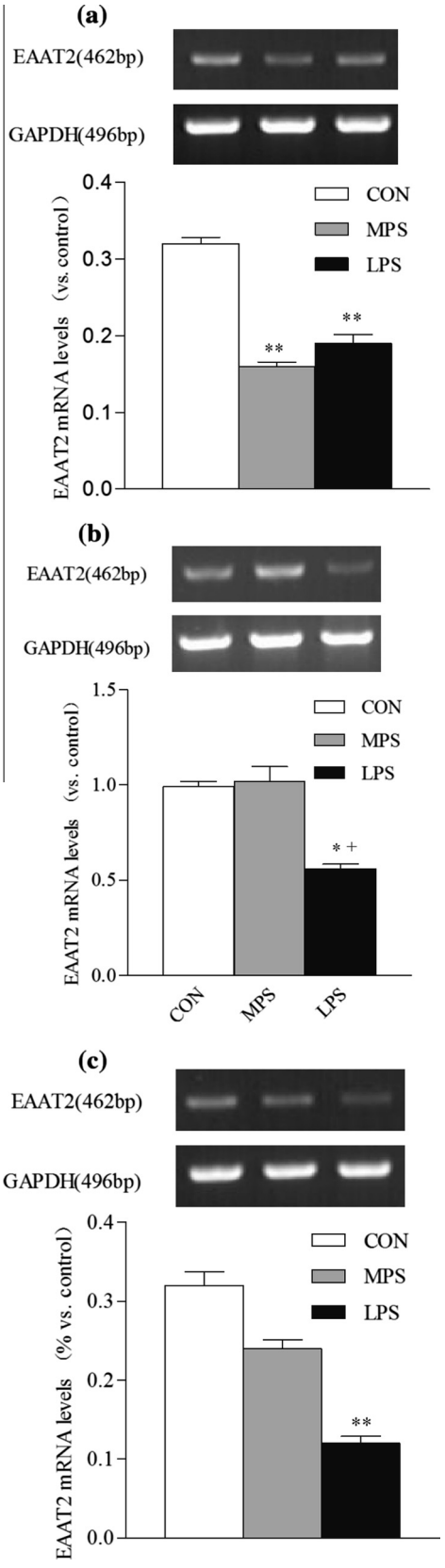

Fig. 3. Effect of prenatal stress on the EAAT2 mRNA expression assessed in the hippocampus (a), striatum (b) and frontal cortex (c) of offspring rats at 1 month of age. The results, expressed as % of control/unstressed rats, represent the mean ± SEM of 12 independent determinations (six males and six females). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. CON, $^{+}P < 0.05$ vs. MPS.

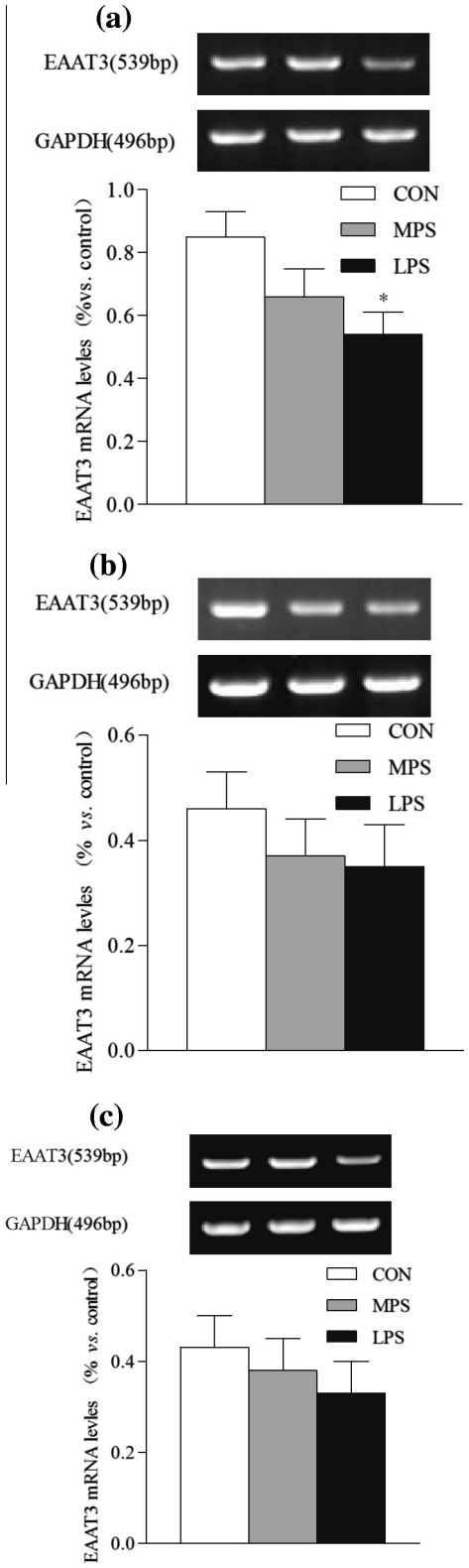

Fig. 4. Effects of prenatal stress on the EAAT3 mRNA expression assessed in the hippocampus (a), striatum (b) and frontal cortex (c) of offspring rats at 1 month of age. The results, expressed as % of control/unstressed rats, represent the mean ± SEM of 12 independent determinations (six males and six females). $^{*}P < 0.05$ vs. CON.

to the significant reduction of the LPS group compared to the CON group ($P < 0.05$) (Fig. 4a). Besides, we did not

observe a significant main effect of sex ($F(1,34) = 0.828$; $P > 0.05$) and PS ($F(2,33) = 0.656$; $P > 0.05$) as well as the interaction between stress and sex ($F(2,30) = 1.308$; $P > 0.05$) on EAAT3 mRNA level in the striatum. (Fig. 4b). In the frontal cortex, EAAT3 mRNA expression were neither effected by PS ($F(2,33) = 0.576$; $P > 0.05$), sex ($F(1,34) = 2.972$; $P > 0.05$) nor the interaction between PS and sex ($F(2,30) = 0.139$; $P > 0.05$). (Fig. 4c). Thus we predicted that the decline of EAAT2 expression and in the three brain region and EAAT3 reduction in the hippocampus might lead to the accumulation of glutamate in the synaptic cleft. These results also indicated the complexity of the impact of PS on EAAT2 and EAAT3 mRNA expression.

DISCUSSION

The results of our present study demonstrate that prenatal restraint stress induced depression-like behaviour to 1-month-old rat offspring, and this may relate to the attenuation of EAAT2 and EAAT3 as well as pGluR1 variation in the hippocampus, striatum and frontal cortex. Our findings provide evidence that aberrations in the glutamatergic system are potentially involved in the behavioural alterations in offspring arising from PS exposure.

Both male and female juvenile offspring rats exposed to mid and late prenatal restraint stress exhibited maladaptive behaviour in TST, including prolonged immobility time and shortened immobility latency. This was consistent with our previous finding that PS induced behavioural despair in forced swim test (Song, 2012). Szymanska M. and colleagues also certificated that 21-day-old male rat offspring exhibited prolonged immobile time in forced swim test when exposed to PS (Szymanska et al., 2009). Furthermore, research has discovered that prenatal restraint stress produced an increase in immobility time in female mice offspring (Alonso et al., 2000). Our results are also in accordance with the study which stated that animal born from pregnant rats subjected to restraint stress showed increased immobility duration in the constrained swim test, which is an experimental model of depression (Drago et al., 1999). All of these together allow us to conclude that PS could cause depression-like behaviour to offspring rats.

Based on the hypothesis that glutamate signalling might play a role in the behavioural despair of the stressed rat offspring, we conducted our molecular manipulation of glutamate receptors and transporters. It has been reported that PS causes no effect on the total level of GluR1 in the hippocampus and frontal cortex of male offspring (Fumagalli et al., 2009). These implied that PS might cause the changes of GluR1 phosphorylation. GluR1 subunit has two distinct phosphorylation sites, one at Ser831 site and another at Ser845 site (Roche et al., 1996). Previous study showed that antidepressant fluoxetine can increase phosphorylation of the AMPAR subunit GluR1, preferentially at the Ser-845 PKA site (Svenningsson et al., 2002). It has also been reported that the phosphorylation of Ser845-GluR1 accompanies surface insertion of AMPARs receptors at extra synaptic sites, which provides a pool of receptors available for long-term potentiation (LTP) induction (Oh et al., 2006). Indeed, our results indicated that exposure to mid prenatal restraint stress caused a decreased phosphorylation level of GluR1 at Ser-845 site in the hippocampus of both male and female rat offspring, which implicated that PS might inhibit the trafficking of AMPAR from cytoplasm to synapses, thus to decline AMPAR activity. So we speculated that the decrease of Ser-845 phosphorylation of GluR1 may inhibit the trafficking of AMPAR to postsynaptic membrane, and then partially affect the binding of glutamate to its receptor. Our results also showed the increase of pGluR1 in the female frontal cortex, although slight, as well as significant sex discrepancy of pGluR1 expression in this brain region. The differences may be due to: firstly, oestrogen in female rats might influence the activity of AMPAR in the frontal cortex (Cyr et al., 2001), which provided a protection to PS; secondly, these brain regions might play a different role in the pathophysiological processing of depression. As seen from the review of Jonathan Savitiz and Wayne C. Drevets that there is a loss of cortices (especially mPFC) control over limbic structures such as the hippocampus and amygdala; or alternatively, the disinhibited limbic drive which overrides cortical regulation in the regulation of affective illness (Savitz and Drevets, 2009).

Post-mortem studies of BPD and MD patients had verified reduced EAAT3 and EAAT4 transcripts expression (McCullumsmith and Meador-Woodruff, 2002), and this is also supported by Zinc's study that EAAT2 mRNA was attenuated in the hippocampus and cortex of male rat of learned helpless animal models, an animal model of depression (Zink et al., 2010). These findings inspired us that the glutamate transporters might also be involved in PS induced depression-like behaviour in offspring rats. Our research demonstrated significantly reduced EAAT2 mRNA level in the hippocampus, striatum and frontal cortex of both male and female rats of stress group. Significantly reduction of EAAT3 mRNA expression was also found in the hippocampus of the LPS group. This is also consistent with the study which demonstrated that EAAT2 gene knock-out mice showed a significant reduction of the ability to reuptake glutamate, and the enhancement of the sensitivity to stress stimulus (Robinson, 1998). Moreover, EAAT2 mRNA reduction was also in accordance with the finding that PS induced glial cell reduction in animal models and human post-mortem research (Lee et al., 2007; Behan et al., 2011). We thus inferred that the decrease of EAATs level can result in the decrease of glutamate reuptake and then potentially induce the accumulating of glutamate in the synaptic cleft, which might be a risk factor of the increase of synaptic glutamate level.

Recent research disputed a lot about the time window of offspring depressive behaviour. Mueller and Bale argued that early PS showed maladaptive stress

responsivity in TST and sucrose preference test, while mice exposed to mid and LPS have no effect on offspring depression-like behaviour (Mueller and Bale, 2008). Other research showed stress during late gestation caused behavioural despair to offspring rats (Morley-Fletcher et al., 2003). In the present study, we did not find a significant difference between LPS and MPS groups of behavioural responses and glutamatergic system, except for reduced EAAT2 mRNA expression in the striatum in the LPS group compared to the MPS group. Such disagreement might be due to the intensity and duration of stress and the animal species.

Among the three molecules, EAAT2 is the predominant glutamate transporter and is in charge of 90% glutamate reuptake, and EAAT3 expression at postsynaptic membrane is 15 times the density of AMPA receptors (Meldrum, 2000; Choudhury et al., 2012). Our results also showed that EAAT2 mRNA reduction is more distinct than EAAT3. So we speculate that EAAT2 might play a more crucial role among the three molecules examined in the process of depression-like behaviour.

Finally, it is important to note that the changes of pGluR1, EAAT2 and EAAT3 are not fully consistent with the behavioural changes in TST, the underlying reasons might be that other glutamate receptors or glutamate transporters might also be involved in regulating the glutamate level in the synaptic cleft besides the molecules that we examined, and different brain regions also have different responsiveness to the PS. Besides, except for the sex difference on pGluR1 level in the frontal cortex in stress group, we did not find any other significant sex difference on the EAAT2, EAAT3 and pGluR1 expression, which is in line with our behavioural tests. We guess that some other factors might be also involved in the impact of pGluR1 on depression-like behaviour induced by PS. In other words, the variations of pGluR1 in frontal cortex were not sufficient to cause sex difference on offspring depression-like behaviour.

CONCLUSION

Our results indicate that mid and late gestational stress can induce depression-like behaviour to juvenile rat offspring, and the decrease of EAAT2 and EAAT3 as well as pGluR1 variation might be involved in depression symptoms. However, further research needs to be done on the other glutamate receptors and its downstream signalling pathways such as extracellular regulated protein kinases (ERK)–mitogen-activated protein kinase (MAPK)–cAMP response element-binding protein (CREB) signalling to confirm that glutamatergic abnormity might participate in the juvenile depressive behaviour induced by PS.

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