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PKA-mediated phosphorylation of CREB and NMDA receptor 2B in the hippocampus of offspring rats is involved in transmission of mental disorders across a generation

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ABSTRACT

This study is aimed at the mechanism of transmission of mental disorders across a generation. We used 10 different stressors to establish an animal model of chronic unpredictable stress (CUS) before pregnancy. Forced swimming test (FST) and open field test (OFT) were used to analyze the behavior of 30-day-old adolescent offspring rats born to stress mothers. Magnetic resonance spectroscopy was used to measure glutamate, gamma-aminobutyric acid (GABA), and glutamine. Phosphate-activated glutaminase (PAG), glutamate decarboxylase (GAD), GABA-transaminase (GABA-T), protein kinase A (PKA), cAMP response element-binding protein (CREB), and N-methyl-D-aspartate (NMDA) receptor 2B (NR2B) were detected by western blot. Adolescent offspring rats in the CUS group exhibited depressive-like behavior in the FST and anxious behavior in the OFT. GAD was increased and GABA-T was decreased, which resulted in an increase in GABA levels and decrease of the glutamate/GABA ratio in the hippocampus of CUS offspring rats. Disruption of the glutamate/GABA–glutamine cycle was related to decrease PKA-mediated phosphorylation of CREB and NR2B in the hippocampus. These findings highlight the importance of mental health of females before pregnancy and suggest that CUS before pregnancy reduces p-CREB and p-NR2B in the offspring hippocampus, which could be responsible for behavioral disorders in the adolescent offspring.

1. Introduction

Increasing evidence indicates that maternal stress may increase the risk of behavioral disorders in offspring (Barbie-Shoshani et al., 2016; Gur et al., 2017; Scheinost et al., 2017). Most of the studies focused on the effects of stress exposure during pregnancy on the offspring. The fetus receives signals of maternal stress. Depending on the timing of these exposures, more or less persisting effects occur on tissue structure and function, brain development, and metabolism (De Weerth, 2018). In fact, female stress exists in daily life outside of pregnancy. There are currently few experiments that examine the effect of stress before pregnancy on the offspring. In a prior study, we found that female rats, suffering from chronic unpredictable stress (CUS) before pregnancy, display increased corticotropin-releasing hormone (CRH) and corticosterone (COR) levels during pregnancy (Huang et al., 2013) compared with a non-CUS group. High levels of glucocorticoids (GCs) in pregnant mothers could affect gene programming of the hypothalamus-pituitary-adrenal (HPA) axis through the maternal-placental-fetal interface, which could lead to abnormal regulation of the HPA axis in the offspring (Moisiadis and Matthews, 2014). The HPA axis is modulated by complex feedback mechanisms involving local hypothalamic inhibitory and excitatory systems, as well as projections from stressor-sensitive brain regions, such as the hippocampus (Herman et al., 2016). These stressor-sensitive regions can modulate corticotropin-releasing-factor release through interaction with gamma-aminobutyric acid (GABA)-, glutamate- and peptide-containing neurons located in the hypothalamus (Herman et al., 2016). We recently discovered that offspring rats, whose mothers were exposed to CUS before pregnancy, are more vulnerable to stressful conditions in adolescence, based on abnormal behavior in the force swimming test (FST). However, the mechanism of transmission of mental disorder across generations is unknown.

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Many studies have highlighted the central role of the glutamate/GABA–glutamine cycle in the modulation of stress responses (Hasler et al., 2009). Most glutamate is converted to glutamine by glutamine synthase, and then glutamine is subsequently transferred to the neuronal compartment and converted to glutamate by phosphate-activated glutaminase (PAG) (Adrover et al., 2015). GABA is synthesized in the presynaptic terminals and cell bodies of GABAergic interneurons from glutamate by the action of glutamate decarboxylase (GAD), which modulates a variety of physiological and pathological conditions (Muller et al., 2014). One way of inactivating GABA in the brain is conversion to succinic semi-aldehyde and glutamate by GABA-transaminase (GABA-T) in neuronal mitochondria (Radley et al., 2009). The N-methyl-D-aspartate (NMDA) receptor 2B (NR2B) is a glutamate-gated ion channel strategically positioned to play a crucial role in the regulation of synaptic function in the hippocampus (Gray et al., 2011). In a prior study, we found that stress before pregnancy leads to both decreased learning and memory, in the water maze test, and decreased NR2B protein levels in the hippocampus of offspring rats (Huang et al., 2010). Synaptic NR localizes to the postsynaptic density where they are structurally organized and spatially restricted in a large macromolecular signaling complex comprised of scaffolding and adaptor proteins, which physically link receptors to kinases, phosphatases, and other downstream signaling proteins (Sheng and Hoogenraad, 2007). Protein kinase A (PKA) is a major kinase known to regulate NR trafficking and gating. At synaptic sites, PKA is kept in close proximity to NRs (Sanderson and Dell'Acqua, 2011). Phosphorylation of ser1166 on NR2B by PKA is critical for Ca^{2+} signaling in spines (Murphy et al., 2014). PKA is activated by cAMP production and translocates to the nucleus where it phosphorylates cAMP response element-binding protein (p-CREB), which activates transcription of target genes in response to a diverse array of stimuli, such as peptide hormones and neuronal activity (Halt et al., 2012).

Therefore, we hypothesized that disruption of the glutamate/GABA – glutamine cycle, abnormal expression of PAG, GAD, and GABA-T, and alteration of PKA-mediated phosphorylation of CREB and NR2B may be involved in transmission of mental disorders across a generation. In this study, we used FST and open field test (OFT) to examine the effect of CUS before pregnancy on the behavioral response of adolescent offspring rats. In addition, we use magnetic resonance spectroscopy (MRS) to measure the levels of glutamate, GABA, and glutamine in the hippocampus, and western blotting to detect the protein expressions of PAG, GAD, GABA-T, PKA, p-CREB, and p-NR2B in the hippocampus in order to identify changes by which pre-pregnancy stress could increase risk of behavioral disorders in adolescent offspring.

2. Materials and methods

2.1. Statement of ethics

All animal experiments were reviewed and approved by the Medical Animal Care and Welfare Committee of Shantou University Medical College (Shantou, China). All studies were carried out in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23 revised 1996). Every effort was made to minimize the number of animals used and to reduce suffering.

2.2. Establishment and assessment of the animal model of CUS before pregnancy

An animal model of CUS for female rats was established and assessed. Twelve female SD rats (approximately 8 weeks old; 200 g–250 g) were provided by the Animal Center of Shantou University. Before the beginning of any stress procedures, rats were randomly assigned into the following groups: control ($n = 6$) and CUS ($n = 6$). Rats were housed singly in nontransparent cages (60 cm × 40 cm × 25 cm) in a separate room under controlled 12 h light/12 h dark conditions (lights on at 8:00 AM) and temperature (24 °C). All rats had free access to food and water throughout the experiment, unless specified by the experimental procedure. The CUS procedure included 10 different stressors and lasted 3 weeks. The stressors were comprised of food or water deprivation for 24 h, cage tilting at a 45° for 24 h, swimming in cold (4 °C) water for 5 min, electric foot stimulation (1.0 mA, each time for 1 s, 10 times per minute) for 5 min, cage rocking (5 times per second) for 15 min, lights on overnight, elevation of temperature to 45 °C for 15 min, restraint for 12 h, and a soiled cage (250 ml of tap water into the sawdust bedding) for 24 h followed by cage cleaning. Each day, one stressor was selected randomly and executed at 9:00 AM. Consecutive selection of the same stressor was avoided. We used an open field test (OFT) and sucrose intake test (SIT) to assess the effectiveness of the CUS model. OFT was performed before (W0) and after (W3) establishment of the CUS model. Sucrose intake and body weight of rats were measured before the start of the CUS procedure (W0) and once a week (W1, W2, W3) during the CUS procedure. The CUS, OFT, and SIT were performed as previously described (Huang et al., 2012).

2.3. Pregnancy of female rats

When the CUS procedure was finished (24 h after the last stressor), all female rats were housed in pairs with a male rat for 1 week of mating. The day sperm was observed in vaginal smears was designated as embryonic day 0. The mean time from stopping the CUS procedure to pregnancy (embryonic day 0) was 2.5 days. All rats became pregnant. Nest material was provided for the gestational rats, which were housed singly and undisturbed. There were 64 offspring (30 females and 34 males) from mother rats of the control group and 67 offspring (34 females and 33 males) from the CUS group. In order to minimize the number of animals used and to reduce suffering, we randomly selected 36 offspring from each group (3 females and 3 males from each mother) for the following experiments.

2.4. Use of FST and OFT to assess the behavior of adolescent offspring

The behavior of offspring was assessed by FST and OFT when they were 30 days old. Adolescent rats were individually placed in a cylindrical tank measuring 60 cm × 38 cm. The tank was filled with water (24 ± 1 °C) to a height of 40 cm. Animals were forced to swim for 15 min (pre-test) and 24 h later they were subjected to a 5 min swimming (test). The total duration of floating and active behavior (swimming or climbing) was measured during the test. After the FST, offspring were removed from the tank, carefully dried in heated cages and then put back into their home cages.

Twenty-four hours after the FST, offspring were assessed by the OFT. The open field consisted of a square arena (60 cm × 60 cm), with a white floor divided into 36 squares (10 cm × 10 cm), enclosed by 25 cm-high walls made of black plastic. In this test, the 20 squares adjacent to the wall represented a protected field, named the “peripheral arena”, and the other 16 squares represented an exposed field or “central arena”. The test was initiated by placing a single rat in the middle of the arena and letting it move freely for 5 min. Offspring movement was continuously videotaped by a video camera placed over the structure and then recorded using a continuous sampling method. Videotapes were analyzed by a video tracking system (Ethovision, Noldus Information Technology, Netherlands). The arena was carefully cleaned with alcohol and rinsed with water after each test.

2.5. Measurement of glutamate, GABA, and glutamine levels by MRS

Twenty-four hours after OFT assessment, offspring were anesthetized with phenobarbital by intramuscular injection in the left leg (160 mg/kg), and placed in a prone position for MRI scanning (7T/160/

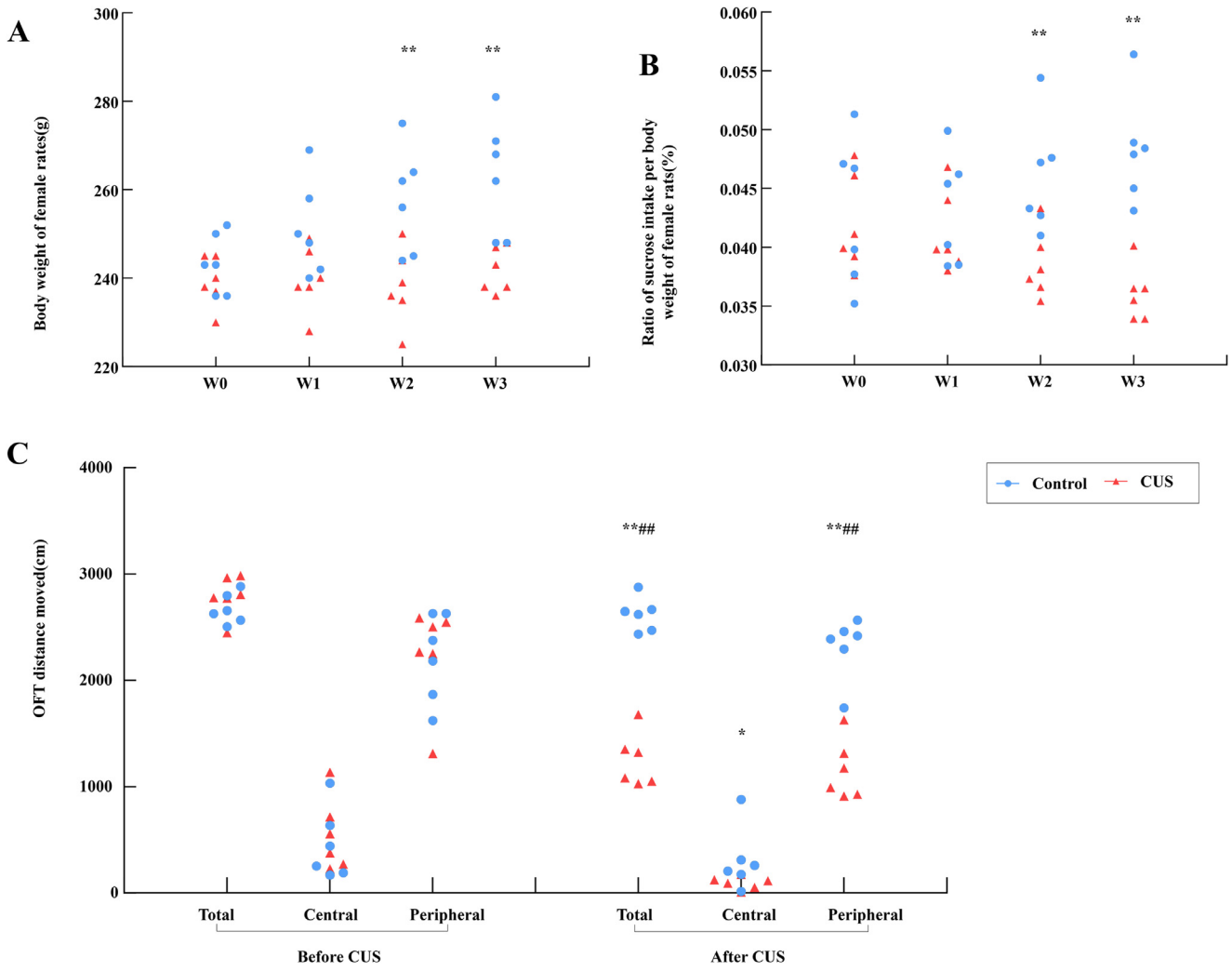

Fig. 1. *Effect of CUS on body weight, ratio of sucrose intake per body weight, and OFT movement of female rats prior to insemination.*
Body weight (A) and sucrose intake/body weight (B) of female rats from week 0 to week 3, and movement of female rats in the OFT (C) before and after CUS are shown in Fig. 1. Data represent the mean ± S.D. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared with the control group in the same week. ## indicates $p < 0.01$, the movement of female rats of CUS group in the OFT after CUS compared with that before CUS.

AS small animal imaging system, Agilent Technologies, CA) with a 9563 quadrature volume coil. The room temperature of the scanning process was controlled in a range from 20 to 22 °C, and the breathing rate of the animal was 65–75 times/min, as monitored using an MRI-compatible small animal monitoring system (SA Instruments, U.S.). MRS was performed as previously described (Huang et al., 2016).

2.6. Protein expression of PAG, GAD, GABA-T, p-NR2B, PKA, and p-CREB in the hippocampus of offspring rats was detected by western blot

Offspring hippocampi were frozen in liquid nitrogen and stored at −70 °C until western blot analysis. Brain tissue was homogenized in cold RIPA lysis buffer (Beyotime, China) and the protein concentrations were determined by a BCA assay kit (Beyotime, China). Equal amounts of protein (20 μg) were separated on 5–10% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (pore size, 0.22 μm; Millipore). The membrane was blocked in Tris-buffered saline (TBST: 20 mM Tris, pH 7.6, 135 mM NaCl, and 0.05% Tween) containing 5% nonfat dry milk at room temperature for 1 h, and then incubated with one of the following primary antibodies overnight at 4 °C: β-tubulin (49 kDa, 1:8000, Abcam, USA), PAG (45 kDa, 1:5000, Abcam, USA), GAD (65 kDa, 1:2000, Abcam, USA), GABA-T (67 kDa, 1:5000, Abcam, USA), PKA (40 kDa, 1:20000, Abcam, USA), p-NR2B (Ser1166) (180 kDa, 1:5000, Abcam, USA), and p-CREB (Ser133) (37 kDa, 1:1000, Abcam, USA). After washing the membrane 3 × with TBST, secondary HRP-labeled goat-anti-rabbit lgG was added to the membrane according to the vendor's recommendation (1:5000, Abcam, USA) and incubated for 1 h at room temperature. The membrane was then washed again as described previously. Band intensity (the sum of the pixels within the band of interest minus the sum of the background pixels) was quantified using BandScan 5.0 (Glyko Bandscan software). Protein levels of PAG, GAD, GABA-T, PKA, p-NR2B, and p-CREB were normalized relative to β-tubulin.

2.7. Statistical analysis

Data are presented as the mean ± S.D. Differences in motion of maternal rats in the OFT between the two groups (control versus CUS), and between before and after CUS in the same group, were analyzed using an independent sample *t*–test and a paired sample *t*–test, respectively. The effect of CUS on body weight and sucrose intake of rats during the CUS procedure was analyzed using a one-way repeated measure analysis of variance (ANOVA), with one factor (rats exposed to CUS) and one within-subject factor of time (weeks 0–3). Two-way ANOVA with two between-subject factors—maternal rats exposed to CUS before pregnancy (yes versus no) and gender of offspring rats

(female versus male)—was performed for the behavioral test of offspring rats. Three-way ANOVA with three between-subject factors—maternal rats exposed to CUS before pregnancy (yes versus no), gender of offspring rats (female versus male), and two hippocampi (left versus right)—was performed for the MRS and western blot data. Separate one-way ANOVAs were performed, when needed, in order to elucidate specific differences between groups. The correlations between the behavioral test of offspring rats and protein expression of GAD, PAG, GABA-T, PKA, p-CREB, and p-NR2B were analyzed by Spearman correlation analysis. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Body weight, sucrose intake, and moving path of rats are decreased after CUS

The OFT is used to measure the activity of rats in a novel environment (Willner, 1997). SIT is an additional, objective indicator to evaluate the reward value of a sucrose solution in rodent CUS models (Willner et al., 1992). If an animal is depressed, its moving path in the OFT and sucrose solution intake in the SIT should both decrease. In order to exclude the impact of body weight on sucrose intake which is related to changes in body weight, the ratio of sucrose intake per body weight was calculated.

Fig. 1A and B shows the body weight and the ratio of sucrose intake per body weight of mothers before, during, and after the CUS procedure. Body weight and the ratio of sucrose intake per body weight were no different between control and CUS groups before the procedure. At weeks 2 and 3 during and following CUS, both body weight and ratio of sucrose intake per body weight were decreased in CUS rats compared with controls. These findings demonstrate that the reward value of the sucrose solution in rats is reduced after exposure to CUS.

Results of the OFT for maternal rats is shown in Fig. 1C. There was no difference in the moving path of maternal rats in the randomized groups before the CUS. At termination of the CUS treatment, the moving path of CUS group in the OFT was reduced compared with the control group. The OFT total and peripheral paths of the CUS group were reduced from before initiation of the CUS. The above results show that the activity of maternal rats in the novel environment was reduced by the CUS treatment.

3.2. Adolescent offspring, from mothers exposed to CUS before pregnancy, exhibit depressive-like behavior in the FST and anxiety in the OFT

We used the FST and OFT to assess the behavior of adolescent offspring at 30 days of age. Rats were considered as floating (immobile) when they did not struggle in the FST, i.e. they only made movements necessary to keep their heads above the water. Increased passive behavioral responses in the FST, such as immobility, are considered an indication of depressive-like symptomatology (Jans et al., 2010). The OFT was used to assess the anxious behavior of offspring. In the OFT, the central arena of the open field is novel and potentially threatening, whereas the peripheral arena is relatively safe. If the animal possesses anxiety, it will tend to stay in the peripheral arena. Therefore, the parameter for the anxious behavior of offspring rats is percentage of time spent in the peripheral arena. Fig. 2 shows the offspring behavior in the FST and OFT. The floating time of CUS offspring in the FST was longer than that in the control group for both females and males. Female CUS offspring exhibited longer floating time than their male counterparts. These results show that CUS offspring are affected by CUS before pregnancy, and they exhibit depressive-like behavior in the FST, with female offspring displaying greater severity of abnormality than corresponding male offspring.

The percentage of the time spent in the peripheral arena in the OFT by CUS offspring was longer than that in the control group for both females and males. The percentage of the time spent in the peripheral arena of male CUS offspring was longer than their female counterpart. The results suggest CUS offspring exhibit more anxious behavior. Moreover, male CUS offspring displayed a more severe phenotype than corresponding female CUS offspring.

3.3. CUS before pregnancy results in an altered glutamate/GABA–glutamine cycle in the hippocampus of adolescent offspring

To examine the impact of CUS before pregnancy on glutamate/GABA–glutamine cycle metabolism in the hippocampus of adolescent CUS offspring, the animals underwent a 13 min MRI scan after the FST. 1H-MRS values for 18 metabolites in the hippocampus of offspring rats were acquired using LC Model. Twelve of the 18 metabolites included in the LC Model basis set were reliably quantified (Cramer-Rao lower bounds [CRLB] < 25%) from each spectrum and only those were used for further analysis. The signals of glutamine, glutamate, and GABA could be readily identified. Because the creatine (Cr) is relatively constant in various physiological and pathological conditions, it was used as an internal standard. MRS analysis of 18 metabolites in the hippocampus of offspring rats was performed using LC Model. A representative measurement of a single animal is shown in Fig. 3, and the green rectangles indicate the two hippocampi in the offspring rat.

Three-way ANOVA showed that the Cr + PCr was not different between the groups. The 1H-MRS values for glutamine, glutamate, and GABA were normalized to the Cr + PCr, and all relative values are shown in Fig. 4. The relative value of glutamine in the hippocampus was higher in the offspring whose mothers were exposed to chronic stress before pregnancy than those in the control group for both females and males. The relative value of glutamine in the right hippocampi of male CUS offspring was lower compared with their corresponding left hippocampi. The relative value of glutamate in the hippocampus of CUS offspring was higher than those in the control group for both females and males. In addition, the relative value of glutamate in the right hippocampi of CUS offspring was higher compared with their corresponding left hippocampi. The relative value of glutamate in the right hippocampi of female CUS offspring rats was lower than that of male offspring in the same group. The relative value of GABA in the hippocampus of CUS offspring was higher than those in the control group for both females. The relative value of GABA in the right hippocampi of female CUS offspring was higher compared with their corresponding left hippocampi and that of male CUS offspring in the same group. The glutamate/glutamine ratio in the right hippocampi of male CUS offspring was higher than that in the control group, and higher compared with their corresponding left hippocampi. The glutamate/GABA ratio in the hippocampus of CUS offspring was lower compared with those in the control group in both females and males. The glutamate/GABA ratio in the right hippocampi of female CUS offspring was lower than that of male offspring rats in the same group. These findings show that stress before pregnancy alters the metabolic balance of the glutamate/GABA–glutamine cycle in the hippocampus of adolescent offspring rats, and the sex differences for the above changes exist only in the right hippocampi.

3.4. Disruption of the glutamate/GABA–glutamine cycle may be due to changes in PAG, GAD, and GABA-T expression in the hippocampus of adolescent offspring

To examine the influence of stress before pregnancy on the protein expression of PAG, GAD, GABA-T, p-NR2B, PKA, and p-CREB in the hippocampus, brains were rapidly removed after the last MRI scan. Representative western blots and the relative levels of protein are shown in Figs. 5 and 6, respectively.

PAG expression in the left hippocampi of CUS offspring was higher than in the control group for both females and males. PAG levels in the right hippocampi of female CUS offspring were higher than those in the control group. Among CUS offspring rats, PAG levels in the right

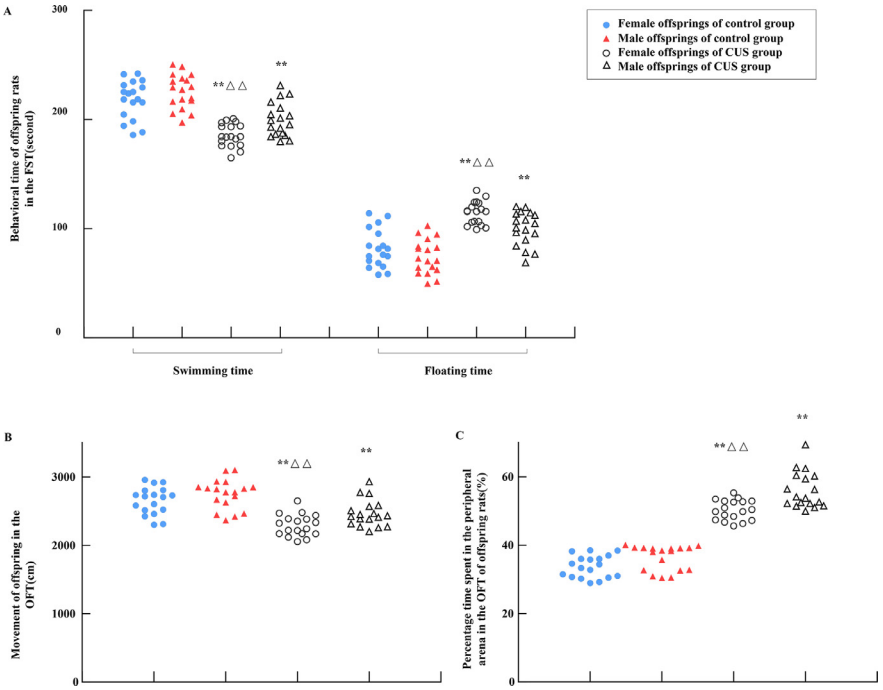

Fig. 2. *Behavioral performance of offspring rats in the FST and OFT.*
Fig. 2A shows the swimming and floating times of offspring rats in the FST. Fig. 2B shows the moving path of offspring rats in the OFT. Fig. 2C shows the percentage of time spent in the peripheral arena in the OFT. Data represent the mean ± S.D. ** and △△ indicate $p < 0.01$, compared with offspring rats of the same gender in the control group and compared with the male offspring rats in the same group, respectively.

hippocampi were higher in females than males. GAD expression in the hippocampus of CUS offspring was higher than those in the control group. Hippocampal GAD levels were higher in female CUS offspring than male CUS offspring. GAD levels were higher in the right hippocampi than in the left hippocampi of CUS offspring. GABA-T expression in the hippocampus of CUS offspring was lower than those in the control group. GABA-T levels in the right hippocampi was lower in the female CUS offspring than male CUS offspring, and was also lower in the right hippocampi than in the left hippocampi of female CUS offspring.

The above findings revealed that changes in the right hippocampal PAG levels in male CUS offspring may lead to alterations in the metabolic balance of glutamate-glutamine cycle. Moreover, GAD expression in the hippocampus was increased and GABA-T expression was decreased, which can account for the increase in GABA levels and decrease of the glutamate/GABA ratio observed in the hippocampus of CUS offspring rats.

3.5. Changes in the metabolic enzymes of the glutamate/GABA–glutamine cycle correlate with decreased PKA-mediated phosphorylation of CREB and NR2B in the hippocampus of CUS offspring

Negative correlations emerged between GABA-T and GAD expression ($r = -0.823$, $p < 0.001$) or PAG ($r = -0.427$, $p < 0.001$). GABA-T expression was positively associated with p-NR2B ($r = 0.659$, $p < 0.001$) and p-CREB ($r = 0.753$, $p < 0.001$) expression, but GAD and PAG expression were negatively related with the expression of p-NR2B (GAD: $r = -0.496$, $p < 0.001$; PAG: $r = -0.249$, $p = 0.003$) and p-CREB (GAD: $r = -0.63$, $p < 0.001$; PAG: $r = -0.418$, $p < 0.001$). Decreased expression of PKA exhibited a close correlation with lower protein expression of p-NR2B ($r = 0.657$, $p < 0.001$) and p-CREB ($r = 0.495$, $p < 0.001$) in the hippocampus of offspring rats.

PKA expression in the right hippocampi of CUS offspring was lower than that in the control group, and that in the left hippocampi of male CUS offspring was lower than that in the control group. PKA levels in the hippocampus were higher in female CUS offspring than the counterpart male CUS offspring, and it was lower in the right hippocampi than in the left hippocampi of male CUS offspring rats. Expression of p-NR2B in the hippocampus of CUS offspring was lower than those in the control group, and it was higher in female CUS offspring than the counterpart male CUS offspring. Phospho-NR2B was lower in the right hippocampi than in the left hippocampi of male CUS offspring. Expression of p-CREB in the hippocampus of CUS offspring was also lower than those in the control group, and that in the right hippocampi was higher in the CUS female offspring than in the counterpart male CUS offspring. The p-CREB was lower in the right hippocampi than in the left hippocampi of male CUS offspring.

The above findings suggest that decreased protein of PKA results in a decreased phosphorylation of CREB and NR2B in the hippocampus of adolescent offspring whose mothers were exposed to CUS before pregnancy. Moreover, decreased protein expression of p-CREB and p-NR2B in the hippocampus corresponded to the change in the metabolic enzymes of glutamate/GABA–glutamine cycle in the hippocampus.

3.6. Correlations between adolescent offspring behavior in the FST and OFT and protein expression of GAD, PAG, GABA-T, p-NR2B and p-CREB in the hippocampus

Spearman correlations demonstrated correlations between adolescent offspring behavior in the FST and OFT and protein expression of GAD, PAG, GABA-T, p-NR2B and p-CREB in the hippocampus (see Table 1). Expression of GAD and PAG were positively correlated with anxious behavior (percentage of the time spend in the peripheral arena) in the OFT and depressive-like behavior (floating time) in the FST. Decreased protein expression of GABA-T, p-NR2B, and p-CREB in the hippocampus of offspring rats correlated with more anxious behavior in the OFT and depressive-like behavior in the FST.

4. Discussion

The present study was designed to elucidate whether stress before pregnancy could increase the anxious or depressive behavior in the adolescent offspring, and if so, whether such effect is related to the PKA-mediated phosphorylation of NR2B and CREB. Our findings show that reductions in body weight, sucrose intake, and movement activity of mother rats occurred, which demonstrates the onset of depression-like behavior following CUS. In addition, the adolescent offspring, whose mothers were exposed to CUS before pregnancy, exhibited more

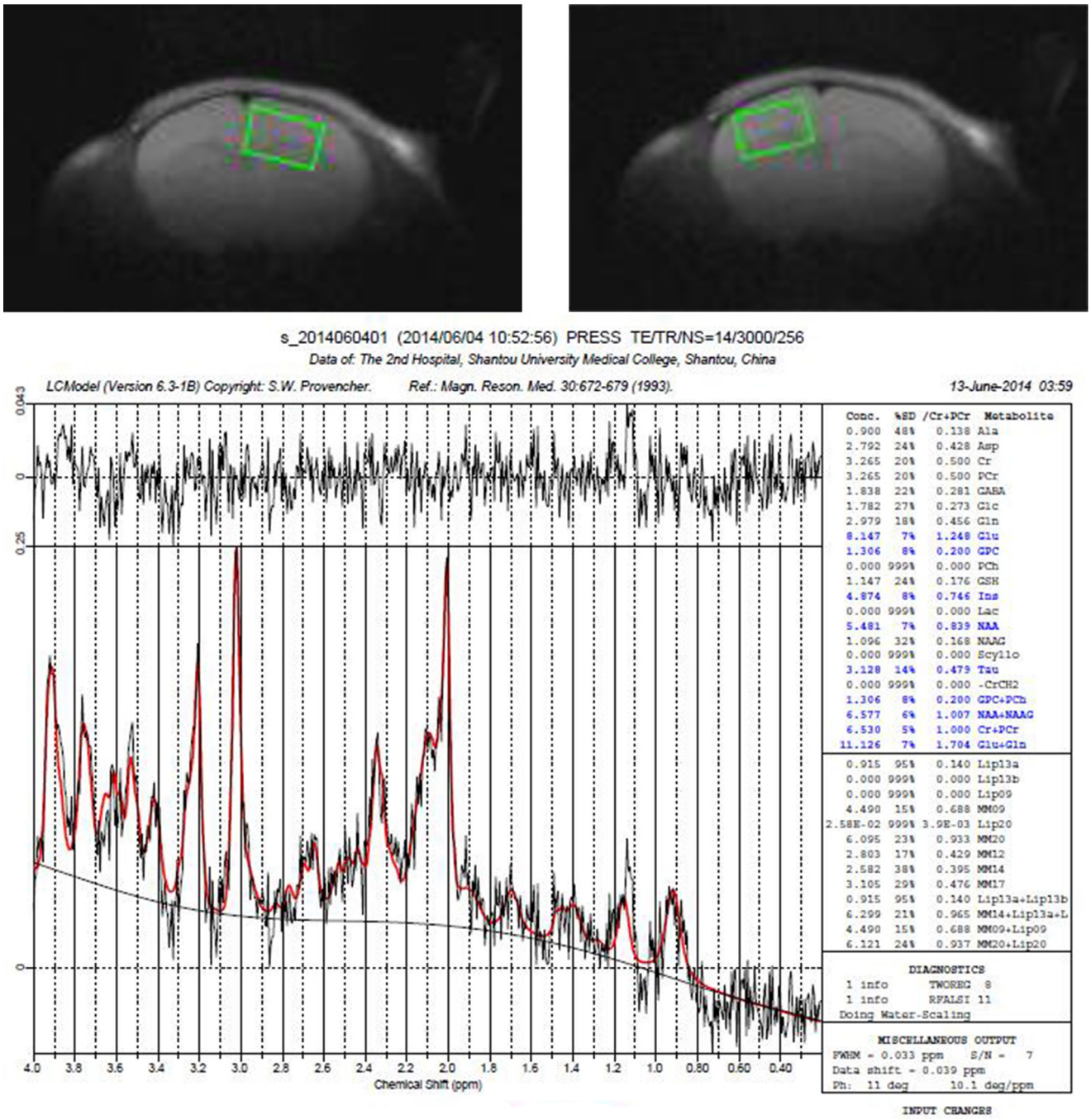

Fig. 3. *MR spectra of the hippocampus of an offspring rat.*
MRS analysis of 18 metabolites in the hippocampus of offspring rats was performed using LC Model. A representative measurement of a single animal is shown. The green rectangles point out two hippocampi in the offspring rat. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

anxious behavior in the OFT and more depressive-like behavior in the FST. There is considerable interest in the transmission of mental disorders across generations. We think there are two mechanisms involved in transmission of mental disorders across a generation. Firstly, imposition of CUS on animals a short period prior to pregnancy may affect offspring in the early embryonic environment and thus impact brain development. Secondly, stress before pregnancy may increase the risk of postnatal depression, which could affect brain development of offspring throughout childhood (Goodman et al., 2011). Because of the timeliness of the CUS animal model, and no observation of biting pups by the mother rats of CUS group in this study, we cannot be sure there was a difference in the quality of pup care between the CUS group and control group. Therefore, we mainly discuss the first mechanism mentioned above.

Imposition of CUS on animals a short period prior to pregnancy may have effects on offspring in early embryonic environment. During fetal development, the brain undergoes rapid growth that is characterized by a high turnover of neuronal connections (Dubois et al., 2014). This makes the fetal brain especially vulnerable to GCs that may reach it in excess amounts from the maternal circulation as a result of stress (Kapoor et al., 2009). In a prior study, we found CUS before pregnancy not only leads to an increase in GC levels of animals following CUS, but

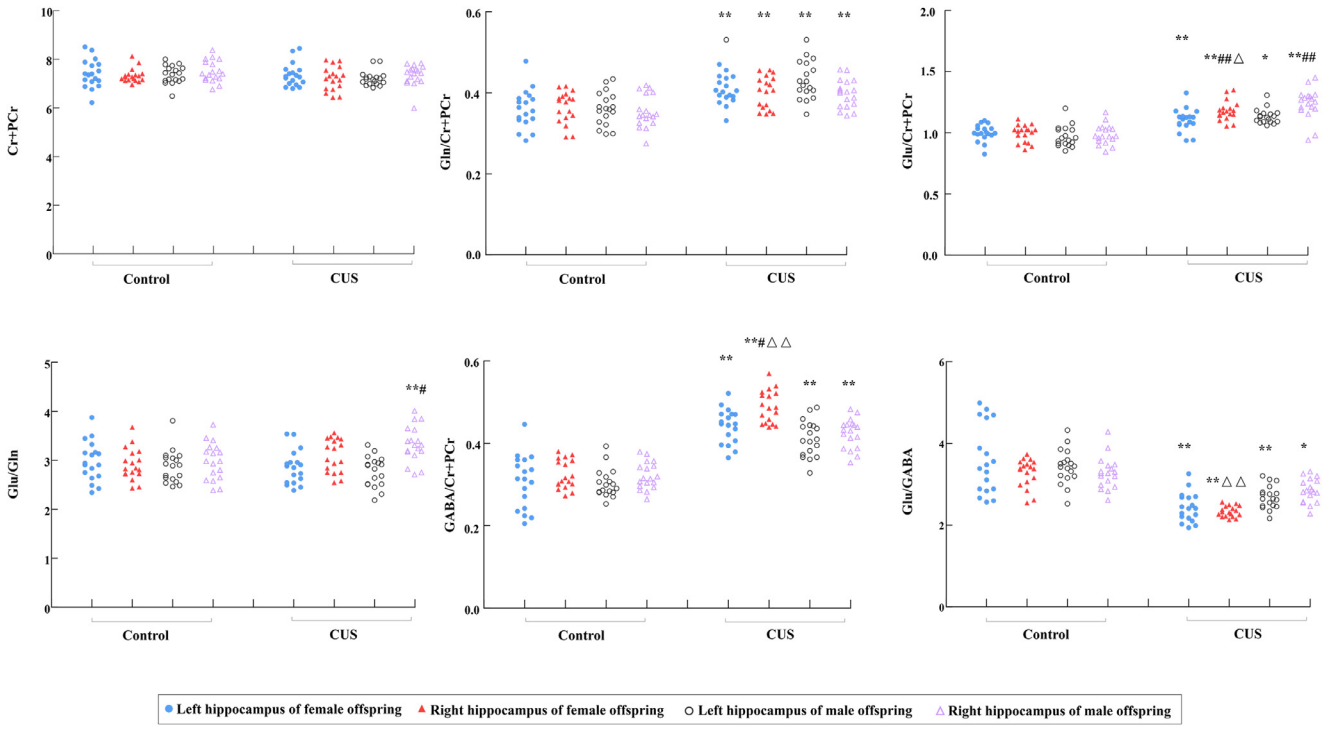

Fig. 4. *1H-MRS values of Cr+PCr, GABA, glutamine, glutamate, glutamate/GABA and glutamate/glutamine in the hippocampus of offspring rats.*
Data represent the mean ± S.D. * and ** indicate $p < 0.05$ and $p < 0.01$, compared with the same side hippocampus of the same sex offspring rats in the control group. # and ## indicate $p < 0.05$ and $p < 0.01$, compared with the left hippocampus of the same sex in offspring rats in the same group. △ and △△ indicate $p < 0.05$ and $p < 0.01$, compared with the same side hippocampus of male offspring rats in the same group.

also results in higher GC levels than non-CUS during pregnancy (Huang et al., 2013, 2012). A review concluded that exposure of embryonic neurons to maternal high levels of GCs affects the HPA axis, resulting in stress-related behavioral disorders in the offspring (Moisiadis and Matthews, 2014).

Clinical research has shown alterations in levels, clearance and metabolism of glutamate in mood and anxiety disorders (Gorman and Docherty, 2010), and glutamate neurotransmission can be affected in prenatally stressed offspring (Wu et al., 2015). In our study, the levels of glutamate and glutamine in the hippocampus of CUS offspring are higher than in control offspring when subjected to OFT and FST in adolescence. In a prior experiment, we found the metabolic balance in the glutamate-glutamine cycle of CUS offspring is similar to non-CUS offspring under normal conditions, but the glutamate-glutamine cycle of CUS offspring is more vulnerable to acute stress in early adolescence. In the present study, FST can be regarded as an acute stress. PAG expression in the right hippocampus of CUS male offspring did not increase following the increase in glutamine, indicating disruption of the metabolic balance of the glutamate–glutamine cycle in male CUS offspring after acute stress in early adolescence. The above changes may affect male offspring rats in at least two ways. On the one hand, accumulation of glutamine in presynaptic neurons may inhibit the conversion of glutamate to glutamine in astrocytes through negative feedback of the glutamate–glutamine cycle (Munakata et al., 2014; Schousboe et al., 2013), which could result in the accumulation of glutamate in astrocytes. Studies show that the morphology of astrocytes is changed and neurotransmission loses spatial fidelity when glutamate levels are extremely high in astrocytes (Hanson et al., 2015; Hinzman et al., 2012). On the other hand, decreased conversion of glutamine to glutamate in presynaptic neurons may result in decreased glutamate being released into the synaptic cleft, leading to hampered neurotransmission. Brain GABA levels reflect the dynamic balance between GABA synthesis and reuptake, as well as the regulation of GAD and GABA-T (Schousboe et al., 2013). We show GAD expression in the

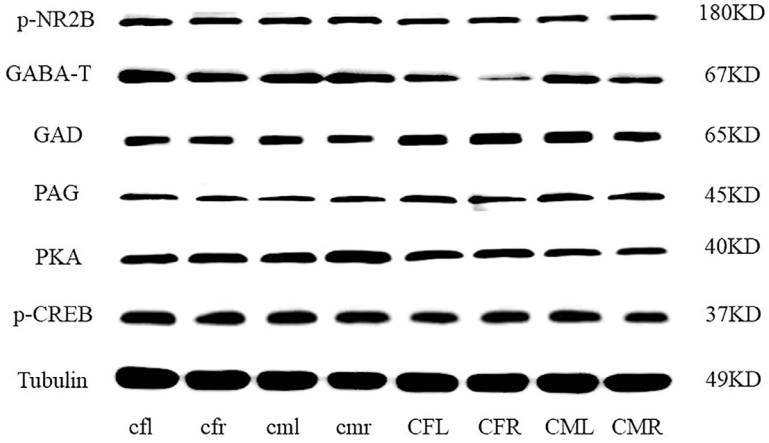

Fig. 5. *Representative western blots of PAG, GAD, GABA-T, p-NR2B, PKA, and p-CREB.*
Lane 1 (cfl): left hippocampus of female offspring in the control group; Lane 2 (cfr): right hippocampus of female offspring in the control group; Lane 3 (cml): left hippocampus of male offspring in the control group; Lane 4 (cmr): right hippocampus of male offspring in the control group; Lane 5 (CFL): left hippocampus of female offspring in the CUS group; Lane 6 (CFR): right hippocampus of female offspring in the CUS group; Lane 7 (CML): left hippocampus of male offspring in the CUS group; Lane 8 (CMR): right hippocampus of male offspring in the CUS group.

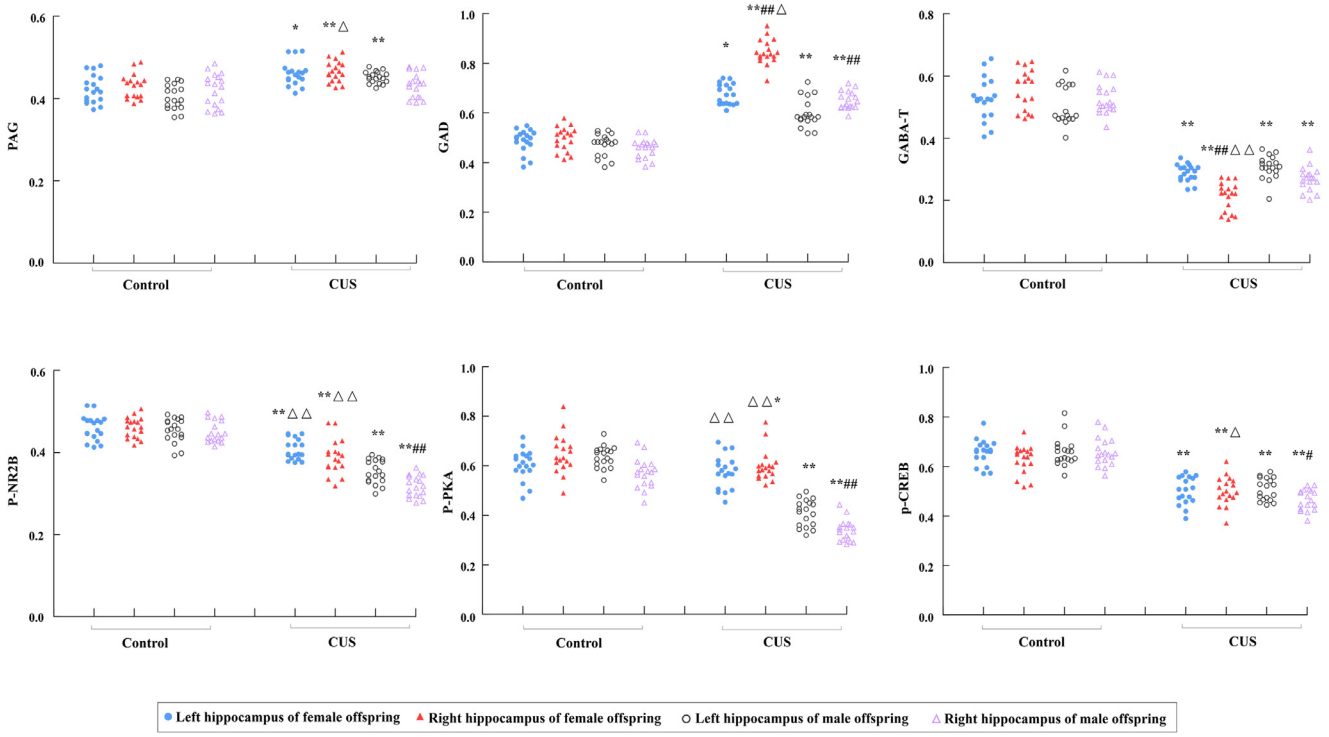

Fig. 6. *Relative protein levels of PAG, GAD, GABA-T, p-NR2B, PKA, and p-CREB in the offspring hippocampus.*
Data represent the mean ± S.D. * and ** indicate $p < 0.05$ and $p < 0.01$, compared with the same side hippocampus of the same sex offspring rats in the control group. # and ## indicate $p < 0.05$ and $p < 0.01$, compared with the left hippocampus of the same sex offspring rats in the same group. △ and △△ indicate $p < 0.05$ and $p < 0.01$, compared with the same side hippocampus of male offspring rats in the same group.

hippocampus is increased and GABA-T is decreased, which may cause an increase of GABA levels and an imbalance in the glutamate-GABA cycle in CUS offspring. This change is most obvious in the right hippocampus of female CUS offspring. Some studies have shown decreased expression of GABA-T in mood disorders (Boddum et al., 2016). In support of this, our results suggest that decreased protein expression of GABA-T in the hippocampus corresponds to the depression–like behavior of female CUS offspring.

Altogether, the above changes show the glutamate/GABA – glutamine cycle is altered in the hippocampus of CUS offspring when they are subjected to acute stress (FST). When glutamate is released into the synaptic cleft, it binds to postsynaptic NRs, thus, mediating and modulating synaptic transmission (Tigaret et al., 2016). Consistent with this, extracellular signals regulate Ca^{2+} permeation through NR-mediated Ca^{2+} influx in a PKA-dependent manner in response to acute stress (Whitehead et al., 2013). In general, postsynaptic potentiation requires both Ca^{2+} influx through NRs and the ensuing CREB activation. Ca^{2+} influx stimulates not only CREB activity but also CREB binding to the NR and CREB accumulation at postsynaptic sites. CREB binding to NR2B is critical for the postsynaptic signal (Ko et al., 2018). This mechanism supports selective enrichment of CREB at synapses that undergo potentiation upon repeated release of glutamate. Phosphorylation of Ser1166 on NR2B is a molecular and functional target of PKA essential for Ca^{2+} signaling in spines, and is regulated by emotional response to stress (Murphy et al., 2014). Moreover, both human and experimental studies supported the link of PKA-dependent CREB and PKA-dependent NR signaling to depression (Ates-Alagoz and Adejare, 2013; Liu et al., 2016; Zou et al., 2017). In this study, we found decreased PKA and subsequent decreased protein expression of p-NR2B and p-CREB in the hippocampus of adolescent CUS offspring. These findings add to our understanding of how stress before pregnancy influences the metabolic enzymes in the glutamate/GABA–glutamine cycle and PKA-mediated phosphorylation of CREB and NR2B in the hippocampus of offspring rats, which may be involved the mechanism of behavioral disorders in the adolescence.

Another novel finding of the present study is that the above effects are preferentially enhanced in the right hippocampus of CUS offspring. Hemispheric asymmetries play an important role in almost all cognitive functions. Investigators have proposed that abnormal asymmetry may serve as a risk factor, or marker of genetic liability in behavioral disorders (Ocklenburg and Gunturkun, 2012). Many studies demonstrate universal left-right asymmetry of hippocampal synapses with a fundamental relationship between synaptic area and the expression of NRs (Kawahara et al., 2013; Shinohara et al., 2008). Patients with damage

Table 1
Correlations between adolescent offspring rat behavior in the FST and OFT and protein expression of GAD, PAG, GABA-T, p-NR2B and p-CREB in the hippocampus.

	GAD		GABA-T		PAG		p-NR2B		p-CREB	
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
Percentage of peripheral arena time in the OFT										
R	0.705	0.684	−0.806	−0.818	0.537	0.242	−0.771	−0.799	−0.754	−0.749
p	<0.001	<0.001	<0.001	<0.001	<0.001	0.041	<0.001	<0.001	<0.001	<0.001
Floating time in the FST										
r	0.641	0.715	−0.628	−0.657	0.387	0.248	−0.45	−0.477	−0.561	−0.536
p	<0.001	<0.001	<0.001	<0.001	0.01	0.036	<0.001	<0.001	<0.001	<0.001

to the right hippocampus perform worse on positional memory than patients with lesions in the left hippocampus (Woolard and Heckers, 2012). Depression is associated with an inter-hemispheric imbalance; a hyperactive right-hemisphere (RH) and a relatively hypoactive left-hemisphere (Hecht, 2010). Here we report stress before pregnancy alters the left-right hippocampal asymmetry of the glutamate/GABA–glutamine cycle and PKA-mediated phosphorylation of NR2B and CREB in the offspring rats, which correlates with their abnormal behavior in the FST and OFT.

In summary, our findings complement the increasing literature that draws attention to the importance of the mental condition of females before pregnancy on brain development of offspring, and suggest that adolescents, whose mothers are exposed to CUS before pregnancy, and who have an altered glutamate/GABA–glutamine cycle and PKA-mediated phosphorylation of CREB and NR2B in the hippocampus, may be at increased risk for behavioral disorders. However, our study design limits our ability to dissect the events occurring after pregnancy (e.g. changes in maternal care). We recognize the influences of postnatal maternal mental conditions, which may affect brain development of offspring throughout childhood. We will add experiments involving cross-fostering to analyze the effect of postnatal maternal mental conditions on the brain development of offspring in our future study.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psychres.2019.112497.

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