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Behavioral and neurobiological studies on the male progeny of maternal rats exposed to chronic unpredictable stress before pregnancy

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ABSTRACT

Studies have shown that maternal chronic stress or depression is linked to an increased risk for affective disorders in progeny. However, the impact of maternal chronic stress before pregnancy on their progeny in animal models has not been well studied. We investigated the behaviors and the neurobiology in 60-day-old male progeny of maternal rats exposed to a 21-day chronic unpredictable stress (CUS) before pregnancy, with male progeny of unstressed maternal rats as the control. Sucrose consumption test showed that both sucrose intake and sucrose consumption percentage of the CUS progeny were lower than those of the control progeny ($P < 0.05$). The number of times crossing the removed hidden platform in the CUS progeny was significantly fewer than that in the control progeny in Morris water maze test ($P < 0.05$). The level of 5-hydroxytryptamine (5-HT) in the hypothalamus was reduced but the level of norepinephrine (NE) in the hippocampus was increased in CUS progeny when compared to the control ($P < 0.05$). Western blotting showed that the relative level of phosphorylated CREB (P-CREB) in the CUS progeny was lower than that in the control progeny ($P < 0.05$). There were significant positive correlations between sucrose consumption percentage and the level of 5-HT in hypothalamus $P < 0.05$) or the level of P-CREB in hippocampus ($P < 0.05$). In conclusion, depression or stressful events before pregnancy was also associated with high risk of depression in progeny, and the down-regulation of P-CREB in the hippocampus might be one of the mechanisms underlying depression in the CUS progeny.

Maternal stress or depression has a negative impact on the behavior and is linked to an increased risk for affective disorders in their progeny [3,4,8,22]. Follow-up on the progeny of depressed and non-depressed parents shows that parental depression is a strong and consistent risk factor for progeny's major depressive disorder, and the risks for anxiety disorders, major depression, and substance dependence in the progeny of depressed parents are about three times as high as that in the progeny of non-depressed parents [22]. Multiple animal experiments have demonstrated that prenatal stress is related to the increased depression-like and anxiety-like behaviors in progeny [8,14]. However, only a few studies have investigated the impact of maternal depression before pregnancy on their offspring and the related neurobiological mechanisms in animal models.

In this study, we have investigated male progeny of maternal rats exposed to chronic unpredictable stress (CUS) before pregnancy for the symptom of anhedonia by sucrose consumption test, spatial cognitive ability by Morris water maze test, neurotransmitters's level in different brain regions by high performance liquid chromatography (HPLC), and protein expression of phosphorylated Cyclic AMP responsive element-binding protein (P-CREB), a molecular convergence point in the pathophysiology of depression, by western blot analysis, as well as the correlation between behavior and brain measures. Our results showed that depression or stressful events before pregnancy was also associated with high risk of depression in progeny, and the down-regulation of P-CREB in the hippocampus might be one of the mechanisms underlying depression in the CUS progeny.

Virgin female Sprague-Dawley rats (SD, 200–220 g), were brought into the laboratory from the Lab Animal Center of Shantou University Medical College (Shantou, China) one week before the experiment and housed individually. The room temperature was maintained at $22 \pm 1\,^{\circ}C$ with low humidity and food and water freely available. Sixteen rats were randomly assigned to two groups: chronic unpredictable stress group (CUS group, $n = 8$) and the non-stressed control group (Control group, $n = 8$). Rats assigned to the CUS group were subjected to a 21-day CUS according to a previously reported protocol [9]. To be completely unpredictable to the rats, the stressors were applied randomly with the last stressor being food and water deprivation. The control group rats were housed in separate individual rooms without any contact with the CUS group. Ten days after the 21-day CUS, females were caged

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with sexually experienced males of the same strain (ratio 4:1). Pregnancy was confirmed by sperm-positive vaginal smear. The pregnant female rats were housed individually for the whole gestation. The pups were weaned at the age of 22 days and then housed in groups of three to four according to sex. All the subsequent experiments described below were performed on the male progeny aged two months. Twelve progeny from the control mothers and eleven from the CUS mothers (with one or two pups from each mother) were used. After the behavioral test, the rats were anesthetized with ether and were decapitated. The brains were removed from the skull and placed on ice. With the reference landmark of the bregma, the hippocampus, hypothalamus and prefrontal cortex were dissected according to the Paxinos and Watson [15]. The hypothalamus, left hippocampus, and left prefrontal cortex were used to detect the monoamine neurotransmitters by HPLC, and the right hippocampus was used to detect the P-CREB protein expression by western blotting. All the experimental protocols and procedures were approved by the Laboratory Animals Care and Use committee of Shantou University Medical College (Shantou, China).

The sucrose consumption test was conducted using a two-bottle choice procedure according to a previously described method [10] with some modifications. Initially, the rats were trained to habituate to 1% sucrose solution and tap water for 3 days. During the training time, the positions of bottles were changed several times. Subsequently, the rats were food and water deprived for 21 h before testing. Then they were allowed to consume 1% sucrose solution and tap water for 3 h. The positions of bottles were counterbalanced across the left or right side of the testing cages. Sucrose consumption was monitored by weighing the bottles at the beginning and end of the test. Sucrose consumption percentage was calculated as follows: sucrose consumption percentage = sucrose consumption/(sucrose consumption + water consumption) × 100%.

The second day after the sucrose consumption test, the spatial memory (acquisition or retention) of the rats was tested using Morris water maze (MWM) according to a previously described method [6] with some modifications. The black water maze (1.8 m in diameter × 0.7 m in height) was filled with tap water and divided into four quadrants. In the center of the 2nd quadrant was a removable escape platform (10 cm diameter), which was 1.5 cm below the water level and covered with black plastic to disguise its presence and to create traction for the rat to climb onto the top of the platform. The water maze was located in a well-lit white room with several distal visual stimuli hanging on the walls to provide spatial cues.

The acquisition training lasted four days, with two trials per day. In each trial, each rat was placed in the water facing the wall of the pool at a randomly selected quadrant. For each trial, the rat swam in the pool for 120 s or until climbing onto the platform. If the rat did not find the platform within 120 s, it was manually guided to the platform. The rat remained on the platform for 15 s in all trials and was then removed from the pool, dried in a clean cage, and returned to its home cage. The pool was strained of feces and stirred gently between each swim session to disrupt any scent left behind from the rat's path taken to find the platform. On the fifth day of MWM test, there was one probe trial in which the platform was removed from the pool and the animal swam for 120 s.

All swim sessions were videotaped and recorded with DigBehav-Morris water maze Video Analysis System (Mobile datum Software Technology Co. Ltd., Shanghai, China).

The hypothalamus, left hippocampus, and left prefrontal cortex of the rats were homogenized in 0.1 M perchloric acid containing 0.02% ascorbic acid and 0.2 mM EDTA–2Na, centrifuged at 10,800 g for 20 min at 4 °C. Then, a 20 μl supernatant aliquot was injected directly into the HPLC column.

The HPLC procedure was performed according to the method previously described [2] with some modifications. For monoamine analysis, an Agilent HC-C18 analytical column (250 mm × 4.6 mm, 5 μm; Agilent, USA) was used. The mobile phase consisted of 20% methanol and 80% aqueous solution, containing 30 mM citric acid, 40 mM sodium acetate, 0.2 mM ethylenediamine tetraacetic acid (EDTA) disodium salt and 0.5 mM octanesulfonic acid sodium salt, at a flow rate of 1.0 ml/min and at pH value of 3.8. The level of 5-hydroxytryptamine (5-HT) and norepinephrine (NE) were detected using a Waters 474 scanning fluorescence detector (Waters, USA) with the excitation and emission wavelengths set at 280 nm and 330 nm, respectively.

The amount of monoamines in the supernatants were calculated by comparing their elution times and peak areas with those of standards, and reported as ng/g tissue.

The right hippocampus of the rats was homogenized in cell lysis buffer for western blotting (Beyotime, Jiangsu, China). Protein (50 μg/lane) separated by SDS-PAGE were blotted onto nitrocellulose (NC) membranes by electrophoretic transfer. Blots were incubated in blocking buffer (10% nonfat dry milk powder in tris–buffered saline containing 0.5% Tween-20, TBS-T) for 1 h at room temperature and washed three times with TBS-T for 10 min each. Blots were then incubated at 4 °C with 1:1000 diluted primary antibodies, mouse anti-β-actin or rabbit anti-P-CREB (cell signaling, USA), and washed three times for 10 min each in TBS-T. Blots were incubated with appropriate HRP-labelled secondary IgG antibody for 2 h at room temperature, washed three times for 10 min each in TBS-T, treated with BeyoECL reagents (Beyotime, Jiangsu, China), and exposed to film (Koda, USA). Band intensity was quantified by Bandscan 5.0 analysis software (Glyko Bandscan software). The relative level of each signal protein was calculated as the ratio of total gray of P-CREB/β-actin.

The statistical analysis was performed using the "Statistical Package for Social Sciences" (SPSS, Version 11.5). Group comparisons in escape latency and swimming velocity during the acquisition training of MWM were analyzed with repeated measures analysis of variance (ANOVA) (group × trial) and multivariate ANOVA (group × trial) of the general linear model. Group differences for probe trial of MWM, sucrose intake, sucrose consumption percentage, contents of monoamine, and the relative expression level in western blot were determined using independent sample *t* tests (two-tailed). Pearson correlation tests (two-tailed) were used for behavior and brain measure correlation. Only $P < 0.05$ was considered to be statistically significant. All data were reported as mean ± SD.

The sucrose intake and sucrose consumption percentage in the CUS progeny were lower than those in the control progeny. The difference in the sucrose intake (10.23 ± 4.12 g vs. 6.48 ± 3.19 g, $t(21) = 2.424$, $P < 0.05$) (Fig. 1A) and in the sucrose consumption percentage (85.43 ± 20.15% vs. 60.98 ± 24.65%, $t(21) = 2.614$, $P < 0.05$)

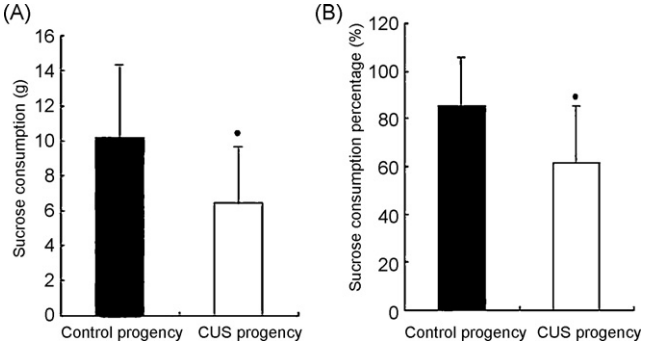

Fig. 1. Sucrose intake and sucrose consumption percentage. The significant difference in the sucrose intake ($t(21) = 2.424$, $P < 0.05$, A) and the sucrose consumption percentage ($t(21) = 2.614$, $P < 0.05$, B) between CUS progeny and control progeny. *$P < 0.05$ vs. control.

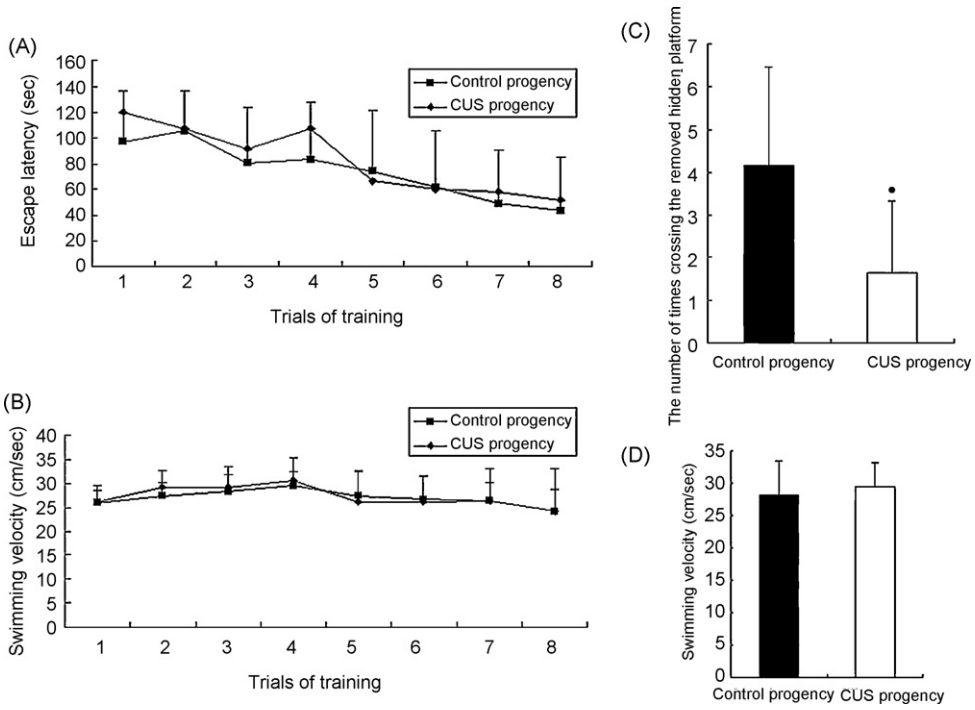

Fig. 2. Performance in the spatial memory acquisition and retention. There was no significant difference in the escape latency ($F(1, 147) = 1.346$, $P > 0.05$, A) and in the swimming velocity ($F(1, 147) = 0.130$, $P > 0.05$, B) during acquisition trail. The number of times crossing the removed hidden platform in the CUS progeny was significantly fewer than that in the control progeny during the probe trial ($t(21) = 2.99$, $P < 0.05$, C). There was no significant difference in the swimming velocity during the probe trial between the two progeny ($t(21) = 0.71$, $P > 0.05$, D).

(Fig. 1B) was significant between the control progeny and CUS progeny.

The escape latency for both the control progeny and the CUS progeny decreased with increasing trials. When compared through repeated measures ANOVA, there was no significant effect on the escape latency between group during the acquisition trail in the MWM ($F(1, 147) = 1.346$, $P > 0.05$) (Fig. 2A). Also, there was no significant effect on the swimming between the groups velocity during acquisition trial in the MWM ($F(1, 147) = 0.130$, $P > 0.05$) (Fig. 2B).

During the probe trial, the number of times that rats crossed the removed hidden platform in the CUS progeny was significantly fewer than that in the control progeny (1.64 ± 1.69 vs. 4.17 ± 2.29, $t(21) = 2.99$, $P < 0.05$) (Fig. 2C). There was no significant difference in the swimming velocity between the two progeny ($t(21) = 0.71$, $P > 0.05$) (Fig. 2D).

The contents of NE and 5-HT can be detected in the hippocampus, hypothalamus and prefrontal cortex by HPLC in the two progeny. The level of 5-HT in the hypothalamus of the CUS progeny and control progeny was 500.17 ± 80.94 ng/g tissue and 569.63 ± 50.91 ng/g tissue respectively, and there was significant difference between the two progeny ($t(21) = 2.436$, $P < 0.05$) (Fig. 3A). Compared with the control, the level of NE in the hippocampus of the CUS progeny was significantly higher (907.56 ± 207.27 ng/g tissue vs. 2315.01 ± 1397.12 ng/g tissue, $t(21) = 3.456$, $P < 0.05$) (Fig. 3B). The levels of NE and 5-HT in other brain regions showed no statistical difference between the two progeny (Fig. 3).

The signals of β-actin and P-CREB can be detected in the hippocampus (Fig. 4A and B). There was a significant difference in the relative level of P-CREB/β-actin between the control progeny and CUS progeny ($33.24 \pm 9.36\%$ in the control progeny vs. $20.56 \pm 7.78\%$ in the CUS progeny, $t(21) = 3.514$, $P < 0.05$) (Fig. 4)

There were significant positive correlations between sucrose consumption percentage and the level of 5-HT in hypothalamus ($r = 0.476$, $P < 0.05$) or the level of P-CREB in hippocampus ($r = 0.548$,

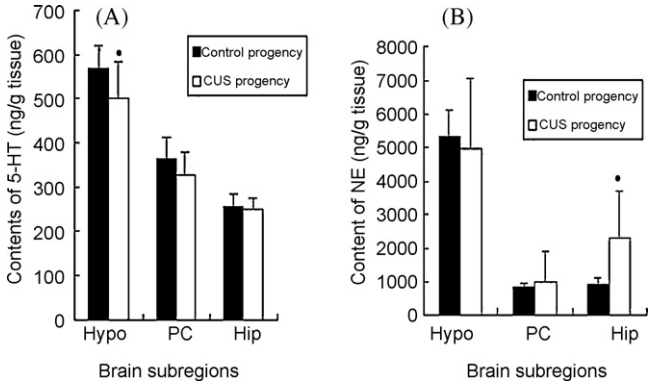

Fig. 3. The levels of NE and 5-HT in different brain regions. The level of 5-HT in the hypothalamus of CUS progeny was lower than that of the control progeny ($t(21) = 2.436$, $P < 0.05$, A), while the level of NE in the hippocampus of CUS progeny was higher than that of the control progeny ($t(21) = 3.456$, $P < 0.05$, B).*$P < 0.05$ vs. control.

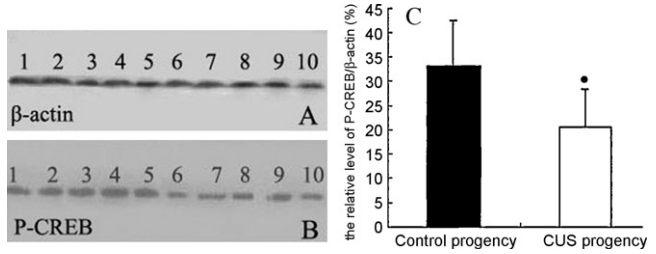

Fig. 4. Western blots showing the expression of β-actin and P-CREB, and the relative level of P-CREB/β-actin in hippocampus. Protein expression of β-actin (A) and P-CREB (B) in the hippocampus of the control progeny (lanes 1–5) and CUS progeny (lanes 6–10) was detected by western blot analysis, and the relative level of P-CREB/β-actin in CUS progeny was lower than that of the control progeny ($t(21) = 3.514$, $P < 0.05$,C). *$P < 0.05$ vs. control.

$P < 0.05$). However, there was no significant correlation between other data.

The effects of prenatal maternal stress in offspring have been investigated extensively in humans and in animals [8,14]. Progeny exposure to prenatal stress is reportedly associated with adverse neurodevelopment and behavioral impairments during the juvenile phase, and cognitive impairments and increased anxiety-like and depression-like behavior when grown into adult [8,14]. However, only studies by Ryzhavskii et al. reported the effects of maternal stress before pregnancy on their progeny in animal models in 2002–2003 [19,20]. They found that the progeny of female exposed to chronic emotional stress before pregnancy differed from the control by having reduced weight of the brain and cerebral hemispheres, decreased thickness of the parietal cortex, small cerebellum and Purkinje cells, increased percentage of dark Purkinje cells, enlarged layer V neuronal nuclei, and low exploratory activity in the plus-maze test [19,20]. In this study, we investigate the effects of maternal depression in their two months male progeny by sucrose consumption test, referenced spatial memory test and monoamine neurotransmitter detection.

The CUS progeny showed decreased sucrose intake and consumption percentage. The decrease in sucrose intake or in the sucrose consumption percentage signifies loss of preference for the palatable sucrose solution [7] and is an indication of the core symptom of depression, anhedonia [7,17]. Therefore, our results from sucrose consumption test denote an increased likelihood of depression in the progeny of the depressed maternal rat.

Spatial memory test showed normal acquisition but impaired retention in the MWM. That is, although the CUS progeny found the escape platform in the same amount of time as did the control progeny, they showed a markedly reduced benefit from prior learning during the long-term retention test. There were no significant difference in the swimming velocity between the two progeny both in the spatial memory acquisition and retention, so the differences observed in water maze performance was not because of the different of motor ability. Although we did not investigate on the dissociation of memory acquisition and retention in the spatial memory test, a previous study has suggested that the long-term retention of information is more critically dependent upon entorhinal–hippocampal connections than information acquisition [21].

Serotonergic system and adrenergic system played critical roles in modulating functional neural circuits in brain, and were implicated in hippocampus-dependent memory [1,12,18]. The brain regions, such as hippocampus, hypothalamus, and prefrontal cortex, are involved in the response to stress and are the areas most relevant to depression [9,13]. We observed the reduced level of 5-HT in the hypothalamus but the increased level of NE in the hippocampus of CUS progeny compared to the control. Abnormalities in NE and 5-HT have been demonstrated in numerous depressed humans and animal models [1,12,18]; abnormally reduced 5-HT and NE or decreased 5-HT with increased NE have been documented [1,12,18]. In our studies, the results were more prone to support the latter.

Monoaminergic signaling pathways mainly act via G-proteins that in turn activation of adenylyl cyclase activity, protein kinase A (PKA), and transcription factor cAMP response element-binding protein (CREB) [5]. Phosphorylated CREB can regulate multiple target genes involved in the pathophysiology of depression [5,17]. In our studies, the relative level of P-CREB/β-actin in the CUS progeny was lower than that in the control progeny, which was similar to our previous studies in male rats between the CUS group and the control group [9].

We found significant correlations between the animal behavior and brain measures. Hypothalamal 5-HT level was positively correlated with the sucrose consumption percentage, suggesting the role of 5-HT playing in ingestive behavior [11]. Also there was a positive correlation between the sucrose consumption percentage and the hippocampal P-CREB level, we therefore could infer that down-regulation of P-CREB in the hippocampus might be one of the mechanisms underlying depression in the CUS progeny.

Taken together, the male progeny of the depressed maternal rat in our study showed behaviors consistent with depression, such as anhedonia, deficit in spatial memory and monoamine neurotransmitter disturbance. These findings in rat models support the notion that children of depressed mothers are at high risk for depressive and anxiety disorders in human [3,4]. Studies in human have also shown that when maternal depression is untreated, psychiatric disorders in their progeny are less likely to improve, while when maternal depression is treated until remission, decreased psychiatric symptoms and improved functioning in the progeny could be achieved [16,22]. The key signal transduction molecular P-CREB may also play important roles in the development of depression in the progeny of CUS mother. However, many questions still need to be answered include the effects maternal depression on the progeny development, the specific signal transduction pathways and molecular mechanism, and the effects of medical intervention. We will address these aspects in our forthcoming studies.

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