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Environmental Enrichment during Gestation Improves Behavior Consequences and Synaptic Plasticity in Hippocampus of Prenatal-Stressed Offspring Rats

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Prenatal stress can result in various behavior deficits in offspring. Here we tested the effects of environmental enrichment during gestation used as a preventive strategy on the behavior deficits of prenatal-stressed offspring rats as well as the underlying structure basis. We compared the effect size of environmental enrichment during gestation on prenatal-stressed offspring to that of environmental enrichment after weaning. Our results showed that environmental enrichment during gestation partially prevented anxiety and the damage in learning and memory in prenatal-stressed offspring as evaluated by elevated plus-maze test and Morris water maze test. At the same time, environmental enrichment during gestation inhibited the decrease in spine density of CA1 and dentate gyrus neurons and preserved the expression of synaptophysin and glucocorticoid receptors (GRs) in the hippocampus of prenatal-stressed offspring. There was no significant difference in offspring behavior between 7-day environmental enrichment during gestation and 14-day offspring environmental enrichment after weaning. These data suggest that environmental enrichment during gestation effectively prevented the behavior deficits and the abnormal synapse structures in prenatal-stressed offspring, and that it can be used as an efficient preventive strategy against prenatal stresses.

Key words: environmental enrichment, synaptic plasticity, prenatal stress, hippocampus, rat

I. Introduction

The gestation is a sensitive period vulnerable to various external stimuli, known as prenatal stress. High-level prenatal stress exerts profound impacts on the postnatal development of central nervous system in offspring and thus induces neurological deficits [2, 9, 20, 29]. Previous retrospective studies have shown that offspring from pregnant women exposed to natural and man-made unpleasant experience, such as earthquakes, wars or interpersonal relation disturbances, suffered from mental retardation, language hypoevolution, attention deficit, and neuropsychiatric impairment symptoms including schizophrenia, anxiousness and depression [17, 18, 30–32, 34]. Since multiple pathways are the underlying mechanisms mediating the effects of prenatal stress on different organs in the offspring, it is challenging to use single medication to antagonize all the damages caused by prenatal stress [34].

Environmental enrichment refers to modified experimental housing conditions with enhanced sensory, cognitive and motor stimulation, and sometimes combined with social stimulation. The neuroprotective effects of environmental enrichment have been proved in various experimental brain injury models including stroke, epilepsy, Alzheimer's disease, Parkinson's disease, Hungtington disease and so on. The effects of environmental enrichment on rats challenged with maternal separation and prenatal stresses were

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also investigated. It appears that adolescent exposure to an enriched environment reduced the activation of HPA axis in Long-Evans rats encountering maternal separation [12]. Enriched environment was also tested as potential therapy for postnatal brain damages [8]. Morley-Fletcher and colleagues found that prenatal-stressed rats with an adolescent exposure to enriched environment displayed improved performance in social behavior, which was accompanied by less activation of the HPA axis, possibly through beneficially affecting the basal immune parameters [19, 24]. Therefore, exposing the filial generation with enriched environment during adolescent was an effective way to reduce the brain impairment induced by prenatal stress. However, it remains to be determined whether environmental enrichment during gestation can be used as an efficient preventive strategy against prenatal stress. A recent study showed that the fetal development of visual system was accelerated by maternal exposure to enriched environment [28]. Therefore, in the present study we tested the effects of environmental enrichment during gestation on the behavior deficits of prenatal-stressed offspring. In addition, we compared the effects of 7-day environmental enrichment during gestation on prenatal-stressed offspring rats to that of 14-day environmental enrichment after weaning. Finally, we investigated possible underlying structural basis. Our results clearly show that environmental enrichment applied during gestation effectively prevents behavior deficits and abnormal synapse structures in prenatal-stressed offspring, and that it can be used as an efficient preventive strategy against prenatal stress.

II. Materials and Methods

1. Animals and experimental design

12 pregnant Sprague-Dawley (SD) rats (the day of plug was considered as gestation day 1) were provided by the department of experimental animals, Central South University. The rats were randomly divided into three groups: 1) Pregnant rats received no intervention and were raised under standard pregnant condition (n=4); 2) Pregnant rats received three times of restraint stress (45 min each session) per day from gestation day 13 (GD 13) to 19 (GD 19) and were raised under standard pregnant condition (n=4); 3) Pregnant rats received three times of the restraint stress per day from GD 13 to GD 19 and were raised under enriched pregnant condition (n=4) from GD 14 to GD 20. After birth, litters were culled to 8 pups within 24 h of birth, preferably with equal numbers of males and females [15]. Pups were raised by their own mother under the standard condition for 21 days. All offspring were weaned on postnatal day 21 (P21). Then, 12 offspring (6 male and 6 female, 3 offspring/litter) born to the rats in group 1 were randomly selected and regarded as control group (Ctrl); 12 offspring (6 male and 6 female, 3 offspring/litter) born to the rats in group 2 were randomly selected were regarded as prenatal stress group (PS); 12 offspring (6 male and 6 female, 3 offspring/litter) born to the rats in group 3 were randomly selected and regarded as prenatal stress plus maternal enrichment group (PS+ME). All rats in Ctrl, PS, and PS+ME group were raised under the offspring standard condition from P21 to P34. In addition, 12 offspring (6 male and 6 female, 3 offspring/litter) born to the rats in group 2 were randomly selected and were raised under the offspring enriched condition from P21 to P34. The 12 rats were regarded as prenatal stress plus offspring enrichment group (PS+OE). On P35, the above 48 rats started to be subjected to elevated plus-maze test and Morris Water Maze test. On P42, all of the 48 rats were sacrificed and brain samples were saved for structural and protein studies.

2. Prenatal stress protocol and raise conditioning

2.1 Prenatal stress protocol

The prenatal stress was applied from GD13 to GD19. The pregnant rat was placed in a plastic transparent restrainer (6.8 cm in diameter) with vent holes. Each stressed animal received three restraint sessions (45 min each) with a 15-min break between each session. Gentle and careful handling was practiced to avoid abortion. One of the following three time segments (8:00–11:00 a.m., 11:00 a.m.–2:00 p.m., and 4:00–7:00 p.m.) was randomly selected each day to minimize the adaptation in the stressed animals.

2.2 Raise conditioning

2.2.1 Standard conditions for pregnant rats and offspring

Pregnant rat was housed in a 16×12×8 cm cage alone. Four offspring were housed in a 30×20×15 cm cage after weaning. All cages of standard conditions had no treadmills, cartoon houses or toys. Food and water were available *ad libitum* and a 12-hr light and darkness cycle was used.

2.2.2 Enriched conditions of pregnant rats and offspring rats

Two pregnant rats were kept in a 40×25×30 cm cage at the same time. Four offspring rats were kept in a 40×25×30 cm cage. All cages of enriched conditions were equipped with treadmills, cartoon houses, and plastic toys. The plastic toys were replaced every day. Food and water were available *ad libitum* and a 12-hr light and darkness cycle was provided.

3. Behavioral tests

3.1 Elevated plus-maze test

Elevated plus-maze test was performed according to Pellow *et al.* [27]. The plus-maze used in the present study consists of two open arms (L×W, 50×10 cm) and two closed arms (L×W×H, 50×10×40 cm) connected to a central platform. The plus-maze was fixed on the scaffold and elevated to 50 cm high. Each animal was placed on the platform gently and the activity was recorded by a video camera for 5 min. The number of entries to each arm and the time spent in the open arms in each rat were counted.

3.2 Morris Water Maze

Rats were tested during the day (light phase). A 60 cm-high and 150 cm-diameter round tank was filled with

water at 25±1°C to a depth of 50 cm. A thin-layer of tiny white plastic foam was placed on the water surface. According to Notenboom *et al.* [26], the behavior test was composed of training for 4 days and probe trial. During the training, the water tank was divided into 4 quadrants and a hidden platform beneath the water surface was placed in the center of one of the quadrants. Each animal was placed in the water tank in every quadrant each day with head facing the wall of the tank, respectively. Each animal received four acquisition trials per day. The time for the rat to find the hidden platform was measured as the indicator of learning ability. The maximum trial length was 60 s with an approximately 15-min intertribal interval. If a rat failed to find the platform within the 60 s, it would be placed on the platform and remain on the platform for 10 s. For the probe trial on the 5th day, the platform was removed and each rat was allowed to swim for 60 s. Percent time spent in the target quadrant was utilized as the measure of learning in the probe trial. The rats' behavior was recorded by a video camera overhead and analyzed using video tracking software.

4. Tissue preparations

All experimental offspring were sacrificed under chloral hydrate anesthesia the day after Morris water maze test. The brain was dissected out rapidly and cut into two halves. The right half of the brain was subjected to Golgi staining, while the left half subjected to western blot and immunohistochemical staining.

5. Golgi staining and spine analysis

Golgi staining was carried out using FD Rapid GolgiStain TM kit (FD Neuro-Technologies Inc.). Briefly, the half brain was immersed in the mixture of Solution A and B and kept there for 14 days, followed by incubation in Solution C for 48 hr at 4°C. The tissue was then embedded and cryosectioned at a thickness of 100 μm. The sections were collected on gelatin-coated slides and incubated in the mixture consisting of Solution D and E and distilled water (Volume: 1:1:2) for 10 min at room temperature. All slides were dehydrated and covered with resinene.

For spine analysis, the criteria [37] for the inclusion of dendrites include cell body located in the region of interest (CA1 or granular cell layer), relatively intact dendritic tree, and no overlapping with neighboring dendrites. At least 3 cells were studied for each animal. In apical dendrites, the spine number was counted on five segments (>15 μm) randomly selected from the 3–5 tertiary apical dendrites with at least one branch point in a single neuron. In basal dendrites, the spine number was counted on five segments (>15 μm) randomly selected from the 2–4 tertiary basal dendrites with at least one branch point in a single neuron. The spine density was represented as number/10 μm.

6. Western blot

The hippocampus was quickly dissected out from the brain and stored at −70°C until use. The frozen tissue was homogenized in lysis buffer (Sigma, MO, USA) containing the following protease inhibitors: phenylmethylsulfonyl fluoride (100 μg/mL), leupeptin (1 μg/mL), pepstatin (1 μg/mL), and aprotinin (1 μg/mL). Lysates were cleared by centrifugation (16 000 g at 4°C for 20 min) and protein concentration was determined by BCA assay (Pierce, IL, USA). An aliquot of 20 μg protein was run on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to polyvinyldifluoride membranes (Biorad, CA, USA). The membrane was blocked in TBST (Tris-base buffer with 0.1% Tween-20) containing 5% skim milk and incubated with the primary antibody against synaptophysin (1:5000, Sigma, MO, USA) or glucocorticoid receptor (GR, 1:500, Santa Cruz, CA, USA) at room temperature for 2 hr, followed by 1 hr incubation with secondary antibodies conjugated to horse radish peroxidase (Biorad, CA, USA). The immunoreactive bands were detected by a chemoluminescent western blot kit (LumiGLO, USA) and exposed to Kodak Biomax film (Eastman Kodak, NY, USA). Bands were quantified by the mean gray value using NIH Image J 7.0. β-actin was used as internal loading control.

7. Immunohistochemistry

The brain samples were fixed in 4% paraformaldehyde overnight and placed in 30% sucrose solution at 4°C for 48 hr. The brain was then cryosectioned coronally at a thickness of 30 μm. The sections were rinsed in 0.01 M PBS and incubated in 3% hydrogen peroxide solution to neutralize endogenous peroxydases. The sections were then blocked in 5% bovine serum solution for 1 hr at room temperature. After rinsing with 0.01 M PBS, the sections were incubated in primary antibody containing either anti-synaptophysin (1:2000) or anti-GR (1:500) at 4°C overnight and biotinylated goat anti-mouse antibody (1:200, vector, CA, USA) for 1 hr at room temperature, followed by incubation with avidin conjugated to horse radish peroxidase for 1 hr at room temperature. Finally, the sections were incubated in 0.01 M PBS solution containing 0.05% diaminobenzidine and 0.3% hydrogen peroxide. The reaction was stopped when the appropriate. Sections were dehydrated and covered and stored at 4°C until studied.

8. Statistic analyses

Quantitative data are expressed as mean±SEM. Statistical analyses were performed using SPSS 13.0. Three-way ANOVA and one way ANOVA were used as indicated. Student-Newman-Keuls test (SNK-q) was used to test homogeneity of variance and Kruskal Wallish Test was used to analyze the data with heterocedasticity. A $P<0.05$ was considered significant.

III. Results

1. Environmental enrichment during gestation partially prevented anxiety-like behavior in prenatal-stressed offspring

In the elevated plus-maze test, the longer time in open arms and the higher frequency entering the open arms

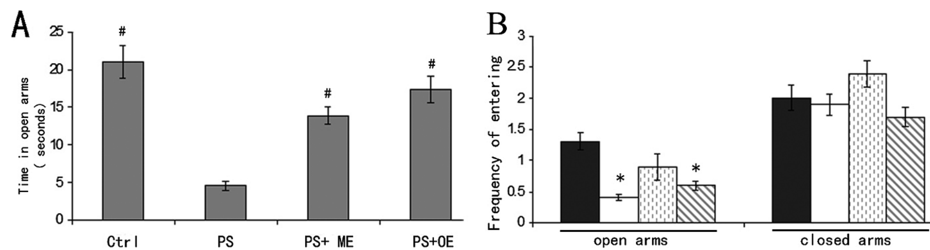

Fig. 1. Environmental enrichment during gestation reduced anxiety-like behavior in prenatal-stressed offspring. Compared to the Ctrl group, the time in open arms (**A**) and the frequency entering the open arms (**B**) were both decreased in the PS group ($P<0.05$). Environmental enrichment during gestation and after weaning both increased the time in open arms (**A**) in offspring rats ($P<0.05$ vs the PS group). There was no difference between prenatal enrichment and offspring enrichment (**A** and **B**, $P>0.05$). (n=12/group). Black bar: Ctrl group, white bar: PS group, white bar with black dot: PS+ME group, White bar with black Oblique line: PS+OE group. * $P<0.05$, vs the Ctrl group, # $P<0.05$, vs the PS group.

represent less anxiety. In this study, three way ANOVAs analysis showed that prenatal stress significantly reduced the average time staying in the open arm in rats in PS group which were offspring from a stressed rat ($F(1, 36)=19.495$, $P=0.000$). Moreover, Environmental enrichment either during gestation or after weaning exhibited significant effects on time in open arms, respectively ($F(1, 36)=6.264$, $P=0.017$; $F(1, 36)=11.831$, $P=0.001$). As shown in Figure 1A, rats from the Ctrl group, who received no prenatal stress and were raised in the standard condition, spent 20.99±2.23 s in the open arms, while rats in the PS group spent 4.50±0.58 s ($P<0.01$ vs. control). Environmental enrichment significantly increased the time staying in the open arm for the rats in the PS+ME group (13.85±1.08 s) and in the PS+OE group (17.35±1.74 s), as compared to the PS group (Fig. 1A). However, the average time staying the open arms both in the PS+ME group and the PS+OE group was still lower than that in the Ctrl group, respectively (Fig. 1A). Prenatal stress had obvious effects on the frequency of rats entering the open arms in the PS group (Fig. 1B). No changes were observed in the frequency of entering the closed arms among the four groups (Fig. 1B). The above results indicated that prenatal stress greatly increased anxiety-like behavior in the offspring, which could be significantly alleviated by environmental enrichment during gestation or after weaning. However, there was no significant difference in the size of protective effect between 7-d environmental enrichment during gestation and 14-d environmental enrichment after weaning.

2. Environmental enrichment during gestation partially reduced the damage in learning and memory in prenatal-stressed offspring

The Morris Water Maze is widely used to test the spatial learning capability and memory in experimental animals. We recorded the latency in finding the hidden platform in the four groups of rats for 4 consecutive days. On day 1, rats in the PS group showed the longest latency ($F(1, 36)=13.408$, $P=0.001$, Fig. 2A). The prenatal tress also significantly increased the latency in the PS group on day 3 and 4, respectively (Day 2, $F(1, 36)=2.479$, $P=0.124$; Day 3, $F(1, 36)=9.301$, $P=0.004$; Day 4, $F(1, 36)=8.992$, $P=0.005$, vs. the Ctrl group). No significant difference in the latency was observed either in the PS+ME group or in the PS+OE group compared to the PS group (Day 1: ME $F(1, 36)=5.034$, $P=0.031$, OE $F(1, 36)=4.972$, $P=0.032$; Day 2: ME

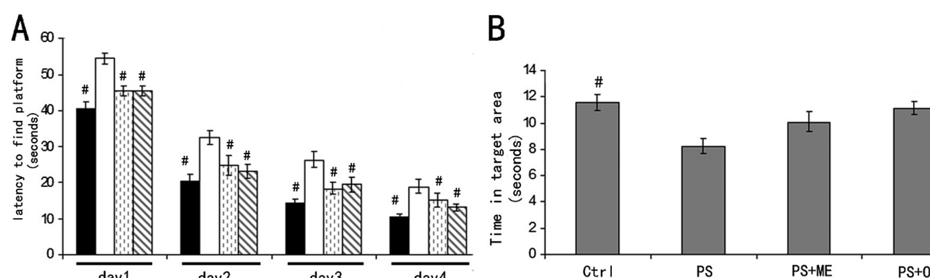

Fig. 2. Environmental enrichment during gestation partially reduced the damage in learning and memory of prenatal-stressed offspring. **A:** The latency to find the hidden platform gradually decreased in the four groups during the study process. In the first four days, the latency of PS group was significantly longer than other three groups ($P<0.05$). **B:** The time in the target area was significantly decreased in the PS group, compared to the Ctrl group ($P<0.05$). The time for the PS+ME and the PS+OE groups both was partially increased compared to the PS group (n=12 rats per group). Black bar: Ctrl group, white bar: PS group, white bar with black dot: PS+ME, White bar with black Oblique line: PS+OE. # $P<0.05$, vs. with the PS group.

F (1, 36)=0.543, P=0.466, OE F (1, 36)=2.838, P=0.101; Day 3: ME F (1, 36)=4.168, P=0.049, OE F (1, 36)=3.045, P=0.090; Day 4: ME F (1, 36)=1.803, P=0.188, OE F (1, 36) =5.682, P=0.023) in the late stage of study. The data showed that prenatal stress greatly impaired the learning ability in the offspring, while environmental enrichment during gestation or after weaning both were able to reduce the impairment of the learning ability.

The time that each animal spent in the target quadrant where the hidden platform was previously placed was summarized in Fig. 2B. There was significant decrease in the time for that rats in the PS group as compared to that in the Ctrl group (P<0.05). The time in the area of interest increased in the PS+ME group when compared to the PS group (P>0.05). The data suggest environmental enrichment during gestation partially reduced the memory damage caused by the prenatal stress.

3. Environmental enrichment during gestation partially inhibited the damage of synaptic plasticity in hippocampus of prenatal-stressed offspring

To study the dendritic and synaptic morphology of hippocampal neurons, we used Golgi staining to outline the spines on neuronal processes as described in previous study [37]. Figure 3A–D showed the representative Golgi-stained pyramidal neurons in the hippocampal CA1 from the Ctrl, the PS, the PS+ME and the PS+OE group, respectively. The quantitative analysis showed that prenatal stress greatly decreased the spine density on apical dendrites in the PS group as compared to controls (P<0.05, Fig. 3E). The spine density on apical dendrites in both the PS+ME group and the PS+OE group was significantly higher than that in the PS group (P<0.05, Fig. 3E), but still lower than that in the Ctrl group (Fig. 3E). In basal dendrites, no significant change in the spine density was observed among the four different groups (P>0.05, Fig. 3F).

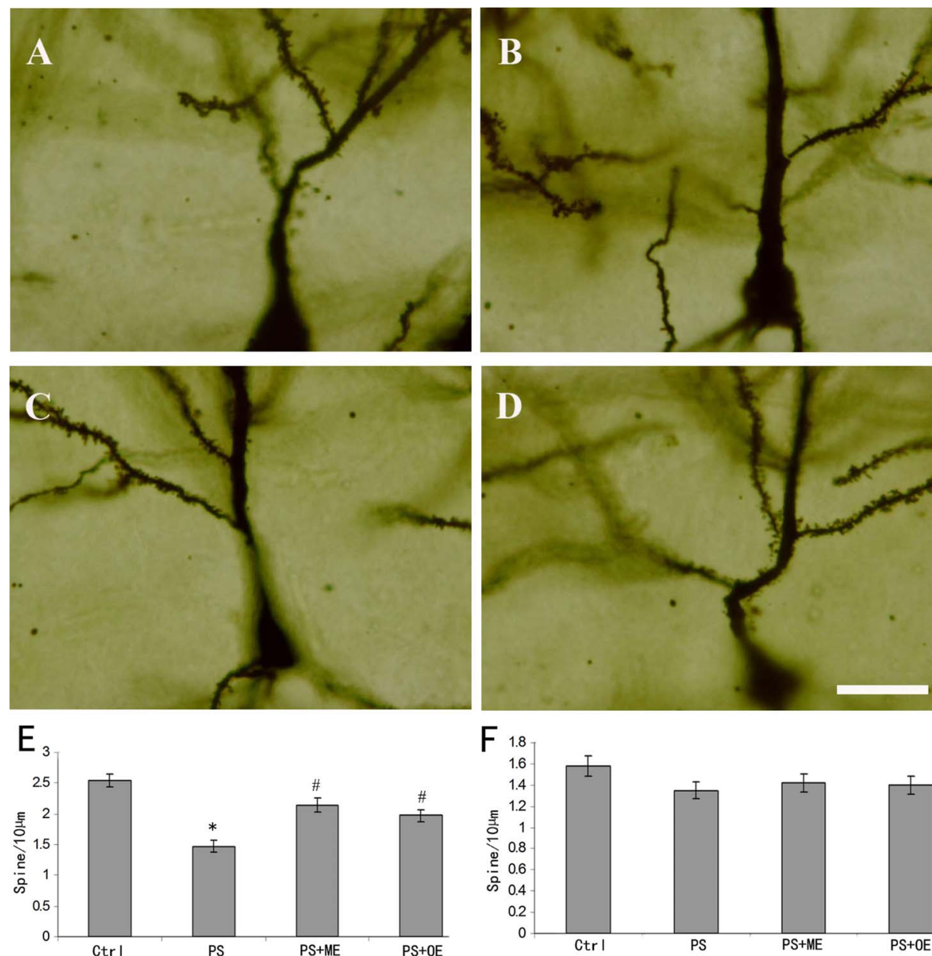

Fig. 3. Environmental enrichment during gestation increased the spine density of hippocampal CA1 neurons in prenatal-stressed offspring. **A–D**: Representative Golgi-stained neurons in hippocampal CA1 from the Ctrl, the PS, the PS+ME and the PS+OE groups, respectively. The black process in neuronal branches is the spine. **E**: The spine density of apical dendrites in the PS group was significantly lower than that of the Ctrl group (P<0.05). The spine density of apical dendrites in the PS+ME group and the PS+OE group were significantly increased when compared to the PS group (P<0.05). **F**: In basal dendrites, no significant change in the spine density was observed among the four different groups (n=6/group). * P<0.05, vs the Ctrl group, $^{\#}$ P<0.05, vs the PS group. Bar=20 μm.

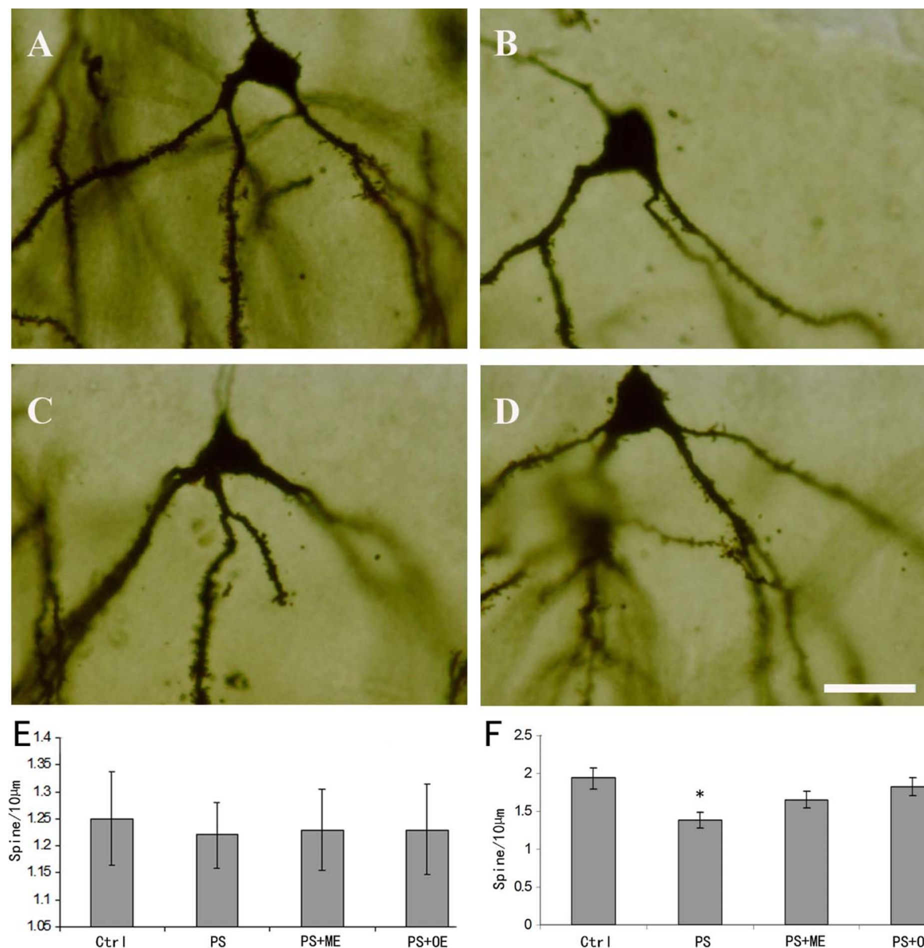

Fig. 4. Maternal environmental enrichment during gestation increased the spine density of granular cells of the dentate gyrus of prenatal-stressed offspring. **A–D**: Representative Golgi-stained neurons in dentate gyrus of the Ctrl, the PS, the PS+ME and the PS+OE groups, respectively. **E**: In granular cell dendrites of the inner part of dentate gyrus, no change was detected in the spine density among the four groups. **F**: The spine density in the granular cells of the outer part of dentate gyrus in the PS group was greatly reduced when compared to the Ctrl group ($P<0.05$, *) and the PS+MS group and the PS+OS group ($P>0.05$) (n=6/group). Bar=20 μm.

Figure 4A–D are typical morphological images of the spines on dendrites of granular cells in the dentate gyrus in all four groups. Quantitative data showed there was no change in the spine density in inner granular cell layer among the four different groups ($P>0.05$, Fig. 4E). In contrast, for the dendrites of granular cells in the outer granular cell layer, the spine density in the PS group was greatly reduced by the prenatal stress ($P<0.05$ vs. the Ctrl group, Fig. 4F). Rats in the PS+ME group and the PS+OE group displayed a tendency for higher average spine density than that in the PS group, although the changes were not significant ($P>0.05$, Fig. 4F). The data suggested that environmental enrichment during gestation or after weaning both increased the spine density in the CA1 and the dentate gyrus in the prenatal-stressed rats.

To confirm the changes in synaptic structure in the hippocampus, we studied the expression of synaptophysin, a synaptic vesicle protein, in the hippocampus of four groups. Synaptophysin staining in the hilus of PS groups (Fig. 5B) was weaker compared to that of the Ctrl, the PS+ME and the PS+OE groups (Fig. 5A, C, and D). Furthermore, the expression of synaptophysin in hippocampus was quantitated by western blot, showing decrease in the PS group ($P<0.05$ vs. the controls, Fig. 5E and F). The decreased expression of synaptophysin in hippocampus was rescued in both the PS+ME group and the PS+OE group ($P<0.05$, Fig. 5E and F). In summary, the restoration of spine density and synaptophysin expression indicated that the maternal environmental enrichment was able to reduce synaptic structure damage in hippocampus induced by prenatal stress.

4. Environmental enrichment during gestation partially inhibited the decrease of GR expression in the hippocampus of prenatal-stressed offspring

GRs play a critical role in the modulation of hippocampus synaptic function [14, 36]. Therefore, the expression of GRs in the hippocampus was measured by immunohistochemistry and western blot. As shown in Figure 6, the staining of GR in the CA1 of PS group (Fig.

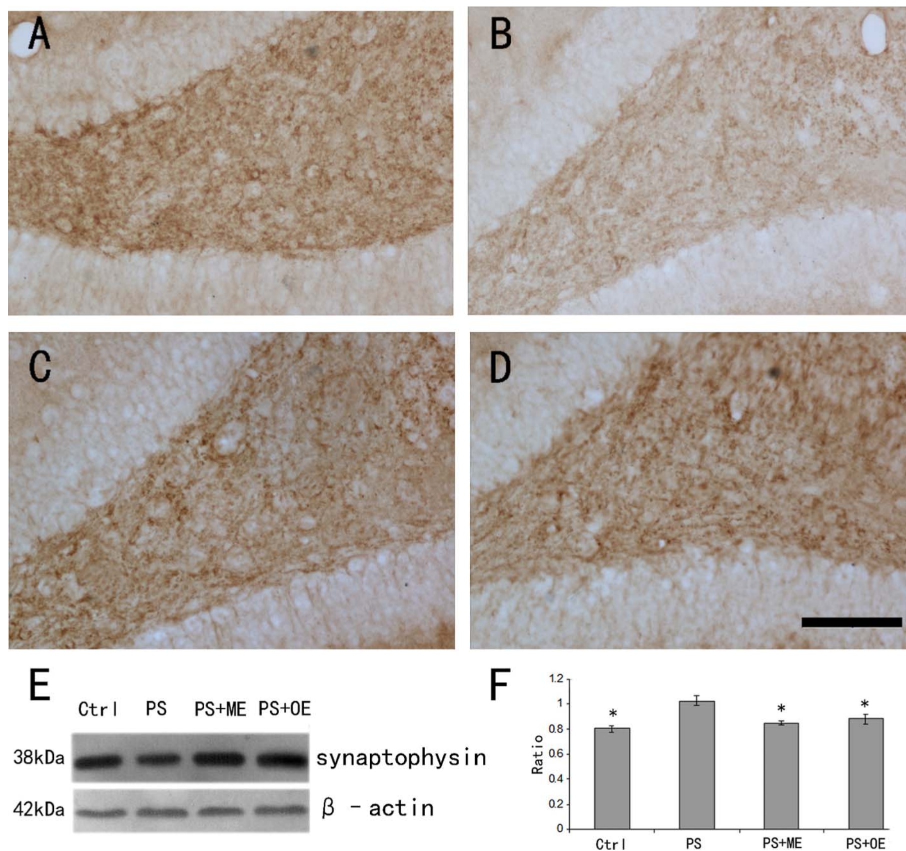

Fig. 5. Environmental enrichment during gestation increased synaptophysin expression in hippocampus in prenatal-stressed offspring. **A–D**: Representative synaptophysin staining in the hilus of the Ctrl, the PS, the PS+ME and the PS+OE groups, respectively. Synaptophysin staining in PS group (Panel **B**) was weaker than that of the other three groups (Panel **A, C**, and **D**). **E**: Synaptophysin expression in hippocampus was detected by western blot. The relative mean grey value ratio in the PS group was significantly higher than that of the other three groups (*: P<0.05, vs the PS group, n=6/group). Bar=50 μm.

6B) was weaker than that of the other three groups (Fig. 6A, C, and D). Similar changes in GRs expression in hippocampus were confirmed by western blot (Fig. 6E and F). The expression of GRs in the PS group was significantly lower than those in the other three groups (P<0.05 Fig. 6E and F). There was no significant difference in the GRs expression between PS+ME group and the PS+OE group (P>0.05) (Fig. 6E and F). The above data indicated that prenatal stress was able to decrease the expression of GRs in the hippocampus in the offspring, while environmental enrichment during gestation as well as after weaning showed a rescuing effect.

IV. Discussion

The present study was designed to test the effects of environmental enrichment during gestation on behavior deficits of prenatal-stressed offspring as well as the underlying structural basis and eventually to be used as a preventative strategy. Our results demonstrated that environmental enrichment during gestation effectively prevented the anxiety behavior, as well as the impairment in learning and memory in prenatal-stressed rats. Environmental enrichment during gestation also partially alleviated the decrease in spine density and the expression of synaptophysin and GRs in the hippocampus of prenatal-stressed rats.

1. Environmental enrichment during gestation can be an efficient preventive strategy against behavior deficits in prenatal-stressed rats

Prenatal stress is one of the critical factors affecting the brain development in offspring [7]. In the present study, restraint stress three times a day for 7 days was used to mimic maternal stress in female pregnant rats and several behavior tests were performed in the offspring. Our data showed that restraint stress caused reduced entries in the elevated plus-maze test, longer latency and reduced time spent in the target quadrant in the Morris Water Maze. All the above behavioral changes indicated that prenatal stress increased level of anxiety and impaired spatial learning and memory in offspring. The finding is consistent with previous studies showing that prenatal stress greatly increased anxiety-like and depression-like activities and compromised learning and memory abilities in rodents [6, 11, 12, 35].

To antagonize the negative effects of prenatal stress on offspring, the environmental enrichment during gestation

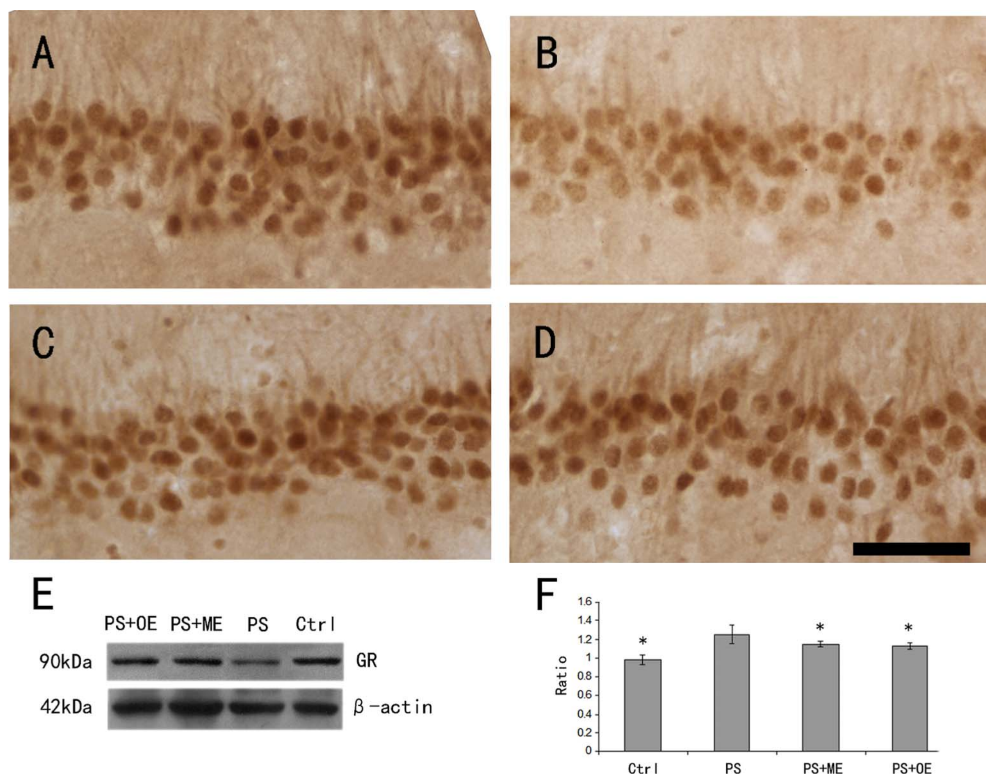

Fig. 6. Environmental enrichment during gestation partially inhibited the decrease of GR expression in the hippocampus of prenatal-stressed offspring. **A–D**: Representative GR staining in the CA1 of the Ctrl, the PS, the PS+ME and the PS+OE groups respectively. GR staining in PS group (Panel **B**) was weaker than that of the other three groups (Panel **A, C**, and **D**). **E**: The expression of GRs in hippocampus was detected by western blot. **F**: The relative mean grey value ratio in PS group was significantly higher than that of the other three groups (*: $P<0.05$, n=6/group). Bar=50 μm.

was studied in the present study. Our results showed that environmental enrichment from GD14 to GD20 dramatically increased the number of entries in the open arms and the time spent there in the offspring, reduced the latency in finding the platform and prolonged the stay in the target quadrant in the Morris Water Maze, alleviating behavioral changes induced by prenatal stress. Bastani and co-workers reported [3] that in clinical settings, the application of relaxation training to pregnant women reduced anxiety and perceived stress and decreased the occurrence of low birth weight infant. Both Bastani's data and our present data proved that maternal intervention could be a preventive and therapeutic strategy to effectively reduce the developmental deficits caused by prenatal stress.

Moreover, we compared the beneficial effects of environmental enrichment during gestation on offspring with that of the offspring environmental enrichment to achieve better outcome. Our results did not show any difference in the elevated plus-maze test and the Morris Water Maze (Figs. 1 and 2). However, the maternal environmental enrichment only lasted 7 days, whereas the offspring environmental enrichment lasted 14 days in the present study. This argues in favor of maternal environmental enrichment during gestation, since it achieved similar end effects with a shorter period of intervention. The maternal intervention was applied before any of the behavioral abnormalities actually showed up so that it could be regarded as a more powerful preventive strategy than the offspring environmental enrichment, which functioned more in a damage control fashion.

2. The effects of environmental enrichment during gestation on the synaptic plasticity of offspring hippocampus underlied the improvement in the behavior deficits of prenatal-stressed offspring

The hippocampus is the major structure regulating the mood and the formation of spatial memory. Most external input comes from the adjoining entorhinal cortex (EC), via the axons of the so-called perforant path and terminates in the dentate gyrus and CA3. In the end of the loop, pyramidal cells of CA1 send their axons to the subiculum and deep layers of the EC. This loop is named the trisynaptic circuit by Per Andersen [1]. Therefore, the granular cells receiving most input in the hippocampus and the pyramidal cells in charge of the output of hippocampus in the CA1 region were studied by Golgi staining. The expression of synaptophysin in hippocampus was tested to confirm the staining result. We showed that prenatal stress significantly reduced the spine density of CA1 pyramidal cells and granular cells located in the outer part of the granular cell layer. Spine is

a specialized postsynaptic protrusion in dendrites where the signal is received by postsynaptic neurons. Synaptophysin, a 38 kD transmembrane glycoprotein, is located in the presynaptic site in all kinds of neurons. Synaptophysin is a key protein in regulating the release of neurotransmitter in the presynaptic site [5]. Our immunohistochemical staining data revealed decreased expression of synaptophysin in the hippocampus. Our findings suggest that the prenatal stress undermines the hippocampal trisynaptic connections. The decreased spine number indicated impaired signal receiving, while reduced expression of synaptophysin suggested impaired output in hippocampal circuits. The combination leads to bigger damage to learning and memory. It was reported that the prenatal stress reduced the spine density in the prefrontal cortex, the anterior cingulate cortex [25] and hippocampus [13]. Previous microarray studies found that a large number of genes encoding pre- and post-synaptic proteins (including synatophysin) were downregulated by prenatal stress [4, 16], which may explain the anxiety behavior induced by prenatal stress [4]. More interestingly, the data in the present study found that environmental enrichment during gestation reversed the decreases in the spine density of CA1 pyramidal cells and granular cells located in the outer part of the granular cell layer, as well as the expression of synaptophysin in prenatal-stressed rats. Yang and co-workers had found that environmental enrichment in the early postnatal days restored long-time potential in the CA1 region and consequently improved learning and memory [35]. It was likely that the better performance in behavior induced by environmental enrichment that we observed could also be explained by improved synaptic function in the hippocampus.

Corticosterone is the counterpart hormone of cortisol in rodents. Excess corticosterone was thought to be a major stress hormone mediating the effects of prenatal stress [10, 33] Corticosterone acts through binding to two types of corticosteroid receptors: mineralocorticoid receptors (MRs) and GRs. GRs were considered to play a more important part than MRs during hypercortisolemia [23]. Therefore, we tested the expression of GRs in hippocampus in the present study. As previously reported [24], our results found that the prenatal stress greatly reduced the expression of GRs in the CA1 field as well as in the whole hippocampus. Prenatal stress also reduced the local expression of GRs in CA2 field (data not shown). Importantly, environmental enrichment during gestation prevented the decrease of GRs expression in the prenatal-stressed rats. The importance of GRs in the modulation of hippocampus synaptic function was well investigated in the CA1 subregion [36] and the dentate gyrus [14]. For example, RU 38486, an antagonist of GRs, completely blocked the stress-induced synaptic depression, such as long-term depression, in the hippocampal slice [36]. However, whether the improvement in synaptic morphology and function by environmental enrichment during gestation was GR-dependent or not needs to be further explored. In addition, Lee *et al.* found that prenatal stress induced long-term dysregulation of nongenomic glucocorticoid action in the amygdala of adult offspring, which resulted in abnormal behaviors of offspring [21]. Thus further study is also needed to explore whether glucocorticoid and its receptors in the amygdale are involved in the prevention effects of environmental enrichment during gestation on prenatal-stressed offspring.

In conclusion, our study proved that environmental enrichment during gestation decreased anxiety-like behavior and partially prevented the impairment in learning and memory in prenatal-stressed rats. Our finding supported the notion that environmental enrichment during gestation could serve as an effective preventive strategy against the damage induced by prenatal stress. The beneficial effects of environmental enrichment during gestation might be due to the preservation of synaptic morphology and function in the hippocampus. The underlying mechanism merits worth further investigation.

V. References

1. Anderson, P., Bliss, T. V. and Skrede, K. K. (1971) Lamellar organization of hippocampal pathways. *Exp. Brain Res.* 13; 222–238.
2. Bale, T. L. (2011) Sex differences in prenatal epigenetic programing of stress pathways. *Stress* 14; 348–356.
3. Bastani, F., Hidarnia, A., Kazemnejad, A., Vafaei, M. and Kashanian, M. (2005) A randomized controlled trial of the effects of applied relaxation training on reducing anxiety and perceived stress in pregnant women. *J. Midwifery Womens Health* 50; e36–40.
4. Bogoch, Y., Biala, Y. N., Linial, M. and Weinstock, M. (2007) Anxiety induced by prenatal stress is associated with suppression of hippocampal genes involved in synaptic function. *J. Neurochem.* 101; 1018–1030.
5. Calhoun, M. E., Jucker, M., Martin, L. J., Thinakaran, G., Price, D. L. and Mouton, P. R. (1996) Comparative evaluation of synaptophysin-based methods for quantification of synapses. *J. Neurocytol.* 25; 821–828.
6. Chapillon, P., Patin, V., Roy, V., Vincent, A. and Caston, J. (2002) Effects of pre- and postnatal stimulation on developmental, emotional, and cognitive aspects in rodents: a review. *Dev. Psychobiol.* 41; 373–387.
7. Charil, A., Laplante, D. P., Vaillancourt, C. and King, S. (2010) Prenatal stress and brain development. *Brain Res. Rev.* 65; 56–79.
8. Francis, D. D., Diorio, J., Plotsky, P. M. and Meaney, M. J. (2002) Environmental enrichment reverses the effects of maternal separation on stress reactivity. *J. Neurosci.* 22; 7840–7843.
9. Gerecke, K. M., Kishore, R., Jasnow, A., Quadros-Menella, P., Parker, S., Kozub, F. J., Lambert, K. G. and Kinsley, C. H. (2012) Alterations of sex-typical microanatomy: Prenatal stress modifies the structure of medial preoptic area neurons in rats. *Dev. Psychobiol.* 54; 16–27.
10. Glavas, M. M., Yu, W. K. and Weinberg, J. (2006) Effects of mineralocorticoid and glucocorticoid receptor blockade on hypothalamic-pituitary-adrenal function in female rats prenatally exposed to ethanol. *Alcohol Clin. Exp. Res.* 30; 1916–1924.
11. Guilarte, T. R., Toscano, C. D., McGlothan, J. L. and Weaver, S. A. (2003) Environmental enrichment reverses cognitive and molecular deficits induced by developmental lead exposure. *Ann. Neurol.* 53; 50–56.
12. Hellemans, K. G., Benge, L. C. and Olmstead, M. C. (2004) Adolescent enrichment partially reverses the social isolation syndrome. *Brain Res. Dev. Brain Res.* 150; 103–115.

13. Ishiwata, H., Shiga, T. and Okado, N. (2005) Selective serotonin reuptake inhibitor treatment of early postnatal mice reverses their prenatal stress-induced brain dysfunction. *Neuroscience* 133; 893–901.
14. Karst, H. and Joels, M. (2003) Effect of chronic stress on synaptic currents in rat hippocampal dentate gyrus neurons. *J. Neurophysiol.* 89; 625–633.
15. Keshet, G. I. and Weinstock, M. (1995) Maternal naltrexone prevents morphological and behavioral alterations induced in rats by prenatal stress. *Pharmacol. Biochem. Behav.* 50; 413–419.
16. Kinnunen, A. K., Koenig, J. I. and Bilbe, G. (2003) Repeated variable prenatal stress alters pre- and postsynaptic gene expression in the rat frontal pole. *J. Neurochem.* 86; 736–748.
17. Koenig, J. I., Elmer, G. I., Shepard, P. D., Lee, P. R., Mayo, C., Joy, B., Hercher, E. and Brady, D. L. (2005) Prenatal exposure to a repeated variable stress paradigm elicits behavioral and neuroendocrinological changes in the adult offspring: potential relevance to schizophrenia. *Behav. Brain Res.* 156; 251–261.
18. Kofman, O. (2002) The role of prenatal stress in the etiology of developmental behavioural disorders. *Neurosci. Biobehav. Rev.* 26; 457–470.
19. Laviola, G., Rea, M., Morley-Fletcher, S., Di Carlo, S., Bacosi, A., De Simone, R., Bertini, M. and Pacifici, R. (2004) Beneficial effects of enriched environment on adolescent rats from stressed pregnancies. *Eur. J. Neurosci.* 20; 1655–1664.
20. Lazinski, M. J., Shea, A. K. and Steiner, M. (2008) Effects of maternal prenatal stress on offspring development: a commentary. *Arch. Womens Ment. Health* 11; 363–375.
21. Lee, E. J., Son, G. H., Chung, S., Lee, S., Kim, J., Choi, S. and Kim, K. (2011) Impairment of fear memory consolidation in maternally stressed male mouse offspring: evidence for non-genomic glucocorticoid action on the amygdala. *J. Neurosci.* 31; 7131–7140.
22. Lister, R. G. (1990) Ethologically-based animal models of anxiety disorders. *Pharmacol. Ther.* 46; 321–340.
23. Lu, N. Z., Wardell, S. E., Burnstein, K. L., Defranco, D., Fuller, P. J., Giguere, V., Hochberg, R. B., McKay, L., Renoir, J. M., Weigel, N. L., Wilson, E. M., McDonnell, D. P. and Cidlowski, J. A. (2006) International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. *Pharmacol. Rev.* 58; 782–797.
24. Morley-Fletcher, S., Rea, M., Maccari, S. and Laviola, G. (2003) Environmental enrichment during adolescence reverses the effects of prenatal stress on play behaviour and HPA axis reactivity in rats. *Eur. J. Neurosci.* 18; 3367–3374.
25. Murmu, M. S., Salomon, S., Biala, Y., Weinstock, M., Braun, K. and Bock, J. (2006) Changes of spine density and dendritic complexity in the prefrontal cortex in offspring of mothers exposed to stress during pregnancy. *Eur. J. Neurosci.* 24; 1477–1487.
26. Notenboom, R. G., Ramakers, G. M., Kamal, A., Spruijt, B. M. and de Graan, P. N. (2010) Long-lasting modulation of synaptic plasticity in rat hippocampus after early-life complex febrile seizures. *Eur. J. Neurosci.* 32; 749–758.
27. Pellow, S., Johnston, A. L. and File, S. E. (1987) Selective agonists and antagonists for 5-hydroxytryptamine receptor subtypes, and interactions with yohimbine and FG 7142 using the elevated plus-maze test in the rat. *J. Pharm. Pharmacol.* 39; 917–928.
28. Sale, A., Cenni, M. C., Ciucci, F., Putignano, E., Chierzi, S. and Maffei, L. (2007) Maternal enrichment during pregnancy accelerates retinal development of the fetus. *PLoS One* 2; e1160.
29. Solano, M. E., Jago, C., Pincus, M. K. and Arck, P. C. (2011) Highway to health; or How prenatal factors determine disease risks in the later life of the offspring. *J. Reprod. Immunol.* 90; 3–8.
30. Watson, J. B., Mednick, S. A., Huttunen, M. and Wang, X. (1999) Prenatal teratogens and the development of adult mental illness. *Dev. Psychopathol.* 11; 457–466.
31. Weinstock, M. (1997) Does prenatal stress impair coping and regulation of hypothalamic-pituitary-adrenal axis? *Neurosci. Biobehav. Rev.* 21; 1–10.
32. Weinstock, M. (2001) Alterations induced by gestational stress in brain morphology and behaviour of the offspring. *Prog. Neurobiol.* 65; 427–451.
33. Weinstock, M. (2005) The potential influence of maternal stress hormones on development and mental health of the offspring. *Brain Behav. Immun.* 19; 296–308.
34. Weinstock, M. (2008) The long-term behavioural consequences of prenatal stress. *Neurosci. Biobehav. Rev.* 32; 1073–1086.
35. Yang, J., Hou, C., Ma, N., Liu, J., Zhang, Y., Zhou, J., Xu, L. and Li, L. (2007) Enriched environment treatment restores impaired hippocampal synaptic plasticity and cognitive deficits induced by prenatal chronic stress. *Neurobiol. Learn Mem.* 87; 257–263.
36. Yang, Y., Zheng, X., Wang, Y., Cao, J., Dong, Z., Cai, J., Sui, N. and Xu, L. (2004) Stress enables synaptic depression in CA1 synapses by acute and chronic morphine: possible mechanisms for corticosterone on opiate addiction. *J. Neurosci.* 24; 2412–2420.
37. Yau, S. Y., Lau, B. W., Tong, J. B., Wong, R., Ching, Y. P., Qiu, G., Tang, S. W., Lee, T. M. and So, K. F. (2011) Hippocampal neurogenesis and dendritic plasticity support running-improved spatial learning and depression-like behaviour in stressed rats. *PLoS One* 6; e24263.

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