Research Report

Influenza A(H1N1) vaccination during early pregnancy transiently promotes hippocampal neurogenesis and working memory. Involvement of Th1/Th2 balance

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ABSTRACT

The 2009 influenza A(H1N1) pandemic led to a particularly high risk of morbidity and mortality among pregnant women. Therefore, inactivated influenza vaccines have been widely recommended for women in any period of gestation. Recent studies have shown that the peripheral adaptive immune system plays an important role in the function of the central nervous system (CNS). The present study was conducted to explore if influenza vaccination, aiming to induce protective immune activation, affects maternal neurogenesis and cognitive ability. The results showed that A(H1N1) pregnant mice (AIV+Pre) had superior spatial working memory performance compared with pregnant controls (Pre). At the cellular level, a transient increase in both cell proliferation and neuronal differentiation in the dentate gyrus (DG) was found in the AIV+Pre group compared with the Pre group when BrdU was injected on gestational day 14 (G14). However, there were no obvious differences between A(H1N1) virgin mice (AIV+Vir) and virgin controls (Vir) in both hippocampal neurogenesis and working memory. Our findings further indicated that prolactin (PRL) concentrations were not overtly different between the AIV+Pre group and the Pre group at any time. Interestingly, IL-4 and IFN-γ levels were obviously increased both in the serum and hippocampus of the AIV+Pre group (with a T helper-1 like response; Th1) compared with the Pre group (with a T helper-2 like response; Th2) at G14, whereas the expression of IL-6 and TNF-α, the proinflammatory factors, was significantly reduced. Altogether, the results suggest that A(H1N1) vaccination during early pregnancy may contribute to adult hippocampal neurogenesis and spatial working memory and that the improvements were, at least in part, associated with Th1/Th2 balance.
1. Introduction

Brain plasticity allows the brain to adapt structurally to a changing environment. The peripheral immune response as a neuroethology relevant stressor (Dantzer et al., 2008) is important to the performance (Sapolsky, 2000a; Sapolsky et al., 2000b). One prime example of brain plasticity is adult neurogenesis, in which new neurons are generated in a sustained manner throughout life at a relatively low rate (Kuhn et al., 1996; Cameron and McKay, 1999). It is still unclear how this process is regulated physiologically. One of the two regions involved in adult neurogenesis is the hippocampal dentate gyrus (DG), a brain region that is centrally involved in spatial learning and memory (Eriksson et al., 1998; Neves et al., 2008). It has been reported that immune activation signals the CNS through cytokines, which can penetrate the blood-brain barrier (BBB) and play a role in brain development (Deverman and Patterson, 2009; Diz-Chaves et al., 2012). For instance, peripheral lipopolysaccharide (LPS) application activates an innate immune response, which robustly decreases adult hippocampal neurogenesis (Ekdahl et al., 2003; Monje et al., 2003). The association between the CNS and the adaptive immune system is also reflected in the performance of hippocampal-dependent spatial ability, such as in the radial arm maze task (Karkada et al., 2012) and the Morris Water Maze task (Kipnis et al., 2004).

Previous studies have shown that pregnant and postpartum women are vulnerable to influenza pandemics (Jamieson et al., 2009). Maternal infection during pregnancy can also increase the incidence of neurodevelopmental disorders such as schizophrenia and autism in the offspring (Ebert and Kotler, 2005; Boksa, 2008). Therefore, the Advisory Committee on Immunization Practices recommends that pregnant women should receive inactivated influenza vaccine at any stage of gestation to prevent the virus. Although immunogenicity and the safety have been confirmed, influenza vaccination, which inducing strong immune activation, is very likely to affect adult neurogenesis and cognitive function (Ziv and Schwartz, 2008; Gonzalez-Perez et al., 2010).

Th1/Th2 cytokine balance has proven to be a main mediator of structural alterations and neurogenesis in animal models of chronic stress (McEwen, 2001). In particular, recent studies indicated a positive correlation between hippocampal neurogenesis and the Th1/Th2 balance. Researchers have reported decreased hippocampal neurogenesis is associated with decrease in the Th1/Th2 balance in animal studies of aging (Baruch et al., 2013), whereas a correlation between increased hippocampal neurogenesis and an increase in the Th1/Th2 balance has also been reported in studies of Glatiramer Acetate (Palumbo et al., 2012) and moderate exercise (Gholamnezhad et al., 2014; Zhao et al., 2012).

The present study was designed to explore the influence of A(H1N1) vaccination during early pregnancy on maternal hippocampal neurogenesis and spatial memory ability. It may unveil another beneficial effect of A(H1N1) vaccination other than infection prevention.

2. Results

2.1. Maternal vaccination and antibody levels

The serum antibody titers of all of the pregnant mice were at baseline level before vaccination. 14 d after vaccination, however, the HI antibody titers were significantly increased in the AIV+Pre group compared with the Pre group, demonstrating successful immunization (Fig. 1; n=6, p<0.001). In another set of experiments, the HI antibody titers of the virgin mice were also measured, as shown in Fig. 1 (n=6, p<0.001).

2.2. Maternal vaccination improves spatial working memory

Fig. 2A and B represents the effects of vaccination on maternal working memory errors and reference memory errors, respectively, during the training phase. The number of working memory errors was obviously less in the AIV+Pre group than those in the Pre group (Fig. 2A; groups: F1, 18=7.67, p<0.01, time: F6, 108=67.34, p<0.001, groups × time: F6, 108=0.73, p>0.05; n=10, RM-ANOVA). Statistical analysis did not show any difference in reference memory errors between the two groups (Fig. 2B; groups: F1, 18=0.36, p>0.05, time: F6, 108=26.23, p<0.001, groups × time: F6, 108=0.86, p>0.05; n=10, RM-ANOVA).

We further evaluated the influence of vaccination on the homogeneous virgin mice. Interestingly, there were no significant differences between the AIV+Vir group and the Vir group in either working memory errors or reference memory errors during the training phase (data not shown). It was suggested that the pregnant condition might undertake a prerequisite for the increase of spatial working memory induced by immune interference.

2.3. Maternal vaccination increases hippocampal neurogenesis

In recent years, it has been demonstrated that adult hippocampal neurogenesis is highly relevant to spatial learning and memory (van Praag et al., 1999). In this scenario, pregnant mice received one i.p. injection of bromodeoxyuridine (BrdU,
50 mg/kg) during different stages of pregnancy (G7, G14) and postpartum (P7). Brains were examined 2 d after BrdU injection. To assess cell proliferation, BrdU+ cells were counted unilaterally in the DG (Fig. 3A). Our results showed that there were significantly more BrdU+ cells in the AIV+Pre group than in the Pre group (Fig. 3D; \( p < 0.05; n = 6, t\)-test) when BrdU was injected at G14. However, we found no significant alteration in cell proliferation at G7 and P7.

The effect of maternal vaccination on the neuronal differentiation of newly generated cells is shown in Fig. 3B and C. Pregnant mice were given injections of BrdU once a day for 4 successive days at all of the above time points (G7, G14 and P7). Then, their hippocampi were analyzed for the presence of proliferating cells (BrdU+). AIV+Pre group displayed more BrdU+/DCX+ cells were observed in the DG of the AIV+Pre group than in the Pre group (Fig. 3D; \( p < 0.05; n = 6, t\)-test) when BrdU was injected at G14. However, we found no significant alteration in cell proliferation at G7 and P7.

2.4. Maternal vaccination regulates the expression of prolactin

The production of prolactin during gestation has been shown to improve neurogenesis during both pregnancy and lactation in the subventricular zone (SVZ) but not in the DG (Bridges and Grattan, 2003; Shingo et al., 2003; Rolls et al., 2008). Our results showed that the levels of prolactin were not overtly different between the AIV+Pre group and the Pre group at any time point (G7, G14 and P7) (Fig. 4A, \( p > 0.05; n = 10\) per group, \( p > 0.05\)).

2.5. Maternal vaccination regulates cytokine and growth factor levels

Cytokines are implicated both in the immune response and in mediating various events in the CNS. That is, they are vital immunoregulators of neural functions and neuronal survival (Vizi and Kiss, 1998). Our data showed that the levels of IL-4 and IFN-\( \gamma \) were obviously up-regulated in the serum of the AIV+Pre group compared with the Pre group at G14 (Fig. 5A; IL-4, \( p < 0.05; \text{IFN-} \gamma, p < 0.01; n = 6, t\)-test). In contrast, the levels of the inflammatory factors IL-6 and TNF-\( \alpha \) were significantly decreased in the AIV+Pre group (Fig. 5A; IL-6, \( p < 0.05; \text{TNF-} \alpha, p < 0.01; n = 6, t\)-test). Interestingly, the immunization-induced changes in the levels of the cytokines above in the hippocampus at G14 showed a similar tendency to those in the serum (Fig. 5B; IL-4, \( p < 0.05; \text{IFN-} \gamma, p < 0.05; \text{IL-6, } p < 0.05; \text{TNF-} \alpha, p < 0.01; n = 6, t\)-test).

TGF-\( \beta \), an anti-inflammatory cytokine, is considered a significant cytokine in cell growth, differentiation and immune function regulation (Graciarena et al., 2010). We assessed the TGF-\( \beta \) protein level in the hippocampus for each group at G14, and there was a significantly higher concentration in the AIV+Pre group than in the Pre group (Fig. 4B; \( p < 0.05; n = 6, t\)-test).

As brain-derived neurotrophic factor (BDNF) is considered an essential modulator for some hippocampal activities, such as spatial learning ability (Sakata et al., 2013) and neurogenesis (Scharfman et al., 2005), the expression of BDNF in the DG was also measured (Fig. 6A). Relative to the Pre group, the AIV+Pre group has a significantly higher level of BDNF immunoreactivity (Fig. 6B, \( p < 0.05; n = 6, t\)-test).
Fig. 3 – A(H1N1) vaccination during early pregnancy induces a transient increase in hippocampal neurogenesis. (A) Representative confocal micrographs of the newly formed cells (BrdU+) in the DG. (B) Representative photographs in the DG that were double stained for BrdU (red) and DCX (green). (C) Representative photographs in the DG that were double stained for BrdU (red) and NeuN (green). (D) Mice were killed 2, 7 or 21 d after the first BrdU injection at G14. Their unilateral DG were analyzed for BrdU+ cells on day 2 (*p<0.05, Student's t-test; n=6 per group), BrdU+/DCX+ cells on day 7 (n=6 per group) and BrdU+/NeuN+ cells on day 21 (n=6 per group). Scale bar, 100 μm in A, B and C.
2.6. IFN-γ/IL-4 ratio and correlation with BDNF level

We investigated the relationship between the Th1/Th2 ratio and hippocampal neurogenesis. The Th1/Th2 ratio was represented by the IFN-γ (a classical Th1-derived cytokine) protein level/the IL-4 (a classical Th2-derived cytokine) protein level. Both in the serum and the hippocampus, the IFN-γ/IL-4 ratio in the AIV+Pre group was significantly un-regulated compared with the Pre group (Fig. 7A and B, p < 0.05; n = 6). This association of the IFN-γ/IL-4 ratio with BDNF level, and thus with the hippocampal neurogenesis, was further demonstrated by the positive correlation between the IFN-γ/IL-4 ratio and BDNF levels in the hippocampus of Pre mice with and without AIV treatment (Fig. 7C, p < 0.01; r² = 0.6147).

3. Discussion

Here, we show that working memory and hippocampal neurogenesis are elevated in mice treated with the A(H1N1) influenza vaccine, which induces a neurobeneficial immune response, during early pregnancy. We further demonstrate that the Th1/Th2 cytokine balance shifts toward a Th1-like response, with beneficial consequences for brain function.

A recent study has demonstrated that peripheral immune activation is associated with a transient increase in hippocampal neurogenesis (Wolf et al., 2009). To assess the cytological changes, hippocampal granular layer cells were quantified via unbiased stereological system. Our data reveal that prenatal immunization increased the number of newborn cells, proliferating DCX-positive precursor cells and mature neurons in the

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Fig. 4 – Prolactin (PRL) concentration in the serum and TGF-β levels in the hippocampus are correlated with neurogenesis. (A) No significant difference was observed between the two groups over time (p > 0.05; n = 6 per group). (B) There was a significant increase in the TGF-β levels in the hippocampus of the AIV+Pre group compared with the Pre group (*p < 0.05, Student’s t-test; n = 6 per group).

Fig. 5 – A(H1N1) vaccination during early pregnancy regulates cytokine production. The levels of IL-4, IL-6, IFN-γ and TNF-α in the serum (A and C) and the hippocampus (B and D) of each group were quantitatively analyzed using ELISA kits. The values are presented as the mean ± SEM (*p < 0.05, Student’s t-test; n = 6 per group).
DG (2 d, 7 d and 21 d after the first BrdU injection, respectively) in the AIV+Pre group compared with the Pre controls. Many newly generated cells that emerge from the SGZ migrate to form new neurons in the granular layer, while some of the newly divided SGZ cells either die (Morshead and van der Kooy, 1992) or generate glial cells that migrate into the corpus callosum (Nait-Oumesmar et al., 1999). In this scenario, more surviving neurons support superior working memory performance in the radial arm maze task. These observations indicate that A(H1N1) vaccination during early pregnancy benefits adult hippocampal neurogenesis and spatial cognitive ability.

However, we wondered whether the interesting benefits induced by the vaccine were specific to pregnant mice. Therefore, another independent experiment with the same procedures was performed in homologous virgin mice (with all other conditions being the same). Quantitative analysis of hippocampal neurogenesis revealed that immunization did not induce overt differences between the AIV+Vir group and the Vir group. This may be an indication that an adaptive peripheral immune response can transiently affect hippocampal neurogenesis in pregnant mice rather than in virgin mice.

Th1/Th2 balance is involved in behavior and adult neurogenesis, which are reflected by cytokine levels (Palumbo et al., 2012; He et al., 2014). We found evidence of beneficial regulation of adult hippocampal neurogenesis in AIV+Pre mice. Namely, a higher Th1/Th2 ratio in the AIV+Pre group and a lower Th1/Th2 ratio in the Pre group suggest that vaccination transiently promotes adult neurogenesis and spatial memory ability probably via inducing Th1-shift of systematic cytokines.
On the other hand, cytokines play a critical role in complex cognitive processes, such as synaptic plasticity, neurogenesis and neuromodulation. More specifically, the inflammatory cytokines TNF-α, IL-6 and IL-1β are associated with cognitive decline (Dik et al., 2005), whereas IL-4 and IFN-γ have been demonstrated to be neuroprotective (Butovsky et al., 2006, Baron et al., 2008). These cytokines perform immunomodulation not only via direct ligand receptor response but also via their cross-talk with resident microglia (Vitkovic et al., 2000; Butovsky et al., 2006; Schwartz and Shechter, 2010). Here, we found that A(H1N1) vaccination during early pregnancy increased IFN-γ, IL-4 and TGF-β levels and decreased IL-6, TNF-α and IL-1β levels. These data indicate that immunization creates neurobeneficial milieu both in the periphery and in the brain.

It is worth noting why vaccination has different effects on hippocampal neurogenesis and cytokines levels in pregnant and virgin mice. A recent study by Schreurs et al. (2012) might be able to explain the differences based on increased blood–brain barrier (BBB) permeability in pregnancy. The peripheral cytokines changes are consistent with those in the brain, supporting an increased permeability of the BBB in pregnant mice. We therefore deduce that during pregnancy, cytokines may exert their function on neuronal precursors directly or indirectly through the BBB from the periphery. However, two questions need to be answered in further research. One is whether Th1/Th2 balance can also explain the phenomenon that there is a decrease in hippocampal neurogenesis during pregnancy (Rolls et al., 2008; Piao et al., 2012). The other is whether circulating AIV-special T cells have a role in shaping brain function which can pass the BBB and choroid plexus into brain.

The production of prolactin mediates the pregnancy-induced increase in neurogenesis in the SVZ of mice (Shingo et al., 2003). Prolactin (PRL) regulates a large number of physiological processes (immunity and stress) (Bole-Feyset et al., 1998), reduces anxiety-behavior in the adult (Torner et al., 2001), and counteracts the decrease in hippocampal neurogenesis that is induced by stress (Torner et al., 2009). These observations suggest that higher prolactin level during pregnancy plays a crucial role in postpartum neurogenesis and maternal behaviors. Given that PRL levels are comparable pregnancy plays a crucial role in postpartum neurogenesis and cytokines levels in pregnant and virgin mice. A recent study by Schreurs et al. (2012) might be able to explain the differences based on increased blood–brain barrier (BBB) permeability in pregnancy. The peripheral cytokines changes are consistent with those in the brain, supporting an increased permeability of the BBB in pregnant mice. We therefore deduce that during pregnancy, cytokines may exert their function on neuronal precursors directly or indirectly through the BBB from the periphery. However, two questions need to be answered in further research. One is whether Th1/Th2 balance can also explain the phenomenon that there is a decrease in hippocampal neurogenesis during pregnancy (Rolls et al., 2008; Piao et al., 2012). The other is whether circulating AIV-special T cells have a role in shaping brain function which can pass the BBB and choroid plexus into brain.

In conclusion, our results show that the alteration of the cytokine milieu induced by immunological manipulation influences behavior and adult neurogenesis to some extent. Pregnancy can thus function as a “primer” that increases BBB permeability, resulting in the pro-neurogenic effect of immunological manipulation during this special physiological period (Schreurs et al., 2012; Cipolla et al., 2012). The precise immunoregulatory signature of these processes remains to be further elaborated. And other aspects in the process, including other cytokines and maternal hormones, should also be extended. Our findings should encourage attempts in this direction because pregnancy could offer a valuable way to facilitate beneficial immunological manipulations, such as the A(H1N1) influenza vaccine vaccination.

4. Experimental procedures

4.1. Animals and vaccination

Female C57BL/6j mice (8-wk old) were purchased from the Laboratory Animal Center of Sun Yat-sen University. Half of them were randomly mated with healthy males. The presence of a vaginal plug was designated at gestational day 0 (G0), and the delivery day was designated postnatal day 0 (P0). For the vaccinated group, each pregnant mouse was immunized with a single dose of injection containing 3 μg of the A (H1N1) influenza vaccine in the first trimester (G2.5). The pregnant mice in the control group were treated with sterilized PBS (n=10 per group) in same procedure. The inactivated A(H1N1) influenza vaccine (split virion) was obtained from the Center for Disease Control and Prevention (Guangdong province, China). The efficiency and safety of the vaccine had been confirmed before.

In another set of experiment, the other half of the virgin mice were randomly assigned to two groups and treated with the procedure mentioned above (n=10 per group). All experiments were in accordance with the Guide of the Institutional Animal Ethics Committee of Sun Yat-sen University. Experimental animals were individually maintained on 12 h light/dark cycles with food and water provided ad libitum.

4.2. Hemagglutination inhibition (HI) assay

Mouse blood was collected using the tail vein bleeding method. After incubation at 37 °C for 2 h and then at 4 °C overnight, sera were obtained by centrifugation at 5000 rpm/min for 10 min. The antibody titers of all serum samples were detected using a hemagglutination inhibition (HI) assay with chicken erythrocytes, according to a previous procedure (Zhu et al., 2009). Briefly, sera were pretreated with a receptor-destroying enzyme at 37 °C for 18 h and were then heated at 56 °C for 30 min. They were then diluted with PBS in serial two-fold dilutions in 96-well plates. The highest dilution rate that caused complete hemagglutination inhibition against the four hemagglutination units (HAU) of the virus was recorded as the HI titer.

4.3. Administration of BrdU and tissue preparation

For cell proliferation (BrdU+) analysis, six pregnant mice from each group received one i.p. injection of BrdU (50 mg/kg; Sigma-Aldrich) at G7, G14 or P7, respectively. Then, they were deeply anesthetized and perfused intracardially with 0.9% NaCl followed by 4% paraformaldehyde. Their brains were removed, postfixed overnight at 4 °C and then equilibrated in
30% sucrose. 40-μm-thick frozen sections of the entire hippocampus were collected with a random start using a freezing microtome (Leica SM2000R) and stored at 4 °C before histological analysis. In addition, another set of pregnant mice received a 4-d course of daily BrdU injections (i.p.) and were killed 7 or 21 d after the first injection for double labeling of BrdU⁺/ DCX⁺ (n=6 per group) or BrdU⁺/ NeuN⁺ (n=6 per group), respectively.

4.4. Immunofluorescent staining

For characterization of hippocampal neurogenesis, sections were immunostained for BrdU and other cellular markers using an immunofluorescent procedure as previously described (Bachstetter et al., 2010). For BrdU detection, sections were pretreated with 50% formamide/2 × saline-sodium citrate (SSC) at 65 °C for 2 h, washed in 5 min in 2 × SSC, denatured in 2 N HCl for 40 min at 28 °C and washed for 5 min in 0.1 M borate buffer with a pH of 8.5. They were then incubated for 60 min at 37 °C in 1% BSA blocking solution containing 10% normal goat serum and 0.25% Triton X-100 (Sigma). Sections were then incubated with rat anti-BrdU antibody (1:500; Oxford Biotechnology) combined with goat anti-DCX antibody (1:1000; Santa Cruz Biotechnology) or mouse anti-NeuN antibody (1:1000; sigma) for 2 h at 37 °C and then overnight at 4 °C. On the next day, following washes with PBS, the specimens were incubated for 2 h at 37 °C in blocking solution containing Alexa Fluor 594 donkey anti-rat antibody combined with Alexa Fluor 488 donkey anti-goat antibody or Alexa Fluor 488 goat anti-mouse antibody (all 1:500; Invitrogen). For BDNF staining, sections were blocked in 1% BSA at 37 °C for 1 h, followed by staining with primary antibody rabbit anti-BDNF (1:200; Abcam) at 37 °C for 2 h and an overnight incubation at 4 °C. The secondary antibody used was Alexa Fluor 488 goat anti-rabbit (1:400; Invitrogen).

4.5. Confocal and MBF stereoinvestigator

All cells stained in the dentate gyrus (DG) were observed with a Zeiss LSM 710 confocal laser-scanning microscope. Neurogenesis, including cell proliferation and neuronal differentiation, was assessed by unilateral counting in the DG using the stereo investigator stereology system (MicroBrightField, Williston, USA). Actual section thickness was measured, and appropriate guard zones at the top and bottom of each section was defined to avoid oversampling. Measurements were made in a systematic series of six coronal sections, which were 240 μm apart from one another, with a random start and spanning the whole hippocampus to ensure that the entire structure was sampled.

4.6. Radial-arm maze test

The eight-arm radial maze test was performed in a full-automatic eight-arm radial maze apparatus (RM-200 radial maze, Chengdu TME Technology Co, Ltd) to assess spatial working/reference memory (Fredriksson and Archer, 1996). The maze consisted of an octagonal-shaped central platform that was 26 cm in diameter, with eight enclosed arms radiating outwards (42 cm in length, 11 cm in width and 13 cm in height). Food containers were located at the far end of each arm with pellet sensors, which could automatically record pellet intake. The apparatus was controlled by a PC that was connected to a digital camera mounted directly above the maze for continuous mouse position tracking. Mice were on a restricted diet to keep their body weight at 80% to 85% of the initial level throughout the testing period.

The test procedure was performed as previously described (Dubroqua et al., 2012). In brief, during the habituation period, each mouse was placed in the maze for 10 min. Food pellets were scattered throughout the maze over the first 2 d. Over the next 2 d, food pellets were moved gradually toward the food containers. Over the last 2 d only the food cups were baited. Animals were given six consecutive days to habituate to the maze as described above. Mice that failed to explore the maze or did not consume any reward were dropped from further training. Then, four food-baited arms were randomly selected. A trial began by confining the mouse in the center platform with all of the doors closed. Ten seconds later, the doors of the four selected arms were opened, and the mouse was allowed to explore and consume food freely, and data from the pellets sensors were recorded. A trial was terminated immediately when all four pellets were consumed or after 10 min had elapsed. In the training phase, we evaluated the performance of the mice (n=10 per group) for seven consecutive days. The apparatus was wiped clean with 75% alcohol after each animal trial to avoid olfactory cueing. Performance was evaluated by two types of errors: the number of times the animal entered a non-baited arm (reference memory error) and the number of times the animal re-entered a baited arm on the same training trial (working memory error).

4.7. Enzyme linked immunosorbent assay (ELISA)

Pregnant mice were sacrificed at G14. Their brains were immediately removed and the hippocampal tissues were dissected and dissociated. The hippocampus of each mouse was homogenized in radioimmunoprecipitation assay buffer (RIPA; 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail (Sigma) and 1 mM PMSF), followed by centrifugation for 15 min at 12,000 rpm, 4 °C. The supernatants were collected and stored at −20 °C before detection, and the protein concentrations were measured by BCA assay (Beyotime) and were adjusted to 4.5 mg/ml.

Cytokine: The levels of the cytokines (IL-4, IL-6, IFN-γ, TNF-α and TGF-β) in both the serum and the hippocampus of each mouse were detected in duplicate by commercially available ELISA kits (NeoBioscience Technology Co., Ltd.) according to the manufacturer’s protocol. Six standards (with expected concentrations of 1,000, 500, 250, 125, 62.5, 31.25 and 15.65 pg/ml) were prepared by serial dilution with an assay buffer kit, using a sensitivity (threshold for detection) of 8.0 pg/ml. The prepared plates were analyzed using a microplate reader (BIO-TEK ELx800, USA) at 450 nm.

Prolactin: PRL levels in the serum were determined with an ELISA kit (boster biology, China) following the manufacturer’s instructions.
4.8. Statistical analysis

Evaluations were performed by an examiner who was blinded to the experimental group. Statistical analysis was performed using SPSS 17.0 software. All values are expressed as the mean ± SEM. Data from the Radial-arm maze test were analyzed using two-way repeated measures ANOVA. Statistical significance was evaluated by Student’s t-test for all of the rest of the data in this study, and p values < 0.05 were deemed statistically significant for all comparisons.

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Disclosures

All authors hereby declare that they have no conflicts to disclose.

Author contributions

Zhibin Yao designed the research; Yucen Xia and Fangfang Qi performed the research; Yucen Xia and Juntao Zou analyzed the data; Yucen Xia and Fangfang Qi wrote the paper.

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REFERENCES


Karkada, G., Shenoy, K., Halahalli, H., Karanth, K., 2012. Nardostachys jatamansi extract prevents chronic restraint