The Synergistic Roles of the Chronic Prenatal and Offspring Stress Exposures in Impairing Offspring Learning and Memory

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Accepted 23 March 2016

Abstract. In Alzheimer’s disease (AD), extensive experimental studies have demonstrated a negative impact of chronic stress during various stages of life (including prenatal phase) on some aspects of AD pathology. Nevertheless, presently, few studies have been involved in the learning and memory impairments, as well as neuropathology elicited by the chronic prenatal stress (CPS) and the chronic offspring stress (COS) exposures simultaneously, particularly for the adult male APP\textsubscript{swe}/PS1\textsubscript{dE9} murine offspring. Therefore, the aim of the present study was to investigate the influence of CPS on learning and memory impairments induced by COS in 6-month-old male APP\textsubscript{swe}/PS1\textsubscript{dE9} offspring mice and the related mechanism. Our study firstly demonstrates that 14-day exposure to CPS could exacerbate the learning and memory impairments, as well as neuropathological damages in the CA3 regions of the hippocampus and cortex neurons, which is induced by the 28-day exposure to COS in 6-month-old male APP\textsubscript{swe}/PS1\textsubscript{dE9} offspring mice. In addition, CPS could potentiate the production of A\textsubscript{\textbeta}\textsubscript{PP}, A\textsubscript{\textbeta}\textsubscript{42}, and corticosterone in 6-month-old male APP\textsubscript{swe}/PS1\textsubscript{dE9} offspring mice that also suffer COS. In conclusion, our novel findings strongly implicate the synergistic roles of the CPS and COS exposures in impairing offspring learning and memory. Moreover, CPS potentiating the production of A\textsubscript{\textbeta}\textsubscript{42} might be mediated by glucocorticoids through increasing the expression of APP and BACE1 gene.

Keywords: Alzheimer’s disease, amyloid-\textbeta, chronic offspring stress, chronic prenatal stress, glucocorticoid, learning and memory impairments

INTRODUCTION

Alzheimer’s disease (AD) is an age-related chronic neurodegenerative disorder defined by a progressive loss of memory and cognitive function. The pathogenesis of AD involves increased extracellular deposition of amyloid-\textbeta (A\textbeta) in neuritic plaques [1].
neuronal loss, and synaptic changes in the areas of brain essential for cognitive and memory functions [2]. Although only a minority of AD cases is caused by mutations in three different genes for either the amyloid precursor protein (APP) or Presenilin-1 and -2, the etiology of the more common sporadic AD remains unclear, and a combination of genetic factors and environmental determinants (such as chronic stress) with epigenetic events has been implicated [3].

Whereas acute stress is a necessary adaptive mechanism for survival, chronic stress causes over activation and dysregulation of the hypothalamo-pituitary-adrenal (HPA) axis with negative consequences on brain morphology and function [4]. Presently, it is well documented that chronic stress negatively impacts the learning and memory process in adult individuals. Chronic stress accelerates learning and memory impairments and increases amyloid deposition in AD murine models [5].

The early-life environment is one of the most important factors affecting life-long health [6]. Chronic prenatal stress (CPS) is an important programming factor in brain development and function. A cross-sectional study indicated that approximately 6% of pregnant women reported high levels of chronic psychological stress during their pregnancies that resulted from conditions including depression, panic disorder, or domestic violence [7]. Clinical and preclinical studies have shown that CPS has a major impact on the neurobehavioral development of the offspring [8]. Furthermore, animal studies have shown that CPS alters the behavior of the adult offspring. For example, the offspring of mothers that experienced chronic stress during pregnancy showed learning ability impairment in the Morris water maze at two [9] and three months old [10]. To some extent, this may be because CPS can influence adult behavior by permanently altering HPA function and significantly elevating plasma glucocorticoids concentrations under both basal or stressed conditions in offspring [11]. Physiologically, glucocorticoids play a vital role in many aspects of normal brain development [11] by initiating terminal maturation, remodeling of axons and dendrites, and affecting cell survival [12], but excess glucocorticoids secretion is strongly associated with neuronal atrophy and dysfunction, and impaired cognition. Noteworthy, glucocorticoids could potentiate Aβ-induced learning and memory impairment and pathological damage in CA1 field of hippocampus in Sprague-Dawley rats [13]. Furthermore, we also found that glucocorticoids can increase impairments in learning and memory due to elevated amyloid-β protein precursor (AβPP) expression and neuronal apoptosis in 12-month-old mice [14].

Indeed, some experimental studies have demonstrated a negative impact of chronic stress during various stages of life (including prenatal phase) on some aspects of AD pathology (Postnatal phase [15, 16]; prenatal phase [17–19]). However, presently, few studies have been involved in the learning and memory impairments, and neuropathology elicited by the CPS and the chronic offspring stress (COS) exposures simultaneously, particularly in adult male APPswe/PS1dE9 offspring. Because the CPS could significantly elevate plasma glucocorticoids concentrations under stressed conditions in offspring [11] and the stress is usually unavoidable to a pregnant woman and her offspring, the purpose of the present study was to explore the influence of CPS on learning and memory impairments induced by COS in 6-month-old male APPswe/PS1dE9 murine offspring. And if these impairments are influenced by CPS, then the present study would explore what the underlying mechanism is.

**MATERIALS AND METHODS**

**Experimental conditions and breeding**

The experimental timeline is depicted in Fig. 1. All animals were under standard laboratory conditions: 12-h light/dark cycle (turn on the light at 7 : 00 a.m. and turn off at 7 : 00 p.m., unless specified otherwise), 18 ± 2°C, relative humidity of 42%, and 5 animals per cage. Food and water were provided, and the mice received *ad libitum* throughout the study unless specified otherwise. The mice were habituated to their environment for 2 weeks before the experiments began. All experimental procedures were performed according to NIH guidelines for the care and use of laboratory animals and were reviewed and approved by the Institutional Animal Care and Use Committee of Anhui Medical University. All efforts made were to minimize animal pain, suffering or discomfort, and the number of mice used.

To obtain offspring, virgin female C57BL/6JNju mice (Model Animal Research Center of Nanjing University, strain # J000664, China) were mated with male B6C3-Tg(APPswe,PSEN1dE9)85Db/o MmJNju mice (Model Animal Research Center of Nanjing University, strain # J004462, China) at 10–12 weeks of age, as described previously [17]. Gestational day 1 (G1) was designated by the presence of a
sperm plug, and this was corroborated by the daily weight gains of the pregnant dams. Subsequently, the pregnant dams were housed individually with sawdust bedding. On G7, pregnant females were randomly assigned to either the CPS group \((n = 10)\) or the control group \((n = 10)\).

**Chronic prenatal stress procedure**

The pregnant dams in the CPS group experienced an unpredictable stress procedure during the gestation day 7–20 (G7–G20). Stressors consisted of tail pinch (2 cm apart from the end of the tail) for 5 min, restraint for 2 h, cold swimming at 4–8°C for 5 min and then towel-drying, warm swimming at 28–35°C for 15 min and then towel-drying, reverse day and night cycles, water deprivation for 24 h, food deprivation for 24 h. Each stressor was administered randomly, once every 7 days (two times within 14 days) and twice daily per mouse (at 9:00 a.m., and 3:00 p.m.) except for the 24-h stressors [20, 21]. Control females were left undisturbed for the duration of their pregnancies. Our pilot experiment has demonstrated that this type of stress could result in marked elevation of plasma corticosterone in pregnant mice \((p < 0.05)\). Additionally, stressed animals showed an increased relative adrenal weight, which was significant between stressed and nonstressed dams in the post hoc analyses.

**Offspring**

Since estrogens could prevent the processing of AβPP into Aβ and reduce the tau hyperphosphorylation [22], in this study, only male offspring were used for research. At postnatal day 21 (P21), pups were weaned. At postnatal day 25 (P25), all male pups were ear-punched and marked to enable permanent identification. Scissors were cleaned with ethanol and the tip of murine tail was cut off (<5 mm of tissue) for genotyping using PCR amplification of genomic DNA, as described previously [17]. Male APPswe/PSEN1dE9 pups were housed per 2 littermates. For APPswe/PSEN1dE9 mice began to develop amyloid plaques at 6–7 months of age [23], at 6 months (P180), offspring were randomly divided into the prenatal control-offspring control group (Control, \(n = 19\)), the prenatal control-COS group (COS-treated group, \(n = 18\)), the CPS-offspring control group (CPS-treated group, \(n = 18\)), and the CPS plus COS group (CPS + COS, \(n = 18\)). To achieve offspring chronic multiple stress, adult offspring mice were daily subjected to the same twice (at 9:00 a.m., and 3:00 p.m.) unpredictable stress procedure, as stated above for four weeks (Week0–Week4). Body weights of the offspring were measured at Week0, Week1, Week2, Week3 and Week4, respectively.

**Morris water maze test**

The water maze test is a widely accepted method for learning and memory testing, so we performed this test to measure the memory capacity of adult offspring mice as described by Morris [24]. Briefly, the test was carried out in a circular pool (150 cm in diameter and 60 cm in height) filled with water (22°C) that was opaque due to the addition of milk powder [13]. An escape platform (10 cm in diameter) was hidden 1.5 cm beneath the water surface and placed at the midpoint of one quadrant.

**Place navigation test**

Four trials per day were conducted for 4 consecutive days. Each mouse (part of mice in each group, \(n = 8–9\)) was randomly placed in water, facing the pool wall, in one of the four starting points (the midpoints of the four quadrants in the pool). An automatic tracking system recorded and analyzed the mouse swim paths. Each trial was terminated when the mouse reached the platform or after 90 s, whichever occurred first. If the mouse reached the platform, it was allowed to stay there for 20 s. Alternatively, the
mouse failed to find the platform within 90 s, it was placed on it for 20 s, and assigned a latency of 90 s. The mouse was then wiped dry, returned to its cage, and warmed with a lamp if necessary. The inter-trial interval was 15 min. The latency to find the platform, the total swimming distance, and the average swimming speed were recorded. The average values of the four trials were considered to be the result of that day.

**Spatial probe test**

In order to assess the maintenance ability of memory, a probe trial was performed 24 h after the last trial on the fourth day. The platform was removed from the pool, the mice were allowed to swim freely and the trial was performed with a cutoff time of 90 s. The number of crossing the platform site (NCP) and swimming time in the quadrant of the platform (STP) was taken to indicate the degree of memory consolidation that has taken place after learning [14].

**Tissue preparation**

The blood samples of offspring mice were collected 4 hours after the last stress trial. Then offspring mice were sacrificed by cervical dislocation and the brains were removed. The brains were immediately dissected in half along the sagittal line, half was used to isolate hippocampus and snap frozen in liquid nitrogen for biological analysis, the other half was fixed in 10% formaldehyde in 0.1 M PBS. Serum was separated by centrifugation at 3000×g for 10 min at 4°C, then stored at –80°C until used.

**Histological examination**

Fixed brains were embedded in paraffin. Sections (6 μm) were cut coronally at a site 3 mm behind the bregma using a microtome. Then, two slides were selected from each sample and were stained with hematoxylin and eosin (HE). The numbers of normal and abnormal pyramidal cells in the hippocampal CA3 region of each slide were investigated under a light microscope (Olympus 45, Tokyo, Japan) at a × 400 magnification by 2 pathologists in a blinded manner. The average numbers were taken as the final result of the slide, and the average number of these two slides was the result of the sample.

**Immunohistochemistry**

Brains were embedded in paraffin, 6 μm coronal sections were serially cut (at a site 3 mm behind the bregma using a microtome) and affixed to slides to ensure adhesion. Slides were processed as follows: briefly, slides were deparaffinized in xylene, hydrated in a series of ethanol and deionized water, put in citrate buffer (pH 6.0) for 5 min at 95°C to repair epitope, quenched using a freshly prepared dilute (3%) hydrogen peroxide to inactivate endogenous peroxidase, washed in deionized water followed by 0.01 M PBS, blocked with 2% fetal bovine serum. Then the slides were incubated overnight with one of the following primary antibodies at 4°C: rabbit polyclonal anti-APP (1 : 100; BBI, # AB60097b, Shanghai, China); rabbit polyclonal anti-BACE1 C-Terminal (485-501) (1 : 100; Millipore, # 195111, MA, USA), rabbit polyclonal anti-Ab42 (1 : 50; Abcam, # ab10148, MA, USA), respectively. Immunostaining was visualized by the peroxidase method with a universal anti-IgG HRP polymer and 3,3′-diaminobenzidine oxidation (GBI, # PV-6000, USA). To assure comparable immunostaining, slides were processed together under the same conditions. To evaluate the specificity of the staining, slides from each sample were incubated in the absence of the primary antibodies and in such cases no immunostaining was observed. Non-specific secondary immunostaining was also assessed by incubating slides with the primary and a non-specific secondary antibody, no immunostaining was observed as well. Slides were lightly counterstained with hematoxylin and were resin-mounted. Then, slides were observed with a light microscope (Olympus 45, Tokyo, Japan) at a × 400 magnification by an observer who was blinded to the experimental group. The pyramidal cell layer in the CA3 hippocampal region was placed on the diameter axis of the object lens. The mean optical density of AbPP, BACE1, and Ab42 positive neurons were measured on each vision field by using an Image-Pro Plus Image Analysis Software (Meyer Instruments, Inc., Houston, TX, USA). Two consecutive vision fields were observed on each slide. The average mean optical density of two vision fields was the result of the slide, and the average mean optical density of the two slides was the result of the sample.

**Congo red staining method**

Brains were paraffin embedded, 20 μm coronal sections were serially cut (at a site 3 mm behind
the bregma using a microtome) and affixed to slides to ensure adhesion. Then, slides were deparaffinized, counterstained in hematoxylin, rinsed well, immersed in Congo red solution for 40 min at room temperature (RT) before being rinsed in running water. Subsequently, the slides were dipped in 0.2% alkaline alcohol for 5–10 s to differentiate the non-specific background staining and washed in running tap water for 10 min. Finally, the slides were dehydrated in a series of ethanol, cleared in xylene, and covered with neutral gum.

**Corticosterone measurement**

In order to determine HPA axis responsiveness in the face of stressors, concentrations of corticosterone in serum, hippocampus, and frontal cortex were measured 2 h after last stress session using a commercially available enzyme immunoassay kit (CUSABIO, # CSB-E07969 m, Wuhan, China), following the manufacturer’s protocol. Optical densities were read at 450 nm using the Epoch (BioTek, USA) microplate reader. In addition, protein concentrations were determined by using the Lowry protein assay.

**Aβ42 assay**

The amount of Aβ42 in the hippocampal homogenates were analyzed by using commercially available enzyme-linked immunosorbent assay (ELISA) kits against mouse Aβ42 (CUSABIO, # CSB-E10787 m, Wuhan, China), following the manufacturer’s protocol. Optical densities were read at 450 nm using the Epoch (BioTek, USA) microplate reader. The values were calculated by comparison with standard curve of synthetic of Aβ42. In addition, protein concentrations were determined by using the Lowry protein assay.

**Western blots**

For protein lysate preparation for immunoblot assays, hippocampal tissue (n = 5 per group) was homogenized in ice-cold RIPA buffer containing protease inhibitor cocktail (50 mM Tris-HCl [pH 7.4], 0.25% DOC, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM NaF) and briefly sonicated. After incubation of this mixture for 30 min on ice, the preparation was centrifuged for 30 min in a refrigerated Eppendorf centrifuge (Eppendorf 5417 R, Hamburg, Germany) at 15000 × g 4°C. The supernatant was collected as total protein extracts. The protein concentrations were determined by the Lowry method. Then, the protein samples were mixed in a sodium dodecyl sulfate (SDS) sample buffer, and denatured at 95°C for 5 min and then 30 μg of proteins were electrophoresed on 12% Tris-glycine acrylamide gels and the protein bands then were electrophoretically transferred to Immobilon-P polyvinylidene fluoride (PVDF) membranes, 0.45 μm (Millipore, Billerica, MA, USA). The membranes were blocked with 5% (w/v) non-fat milk in Tris-buffered saline-0.1% Tween (TBS-T) for 1 h at RT. After washing with TBS-T, membranes were incubated overnight at 4°C with the following primary antibodies: 1:4000 rabbit polyclonal anti-APP (BBI, # AB60097b, Shanghai, China); 1:1000 rabbit polyclonal anti-BACE1 C-Terminal (485–501) (Millipore, # 195111, MA, USA); and with 1:800 mouse monoclonal anti-β-actin (Santa Cruz Biotechnology, # sc-81178, Heidelberg, Germany) for normalization. After washing with TBS-T, membranes were incubated for 1 h at RT with horseradish peroxidase-conjugated secondary antibodies in blocking buffer: 1:80000 goat anti-rabbit (BBI, # AB10058, Shanghai, China) and 1:15000 goat anti-mouse (BBI, # AB10087, Shanghai, China), respectively. After that, membranes were washed in TBS-T and visualized by enhanced chemiluminescence (Thermo Scientific, # 34094, USA) using a Tanon 5200 Imager. Images were quantified using ImageJ (http://rsbweb.nih.gov/ij/) software, corrected for background signal and β-actin signal.

For Aβ42 detection, 30 μg of proteins were separated on a 10–20% gradient tricine polyacrylamide gel and transferred to a PVDF membrane, 0.22 μm (Millipore, Billerica, MA, USA). Afterwards, the membrane was incubated overnight at 4°C with the primary antibody: 1:1000 rabbit polyclonal anti-Aβ42 (Abcam, # ab10148, MA, USA). The next day, membrane was washed and further incubated for 1 h at RT with horseradish peroxidase-conjugated secondary antibodies in blocking buffer: 1:80000 goat anti-rabbit (BBI, # AB10058, Shanghai, China). After washing, the protein bands were visualized by enhanced chemiluminescence (Thermo Scientific, # 34094, USA) using a Tanon 5200 Imager. The membrane then were stripped and reprobed with mouse monoclonal β-actin antibody (Santa Cruz Biotechnology, # sc-81178, Heidelberg, Germany; 1: 800 dilution) to assess equal loading amounts. Intensities of specific bands were quantified by using ImageJ.
software, corrected for background signal and β-actin signal.

Statistical analysis

Body weights of the offspring, as well as escape latency in Morris Water Maze test, were analyzed by using repeated measures two-way ANOVA (CPS × COS × time) and also independently at the various time points by using a two-way ANOVA (CPS × COS). Besides, the rest of the experimental data were analyzed by using a two-way ANOVA (CPS × COS). Sources of significant main effects or interactions were then determined by analysis with the Bonferroni’s post hoc test. Data were expressed as means ± standard deviation (SD). Statistical significance was set at an α level of 0.05, and all tests were two-tailed. All statistical analyses were conducted by using the SPSS 15.0 (SPSS Inc., Chicago, IL, USA). All graphs were constructed in GraphPad Prism (version 4, GraphPad Software, San Diego, CA, USA).

RESULTS

Body weight

We first evaluated the influence of CPS on birth weight. Significant difference was observed in birth weight (nonstressed, 6.6 ± 0.2 g, n = 10; stressed, 6.1 ± 0.1 g, n = 9; t = 1.918, p < 0.05). To determine whether CPS and COS affect the body weight in adult offspring, the body weight was measured, and the result was shown in Fig. 2A. Compared with the CPS-treated group and the Control group, the body weight in COS group was markedly decreased (p < 0.05). Further study showed the lightest body weight was in CPS + COS group.

Learning and memory impairments

The effect of CPS and COS on offspring escape latency is shown in Fig. 2B. Escape latency decreased significantly over time during the experiment (F(3, 26) = 90.299, p < 0.001). Post hoc analyses showed that COS alone could significantly increase the escape latency (p < 0.05 versus Control group); CPS + COS could further increase the escape latency (p < 0.05 versus Control group and COS-treated group).

The effect of CPS and COS on offspring swimming distance and swimming speed was shown in Fig. 2C and D. Swimming distance decreased significantly over time during the experiment (F(3, 26) = 80.531, p < 0.001). Both CPS and COS significantly increased the swimming distance (F(3, 26) = 3.738, p < 0.014 for CPS; F(3, 26) = 9.641, p < 0.001 for COS). Post hoc analyses showed that the swimming distance was longest in the CPS + COS group (p < 0.001 versus Control, p = 0.004 versus COS-treated group, p < 0.001 versus CPS-treated group). Whereas, there were no significant differences in swimming speeds among groups.

Moreover, the effect of CPS and COS on offspring STP and NCP was shown in Fig. 2E and F. A significant overall effect of COS was found (F(1, 28) = 17.055, p < 0.001 for STP; F(1, 28) = 28.831, p < 0.001 for NCP). Post hoc analyses showed that CPS + COS group mice had decreased STP and NCP as compared to the Control (p < 0.001 and p < 0.001, for STP and NCP, respectively), CPS-treated group (p = 0.002 and p < 0.001, for STP and NCP, respectively), and COS-treated groups (p = 0.036 and p = 0.017, for STP and NCP, respectively). Furthermore, the mice in COS-treated group also had decreased STP (p = 0.025) and NCP (p = 0.004) as compared to the Control group.

Pathological change in hippocampus

Histological examination (HE stain) was used to examine the neuropathology in the CA3 subfield of hippocampus. No remarkable neuronal abnormalities were observed in the Control and CPS-treated groups. The pyramidal neurons in the CA3 region were arranged neatly and tightly, and no neuron loss was found. Additionally, neurons were round and intact with nuclei stained clear, dark blue (Fig. 3A, C). However, obvious hippocampal histopathological damage was observed in the COS-treated groups and CPS + COS groups. The pyramidal layered structure was disintegrated, and neuronal loss was found in the CA3 region. Neurons with pyknotic nuclei and with shrunken or irregular shape were also observed (Fig. 3B, D). The average number of normal neurons was lowest in the CPS + COS group. Moreover, the neurons in CPS + COS group had overall worse cell morphology than those in the Control, COS-treated, and CPS-treated groups (Fig. 4).

Expression of AβPP, BACE1, and Aβ42 protein detected by immunohistochemistry

We investigated the effect of CPS and COS on the distribution of AβPP, BACE1, and Aβ42 in
Fig. 2. Effects of chronic prenatal stress and chronic offspring stress on body weight, and learning and memory impairments in 6-month-old male APPswe/PS1dE9 mice (Morris water maze). A) Body weights during the experiment (*p < 0.05 versus CPS + COS; #p < 0.01 versus CPS + COS; ℃p < 0.01 versus Control; ∆p < 0.01 versus CPS-treated group). B) The escape latencies in place navigation test (*p < 0.05 versus Control; #p < 0.001 versus Control; ∆p < 0.05 versus COS-treated group; ψp < 0.001 versus CPS-treated group). C) The swimming distances in place navigation test (*p < 0.05 versus Control; #p < 0.01 versus Control; ∆p < 0.05 versus COS-treated group; ψp < 0.05 versus CPS-treated group; ℃p < 0.01 versus CPS-treated group). D) The swimming speed in place navigation test. E) The swimming time in the quadrant of platform (STP) in spatial probe test (*p < 0.05, **p < 0.001). F) The average number of crossing the platform site (NCP) in spatial probe test (*p < 0.05, **p < 0.001). Data are presented as means ± SD. n = 8–10 per group. Control, prenatal control-offspring control group; COS, COS-treated group; CPS, CPS-treated group; CPS+COS, the CPS+COS group.

the hippocampus by immunohistochemical staining (Fig. 5). In the hippocampal CA3 region, both CPS and COS significantly increased the expression of AβPP (F(1,16) = 74.165, p < 0.001 for CPS; F(1,16) = 142.229, p < 0.001 for COS). Post hoc analyses revealed that, in the CA3 region, offspring mice exposed successively to CPS and COS had more expression of AβPP as compared to all other groups (p < 0.001 for Control, p < 0.001 for COS-treated, p < 0.001 for CPS-treated). Further, COS-treated group mice had more expression of AβPP than Control group (p = 0.049).

In addition, the results of BACE1 immunohistochemical staining showed that COS-treated group as well as CPS + COS group mice had increased expression of BACE1 as compared to Control (p < 0.001 and
Fig. 3. Effects of chronic prenatal stress and chronic offspring stress on pathological changes in CA3 subfield of the hippocampus in 6-month-old male APPswe/PS1dE9 mice (HE stain, 400 ×). No remarkable neuronal abnormalities in the hippocampus of the Control and CPS-treated groups were observed, while the COS-treated group and CPS + COS group mice showed neuropathological changes characterized by shrunken eosinophilic cytoplasm, degenerating neurons (no visible nucleolus) (arrowhead), and nuclear condensation and pyknosis (arrow). Control group (A); COS-treated group (B); CPS-treated group (C); CPS + COS group (D). HE, hematoxylin and eosin.

Fig. 4. Effects of chronic prenatal stress and chronic offspring stress on pyramidal cells in CA3 subfield of the hippocampus in 6-month-old male APPswe/PS1dE9 mice (*p < 0.05, **p < 0.001). Data are presented as means ± SD. n = 5 per group.
Fig. 5. Effects of chronic prenatal stress and chronic offspring stress on AβPP, BACE1, and Aβ_{42} contents in CA3 subfield of the hippocampus in 6-month-old male APPswe/PS1dE9 mice. A) Immunohistochemistry analysis of AβPP, BACE1, and Aβ_{42} in the hippocampal tissue (400×). B) Bar graph displays the results from immunohistochemistry analysis with Image-Pro Plus 6.0 (*p < 0.05, **p < 0.001). Data are presented as means ± SD, n = 5 per group.

Similarly, for the expression of Aβ_{42}, post hoc analyses showed that COS-treated group as well as CPS+COS group mice had increased expression of Aβ_{42} as compared to Control (p = 0.003 and p = 0.001, for COS-treated and CPS+COS, respectively) and CPS-treated groups (p = 0.03 and p = 0.006, for COS-treated and CPS+COS, respectively).

**Aβ plaques detected by Congo red staining method**

Congo red was used to stain the amyloid plaques in the brains of mice. The results showed that the frontal cortex of the brain of CPS+COS group mice had a larger percentage of areas that were positive for...
Fig. 6. Effects of chronic prenatal stress and chronic offspring stress on amyloid deposits in the frontal cortex and hippocampus of 6-month-old male APPswe/PS1dE9 mice (Congo red staining, 400×). Congophilic deposits are found both in the cortex (A–D 100×) and in the hippocampus (E, F 400×). Of note, there were few amyloid plaques in the hippocampus of COS-treated group and CPS+COS group, and no amyloid plaque was found in the hippocampus of Control and CPS-treated groups. Control group (A); COS-treated group (B, E); CPS-treated group (C); CPS+COS group (D, F)

amyloid plaques than all other groups (Fig. 6A-D). The amyloid plaques exhibited a light red dispersion without distinct boundaries. Whereas, there were few amyloid plaques in the hippocampus of COS-treated group and CPS + COS groups (Fig. 6E, F), and even no amyloid plaque was found in the hippocampus of Control and CPS-treated groups.

Expression of AβPP, BACE1, and Aβ42 protein detected by western blotting

Analysis of hippocampal AβPP concentrations revealed a significant increase in hippocampal AβPP in the CPS+COS group, compared to all other groups ($p<0.001$ for Control, COS-treated, and CPS-treated). Furthermore, both COS-treated group ($p<0.001$) and CPS-treated group ($p=0.011$) mice had also evident increased AβPP in hippocampus as compared to the Control group (see Fig. 7A for more details).

For BACE1, post hoc analyses showed that COS-treated group as well as CPS+COS group mice had increased expression of BACE1 as compared to Control group ($p<0.001$ and $p<0.001$, for COS-treated group and CPS+COS group, respectively) and CPS-treated group ($p=0.003$ and $p<0.001$, for COS-treated group and CPS+COS group, respectively). Further, there was a non-significant elevation
Fig. 7. Effects of chronic prenatal stress and chronic offspring stress on AβPP, BACE1, and Aβ42 contents in the hippocampus of 6-month-old male APPswe/PS1dE9 mice. A–C) Western blot analysis of AβPP, BACE1, and Aβ42 in the hippocampal tissue. Bar graph displays the results from western blot analysis (*p < 0.05, **p < 0.001). Quantification of protein blots with protein levels normalized against β-actin as a loading control, data are presented as means ± SD. n = 5 per group. D) ELISA analysis of Aβ42 in the hippocampal tissue (*p < 0.05, **p < 0.001). Data are presented as means ± SD. n = 6–7 per group.

Expression of Aβ42 protein detected by ELISA

Since Aβ42 is more cytotoxic and more likely to aggregate to form plaques than Aβ40, we further used ELISA to examine Aβ42. In the hippocampus, Aβ42 protein were 172 ± 20 (Control), 219 ± 17 (COS), 185 ± 31 (CPS), and 242 ± 29 pg/mg protein (COS + CPS), respectively (see Fig. 7D for more details). Two-way ANOVA analyses showed that both CPS and COS significantly increased Aβ42 protein (F(1,22) = 7.307, p = 0.013 for CPS; F(1,22) = 25.202, p < 0.001 for COS). Post hoc analyses revealed a significant increase in hippocampal Aβ42 in the CPS + COS group, compared to all other groups (p < 0.001 for Control, p = 0.020 for COS-treated, p = 0.001 for CPS-treated). Moreover, COS-treated group mice had also evident increased Aβ42 in hippocampus as compared to the Control group (p = 0.006).

Levels of corticosterone detected by ELISA

Concentrations of corticosterone in serum and hippocampus were measured to determine HPA axis
Fig. 8. Effects of chronic prenatal stress and chronic offspring stress on corticosterone contents in serum and hippocampus of 6-month-old male APPswe/PS1dE9 mice (ELISA). A) Corticosterone levels in serum. B) Corticosterone levels in hippocampus. Data are presented as means ± SD. n = 6–8 per group. *p < 0.05, **p < 0.001.

responsiveness for stressors. In the serum (Fig. 8A), concentrations of corticosterone were 6.2 ± 1.2 (Control), 10 ± 1.9 (COS), 6.9 ± 2.1 (CPS), and 13 ± 2.5 ng/ml (COS + CPS), respectively. Two-way ANOVA analyses showed that both CPS and COS significantly increased corticosterone concentrations (F(1,26) = 10.840, p = 0.003 for CPS; F(1,26) = 67.332, p < 0.001 for COS). Post hoc analyses revealed a significant increase in serum corticosterone in the CPS + COS group, compared to all other groups (p < 0.001 for Control, p = 0.010 for COS-treated group, p < 0.001 for CPS-treated group). Furthermore, COS-treated group mice also had evident increased corticosterone in serum as compared to the Control (p < 0.001) and CPS-treated (p = 0.015) groups.

To some extent, the effects in the hippocampus were different (Fig. 8B). Concentrations of corticosterone were 2.5 ± 1.0 (Control), 3.9 ± 1.7 (COS), 2.4 ± 0.9 (CPS), and 6.8 ± 2.0 ng/mg protein (COS+CPS), respectively. Two-way ANOVA analyses showed that there were no effects of CPS (F(1,8) = 5.983, p = 0.40) or COS (F(1,8) = 11.142, p = 0.0) on corticosterone. Post hoc analyses revealed that the CPS + COS group had significantly higher levels of corticosterone than the Control (p = 0.021) and CPS-treated (p = 0.021) groups.

**DISCUSSION**

AD, the most common age-related neurodegenerative disorder and the most common form of dementia, is a progressive disease. Currently, it is widely recognized that we need to develop effective AD fluid biomarkers [25] and imaging techniques [26] to aid the development of disease-modifying treatments, to evaluate disease risk or prognosis, to facilitate early diagnosis, and to improve clinical care. As we know, amyloidogenic cleavage of AβPP by BACE1 and γ secretase leads to the production of different length of Aβ peptides in which Aβ42 is the most fibrillogenic and toxic species contributing to the progression of AD [27, 28]. In fact, Aβ42 really could reduce the membrane fluidity, accelerate the amyloidogenic processing of AβPP [29, 30], form insoluble protein aggregates, and induce neuroinflammatory responses and oxidative stress, therefore affecting synaptic function and then causing progressive neuronal cell death [31, 32]. In AD, neuronal damage and loss is widespread, appearing first in the hippocampus (a region of the brain considered critical for learning and memory) from where it gradually spreads to the associated regions of the neocortex (prefrontal cortex, for instance, is an important area for the regulation of cognition, and shares afferent and efferent connections with the hippocampus) [33].

Previous epidemiological studies have shown that chronic stress might affect the progression of AD-related symptoms and pathologies [34]. Similarly, it has also been demonstrated that immobilization or restraint stress in various AD mouse models during adulthood resulted in higher plaque deposition, elevated concentrations of Aβ42 and Aβ40 in the hippocampus, and these mice displayed augmented neuronal degeneration with concomitant cognitive deficits [5, 15, 35, 36]. What is more, research on animals provided mounting evidence that chronic prenatal stress (CPS) might have long-term effects on offspring development. For instance, Lemaire et al. [37] showed that prenatal stress in rats induced lifespan reduction of neurogenesis in the dentate gyrus and produced impairment in hippocampal-related spatial tasks. However, in this study, we demonstrated that there were no significant differences in escape latencies, swimming distances, and swim-
ming time in the quadrant of platform and average number of crossing the platform site in the Morris water maze between the control and CPS-treated group, suggesting CPS alone may not induce learning and memory impairments in 6-month-old male APPswe/PS1dE9 offspring mice. Further study showed that the mice that were successively exposed to CPS and COS had more escape latencies and swimming distances, less swimming time in the quadrant of platform, and an average number of crossing the platform site than the mice in COS-treated group in the Morris water maze. These results suggest 14-day CPS could exacerbate the impairments of learning and memory which were induced by the 28-day COS in 6-month-old male APPswe/PS1dE9 offspring.

Moreover, the results of our histopathological experiments indicated that four weeks of exposure to COS induced histological damage and pathological changes in the CA3 region of the hippocampus neurons (HE stain), and these effects were exacerbated by CPS, which suggest neuropathological damage exacerbated by CPS may be one of the reasons for learning and memory impairments in CPS plus COS group. Noteworthy, it is known that the hippocampus can be roughly divided into four subfields: DG, CA3, CA2, and CA1, which correlate with the ability of learning and memory, but unexpectedly, the shape and the number of neurons in DG, CA2 and CA1 subfield were not affected significantly. The underlying mechanism is still unclear.

As mentioned above, Aβ42 is the most fibrillogenic and toxic species contributing to the progression of AD, and the expression levels of AβPP and BACE1 affect the production of Aβ. Therefore, the current examined levels of AβPP, BACE1 and Aβ42 showed that the mice that were successively exposed to CPS and COS had the greatest expression of Aβ42 in hippocampus. Consequently, it is plausible that the neuropathological damage, exacerbated by CPS in the CA3 region of the hippocampus may be associated with the excessive expression of Aβ42. Additionally, the mice that were successively exposed to CPS and COS had the greatest expression levels of AβPP in hippocampus. Taken together, our research strongly implicates that CPS increases the production of Aβ42 by promoting the expression of AβPP in 6-month-old male APPswe/PS1dE9 offspring that also suffer from the COS.

To our knowledge, this is the first report that exposure to 14-day CPS could exacerbate the learning and memory impairments, as well as neuropathological damages in the CA3 regions of the hippocampus and cortex neurons, which was induced by the 28-day COS in 6-month-old male APPswe/PS1dE9 murine offspring. Because stress is usually unavoidable to pregnant woman and offspring, and CPS could elevate stress-stimulated HPA activity in offspring, it is necessary to research the learning and memory impairments, and neuropathology pertaining to chronic prenatal and offspring stress exposures simultaneously. In general, although not always, CPS increases depressive and anxious behavior to a greater extent in female offspring, but impairs cognition more in the males [38, 39]. Further, estrogens could prevent the processing of AβPP into Aβ and reduce the tau hyperphosphorylation [22]. Which is why male offspring were used in this study. In addition, G7 was selected to begin the stress paradigm, because: (1) the fetal central nervous system becomes substantially developed at this point [40], (2) to minimize premature termination of the pregnancy as a result of excessive stress, (3) the last two weeks of pregnancy (from G7 to G20) is a critical period in development of the fetal HPA system [41, 42].

In fetus, CPS can downregulate placental 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) [43], the barrier enzyme which protects the fetus from maternal corticosterone by metabolizing it to inactive 11-keto forms (cortisone and 11-dehydrocorticosterone), leaving the fetus less protected and causing increased transplacental transfer of maternal corticosterone to the fetal compartment. In turn, heightened corticosterone concentrations in fetuses are thought to affect their synaptic plasticity and neurotransmitter activity, resulting in subtle or drastic changes in subsequent function, and notably elevating basal and stress-stimulated HPA activity [44]. This is supported by the study that high glucocorticoid exposure during fetal development in rats and primates permanently increases glucocorticoid levels and attenuates hippocampal GR gene expression in offspring [45, 46]. A lot of studies have shown that excess glucocorticoids secretion is strongly associated with neuronal atrophy and dysfunction, and impaired cognition [13, 47]. In this study, the levels of serum corticosterone were significantly higher in the mice that were successively exposed to CPS and COS than the mice of other groups. This result suggested that elevated corticosterone might mediate the learning and memory impairments in CPS plus COS group.

The hippocampus, an important receptor site for glucocorticoids in the central nervous system, devel-
directly affect AβPP or BACE1 has glucocorticoid receptor sites [49–51]. Moreover, Green et al. [49] reported that, in a triple-transgenic model of AD, application of dexamethasone accelerated increases in intraneuronal Aβ42 levels, hinting that corticosterone can directly affect AβPP processing. Similarly, we found some intracellular AβPP and Aβ42 in the CA3 region of the hippocampus of each group; particularly, the mice that were successively exposed to CPS and COS had the greatest expression levels, which was further proved by western blots. It can be speculated that the CPS potentiating Aβ42 production in adult male APP-swe/PS1dE9 offspring might be mediated by glucocorticoids through increasing the expression of APP gene.

Further, intriguingly, the results showed that the frontal cortex in the brain of CPS plus COS group mice had a larger percentage of areas that were positive for amyloid plaques (Congo red staining method) than all other groups; whereas, there were few amyloid plaques in the hippocampus of COS-treated and CPS plus COS groups; even no amyloid plaque was found in the hippocampus of Control and CPS-treated groups. The possible reason may be that the prefrontal cortex differentiates relatively late [52]; therefore, the prefrontal cortex is particularly vulnerable to chronic stress. However, the underlying mechanism still needs to be studied in the future.

CONCLUSION

In summary, the present study firstly demonstrated that 14-day exposure to CPS could exacerbate the learning and memory impairments, as well as neuropathological damages in the CA3 regions of the hippocampus and cortex neurons, which was induced by the 28-day COS in 6-month-old male APP-swe/PS1dE9 offspring. Our novel findings strongly implicate the synergistic roles of the CPS and COS exposures in impairing offspring learning and memory, and that CPS potentiating the production of Aβ42 might be mediated by glucocorticoids through increasing expression of APP and BACE1 gene. Therefore, the prevention of CPS and COS might help to reduce the risk of suffering adult neurodegenerative disorders, such as AD.

ACKNOWLEDGMENTS

This study was supported by grants from the Nature Science Foundation of Anhui Province (grant no.1208085MH145), the Department of Anhui Province Education (grant no. KJ2011Z161), and the Postgraduate Thousand Talents Program of Anhui Province.

Authors’ disclosures available online (http://j-alz. com/manuscript-disclosures/16-0011r1).

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