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Prenatal melamine exposure impairs cognitive flexibility and hippocampal synaptic plasticity in adolescent and adult female rats

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ABSTRACT

Prenatal melamine exposure (PME) affects spatial cognition in adolescent male rats and impairs synaptic functions. Strikingly, these effects can persist into adulthood. The current experiments examined whether PME-induced behavioral defects would be observed in female offspring, and how these effects varied with age (adolescent and adult). After female rats were exposed to melamine through their entire gestational period (GD), their spatial cognition was tested using water maze tasks at postnatal day 36 (PD36) and PD90. Long-term potentiation (LTP) and long-term depression (LTD) were recorded from the hippocampal Schaffer collaterals to the CA1 area. Results indicated that PME led to substantial reversal learning deficits and disrupted LTD at both PD36 and PD90. Additionally, PME did not affect LTP, although a lower fEPSP slope was observed in the adolescent PME-treated group than in the adult PME-treated group. Additionally, PME did not induce a horizontal shift in the synaptic modification threshold but caused a downward shift of the frequency–response curve at the low-frequency stimulation (LFS) of 1.0 Hz. However, the paired-pulse facilitation (PPF) ratio and the input/output function were not affected. Although the expression of both NR1 and NR2B subunits of NMDA receptors were diminished at PD36, the NR1 level was not affected at PD90. These findings suggest that PME impairs spatial memory in adolescent and adult females, and the impairments of hippocampal synaptic function via the inhibition of NMDAR expression may play a role in these effects.

1. Introduction

Melamine (1,3,5-triazine–2,4,6-triamine) is a nitrogen heterocyclic triazine compound (Cook et al., 2005; Ingelfinger, 2008), that has been widely used as an industrial chemical in many plastics, adhesives, glues, and laminated products such as plywood, cement, cleansers, fire retardant paint, and more (Hau et al., 2009; Ingelfinger, 2008). Previous studies have discovered that melamine has been illegally added to milk-derived products. Chronic consumption of melamine is detrimental to a range of body organs. In addition to abundant evidence of nephrotoxicity (Hau et al., 2009; Skinner et al., 2010), melamine is thought to interfere with brain development through its inhibitory action on the glutamatergic system (An and Sun, 2018; Yang et al., 2011). Recently, it has become evident that other non-glutamatergic mechanisms are involved in the developmental neurotoxicity associated with melamine (An et al., 2015; An et al., 2014; An and Zhang, 2014b). In addition, animal studies have indicated that melamine targets neurotransmitter systems, including the glutamatergic and cholinergic systems (An and Sun, 2018; An et al., 2013b).

Data from animal studies suggest a solid association between early-life exposure and neuropathology (An et al., 2012a; An and Sun, 2017b). However, it should not be overlooked that pregnant and lactating women are consuming milk powder and livestock products that may contain melamine (Dong et al., 2010). Notably, melamine can be transferred from mothers to both unborn babies and infants by passing through the placental barrier and being secreted in breast milk (Chu et al., 2010). Maternal melamine consumption has a strong correlation with birth defects, low body weight, and adverse neurobehavioral

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outcomes (An and Zhang, 2014a; Chu et al., 2013; Jingbin et al., 2010). Of particular relevance to these findings are the detrimental effects on spatial cognition and hippocampal synaptic plasticity of male animals observed in adulthood (An and Zhang, 2016). Previous studies have indicated that prenatal melamine may induce permanent alterations in neurodevelopment that lead to neuropathology in adulthood (An and Sun, 2017a; An and Zhang, 2016). Furthermore, melamine can induce age- and gender-related differences in the levels of circulating serum (Silva et al., 2016). Meanwhile, other studies have indicated that melamine might be more toxic to females (Dai et al., 2015; Sun et al., 2016). These effects seemed to be related to age and gender (An and Sun, 2017a). However, to the best of our knowledge, no studies have investigated the age-related effects of prenatal exposure to melamine on the cognitive function of female offspring.

The hippocampus is the center of spatial learning and memory (Bliss and Collingridge, 1993; Morris, 2003). Compared to other brain regions, hippocampal neurons are more sensitive to external factors during their lifetimes (Rosenzweig and Bennett, 1996). Long-term depression (LTD) and long-term potentiation (LTP), which are two major forms of activity-dependent synaptic plasticity in the brain, interplay to permit weakening or strengthening of synapses. Furthermore, these functions allow the refinement of neural circuits and are necessary for adaptive behavior. Recently, it has been recognized that melamine dramatically damages LTP and LTD in the hippocampus (An and Sun, 2017a, 2018). In the present study, we hypothesized that prenatal exposure to melamine would lead to performance deficits in adolescent female rats. These deficits could still be a major cognitive problem in adulthood. Testing separate groups of animals at PD36 and PD90 was necessary in order to examine the effects of PME on age. To investigate the adverse effects of PME on learning and memory maintenance, this study utilized Morris water maze (MWM) tests. Meanwhile, we recorded the local field potential (LFP) in the hippocampus from the Schaffer collaterals to the CA1 area, as well as the expression of NMDAR subunits at both ages. We then attempted to interpret the mechanisms underlying the behavioral results.

2. Materials and methods

2.1. Reagents

Melamine (purity > 99.5%) was purchased from Yingda Sparseness & Nobel Reagent Chemical Factory, Tianjin, PR China. Other reagents were of analytical reagent grade.

2.2. Experimental animals and treatment

Male and nulliparous female Wistar rats aged 10 weeks (Laboratory Animal Center, Academy of Military Medical Science of People's Liberation Army) were reared in plastic cages with ad libitum access to food and water. Rats were housed under a 12:12 h light:dark cycle (lights on at 0700) in a temperature and humidity controlled vivarium. All experiments were conducted during the light phase. After arrival at the facility, rats were given at least 7 days to acclimatize before experiments were initiated. All experiments were performed in accordance with the Care and Use of Animals Committee of Guangzhou University of Chinese Medicine (SCXK-2013-0020).

After an adaptation period of 1 week in our facilities, one or two female rats were housed with one male for mating. Vaginal plug check and vaginal smear observation by microscope were carried out each morning. Day 0 of gestation (GD0) was determined by the presence of a vaginal plug and/or spermatozoids in the vaginal smear. The female rat was then housed separately from the male and body weight (BW) was recorded daily. Pregnant dams were randomly divided into three groups (thirteen rats per group): melamine (M) group in which animals intragastric received 400 mg/kg/day (40 mg/ml); control (C) group in which animals received the same dose of distilled water; chow-fed (CF) group rats were given chow and water *ad libidum*. The doses of melamine were based on the doses used by our previous studies (An and Sun, 2017c; An and Zhang, 2014a, 2016). Gavage was performed and melamine or distilled water was given once a day throughout the whole gestational period. The day of parturition was designated postnatal day (PD) 0.

Litters were left undisturbed until weaning on PD21. Weaned pups from the same litter were housed in cages of 3 or 4 in separate colony rooms. One or two pups from each litter were randomly selected for the present experiment. No more than one rat from each litter was assigned to behavioral, electrophysiological or biochemical test. There were six groups. The PME group rats were selected from dams that were administered with melamine throughout their gestational period. The offspring rats were randomly assigned to the following two groups: (1) Adolescent PME (PME36) group (n = 12); (2) Adult PME (PME90) group (n = 12). The prenatal control (PCE) group rats were selected from dams administered with distilled water. The offspring rats were randomly divided into two groups: (1) Adolescent PCE (PCE36) group (n = 12); (2) Adult PCE (PCE90) group (n = 12). The untreated (PCF) group rats were selected from dams that were given *ad libidum* access to food and water. The offspring rats were randomly divided into two groups: (1) Adolescent PCF (PCF36) group (n = 12); (2) Adult PCF (PCF90) group (n = 12). Briefly, 12 rats per group were used for the behavioral test (total 72 rats), 12 rats per group for the electrophysiological test (total 72 rats) and 6 rats per group for the biochemical test (total 36 rats). Half of these rats were adolescent and half of them were adult.

2.3. Maternal blood melamine concentration measurements

On GD10, GD15 and GD20 during the whole gestation, blood samples were drawn from the tail vein from a separate set of rat dams. Extraction procedures were modified from previous reports (Baynes et al., 2008; Wu et al., 2009). Samples were drawn 3 h after the introduction of the gavage, which was the half-lives of melamine in fetal brain and was also about 2 h after the maximum concentration of melamine in maternal and fetal brains and organs (Chu et al., 2013). Blood melamine samples were assayed using a modification of our previous method (An et al., 2012a). Briefly, plasma samples were obtained by centrifuging collected blood at 1000 × g at 4 °C for 10 min. 100 μL plasma was mixed well with 900 μL 0.02 M PB (phosphate buffer) and the mixture was centrifuged at 13,000 × g at 4 °C for 15 min before extraction and detection. 50 μL Detector Antibody and 50 μL HRP (horseradish peroxidase) Conjugate were added into 50 μL samples. After incubating for 30 min, we discarded the liquid by rigorously flicking and bolted the remaining liquid. The Detection Reagent A and B were added and the liquid was incubated for 15 min in the dark. Finally, we read the optical density absorbance at 450 nm within 3 min of adding the Stop Solution.

2.4. Learning and re-acquisition learning in MWM test

At either PD34 to PD40 or PD88 to PD94, spatial learning and re-acquisition learning were assessed in the MWM using the procedures that described previously by our laboratory (An and Zhang, 2015; Han et al., 2014). The MWM consisted of a 1.5-m-diameter circular tank filled with 45 cm deep water made opaque with black nontoxic ink, maintained at 25 ± 1 °C. The tank was divided into four equal quadrants (I, II, III, and IV), and a 10-cm-diameter platform was submerged 2 cm below the water surface in the center of quadrant III. The experimental environment with moderate light was kept noiseless. A CCD camera suspended above the pool center recorded the swim paths of the animals and video output was digitized by a video tracking system (Ethovision 2.0, Noldus, Wagenigen, Netherlands) connected to a personal computer, through which data were collected for off-line analyzing. The tests consisted of two consecutive periods: initial spatial

training (IT), and spatial re-acquisition training (RT). In IT stage, rats were subjected to four trials per day (10 min intertrial interval) for five consecutive days. In each trial, each rat was placed into the water facing the pool wall from one of four different starting positions. The order of starting points was randomized but the same for all animals on each training day. They were allowed to swim freely until they found the platform. Rats that failed to locate the platform in 60 s were guided to the platform where they stayed for 10 s. Escape latency was recorded. After the initial spatial learning task, a reversal learning protocol was conducted. During RT stage, the platform was placed in the center of quadrant I, which was opposite to quadrant III. Reversal learning entailed 3 additional days with 4 trials per day, similar to initial training.

2.5. In vivo electrophysiological recording

6 rats of each group were chosen for LTP/LTD recording and 6 rats were chosen for the stimulation at 0.1/10 Hz recording. At either PD36 or PD90, electrophysiological tests were performed and the protocols closely followed our published procedures (An et al., 2018; An et al., 2012b; An et al., 2013a). Briefly, the rats were anesthetized with an intraperitoneal injection of 30% urethane with a dosage of 4 ml/kg and placed in a stereotaxic frame (SN-3, Narishige, Japan). Deep body temperature was monitored throughout the experiment, and a heating pad was used to maintain the temperature of the animals at 36.5 ± 0.5 °C. The scalp was opened, and trephine small holes were drilled in the skull for the monopolar recording (insulated platinum iridium wire, AM Systems, WA; hippocampal CA1 region; AP: −3.5 mm, ML: 2.5 mm, DV: −1.8 to −2.5 mm) and tungsten bipolar stimulating electrodes (FHC, ME; hippocampal Schaffer collaterals region; AP: −4.2 mm, ML: 3.5 mm, DV: −2.4 to −3.0 mm), which were based on The Rat Brain in Stereotaxic Coordinates. The head side of each rat was chosen randomly for either LTP or LTD recording. After the electrodes were implanted and located properly in destination positions, test stimuli were delivered to the Schaffer collaterals every 20 s (0.05 Hz). To observe the proper field excitatory postsynaptic potentials (fEPSPs), the advisable intensity (range 0.3–0.8 mA) that evoked a response of 45%–60% of the maximal response size was chosen. About 1 h after the electrodes were located properly in destination positions, input/output (I/O) function were established. Paired-pulse facilitation (PPF) was investigated by applying two pulses in rapid succession (inter-pulse intervals of 15, 30, 60, 120, and 240 ms, respectively) at 5 min intervals. Baseline fEPSPs were recorded under low-frequency test stimulations (0.033 Hz) for 20 min. After recording baseline, high-frequency stimulation (HFS) (ten trains of 10 pulses at 100 Hz every 2 s, 0.2 ms pulse duration) was delivered to induce LTP and low-frequency test stimulations (0.033 Hz) were used again after HFS for a period of 60 min to monitor any change of fEPSPs. During LTD recording, the surgery was performed on the other head side of each rat. After recording baseline, low-frequency stimulation (LFS) (900 pulses of 1 Hz for 15 min) was delivered and low-frequency test stimulations (0.033 Hz) were used to monitor any change of fEPSPs. Initial data measurement was performed in Clampfit 9.0 (Molecular Devices, Sunnyvale, CA, USA). A running average of two fEPSPs was taken for graphic presentation and data analysis. The average amplitudes during the baseline period were normalized to 100%, and the relative amplitudes at every point were normalized relative to the baseline period. The average amplitude between 40 and 60 min after delivery of the conditioning stimulus (HFS/LFS) was used to analyze. To further obtain the relationship between stimulation frequency and synaptic plasticity, LTD was evoked using 900 pulses at 0.1 or 10 Hz in the selective groups of rats. The fEPSPs were recorded after applying the stimulation at the 0.1 Hz in one hemisphere of each rat, and the stimulation at the 10 Hz was given in the other hemisphere. The head side of each rat was chosen randomly. The crossover point between LTP and LTD observed in the frequency–response curve was defined as the synaptic modification threshold (θ).

2.6. Protein preparations and Western blot analysis

As previously described with modifications (Li et al., 2018; Sun et al., 2018a; Sun et al., 2018b), hippocampi were rapidly dissected on ice and stored at −80 °C. Tissues were homogenized in ice-cold lysis buffer (pH 7.4) containing protease and phosphatase inhibitor cocktails (Sigma). The samples were centrifuged at 14,000 r.p.m. for 15 min at 4 °C and the supernatant was collected. Protein concentrations were detected by the bicinchoninic acid (BCA) assay. Twenty micrograms (15 μL) of total protein per lane were resolved in 10–15% SDS-PAGE gels followed by electro-transferring to PVDF membranes (Pall, Pensacola, FL). Non-specific binding of antibodies to membranes was probed with the primary rabbit anti-NR1 (1:1000; Chemicon, CA, USA), rabbit anti-NR2A (1:1000; Millipore, MA, USA), or mouse anti-NR2B antibody (1:1000; Millipore, MA, USA) overnight at 4 °C. Mouse anti-β-actin (1:20,000; Sigma, MA) was used as an internal control. After further incubation in horseradish-peroxidase (HRP)-conjugated secondary goat anti-rabbit IgG (1:3000) and goat anti-mouse IgG (1:1000) antibodies (Southern Biotechnology Associates, AL, USA) for 2 h at room temperature, immunoreactivity was detected by ECL Western Blotting Detection Kit (CWBIO, China).

2.7. Data acquisition and statistical analysis

All data were presented as the mean ± S.E.M.. The data of melamine level were compared using a repeated-measures ANOVA in which testing day was a within subjects measure (i.e., repeated measure) and treatment (control or melamine treatment) was the between subjects factor, with 6 rats in the M group and 8 rat in the C group. The data of the MWM test were subjected to a repeated-measures ANOVA in which training day was a within subjects measure, and age (PD36 or PD90) and treatment (PCE, PME or PCF) were the between-subjects factors, with 12 rats in each group. A two-way ANOVA examined the effects of age (PD36 or PD90) and treatment (PCE, PME or PCF) on the data of fEPSP slopes of LTP/LTD recordings, with 6 rats in each group. A three-way ANOVA examined the effects of age (PD36 or PD90), treatment (PCE, PME or PCF) and stimulation frequency (0.1, 1, 10, or 100 Hz) on the data of the frequency–response test, with 6 rats in each group. The data derived from the western blot tests were subjected to a two-way ANOVA in which age (PD36 or PD90) and treatment (PCE, PME or PCF) were the between-subjects factors. When the ANOVA revealed a significant main effect or significant interaction, the data were further analyzed by Bonferroni post hoc tests. All the analyses were performed using SPSS 16.0 software. A P value of < 0.05 was taken to indicate statistical significance.

3. Results

3.1. PME enhanced melamine concentration in maternal serum

A repeated measure analyses of variance confirmed that there was a significant interaction between the duration of time (GD10, GD15 or GD20) and treatment (control treatment or melamine treatment; Table 1, $F_{(2,\ 24)} = 107.373$, $P < 0.001$), which indicated the concentration of melamine in maternal serum cumulatively increased with chronic exposure. Meanwhile, a statistical effect of treatment was found ($F_{(1,\ 12)} = 163.915$, $P < 0.001$; Bonferroni post-tests, GD10, GD15 and GD20: $P < 0.001$).

3.2. Impaired spatial re-acquisition learning

In order to determine whether PME affected the abilities of spatial cognition, the performance of PME-treated rats was tested by using MWM task. On the IT stage, a repeated measure ANOVA measurement

Table 1
Assay the concentration of melamine.

Group	Maternal blood melamine (μg/ml)		
	GD10^{aaa}	GD15^{aaa}	GD20^{aaa}
C	0.43 ± 0.10	0.33 ± 0.12	0.34 ± 0.16
M	715.78 ± 49.91	952.45 ± 47.60	1025 ± 78.54

The data are represented as the mean levels of melamine in maternal blood serum on GD10, GD15 and GD20. All data are presented as mean ± S.E.M.. ^{aaa}, $P < 0.001$ M group versus C group. $n = 6$ for M group, and $n = 8$ for C group.

on the escape latency revealed a significant main effect of day (testing day 1–5; Fig. 1A, $F_{(4, 264)} = 143.292$, $P < 0.001$) but no significant effect of treatment (PCE, PME or PCF; Fig. 1A, $F_{(2, 12)} = 0.873$, $P > 0.05$) or age (PD36 or PD90; Fig. 1A, $F_{(1, 16)} = 0.271$, $P > 0.05$). Similarly, a repeated measure ANOVA was performed to the escape latency of the RT stage and revealed a significant main effect of day (testing day 6–8; Fig. 1A, $F_{(2, 132)} = 84.985$, $P < 0.001$), treatment (PCE, PME or PCF; Fig. 1A, $F_{(2, 6)} = 23.360$, $P < 0.001$) and age (PD36 or PD90; Fig. 1A, $F_{(1, 8)} = 13.571$, $P < 0.001$). Meanwhile, a repeated measure ANOVA measurement on swimming velocity revealed that there was no significant effect of day (IT testing day 1–5; RT testing 6–8), treatment (PCE, PME or PCF) or age (PD36 or PD90) on training day of the IT or the RT stage (Fig. 1B). Therefore, PME affected the performance of the RT but not the IT stage.

3.3. The PME-induced impairment of synaptic plasticity in hippocampal CA1 by CA3 stimulation

Stimulation of the CA3 region evoked the field potentials in the CA1 region of the hippocampus. We observed that HFS of the CA3 subfield induced reliable LTP concurrently in the hippocampal CA1 (Fig. 2A and B). A two-way measure ANOVA revealed significant effects of age (PD36 or PD90; Fig. 2E, $F_{(1, 30)} = 3.590$, $P < 0.05$) and a significant interaction between age and treatment ($F_{(2, 30)} = 3.493$, $P < 0.05$), but no effect on treatment (PCE, PME or PCF; $F_{(2, 30)} = 0.378$, $P > 0.05$). A post hoc test revealed that the fEPSP slopes were significantly lower in the PME36 group than those in the PME90 group (Fig. 2E, $P < 0.05$), while there was no statistical difference between the PCE36 group and the PCE90 group (Fig. 2E, $P > 0.05$), or between the PCF36 group and the PCF90 group (Fig. 2E, $P > 0.05$). In LTD recording, a two-way measure ANOVA found significant effects of treatment (PCE, PME or PCF; Fig. 2D, $F_{(2, 30)} = 3.648$, $P < 0.05$) and the interaction between age and treatment ($F_{(2, 30)} = 4.403$, $P < 0.05$), but no significant effect on age (PD36 or PD90; $F_{(1, 30)} = 0.671$, $P > 0.05$). A post hoc test showed that there was a significantly inhibited fEPSP slopes in the PME36 group compared to those in the PCE36 group (Fig. 2F, $P < 0.05$) and the PCF36 group (Fig. 2F, $P < 0.05$), and a considerably suppressive effect existed in the PME90 group compared to those in the PCE90 group (Fig. 2F, $P < 0.05$) and the PCF90 group (Fig. 2F, $P < 0.05$). Furthermore, a three-way ANOVA found a significant main effect of treatment (PCE, PME or PCF; Fig. 3A, $F_{(2, 150)} = 3.359$, $P < 0.05$), but no significant main effect of age (PD36 or PD90; $F_{(1, 150)} = 0.719$, $P > 0.05$). The interaction between frequency of the stimulation (0.1, 1, 10 or 100 Hz) and age ($F_{(4, 150)} = 0.546$, $P > 0.05$) or the interaction between frequency of the treatment and

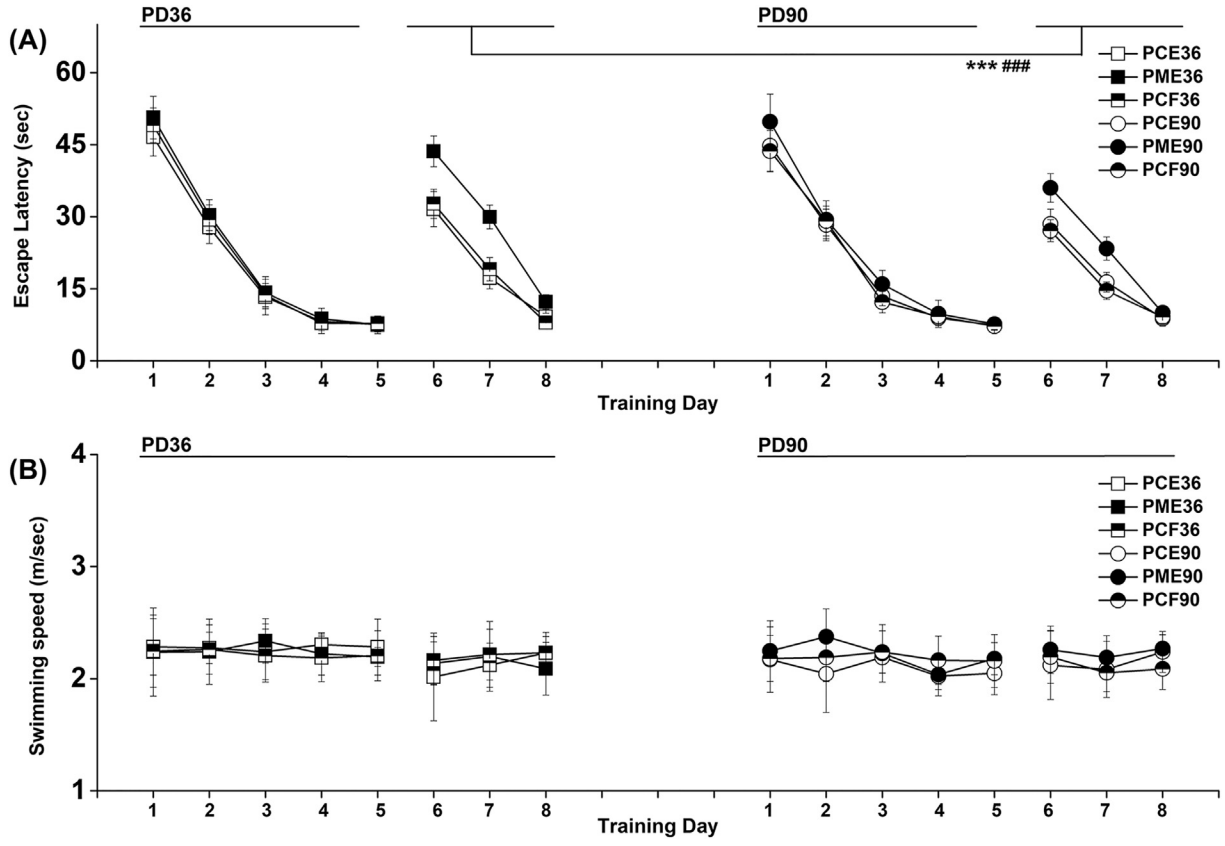

Fig. 1. Performances of rats in IT and RT stages of MWM task.
During consecutive 5 days, the animal is trained with a constant platform position in IT stage. During 6th to 8th day, the platform is installed at a new location (reversal) in RT stage. (**A**) Escape latencies during IT and RT stages. (**B**) Swimming speed during IT and RT stages. Data are expressed as mean ± S.E.M.. ***, $P < 0.001$, treatment effect. $^{###}$, $P < 0.001$, age effect. $n = 12$ for per group.

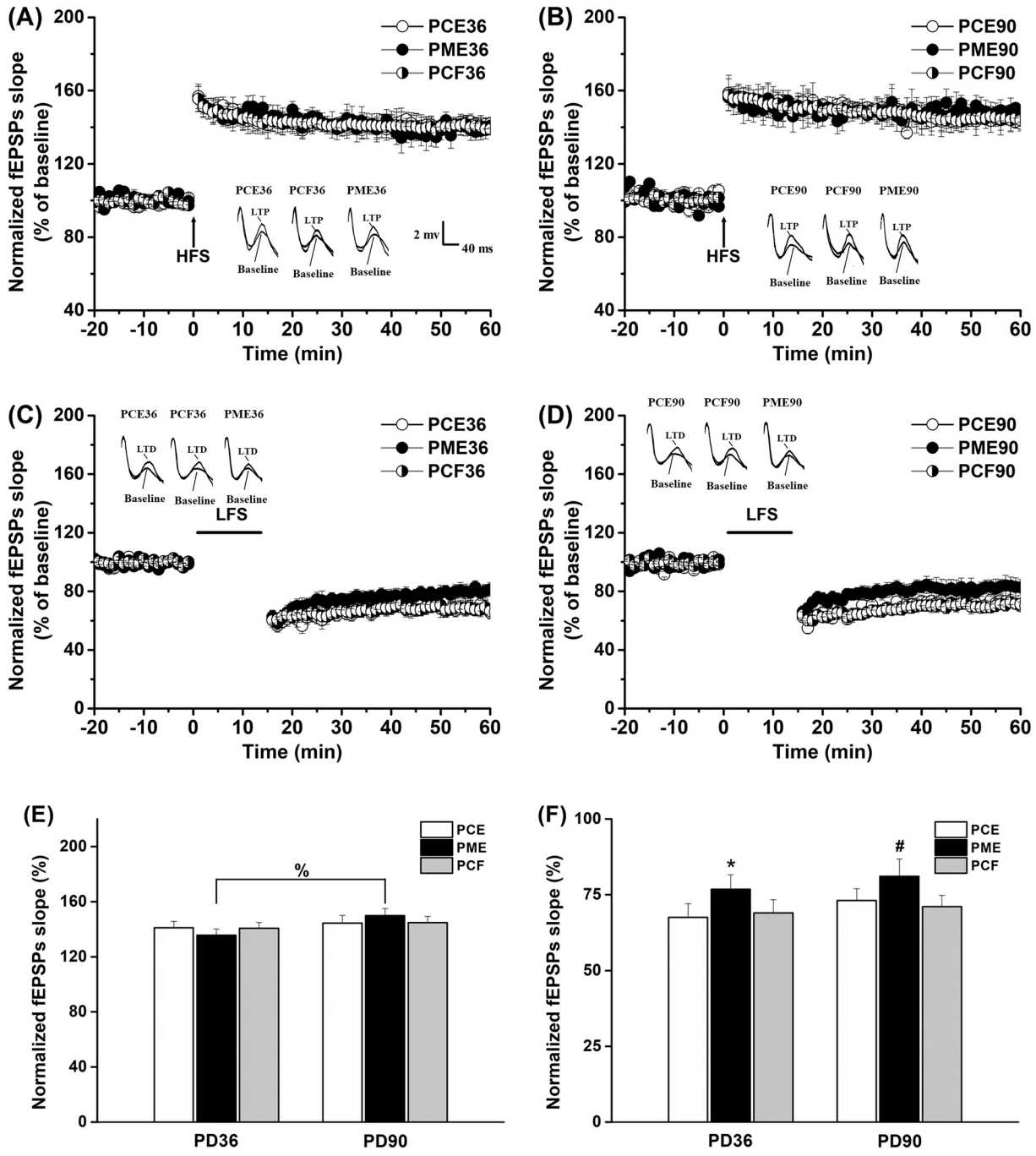

Fig. 2. The effect of PME on synaptic plasticity from Schaffer collateral to CA1 region.
LTP (**A**) and (**B**) and LTD (**C**) and (**D**) were induced by using HFS and LFS after recording a stable baseline for 20 min, respectively. 20 min of evoked responses were normalized and used as the baseline. (**A**) and (**B**) were changes of time coursing in fEPSP slopes of PD36 and PD90 during LTP recordings, respectively. Arrow represents application of HFS. The inserts are the traces of averaged fEPSPs, which are taken at 40 min after HFS. (**C**) and (**D**) were changes of time coursing in fEPSP slopes of PD36 and PD90 during LTD recordings, respectively. Dark black line represents application of LFS. The inserts are the traces of averaged fEPSPs, which are taken at 40 min after LFS. (**E**) Magnitude of LTP was determined as responses between 40 and 60 min after HFS. (**F**) Magnitude of LTD was determined as responses between 40 and 60 min after LFS. Data are expressed as mean ± S.E.M.. *, $P < 0.05$, PME36 group vs. PCE36 group and PCF36 group. $^{\#}$, $P < 0.05$, PME90 group vs. PCE90 group and PCF90 group. $^{\%}$, $P < 0.05$, PME36 group vs. PME90 group. $n = 6$ for per group.

age ($F_{(2,\ 150)} = 0.478$, $P > 0.05$)was also not significant. However, the interaction between frequency of the stimulation and treatment ($F_{(8,\ 150)} = 2.76$, $P < 0.05$; For 1.0 Hz, PME vs. PCE: $P < 0.05$) was also significant. Therefore, PME caused no horizontal shift in the synaptic modification threshold (θ) but produced an apparent downward shift of the frequency–response curve at the LFS of 1.0 Hz. To clarify whether the presynaptic mechanism was involved in the effects of PME on synaptic function, PPF in the hippocampal CA1 region was examined immediately before baseline recording. No significant difference was found in the PME groups (Fig. 3B). No significant differences of synaptic transmission by low frequency stimulation of the Schaffer collateral-CA1 pathway were detected in the PME groups, when compared to vehicle controls (Fig. 3C).

Notably, there was no significant effect of the HFS on the Schaffer collateral-commissural synapses in contralateral CA1 area prior to inducing LTD with LFS (Fig. S1). Therefore, the HFS protocol did not

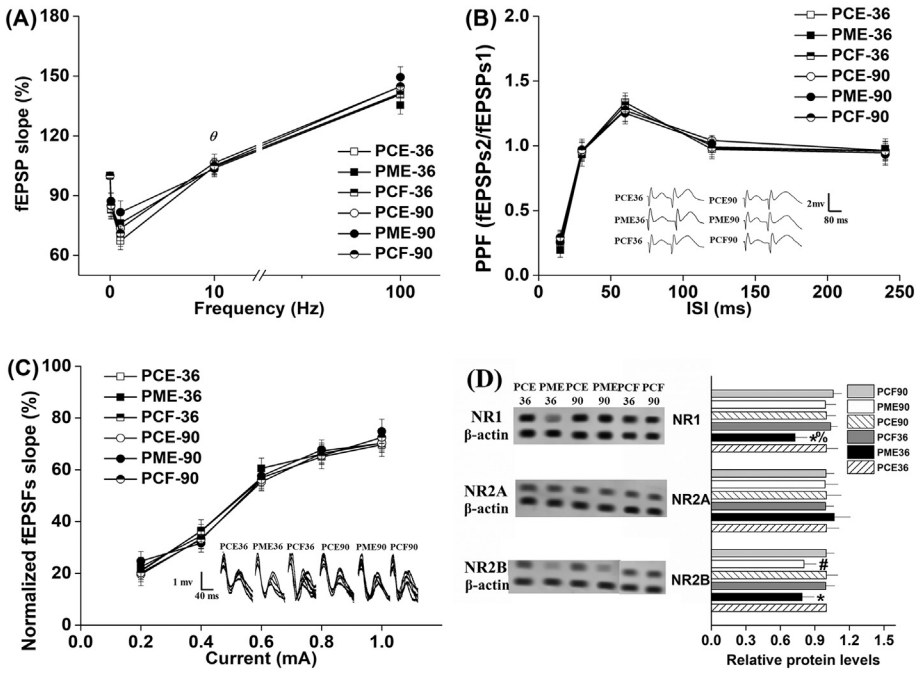

Fig. 3. Effect of PME on frequency–response function and NMDAR subunit levels in the CA1 region of the hippocampus.
(**A**) The shift of synaptic modification threshold (θ). (**B**) PPF, a form of short-term plasticity, was measured and expressed as the ratio of fEPSPs2 to fEPSPs1. The inserts are the mean fEPSP traces (inter-pulse intervals of 30 ms). (**C**) CA1 input–output curves of fEPSP slopes. The inserts are the mean fEPSP traces of each group. (**D-left**) Representative immunoblots showing NR1 (M = 120 kDa), NR2A (M = 170 kDa), NR2B (M = 180 kDa). Each lane shows receptor expression in individual animals from PME and its vehicle groups. (**D-right**) Corresponding densitometric analysis of NR1, NR2A and NR2B subunits. Data are expressed as mean ± S.E.M.. *, $P < 0.05$, PME36 group vs. PCE36 group and PCF36 group. $^{\#}$, $P < 0.05$, PME90 group vs. PCE90 group and PCF90 group. $^{\%}$, $P < 0.05$, PME36 group vs. PME90 group. $n = 6$ for per group.

potentiate LTD in the contralateral hippocampal CA1 region in the current study. Similarly, no significant effect of the stimulation at 900 pulses at 0.1 Hz on the fEPSPs of the other hemisphere was found (Fig. S2).

3.4. PME affected the expression of NMDAR subunits in the hippocampus

Western blot analysis was conducted to assess the expression of NMDA receptor subunits in the hippocampus of the PME groups at PD36 and PD90. A two-way measure ANOVA found a significant effect of treatment (PCE, PME or PCF; Fig. 3D, $F_{(2,\ 30)} = 4.620$, $P < 0.05$) and a significant interaction between age (PD36 or PD90) and treatment ($F_{(2,\ 30)} = 0.701$, $P < 0.05$), on the expression of NMDA-NR1. A post hoc test revealed that, compared to controls, the expression of the NMDA-NR1 subunit was significantly reduced in the PME36 group (Fig. 3D, $P < 0.05$), but not in the PME90 group. Moreover, the level of NR1 subunit in the PME90 rats was higher than the PME36 group ($P < 0.05$). There was no significant effect of age (PD36 or PD90), treatment (PCE, PME or PCF) or their interaction on the level of NMDA-NR2A. A two-way ANOVA conducted on the expression of NMDA-NR2B revealed a significant main effect of treatment (PCE, PME or PCF; Fig. 3D, $F_{(2,\ 30)} = 5.992$, $P < 0.01$), but not of age (PD36 or PD90; $F_{(1,\ 30)} = 0.441$, $P > 0.05$) nor of the interaction between treatment and age ($F_{(2,\ 30)} = 0.340$, $P > 0.05$). PME decreased the expression of the NMDA-NR2B subunit in both the PD36 ($P < 0.05$) and in the PD90 ($P < 0.05$) groups. No difference was found between the PME36 and the PME90 groups.

4. Discussion

Our previous studies have revealed that PME impairs cognitive function in the adolescent female rat. It still remains unknown, however, if the effects of PME vary with age. The present study yielded novel and interesting results. Using a PME animal model, we found that PME-treated adolescent and adult female rats both exhibited the impairment of re-acquisition ability. Additionally, both adolescent and adult animals expressed lower LTD in low-frequency stimulation. Most importantly, the effects of PME on LTP and the levels of NMDAR subunits differed considerably between adolescent and adult rats, suggesting that the effects of PME vary with age.

Several studies concerning the neurotoxicity of melamine have shown that chronic melamine exposure can induce cognitive defects in functions such as spatial learning ability (An et al., 2011), reference memory (An et al., 2012a) and reversal learning ability (An et al., 2013b). In the present study, consistent with our previous observations (An and Sun, 2017c), the data indicated that PME did not affect the learning ability of female adolescent offspring. Furthermore, we did not find that PME caused significant disruption in the performance of spatial learning tasks in female adult offspring, although hippocampal pyramidal cell neurogenesis occurred from PD16 to PD20 (Bayer, 1980), and abnormal melamine concentrations were observed during this period. Additionally, there was no statistical difference between the PCF group and the PCE group, a finding that possibly rules out the effect of prenatal stress in the current study. Taken together, the behavioral tests indicated that PME impaired re-acquisition ability in both adolescent and adult female rats.

It is tempting to infer that affected hippocampal synaptic plasticity results in distorted cognitive function, since previous studies have shown that both hippocampal LTP and spatial cognition are impaired in adolescent males following PME (An and Zhang, 2014a). Unfortunately, we did not obtain significantly altered fEPSP slopes in the CA1 region for either the adolescent or adult PME-treated groups. However, the differences between the PME36 and the PME90 rats suggest the possibility of diverse PME effects for different ages. To our knowledge, we were the first to demonstrate the effects of PME on female offspring. Although we cannot provide a direct explanation for these effects, it is clear that they are specifically related to PME and not prenatal stress effects, since these effects were not observed in either the adolescent or adult PCF groups. Further experiments are necessary to determine the reliability of this observation.

Recently, several studies have demonstrated that hippocampal LTD facilitates reversal learning in behavioral tests (Duffy et al., 2008; Ge et al., 2010; Kemp and Manahan-Vaughan, 2004). Selected evidence has indicated that reversal learning in the water maze is impaired by blocking hippocampal LTD, and enhanced by facilitating hippocampal LTD due to the application of either the selective GluN2B antagonist Ro25-6981 or the AMPAR endocytosis inhibitor Tat-GluA23Y (Dong et al., 2013). LTD may act to increase the signal-to-noise ratio between acquired memory and new learning, subsequently preventing previous traces from interfering with the encoding of new information when the

demands of a task change (An and Zhang, 2013). Given our behavioral findings that melamine-treated rats took longer to find a new platform location during the re-acquisition learning phase, we next determined if PME affected hippocampal CA1 LTD. Our results indicated that PME disrupted LTD in both adolescent and adult rats. These results could explain the fact that PME-treated rats displayed poor performance in the RT stage, and also support the previous conclusion that hippocampal CA1 LTD is necessary for spatial reversal learning in the water maze. An alternative explanation is that LTD inhibited by PME may impair behavioral flexibility by disabling the nervous system from acquiring new information and/or increasing the interference of new learning by previously acquired information via the disruption of critical hippocampal circuitry (Collingridge et al., 2010; Duffy et al., 2008). This possibility was supported by electrophysiological observations indicating that LTP could not be reversed or depotentiated by LFS (An and Sun, 2017c). Meanwhile, no difference was observed between the PME36 and the PME90 groups in the fEPSP slopes of the LTD recordings. Inconsistent with these data, we found both PME36 and PME90 rats performed differently on the first day of the RT test. Given that we observed a significant difference between the LTP recordings of these two age groups, however, these results were consistent with the hypothesis that LTP and LTD are not independent but rather act as a single entity in the regulation of synaptic efficiency and the modulation of spatial cognition (Collingridge et al., 2010; Malenka and Bear, 2004).

The dynamic ranges of depression and potentiation were found to be linked, and the direction of the change in synaptic weight was revealed to be a function of synaptic activity (Yeh et al., 2012). PME did not shift the synaptic modification threshold but caused a vertical shift at the LFS of 1.0 Hz but not at the HFS of 100 Hz, suggesting that PME modulates the induction of LTP and LTD independently. These findings were in line with the changes in the glutamatergic circuits following melamine treatment, indicating that acute melamine is specific to inhibit induction, but not expression, of synaptic plasticity via the mediation of postsynaptic NMDAR activation. Remarkably, the production of nitric oxide (NO) affected NMDAR activation, and then changed the chemical signaling in surrounding synaptic terminals (Montague et al., 1994), thus contributing to reactive nitrogen species formation, a process that had been implicated in our prior research (An et al., 2015). Previously, melamine was found to induce hippocampal neuron apoptosis and disrupt the homeostasis of presynaptic Ca^{2+} (Wang et al., 2011), which is important in the modulation and control of neuronal excitability (Toescu and Vreugdenhil, 2010) as well as the induction of LTP (Schneider et al., 2001; Yashiro and Philpot, 2008) and LTD (Yashiro and Philpot, 2008). However, we did not observe any effects of PME on presynaptic functions, such as PPF and I/O. Interestingly, following melamine treatment, expression of the postsynaptic NMDAR-NR2B subunit was reduced in both age groups, while the reduction in the NMDA-NR1 level was observed in PD36 but not PD90 rats. Previous studies implied that the detrimental effects of prenatal melamine on hippocampal function could persist into adulthood (An and Zhang, 2014a, 2016). One possibility for this is that melamine-related insoluble metabolites cannot impact physiological function once they have been removed from organs or cells (Wang et al., 2011). However, a maturational recovery of function was not thoroughly effective after PME (An and Zhang, 2016), providing compelling evidence that melamine affects the CNS even in adulthood, and contradicting the idea that melamine toxicity is associated only with residual melamine (Tyan et al., 2009). The underlying mechanism for the sustained effects of PME remains to be explored. For low frequency-induced LTD, NR1/NR2B made a larger contribution to total charge transfer than NR1/NR2A, while for high-frequency tetanic stimulation induced LTP, the charge transfer mediated by NR1/NR2A considerably exceeded that of NR1/NR2B (Erreger et al., 2005). Therefore, significant decayed LTD and reduced NMDA-NR1 level were observed in the PME groups. Furthermore, our results strongly suggest that NR2B receptor-dependent LTD is necessary to mediate spatial flexibility (Dong et al., 2013; Tsetsenis et al., 2011) and memory consolidation (An and Sun, 2018; Ge et al., 2010).

Importantly, the maximum melamine allowed in baby formula has been set at 1.0 mg/kg, while a maximum of 2.5 mg/kg is permissible in other foods and animal feed, according to the World Health Organization (WHO, 2008). However, quantities of several hundred mg/kg of melamine have been identified in illegally adulterated milk/food products (Deabes and El-Habib, 2012; Hilts and Pelletier, 2009). Therefore, the chosen dose in the current study is only relevant to the consumption of food products that have been illegally adulterated with melamine.

In summary, the main finding of this study is that PME lead to cognitive deficits in both adolescent and adult female offspring. The adverse effects of PME on long-term synaptic plasticity could explain the abnormal flexibility of cognitive function. Additionally, the differences in the fEPSP slopes of LTP and NMDAR subunit levels between adolescent and adult offspring may highlight the age-related effects of PME. Taken together, these findings provide evidence for the role of PME in cognitive ability and synaptic function in the hippocampus, and enhance the understanding of developmental and age-related deficits after PME.

Author contributions

Conceived and designed the experiments: LA, WS, JL, PDL, CZT; performed the experiments WS, LL, JL; analyzed the data: JL, LL, PDH, LA; wrote the manuscript: LA, WS, PDH, PDL.

Declaration of competing interest

There is not a conflict of interest for authors.

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Appendix A. Supplementary data

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