

Rapamycin Effectively Impedes Melamine-Induced Impairments of Cognition and Synaptic Plasticity in Wistar Rats

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Abstract Our previous investigation demonstrated that autophagy significantly reduced melamine-induced cell death in PC12 cells via inhibiting the excessive generation of ROS. In the present study, we further examine if rapamycin, used as an autophagy activator, can play a significant role in protecting neurons and alleviating the impairment of spatial cognition and hippocampal synaptic plasticity in melamine-treated rats. Male Wistar rats were divided into three groups: control, melamine-treated, and melamine-treated + rapamycin. The animal model was established by administering melamine at a dose of 300 mg/kg/day for 4 weeks. Rapamycin was intraperitoneally given at a dose of 1 mg/kg/day for 28 consecutive days. The Morris water maze test showed that spatial learning and reversal learning in melamine-treated rats were considerably damaged, whereas rapamycin significantly impeded the cognitive function impairment. Rapamycin efficiently alleviated the melamine-induced impairments of both long-term potentiation (LTP) and depotentiation, which were damaged in melamine rats. Rapamycin further increased the expression level of autophagy markers, which were significantly enhanced in melamine rats. Moreover, rapamycin noticeably decreased the reactive oxygen species level, while the

superoxide dismutase activity was remarkably increased by rapamycin in melamine rats. Malondialdehyde assay exhibited that rapamycin prominently reduced the malondialdehyde (MDA) level of hippocampal neurons in melamine-treated rats. In addition, rapamycin significantly decreased the caspase-3 activity, which was elevated by melamine. Consequently, our results suggest that regulating autophagy may become a new targeted therapy to relieve the damage induced by melamine.

Keywords Rapamycin · Melamine · Oxidative stress · LTP · Depotentiation · Rats

Introduction

It is comprehensively known that the developing brain has a distinctive set of characteristics that make it unusually sensitive to damage. Understanding the underlying mechanism of the vulnerability of the immature nervous system to melamine neurotoxicity is essential and often provides new insight into the toxicity of melamine. In fact, its role in the nervous system was fully appreciated since the early twenty-first century. Several *in vitro* neurotoxicity studies have been reported that apoptosis [1], disrupt metabolism [2], and hyperpolarization and spontaneous firing [3] can be induced by melamine. Moreover, electrophysiological studies using brain slice preparations have been performed, by which the effect of melamine on voltage-dependent potassium channels and voltage-gated sodium channels in the hippocampal CA1 pyramidal neurons is investigated by whole-cell patch-clamp technique [4, 5]. *In vivo* neurotoxicity studies have been performed on different animal models in order to examine the potential effects of melamine on the brain. It was found that melamine could pass the blood–brain barrier and in rats approximately 7.5 % of the

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total amount in the plasma enters the brain [6]. Melamine impaired the central nervous system (CNS) and induced deficits of learning and reference memory [7]. In reversal exploring test, melamine-treated rats stubbornly swam towards the original platform position, suggesting that the impairments of cognitive flexibility could not only interfere in suppressing previously acquired behavior strategies but also disturb establishing new strategies [8]. The toxicity of melamine is involved in breaking down redox balance and the oxidation-antioxidation homeostasis [9, 10]. Moreover, the oxidative stress and enhanced apoptosis were effectively rescued and cognitive defects recovered to a large extent, with a predominantly combination vitamins [11]. Nevertheless, compared with focusing on the toxicity of melamine, how to relieve the neural damage induced by melamine is more worthy of our attention.

Synaptic plasticity in the form of long-term potentiation (LTP) and long-term depression (LTD) comprises the cellular basis for learning and memory in the hippocampus [12]. The reversal of LTP which occurs following the low-frequency stimulation in the hippocampus has been termed depotentiation. It is considered as a fundamental mechanism of cognitive flexibility, dominating the process of precise spatial characteristics by the way of directly to hippocampal information storage [12–14]. The major types of ionotropic glutamate receptors, which understood excitatory synaptic function, and LTP contribute to the postsynaptic response at glutamatergic synapses, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPA), and NMDARs [15]. Furthermore, another essential protein for the synaptic plasticity in the hippocampus is the postsynaptic density protein 95 (PSD-95). It is well known that decreasing levels of these proteins may impair plasticity and cognition [16].

Rapamycin is shown to be an inhibitor of S6K1 activation that is an important mediator of PI3 kinase signaling and the activation of autophagy [17]. In some neurodegenerative disorder, rapamycin confers neuroprotection. For instance, in models of Parkinson's disease (PD), rapamycin blocks RTP801 which is induced by PD toxin [18]. Besides, in the rapamycin-treated Alzheimer's disease (AD) mice, mammalian target of rapamycin (mTOR) activity and A β levels were significantly reduced instead of detecting in the hippocampal neurons of AD mice enhanced mTOR signaling significantly [19]. Although mTOR is regarded as 'conciierge' of autophagy, it is a ubiquitous complex serine-threonine protein kinase which directly influences protein synthesis, transcription, autophagy, metabolism, and organelle biogenesis and maintenance [20]. Autophagy is an evolutionarily conserved process of self-digestion and plays an important role in maintaining cellular homeostasis from yeast to mammals [21]. Usually, it can remove degradation of intracellular macromolecules and organelles in order to maintain the cellular metabolism and physiological function. In the nervous system, autophagy is not only

activated by conditions of nutrient deprivation but also has been related with many physiological and pathological processes, such as neuronal development, differentiation, and neural degenerative diseases [22]. The activation of autophagy could protect ischemic hippocampal neuron through removing the abnormal sediment in impaired neurons [23]. Moreover, autophagy can reduce the level of mutant or toxic proteins such as A β and Tau protein in neurons, which may affect neuro-protective role of autophagy in AD [24].

The reactive oxygen species (ROS), which production decreases antioxidant defense and increases lipid peroxidation and membrane degeneration, can generally be believed that oxidative stress plays a significant role in occurrence and development of neurodegenerative diseases [25]. Our previous studies showed that residual melamine concentrations in the hippocampus resulted in enhancing ROS production, and as ROS scavengers [26], vitamin C and vitamin E combination therapy could effectively alleviate apoptosis induced by melamine [11]. It was reported that autophagy could be induced by oxidative damage [27]. In addition, obsolete organelles can be selectively removed into limit ROS amplification by autophagy [28]. Obviously, the interaction between autophagy and ROS plays an essential role in cellular homeostasis. Our recent data show that melamine-induced cell death in PC12 cells was significantly decreased by autophagy via inhibiting the excessive generation of ROS, suggesting that regulating autophagy may become a new targeted therapy to release the damage caused by melamine [29]. Accordingly, we wonder if autophagy is able to alleviate the nerve injury induced by melamine.

This *in vivo* study was aimed to investigate the protective effect of autophagy on oxidative damage induced by melamine in Wistar rats. We hypothesized that autophagy could play an important role in significantly alleviating the impairment of synaptic plasticity and improving cognitive deficits induced by melamine through reducing ROS. This was done by establishing a rat model of melamine treatment and using rapamycin to regulate autophagy. Afterwards, the spatial learning and memory test were performed by the Morris water maze (MWM). Furthermore, both LTP and depotentiation from the hippocampal Schaffer collaterals to CA1 region were recorded. In order to explore the underlying mechanism, both immunofluorescence and Western blot assay were employed to detect if the autophagy level was significantly changed by melamine. Moreover, to determine whether or not upgrading autophagy could improve the impairments of cognitive functions and synaptic plasticity through decreased ROS level, both malondialdehyde (MDA) and total superoxide dismutase (T-SOD) measurements were performed to measure the intracellular ROS level.

Materials and Methods

Reagents

The experimental melamine (purity >99.5 %) was obtained from Yingda Sparse-ness & Nobel Reagent Chemical Factory, Tianjin, PR China. Rapamycin was purchased from Cell Signaling Technology. Melamine assay kit was bought from Huaan Magnech Bio-Tech Co., Ltd., Beijing, P.R. China. Superoxide dismutase (SOD) assay kit (A001-1) and MDA assay kit (A003-1) were purchased from the Nanking Jiancheng Bioengineering Research Institute (Nanking, China). Anti-LC3 IgG (M186) primary antibody was purchased from MBL. Anti-beclin-1ab (ab62577), anti-NR2B ab (ab65783), anti-PSD95 ab (ab18258), anti-synaptophysin (SYP) ab (ab14692), and caspase-3 (CASP3C) assay kit were obtained from Cell Signaling Technology.

Animals and Drugs Treatment

Three-week-old healthy male Wistar rats were purchased from the Beijing HFK Bioscience Co., LTD. Before performing experiments, rats were allowed 3 days of habituation and reared in groups of 4–5 under standard laboratory conditions (24 ± 2 °C room temperature, 12 h light/dark cycle with lights on at 7:00 a.m., and freedom to food and water) in the Medical School of Nankai University. All experiments were conducted during the light phase and performed according to the protocols approved by the Committee for Animal Care at Nankai University and in accordance with the practices outlined in the NIH Guide for the Care and Use of Laboratory Animals. Every effort has been made to minimize animal suffering and the number of animals.

After the habituation, rats were arbitrarily assigned into three groups, which were control group (CON, $n=6$), melamine group (MEL, $n=6$), and melamine + rapamycin group (MEL + RAP, $n=6$). The rats in the MEL group were gavaged with melamine solution (30 mg/mL, dissolved in 1 % carboxymethyl cellulose(CMC)) at a dose of 300 mg/kg/day given once a day for 4 weeks covering day 1 to day 28, while the animals in the CON group were received the same dose of 1 % CMC. In the MEL-RAP group, each rat was administered with melamine solution at a dose of 300 mg/kg/day for 28 consecutive days and intraperitoneal injected with rapamycin solution (1 mg/mL) at a dose of 1 mg/kg/day once a day covering day 1 to day 28.

Morris Water Maze Experiment

The Morris water maze (MWM) test was employed to assess the spatial learning and memory function 1 day after the last treatment. The experimental procedure was carried out and the details could be found in our previous studies [30–34]. The

MWM was a black circular stainless steel pool with a diameter of 150 cm, height of 60 cm, water depth of 45 cm, and temperature of 22 ± 2 °C. Black ink was used to render the water opaque. The maze was divided into four quadrants with two imaginary perpendicular lines crossing in the center of tank. The end of each line demarcates four cardinal points: north (N), south (S), east (E), and west (W). A platform (10 cm in diameter) was submerged 2 cm below the surface of the water and placed in the middle of the same quadrant throughout the training phase.

The MWM test includes four consecutive stages: initial training (IT), space exploring test (SET), reversal training (RT), and reversal exploring test (RET). During the IT (1–5 days) stage, the rats were subjected to four consecutive trials, which start location N, S, E, and W successively, and per trial with intervals of 5 min at least. In each trial, each rat was placed into the pool and permitted to search for the submerged platform for 60 s. If the rat failed to locate the platform within 60 s, it would be gently guided to the platform, and the escape latency was recorded as 60 s. The rat's movement was monitored by a charge-coupled device (CCD) camera connected to a personal computer, through which data were collected and analyzed (Ethovision 2.0, Noldus, Wageningen, the Netherlands). The mean escape latency of four trials was noted as the daily result of learning ability for the animal. On the 6th day of the test, the SET stage was performed using one trial without the platform after the last session of the IT stage at least 24 h later. After the platform was removed, the rats were released individually into the pool from one of the starting points and allowed to explore the pool for 60 s. The frequency with which each rat passed the hidden platform and the resident time that each rat spent in the target quadrant was noted as the result of the spatial memory function, namely platform crossings and time spent in target quadrant. In addition, the other two parameters of swimming speed and distance were also recorded. Finally, the RT stage was conducted for 3 days (7–9 days) in the same way and with the same parameters in the IT stage. The difference was that the platform was moved into the opposite quadrant in the center of the SE quadrant. For the RT stage, the approach and the parameters were the same as those in the SET stage.

In Vivo Electrophysiological Test

Synaptic plasticity between the hippocampal *Schaffer collaterals* and CA1 pyramidal neurons, including LTP and depotentiation, was assessed by in vivo electrophysiological techniques after the MWM test. The protocol was adopted and modified on the basis of our previous study [31]. The animals were anesthetized with 30 % urethane with a dosage of 4 mL/kg by intraperitoneal injection. After the anesthesia, they were placed in a stereotaxic frame (SN-3, Narishige, Japan) for surgery. An incision of about 2-cm long was made and a small

hole in the brain skull was drilled on the left side for both the recording and stimulating electrodes. According to Paxinos and Watson coordinates [31], the bipolar stimulating electrode was implanted into the hippocampus Schaffer collaterals region (4.2 mm posterior to the bregma, 3.5 mm lateral to midline, and 2.5 mm ventral below the dura), and the recording electrode was implanted into the hippocampus CA1 region (3.5 mm posterior to the bregma, 2.5 mm lateral to midline, and 2.0 mm ventral below the dura). Test stimuli were delivered to the Schaffer collaterals every 30 s at an intensity that evoked a response of 70 % of its maximum (range 0.3–0.5 mA). Once the response stabilized, sampling was made under low-frequency stimulations (0.05 Hz) for 20 min as the baseline. After the baseline, a theta burst stimulation (TBS) consisting of 30 trains of 12 pulses (200 Hz) at 5 Hz was applied to induce LTP. Following TBS, the single-pulse stimulation was resumed to sample the evoked response every 60 s for 1 h at the baseline intensity (Scope software, PowerLab; AD Instruments, New South Wales, Australia). The evoked responses of the last stabilized 20 min of LTP were normalized and used as the baseline of depotentiation. Subsequently, low-frequency stimulation (LFS) (900 pulses of 1 Hz for 15 min) was delivered to induce depotentiation. Following LFS, single-pulse recording resumed every 60 s for 1 h. Initial data measurement was performed in Clampfit 9.0 (Molecular Devices, Sunnyvale, CA, USA). The field excitatory postsynaptic potentials (fEPSPs) slope was used to measure synaptic efficacy.

Western Blot Assay

After electrophysiological test, each hippocampus was mashed with a grinder and 200 μ l lysis buffer (Beyotime Biotechnology, Haimen, China) containing a proteinase inhibitor cocktail (1:100 dilutions). Three repeated measurements were performed in each animal ($n=4$ for each group). The lysates were centrifuged at 12,000 r/min for 20 min at 4 °C. And then the protein concentration was determined using the BCA Protein Assay Kits according to the manufacturer's instructions (Beyotime Biotechnology, Haimen, China). The supernatant was mixed with loading buffer (ratio is 4:1) and boiled at 95–100 °C for 20 min.

The method of western blotting was modified on the basis of the previous studies [29, 35]. After the same amount of protein (50 μ g) was electrophoresed by SDS-PAGE 10 % gels, proteins were electro transferred onto 0.45- μ m polyvinylidene difluoride (PVDF) membranes (Millipore Corporation). Subsequently, the membrane was blocked in Tris-buffered saline (TBS) including 5 % skimmed milk for 1.5 h at room temperature and incubated with primary antibody (Anti-LC3, 1:1000 dilution, MBL, JPN; Anti-beclin-1, Abcam, UK; 1:1000 dilution, Anti-NR2B, 1:1000 dilution, Abcam, UK; PSD-95, 1:1000 dilution, Abcam, UK; Anti-

SYP, 1:1000 dilution, Abcam, UK) diluted in blocking buffer overnight at 4 °C. On the next day, the PVDF membranes were washed 3×10 min with Tris-buffered saline/Tween 20 (TBST) and subsequently incubated with secondary antibody (1:3000 dilution; Millipore Corporation, Billerica, MA, USA). After 4×10 min TBST washes, the membranes were used to detect the protein band intensities by using chemiluminescent HRP substrate. Finally, the quantitation analysis was performed by Photoshop 8.0.1 and compared to the loading control proteins β -actin (1:1000, Santa Cruz).

Measurement of Reactive Oxygen Species and Caspase-3

Hippocampus tissues were mashed with a grinder and 200 μ l lysis buffer (Beyotime Biotechnology, Haimen, China) containing a proteinase inhibitor cocktail (1:100 dilutions). The lysates were centrifuged at 12,000 r/min for 20 min at 4 °C. And then the protein concentration was determined using the BCA Protein Assay Kits according to the manufacturer's instructions (Beyotime Biotechnology, Haimen, China).

After exacting total protein, we performed the measurement of reactive oxygen species, including measurement of MDA and T-SOD. The levels of total MDA and superoxide dismutase T-SOD activity in the hippocampus were evaluated by the assay kits (Beyotime Biotechnology, Haimen, China). In addition, caspase-3 test was performed to measure the cell activity of hippocampus by the ELISA assay kits (Sigma Chemical Co., St Louis, MO).

Statistical Analysis

All data were presented as mean \pm SEM. A two-way repeated measure ANOVA was used to measure the time-course data, including escape latencies, distance, and mean swimming speed. Other data, obtained from the SET/RET stage, electrophysiological experiments and Western, were analyzed by a one-way ANOVA. To detect significant differences between groups, ANOVAs were supported by post hoc LSD test. All the analyses were performed using SPSS (17.0) software. Significant differences were taken when $p < 0.05$.

Result

Melamine Content in Hippocampus

The melamine concentrations of the hippocampus in the three groups were measured and the results are presented in Fig. 1. It showed that there was a statistical difference between the groups (Fig. 1, $F_{(2, 6)} = 150.868$, $p < 0.001$). And the level of melamine content was significantly higher in both the MEL and MEL + RAP groups than that in the CON group (Fig. 1, $p < 0.001$). No statistical difference of the melamine content

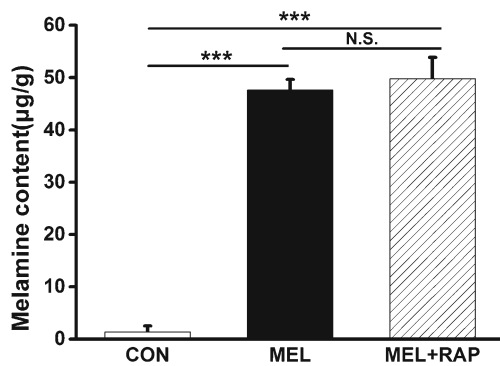


Fig. 1 Melamine content in the hippocampus. Data are presented as mean \pm SEM. *** $p < 0.001$ comparison between the CON group and the MEL group/MEL + RAP group; $n = 6$ in each group

level was found between the MEL group and the MEL + RAP group.

The Performance of Rats in MWM Experiment

In order to examine if the cognitive function of rats was affected after oral administration of melamine, and further assess whether or not the treatment of rapamycin could be used to retard the influence of melamine, MWM test was performed. The data, obtained from the MWM test in all groups on each test day, are presented in Figs. 2 and 3. It can be seen that the escape latencies are visibly decreased along with training during the IT stage (Fig. 2a). There were the statistical differences of day ($F_{(1,618, 24.675)} = 139.01$, $p < 0.001$), day \times group interaction ($F_{(3,237, 24.675)} = 1.558$, $p < 0.05$), and group ($F_{(2, 15)} = 8.047$, $p < 0.05$), as all three groups did improve over 5 days of training. And there were significant differences of the escape latencies between the MEL group and the CON group (Fig. 2a; $p < 0.01$ at the 2nd day, $p < 0.001$ at the 3rd day, $p < 0.01$ at the 4th day, and $p < 0.05$ at the 5th day), signifying that rats in the MEL group needed more time to find the platform compared to that in the CON group. Interestingly, the escape latencies were considerably reduced in the MEL + RAP group compared to that in the MEL group (Fig. 2a; $p < 0.01$ at the 4th day and $p < 0.05$ at the 5th day). In addition, there was no significant difference of swimming speed among these three groups (Fig. 2b, $p > 0.05$). Furthermore, it was found that there were more or less similar results for the swimming distance compared to that of the escape latencies (Fig. 2c).

The reference memory was examined in the SET stage. It was discovered that there were statistical differences of group in the platform crossings (Fig. 2e, $F_{(2, 15)} = 10.263$, $p < 0.05$) and the NW quadrant dwell time (Fig. 2d, $F_{(2, 15)} = 5.421$, $p < 0.05$). Post hoc LSD test showed that the NW quadrant dwell time (Fig. 2d, $p < 0.01$) and the platform crossings (Fig. 2e, $p < 0.001$) were significantly decreased in the MEL group compared to that in the CON group. Interestingly, the

above indicators were considerably increased by rapamycin treatment (Fig. 2d, $p < 0.05$; Fig. 2e, $p < 0.01$). There were no statistical differences of either the NW quadrant dwell time or the platform crossings between the CON group and the MEL + RAP group (Fig. 2d, e, $p > 0.05$).

With respect to the RT stage, the learning flexibility was then examined after the hidden platform was moved into the contralateral quadrant. During the RT stage, the mean escape latency was calculated for each rat on each of 3-day training (Fig. 3a). There were significant differences of day ($F_{(2, 30)} = 62.638$, $p < 0.001$), group ($F_{(2, 15)} = 33.829$, $p < 0.001$), and day \times group interaction ($F_{(4, 30)} = 10.280$, $p < 0.001$). There was a significant effect of rapamycin treatment, in which the escape latencies were significantly shortened in the MEL + RAP group compared to that in the MEL group. As shown in Fig. 3a, rats in the MEL group did show significantly prolonged escape latency compared with that in the CON group (Fig. 3a; $p < 0.001$ for the 1st and 2nd day; $p < 0.01$ for the 3rd day). The results showed that rapamycin-treated rats were able to utilize a spatial strategy to learn the position of the platform (comparison between the MEL group and the MEL + RAP group; Fig. 3a, $p < 0.001$ for the 1st and 2nd day; $p < 0.01$ for the 3rd day). In addition, there were no differences of swimming speed among these three groups throughout the test (Fig. 3b, $p > 0.05$). On the other hand, the reference memory was assessed, revealing that there were statistical differences of group in the SE quadrant dwell time (Fig. 3c, $F_{(2, 15)} = 3.790$, $p < 0.05$) and the platform crossings (Fig. 3d, $F_{(2, 15)} = 10.556$, $p < 0.001$). Post hoc LSD test showed that the quadrant dwell time (Fig. 3c, $p < 0.05$) and the platform crossings (Fig. 3d, $p < 0.001$) were significantly decreased in the MEL group compared to that in the CON group. In addition, the platform crossings in the MEL + RAP group were significantly increased compared to that in the MEL group (Fig. 3d, $p < 0.01$), but there was no statistical difference of the quadrant dwell time between these two groups. There were no statistical differences of either the NW quadrant dwell time or the platform crossings between the CON group and the MEL + RAP group (Fig. 3c, d, $p > 0.05$).

Long-Term Potentiation and Depotentiation from Schaffer Collaterals to CA1 Region

In the LTP test, a basal fEPSPs was evoked by the stimulation of *Schaffer collaterals* in the hippocampus CA1 area, and then theta burst stimulation (TBS) was delivered to induce LTP for 1 h. It can be seen that the fEPSP slopes are increased immediately after TBS, and then more or less stabilized to a level above the baseline period in all the three groups (Fig. 4a, left). The inset in Fig. 4a presents an example of fEPSPs at the baseline-TBS, LTP, and depotentiation of a rat in the CON group. The last 15 min data showed that there was a significant difference of group (Fig. 4b, $F_{(2, 15)} = 10.412$, $p < 0.001$). Moreover, the mean fEPSP slopes were much smaller in the

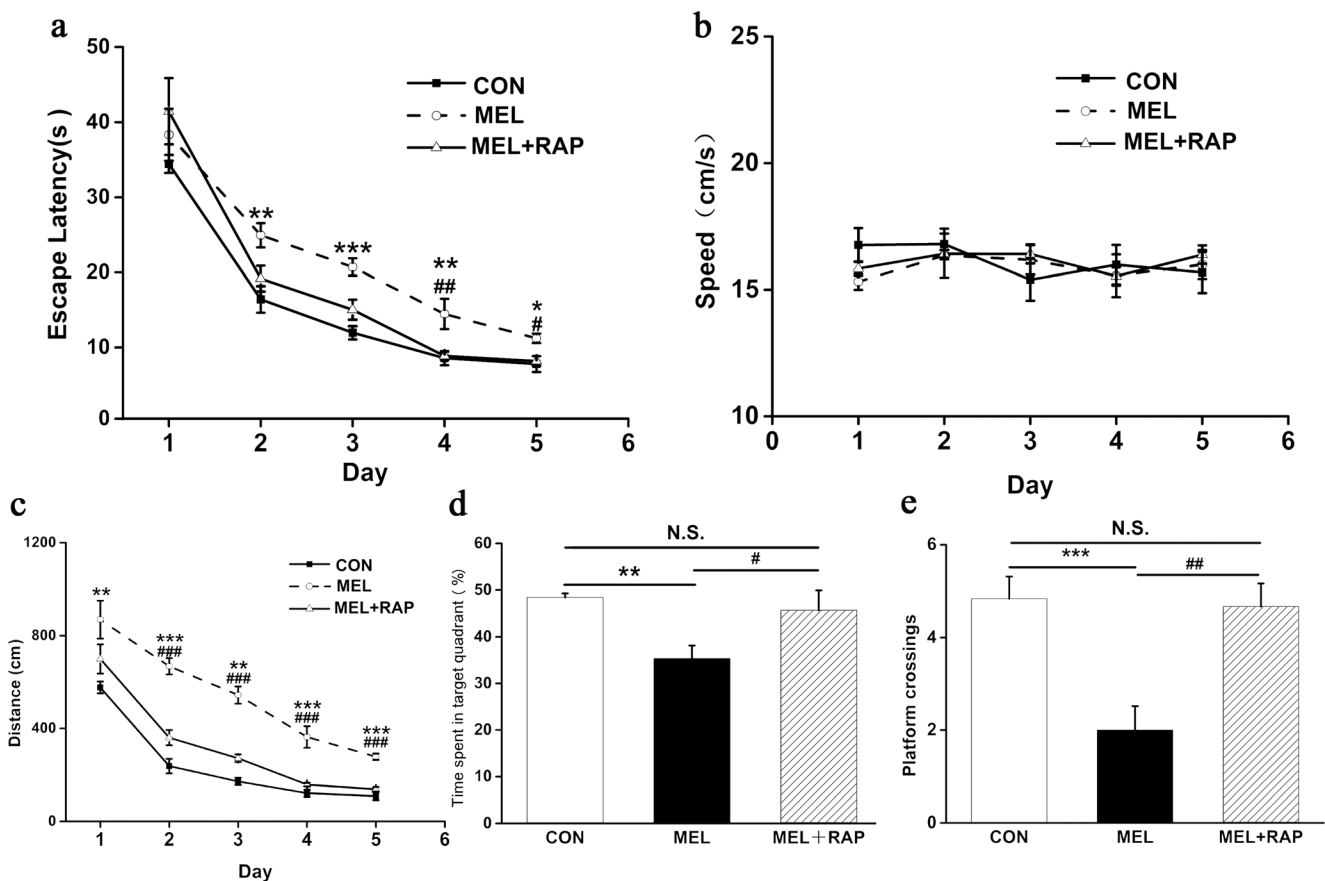


Fig. 2 The performance of rats in the IT & SET stages of MWM test. **a** Mean escape latency was determined for each day in the IT stage among the three groups. **b** Mean swimming speed in the IT stage. **c** Mean distance was measured for each day in the IT stage. **d** Mean percentage of time spent in target quadrant in the SET stage. **e** Mean number of

platform area crossings in the SET stage. Data are expressed as mean \pm SEM. No significant, $p > 0.05$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ comparison between the CON group and the MEL group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ comparison between the MEL group and the MEL + RAP group, $n = 6$ in each group

MEL group than that in the CON group (Fig. 4b, $p < 0.01$); however, they were significantly enhanced by rapamycin in the MEL + RAP group (Fig. 4b, $p < 0.01$). In order to assess if depotentiation, a form of LTP reversal, was effectively involved in the process, an LFS induction protocol was used for eliciting depotentiation (Fig. 4a, right). Since there have been obvious differences of fEPSP slopes between melamine-treated animals and normal ones before the LFS, the observed differences after stimulation cannot be employed to correctly evaluate the effect of melamine or rapamycin treatment on synaptic plasticity. Therefore, LTP-evoked responses in the last 20 min were normalized and used as the baseline of depotentiation (Fig. 4a, left). It is confirmed the statistical differences of group (Fig. 4c, $F_{(2, 15)} = 4.991$, $p < 0.05$). The depotentiation was significantly inhibited in the MEL group compared to that in the CON group (Fig. 4c, $p < 0.05$), but it was considerably strengthened by rapamycin in melamine-treated rats (Fig. 4c, $p < 0.05$). There were no statistical differences of the fEPSP slopes between the CON group and the MEL + RAP group (Fig. 4b, c, $p > 0.05$)

Rapamycin Enhances the Expression of NR2B, PSD-95, and SYP in the Hippocampus

To examine the effects of rapamycin on the expression of NR2B, PSD95, and SYP in melamine-treated rats, three prominent bands at about 180, 95, and 38 kDa were distinguished by NR2B, PSD-95, and SYP antibodies, respectively (Fig. 5a). The data showed that there were statistical differences of the protein levels of PSD-95, NR2B, and SYP among the three groups (NR2B— $F_{(2, 6)} = 19.286$, $p < 0.05$; PSD-95— $F_{(2, 6)} = 61.779$, $p < 0.001$; and SYP— $F_{(2, 6)} = 9.408$, $p < 0.05$). The level of NR2B expression was statistically decreased in the MEL group compared to that in the CON group (Fig. 5b, $p < 0.05$). However, it was found that the NR2B level in the MEL + RAP group was significantly enhanced by rapamycin compared to that in the MEL group (Fig. 5b, $p < 0.05$). There was no statistical difference of the NR2B level between the CON group and the MEL + RAP group (Fig. 5b, $p > 0.05$). Moreover, the expression of hippocampal PSD-95 was considerably reduced in the MEL group compared to that in the

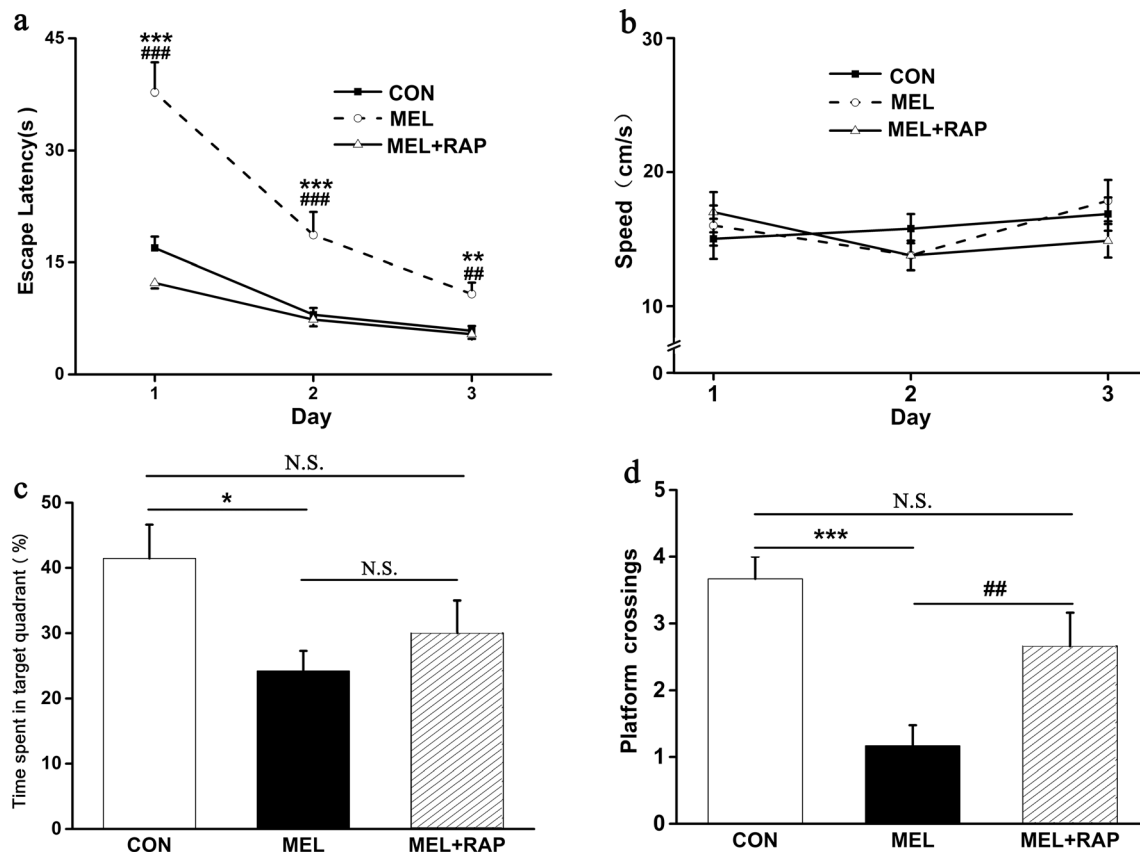


Fig. 3 The performance of rats in the RT & RET stages of MWM test. **a** Mean escape latency was calculated for each day in the RT stage among the three groups. **b** Mean swimming speed in the RT stage. **c** Mean percentage of time spent in new target (SE) quadrant in the RET stage. **d** Mean number of platform area crossings in the RET stage. Data

represent mean \pm SEM. Error bars indicate SEM. ** $p < 0.01$, *** $p < 0.001$ comparison between the CON group and the MEL group; ## $p < 0.01$, ### $p < 0.001$ comparison between the MEL group and the MEL + RAP group; $n = 6$ in each group

CON group (Fig. 5c, $p < 0.001$), while it was significantly enhanced by rapamycin in the MEL + RAP group compared to that in the MEL group (Fig. 5c, $p < 0.01$). The expression of hippocampal SYP was considerably reduced in either the MEL group or the MEL + RAP group compared to that in the CON group (Fig. 5d, $p < 0.05$). However, there is no significant difference of SYP level between the MEL group and the MEL + RAP group (Fig. 5d, $p > 0.05$).

Rapamycin Enhances the Level of Beclin-1 and Ratio of LC3-II/LC3-I

To assess the effect of rapamycin on neuronal autophagy in the melamine-treated rats, both LC3-II/LC3-I ratio and beclin-1 level were measured (Fig. 6a). There were significant differences of the level of Beclin-1 expression and the ratio of LC3-II/LC3-I among the three groups (Beclin-1— $F_{(2, 6)} = 28.063$, $p < 0.05$; ratio of LC3-II/LC3-I = $F_{(2, 6)} = 89.155$, $p < 0.001$). And the data confirmed that both Beclin-1 and the ratio of LC3-II/LC3-I were significantly increased in the MEL group compared to that in the CON group (Fig. 6b, c, $p < 0.05$), while they were further enhanced by rapamycin in the

MEL + RAP group compared to that in the MEL group (Fig. 6b, c, $p < 0.05$). There are also significant differences of either Beclin-1 (Fig. 6b, $p < 0.05$) or the ratio of LC3-II/LC3-I (Fig. 6c, $p < 0.05$) between the CON group and the MEL + RAP group.

ROS Levels Were Altered by Rapamycin in Melamine-Treated Rats

In order to examine if rapamycin effectively reduced the level of ROS in the hippocampal neurons in the melamine-treated rats, the levels of MDA and SOD were measured. The results showed that there were significant differences of T-SOD and MDA levels among the three groups in the hippocampus (Fig. 7, T-SOD; $F_{(2, 15)} = 105.526$, $p < 0.001$ and MDA; $F_{(2, 9)} = 19.626$, $p < 0.05$). The level of T-SOD was significantly lower in both the MEL group and the MEL + RAP group compared to that in the CON group (Fig. 7a, $p < 0.001$); however, it was significantly enhanced by rapamycin in the MEL + RAP group compared to that in the MEL group (Fig. 7a, $p < 0.001$). Furthermore, the level of MDA was significantly higher in the MEL group compared to that in the

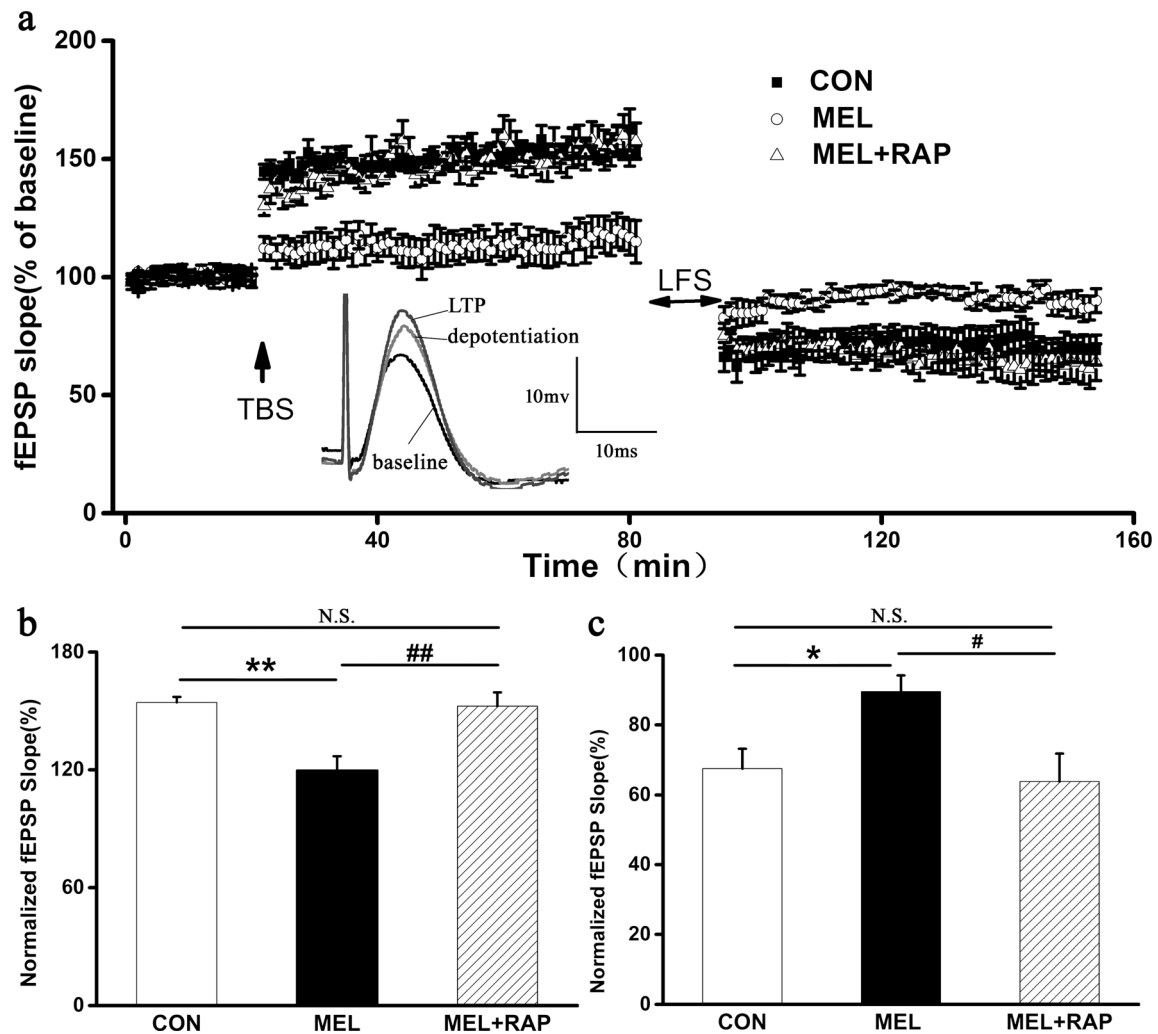


Fig. 4 The effects of melamine and rapamycin on the long-term potentiation and depotentiation from *Schaffer collaterals* to the hippocampal CA1. **a** The changes of time coursing in fEPSPs slopes in both LTP and depotentiation stages in the three groups. The first 20 min of evoked responses were normalized and used as the baseline responses of LTP. The last 15 min of evoked responses during LTP were normalized and used as the baseline responses of depotentiation which was induced by low frequency stimulation (LFS). The inset shows an example of

fEPSPs at baseline-TBS, LTP, and depotentiation. **b** Magnitude of LTP was determined as responses between 40 and 60 min after the TBS. **c** Magnitude of depotentiation was determined as responses between 40 and 60 min after LFS. Data represent mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ comparison between the CON group and the MEL group; # $p < 0.05$, ## $p < 0.01$ comparison between the MEL group and the MEL + RAP group; $n = 6$ in each group

CON group (Fig. 7b, $p < 0.001$), but it was significantly decreased by rapamycin in the MEL + RAP group compared to that in the MEL group (Fig. 7b, $p < 0.001$). There was no significant difference of MDA level between the CON group and the MEL + RAP group (Fig. 7b, $p > 0.05$).

Rapamycin Significantly Reduced Caspase-3 Activity in Melamine-Treated Rats

In order to test if rapamycin efficiently attenuated the hippocampal neuron apoptosis in melamine-treated rats, the caspase-3 activity was measured. A one-way ANOVA showed that the level of caspase-3 activity was significantly different among the three groups in the hippocampus (Fig. 8,

$F_{(2,9)} = 265.877$, $p < 0.001$). The cellular apoptosis was significantly increased in the MEL group compared to that in the CON group ($p < 0.001$, Fig. 8); however, it was dramatically attenuated in the MEL + RAP group compared to that in the MEL group ($p < 0.001$). There was a significant difference of caspase-3 activity between the CON group and the MEL + RAP group ($p < 0.05$).

Discussion

Several previous studies reported that melamine could cause the impairments of cognitive function and synaptic plasticity in rats, and the underlying mechanism was possibly associated

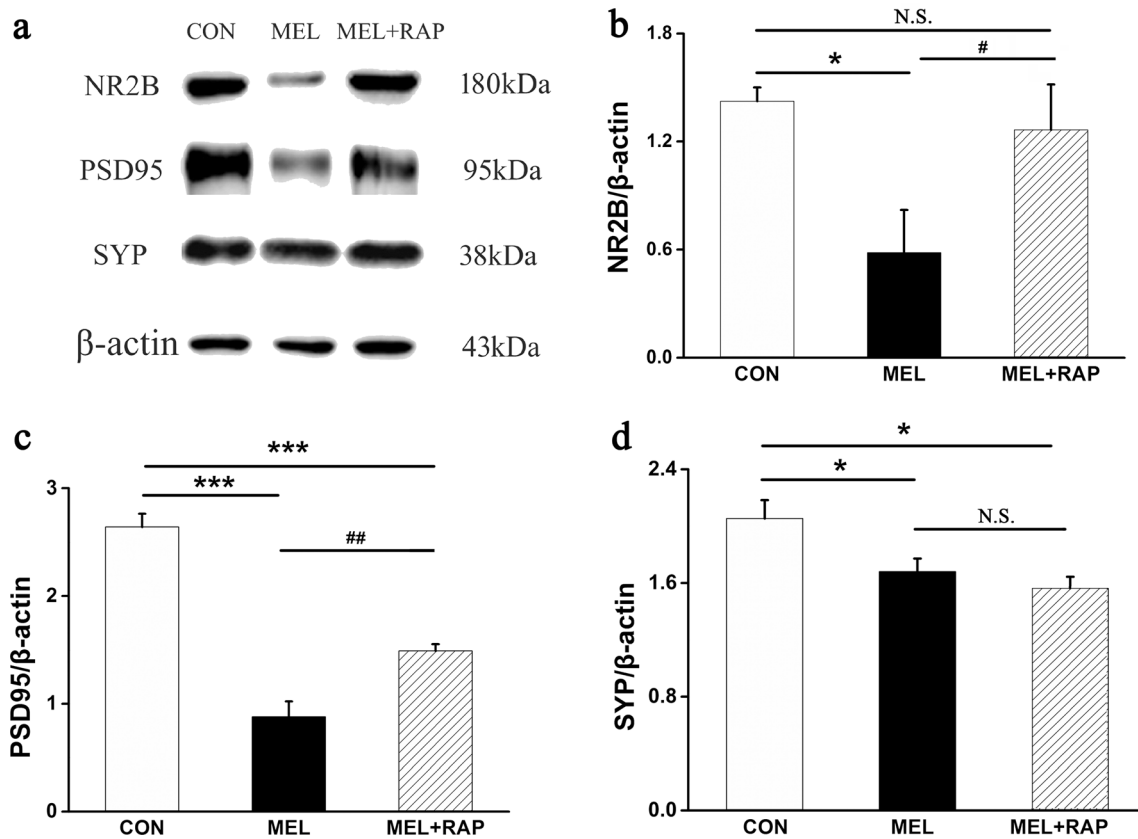


Fig. 5 Rapamycin enhances the expression of NR2B, PSD-95, and SYP in the hippocampus. **a** Results are immunoblots from single representative experiments. The expression values of NR2B (**b**), PSD-95 (**c**), and SYP (**d**) were normalized with β-actin value and then

compared to control. Data are presented as mean ± SEM. * $p < 0.05$ and *** $p < 0.001$ comparison between the CON group and the MEL group/MEL + RAP group; # $p < 0.05$, ## $p < 0.01$ comparison between the MEL group and the MEL + RAP group; $n = 4$ in each group

with oxidative damage [10, 26]. Moreover, the in vitro studies were further found that rapamycin, used as an autophagy activator, was able to alleviate melamine-induced cell death in PC12 cells via decreasing ROS level [29]. In order to investigate the relationship between the cognitive deficits induced by melamine and autophagy and further explore a potential targeted therapy to alleviate the damage caused by melamine, the in vivo experiments were performed in anesthetized rats in the present study. Accordingly, our investigation tried to verify the neuroprotective effect of rapamycin based on the in vivo animal experiments by using behavioral, electrophysiological, and molecular biological approaches. The results showed that rapamycin significantly relieved the impairments of cognitive function and hippocampal neuronal synaptic plasticity in melamine-treated rats. Interestingly, the cognitive improvement was closely associated with both the increase of synaptic protein levels and the decrease of intracellular ROS levels.

In order to clarify if activating autophagy may effectively alleviate the impairment of both spatial learning/memory and spatial reversal learning in melamine-treated rats, the MWM test has been performed [36]. In both the IT and SET stages, the performance was worse in the MEL group compared to

that in the CON group, which is in line with that of our previous studies [7, 31, 33]. Our data further suggest that up-regulation the autophagy level by giving rapamycin may efficiently impede the impairments of cognition induced by melamine, which was consistent with the findings of our previous cell study [29]. In addition, our results also suggested that up-regulating the level of autophagy by intraperitoneally injecting with rapamycin may efficiently improve the cognitive flexibility instead damaged the cognitive flexibility in melamine-treated rats.

As we know that a higher fEPSPs slope is associated with more effective synaptic transmission and better learning and memory in physiology. The LTP measurements in the present study were consistent with the performance of learning period, which was in line with our previous investigations [7, 33, 37]. Fascinatingly, the negative effect of melamine on LTP was remarkably impeded by rapamycin, suggesting that synaptic plasticity should be effectively enhanced in the hippocampus after up-regulating the autophagy level. Moreover, it is well known that the effect of depotentiation could be another important synaptic plasticity to remove memory information by selectively reversing experience-dependent plasticity [33, 38]. Our data show that depotentiation is significantly suppressed

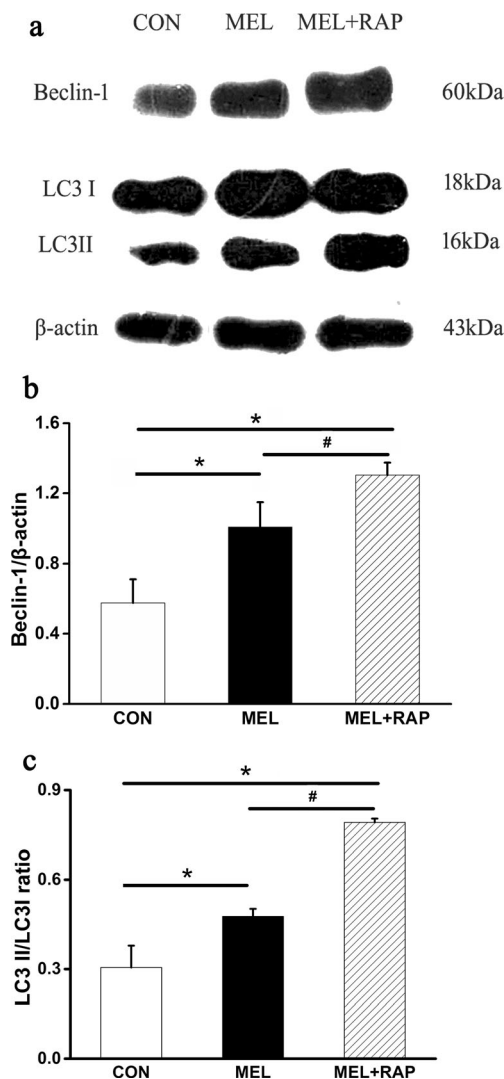


Fig. 6 Rapamycin enhances level of Beclin-1 and ratio of LC3-II/LC3-I. **a** Results are immunoblots from single representative experiments of LC3 and Beclin-1. **b** LC3-II/LC3-I band density ratio was measured in the three groups. **c** Beclin-1/β-actin band density ratio was measured in the three groups. Data are presented as mean ± SEM. * $p < 0.05$; comparison between the CON group and the MEL group/MEL + RAP group; # $p < 0.05$ comparison between the MEL group and the MEL + RAP group; $n = 4$ in each group

in melamine-treated rats, suggesting that the resilience of synaptic structures is seriously damaged. Importantly, the impairment of synaptic plasticity was greatly impeded by rapamycin intervention, which was associated with the performance of rats in reversal learning of MWM.

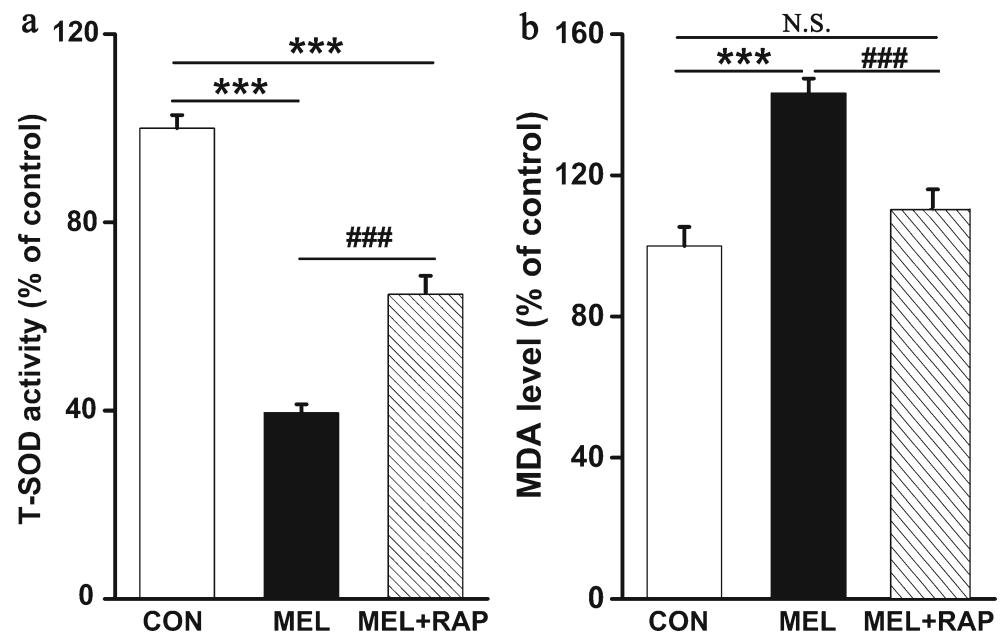
Synaptic dysfunction is a strong correlation of cognitive deficits and synaptic plasticity impairments. The NMDA receptor, as a function subunit, plays a key role in LTP induction. Additionally, the GluN2B subunit-containing NMDA receptor is essential to induce depotentiation by LFS [39]. Moreover, synaptophysin and PSD95 are two important pre-synaptic and postsynaptic structural proteins, respectively, which are closely connected with synaptic plasticity and

cognitive process [32]. Accordingly, the expression of NR2B subunit in the hippocampus was carefully measured in the present study. Our data suggested that there are lower expressions of NR2B and PSD-95 proteins in the melamine-treated animal than that in normal one. Interestingly, both of them are significantly enhanced by rapamycin, suggesting that up-regulating the autophagy level may impede the impairment of synaptic plasticity via increasing the expression of NR2B and PSD-95 proteins. The induction and maintenance of glutamate receptor-dependent plasticity are able to be conserved [40]. The effect of rapamycin on improving synaptic plasticity and resilience may be due to avoiding the change of these two proteins expression in the hippocampus.

In order to detect the effects of both melamine- and rapamycin-induced autophagy in rats, Western blot has been performed to evaluate the levels of autophagy. As we all know that LC3 is post-translationally modified into the LC3-I form, however, it conjugates to phosphatidylethanolamine upon induction of autophagy to form the autophagosome-associated LC3-II form [41]. Beclin-1, as a critical protein involved in the initiation of autophagy, regulates the autophagic pathways including autophagosome formation and maturation [42]. In the present study, the ratio of LC3-II/LC3-I and the level of beclin-1 were significantly enhanced in the MEL + RAP group compared to that in the MEL group, suggesting that rapamycin could activated autophagy in the hippocampus in melamine-treated rats.

Rapamycin was found to possess immunosuppressive and anti-proliferative properties in mammalian cells [17]. Rapamycin-based therapy has shown benefits for patients with renal cancer carcinoma (RCC), tuberous sclerosis complex (TSC), and lymphangioleiomyomatosis (LAM)-related tumors [43–45]. Moreover, rapamycin treatment could reverse learning deficits and enhance late-phase long-term potentiation in TSC2^{+/-} mice [46]. Consequently, it is considered as a potential treatment for nerve injury. In addition, a significant progress has been made in understanding rapamycin regarding as a specific inhibitor of mTOR over the past two decades. A previous study showed that the selective suppression of mTOR rescued memory deficits and enhanced the expression of PSD-95 in the brain of AD mice [47]. Down-regulation of mTOR signaling decreases huntingtin-accumulating neurons in a mouse model of Huntington's disease (HD) [48]. Autophagy is a regulated process of the cellular degradation pathway for the redundant and damaged cytosolic components and organelles, which are associated with a variety of causes of the hippocampal neuronal injury [49, 50]. In addition, ischemic injury attenuates in the hippocampal CA1 neurons through induction of Akt-independent autophagy [51]. On the other hand, autophagy defect may cause the impairments of neural differentiation, axon growth, or neuronal signaling during early neurodevelopment or synaptic activity, resulting in severe synaptic dysfunction. The absence of Atg5 and Atg7

Fig. 7 Measurement of ROS generation in the hippocampal neurons in the three groups. T-SOD activity and MDA level were measured to value ROS of the hippocampus by SOD and MDA kit. Data are presented as mean \pm SEM. *** p < 0.001 comparison between the CON group and the MEL group/MEL + RAP group; ### p < 0.001 comparison between the MEL group and the MEL + RAP group; n = 4 in each group



caused cognitive deficits and neuron loss in mice [52]. As we known that rapamycin could activate autophagy via inhibiting mTOR [12, 13]. There is a protective effect of rapamycin on transgenic animal models of HD [53]. As we known that autophagy is an initial degradation pathway for unfolded or misfolded proteins. Therefore, the levels of these mutant proteins are elevated if autophagy is impaired. Conversely, motivating autophagy would reduce their levels [54]. Additionally, it was reported that the elimination of impaired mitochondria was depend on ubiquitination, suggesting that autophagy could be defective organelles in the post-mitotic neurons [55]. Therefore, enhancing autophagy may be employed as a possible therapeutic strategy in neurodegenerative diseases where the mutant proteins are autophagy substrates [56].

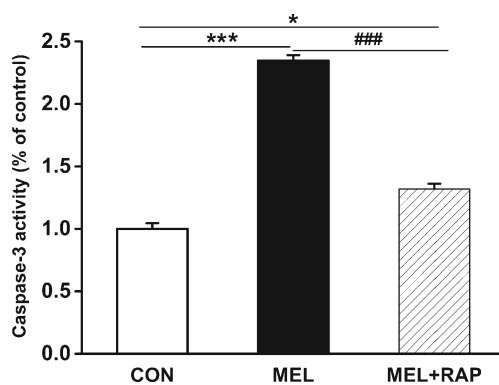


Fig. 8 The effects of rapamycin and melamine on cell viability in the hippocampus. Caspase-3 was measured to value viability of hippocampus by caspase-3 kit. Data are presented as mean \pm SEM. *** p < 0.001 comparison between the CON group and the MEL group; ### p < 0.001 comparison between the MEL group and the MEL + RAP group; n = 4 in each group

A previous in vivo study showed that the chronic hippocampal melamine exposure induced oxidative stress, which was caused by abnormally production and scavenging of ROS and apoptosis [26]. Superoxide dismutase catalyzes transform the superoxide anion to hydrogen peroxide and oxygen molecule [57, 58]. The increasing ROS level in the hippocampus was attributed to the decrease of endogenous antioxidants, which were antioxidants directly or indirectly against superoxide anion radical and hydroxyl radicals [26]. Besides, free radicals attack cellular membranes and result in the formation of the lipid peroxidation byproduct, which is the classic result of oxidative damage [46]. Our data showed that the ROS level was remarkably enhanced by melamine; however, rapamycin significantly decreased it, suggesting that upgrading autophagy was of benefit to the melamine treated rats through decreasing ROS level. Certainly, it is not surprising that there is a significant crosstalk between autophagy and oxidative stress. Lysosomes play a protective role in the early stage of oxidative damage or during mild oxidation [59, 60]. A recent study demonstrated that autophagy was directly related to oxidative stress, involved in some neuronal damage and neurodegenerative disease [61]. The activity of autophagy could protect against H₂O₂-induced apoptosis in PC12 cells [62]. Moreover, it could alleviate irradiation injury through decreasing generation of ROS in mesenchymal stem cells [61]. Alternatively, caspase-3 is one of the most significant members of the caspase family. It drives cells toward apoptosis through a chain of events, called caspase cascade [62, 63]. Several previous studies reported that melamine induced apoptosis in either rat's hippocampus or PC12 cells [2, 34]. Our data showed that there was an increase in caspase-3 in the MEL group. However, it was significantly reduced by rapamycin in the MEL + RAP group, suggesting that

rapamycin might be to inhibit melamine-induced apoptosis to some extent. Nevertheless, the interrelationship between autophagy pathway and apoptotic pathway is extremely complex in the setting of brain pathologies [2].

Furthermore, there are evidences that rapamycin significantly impedes melamine-induced both cognition deficits and synaptic plasticity impairments in the study, which is associated with autophagy pathway. However, it cannot exclude the effect of rapamycin on other signal pathways. It reported that rapamycin not only inhibited mTOR activity but also reduced mTOR phosphorylation, which regulated local protein synthesis during long-term synaptic plasticity [64]. Obviously, with the purpose of examining how autophagy relieves the melamine-induced cognitive impairments, we still need to perform experiments with either mTOR-independent inhibitor including lithium and carbamazepine or autophagic inhibitor such as Wortmannin, 3-MA and chloroquine.

Conclusion

In summary, our in vivo study shows that melamine is able to enhance autophagy in Wistar rats. The data further indicate that the impairments of learning and memory as well as neuronal synaptic plasticity are induced by melamine, which associates with the increase of oxidative stress. However, the above damages are significantly impeded by rapamycin along with inhibiting the excessive generation of ROS and feasibly reducing melamine-induced apoptosis. In conclusion, the results suggest that regulating autophagy may become a new targeted therapy to alleviate melamine-induced neural injury.

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Authors' Contributions JF, HW, and TZ conceived and designed the experiment, JF, HW, JG, MY, RW, and ZY performed the experiments and analyzed the data; and JF and TZ wrote the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they no competing interests.

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