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ORIGINAL ARTICLE

The Neuroprotective Effect of L-Stepholidine on Methamphetamine-Induced Memory Deficits in Mice

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Abstract

Repeated methamphetamine (METH) exposure can cause severe neurotoxicity to the central nervous system, and lead to memory deficits. L-Stepholidine (L-SPD) is a structurally identified alkaloid extract of the Chinese herb *Stephania intermedia*, which elicits dopamine (DA) D1-type receptors partial agonistic activity and D2-type receptors antagonistic activity. In this study, we investigated the effect of L-SPD on METH-induced memory deficits in mice and its underlying mechanisms. We found that repeated exposure to METH (10 mg/kg, i.p., once per day for 7 consecutive days) impaired memory functions in the novel object recognition experiment. Pretreatment of L-SPD (10 mg/kg, i.p.) significantly improved METH-induced memory deficits in mice. Meanwhile, the protein expression of dopaminergic D2 receptors in hippocampus area was significantly increased by repeated METH exposure, while the protein expression of dopamine transporter (DAT) was significantly reduced. Additionally, the protein expression of phospho-protein kinase A (p-PKA) was significantly increased by repeated METH exposure. The hyperpolarization-activated cyclic-nucleotide-gated non-selective cation 1 (HCN1) channel, which was a key regulator of memory functions and could be regulated by p-PKA, was also significantly increased by repeated METH exposure. These changes caused by METH could be prevented by L-SPD pretreatment. Therefore, our data firstly showed that pretreatment of L-SPD exhibited the protective effect against METH-induced memory deficits, possibly through reducing METH-induced upregulation of dopaminergic pathway and HCN1 channels.

Keywords L-Stepholidine · Methamphetamine · Memory deficits · Hyperpolarization-activated cyclic-nucleotide-gated non-selective cation channels · Hippocampus

Introduction

Methamphetamine (METH) belongs to phenethylamine and amphetamine class of psychoactive drugs, which is a worldwide used addictive psychostimulant with a strong action on the central nervous system. Repeated METH exposure not only causes addiction and relapse behavior, but also causes severe neurotoxicity (Rusyniak 2013). One of the neurotoxic effects induced by METH on the brain is memory deficits (Jablonski et al. 2016). Several studies have documented the deficit in memory performance by individuals who have a history of chronic METH abuse (Cadet et al. 2014; Cadet and Bisagno 2015). A better understanding of the molecular mechanism of memory impairment induced by METH will provide effective treatment strategies for curing METH abuse.

Ample studies found that METH could produce abnormal changes in a variety of neurotransmitters, such as the overflow of dopamine (DA), which could cause memory deficits (Nordahl et al. 2003). METH exposure generated persistent effects on the dopaminergic system, including DA release, reuptake, metabolites, and transporters (Moszczynska and Callan 2017; Anneken et al. 2018). A recent study demonstrated that METH exposure led to an excessive release of DA in prefrontal cortex, which caused the activation of neuronal apoptosis pathway, and finally induced damage to memory functions (Long et al. 2017). Therefore, the regulation of the dopaminergic system might improve the memory deficit induced by METH.

L-Stepholidine (L-SPD), a structurally identified alkaloid isolated from the Chinese herb *Stephania intermedia*, which elicits dopaminergic D1-type receptor partial agonistic and

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D2-type receptor antagonistic activities (Natesan et al. 2008), may have potential therapeutic effect on METH-induced memory deficits. Several studies showed that L-SPD displayed a protective effect on addictive behaviors in animal models. L-SPD could inhibit the acquisition, maintenance, and re-acquisition of morphine-induced conditioned place preference (Wang et al. 2007). L-SPD could also attenuate heroin- or METH-induced self-administration behavior (Yue et al. 2014a, b). Meanwhile, L-SPD dose dependently inhibited the hyperlocomotion induced by acute METH exposure and prevented the locomotor sensitization induced by repeating METH exposure (Ma et al. 2014). A recent study demonstrated that L-SPD rescued memory deficits and synaptic plasticity in animal models of Alzheimer's disease via the dopaminergic pathway (Hao et al. 2015). However, little is known about the effect of L-SPD on METH-induced memory deficits. Based on these findings, the present study was aimed to investigate whether L-SPD could improve memory deficits caused by repeated METH exposure and investigate its underlying molecular mechanism.

Materials and Methods

Animals

Male C57BL/6 mice (Laboratory Animal Center, Beijing, China) weighing 20–25 g were housed five per cage with a 12-h light/dark cycle, temperature-controlled environment, and free access to water and food. Animal use procedures were carried out in accordance with the approval of the Ethics Committee in our university. All efforts were made to minimize both the suffering and number of animals used.

Drugs

METH was obtained from Hubei Public Security Bureau (China) and dissolved in 0.9% saline solution (Sal) to a final concentration of 1 mg/ml. L-SPD was provided by the Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, and was dissolved in dimethyl sulfoxide (DMSO) vehicle (Vel, 10%) to a final concentration of 1 mg/ml. METH and L-SPD were administered intraperitoneally (i.p.) at a concentration of 10 mg/kg. The symbol (+) METH or L-SPD in Figs. 5 and 6 indicated that the animals of this group were injected with METH or L-SPD. The symbol (−) indicated that the animals of this group were injected with saline or vehicle.

Experimental Schedule

The experimental procedure was detailed in Fig. 1. Mice were randomly divided into four groups: Vel/Sal, Vel/METH, L-SPD/METH, and L-SPD/Sal. According to the reference (Chen et al. 2012), METH (10 mg/kg, i.p.) was administrated once per day for 7 consecutive days. To study the therapeutic effect of L-SPD, 10 mg/kg L-SPD (i.p.) was chosen according to the reference and administrated 1 h before METH injection (Hao et al. 2015). Behavioral experiments, immunofluorescence staining and western blotting experiment were carried out as described below.

Novel Object Recognition Experiment

Novel objection experiment (NOR) experiment was performed as previously described with slight modification (Long et al. 2017). Briefly, the NOR instrument was a Plexiglas opening box (40 × 40 × 40 cm) with a tracking system XR-XX117 (Shanghai Xinruan Information Technology Co., Ltd. China). The NOR experiment contained three parts: habituation, training, and testing. Twenty-four hours after the last METH or saline injection, the habituation sessions were carried out as described in Fig. 1. During the habituation session, mice were allowed to explore freely in NOR box without objects for 10 min to habituate the environment. Twenty-four hours after the habituation session, two objects were symmetrically placed on the floor of NOR box, and mice were put in the box to explore two objects freely for 5 min (training session). Four hours after the training session, one of the familiar objects was replaced by a novel object, and mice were put back in the box to explore two objects freely for 5 min (testing session). When the distance between mouse head and the object was less than or equal to 2 cm, mice were considered to be exploring the object. The time spent exploring the familiar (F) and the novel (N) object was recorded separately. The discrimination index was calculated ($N-F/N+F$) in the testing session.

Locomotor Activity Experiment

The experimental apparatus consisted of a Plexiglas chamber (40 × 40 × 35 cm) with white floor (YH2018RLA16, Wuhan Yihong Technology Co., Ltd. China). Forty-eight hours after the last METH or saline injection, the locomotor activity experiment was carried out as described in Fig. 1. The speed and total distance of mice movements during 10 min period were recorded by a computer-based image analyzer, in order to investigate the effect of METH and L-SPD exposure on the locomotor activity of mice.

Immunofluorescence Staining

After the last NOR test, mice were immediately anesthetized by pentobarbital sodium and transcardially perfused with 0.9% NaCl solution, followed by 4% paraformaldehyde fixative. Then the brain was removed and sectioned coronally at

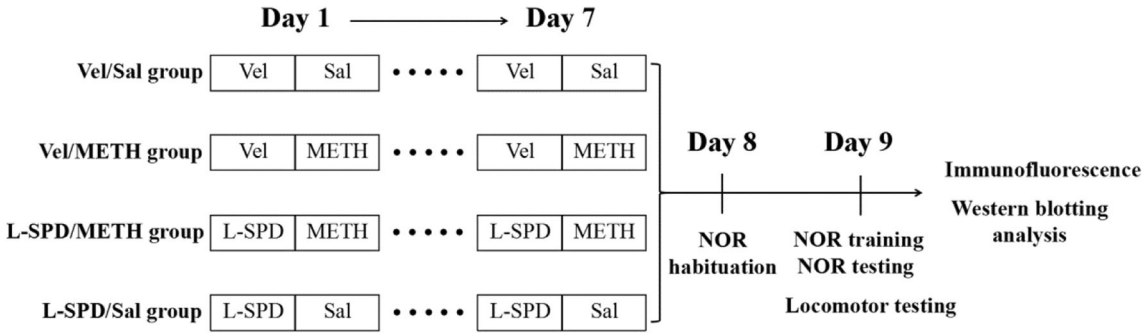

Fig. 1 Experimental procedure. METH (10 mg/kg, i.p.) was administrated once per day for 7 consecutive days. L-SPD (10 mg/kg, i.p.) was administrated 1 h before METH injection. Twenty-four hours after the last METH or saline injection, the habituation session of novel object recognition (NOR) experiment was carried out. Twenty-four hours after the habituation session, the training and testing sessions of NOR experiment were performed. Meanwhile, the locomotor activity experiment was carried out 48 h after the last METH or saline injection. After the last NOR test, immunofluorescence staining and western blotting experiments were performed

10 μm by a freezing microtome (Leica CM1860). Sections of the hippocampus were incubated in citric acid buffer for 20 min at 95 °C. After washed in phosphate buffer, sections were then incubated with 5% BSA at room temperature for 1 h. The sections were sequentially incubated with the NeuN primary antibody solutions (1:500, 12943S, Cell Signaling Technology) overnight at 4 °C. After washed in phosphate buffer, sections were then transferred into the secondary antibodies Alexa Fluor 555 Conjugate Goat Anti-Rabbit IgG (H + L) (4413S, Cell Signaling Technology) at room temperature for 1 h. After bleached with phosphate buffer, sections were analyzed with Olympus BX51 FluoView microscope system (Olympus Corporation, Japan). The immunoreactive signal of NeuN was quantified by NIH ImageJ software.

Western Blotting Experiment

After the last NOR test, brains were immediately quickly removed after anesthetized by pentobarbital sodium and the hippocampus (HIP) tissue was dissected in cold artificial cerebrospinal fluid. The tissue was homogenized in protein extraction buffer containing protease and phosphatase inhibitors. Protein concentration was measured using a Protein Assay kit (Boster, China). Proteins were separated on 10% SDS-PAGE gels and transferred to PVDF membranes (Merck Millipore). After being blocked in 5% milk for 1 h at room temperature, membranes were incubated overnight at 4 °C with the following primary antibodies: anti-D1R (dopamine receptor, 1:10000, Absin), anti-D2R (1:500, Absin), anti-DAT (dopamine transporter, 1:500, Santa Cruz), anti-HCN1 (hyperpolarization-activated cyclic-nucleotide-gated non-selective cation channels, 1:1000, Novus), anti-HCN2 (1:1000, Alomone), anti-PKA (protein kinase A, 1:1000, Cell Signaling Technology), anti-Phospho-PKA (Thr197) (1:500, Cell Signaling Technology), or GAPDH (1:5000, Cwbiotech). After incubation with horseradish peroxidase conjugated secondary antibodies (1:3000; Proteintech Group Inc), bands were incubated with chemiluminescent substrate (Thermo Fisher). Immunoreactive signals were quantified by NIH ImageJ software. The band intensities were corrected to GAPDH intensity and then set as relative to the Vel/Sal group.

Statistical Analysis

Data were presented as means ± SEM and analyzed with one-way ANOVA test. Tukey was applied as a post-hoc test to examine the significance of the data. *P* values less than 0.05 were considered statistically significant.

Results

Neuroprotective Effect of L-SPD on METH-Induced Memory Deficits

To investigate the potential protective effect of L-SPD against memory deficits induced by repeated METH exposure, the NOR experiment was performed as shown in Fig. 2a. During the testing session, saline-treated mice showed the exploratory preference to the novel object. Compared with saline-treated mice, the discrimination index of METH treated mice was significantly reduced (Fig. 2b, $n = 10$, $F(3, 36) = 4.318$, $P = 0.030$), suggesting repeated METH exposure (10 mg/kg, i.p., once per day for 7 consecutive days) impaired the memory function. Pretreatment with L-SPD (10 mg/kg, i.p., 1 h before METH injection) significantly ameliorated METH-induced reduction of discrimination index (Fig. 2b, $n = 10$, $P = 0.021$, vs. Vel/METH group), and did not affect the memory function of normal mice. These findings indicated that pretreatment of L-SPD produced a protective effect against METH-induced memory deficits.

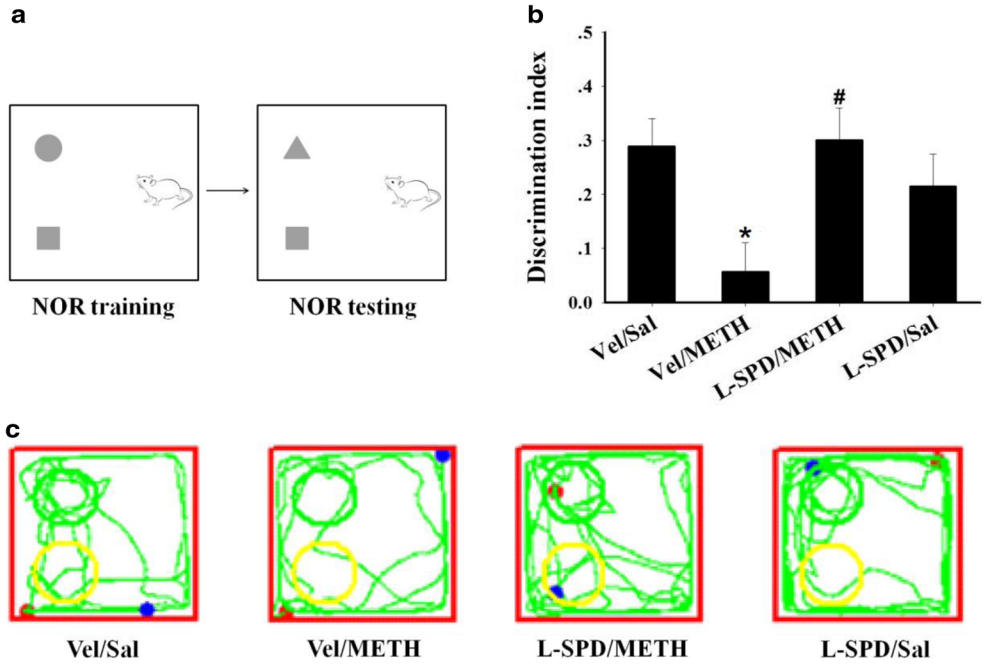

Fig. 2 Effects of L-SPD on METH-induced memory deficits in mice. **a** Pattern diagrams of NOR training and testing. **b** Discrimination index to explore a novel object on NOR testing. Compared with saline treated mice, the discrimination index of METH-treated mice was significantly reduced, suggesting that repeated METH exposure impaired memory functions. L-SPD pretreatment significantly ameliorated METH-induced reduction of the discrimination index. **c** Representative graphs of trajectories during the NOR testing. The green circle represented a circle marking where the new object was. The yellow circle represented a circle marking where the familiar object was. Meanwhile, the different colored dots represented the starting and ending points of the animal. Data were presented as means ± SEM. $n = 10$ for per group, $^{*}P < 0.05$ vs. Vel/Sal group, $^{\#}P < 0.05$ vs. Vel/METH group

Locomotor Activity of Mice with METH Exposure and L-SPD Treatment

To investigate the effect of METH and L-SPD exposure on the locomotion of mice, the locomotor activity experiment was performed as shown in Fig. 3. As shown in Fig. 3a, the speed among the different treatment groups exhibited no significant differences ($n = 8$, $F\ (3,\ 28) = 0.796$, $P > 0.05$). Meanwhile, the total distance of movements among the different treatment groups also exhibited no significant differences (Fig. 3b, $n = 8$, $F\ (3,\ 28) = 1.087$, $P > 0.05$), which suggested that METH and L-SPD exposure did not affect the locomotor activity of mice.

NeuN Expression in HIP Area of Mice with METH Exposure and L-SPD Treatment

Changes of neurons in HIP area play an important role in the memory function. NeuN, a neuron-specific marker protein, was evaluated to explore the mechanism underlying the protective effect of L-SPD on METH-induced memory deficits. There

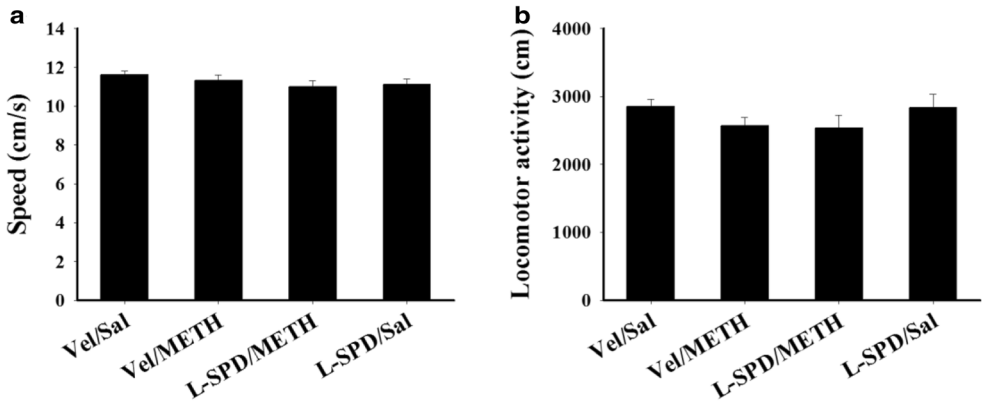

Fig. 3 Effects of repeated METH exposure or L-SPD pretreatment on the locomotor activity of mice. **a** Speeds in the locomotor activity test. **b** Total distance of movements in locomotor activity test. The speed and total distance of movements among the different treatment groups exhibited no significant differences, which suggested that METH and L-SPD exposure did not affect the locomotor activity of mice. Data were presented as means ± SEM. $n = 8$ for per group, $P > 0.05$ vs. Vel/Sal group, $P > 0.05$ vs. Vel/METH group

was no significant difference in the expression of NeuN in HIP area of mice with saline or repeated METH exposure (Fig. 4a, b $n = 4$, $F(3, 12) = 0.628$, $P = 0.938$, vs. saline group). Compared with METH-treated mice or saline-treated mice, the expression of NeuN in HIP area of mice with L-SPD treatment also exhibited no changes (Fig. 4a, b $n = 4$, $P = 0.859$).

L-SPD Prevented METH-Induced Changes of DA Receptors and DAT Protein Expressions

DA neurotransmitter system plays an important role in drug addiction, which also is closely associated with memory function (Berke and Hyman 2000). In this study, we found that the protein level of dopaminergic D2 receptors in HIP area was significantly increased by repeated METH exposure (Fig. 5c, $n = 4$, $F(3, 12) = 10.368$, $P = 0.011$, vs. Vel/Sal group), while the protein expression of D1 receptors remained unchanged in comparison to control group (Fig. 5b, $n = 4$, $F(3, 12) = 0.235$, $P = 0.867$). Meanwhile, the protein expression of DAT in HIP area was significantly reduced by repeated METH exposure (Fig. 5d, $n = 4$, $F(3, 12) = 5.399$, $P = 0.039$, vs. Vel/Sal group). Furthermore, pretreatment with 10 mg/kg L-SPD could prevent METH-induced changes of D2 receptors and DAT protein expressions in HIP area (Fig. 5c, d, $n = 4$, D2R: $P = 0.041$, DAT: $P = 0.015$, vs. Vel/METH group).

L-SPD Prevented METH-Induced Changes of p-PKA and HCN Channels Protein Expressions

p-PKA is an important intracellular signaling molecule for DA receptors, which also plays a key role in the regulation of HCN channels (St Clair et al. 2013). As shown in Fig. 6a and b, the protein level of p-PKA was markedly increased in HIP area of mice with repeated METH exposure ($n = 4$, $F(3, 12) = 7.515$, $P = 0.016$, vs. Vel/Sal group), while the protein expression of PKA showed no significant difference among the different treatment groups. The protein expression of HCN1 channels in HIP area was also increased by repeated METH exposure (Fig. 6c, $n = 4$, $F(3, 12) = 3.489$, $P = 0.039$, vs. Vel/Sal group), while no significant difference was

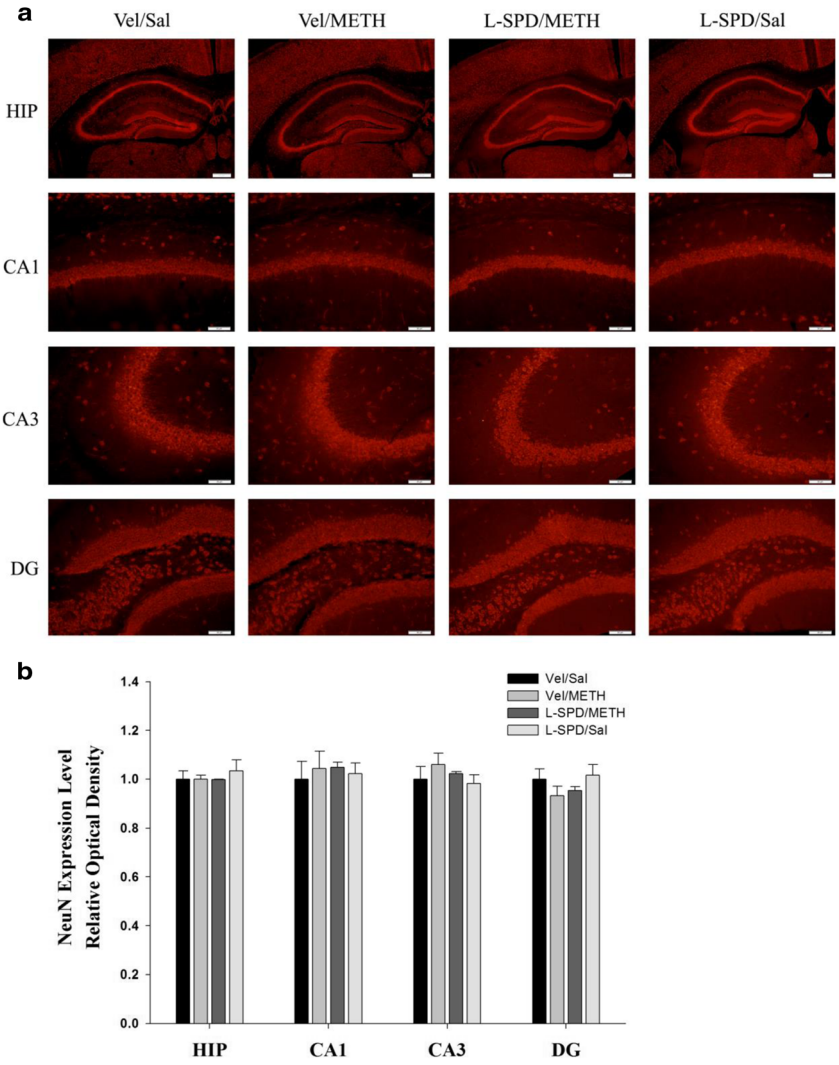

Fig. 4 Effects of repeated METH exposure or L-SPD pretreatment on the neuronal density in HIP area of mice. **a** Representative photomicrographs of Immunofluorescence staining with an anti-NeuN antibody in HIP area (× 40, scale bar, 200 μm). Meanwhile, the photomicrographs of NeuN in HIP area were magnified, including CA1 (× 200, scale bar, 50 μm), CA3 (× 200, scale bar, 50 μm), and DG (× 200, scale bar, 50 μm) areas. **b** Quantitative analysis of NeuN staining. The expression levels of NeuN among the different treatment groups showed no significant difference in HIP area, CA1 area, CA3 area, and DG area. Data were presented as means ± SEM. $n = 4$ for per group, $P > 0.05$ vs. Vel/Sal group, $P > 0.05$ vs. Vel/METH group

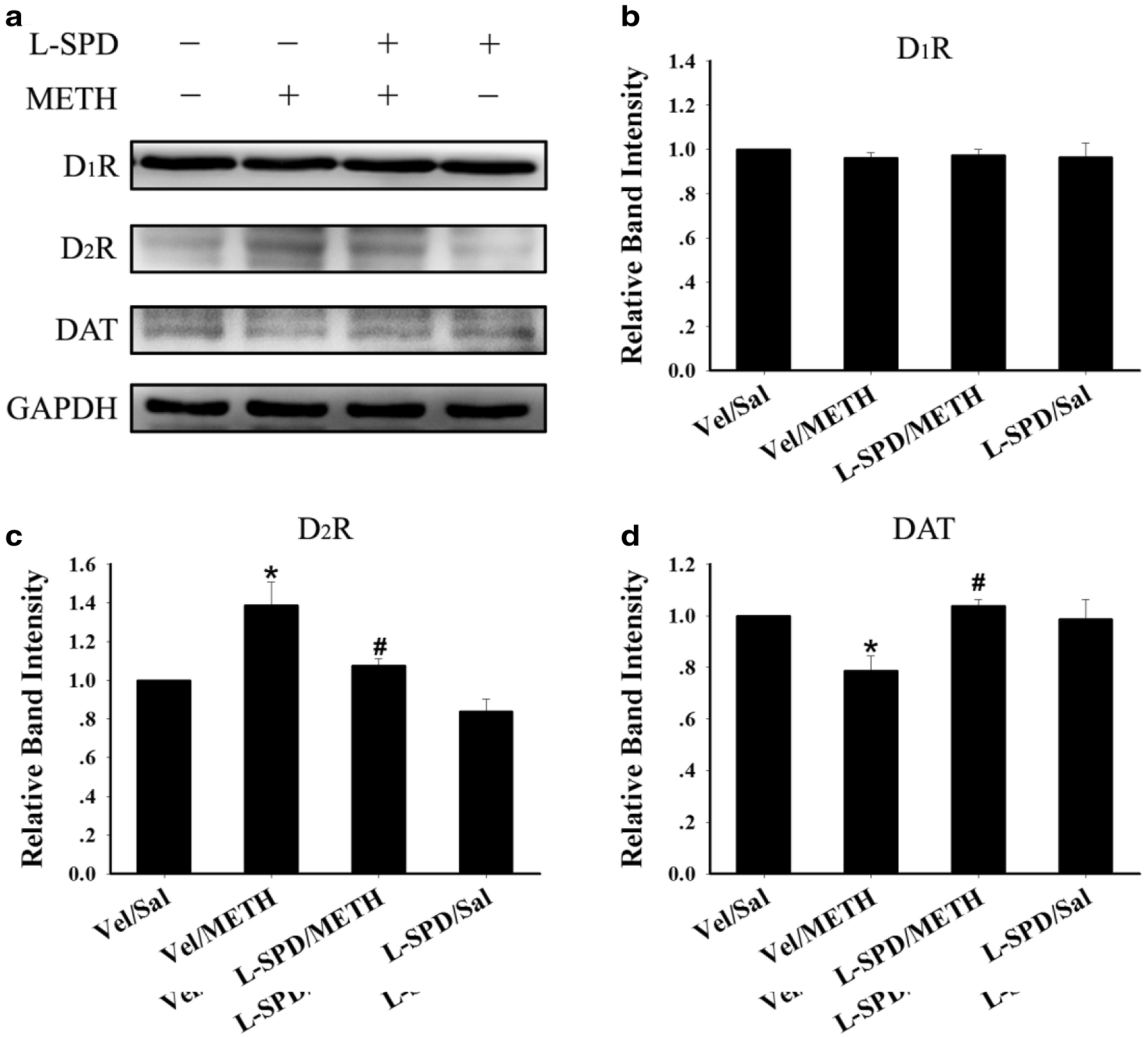

Fig. 5 Effects of L-SPD on METH-induced changes of DA receptors and DAT protein expressions. **a** Representative bands of DA receptors and DAT. **b** D1R protein expression in HIP area showed no significant difference among the different treatment groups. **c** Pretreatment with L-SPD prevented METH-induced increase of D2R protein expression in HIP area. **d** DAT protein expression was significantly decreased in HIP area of METH-treated mice. L-SPD pretreatment could reverse the reduction of DAT protein expression induced by METH. The symbol (+) indicated that the animals of this group were injected with METH or L-SPD. The symbol (−) indicated that the animals of this group were injected with saline or vehicle. Data were presented as means ± SEM. $n = 4$ for per group, $^{*}P < 0.05$ vs. Vel/Sal group, $^{\#}P < 0.05$ vs. Vel/METH group

observed in HCN2 protein expression (Fig. 6d, $n = 4$, F (3, 12) = 1.094, $P = 0.927$, vs. Vel/Sal group). Moreover, pretreatment with 10 mg/kg L-SPD could prevent METH-induced increase of p-PKA and HCN1 protein expressions in HIP area (Fig. 6, $n = 4$, p-PKA: $P = 0.031$, HCN1: $P = 0.040$, vs. Vel/METH group).

Discussion

In this study, we firstly found that L-SPD pretreatment significantly improved memory deficits induced by repeated METH exposure. Repeated METH exposure increased the protein expression of D2 receptors and decreased the protein expression of DAT in HIP area. Meanwhile, the protein expressions of p-PKA and HCN1 channels were enhanced in HIP area induced by repeated METH exposure. These changes of protein expressions caused by METH could be prevented by L-SPD pretreatment. Therefore, these findings indicated that L-SPD pretreatment displayed a neuroprotective effect, probably via reducing METH-induced upregulation of dopaminergic pathway and HCN1 channels.

METH abuse causes a variety of neuropsychiatric disorders, such as anxiety and behavior and cognitive impairments. The NOR task is designed basing on the spontaneous tendency of rodents to explore new things without any punishment or reward, which becomes a widely used model for the investigation of memory alterations (Antunes and Biala 2012). Previous studies have found that chronic METH administration to rodents induce the impaired memory in the NOR task

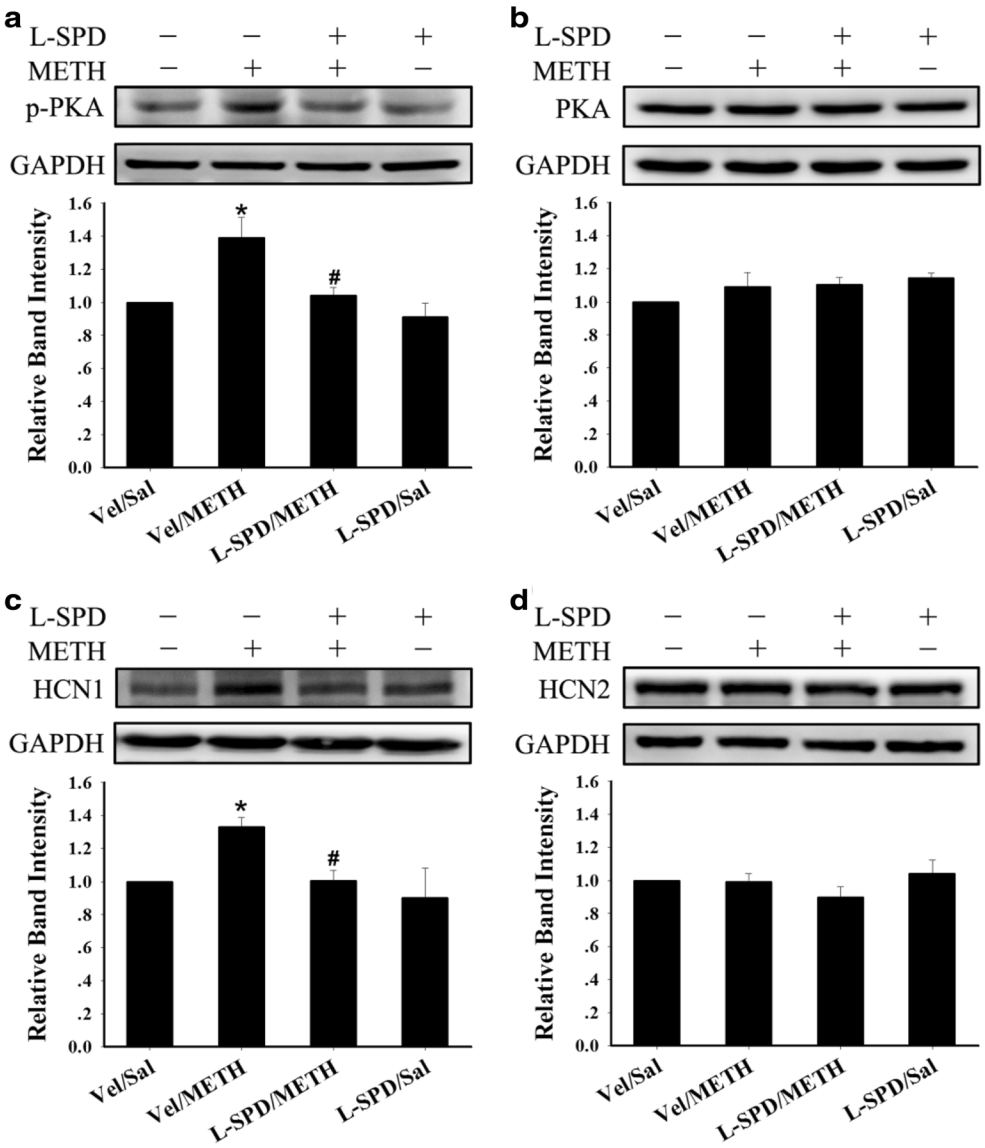

Fig. 6 Effects of L-SPD on METH-induced changes of p-PKA and HCN channel protein expressions. **a** p-PKA protein expression was significantly increased in HIP of METH-treated mice. Pretreatment with L-SPD could inhibit the increase of p-PKA protein expression induced by METH. **b** PKA protein expression in HIP showed no significant difference among the different treatment groups. **c** Pretreatment with L-SPD could prevent METH-induced increase of HCN1 protein expression in HIP. **d** No significant difference was observed in HCN2 protein expression in HIP among the different treatment groups. The symbol (+) indicated that the animals of this group were injected with METH or L-SPD. The symbol (−) indicated that the animals of this group were injected with saline or vehicle. Data were presented as means ± SEM. $n = 4$ for per group, $^{*}P < 0.05$ vs. Vel/Sal group, $^{\#}P < 0.05$ vs. Vel/METH group

(O'Dell et al. 2011; Reichel et al. 2011; Reichel et al. 2012). Repeated METH exposure (1 mg/kg, s.c., once a day for 7 days) in mice showed impairments in memory retention in the NOR task, possibly by its detrimental effects on the function of medial prefrontal cortex (Gonzalez et al. 2014, 2018). In the present study, the NOR task was applied to explore the effect of L-SPD on memory deficits caused by repeated METH exposure. For the first time, we found that L-SPD pretreatment significantly improved the memory deficit induced by repeated METH exposure in NOR task.

The loss of neurons in the hippocampus was observed in many animal models of memory deficits (Li et al. 2014; Ramirez et al. 2018). A single injection of METH (30 mg/kg; i.p.) induced a neuronal loss in the striatum (Zhu et al. 2006). The combined administration of ethanol and METH (2 mg/kg; i.p., once a day for 14 days) induced a neuronal loss in the amygdale and dentate gyrus (Chuang et al. 2011). Since the loss of neurons was the structural neurobiological mechanism underlying memory deficits (Ishikawa et al. 2014), we investigated the expression of neuron-specific marker NeuN to explore the mechanism underlying the protective effect of L-SPD on METH-induced memory deficits. In the present study, we found that the expression of NeuN in HIP area of mice with repeated METH exposure or L-SPD pretreatment exhibited no changes by the Image J–based analysis in the immunofluorescence experiment. The preliminary result of the NeuN level by the Image J analysis suggested that there was not a loss of neurons after METH. In addition to the Image J analysis, there are other methods, such as the stereological analysis, to detect the neuronal loss. And we will use other methods to consolidate this finding in our follow-up study. This result suggested that the protective effect of L-SPD on METH-induced memory deficits might not be related to the loss of neurons, possibly associated with changes in the

function of neurons, such as the alterations of receptors or ion channels on neurons.

METH-induced neurotoxicity is evident in several neurotransmitter systems, such as the brain DA and 5-hydroxtryptamine systems (Seiden et al. 2001; Ares-Santos et al. 2013). Ricaurte and colleagues found that endogenous DA was not essential for the development of METH-induced dopaminergic neurotoxicity and suggested that other factors such as neurotransmitter transporters might be involved (Yuan et al. 2010). METH has a chemical structure similar to DA and acts as a pseudo-transmitter, which binds to DAT to generate an imbalance in the release and reuptake of DA, and finally increases DA levels in synaptic clefts (Kelley and Berridge 2002). Then, DA stimulates its receptors, causing complex adaptive changes in the signal transduction systems in specific brain areas, which leads to several neurotoxic effects such as memory deficits (Fortuna et al. 2017; Gutierrez et al. 2017). HIP is known to be a critical area associated with memory functions, which also plays a key role in drug addiction (Kutlu and Gould 2016). In this study, we found that the protein level of dopaminergic D2 receptors in HIP area was significantly increased by repeated METH exposure, while the protein expression of D1 receptors remained unchanged. Our findings were similar to the observation from Okita K and colleagues, in which D1 receptors availability did not differ in METH users compared with controls (Okita et al. 2018). Meanwhile, the protein expression of DAT in HIP area was significantly reduced by repeated METH exposure. This result was similar to the observation from Gibb and colleagues, in which repeated METH administration could cause dopaminergic deficits such as the reduction in striatal DAT density (Hanson et al. 2009). L-SPD exerted partial agonistic action on dopaminergic D1-type receptors and antagonistic action on D2-type receptors (Natesan et al. 2008). We found that pretreatment with L-SPD could prevent METH-induced changes of D2 receptors and DAT protein expressions in HIP area, without affecting the protein expression of D1 receptors. These findings suggested that L-SPD displayed a neuroprotective effect against METH-induced memory deficits possibly associated with the regulation of D2 receptors and DAT protein expressions, which might affect downstream intracellular molecules and memory functions.

DA is an important reward neurotransmitter in the brain, which is closely related to addiction and memory functions (Chu and Zhen 2010; Thurm et al. 2016). D1 receptors are Gs protein–coupled receptors whose activation leads to the stimulation of adenylate cyclase (AC), resulting in the increased cyclic adenosine 3′, 5′-monophosphate (cAMP) level and PKA activity. D2 receptors are Gi/o protein–coupled receptors which produce the opposite effect and block the activity of AC which may reduce intracellular cAMP level and inhibit PKA activity (Zhao et al. 2013). DAT is a membrane-spanning protein that removes dopamine from the synaptic cleft and deposits it into the cytosol, thus terminating the cAMP-PKA signaling (Fricks-Gleason et al. 2016). In this study, we found that the protein expression of p-PKA in HIP area was significantly increased by repeated METH exposure. p-PKA participated in a number of signal transduction systems, which led to various behavioral effects (Plattner et al. 2015; Jiang et al. 2017). Several studies documented that p-PKA played an important role in the regulation of ion channels, such as calcium-activated potassium channels and voltage-gated L-type Ca^{2+} channels (Cantero Mdel et al. 2015; Abiraman et al. 2016; Xu et al. 2016). The phosphorylation of PKA was also involved in the regulation of HCN channels, which played an important role in memory functions (Cordeiro Matos et al. 2015; St Clair et al. 2017).

HCN channels belong to the superfamily of voltage-gated pore loop channels, which are activated by membrane hyperpolarization and permeable to Na^+ and K^+. HCN channels exhibit several physiological functions, such as neurotransmitter release (Biel et al. 2009), synaptic plasticity (Bender and Baram 2008), and resting membrane potential (Shah 2016). These physiological functions serve as important neurological bases underlying the memory process. HCN1 and HCN2 subunits are the most abundant HCN channels in HIP area (Brewster et al. 2005). Gonzalez and colleagues found that withdrawal from repeated METH administration (1 mg/kg, s.c., once a day for 7 days) increased the current amplitude of HCN channels, and the mRNA expression of *Hcn1* in the prefrontal cortex was decreased, while the mRNA expression of *Hcn2* was increased (Gonzalez et al. 2016). Our previous study found that low-dose METH re-exposure caused an enhancement of working memory, and a decrease in the HCN1 channels protein expression in both HIP and prefrontal cortex area (Zhou et al. 2019). In this study, we found that the protein level of HCN1 channels in HIP area was significantly increased by repeated METH exposure, but no statistical difference was detected in the protein expression of HCN2 channels. Meanwhile, pretreatment with L-SPD could reverse the increased protein expression of HCN1 channels induced by METH, which might contribute to the protective effect of L-SPD on memory deficits induced by METH.

Several studies documented that alteration of HCN channels could regulate synaptic plasticity, which was important for memory functions (Benarroch 2013; Maroso et al. 2016). The upregulation of HCN channels in prefrontal cortex reduced the membrane resistance and synaptic inputs, and finally was associated with age-related decline in working memory (Moore et al. 2005; Wang et al. 2011). HCN1 channels acted as the inhibitory constraint of both spatial learning and long-term plasticity in distal dendrites of HIP by interfering with postsynaptic Ca^{2+} influx (Tsay et al. 2007). The knockout of HCN1 channels in the forebrain could significantly enhance the long-term potentiation amplitude in HIP, which led to an increase in short-term and long-term spatial memory (Nolan et al. 2004). These studies suggested that the alteration of HCN1 channels was

associated with the pathological changes of memory functions. Therefore, we assumed that the downregulation of HCN1 channels in HIP area might be related to the protective effect of L-SPD on the memory impairment induced by METH. Additionally, p-PKA could promote the phosphorylation of adaptor proteins, which was a regulatory protein of HCN channels, and therefore it was capable of facilitating the expression of HCN channels (Ricotta et al. 2002; He et al. 2014). In this study, we found that pretreatment with L-SPD could reverse the increased protein expressions of p-PKA and HCN1 channels induced by repeated METH exposure, which might be the underlying mechanism of the neuroprotective effect of L-SPD on METH-induced memory deficits.

Conclusion

METH abuse is a complex neuropsychiatric disorder that causes serious damages to human health, especially memory deficits. However, little is known about the effect of L-SPD on METH-induced memory deficits. In the present study, we firstly found that L-SPD pretreatment significantly improved memory deficits induced by repeated METH exposure. Repeated METH exposure increased the protein expression of D2 receptors and decreased the protein expression of DAT in HIP area. Meanwhile, both the protein expressions of p-PKA and HCN1 channels were increased in HIP area induced by repeated METH exposure. These changes of protein expressions caused by METH could be prevented by L-SPD pretreatment. Thus, these results indicated that L-SPD pretreatment displayed a neuroprotective effect, probably via reducing METH-induced upregulation of dopaminergic pathway and HCN1 channels, suggesting L-SPD might be an effective therapeutic agent for memory deficits associated with METH abuse.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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