LiCl Pretreatment Ameliorates Adolescent Methamphetamine Exposure-Induced Long-Term Alterations in Behavior and Hippocampal Ultrastructure in Adulthood in Mice

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Abstract

Background: Adolescent methamphetamine exposure causes a broad range of neurobiological deficits in adulthood. Glycogen synthase kinase-3β is involved in various cognitive and behavioral processes associated with methamphetamine exposure. This study aims to investigate the protective effects of the glycogen synthase kinase-3β inhibitor lithium chloride on adolescent methamphetamine exposure-induced long-term alterations in emotion, cognition, behavior, and molecule and hippocampal ultrastructure in adulthood.

Methods: A behavioral test battery was used to investigate the protective effects of lithium chloride on adolescent methamphetamine exposure-induced long-term emotional, cognitive, and behavioral impairments in mice. Western blotting and immunohistochemistry were used to detect glycogen synthase kinase-3β activity levels in the medial prefrontal cortex and dorsal hippocampus. Electron microscopy was used to analyze changes in synaptic ultrastructure in the dorsal hippocampus. Locomotor sensitization with a methamphetamine (1 mg/kg) challenge was examined 80 days after adolescent methamphetamine exposure.

Results: Adolescent methamphetamine exposure induced long-term alterations in locomotor activity, novel spatial exploration, and social recognition memory; increases in glycogen synthase kinase-3β activity in dorsal hippocampus; and decreases in excitatory synapse density and postsynaptic density thickness in CA1. These changes were ameliorated by lithium chloride pretreatment. Adolescent methamphetamine exposure-induced working memory deficits in Y-maze spontaneous alternation test and anxiety-like behavior in elevated-plus maze test spontaneously recovered after long-term methamphetamine abstinence. No significant locomotor sensitization was observed after long-term methamphetamine abstinence.

Conclusions: Hyperactive glycogen synthase kinase-3β contributes to adolescent chronic methamphetamine exposure-induced behavioral and hippocampal impairments in adulthood. Our results suggest glycogen synthase kinase-3β may be a potential target for the treatment of deficits in adulthood associated with adolescent methamphetamine abuse.
Significance Statement

Current methamphetamine (METH)-related studies tend to focus on adults, ignoring adolescent drug exposure. This study investigated the protective effects of the GSK3β inhibitor lithium chloride (LiCl) on adolescent METH exposure-induced long-term alterations in emotion, cognition, behavior, and hippocampal ultrastructure. Adolescent METH exposure significantly increased GSK3β activity in the medial prefrontal cortex (mPFC) and dorsal hippocampus (dHIP), but this effect persisted only in the dHIP in adulthood. Treatment with LiCl before METH administration prevented adolescent METH exposure-induced long-term behavioral alterations, including mild hyperactivity, reduced novel spatial exploration, and impaired social recognition memory as well as GSK3β hyperactivity and ultrastructural alterations in the dHIP in adulthood. Adolescent METH exposure-induced working memory deficits in the Y-maze spontaneous alternation test and anxiety-related behavior in the elevated-plus maze test spontaneously recovered after long-term METH abstinence. Thus, our data suggest GSK3β may be a potential target to improve adolescent METH exposure-induced long-term behavioral and hippocampal impairments.

Keywords: methamphetamine, adolescent drug exposure, glycogen synthase kinase 3 beta, behavior, hippocampus

Introduction

Methamphetamine (METH) is a widely abused psychostimulant with long-lasting neurobiological consequences (Moszczynska and Callan, 2017). Adolescence is a sensitive, vulnerable period for the effects of drug use, and the prevalence of chronic METH use among teenagers is increasing and occurring at earlier ages (Spear, 2016; UNODC, 2017). However, current METH-related studies tend to focus on adult populations, ignoring adolescent drug exposure (Luikinga et al., 2018). Thus, studying how adolescent METH exposure affects emotion, cognition, and behavior in the both adolescence and adulthood is necessary and important.

Previous adolescent studies on the neurotoxic effects of METH tend to focus on brain regions that are associated with dopaminergic reward pathways (Buck and Siegel, 2015). An increasing number of studies have demonstrated that the medial prefrontal cortex (mPFC) and hippocampus are sensitive to adolescent drug exposure-induced nerve damage (Smith, 2003; Spear, 2016; Renard et al., 2017; Luinkinga et al., 2018). However, very few studies have reported on the long-term consequence of adolescent METH exposure on the mPFC, hippocampus, and related behaviors. Vorhees et al. suggested that METH exposure in postnatal hyperactivity (PND) 41 to 50 rats significantly impairs spatial learning/reference memory and sequential learning, indicating impaired mPFC and hippocampal functions in adulthood (PND 80) (Vorhees et al., 2005). In another study in adolescent mice exposed to METH on PND 28 to 42, at 21 days after the last METH administration, mice exhibited a decrease in hippocampal plasticity, which was not observed 23 hours after the last METH injection (North et al., 2013). In addition, the PFC and hippocampus show a biphasic pattern in volume changes across adolescence, and adolescent METH exposure may disorganize the normal pattern of growth and maturation in these brain regions and result in long-term neurobiological alterations (Wierenga et al., 2014; Jablonskiwski et al., 2017). This evidence highlights the need for further research to uncover the long-term effects of adolescent METH exposure on the mPFC and hippocampus.

Glycogen synthase kinase-3β (GSK3β) is a key regulator in neurodevelopmental and neuronal plasticity and a master downstream mediator in phosphoinositide-3-OH kinase/Akt pathway and canonical Wnt pathway, which play crucial roles in METH dependence (Chen et al., 2007; Kishimoto et al., 2008; Salcedo-Tello et al., 2011). Moreover, increased GSK3β activity is implicated in METH-induced hyperactivity, locomotor sensitization, and neurotoxicity (Xu et al., 2011; Wang et al., 2012; Wu et al., 2015; Xing et al., 2015). The GSK3β inhibitor lithium is one of the most common prescription medicines for the treatment of bipolar disorder and has neuroprotective effects (Klein and Melton, 1996; Phiel and Klein, 2001; King et al., 2014). Lithium treatment ameliorates METH-induced neurotoxicity in vitro and METH-induced locomotor sensitization in vivo (Xu et al., 2011; Wu et al., 2015). However, thus far, the effectiveness of lithium on long-term changes in nerve and behavior following adolescent METH exposure remains unknown.

Therefore, in the present study, we aimed to investigate the possible protective effects of lithium chloride (LiCl) on adolescent METH exposure-induced long-term emotional, cognitive, behavioral, molecular, and ultrastructural alterations in adulthood.

MATERIALS AND METHODS

Study Design

Experiment 1: Effects of METH Exposure on GSK3β Activity in the mPFC and Dorsal Hippocampus (dHIP) in Adolescence

Adolescent mice were randomly divided into the saline and chronic METH groups (n = 12 per group) and received 1 daily (o.d.) i.p. injection of METH (1 mg/kg) or saline (10 mL/kg) for 7 days from PND 45 to 51. The dose selection for the drugs was based on a previous study demonstrating that METH treatment at a dose of 1 mg/kg in mice for 7 days impairs recognition memory (Kamei et al., 2006). Twenty-four hours after the last METH or saline injection, mice were killed by decapitation, and the mPFC and dHIP were extracted to determine GSK3β activity by western blotting. Adolescence in Mus musculus is defined as the period from PND 22 to PND 60 (Brust et al., 2015; Spear, 2016). An overview of the experimental timing is provided in Figure 1 (Experiment 1).
× METH, and LiCl × METH. In each group, daily METH (1 mg/kg, i.p., o.d.) or saline (10 mL/kg, i.p., o.d.) administration 30 minutes after LiCl (100 mg/kg, i.p., o.d.) or saline (10 mL/kg, i.p., o.d.) injection was carried out for 7 days from PND 45 to 51. The LiCl dose and interval were chosen based on studies showing that this dose inhibits METH exposure-induced increases in GSK3β activity (Beaulieu et al., 2004; Xu et al., 2011). The day after the last drug administration, mice were killed by decapitation, and the mPFC and dHIP were extracted to determine GSK3β activity by western blotting. An overview of the experimental timing is provided in Figure 1 (Experiment 2).

Experiment 3: Effects of LiCl Pretreatment on Adolescent METH Exposure-Induced Long-Term Emotional, Cognitive, Behavioral and Ultrastructural Alterations in Adulthood

Adolescent mice were also randomly divided into the following groups (n = 14 per group): saline × saline, LiCl × saline, saline × METH, and LiCl × METH. Drug administration in each group was the same as that in Experiment 2. Following a 38-day drug-free period, a behavioral test battery was initiated on PND 90 to investigate the protective effects of LiCl on adolescent METH exposure-induced long-term emotional, cognitive, and behavioral impairments in adulthood. In view of each mouse...
participating in multiple behavioral tests, a total of 6 mild, non-invasive behavioral tests were selected to form the behavioral test battery, which was performed in the following sequence: Y-maze spontaneous alternation test for detecting spatial working memory, open field test (OFT) for detecting locomotor activity, elevated-plus maze (EPM) test for detecting anxiety-related behavior, sucrose preference test (SPT) for detecting anhedonia-like behavior, novel spatial exploration test for detecting novel spatial exploration behavior, and social interaction assay for detecting sociability and social recognition memory. To decrease carryover effects from prior tests, we allowed mice to rest for several days between each test. On PND 132, mice were killed, and their brains were harvested for further western blotting, immunohistochemical, and ultrastructural analyses. To investigate the effect of anxiety levels on our novel spatial exploration test, we randomly divided a separate cohort of adolescent mice into the saline and chronic METH groups (n = 8 per group), and drug administration in each group was the same as that in Experiment 1. On PND 118, all mice were subjected to a light-dark box test. An overview of the experimental timing is provided in Figure 1 (Experiment 3).

Experiment 4(1).—Effects of METH Exposure on Emotion, Cognition, and Behavior in Adolescence
Two separate cohorts of adolescent mice were used. Each cohort was randomly divided into the saline and METH groups (n = 10 per group), and drug administration in each group was the same as that in Experiment 1. Behavioral tests were performed after METH exposure in adolescence. One cohort was subjected to the Y-maze spontaneous alternation test, EPM test, and SPT. The other cohort was subjected to the OFT, novel spatial exploration test, and social interaction assay (Figure 1, Experiment 4(1)).

Experiment 4(2).—Effects of Adult METH Exposure on the Locomotor Activity, Novel Spatial Exploration, and Social Interaction after Long-Term METH Abstinence
Adult mice received METH (1 mg/kg, i.p., o.d) or saline (10 ml/kg, i.p., o.d.) (n = 10 per group) for 7 days from PND 80 to 86. All animals were subjected to the OFT, novel spatial exploration test, and social interaction assay on PND 132, 139, and 146, respectively (Figure 1, Experiment 4(2)).

Experiment 5: Effects of Adolescent METH Exposure on the Expression of METH-Induced Locomotor Sensitization in Adulthood
Adolescent mice were randomly divided into the saline and METH groups (n = 8 per group). Drug administration in each group was the same as that in Experiment 1. On PND 132, the possible expression of METH-induced locomotor sensitization was investigated (Figure 1, Experiment 5).

Animals
All animal procedures were conducted according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Xi’an Jiaotong University. Adolescent male C57BL/6J mice were obtained at PND 30 and were maintained with unrestricted access to food and water on a 12-hour-light cycle, and experiments were conducted during the light portion.

Drug Preparation and Administration
METH hydrochloride and LiCl were dissolved in 0.9% saline to a final concentration of 0.1 and 10 mg/mL, respectively. All drugs were freshly prepared before use.

Behavioral Tests
Y-Maze Spontaneous Alternation (Spatial Working Memory)
Mice were randomly placed at the end of one of the 3 arms to avoid placement bias and allowed to freely explore the Y-maze for 5 minutes. Spontaneous alternation (%), total arm entries, and total distance were measured.

OFT (Locomotor Activity)
Exploration of an open field arena was assessed during a 60-minute test. The center area and 4 corner areas were marked by using Any-maze 5.2 software (Stoelting Co., Wood Dale, IL). The distance moved, movement duration, and movement in the center 4 four corners were measured.

EPM (Anxiety-Related Behavior)
Mice were allowed to freely explore the apparatus for 5 minutes. The time spent in open arms (%), entries in open arms (%), head dips, total distance, and total arm entries were measured.

SPT (Anhedonia-Like Behavior)
Mice were given a 2-bottle choice (one with a 1% sucrose solution and another with tap water) for 3 days. Sucrose preference (%) and liquid ingested (mL/g) in the third day of the SPT were analyzed.

Novel Spatial Exploration Test
The procedure for this test was adapted from Melnikova et al. and Wolf et al. (Melnikova et al., 2006; Wolf et al., 2016). This test was conducted in the same Y-maze (spontaneous alternation test apparatus) rotated by 45° with different distal cues. The wall of 1 of the 3 arms was marked with a black and white pattern, which was defined as the novel arm. This test had 2 phases with a 5-minute intertrial interval. During the T1 phase, the novel arm was blocked, and the mice were allowed to freely explore the Y-maze for 5 minutes. During the T2 phase (a second 5-min exploration), the novel arm was opened. The time spent in the novel arm (%), entries in the novel arm (%), latency to first entry, longest single visit to the novel arm, total arm entries, and distance moved were measured. The apparatus and placements of mice for this test are shown in supplementary Figure 1A.

Light and Dark Box (Anxiety-Related Behavior)
Mice were placed in the center of the light chamber and allowed to freely explore the apparatus for 5 minutes. The time spent in the light chamber (%), the number of total transitions, and the total distance were measured.

Social Interaction Assay
The test apparatus was rectangular and 3-chambered. Mice were placed in the central chamber to habituate to the box for 5 minutes, followed by 2 successive test phases with a 5-minute intertrial interval. In the T1 phase (sociability test), an unfamiliar mouse (stranger) was placed in 1 of the 2 lateral chambers and enclosed in a circular acrylic cage. Another empty cage of the same design was placed in the other lateral chamber. Mice were allowed to freely explore the test apparatus for 10 minutes. In the T2 phase (social recognition memory test) (a second 10-min exploration), a second unfamiliar mouse (novel) was placed in the circular acrylic cage that was empty in the T1 phase. The sociability scores, time in the stranger chamber (%), entries in the stranger chamber (%), social recognition scores, time in the novel chamber (%), entries in the novel chamber (%), total lateral chamber entries, and distance moved were measured. The
apparatus and placements of mice for this test are shown in supplementary Figure 1B.

Locomotor Sensitization
The locomotor sensitization was examined as previously described (Good and Radcliffe, 2011). On PND 132, mice were challenged with METH (1 mg/kg, i.p.), and their activities were recorded immediately for 120 minutes.

Molecular and Histological Analyses

Western Blotting Analyses
GSK3β activity depends on site-specific phosphorylation. Phosphorylation of Tyr216 (Y216) on GSK3β activates GSK3β, whereas phosphorylation of Ser9 (S9) on GSK3β inhibits its activity. Thus, the increased ratio of Y216-phosphorylated GSK3β (pGSK3β-Y216) to total GSK3β (t-GSK3β) and/or the expression level of pGSK3β-Y216 imply increased GSK3β activity. In contrast, the increased ratio of S9-phosphorylated GSK3β (pGSK3β-S9) to t-GSK3β and/or the expression level of pGSK3β-S9 imply decreased GSK3β activity. The western-blotting procedure was conducted as described in our previous study (Wang et al., 2017). The dilutions of primary antibodies were as follows: phosphorylated pGSK3β-Y216 (1:1000), pGSK3β-Ser9 (1:1000), t-GSK3β (1:2000), and GAPDH (internal control, 1:2000). All species-appropriate horseradish peroxidase-conjugated secondary antibodies were used at a dilution of 1:10 000.

Immunohistochemistry
Following anesthetization with 10% chloral hydrate, mice were perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were postfixed in 4% paraformaldehyde for 3 hours. After cryopreserving in 30% sucrose, brains were sectioned on a freezing microtome at 30 μm. Immunohistochemical staining was performed according to the manufacturer’s protocol using Biotin-Streptavidin HRP Detection Systems (SP-9001, ZSCB-BIO, Beijing, China). The dilution of rabbit anti-phospho-GSK3β-Ser9 was 1:100. The integrated optical density in the mPFC, CA1, CA3, and dentate gyrus (DG) were evaluated.

Electron Microscopic Analysis
Following anesthetization with 10% chloral hydrate, mice were perfused transcardially with saline, followed by 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde and 0.25% glutaraldehyde. Next, brains were immediately removed and stored in 0.1 M phosphate buffer (pH 7.4) with 4% paraformaldehyde and 2.5% glutaraldehyde at 4°C. The dHIP subregions CA1, CA3, and DG were extracted and dissected into ~1 mm3 pieces. Electron microscopy was conducted as described in a previous report (Tian et al., 2009). The thickness of the postsynaptic density (PSD) at the thickest part, the width of the synaptic cleft, the length of the active zone, and the number of Gray’s type-1 asymmetric synapses (excitatory synapses) were measured. More than 60 randomly chosen asymmetric synapses per subregion were analyzed.

Statistical Analysis
Statistical analyses were conducted using GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA). The results are presented as the mean ± SEM. The parametric tests were applied when normality and homogeneity of variance assumptions were satisfied; otherwise the nonparametric tests were used (Mann-Whitney or Kruskal-Wallis). Kruskal-Wallis was followed by Dunn’s post hoc test where appropriate. For parametric tests, METH exposure, LiCl pretreatment, and test time were considered independent effects and were analyzed accordingly; that is, when there was only one effect, unpaired t tests were used and when there were 2 or 3 effects, 2- or 3-way ANOVA was used to determine main effects of METH exposure, LiCl pretreatment, or test time and interaction between these effects. Only when there was a statistical interaction were between-group comparisons done (Bonferroni’s post hoc test). Differences were defined as statistically significant at P < .05.

RESULTS

Experiment 1: METH Exposure Significantly Increased GSK3β Activity in the mPFC and dHIP in Adolescent Mice
Based on a comparison of adolescent METH-exposed and saline-treated mice, western-blot analysis revealed that the ratio of pGSK3β-Y216 to t-GSK3β was increased in the dHIP (F(22) = 2.191, P < .05) (Figure 2F) but not in mPFC (Figure 2B); the ratio of pGSK3β-Ser9 to t-GSK3β was decreased in both the mPFC (F(22) = 2.698, P < .05) (Figure 2C) and dHIP (F(22) = 3.142, P < .01) (Figure 2G), and the expression level of t-GSK3β was unchanged in both the mPFC (Figure 2D) and dHIP (Figure 2H) after 7 days of METH exposure.

Experiment 2: LiCl Pretreatment Inhibited the METH Exposure-Induced Increase in GSK3β Activity in the mPFC and dHIP in Adolescence
Two-way ANOVA for the western-blot data of the ratios of pGSK3β-Y216 to t-GSK3β in dHIP (Figure 3F) and pGSK3β-Ser9 to t-GSK3β in mPFC (Figure 3C) and dHIP (Figure 3G) revealed a significant effect of the interaction of METH exposure × LiCl pretreatment (F(1,34) = 4.287, P < .05; F(1,34) = 4.912, P < .05; F(1,34) = 4.558, P < .05), METH exposure (F(22) = 5.025, P < .05; F(22) = 9.141, P < .01; F(22) = 10.01, P < .01), and LiCl pretreatment (F(2,34) = 4.809, P < .05; F(2,34) = 12.86, P < .01; F(2,34) = 7.309, P < .05). Bonferroni’s post hoc tests revealed that pretreatment with LiCl inhibited the METH exposure-induced increase in the ratio of pGSK3β-Y216 to t-GSK3β in the dHIP (Figure 3F) and the decrease in the ratio of pGSK3β-Ser9 to t-GSK3β in the mPFC (Figure 3C) and dHIP (Figure 3G).

Experiment 3
LiCl Pretreatment Prevented Adolescent METH Exposure-Induced Mild Hyperactivity, Reduced Novel Spatial Exploration, and Impaired Social Recognition Memory in Adulthood
In this section, we report only the most relevant results that are important for the interpretation of the behavioral changes, and the others are shown in supplementary Table 1. First, all the tested animals showed similar characteristics in the Y-maze spontaneous alternation test (Figure 4A; supplementary Table 1).

For the OFT, 2-way ANOVA for the data of total distance traveled revealed a significant effect of the interaction of METH exposure × LiCl pretreatment (F(2,35) = 4.309, P < .05), METH exposure (F(1,35) = 6.633, P < .05), and LiCl pretreatment (F(2,35) = 4.689, P < .05). Bonferroni’s post hoc tests revealed that, in adulthood, adolescent METH-exposed mice (saline × METH) were markedly
more active than the control mice (saline × saline) (P < .01) and adolescent LiCl-pretreated and METH-exposed mice (LiCl × METH) (P < .05) (Figure 4B).

All the tested animals showed similar characteristics in the EPM test (Figure 4C,D; supplementary Table 1) and the 3rd day of the SPT (Figure 4E,F; supplementary Table 1).

Regarding the novel spatial exploration test, 2-way ANOVA for the data of the time spent (%) and latency to first entry in the novel arm revealed a significant effect of the interaction of METH exposure × LiCl pretreatment (F_{1,12} = 15.58, P < .001 and F_{1,12} = 8.865, P < .01, respectively), METH exposure (F_{1,12} = 7.083, P < .01 and F_{1,12} = 7.236, P < .01, respectively), and
LiCl pretreatment \( (F_{(1,52)} = 6.713, P < .05 \) and \( F_{(1,52)} = 14.95, P < .001 \), respectively). Bonferroni’s post hoc tests revealed that, in adulthood, saline × METH mice showed decreased time spent (%) and increased latency to first entry in the novel arm than did saline × saline mice \( (P < .001 \) and .01, respectively) and LiCl × METH mice \( (P < .001 \) and 0.0001, respectively) (Figure 4G,H). In addition, with the same withdrawal time in the novel spatial exploration test, adolescent saline- and METH-treated mice showed similar characteristics in the light-dark box test (Figure 4I,J). For the sociability test, the results revealed that all tested animals showed similar sociability characteristics (Figure 4K; supplementary Table 1). However, for the social recognition memory test, 2-way ANOVA showed no significant effect of the interaction of METH exposure × LiCl pretreatment \( (F_{(1,52)} = 4.727, P < .05 \) and \( F_{(1,52)} = 4.696, P < .05 \), respectively), METH exposure \( (F_{(1,52)} = 10.44, P < .01 \) and \( F_{(1,52)} = 10.63, P < .01 \), respectively), and LiCl pretreatment \( (F_{(1,52)} = 5.732, P < .05 \) and \( F_{(1,52)} = 6.643, P < .05 \), respectively). Bonferroni’s post hoc tests revealed that saline × METH mice obtained a lower average social recognition score and decreased time spent in the novel chamber (%) than did saline × saline mice \( (P < .01 \) and .01, respectively) and LiCl × METH mice \( (P < .01 \) and .01, respectively) (Figure 4L; supplementary Table 1).

LiCl Pretreatment Prevented the Adolescent METH Exposure-Induced Long-Term Increase in GSK3β Activity in the dHIP in Adulthood

The results regarding the long-term effect of adolescent METH exposure on the activity of GSK3β in the mPFC and dHIP are shown in supplementary Figure 2 and Figure 5, respectively. We did not find any difference in the ratio of pGSK3β-Y216 to t-GSK3β or pGSK3β-Ser9 to t-GSK3β, the expression of t-GSK3β protein levels, or the distribution of pGSK3β-Ser9 in the mPFC among all 4 groups (supplementary Figure 2B–D, F). For the dHIP, western-blot analysis revealed no significant difference in the ratio of pGSK3β-Y216 to t-GSK3β or the expression of t-GSK3β protein levels among all 4 groups (Figure 5B,D), whereas 2-way ANOVA for the ratio of pGSK3β-Ser9 to t-GSK3β revealed a significant effect of the interaction of METH exposure × LiCl pretreatment...
METH exposure ($F_{(1,28)} = 5.063, P < .05$), METH exposure ($F_{(1,28)} = 4.450, P < .05$) and LiCl pretreatment ($F_{(1,28)} = 5.544, P < .05$).

Bonferroni’s post hoc tests revealed that, in adulthood, the ratio of pGSK3β-Ser9 to t-GSK3β was reduced by ~40% in saline × METH mice compared with that in control mice ($P < .05$) (Figure 5C). Next, the immunochemical analysis was performed to compare the expression patterns and distribution of pGSK3β-Ser9 in the CA1, CA3, and DG subregions of the dHIP. Two-way ANOVA for the integrated optical density (IOD) of pGSK3β-Ser9 in CA1 and CA3 subregions revealed a significant effect of METH exposure ($F_{(1,8)} = 10.62, P < .05$ and $F_{(1,8)} = 9.362, P < .05$, respectively) and LiCl pretreatment ($F_{(1,8)} = 20.14, P < .01$ and $F_{(1,8)} = 31.48, P < .001$, respectively), but no interaction between METH exposure and LiCl pretreatment (Figure 5F,G).

LiCl Pretreatment Prevented the Adolescent METH Exposure-Induced Long-Term Increase in Excitatory Synapse Density and Postsynaptic Density Thickness in the dHIP CA1 Subregion in Adulthood

Then, we further examined the ultrastructural changes in the excitatory synapses of the dHIP CA1, CA3, and DG subregions by using electron microscopy. For the CA1 subregion, electron microscopy revealed no significant differences in the width of synaptic cleft or the length of active zone (Figure 6D,E). However, the 2-way ANOVA for the density of excitatory synapses and the thickness of PSD revealed a significant effect of the interaction of METH exposure × LiCl pretreatment ($F_{(1,8)} = 9.284, P < .05$ and $F_{(1,8)} = 5.651, P < .05$, respectively), METH exposure ($F_{(1,8)} = 37.14, P < .001$ and $F_{(1,8)} = 9.016, P < .05$, respectively), and LiCl pretreatment ($F_{(1,8)} = 18.95, P < .01$ and $F_{(1,8)} = 10.46, P < .05$, respectively). Bonferroni’s post hoc tests revealed that, in adulthood, excitatory synapse density and PSD thickness in the CA1 subregion were reduced by approximately 30% and 15%, respectively, in saline × METH mice compared with those in control mice ($P < .01$ and $P < .05$, respectively) (Figure 6B, C). Additionally, compared with saline × METH mice, LiCl × METH mice displayed a significant increase in excitatory synapse density and PSD thickness in the CA1 subregion ($P < .01$ and $P < .05$, respectively) (Figure 6B, C). For the CA3 and DG subregions, there were no strong differences in the investigated structural parameters, including excitatory synapse density, PSD thickness, synaptic cleft width, or active zone length among all 4 groups (Figure 6F–M).

Experiment 4: METH Exposure Led to Alterations in Working Memory, Anxiety Behavior, and Novel Spatial Exploration in Adolescence

The most relevant results are shown in Figure 7 and the others are shown in supplementary Table 2. In adolescence, compared with saline-treated mice, METH-exposed mice showed...
Figure 6. Effects of lithium chloride (LiCl) pretreatment on alterations in synaptic ultrastructure within different subregions of the adult dorsal hippocampus (dHIP) induced by adolescent methamphetamine (METH) exposure. Representative electron micrographs of the CA1, CA3, and dentate gyrus (DG) of the tested mice; the straight arrows indicate Gray’s type-1 asymmetric synapses (excitatory synapses), whereas the curved arrows indicate the length of the presynaptic active zone; the boxes indicate regions shown at higher magnification in the lower panels, and scale bars represent 500 nm under low magnification and 100 nm under high magnification (A). Histograms show relative changes in the total number of excitatory synapses, thickness of postsynaptic density (PSD) at the thickest part, width of the synaptic cleft, and length of the active zone in CA1 (B–E), CA3 (F–I), and DG (J–M). More than 60 randomly chosen excitatory synapses from each subregion were analyzed. Data are expressed as the mean ± SEM; n = 3/group; *P < .05 and **P < .01, compared with the saline × saline group; #P < .05 and ##P < .01, comparison between the 2 indicated groups; 2-way ANOVA followed by the Bonferroni post hoc test.
decreased spontaneous alternation (%) in the Y-maze spontaneous alternation test ($t_{18} = 2.533, P < .05$) (Figure 7A), decreased time spent and number of entries in the open arms (%) in the EPM test ($t_{17} = 2.360, P < .05$ and $t_{17} = 2.727, P < .05$, respectively) (Figure 7C,D), and decreased time spent (%) and longest single visiting time in the novel arm in the novel spatial exploration test ($t_{18} = 2.781, P < .05$ and $t_{18} = 2.717, P < .05$, respectively) (Figure 7G,H). All tested mice displayed similar characteristics in the OFT, SPT, and social interaction assays (Figure 7; supplementary Table 2).

Adult METH Exposure Led to Long-Term Alterations in Social Recognition Memory but Not Locomotor Activity or Novel Spatial Exploration

The most relevant results are shown in Figure 8 and the others are shown in supplementary Table 3. Following long-term drug abstinence, adult METH-exposed mice showed a lower average social recognition score and decreased time spent in the novel chamber (%) than did adult saline-treated mice in the social interaction assay ($t_{17} = 2.425, P < .05$ and $t_{17} = 2.553, P < .05$,

![Figure 7](image-url) Effects of methamphetamine (METH) exposure on emotion, cognition, and behavior in adolescence. Histograms show spontaneous arm alteration (%) in the Y-maze spontaneous alternation test (A), total distance moved in the open field test (OFT) (B), time spent and number of entries in the open arms (%) in the elevated-plus maze (EPM) test (C and D, respectively), sucrose preference (%) and total liquid ingested in the 3rd day of the sucrose preference test (SPT) (E and F, respectively), time spent (%) and longest single visiting time in the novel arm in the novel spatial exploration test (G and H, respectively), and the sociability scores and social recognition scores in the social interaction assay (I and J, respectively). Data are expressed as the mean ± SEM; n = 10/group; *$P < .05$, comparison between the 2 indicated groups; unpaired t tests.
respectively) (Figure 8C, supplementary Table 3). All tested mice displayed no significant differences in the OFT and novel spatial exploration test (Figure 8; supplementary Table 3).

**Experiment 5: Adolescent METH-Exposed Mice Did Not Display METH-Induced Locomotor Sensitization after Long-Term Drug Abstinence**

The results are shown in Figure 9, and we did not find any significant differences in the distance traveled in each interval time (Figure 9A) or in total (Figure 9B) between the METH and saline groups.

**Discussion**

In the present study, we demonstrated the protective effects of pretreatment with LiCl on adolescent METH exposure-induced long-term alterations in behaviors and hippocampal ultrastructure in adults.

Adolescent METH exposure can induce emotional, behavioral, and cognitive deficits, but not all the deficits last for a long time (Hayase et al., 2005; McGregor et al., 2005; Haidar et al., 2016; Fonseca et al., 2017; Thompson et al., 2017). Based on our results, adolescent METH exposure-induced working memory deficits in the Y-maze spontaneous alternation test and anxiety-like behavior in the EPM test spontaneously recovered after long-term METH abstinence; reduced novel spatial exploration was observed both in adolescence and subsequent adulthood; and mild hyperactivity and impaired social recognition memory were found only in subsequent adulthood. In addition, adult METH exposure also led to long-term social recognition memory impairment. Further studies are needed to confirm these results. In addition, we did not observe METH-induced locomotor sensitization after 80 days of drug abstinence (Good and Radcliffe, 2011). This may be due to the fact that the mice were only received METH injection for 1 week and only with 1 mg/kg in adolescence in our locomotor sensitization test.

Our novel spatial exploration test was adapted from the Y-maze forced alternation test, which was used to detect novelty exploration and recognition memory (Dellu et al., 2000; Melnikova et al., 2006; Wolf et al., 2016). In contrast to previous studies, to increase recognition and navigation in the novel space, we marked the wall of the novel arm with a black and white pattern that was entirely different from the other 2 arms (Dellu et al., 2000; Melnikova et al., 2006; Wolf et al., 2016). If the 3 arms are the same (3 arms with no differences or mice have impaired spatial memory), mice could explore all arms equally (Dellu et al., 2000). Here, the average time spent in the novel arm (%) in each group was less than one-third, indicating that animals avoided the novel arm. Thus, our novel spatial exploration test may reflect anxiety more than memory. However, the changes in the EPM test spontaneously recovered after long-term METH abstinence. We further performed a light-dark box test to detect possible anxiety behavior, and no group differences were detected. Explaining the differences between these behavioral results is difficult, perhaps because different behavioral tests assess different anxiety profiles. In any case, adolescent METH exposure led to alterations in novel spatial exploration in adulthood.

Novel spatial exploration and social interaction behaviors critically depend on the intact function of the mPFC and dHIP, which are also vital for locomotion (Thimus-Blanc et al., 1996; Dellu et al., 2000; Adams et al., 2009; Casanova et al., 2013; Tanimizu et al., 2017). Our western-blot analysis in these brain regions indicates that adolescent METH exposure increases GSK3β activity by regulating the phosphorylated pattern of GSK3β. Specifically, S9-phosphorylated GSK3β (inhibitory form) rather than Y216-phosphorylated GSK3β (active form) contributed to the adolescent METH exposure-induced increase GSK3β activity in the mPFC, and this increased activity can be diminished with development. GSK3β activity in the mPFC showed almost no fluctuations from PND 45 to PND 51 (Xing et al., 2016). Therefore, we hypothesize that the relatively stable GSK3β activity is temporarily affected by METH exposure but would recover with METH abstinence. Regarding the dHIP, the adolescent METH exposure-induced increase in GSK3β activity was observed in adolescence and remained in adulthood (80 days after METH exposure, PND 132). S9-phosphorylated GSK3β (inhibitory form) may play a more important role in adolescent METH exposure-induced long-term deficits than does Y216-phosphorylated GSK3β (active form) in the dHIP. Compared with Y216-phosphorylated GSK3β, S9-phosphorylated GSK3β showed large fluctuations in its activity levels from PND45 to PND51 in the dHIP (Beurel et al., 2012). METH exposure during this period could significantly disturb the developmental change in S9-phosphorylated GSK3β, which may result in a long-term
change in GSK3β activity in dHIP. In addition, the alteration in expression of S9-phosphorylated GSK3β was prominent in the CA1 and CA3 subregions of the dHIP, but the changes in synaptic ultrastructure were limited to the CA1 subregion. These results suggest that adolescent METH exposure-induced long-term dHIP damage is predominately located in the CA1 subregion and support that hyperactivation of GSK3β causes significant changes in synaptic plasticity (Salcedo-Tello et al., 2011; Nelson et al., 2013). METH exposure can attenuate brain tissue oxygen pressure, which often induces delayed neuronal damage (Kousik et al., 2011; Weaver et al., 2014). Additionally, among the various brain regions, the hippocampus is more vulnerable to hypoxia, especially in the CA1 subregion, but the DG subregion is relatively resistant (Gorter et al., 1997; Ouyang et al., 2007; Zhu et al., 2012). These reasons may explain why the CA1 subregion is more vulnerable to adolescent METH exposure-induced long-term hippocampal damage.

LiCl has neuroprotective effects, and more direct evidence indicates that LiCl attenuates METH-induced neurotoxicity and behavioral sensitization (Phiel and Klein, 2001; Xu et al., 2011; Wu et al., 2015). However, to the best of our knowledge, no study has investigated the potential role of LiCl in response to adolescent METH exposure-induced long-term consequences. In the present study, pretreatment with LiCl ameliorated adolescent METH exposure-induced mild hyperactivity, reduced novel spatial exploration, impaired social recognition memory, and increased GSK3β activity and changes in synaptic ultrastructure in the dHIP in adulthood. These results extend the findings of previous studies and indicate that LiCl can produce neuroprotection to resist adolescent METH exposure-induced long-term deficits (Phiel and Klein, 2001).

Our study has 3 limitations that must be addressed. First, the alterations in molecular and synaptic plasticity may not perfectly reflect behavioral alterations. Second, lithium directly inhibits GSK3α and GSK3β. The effect of GSK3α cannot be eliminated in our present study. Third, only one cognitive test was included in our present study. Thus, investigating other aspects of cognition function is necessary in future studies.

This study reveals that GSK3β is a key factor in adolescent chronic METH exposure-induced behavioral impairments. The CA1 region is more vulnerable to adolescent METH exposure. In addition, pretreatment with LiCl produced neuroprotection to prevent adolescent METH exposure-induced alterations in behavior and hippocampal ultrastructure in adulthood. Furthermore, our study suggests that preventing dysregulation of GSK3β activity may be beneficial for adult individuals who are suffering from behavioral dysfunction induced by adolescent chronic METH exposure.

**Supplementary Materials**

Supplementary data are available at *International Journal of Neuropsychopharmacology* (IJNPPY) online.

**Funding**

This work was supported by the National Natural Science Foundation of China (grant nos. 81571856 and 31271340).

**Acknowledgments**

We are grateful to Dr Qinru Sun for his excellent technical support.

**Interest Statement**

None.

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