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Juvenile treatment with mGluR2/3 agonist prevents schizophrenia-like phenotypes in adult by acting through GSK3 β

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Abstract

Prodromal memory deficits represent an important marker for the development of schizophrenia (SZ), in which glutamatergic hypofunction occurs in the prefrontal cortex (PFC). The mGluR2/3 agonist LY379268 (LY37) attenuates excitatory N-methyl-D-aspartate receptor (NMDAR)-induced neurotoxicity, a central pathological characteristic of glutamatergic hypofunction. We therefore hypothesized that early treatment with LY37 would rescue cognitive deficits and confer benefits for SZ-like behaviors in adults. To test this, we assessed whether early intervention with LY37 would improve learning outcomes in the Morris Water Maze for rats prenatally exposed to methylazoxymethanol acetate (MAM), a neurodevelopmental SZ model. We found that a medium dose of LY37 prevents learning deficits in MAM rats. These effects were mediated through postsynaptic mGluR2/3 via improving GluN2B-NMDAR function by inhibiting glycogen synthase kinase-3 β (GSK3 β). Furthermore, dendritic spine loss and learning and memory deficits observed in adult MAM rats were restored by juvenile LY37 treatment, which did not change prefrontal neuronal excitability and glutamatergic synaptic transmission in adult normal rats. Our results provide a mechanism for mGluR2/3 agonists against NMDAR hypofunction, which may prove to be beneficial in the prophylactic treatment of SZ.

Keywords

mGluR2/3 agonist; NMDA receptor; prefrontal cortex; neurodevelopment; animal model; schizophrenia

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Conflict of Interest

The authors declare no competing financial interests.

Author Contributions

B.X. and W.J.G. conceived the project, designed the experiments, and wrote the manuscript. B. X. conducted western blot, electrophysiological recording, and Golgi staining, and analyzed the related data; G.H. did the behavioral tests and analyzed related data; M.J.W. and M.A.S. conducted the water maze test, and analyzed the data.

1. Introduction

Schizophrenia (SZ) is a debilitating psychiatric disorder that affects ~ 1% of the population (Marin, 2016). Current pharmacological treatments for SZ are often limited in efficacy, especially for cognitive symptoms. Although the neural basis of SZ is poorly understood, emerging evidence suggests that disturbances in glutamate synaptic signaling in the prefrontal cortex (PFC) stand out as a strong possible mechanism underlying cognitive impairments (Snyder and Gao, 2013). Specifically, in early brain development, pathological genetic variation combined with environmental risk factors trigger NMDAR hypofunctioning, which may eventually compromise cortical function and delay the onset of cardinal symptoms until adulthood (Murray et al., 2014). This neurodevelopmental model proposes that cognitive impairments are central to the pathophysiology of SZ, resulting from aberrant brain maturational processes (Cannon, 2015; Murray et al., 2004).

Indeed, SZ-like phenotypes are generated in neurodevelopmental animal models, providing useful tools to assess the alterations of normal brain developmental trajectories. For example, prenatal exposure to methylazoxymethanol acetate (MAM) reproduces some of the core features of SZ, including NMDAR dysfunction, GABAergic deficits, as well as cognitive impairments (Du and Grace, 2013; Moore et al., 2006; Snyder et al., 2013). Importantly, NMDAR hypofunction and cognitive impairments in MAM model begin during peri-pubertal stage (Gulchina et al., 2017; Le Pen et al., 2006) (Snyder and Gao, 2013). It is conceivable that this early alteration represents a critical window for therapeutic interventions considering the importance of NMDARs in the refinement of excitatory synapses during postnatal development (Marin, 2016; Monaco et al., 2015); and if this period has been missed, interventions targeting NMDAR hypofunction in SZ would likely be compromised. However, targeting NMDARs in the early stage of development has potentially risky side effects due to the neurotoxic effects of glutamate (Herndon and Coyle, 1977; Liu et al., 1996).

Group II metabotropic glutamate receptors (mGluR2/3) have received significant interest as potential non-dopaminergic SZ drug targets (Delevich et al., 2015; Muguruza et al., 2016; Xing et al., 2016). Specifically, activation of mGluR2/3 negatively modulates synaptic transmission by reducing presynaptic glutamate release (Mateo and Porter, 2007). In contrast, postsynaptic mGluR2/3 enhances neuronal excitability (Jin et al., 2017) and AMPARs and NMDARs function directly (Li et al., 2017; Li et al., 2015a; Li et al., 2015b; Tyszkiewicz et al., 2004; Wang et al., 2013; Xi et al., 2011). Although clinical trials of mGluR2/3 agonists in adult patients with SZ have yielded controversial results (Downing et al., 2014; Patil et al., 2007), emerging data suggest that pharmacological activation of mGluR2/3 modulates glutamate neurotransmission and affects NMDAR function, holding the promise of this treatment strategy for the glutamate hypofunction hypothesis (Li et al., 2015a). Intriguingly, previous studies have revealed that mGluR2/3 levels undergo marked fluctuations in the developing brain (Catania et al., 1994; Defagot et al., 2002), which may have ramifications for the action of mGluR2/3 agonists during the early postnatal critical period (Ross et al., 2000).

In our recent study (Li et al., 2017), we found that targeting mGluR2/3 in the early stage can correct NMDAR hypofunction and has a beneficial effect for cognition deficits observed in adult MAM rats. However, the mechanism of how it works was untested. Using the MAM model, here we test the hypothesis that early interventions with mGluR2/3 agonist prevent SZ-like electrophysiological, morphological, and cognitive deficits in adults via restoring NMDAR signaling during a critical postnatal period.

2. Materials and Methods

2.1. Animals

Adult pregnant Sprague–Dawley rats were purchased from the Charles River Laboratories International (Wilmington, MA) and were intraperitoneally (i.p.) injected with 25 mg/kg MAM or saline control (SAL) on gestational day 17 (GD 17) as described previously (Goto and Grace, 2006; Lodge et al., 2009; Snyder et al., 2013). Pups were weaned on a postnatal day 21 (P21) and rehoused 2–4 per cage under a light/dark cycle of 12hrs, with food and water *ad libitum*. For all experiments, rats aged P12–21 as juveniles, P28–45 as adolescents based on others' (Spear, 2000) and our previous studies (Snyder et al., 2013; Wang and Gao, 2009, 2010; Xing et al., 2016). All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Drexel University College of Medicine Animal Care and Use Committee.

2.2 Drugs and treatment

LY379268 (LY37, Cat. No. 2453), Ro 25-6981 (Cat. No. 1594/1), and tetrodotoxin (TTX) (Cat. No. 1078/1) were obtained from Tocris Bioscience. DNQX (6,7-dinitroquinoxaline-2,3-dione, Cat. No. 2379-57-9), Picrotoxin (Cat. No. 124-87-8) and all other chemicals were purchased from Sigma-Aldrich.

For LY37 treatment, the drug was dissolved in saline (10 mg/ml) as the stock solution and stored at -20°C . The stock solution was freshly diluted to 2 mg/ml with saline on the days of injection. Final concentration for each rat was 0.1, 0.3 or 1.0 mg/kg. SAL- or MAM rats were treated with either control saline or LY37 intraperitoneally every other day from P21 to P27 at 10 a.m.

2.2 Morris water maze

Spatial learning and memory were assessed by the Morris water maze (MWM) as described before (Snyder et al., 2013). Briefly, male SAL and MAM rats were i.p. injected with different doses of LY37 (0.1, 0.3 and 1.0 mg/kg, once daily for five consecutive days) or saline vehicle control 1h before each MWM training day beginning at age P17. The different groups of rats were required to locate a fixed hidden platform using visual cues, and training consisted of four trials each day for five consecutive days before the probe memory test. Each rat was allowed to swim until it found the submerged platform in a 1-meter diameter water maze or until 60 s elapsed. The average of the four trials was used as the mean latency to reach the platform on each training day. The probe memory test was performed 24 h after the last training session and was used to evaluate the animals' ability to recall the learned

platform location. During probe testing, the platform was removed from the pool, and the rats were allowed to swim for 60 s while being tracked by a computerized video system. The amount of time spent swimming in the previous platform location quadrant minus the time spent swimming along the pool edge was calculated and compared among different groups.

2.3 Tissue collection and protein preparation

After deeply anesthetized with Euthazol (Virbac Animal Health, Fort Worth, Texas), rats were sacrificed by decapitation, and the brains were quickly removed. The forebrain tissue containing medial PFC (mPFC) was dissected and the whole-cell lysates were processed as before (Li et al., 2015b; Xi et al., 2011; Xing et al., 2016). Briefly, tissue was homogenized in ice-cold lysis buffer [in mM: 20 Tris-HCl, pH 7.4, 150 NaCl, 1 EDTA, 1 sodium orthovanadate, 0.1 phenylmethylsulfonyl fluoride, 50 sodium fluoride, 10 sodium pyrophosphate, 20 glycerophosphate], with 0.1% sodium dodecyl sulfate (m/V), 0.01% Triton X (V/V), 0.25% sodium deoxycholate (m/V), 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 µg/ml pepstatin]. After centrifugation at 13'000 *g* for 15 min at 4 °C, the supernatant was transferred into new tubes and stored in –80 °C for future use. For some experiments, synaptic membrane protein was prepared as described before (Li et al., 2017; Li et al., 2015b; Snyder et al., 2013). After perfused with ice-cold buffer (in mM: 320 sucrose, 4 HEPES-NaOH buffer, pH 7.4, 2 EGTA, 1 sodium orthovanadate, 0.1 phenylmethylsulfonyl fluoride, 50 sodium fluoride, 10 sodium pyrophosphate, 20 glycerophosphate, with 1 mg/ml leupeptin and 1 mg/ml aprotinin), the mPFC tissue was dissected and homogenized in sucrose buffer and then centrifuged at 1000 *g* for 10 min to remove large cell fragments and nuclear materials. The resulting supernatant was centrifuged at 17'000 *g* for 15 min to obtain cytoplasmic proteins. The pellet was resuspended in homogenization buffer and centrifuged at 17'000 *g* for 15 min to produce synaptosomes. The synaptosomal fraction then was hypoosmotically lysed and centrifuged at 25'000 *g* for 20 min to yield synaptosomal plasma membranes. Homogenization buffer was added to the pellet to make the final samples, which were then stored at –80 °C for future use.

2.4 Western blotting

A bicinchoninic acid (BCA) protein assay was performed to determine protein concentration. The protein sample was mixed with laemmli sample buffer, boiled for 5 minutes, and separated on a 7.5% SDS-PAGE gel. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Billerica, MA, USA). The membranes were blocked for 1 h with 5% fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) and incubated overnight with primary antibodies at 4°C. Blots were probed with anti-mouse NR1 (Thermo Fisher Scientific Cat# 32-0500, RRID: AB_2533060, 1:5000), anti-rabbit NR2A (Millipore Cat# 04-901, RRID: AB_1163481, 1:4000), anti-mouse NR2B (Millipore Cat# 05-920, RRID: AB_417391, 1:2000), anti-rabbit mGluR2 (Millipore Cat# 07-261, RRID: AB_2116167, 1:2000), anti-rabbit mGluR3 (Abcam, Cat# ab140741, RRID: AB_2716689, 1:4000), anti-mouse GluR1 (Millipore Cat# MAB2263, RRID: AB_11212678, 1:2000), anti-mouse GluR2 (Millipore Cat# MABN71, RRID: AB_10806492, 1:2000), anti-phosphor-AktSer473 (Cell Signaling Technology Cat# 4060S, RRID: AB_2315049, 1: 1,000), anti-Akt1 (Cell Signaling Technology Cat# 2938S, RRID: AB_915788 1:2,000), anti-phosphor-GSK3βSer9 (Cell Signaling Technology Cat#

9336S, RRID: AB_331405, 1:10,000), anti-GSK3 β (Cell Signaling Technology Cat# 9315S, RRID: AB_490890, 1:20,000), anti-phosphor-mTORSer2448 (Cell Signaling Technology Cat# 5536S, RRID: AB_10691552, 1:1,000), anti-mTOR (Cell Signaling Technology Cat# 2983S, RRID: AB_2105622, 1:2,000). Blots were also probed with anti-mouse actin (Sigma-Aldrich Cat# A5316, RRID: AB_476743, 1:100,000) as a loading control. After overnight incubation with primary antibodies, the membranes were washed three times with TBST and incubated with horseradish peroxidase-coupled anti-rabbit (Vector Laboratories Cat# PI-1000, RRID: AB_2336198, 1:5000) or anti-mouse IgG secondary antibody (Vector Laboratories Cat# PI-2000, RRID: AB_2336177, 1:5000), and proteins were visualized using enhanced chemiluminescence (Amersham ECL Western Blotting Detection Reagent, product code RPN2106). Two to three primary antibodies with different molecular weight of target proteins and host animals were probed for each PVDF membrane. And the membrane was stripped at most twice by the Restore Western Blot Stripping Buffer (ThermoFisher Scientific, Cat. No. 21059) for 10 min at room temperature and then probed for other primary antibodies. Protein expression of each subunit was evaluated by densitometry using Quantity One 1-D Analysis Software (RRID: SCR_014280). Additionally, samples from each animal were run at least 3 times to minimize interblot variance.

2.5 Slice preparation

Animals were anesthetized with Euthasol (0.2 ml/kg, i.p.), the brains were rapidly removed, and coronal slices through the mPFC were cut (300 μ m thick) using a vibratome (VT1200S, Leica Microsystems) and placed into bath of oxygenated ice-cold cutting solution (in mM: 2.5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 0.5 CaCl₂, 7.0 MgSO₄, 213 sucrose, pH 7.4). Slices were transferred to a holding chamber, submerged in oxygenated artificial cerebrospinal fluid (aCSF; in mM: 124 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgSO₄, 26 NaHCO₃, and 10 dextrose, pH 7.4) at 35°C for one hour and then held at room temperature until used for recording. Slices were placed into a recording chamber mounted on an Olympus upright microscope (BX51), where they were bathed in oxygenated aCSF. Neurons were visualized with optics of infrared differential interference contrast (IR-DIC) and a digital video camera system.

2.6 Electrophysiology

Whole-cell recordings in brain slices were performed as previously described (Xing et al., 2016). In voltage-clamp experiments, evoked excitatory postsynaptic currents (EPSCs) in layer 5 pyramidal neurons were elicited by stimulating layer 2/3 with either a single pulse or paired pulses at 10 Hz (0.1 ms, 10–100 μ A, 20 s inter-stimulus interval) through a bipolar electrode and recorded with a holding potential of +60 mV in aCSF containing the GABA_A receptor antagonist picrotoxin (50 μ M) and the AMPAR antagonist DNQX (6,7-dinitroquinoxaline-2,3-dione; 20 μ M). The miniature EPSCs (mEPSCs) or spontaneous EPSCs (sEPSCs) were recorded in the presence of DNQX (20 μ M) and picrotoxin (50 μ M) with or without TTX (0.5 μ M). EPSCs were recorded using patch electrodes with an open tip resistance of 5–7 M Ω (internal solution, in mM: 110 Dgluconic acid, 110 CsOH, 10 CsCl₂, 1 EGTA, 1 CaCl₂, 5 QX-314, 1 ATP-Mg, 10 HEPES, 5 TEA-Cl at pH 7.4 adjusted with CsOH).

All experiments were conducted with Axon MultiClamp 700B amplifier (Molecular Devices), and data were acquired using pCLAMP 9.2 software and analyzed using Clampfit 10.2 (Molecular Devices). A typical EPSC was selected to create a sample template for the event detections within a 5-min data period. The frequency (number of events) and amplitude of the individual events were examined with the threshold set at the medium level (i.e., 5) in Clampfit. The detected events were then visually inspected to ensure specificity. The evoked EPSC amplitudes were measured by averaging 30 traces from the onset to peak of EPSCs. Only the connections giving stable EPSCs without rundown for at least 5 min were considered for further analysis of drug and stimulus effects. The amplitudes of 2nd and following EPSCs were measured from onset to peak. The normalized ratios of the 2nd divided by the 1st EPSC were calculated to show the paired-pulse ratio (PPR) of synaptic transmission at 20 Hz train. The coefficient of variation (CV) was calculated by dividing the standard deviation by the EPSC mean amplitude. Decay was fit using a double-exponential function as published previously (Wang et al., 2008), using the following formula: $EPSC(t) = w_1 \exp(-t/\tau_1) + w_2 \exp(-t/\tau_2)$. The weighted time constant was defined as $\tau_w = (w_1 \tau_1 + w_2 \tau_2)/(w_1 + w_2)$. The contribution of NMDAR subunit GluN2B to the overall NMDAR-mediated current was estimated from measurements in the baseline condition and with the application of the NR2B antagonist Ro 25-6981, i.e., $(\text{Baseline trace} - \text{Ro trace})/\text{control} \times 100$.

In current-clamp experiments, the intracellular recording solution consisted of the following (mM): 120 K-gluconate, 20 KCl, 4 ATP-Na-ATP, 0.3 Na₂GTP, 5 Na-phosphocreatine, 0.1 EGTA, 10 HEPES, pH 7.3. Whole-cell recording in current clamp mode was used to record action potentials (APs) in response to a series of step currents (–300 to 400 pA, 50 pA steps) to determine evoked firing frequencies (Hz), input resistance (M Ω), resting membrane potential (mV), AP threshold (mV), afterhyperpolarization (AHP) amplitude (mV) and spike half-width (ms), 20–80% rise time (ms), and peak AP amplitude (mV). In some experiments, evoked excitatory postsynaptic potentials (EPSPs) were elicited by a train of 10 stimuli delivered at 20 Hz with the membrane potentials of the recording cells adjusted to about –70 mV through a small holding current (< 100 pA). The locations of the bipolar stimulating electrode and the recording electrode were the same as described above. The peak of each EPSP and the area under the EPSPs were analyzed using Clampfit 10.2 (Molecular Devices) to determine the temporal summation in response to the 10-pulse stimuli.

2.7. Behavioral tests in adult

Behavioral tests including spontaneous alternation T-maze and MK-801-induced locomotor activity were conducted in young adult rats (P60 to P90). The interval between behavioral tests was 3 days.

2.7.1. Spontaneous alternation T-maze—This task was used to measure spatial memory performance in rats as described before (Deacon and Rawlins, 2006). The T-shape maze consisted of three equally sized arms (50 cm $\times$ 10 cm $\times$ 30 cm high) equipped with guillotine doors. During the test, rats were first placed at the end of the start arm and allowed to choose a goal arm. Once the whole body entered into the goal arm, the rat was confined in

the chosen arm for 30 s and removed into a clean cage. After 30 s, the rat was replaced in the start area facing away from the goal arms and allowed to freely choose between the two open goal arms. The protocol was repeated until the rat had made 13 tests. The interval between adjacent tests was 15 min for each rat. To analyze spatial memory performance, the number of alternations between the target arms were counted.

2.7.2. MK801-induced hyperlocomotor activity—MK-801-induced activity in the open field was assessed over a 90 min period using a novel 43.2 cm × 43.2 cm activity test chamber with infrared beams (Med Associates, Inc., St. Albans, VT, USA). Briefly, after habituation in the chambers for 30 min, rats received MK-801 (0.05 mg/kg) injections intraperitoneally in the home cages and then placed back into the chamber while horizontal counts (number of infrared beam breaks) were automatically recorded over 60 min.

2.8. Dendritic spine analysis

One week after the last behavioral test, rats were deeply anesthetized using Euthasol and then decapitated. The brain block containing mPFC was quickly removed, and Golgi-Cox impregnation was performed according to the protocol outlined in the FD Rapid GolgiStain Kit (FD NeuroTechnologies, Inc., Ellicott City, MD) as described before (Xing et al., 2016). Coronal sections of the mPFC were cut at 100 µm and mounted. Images of apical dendritic branches of layer 5 pyramidal neurons were acquired using a Leica light microscope with a 60× water immersion objective. Only neurons with clear cell bodies and primary dendrites that could be easily distinguished from neighboring cell bodies and their dendrites were selected for pictures and analysis. Dendritic spines were sampled from either secondary apical dendrites (branching point to about 80–120 µm from the apical trunk). Eight neurons per rat with four rats per group were included in the analysis. The length of branch segments was determined by using Neurolucida 9 (MicroBrightField, Williston, VT). Automated spine density analysis was performed by using Neurolucida Explorer (version 9) on raw image stacks. The number of dendritic protrusions was counted manually by an investigator blind to treatment. Spine density was calculated with reference to the length of the dendrite, and the results were expressed as spine number per 10 µm.

2.9. Experimental design and statistical analysis

We used a between-subject experimental design for all experiments. Rats with prenatal MAM exposure were controlled by the age-matched prenatal saline exposure group, and experimental groups were randomly divided into two groups: LY37 treatment and saline controls during juvenile. In water maze training, the data were analyzed with a fully factorial ANOVA with group (SAL vs MAM) and drug treatment (saline, LY37-0.1 mg/kg, LY37-0.3 mg/kg and LY37-1.0 mg/kg) as between-subjects factors and training day as a within-subjects factor. The probe test and following western blot data were analyzed with a fully factorial ANOVA with group and drug treatment as between-subjects factors. Overall treatment (SAL-SAL, SAL-LY37, MAM-SAL, and MAM-LY37) was analyzed as a between-subject factor in all other experiments. In some cases, repeated measures (RM) ANOVA was used for comparison of baseline and drug application effect, different stimuli intensities, or different time points (locomotion, long-term side effect of LY37).

The number of animals assigned per group was determined from previous experiences in the field and included into the results section for each experiment. Statistical analyses were performed using the IBM SPSS statistics 24 (RRID: SCR_002865) and GraphPad Prism 7.00 software (RRID: SCR_002798). For all the data, D'Agostino & Pearson normality test was first used to test the normality of values distribution. Parametric testing was used for statistical comparison of experimental groups (ANOVA with Bonferroni's/ Tukey's multiple comparisons test for comparisons of > 2 groups). Data were presented as mean $\pm$ SEM. If the criterion for a normal distribution is not met, Kruskal-Wallis ANOVA was performed followed by Dunn's test for comparisons of > 2 groups. Data are presented as median $\pm$ interquartile range. * p < 0.05, ** p < 0.01, *** p < 0.001 was considered significant.

3. Results

3.1 Early LY37 treatment rescues learning and memory deficits of juvenile MAM rats in water maze

In our previous study (Snyder et al., 2013), we have shown that juvenile MAM exhibited significant learning and memory deficits compared with age-matched SAL controls. We first evaluate the effect of juvenile LY37 treatment on learning and memory in SAL and MAM rats. Male juvenile SAL and MAM rats received daily LY37 treatment at a low (0.1 mg/kg), medium (0.3 mg/kg) or high dose (1.0 mg/kg) during P17 to P21 before Morris water maze (MWM) training, as shown in Fig. 1A and 1B. By three-way RM-ANOVA, there were significant between-subject effects of day ($F_{(4, 228)} = 57.70$, $p < 0.0001$), day * group (SAL vs MAM) interaction ($F_{(4, 228)} = 3.31$, $p < 0.0001$), day * group * treatment (saline vs LY37) interaction ($F_{(12, 228)} = 2.68$, $p = 0.002$). A significant group ($F_{(1, 57)} = 2.68$, $p < 0.0001$), but not treatment ($F_{(3, 57)} = 1.34$, $p = 0.27$) or group * treatment ($F_{(3, 57)} = 1.26$, $p = 0.30$), within-subject effect was found. These data suggest that, although the learning performance of animals improved with time, the learning abilities varies with group and treatment. *Post-hoc* analysis revealed a significant difference in escape latencies on training days 3 to 5 (all $p < 0.0001$, Bonferroni's test) between SAL-saline and MAM-saline rats (Fig. 1C). A significant decrease on day 4 ($p = 0.002$) and day 5 ($p = 0.014$) between MAM-LY37 (0.3 mg/kg) and MAM-SAL rats was found (Fig. 1B), but no difference of different treatment was detected among SAL groups (all $p > 0.05$; Fig. 1A).

After training, memory for the platform location was tested 24 hrs later using a probe trial in which the hidden platform is removed and the amount of time the animal spends swimming in the quadrant of the previous platform location is recorded. Two-way ANOVA revealed a significant effect of group ($F_{(1, 64)} = 45.35$, $p < 0.0001$), treatment * group ($F_{(3, 64)} = 3.04$, $p < 0.036$), but not treatment ($F_{(3, 64)} = 1.83$, $p = 0.15$). As shown in Fig. 1D, *post-hoc* analysis revealed that the MAM-SAL rat spent significantly less time in the target quadrant compared with SAL-SAL controls ($p < 0.0001$, Bonferroni's test). MAM-LY37 (0.3 mg/kg) rats spent significantly more time swimming in the previous target quadrant compared with MAM-SAL ($p = 0.004$). Together, these results indicate an improvement of learning and memory performance of MAM rats received a medium dose of LY37 treatment during juvenile.

3.2. LY37 treatment restores GluN2B and serine 9-phosphorylated GSK3 β levels in the PFC of MAM rats

The glutamate system and, in particular, N-methyl-D-aspartate receptors (NMDARs) within hippocampus and prefrontal cortex (PFC) are important for disease symptoms and the progression of SZ (Cannon, 2015). We have previously shown NMDAR deficits in hippocampus from juvenile MAM (Snyder et al., 2013). To determine the potential mechanisms that may underlie the effect of LY37 (0.3 mg/kg), we collected the medial PFC (mPFC) tissues and examined the protein levels of NMDAR subunits (GluN1, GluN2A and GluN2B), mGluR2/3, as well as Akt-GSK3 β -mTOR signaling pathway per our recent report (Xing et al., 2016) 24 hrs after the probe tests. By two-way ANOVA and Bonferroni *post-hoc* test, we found significantly decreased levels of GluN2B ($F_{\text{group (1,19)}} = 5.21$, $p = 0.034$, $F_{\text{treatment (1,19)}} = 16.79$, $p = 0.001$, $F_{\text{interaction (1,19)}} = 9.43$, $p = 0.006$; SAL-SAL vs. MAM-SAL, $p = 0.047$) and mGluR2 ($F_{\text{group (1,15)}} = 19.83$, $p < 0.0001$, $F_{\text{treatment (1,15)}} = 0.013$, $p = 0.91$, $F_{\text{interaction (1,15)}} = 0.13$, $p = 0.73$; SAL-SAL vs. MAM-SAL, $p = 0.022$) in MAM-SAL rats, as shown in Fig. 2A), and LY37 treatment rescued GluN2B (MAM-SAL vs. MAM-LY37, $p = 0.0034$), but not mGluR2 levels (MAM-SAL vs. MAM-LY37, $p = 0.99$). There is no significant difference of NR1 ($F_{\text{group (1, 19)}} = 0.56$, $p = 0.47$, $F_{\text{treatment (1,15)}} = 3.62$, $p = 0.07$, $F_{\text{interaction (1,19)}} = 0.02$, $p = 0.90$), NR2A ($F_{\text{group (1, 19)}} < 0.001$, $p = 0.98$, $F_{\text{treatment (1,15)}} = 0.47$, $p = 0.50$, $F_{\text{interaction (1,19)}} = 0.26$, $p = 0.62$) and mGluR3 ($F_{\text{group (1, 19)}} = 0.15$, $p = 0.70$, $F_{\text{treatment (1,15)}} = 0.44$, $p = 0.51$, $F_{\text{interaction (1,19)}} = 2.53$, $p = 0.13$) among groups. For Akt-GSK3 β -mTOR signaling, only a significant decrease of pGSK3 β ($F_{\text{group (1,19)}} = 13.67$, $p = 0.0015$, $F_{\text{treatment (1,19)}} = 10.73$, $p = 0.004$, $F_{\text{interaction (1,19)}} = 1.27$, $p = 0.27$; SAL-SAL vs. MAM-SAL, $p = 0.017$, Fig. 2B) in MAM-SAL group was observed, and the level of pGSK3 β was rescued by LY37 treatment (MAM-SAL vs. MAM-LY37, $p = 0.023$). No significant difference of Akt1 ($F_{\text{group (1, 19)}} = 0.60$, $p = 0.45$, $F_{\text{treatment (1,15)}} = 0.03$, $p = 0.86$, $F_{\text{interaction (1,19)}} = 0.72$, $p = 0.41$), GSK3 β ($F_{\text{group (1, 19)}} = 0.60$, $p = 0.45$, $F_{\text{treatment (1,15)}} = 3.05$, $p = 0.10$, $F_{\text{interaction (1,19)}} = 0.08$, $p = 0.78$), mTOR ($F_{\text{group (1, 19)}} = 0.17$, $p = 0.69$, $F_{\text{treatment (1,15)}} = 1.30$, $p = 0.27$, $F_{\text{interaction (1,19)}} < 0.01$, $p = 0.97$), pAkt ($F_{\text{group (1, 19)}} = 0.45$, $p = 0.51$, $F_{\text{treatment (1,15)}} = 1.28$, $p = 0.27$, $F_{\text{interaction (1,19)}} = 2.80$, $p = 0.11$) and pmTOR ($F_{\text{group (1, 19)}} = 0.08$, $p = 0.78$, $F_{\text{treatment (1,15)}} = 0.99$, $p = 0.33$, $F_{\text{interaction (1,19)}} = 0.03$, $p = 0.86$) was detected. Together, these results suggest that GluN2B, mGluR2, and pGSK3 β protein levels are decreased in juvenile MAM rats, while LY37 treatment restores GluN2B and pGSK3 β levels that may underlie improved spatial learning and memory abilities.

3.3. MAM-exposed rats show blunted response to LY37 due to reduced presynaptic mGluR2 levels

Previous studies suggest that mGluR2 contributes to the presynaptic inhibition of glutamate release while mGluR3 is mainly located in postsynaptic sites in spine synapses (Jin et al., 2017; Li et al., 2017; Li et al., 2015b; Petralia et al., 1996; Tamaru et al., 2001; Xi et al., 2011). To examine whether the decreased mGluR2 levels lead to alteration in presynaptic response to LY37 in MAM rats (Ferraguti and Shigemoto, 2006), we compared the paired-pulse ratio (PPR), an indicator of the probability of vesicle release from presynaptic terminals, of evoked NMDAR-mediated currents with LY37 bath application between SAL and MAM rats. As shown in Fig. 3A and 3B, a two-way RM ANOVA ($F_{(1,18) \text{ treatment}} = 0.90$, $p = 0.0077$, $F_{(1,18) \text{ group}} = 0.71$, $p = 0.41$, $F_{(1,18) \text{ interaction}} = 4.83$, $p = 0.04$) revealed that

LY37 [300 nM, based on our previous study (Xi et al., 2011)] significantly increased the PPR of layer 5 pyramidal neurons in mPFC slices from SAL rats (SAL_{baseline} vs. SAL_{LY37}, $p = 0.0035$, Bonferroni's test), but this effect was absent in MAM group (MAM_{baseline} vs. MAM_{LY37}, $p > 0.99$, Bonferroni's test). Additionally, the inverse square of the coefficient of variation (CV, standard deviation/mean), computed from the first EPSC amplitude recorded in individual neurons from SAL rats, was also significantly decreased ($F_{(1,17)} \text{ treatment} = 5.39$, $p = 0.033$, $F_{(1,17)} \text{ group} = 3.89$, $p = 0.065$, $F_{(1,17)} \text{ interaction} = 4.25$, $p = 0.055$; SAL_{baseline} vs. SAL_{LY37}, $p = 0.011$, Bonferroni's test) following LY37 application, in agreement with a decrease in glutamate release probability. However, there was no change of CV⁻² in MAM rats (MAM_{baseline} vs. MAM_{LY37}, $p > 0.99$, Bonferroni's test). Altogether, these results suggest a blunted presynaptic glutamate release in MAM rats in response to LY37 that is attributable to the decreased presynaptic mGluR2 levels. Thus, the beneficial effect of LY37 on MAM rats is thus likely to be dependent on postsynaptic mechanisms.

3.4. LY37 corrects postsynaptic NMDAR hypofunction via GSK3 β -GluN2B signaling in MAM rats

We then asked whether MAM rats showed NMDAR dysfunction and whether *in vivo* LY37 treatment could enhance NMDAR function, and improve learning ability of MAM rats. As shown in Fig. 4A and 4B, the amplitudes of evoked NMDAR-EPSCs induced by a series of stimulus intensities were significantly decreased ($F_{\text{treatment} (3, 33)} = 3.88$, $p = 0.018$, $F_{\text{stimulus intensity} (2, 66)} = 3.88$, $p < 0.0001$, $F_{\text{interaction} (6, 66)} = 1.44$, $p = 0.22$, two-way RM ANOVA) in layer 5 prefrontal pyramidal neurons from MAM-SAL rats compared with that from SAL-SAL controls (Bonferroni test, $p < 0.05$ at all stimulus intensities). However, the MAM-LY37 (0.3 mg/kg/day, from P21 to P27) rats showed a comparable amplitude of NMDAR-EPSCs (Bonferroni test, $p > 0.05$ at all stimulus intensities) to SAL-SAL controls, and significantly greater than MAM-SAL group ($p < 0.05$ at all stimulus intensities). These results suggest that early LY37 treatment reversed prefrontal NMDAR hypofunction in MAM model.

GluN2B-rich synapses in the PFC are critically implicated in online cognitive computations and plasticity in learning, as well as psychiatric disorders such as SZ (Monaco et al., 2015; Wang et al., 2008). To determine whether GluN2B subunits of NMDARs were targeted by LY37 treatment, we bath-applied Ro25-6981 (Ro, 1 μ M, a selective NMDAR-GluN2B antagonist, to examine its influence on the NMDA-eEPSCs. As shown in Fig. 4C–4E, Ro25-6981 caused a ~40% reduction of the currents in prefrontal synapses from SAL-SAL ($41.2\% \pm 5.2\%$), SAL-LY37 ($46.2\% \pm 7.0\%$), and MAM-LY37 ($41.9\% \pm 6.9\%$) groups, compared with a ~20% decrease from MAM-SAL rats ($17.3\% \pm 4.3\%$). The GluN2B ratio of the MAM-SAL group was significantly lower than SAL-SAL and MAM-LY37 group ($F_{(3, 40)} = 4.89$, $p = 0.0055$, one-way ANOVA, $p < 0.05$, Bonferroni test, Fig. 4E). Furthermore, as shown in the EPSC traces scaled by peak amplitude (Fig. 4C, lower panel), a RM ANOVA ($F_{(3,40)} \text{ treatment} = 0.71$, $p = 0.55$, $F_{(1,40)} \text{ Ro treatment} = 78.2$, $p < 0.001$, $F_{(3,40)} \text{ interaction} = 4.18$, $p = 0.012$) revealed that Ro25-6981 caused a significant decrease in the NMDA current decay in PFC from SAL-SAL ($\tau_{\text{baseline}} = 77.4 \pm 5.9$ ms vs. $\tau_{\text{Ro}} = 47.5 \pm 5.7$ ms, $p < 0.0001$, Bonferroni's test), SAL-LY37 ($\tau_{\text{baseline}} = 81.9 \pm 7.2$ ms vs. $\tau_{\text{Ro}} = 46.1 \pm 4.1$ ms, $p < 0.0001$), as well as MAM-LY37 group ($\tau_{\text{baseline}} = 83.4 \pm 5.7$ ms vs. $\tau_{\text{Ro}} = 50.2$

± 7.7 ms, $p < 0.0001$) compared with that of MAM-SAL control ($\tau_{\text{baseline}} = 41.9 \pm 4.1$ ms vs. $\tau_{\text{Ro}} = 35.6 \pm 2.4$ ms, $p = 0.62$). Altogether, these results suggest that MAM rats exhibited GluN2B hypofunction in PFC that could be rescued by *in vivo* LY37 treatment.

We next tested the hypothesis that LY37 treatment improved postsynaptic NMDAR-GluN2B mediated currents in MAM animals through inhibiting GSK3 β activity. Anti-pGSK3 β Ser9 antibody (10 mg/ml) was loaded into the recording pipette to selectively block the postsynaptic pGSK3 β Ser9, and evoked NMDA-EPSCs were recorded in prefrontal layer 5 pyramidal neurons. To allow diffusion of the antibody into the recording cell, we waited at least 10 min after membrane break-in. A two-way RM ANOVA analysis showed that there was significant effect of LY37 application ($F_{\text{LY37}}(1,42) = 24.07$, $p < 0.0001$), but not pGSK3 β Ser9 antibody treatment ($F_{\text{treatment}}(3,42) = 2.59$, $p = 0.066$) nor interaction ($F_{\text{interaction}}(3,42) = 2.09$, $p = 0.12$) in amplitude of NMDA-eEPSCs. For decay time, the effects of pGSK3 β Ser9 antibody treatment and LY37 application were revealed significant ($F_{\text{treatment}}(3,39) = 3.05$, $p = 0.040$, $F_{\text{LY37}}(1,39) = 6.17$, $p = 0.017$, $F_{\text{interaction}}(3,39) = 2.07$, $p = 0.12$). In SAL rats, LY37 (300 nM) caused significant decreased amplitudes ($p = 0.0002$, Bonferroni's test) and increased decay time of NMDA-eEPSCs ($p = 0.035$) with regular intracellular solution (Fig. 5A), suggesting a possible combined action in both presynaptic inhibition of glutamate release (reducing amplitude) and postsynaptic increase of GluN2B function (increasing decay time) caused by LY37. However, with anti-pGSK3 β Ser9 antibody in the recording pipette, LY37 remained effective in reducing the current amplitudes ($p = 0.034$) but did not affect the decay time ($p > 0.99$), indicating a blockade of the postsynaptic effect of LY37 by inhibiting pGSK3 β Ser9 without affecting the presynaptic glutamate release. Similarly, in MAM rats (Fig. 5B), LY37 significantly increased the decay time of NMDA-eEPSCs with a regular intracellular solution ($p = 0.048$), but not with the anti-pGSK3 β Ser9 antibody ($p > 0.99$). The amplitudes of NMDA-eEPSCs were not significantly affected by LY37 in both conditions of with ($p = 0.51$) or without anti-pGSK3 β Ser9 antibody ($p > 0.99$), suggesting a blunted presynaptic response to LY37 in MAM model. Altogether, our results indicated that LY37 strengthened postsynaptic GluN2B function through interaction with the GSK3 β signaling pathway.

3.5. Juvenile LY37 treatment prevents dendritic spine loss in PFC from adult MAM rats

One important observation in SZ subjects is the reduction of dendritic spine density in adult PFC, which may be due to excessive synaptic pruning during the critical period (Kolluri et al., 2005; Selemon and Zecevic, 2015). We previously showed that dendritic spine density was reduced in the mPFC of adult MAM rats but rescued by a treatment in juvenile with GSK3 β inhibitor (Xing et al., 2016). Since LY37 increased postsynaptic inhibitory pGSK3 β Ser9 levels in juvenile MAM PFC, LY37 treatment could theoretically have a similar preventive effect on the decline of spine density in MAM animals. To test this prediction, juvenile MAM rats received LY37 (0.3 mg/kg from P21 to P27), the Golgi staining and analysis of apical dendritic spines were performed when the rats reached young adulthood. We did not find any significant changes in body weight of rats from the different groups during the treatment period ($F_{\text{treatment}}(3, 40) = 1.76$, $p = 0.17$, $F_{\text{day}}(6, 240) = 1642$, $p < 0.0001$, $F_{\text{interaction}}(18, 240) = 0.56$, $p = 0.92$, two-way RM ANOVA, Fig. 6A). However, compared with SAL-SAL rats, the density of apical dendritic spines in the mPFC was

significantly decreased in MAM-SAL group ($F_{(3, 28)} = 14.28$, $p < 0.0001$, one-way ANOVA, SAL-SAL vs. MAM-SAL, $p < 0.0001$, Tukey's test, Fig. 6B and 6C), but the reduction was not observed in MAM-LY37 group (MAM-SAL vs. MAM-LY37, $p = 0.0003$, SAL-SAL vs. MAM-LY37, $p = 0.81$, Tukey's test, Fig. 6B and 6C), suggesting that early LY37 treatment prevented the loss of dendritic spines induced by gestational MAM exposure. Additionally, the spines were further classified as mushroom, stubby, and thin spines as previous studies reported (Xing et al., 2016), and we did not observe a significant difference (mushroom spine, $F_{(3, 28)} = 0.65$, $p = 0.59$, $F_{(3, 28)} = 1.42$, $p = 0.26$, $F_{(3, 28)} = 0.70$, $p = 0.56$, one-way ANOVA; Fig. 6D) in changes of spine morphology among the groups.

3.6. Juvenile LY37 treatment enhances temporal summation of excitatory synaptic potentials in PFC pyramidal neurons and rescues spatial memory deficits in MAM rats

Prefrontal pyramidal neurons exhibit temporal summation of excitatory synaptic potentials that is critical for cognitive functions (Wang, 1999). To investigate the effect of early LY37 treatment on the temporal summation of PFC pyramidal neurons in MAM model (Xing et al., 2016), the membrane potentials of layer 5 pyramidal neurons were held to -70 mV, and 10 stimuli at 20 Hz (50 ms intervals) were applied to layers I–II border on PFC slices. The peaks of evoked EPSPs and the area under the EPSPs were measured to determine the temporal summation in response to the 10-pulse stimuli (Fig. 7A). By two-way RM ANOVA for the peak of eEPSPs, there was a significant effect of the number of stimuli ($F_{(9, 297)} = 8.91$, $p < 0.0001$), treatment ($F_{(3, 33)} = 3.62$, $p = 0.023$), and an interaction ($F_{(27, 297)} = 2.55$, $p < 0.0001$, Fig. 7B). *Post-hoc* analysis revealed a significant difference in the peak of eEPSPs on stimuli 7–10 between SAL-SAL and MAM-SAL groups (Bonferroni's test, stimuli 7 $p = 0.021$, stimuli 8 $p = 0.0003$, stimuli 9 $p < 0.0001$, stimuli 10 $p < 0.0001$). Compared with MAM-SAL, MAM-LY37 group showed significant increase of eEPSPs amplitude on stimuli 8 ($p = 0.030$), stimuli 9 ($p = 0.026$), and stimuli 10 ($p = 0.018$). For the area under the EPSPs, a significant decrease was found in MAM-SAL group compared with SAL-SAL control and MAM-LY37 ($p = 0.0029$, Kruskal-Wallis test, MAM-SAL vs. SAL-SAL, $p = 0.0056$, MAM-SAL vs. MAM-LY37, $p = 0.010$, Dunn's test, Fig. 7C). These results suggested that the reduced temporal summation of excitatory synaptic potentials could be corrected by juvenile LY37 treatment in MAM model.

One of the distinguishing characteristics of cognitive impairments in SZ patients is cognitive deficits, which are highly related to PFC dysfunction and commonly observed in MAM model. In this study, we evaluated the effect of early LY37 treatment on spatial memory in MAM model by using spontaneous alternation T-maze. MAM-SAL rats exhibited deficient spontaneous alternation with a 30 seconds intra-trial delay compared with SAL-SAL controls ($p = 0.0064$, Kruskal-Wallis test, $p = 0.0055$, Dunn's test, Fig. 8A), and the impairment was prevented by juvenile LY37 treatment (SAL-SAL vs. MAM-SAL, $p = 0.0078$, MAM-SAL vs. MAM-LY37, $p = 0.02$, Dunn's test, Fig. 8A).

MAM rats have increased response to the open channel NMDAR antagonists that is relevant to an NMDAR hypofunction model of SZ (Le Pen et al., 2011; Phillips et al., 2012). Thus, we examined whether early LY37 treatment would prevent the MK801 (0.05 mg/kg)-induced hyperactivity in MAM animals (Fig. 8B). We measured the locomotor activity of

male rats in the novel open field for 30 minutes (spontaneous locomotion), then i.p. injected MK801 (0.05 mg/kg) and recorded the locomotion for 60 minutes (MK801-induced hyperlocomotion). By two-way RM ANOVA, there was a significant effect of time ($F_{(17, 578)} = 16.38, p < 0.0001$), treatment ($F_{(3, 34)} = 2.93, p = 0.048$), but not an interaction ($F_{(51, 578)} = 0.98, p = 0.51$, Fig. 8B, $n = 7-12$). The *post hoc* analysis revealed that MAM-SAL group exhibited increased spontaneous locomotor activity in response to a novel context in comparison to SAL-SAL controls after first 5 minutes ($p < 0.05$, Bonferroni's test), whereas MAM-LY37 rats showed significantly increased locomotor activity only in the 5–10 minute period ($p < 0.05$, Bonferroni's test). After MK801 injection, significant hyperlocomotion was observed in MAM-SAL animals from 75–90 min ($p < 0.05$, Bonferroni's test). These results suggest that juvenile LY37 treatment ameliorated not only MAM exposure-induced spontaneous hyperactivity but also prevented MK801-induced hyperlocomotion in the MAM model.

3.7. Juvenile LY37 treatment does not change the electrophysiological properties and basal excitatory neurotransmission of layer 5 pyramidal neurons from PFC of normal rats

Early LY37 treatment during this critical period may have a deleterious influence on prefrontal pyramidal neurons. To test the side effect possibility, normal rats were used to receive LY37 treatment (0.3 mg/kg/day, i.p., once daily) during juvenile. After 1 hour, 1 week, 5 weeks and 10 weeks from the last treatment, we recorded action potentials (AP), in slices from prefrontal cortical layer 5 pyramidal neurons in response to varying step currents from -300 pA to $+550$ pA. Only one hour after the last LY37 injection, we found an increase in spike number with high current injection ($+400$ to $+500$ pA) in cells from LY37 treated rats ($F_{\text{current}}(11, 275) = 63.17, p < 0.0001$, $F_{\text{treatment}}(1, 25) = 1.72, p < 0.0001$, $F_{\text{interaction}}(11, 275) = 6.23, p < 0.0001$, two-way RM ANOVA, SAL treatment vs. LY37 treatment $p < 0.05$, Bonferroni's test, Fig. 9A). There was no difference in spike number, resting membrane potential, AP threshold, AP peak amplitude, AP 1/2 width, 20–80% rise time, and the afterhyperpolarization between LY37- and SAL-rats at 1 week, 5 weeks and 10 weeks after LY37 treatment (Fig. 9B–D and Table 1). These results suggested that repeated LY37 treatment during late juvenile has little long-lasting effect on physiological properties in prefrontal pyramidal neurons.

Furthermore, we also recorded the spontaneous EPSCs to examine the excitatory synaptic transmission following developmental LY37 treatment. One hour after the last treatment of 0.3 mg/kg LY37, we found significantly increased amplitude ($t(27) = 2.33, p = 0.028$, unpaired t-test), but not frequency ($p = 0.43$, Mann Whitney test), of sEPSCs compared with SAL controls (Fig. 9E and 9F). However, 1 week, 5 weeks and 10 weeks after repeated treatment with LY37 during juvenile, we did not find any significant alterations in sEPSC frequency and amplitude (Fig. 9E and 9F) when compared with saline-treated controls. Altogether, our results suggest no obvious side effect of repeated LY37 treatment on neuroexcitatory and synaptic transmission in normal developing PFC.

4. Discussion

We report here that treatment with mGluR2/3 agonist LY37 during juvenile period prevents prefrontal hypofunction of NMDAR and rescues dendritic spine deficits and memory impairments in the neurodevelopmental MAM model of SZ without obvious lasting effects on the physiological properties of layer 5 PFC pyramidal neurons. Specifically, at the molecular level, LY37 treatment restores postsynaptic GluN2B-NMDARs functions in developing PFC by enhancing GSK3 β phosphorylation at Ser9 (Fig. 10).

Memory impairment is one of the common cognitive deficits and prodromal symptoms prior to psychosis onset in SZ patients (Haut et al., 2015). It is hypothesized that progressive loss of connectivity of mPFC (indicated by reduction in cortical thickness by MRI) from the prodromal to fully psychotic phases, leading to aberrant monitoring beliefs and episodic memories and the subsequent development of false episodic memories and delusions (Cannon, 2015). Two recent studies with magnetic resonance imaging support this hypothesis: 1) a steeper rate of gray matter thinning in the PFC from youth at clinical high risk progressing to full psychosis compared to individuals who did not converted to psychosis (Cannon et al., 2015); 2) a positive correlation was found between unusual thought content at baseline and the rate of gray matter loss in PFC among clinically high risk youth eventually developing psychotic disorders (Chung et al., 2015). Consistent with our previous report (Snyder et al., 2013), poor spatial learning and memory performance was observed in juvenile MAM rats, reminiscent of the episodic memory deficits that observed among at-risk subjects who later display psychosis (Allen et al., 2011; Lencz et al., 2006). Memory deficits are considered as an important therapeutic target for improving outcomes in SZ (Ragland et al., 2012; Ragland et al., 2009). Water maze is typically considered a hippocampus-dependent task, previous studies have shown the PFC impairment in rodent also induces poor performance in water maze (de Bruin et al., 1997; Mogensen et al., 1995; Woolley et al., 2013). Although we cannot rule out the possibility that LY37 improves the water maze impairments via restore hippocampal NMDAR hypofunction (Snyder et al., 2013), our data have indicated that learning deficits can be corrected by juvenile LY37 treatment, which correlated with spatial memory improvement in adult MAM rats.

In this study, we investigated mGluR2/3 agonists for the potential to treat juvenile learning and memory deficits because of their actions in modulating NMDARs. Pharmacological blocking NMDARs exacerbates psychotic symptoms of SZ patients and manifest behavioral anomalies related to phenomena observed in SZ. LY37 treatment can prevent ketamine- and MK-801-induced hyperlocomotion probably via increasing both GluN2A and GluN2B expression (Imre et al., 2006; Xi et al., 2011). In the MAM model, NMDAR dysfunction is evident during juvenile and adolescence that underlie long-term potentiation (LTP) and memory deficits (Snyder et al., 2013). We found LY37 at 0.3 mg/kg as a pretreatment 30 min before training session improved water maze performance in MAM rats. Among NMDAR subunits, MAM rats exhibited a significant GluN2B decrease in mPFC during juvenile (Li et al., 2017), suggesting LY37 would primarily target on GluN2B subunits, which is confirmed by our western blot results.

We also observed decreased inhibitory pGSK3 β Ser9 levels (hyperactive GSK3 β) in MAM PFC, and these changes were restored by repeated LY37 treatment. Under normal conditions, inhibition of GSK3 can facilitate the internalization of GluN2B-containing NMDARs (Chen et al., 2007; Wildburger and Laezza, 2012). However, in a hyperdopaminergic condition, increased internalization and decreased protein synthesis of GluN2B-NMDARs were reported in the PFC through GSK3 β over-activation (Li et al., 2009). Previous studies have shown a hyperdopaminergic state in MAM model that consisting of a greater number of spontaneously firing in ventral tegmental DA neurons (Gill et al., 2011; Lodge and Grace, 2007). Thus, inhibition of GSK3 β activity by LY37 treatment potentiates prefrontal GluN2B-NMDARs function in juvenile MAM rats. Notably, in our recent study, we found that direct inhibition of GSK3 β during the early stage of postnatal development could reverse memory deficits in MAM model by correcting synaptic plasticity imbalance (Xing et al., 2016). However, we also observed an obvious memory impairment in SAL rats treated with GSK3 β inhibitor SB216763 in juvenile that was not found with juvenile LY37 treatment.

Remarkably, low (0.1 mg/kg) and high doses (1.0 mg/kg) of LY37 did not improve learning and memory in MAM rats. It is well known that mGluR2/3 agonists reduce glutamate release and decrease excitation of the postsynaptic neuron, whereas stimulation of postsynaptic mGluR2/3 results in enhancement of AMPARs and NMDARs function (Li et al., 2015a). The minimal effect of 0.1 mg/kg LY37 may be ascribed to insufficient mGluR2/3 activation, while excessive presynaptic mGluR2/3 stimulation leading to a lack of glutamate release may account for the insufficiency of a high dose of LY37. This explanation is in line with a recent report that infusion of APDC, a highly selective and potent mGluR2/3 agonist, into rat PFC enhanced T-maze performance at only low, but not high, doses (Jin et al., 2016). The inverted-U dose-response of mGluR2/3 agonists may explain why no beneficial effects of mGluR2/3 agonists LY354740 (3–10 mg/kg) were detected on SZ-like cognition impairments (Schlumberger et al., 2009). Intriguingly, juvenile MAM rats showed a decreased level of mGluR2 in PFC, probably located at presynaptic sites. However, these deficits seem less likely to be involved in the action of LY37 on learning improvement. These data further suggest that the effect of beneficial dose of mGluR2/3 agonists is dependent on postsynaptic mechanisms.

We have recently shown a normal basal AMPAR-mediated neurotransmission in juvenile MAM rats (Xing et al., 2016). Thus, the major impairment of excitatory neurotransmission caused by prenatal MAM exposure is NMDAR-mediated neurotransmission, especially GluN2B-NMDARs function as shown in this study. During normal postnatal development, GluN2B-to-GluN2A subunit switch appears to be absent in the PFC local circuitry, and the levels of GluN2B remain persistently high until adulthood in PFC (Wang et al., 2008). This enrichment of GluN2B subunits endows the PFC with the enhanced temporal summation properties required for working memory because of its slower decay time compared to GluN2A (Monaco et al., 2015). Given the depressed GABAergic synaptic transmission in the developing PFC of MAM rats (Xing et al, unpublished observations), decreased levels of GluN2B levels may result in restored excitatory/inhibitory balance at a low level, leading to cortical network distortion and inefficiency (Murray et al., 2014).

Previous preventive therapy studies on animal models can reduce SZ-like behaviors in adults and most of the studies selected the juvenile period as an intervention window (Marin, 2016). Here, we report that spatial memory deficits, spontaneous hyperlocomotion and MK801-induced hyperactivity in MAM rats are prevented by juvenile LY37 treatment. Behavioral deficits in MAM model were rescued by juvenile LY37 treatment, reminiscent of the preventive effect of GSK3 β inhibitor in our recent study (Xing et al., 2016). Because LY37 reversed the decreased pGSK3 β levels in PFC at an early stage, the restored dendritic spine density and spatial memory ability are likely attributable to the inhibition of GSK3 β activity. Notably, the inhibition of GSK3 β activity by SB216763 during the juvenile period was reported to improve functional connectivity between hippocampus and PFC and reverse working memory impairments in Df(16)A^{+/-} mice, a genetic model of SZ derived from genome-wide association studies (Tamura et al., 2016). It would be interesting to test whether LY37 can rescue cognitive deficits accompanied by developmental hyperactive GSK3 β in SZ animal models such as the Df(16)A^{+/-} mice.

MAM rats exhibited MK801-induced hyperactivity that mimicked exacerbation of psychotic symptoms in subjects with SZ driven by NMDAR antagonism (Le Pen et al., 2011; Phillips et al., 2012). Previous studies reported a dose difference of MK801 in evoking hyperactivity. A low- (0.05 mg/kg) but not high dose (0.2 mg/kg) of MK801 produced a notable psychostimulating effect on MAM rats, whereas the higher dose (0.2 mg/kg) of MK801 exerted the strongest hyperactivity in SAL controls (Le Pen et al., 2011). This dose difference was probably due to the high basal level of locomotion observed in the MAM model. Our findings indicated that juvenile LY 37 administration prevented both spontaneous hyperactivity and hypersensitivity to MK-801-induced hyperactivity. A similar effect was elicited using diazepam as a pharmacological intervention during the juvenile stage (Du and Grace, 2013), suggesting that enhancement of GABAergic tone may account for the actions of LY37 in reducing hyperlocomotion in adult MAM rats.

One of the caveats of early intervention for SZ is the difficulty of assessing the impact of treatments on non-syndromic conditions. Especially, we found that treatment with GSK3 β inhibitor in early stages appears to be problematic, with significant alteration of morphological changes in dendritic spines of mPFC pyramidal neurons (Xing et al., 2016). Thus, we assessed the long-term outcome of juvenile LY37 treatment in normal rats. Importantly, we observed little influence on neuronal excitation and excitatory neurotransmission even 10 weeks after LY37 administration, and no changes in spine morphology, suggesting a relatively safe early intervention.

However, our study also had several limitations. First, only a single dose of LY37 treatment during juvenile was shown to improve behavioral deficits in adult, it is not clear how wide the window of the efficacy of LY37 would be, thus finding appropriate dosing could represent a significant challenge in further investigations. Second, the safety of the juvenile LY37 treatment in control rats is only based on physiological experiments, conducted only in layer 5 pyramidal neurons from the mPFC. There may be many other functional changes during LY37 treatment that deserves further studies, such as protein expression, connectivity in PFC and other brain regions that are not accounted for by the present study. Third, although spontaneous alteration T maze performed in this study were recognized as PFC-

dependent in some cases, it is widely viewed as a spatial memory task that is mediated by distributed circuits including the hippocampus. Thus, it remains unclear to what extent any of behavioral alterations in MAM rats are mediated by the PFC versus hippocampus or other regions. Therefore, further studies are warranted to unravel the potential side effects of mGlu2/3 agonists in other brain regions related to cognitive functions.

In conclusion, our results suggest that prefrontal NMDAR hypofunction and PFC-dependent behavioral anomalies in a neurodevelopmental SZ rat model can be prevented by juvenile LY 37 treatment, highlighting mGluR2/3 as a potential early intervention target for SZ.

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Highlights

1. Juvenile LY37 treatment alleviates learning deficits in MAM rats.
2. LY37 corrects prefrontal GSK3 β and GluN2B levels in MAM rats.
3. LY37 prevents working memory deficits in adult MAM rats.
4. LY37 has limited effect on normal developing PFC.

Figure 1. Repeated LY37 treatment during juvenile restores spatial learning and memory deficits in MAM rats

A. High (1.0 mg/kg), medium (0.3 mg/kg) and low (0.1 mg/kg) doses of LY37 treatment have no effect on escape latencies in SAL rats ($n = 6-12$ for each group). **B.** only 0.3 mg/kg LY37 treatment improves learning ability in MAM rats. There are significant shorter latencies on day 4 and day 5 in MAM-LY37 (0.3 mg/kg) rats compared with MAM-SAL rats ($n = 6-16$ for each group). **C.** MAM rats exhibited significantly longer escape latencies from training day 3 to day 5 in water maze (replotted from A and B). **D.** In probe test, MAM rats show decreased time in the target quadrant compared with SAL rats. There is a significant increase in time spent on target quadrant in MAM-LY37 (0.3 mg/kg) rats compared with their saline controls. **A–C**, three-way repeated measure ANOVA followed by Bonferroni's test. **D**, two-way ANOVA followed by Bonferroni's test.

Figure 2. LY37 treatment rescues GluN2B-NMDAR and phosphorylated GSK3β at Ser9 protein levels in the mPFC of juvenile MAM rats

A. Representative western blots and summary histograms show the NMDAR subunit and mGluR2/3 protein levels in the mPFC from SAL-SAL, SAL-LY37 (0.3 mg/kg), MAM-SAL, and MAM-LY37 rats 24 hours after probe test of water maze ($n = 6$). MAM exposure resulted in significant decreases of GluN2B and mGluR2, and the levels of GluN2B, but not mGluR2, are rescued by LY37 treatment. **B.** Representative western blots and summary histograms show the levels of Akt-GSK3β-mTOR signaling after probe test. No significant difference was detected for the Akt1, pAktSer473, mTOR or pmTORSer2448 among groups. MAM exposure resulted in reduced pGSK3βSer9 levels, which are restored by LY37 treatment. * $p < 0.05$, ** $p < 0.01$ two-way ANOVA followed by Bonferroni's test.

Figure 3. Juvenile MAM rats exhibit blunted presynaptic response to LY37

A. LY37 (300 nM, bath application) significantly increased PPR and decreased CV^{-1} of evoked NMDA-EPSCs in SAL group ($n = 9$ from 3 rats), indicating a decrease of glutamate release. **B.** In contrast, LY37 had no effect on both PPR and CV^{-1} in MAM rats ($n = 9$ from 3 rats), suggesting a blunted presynaptic response to mGlu2/3 agonists. * $p < 0.05$, ** $p < 0.01$ two-way ANOVA followed by Bonferroni's test

Figure 4. Subchronic treatment with a medium dose of LY37 prevents GluN2B-NMDAR hypofunction caused by MAM exposure

A and B. Representative eEPSC traces (**A**) and summarized input-output curves (**B**) of NMDAR-mediated EPSC in response to a series of stimulation intensities in SAL-SAL, SAL-LY37, MAM-SAL and MAM-LY37 rats ($n = 9-10$ cells from 3 rats for each group). In all three intensities, MAM rats show decreased NMDAR-eEPSCs compared with SAL-SAL and MAM-LY37 groups. * $p < 0.05$ vs. SAL-SAL, # $p < 0.01$ vs. MAM-LY37, two-way RM ANOVA followed by Bonferroni's test. **C.** Representative NMDAR-eEPSC traces at baseline and after Ro25-6981 (Ro, 1 μ M) bath application. The lower panel of scaled traces shows the reduced decays caused by Ro application as indicated by shadow area. **D.** Summary

histograms show that there is significant decrease of NMDAR-eEPSC amplitude in SAL-SAL (n = 12 cells from 4 rats), SAL-LY37 (n = 10 cells from 3 rats) and MAM-LY37 (n = 11 cells from 4 rats) groups after Ro application, but not that in MAM-SAL rats (n = 11 cells from 4 rats for each group). **E.** Summary graph shows a significant decrease of Ro-sensitive currents of NMDA channels in MAM-SAL but not in MAM-LY37 rats. * $p < 0.05$, ANOVA followed by Bonferroni's test. Data are mean $\pm$ SEM.

Figure 5. The effect of LY37 on postsynaptic NMDARs is dependent on pGSK3β activity
A and B. Representative NMDAR-EPSC original (upper) and scaled (lower) traces in SAL (A) and MAM group (B). Summary histograms show the decreases of amplitudes caused by LY37 are less affected by loading pGSK3βSer9 antibody (pGSK3β Ab, 10 mg/ml) into the recording pipettes in SAL group (n = 13 from 5 rats without pGSK3βAb, n = 12 from 5 rats with pGSK3βAb), but the increased decay time is blocked by postsynaptic pGSK3βSer9 inhibition. For MAM rats, No significant difference of amplitudes before and after LY37 with or without pGSK3βSer9 Ab (n = 13 from 5 rats without pGSK3βAb, n = 13 from 5 rats with pGSK3βAb), whereas increased decay time is blocked by postsynaptic pGSK3βSer9

inhibition. * $p < 0.05$, *** $p < 0.001$, two-way RM ANOVA followed by Bonferroni's test.
Data are mean $\pm$ SEM.

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Figure 6. Juvenile LY37 administration prevents prefrontal dendritic spine loss in adult MAM rats

A. During the LY37 treatment (P21 to P27), no difference in the body weight among SAL-SAL, SAL-LY37, MAM-SAL and MAM-LY37 ($n = 9$ for each group) is found. Represent images (**B**) of Golgi staining of mPFC showing that the decreased apical dendritic spine density in layer 5 pyramidal neurons from MAM rats is rescued by early LY37 treatment (**C**). Scale bars = $50 \mu\text{m}$ for the upper panel and $10 \mu\text{m}$ for the lower panel. **D.** No difference was found in mushroom, stubby, and thin spines fraction among SAL-SAL, MAM-SAL, SAL-LY37 and MAM-LY37 groups ($n = 8$ neurons from 4 rats). *** $p < 0.001$, ANOVA followed by Bonferroni's test. Data are mean $\pm$ SEM.

Figure 7. Juvenile LY37 treatment rescues prefrontal temporal potential summation of layer 5 pyramidal neurons in MAM rats

A. Representative traces of 10 stimuli evoked EPSPs from SAL-SAL (n=10 cells from 3 rats), SAL-LY37 (n=9 cells from 3 rats), MAM-SAL (n=9 cells from 3 rats), and MAM-LY37 (n=9 cells from 3 rats) groups. The scaled traces show the area under the EPSPs, the differences between saline-treatment and LY37 treatment are highlighted by the shadow area. **B.** Summary line graphs show that there is a decrease of eEPSP amplitude in MAM-SAL rats that can be correct by juvenile LY37 treatment. * $p < 0.05$, *** $p < 0.001$ vs. SAL-SAL, # $p < 0.05$ vs. MAM-LY37, two-way RM ANOVA followed by Bonferroni's test. Data are mean $\pm$ SEM. **C.** The area under EPSPs of MAM-SAL rats is significantly smaller than SAL-SAL group and MAM-LY37 group. * $p < 0.05$, ** $p < 0.01$, Kruskal-Wallis test followed by Dunn's test, Data are median $\pm$ interquartile range.

Figure 8. LY37 treatment in juvenile prevents spatial memory deficits and ameliorates MK801-induced hyperlocomotion in adult MAM rats

A. MAM rats showed impaired spatial memory assessed by spontaneous T-maze. These deficits were absent in the MAM rats with juvenile LY37 treatment. ** $p < 0.01$, # $p < 0.05$, Kruskal-Wallis test followed by Dunn's test, Data are median $\pm$ interquartile range. **B.** MAM animals exhibited a significantly increased spontaneous locomotion activity (arrowhead) in response to MK801 administration (0.05 mg/kg). Juvenile LY37 treatment normalized the locomotor activity in response to either novel environment (first 30 min) or MK801 (rest 60 min). * $p < 0.05$ MAM-SAL vs. SAL-SAL, # $p < 0.05$ MAM-LY37 vs. SAL-SAL, two-way RM ANOVA followed by Bonferroni's test. Data are mean $\pm$ SEM.

Figure 9. Juvenile LY37 treatment has little effect on neuronal excitability and excitatory neurotransmission in layer 5 pyramidal neurons in normal mPFC

Summary graphs of action potentials from saline- and LY37-treated rats 1 hour (A), 1 week (B), 5 weeks (C) and 10 weeks (D) ($n = 13$ to 20 from $6-7$ rats) after the last injection. Summary histograms of amplitude (E) and frequency (F) of spontaneous EPSCs recorded at -70 mV in layer 5 pyramidal neurons from normal juvenile rats treated with LY37 (0.3 mg/kg/day, i.p., once daily, P21–27) 1 hour, 1 week, 5 weeks and 10 weeks after the last injection. A–D, * $p < 0.05$, two-way RM ANOVA followed by Bonferroni's test. Data are mean $\pm$ SEM. E–F, * $p < 0.05$, unpaired student t-test.

Figure 10. Schematic graph explaining the mechanisms of mGluR2/3 agonists LY37 on prefrontal NMDAR hypofunction in MAM model

In normal condition, glutamate release from the presynaptic site, and activation of presynaptic mGluR2/3 inhibits glutamate release. Postsynaptic mGluR2/3, however, activates GSK3 β by decreasing the inhibitory pGSK3 β Ser9, leading to an increase of an NMDA-GluN2B function. The overall effect of LY37 depends on the dosage. In contrast, the reduction of presynaptic mGluR2 in MAM animals reduces the effect of LY37 on inhibition of glutamate release, whereas the postsynaptic enhancement of NMDAR-GluN2B modulated by postsynaptic mGluR2/3 retains. Treatment with LY37 during juvenile period rescues the GluN2B-GSK3 β signaling, improves the NMDAR hypofunction, and thus prevents the consequential morphological changes and behavioral deficits.

Table 1
Physiological Properties of layer V Pyramidal Neurons in normal rats after repeated SAL- or LY37 exposure

	Resting membrane potential (mV)	Input Resistance (M Ω)	Spike threshold (mV)	Peak spike amplitude (mV)	Afterhyperpolarization (mV)	Spike half-width (ms)
1hr after SAL	-63.8 $\pm$ 2.2	107.7 $\pm$ 9.6	-37.2 $\pm$ 2.4	64.3 $\pm$ 2.2	4.8 $\pm$ 0.4	2.8 $\pm$ 0.2
1hr after LY37	-56.6 $\pm$ 1.3	115.4 $\pm$ 11.3	-33.6 $\pm$ 2.6	71.8 $\pm$ 2.2	4.8 $\pm$ 0.4	2.8 $\pm$ 0.2
1 wk after SAL	-59.3 $\pm$ 1.2	88.1 $\pm$ 14.9	-34.9 $\pm$ 1.8	64.4 $\pm$ 3.6	2.8 $\pm$ 0.3	2.4 $\pm$ 0.3
1 wk after LY37	-55.3 $\pm$ 1.2	112.1 $\pm$ 7.8	-29.4 $\pm$ 2.6	61.3 $\pm$ 2.3	3.0 $\pm$ 0.3	3.1 $\pm$ 0.2
5 wks after SAL	-60.8 $\pm$ 1.9	104.2 $\pm$ 8.8	-46.4 $\pm$ 2.5	62.1 $\pm$ 2.1	2.8 $\pm$ 0.3	2.0 $\pm$ 0.1
5 wks after LY37	-59.8 $\pm$ 1.7	105.5 $\pm$ 10.0	-45.0 $\pm$ 1.8	63.6 $\pm$ 2.8	3.1 $\pm$ 0.4	2.1 $\pm$ 0.2
10 wks after SAL	-64.0 $\pm$ 1.1	105.7 $\pm$ 8.5	-40.8 $\pm$ 1.9	58.2 $\pm$ 2.4	2.3 $\pm$ 0.3	2.3 $\pm$ 0.2
10 wks after LY37	-60.9 $\pm$ 1.6	109.6 $\pm$ 7.3	-43.1 $\pm$ 1.7	65.5 $\pm$ 2.3	2.0 $\pm$ 0.2	2.2 $\pm$ 0.1

There were no significant differences between cells from SAL- and LY37-treated rats at different time points (all $p > 0.05$).