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LY395756, an mGluR2 agonist and mGluR3 antagonist, enhances NMDA receptor expression and function in the normal adult rat prefrontal cortex, but fails to improve working memory and reverse MK801-induced working memory impairment

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Abstract

Targeting group II metabotropic glutamate receptors (mGluR2/3) has been proposed to correct the dysfunctional glutamatergic system, particularly NMDA receptor (NMDAR) hypofunction, for treatment of schizophrenia. However, how activation of mGluR2/3 affects NMDAR function in adult animals remains elusive. Here we show the effects of LY395756 (LY39), a compound acting as both an mGluR2 agonist and mGluR3 antagonist, on the NMDAR expression and function of normal adult rat prefrontal cortex (PFC) as well as working memory function in the MK801 model of schizophrenia. We found that *in vivo* administration of LY39 significantly increased the total protein levels of NMDAR subunits and NR2B phosphorylation in the PFC, along with the amplitude of NMDAR-mediated miniature excitatory postsynaptic currents (mEPSC) in the prefrontal cortical neurons. Moreover, LY39 also significantly increased mTOR and pmTOR expression, but not ERK1/2, Akt, and GSK3 β , suggesting an activation of mTOR signaling. Indeed, the mTOR inhibitor rapamycin, and actinomycin-D, a transcription inhibitor, blocked the enhanced effects of LY39 on NMDAR-mEPSCs. These results indicate that LY39 regulates NMDAR expression and function through unidentified mTOR-mediated protein synthesis in the normal adult rat PFC. However, this change is insufficient to affect working memory function in normal animals, nor to reverse the MK801-induced working memory deficit. Our data provide the first evidence of an *in vivo* effect of a novel compound that acts as both an mGluR2 agonist and mGluR3 antagonist on synaptic NMDAR expression and function in the adult rat PFC, although its effect on PFC-dependent cognitive function remains to be explored.

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Conflict of interest

The authors claim no financial conflict of interests.

Keywords

mGluR2 agonist and mGluR3 antagonist; NMDA receptor; mTOR signaling; protein synthesis; prefrontal cortex; cognitive function; animal model; schizophrenia

Introduction

Cognitive dysfunction in schizophrenia (SCZ) usually persists into asymptomatic periods and is not reversed by dopaminergic antipsychotic treatments (Kahn and Sommer, 2015; Millan et al., 2012; Weinberger and Gallhofer, 1997). Although the mechanism associated with cognitive impairment of SCZ remains unclear, the N-methyl-D-aspartate receptor (NMDAR) hypofunction hypothesis is widely linked with cognitive deficits (Lisman et al., 2008; Snyder and Gao, 2013). Unfortunately, current antipsychotic drugs almost exclusively target dopaminergic and serotonergic systems without direct effects on glutamatergic receptors (Howes and Kapur, 2009; Miyamoto et al., 2005; Seeman, 2011). Therefore, the searching for novel treatments that can recover the dysfunctional glutamate system is a promising strategy in drug development for treatment of SCZ, especially for those patients whose symptoms are unrelated to dopaminergic dysfunction (Howes and Kapur, 2014).

We are particularly interested in the group II metabotropic glutamate receptors (mGluRs) because mGluR2/3 agonists have exhibited therapeutic potential for different types of neurological and psychiatric disorders, particularly SCZ, due to their regulatory effects on the glutamatergic system (Conn et al., 2009; Fell et al., 2012; Li et al., 2015; Mezler et al., 2010; Moghaddam and Adams, 1998).

Activation of mGluR2/3 can directly decrease presynaptic glutamate release via $G_{\alpha i/o}$ -mediated inhibition of the adenylate cyclase-cyclic AMP-Protein Kinase A pathway (Cartmell and Schoepp, 2000; Moghaddam, 2004; Schoepp et al., 1999). However, recent studies have also provided evidence that mGluR2/3 agonists enhance postsynaptic excitatory receptor function, including both α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) and NMDARs. In particular, we found that selective mGluR2/3 agonist LY379268 reverses dizocilpine (MK-801)-induced postsynaptic NMDA dysfunction via activation of the glycogen synthase kinase 3 β (GSK3 β) pathway (Xi et al., 2011). In addition, LY379268 also increases surface expression and function of AMPARs through activation of extracellular signal-regulated kinase1/2 (ERK1/2) and GSK3 β signaling pathways in cultured prefrontal cortical neurons (Wang et al., 2013).

LY395756 (LY39) is a novel compound that serves as both an mGluR2 agonist and mGluR3 antagonist ($K_i = 0.165$ and $0.302 \mu\text{M}$, respectively) (Ceolin et al., 2011; Lucas et al., 2013). We recently observed that LY39 also exhibited a strong effect on AMPAR expression in cultured neurons (Wang et al., 2013). Given that mGluR2 and mGluR3 display distinct regional, cell-type, and synaptic specificities (Petralia et al., 1996; Tamaru et al., 2001; Wright et al., 2013), we speculate that mGluR2 and mGluR3 receptors may play different regulatory roles in synaptic transmission, and consequently have differential clinical implications. Indeed, evidence suggests that mGluR2 mainly mediates antipsychotic activity in SCZ (Fell et al., 2008; Woolley et al., 2008), while mGluR3 exerts neuroprotective effects

(Durand et al., 2013). These findings prompt us to further examine whether targeting mGluR2 with LY39 would have an effect on NMDAR function in adult rat PFC and whether this action is sufficient to affect cognitive function. Our results showed that LY39 significantly increased NMDAR expression in the PFC and function of the prefrontal cortical neurons, and this enhancement was mediated by activation of mammalian target of rapamycin (mTOR)-dependent protein synthesis. However, this effect is insufficient to reverse MK801-induced working memory deficits.

Materials and Methods

Animals and Treatments

Adult male Sprague Dawley rats (P90, 330–360g) were purchased from Charles River Laboratories (Wilmington, MA). They were housed under conditions of constant temperature (21–23°C) and humidity on a reverse 12 hour light/dark cycle with food and water available *ad libitum*. Animals were allowed to adapt to the new environment for 2 days before the experiments. The animal procedures were performed in accordance with the National Institutes of Health (NIH) animal use guidelines and were approved by the Institutional Animal Care and Use Committee of Drexel University College of Medicine.

LY39, (+)-MK801 maleate, and Actinomycin-D were purchased from Tocris Bioscience (Minneapolis, MN) and Rapamycin was purchased from LC Laboratories (Woburn, MA).

Acute treatment for Western blot assay

LY39 at a dose of 0.3, 1.0, or 3.0 mg/kg (single dose, intraperitoneally, I.P.) was administered one hour before the rats were sacrificed for tissue collection as a peak brain concentration of a similar compound LY379268 could be reached within 30 min post I.P. administration (Bond et al., 2000). For electrophysiological recording, a single injection of LY39 (3.0 mg/kg, I.P.) was administered one hour before the animals were sacrificed for *ex vivo* slices. In both cases, saline solution (0.9 % sodium chloride) was used as a vehicle control with 5 animals in each treatment group. In additional experiments, rapamycin (1 mg/kg) (Autry et al., 2011), actinomycin-D (act-D, 0.5 mg/kg) (Miller et al., 2014), or saline was administered 30 min before LY39 or saline injection. All animals were deeply anesthetized with Euthasol (0.2 ml/kg, Virbac Animal Health) and were decapitated per the approved IACUC protocol.

Adult rat perfusion

To preserve brain tissue quality, adult rats were anesthetized with Euthasol and then rapidly perfused with intracardiac injection of 60 ml ice-cold sucrose buffer (in mM: 320 sucrose, 4 HEPES-NaOH buffer, pH 7.4, 2 EGTA, 1 sodium orthovanadate, 0.1 phenylmethylsulfonyl fluoride, 10 sodium fluoride, 10 sodium pyrophosphate) for Western blotting, or of 60 ml cold 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) for electrophysiology.

Synaptic membrane protein collection

The animals were decapitated, and the brains were quickly removed. The forebrain containing the prelimbic area was dissected, homogenized in cold lysis buffer (in mM: 320 sucrose, 4 HEPES-NaOH buffer, pH 7.4, 2 EGTA, 1 sodium orthovanadate, 0.1 phenylmethylsulfonyl fluoride, 10 sodium fluoride, 10 sodium pyrophosphate, with 1 µg/ml leupeptin and 1 µg/ml aprotinin). The tissues were centrifuged at 1,000g for 10 minutes at 4°C to remove large cell fragments and nuclear materials, and the resulting supernatant was centrifuged again at 15,000g for 15 minutes at 4°C to harvest cytoplasmic proteins in the supernatant. The pellet from this spin was resuspended in lysis buffer and centrifuged at 15,000g for an additional 15 minutes at 4°C to produce synaptosomes. The synaptosomal fraction was then hypoosmotically lysed and centrifuged at 25,000g for 30 minutes at 4°C to collect the crude synaptosomal pellet. Lysis buffer was added to the pellet to make the final samples, which were then stored in -80°C for future use, or aliquots were made and stored at -20°C for immediate use.

Western blots

A bicinchoninic acid (BCA) protein assay was performed to determine protein concentration. The protein sample was mixed with 4× laemmli and lysis buffer, boiled for 5 minutes, and separated on a 7.5% SDS-PAGE gel. After electrophoresis, proteins were transferred to Immobilon PVDF membranes (Millipore, IPVH00010). The membrane was blocked in 5% nonfat milk and probed with primary antibodies at 4°C overnight. Each blot was used to probe multiple antibodies, including anti-mouse NR1 (Invitrogen, 32-0500, 1:5000), anti-rabbit NR2A (Millipore, 04-901, 1:2,500), anti-mouse NR2B (Millipore, 05-920, 1:2000), anti-rabbit pNR2B-Tyr1472 (CALBIOCHEM, 1:1000), anti-rabbit pNR2B-Ser1303 (Millipore, 07-398, 1:1000), anti-rabbit Akt (Cell Signaling Technology, 2938S, 1:1000), anti-rabbit pAkt-Ser473 (Cell Signaling Technology, 4060S, 1:1000), anti-rabbit ERK1/2 (Cell Signaling Technology, 4695S, 1:10,000), anti-rabbit pERK1/2 (Cell Signaling Technology, 4370S, 1:10,000), anti-rabbit GSK3b (Cell Signaling Technology, 9315S, 1:20,000), anti-rabbit pGSK3b-Ser9 (Cell Signaling Technology, 9336S, 1:2000), anti-rabbit mTOR (Cell Signaling Technology, 2983S, 1:2000), anti-rabbit pmTOR-S2448 (Cell Signaling Technology, 2983S, 1:1000), anti-4E-BP1 (Cell Signaling Technology, 9452, 1:1000), anti-p4E-BP1 (Cell Signaling Technology, 236B4, 1:500-1000), and anti-mouse actin (Sigma, A5316, 1:100,000) served as a loading control. Membranes were stripped for 30 minutes with Restore Western Blot Stripping Buffer (Thermo Scientific, 21063) between each different set of primary antibodies. Specifically, we probed phospho-antibodies first and then probed antibodies against each corresponding total protein. The blots were incubated with horseradish peroxidase-coupled anti-rabbit or anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at room temperature for 1 hour. After primary and secondary antibody incubation, Tris buffered saline with Tween 20 (TBST) was used to rinse the membranes three times for 20 minutes each. Proteins were visualized using enhanced chemiluminescence kit (ECL Plus, Amersham Biosciences). Each group included at least 5 animals and samples from each animal were run at least 4 times to minimize inter-blot variance. The data were analyzed with Image J (NIH) and Student's t-test was used for statistical analysis. The data were presented as mean ± SEM.

Electrophysiological recording in prefrontal cortical slices

One hour after saline or LY39 injection, the animals were anesthetized and brains were removed. Coronal slices of the forebrain containing the prelimbic area (mPFC) were cut at 300 μm within an ice-cold oxygenated sucrose solution (in mM: 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 25 NaHCO_3 , 75 sucrose, 25 glucose, 0.5 CaCl_2 , 7.0 MgSO_4 , pH 7.4) using a Leica VT 1200S Vibratome (Leica Microsystems Inc., Buffalo Grove, IL). Slices were transferred to a holding chamber, submerged in oxygenated Ringer's solution (in mM: 124 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 2 CaCl_2 , 1 MgSO_4 , 26 NaHCO_3 , and 10 dextrose, pH 7.4) at 35°C for one hour. The PFC slices were transferred into a heated (36°–37°C) recording chamber mounted on an Olympus BX51 upright microscope (Olympus America Inc., Center Valley, PA), where the slices were bathed in oxygenated Ringer's solution. Neurons were visualized with the assistance of infrared differential interference contrast via a Panasonic CCD video camera. Borosilicate glass pipettes purchased from the Harvard Apparatus (Holliston, MA) were pulled with a P-97 puller (Sutter Instruments, Novato, CA) to create patch electrodes with a resistance of 5–7 M Ω . The electrical signals were amplified and filtered at 1 kHz with a MultiClamp 700B amplifier, converted through a DigiData 1322A, and recorded through pCLAMP 9.2 software (Molecular Devices, Sunnyvale, CA).

To record NMDAR- and AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs), a Cs^+ -intracellular solution (in mM: 110 D-gluconic acid, 110 CsOH, 10 CsCl_2 , 1 EGTA, 1 CaCl_2 , 10 HEPES, 1 Mg-ATP at pH 7.3 adjusted with CsOH and 5 QX-314) was filled into the pipette in order to block sodium and potassium channels. Under voltage clamp mode, AMPAR-mEPSCs were recorded with membrane potential held at -70 mV in the presence of sodium channel blocker tetrodotoxin (TTX, 0.5 μM) and GABA_A receptor antagonist picrotoxin (100 μM), whereas NMDAR-mEPSCs were recorded with membrane potential held at $+60$ mV in the presence of TTX (0.5 μM), picrotoxin (100 μM) and AMPAR antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX, 20 μM). A 5-min stable baseline EPSC recording was recorded for each cell to ensure the reliability of the recording, and then the recording was continued for another 5 min under each condition. The series resistance was compensated and constantly monitored by injection of -100 pA current pulse (150 ms duration).

Data analysis

For the Western blot assay, the relative expression of proteins was evaluated by measuring the optical density of each band with Image J software. The densitometry of total protein was normalized to actin, whereas phosphorylation of a protein was normalized to its respective total protein level.

For electrophysiological recording, data were analyzed with Clampfit 9.2 (Molecular Devices, Sunnyvale, CA). Cells without a stable baseline recording and cells with a series resistance change of $>20\%$ were excluded from data analysis. To analyze the mEPSC events, a typical AMPAR-mediated mEPSC or an NMDAR-mediated mEPSC was selected to create a template, and then mEPSC events within a 5-min recording were detected by the Clampfit software with a threshold set at 5. All detected events were checked to ensure reliability of

the method. The mEPSC frequency (number of events) was normalized to events per second (Hz) and the mEPSC amplitude was measured from the onset to the peak of the events.

All data were presented as mean $\pm$ SEM. Data between two groups were analyzed with unpaired Student's t-tests, and comparisons of multiple groups were carried out with ANOVA followed by Tukey's post hoc test to compare between individual groups. Statistical analysis was performed using GraphPad Prism (GraphPad software Inc., La Jolla, CA) and a p-value <0.05 was considered statistically significant.

Assessment working memory test using cross maze

Treatment schedule

Thirty two male adult Sprague Dawley rats (P90, 300–350g) were randomly assigned to four treatment groups: Saline+Saline (n=8), Saline+LY39 (n=8), MK801+Saline (n=8) and MK801+LY39 (n=8). The injection procedure was identical for each animal. Briefly, on the test day (set-shifting day), the first injection was given 90 min before training started. Different groups of rats received saline (0.5 ml, 0.9%, i.p.) or an NMDAR antagonist MK801 (0.1mg/kg dissolved in 0.9% saline, i.p.; Ascent Scientific, Bristol, UK). The dose of MK801 was in accordance with previous work (Antonios Boultsadakis and Pitsikas, 2010) and our own study (Xi et al., 2011). Thirty min after the first injection, each respective group received their second injection of either saline (0.5 ml, i.p.) or LY39 (3.0 mg/kg).

Attentional set-shifting task

The procedure of the behavioral experiment was similar to that described in previous publications (Block et al., 2007; Jeevakumar et al., 2015). Briefly, the set-shifting task was processed in a wooden cross maze that was painted white, with four $14.5 \times 4.5 \times 9.0$ inch arms. Prior to the behavioral test, rats were food-restricted to 85% of their free-feeding weight and handled 5 min daily for 5 days to become familiar with the experimenter and the local environment. Broken pieces of Frosted Cheerios were used as the food reward and were given to the animals in their home cage to habituate them to the taste and texture.

On the first day of habituation, four pieces of food reward were placed throughout each arm. Then, rats were put into the center of the cross maze with their cage-mates to freely explore the maze. If all 16 bits of food reward were consumed within 5 min, the maze was rebaited, while rats were kept in the holding cage. Then they were placed back into the center of the maze, and allowed to freely explore for an additional 5 minutes. On the second day, rats were placed individually into the maze with only one Cheerios bit in the well of each arm. Rats were required to consume all of the food reward three times within 10 mins to reach criterion and continue to Day 3. If they did not, this procedure would be repeated on the next day.

The turn bias was determined on the following day. One arm of the cross maze was blocked off to form a T-maze. Each animal was placed into the stem arm and allowed to turn either left or right to retrieve the food reward. After the reward was consumed, the rat was returned back to the stem arm. If the rat chose the same arm into which it initially turned, it was placed back into the stem arm until the animal turned to the opposite direction. This was

repeated for 7 trials. Any direction initially chosen four times or more out of seven was considered the animal's turn bias.

The next day, rats began the Response Discrimination day. On this day, they needed to turn the opposite direction of their turn bias (as determined on the previous day) to get the food reward. Rats pseudorandomly started from either the North, East, or West arm. A visual cue, which was a (10 × 4 inches) plastic sheet with diagonal black and white stripes, was placed on the floor of either goal arm. Both the placement of the visual cue and the location of the start arm varied pseudorandomly in order to balance the frequency of occurrences across blocks of 12 consecutive trials. Between trials, there was a 15 second inter-trial delay. Criterion was reached when a rat made 9 correct choices out of 10 consecutive trials. Then, a probe trial was given in which rats were placed into the fourth arm (the unused South arm) and the visual cue was always opposite to the correct arm. Performing correctly on the probe trial was used as an indicator to stop response discrimination. Otherwise, if an error was made, the training continued until rats made another 5 consecutive correct trials, followed by another probe trial. This was repeated until rats made a correct choice on the probe trial.

On the attentional set-shifting day, rats were trained to use the visual cue to guide their choices. The location of the start arm and visual cue were identical to those used the previous day. The training and response criteria on the set-shifting day were also the same as those used on the Response Discrimination day. Rats needed to make 9 correct choices out of 10 consecutive trials to reach criterion, and the probe trial was conducted similar to the previous day. However, the visual cue was always placed into the arm opposite to the direction that was rewarded during Response Discrimination.

For each day, we analyzed the total number of trials to criterion and the number of probe trials. On the set-shifting day, we also analyzed the number of errors defined as entries into arms without the visual cue. For investigating the shift in strategies, errors were further divided into three types. Type I errors were perseverative errors, by which the rat persistently used the old strategy to make choices, continuing to return to the side which was acquired on the response discrimination day, even when the visual cue was absent. Six trials in each block of 12 allowed for these errors. These trials were further divided into blocks of 4 consecutive trials. Perseverative errors were recorded if the rat turned to the same side as response discrimination 3 times or more in one block of 4 trials. Once the rat made less than 3 perseverative errors within one block of 4 trials, all of the subsequent errors were recorded as regressive errors. This is because now the rat appeared to have utilized a different strategy in 50 % of the trials. Type III errors, never reinforced errors, were recorded when the rat turned to the arm that did not contain the cue and was opposite to the side of response discrimination.

Results

In vivo treatment with LY39 induces a significant enhancement of NMDAR synaptic membrane protein expression in the adult rat PFC in a dose-dependent manner

To determine whether LY39 can directly modulate the expression of NMDAR subunits *in vivo*, the rats were treated with three different doses of LY39 (single i.p. injection) with

saline as a vehicle control. As shown in Figure 1, LY39 at a dose of 0.3 mg/kg had no effects on the total protein levels of NR1, NR2A, and NR2B, as well as phosphorylation levels of pNR2BTyr1472 (Y1472) and pNR2BSer1303 (S1303) (n=5 for both saline and LY39, p=0.488, 0.793, 0.540, 0.704, 0.208, respectively; Fig. 1A). However, when we increased the LY39 dose to 1.0 mg/kg, there was a significant increase in the total protein level of NR2B, but no significant changes in NR1 and NR2A, as well as the pNR2B site Y1472 and S1303 compared with saline-treated rats (n=5 for both saline and LY39, p=0.020 for NR2B; Fig. 1B). More importantly, when a dose of 3.0 mg/kg LY39 was administered, there were significant increases in NR1, NR2A, NR2B, and pNR2BTyr1472, but not pNR2BSer1303 (n=5 for both saline and LY39, p=0.011, 0.005, 0.011, 0.013, 0.458, respectively; Fig. 1C). Together, these data indicated that *in vivo* treatment with LY39 induced a significant enhancement of NMDAR expression in the normal adult PFC in a dose-dependent manner.

In vivo treatment with LY39 enhances NMDAR post-synaptic function in adult rat PFC neurons

Next we examined whether the enhanced NMDAR expression results in functional changes and whether LY39 affects NMDAR expression in postsynaptic sites. We used whole-cell patch clamp recording of NMDAR-mEPSCs in layer 5 pyramidal neurons by holding the membrane potential at +60 mV in the presence of TTX (0.5 μ M), picrotoxin (50 μ M), and DNQX (20 μ M). As shown in Figure 2, *in vivo* treatment with 3.0 mg/kg LY39 significantly enhanced NMDAR-mEPSC amplitude, but not frequency (n=7 cells for saline; n=7 for LY39, p=0.002 for amplitude and p=0.403 for frequency; Fig. 2A and B), suggesting a significant increase in postsynaptic NMDAR expression. Moreover, LY39 also significantly increased the time constant decay of NMDAR-mEPSCs compared with that of saline control (p=0.032; Fig. 2C). As NR2B has a slower decay time relative to NR2A (Cull-Candy et al., 2001), LY39's effect on the NMDAR-mEPSC decay time is likely derived from the upregulation of NR2B, consistent with the increased expression of the total NR2B protein seen in our Western blot experiment.

Treatment with LY39 may regulate NMDAR expression through activation of mTOR signaling in normal adult rat PFC

Previous studies have indicated that activation of group II mGluR with different agonists could regulate NMDAR expression and function under various conditions through several intracellular signaling pathways, including ERK, mTOR, Akt, and GSK3 β (Sanchez-Perez et al., 2006; Wang et al., 2013; Xi et al., 2011). Therefore, we screened these signaling proteins and their phosphorylation states one hour after administration of LY39.

As shown in Figure 3, LY39 treatment at a dose of 0.3 mg/kg resulted in no significant changes in mTOR and pmTOR expression, or in eIF4E, an up-stream regulatory molecule of mTOR and pmTOR (n=5 for each group, p=0.214 for mTOR, p=0.905 for pmTOR, p=0.604 for eIF4E; Fig. 3A). However, at a dose of 1.0 mg/kg, LY39 significantly increased the total protein level of mTOR, but not others, including ERK, Akt, and GSK3 β total protein and their phosphorylation sites (n=5 for each group, p=0.013 for total mTOR and p>0.05 for all others; Fig. 3B). Similarly, when the dosage of LY39 was increased to 3.0 mg/kg, there was a significant decrease in total protein levels of mTOR and in pmTOR-Ser2448, as well as a

significant decrease of phosphorylation at an inhibitory site of GSK3 β , i.e., pGSK3 β -Ser9 (n=5 for each group, p=0.015 for total mTOR, p=0.039 for pmTOR-Ser2448, p=0.001 for pGSK3 β -Ser9, and p>0.05 for others; Figure 3C). These data suggest that administration of LY39 may regulate NMDAR expression via activation of either the mTOR or GSK3 β signaling pathways in a dose-dependent manner, but has no effects on ERK or Akt signaling. However, since pGSK3 β -Ser9 is an inhibitory site, a decrease would result in a relative increase of GSK3 β activity, which would eventually reduce NMDAR expression, as previous studies reported (Li and Gao, 2011; Li et al., 2009). This seems conflict with our finding of enhanced NMDAR expression by LY39 treatment. Therefore, we hypothesized that treatment with LY39 could likely regulate NMDAR expression through activation of mTOR signaling by phosphorylating site serine 2448 in the normal adult rat PFC.

In addition, we tested the expression of 4E-BP1 and its phosphorylated form p4E-BP1. 4E-BP1 is a repressor of mRNA translation and is phosphorylated and inactivated by mTOR. We found that administration of LY39 at a dose of 3.0 mg/kg did not induce significant changes in total protein levels of 4E-BP1 (p=0.978) and p4E-BP1 (n=4 for vehicle group and n = 5 for LY39 group, p=0.206; Figure 4).

LY39 regulates postsynaptic NMDAR function via mTOR-mediated protein synthesis in layer 5 pyramidal neurons of normal adult rat PFC

Because the protein levels of mTOR and pmTOR were significantly increased by LY39 treatment, we tested whether the enhancement in expression and function of NMDARs was mediated by the activation of mTOR signaling. The mTOR signaling pathway is critically involved in regulating not only NMDAR expression and function (Autry et al., 2011; Miller et al., 2014), but also rapid local protein synthesis and synaptic plasticity (Tsokas et al., 2005). A previous study has reported that pre-treatment with rapamycin (i.p.) reduced the phosphorylation of ribosomal protein s6 kinase in the cortex and hippocampus 30 min after ketamine administration, indicating that rapamycin had penetrated brain tissue (Autry et al., 2011). We therefore used mTOR inhibitor rapamycin (1.0 mg/kg, i.p.) to inhibit mTOR function and assess whether LY39-induced increases in NMDAR expression were blocked. Rapamycin was administered 30 min before LY39 (3.0 mg/kg, i.p.) or saline injection, as previously reported (Autry et al., 2011). One hour later, animals were sacrificed and living brain slices containing the mPFC were made for physiological recording of NMDAR-mEPSCs. As shown in Figure 5, rapamycin effectively and significantly blocked LY39-induced increase of NMDAR-mEPSC amplitude (n=7 for saline and n=7 for LY39; n=6 for rapamycin+saline and n=8 for rapamycin+LY39; p=0.0004 between LY39 and rapamycin+LY39; Figure 5A and 5B), but not frequency (p>0.05 for all comparisons). Moreover, the increased decay time of NMDAR-mEPSCs induced by LY39 was also significantly reduced by 65.97 $\pm$ 14.92 ms with the administration of rapamycin (p=0.048, Figure 4C). These results suggest that the LY39-induced increase of NMDAR expression requires the activation of mTOR-mediated signaling in postsynaptic sites.

To further confirm that LY39 was indeed affecting protein synthesis, we treated animals with the transcription inhibitor actinomycin-D (act-D, 0.5 mg/kg, i.p.) 30 min before administration of LY39, and again, saline was used as a control. As shown in Figure 5A and

5B, there was a significant decrease in amplitude by about 35% and frequency by about 50% when comparing act-D+saline with saline controls (n=10 for act-D+saline, $p < 0.01$ for both amplitude and frequency), indicating a significant decrease of postsynaptic NMDAR numbers. Moreover, act-D significantly decreased the NMDAR-mEPSC amplitude by about 35% (n=9 for act-D+LY39, $p = 0.0359$ between LY39 and act-D+LY39) and frequency by about 30% ($p < 0.01$) compared to the LY39 group. This result not only suggests that LY39 enhanced NMDAR expression and function by regulating protein synthesis via activation of mTOR signaling, but also indicates that the change is associated with transcriptional regulation of protein synthesis.

LY39 exhibited no significant effect on working memory in naïve animals and could not reverse MK801-induced working memory impairment

We utilized a maze-based procedure to measure the effect of treatment in an attentional set-shifting task. On the response discrimination day, ANOVA analysis, which was performed with GraphPad with treatment as the independent-factor and number of trials to criterion and number of probe trials to complete the task as dependent variables, revealed that all rats in all four groups took an equivalent number of trials to reach criterion (Saline+Saline, 70.00 ± 6.04 ; Saline+LY39, 86.88 ± 13.18 ; MK801+Saline, 69.25 ± 13.00 ; MK801+LY, 70.75 ± 16.35 ; $F(3,27) = 0.42$, $p = 0.7372$). The differences in the number of probe trials were also not significant (Saline+Saline, 1.49 ± 0.20 ; Saline+LY39, 1.50 ± 0.19 ; MK801+Saline, 1.57 ± 0.37 ; MK801+LY, 1.63 ± 0.32 ; $F(3,26) = 0.09$, $p = 0.9636$).

However, the following day, when animals had to shift their strategy from an egocentric to a cue-based approach, a Kruskal Wallis nonparametric ANOVA analysis of the number of trials for animals to reach criterion revealed a significant difference ($F(3,28) = 3.02$, $p = 0.031$). A *post hoc* analysis showed that the Saline+Saline and Saline+LY groups were significantly different from MK+Saline ($p < 0.01$) but not MK+LY ($p > 0.05$; Figure 6A). Mann-Whitney U test for MK+Saline vs MK+LY was also not significant ($p = 0.798$; Saline+Saline, 104.50 ± 8.21 ; Saline+LY39, 102.30 ± 6.38 ; MK801+Saline, 162.00 ± 17.31 ; MK801+LY39, 145.50 ± 27.84). Animals in all groups required an equivalent number of probe trials to complete the task (Saline+Saline, 1.14 ± 0.14 ; Saline+LY39, 1.25 ± 0.16 ; MK801+Saline, 1.25 ± 0.16 ; MK801+LY, 1.29 ± 0.18 ; $F(3,26) = 0.13$, $p = 0.940$; Figure 6B). Two-way ANOVA conducted with treatment and type of error as independent-factors and number of errors as the dependent variable revealed that the main effect of error type was significant ($F(2, 82) = 40.66$, $p < 0.0001$) and the interaction of treatment $\times$ error type was significant ($F(6,82) = 2.28$, $p = 0.0436$), but the main effect of treatment was non-significant ($F(3,82) = 1.41$, $p > 0.05$). The *post hoc* test indicated that compared with the control group, animals in MK801+Saline group and MK801+LY39 group made more perseverative errors (Saline+Saline, 26.63 ± 6.96 vs MK801+Saline, 50.25 ± 7.34 , $t = 3.07$, $p < 0.01$ and vs MK801+LY39, 49.63 ± 8.12 , $t = 2.99$, $p < 0.05$; Figure 6C). This suggests that these animals had a deficit specifically in shifting away from the original egocentric strategy to the cue-based strategy which was necessary to perform the task optimally. While compared with the Saline+LY39 (27.63 ± 6.96) group, animals injected MK801 (MK801+Saline and MK801+LY) also made significantly more perseverative errors (vs MK801+Saline, $t = 2.94$, $p < 0.05$; MK801+LY39, $t = 2.86$, $p < 0.05$). There was no significant

difference between Saline+Saline and Saline+LY ($t=0.13$, $p>0.05$) nor between the MK801+Saline and MK801+LY39 ($t=0.08$, $p>0.05$), indicating that LY39 alone and as a potential rescue had no significant effects on set-shifting performance. Finally the *post hoc* test did not demonstrate any significant difference between groups in regressive errors (Saline+Saline, 11.50 ± 3.50 ; Saline + LY39, 12.00 ± 4.54 ; MK801+Saline, 11.00 ± 2.66 ; MK801+LY, 7.43 ± 2.62 ; $p>0.05$ for all; Figure 6D), or never-reinforced errors (Saline + Saline, 8.00 ± 2.06 ; Saline+LY39, 6.38 ± 1.76 ; MK801+Saline, 7.25 ± 1.31 ; MK801+LY, 4.63 ± 1.16 ; $p>0.05$ for all; Figure 6E), suggesting that both LY39 and MK801 did not affect animal's ability in maintaining and identifying a novel strategy respectively (Block et al., 2007).

Discussion

In this study, we have investigated the effects of *in vivo* administration of LY39 on NMDAR expression and function in the adult rat PFC. We found that LY39 (3.0 mg/kg dose) significantly increased the total protein levels of NMDAR subunits and pNR2B-Y1472, a phosphorylation site that promotes receptor insertion. These changes are accompanied by a significant increase of mTOR and pmTOR expression, but unaltered ERK1/2, Akt and GSK3 β expression. Furthermore, NMDAR-mEPSC amplitude was also significantly increased by LY39 and this effect was blocked by mTOR inhibitor rapamycin and transcription inhibitor actinomycin-D, suggesting that LY39 regulates NMDARs through mTOR-mediated protein synthesis. However, the enhancing effect was neither sufficient to affect working memory function in naïve animals, nor to reverse MK801-induced working memory impairment.

Targeting mGluR2/3 in the dysfunctional glutamatergic system in schizophrenia

The mGluR2/3 subtypes of glutamate receptors have long been linked with SCZ (Li et al., 2015; Moghaddam, 2004), and mGluR2/3 agonism has been proposed to target the dysfunctional glutamatergic system, particularly NMDAR hypofunction. However, in patients with SCZ and animal models for SCZ, direct evidence of whether and how activation of mGluR2/3 affects NMDAR expression and function in the normal adult brain remains unclear and under-studied. In a previous study, we reported that the selective mGluR2/3 agonist LY379268 significantly increased the expression of NMDARs and reversed MK801-induced NMDAR dysfunction and hyperlocomotor activity in this NMDAR antagonism model of SCZ (Xi et al., 2011). LY379268 also strongly regulated AMPAR expression and trafficking in cultured prefrontal cortical neurons (Wang et al., 2013).

However, recent studies suggest that mGluR2 and mGluR3 have differential effects on synaptic function due to their distinct regional and cellular distributions. Specifically, although both mGluR2 and mGluR3 are expressed in the PFC (Ferraguti and Shigemoto, 2006; Marek, 2010), mGluR2 is more enriched in the PFC region, whereas mGluR3 is more localized to the hippocampal area (Wright et al., 2013). At a cellular level, mGluR2 is located in both pre- and postsynaptic sites of individual neurons, whereas mGluR3 receptors are mainly found in postsynaptic sites of neurons and in glia cells (Petralia et al., 1996;

Tamaru et al., 2001). These properties may endow mGluR2 and mGluR3 receptors with differential actions in synaptic functions and in treatment. Specifically, activation of mGluR2 exhibits stronger antipsychotic effects (Fell et al., 2008; Woolley et al., 2008), while activation of mGluR3 exerts more neuroprotective actions (Durand et al., 2013). Therefore, compounds with higher selectivity for mGluR2, such as LY39, would be expected to have stronger effects in treatment of SCZ compared to those targeting both mGluR2 and mGluR3 with equal affinity, such as LY379268. Indeed, a recent study differentiated the roles of mGluR2 and mGluR3 receptors by using LY541850, an mGluR2 agonist/mGluR3 antagonist, and reported that systemic administration of LY541850 to wild-type mice reduced the increase in locomotor activity following both phencyclidine and amphetamine administration, supporting the hypothesis that mGluR2 mediates the antipsychotic effects of mixed group II agonists (Hanna et al., 2013).

In this study, we found that *in vivo* administration of LY39 significantly increased the expression of NMDAR subunits, including NR1, NR2A and NR2B, as well as pNR2B at Y1472 in the adult rat PFC. Moreover, the protein changes in PFC are accompanied by a direct enhancing effect on the NMDAR-mEPSC amplitude. These findings are not only in support of our previous studies of LY379268, a selective agonist targeting both mGluR2 and mGluR3, on glutamate receptors (Wang et al., 2013; Xi et al., 2011), but in agreement with several other similar reports, in which mGluR2/3 agonists also show an enhancing effect on NMDAR function (Cheng et al., 2013; Li et al., 2015; Trepanier et al., 2013; Tyszkiewicz et al., 2004). Clearly, LY39 has strong effects on NMDAR expression and function, although it remains to be determined whether this enhancement is derived by the activation of mGluR2 or antagonistic effect of mGluR3 subunit.

mTOR signaling mediates the regulation of NMDAR expression and function

In our previous report, we have shown that both Akt and GSK3 β signaling were involved in the regulatory effects of mGluR2/3 agonist LY379268 on NMDAR function in the MK801 model of SCZ (Xi et al., 2011). However, Akt, GSK-3 β , and ERK were not altered by LY39 treatment. In contrast, protein levels of mTOR and pmTOR were significantly increased. We therefore further determined whether activation of mTOR signaling-mediated protein synthesis is involved in the regulation of NMDAR expression and function. We found that the effect of LY39 on NMDARs was effectively blocked by both mTOR inhibitor rapamycin and transcription inhibitor actinomycin-D. These findings are consistent with previous studies in which mTOR is found to mediate protein synthesis of NMDARs (Li et al 2010, Miller et al 2014), indicating the involvement of local protein synthesis (Gong et al., 2006; Hanus and Schuman, 2013; tom Dieck et al., 2014; Wang et al., 2013). To test this possibility, we examined the expression of 4E-BP1, an mRNA suppressor downstream of mTOR, and its phosphorylated form, p4E-BP1. We expected that increased mTOR activity would result in an increase of phosphorylated 4E-BP1 to affect translation and protein synthesis of NMDARs directly. However, we found that administration of LY39 did not alter the protein levels of 4E-BP1 and p4E-BP1. At this point, how activation of mTOR via LY39 treatment affects protein synthesis remains unclear. Recent studies have suggested mTOR signaling controls multiple steps in ribosome biogenesis, which is a critical step in the cellular generation of ribosomes that are necessary for protein synthesis. Activation of

mTOR signaling positively regulates several steps in this process, including ribosomal RNA transcription, synthesis of ribosomal proteins and other components required for ribosome assembly. It is thus possible that activation of mTOR signaling promotes ribosome production, which bypasses the regulation of protein synthesis by translation factors, such as 4E-BP1 (Iadevaia et al., 2014). This assumption needs further exploration because, despite the important advances in our understanding of mTOR signaling, major questions remain about the molecular mechanisms by which it regulates ribosome biogenesis (Iadevaia et al., 2012; Iadevaia et al., 2014).

The effects of mGluR2/3 agonism on cognitive function appear to be limited, at least in acute treatment

Our study, although conducted in adult animals compared to others in cell culture or brain slices from juvenile animals (Cheng et al., 2013; Trepanier et al., 2013; Tyszkiewicz et al., 2004), reflects the acute effects of mGluR2 activation or mGluR3 antagonism. It is still unclear how chronic treatment with LY39 would affect synaptic function and synaptic plasticity by modulating NMDAR expression. Furthermore, it is also unknown whether the effects of mGluR2/3 agonist or mGluR2 agonism/mGluR3 antagonism, such as the mechanistic action of LY39, on NMDAR expression and function are sufficient to affect cognitive function, especially prefrontal-dependent working memory, cognitive flexibility, and learning in animal models for SCZ. Although mGluR2/3 agonists reversed certain cognitive deficits induced by NMDAR blockade in NMDAR antagonism models of SCZ (Moghaddam and Adams, 1998; Nikiforuk et al., 2010), in normal animals these agents either had no effects or even impaired cognitive function (Aultman and Moghaddam, 2001; Higgins et al., 2004; Schlumberger et al., 2009). Indeed, our results add additional evidence indicating that despite the strong acute effect in modulation of NMDAR subunit expression and function in the PFC, these effects are insufficient to affect working memory function in naïve animals. A previous study suggested the possibility that the efficacy of mGluR2/3 agonists on cognitive function may be limited to conditions associated with a prominent NMDAR dysfunction or a disrupted glutamatergic system in a disease state (Nikiforuk et al., 2010). This seems to be a practical assumption because neither mGluR2/3 agonists nor selective mGluR2 agonists have ever exhibited any potential for cognitive enhancement under normal circumstances. However, we observed that LY39 was unable to reverse MK801-induced working memory impairment. Furthermore, although LY39 as an mGluR2 agonist exerts significant effects on NMDAR expression, it also functions as an mGluR3 antagonist. It is unknown whether a more selective agonist that targets mGluR2 only or an mGluR2 positive allosteric modulator (PAM) would be more effective compared with mGluR2/3 agonists or a compound like LY39, which acts as both an agonist of mGluR2 and an antagonist of mGluR3. Nevertheless, our findings provide solid evidence indicating that activation of mGluR2 has a strong effect on regulating NMDAR function and thus potentially could affect NMDAR-dependent synaptic plasticity. Whether this effect could eventually enhance PFC-dependent learning and memory after chronic treatment is still an open question. In addition, further studies should focus on LY39-like compounds' potential effect in other animal models for SCZ such as genetic (e.g., dysbindin or neuregulin) or neurodevelopmental models (e.g., methylazoxymethanol, MAM). Furthermore, although mGluR2/3 agonist LY2140023 (pomaglumetad methionil) failed to meet the primary

endpoint based on the positive and negative syndrome scale (PANSS) in Phase III clinical trials (Adams et al., 2013; Adams et al., 2014), mGluR2/3 still serves as a promising avenue in the search for a viable therapeutic target for cognitive deficits in SCZ. It is possible that mGluR2 agonists would be more effective in a subpopulation of patients without prominent hyperdopaminergic symptoms, i.e., patients with normal dopaminergic function (Howes and Kapur, 2014). Especially during early stage of SCZ, when NMDAR dysfunction occurs, selective mGluR2 agonists or PAMs (Galici et al., 2005) would be more efficacious than in the general adult SCZ population. This assumption, however, remains to be tested and is warrants further investigation.

In summary, we have provided the first evidence of a direct regulatory effect of LY39 on NMDAR expression and function in the adult rat PFC, and of the role of mTOR signaling-mediated protein synthesis in this regulation. Our findings indicate that targeting mGluR2 may be a strategy for the dysfunctional NMDARs or more generally, the glutamate system in SCZ, as well as other psychiatric disorders. Further studies are needed to expound upon the role of such compounds in cognitive deficits, which currently remain limited.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

3,5-DHPG	3,5-dihydroxyphenylglycine
AC	adenylate cyclase
ACPD	1-amino-1,3-dicarboxycyclopentane
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPH	amphetamine
APDC	dihydroxyphenylglycine
cAMP	cyclic-adenosine monophosphate
DCG-IV	(2S,1'R,2' R,3'R)-2-(2,3-dicarboxycyclopropyl) glycine
ERK1/2	Extracellular signal-regulated kinase1/2
GABA	gamma-aminobutyric acid
GSK3β	glycogen synthase kinase 3 β
I.P.	intraperitoneal
mGluRs	metabotropic glutamate receptors

NMDAR	N-methyl-D-aspartate receptor
PKA	protein kinase A
PKC	protein kinase C
SNAP	soluble NSF activating protein
SNARE	soluble NSF activating protein receptor
SNP	single nucleotide polymorphism
SCZ	schizophrenia
VAMP	vesicle-associated membrane protein

References

- Adams DH, Kinon BJ, Baygani S, Millen BA, Velona I, Kollack-Walker S, Walling DP. A long-term, phase 2, multicenter, randomized, open-label, comparative safety study of pomaglumetad methionil (LY2140023 monohydrate) versus atypical antipsychotic standard of care in patients with schizophrenia. *BMC Psychiatry*. 2013; 13:143. [PubMed: 23694720]
- Adams DH, Zhang L, Millen BA, Kinon BJ, Gomez JC. Pomaglumetad Methionil (LY2140023 Monohydrate) and Aripiprazole in Patients with Schizophrenia: A Phase 3, Multicenter, Double-Blind Comparison. *Schizophrenia Research and Treatment*. 2014; 2014:758212. [PubMed: 24772351]
- Boultadakis, Antonios; Pitsikas, N. Effects of the Nitric Oxide Synthase Inhibitor L-NAME on Recognition and Spatial Memory Deficits Produced by Different NMDA Receptor Antagonists in the Rat. *Neuropsychopharmacology*. 2010
- Aultman JM, Moghaddam B. Distinct contributions of glutamate and dopamine receptors to temporal aspects of rodent working memory using a clinically relevant task. *Psychopharmacology (Berl)*. 2001; 153:353–364. [PubMed: 11271408]
- Autry AE, Adachi M, Nosyreva E, Na ES, Los MF, Cheng P-f, Kavalali ET, Monteggia LM. NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature*. 2011; 475:91–95. [PubMed: 21677641]
- Block AE, Dhanji H, Thompson-Tardif SF, Floresco SB. Thalamic–prefrontal cortical–ventral striatal circuitry mediates dissociable components of strategy set shifting. *Cereb Cortex*. 2007; 17:1625–1636. [PubMed: 16963518]
- Bond A, Jones NM, Hicks CA, Whiffin GM, Ward MA, O'Neill MF, Kingston AE, Monn JA, Ornstein PL, Schoepp DD, Lodge D, O'Neill MJ. Neuroprotective effects of LY379268, a selective mGlu2/3 receptor agonist: investigations into possible mechanism of action in vivo. *J Pharmacol Exp Ther*. 2000; 294:800–809. [PubMed: 10945827]
- Cartmell J, Schoepp DD. Regulation of neurotransmitter release by metabotropic glutamate receptors. *J of Neurochem*. 2000; 75:889–907. [PubMed: 10936169]
- Ceolin L, Kantamneni S, Barker GR, Hanna L, Murray L, Warburton EC, Robinson ES, Monn JA, Fitzjohn SM, Collingridge GL, Bortolotto ZA, Lodge D. Study of novel selective mGlu2 agonist in the temporo-ammonic input to CA1 neurons reveals reduced mGlu2 receptor expression in a Wistar substrain with an anxiety-like phenotype. *J Neurosci*. 2011; 31:6721–6731. [PubMed: 21543601]
- Cheng J, Liu W, Duffney LJ, Yan Z. SNARE proteins are essential in the potentiation of NMDA receptors by group II metabotropic glutamate receptors. *J Physiol*. 2013; 591:3935–3947. [PubMed: 23774277]
- Conn PJ, Lindsley CW, Jones CK. Activation of metabotropic glutamate receptors as a novel approach for the treatment of schizophrenia. *Trends in pharmacological sciences*. 2009; 30:25–31. [PubMed: 19058862]

- Cull-Candy S, Brickley S, Farrant M. NMDA receptor subunits: diversity, development and disease. *Curr Opin Neurobiol.* 2001; 11:327–335. [PubMed: 11399431]
- Durand D, Carniglia L, Caruso C, Lasaga M. mGlu3 receptor and astrocytes: Partners in neuroprotection. *Neuropharmacology.* 2013; 66:1–11. [PubMed: 22564439]
- Fell MJ, McKinzie DL, Monn JA, Svensson KA. Group II metabotropic glutamate receptor agonists and positive allosteric modulators as novel treatments for schizophrenia. *Neuropharmacology.* 2012; 62:1473–1483. [PubMed: 21704048]
- Fell MJ, Svensson KA, Johnson BG, Schoepp DD. Evidence for the role of metabotropic glutamate (mGlu)2 not mGlu3 receptors in the preclinical antipsychotic pharmacology of the mGlu2/3 receptor agonist (–)-(1R,4S,5S,6S)-4-amino-2-sulfonylbicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY404039). *The Journal of pharmacology and experimental therapeutics.* 2008; 326:209–217. [PubMed: 18424625]
- Ferraguti F, Shigemoto R. Metabotropic glutamate receptors. *Cell Tissue Res.* 2006; 326:483–504. [PubMed: 16847639]
- Galici R, Echemendia NG, Rodriguez AL, Conn PJ. A selective allosteric potentiator of metabotropic glutamate (mGlu) 2 receptors has effects similar to an orthosteric mGlu2/3 receptor agonist in mouse models predictive of antipsychotic activity. *J Pharmacol Exp Ther.* 2005; 315:1181–1187. [PubMed: 16123306]
- Gong R, Park CS, Abbassi NR, Tang SJ. Roles of glutamate receptors and the mammalian target of rapamycin (mTOR) signaling pathway in activity-dependent dendritic protein synthesis in hippocampal neurons. *J Biol Chem.* 2006; 281:18802–18815. [PubMed: 16651266]
- Hanna L, Ceolin L, Lucas S, Monn J, Johnson B, Collingridge G, Bortolotto Z, Lodge D. Differentiating the roles of mGlu2 and mGlu3 receptors using LY541850, an mGlu2 agonist/mGlu3 antagonist. *Neuropharmacology.* 2013; 66:114–121. [PubMed: 22445601]
- Hanus C, Schuman EM. Proteostasis in complex dendrites. *Nat Rev Neurosci.* 2013; 14:638–648. [PubMed: 23900412]
- Higgins GA, Ballard TM, Kew JN, Richards JG, Kemp JA, Adam G, Woltering T, Nakanishi S, Mutel V. Pharmacological manipulation of mGlu2 receptors influences cognitive performance in the rodent. *Neuropharmacology.* 2004; 46:907–917. [PubMed: 15081787]
- Howes OD, Kapur S. The dopamine hypothesis of schizophrenia: version III—the final common pathway. *Schizophr Bull.* 2009; 35:549–562. [PubMed: 19325164]
- Howes OD, Kapur S. A neurobiological hypothesis for the classification of schizophrenia: type A (hyperdopaminergic) and type B (normodopaminergic). *Br J Psychiatry.* 2014; 205:1–3. [PubMed: 24986384]
- Iadevaia V, Huo Y, Zhang Z, Foster LJ, Proud CG. Roles of the mammalian target of rapamycin, mTOR, in controlling ribosome biogenesis and protein synthesis. *Biochem Soc Trans.* 2012; 40:168–172. [PubMed: 22260684]
- Iadevaia V, Liu R, Proud CG. mTORC1 signaling controls multiple steps in ribosome biogenesis. *Semin Cell Dev Biol.* 2014; 36:113–120. [PubMed: 25148809]
- Jeevakumar V, Driskill C, Paine A, Sobhanian M, Vakil H, Morris B, Ramos J, Kroener S. Ketamine administration during the second postnatal week induces enduring schizophrenia-like behavioral symptoms and reduces parvalbumin expression in the medial prefrontal cortex of adult mice. *Behavioural brain research.* 2015; 282:165–175. [PubMed: 25591475]
- Kahn RS, Sommer IE. The neurobiology and treatment of first-episode schizophrenia. *Mol Psychiatry.* 2015; 20:84–97. [PubMed: 25048005]
- Li ML, Hu XQ, Li F, Gao WJ. Perspectives on the mGluR2/3 agonists as a therapeutic target for schizophrenia: Still promising or a dead end? *Prog Neuropsychopharmacol Biol Psychiatry.* 2015; 60:66–76. [PubMed: 25724760]
- Li YC, Gao WJ. GSK-3 β activity and hyperdopamine-dependent behaviors. *Neurosci Biobehav Rev.* 2011; 35:645–654. [PubMed: 20727368]
- Li YC, Xi D, Roman J, Huang YQ, Gao WJ. Activation of glycogen synthase kinase-3 β is required for hyperdopamine and D2 receptor-mediated inhibition of synaptic NMDA receptor function in the rat prefrontal cortex. *J Neurosci.* 2009; 29:15551–15563. [PubMed: 20007479]

- Lisman JE, Coyle JT, Green RW, Javitt DC, Benes FM, Heckers S, Grace AA. Circuit-based framework for understanding neurotransmitter and risk gene interactions in schizophrenia. *Trends in Neurosci.* 2008; 31:234–242.
- Lucas SJ, Bortolotto ZA, Collingridge GL, Lodge D. Selective activation of either mGlu2 or mGlu3 receptors can induce LTD in the amygdala. *Neuropharmacology.* 2013; 66:196–201. [PubMed: 22531751]
- Marek GJ. Metabotropic glutamate2/3 (mGlu2/3) receptors, schizophrenia and cognition. *European Journal of Pharmacology.* 2010; 639:81–90. [PubMed: 20371229]
- Mezler M, Geneste H, Gault L, Marek GJ. LY-2140023, a prodrug of the group II metabotropic glutamate receptor agonist LY-404039 for the potential treatment of schizophrenia. *Curr Opin Investig Drugs.* 2010; 11:833–845.
- Millan MJ, Agid Y, Brüne M, Bullmore ET, Carter CS, Clayton NS, Connor R, Davis S, Deakin B, DeRubeis RJ, Dubois B, Geyer MA, Goodwin GM, Gorwood P, Jay TM, Joëls M, Mansuy IM, Meyer-Lindenberg A, Murphy D, Rolls E, Saletu B, Spedding M, Sweeney J, Whittington M, Young LJ. Cognitive dysfunction in psychiatric disorders: characteristics, causes and the quest for improved therapy. *Nat Rev Drug Discov.* 2012; 11:141–168. [PubMed: 22293568]
- Miller OH, Yang L, Wang CC, Hargroder EA, Zhang Y, Delpire E, Hall BJ. GluN2B-containing NMDA receptors regulate depression-like behavior and are critical for the rapid antidepressant actions of ketamine. 2014
- Miyamoto S, Duncan GE, Marx CE, Lieberman JA. Treatments for schizophrenia: a critical review of pharmacology and mechanisms of action of antipsychotic drugs. *Mol Psychiatry.* 2005; 10:79–104. [PubMed: 15289815]
- Moghaddam B. Targeting metabotropic glutamate receptors for treatment of the cognitive symptoms of schizophrenia. *Psychopharmacol (Berl).* 2004; 174:39–44.
- Moghaddam B, Adams BW. Reversal of phencyclidine effects by a group II metabotropic glutamate receptor agonist in rats. *Science.* 1998; 281:1349–1352. [PubMed: 9721099]
- Nikiforuk A, Popik P, Drescher KU, van Gaalen M, Relo AL, Mezler M, Marek G, Schoemaker H, Gross G, Bespalov A. Effects of a positive allosteric modulator of group II metabotropic glutamate receptors, LY487379, on cognitive flexibility and impulsive-like responding in rats. *J Pharmacol Exp Ther.* 2010; 335:665–673. [PubMed: 20739457]
- Petralia RS, Wang YX, Niedzielski AS, Wenthold RJ. The metabotropic glutamate receptors, MGLUR2 and MGLUR3, show unique postsynaptic, presynaptic and glial localizations. *Neuroscience.* 1996; 71:949–976. [PubMed: 8684625]
- Sanchez-Perez AM, Llansola M, Felipe V. Modulation of NMDA receptors by AKT kinase. *Neurochemistry international.* 2006; 49:351–358. [PubMed: 16621159]
- Schlumberger C, Schafer D, Barberi C, More L, Nagel J, Pietraszek M, Schmidt WJ, Danysz W. Effects of a metabotropic glutamate receptor group II agonist LY354740 in animal models of positive schizophrenia symptoms and cognition. *Behav Pharmacol.* 2009; 20:56–66. [PubMed: 19179851]
- Schoepp DD, Jane DE, Monn JA. Pharmacological agents acting at subtypes of metabotropic glutamate receptors. *Neuropharmacology.* 1999; 38:1431–1476. [PubMed: 10530808]
- Seeman P. All roads to schizophrenia lead to dopamine supersensitivity and elevated dopamine D2High receptors. *CNS Neuroscience & Therapeutics.* 2011; 17:118–132. [PubMed: 20560996]
- Snyder MA, Gao WJ. NMDA hypofunction as a convergence point for progression and symptoms of schizophrenia. *Frontiers in Cellular Neuroscience.* 2013; 7:31. [PubMed: 23543703]
- Tamaru Y, Nomura S, Mizuno N, Shigemoto R. Distribution of metabotropic glutamate receptor mGluR3 in the mouse CNS: differential location relative to pre- and postsynaptic sites. *Neuroscience.* 2001; 106:481–503. [PubMed: 11591452]
- tom Dieck S, Hanus C, Schuman EM. SnapShot: Local Protein Translation in Dendrites. *Neuron.* 2014; 81:958–958.e951. [PubMed: 24559682]
- Trepanier C, Lei G, Xie YF, Macdonald JF. Group II metabotropic glutamate receptors modify N-methyl-D-aspartate receptors via Src kinase. *Scientific reports.* 2013; 3:926. [PubMed: 23378895]
- Tsokas P, Grace EA, Chan P, Ma T, Sealfon SC, Iyengar R, Landau EM, Blitzner RD. Local protein synthesis mediates a rapid increase in dendritic elongation factor 1A after induction of late long-

- term potentiation. *The Journal of neuroscience: the official journal of the Society for Neuroscience*. 2005; 25:5833–5843. [PubMed: 15958750]
- Tyszkiewicz JP, Gu Z, Wang X, Cai X, Yan Z. Group II metabotropic glutamate receptors enhance NMDA receptor currents via a protein kinase C-dependent mechanism in pyramidal neurones of rat prefrontal cortex. *J Physiol*. 2004; 554:765–777. [PubMed: 14645456]
- Wang MJ, Li YC, Snyder MA, Wang H, Li F, Gao WJ. Group II metabotropic glutamate receptor agonist LY379268 regulates AMPA receptor trafficking in prefrontal cortical neurons. *PLoS ONE*. 2013; 8:e61787. [PubMed: 23593498]
- Weinberger DR, Gallhofer B. Cognitive function in schizophrenia. *Int Clin Psychopharmacol*. 1997; 12(Suppl 4):S29–36. [PubMed: 9352344]
- Woolley ML, Pemberton DJ, Bate S, Corti C, Jones DN. The mGlu2 but not the mGlu3 receptor mediates the actions of the mGluR2/3 agonist, LY379268, in mouse models predictive of antipsychotic activity. *Psychopharmacology*. 2008; 196:431–440. [PubMed: 18057917]
- Wright RA, Johnson BG, Zhang C, Salhoff C, Kingston AE, Calligaro DO, Monn JA, Schoepp DD, Marek GJ. CNS distribution of metabotropic glutamate 2 and 3 receptors: transgenic mice and [(3)H]LY459477 autoradiography. *Neuropharmacology*. 2013; 66:89–98. [PubMed: 22313530]
- Xi D, Li YC, Snyder MA, Gao RY, Adelman AE, Zhang W, Shumsky JS, Gao WJ. Group II metabotropic glutamate receptor agonist ameliorates MK-801-induced dysfunction of NMDA receptors via Akt/GSK-3beta pathway. *Neuropsychopharmacol*. 2011; 36:1260–1274.

Figure 1.

In vivo treatment with LY39 induces a significant enhancement of synaptic membrane protein expression of NMDARs in the adult rat PFC in a dose-dependent manner. A–C, Representative images of immunoblots on the left panel show the synaptic membrane proteins of NMDAR subunits and NR2B phosphorylation sites with actin as a control for the total protein levels. Summary bar graphs on the right show the relative changes in NMDAR subunits and changes at NR2B phosphorylation sites induced by LY39 treatment at doses of 0.3, 1.0, and 3.0 mg/kg, respectively. There were no significant changes in any subunits with 0.3 mg/kg treatment, a significant increase of NR2B in 1.0 mg/kg treatment, and significant increases in total proteins of all subunits as well as a significant increase of pNR2BTyr1472 but not pNR2BSer1303 in 3.0 mg/kg treatment ($n=5$ for each group, * $p<0.05$, ** $p<0.01$).

Figure 2.

Treatment with a high dose of LY39 (3.0 mg/kg) significantly increases the amplitude and decay time of NMDAR-mEPSCs in the adult rat PFC neurons. A, Representative NMDAR-mEPSC events recorded from the PFC layer 5 pyramidal neurons of both saline- and LY39-treated adult rats. B, Summary graphs show that *in vivo* treatment of LY39 resulted in a significant increase of NMDAR-mEPSC amplitude, but not frequency, compared with that of saline-treated animals. C, Sample traces of mEPSCs (left) and summary graph (right) of

the 63% decay time. LY39 treatment effectively increased the decay time (* $p < 0.05$, ** $p < 0.01$).

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Figure 3.

Treatment with LY39 significantly increases total protein levels of mTOR in a dose-dependent manner in the adult rat PFC. A, LY39 at a low dose of 0.3 mg/kg had no effects on the expression of eIF4E, mTOR and pmTOR. B, Total protein levels of mTOR, but not others, was significantly increased at a dose of 1.0 mg/kg treatment with LY39. C, At a higher dose of 3.0 mg/kg, LY39 treatment significantly increased the total protein level of mTOR, but decreased pGSK3 β -Ser9 (* $p < 0.05$, ## $p < 0.05$).

Figure 4.

A single administration of LY39 (3.0 mg/kg, i.p.) did not affect the total protein level of 4E-BP1 and its phosphorylation in the PFC. A, Representative images of 4E-BP1 and p4E-BP1 from the PFC tissues of saline- and LY39-treated animals. B, Summary histogram shows the average levels of 4E-BP1 and p4E-BP1. Treatment with LY39 did not induce significant changes in either total 4E-BP1 or phosphorylated 4E-BP1 in comparison to saline-treated animals (n = 4 for vehicle group and n = 5 for LY39 group, $p > 0.05$ for both).

Figure 5.

mTOR inhibitor rapamycin and transcription inhibitor actinomycin-D significantly blocked the enhancing effects of LY39 on the NMDAR-mEPSC in layer 5 pyramidal neurons of normal adult rat PFC. A, Representative NMDAR-mEPSC traces of *in vivo* inhibitor+saline and inhibitor+LY39 treatment in adult rats. B, Summary bar graphs of NMDAR-mEPSC amplitude and frequency. Both rapamycin and act-D significantly reduced NMDAR-mEPSC amplitude compared with LY39 treatment group. Act-D administration alone also significantly decreased NMDAR-mEPSC amplitude versus saline. No significant differences

were observed among all groups in NMDAR-mEPSC frequency in adult rats with exception of actd+saline and actd+LY39; both caused significant decreases (* $p<0.05$, ** $p<0.01$, # $p<0.05$, ## $p<0.01$).

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Figure 6.

LY39 exhibited no significant effect on working memory in saline-treated animals and failed to reverse MK801-induced working memory impairment. A, ANOVA analysis of the number of trials for animals to reach criterion revealed a significant difference ($F(3,28) = 3.02$, $p = 0.031$). A *post hoc* test showed that the Sal+Sal and Sal+LY groups were significantly different from MK+Sal (** $p < 0.01$), but not MK+LY ($p > 0.05$), and Mann-Whitney U test for MK+Sal vs MK+LY was not significant ($p = 0.798$). B, Animals in all groups required equivalent number of probe trials to complete the task ($F(3,26) = 0.13$, $p =$

0.940). C, The *post hoc* test indicated that compared with the control group, animals in the MK801+Sal group and MK801+LY39 group made more perseverative errors (Sal+Sal vs MK801+Sal, $t = 3.07$, $p < 0.01$ and vs MK801+LY39, $t = 2.99$, $p < 0.05$). While compared with the Sal+LY39 group, animals injected MK801 (MK801+Sal and MK801+LY) made significant more perseverative errors (vs MK801+Sal, $t = 2.94$, $p < 0.05$; MK801+LY39, $t = 2.86$, $p < 0.05$). There was no significant difference between Sal+Sal and Sal+LY ($t = 0.13$, $p > 0.05$) nor between the MK801+Sal and MK801+LY39 ($t = 0.08$, $p > 0.05$), indicating that LY39 alone and as a potential rescue had no significant effects on set-shifting performance. D and E, The *post hoc* test did not demonstrate any significant difference between groups in regression errors ($p > 0.05$ for all; D), or never-reinforced errors ($p > 0.05$ for all; E), suggesting that both LY39 and MK801 did not affect animals' ability in maintaining and identifying a novel strategy respectively.