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Group II metabotropic glutamate receptor agonist ameliorates MK801-induced dysfunction of NMDA receptors via the Akt/GSK-3 β pathway in adult rat prefrontal cortex

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Abstract

Pharmacological intervention targeting mGluRs has emerged as a potential treatment for schizophrenia, whereas the mechanisms involved remain elusive. We explored the antipsychotic effects of a mGluR2/3 agonist in the MK-801 model of schizophrenia in the rat prefrontal cortex. We found that the mGluR2/3 agonist LY379268 effectively recovered the disrupted expression of NMDA receptors induced by MK-801 administration. This effect was attributable to the direct regulatory action of LY379268 on NMDA receptors via activation of the Akt/GSK-3 β signaling pathway. As occurs with the antipsychotic drug clozapine, acute treatment with LY379268 significantly increased the expression and phosphorylation of NMDA receptors, as well as Akt and GSK-3 β . Physiologically, LY379268 significantly enhanced NMDA-induced current in prefrontal neurons and a GSK-3 β inhibitor occluded this effect. In contrast to the widely proposed mechanism of modulating presynaptic glutamate release, our results strongly argue that mGluR2/3 agonists modulate the function of NMDA receptors through postsynaptic actions and reverse the MK-801-induced NMDA dysfunction via the Akt/GSK-3 β pathway. This study provides novel evidence for postsynaptic mechanisms of mGluR2/3 in regulation of NMDA receptors and presents useful insights into the mechanistic actions of mGluR2/3 agonists as potential antipsychotic agents for treating schizophrenia.

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Author Contributions

W.J.G. conceived the study, supervised the project, and wrote the manuscript. D.X. carried out most of the experiments and analysis of western blots and immunoprecipitation. Y.C.L. carried out the electrophysiologic experiments, western blots and immunoprecipitation. M.A.S., R.Y.G., and A.E.A. conducted the behavioral test. J.S.S. supervised the behavioral test. M.A.S. also designed and supervised the entire process of behavioral test, carried out western blots, and proof-read the manuscript.

Conflict of Interest

The authors claim no conflict of interest.

Keywords

Antipsychotics; metabolic glutamate receptors; NMDA receptors; NMDA antagonism; signaling pathway; schizophrenia

INTRODUCTION

Pharmacological intervention targeting metabotropic glutamate receptors (mGluRs) has emerged as a potential treatment for schizophrenia (Conn *et al*, 2009; Imre, 2007; Moghaddam, 2004; Patil *et al*, 2007; Weinberger, 2007). Recent studies indicate that mGluR2/3 agonists reverse the behavioral effects of the N-methyl-D-aspartic acid (NMDA) receptor antagonists phencyclidine (PCP) and dizocilpine (MK-801) in animal models and in patients with schizophrenia (Benneyworth *et al*, 2007; Cartmell *et al*, 2000a; Fell *et al*, 2008; Moghaddam and Adams, 1998; Olszewski *et al*, 2008; Patil *et al*, 2007; Schlumberger *et al*, 2009; Swanson and Schoepp, 2002; Woolley *et al*, 2008). These studies suggest that mGluR2/3 agonists have antipsychotic properties and may offer promise for a new class of non-dopaminergic therapeutics for the treatment of schizophrenia (Conn *et al*, 2008; Weinberger, 2007). The findings are exciting, but important questions remain: Why do mGluR2/3 agonists have the same therapeutic efficacy as D2 receptor antipsychotic agents and by what mechanisms do mGluR2/3 agonists ameliorate schizophrenic behaviors?

The hypothesis of NMDA hypofunction for schizophrenia is based on the observations that NMDAR antagonists, such as phencyclidine, ketamine, and dizocilpine (MK-801), are able to produce a pattern of neurochemical and behavioral changes that are similar to those seen in schizophrenia in both animal models and human subjects (Farber, 2003; Javitt, 2004; Javitt and Zukin, 1991; Jentsch and Roth, 1999; Krystal *et al*, 1999; Krystal *et al*, 1994; Lahti *et al*, 1995; Marino and Conn, 2002; Moghaddam, 2003; Olney and Farber, 1995). In a recent study, we reported an inverted-U dose-dependent alterations of NMDAR mRNA and differential protein expressions of NR2A and NR2B subunits in MK-801-treated rat prefrontal cortex (PFC) (Xi *et al*, 2009b). Our results reconciled many discrepancies reported in previous studies in the MK-801 model, suggesting that MK-801 treatment increased the expression of NMDARs at low concentrations (Gao and Tamminga, 1995; Rujescu *et al*, 2006; Wang *et al*, 1999) but decreased their expression at high doses (Harris *et al*, 2003; Lindahl and Keifer, 2004). We reasoned that mGluR2/3 agonists may reverse the behavioral effects of NMDAR antagonists by directly affecting NMDAR expression and function. Recent studies provide some evidence for this and indicated that mGluR2/3 agonists significantly enhanced NMDA-induced current in dissociated culture neurons (Tyszkiewicz *et al*, 2004) and in prefrontal cortical slices under conditions of blockade of glutamate transporters (Oliveira *et al*, 2008). However, it remains unknown how the mGluR2/3 agonist affects the expression and function of NMDA receptors in the animal model of NMDA receptor antagonist. Phosphorylation of NR2B on Tyr1472 disrupts the interaction between NMDA receptors and the AP2-clathrin endocytic complex, leading to stabilization of the receptor on the cell surface (Barki-Harrington *et al*, 2009; Zhang *et al*, 2008); whereas phosphorylation of NR2B at Ser1303 results in either disruption of NR2B-CaMKII complex and inhibition of NMDA currents (Liu *et al*, 2006) or potentiation of

NMDA current by PKC (Liao *et al*, 2001). Does mGluR2/3 agonists directly interact with NMDA receptors or are their effects mediated indirectly via activation of an intracellular signaling pathway?

One candidate signaling pathway is the AKT/GSK-3 (glycogen synthase kinase-3) pathway. Previous work suggested that GSK-3 (particularly GSK-3 β) plays an important role in the pathogenesis of schizophrenia (Emamian *et al*, 2004; Jope and Roh, 2006) and acts as a common signaling pathway for diverse psychotomimetic agents, including D-amphetamine, D-lysergic acid diethylamide (LSD), and PCP (Svenningsson *et al*, 2003). Akt is one of the principal kinase inhibitors of GSK-3 β . Increased activity of either Akt or its phosphorylation of Ser473 will inhibit GSK-3 β kinase activity. In addition, N-terminal serine phosphorylation of GSK-3 β also inactivate GSK-3 β kinase and thus decrease the activity (Jope *et al*, 2006; Koros and Dorner-Ciossek, 2007). Furthermore, it has been reported that stimulation of NMDA receptors with NMDA can activate GSK-3 β by decreasing the phosphorylation of Akt (Luo *et al*, 2003). Moreover, converging evidence suggests impaired Akt/GSK-3 β signaling in schizophrenia (Emamian *et al*, 2004; Kozlovsky *et al*, 2002; Kozlovsky *et al*, 2005). We therefore hypothesized that mGluR2/3 agonists restore the disrupted NMDA receptor function induced by the MK-801 blockade in vivo through regulation of NMDA receptors in the PFC to improve schizophrenic symptoms and to change behaviors. We tested this hypothesis and demonstrated that mGluR2/3 agonists may modulate the function of NMDA receptors through postsynaptic actions and reverse the MK-801-induced NMDA dysfunction via activation of the Akt/GSK-3 β pathway.

MATERIALS AND METHODS

Animals and treatments

We used 144 female Sprague-Dawley rats at 90 ± 2 days (250–278 g). The animals were cared for under the animal use guidelines of the National Institutes of Health, and the experimental protocol was approved by the Institutional Animal Care and Use Committee at Drexel University College of Medicine. Female young adult Sprague-Dawley rats (3 months) were selected because PCP-, MK-801-, and ketamine-induced cortical injury is more reproducible in female and adult animals (Dickerson and Sharp, 2006; Farber *et al*, 1995; Nakki *et al*, 1996). Animals were allowed to adapt to the new environment for 2 days before we began the experiments. Acute treatment (single-dose, intraperitoneal, i.p.): (1) clozapine (5 mg/kg), mGluR2/3 agonist LY379268, 0.3 or 3.0 mg/kg, MK-801 (0.033 or 1 mg/kg); (2) single-dose of MK-801 (0.033, 0.1, or 1 mg/kg, i.p.) followed by LY379268 (0.3, 1, or 3 mg/kg, i.p.) 50 min later; or LY379268 as pre-treatment 30 min prior to MK-801 administration. GSK inhibitor 3-(2, 4-dichlorophenyl)-4-(1-methyl-1H-indol-3yl)-1H-pyrrole-2, 5-dione (SB216763, 1 mg/kg, i.p.) was applied 30 min prior to MK-801 or LY379268. Saline solution (0.9 % sodium chloride) was used as the vehicle control. Subchronic administration of MK-801 was performed by 5 consecutive daily injections at 0.033 mg/kg or 1.0 mg/kg, followed by LY379268 at 0.3 mg/kg or 3.0 mg/kg 50 min after each MK-801 injection, with 0.9% saline as the vehicle control. We chose the dosage and time interval for MK-801 and LY354740 injections on the basis of previous electrophysiological and behavioral findings (Cartmell *et al*, 1999; Homayoun *et al*, 2005;

Moghaddam *et al.*, 1998) and those from our recent report (Xi *et al.*, 2009b). Clozapine and MK-801 were obtained from Sigma-Aldrich; LY379268 and SB216763 were purchased from Tocris Bioscience. All animals were deeply anesthetized with Euthasol (0.2 ml/kg, Virbac Animal Health) and were immediately decapitated. Brain tissues containing medial and dorsal frontal cortex (with striatum excluded) were collected for Western blot and medial PFC (prelimbic area) for electrophysiological recording. The tissues were taken 50 min after the drug injection.

Western blotting

The PFC tissues were trimmed, weighed immediately, and homogenized in lysis buffer (5 μ l/mg, 20 mM Tris-HCl with pH 7.4, 200 mM NaCl, 1 mM Na₃VO₄, 10 mM NaF, and protease inhibitor cocktail). After centrifugation at 13,000g for 15 min at 4 °C, the supernatant was transferred into new tubes, and the protein concentration was measured using a protein assay kit (Bio-Rad Laboratories). Each sample contained 10 μ g of protein that was dissolved in 10 μ l lysis buffer solution with 5 μ l 6 \times sample buffer and 0.5 μ l β -mercaptoethanol. The protein samples were boiled at 95 °C for 3 min, subjected to SDS-PAGE gel electrophoresis, and then transferred to nitrocellulose membranes (Bio-Rad) overnight. The membranes were blocked with 5% nonfat dry milk in TBST (0.05% Tween-20 in 1 \times Tris-buffered saline) for 1 h and were incubated in the following dilutions of primary antibodies for 1 h: monoclonal mouse anti-NR2A and anti-NR2B (1:4,000, Zymed/Invitrogen), monoclonal mouse anti-actin (1:20,000, Sigma-Aldrich), rabbit anti-pNR2B Tyr1472 (1:2,000, Millipore), rabbit anti-pNR2B Ser1303 (1:2,000, Millipore), and rabbit anti-GSK-3 β , rabbit anti-pGSK Ser9, rabbit anti-Akt, rabbit anti-pAkt Ser473 at 1:500-1,000 (Cell Signaling). After several rinses with TBST, the membranes were incubated in horseradish peroxidase-conjugated goat anti-mouse or rabbit IgG (Jackson ImmunoResearch Laboratories) at 1:8,000 for 2 h. The immunopositive protein bands were detected with ECL Western Blotting System (Amersham Bioscience). After exposure of the membranes to film, the band densities were measured with Image J (NIH). Final data were normalized to the levels of β -actin or total proteins (for phosphorylation) and then to the control levels with control as 1. To minimize interblot variability, each group included 4–5 rats and each sample from an animal was analyzed 4 times. The mean value for each sample was calculated from all of the replicates in the different animals, and the results were presented as mean \pm standard error. Significance was determined with the Student *t* test or ANOVA.

Immunoprecipitation

Tissues containing PFC were microdissected and then homogenized in ice-cold NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, and protease inhibitor cocktail), centrifuged at 13,000 g for 10 min at 4°C. Supernatant fractions (500 μ g proteins) were incubated overnight with 2.5 μ g of monoclonal anti-mGluR2/3 (Millipore) or anti-NR2B (BD Bioscience). The immuno-complexes were isolated by addition of 25–100 μ l of protein G-sepharose beads (GE Healthcare Bio-Sciences AB), followed by incubation for 3 h to 4 h at 4°C. The pellets were then washed four times with lysis buffer, resuspended in Laemmli sample buffer, and boiled for 10 min. After they were centrifuged at 10,000 g for 5 min, the supernatant was collected. The immunoprecipitated proteins were analyzed by Western blot

with antibodies against mGluR2/3 or NR2B. Wash-in supernatants that were treated with pellets of IP-NR2B or IP-mGluR2/3 after the IP samples were centrifuged were used as negative controls to avoid a false positive response.

Electrophysiological Recoding in Prefrontal Cortical Slices

Sprague-Dawley rats at postnatal day 12 to 30 were used for this study. The detailed procedure can be found in the reports of our previous studies (Li *et al*, 2009). The animals were deeply anesthetized with Euthasol (0.2 ml/kg), and the brains were removed immediately. The blocks of neocortex containing medial PFC were trimmed and were sectioned using Leica VT1000s Vibratome (Leica Microsystems USA). Horizontal brain slices at a thickness of 300 μm were incubated in oxygenated Ringer's solution at 35 °C for 1 h. The Ringer's solution contained the following ingredients (in mM): 128 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 2 CaCl_2 , 1 MgSO_4 , 26 NaHCO_3 , and 10 dextrose, pH 7.4. Whole-cell patch clamp recordings were conducted in the medial PFC through an upright Zeiss Axioskop 2 microscope (Carl Zeiss, Inc.) equipped with optics of infrared-differential interference contrast and a digital video camera system. The recordings were conducted at ~ 35°C. The resistance of the recording pipette was 7–9 M Ω . A Cs⁺-solution containing (in mM) Cs-gluconate 120, lidocaine 5 (QX-314), 6 CsCl_2 , 1 ATP-Mg, 0.2 Na_2GTP , and 10 Hepes at pH 7.3 (adjusted with CsOH) was used to block sodium and potassium channels. NMDA was applied either by pressure puff (100 μM) through a glass peptide (~ 2 M Ω) connected to a microinjector (PMI-100, Cornerstone series, Dagan Corp.) or by bath (15 μM), NMDA-induced inward currents were recorded at –60 mV in the presence of CNQX (20 μM) and picrotoxin (50 μM). All chemicals were purchased from Sigma-Aldrich unless otherwise specified (Sigma-Aldrich Inc.). The electric signals were amplified and filtered at 2 kHz in voltage clamp mode with a MultiClamp 700B (Molecular Devices) and acquired through a DigiData 1322A (data acquisition system) and pCLAMP 9.2 software (Molecular Devices). The series resistances were compensated and were constantly monitored. The amplitudes of the NMDA-induced currents were measured with Clampfit 9.2 software (Molecular Devices).

Total Activity Testing

A SmartFrame open field activity box (Kinder Scientific, Poway, CA) containing 32 infrared photobeams in both the X and Y plane was used to assess the animal's activity level for five minutes starting at 60 minutes after the last injection. This system recorded each time any part of the animal's body crossed a photobeam and allowed us to determine the total activity level for each animal as the sum of its horizontal, vertical, and repetitive crossings. Female adult rats (PD90, n=20) were tested for baseline total activity, then randomly divided into two groups (n=10 each). One group received 5 daily IP injections of 0.033 mg/kg MK-801 and the control group received 5 daily IP injections of saline prior to retesting at the same time of day as baseline testing. Total activity data are presented as the percent decrease from baseline. Data that were multiple SD from the mean were considered to be outliers and excluded from the data set, which resulted in n=9 per group. Data were analyzed by Student's t-test (unpaired, two-tailed) at a significance level of 0.05.

RESULTS

The mGluR2/3 agonist LY379268 recovered the disrupted expressions of NMDA receptors induced by subchronic MK-801 administration

Although a clinical trial has reported that mGluR2/3 agonists could be a nondopaminergic antipsychotic agent to treat schizophrenia, the mechanisms by which they operate remain unclear. To study whether the mGluR2/3 agonist LY379268 could reverse the disrupted functions of NMDA receptors induced by MK-801 treatment, we gave rats MK-801 at two different intraperitoneal (i.p.) doses (low dose, 0.033 mg/kg; high dose, 1 mg/kg) once a day for 5 days (Xi *et al.*, 2009b). We chose a relatively low dose of MK-801 at 0.033 mg/kg and high dose of 1 mg/kg because we previously reported that these two doses induced opposite changes of NMDA receptor subunits and 0.033 mg/kg was the most effective dosage in inducing alterations of NMDA receptor subunits compared to the intermediate dose of 0.1 or 0.33 mg/kg (Xi *et al.*, 2009b). In addition, this relative low dosage is effective in affecting the locomotor activity as previous studies reported (Jackson *et al.*, 2004; Tsukada *et al.*, 2005; Winger *et al.*, 1989). Indeed, daily treatment with MK-801 (0.033 mg/kg, once a day for 5 days) resulted in a significant reduction in total activity compared with saline treatment, relative to the animals' pre-drug baseline ($n=20$, $P < 0.05$). No other motor effects were observed in the animals (Supplemental Fig. 1).

LY379268 at 0.3 or 3.0 mg/kg i.p., respectively, was administered 50 min after each injection of MK-801. We first examined the expression levels of NR2A and NR2B subunits in response to subchronic treatment with MK-801. Note that the expression levels of NR2A and NR2B were normalized to those of actin controls, whereas the expression levels of phospho-NR2B Tyr1472 and Ser1303 were normalized to the total protein level of NR2B. As shown in Figure 1, treatment with 0.033 mg/kg MK-801 significantly increased expression levels of NR2A and NR2B subunits ($P < 0.05$) without significant effect on the NR2A/NR2B ratio ($P > 0.05$). In contrast, at a high dose of 1 mg/kg, MK-801 induced significant decreases in the levels of both NR2A and NR2B proteins ($P < 0.05$) and in the NR2A/NR2B ratio ($P < 0.05$). These data indicate that low and high doses of MK-801 induce opposite changes in NMDA receptors in the PFC, consistent with the results of our recent study (Xi *et al.*, 2009b). In addition, subchronic administration of MK-801 at both doses significantly decreased the ratios of pNR2B/NR2B at Ser1303 and Tyr1472 ($P < 0.05$).

We found that low-dose treatment with LY379268 (0.3 mg/kg) led to complete recovery of the disrupted expression of NMDA receptors induced by low-dose MK-801. Both total protein expression and NR2B phosphorylation were recovered to control levels ($P > 0.05$, Fig. 1A). In contrast, while treatment with LY379268 (3 mg/kg) completely restored the pNR2B/NR2B ratio at the phosphorylation sites of Ser1303 and Tyr1472, it only partially rescued the total proteins of NR2A and NR2B subunits induced by high-dose MK-801 (ANOVA, MK-801 at 0.033 mg/kg and LY at 0.3 mg/kg: NR2A, $F=47.5$, $P<0.001$; NR2B, $F=23.39$, $P<0.001$; pNR2BTyr1472, $F=25.91$, $P<0.001$; pNR2BSer1303, $F=46.98$, $P<0.001$. MK-801 at 1.0 mg/kg and LY at 3.0 mg/kg: NR2A, $F=64.17$, $P<0.001$; NR2B, $F=13.58$, $P<0.001$; pNR2BTyr1472, $F=2.78$, $P=0.085$; pNR2BSer1303, $F=42.93$, $P<0.001$;

Fig. 1B). These data indicate that, as we proposed, the mGluR agonist can restore the disrupted NMDA receptor functions induced by subchronic MK-801 treatment by readjusting subunit expression and phosphorylation of NMDA receptors in the PFC.

Acute administration of LY379268 reverses the disrupted NMDA receptor expression induced by MK-801 treatment

To investigate the mechanisms associated with LY379268 in the modulation of NMDA receptors, we tested acute effects of MK-801 and LY379268 on the NR2A and NR2B expressions. As shown in Figure 2, a single low-dose injection of MK-801 (0.033mg/kg, acute) induced a significant increase of NR2B subunit ($P < 0.05$) and a decrease of pNR2B Tyr1472 ($P < 0.05$), but had no effect on NR2A ($P > 0.05$). A single, low dose injection of LY379268 (0.3 mg/kg), either prior to or after MK-801 administration, partially reversed the MK-801 effects on NR2B and pNR2BTyr1472 expressions. In contrast, high-dose MK-801 (1 mg/kg) administration significantly decreased the total protein levels of both NR2A and NR2B subunits, as well as pNR2BTyr1472 expression ($P < 0.05$). LY379268 (3 mg/kg), when applied 50 min after, but not 30 min prior to, MK-801 administration, partially reversed NR2A but not NR2B expression, and completely recovered pNR2BTyr1472 to control levels (ANOVA, MK-801 at 0.033 mg/kg and LY at 0.3 mg/kg: NR2A, $F=2.95$, $P > 0.05$; NR2B, $F=3.56$, $P < 0.001$; pNR2BTyr1472, $F=5.42$, $P < 0.005$. MK-801 at 1.0 mg/kg and LY at 3.0 mg/kg: NR2A, $F=19.70$, $P < 0.001$; NR2B, $F=2.16$, $P=0.110$; pNR2BTyr1472, $F=13.73$, $P < 0.001$). This data indicates that acute LY379268 is able to restore the disrupted NMDAR functions induced by acute MK-801 administration but may not prevent the effects of MK-801 at high dose.

Similar to clozapine, the mGluR2/3 agonist LY379268 increases the expression of NMDA receptors in normal rat PFC

Several lines of evidence implicate an NMDA receptor hypofunction in the cognitive deficits of patients with schizophrenia, suggesting that pharmacological manipulation of NMDA receptors may be a feasible therapeutic strategy for treatment of this devastating disorder (Coyle *et al*, 2003; Farber *et al*, 1998; Millan, 2005; Moghaddam, 2003). Indeed, previous studies have indicated that functions of NMDA receptors are effectively regulated by D2 antipsychotic agents (Arvanov *et al*, 1997; Ninan *et al*, 2003; Wittmann *et al*, 2005). We therefore examined the effects of acute LY379268 (single dose injection, i.p.) on NMDA receptor subunit expression and on NR2B phosphorylation in the PFC compared with those of the D2 antipsychotic drug clozapine (5 mg/kg, i.p.). We found that, as shown in Figure 3, similar to the effects of clozapine, LY379268 significantly increased the expression of both NR2A and NR2B subunits as well as the phosphorylation of pNR2B Ser1303 in the PFC (ANOVA, NR2A, $F=21.10$, $P < 0.001$; NR2B, $F=6.50$, $P < 0.005$; pNR2BSer1303, $F=6.59$, $P < 0.005$). The representative Western blot images and the summary histogram (Fig. 3A and B) showed the comparison of NR2A, NR2B, and pNR2B Ser1303 expressions between control and drug-treated groups. Overall, NR2A protein was increased to higher levels than NR2B for all drug treatments ($P < 0.05$). The total NR2A protein level was particularly upregulated by a high-dose of LY379268 (3.0 mg/kg) compared with that of a low dose (0.3 mg/kg, $P < 0.05$). Our data support previous studies indicating that clozapine can directly regulate NMDA receptors (Arvanov *et al*, 1997; Ninan

et al, 2003; Wittmann *et al*, 2005). Most importantly, the enhancing effects of LY379268 on NMDA receptors support our hypothesis and are in agreement with recent findings reported in vitro (Oliveira *et al*, 2008; Tyszkiewicz *et al*, 2004).

The mGluR2/3 agonist LY379268 enhances NMDA-mediated current in layer 5 pyramidal neurons

To confirm the effects of the mGluR2/3 agonist on NMDA receptor function, we investigated the effects of LY379268 on NMDA-induced currents in PFC layer 5 pyramidal neurons. As shown in Figure 4A, bath-applied NMDA (15 μ M) induced stable inward current when recorded at -60 mV in the presence of AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (20 μ M) and GABA_A receptor antagonist picrotoxin (50 μ M) with regular Ringer's solution. LY379268 (300 nM) dramatically and significantly increased the NMDA-induced current by an average of $48.6 \pm 14.7\%$ ($n = 4$, $P < 0.05$). This enhancement was further verified in another set of experiments in which NMDA was applied through pressure puffs (100 μ M puff, 0.2–0.3 s, 1–2 psi, every 90 s). The puff-NMDA-induced currents were also dramatically and significantly increased by LY379268 (300 nM bath) for $53.1 \pm 13.0\%$ ($n = 5$, $P < 0.01$, Fig. 4B), and this effect was long lasting with little recovery in washout. Again, the NMDA enhancement was consistent with results from previous studies conducted in dissociated prefrontal neurons (Tyszkiewicz *et al*, 2004) and in cortical slices under conditions of blocking glutamate transporters (Oliveira *et al*, 2008).

The mGluR2/3 and NMDA receptors are not co-localized in the PFC

Our results provide strong evidence that mGluRs regulate NMDAR subunit expression, phosphorylation, and function. To examine how this occurs, we first tested whether the effects involve protein-protein interactions because previous studies indicated that mGluRs could potentially interact with other receptor proteins (Enz, 2007; Gonzalez-Maeso *et al*, 2008). As shown in Figure 5, the representative western blot images of co-immunoprecipitation showed that the immunoprecipitated protein of mGluR2/3 did not contain NR2B (Fig. 5A), and the immunoprecipitated protein of NR2B also did not contain mGluR2/3 (Fig. 5B). Because NR2B subunits usually form heteromer with NR2A and NR1 in the central nervous system (Cull-Candy and Leszkiewicz, 2004), these results suggest that direct protein-protein interaction between these two receptors may not occur in the prefrontal neurons.

The mGluR2/3 agonist LY379268 shares a common signaling pathway (Akt/GSK pathway) with clozapine in the normal rat PFC

How does the mGluR2/3 agonist affect the expression and function of NMDA receptors if not via direct protein-protein interaction? Because mGluR2/3 agonists share similar antipsychotic efficacy with D2 antipsychotic agents, we tested whether they share this same signaling pathway, as previously reported (Svenningsson *et al*, 2003), in the PFC by examining the acute effects of LY379268 on the Akt and GSK-3 β signaling. To detect the phosphorylation of Akt and GSK-3 β , we injected the drugs intraperitoneally 60 min prior to anesthesia and decapitation for immunoblot analysis. Acute intraperitoneal administration of

LY379268 (0.3 and 3.0 mg/kg) or clozapine (5 mg/kg) similarly affects the expression of Akt and GSK-3 β (Fig. 6). The total protein levels of GSK-3 β in all drug-treated groups were stable, without significant changes ($P > 0.05$, Fig. 6). In contrast, LY379268 at 0.3 and 3.0 mg/kg, and clozapine significantly increased the expression of GSK-3 β Ser9 phosphorylation by 1.36-, 1.22-, 1.79-, and 1.50-fold, respectively ($P < 0.05$). Moreover, Akt, an upstream inhibitor of GSK3 β , was significantly increased by LY379268 at 3.0 mg/kg and by clozapine treatment ($P < 0.05$) but was not altered by LY379268 at 0.3 mg/kg. The phosphorylation of pAkt Ser473, which would activate Akt, was, however, differentially and significantly changed by LY379268 at 0.3 mg/kg (increased almost 2-fold, $P = 0.010$) and 3.0 mg/kg (decreased, $P = 0.007$), and decreased but not significantly by clozapine ($P = 0.057$). ANOVA analysis indicates significant changes of pGSK-3 β Ser9, Akt and pAkt but not GSK-3 β (GSK-3 β , $F=0.68$, $P=0.574$; pGSK-3 β Ser9, $F=5.42$, $P<0.01$; Akt, $F=53.23$, $P<0.001$; pAktSer473, $F=6.80$, $P<0.005$). These results indicated that LY379268 and D2 antipsychotic agents similarly decreased GSK-3 β activity by increasing pGSK-3 β Ser9 and Akt or pAkt Ser473 expressions, consistent with those from previous studies (Emamian *et al*, 2004; Li *et al*, 2007).

Regulation of GSK-3 β activity contributes to the postsynaptic effects of mGluR2/3 agonist LY379268 on disrupted NMDA receptors induced by MK-801

Previous studies indicated that phosphorylation of molecules associated with the GSK-3 β signaling pathway in rat brain was dose-dependently altered by injection with PCP/MK-801 (Ahn *et al*, 2005; Seo *et al*, 2007), with significant alterations of pAktSer473 and pGSK-3 β Ser9 levels. These changes peaked at 30 min and remained steady until 90 min after injection. The question is whether treatment with LY379268 could restore the altered Akt-GSK-3 β signaling induced by acute and subchronic MK-801 injection *in vivo*. As shown in Figure 7, acute MK-801 at a low-dose (0.033 mg/kg) induced negligible effects on total protein levels of GSK-3 β and Akt ($P > 0.05$) and of pGSK-3 β Ser9 ($P > 0.05$) but led to a significant decrease of protein levels of pAkt Ser473 ($P < 0.05$), indicating a possible increase in GSK-3 β activity. Treatment with LY379268 rescued the pAkt Ser473 to vehicle control levels. In contrast, high-dose MK-801 (1 mg/kg) significantly decreased the total proteins of GSK-3 β and Akt ($P < 0.05$) as well as the ratios of pGSK-3 β Ser9/GSK-3 β and pAkt Ser473/Akt ($P < 0.05$) (Fig. 7A and B); these results agree with those of Lei *et al* (Lei *et al*, 2008) and differ slightly from those of other researchers (Ahn *et al*, 2005; De Sarno *et al*, 2006; Seo *et al*, 2007). LY379268 treatment partially but significantly reversed the alterations of Akt and pAkt Ser473 induced by MK-801 when it (LY379268) was applied after but not prior to MK-801. It did, however, completely recover the expressions of GSK-3 β and pGSK-3 β Ser9 (ANOVA, MK-801 at 0.033 mg and LY at 0.3 mg/kg: GSK-3 β , $F=0.94$, $P=0.432$; pGSK-3 β Ser9, $F=0.46$, $P=0.712$; Akt, $F=0.32$, $P=0.812$; pAktSer473, $F=3.72$, $P=0.028$. MK-801 at 1 mg and LY at 3 mg/kg: GSK-3 β , $F=3.18$, $P=0.036$; pGSK-3 β Ser9, $F=9.16$, $P<0.001$; Akt, $F=27.63$, $P<0.001$; pAktSer473, $F=4.29$, $P=0.017$). These data suggest that LY379268 reversed the effects of MK-801 by modulating the Akt/GSK-3 β pathway.

We next examined the effects of MK-801 and LY379268 on the Akt/GSK-3 β pathway under conditions of subchronic treatments to see whether LY379268 could also recover subchronic

MK-801-induced disruption of NMDA receptors in the PFC via this signaling pathway. As shown in Figure 7C, the upper panel represents the immunoblots of GSK-3 β and pGSK-3 β Ser9 in control, MK-801-treated, MK-801/LY379268-treated groups, whereas the lower panel shows the summary data. MK-801 at 0.033 mg/kg significantly decreased the protein of pGSK-3 β Ser9 ($P < 0.05$) but not the total protein of GSK-3 β ($P > 0.05$); this decrease was reversed by LY379268 at 0.3 mg/kg ($P > 0.05$). In contrast, both GSK-3 β and pGSK-3 β Ser9 were significantly decreased by subchronic MK-801 at 1.0 mg/kg ($P < 0.05$), and LY379268 at 3.0 mg/kg restored the pGSK-3 β Ser9 level ($P > 0.05$) but had little effect on total GSK-3 β expression ($P < 0.05$, ANOVA analysis of MK-801 at 0.033 mg and LY at 0.3 mg/kg: GSK-3 β , $F=1.09$, $P=0.350$; pGSK-3 β Ser9, $F=11.83$, $P<0.001$. MK-801 at 1 mg and LY at 3 mg/kg: GSK-3 β , $F=2.24$, $P=0.135$; pGSK-3 β Ser9, $F=9.13$, $P<0.005$. Fig. 7C), suggesting a complex action.

Intermediate dose of LY379268 reversed the effects of medium dose MK-801 on both NMDA receptors and GSK-3 β activity

Because of the complicated actions of low and high doses of LY379268 on the effects of low (0.033 mg/kg) and high (1.0 mg/kg) doses of Mk-801-induced expression of GSK-3 β activity, we tested the effects of an intermediate dose of LY379268 (1.0 mg/kg) on medium-dose (0.1 mg/kg, single dose) MK-801-induced expression of NR2 subunits and the Akt/GSK-3 β pathway. As exhibited in Figure 8, LY379268 also recovered the MK-801 effects on the expressions of both the NR2A subunit and Akt/GSK-3 β activity. The medium dose of MK-801 induced a significant increase of NR2A ($P < 0.05$), but not NR2B subunit ($P > 0.05$) levels and an intermediate dose of LY379268 recovered the NR2A expression, without a clear effect on NR2B (ANOVA, NR2A, $F=3.33$, $P=0.045$; NR2B, $F=1.44$, $P=0.26$). In addition, the medium dose of MK-801 induced significant decreases of both pGSK-3 β /GSK-3 β and pAkt/Akt ratios ($P < 0.05$), but had no significant effects on the total protein levels of GSK-3 β and Akt ($P > 0.05$). Further, the medium dose of LY379268 reversed the pGSK-3 β /GSK-3 β and pAkt/Akt ratios and exhibited no effects on the total protein expressions of GSK-3 β and Akt (ANOVA, GSK-3 β , $F=2.87$, $P=0.080$; pGSK-3 β Ser9, $F=8.93$, $P=0.003$; Akt, $F=0.67$, 2.87 , $P=0.537$; pAktSer473, $F=7.71$, $P=0.004$). These data indicate that although a higher dose of MK-801 might mimic the psychosis of schizophrenia, its action on the NMDA receptors and Akt/GSK-3 β pathway is more complicated.

Pre-treatment with GSK-3 β inhibitor blocks the effects of LY379268 and MK-801 on the expression and function of NMDA receptors

If GSK-3 β is a critical intracellular mediator in the regulation of NMDA receptor expressions in MK-801 and LY379268 treatment, inhibition of GSK-3 β should block the effects of these drugs. We therefore examined the expression and phosphorylation of NMDA receptors under conditions of applying the potent, selective GSK-3 β inhibitor SB216763 (3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3yl)-1H-pyrrole-2,5-dione) (1 mg/kg, i.p.) 30 min prior to MK-801 or LY379268 injection. This dose was previously reported to be effective in protecting both small intestine epithelium and hippocampal neurons in vivo from radiation-induced apoptosis and can stabilize β -catenin, the downstream substrate of GSK-3 β (Thotala *et al*, 2010; Thotala *et al*, 2008). As shown in Figure 9A and B, an acute high-dose of MK-801 (1.0 mg/kg) dramatically and significantly

decreased the expressions of NR1, NR2A, NR2B, and pNR2B Ser1303 to almost undetectable levels ($P < 0.01$), whereas LY379268 (3.0 mg/kg) significantly increased the expressions and phosphorylation of NR2B, as well as NR1 and NR2A ($P < 0.05$, ANOVA, NR1, $F=18.47$, $P < 0.001$; NR2A, $F=13.56$, $P < 0.001$, NR2B, $F=23.02$, $P < 0.001$, pNR2B Ser1303, $F=76.62$, $P < 0.001$). Pre-administration of the GSK-3 β inhibitor SB216763 successfully blocked the effects of both MK-801 and LY379268 on the expression of NMDA receptor subunits and pNR2B Ser1303 ($P > 0.05$). To further confirm the effects of the GSK-3 β inhibitor, we used another highly selective but structurally different GSK-3 β inhibitor 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD, 10 μ M) to examine the role of LY379268 in NMDA-induced current in layer 5 prefrontal pyramidal neurons. We recently showed that both inhibitors have similar efficacy in hyperdopamine and D2 receptor-mediated action in NMDA receptor internalization (Li *et al*, 2009), as previously reported in cultured cortical neurons (Chen *et al*, 2007b). In addition, we found that both GSK inhibitors themselves did not show clear effects on the basal NMDA receptor-mediated transmissions (Li *et al*, 2009). Similar findings were also reported in other studies (Peineau *et al*, 2008; Peineau *et al*, 2007; Zhu *et al*, 2007). The amplitudes of puff-NMDA-induced currents (100 μ M puff, 0.2–0.3 s, 1–2 psi, every 90 s) were significantly increased by about 50% in bath-applied LY379268 (300 nM, Fig. 9C, left panel and right histogram), and in some cases, spikes were induced due to enhanced NMDA functions. The GSK-3 β inhibitor TDZD itself did not show significant effects on the puff-NMDA-induced currents (data not shown) but was effective in occluding the action of LY379268 when it was applied prior to LY379268 or co-applied with LY379268 (Fig 4 and Fig 9C, middle panel). The average amplitude of NMDA-induced currents in TDZD was almost identical to that in TDZD + LY379268 ($0.17 \pm 0.06\%$, $P > 0.05$, Fig. 9C right histogram), suggesting the clear involvement of the Akt/GSK-3 β pathway in the regulation of the NMDA receptor function.

DISCUSSION

In this study, we utilized the MK-801 model and investigated the antipsychotic effects of the mGluR2/3 agonist LY379268 on NMDAR functions in adult rat PFC. There are several important findings (see Supplemental Table 1). First, we found that MK-801 induced distinct changes of NMDA receptor subunits under both conditions of acute and subchronic treatment. Both the NR2A and NR2B subunits, and particularly NR2A, were significantly increased by low-dose MK-801 but decreased by high-dose treatment. This result was consistent with our recent report of an inverted-U dose dependent alteration of NR2 subunits in response to MK-801 administration (Xi *et al*, 2009b). In addition, both pNR2B Tyr1472 and Ser1303 were significantly decreased by MK-801. Most importantly, LY379268 effectively and bi-directionally recovered the disrupted expression of NMDA receptors induced by MK-801 administration, and this effect was attributable to the direct regulatory action of LY379268 on NMDA receptors via postsynaptic GSK-3 β signaling pathway. These results provide useful insights into the mechanistic actions of mGluR2/3 agonists as potential antipsychotic agents for treating schizophrenia.

It is generally proposed that hypofunction of NMDA receptors on GABAergic interneurons disrupts the functional integrity of the corticolimbic circuit, which in turn results in disinhibition of glutamatergic pyramidal neurons and increased glutamate release (Lisman *et*

al, 2008; Olney *et al*, 1995). Furthermore, numerous studies have reported changes in expression of NR1, NR2A and NR2B in the animal models of NMDA antagonists (Barbon *et al*, 2007; Gao *et al*, 1995; Harris *et al*, 2003; Lindahl *et al*, 2004; Rujescu *et al*, 2006; Wang *et al*, 1999). There is, however, little consensus on what alterations in NMDA receptor subunits might contribute to the NMDA hypofunction observed in patients with schizophrenia, and some of these data are inconsistent (Akbarian *et al*, 1996; Kristiansen *et al*, 2007; Kristiansen *et al*, 2010; Luthi *et al*, 2001; Matthews *et al*, 2000; Meador-Woodruff and Healy, 2000; Moghaddam, 2003). How do the disrupted expressions of NR2A and NR2B subunits, as well as the alteration of phosphorylation, affect brain functions and thus schizophrenia symptoms? NR2A and NR2B are the principal NR2 subunits in neurons of the PFC (Wang *et al*, 2008) and NR2A and NR2B subunits are differentially distributed on pyramidal neurons and GABAergic interneurons (Wang and Gao, 2009). Furthermore, NR2A and NR2B-containing receptors also have different roles in synaptic modulation (Barria and Malinow, 2005). NR2B overexpression in the frontal cortex enhances cognitive functions (Tang *et al*, 1999); whereas mice lacking the NR2A subunit (Itokawa *et al*, 2003) or rats reared in isolation with increased NR2A expression in the PFC (Turnock-Jones *et al*, 2009) display abnormal behaviors, similar to schizophrenic symptoms. Indeed, we found that MK-801 administration induced significant changes of both NR2A and NR2B subunits in the PFC. NMDA receptor blockade has been found to reduce the number of parvalbumin (PV)-containing neurons and disruption in gamma rhythms in the entorhinal cortex (Cunningham *et al*, 2006). Interestingly, these effects may be mediated primarily by NR2A subunit because NR2A is particularly enriched in PV-containing GABA cells at both transcript and protein levels, as we reported (Wang *et al*, 2009; Xi *et al*, 2009a; Xi *et al*, 2009b). Because PV-containing GABA cells are selectively reduced in schizophrenia (Hashimoto *et al*, 2003), it is likely that alterations of NR2 subunits, particularly NR2A, may dramatically change the functions of inhibitory circuitry, as proposed in the NMDA hypofunction hypothesis. Indeed, a recent study suggests that the activity of NR2A-containing NMDA receptors plays a pivotal role in the maintenance of the GABAergic function in PV-containing interneurons (Kinney *et al*, 2006). The alteration of NR2A and NR2B subunits induced by MK-801, as observed in this study, could thus change the balance of neuronal activity between pyramidal neurons and GABAergic interneurons, and consequently affect brain functions.

The molecular mechanisms underlying the functional regulation of NMDA receptors have been the subject of extensive studies. In particular, phosphorylation has emerged as a fundamental mechanism that regulates NMDA receptor trafficking and channel properties (Chen and Roche, 2007a), and consequently, results in schizophrenia (Lau and Zukin, 2007). It follows then, that alterations in NMDAR phosphorylation states could play an important role in NMDAR hypofunction and the pathophysiology of schizophrenia. We found that both phosphorylation sites of NR2B Tyr1472 and Ser1303 were significantly decreased by MK-801. Although D2R-NR2B interaction was found to reduce NR2B phosphorylation at Ser1303 and to inhibit NMDA currents in the striatal neurons (Liu *et al*, 2006), it is unlikely that the change of pNR2BSer1303 observed in our study is mediated by D2 receptors because previous study indicated that mGluR2/3 did not interact with D2 receptors in both *in vivo* and *in vitro* (Fell *et al*, 2009). Nevertheless, the decrease of phosphorylations of

Tyr1472 and Ser1303 in NR2B subunits in the MK-801 model may suggest a reduction of NR2B trafficking or dynamic although how the simultaneous decrease of these two phosphorylation sites affects the brain functions remains to be explored.

A clear picture of how NMDAR expression is disrupted in schizophrenia has not emerged and thus we can not directly compare our current data. Still, the present observation of altered NR2 subunit expression in the PFC is suggestive of NMDA receptor dysfunction in a brain region involved in cognitive functions and therefore provides indirect support for the validity of NMDAR antagonism as a preclinical model of schizophrenia. Our data strongly suggest that alternation of NR2 subunits in the corticolimbic region play a critical role in the regulation of normal cortical functions as well as schizophrenic behaviors. We believe that, as previously reported (Jackson *et al*, 2004), a low dose MK-801-induced increase of NR2A and NR2B would result in dysfunctions of cognitive functions, whereas decreased NR2 subunits in high-dose of MK-801 would induce positive symptoms, as NR2A subunit is significantly decreased in brain tissues in patients with schizophrenia (Woo *et al*, 2004). These studies have thus provided strong evidence for the network effects of NMDA antagonism and established the theoretical basis for the NMDA hypofunction hypothesis for schizophrenia pathogenesis (Lisman *et al*, 2008).

An intriguing question that we explored is whether mGluR2/3 agonist can reverse disrupted NMDA receptor expressions in the MK-801 model. In our view, any antipsychotic agents used to treat schizophrenia should improve the functionality of NMDA receptors if NMDA hypofunction is critical for schizophrenia. We sought to determine whether and how mGluR2/3 agonists could improve the functions of the NMDA receptors. Many researchers speculate that mGluR2/3 agonists block NMDA antagonist-mediated behavior by reducing excessive glutamate release through presynaptic mechanisms (Moghaddam, 2004; Patil *et al*, 2007; Recasens *et al*, 2007; Schoepp, 2001), but how could a presynaptic inhibitor improve the function of NMDA receptors disrupted by noncompetitive antagonists? The mGluR2/3 receptors are localized not only in presynaptic terminals but also in postsynaptic sites and glia in the cerebral cortex and limbic regions (Blumcke *et al*, 1996; Cartmell and Schoepp, 2000b; Liu *et al*, 1998; Moghaddam, 2004; Petralia *et al*, 1996a; Petralia *et al*, 1996b). The highly speculative role of the mGluR2/3 agonist in reducing presynaptic glutamate release ignores the possible modulatory effects of mGluR agonists in postsynaptic sites for both excitatory and inhibitory transmissions. In addition, mGluR2/3 agonists are also effective in attenuating amphetamine-induced hyperlocomotion (Cartmell *et al*, 1999) and LSD- or hallucinogenic drug-induced symptoms (Gonzalez-Maeso *et al*, 2008; Winter *et al*, 2004). The ability of mGluR2/3 agonists to reverse behaviors across psychostimulants and psychomimetics is an interesting feature that strongly suggests a mechanistic commonality between D2 antipsychotic drugs and mGluR2/3 agonists.

Indeed, we found that the mGluR2/3 agonist LY379268 was able to effectively and bi-directionally reverse the disrupted subunit expressions of NMDA receptor subunits induced by both low- and high-dose of MK-801 administration. Interestingly, the total protein level of NR2A was also particularly upregulated by LY379268. An important issue is determining how LY379268 regulates NMDA receptors: by pre- or postsynaptic mechanisms. Our data strongly suggest that the effects of mGluR2/3 agonist on NMDA receptor regulations are

likely attributable to a direct postsynaptic action because 1) the NMDA-induced currents, which do not require presynaptic glutamate release, were significantly enhanced by mGluR2/3 agonist; 2) the large amount of protein expressions and phosphorylations in response to LY379268 treatment also indicated a direct postsynaptic modulation of NMDA receptor function; 3) the LY effects were effectively blocked by GSK-3 β inhibitors under both in vitro and in vivo conditions.

How is this effect achieved and how is the NMDA receptor function regulated by mGluR2/3 agonist? The mGluR2/3 agonists are highly selective, without significant affinity for any other receptors, including dopamine receptors and ionotropic glutamate receptors (Chartoff *et al*, 2005; Fell *et al*, 2009; Harrison, 2008; Patil *et al*, 2007; Swanson *et al*, 2002). We also did not find a direct protein interaction between NR2 and mGluR2/3. Thus, it seems likely that mGluR2/3 agonists function through an intracellular signaling pathway to reverse MK801 induced disruption of NMDARs. However, despite compelling behavioral data and results from a recent preclinical trial (Patil *et al*, 2007), the cellular mechanisms by which activation of mGluR2/3 attenuates the effects of NMDA receptor antagonism remained an open question.

A likely candidate pathway, which we investigated, is the GSK3/AKT pathway. Several lines of evidence converge in implicating abnormal Akt and GSK-3 β activity in the pathogenesis of schizophrenia (Arguello and Gogos, 2008; Emamian *et al*, 2004; Koros *et al*, 2007; Tan *et al*, 2008). Changes in GSK-3 β and Akt have also been identified in several animal models that mimic some of the symptoms of schizophrenia, including the ventral hippocampal lesion model and the dopamine transporter knockout mouse, and following challenge with psychotomimetic, amphetamine, and LSD (Ahn *et al*, 2005; Nadri *et al*, 2003; Svenningsson *et al*, 2003). Collectively, these studies suggest that the Akt/GSK-3 β pathway may play a prominent role in schizophrenia and that antipsychotic agents may alleviate psychosis by directly reversing deficits through the cellular functions associated with GSK-3 β . Because mGluR2/3 agonists and D2-related antipsychotic agents have similar therapeutic efficacies in reducing behavioral alterations in patients with schizophrenia and in animal models (Patil *et al*, 2007), we hypothesize that mGluR2/3 agonists and D2-related antipsychotic agents may share the same intracellular signaling pathway, i.e., Akt/GSK-3 β , in the regulation of NMDA receptor functions. Indeed, we demonstrated that MK-801 at a low dose of 0.033 mg/kg decreased the Akt activity by reducing pAktSer473; at medium dose of 0.1 mg/kg, it increased the activity of GSK-3 β by decreasing both pGSK-3 β Ser9 and pAktSer473; whereas at high dose of 1.0 mg/kg, it not only dramatically reduced the levels of pAktSer473 and pGSK-3 β Ser9, but it also reduced the total protein levels of Akt and GSK-3 β . These data may help explain findings reported in previous studies that both pGSK3 and total GSK3 were reduced in schizophrenia postmortem brain (Emamian *et al*, 2004; Kozlovsky *et al*, 2000, 2002; Nadri *et al*, 2004).

Importantly, similar to clozapine treatment, LY379268 reversed the actions of MK-801 on Akt and GSK-3 β activities. Acute LY379268 at low dose of 0.3 mg/kg significantly increased the levels of pGSK-3 β Ser9 and pAktSer473, indicating a decrease of GSK-3 β activity. By doing so, it can offset the increased Akt and GSK-3 β activity induced by low and medium doses of MK-801. Furthermore, LY379268 significantly enhanced NMDA-

induced current in the prefrontal cortical neurons, and the GSK-3 β inhibitor occluded this effect under both in vitro and in vivo conditions. These data suggest that LY379268 at a relatively low dose may regulate the expression and phosphorylation of NMDA receptors by directly decreasing the activity of GSK-3 β through enhancing the pAktSer473 and/or pGSK-3 β Ser9. Our results are consistent with recent evidence showing that antipsychotic agents can decrease GSK-3 β activity by increasing the phosphorylation of Akt and GSK-3 β Ser9 (Emamian *et al.*, 2004; Li *et al.*, 2007). However, at a higher dosage, LY379268's effects on the Akt and GSK activity seemed to be more complicated and, probably unspecific, although it also effectively reversed the expression of NMDA receptors. At a dose of 3.0 mg/kg, it not only significantly increased the pGSK-3 β Ser9 but also increased the total protein level of Akt, suggesting a clear reduction of GSK-3 β activity. This seems to be odd because a high dose of MK-801 (1.0 mg/kg) also significantly decrease the activities of both Akt and GSK-3 β . Obviously other mechanism(s) may be involved in the regulation of NMDA receptor functions under high dose LY379268 application and further study is needed. Particularly, it is necessary to examine a clinic-relevant dose of mGluR2/3 agonist in the treatment of schizophrenia behaviors and to identify whether behavioral effects of mGluR2/3 agonists are directly associated with NMDA receptor dysfunctions.

It is known that GSK-3 β can be indirectly inhibited via regulation of several intracellular signaling cascades, including the canonical Wnt, Reelin and tyrosine kinase receptor (Trk)-phosphoinositide 3'-kinase (PI3k)-Akt. Recently, direct inhibition of GSK-3 β has emerged as a possible option in the pharmacotherapy of several neuropsychiatric disorders, including schizophrenia. We have demonstrated that the mGluR2/3 agonist, like the D2 antipsychotic agents, is able to reverse the effects of MK-801 by increasing the expression of NMDA receptors in the prefrontal neurons via compensatory regulation of the impaired function of the Akt/GSK-3 β signaling pathway. The Akt/GSK-3 β signaling cascade was recently identified as being particularly important in mediating D2-associated behaviors and prefrontal cortical functions (Arguello *et al.*, 2008; Beaulieu *et al.*, 2007a; Beaulieu *et al.*, 2007b; Beaulieu *et al.*, 2005; Tan *et al.*, 2008) and in hyperdopamine/D2 receptor-mediated regulation of NMDA receptor trafficking (Li *et al.*, 2009). This study thus provides novel evidence for the cellular and molecular mechanisms of mGluR2/3 regulation of NMDA receptors and presents useful insights into the mechanistic actions of mGluR2/3 agonists as potential antipsychotic agents for the treatment of schizophrenia. Specifically, in contrast to the widely proposed mechanism of modulating presynaptic glutamate release, our results strongly argue that mGluR2/3 agonists modulate the function of NMDA receptors through postsynaptic actions and reverse the MK-801-induced NMDA dysfunction via the Akt/GSK-3 β pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
GSK-3β	glycogen synthase kinase-3 β
LSD	lysergic acid diethylamide
LY379268	(-)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylic acid
mGluRs	metabotropic glutamate receptors
MK-801	dizocilpine
NMDA	N-methyl-D-aspartate
PCP	phencyclidine
PFC	prefrontal cortex
RT-PCR	reverse transcription PCR
SB216763	3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3yl)-1H-pyrrole-2,5-dione
TDZD	4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione

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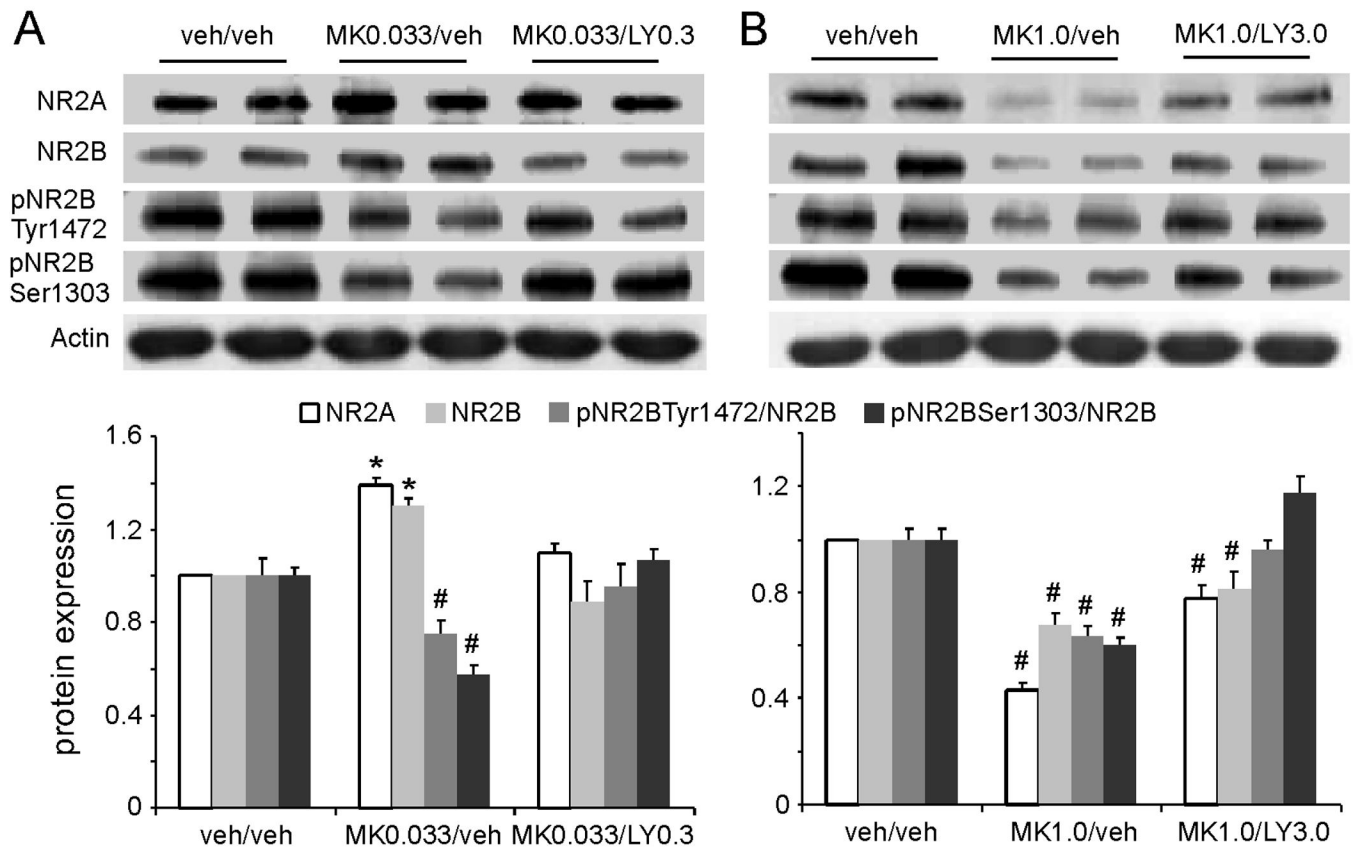


Figure 1.

The mGluR2/3 agonist LY379268 recovered the disrupted expression of NMDA receptors induced by subchronic MK-801 administration. The drugs were administered once a day for 5 days (i.p.). LY379268 was applied 50 min after MK-801 injection and brain tissues were collected for western blotting 50 min after the last injection. (A) Subchronic administration of MK-801 at 0.033mg/kg significantly increased the total proteins of NR2A and NR2B subunits and decreased NR2B phosphorylation at Ser1303 and Tyr1472 ($P < 0.05$). LY379268 treatment (0.3 mg/kg) completely recovered the disrupted expression of NMDA receptors induced by MK-801. Both total protein and NR2B phosphorylation were recovered to control levels ($P > 0.05$). (B) In contrast, MK-801 at 1.0 mg/kg dramatically and significantly decreased the total proteins of NR2A and NR2B subunits, as well as phosphorylation of Ser1303 and Tyr1472 ($P < 0.05$). LY379268 treatment (3 mg/kg), however, only partially but significantly restored the total protein levels of NR2A and NR2B subunits ($P < 0.05$), although the phosphorylation was completely recovered to control levels.

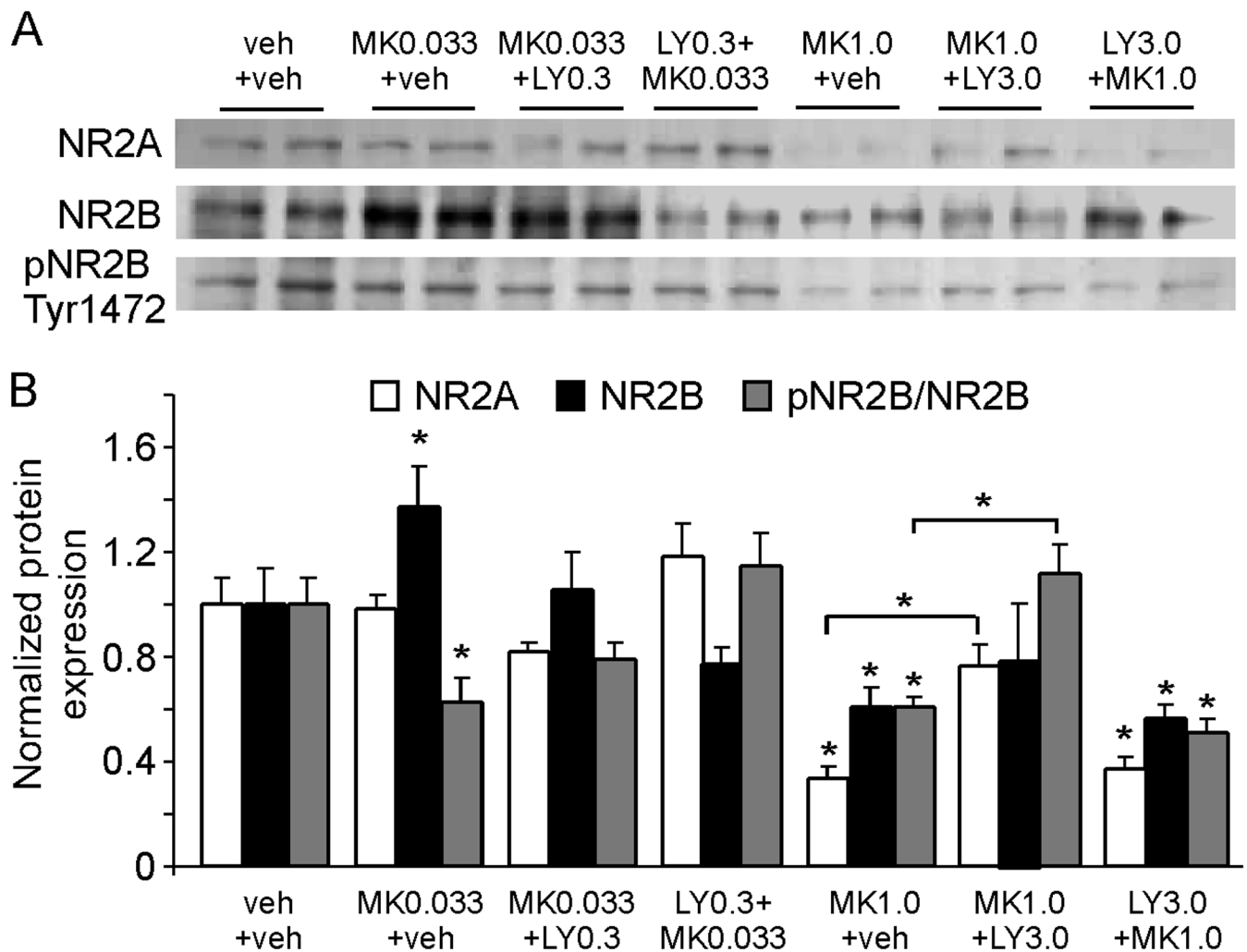


Figure 2.

Acute administration of LY379268 reverses the NMDA receptor expression induced by MK-801 treatment. (A) Representative western blot showing the expressions of NR1, NR2B, and pNR2BTyr1472 under different treatment groups. (B) Summary histogram showing the comparisons of altered NR2A, NR2B, and pNR2BTyr1472. Single low-dose MK-801 (0.033mg/kg, acute, i.p) induced a significant increase of NR2B ($P < 0.05$) and a decrease of pNR2BTyr1472 ($P < 0.05$), but had effect on NR2A ($P > 0.05$). Acute LY379268 (0.3 mg/kg) treatment at low dose, either prior to or after MK-801 administration, partially reversed the MK-801 effects on NR2B and pNR2BTyr1472 expressions. In contrast, high-dose MK-801 (1 mg/kg) significantly decreased both NR2A and NR2B, as well as pNR2BTyr1472 expression ($P < 0.05$). LY379268 (3 mg/kg), when applied 50 min after, but not 30 min prior to, MK-801 administration, partially reversed NR2A but not NR2B, and completely recovered pNR2BTyr1472 to control level.

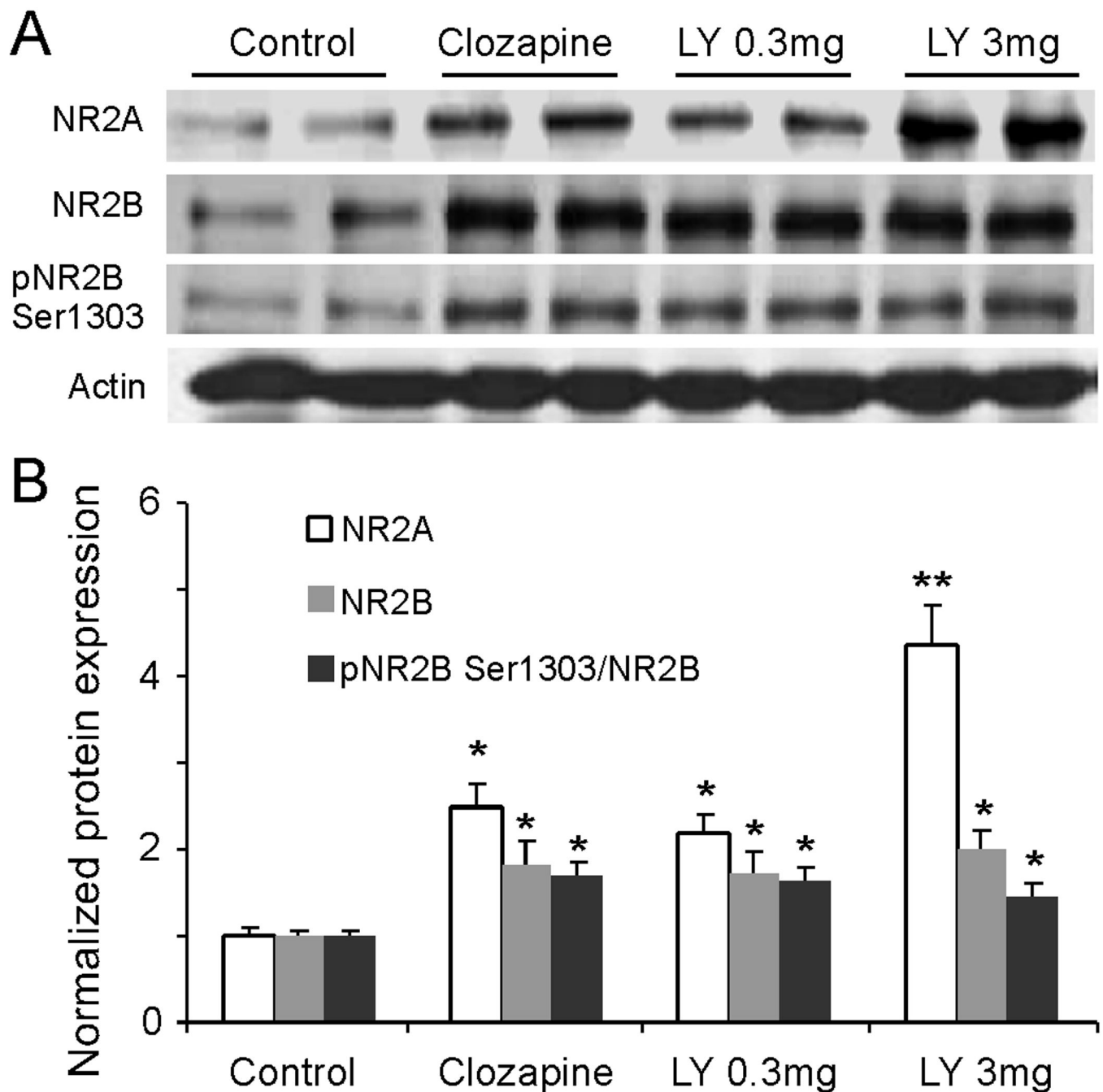


Figure 3.

Similar to clozapine, acute treatment of the mGluR2/3 agonist LY379268 enhances NMDA receptor functions in normal rat PFC. (A) Representative Western blot images of the expression of NR2A, NR2B, pNR2B Ser1303, and actin. (B) Summary histogram showing the similar and significant enhancing effects of clozapine (5 mg/kg, i.p) and LY379268 on the expressions of NR2 subunits and pNR2B Ser1303 ($P < 0.05$). Overall, it appeared that the increases in NR2A proteins were more than those in NR2B for all drug treatments ($P < 0.05$). The total protein level of NR2A was particularly upregulated by high-dose LY379268

(3.0 mg/kg) compared with that of low-dose LY379268 (0.3 mg/kg) and clozapine ($P < 0.05$).

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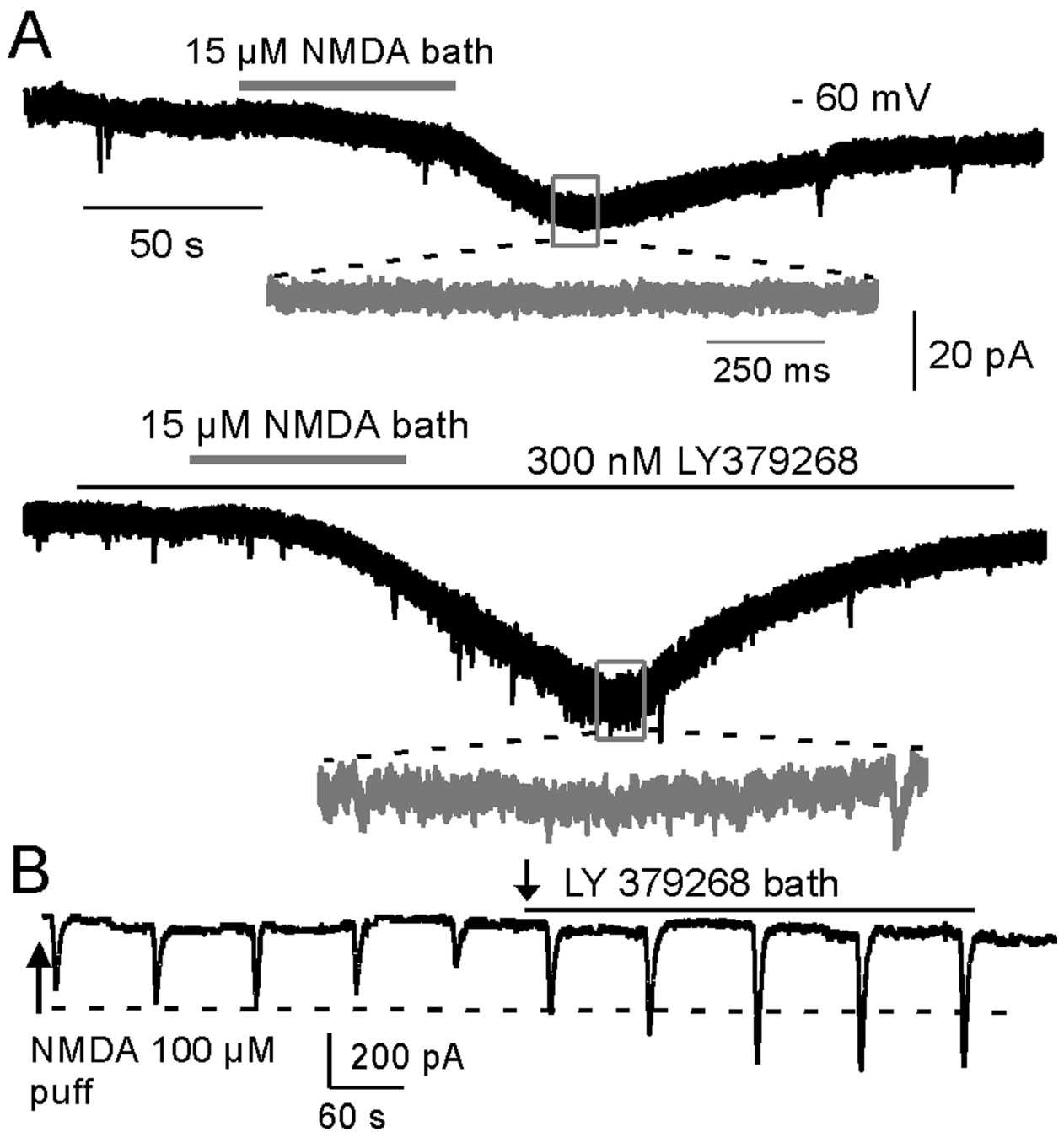


Figure 4.

LY379268 enhances NMDA-mediated current. (A) Bath-applied NMDA (15 μM) induced stable inward current when recorded at -60 mV in the presence of CNQX (20 μM) and picrotoxin (50 μM) with regular Ringer's solution. Bath application of LY379268 (300 nM) dramatically and significantly increased the NMDA-induced current by an average of $48.6 \pm 14.7\%$ ($n = 4$, $P < 0.05$). (B) Puff-NMDA-induced currents were also dramatically and significantly increased by LY379268 (300 nM bath) for $53.1 \pm 13.0\%$ ($n = 5$, $P < 0.01$).

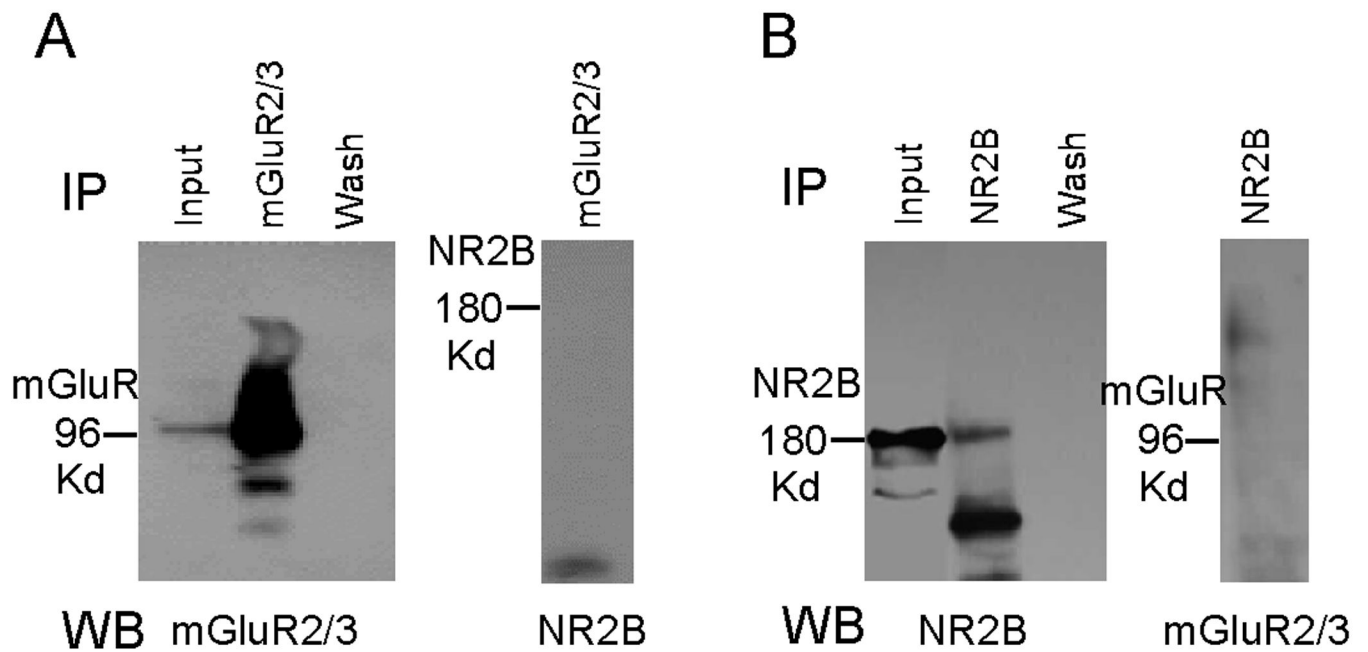


Figure 5.

The mGluR2/3 and NMDA receptors are not co-localized in the PFC. Both mGluR2/3 (A, 96 kD) and NR2B (B, 180 kD) were immunoprecipitated with anti-mGluR2/3 and anti-NR2B antibodies, respectively, and were confirmed by western blot (A and B; left panel). The immunoprecipitated mGluR2/3 proteins did not express NR2B (A; right panel) and immunoprecipitated NR2B proteins did not express mGluR 2/3 (B; right panel), confirming that co-localization is not found between these two receptors. The inputs represented the mGluR2/3 (A; left panel) or NR2B (B; left panel) signal. Wash denotes the negative control, which was treated with the wash-in supernatant of IP-mGluR2/3 (A; left panel) or IP-NR2B (B; left panel) after the immunoprecipitated samples were centrifuged.

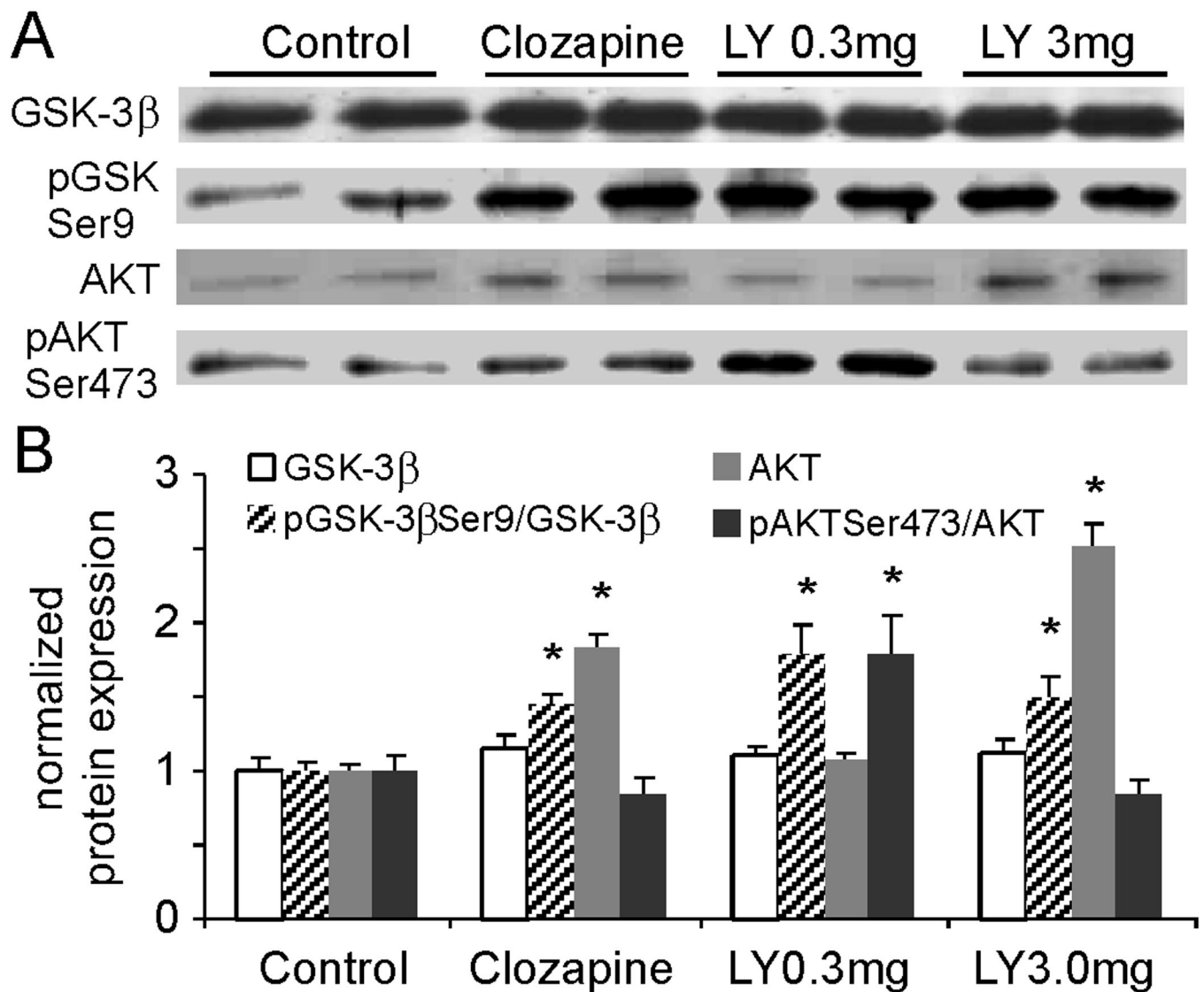


Figure 6.

Clozapine shares a common signaling pathway (Akt/GSK pathway) with the mGluR2/3 agonist in normal rat PFC. Acute intraperitoneal administration of LY379268 (0.3 and 3.0 mg/kg) or clozapine (5 mg/kg) similarly affects the expression of GSK-3 β and Akt. (A and B) The total protein levels of GSK-3 β in all drug-treated groups were stable, without significant changes ($P > 0.05$). In contrast, clozapine and both doses of LY379268 significantly increased the expression of GSK-3 β Ser9 phosphorylation ($P < 0.05$). Moreover, Akt was significantly increased in response to treatment with LY379268 (3.0 mg/kg) or clozapine ($P < 0.05$) but was not altered by treatment with LY379268 at 0.3 mg/kg. The phosphorylation of was, however, differentially and significantly changed by LY379268 at 0.3 mg/kg (increased almost 2-fold, $P = 0.010$) and at 3.0 mg/kg (decreased, $P = 0.007$) and decreased but not significantly by clozapine treatment ($P = 0.057$).

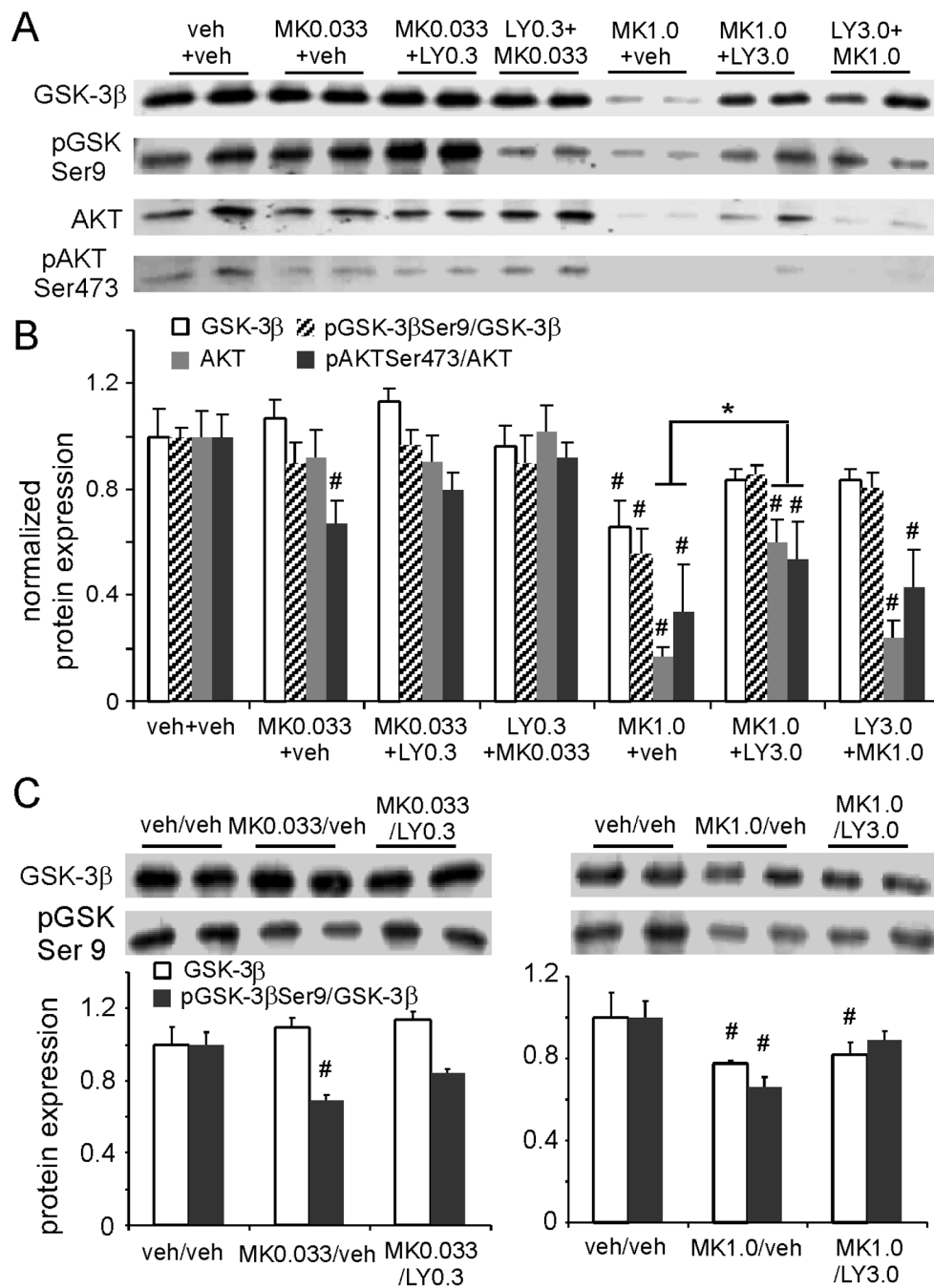


Figure 7.

Activation of the Akt/GSK pathway contributes to the postsynaptic effects of the mGluR2/3 agonists. (A and B) Immunoblots and summary histogram show the expressions of GSK-3β, pGSK3βSer9, Akt, and pAktSer473 in control and six acute treated groups. Acute MK-801 at low-dose (0.033 mg/kg) induced negligible effects on total protein levels of GSK-3β and ($P > 0.05$) and of pGSK-3β Ser9 level ($P > 0.05$), but a significant decrease of pAkt Ser473 ($P < 0.05$), indicating a possible increase of GSK-3β activity. LY379268 rescued the ratio of pAkt Ser473/Akt to vehicle control levels. In contrast, high-dose MK-801 (1 mg/kg)

significantly decreased the total proteins of GSK-3 β and Akt ($P < 0.05$), as well as the ratios of pGSK-3 β Ser9/GSK-3 β and pAkt Ser473/Akt ($P < 0.05$). LY379268 treatment partially but significantly reversed the alterations of Akt and pAkt Ser473/Akt ($P < 0.05$) when it was applied after but not prior to MK-801 administration. The expressions of GSK-3 β and pGSK-3 β Ser9/GSK-3 β were, however, similarly recovered by treatment with LY379268. (C) Subchronic MK-801 at 0.033 mg/kg significantly decreased pGSK Ser9 ($P < 0.05$), but not total protein of GSK-3 β ($P > 0.05$), and this decrease was reversed by LY379268 at 0.3 mg/kg ($P > 0.05$). In contrast, both GSK-3 β and pGSK Ser9 were significantly decreased by subchronic MK-801 at 1.0 mg/kg ($P < 0.05$), and LY379268 at 3.0 mg/kg restored pGSK-3 β Ser9 levels ($P > 0.05$) but had minimal effects on total GSK-3 β expression ($P < 0.05$).

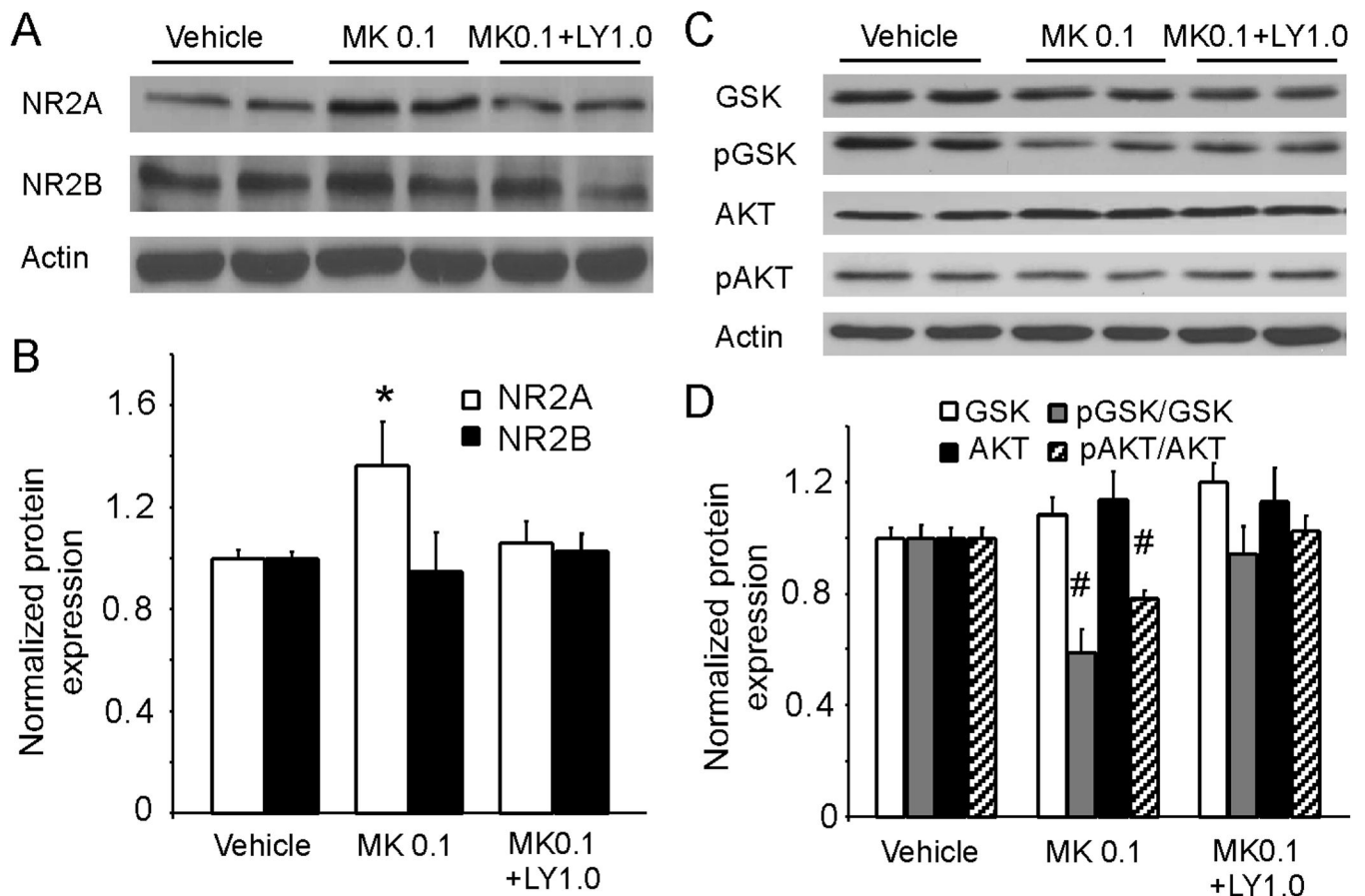


Figure 8.

LY379268 also recovered the acute MK-801 effects on the expressions of both NR2A subunit and Akt/GSK-3 β activity at medium dose. (A) Medium dose of MK-801 (0.1 mg/kg, single dose) induced significant increase of NR2A ($P < 0.05$), but not NR2B subunit and intermediate dose of LY379268 (1.0 mg/kg) recovered the NR2A expression without a clear effect on NR2B. (B) Medium dose of MK-801 induced significant decreases of both pGSK/GSK and pAkt/Akt ratios ($P < 0.05$) but had no clear effects on the total protein levels of GSK-3 β and Akt. Medium dose of LY379268 reversed the pGSK/GSK and pAkt/Akt ratios and exhibited no effects on the total protein expressions of GSK-3 β and Akt.

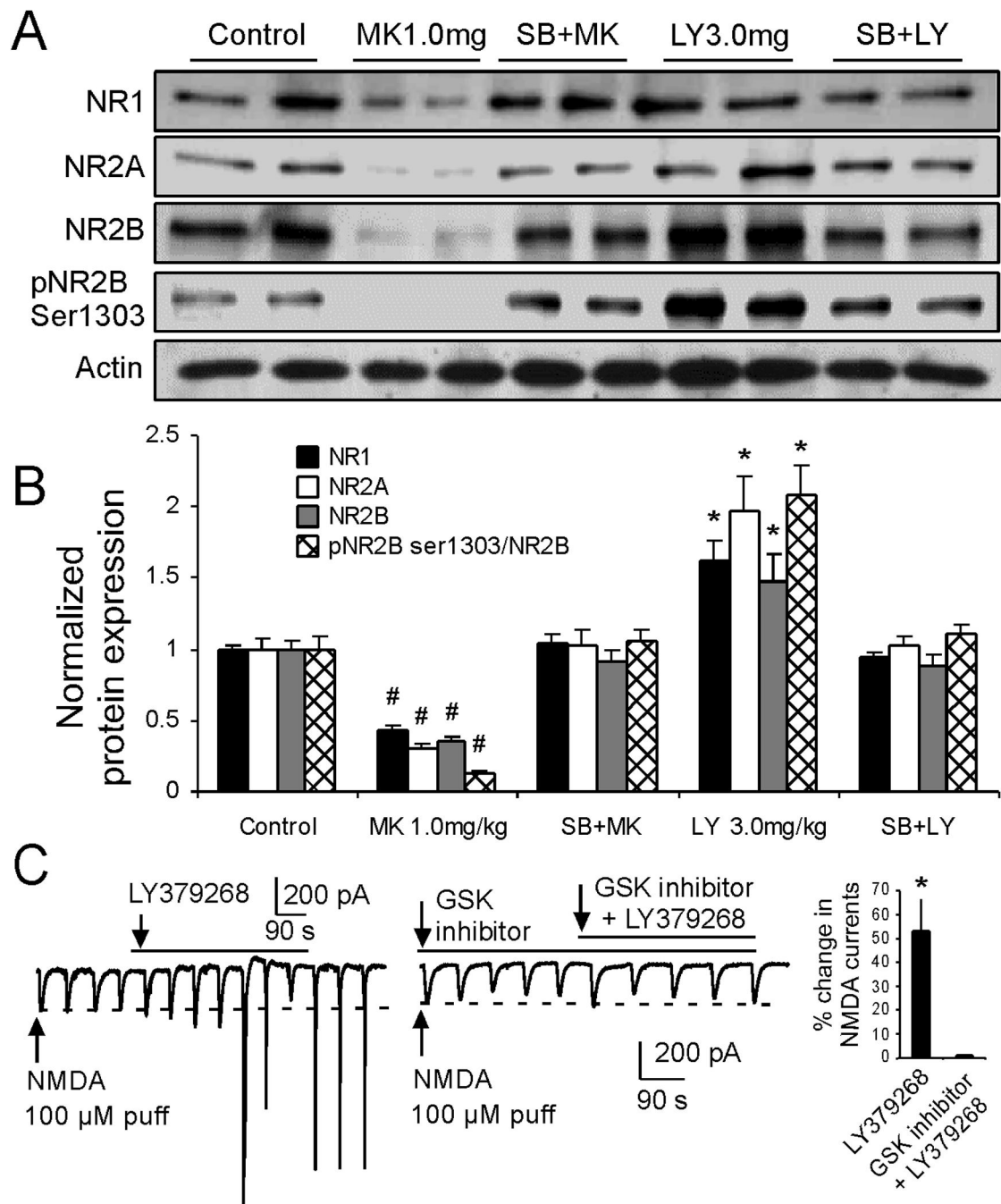


Figure 9. Effects of mGluR2/3 agonists on NMDA receptors are blocked by GSK inhibitor. (A and B) High-dose MK-801 (1.0 mg/kg, single dose) dramatically and significantly decreased the expression of NR1, NR2A, NR2B, and pNR2B Ser1303 to almost undetectable levels ($P < 0.01$), whereas LY379268 (3.0 mg/kg, single dose) significantly increased the expression and phosphorylation of NR2B, NR1, and NR2A ($P < 0.05$). Pre-administration of the GSK-3 β inhibitor SB216763 (1 mg/kg, i.p, 30 min prior to MK-801 or LY379268) successfully blocked the effects of both MK-801 and LY37926 on the expression of NMDA

receptor subunits and pNR2B Ser1303 ($P > 0.05$). (C) Left panel, puff-NMDA-induced currents (100 μ M puff, 0.2–0.3 s, 1–2 psi, every 90 s) were significantly increased by LY379-268 (300 nM bath) and in some cases, spikes were induced by the enhanced NMDA current. Middle panel, selective GSK-3 β inhibitor TDZD (10 μ M bath) itself did not show significant effects on the puff-NMDA-induced currents but was very effective in occluding the action of LY379268 (300 nM bath) when it was applied prior to LY379268 or co-applied with LY379268. Right panel: summary histogram showing that the NMDA current was significantly increased by about 50% in LY379-268 (* $P < 0.05$) whereas the average amplitude of NMDA-induced currents in TDZD was almost identical to that in TDZD + LY379268 ($0.17 \pm 0.06\%$, $P > 0.05$).