JPn

Neuroscience

The Journal of Physiology

SNARE proteins are essential in the potentiation of NMDA receptors by group II metabotropic glutamate receptors

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Key points

- Activation of group II metabotropic glutamate receptors (mGluRs) enhances NMDA receptor (NMDAR)-mediated currents in cortical pyramidal neurons.
- In this study, we found that group II mGluR-induced enhancement of NMDAR currents was associated with increased NMDAR surface expression and synaptic localization.
- Inhibition of SNAP-25 or knockdown of syntaxin 4 blocked the enhancement of NMDAR currents by group II mGluRs.
- Group II mGluRs increase the activity of Rab4 small GTPase. Rab4 knockdown or dominant negative Rab4 abolished the enhancing effect of Group II mGluRs on NMDAR currents.
- These results suggest that SNARE proteins and Rab4 are key molecules involved in the enhancement of NMDAR exocytosis and function by group II mGluRs. Identification of key molecules involved in NMDAR up-regulation could provide novel drug targets for schizophrenia treatment.

Abstract The group II metabotropic glutamate receptors (group II mGluRs) have emerged as the new drug targets for the treatment of mental disorders like schizophrenia. To understand the potential mechanisms underlying the antipsychotic effects of group II mGluRs, we examined their impact on NMDA receptors (NMDARs), since NMDAR hypofunction has been implicated in schizophrenia. The activation of group II mGluRs caused a significant enhancement of NMDAR currents in cortical pyramidal neurons, which was associated with increased NMDAR surface expression and synaptic localization. We further examined whether these effects of group II mGluRs are through the regulation of NMDAR exocytosis via SNARE proteins, a family of proteins involved in vesicle fusion. We found that the enhancing effect of APDC, a selective agonist of group II mGluRs, on NMDAR currents was abolished when botulinum toxin was delivered into the recorded neurons to disrupt the SNARE complex. Inhibiting the function of two key SNARE proteins, SNAP-25 and syntaxin 4, also eliminated the effect of APDC on NMDAR currents. Moreover, the application of APDC increased the activity of Rab4, a small Rab GTPase mediating fast recycling from early endosomes to the plasma membrane, and enhanced the interaction between syntaxin 4 and Rab4. Knockdown of Rab4 or expression of dominant-negative Rab4 attenuated the effect of APDC on NMDAR currents. Taken together, these results have identified key molecules involved in the group II mGluR-induced potentiation of NMDAR exocytosis and function.

Abbreviations APDC, $(2R, 4R)$-4-aminopyrrolidine-2,4-dicarboxylate; BoTx, botulinum toxin; GFP, green fluorescent protein; mGluR, metabotropic glutamate receptor; NMDAR, *N*-methyl-D-aspartate receptor; PKC, protein kinase C; PSD-95, postsynaptic density protein 95; siRNA, small interference RNA; shRNA, short hairpin RNA; SNAP-25, synaptosomal-associated protein 25; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor.

Introduction

The metabotropic glutamate receptors (mGluRs) are G-protein-coupled receptors playing an important role in the regulation of synaptic functions (Conn & Pin, 1997; Anwyl, 1999). Currently, mGluRs have emerged as the promising drug targets for several neurological and psychiatric disorders (Krystal *et al.* 2010). In particular, group II mGluRs, which comprise mGluR2 and mGluR3, have been implicated in the treatment of schizophrenia, anxiety disorders and depression (Krystal *et al.* 2010). Rodent studies suggest that the agonists of group II mGluRs reduce the disruption of working memory and abnormal locomotor activity in schizophrenia models (Moghaddam & Adams, 1998; Harich *et al.* 2007), exhibit anxiolytic efficacy in several stress and anxiety models (Schoepp *et al.* 2003; Nordquist *et al.* 2008), and inhibit ischaemia-induced neuronal death in hippocampus (Pizzi *et al.* 1996). Furthermore, preliminary human studies have shown that the agonists of group II mGluRs effectively alleviate schizophrenia symptoms (Patil *et al.* 2007).

Group II mGluRs are highly expressed in forebrain regions including frontal cortex (Ohishi *et al.* 1993*a,b*; Petralia *et al.* 1996). The mGluR2 is expressed in neurons not only presynaptically but also postsynaptically, while mGluR3 is localized pre- and postsynaptically in both neurons and glial cells (Neki *et al.* 1996; Petralia *et al.* 1996; Tamaru *et al.* 2001). The physiological roles of group II mGluRs in inhibition the release of glutamate or other neurotransmitters have been well studied in presynaptic terminals (Kamiya *et al.* 1996; Schoepp, 2001). However, the function of group II mGluRs in postsynaptic neurons is still elusive. There have been reports showing that the activation of the postsynaptic group II mGluRs fine-tune baroreceptor signal transmission in nucleus tractus solitarius (Sekizawa *et al.* 2009), depolarize neurons and enhance network activity in hippocampus (Ster *et al.* 2011), and reduce calcium currents in cerebellar granule cells (Chavis *et al.* 1994). In addition, a previous study in our lab revealed that the activation of group II mGluRs potentiated NMDA receptor currents in frontal cortical pyramidal neurons (Tyszkiewicz *et al.* 2004).

NMDA receptors (NMDARs) are ionotropic glutamate receptors that play a critical role in regulating synaptic plasticity and cognitive processes. NMDAR dysfunction has been associated with psychiatric disorders, neurodegenerative diseases, and neurodevelopmental illnesses (Gonda, 2012). In particular, NMDAR hypofunction is considered as a fundamental pathophysiology in schizophrenia (Kantrowitz and Javitt, 2012). Treating animals with NMDAR antagonists produces abnormal behaviours that resemble the positive, negative and cognitive symptoms of schizophrenia (Rung *et al.* 2005; Bubenikova-Valesova *et al.* 2008). Thus, pharmacological agents, such as group II mGluRs agonists, that enhance NMDAR function could potentially be used as antipsychotics.

The trafficking of NMDARs between intracellular compartments is dynamically regulated by various proteins (Wenthold *et al.* 2003; Prybylowski & Wenthold, 2004; Lau & Zukin, 2007). Recently, the SNARE proteins (comprising families of membrane-associated proteins, i.e. the synaptobrevin/VAMP, syntaxin and SNAP-25 families), which regulate vesicle transport and docking (Gerst, 1999) and mediate membrane fusion (Jahn & Scheller, 2006), have been implicated in the delivery of NMDAR vesicles at postsynaptic sites (Lau & Zukin, 2007; Suh *et al.* 2010). In this study, we examined the involvement of SNAREs and interacting proteins in the group II mGluR-induced regulation of NMDARs. Identification of key molecules involved in NMDAR up-regulation could provide novel drug targets for schizophrenia treatment.

Methods

Animals and reagents

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of the State University of New York at Buffalo. Pregnant rats were anaesthetized with isoflurane vapour and immediately killed. The frontal cortex was dissected from rat embryos (E18) and used for the cortical culture preparation.

The mGluR ligand $(2R, 4R)$-4-aminopyrrolidine-2, 4-dicarboxylate (APDC) was from Tocris (Ballwin, MO, USA). It was made up as concentrated stocks and stored at $-20°C$. The final DMSO concentration in all applied solutions was less than 0.1%. Stocks were thawed and diluted immediately prior to use. SNAP-25 (synaptosomal-associated protein 25) blocking peptide was designed as QSFFSGLFGGSSKIEEACE, which mimics the NH_2-terminal 19aa of SNAP-25 (Lledo *et al.* 1998). SNAP-23 blocking peptide was designed as MDDLSP-EEIQLRAHQVTD, which mimics the NH_2-terminal domain of SNAP-23 (Vaidyanathan *et al.* 2001). The scrambled peptide (GFAESLFQSIEKESGFSCG) serves as a negative control (Lledo *et al.* 1998).

Transfection and DNA constructs

To suppress the expression of various proteins, small interfering RNA (siRNA) or small hairpin RNA (shRNA) was transfected into cultured neurons using the Lipofectamine 2000 method (Invitrogen, Carlsbad, CA, USA). The shRNA oligonucleotide targeting rat syntaxin 4 (CCTGCGAGAGGAGATCAAA; Kennedy *et al.* 2010) was cloned into pLKO.3G vector (Addgene, Cambridge, MA, USA), which contains an enhanced green fluorescent protein marker (eGFP). The siRNA (20 nM) targeting Rab4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was co-transfected with EGFP (0.3 ng μl^{-1}). Dominant-negative Rab4 (DN-Rab4, Rab4-S27N; Odley *et al.* 2004) was constructed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). All constructs were verified by DNA sequencing. At 2–3 days after transfection, electrophysiological recording was conducted in GFP-positive neurons.

Electrophysiological recordings in cultured neurons

Cortical cultures from E18 embryos were prepared as previously described (Yuen *et al.* 2005, 2011, 2012). Cultures were maintained in Neurobasal with B27 supplement (Invitrogen Grand Island, NY, USA). Recordings of whole-cell NMDA-elicited currents employed similar techniques to those described previously (Gu *et al.* 2005, 2012; Yuen *et al.* 2005). The internal solution contained (in mM): 180 *N*-methyl-D-glucamine, 40 Hepes, 4 MgCl$_2$, 0.1 BAPTA, 12 phosphocreatine, 3 Na$_2$ATP, 0.5 Na$_2$GTP, and 0.1 leupeptin (pH 7.2–7.3, 265–270 mosmol l^{-1}). The external solution contained (in mM): 127 NaCl, 20 CsCl, 10 Hepes, 5 BaCl$_2$, 12 glucose, 1 CaCl$_2$, 20 glycine, and 0.001 TTX (pH 7.3–7.4, 300–305 mosmol l^{-1}). Recordings were obtained with the Axopatch 200B amplifier (Molecular Device, Sunnyvale, CA, USA) controlled and monitored with a PC running pCLAMP with a DigiData 1322A series interface. Electrode resistances were typically 3–5 MΩ in the bath. After sealing rupture, series resistance (4–10 MΩ) was compensated (60–70%) and periodically monitored. The cell membrane potential was held at −60 mV. NMDA (100 μM) was applied for 2 s every 30 s to minimize desensitization-induced decrease of current amplitude. Drugs were applied with a gravity-fed 'sewer pipe' system. Solution changes were affected by the SF-77B fast-step solution stimulus delivery device (Warner Instruments, Hamden, CT, USA). Data analyses were performed with Clampfit (Molecular Device) and KaleidaGraph (Albeck Software, Reading, PA, USA).

Biochemical measurement of surface and total proteins

The surface and total NR2A, NR2B and NR1 receptors were detected as previously described (Yuen *et al.* 2009, 2012). In brief, after treatment, rat cortical slices were incubated with PBS containing 1 mg ml^{-1} sulfo-*N*-hydroxysuccinimide-LC-biotin (Pierce Chemical Co., Rockford, IL, USA) for 20 min on ice. Slices were then rinsed three times in Tris-buffered saline to quench the biotin reaction, followed by homogenization in modified radioimmunoprecipitation assay buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 50 mM Na$_3$PO$_4$, 150 mM NaCl, 2 mM EDTA, 50 mM NaF,10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 mg ml^{-1} leupeptin). The homogenates were centrifuged at 14,000 *g* for 15 min at 4°C. Protein (15 μg) was removed to measure total NR2A, NR2B and NR1. For surface protein, 150 μg of protein was incubated with 100 μl of 50% NeutrAvidin Agarose (Pierce Chemical Co.) overnight at 4°C. Bound proteins were resuspended in 100 μl of 2× loading buffer and boiled. Western blots were performed on both total and biotinylated (surface) proteins using anti-NR2A (1:500, EMD Millipore CO., Billerica, MA, USA, 07–632), anti-NR2B (1:500, , 06–600), or anti-NR1 antibody (1:1000, Cell Signaling Technology Inc., Danvers, MA, USA, 5704).

Co-immunoprecipitation

After treatment, rat cortical slices were collected and homogenized in NP-40 lysis buffer (0.5% NP-40, 10% glycerol, 50 mM Tris, pH 7.6, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 0.1 mM EDTA, 0.1 mM Na$_3$VO$_4$, and 1 mM phenylmethylsulfonyl fluoride, with protease inhibitor tablet). Lysates were ultracentrifuged (100,000 *g*) at 4°C for 1 h. Supernatant fractions were incubated with anti-Rab4 antibody (1:200, BD Bioscience, San Jose, CA, USA, 610889) or anti-rabaptin-5 antibody (2 μg, Santa Cruz Biotechnology, sc-15351) overnight at 4°C, followed by incubation with 50 μl of protein A/G plus agarose (Santa Cruz Biotechnology) for 1 h at 4°C. Immunoprecipitates were washed three times with lysis buffer, then boiled in 2x SDS loading buffer for 5 min, and separated on 10% SDS-polyacrylamide gels. Western blotting experiments were performed with anti-syntaxin 4 antibody (1:1000, Millipore, AB5330) or anti-Rab4 antibody (1:1000, BD Biosciences, 610889).

Immunocytochemical staining

Neurons grown on coverslips were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and then washed three times with PBS. For the detection of surface NR1 receptors, cortical cultures were blocked and incubated with the anti-NR1 antibody (1:500, NeuroMab, Davis, CA, USA, 75–272) at 4°C overnight. After washing, cultures were incubated with an Alex568-conjugated secondary antibody (1:500, Invitrogen) for 1 h at room

temperature. To co-stain NR1 with PSD-95, neurons were fixed and permeabilized with 0.1% Triton X-100 in PBS for 20 min, followed by 1 h incubation with 5% bovine serum albumin (BSA) to block non-specific staining. Neurons were then incubated with the anti-PSD-95 antibody (1:1000, NeuroMab, 2507) and anti-NR1 antibody (1:300, Cell Signalling, 5704) at 4°C overnight. After three washes, they were incubated with an Alex568 (red) or Alex488 (green) conjugated secondary antibody (1:1000, Invitrogen) at room temperature for 1 h. To detect syntaxin 4, neurons were fixed and permeabilized with 0.1% Triton X-100 and then incubated with anti-syntaxin 4 (1:500, Millipore, AB5330), followed by Alex568 (red) conjugated secondary antibody (1:500, Invitrogen). After washing in PBS, the coverslips were mounted on slides with VECTASHIELD mounting media.

Fluorescent images were obtained using a 100× objective with a cooled CCD camera mounted on a Nikon microscope. The surface NR1 clusters, total NR1, total PSD-95 and their localizations were measured using Image J software as previously described (Yuen *et al.* 2011; Gu *et al.* 2012). All specimens were imaged and analysed under identical conditions and parameters. To define dendritic clusters, a single threshold was chosen manually, so that clusters corresponded to puncta of at least twofold greater intensity than the diffuse fluorescence on the dendritic shaft. Three to four independent experiments for each of the treatments were performed. On each coverslip, the cluster density, size, and fluorescence intensity of four to six neurons (2–3 dendritic segments of at least 50 μm length per neuron) were measured. Quantitative analyses were conducted blind (without knowledge of experimental treatment).

Statistics

All data are expressed as the mean ± SEM. Experiments with two groups were analysed statistically using unpaired Student's *t* tests. Experiments with more than two groups were subjected to one-way ANOVA, followed by *post hoc* Tukey tests.

Results

Group II mGluRs regulate NMDAR surface expression and synaptic localization

To study the regulation of NMDA receptors by group II mGluRs, we firstly examined the effect of APDC, a highly selective mGluR2/3 agonist (Schoepp *et al.* 1996; Carlton *et al.* 2011), on NMDAR currents in cultured cortical neurons. As shown in Fig. 1*A–C*, application of APDC (50 μM) caused a significant and reversible enhancement of NMDAR currents ($19.2 \pm 0.6\%$, $n = 20$, $P < 0.001$, ANOVA). To identify the receptors involved, we

examined the ability of LY341495, a selective antagonist of group II mGluRs (Kingston *et al.* 1988), to prevent the action of APDC. Application of LY341495 alone (0.5 μM) had no effect on the amplitude of NMDAR currents ($-1.3 \pm 1.5\%$, $n = 16$, $P > 0.05$, ANOVA). However, the potentiation of NMDAR currents by APDC (50 μM) was effectively abolished in the presence of LY341405 ($1.2 \pm 1.1\%$, $n = 16$, $P < 0.001$, ANOVA). These results are consistent with our previous study showing that group II mGluRs mediate the enhancing effect of APDC on NMDAR currents in acutely dissociated neurons (Tyszkiewicz *et al.* 2004).

The group II mGluR-induced enhancement of NMDAR currents could be a result of increased level of NMDARs on the plasma membrane. To test this possibility, we examined the surface expression of NMDAR subunits in cortical slices in the absence or presence of APDC (50 μM, 10 min), using the surface biotinylation and Western blotting methods (Yuen *et al.* 2011, 2012). As shown in Fig. 1*D* and *E*, surface levels of NR2A and NR1 subunits were significantly increased by APDC treatment (NR2A: $76.2 \pm 6.9\%$, $n = 5$, $P < 0.001$, *t* test; NR1: $36.4 \pm 7\%$, $n = 5$, $P < 0.01$, *t* test), whereas surface NR2B subunits remained unchanged ($-2 \pm 8\%$, $n = 5$, $P > 0.05$, *t* test). The total protein levels of NMDAR subunits in cortical slices were not altered by APDC treatment (Fig. 1*D* and *F*). These data indicate that group II mGluRs primarily regulate the trafficking of NR1/NR2A-containing NMDARs. Consistently, our previous electrophysiological and pharmacological studies have shown that ifenprodil, a selective NR2B inhibitor, failed to block the enhancing effect of APDC on NMDAR currents (Tyszkiewicz *et al.* 2004), suggesting that NR1/NR2A channels are the main targets of group II mGluRs (Tyszkiewicz *et al.* 2004).

Next, immunocytochemical experiments were performed to confirm the effect of APDC on NMDAR trafficking. As shown in Fig. 1*G*, APDC (50 μM, 10 min) caused a large increase in the surface NR1 cluster density (number of clusters per 30 μm dendrite; control: 12.6 ± 0.5, $n = 31$; APDC: 15.3 ± 0.5, $n = 31$, $P < 0.05$, *t* test). The cluster size (μm^2) of surface NR1 was also up-regulated by APDC treatment (control: 0.42 ± 0.02, $n = 31$; APDC: 0.50 ± 0.03, $n = 31$, $P < 0.05$, *t* test). Fluorescence intensity of surface NR1 cluster was unchanged (control: 159.5 ± 5.0, $n = 31$; APDC: 154.0 ± 5.0, $n = 31$, $P > 0.05$, *t* test). These results further suggest that NMDAR clusters on the plasma membrane are increased by the activation of group II mGluRs.

Since NMDARs are expressed both synaptically and extrasynaptically, we investigated the effect of APDC on NMDARs at synapses. Synaptic NMDAR clusters were measured by detecting NR1 colocalized with the synaptic marker PSD-95. As shown in Fig. 1*H*, APDC treatment (50 μM, 10 min) induced a remarkable enhancement of
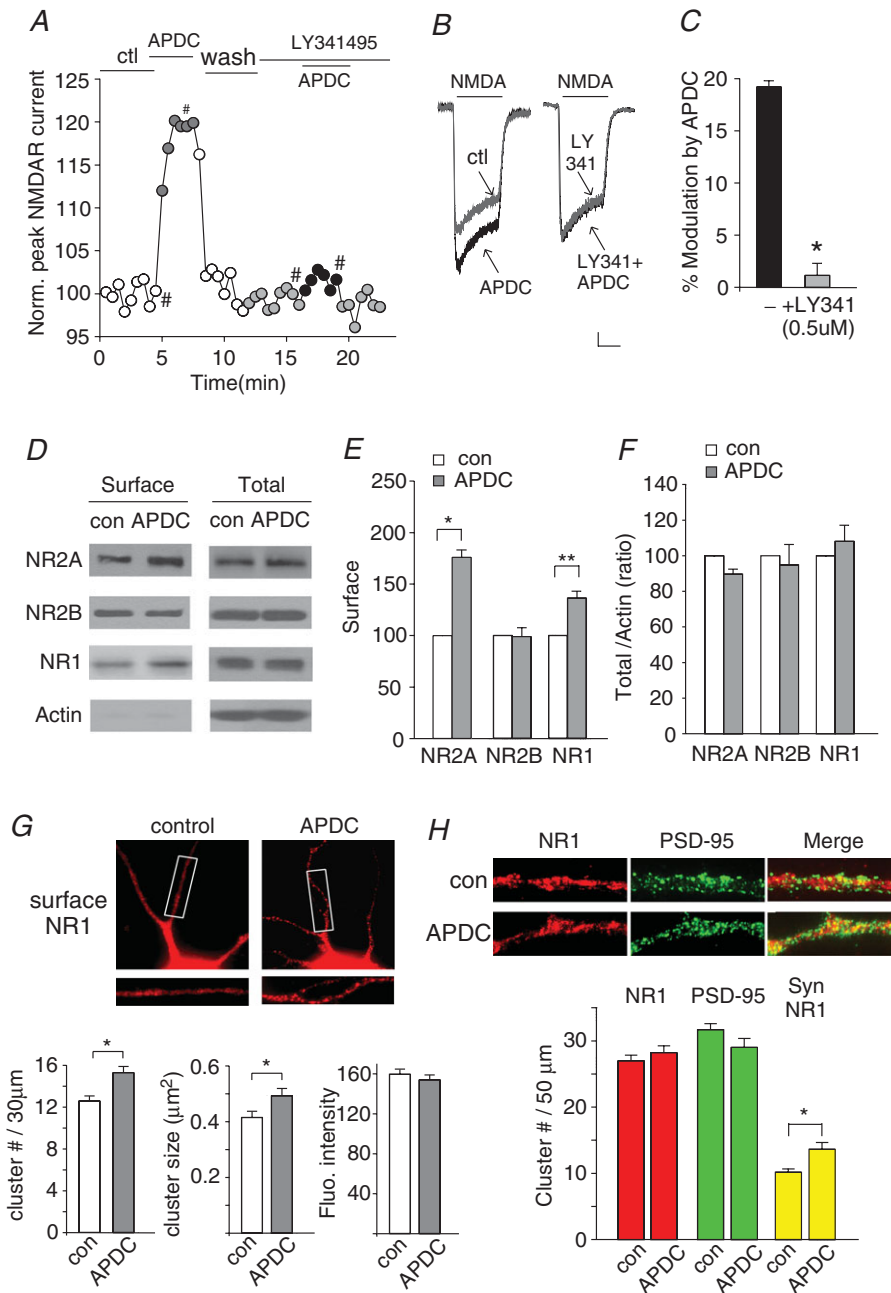
Figure 1. Activation of Group II mGluRs increases NMDAR currents and NMDAR surface expression and synaptic localization

A, plot of peak NMDAR currents as a function of time and drug application in cultured cortical pyramidal neurons. APDC (50 μM): a selective group II mGluR agonist; LY341495 (0.5 μM): a selective group II mGluR antagonist. *B*, representative current traces taken from the recordings used to construct panel *A* (at time points denoted by #). Scale bars: 0.1 nA, 1 s. *C*, cumulative data (mean ± SEM) showing the percentage modulation of NMDAR currents by APDC in the absence or presence of LY341495. *$P < 0.001$, ANOVA. *D–F*, immunoblots (*D*) and quantitative analysis (*E* and *F*) of the surface and total NMDAR subunits in cortical slices treated without (con) or with APDC (50 μM, 10 min). *$P < 0.001$, **$P < 0.01$, *t* test. *G*, immunocytochemical images and quantitative analysis of surface NR1 in cortical cultures treated without (control) or with APDC (50 μM, 10 min). Enlarged versions of the boxed regions of dendrites are shown beneath each of the images. Surface NR1 cluster density, size and fluorescence intensity were analysed. *$P < 0.05$, *t* test. *H*, immunocytochemical images and quantitative analysis (cluster size) of total NR1 clusters (red puncta), PSD-95 clusters (green puncta) and synaptic NR1 (PSD-95 colocalized, yellow puncta) along dendrites of cortical cultures treated without or with APDC (50 μM, 10 min). *$P < 0.05$, *t* test.

synaptic NR1 (colocalized with PSD-95) cluster density (number of clusters per 50 μm dendrite) in cultured cortical neurons (control: 10.2 ± 0.5, $n = 48$; APDC: 13.6 ± 1.0, $n = 30$, $P < 0.05$, t test). The total NR1 and PSD-95 cluster intensity remained unchanged by APDC. These results indicate that APDC increases the number of synaptic NMDARs.

SNAP-25 is involved in group II mGluR-induced enhancement of NMDAR currents

The group II mGluR-induced potentiation of NMDAR currents is accompanied by increased surface NMDARs at synapses, suggesting that the activation of group II mGluRs might influence the membrane delivery of NMDARs. It is known that SNAREs are the key protein family driving membrane fusion in all eukaryotic cells

(Jahn & Scheller, 2006). During intracellular vesicle fusion, SNAREs, including SNAP-25, syntaxins (t-SNARE, located at target membrane) and VAMP/synaptobrevin (v-SNARE, located at vesicle membrane), assemble into stable complexes that force membranes to link tightly together (O'Connor *et al.* 1994; Jahn & Scheller, 2006). It has been well characterized that SNAREs regulate synaptic vesicle exocytosis in presynaptic terminals (O'Connor *et al.* 1994). However, recent studies have also shown that SNAREs mediate membrane fusion events in postsynaptic neurons and are critical in the control of synaptic strength (Lledo *et al.* 1998; Kennedy *et al.* 2010; Suh *et al.* 2010).

To study the involvement of SNAREs in the NMDAR trafficking regulated by group II mGluRs, we dialysed neurons with botulinum toxin (BoTx), which disrupts the SNARE complex by cleaving the v-SNAREs of VAMP/synaptobrevin family proteins (Schiavo *et al.*
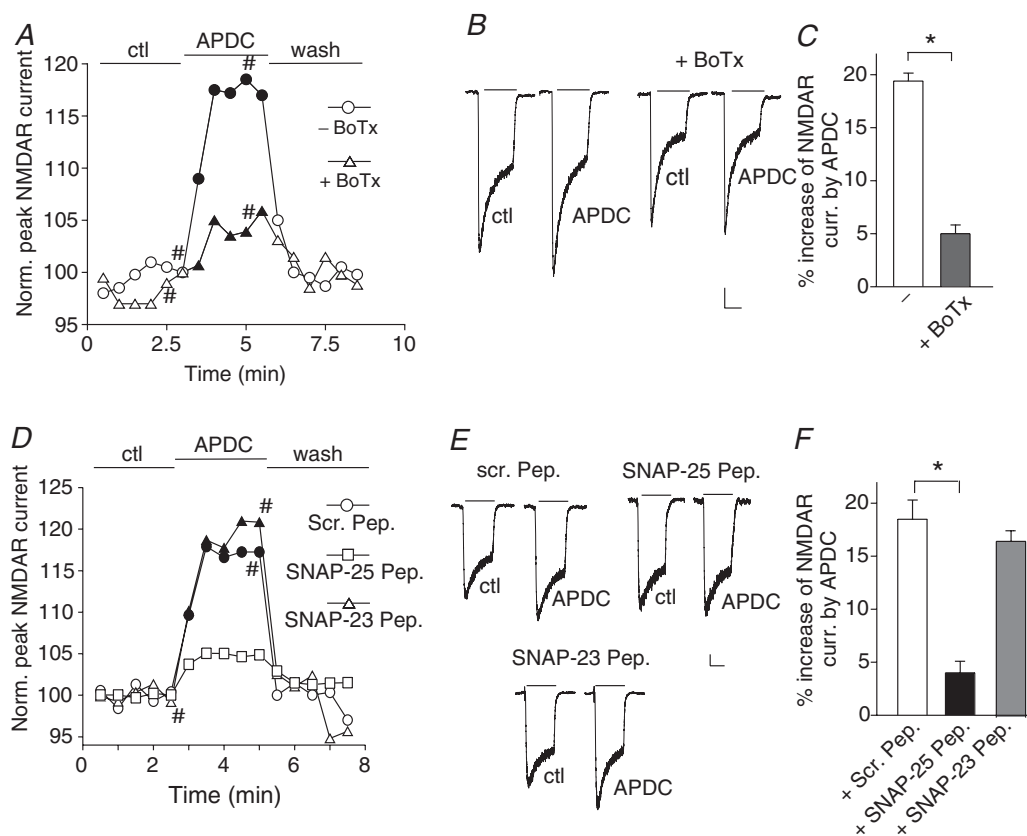

Figure 2. Inhibiting the SNARE protein SNAP-25 blocks group II mGluR-induced enhancement of NMDAR currents
A, plot of normalized peak NMDAR currents showing the effect of APDC (50 μM) in neurons dialysed without or with botulinum toxin (BoTx, 0.5 μM). *B*, representative current traces taken from the records used to construct panel *A* (at time points denoted by #). Scale bars: 0.1 nA, 1 s. *C*, cumulative data (mean ± SEM) showing the percentage modulation of NMDAR currents by APDC in the absence or presence of BoTx. *P < 0.01, *t* test. *D*, plot of normalized peak NMDAR currents showing the effect of APDC (50 μM) in neurons dialysed with a scrambled peptide (1 mM), a SNAP-25 blocking peptide (1 mM) or a SNAP-23 blocking peptide (1 mM). *E*, representative current traces taken from the records used to construct *D* (at time points denoted by #). Scale bars: 0.1 nA, 1 s. *F*, cumulative data (mean ± SEM) showing the percentage modulation of NMDAR currents by APDC in the presence of various peptides. *P < 0.01, ANOVA.

1993). As shown in Fig. 2*A–C*, dialysis with BoTx (0.5 μM) abolished the enhancing effect of APDC on NMDAR currents (control: $19.0 \pm 1.2\%$, $n = 10$; +BoTx: $6.0 \pm 2.5\%$, $n = 7$, $P < 0.01$, *t* test), indicating the participation of SNAREs in the group II mGluR-induced potentiation of NMDARs.

Next, we investigated the role of SNAP-25, a key member of the SNARE complex (Jahn & Scheller, 2006). SNAP-25 is predominately expressed in neuronal systems (Oyler *et al.* 1989) and is involved in protein kinase C (PKC)-dependent incorporation of surface NMDARs (Lau *et al.* 2010). We dialysed neurons with a blocking peptide that mimics the N-terminal domain of SNAP-25, which disrupts the interaction of SNAP-25 with *N*-ethylmaleimide-sensitive factor (NSF) that is required for the assembly–disassembly cycle of SNAREs (Lledo *et al.* 1998; Jahn & Fasshauer, 2012). The scrambled peptide serves as a negative control (Lledo *et al.* 1998). As shown in Fig. 2*D–F*, dialysis with the SNAP-25 blocking peptide (1 mM, Lledo *et al.* 1998) significantly attenuated the enhancing effect of APDC on NMDAR currents (scrambled peptide: $19.4 \pm 0.8\%$, $n = 12$; SNAP-25 peptide: $4.1 \pm 1.1\%$, $n = 11$, $P < 0.01$, ANOVA).

SNAP-23, a homologue of SNAP-25 (Vaidyanathan *et al.* 2001), associates with syntaxin 4 in the SNARE complexes (St-Denis *et al.* 1999) and regulates postsynaptic glutamate receptors (Suh *et al.* 2010). We next examined the participation of SNAP-23 by dialysing neurons with a blocking peptide that mimics the N-terminal domain of SNAP-23. This peptide disrupts the interaction of SNAP-23 with syntaxin 4 (Vaidyanathan *et al.* 2001), which is required for vesicle exocytosis (Predescu *et al.* 2005; Kawaguchi *et al.* 2010). As shown in Fig. 2*D–F*, the SNAP-23 blocking peptide (1 mM) failed to abolish the enhancing effect of APDC on NMDAR currents ($16.6 \pm 1.0\%$, $n = 12$). These data suggest that SNAP-25, but not SNAP-23, plays a role in the group II mGluR-induced increase of functional NMDARs.

Syntaxin 4 is required for the group II mGluR-induced enhancement of NMDAR currents

The syntaxin family of proteins is another key component of SNARE complex. Among syntaxin members, syntaxin 4 (Stx4) is localized at the plasma membrane in the postsynaptic terminals (Sherry *et al.* 2006; Kennedy *et al.* 2010). Previous studies have shown that syntaxin 4 binds to SNAP-25 (Hu *et al.* 2007) and is critical for the activity-dependent exocytosis of AMPA receptors in dendritic spines (Kennedy *et al.* 2010). To examine whether syntaxin 4 is involved in the group II mGluR-induced regulation of NMDARs, we used a shRNA (Kennedy *et al.* 2010) to knockdown the endogenous

syntaxin 4 in cultured cortical neurons. Previous studies have demonstrated that the syntaxin 4 shRNA led to a significant suppression of syntaxin 4 expression in 3T3 cultures and hippocampal neurons (Kennedy *et al.* 2010). In this study, the knockdown efficacy of syntaxin 4 shRNA was also confirmed in cultured cortical neurons (Fig. 3*A*; ~90% suppression in GFP-positive neurons, $n = 12$). Cellular knockdown of syntaxin 4 abolished the enhancing effect of APDC on NMDAR currents (Fig. 3*B–D*; GFP: $16.3 \pm 0.8\%$, $n = 12$; Stx4 shRNA: $5.3 \pm 0.6\%$, $n = 12$, $P < 0.01$, *t* test). These results indicate that syntaxin 4 is required for the group II mGluR-induced potentiation of NMDARs.

Rab4-mediated NMDAR recycling underlies the group II mGluR-induced enhancement of NMDAR currents

In addition to SNAREs, several protein families are suggested to be involved in vesicle trafficking and fusion. Among them, Rab family of small GTPases has been shown to specifically regulate the vesicle transport
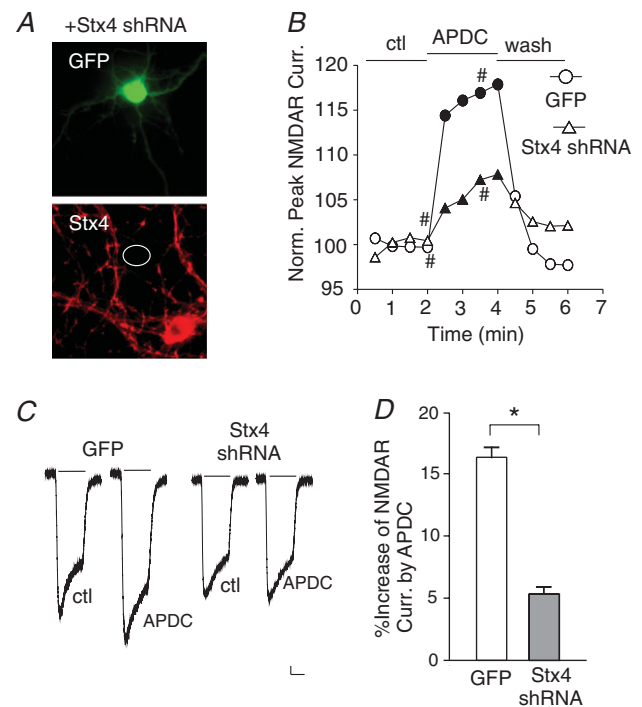
Figure 3. Knockdown of syntaxin 4 blocks group II mGluR-induced enhancement of NMDAR currents
A, immunocytochemical staining of Stx4 in cultured cortical neurons co-transfected with syntaxin 4 shRNA (Stx4 shRNA) and GFP. *B*, plot of normalized peak NMDAR currents showing the effect of APDC (50 μM) in neurons transfected with GFP or Stx4 shRNA. *C*, representative current traces taken from the records used to construct panel *B* (at time points denoted by #). Scale bars: 0.1 nA, 1 s. *D*, cumulative data (mean $\pm$ SEM) showing the percentage modulation of NMDAR currents by APDC in neurons with different transfections. *P < 0.01, *t* test.

between organelles and acts as the upstream molecule of SNAREs (Gerst, 1999; Zerial M, 2001). In particular, Rab4 controls a rapid direct recycling route from early endosomes to cell surface (van der Sluijs *et al.* 1992), which could potentially be involved in the group II mGluR-induced regulation of NMDAR trafficking. To test this hypothesis, we performed siRNA knockdown of Rab4 in cortical neurons. The knockdown efficacy of the Rab4 siRNA has been demonstrated in our previous study (Yuen *et al.* 2011). As shown in Fig. 4A and B, Rab4 knockdown remarkably eliminated the APDC-induced increase of NMDAR currents (scrambled siRNA: $19.2 \pm 1.0\%$, $n = 10$; Rab4 siRNA: $3.4 \pm 0.5\%$, $n = 12$, $P < 0.01$, ANOVA), but had no effect on basal

NMDAR current density (pA pF^{-1}; scrambled siRNA: 31.7 ± 6.1, $n = 8$; Rab4 siRNA: 34.5 ± 4.7, $n = 8$, $P > 0.05$, t test). To further confirm the involvement of Rab4, a dominant-negative Rab4 (DN-Rab4, Rab4-S27N) was used (Odley *et al.* 2004). As shown in Fig. 4C, APDC failed to potentiate NMDAR currents in neurons transfected with DN-Rab4 ($5.8 \pm 1.7\%$, $n = 8$, $P < 0.01$, ANOVA). These data suggest that Rab4 is critical for the potentiation of NMDAR currents by group II mGluRs.

Next we examined the impact of group II mGluRs on Rab4 activity. Since Rabaptin-5 only binds to the GTP-bound active Rab4 at its N terminus (Vitale *et al.* 1998), co-immunoprecipitation experiments were performed to measure the Rabaptin-5-bound active
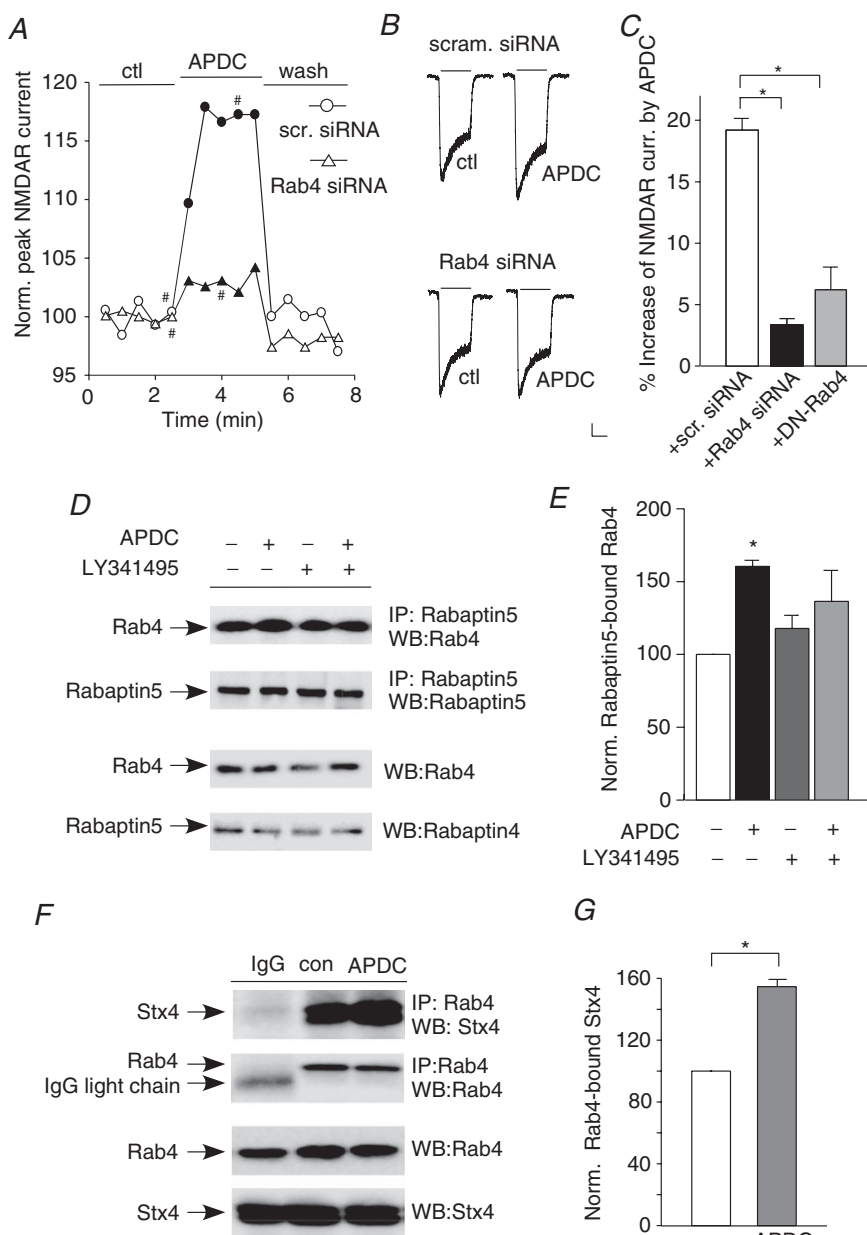

Figure 4. Rab4 is required for APDC enhancement of NMDAR currents
A, plot of normalized peak NMDAR currents showing the effect of APDC (50 μM) in neurons transfected with a scrambled siRNA or Rab4 siRNA. *B*, representative current traces taken from the records used to construct panel *A* (at time points denoted by #). Scale bars: 0.1 nA, 1 s. *C*, cumulative data (mean $\pm$ SEM) showing the percentage modulation of NMDAR currents by APDC in neurons transfected with a scrambled siRNA, Rab4 siRNA or dominant-negative Rab4 (DN-Rab4). *P < 0.01, ANOVA. *D*, representative co-immunoprecipitation blots showing the effect of APDC (50 μM, 10 min) on active (Rabaptin5-bound) Rab4 in cortical slices pretreated without or with the mGluR2/3 antagonist LY341495 (0.5 μM, 10 min). *E*, quantitative analysis (mean $\pm$ SEM) showing the level of active (Rabaptin5-bound) Rab4 with different treatments. *P < 0.01, ANOVA. *F*, representative co-immunoprecipitation blots showing the effect of APDC (50 μM, 10 min) on the interaction between syntaxin 4 and Rab4 in cortical slices. *G*, quantitative analysis (mean $\pm$ SEM) showing the level of Rab4-bound syntaxin 4 in control *vs.* APDC-treated cortical slices. *P < 0.01, *t* test.

Rab4. As shown in Fig. 4D and E, APDC treatment ($50 \mu M$, 10 min) induced a significant enhancement of active (Rabaptin-5-bound) Rab4 in rat cortical slices (1.62 ± 0.09-fold of control, $n = 4$, $P < 0.01$, ANOVA), and this effect was significantly attenuated by LY341495 ($0.5 \mu M$, LY: 1.18 ± 0.10-fold of control; APDC+LY: 1.36 ± 0.21-fold of control, $n = 4$, $P > 0.05$, ANOVA). These results indicate that group II mGluRs enhance Rab4 activity, which could facilitate the Rab4-mediated protein recycling to the plasma membrane.

Previous studies have shown that Rab4 directly binds to syntaxin 4 when it adopts an active open conformation, which allows syntaxin 4 to enter the SNARE complexes leading to the membrane fusion (Li *et al.* 2001). We then examined whether Rab4–syntaxin 4 interaction is regulated by group II mGluRs. Co-immunoprecipitation experiments indicated that Rab4 was bound to syntaxin 4 in cortical slices, and their association was significantly increased by APDC treatment (Fig. 4F and G; $54.6 \pm 4.4\%$, $n = 5$, $P < 0.001$, *t* test). It further suggests that the activation of group II mGluRs facilitates Rab4–SNARE-mediated exocytosis of target proteins.

Discussion

In this study, we have revealed the key molecules involved in the regulation of NMDA receptors by group II mGluRs (Fig. 5). Previous studies suggested that the agonists of group II mGluRs could be potential antipsychotics due to their inhibition of presynaptic glutamate release (Kamiya *et al.* 1996; Schoepp, 2001). In this study, we found that activation of group II mGluRs in postsynaptic neurons significantly increased the surface and synaptic NR1/NR2A-containing NMDAR clusters. NR2A subunit is mainly expressed in the synaptic sites of mature neurons and shows different properties from NR2B subunit (Yashiro & Philpot, 2008). It has been found that NR2A plays a dominant role in the phencyclidine-induced apoptosis and the development of schizophrenia-like behaviours (Anastasio *et al.* 2009). NR2A is selectively altered in the medial prefrontal cortex of rats reared in isolation, a preclinical model of schizophrenia (Turnock-Jones *et al.* 2009). Moreover, the aberrant gamma activity in the schizophrenia model induced by NMDAR antagonist is primarily mediated by NR2A-containing NMDARs (Kocsis, 2012). Hence, the selective up-regulation of NR2A-containing NMDARs by group II mGluRs agonists may account for their beneficial effects as potential antipsychotics.

Mounting evidence suggests that diverse protein families are involved in the regulation of NMDAR trafficking (Wenthold *et al.* 2003; Prybylowski & Wenthold, 2004; Lau & Zukin, 2007). Dendritic SNARE proteins play a critical role in the late stage of synaptic vesicle exocytosis (Ovsepian & Dolly, 2011), including the regulation of postsynaptic glutamate receptor trafficking (Lledo *et al.* 1998). SNAP-25, a key component of SNARE complex, is expressed in excitatory neurons and localized at both presynaptic and postsynaptic terminals (Schwab *et al.* 2001). Previous studies have shown that SNAP-25 participates in the postsynaptic fusion events contributing to LTP (Lledo *et al.* 1998) and PKC-dependent insertion of synaptic NMDARs (Lan *et al.* 2001; Lau *et al.* 2010). In agreement with these results, we revealed that SNAP-25 is involved in the group II mGluR-induced potentiation of NMDAR currents.

A homologue of SNAP-25, SNAP-23 (59% identical with SNAP-25), is expressed in the brain (Chen *et al.* 1999) and binds to multiple syntaxins and synaptobrevins (Ravichandran *et al.* 1996). Recent studies indicate that SNAP-23 is predominantly localized at dendritic spines (Suh *et al.* 2010). Loss of SNAP-23 led to a marked decrease of surface NMDARs (Suh *et al.* 2010). However, we did not find any involvement of SNAP-23 in the group II mGluR-induced regulation of NMDAR currents. The underlying reason for this is unclear. Probably it is because SNAP-23 is more involved in the basal recycling of NMDARs (Suh *et al.* 2010), or because SNAP-23 binds

Figure 5. Schematic diagram showing the potential mechanism underlying the regulation of NMDARs by group II mGluRs

Activation of group II mGluRs increases Rab4 activity and the interaction between Rab4 and syntaxin 4, facilitating the formation of SNARE complexes composed of SNAP-25, syntaxin 4 and VAMP at postsynaptic sites, which leads to the increased exocytosis of NMDARs. Consequently, NMDAR surface expression and synaptic function are up-regulated by the activation of group II mGluRs.
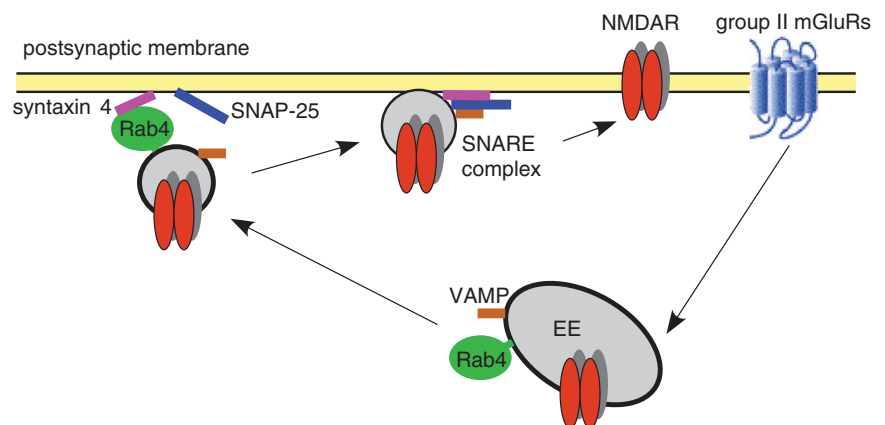
to other SNARE family members less efficiently than SNAP-25 (Vaidyanathan *et al.* 2001).

Syntaxins, a key component of the SNARE complex, contain 16 members in mammalian cells (Gerst, 1999). Among them, syntaxins 1–4 are localized to the plasma membrane (Bennett *et al.* 1993), and only syntaxin 1 and syntaxin 4 are highly expressed in the brain. While syntaxin 1 is present presynaptically and its role in regulating transmitter release has been well studied, the specific syntaxin member involved in postsynaptic membrane fusion is unclear. Syntaxin 4, which had been shown to be present postsynaptically (Sherry *et al.* 2006; Kennedy *el al.* 2010), was recently shown to direct the membrane fusion of AMPAR-containing recycling vesicles at hippocampal synapses (Kennedy *et al.* 2010; Mohanasundaram & Shanmugam, 2010). In this study, we showed that syntaxin 4 was required for the group II mGluR-induced enhancement of NMDAR currents, suggesting that syntaxin 4 plays an important role in regulating the exocytosis of NMDARs.

Rab family GTPases, suggested as the SNARE regulators acting upstream of SNARE complex assembly, mediate directional transport of vesicles between different organelles (Gerst, 1999; Pfeffer, 2001). Rab proteins associate with membranes in their GTP-bound active form and are dissociated in their GDP-bound inactive form. This dynamic cycling is essential for their functioning (Fukuda, 2008). Among Rab proteins, Rab4 is the central player in the fast recycling of μ-opioid receptor (Roman-Vendrell *et al.* 2012), β_2-adrenergic receptor (Yudowski *et al.* 2009) and glutamate receptors (Yuen *et al.* 2011). Rab4 binds to syntaxin 4 when it adopts an active open conformation (Li *et al.* 2001). The binding of Rab proteins to SNAREs leads to the dissociation of negative regulators for SNARE complex formation (Gerst, 1999). Here, we found that knockdown of Rab4 or expression of dominant-negative Rab4 blocked the group II mGluR-induced enhancement of NMDAR currents, indicating that Rab4 is important for the regulation of NMDAR exocytosis. Furthermore, we found that the interaction between Rab4 and syntaxin 4 was increased by group II mGluRs agonist, suggesting that group II mGluRs activation may facilitate the formation of stable SNARE complexes mediating membrane fusion.

In addition to coupling to the Gi–PKA pathway, group II mGluRs are also linked to phospholipase C (Klein *et al.* 1997; Otani *et al.* 2002), which will lead to the activation of PKC. Our previous study has shown that APDC enhances NMDAR currents via a PKC-dependent mechanism (Tyszkiewicz *et al.* 2004). Since Rab4, SNAP25 and syntaxin 4 are all regulated by PKC (Chung *et al.* 2000; Lan *et al.* 2001; Imamura *et al.* 2003; Lau *et al.* 2010), group II mGluRs may enhance the SNARE-mediated NMDAR exocytosis through PKC signalling.

In summary, our findings have shown that SNARE complexes, composed of SNAP-25 and syntaxin 4, and the Rab4 protein are critically involved in the increased membrane delivery of NMDA receptors by group II mGluRs in cortical pyramidal neurons. Given the role of NMDAR hypofunction in the pathology of schizophrenia (Kantrowitz & Javitt, 2012), the up-regulation of NMDAR function could be one of the mechanisms underlying the antipsychotic effects of group II mGluRs.

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Additional information

Competing interests

The authors declare no conflict of interest.

Author contributions

All the experiments were performed in the research laboratory of Z.Y. in the Department of Physiology and Biophysics, State University of New York at Buffalo. Conception and design of the experiments: Z.Y., and J.C.; collection, analysis and interpretation of data: J.C., W.L. and L.D.; drafting the article or revising it critically for important intellectual content: Z.Y. and J.C. All authors approved the final version of the manuscript. The authors declare no conflict of interest.