0026-895X/07/7104-1030–1039$20.00
MOLECULAR PHARMACOLOGY
Vol. 71, No. 4
32490/3190524
Mol Pharmacol 71:1030–1039, 2007
Printed in U.S.A.

RGS4 Modulates Serotonin Signaling in Prefrontal Cortex and Links to Serotonin Dysfunction in a Rat Model of Schizophrenia

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Received November 8, 2006; accepted January 12, 2007

ABSTRACT

Regulator of G protein signaling 4 (RGS4) has recently been identified as one of the genes linked to the susceptibility of schizophrenia. However, the functional roles of RGS4 and how it may be involved in the pathophysiology of schizophrenia remain largely unknown. In this study, we investigated the possible impact of RGS4 on the function of serotonin and dopamine receptors, two main targets for schizophrenia treatment. Activation of serotonin 5-HT_{1A} receptors or dopamine D_4 receptors down-regulates the function of NMDA receptor (NMDAR) channel, a key player controlling cognition and emotion, in pyramidal neurons of prefrontal cortex (PFC). Blocking RGS4 function significantly potentiated the 5-HT_{1A} regulation of NMDAR current; conversely, overexpression of RGS4 attenuated the 5-HT_{1A} effect. In contrast, the D_4 regulation of NMDAR current was not altered by RGS4 manipulation. Moreover, the 5-HT_{1A} regulation of NMDA receptors was significantly enhanced in a subset of PFC pyramidal neurons from rats treated with subchronic phencyclidine, an animal model of schizophrenia, which was found to be associated with specifically decreased RGS4 expression in these cells. Thus, our study has revealed an important coupling of RGS4 to serotonin signaling in cortical neurons and provided a molecular and cellular mechanism underlying the potential involvement of RGS4 in the pathophysiology of schizophrenia.

Schizophrenia is a psychiatric disorder with high heritability (Cardno and Gottesman, 2000; Sullivan et al., 2003). Molecular genetic studies suggest that schizophrenia is a complex genetic disorder involving multiple susceptibility genes (Harrison and Owen, 2003). Regulator of G protein signaling 4 (RGS4) is one of such putative genes identified recently from association and linkage analyses (Chowdari et al., 2002; Morris et al., 2004; Williams et al., 2004). Moreover, a microarray study has found that the expression of *RGS4* gene, which is located at the major schizophrenia susceptibility locus chromosome 1q21-q22 (Brzustowicz et al., 2000), is consistently and significantly decreased in cortical areas of patients with schizophrenia (Mirnics et al., 2001).

RGS proteins are known to function as GTPase-activating proteins to dampen or negatively regulate G protein-coupled receptor (GPCR) signaling pathways (Berman et al., 1996; Vries et al., 2000), and have been proposed as new central nervous system drug targets (Neubig and Siderovski, 2002). More than 20 mammalian RGS proteins have been cloned, but how RGS proteins achieve their specificity in regulating numerous G-protein signaling pathways is not fully understood. RGS4 is one of the five members enriched in human brain (Larminie et al., 2004), with the highest expression in prefrontal cortex (Erdely et al., 2004), a major locus of dysfunction in schizophrenia (Weinberger et al., 1986; Andreasen et al., 1997). RGS4 has been shown to modulate both $G_{i/o}$- and G_q-mediated signaling in transfected cell lines (Yan et al., 1997; Ghavami et al., 2004). It remains largely unknown how RGS4 may regulate the signaling of different neuromodulators in cortical neurons and how it could contribute to the pathophysiology of schizophrenia.

Converging evidence has implicated the prefrontal cortex (PFC) as a major locus of dysfunction in schizophrenia (Weinberger et al., 1986; Andreasen et al., 1997). Aberrant dopa-

This work was supported by National Institutes of Health grants MH63128 and NS48911 and an Independent Investigator Award from NARSAD: The Mental Health Research Association (to Z.Y.).

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.106.032490.

ABBREVIATIONS: RGS, regulator of G protein signaling; GPCR, G protein-coupled receptor; PFC, prefrontal cortex; NMDAR, *N*-methyl-D-aspartate receptor; PCP, phencyclidine; DA, dopamine; NMDA, *N*-methyl-D-aspartate; EPSC, excitatory postsynaptic current; QX-314, (2,6-dimethylphenyl)carbamoylmethyl-triethyl-azanium; ANOVA, analysis of variance; GFP, green fluorescent protein; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; bp, base pair(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 8-OH-DPAT, 8-hydroxy-2-di-*n*-propylamino-tetralin; PD168077, *N*-methyl-4-(2-cyanophenyl)piperazynil-3-methylbenzamide maleate.

mine and serotonin signaling in PFC plays a central role in the etiology of schizophrenia (Lewis and Lieberman, 2000; Sawa and Snyder, 2002). Antipsychotic drugs derive their therapeutic efficacy mainly by acting on dopamine and serotonin receptors in limbic areas, including PFC (Creese et al., 1976; Lieberman et al., 1998; Meltzer, 1999; Kapur and Remington, 2001). One of the important targets of dopamine and serotonin receptors that could be involved in schizophrenia is the NMDAR channel; NMDAR hypofunction is critically associated with the symptom manifestation of this disease (Tsai and Coyle, 2002; Moghaddam, 2003). The noncompetitive NMDA receptor antagonist phencyclidine (PCP) has been found to produce a variety of behavior abnormalities resembling schizophrenia in humans and animals (Allen and Young, 1978; Snyder, 1980; Javitt and Zukin, 1991). Thus, systemic administration of PCP represents the most widely used and best-established model of schizophrenia (Jentsch et al., 1997; Moghaddam and Adams, 1998; Jentsch and Roth, 1999). It has been shown that PFC glutamatergic inputs excite dopamine (DA) neurons in the ventral mesencephalon that project back to PFC but inhibit DA neurons that project to striatum through GABAergic interneurons. Therefore, hypofunctional NMDA receptors in PFC could result in decreased activity of DA neurons projecting to PFC and increased activity of DA neurons projecting to striatum (Sesack and Carr, 2002). Reduced DA inputs in PFC and increased DA inputs in striatum might be responsible for some of the negative and positive symptoms of schizophrenia, respectively (Lewis and Lieberman, 2000).

Our recent studies have shown that D_4 and 5-HT_{1A} receptors, two dopamine and serotonin receptor subtypes highly enriched in PFC (Wedzony et al., 2000; Feng et al., 2001), inhibit NMDA receptor function via both convergent and distinct signaling mechanisms (Wang et al., 2003; Yuen et al., 2005). In this study, we sought to determine whether RGS4 is involved in the modulation of D_4 and 5-HT_{1A} signaling in PFC pyramidal neurons and whether RGS4 aberration occurs in the rat PCP model of schizophrenia.

Materials and Methods

Electrophysiological Recordings in Slices. To evaluate the regulation of NMDAR-EPSC in pyramidal neurons located in deep layers (V–VI) of PFC slices from juvenile (3–5 weeks postnatal) Sprague-Dawley rats, the whole-cell voltage-clamp recording technique was used. Electrodes were filled with the following internal solution: 130 mM cesium methanesulfonate, 10 mM CsCl, 4 mM NaCl, 10 mM HEPES, 1 mM $MgCl_2$, 5 mM EGTA, 2.2 mM QX-314, 12 mM phosphocreatine, 5 mM MgATP, 0.5 mM Na_2GTP, and 0.1 mM leupeptin, pH 7.2 to 7.3; 265–270 mOsM. The brain slice (300 μm) was placed in a perfusion chamber attached to the fixed-stage of an upright IR differential interference contrast microscope (Olympus, Tokyo, Japan) and submerged in continuously flowing oxygenated artificial cerebrospinal fluid (130 mM NaCl, 26 mM $NaHCO_3$, 3 mM KCl, 5 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 1 mM $CaCl_2$, and 10 mM glucose) containing 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (20 μM) and bicuculline (10 μM) to block α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate receptors and $GABA_A$ receptors. Cells were visualized with a 40× water-immersion lens and illuminated with near-IR light, and the image was detected with an IR sensitive charge-coupled device camera. A Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, CA) was used for these recordings. Tight seals (2–10 GΩ) from visualized pyramidal neurons were obtained by the "blow and seal" technique (Stuart et al., 1993). In brief, positive pressure was applied to the patch pipette as it was advancing through the slice to the targeted cell. The positive pressure was released when the pipette tip touched the cell membrane, and a slight negative pressure was then applied to obtain a tight seal. The membrane was disrupted with additional suction, and the whole-cell configuration was obtained. The access resistances ranged from 13 to 18 MΩ and were compensated 50 to 70%. Evoked currents were generated with a 50-μs pulse from a stimulation isolation unit controlled by a S48 pulse generator (Astro-Med, Inc., West Warwick, RI), and the interstimulus interval was 30 s. A bipolar stimulating electrode (FHC Inc., Bowdoinham, ME) was positioned ~100 μm from the neuron under recording. Before stimulation, cells (clamped at −70 mV) were depolarized to +60 mV for 3 s to fully relieve the voltage-dependent Mg^{2+} block of NMDAR channels. The Clampfit program (Molecular Devices) was used to analyze evoked synaptic activity. The peak of NMDAR-EPSC was calculated by taking the mean of a 2- to 4-ms window around the peak and comparing with the mean of a 4- to 8-ms window immediately before the stimulation artifact.

Whole-Cell Recordings in Dissociated or Cultured Neurons. Acutely dissociated PFC pyramidal neurons from 4-week-old rats were prepared using procedures described previously (Feng et al., 2001). Some experiments were performed in PFC primary cultures from embryonic day 18 rat embryos that have grown for 3 weeks in vitro (Gu et al., 2005). Recordings of whole-cell, ligand-gated ion channel currents employed standard voltage-clamp techniques. Recording pipette was filled with internal solution consisting of 180 mM *N*-methyl-D-glucamine (NMG), 40 mM HEPES, 4 mM $MgCl_2$, 0.1 mM BAPTA, 12 mM phosphocreatine, 3 mM Na_2ATP, 0.5 mM Na_2GTP, and 0.1 mM leupeptin, pH 7.2 to 7.3, 265 to 270 mOsM. The external solution consisted of 127 mM NaCl, 20 mM CsCl, 10 mM HEPES, 1 mM $CaCl_2$, 5 mM $BaCl_2$, 12 mM glucose, 0.001 mM tetrodotoxin, and 0.02 mM glycine, pH 7.3 to 7.4, 300 to 305 mOsM. Recordings were obtained with a Molecular Devices 200B patch clamp amplifier that was controlled and monitored with an IBM PC running pCLAMP (ver. 8) with a DigiData 1320 series interface (Molecular Devices). Electrode resistances were typically 2 to 4 MΩ in the bath. After seal rupture, series resistance (4–10 MΩ) was compensated (70–90%) and periodically monitored. The cell membrane potential was held at −60 mV. The application of NMDA (100 μM) evoked a partially desensitizing inward current. NMDA was applied for 2 s every 30 s to minimize desensitization-induced decrease of current amplitude. Peak values were measured for generating the plot as a function of time and drug application. Drugs were applied with a gravity-fed "sewer pipe" system. The array of application capillaries (~150 μm i.d.) was positioned a few hundred micrometers from the cell under study. Solution changes were effected by the SF-77B fast-step solution stimulus delivery device (Warner Instrument Co., Hamden, CT).

To test the impact of RGS proteins on GPCR signaling, antibodies raised against their highly specific regions were dialyzed into neurons via the patch electrode for at least 10 min before electrophysiological recordings started. Antibodies used in this study include the N-terminal goat anti-RGS4 (Santa Cruz Biotechnology, Santa Cruz, CA), the chicken polyclonal anti-RGS4 (Abcam Inc., Cambridge, MA), the anti-RGS4 rabbit antibody U1079 (Krumins et al., 2004), the N-terminal rabbit anti-RGS2 (Santa Cruz Biotechnology), and the anti-RGS6/7 rabbit antibody (Santa Cruz Biotechnology). All of the antibodies were diluted at 1:50 to the internal solution.

Data analyses were performed with AxoGraph (Molecular Devices) and Kaleidagraph (Abelbeck/Synergy Software, Reading, PA). ANOVA tests were performed to compare the differential degrees of current modulation between groups subjected to different treatments. The summary data were indicated with mean ± S.E.M.

PCP Administration. The rat PCP model of schizophrenia was generated as we reported recently (Wang et al., 2006). In brief, PCP (Sigma, St. Louis, MO) was administered at a dose of 3 mg/kg in sterile saline. This dose of PCP administration to rats has been

shown to induce a variety of behavioral and biochemical changes reminiscent of schizophrenia in humans (Jentsch et al., 1997, 1998; Adams and Moghaddam, 1998; Moghaddam and Adams, 1998). Injections were given i.p. at a volume of 1 ml/kg once daily for various days, and rats were sacrificed 24 h after the last injection.

Transfection. Cultured PFC neurons (11 DIV) were transfected with green fluorescent protein (GFP) or GFP-tagged RGS4 plasmids. GFP was fused at the N terminus of RGS4, which has been shown not to interfere with RGS4 cellular localization or binding to G proteins (Chatterjee and Fisher, 2000; Roy et al., 2003). RGS4 cDNA (GenBank accession number NM017214) was amplified by RT-PCR from rat brain total RNA. The primers used for PCR were 5′-CCGGATCCCACAGTTAAACAAGATGTGC and 5′-CCGCGGCCGCTGTGTG AGAATTAGGCACAC. The PCR product was then cloned to PCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and further subcloned to pcDNA3.1 and pEGFP-C2 vector (Clontech, Mountain View, CA) to get GFP-tagged RGS4. Transfection of GFP or GFP-tagged RGS4 constructs to primary cultured PFC neurons was conducted with Lipofectamine 2000 reagents (Invitrogen). Two to three days after transfection, electrophysiological recordings were performed on GFP-positive neurons.

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. Neurons were then permeabilized with 0.1% Triton X-100 in PBS for 5 min, followed by 1-h incubation with 5% BSA to block nonspecific staining. Neurons were then incubated with anti-RGS4 antibody (1:200; Santa Cruz Biotechnology) at 4°C overnight. After washing off the nonbinding primary antibodies, the cells were incubated with a rhodamine-conjugated secondary antibody (1:200; Sigma) for 1 h at room temperature. After washing in PBS three times, the coverslips were mounted on slides with mounting media. Fluorescent images were obtained using a 40× objective with a cooled charge-coupled device camera mounted on a Nikon microscope (Nikon, Tokyo, Japan).

Western Blotting. Procedures were similar to those described previously (Gu and Yan, 2004). Primary antibodies used for blotting include anti-RGS4 (1:2000; Abcam), anti-NR1 (1:5000; Upstate Biotechnology, Lake Placid, NY), anti-NR2A (1:1000; Upstate), anti-NR2B (1:1000; Upstate) and anti-actin (1:1000; Santa Cruz Biotechnology). Quantification was obtained from densitometric measurements of immunoreactive bands on films using NIH Image software (http://rsb.info.nih.gov/nih-image/).

Quantitative Real-Time RT-PCR and Single-Cell RT-PCR. Total RNA was isolated from rat PFC using TRIzol reagent (Invitrogen) and treated with DNase I (Invitrogen) to remove genomic DNA. Then SuperScript III first-strand synthesis system for RT-PCR (Invitrogen) was used to obtain cDNA from the tissue mRNA, followed by the treatment with RNase H (2 U/μl) for 20 min at 37°C. Quantitative real-time RT-PCR was carried out using the iCycler iQ Real-Time PCR Detection System and iQ Supermix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. In brief, GAPDH was used as the housekeeping gene for quantitation of the expression of target genes (RGS4 and 5-HT_{1A}) in samples from saline- versus PCP-treated rats. -Fold changes in the target gene relative to the GAPDH endogenous control gene was determined by: -Fold change = $2^{-\Delta(\Delta C_T)}$, where $\Delta C_T = C_{T,\ target} - C_{T,\ GAPDH}$, and $\Delta(\Delta C_T) = \Delta C_{T,\ PCP} - \Delta C_{T,\ saline}$. C_T (threshold cycle) is defined as the fractional cycle number at which the fluorescence reaches 10× the S.D. of the baseline. The primers used for RGS4 were 5′-TGCAGGCAACAAAAGAGGTGAA and 5′-CCCCGCAGCTGGAAGGAT (192 bp); for 5-HT_{1A}, 5′-TCTGTACCAGGTGCTCAACAAG and 5′-AGAGGAAGGTGCTCTTTGGAGT (638 bp); and for GAPDH, 5′-GATGACATCAAGAAGGTGGTGAAG and 5′-GGTGCAGCGAACTTTATTGATGGT (440 bp). A total reaction mixture of 20 μl was amplified in a 96-well thin-walled PCR plate (Bio-Rad Laboratories) using the following PCR cycling parameters: 95°C for 5 min followed by 25 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 60 s. PCR products were detected with 2% agarose gels. Quantitative real-time RT-PCR was performed in duplicate reactions.

Single-cell RT-PCR was performed as described previously (Yan and Surmeier, 1997; Feng et al., 2001). In brief, single neuron was aspirated into the electrode (containing ~5 μl of sterile recording solution) by negative pressure after recording. After aspiration, the tip of the electrode was broken, and contents were ejected into 0.5-ml Eppendorf tube containing 3 μl of diethyl pyrocarbonate-treated water, 1 μl oligo(dT) (0.5 μg/μl), 0.5 μl of RNAsin (40 U/μl), 0.5 μl of dithiothreitol (0.1 M). The mixture was heated to 70°C for 10 min and then incubated on ice for 1 min. First-strand cDNA was synthesized from the single-cell mRNA by adding Superscript III RT (1 μl of 200 U/μl), 2 μl of buffer (10×), 1 μl of dithiothreitol (0.1 M), 1 μl of dNTPs (10 mM each), and 4 μl of $MgCl_2$ (25 mM). The mixture was then incubated at 42°C for 50 min and terminated by heating the mixture to 70°C for 15 min and then icing. The RNA strand was removed by adding 1 μl of RNase H (2 U/μl) and incubated for 20 min at 37°C. The cDNA was then subjected to 45 cycles of PCR amplification. Quantitation was performed as described above.

Results

Selective Modulation of the 5-HT_{1A} Effect on NMDAR Function by RGS4 in PFC Neurons. To test the impact of RGS4 on serotonin or dopamine signaling, we examined the influence of RGS4 on serotonin or dopamine receptor-mediated regulation of NMDAR function. To do so, we infused an anti-RGS4 antibody into neurons through the recording pipette to achieve an effective inhibition of RGS4 function and then tested whether the RGS4 antibody could affect 5-HT_{1A} or D_4 regulation of NMDAR-mediated synaptic transmission (Wang et al., 2003; Yuen et al., 2005). We first used the anti-RGS4 (Santa Cruz Biotechnology) raised against the highly specific N terminus of RGS4, which has been shown to recognize recombinant RGS4 in transfected cell lines (Wang et al., 1998) and affect GPCR regulation of voltage-dependent Ca^{2+} channels in neurons (Diverse-Pierluissi et al., 1999). As shown in Fig. 1, A and B, bath application of the 5-HT_{1A} agonist 8-OH-DPAT (20 μM) reduced the amplitude of NMDAR-EPSC in a reversible manner in PFC slices. When anti-RGS4 (Santa Cruz Biotechnology) was included in recording pipette, 8-OH-DPAT caused a substantially bigger reduction of NMDAR-EPSC. Although the RGS4 antibody caused a significant increase (~70%) in the effect of 8-OH-DPAT on NMDAR-EPSC (Fig. 1C), it did not alter the reduction of NMDAR-EPSC by the dopamine D_4 receptor agonist PD168077 (20 μM) in PFC slices (control, 36.8 ± 5.9%, $n = 10$; with RGS4 antibody, 37.3 ± 6.6%, $n = 8$).

To further test the specific involvement of RGS4 in regulating serotonin signaling, we examined two other RGS4 antibodies, one from Abcam and U1079 (Krumins et al., 2004). Moreover, we dialyzed neurons with the anti-RGS2 or anti-RGS6/7 antibody as a control. As shown in Fig. 1D, 8-OH-DPAT induced a much bigger depression of NMDAR-EPSC in the neuron injected with anti-RGS4 (Abcam), but not in the cell loaded with anti-RGS2. As summarized in Fig. 1E, the effect of 8-OH-DPAT on NMDA-EPSC was significantly enhanced by various RGS4 antibodies (control, 34.2 ± 4.6%, $n = 15$; sc, 58.3 ± 9.4%, $n = 12$; Abcam, 54.1 ± 8.7%, $n = 10$; U1079, 51.3 ± 9.2%, $n = 8$; $p < 0.001$, ANOVA), although it was not altered by anti-RGS2 (38.1 ± 7.1%, $n = 8$) or anti-RGS6/7 (31.9 ± 6.6%, $n = 8$). The specificity of the RGS4 antibody (Santa Cruz Biotechnology) was further confirmed with the heat-inactivated RGS4 antibody (60°C for 30

min), which caused little change on the 8-OH-DPAT regulation of NMDAR-EPSC (35.2 ± 4.8%, $n = 8$) in PFC pyramidal neurons.

To confirm the specific RGS4 regulation of 5-HT_{1A} effects on NMDA receptors, we further examined the involvement of RGS4 in 5-HT_{1A} or D_4 regulation of NMDA (100 μM)-evoked ionic current in dissociated PFC pyramidal neurons (Wang et al., 2003; Yuen et al., 2005). As shown in Fig. 2, A and B, bath application of 8-OH-DPAT (20 μM) reduced the amplitude of NMDAR current, whereas in the neuron dialyzed with anti-RGS4 (Santa Cruz Biotechnology), the regulation of NMDAR current by 8-OH-DPAT was substantially enhanced. By contrast, bath application of PD168077 (20 μM) reduced the amplitude of NMDAR current to a similar extent with or without anti-RGS4 (Fig. 2C; control, 15.2 ± 2.3%, $n = 14$; with RGS4 antibody, 15.3 ± 2.9%, $n = 12$), suggesting the lack of involvement of RGS4 in regulating D_4 signaling. The basal NMDAR current was not altered by anti-RGS4 (control cells, 1235 ± 273 pA, $n = 14$; anti-RGS4-dialyzed cells, 1256 ± 288 pA, $n = 12$). As summarized in Fig. 2D, all three RGS4 antibodies caused a significant increase (40–50%) in the effect of 8-OH-DPAT on NMDAR current (control, 19.3 ± 2.5%, $n = 42$; Santa Cruz Biotechnology, 28.6 ± 4.9%, $n = 32$; Abcam, 27.5 ± 4.6%, $n = 22$; U1079, 26.7 ± 5.1%, $n = 18$; $p < 0.001$, ANOVA), whereas the heat-inactivated RGS4, RGS2, or RGS6/7 antibody failed to change the 8-OH-DPAT effect on NMDAR current (inactive anti-RGS4, 19.9 ± 2.8%, $n = 18$; anti-RGS2, 20.1 ± 3.3%, $n = 12$; anti-RGS6/7, 18.5 ± 2.9%, $n = 12$). Taken together, these results suggest that RGS4 is specifically involved in regulating cortical serotonin signaling.

Because the 5-HT_{1A} effect on NMDA receptors is potentiated by the RGS4 antibody that is supposed to scavenge endogenous RGS4, we further tested whether enhanced RGS4 signaling could suppress the 5-HT_{1A} effect on NMDAR current. To do so, we overexpressed the GFP-fused RGS4 protein that has been shown to be functional (Roy et al., 2003) in cultured PFC pyramidal neurons. Neurons were transfected with GFP alone or GFP-fused RGS4. Immunocytochemical staining was first performed to verify the overexpression of RGS4. As shown in Fig. 3, A and B, in the neuron transfected with GFP-fused RGS4, RGS4 staining was much higher than surrounding nontransfected neurons, whereas in the neuron transfected with GFP alone, RGS4 staining was similar to surrounding nontransfected neurons. These results indicated that transfection with the GFP-fused RGS4 significantly increased the RGS4 protein level, whereas GFP alone did not change the RGS4 expression. Two days after transfection, GFP-positive neurons were recorded. The basal NMDAR current was not altered by RGS4 overexpression (GFP-transfected cells, 1336 ± 231 pA, $n = 15$; RGS4-transfected cells, 1308 ± 262 pA, $n = 15$). In neurons transfected with GFP alone, 8-OH-DPAT reduced NMDAR current by 22.1 ± 4.3% ($n = 15$), which was similar to nontransfected control neurons (22.4 ± 3.8%, $n = 12$). In neurons overexpressed with the GFP-fused RGS4, 8-OH-DPAT produced a significantly ($p < 0.005$, ANOVA) smaller reduction of NMDAR current (14.3 ± 3.4%, $n = 15$). The time course and current traces from representative cells transfected with GFP alone or GFP-fused RGS4 are shown in Fig. 3, C and D. Whereas overexpression of RGS4 resulted in ~35% smaller effect of 8-OH-DPAT on NMDAR current (Fig. 3E), the PD168077 reduction of NMDAR current was not changed in neurons transfected with either GFP alone (15.4 ± 2.8%, $n =$

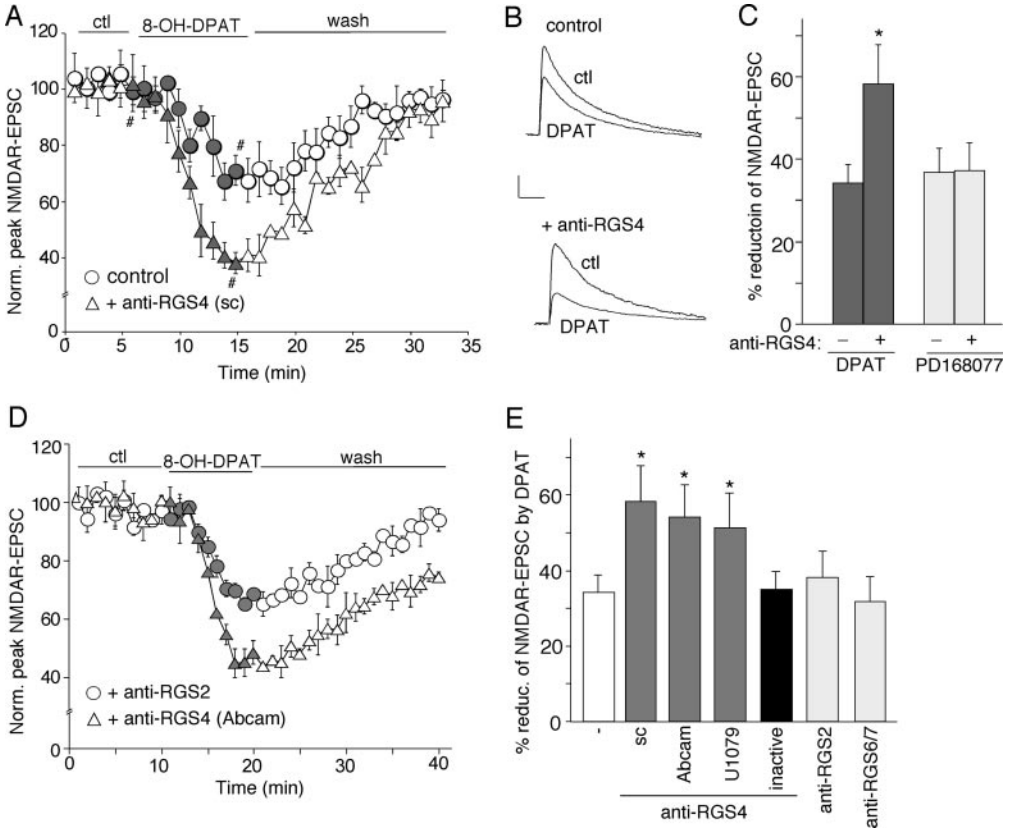

Fig. 1. Blocking RGS4 function potentiated 5-HT_{1A} regulation of NMDAR-mediated synaptic responses in PFC slices. A and D, plot of normalized peak NMDAR-EPSCs showing the effect of 8-OH-DPAT (a selective 5-HT_{1A} agonist, 20 μM) on the amplitude of evoked NMDAR-EPSC with different RGS antibodies included in the recording pipette. B, representative current traces taken from the records used to construct A (at time points denoted by #). Scale bars: 50 pA, 100 ms. C, cumulative data (mean ± S.E.M.) showing the percentage reduction of NMDAR-EPSC by 8-OH-DPAT or PD168077 (a selective D_4 agonist, 20 μM) with or without injection of anti-RGS4 (Santa Cruz). E, cumulative data (mean ± S.E.M.) showing the percentage reduction of NMDAR-EPSC by 8-OH-DPAT in a sample of neurons loaded with different RGS antibodies. *, $p < 0.001$, ANOVA.

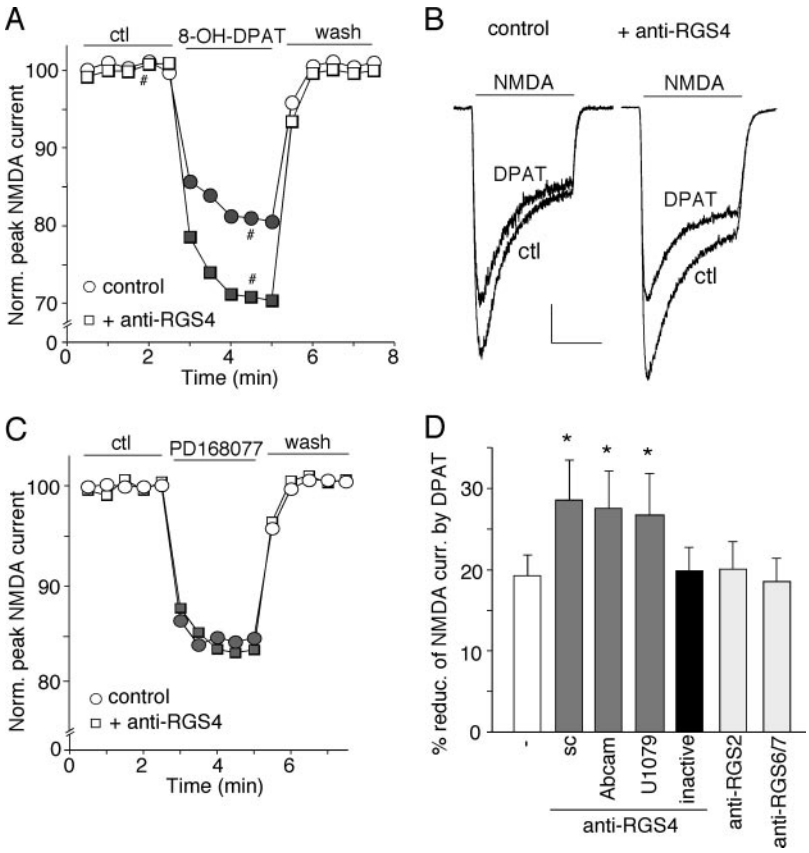

Fig. 2. Blocking RGS4 function enhanced 5-HT_{1A} regulation of NMDAR-mediated ionic current in acutely dissociated PFC pyramidal neurons. A and C, plot of normalized peak NMDAR current showing the effect of 8-OH-DPAT (20 μM, A) or PD168077 (20 μM, C) on NMDA (100 μM)-elicited current in representative PFC neurons with or without RGS4 antibody (Santa Cruz Biotechnology) included in the recording pipette. B, representative current traces taken from the records used to construct A (at time points denoted by #). Scale bars, 0.2 nA, 0.5 s. D, cumulative data (mean ± S.E.M.) showing the percentage reduction of whole-cell NMDAR current by 8-OH-DPAT in a sample of neurons loaded with different RGS antibodies. *, $p < 0.001$, ANOVA.

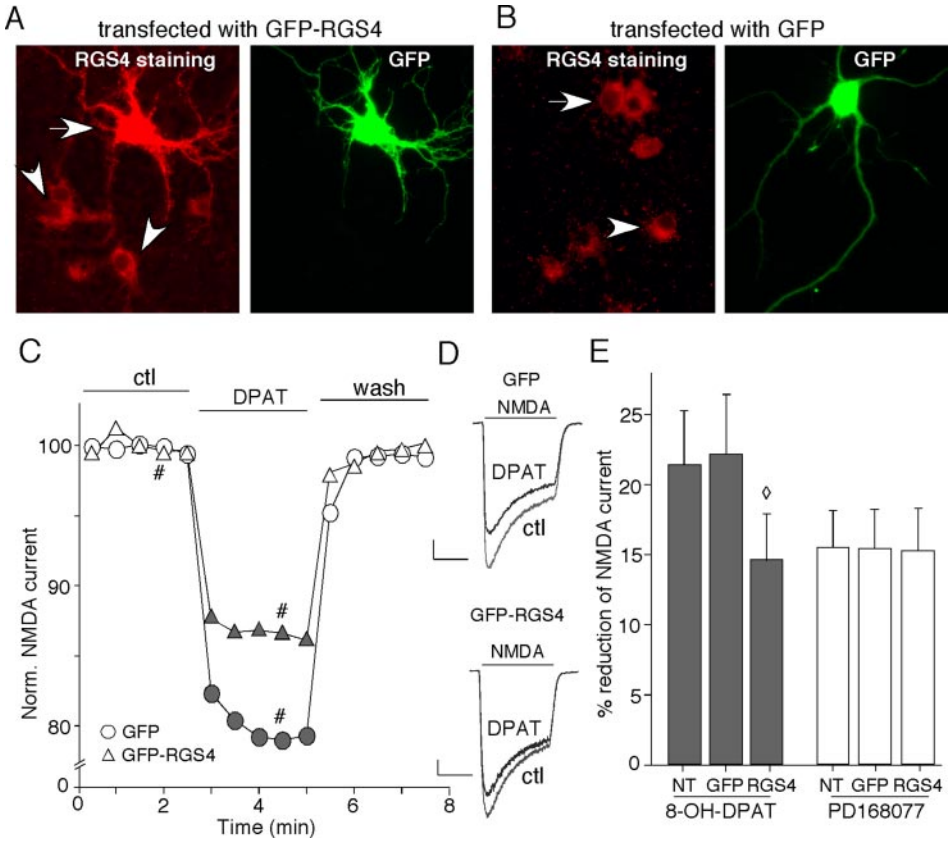

Fig. 3. RGS4 overexpression suppressed 5-HT_{1A} regulation of NMDAR current in cultured PFC neurons. A and B, immunocytochemical staining showing enhanced RGS4 expression in neurons transfected with the GFP-fused RGS4 (A) and normal RGS4 expression in neurons transfected with GPF alone (B). Arrows indicate GFP-positive neurons, and arrowheads indicate GFP-negative neurons. C, plot of normalized peak NMDAR current showing the effect of 8-OH-DPAT in representative GFP-positive neurons transfected with GFP alone or GFP-fused RGS4. D, representative current traces taken from the records used to construct C (at time points denoted by #). Scale bars, 0.2 nA, 1 s. E, cumulative data (mean ± S.E.M.) showing the percentage reduction of NMDAR current by 8-OH-DPAT or PD168077 in nontransfected (NT) neurons or neurons transfected with GFP alone or GFP-fused RGS4. ◊, $p < 0.005$, ANOVA.

12) or GFP-fused RGS4 (15.2 ± 3.1%, $n = 12$), compared with nontransfected control neurons (15.5 ± 2.6%, $n = 12$). Taken together, these results indicate that RGS4 is a selective negative regulator of serotonin signaling in PFC neurons.

Enhanced 5-HT$_{1A}$ Regulation of NMDAR Function in PFC Neurons from PCP-Treated Rats. To test whether aberrant RGS4 and serotonin signaling could be implicated in schizophrenia, we examined these molecules in rats with systemic administration of PCP (Jentsch and Roth, 1999), which represents a more thorough model of schizophrenia than other existing pharmacological or genetic models (Gainetdinov et al., 2001). The 5-HT$_{1A}$ modulation of NMDAR-EPSC in PFC slices was first tested in PFC neurons from rats after subchronic PCP exposure (i.p. for 7 days). As shown Fig. 4, A and B, neurons from PCP-treated rats showed two different kinds of response to 8-OH-DPAT. Significantly ($p < 0.001$, ANOVA) enhanced 8-OH-DPAT inhibition of NMDAR-EPSC (63.3 ± 4.5%, $n = 10$) was observed in ~36% of the total PFC neurons we tested ($n = 28$) from PCP-treated rats, compared with the effect of 8-OH-DPAT in neurons from saline-treated rats (36.0 ± 8.8%, $n = 28$). In the remaining 64% of neurons from PCP-treated rats, the 8-OH-DPAT effect on NMDAR-EPSC was unaltered (38.2 ± 6.5%, $n = 18$). No obvious differences in morphology or electrophysiological features (e.g., NMDAR-EPSC amplitude, holding current, and input resistance) were observed between the two groups of neurons showing distinct responses to 8-OH-DPAT (data not shown).

We further examined the effect of PCP treatment on 5-HT$_{1A}$ regulation of NMDAR current in dissociated PFC neurons. As shown in Fig. 4C, neurons from PCP-treated rats also exhibited two different kinds of response to 8-OH-DPAT. In ~35% of the total PFC neurons we examined ($n = 40$) from PCP-treated rats (i.p. for 7 days), significantly ($p < 0.001$, ANOVA) enhanced 8-OH-DPAT modulation of NMDAR current (31.9 ± 4.2%, $n = 14$) was observed, compared with neurons from saline-treated rats (19.4 ± 2.8%, $n = 30$). In the remaining 65% of neurons from PCP-treated rats, the 8-OH-DPAT modulation of NMDAR current was normal (19.5 ± 3.3%, $n = 26$). Representative cells from saline-treated rats and from PCP-treated rats with the enhanced 8-OH-DPAT effect are shown in Fig. 4D.

We then examined whether RGS4 was involved in the enhanced 5-HT$_{1A}$ effect on NMDR current in a subset of

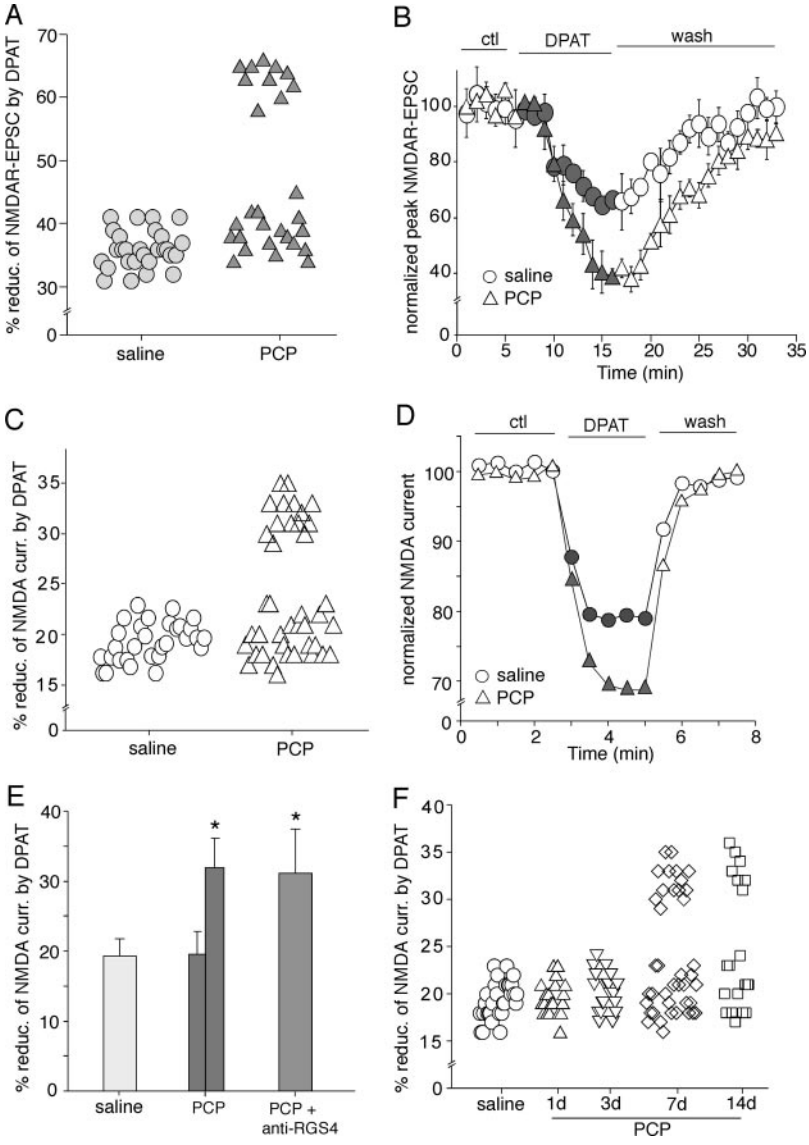

Fig. 4. PCP administration enhanced the 5-HT$_{1A}$ effect on NMDA receptors in a subset of PFC pyramidal neurons. A and C, plot of distribution of the percentage reduction of NMDAR-EPSC (A) or whole-cell NMDAR current (C) by 8-OH-DPAT in PFC neurons from saline- or PCP-treated (3 mg/kg i.p. for 7 days) rats. Note that two groups of cells from PCP-treated rats responded differently to 8-OH-DPAT. B and D, plot of normalized peak NMDAR-EPSCs (B) or whole-cell NMDAR current (D) showing the effect of 8-OH-DPAT in representative neurons from saline-treated rats or the subset of neurons from PCP-treated rats with enhanced 8-OH-DPAT effects. E, cumulative data (mean ± S.E.M.) showing the percentage reduction of NMDAR current by 8-OH-DPAT in neurons from saline- or PCP-treated rats with or without the RGS4 antibody included in recording pipette. Note that the effects of 8-OH-DPAT in neurons from PCP-treated rats are analyzed separately for the two distinct groups. *, $p < 0.001$, ANOVA. F, plot of distribution of the percentage reduction of NMDAR current by 8-OH-DPAT after different periods of PCP treatment.

neurons from PCP-treated rats. As shown in Fig. 4E, when the RGS4 antibody was included in recording pipette, all neurons from PCP-treated rats showed significantly ($p < 0.001$, ANOVA) potentiated responses to 8-OH-DPAT, reducing NMDAR current by 31.1 ± 6.3% ($n = 28$). This RGS4 antibody-induced increase (~60%) in the 5-HT_{1A} effect was comparable with the increase (~64%) seen in the subset of neurons from PCP-treated rats with enhanced responses when no RGS4 antibody was infused. These results suggest that the effect of the RGS4 antibody is occluded in those neurons from PCP-treated rats with enhanced 5-HT_{1A} responses.

To test the length of PCP treatment needed to induce the potentiated 5-HT_{1A} effect on NMDR current, we examined rats with different durations of PCP exposure. As shown in Fig. 4F, the 8-OH-DPAT modulation of NMDAR current was not altered after 1 ($n = 20$) or 3 days ($n = 20$) of PCP administration but was significantly changed after 7 ($n = 40$) or 14 days ($n = 20$). Taken together, these data suggest that subchronic PCP treatment in a population of PFC pyramidal neurons causes potentiated serotonin signaling.

Decreased RGS4 Expression in PFC Neurons after PCP Treatment. To further confirm the involvement of RGS4 in the enhanced 5-HT_{1A} signaling after PCP treatment, we examined the changes of RGS4 expression in PCP-treated rats. As shown in Fig. 5,A and B, RGS4 protein levels detected with Western blotting were significantly lower in PFC slices from rats exposed to PCP for 7 days (72 ± 12% of control, $n = 10$) or 14 days (68 ± 11% of control, $n = 8$), compared with saline-treated rats, whereas there was no change from rats treated with PCP for 1 day (98 ± 11% of control, $n = 8$) or 3 days (96 ± 13% of control, $n = 8$). The time course of RGS4 expressional changes coincided with the onset of 5-HT_{1A} functional changes, both occurred after 7 days but not 3 days of PCP exposure. Moreover, we examined the expression of NMDA receptors in PCP-treated rats. No significant changes in the protein levels of NMDAR subunits, such as NR1, NR2A, or NR2B, could be observed in PFC from rats exposed to different durations of PCP (Fig. 5, A and B). In addition, no change in RGS4 protein levels was found in other brain areas, including striatum, hippocampus, and cerebellum (Fig. 5C). These results indicate that subchronic PCP treatment induces a selective loss of RGS4 expression in PFC.

We further examined the RGS4 mRNA in PFC slices after PCP treatment with quantitative real-time RT-PCR. As shown in Fig. 6, A and B, PCP (7-day) treatment significantly decreased the RGS4 mRNA level in PFC (67.3 ± 14.3% of control, $n = 8$), whereas the 5-HT_{1A} receptor mRNA level was not changed (96.8 ± 11.6% of control, $n = 8$). GAPDH, which was used as a control, was also not changed by PCP treatment (102.1 ± 9.7% of control, $n = 8$).

To verify that the decreased RGS4 mRNA level in PFC slices from PCP-treated rats was from the subset of PFC neurons with enhanced 5-HT_{1A} effects on NMDAR current, single-cell RT-PCR (Yan and Surmeier, 1997) was conducted to detect RGS4 after whole-cell recording. RGS4 mRNA levels were compared in the two groups of PFC neurons from PCP-treated rats with or without enhanced 5-HT_{1A} effects on NMDAR current. We found that PFC neurons with enhanced 8-OH-DPAT effects showed significantly decreased RGS4 mRNA levels (Fig. 6, C and D; 40.3 ± 15.5% of control, $n = 12$; $p < 0.001$, ANOVA), whereas neurons with normal 8-OH-DPAT effects showed no significant changes in RGS4 mRNA levels (Fig. 6, C and D; 92.5 ± 18.0% of control, $n = 16$). These results suggest that the enhanced 5-HT_{1A} signaling in a subset of PFC neurons from PCP-treated rats could be due to the loss of RGS4-mediated negative control in these cells.

Discussion

Although several neurotransmitter systems, including dopamine, serotonin, glutamate, and GABA, are implicated in the pathogenesis of schizophrenia (Carlsson et al., 2001), putative susceptibility genes with no obvious direct functional links to these systems are emerging (Harrison and Owen, 2003). Elucidating the interaction among these contributing factors is of great importance to understanding the

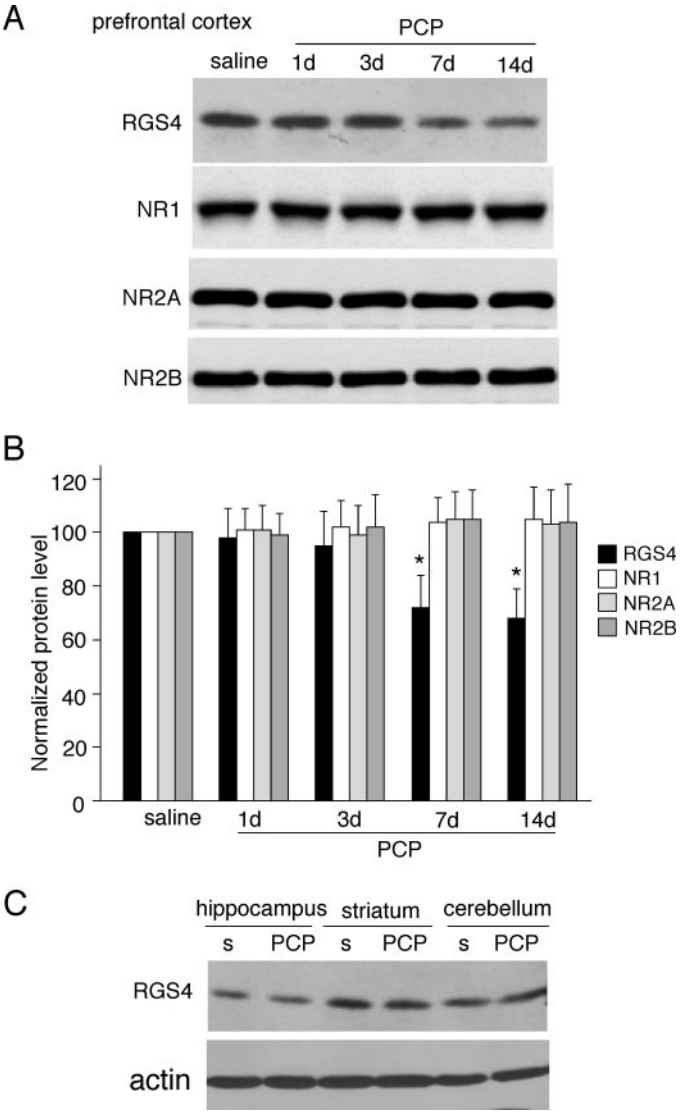

Fig. 5. PCP administration selectively reduced the RGS4 protein level in PFC. A, Western blots showing the protein levels of RGS4, NR1, NR2A, and NR2B in PFC slices from saline- or PCP-treated (3 mg/kg, i.p. for 7 days) rats. B, quantitative analysis showing that the RGS4 protein level was significantly lower after PCP administration, whereas the protein levels of NR1, NR2A, and NR2B had no significant changes. *, $p < 0.001$, ANOVA. C, Western blot showing the protein levels of RGS4 and actin in striatum, hippocampus, and cerebellum from saline- (s) or PCP-treated rats (3 mg/kg i.p. for 7 days).

pathophysiology of schizophrenia. It is speculated that these susceptibility genes may converge functionally upon the risk of schizophrenia via influencing synaptic plasticity of cortical circuitry, which is controlled by NMDA receptor-mediated glutamate transmission (Harrison and Weinberger, 2005). Given the key involvement of aberrant NMDAR signaling in schizophrenia (Tsai and Coyle, 2002), it is thought that dysregulation of NMDA receptors underlies the major action of plausible susceptibility genes for this disease (Moghaddam, 2003). In the present study, we provide evidence showing that one of the genes linked to schizophrenia, RGS4, is selectively involved in the negative control of the 5-HT_{1A} regulation of NMDAR function in PFC pyramidal neurons. Moreover, in the PCP model of schizophrenia, activation of 5-HT_{1A} receptors abnormally inhibits NMDAR current in a subset of PFC neurons, which is associated with the diminished expression of RGS4 in these cells. Thus, these results have demonstrated a functional interaction among RGS4, 5-HT_{1A}, and NMDA signaling in PFC neurons and the potential aberrations of this interaction in schizophrenia.

GPCR signaling pathways, which mediate intracellular responses to extracellular stimuli, need to be tightly regulated. The RGS protein provides a key mechanism for short-term regulation of G proteins (Vries et al., 2000). RGS4 was initially shown to negatively regulate GPCR signaling by accelerating the rate of GTP hydrolysis through the $G\alpha_i$ subfamily of G protein subunits (Berman et al., 1996). Later, RGS4 was found to be relatively promiscuous, attenuating both G_i-mediated inhibition of cAMP synthesis and G_q-mediated activation of phospholipase Cβ and mitogen-activated protein kinase activation via serving as a GTPase-activating protein and/or an effector antagonist (Hepler et al., 1997; Huang et al., 1997; Yan et al., 1997). However, most of the studies were in vitro assays in cell lines overexpressing RGS4 along with certain receptors and G protein subunits. The specificity of endogenous RGS proteins for different GPCR signaling pathways may be different in native neurons. In this study, we found that 5-HT_{1A} and D_4 receptors, both of which couple to G_i-mediated signaling to regulate NMDA receptors (Wang et al., 2003; Yuen et al., 2005), are differentially influenced by RGS4 in prefrontal cortical neurons. The selective coupling of RGS4 to 5-HT_{1A} signaling is consistent with previous reports showing that RGS4 attenuates $G_{\alpha i}$-mediated cAMP production in 5-HT_{1A}, but not dopamine D_2, receptor-expressing cell lines (Ghavami et al., 2004), and RGS4 is involved in 5-HT_{1A}-mediated neurotransmitter release (Beyer et al., 2004). Because RGS4 is capable of discriminating between specific G_q-coupled receptors (Xu et al., 1999), we speculate that different specificity of RGS4 in regulating 5-HT_{1A} versus D_4/D_2 signaling is probably conferred by distinct interaction of the N-terminal domain of RGS4 with these receptor complexes. The detailed mechanisms underlying the preference of RGS4 for serotonin signaling await to be further investigated.

RGS4 is associated with schizophrenia susceptibility (Harrison and Owen, 2003), so it is important to know whether RGS4 aberration occurs in the pathological condition. Given the preferential link of RGS4 to serotonin signaling, we first examined the 5-HT_{1A} regulation of NMDA receptors in the PCP model of schizophrenia. It is noteworthy that a significant potentiation (65–75%) of the 5-HT_{1A} regulation of NMDAR current was found in a subpopulation (~1/3) of PFC neurons from rats with subchronic (>7 days) PCP treatment. Because RGS4 is specifically involved in the negative control of 5-HT_{1A} effects on NMDA receptors, we suspected that the potentiated 5-HT_{1A} regulation of NMDAR current in the subset of PFC neurons from PCP-treated rats could be due to the loss of RGS4 expression in these cells. Indeed, we detected a decreased RGS4 protein level specifically in the PFC area from rats exposed to subchronic PCP. Moreover, blocking RGS4 function with the RGS4 antibody failed to further increase the 5-HT_{1A} effect on NMDA receptor current in the group of PFC neurons with elevated 5-HT_{1A} responses, suggesting the lack of endogenous functional RGS4 in these cells

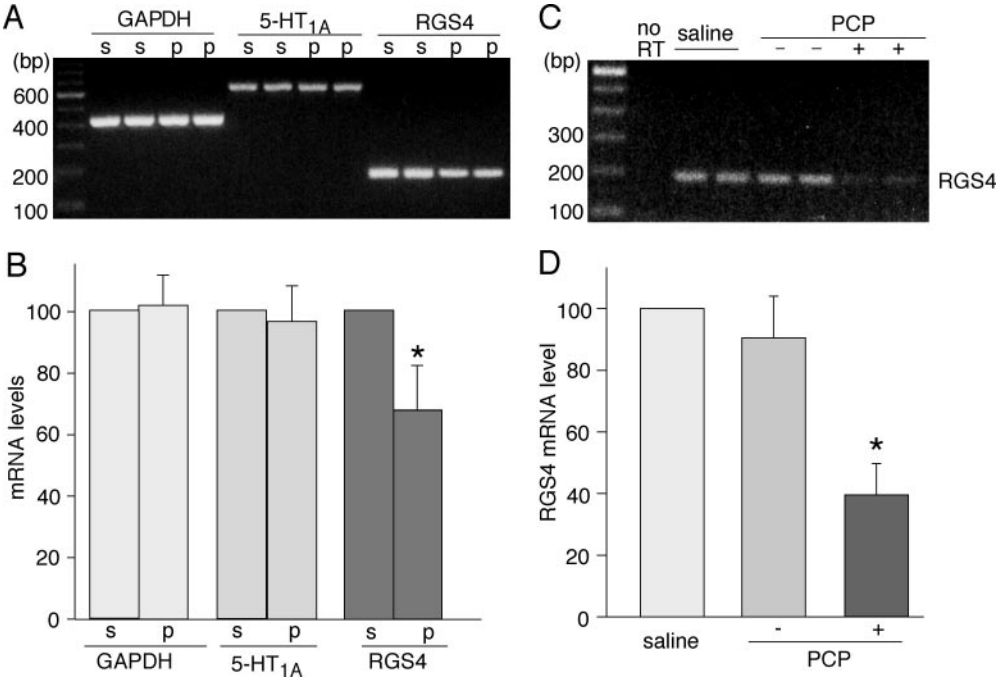

Fig. 6. PCP administration reduced RGS4 mRNA level in the subset of PFC neurons showing enhanced 5-HT_{1A} signaling. A, agarose gel electrophoresis showing RT-PCR products of RGS4, 5-HT_{1A} receptor and GAPDH in PFC areas from saline-treated (s) or PCP- treated (p) rats (3 mg/kg, i.p. for 7 days). B, plot of normalized semiquantitative RT-PCR results showing mRNA levels of RGS4, 5-HT_{1A}, and GAPDH in PFC from saline- (s) or PCP-treated (p) rats. C, agarose gel electrophoresis showing single-cell RT-PCR products of RGS4 in neurons from saline- or PCP-treated rats with (+) or without (−) enhanced 8-OH-DPAT effects on NMDAR current. D, plot of normalized single-cell RT-PCR results showing that the RGS4 mRNA level was significantly reduced in those cells from PCP-treated rats with enhanced responses to 8-OH-DPAT (+), but not in those neurons without enhanced effects of 8-OH-DPAT (−). *, $p < 0.001$, ANOVA.

after PCP treatment. By using single-cell RT-PCR, we confirmed that the RGS4 mRNA level was indeed selectively diminished in the subset of PFC neurons with elevated 5-HT_{1A} responses after subchronic PCP exposure. Hence, these results suggest that in the PCP model of schizophrenia, loss of RGS4 expression results in the potentiated 5-HT_{1A} inhibition of NMDAR current in a pool of PFC pyramidal neurons. This effect of PCP treatment on 5-HT_{1A} signaling is unique and differs from that on dopamine signaling. We have found that rats exposed to PCP show attenuated D_4 inhibition of NMDAR current as a result of the loss of D_4 regulation of Ca^{2+}/camodulin-dependent protein kinase II and unchanged D_1 enhancement of NMDAR current (Wang et al., 2006).

Levels of RGS proteins or mRNAs are tightly regulated by neuronal stimuli, which provides an important mechanism for influencing GPCR signaling pathways (Burchett et al., 1998; Ingi et al., 1998). The specific alteration of RGS4 expression in PFC, a prominent area affected in schizophrenia (Weinberger et al., 1986; Andreasen et al., 1997), hints that RGS4 in PFC neurons may have specialized roles in schizophrenia. The mechanisms underlying this altered prefrontal cortical RGS4 expression in the PCP model of schizophrenia are unclear, but dopamine signaling may play a key role. Previous studies have shown that selective activation of dopamine D_2 receptors with specific agonists causes the upregulation of RGS4 expression in rat striatum (Geurts et al., 2002; Taymans et al., 2003). The functional deficit of dopamine neurotransmission in the prefrontal cortex in schizophrenia (Lewis and Lieberman, 2000) may cause the downregulation of RGS4 expression in PFC neurons.

Taken together, our study has revealed one of the biological roles of RGS4 in cortical neurons and, more importantly, this function of RGS4 is related to 5-HT_{1A} regulation of glutamatergic network, which is thought to be important for schizophrenia and antipsychotic drug treatment (Bantick et al., 2001). The alteration of RGS4 expression in PFC and the subsequent change in RGS4 modulation of 5-HT_{1A} signaling on NMDAR function in the PCP model of schizophrenia provides a potential mechanism for the synaptic dysfunction in schizophrenia (Frankle et al., 2003). It is perceivable that impaired RGS4 function may induce oversuppression of NMDAR response by 5-HT_{1A} receptors, which could lead to schizophrenia-like behavioral manifestations because of NMDAR hypofunction (Tsai and Coyle, 2002).

Acknowledgments

We thank Xiaoqing Chen for technical support. We are grateful to Dr. Stephen J. Gold for providing the RGS4 antibody U1079.

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