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Phosphorylation-independent Regulation of Metabotropic Glutamate Receptor 5 Desensitization and Internalization by G Protein-coupled Receptor Kinase 2 in Neurons*

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The uncoupling of metabotropic glutamate receptors (mGluRs) from heterotrimeric G proteins represents an essential feedback mechanism that protects neurons against receptor overstimulation that may ultimately result in damage. The desensitization of mGluR signaling is mediated by both second messenger-dependent protein kinases and G protein-coupled receptor kinases (GRKs). Unlike mGluR1, the attenuation of mGluR5 signaling in HEK 293 cells is reported to be mediated by a phosphorylation-dependent mechanism. However, the mechanisms regulating mGluR5 signaling and endocytosis in neurons have not been investigated. Here we show that a 2-fold overexpression of GRK2 leads to the attenuation of endogenous mGluR5-mediated inositol phosphate (InsP) formation in striatal neurons and siRNA knockdown of GRK2 expression leads to enhanced mGluR5-mediated InsP formation. Expression of a catalytically inactive GRK2-K220R mutant also effectively attenuates mGluR5 signaling, but the expression of a GRK2-D110A mutant devoid in $G\alpha_{q/11}$ binding increases mGluR5 signaling in response to agonist stimulation. Taken together, these results indicate that the attenuation of mGluR5 responses in striatal neurons is phosphorylation-independent. In addition, we find that mGluR5 does not internalize in response to agonist treatment in striatal neuron, but is efficiently internalized in cortical neurons that have higher levels of endogenous GRK2 protein expression. When overexpressed in striatal neurons, GRK2 promotes agonist-stimulated mGluR5 internalization. Moreover, GRK2-mediated promotion of mGluR5 endocytosis does not require GRK2 catalytic activity. Thus, we provide evidence that GRK2 mediates phosphorylation-independent mGluR5 desensitization and internalization in neurons.

Glutamate is the major excitatory neurotransmitter in the mammalian brain and functions to activate two distinct classes of receptors (ionotropic and metabotropic) to regulate a variety of physiological functions (1–3). Ionotropic glutamate receptors, such as NMDA, AMPA, and kainate receptors, are ligand-gated ion channels, whereas metabotropic glutamate receptors (mGluRs)[5] are members of the G protein-coupled receptor (GPCR) superfamily (4–7). mGluRs modulate synaptic activity via the activation of heterotrimeric G proteins that are coupled to a variety of second messenger cascades. Group I mGluRs (mGluR1 and mGluR5) are coupled to the activation of $G\alpha_{q/11}$ proteins, which stimulate the activation of phospholipase $C\beta1$ resulting in diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3) formation, release of Ca^{2+} from intracellular stores and subsequent activation of protein kinase C.

The attenuation of GPCR signaling is mediated in part by G protein-coupled receptor kinases (GRKs), which phosphorylate GPCRs to promote the binding of β-arrestin proteins that uncouple GPCRs from heterotrimeric G proteins (8–10). GRK2 has been demonstrated to contribute to the phosphorylation and desensitization of both mGluR1 and mGluR5 in human embryonic kidney (HEK 293) cells (11–17). GRK4 is also implicated in mediating the desensitization of mGluR1 signaling in cerebellar Purkinje cells, but does not contribute to the desensitization of mGluR5 (14, 15). In addition, GRK4 plays a major role in mGluR1 internalization (13, 14). A role for GRK2 in promoting mGluR1 internalization is less clear as different laboratories have obtained discordant results (11, 14, 15, 16). However, the only study examining the role of GRK2 in regulating mGluR1 endocytosis in a native system reported that GRK2 knockdown had no effect upon mGluR1 internalization in cerebellar Purkinje cells (14).

GRK2 is composed of three functional domains: an N-terminal regulator of G protein signaling (RGS) homology (RH) domain, a central catalytic domain, and a C-terminal $G_\beta\gamma$ binding pleckstrin homology domain (18). In HEK 293 cells,

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[5] The abbreviations used are: mGluR, metabotropic glutamate receptor; DHPG, (*S*)-3,5-dihydroxylphenylglycine; DIV, days *in vitro*; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HEK, human embryonic kidney; InsP, inositol phosphate; NC, non-coding; PKC, protein kinase C; RGS, regulator of G protein signaling; RH, regulator of G protein signaling homology; siRNA, small interfering RNA; PBS, phosphate-buffered saline.

mGluR1 desensitization is not dependent on GRK2 catalytic activity. Rather the GRK2 RH domain interacts with both the second intracellular loop domain of mGluR1 and the α-subunit of $G\alpha_{q/11}$ and attenuates second messenger responses by disrupting the mGluR1/$G\alpha_{q/11}$ signaling complexes (12, 19–21). Although the molecular mechanism underlying GRK2-mediated attenuation of mGluR1 signaling is relatively well established in HEK 293 cells, the role of GRK2 in regulating the desensitization of mGluRs in neurons remains to be determined. Moreover, it is not known whether GRK2-dependent attenuation of mGluR5 signaling is mediated by the same phosphorylation-independent mechanism that has been described for mGluR1. In a previous study, GRK2-mediated mGluR5 desensitization was reported to be phosphorylation-dependent, based on the observation that the overexpression of a catalytically inactive GRK2 (K220R) did not attenuate mGluR5 signaling (15). In the present study, we examined whether a 2-fold overexpression of GRK2 in primary mouse striatal neurons to match GRK2 expression levels found in the cortex results in increased agonist-stimulated desensitization and internalization of endogenous mGluR5. We report here that GRK2 mediates phosphorylation-independent mGluR5 desensitization and internalization. Furthermore, GRK2 knockdown causes an increase in mGluR5 signaling, demonstrating that endogenous GRK2 plays a role in mGluR5 desensitization.

EXPERIMENTAL PROCEDURES

Materials—(*S*)-3,5-Dihydroxylphenylglycine (DHPG) was purchased from Tocris Cookson Inc. (Ellisville, MO). *myo*-[^{3}H]Inositol and [^{32}P]orthophosphate were acquired from PerkinElmer Life Sciences (Waltham, MA). The Dowex 1-X8 (formate form) resin with 200–400 mesh was purchased from Bio-Rad (Mississauga, ON). Bovine serum albumin was obtained from BioShop Canada Inc. (Mississauga, ON). Horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody was from Bio-Rad. ECL Western blotting detection reagents were from GE Healthcare (Oakville, Ontario, Canada). EZ-Link Sulfo-NHS-SS-Biotin and immobilized NeutrAvidin beads were from Pierce Biotechnology. Hiperfect transfection reagent was purchased from Qiagen (Mississauga, ON). QuikChange^{TM} site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Rabbit anti-mGluR1 and anti-mGluR5 antibodies were from Upstate/Millipore (Billerica, MA). Mouse anti-NeuN monoclonal antibody, Alexa Fluor 568 goat anti-mouse, and Alexa Fluor 488 goat anti-rabbit secondary antibodies were purchased from Invitrogen/Molecular Probes (Burlington, ON). Rabbit anti-GRK2 and anti-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-clathrin antibody was from BD Biosciences (Mississauga, ON). Rabbit anti-FLAG antibody and all other biochemical reagents were purchased from Sigma-Aldrich.

mGluR5 Knock-out Mice—B6;129-Grm5^{tm1Rod}/J (mGluR5$^{-/-}$) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed in an animal care facility at 23 °C on a 12 h light/12 h dark cycle with food and water provided *ad libitum*. Animal care was in accordance with the University of Western Ontario Animal Care Committee.

Adenovirus Construction—The mouse GRK2 cDNA used for adenovirus construction was FLAG-tagged in the N-terminal region (22). Mutations (K220R and D110A) introduced to mouse GRK2 construct were created using the QuikChange^{TM} site-directed mutagenesis kit. cDNA encoding FLAG-tagged GRK2, GRK2-K220R, and GRK2-D110A or green fluorescent protein (GFP) were used to generate adenoviral constructs (AdMax) as per the manufacturer's instructions (Microbix Biosystems, Toronto, ON).

Neuronal Primary Culture Preparation—Neuronal cultures were prepared from the striatal region of E15 mouse embryo brains. Animal procedures were approved by The University of Western Ontario Animal Care Committee. After dissection, either striatal or cortical tissue was submitted to trypsin digestion followed by cell dissociation using a fire-polished pasteur pipette. Cells were plated on poly-L-ornithine-coated dishes in neurobasal media supplemented with N2 and B27 supplements, 2 mM glutamax, 50 μg/ml penicillin, and 50 μg/ml streptomycin. Cells were incubated at 37 °C and 5% CO_2 in a humidified incubator and cultured for 12 days *in vitro* (DIV) with media replenishment every 4 days. The cultures were infected with the different adenoviruses (GFP, wild-type GRK2, GRK2-K220R, or GRK2-D110A) at DIV9 and used for experiments at DIV12.

Small Interfering RNA (siRNA) Transfection—Striatal neurons seeded in 12-well plates were transfected in cultured medium using Hiperfect transfection reagent. Neurons were transfected with either non-coding (NC) or GRK2 siRNA as per the manufacturer's instructions. siRNA (20 nM final concentration) was added to 100 μl of Neurobasal medium, followed by addition of 6 μl of Hiperfect. Transfection mix was vortexed, incubated for 5–10 min at room temperature, and added to neuronal cultures. Neurons were transfected twice at DIV5 and DIV7 and used for experiments at DIV9. Both the GRK2 siRNA and NC siRNA (siGENOME control siRNA-non-targeting siRNA 1) were purchased from Dharmacon Research (Lafayette, CO). The GRK2 mouse antisense oligonucleotide had the following sequence: 5′-AAGAAATATGAGAAGCTGGAG-3′ (23). The effect of siRNA transfection was assessed by measuring the total GRK2 protein expression by Western blotting analysis.

Inositol Phosphate Formation—Inositol lipids were radiolabeled by incubating the striatal neurons infected with different adenoviruses (GFP, GRK2, GRK2-K220R, or GRK2-D110A) overnight with 1 μCi/ml *myo*-[^{3}H]inositol in Neurobasal medium. Unincorporated *myo*-[^{3}H]inositol was removed by washing cells with Hank's balanced salt solution (HBSS). Cells were preincubated for 1 h in HBSS at 37 °C and then preincubated in 500 μl of the same buffer containing 10 mM LiCl for an additional 2 min at 37 °C. Cells were then incubated in the presence of either DHPG or carbachol for 5 min at 37 °C. Drug concentration is indicated in the figures. The reaction was stopped on ice by the addition of 500 μl of perchloric acid and then neutralized with 400 μl of 0.72 M KOH, 0.6 M $KHCO_3$. Total [^{3}H]inositol incorporated into cells was determined by counting the radioactivity present in 50 μl of cell lysate. Total inositol phosphate was purified from cell extracts by anion exchange chromatography using Dowex 1-X8 (formate form)

200–400 mesh anion exchange resin. [^{3}H]Inositol phosphate formation was determined by liquid scintillation using a Beckman LS 6500 scintillation system (20, 21).

Co-immunoprecipitation—Striatal neurons infected with either GFP or GRK2 adenovirus were incubated at 37 °C in HBSS in the presence of 10 μM DHPG for 5 min. Cells were washed with ice-cold HBSS and solubilized in lysis buffer (25 mM HEPES, pH 7.5, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.1% Triton X-100) containing protease inhibitors (1 mM AEBSF and 10 μg/ml of both leupeptin and aprotinin). mGluR5 was immunoprecipitated from 500–1000 μg of total cell lysate using anti-mGluR5 antibody and protein G-Sepharose beads by 2 h rotation at 4 °C. Afterward, beads were washed one time with lysis buffer and two times with PBS, and proteins were eluted in SDS-PAGE loading buffer by warming the samples at 55 °C for 5 min. Eluted samples were subjected to SDS-PAGE, followed by electroblotting onto nitrocellulose membranes.

Internalization Assay—To evaluate the effect of GRK2 overexpression on mGluR5 internalization, either cortical or striatal neurons infected with different adenoviruses (GFP, GRK2, GRK2-K220R, or GRK2-D110A) were incubated at 37 °C in HBSS in the presence of 10 μM DHPG for varying times (0, 5, or 10 min). Cells were washed with HBSS and incubated on ice. Plasma membrane proteins were biotinylated with sulfo-NHS-SS-Biotin for 1 h on ice, as described previously (24). To quench the biotinylation reaction, cells were washed and incubated for 30 min with cold 100 mM glycine in HBSS, followed by three washes with cold HBSS. Cells were then lysed in radioimmune precipitation assay buffer (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2, 0.05 M EDTA, 1% nonidet P40, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (1 mM AEBSF and 10 μg/ml of both leupeptin and aprotinin). Biotinylated proteins were separated from non-biotinylated proteins by Neutravidin bead pull-down from equivalent amounts of total cellular protein from each sample (100 μg per sample). Biotinylated proteins were subjected to SDS-PAGE, followed by electroblotting onto nitrocellulose membranes.

Recycling Assay—To evaluate the effect of GRK2 overexpression on mGluR5 recycling, striatal neurons infected with either GFP or GRK2 adenovirus were incubated at 37 °C in HBSS in the presence of 10 μM DHPG for 10 min to stimulate internalization of plasma membrane mGluR5. Agonist was washed out, and cells were incubated in HBSS at 37 °C for 10 or 30 min to allow internalized mGluR5 to recycle to the plasma membrane. Cells were washed with HBSS and incubated on ice. Plasma membrane proteins were biotinylated with sulfo-NHS-SS-Biotin, and subsequent steps were conducted as described in internalization assay section.

Immunoblotting—Membranes were blocked with 10% milk in wash buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.0, and 0.05% Tween 20) for 1 h and then incubated with rabbit anti-GRK2 (1:2000), rabbit anti-mGluR5 (1:4000), rabbit anti-mGluR1 (1:1000), mouse anti-clathrin (1:1000), or rabbit anti-actin (1:10,000) antibodies in wash buffer containing 3% milk overnight. Membranes were rinsed three times with wash buffer and then incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG diluted 1:10,000 in wash buffer containing 3% skim milk for 1 h. Membranes were rinsed three times with wash buffer and incubated with ECL Western blotting detection reagents.

Whole Cell Phosphorylation—Whole cell phosphorylation experiments were performed as described previously (25). To label the intracellular ATP pool with [^{32}P]orthophosphate, striatal neurons infected with either GFP or GRK2 adenoviruses were incubated for 1 h at 37 °C in HBSS containing [^{32}P]orthophosphate (100 μCi/ml). Subsequently, the neurons were incubated at 37 °C in the presence of 10 μM DHPG for varying times (0, 5, or 10 min). Cells were solubilized in radioimmune precipitation assay buffer containing protease and phosphatase inhibitors (1 mM AEBSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 mM NaF, 10 mM sodium pyrophosphate, and 0.1 mM phenylmethylsulfonyl fluoride). Samples were normalized to total protein content. mGluR5 was immunoprecipitated using a rabbit anti-mGluR5 antibody and subsequently subjected to SDS-PAGE followed by autoradiography. The extent of receptor phosphorylation was quantitated by densitometry of the resulting autoradiographs.

Immunofluorescence and Confocal Imaging—Striatal neurons either uninfected or infected with FLAG-GRK2 adenovirus were washed twice in PBS and fixed with 3% formaldehyde in PBS for 20 min. After fixation, cells were washed with PBS and preincubated with a permeabilization solution (PBS, 0.05% Triton, and 3% bovine serum albumin) for 10 min. Subsequently, rabbit anti-FLAG (1:500) and mouse anti-NeuN (1:1000) antibodies were added to cells and incubated for 1 h in permeabilization solution. Cells were washed and incubated with goat anti-mouse and anti-rabbit antibodies conjugated to Alexa Fluor 568 and Alexa Fluor 488, respectively for 40 min in permeabilization solution at 1:1000 dilution. Detection of immunolabeled proteins was performed using dual excitation (488 and 543 nm) and emission (505–530 nm for Alexa Fluor 488-labeled anti-FLAG antibody and GFP, and 590–610 nm for Alexa Fluor 555-labeled anti-NeuN antibody) filter sets. Confocal microscopy was performed on a Zeiss LSM-510 laser scanning microscope using a Zeiss 63 × 1.3 NA oil immersion lens.

Data Analysis—Non-saturated, immunoreactive mGluR5 bands from both internalization and whole cell phosphorylation assays were quantified by scanning densitometry using Scion Image software. Means ± S.E. are shown for the number of independent experiments indicated in the figure legends. GraphPad Prism software was used to analyze data for statistical significance and for curve fitting. Statistical significance was determined by analysis of variance (ANOVA) testing followed by post-hoc multiple comparison testing.

RESULTS

Expression of mGluR5 in Striatal Neurons—Because the role of GRK2 in regulating Group I mGluR desensitization and internalization in neurons has not been investigated, we first examined which neuronal culture preparation represented the most appropriate model to study GRK2-dependent regulation of endogenous mGluR5 signaling. Primary striatal neurons obtained from E15 mouse embryos cultured 12 days *in vitro* expressed high levels of mGluR5 protein, whereas cortical neurons cultured under the

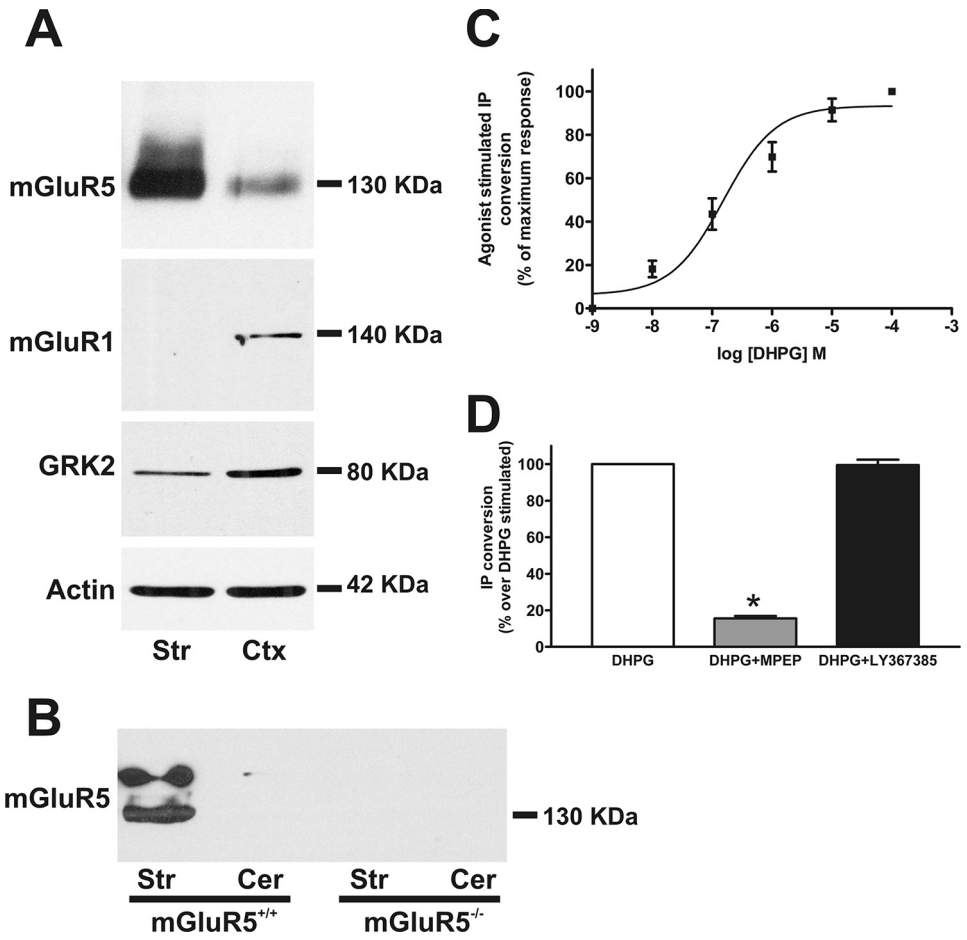

FIGURE 1. **Striatal neurons as a model for mGluR5 signaling studies.** *A*, shown are representative immunoblots for mGluR5, mGluR1, GRK2, and actin protein expression in either striatal (*Str*) or cortical (*Ctx*) neuronal cultures. 50 μg of cell lysate were used for each sample. *B*, shown is a representative immunoblot for mGluR5 protein expression in either striatal (*Str*) or cerebellum (*Cer*) from either wild-type or mGluR5$^{-/-}$ mice. 50 μg of cell lysate were used for each sample. *C*, shown is DHPG-stimulated inositol phosphate formation in striatal neurons stimulated with increasing concentrations of agonist for 5 min at 37 °C. The data points represent the means ± S.E. of five independent experiments, normalized to the maximum DHPG-stimulated response. *D*, shown is DHPG-stimulated inositol phosphate formation in response to 10 μM agonist for 5 min at 37 °C, either in the presence or absence of mGluR1- (LY36785) or mGluR5-antagonists (MPEP). Data represent the means ± S.E. of four independent experiments. The *asterisk* indicates a significant difference compared with control ($p < 0.05$).

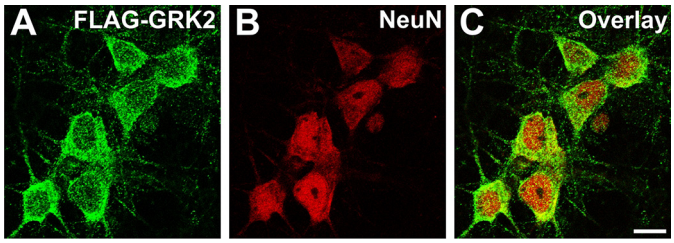

FIGURE 2. **GRK2 desensitizes mGluR5 in neurons.** Shown are representative laser-scanning confocal micrographs demonstrating the presence of both Alexa Fluor 555-conjugated anti-NeuN antibody (*A*) and Alexa Fluor 488-conjugated anti-FLAG antibody-labeled FLAG-GRK2 (*B*) in the same striatal neurons (*C*). Data are representative images of multiple cells from three independent experiments. *Bar*, 10 μM.

same conditions expressed lower levels of mGluR5, but also expressed mGluR1 (Fig. 1*A*). In contrast, mGluR1 protein expression was not detected in striatal cultures (Fig. 1*A*). GRK2 protein expression levels were also ~2-fold higher in the cortical neuronal cultures as compared with the striatal neuronal cultures (Fig. 1*A*).

To determine whether anti-mGluR5 antibody was specific to mGluR5 protein, we compared whole cell lysates obtained from the cerebellum and cortex of either wild-type or mGluR5$^{-/-}$ mice. Anti-mGluR5 antibody detected a 130-kDa band only in wild-type striatal lysate (Fig. 1*B*). No band was detected in the cerebellum, which does not express mGluR5, or in the striatum of mGluR5$^{-/-}$ lysates (Fig. 1*B*). DHPG, a specific group I mGluR agonist, promoted a dose-dependent increase in InsP formation in striatal neuronal cultures, demonstrating the expected signaling function of endogenous group I mGluRs (Fig. 1*C*). Based on the results from the dose response curve, in all subsequent experiments either 1 or 10 μM DHPG were used. The specificity of the group I mGluR-mediated InsP formation in striatal neurons was further established by the pretreatment of cells with specific mGluR5 (10 μM MPEP) and mGluR1 (100 μM LY367385) antagonists. The pretreatment of cells with MPEP reduced DHPG-stimulated InsP formation to 15.5 ± 1.3% of control and LY367385 pretreatment had no effect on DHPG-stimulated InsP formation (Fig. 1*D*). Taken together, these data indicated that mGluR5 was the primary group I mGluR expressed and activated by DHPG in embryonic-derived striatal cultures. Consequently, in all subsequent experimentation striatal neurons were used to examine GRK2-mediated regulation of mGluR5 desensitization.

GRK2-mediated Attenuation of mGluR5 Signaling—GRK2 protein expression in cortical neurons (which express mGluR5) was 2-fold higher than that of striatal neurons (Fig. 1*A*). Therefore, we tested whether a physiologically similar 2-fold increase in GRK2 expression would result in attenuated mGluR5 signaling in striatal neurons. GRK2 was overexpressed in neurons using a FLAG epitope-tagged GRK2 adenovirus. To determine whether the FLAG-GRK2 adenovirus efficiently infected neurons, we immunolabeled striatal neuronal cultures with both an antibody that recognizes the FLAG epitope tag and an antibody directed against the neuron-specific marker NeuN (neuronal nuclei) (26). We found that the FLAG-GRK2 adenoviral construct was able to effectively infect striatal neurons as the NeuN-positive neurons also exhibited FLAG-positive labeling (Fig. 2). Infection of striatal neurons with a GRK2 adenovirus increased GRK2 expression to 193 ± 7% of control neurons

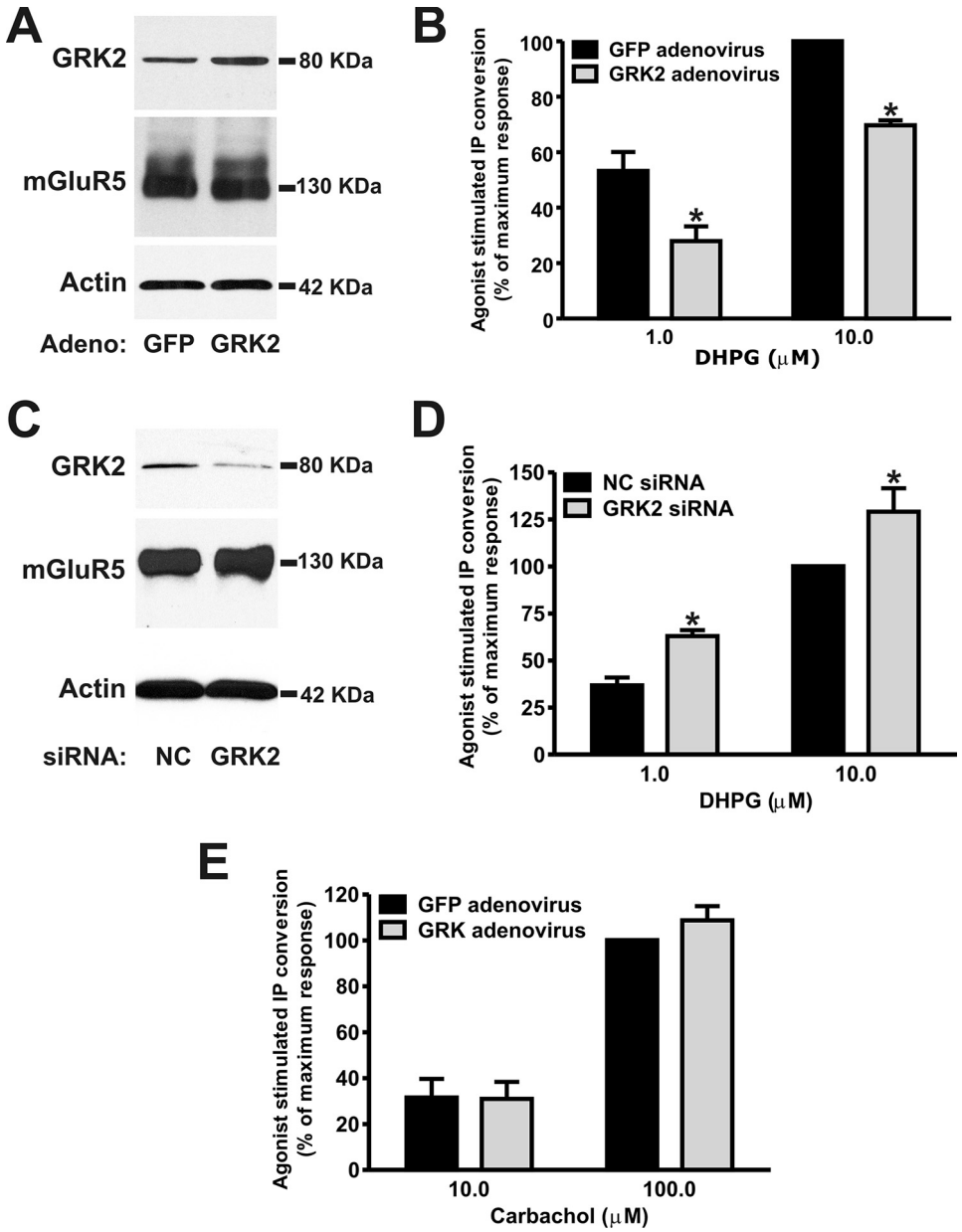

FIGURE 3. *A*, shown are representative immunoblots for mGluR5, GRK2, and actin protein expression in neurons infected with either GFP or GRK2 adenovirus. 50 μg of cell lysate were used for each sample. *B*, shown is mGluR5-stimulated inositol phosphate formation in response to either 1 or 10 μM DHPG for 5 min at 37 °C in striatal neurons infected with either GFP or GRK2 adenovirus. Data represent the means ± S.E. of four independent experiments, normalized to DHPG-stimulated GFP-infected neurons maximum response. *Asterisks* indicate significant differences compared with GFP adenovirus infected neurons at the corresponding agonist concentration ($p < 0.05$). *C*, shown are representative immunoblots for mGluR5, GRK2, and actin protein expression in neurons transfected with either NC or GRK2 siRNA. 50 μg of cell lysate were used for each sample. *D*, shown is mGluR5-stimulated inositol phosphate formation in response to either 1 or 10 μM DHPG for 5 min at 37 °C in striatal neurons transfected with either NC or GRK2 siRNA. Data represent the means ± S.E. of five independent experiments, normalized to DHPG-stimulated NC siRNA-transfected neurons maximum response. *Asterisks* indicate significant differences compared with NC siRNA transfected neurons at the corresponding agonist concentration ($p < 0.05$). *E*, shown is mGluR5-stimulated inositol phosphate formation in response to either 10 or 100 μM carbachol for 5 min at 37 °C in striatal neurons infected with either GFP or GRK2 adenovirus. Data represent the means ± S.E. of four independent experiments, normalized to carbachol-stimulated GFP-infected neurons maximum response.

infected with GFP adenovirus (Fig. 3*A*). Neurons infected with either GRK2- or GFP adenovirus were submitted to InsP formation assay and stimulated with DHPG for 5 min. GRK2 overexpression resulted in a significant attenuation of InsP formation in striatal neurons following stimulation with either 1 or 10 μM DHPG (Fig. 3*B*). Because the expression of mGluR5 was not altered by the moderate overexpression of GRK2 (Fig. 3*A*), the observed decrease in DHPG-stimulated InsP formation was the result of GRK2-mediated attenuation of mGluR5 responsiveness. To further establish the role of endogenously expressed GRK2 on mGluR5 desensitization, GRK2 siRNA was used to knockdown GRK2 protein expression in striatal neurons. Transfection of GRK2 siRNA into striatal neurons decreased GRK2 protein expression to 37 ± 9% of that in control neurons transfected with non-coding (NC) siRNA (Fig. 3*C*). siRNA transfection did not alter mGluR5 expression (Fig. 3*C*), but siRNA-mediated GRK2 knockdown led to an increase in DHPG-stimulated InsP formation (Fig. 3*D*). Thus, providing evidence that endogenously expressed GRK2 regulates mGluR5 activity in striatal neurons. A 2-fold increase in GRK2 expression was not sufficient to promote muscarinic receptor desensitization, as InsP formation stimulated by carbachol was not different between neurons infected with GFP or GRK2 adenovirus (Fig. 3*E*). However, it is possible that higher levels of GRK2 expression might lead to muscarinic receptor desensitization. Taken together, these data indicate that at the level of GRK2 expression tested mGluR5 but not muscarinic receptor signaling was desensitized by GRK2.

Assessment of mGluR5 Phosphorylation—The most common mechanism underlying GPCR desensitization is GRK-mediated phosphorylation followed by binding of β-arrestin proteins, which function to uncouple GPCRs from heterotrimer G proteins (8–10). Therefore, we examined whether GRK2 plays a role in mGluR5 phosphorylation. DHPG stimulation of neurons led to a small increase in mGluR5 phosphorylation when compared with control untreated neurons at both 5 and 10 min of agonist stimulation (Fig. 4, *A* and *B*). This increase in DHPG-stimulated mGluR5 phosphorylation was enhanced in GRK2-overexpressing neurons as compared with GFP-infected neurons. However, the apparent increase in

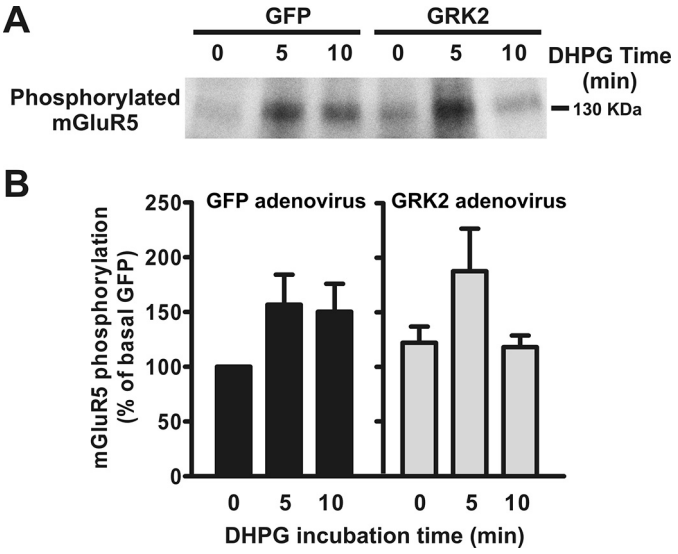

FIGURE 4. **DHPG-induced mGluR5 phosphorylation in striatal neurons.** *A*, shown is a representative autoradiograph demonstrating mGluR5 phosphorylation in striatal neurons infected with either GFP or GRK2 adenovirus and stimulated with 10 μM DHPG for 0, 5, or 10 min. *B*, graph shows the densitometric analysis of mGluR5 phosphorylation autoradiograms. Data represent the means ± S.E. of seven independent experiments, normalized to mGluR5 phosphorylation in GFP-infected neurons not treated with agonist.

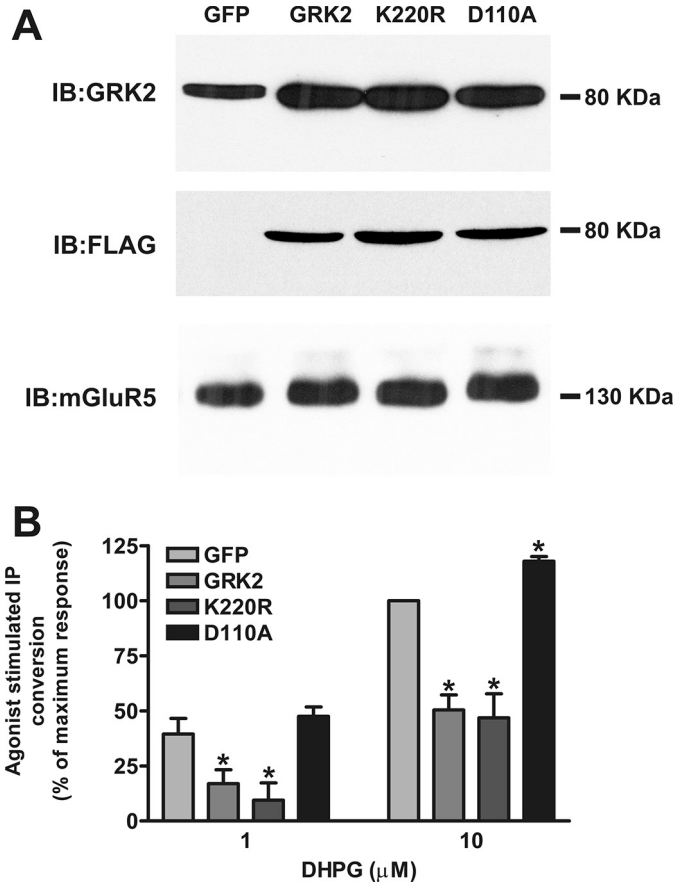

FIGURE 5. **GRK2-mediated mGluR5 desensitization in striatal neurons.** *A*, shown are representative immunoblots for mGluR5 and GRK2 protein expression in neurons infected with GFP, GRK2, GRK2-K220R, or GRK2-D110A adenovirus. 50 μg of cell lysate were used for each sample, and the samples were immunoblotted for the expression of GRK2, FLAG, and mGluR5 protein using anti-GRK2, anti-FLAG, and anti-mGluR5 antibodies. *B*, shown is mGluR5-stimulated inositol phosphate formation in response to either 1 or 10 μM DHPG for 5 min at 37 °C in striatal neurons infected with GFP, GRK2, GRK2-K220R, or GRK2-D110A adenovirus. Data represent the means ± S.E. of five independent experiments, normalized to DHPG-stimulated GFP-infected neurons maximum response. *Asterisks* indicate significant differences compared with GFP adenovirus-infected neurons at the corresponding agonist concentration ($p < 0.05$).

mGluR5 phosphorylation was again not statistically significant different between control and GRK2-infected cultures at any time point tested (Fig. 4*B*).

Phosphorylation-independent GRK2-mediated Attenuation of mGluR5 Signaling—Because we did not observe a significant increase in GRK2-mediated mGluR5 phosphorylation, we sought to investigate whether phosphorylation was required for GRK2-dependent attenuation of mGluR5 signaling in striatal neurons. To address this, we developed adenoviral constructs to express two GRK2 mutants in striatal neurons: a catalytically inactive GRK2 mutant (K220R) (27) and a GRK2 mutant impaired in $G\alpha_{q/11}$ binding (D110A) (28, 29). Adenoviral titers were chosen that resulted in a 2-fold above basal overexpression of wild-type GRK2, GRK2-K220R, and GRK2-D110A protein (Fig. 5*A*). Expression of mGluR5 in the striatal neuronal cultures was not altered by infection with any of the adenoviral constructs (Fig. 5*A*). InsP formation assays were performed to determine whether either GRK2 mutant retained the capacity to attenuate mGluR5 signaling. Overexpression of either wild-type GRK2 or GRK2-K220R resulted in a significant reduction of DHPG-stimulated InsP formation at both 1 μM and 10 μM concentrations tested (Fig. 5*B*). In contrast, mGluR5-stimulated InsP formation in response to 1 μM DHPG treatment was not attenuated in striatal neurons overexpressing GRK2-D110A (Fig. 5*B*). However, DHPG-mediated InsP formation in neurons infected with GRK2-D110A adenovirus was significantly higher than that of GFP adenovirus infected neurons following treatment of cells with 10 μM DHPG (Fig. 5*B*). Thus, unlike what was previously reported for GRK2-dependent regulation of mGluR5 signaling in HEK 293 cells (15), GRK2-dependent attenuation of mGluR5 signaling was mediated by a phosphorylation-independent mechanism in striatal neurons.

GRK2 Regulation of mGluR5 Trafficking—Internalization of a number of GPCRs can be altered by the expression of GRK2 (30, 31). Therefore, we examined mGluR5 internalization in striatal neurons overexpressing GRK2 and cortical neurons. To measure mGluR5 internalization, a modified cell surface biotinylation assay was utilized (24). Neuronal cultures were first stimulated with DHPG at varying times (0, 5, and 10 min) and then biotinylated on ice. Cell surface loss of biotinylated mGluR5 protein indicated receptor internalization. We found that DHPG treatment (10 μM) of striatal neurons expressing endogenous levels of GRK2 did not result in mGluR5 internalization at either the 5- or 10-min time points of agonist stimulation tested (Fig. 6, *A* and *B*). However, mGluR5 internalization was observed both in striatal neurons that were infected with adenovirus to overexpress GRK2 and in cortical neurons expressing endogenous levels of GRK2 protein (Fig. 6, *A* and *B*). Thus, the overexpression of GRK2 in striatal neurons at levels equivalent to endogenous GRK2 expression levels found in cortical neurons (Fig. 6*C*) established a striatal neuron mGluR5 internalization phenotype that resembled mGluR5 internalization in cortical neurons. These data suggest that different levels

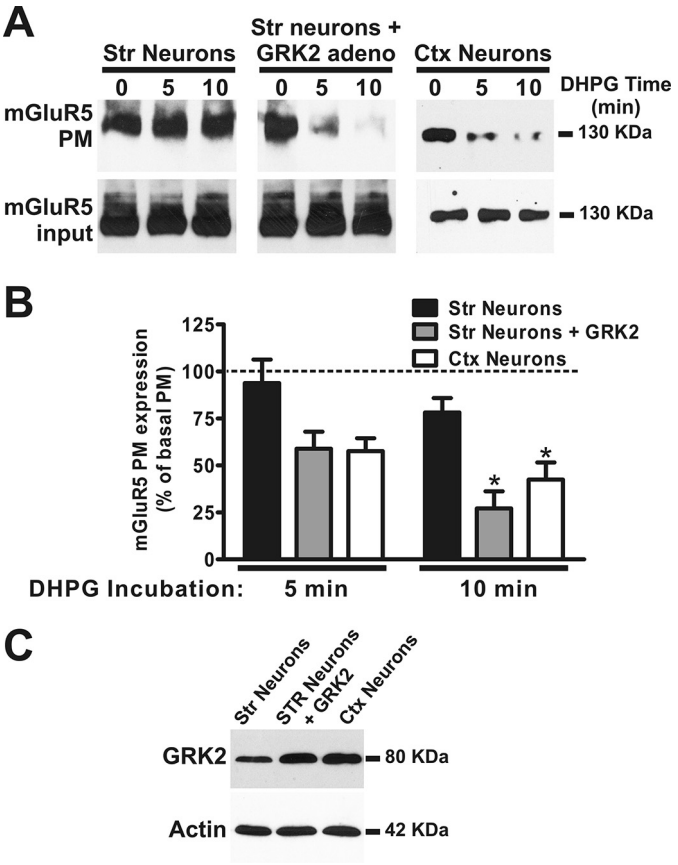

FIGURE 6. **GRK2-mediated mGluR5 internalization in striatal neurons.** *A*, shown is a representative immunoblot for cell surface biotin-labeled mGluR5 (*upper panel*) in cortical (*Ctx*) and striatal (*Str*) neurons infected with either GFP or GRK2 adenovirus and stimulated with 10 μM DHPG for 0, 5, or 10 min. Total cell lysates (50 μg) are used to determine mGluR5 total cell expression (*input*) for each sample (*lower panel*). *B*, graph shows the densitometric analysis of biotin-labeled cell surface mGluR5 immunoblot. Data represent the mean ± S.E. of four independent experiments, normalized to cell surface mGluR5 in neurons not treated with agonist. *Asterisks* indicate significant difference as compared with mGluR5 cell surface expression in striatal neurons expressing endogenous levels of GRK2 ($p < 0.05$). *C*, shown are representative immunoblots for GRK2 and actin protein expression in cortical and striatal neurons infected with either GFP or GRK2 adenovirus. 50 μg of cell lysate were used for each sample.

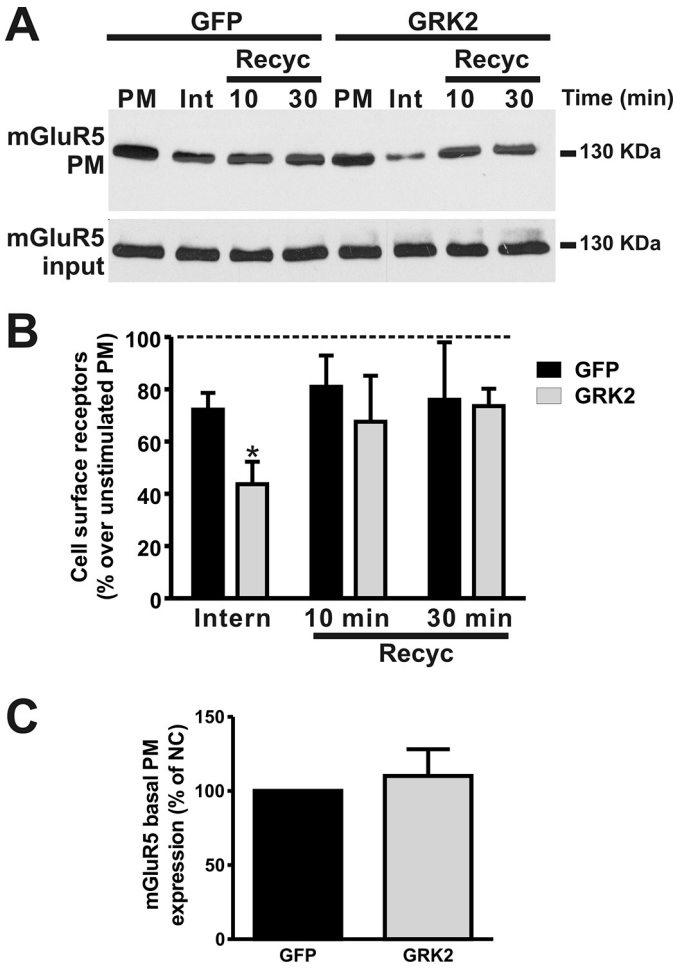

FIGURE 7. **GRK2 does not affect recycling or basal plasma membrane expression of mGluR5.** *A*, shown is a representative immunoblot for cell surface biotin-labeled mGluR5 (*upper panel*) in striatal neurons infected with either GFP or GRK2 adenovirus and stimulated with 10 μM DHPG for 10 min and allowed to recover for 10 or 30 min. Total cell lysates (50 μg) were used to determine mGluR5 total cell expression (*input*) for each sample (*lower panel*). *B*, graph shows the densitometric analysis of biotin-labeled cell surface mGluR5 immunoblot. Data represent the mean ± S.E. of four independent experiments, normalized to cell surface mGluR5 in neurons not treated with agonist. *Asterisk* indicates significant difference as compared with mGluR5 cell surface expression in striatal neurons expressing endogenous levels of GRK2 ($p < 0.05$). *C*, graph shows the densitometric analysis of biotin-labeled cell surface mGluR5 immunoblot from unstimulated neurons. Data represent the mean ± S.E. of five independent experiments, normalized to total mGluR5 expression for each sample and expressed as percentage of cell surface mGluR5 in neurons infected with GFP adenovirus.

of endogenous expression of GRK2 protein in distinct tissues may differentially affect mGluR5 internalization accordingly.

To determine whether GRK2 could affect mGluR5 recycling, we performed a modified cell surface biotinylation assay. Neuronal cultures were first incubated with DHPG for 10 min to stimulate mGluR5 internalization. Agonist was washed out and cells were incubated at 37 °C to allow internalized mGluR5 to recycle to the plasma membrane, and cells were then biotinylated on ice. mGluR5 internalization was increased in GRK2-infected neurons, as compared with GFP-infected neurons (Fig. 7, *A* and *B*). However, when agonist was washed out and neurons were allowed to recover for 10 and 30 min, the same levels of mGluR5 plasma membrane expression were observed in neurons infected with either GFP or GRK2 adenovirus, indicating that mGluR5 recycling is not affected by GRK2 overexpression (Fig. 7, *A* and *B*). Furthermore, basal levels of mGluR5 plasma membrane expression were the same in GFP- and GRK2-infected neurons, indicating that GRK2 overexpression does not alter the potential constitutive internalization and recycling of mGluR5 (Fig. 7*C*).

Previous studies demonstrated that GRK2-mediated receptor phosphorylation contributed to GPCR endocytosis (30, 31, 32, 33). Furthermore, GRK2-mediated mGluR1 internalization appears to be phosphorylation-dependent, because GRK2-K220R overexpression was reported to decrease mGluR1 internalization in HEK 293 cells (28, 29). Therefore, we examined whether GRK2-promoted endocytosis of mGluR5 in striatal neurons requires GRK2 catalytic activity. Following treatment with DHPG for either 5 or 10 min, mGluR5 internalization in striatal neurons was increased following the overexpression of either wild-type GRK2, GRK2-K220R, or GRK2-D110A (Fig. 8, *A* and *B*). These data indicated that, unlike what was previously

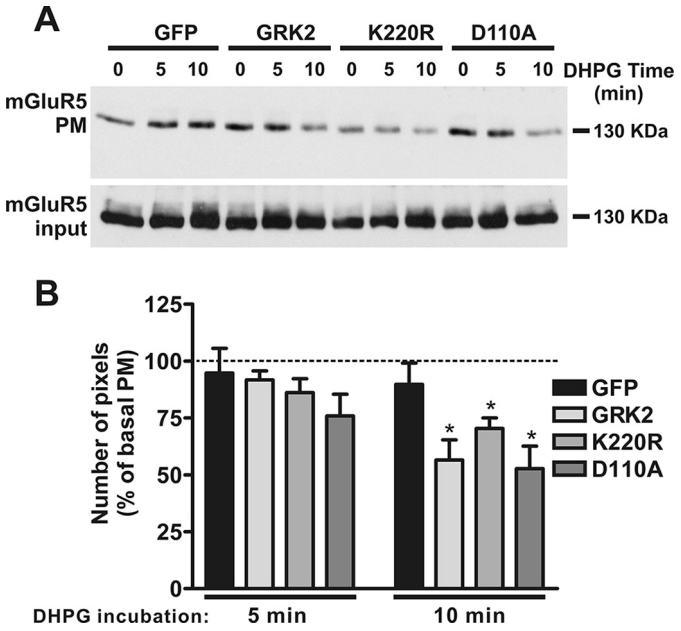

FIGURE 8. **GRK2 mutants promote mGluR5 internalization.** *A*, shown is a representative immunoblot for cell surface biotin-labeled mGluR5 (*upper panel*) in striatal neurons infected with GFP, GRK2, GRK2-K220R, or GRK2-D110A adenovirus and stimulated with 10 μM DHPG for 0, 5, or 10 min. Total cell lysates (50 μg) were used to determine mGluR5 total cell expression (*input*) for each sample (*lower panel*). *B*, graph shows the densitometric analysis of biotin-labeled cell surface mGluR5 immunoblot. Data represent the mean ± S.E. of five independent experiments, normalized to cell surface mGluR5 in neurons not treated with agonist. *Asterisks* indicate significant differences as compared with untreated matched controls ($p < 0.05$).

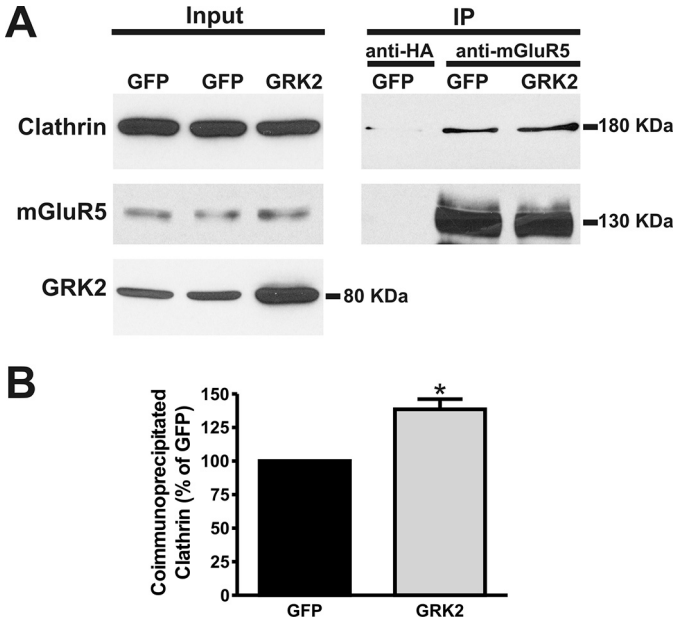

FIGURE 9. **GRK2 overexpression leads to an increase in clathrin recruitment to mGluR5.** *A*, shown is a representative immunoblot for the co-immunoprecipitation of clathrin with mGluR5 in striatal neurons infected with either GFP or GRK2 adenovirus and stimulated with 10 μM DHPG for 5 min (*right panel*). mGluR5 immunoprecipitation (*IP*) was performed by using either anti-mGluR5 or anti-HA (negative control) antibodies from an equivalent amount of protein (500–1000 μg of protein). Total cell lysates (30 μg) were used to determine clathrin, mGluR5, and GRK2 total cell expression (*input*) for each sample (*left panel*). *B*, graph shows the densitometric analysis of immunoblots for clathrin that was co-immunoprecipitated with mGluR5. Data represent the mean ± S.E. of three independent experiments, normalized to clathrin co-immunoprecipitated in GFP-infected neurons. The *asterisk* indicates a significant difference as compared with GFP-infected neurons ($p < 0.05$).

reported for mGluR1 internalization in HEK 293 cells (11, 14, 15, 16), GRK2-mediated mGluR5 endocytosis does not require GRK2 catalytic activity in striatal neurons.

GRK2 can function as a clathrin adaptor, facilitating the internalization of a number of receptors in a mechanism that is β-arrestin-independent (34, 35, 36, 37). To investigate the GRK2-mediated facilitation of mGluR5 endocytosis, we tested whether GRK2 could modulate clathrin-mGluR5 interaction. GRK2 overexpression led to an increase in the recruitment of clathrin to mGluR5 (Fig. 9). The recruitment of clathrin by GRK2 might represent the mechanism underlying the phosphorylation-independent internalization of mGluR5.

DISCUSSION

GPCR desensitization represents an important regulatory mechanism by which acute and/or chronic receptor overstimulation is avoided. For many GPCRs, receptor desensitization correlates with GRK-mediated receptor phosphorylation followed by the binding of β-arrestin proteins (8–10). However, this simple correlation does not necessarily hold for all GPCRs. In the present study, we provide evidence that GRK2 contributes to the phosphorylation-independent desensitization of endogenously expressed mGluR5 in primary mouse striatal neurons. Overexpression of GRK2 by ~2-fold to match GRK2 expression levels found in other brain regions that express mGluR5 (*e.g.* cortical neurons) leads to pronounced attenuation of mGluR5-stimulated InsP formation in response to DHPG stimulation. Furthermore, mGluR5-stimulated InsP formation is increased following the siRNA knockdown of GRK2 protein demonstrating a role for endogenous GRK2 in the regulation of mGluR5 activity. In addition, agonist treatment induces little mGluR5 internalization in striatal neurons when compared with cortical neurons. In contrast, agonist-stimulated mGluR5 internalization is significantly increased in striatal neurons expressing levels of GRK2 that are comparable to cortical neurons. Furthermore, expression of a catalytically inactive GRK2 promotes mGluR5 desensitization and internalization, indicating that GRK2-dependent mGluR5 desensitization and internalization are mediated by a phosphorylation-independent mechanism.

We find that GRK2-dependent mGluR5 desensitization is independent of GRK2 catalytic activity. Rather, intact interactions between the GRK2 RH domain and $G\alpha_{q/11}$ are essential for the attenuation of mGluR5 signaling. Thus, the mechanism underlying GRK2-mediated mGluR5 desensitization in striatal neurons is similar to what we previously reported for mGluR1 expressed in HEK 293 cells (12, 20, 21, 28). The present findings are distinct from a previous report that GRK2-dependent mGluR5 desensitization in HEK 293 cells requires GRK2 catalytic activity (15). In the previous study, overexpression of GRK2-K220R led to enhanced mGluR5-mediated activation of a G protein-coupled inward rectifier potassium channel and also resulted in altered mGluR5 expression. In contrast, we find that GRK2-K220R expression not only effectively attenuates mGluR5 signaling but it does not affect the level of endogenous mGluR5 protein expression in striatal neurons. Unexpectedly, a

2-fold overexpression of GRK2-D110A resulted in a statistically significant increase in InsP formation in striatal neurons. Thus, it is likely that the GRK2-D110A mutant functions to compete with endogenous GRK2 to bind mGluR5, but does not functionally uncouple the receptor from $G\alpha_{q/11}$. Thus, it appears that the interaction of the GRK2 RH domain with $G\alpha_{q/11}$ represents the primary mechanism by which GRK2 functions to attenuate mGluR5 signaling. The observed difference in GRK2-mediated regulation of mGluR5 activity in HEK 293 cells and primary striatal neurons highlights the importance of studying the regulation of GPCR in their natural environment.

We found that in cultured striatal neurons, agonist treatment did not promote internalization of endogenously expressed mGluR5. However, mGluR5 endocytosis was significantly higher in cortical neurons, which express twice as much GRK2 protein than striatal neurons (38). Overexpression of GRK2 in striatal neurons to match GRK2 expression levels found in cortical neurons increased mGluR5 internalization to the same level as that of cortical neurons. These data demonstrate that GRK2 protein expression level in different tissues might result in altered rates of mGluR5 endocytosis. The observed differences in GRK2 protein expression and mGluR5 endocytosis may be important physiologically in determining the sensitivity of neurons to excitotoxic cell death. For example, heterozygous GRK2 knock-out mice exhibit increased neuronal cell loss following unilateral carotid artery occlusion and hypoxia (39). Moreover, hippocampal slices and cerebellar granular neurons derived from heterozygous GRK2 knock-out mice are more sensitive to glutamate-induced death. GRK2 protein is expressed at levels in the hippocampus and cerebellum that are comparable to the cortex and are twice the expression levels found in the striatum (38). Consequently, the relatively reduced GRK2 protein expression found in the striatum may make striatal neurons more susceptible to neuronal loss following either injury or in diseases associated with excitotoxic cell death such as Huntington disease. Interestingly, mutant huntingtin has also previously been demonstrated to regulate Group I mGluR signaling via its association with optineurin (38).

Internalization of muscarinic (M2-M5 receptors) (40), β2 adrenergic (41), and dopamine D2 receptors (30) is also enhanced by expression of specific GRKs. Furthermore, co-expression of arrestins and GRKs seems to synergistically improve internalization of some GPCRs (23, 31, 42). These data are consistent with findings that arrestins seem to preferentially bind to phosphorylated receptors and facilitate clathrin-mediated endocytosis (8–10). Although mGluR1 and mGluR5 share the same mechanism of phosphorylation-independent GRK2 desensitization, the mechanism by which GRK2 regulates the internalization of these two receptors appears to be different. We demonstrate here that GRK2 plays a crucial role in promoting mGluR5 endocytosis. We have reported that the co-expression of GRK2 with β-arrestin 1 leads to increased agonist-stimulated mGluR1 internalization, suggesting that GRK2-mediated mGluR1 phosphorylation may be important for its internalization (16). In contrast to what is reported for mGluR1, we demonstrate here that a 2-fold increase in either GRK2 or GRK2-K220R protein expression is sufficient to support mGluR5 internalization in striatal neurons. These data suggest that receptor phosphorylation is not required for mGluR5 endocytosis. This is different from what is observed for mGluR1, where GRK2-K220R overexpression is reported to decrease mGluR1 internalization in HEK 293 cells and GRK2 knockdown in cerebellar Purkinje neurons did not alter the extent of mGluR1 internalization (11, 14, 17). Furthermore, we show here that GRK2 overexpression leads to a modest increase in clathrin recruitment to mGluR5. Although the mechanism by which GRK2 targets mGluR5 for endocytosis in striatal neurons remains to be determined, these data suggest that clathrin is involved, even though phosphorylation of the receptor does not play a role. Previously, it has been shown that GRK2 can associate directly with clathrin via a clathrin binding box that is found in many endocytic adaptor proteins including β-arrestins (34). The mutation of the GRK2 clathrin binding motif prevents GRK2-mediated phosphorylation and internalization of the β_2-adrenergic receptor (35). GRK2 has also been reported to facilitate the β-arrestin-independent internalization of the β_1-adrenergic, adrenocorticotropin, and leukotriene B4 receptors (34, 36, 37). Thus, it is possible that GRK2 replaces β-arrestin as an endocytic adaptor protein for mGluR5 endocytosis.

In summary, we have shown here that, similar to what is observed for mGluR1, GRK2-mediated attenuation of mGluR5 signaling in striatal neurons is independent of receptor phosphorylation. It is rather mediated by GRK2 RH domain interactions with $G\alpha_{q/11}$. Moreover we show that GRK2-mediated endocytosis of endogenous mGluR5 in striatal neurons is also independent of GRK2 catalytic activity and $G\alpha_{q/11}$ binding.

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