Macromolecular Composition Dictates Receptor and G Protein Selectivity of Regulator of G Protein Signaling (RGS) 7 and 9-2 Protein Complexes in Living Cells*

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Background: RGS7 and RGS9-2 regulate G protein signaling in the striatum, but the selectivity of their action is largely unknown.

Results: RGS protein complexes show distinct patterns of receptor and G protein selectivity.

Conclusion: Macromolecular composition dictates receptor and G protein selectivity of the RGS7 and RGS9-2 protein complexes.

Significance: These data demonstrate novel mechanisms contributing to the regulation of striatal G protein signaling.

Regulator of G protein signaling (RGS) proteins play essential roles in the regulation of signaling via G protein-coupled receptors (GPCRs). With hundreds of GPCRs and dozens of G proteins, it is important to understand how RGS regulates selective GPCR-G protein signaling. In neurons of the striatum, two RGS proteins, RGS7 and RGS9-2, regulate signaling by μ-opioid receptor (MOR) and dopamine D2 receptor (D2R) and are implicated in drug addiction, movement disorders, and nociception. Both proteins form trimeric complexes with the atypical G protein β subunit Gβ5 and a membrane anchor, R7BP. In this study, we examined GTPase-accelerating protein (GAP) activity as well as Go and GPCR selectivity of RGS7 and RGS9-2 complexes in live cells using a bioluminescence resonance energy transfer-based assay that monitors dissociation of G protein subunits. We showed that RGS9-2/Gβ5 regulated both Gi and Go with a bias toward Go, but RGS7/Gβ5 could serve as a GAP only for Go. Interestingly, R7BP enhanced GAP activity of RGS7 and RGS9-2 toward Go and Gi and enabled RGS7 to regulate Gi signaling. Neither RGS7 nor RGS9-2 had any activity toward Gz, Gs, or Gq in the absence or presence of R7BP. We also observed no effect of GPCRs (MOR and D2R) on the G protein bias of R7 GRS proteins. However, the GAP activity of RGS9-2 showed a strong receptor preference for D2R over MOR. Finally, RGS7 displayed an 4 times greater GAP activity relative to RGS9-2. These findings illustrate the principles involved in establishing G protein and GPCR selectivity of striatal RGS proteins.

Signal transduction through G protein-coupled receptors (GPCRs)2 regulates fundamental processes in the nervous system, including neuronal excitability and neurotransmitter release (1). GPCRs activate heterotrimeric G proteins, which in turn engage a wide range of the intracellular effectors to produce a cellular response. Activation of G proteins entails their binding to GTP and resulting dissociation into Gα-GTP and Gβγ subunits. The extent and duration of signaling in GPCR pathways is critically controlled by the regulator of G protein signaling (RGS) proteins that limit G protein activity (2, 3). RGS proteins bind directly to activated Gα and facilitate the GTP hydrolysis, thus serving as GTPase-accelerating proteins (GAPs).

In humans, 17 Gα, about 865 GPCR, and ~30 RGS genes have been identified (4–6). With most of the components expressed in the nervous system, this forms a formidable array of possible combinations. However, activation of individual GPCR pathways often produces unique cellular and behavioral responses. Understanding the mechanisms of this signaling selectivity is one of the biggest challenges in studying neuronal GPCR pathways.

Neurons of the striatum, a nucleus that plays a major role in reward behavior and motor control, express a number of GPCRs that respond to many neurotransmitters, including dopamine, opioids, serotonin, and acetylcholine (7, 8). Several studies have demonstrated that the long splice isoform of RGS9 (RGS9-2) serves as a critical GAP in these neurons (9, 10). In particular, RGS9-2 has been shown to regulate signaling downstream from D2 dopamine (D2R) and μ-opioid (MOR) receptors and has been implicated in drug addiction and movement disorders (11–15). However, no studies directly examined the impact of RGS9-2 on G protein dynamics activated by D2R and MOR.

Recent behavioral studies implicated another RGS protein in the striatum, RGS7, in controlling the effects of addictive drugs and suggested that it may be differentially involved in controlling MOR and D2R signaling (16). Both RGS9-2 and RGS7 share extensive homology in their macromolecular organization. In addition to the catalytic RGS domain, they possess the N-terminal Dishevelled, EGL-10, Pleckstrin/Dishevelled, EGL-10, Pleckstrin helical extension module and a G protein γ sub-
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unit-like domain (10). The G protein γ subunit-like domain forms a constitutive complex with the atypical G protein β subunit, Gβ5 (17, 18), and both RGS7 and RGS9-2 exist as oblig-atory dimers with Gβ (19). In the striatum, RGS/Gβ5 dimers associate with membrane anchor R7BP (20) that recruits them to the plasma membrane and potentiates the GAP activity (21, 22). What remains completely unexplored is the relative activity and selectivity of the RGS7 and RGS9-2 complexes, as well as the role of R7BP in this process.

In this study, we examined the ability of the RGS7/Gβ5 and RGS9-2/Gβ5 complexes to regulate G protein signaling by MOR and D2R under the native environment of living cells using a bioluminescence resonance energy transfer (BRET)-based assay. We report a marked differences in the catalytic activity of complexes as well as their G protein and GPCR selectivity, depending on their macromolecular composition. Our findings illustrate mechanisms for establishing the selective regulation of striatal GPCR signaling pathways.

EXPERIMENTAL PROCEDURES

DNA Constructs, Antibodies, and Recombinant Proteins—Construction of RGS9-2, Gβ5S, and R7BP in pcDNA3.1 was described previously (20). RGS7, Gβ1, Gγ2, and Gαz in pcDNA3.1+ were purchased from the Missouri S&T cDNA Resource Center. Generation of sheep anti-RGS9-2 (20) and rabbit anti-RGS7 (23) were described previously. Rabbit anti-4-1, Gβ5 and rabbit anti-R7BP were gifts from Dr. William Simonds (NIDDK, National Institutes of Health, Bethesda, MD). Mouse anti-β-actin (AC-15) (Sigma-Aldrich), rabbit anti-Gro (K-20) (Santa Cruz Biotechnology), rabbit anti-Gα1/2 (Affinity BioReagents), rabbit anti-D2R (H-50) (Santa Cruz Biotechnology), mouse anti-GFP (clones 7.1 and 13.1) (Roche Applied Science) and rabbit anti-Renilla luciferase (GeneTex) were purchased. Recombinant His-tagged RGS7 and RGS9-2 were coexpressed with Gβ5S in S9 insect cells, and the complexes were purified as described previously (24).

Cell Culture and Transfection—HEK293T/17 cells were grown in DMEM supplemented with 10% FBS, minimum Eagle’s medium non-essential amino acids, 1 mM sodium pyruvate, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified incubator containing 5% CO2. For transfection, cells were seeded into 6-cm dishes at a density of 4 × 106 cells/dish. After 4 h, expression constructs (total 5 μg/dish) were transfected into the cells using PLUS (5 μl/dish) and Lipofectamine LTX (8 μl/dish) reagents. The GPCR (μ-opioid receptor or dopamine D2 receptor), Go (Gαo, Gai1, Gαα, Gqα, or Grαs), Venus 1–155–Gβ, masGRK3ct-Rlu8, Gβ5S, and R7BP constructs were transfected at a 1:2:1:1:1:1:1 ratio with different amounts of R7 RGS (RGS5 or RGS9-2). An empty vector was used to normalize the amount of transfected DNA.

Fast Kinetic BRET Assay—Agonist-dependent cellular measurements of BRET between masGRK3ct-Rlu8 and Gβ1γ2-Venus were performed to visualize the action of G protein signaling in living cells, as described previously, with slight modifications (25). 16 to 24 h post-transfection, HEK293T/17 cells were washed once with PBS containing 5 mM EDTA (EDTA/PBS) and detached by incubation in EDTA/PBS at room temperature for 10 min. Cells were harvested with centrifugation at 500 g for 5 min and resuspended in PBS containing 0.5 mM MgCl2 and 0.1% glucose (BRET buffer). Approximately 50,000–100,000 cells/well were distributed in 96-well flat-bottomed white microplates (Greiner Bio-One). The Rluc substrate, coelenterazine-h (Nanolight Technologies), was dissolved in acified alcohol at a final concentration of 5 mM and stored at −20 °C. Acidified alcohol was prepared by adding 200 μl of 3N HCl to 10 ml of ethanol. Aliquots were dissolved in BRET buffer immediately before use and added to cell suspension at a final concentration of 5 mM. BRET measurements were made using a microplate reader (POLARStar Omega, BMG Labtech) equipped with two emission photomultiplier tubes, allowing us to detect two emissions simultaneously with the highest possible resolution of 50 milliseconds for every data point. All measurements were performed at room temperature. The BRET signal is determined by calculating the ration of the light emitted by Gβ1γ2-Venus (535 nm) over the light emitted by masGRK3ct-Rlu8 (475 nm). The average base-line value (basal R) recorded prior to agonist stimulation was subtracted from BRET signal values, and the resulting difference (ΔR) was normalized against the maximal ΔR value (Rmax) recorded upon agonist stimulation. The rate constants (1/τ) of the activation and deactivation phases were obtained by fitting a single exponential curve to the traces. kGAP rate constants were determined by subtracting the basal deactivation rate (kapp) from the deactivation rate measured in the presence of exogenous RGS protein. Obtained kGAP rate constants were used to quantify GAP activity.

Western Blotting—For each sample, ~5,000,000 cells were lysed in 500 μl of sample buffer (125 mM Tris (pH 6.8), 4 mM urea, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.16 mg/ml bromphenol blue). Western blot analysis of proteins was performed following SDS-PAGE. Blots were blocked with 5% skim milk in PBS containing 0.1% Tween 20 (PBST) for 30 min at room temperature, followed by a 90-min incubation with specific antibodies diluted in PBST containing 1% skim milk. Blots were washed in PBST and incubated for 45 min with a 1:10,000 dilution of secondary antibodies conjugated with horseradish peroxidase in PBST containing 1% skim milk. Proteins were visualized on x-ray film by SuperSignal West Femto substrate (Pierce). Band densities were quantified using ImageJ software by measuring the integrated intensity. The relative expression level of RGS proteins was determined by subtracting the background densities in the absence of exogenous RGS proteins and normalizing the resulting value as a fraction of the brightest band intensity expressing the maximal amount of RGS protein.

RGS-Ga Pull-down Assay—Mouse brain membranes (1 mg protein) in 0.5 ml of binding buffer (20 mM HEPES (pH 8.0), 380 mM NaCl, 5 mM MgCl2, 2 mM 1-mercaptoethanol, protease inhibitors) containing GDP (10 μM) and AlF4− (20 μM AlCl3 plus 10 mM NaF) were incubated for 1 h at room temperature with His-tagged RGS proteins (10 μg). Membranes were solubilized with 1% Nonidet P-40 for 1 h of incubation on ice and centrifuged at 20,000 × g for 1 h at 4 °C. The detergent-soluble extracts were incubated with nickel-nitrilotriacetic acid beads for 30 min at 4 °C, washed five times with wash buffer (20 mM HEPES (pH 8.0), 380 mM NaCl, 5 mM MgCl2, 0.1% (w/v) C12E10, 20 mM HEPES (pH 8.0), 380 mM NaCl, 5 mM MgCl2, 0.1% (w/v) C12E10,
agonist promotes the interaction of the Venus-tagged G with increasing amounts of D2R construct and stimulated cells encoded by changes in GPCR activity. First, we transfected cells with treatment of the RGS proteins with Go versus Gi (Fig. 5) and their activities in the absence versus presence of R7RGS at a 1:1:1:1 ratio between cDNA constructs using Lipofectamine LTX reagent in 96-well plate. 16 h after transfection, cells were treated with 50 mM isoproterenol together with serial doses of morphine for 5 h. The level of expressed luciferase were determined using a Bright-Glo luciferase assay kit (Promega) according to the instructions of the manufacturer.

Statistical Analysis—Linear regression was used to relate the $k_{\text{GAP}}$ value to the expression level of RGS7 or RGS9-2 proteins. To compare the activities of the RGS proteins on Go versus Gi (Fig. 5) and their activities in the absence versus presence of R7RGS at a 1:1:1:1 ratio between cDNA constructs using Lipofectamine LTX reagent in 96-well plate. 16 h after transfection, cells were treated with 50 mM isoproterenol together with serial doses of morphine for 5 h. The level of expressed luciferase were determined using a Bright-Glo luciferase assay kit (Promega) according to the instructions of the manufacturer.

**RESULTS**

Live Cell Receptor-based Assays Allow Examination of the GAP Activity of R7 RGS Proteins in a Physiological Context—To visualize RGS action in living cells, we reconstituted HEK293T cells with GPCRs, Go subunits, and BRET sensors (Gβγ-Venus and masGRK3ct-Rluc8, recently developed by Hollins et al. (25) (Fig. 1A)). In this assay system, activation of a GPCR by an agonist promotes the interaction of the Venus-tagged Gβγ subunits with the Rluc8-tagged masGRK3ct reporter producing the BRET signal. Conversely, application of an antagonist quenches GPCR-driven G protein activation and results in BRET signal decay. Indeed, activation of the heterologously expressed D2R by dopamine resulted in generation of the BRET signal. Conversely, application of an antagonist produced a typical sigmoidal dose-response curve of the response amplitude ($E_{50} = 1.91 \times 10^{-7} \pm 1.67 \times 10^{-7}$ m), indicating that an increase in the agonist concentration results in an increase in the pool of the activated G proteins. As expected, enhanced GPCR activation also resulted in the increase in the rate of G protein activation (Fig. 1H). However no changes in the deactivation phase of the response were noted (Fig. 1I). These data indicate that the amount of active G protein also does not change the deactivation rates. Finally, we tested the effect of increasing the RGS concentration on the deactivation kinetics of D2R-Go signaling (Fig. 1J–L). In contrast to the manipulations with GPCR and G protein concentration, an increase in the RGS7 concentration substantially accelerated the deactivation rates of the response (Fig. 1J and K). In fact, within the concentration range tested, the calculated GAP activity of RGS7 showed a clear linear relationship with its expression level (Fig. 1L). The final set of control experiments ensured that the expression of components of the RGS complex did not affect the levels of the receptor, Go subunits, or BRET sensors in cells (Fig. 2). We thus conclude that, under the assay conditions, GTP hydrolysis by G proteins is the rate-limiting step that dictates the deactivation phase of the response. The function of the R7 RGS proteins can thus be quantitatively analyzed by measuring the deactivation kinetics that show no sensitivity to fluctuations in GPCR and/or G protein concentration.

RGS7 and RGS9-2 Complexes Selectively Regulate the Ga/o Subfamily in the absence or presence of R7BP—Members of the R7 RGS family have been shown previously to be selective GAPs for the Ga/o proteins in the in vitro biochemical assays (26). However, their selectivity was never examined in the physiological context of living cells. Furthermore, on the basis of the genetic evidence from Caenorhabditis elegans, it was proposed recently that R7BP might unlock the GAP activity of the R7 RGS proteins toward other Go subunits, e.g. Gaq (27). We therefore used the in-cell BRET assay to re-examine the regulation of the Ga GTPase by R7 RGS complexes in living cells. In these experiments, we chose a panel of representative GPCRs well known to activate each of the Go subunits examined, e.g. D1R for Gas, M1R for Gaq, D2R for Gaq and MOR for Gao. Consistent with the in vitro studies, we found that, in cells, both RGS7/Gβ5 and RGS9-2/Gβ5 potently terminated a D2R-driven response via Gaq, a representative member of the Ga/o family (Fig. 3, A, D, E, and H). However, we observed no regulation of the M1R-Gaq or D1R-Gas response termination by either RGS7/Gβ5 (Fig. 3, B, C, and D) or RGS9-2/Gβ5 (F, G, and H). These data indicate that the RGS7/Gβ5 and RGS9-2/Gβ5 dimers are Ga/o subfamily-selective regulators in living cells.
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A

B

C

D

E

F

G

H

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K

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Next, we examined the possibility that R7BP may enable Gaq or Gas regulation by RGS7 or RGS9-2 complexes. Again, we found that trimeric RGS7/Gβ5/R7BP and RGS9-2/Gβ5/R7BP complexes potently regulated Gaq deactivation but had no effect on the deactivation rates of Gaq and Gas (Fig. 3, I–P). Because expression levels of RGS7 or RGS9-2 were found to be similar across cells reconstituted with various G protein subunits (data not shown), the lack of GAP activity toward Gaq and Gas cannot be attributed to a lower expression of RGS7 and RGS9-2. Thus, we conclude that complex formation with R7BP does not influence subfamily selectivity and that the RGS7/Gβ5/R7BP and RGS9-2/Gβ5/R7BP trimers are also Gi/o subfamily-selective GAPs.

Varying G Protein Selectivity of the RGS7 and RGS9-2 Complexes within the Gaq/o Family—In addition to Gaq, the Gaq/o family contains the highly homologous proteins Ga1-3 and the atypical subunit Gaz, which is characterized by an extremely slow intrinsic GTP hydrolysis rate (28). Because no previous studies examined the regulation of Gaz GTPase by R7 RGS proteins, we addressed this question using cell-based BRET assays. Morphine application induced a robust BRET signal from the cells reconstituted with MOR and Gaz. Consistent with biochemical measurements, Gaz responses showed very slow deactivation kinetics upon termination of signaling at MOR, with a rate constant of $3.7 \pm 0.1 \times 10^{-3}$ s$^{-1}$. These slow deactivation kinetics were completely unaffected by the addition of either the RGS9-2/Gβ5 or RGS7/Gβ5 complex both in the presence or absence of R7BP (Fig. 4). These observations suggest that R7 RGS complexes do not regulate Gaz signaling.

We next examined the impact of RGS9-2 and RGS7 on Ga1 deactivation in comparison to Gaq. In these experiments, we titrated the amount of RGS proteins and normalized their expression levels by post hoc quantitative Western blotting. We used the $k_{\text{GAP}}$ versus RGS concentration slope as a measure of RGS catalytic efficiency. As evident from Fig. 5A, RGS9-2/Gβ5 can regulate both Gaq and Ga1 in a system containing D2R. Comparison of the two slopes indicates that RGS9-2/Gβ5 shows an ~3-fold greater preference for Gaq over Ga1. In contrast, although RGS7/Gβ5 also effectively terminated Gaq-mediated responses, it had no detectable activity toward Ga1 in a D2R-based system (Fig. 5B). We confirmed that the expression levels of Gaq and Ga1 are similar (Fig. 5, E and F), indicating that the G protein selectivity of these two RGS proteins is not due to different levels of G protein expression. Because in...
vivo R7 RGS proteins also regulate MOR signaling, we next tested the possible effect of GPCR on Gα selectivity of RGS proteins. As with D2R signaling, RGS9-2/Gα5 exerted a more efficacious GAP activity toward Gαo relative to Gαi1 (3-fold) (Fig. 5C). Again, RGS7/Gβ5 selectively regulated Gαo but not Gαi signaling when MOR was used instead of D2R to drive the responses (Fig. 5D). These data indicate that the RGS9-2/Gβ5 dimer is a GAP for both Gαo and Gαi, with a preference toward Gαo, that the RGS7/Gβ5 dimer is strictly selective for Gαo and does not regulate Gαi1, and that G protein selectivity of these RGS proteins does not depend on the GPCR.

**R7BP Potentiates the GAP Activity of RGS7 and RGS9-2 and Enables the Regulation of Gαi by RGS7 with Both D2R and MOR**—We examined the effects of R7BP on the activity of RGS7 and RGS9-2 by comparing their GAP activity toward...
Gαo and Gαi1 in the D2R receptor system. Coexpression with R7BP potentiated the GAP activity of both RGS7 and RGS9-2 for Gαo. The extent of the regulation was similar and reached ~2-fold (Fig. 6, A and B). This increase in activity was independent from the regulation of the RGS9-2 expression levels by R7BP because calculated $k_G$ values were normalized to the relative expression levels of the proteins. A similar stimulatory effect of R7BP (2.5-fold) was observed for RGS9-2 when Gαi was used in the assay instead of Gαo (Fig. 6, C). Strikingly, coexpression with R7BP dramatically affected the ability of the RGS7/Gβ5 complex to regulate Gαi, essentially resulting in an all-or-nothing effect (Fig. 6, D). Although, in the absence of R7BP, we detected no GAP activity of RGS7/Gβ5 toward Gαi, the RGS7/Gβ5/R7BP trimer was capable of efficiently regulating Gαi inactivation, with the $k_G$ values approaching those for the RGS9-2/Gβ5/R7BP complex.

We next tested the possible effect of GPCRs on the function of R7BP. For this purpose, we switched receptors from D2R to MOR (Fig. 7). With MOR, we observed similar effects of R7BP on GAP activity as with the cells transfected with D2R. R7BP potentiated RGS9-2 GAP activity toward Gαo and Gαi ~2-fold (Fig. 7, A and C). Likewise, R7BP enhanced RGS7 activity toward Gαo 2.5-fold (Fig. 7, B) and played a permissive role in enabling the regulation of Gαi1 GTPase activity (D). Thus, we conclude that GPCRs do not have a significant effect on the ability of R7BP to augment the GAP activity of both RGS7 and RGS9-2.

RGS9-2 Is Less Efficacious but More Selective GAP Relative to RGS7—Next, we addressed questions pertaining to the relative efficiencies of RGS7 versus RGS9-2 in G protein deactivation and the possible GPCR selectivity of their effects (Fig. 8). To enable such comparisons, we obtained absolute quantitative values for the RGS activity. Direct comparison of RGS7/Gβ5 and RGS9-2/Gβ5 indicate that they accelerate deactivation rates in D2R- and MOR-based systems to a different extent (Fig. 8, A and B). The expression levels of RGS7 and RGS9-2 were quantified by Western blot analysis with purified RGS proteins as standards (Fig. 8, C and D). Given the linear relationship between RGS concentration and G protein deactivation rates, these values were used to normalize $k_G$ values to derive the specific activity for each RGS protein in each GPCR system. The results allow direct comparisons of RGS efficiencies between MOR and D2R. As evident from such an analysis, RGS7 produced a 3- and 4.5-fold higher activity than RGS9-2 on D2R and MOR signaling, respectively (Fig. 8, E). A more efficacious GAP activity of RGS7 is likely explained by its higher affinity toward the transition state of the Gαo, as evidenced by the pull-down assay between recombinant R7 RGS7/Gβ5 complexes and native brain lysates (Fig. 8, F). Thus, we conclude that RGS7 is a more potent GAP than RGS9-2, irrespective of the GPCR used in the assay. However, although RGS7 exerted a similar GAP activity in both the D2R and MOR systems, RGS9-2 was about 2-fold more effective in deactivating D2R relative to MOR signaling (Fig. 8, E). Thus, RGS9-2 exhibits GPCR selectivity and preferentially regulates D2R signaling.

RGS7 and RGS9-2 Deferentially Control the Gαi-mediated Inhibition of Adenylate Cyclase Activity—To examine how the selectivity of RGS7 and RGS9-2 on G protein inhibition propagates the regulation of downstream effector signaling, we chose...
an adenylyl cyclase (AC) system for its central role in cellular signaling. AC is stimulated by Gs and inhibited by Gi, thus integrating G protein inputs (Fig. 9A). Using this system, we analyzed the effects of RGS9-2 and RGS7 on the ability of MOR to suppress cAMP production using a CRE-luciferase reporter construct. Stimulation of MOR with morphine caused a dose-dependent inhibition of 2AR-agonist isoproterenol-mediated CRE-luciferase induction (Fig. 9B). Cotransfection of RGS9-2 resulted in the rightward shift of the dose-response curve, increasing the IC50 values ~3-fold (8.38 ± 0.68 nM to 25.82 ± 2.39 nM) (Fig. 9C). This indicates that RGS9-2 reduces the potency of MOR-Gi-AC signaling and is consistent with the action of RGS9-2 as a negative regulator of Ga. In contrast, cotransfection of RGS7 did not significantly

**FIGURE 5.** RGS9-2/Gi and RGS7/Gi discriminate between Go and Ga. Gi (○) and Go (●) were reconstituted in HEK293T cells with D2R (A and B) or MOR (C and D). The deactivation rate constant measured in the absence of R7 RGS was subtracted from the value measured in the presence of R7 RGS, and the resulting kGAP values were plotted against the expression level of R7 RGS determined by Western blotting. The slope values obtained from linear regression analysis are 2.1 × 10^{-8} ± 5.5 × 10^{-8} for D2R-Go-RGS9-2, 7.6 × 10^{-8} ± 1.4 × 10^{-8} for D2R-Gi-RGS9-2, 1.6 × 10^{-8} ± 2.2 × 10^{-8} for D2R-Go-RGS7, 9.4 × 10^{-8} ± 5.0 × 10^{-8} for D2R-Gi-RGS7, 1.9 × 10^{-8} ± 8.0 × 10^{-8} for MOR-Go-RGS9-2, 5.8 × 10^{-8} ± 7.0 × 10^{-8} for MOR-Gi-RGS9-2, 2.2 × 10^{-8} ± 2.2 × 10^{-8} for MOR-Go-RGS7, and 7.4 × 10^{-8} ± 8.2 × 10^{-8} for MOR-Gi-RGS7. Four to 12 replicate samples were used for obtaining each data point. The data shown are representative of at least two independent experiments. E and F, comparison of expression levels of transfected Go and Gi trimers. E, given the interdependence of Go and Gβγ expression, we used the abundance of Venus 156–239-tagged Gβγ to estimate the expression levels of Go and Ga1. Increasing amounts of Go subunits for transfection increase the expression levels of Gβγ as expected. The conditions in the second samples from the left in experiments with Go and Ga1 were used in all experiments with varying Go subunits and show similar expression levels of Gβγ. F, basal BRET measured before agonist application. Basal BRET reports the extent of Go-Gβγ heterotrimer formation.

**G Protein and GPCR Selectivity of RGS Complexes**

**A**

![Graph A](image1)

**B**

![Graph B](image2)

**C**

![Graph C](image3)

**D**

![Graph D](image4)

**E**

![Graph E](image5)

**F**

![Graph F](image6)}
affect MOR signaling to AC (Fig. 9, B and C), consistent with the lack of the RGS7 activity on Goi revealed in the BRET assays. Thus, these data, together with data from the BRET assays, indicate that RGS proteins differentially control GPCR-mediated signaling to downstream effectors, consistent with their G protein selectivity profile.

**DISCUSSION**

The main result of this study is the establishment of the selectivity for two major striatal RGS proteins in their ability to regulate physiologically relevant GPCRs in the native environment of a living cell. The GAP activity of RGS proteins is usually assayed in *in vitro* systems with purified components where RGS proteins, and often only their catalytic domains, are studied in isolation from protein-protein interactions, receptors, and the membrane environment. Under those conditions, RGS proteins display very few differences in their substrate selectivity and specific activity. However, it is becoming increasingly appreciated that, *in vivo*, RGS proteins function in a tight association with other components of the GPCR signaling cascades and exist in larger macromolecular complexes (29). For example, both RGS7 and RGS9 form complexes with a range of partners that include Gα5; the membrane anchors R7BP and R9AP (30); and the GPCRs mGlur6 (31), D2R (13, 32), MOR (33–35), m3 muscarinic (36), and GPR158/179 (37). Nevertheless, how these interactions shape RGS action in cells is poorly understood. We used a cell-based BRET assay system to study the influence of multisubunit RGS complexes on the kinetics of G protein subunit reassociation following termination of GPCR activity. We developed quantitative measures of RGS protein GAP activity in this system and applied it to investigate the
activity and selectivity of the striatal RGS proteins RGS7 and RGS9-2.

The following are the key conclusions of our study (Fig. 10). First, we show that in the cellular environment RGS9-2/Gαi2/5 and RGS7/Gαo display a strong preference for Gαi1 over Gαi2. These results are in overall agreement with published in vitro data (26, 38). The RGS7 complex showed the greatest selectivity for Gαi1 and was completely unable to inactivate Gαi2 in the absence of R7BP. Second, we demonstrate that the GAP activity of the RGS9-2/Gαi2/5 complex shows a receptor preference for D2R over MOR in regulation of the Gαi deactivation. To our knowledge, this is the first clear example of GPCR selectivity of the R7 RGS action. Although the mechanisms behind this receptor preference of the RGS9-2 complex need to be established, we speculate that they are likely determined by selective interactions of RGS9-2 with the receptors, as suggested from the studies on RGS4, that selectively interacted with the δ-opioid receptor over the μ-opioid receptor (39). Interestingly, no receptor preference was revealed for the RGS7/Gαi5 action. Third, we found that R7BP acted universally to potentiate the action of both RGS7 and RGS9-2 on both Gαi1 and Gαo and with both D2R and MOR. It had the most pronounced all-or-nothing effect on the ability of RGS7 to regulate Gαi, essentially switching it on. Although the design of our study did not allow distinguishing between allosteric effects and the general effects of positioning RGS complexes on the plasma membrane, we think that both mechanisms are likely involved in the action of R7BP, as exemplified in the studies on related membrane anchor R9AP (24, 41). Finally, we report that in living cells, RGS7 shows a much more potent activity relative to the RGS9-2 complex. In contrast, previous in vitro observations with purified proteins reported approximately equal catalytic activities of these two proteins (26). This illustrates the importance of considering a native, physiologically relevant

FIGURE 7. R7BP augments the GAP activity of RGS7 and RGS9-2 toward Gαi1 and Gαo in the MOR-based system. Gi1 and Go signaling were reconstituted in HEK293T cells with MOR, and the GAP activity of RGS7 and RGS9-2 was examined in the absence (○) or presence (●) of R7BP. Changes in the kGAP values are plotted as a function of RGS concentration. Representative of two to three independent experiments yielding similar results are shown. The slope values obtained from linear regression analysis are 1.7 × 10⁻³ ± 3.4 × 10⁻⁴ for MOR-Go-RGS9-2-R7BP, 6.0 × 10⁻⁴ ± 1.0 × 10⁻⁴ for MOR-Go-RGS9-2, 3.6 × 10⁻⁵ ± 6.5 × 10⁻⁶ for MOR-Go-RGS7-R7BP, 1.8 × 10⁻⁴ ± 2.9 × 10⁻⁵ for MOR-Go-RGS7, 6.7 × 10⁻⁴ ± 4.2 × 10⁻⁵ for MOR-Gi-RGS9-2-R7BP, 3.7 × 10⁻⁴ ± 3.7 × 10⁻⁵ for MOR-Gi-RGS9-2, 3.0 × 10⁻⁴ ± 5.9 × 10⁻⁵ for MOR-Gi-RGS7-R7BP, and 1.2 × 10⁻⁴ ± 6.8 × 10⁻⁵ for MOR-Gi-RGS7. Four to six replicate samples were used for obtaining each data point.
It is interesting to consider the observed differences in selectivity and activity of RGS complexes in the context of striatal G protein signaling regulation. We have reported recently that changes in neuronal excitability and oxygenation trigger a remodeling of RGS complexes in the striatum (40). During this remodeling, RGS9-2 undergoes degradation, and vacated R7BP.
recruits RGS7 to the plasma membrane compartments. Furthermore, multiple studies demonstrated that exposure to addictive drugs (e.g., cocaine, morphine, amphetamine) that influence D2R and MOR signaling also changes RGS9-2 expression (9) and, thus, likely influences the composition of RGS complexes in striatal neurons. Taken together with the results of this study, these observations suggest a model where remodeling of RGS complexes is used to adjust the strength and selectivity of striatal G protein signaling. For example, an increase in dopamine and opioid signaling may be counteracted by tweaking the GAP complex (41) to substitute less efficient RGS9-2 for the stronger RGS7 catalytic subunit. Substituting a more selective GAP for a less selective one will likely also affect the relative balance of D2R versus MOR signaling in the striatum. It thus appears that RGS protein complexes are more than just blunt indiscriminate tools and rather contribute to the homeostatic scaling of G protein signaling in a GPCR- and G protein-selective fashion.

FIGURE 9. Differential effects of RGS7 and RGS9-2 on Gβi-mediated inhibition of adenylate cyclase activity. A, schematic cross-talk signaling pathway of endogenous β2-adrenergic receptor (β2AR) and MOR to the firefly luciferase gene regulated by the CRE response element. B, HEK293T cells were transfected with CRE-luc2P reporter, MOR, Gnr1, Gβ1, Gγ2, and Gβ5 with or without R7 RGS. After 5 h of treatment with 50 nM isoproterenol (ISO) together with serial doses of morphine, luciferase activity was measured. The highest dose of morphine treatment of cells transfected without RGS or with RGS7 or RGS9-2 inhibited ISO-induced luminescence by 68.5 ± 1.3%, 62.9 ± 2.5%, and 61.2 ± 4.7% (S.E., n = 4), respectively. The average luminescence at the highest dose of morphine treatment was subtracted as background, and the resulting difference (ΔR) was normalized against the maximal value upon stimulation by ISO only (Rmax). C, IC50 values were obtained by fitting a four-parameter logistic curve to the inhibition data using GraphPad Prism 5. **, p < 0.01, one-way analysis of variance followed by Tukey’s post hoc test.

FIGURE 10. A model for GPCR and G protein selectivity of striatal R7 RGS complexes. RGS7 and RGS9-2 differentially regulate D2R- and MOR-mediated signaling to Gβi and Gαo in the absence (A) or presence (B) of R7BP. The thickness of the T-shaped arrows indicates the relative strength of GAP activity observed in this study. A thicker line represents stronger activity. RGS7 is a stronger GAP than RGS9-2. RGS9-2, but not RGS7, is capable of regulating Gβi in the absence of R7BP. Both RGS9-2 and RGS7 preferentially regulate Gαo in the absence or presence of R7BP. Although RGS7 does not show a GPCR preference, RGS9-2 complexes selectively regulate D2R over MOR.
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