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The Complex of G Protein Regulator RGS9-2/Gβ5 Controls Sensitization and Signaling Kinetics of Type 5 Adenylyl Cyclase

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Abstract

Multiple neurotransmitter systems in the striatum converge to regulate the excitability of striatal neurons by activating several G protein coupled receptors (GPCRs) that signal to the type 5 adenylyl cyclase (AC5), the key effector enzyme that produces the intracellular second messenger cyclic adenosine monophosphate (cAMP). Plasticity of the cAMP signaling in the striatum is thought to play an essential role in the development of drug addiction. Here, we show that the complex between ninth regulator of G protein signaling (RGS9-2) and type 5 G protein β subunit (Gβ5), critically controls dopamine and opioid GPCRs signaling to AC5 in the striatum. We found that RGS9-2/Gβ5 suppressed basal and Gβγ sensitized activity of AC5 by a mechanism that involved both a direct RGS9-2/Gβ5-AC5 protein-protein interaction and the GTPase stimulatory action of the RGS9-2/Gβ5 complex. Furthermore, RGS9-2/Gβ5 increased the deactivation rate of the inhibitory G protein signaling on AC5. Mice lacking Gβ5-RGS complexes showed increased cAMP production and enhanced sensitization of AC5 upon withdrawal from opioid administration. Our findings establish RGS9-2/Gβ5 complexes as regulators of three key aspects of cAMP signaling: the basal activity, sensitization and temporal kinetics of adenylyl cyclase, thus highlighting the role of these RGS proteins in regulating both inhibitory and stimulatory GPCRs that shape cAMP signaling in the striatum.

Introduction

Signaling through G protein-coupled receptors (GPCRs) regulates many processes in the nervous system, including neurotransmitter release and excitability, and is implicated in the pathogenesis of many neuropsychiatric disorders including schizophrenia, hyperactivity and drug addiction (1). Activation of a GPCR by neurotransmitters promotes GTP binding to the α subunits of the downstream G proteins resulting in their dissociation into α -GTP and $\beta\gamma$ subunits. When dissociated, G protein subunits modulate the activity of downstream effector proteins (2, 3). A key G protein effector is adenylyl cyclase, an enzyme that catalyzes the synthesis of the second messenger cAMP. Many adenylyl cyclase isoforms are stimulated by G α_s , inhibited by G α_i and differentially regulated by $\beta\gamma$ subunits (4). Modulation of the adenylyl cyclase activity is essential for shaping many processes including learning and memory, drug dependence and motor control (5).

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The regulator of G protein signaling (RGS) proteins are critical elements of the G protein pathways that limit the extent of the GPCR signaling by acting as GTPase activating proteins (GAP) for the G α subunits thereby promoting their inactivation (6). The R7 family of RGS proteins (R7 RGS), comprising RGS6, RGS7, RGS9 and RGS11, play particularly important roles in the nervous system, where they control vision, nociception and reward behavior (7). All R7 RGS proteins exist as obligate complexes with the type 5 G protein β subunit (G β 5), which is essential for the structural integrity and proteolytic stability of the R7 RGS complex (7).

The striatum is a structure in the brain that heavily relies on GPCR signaling, and is a gateway nucleus of the mesolimbic system that plays essential role in motor coordination, procedural learning and drug addiction (8, 9). In striatal neurons multiple GPCR pathways, including signaling through dopamine and opioid receptors, converge at the regulation of the type 5 adenylyl cyclase (AC5), a major G protein effector that accounts for approximately 80% of adenylyl cyclase activity in the striatum (10) and mediates many of the striatum-dependent behavioral responses (10–13). However, our understanding of how the G protein signaling to AC5 in the striatum is regulated is fragmented at best.

Here we report that the striatal R7 RGS complex RGS9-2/G β 5 is the key regulator of three most fundamental aspects of AC5 action: basal activity, sensitivity and timing of cAMP signaling. We found that RGS9-2, through its unique C-terminus, forms a complex with AC5 in the striatum, inhibits AC5 activity, and accelerates the recovery of AC5 activity from inhibitory G protein regulation. Furthermore, we show that RGS9-2/G β 5 inhibits AC5 sensitization by a mechanism that involves direct allosteric effects and GAP activity and that reduces stimulatory action of the G protein $\beta\gamma$ subunits. As a result, mice lacking RGS9-2 produce more cAMP in the striatum during withdrawal from opioids.

Results

Elimination of RGS/G β 5 complexes increases cAMP signaling in the striatum

We previously reported that elimination of the RGS/G β 5 complexes results in marked behavioral hyperactivity and enhanced signaling through several Gi/o-coupled GPCRs in the brain (14). Because G β 5-RGS proteins are GAPs for the Gi/o class of G proteins (15) which inhibit cAMP production, we first examined the effect of G β 5-RGS elimination on cAMP signaling.

Unexpectedly, we found that cAMP concentration in striatum of *G β 5*^{-/-} mice that lack all R7 RGS proteins was substantially increased as compared to wild-type littermates (Fig. 1A). Analysis of adenylyl cyclase activity in purified striatal membrane preparations revealed that increased cAMP content in *G β 5*^{-/-} tissues resulted from significantly higher basal activity (Fig. 1B). The higher cAMP production was accompanied by more efficient cAMP-dependent activation of PKA (Fig. 1C), and increased phosphorylation of its targets including key striatal regulator DARPP-32 (Fig. 1D). We did not observe changes in the protein expression of striatal AC5, indicating that the increase in adenylyl cyclase activity cannot be explained by changes in its abundance. We next reconstituted striatal membranes with the individual recombinant RGS/G β 5 complexes and found that they inhibited basal adenylyl cyclase activity. Out of the three proteins tested, the RGS9-2/G β 5 complex, which is enriched in the striatum, displayed the highest inhibitory effect; RGS6/G β 5 had a weaker inhibitory effect and RGS7/G β 5 showed no significant regulation (Fig. 1E). These observations suggest that RGS/G β 5 complexes suppress adenylyl cyclase activity in the striatum.

RGS9-2/Gβ5 desensitizes AC5 to the stimulatory action of Gas

Increased adenylyl cyclase activity upon elimination of Gβ5-RGS complexes is paradoxical, because these RGS proteins inhibit the Gαi/o class of G proteins (15, 16) which inhibit adenylyl cyclase. Therefore, it would be expected that Gβ5 knockout would suppress cAMP production due to an increase in Gαi-mediated inhibitory influence. However, the striatally-enriched AC5 isoform, is also allosterically modulated by the direct interaction with RGS proteins (e.g. RGS2), which inhibit AC5 stimulation by Gαs and βγ subunits (17). Members of the Gi/o family readily release βγ subunits upon activation, making them available for regulation of adenylyl cyclases. The GAP activity of RGS proteins promotes heterotrimer reassembly; hence elimination of RGS/Gβ5 proteins may also sensitize adenylyl cyclases by increasing the availability of free βγ subunits in addition to removing the potential direct inhibitory influence. Indeed, activation of the Gαs-coupled D1 receptor produced greater adenylyl cyclase stimulation in membranes from the striatum of Gβ5 knockout mice as compared to those from wild-type animals (Fig. 2A). To provide more mechanistic insight into observed sensitization we analyzed the dose-response relationship of adenylyl cyclase stimulation by activated Gαs in striatal membrane preparations (Fig. 2B). Addition of the Gβγ subunits caused a robust leftward shift indicating a sensitizing action (Fig. 2B). In contrast, addition of the RGS9-2/Gβ5 complex shifted the curve to the right, indicating a desensitizing effect (Fig. 2B). To discriminate between direct inhibitory effects and βγ mediated effects we next tested the impact of the GAP-deficient N364H mutant of RGS9-2 (18) which eliminated the effect of RGS9-2 on Gβγ signaling (Supplemental Fig. S1). The N364H mutant was as effective in desensitizing adenylyl cyclase activity as wild-type indicating that RGS9-2 can inhibit adenylyl cyclase activity by a mechanism independent of its effect on the G protein cycle (Fig. 2B). Similarly, the inhibitory effect of both wild type recombinant RGS9-2/Gβ5 complex and its N364H mutant (at 0.5 μM) was observed upon stimulation of adenylyl cyclase through the activation of the D1 receptors by DHX (Fig. 2C).

Because AC5 is the predominant adenylyl cyclase isoform in the striatum we next studied the effects of the RGS9-2/Gβ5/R7BP complex on ectopically expressed AC5 in transfected HEK293T cells. The RGS complex significantly diminished both forskolin- and isoproterenol-stimulated AC5 activity in the transfected cells, without affecting the abundance of AC5 (Fig. 2D). RGS9-2/Gβ5 had no effect on the cAMP production catalyzed by the endogenous AC in these cells (Fig. 2D). Furthermore, purified recombinant RGS9-2/Gβ5 complex inhibited the basal activity of recombinant AC5 expressed in insect Sf9 cells in a dose-dependent manner, an effect that was also seen with the GAP-deficient N364H mutant (Fig. 2E). Overall, these observations suggest that RGS-Gβ5 complexes inhibit the activity of the AC5 isoform.

RGS9-2/Gβ5 forms a physical complex with AC5

To understand why RGS9-2 was the most effective isoform of Gβ5-binding RGS proteins in regulating AC5 activity and could act in a GAP-independent fashion, we tested the possibility that RGS9-2 may form a physical complex with AC5 in the striatum. Indeed, when RGS9-2 was immunoprecipitated from wild-type striatum, it co-eluted with a considerable amount of adenylyl cyclase activity (Fig. 3A), whereas control experiments performed with RGS9 knockout striatal tissues showed virtually no AC activity in the eluates. Conversely, Flag-tagged AC5 retained on the beads captured from transfected cells effectively pulled-down RGS9-2 from total striatal lysates (Fig. 3B). In addition, AC5 and RGS9-2 cotransfected in HEK293T cells robustly co-immunoprecipitated each other (Fig. 3C).

We next delineated the binding determinants of the interaction using recombinant proteins (Fig. 3D). The RGS9-2/G β 5 complex bound to the reconstituted cytoplasmic C1/C2 domains but not to the N-terminus of AC5 (Fig. 3E). Deletional mutagenesis of RGS9-2 further showed that full-length AC5 expressed in HEK293T cells could pull-down only the long splice isoform, RGS9-2 or its ~200 amino acid C-terminal domain but not the short isoform RGS9-1 or the core G β 5-GGL module, both of which lack the C-terminal region (Fig. 3F). These observations suggest that RGS9-2, through its unique C-terminal domain, directly interacts with the catalytic domains of AC5.

μ -opioid receptor mediated superactivation of AC5 is attenuated by the RGS9-2/G β 5 complex

Our findings suggest that RGS9-2 action in the striatum is critical for determining the extent of sensitization of AC5. Persistent stimulation of the Gi/o coupled receptors that produce chronic activation of the G proteins leads to the homeostatic sensitization of the adenylyl cyclase, a phenomenon known as “superactivation” (19). A manifestation of superactivation is increased cAMP production in striatal neurons upon withdrawal from chronic opioid administration (20–22). Most of the effects of morphine are mediated by the Gi/o coupled μ -opioid receptors (μ -OR) (23, 24). μ -ORs are abundant in the striatum where they are inhibited by the RGS9-2 complex (25, 26), and mice lacking RGS9-2 show exacerbated morphine withdrawal symptoms (26). We therefore asked whether RGS9-2 is involved in controlling superactivation of AC5 downstream from chronic μ -OR activation.

Striatums of RGS9 knockout mice of mice undergoing acute withdrawal from chronic morphine administration had higher cAMP concentrations than striatums of wild type mice (Fig. 4A). Next, we modeled AC5 superactivation in transfected HEK293T cells transfected with AC5 and μ -OR. Removing morphine after overnight treatment induced increased cAMP concentrations, suggesting increased adenylyl cyclase activation (Fig. 4B). This sensitization was not observed in untransfected cells confirming key role of exogenously supplied AC5 in the process (Supplemental Fig. S2). Co-transfection of RGS9-2 complex reduced this effect by nearly an order of magnitude, consistent with its role as an inhibitor of AC5 sensitization (Fig. 4B). The GAP-deficient mutant of RGS9-2, while expressed at a similar amount as wild type protein (Supplemental Fig. S3), produced only partial blockade suggesting that the effects mediated by G protein activation play a substantial role in the development of the sensitization (Fig. 4B). Indeed, treatment of cells with pertussis toxin, which blocks G protein activation and the resulting release of G β γ subunits, completely prevented morphine-induced superactivation (Fig. 4C). Similarly, blocking G β γ by a GRK3CT scavenger prevented superactivation of AC5 (Fig. 4C). In contrast, transfecting cells with G β γ subunits produced the opposite outcome (Fig. 4C). Overexpressing the G α o subunit did not prevent the development of superactivation but significantly reduced the stimulatory effect of G β γ overexpression.

To obtain a better mechanistic picture of the AC5 superactivation we analyzed the real-time kinetics of this process in intact cells by imaging cAMP dynamics (Fig. 4D). Termination of μ -OR signaling caused a rapid increase in the concentration of cAMP, which plateaued within 2–3 min (Fig. 4E). Addition of the RGS9-2 complex substantially slowed the speed of the cAMP accumulation and reduced the maximal extent of the response (Fig. 4E, F). Overall, these observations indicate that RGS9-2 complex regulates the extent of superactivation of AC5, not only by directly interacting with AC5 but also by controlling the availability of the G β γ subunits through its GAP activity.

RGS9-2 inhibits signaling through Gβγ subunits by preferentially accelerating the GTPase activity of Gao

Our results suggest that the GAP activity of RGS9-2 plays an important role in antagonizing the sensitizing action of the Gβγ subunits upon chronic stimulation of μ-ORs. We therefore next tested the hypothesis that the RGS9-2/Gβ5 complex inhibits Gβγ signaling indirectly by speeding up the inactivation of the Gα subunits and thus facilitating their re-association with Gβγ to form inactive heterotrimers. Stimulation of the Gi/o coupled GPCRs, including μ-OR, in addition to releasing Gβγ, results in the activation of both Gai and Gao subunits that have differential effects on adenylyl cyclase activity. We therefore examined the relative impact of RGS9-2 complex on Gβγ signaling mediated by Gai and Gao. We used a cell-based system in which Gβγ interaction with its model effector GRK was monitored by changes in the signal from a BRET reporter (Fig. 5A). Stimulation of μ-ORs with morphine resulted in robust release of the Gβγ subunits when either Gai or Gao was used in the assay. Virtually no signal was detected in the absence of exogenously supplied Gα subunits or μ-ORs indicating that endogenous Gα and GPCRs do not substantially contribute to the Gβγ response being monitored (Supplemental Fig. S4). In fact, the speed of the Gβγ mobilization was the same for both Gα subunits (Fig. 5B,C). Inactivation of μ-ORs by the addition of the antagonist naloxone resulted in fast termination of the Gβγ-effector interaction as reported by the reduction in the BRET signal (Fig. 5B,D). Termination of Gβγ signaling followed similar kinetics when either Gai or Gao was used in the assay. Introduction of the RGS9-2/Gβ5/R7BP complex resulted in substantial acceleration of the Gβγ deactivation kinetics (Fig. 5B,D) without affecting the abundance of the G proteins or reporter constructs in the assay (Supplemental Fig. S5). However, the RGS9-2 complex differentially affected termination of Gβγ signaling by Gai or Gao, making responses in the Gao-based system substantially more transient. This result is consistent with the ability of RGS9-2 to accelerate the GTPase activity of Gao to a greater extent than that of Gai, which has been previously demonstrated in vitro (15) and suggests a mechanism for why RGS9-2 more strongly affects stimulatory Gβγ action compared to inhibitory Gai actions on the adenylyl cyclase activity upon persistent activation of Gi/o coupled GPCRs.

Temporal resolution of AC5-mediated cAMP signaling is shaped by RGS activity

RGS proteins accelerate the response kinetics in the G protein pathways that signal to ion channels (27–29). However, the effects of RGS proteins on the kinetics of adenylyl cyclase signaling are not known. Given the physical association between RGS9-2 and AC5 we hypothesized that, in addition to modulating AC5 sensitization, the RGS9-2 complex may also be involved in controlling the temporal characteristics of GPCR signaling to AC5. To test this possibility we employed a model of antagonistic adenylyl cyclase regulation by Gαs and Gai during acute GPCR signaling (Fig. 6A). We reasoned that although Gai is not the preferred substrate for the RGS9-2 complex as compared to Gao (15, 16), its direct effect on adenylyl cyclase activity presents a convenient way to study the regulatory influence of RGS proteins that are common for both Gai and Gao subunits.

Stimulation of cells with the agonist of the Gαs-coupled β-adrenergic receptor (β-AR) resulted in a rapid increase in cAMP concentration that plateaued in about 5 minutes (Fig. 6B) and persisted for at least 25 minutes (Supplemental Fig. S6). However, concurrent application of morphine to stimulate the μ-OR caused progressive inhibition of AC5 activity by activating Gai overexpressed in the cells and resulted in a decrease in cAMP concentration to baseline values (Fig. 6B). Inactivation of the μ-OR with the antagonist naltrexone while continuing to stimulate β-AR reversed the inhibitory effect and restored the cAMP concentration (Fig. 6B). Transfection of the RGS9-2 complex substantially accelerated the recovery of cAMP concentration back to the peak values following termination of the μ-OR-Gai signaling (Fig. 6C). At the same time, the extent of the

response or the onset phase of the μ -OR mediated inhibition was not affected by RGS9-2 in this acute signaling paradigm (Fig. 6D). These effects of RGS9-2 complex appear to be mediated exclusively by its GAP activity because the N364H mutant failed to alter the kinetics of the AC5 disinhibition (Fig. 6D). These findings indicate that RGS-G β 5 complexes regulate the temporal characteristics of GPCR-mediated regulation of AC5 in addition to controlling its sensitization upon chronic stimulation of G protein pathways.

Discussion

The key result from our study is the finding that RGS/G β 5 complexes play a major role in controlling adenylyl cyclase activation and sensitization. Many of the adenylyl cyclase isoforms show dual regulation by G proteins: they are activated by G α s but are acutely inhibited by G α i (30). However, persistent activation of many inhibitory Gi/o-coupled GPCRs results in paradoxical increases in adenylyl cyclase activity, a phenomenon referred to as sensitization or “superactivation” (19). This plasticity mechanism has been proposed to play an important role in mediating adaptive changes in neural signaling in various processes that demonstrate a sensitization component, such as sexual behavior (31), locomotor potentiation (32) and drug addiction (33). The molecular changes leading to adenylyl cyclase sensitization are not well understood. However, most studies agree that the sensitization involves liberation of the $\beta\gamma$ subunits of Gi/o proteins which enhance modulation of adenylyl cyclase by G α s either directly by $\beta\gamma$ binding or indirectly through the action of stimulatory protein kinases such as Raf-1 (19). Notably, the activity of the striatal adenylyl cyclase isoforms AC5/6 is potentiated by both phosphorylation by Raf-1 (34, 35) and binding to $\beta\gamma$ (17).

Our study shows that the elimination of RGS/G β 5 proteins markedly enhances the activity of adenylyl cyclase in the striatum. Although we cannot rule out the impact of possible adaptations on cAMP homeostasis in a mouse model that lacks all R7 RGS proteins, our dissection of potential mechanisms suggests that the key role is played by the RGS9-2/G β 5 complex which employs a dual mechanism to control AC5 activity. First, direct physical association of RGS9-2/G β 5 with AC5 inhibits its basal activity and reduces the sensitivity to the activation by the G α s. Second, during persistent activation of the G α i/o coupled receptors, the GAP activity of the RGS9-2/G β 5 additionally promotes heterotrimer reformation and thus reduces the pool of free $\beta\gamma$ subunits that promote AC5 sensitization. In addition to RGS9-2, other R7 RGS isoforms are likely contributing to setting AC5 activity as the surge in basal cAMP concentration seen in G β 5 knockout is not present in striatum of mice lacking only RGS9-2. Our model proposes that R7 RGS protein complexes are effective inhibitors of adenylyl cyclase activity, making it possible to achieve changes in cAMP concentration by modulating the abundance or activity of the R7 RGS proteins. Indeed, drugs of abuse alter the abundance of RGS9-2 in the striatum (36), suggesting that this mechanism likely contributes to the development of dependence by regulating the extent of adenylyl cyclase superactivation.

Enhanced dissociation of the heterotrimers upon elimination of RGS/G β 5 should, in addition to increasing the pool of free G $\beta\gamma$, also result in the increased availability of activated G α i-GTP, which inhibits adenylyl cyclase activity. This could theoretically negate the stimulatory effect of the G $\beta\gamma$ or possibly lead to inhibition of adenylyl cyclases. The latter is seen upon acute stimulation of Gi/o coupled GPCRs. We think the reason why the sensitization of adenylyl cyclase by G $\beta\gamma$ prevails over its inhibition by G α i upon loss of RGS is that R7 RGS proteins more potently regulate the activity of the G α o isoform, which does not exert regulatory effects on AC5 (37, 38). Indeed, we show that the RGS9-2/G β 5/R7BP complex exerts a much stronger regulatory effect on G $\beta\gamma$ signaling with G α o rather than G α i subunits in the μ -OR signaling pathway reconstituted in living cells. This

observation is consistent with a reported preference of Gβ5-RGS complexes for Gαo over the Gαi during stimulation of GTP hydrolysis (15, 16). This model is also supported by findings in transfected C6 cells in which endogenous RGS proteins suppress superactivation of adenylyl cyclases by specifically regulating Gαo proteins (39).

Our findings suggest that inhibition of adenylyl cyclase activity by RGS proteins may be a general phenomenon. Previous studies identified RGS2 as a binding partner of several adenylyl cyclase isoforms including AC5 (40). The N-terminus of RGS2 binds to the catalytic C1/C2 domains of AC5 and inhibits its activity (41). Analysis of the sequence features of the N-terminus of RGS2 and C-terminus of RGS9-2 reveal that both of these regions are predicted to be intrinsically disordered. Intrinsic disorder elements in proteins are thought to underlie their functional flexibility and are hot-spots for protein-protein interactions (42, 43). This observation suggests that binding to intrinsically disordered proteins, including RGS proteins, may be a common feature that adenylyl cyclase isozymes utilize for fine-tuning their activity as well as for the modulating the regulation by subunits of the heterotrimeric G proteins. The RGS/Gβ5 complexes as well as other RGS proteins that specifically regulate GTPase activity of Gi/o proteins are not traditionally thought to affect signaling through Gs-coupled GPCRs. However, our findings introduce a mechanism by which RGS proteins can act as inhibitors of signaling through Gs-coupled GPCRs as well. By reducing the availability of the stimulatory Gβγ subunits and limiting the sensitivity of the adenylyl cyclase regulation by Gαs, RGS proteins act to reduce the extent of cAMP formation induced by the activation of Gs-coupled GPCRs. From the perspective of the striatal GPCR signaling this mechanism suggests that the RGS9-2/Gβ5 complex, in addition to regulating Gi/o-coupled μ-OR and D2 dopamine receptors, is also likely to be involved in controlling signaling through the Gs-coupled D1 receptor.

Finally, we would like to stress functional importance of scaffolding of adenylyl cyclase with RGS proteins which we found played a prominent role in increasing the temporal resolution of cAMP signaling. It was previously demonstrated that RGS protein action is required to reconstitute rapid gating of the ion channel by the G proteins (44). Here we extend these observations and demonstrate that temporal characteristics of the cAMP signaling pathway are also controlled by RGS proteins. Although the regulation of cAMP signaling occurs on a slower timescale relative to the ion channels, RGS activity nevertheless substantially accelerates the ability of adenylyl cyclases to recover from the regulatory influence imposed by G proteins thus enabling greater frequency of cAMP fluctuations.

Materials and Methods

Mice and DNA constructs

The Gβ5^{-/-} (45) and RGS9^{-/-} (46) mice were generated previously and were back-crossed with C57/Bl6 mice for six generations. Littermates for Gβ5^{-/-} (2–4 months old) derived from heterozygous breeding pairs were used for all experiments. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at The Scripps Research Institute.

Cloning of full-length Gβ5, R7BP, RGS9-2, full-length AC5 and fragments were described previously (47–49) (50) (17). The N364H mutation in mouse RGS9-2 was introduced by QuickChange site directed mutagenesis kit (Stratagene). BRET reporter constructs Venus155-239-Gβ1, Venus1-155-Gγ2, masGRKct-Rluc8 were gifts from Dr. Nevin A. Lambert (Medical College of Georgia), and HA tagged μ-opioid receptor (HA-MOR) was a gift from Dr. Ping-Yee Law (University of Minnesota). The cAMP FRET sensor mTurquoise-EPAC-cp173Venus-Venus was provided by Dr. Kees Jalink (The Netherlands

Cancer Institute). Rat cDNA mammalian expression clones for Gai1, Gai2 and Gao in pCMV5 were gifts from Dr. Hiroshi Itoh (Nara Institute of Science and Technology).

Recombinant proteins and membrane preparations

Recombinant complexes of Gβ5 with His-tagged full-length RGS9-2, RGS9-2(N364H), RGS9-1, RGS6, RGS7 or GGL fragment of RGS9 (aa 193-284) were expressed in Sf9/ baculovirus system and purified as described (51, 52). Similarly, expression and purification of His-tagged C-terminal fragment of RGS9-2 (aa 467-675), Gβ1γ2, as well as C1 and C2 domains of canine AC5 were also described (53) (54). GST-tagged N-terminal domains of AC5 were expressed in BL21 (DE3) *E. coli* strain and purified by affinity chromatography on glutathione-Sepharose 4B beads (GE healthcare). The purity of recombinant proteins was assessed by Coomassie staining following gel separation and was found to be at least 70%.

For membrane preparation, striatal tissues or Sf9 cell membranes were homogenized in buffer containing 250 mM sucrose, 20 mM Hepes pH 8.0, 1 mM EDTA, 2 mM MgCl₂, 1 mM DTT, and protease inhibitors. The homogenate was centrifuged at 2,000×g to remove nuclei, followed by centrifugation at 25,000 rpm in Beckman SW28.1 rotor for 35 min in 23/43% sucrose gradient to isolate membrane fraction. The plasma membranes were carefully collected from the layer at the 23/43% interface.

Determination of cAMP concentration and assays for adenylyl cyclase and PKA activity

Brain tissue or HEK293T cells were homogenized in 0.1 N HCl. For striatum tissues, 20 μL HCl was added per mg tissue and samples were homogenized by passing through a series of needles with decreasing gauges. 24 hours post-transfection cells were stimulated with forskolin (5 μM and 1 μM for without AC5 and with AC5 transfection, respectively) or 100 nM isoproterenol for 5 min. For cAMP superactivation assays, cells transfected with μ-OR, AC5 with or without indicated components were pretreated with 1 μM morphine overnight. μ-OR signaling was terminated by abruptly changing the medium followed by 5 min incubation in fresh medium. Cells were then lysed in 400 μL HCl per well in 48-well plate (2×10⁵ cells) for 30 min. Lysates were centrifuged at 600×g for 10 min. Supernatants were collected, diluted 50-fold and cAMP concentrations were quantitated using a cAMP enzyme immunoassay kit following acetylated version protocol (Assay Designs) according to the manufacturer's instructions. The activity of full-length adenylyl cyclase in membrane preparations (1 μg total protein/reaction) was determined in membrane preparations as described previously (55). Striatal membranes (1 μg protein) prepared from Gβ5 knockouts (Gβ5^{-/-}) and their wild-type littermates (Gβ5^{+/+}) were transiently stimulated with 10 μM DHX. For basal adenylyl cyclase activity assays, striatal membranes or Sf9 membranes expressing AC5 (1 μg) was treated with purified RGS protein complexes at indicated concentrations for 20 min on ice. For dose response curves of Gαs-GTPγS stimulated adenylyl cyclase activity experiments, striatal membranes were pre-incubated with 0.5 μM RGS9-2/Gβ5 wild-type or N364H mutant protein, or 0.2 μM Gβ1γ2 for 20 min on ice and then subjected to adenylyl cyclase activity assay transiently stimulated with increasing doses of Gαs-GTPγS as indicated. The resulting cAMP in the sample was determined by cAMP Direct EIA Kit. PKA activity was determined by the MESACUP protein kinase assay kit (MBL, Ltd., Nagoya, Japan).

Preparation of protein extracts and Western blotting

Striatal tissue (~15 mg) or transfected HEK293T cells were homogenized by sonication in ~300 μl detergent buffer (1×PBS, 150 mM NaCl, 1% Trion X-100 containing complete protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail 1 and 2 (Sigma). The homogenate was centrifuged at 15,000×g, 15 min at 4°C. The resulting supernatant was resolved on SDS-PAGE gel, transferred onto PVDF membrane and subjected to Western

blot analyses by probing with the following pan-specific antibodies: AC5/6 (Santa Cruz Biotechnology), DARPP32 (Chemicon), Flag (M2, sigma), RGS9-2 (CT) (56), anti-G β 5 (ATDG)(57) or phospho-specific: p-DARPP32 (T34, Cell Signaling Technology), p-PKA substrate (Cell Signaling Technology).

Immunoprecipitation

The interaction between RGS9-2 and adenylyl cyclase in native tissues was studied by probing adenylyl cyclase activity following immunoprecipitation of RGS9-2 from striatal extracts. Fresh dissected striatal tissue (~50 mg) was homogenized by passing through a series of needles with decreasing gauges in 600 μ L homogenization buffer (50 mM HEPES pH 7.4, 5 mM EDTA, 100 mM NaCl, 1 mM DTT and 10% glycerol) containing proteinase inhibitor cocktail. The non-ionic detergent C₁₂E₉ (0.6%) was added to the buffer, and the lysate was homogenized and centrifuged at 16,000 $\times g$ for 15 min. The supernatant was incubated with 5 μ g RGS9-2 antibodies and 10 μ L of protein G beads for 2 hours at 4°C. Beads were washed 3 times with 500 μ L wash buffer (50 mM HEPES pH7.4, 1 mM EDTA, 1 mM MgCl₂, 150 mM NaCl, 0.05% C₁₂E₉). After the final wash, beads were re-suspended in 240 μ L of the assay buffer (50 mM HEPES pH7.4, 1 mM EDTA, 1 mM MgCl₂, 0.04% C₁₂E₉) containing 100 μ M forskolin and proteinase inhibitors and incubated for 10 min at 30°C and processed for determination of cAMP concentration. In reciprocal immunoprecipitation assays, Flag-tagged full-length AC5 expressed in HEK293T cells was immobilized on protein G beads (GE healthcare) coated with anti-Flag antibody. The beads were mixed with striatal tissue lysate and the mixture was incubated at 4°C for 1 hour. RGS9-2 and Flag-tagged AC5 were co-immunoprecipitated upon their co-expression in HEK293T cells following similar procedures.

Measurement of real-time cAMP dynamics by ratiometric FRET imaging

Real-time changes in cAMP concentration were studied in live HEK293T cells using FRET-based mTurquoise-EPAC-cp173Venus-Venus reporter (58). 24–48 hours after transfection cells grown on coverslips, were washed with HEPES-Hanks' buffer (137 mM NaCl, 5.4 mM KCl, 0.4 mM KH₂PO₄, 0.3 mM Na₂HPO₄, 3 mM NaHCO₃, 0.5 mM MgCl₂, 0.4 mM MgSO₄, 1.3 mM CaCl₂, 20 mM HEPES, 5.6 mM Glucose, pH 7.4), mounted in a POCmin perfusion chamber (PeCon GmbH, Germany), and imaged by a Leica CTR6000 inverted microscope with EM CCD camera (Hamamatsu Corporation) using MetaFluor software (version 7.6.1.0, MDS Analytical Technologies). The chamber was constantly perfused with HEPES-Hanks' buffer by gravity flow (~2 mL/min). Several healthy-looking cells were identified in a field of view using 20X objective and defined as regions of interest by outlining their soma. Subsequent analysis was performed for these regions only. Cells were excited by 30 ms flashes of light using 430/24 nm excitation filter. Emitted light was split by a DV2 dual-view image splitter (MAG Biosystems) into two simultaneously recorded channels with band-pass filters of 470/24 and 535/30 nm respectively. The inter-flash interval for image acquisition was set to 5 s. For each recording, the inverse fluorescence resonance energy transfer (FRET) ratios were calculated by dividing the fluorescence intensity in the 470 nm channel by that recorded in 535 nm channel. The resulting value (R) was normalized against the average baseline value from 10–20 frames recorded prior to stimulation (R₀). Alternatively, the R₀ value was subtracted from as a background, and the resulting difference was normalized against the maximal value recorded upon cellular response (R_{max}). For measuring the acute inhibitory effects of G α i, cells were transfected with mTurquoise-EPAC-cp173Venus-Venus reporter, AC5, μ -OR, G α i2, G β 1, G γ 2, with wild-type or N364H mutant RGS9-2/G β 5/R7BP complex at a 2:1:1:1:1:1 ratio between cDNA constructs. For studying chronic morphine effects cells were transfected with mTurquoise-EPAC-cp173Venus-Venus reporter, AC5, μ -OR, with or without wild-type RGS9-2/G β 5/R7BP complex at a 2:1:1:1:1 cDNA ratio.

For the sensitization studies, transfected cells were pretreated with morphine (1 μ M) or vehicle for 16–18 hours. Cells were then washed and equilibrated in HEPES-Hanks' buffer containing morphine (1 μ M) or vehicle followed by the perfusion of 50 nM naltrexone in HEPES-Hanks. Cells were first stimulated with 50 nM β -adrenergic receptor agonist isoproterenol for 2 min. 10 μ M morphine was then applied to cells for 20 min followed by 5 min perfusion of 10 μ M naltrexone while keeping isoproterenol in the media. Blocking $G\beta\gamma$ signaling was achieved by using a construct encoding C-terminal sequence of $G\beta\gamma$ effector molecule GRK3 (GRK3CT) GRK3 (59) which was transfected into cells together with HA-MOR and Flag-AC5 at 2:3:1 ratio. G protein signaling was blocked by pertussis toxin (PTX; 100 ng/ml) treatment for 16–18 hours.

Pull down assays

Pull-down assays were performed in two ways. In the first method, the reaction consisted only of purified recombinant proteins. These assays were performed as described previously (60). Briefly, 100 pmol of purified GST tagged N-terminal of human AC5 and 100 pmol of purified RGS9-2/ $G\beta 5$ were co-incubated with 20 μ L glutathione agarose beads (GE Healthcare). Alternatively, RGS9-2/ $G\beta 5$ proteins were incubated with purified recombinant C1 and C2 proteins (100 pmol each), specific RGS9-2 antibody and 10 μ L of protein G beads (GE healthcare). In both cases incubations were performed in 500 μ L of the binding buffer (20 mM Tris, pH 7.2, 300 mM NaCl, 0.25% n-dodecanoylsucrose, 50 μ g/mL bovine serum albumin) for 30 min at 4°C. The beads were washed three times by binding buffer and retained proteins were eluted by the SDS sample buffer.

In the second method, we immobilized Flag-tagged full-length AC5 and studied its ability to retain recombinant RGS9-2 mutants. HEK293T cells transfected with Flag-tagged full length AC5 were lysed in the lysis buffer (1 $\times$ PBS buffer + 150 mM NaCl + 0.5% *n*-Dodecanoylsucrose). The homogenate was centrifuged at 15,000 $\times$ g for 15 min at 4°C. Supernatant was incubated with anti-Flag antibody and protein G beads (GE healthcare) for 1 hr at 4°C. After 2 washes with the lysis buffer, the beads were washed once and incubated with purified RGS proteins (100 pmol) previously pre-cleared with anti-Flag antibodies and protein G beads and incubated at 4°C for 1 hour. As before, proteins were eluted from the beads with SDS sample buffer.

Morphine treatment and withdrawal

Morphine withdrawal paradigm was implemented as previously described (26). Briefly, mice were injected intraperitoneally with escalating doses of morphine (20, 40, 60, 80, 100, and 100 mg/kg) every 8 hours. Control mice were injected with saline following the same schedule. Two hours after the last morphine/saline injection, 1 mg/kg naloxone was administered by subcutaneous injection. All mice that received morphine showed prominent signs of withdrawal (including wet dog shakes, back walking, and paw shaking) 30 min following naloxone injection. At that point, mice were euthanized; their striata were dissected and processed for the analysis of cAMP content as described above.

Monitoring real-time kinetics of $G\beta\gamma$ signaling by fast kinetic BRET assay

Agonist-dependent cellular measurements of bioluminescence resonance energy transfer (BRET) between masGRKct-Rluc8 and $G\beta 1\gamma 2$ -Venus were performed to visualize the action of G protein signaling in living cells as previously described with slight modifications (61, 62). HEK293T/17 were transfected using Lipofectamine LTX (8 μ L/dish) and PLUS (5 μ L/dish) reagents. μ -OR, Ga ($G_{\alpha o}$, $G_{\alpha i 1}$), Venus155-239- $G\beta 1$, Venus1-155- $G\gamma 2$, masGRKct-Rluc8, RGS9-2, $G\beta 5$, and R7BP constructs were used at a 1:2:1:1:1:1:1 ratio. BRET measurements were made using a micro plate reader (POLARstar Omega; BMG Labtech) equipped with two emission photomultiplier tubes, allowing us to detect two

emissions simultaneously with 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 the G β 1 γ 2-Venus (535 nm) over the light emitted by the masGRKct-Rluc8 (475 nm). The average baseline value recorded prior to agonist stimulation was subtracted from BRET signal values, and the resulting difference (R) was normalized against the maximal value (Rmax) recorded upon agonist stimulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Elimination of Gβ5-RGS complexes increases cAMP signaling in the striatum

A, Total cAMP concentration determined in the striata of Gβ5 knockout mice (Gβ5^{-/-}) and their wild-type littermates (Gβ5^{+/+}). **B**, Adenylyl cyclase (AC) activity measured in mouse striatal tissues. **C**, Enzymatic activity of PKA in extracts from mouse striatum. **D**, Analysis of PKA substrate phosphorylation by Western blotting. Equal amounts of striatal tissue lysates were probed with the indicated phospho-specific antibodies. **E**, Measurement of basal adenylyl cyclase activity in striatal membranes in the presence of recombinant RGS-Gβ5 complexes. Statistical significance was evaluated by either Student's t-test (panels A, B), two-way ANOVA with Tukey's post hoc test (panel C), non-parametric Wilcoxon test (panel D), or one-way ANOVA with Tukey's post hoc test (panel E). *, $p < 0.05$; **, $p < 0.01$, $n=3-4$ mice). Error bars are SEM values.

Figure 2. The RGS9-2 complex regulates activity of AC5

A, Effect of D1 receptor stimulation by the agonist dihydrexidine (DHX) on adenylyl cyclase activity in striatal membranes. Adenylyl cyclase activity was normalized against the activity without DHX for each genotype. *, $p < 0.05$ (one-way ANOVA followed by post hoc Tukey's test, $n = 3$ mice). **B**, Effects of purified recombinant G β ₁ γ ₂ and RGS9-2/G β 5 complexes on the dose response of Gas-mediated activation of adenylyl cyclases in striatal membranes. The EC₅₀ for Gas-GTP γ S in the control reaction was 164 ± 45 nM, for G β ₁ γ ₂: 27 ± 5 nM, 584 ± 90 nM for wild type RGS9-2/G β 5 and 560 ± 70 nM for N364H mutant ($n = 3$ reactions). **C**, Inhibition of DHX-stimulated adenylyl cyclase activity by the addition of recombinant RGS9-2/G β 5 complexes to striatal membranes. *, $p < 0.05$; **, $p < 0.01$ comparing with unstimulated (basal) control; #, $p < 0.05$ comparing with DHX treated group (one-way ANOVA followed by post hoc Tukey's test, $n = 3$ reactions). **D**, Inhibition of AC5-mediated cAMP production by RGS9-2/G β 5 in HEK293T cells. Cells were transfected with RGS9-2, G β 5 and R7BP with or without AC5. *, $p < 0.05$ (t-test; $n = 3$ reactions). The abundance of AC5 and RGS9-2 in the samples was analyzed by Western blotting (right panel). **E**, Dose-dependent inhibition by recombinant RGS9-2/G β 5 complexes of basal adenylyl cyclase activity in membranes from Sf9 cells expressing AC5. $n = 3$ reactions.

Figure 3. Interaction of RGS9-2 with AC5

A, Co-precipitation of adenylyl cyclase activity with RGS9-2 from native striatal extracts. RGS9-2 was immunoprecipitated from the striatal extracts and the eluates were assayed for the adenylyl cyclase activity. Samples from both wild type (RGS9^{+/+}) and RGS9 knockout (RGS9^{-/-}) animals were used. ***, $p < 0.001$ by t-test, $n=3$ mice per genotype. **B**, Pull-down of native RGS9-2 from striatal lysates by Flag-tagged full-length AC5 expressed in HEK293T and immobilized on beads. In the control experiment untransfected cells were incubated with the same striatal lysate. **C**, Co-immunoprecipitation of RGS9-2 and AC5 from transfected HEK293T cells followed by Western blotting. **D**, The domain composition of AC5 and RGS9/Gβ5 complexes. **E**, RGS9-2 binds to the cytoplasmic C1/C2 but not the NT domain of AC5. *Upper*, Pull-down of recombinant RGS9-2/Gβ5 complex by GST-

AC5NT fusion protein or GST protein alone. *Lower*, Pull-down of recombinant RGS9-2/Gβ5 with purified His-tagged C1/C2 proteins using RGS9-2 antibodies conjugated to beads. *F*, C-terminal region of RGS9-2 is necessary and sufficient for the binding between RGS9-2 and AC5. Ectopically expressed Flag-tagged AC5 was precipitated with Flag antibody and incubated with purified recombinant RGS9 proteins. All of the experiments were repeated at least twice.

Figure 4. RGS9-2 inhibits Gβγ-mediated AC5 sensitization by chronic stimulation of μ-opioid receptors

A, cAMP accumulation in striatal tissues after naloxone-precipitated withdrawal from chronic morphine administration in RGS9 knockouts (RGS9^{-/-}) as compared to wild type (RGS9^{+/+}) subjects. Control group received saline instead of morphine and was analyzed separately. *, $p < 0.05$ ($n=4$ mice, one way ANOVA followed by post hoc Tukey's test). **B**, Effects of RGS9-2 complexes on adenylyl cyclase superactivation induced by morphine withdrawal in transfected HEK293T cells. *, $p < 0.05$; **, $p < 0.01$ comparison with respective no RGS groups, #, $p < 0.05$ comparison between indicated groups (one-way ANOVA followed by post hoc Tukey's test; $n=5$). **C**, The effect of Gβγ blockade on adenylyl cyclase superactivation in transfected HEK293T cells. Cells were treated with pertussis toxin (PTX) and morphine. *, $p < 0.05$ for morphine effect on cAMP induction, #, $p < 0.05$ for the effect of Gβγ and †, $p < 0.05$ for the effect of Gαo (one-way ANOVA followed by post hoc Tukey's test; $n=3$ reactions). **D**, The assay design for monitoring real-time changes in cAMP concentration. **E**, Time course of cAMP change upon AC5 superactivation. The μ-OR antagonist naltrexone was added to cells pre-treated with morphine at the indicated timepoint and the normalized change in FRET ratio, which reflects the change in cAMP concentration, was continuously recorded in live cells. **F**, Quantification of the effect of the RGS9-2 complex on the maximum amplitude of the cAMP change (peak FRET ratio) and the speed of the increase in cAMP concentration ($1/\tau$). To determine τ , the response curve was fitted by a single exponential function. **, $p < 0.01$ (t-test, $n=8$ cells).

Figure 5. RGS9-2 complex preferentially inhibits Gβγ signaling through Gαo

A, The assay design. Activation of the μ-OR causes the G protein heterotrimer to dissociate into Gα and Gβγ subunits. Released Venus-tagged Gβγ subunits interact with luciferase (Rluc)-tagged reporter GRK to produce a BRET signal. Upon termination of μ-OR activation, Gαo subunit hydrolyses GTP and re-associates with Gβγ subunits quenching the BRET signal. **B**, Effect of RGS9-2/Gβ5/R7BP on kinetics of the Gβγ-mediated signaling triggered by μ-OR. Cells were transfected with μ-OR, Gβγ and GRK reporter constructs, as well as indicated Gα subunits, with or without RGS9-2/Gβ5/R7BP complex. The BRET signals averaged from 6 experiments is plotted as individual data points. Solid lines show fits of the deactivation phases by exponential function. **C**, Quantification of the activation time constant following the addition of morphine. Exponential fits of the response onset were used to derive the time constant τ. **D**, Quantification of the deactivation time constant following the addition of naltrexone. Exponential fits of the data shown in panel B were used to derive the time constant τ. Two-way ANOVA (Holm-Sidak method) indicates a significant difference (***, p<0.001; n=6 reactions).

Figure 6. RGS9-2 facilitates kinetics of AC5-mediated cAMP production in living cells

A, Schematic representation of integration of the GPCR signaling to AC5 through Gai/o coupled μ -OR and Gas-coupled β -AR respectively. **B**, Normalized time course of changes in the cAMP concentration in transfected HEK293T cells determined by the ratiometric imaging during the treatments as shown in the protocols by the upper bars. cAMP FRET sensor, μ -OR, AC5, Gai-2, G β 1, and G γ 2 were co-transfected with or without RGS9-2/ G β 5/R7BP. The response before isoproterenol application was set as 0 and changes in cAMP concentration were then normalized to the percentage of maximal response. Traces are averages from 6–9 cells. **C**, Quantification of the effects of the RGS9-2 complexes on kinetics of the cAMP change during Gai deactivation (OFF phase). Time from the baseline to the peak after naltrexone application, and the speed of the cAMP induction ($1/\tau$) were quantified. To determine τ , the response curve was fitted by a single exponential function. RGS9-2 significantly decreased the time to reach peak cAMP concentrations following Gai inactivation. *, $p < 0.01$ (one-way ANOVA followed by post hoc Tukey's test comparison with no RGS control groups, $n=6-9$ cells). **D**, Quantification of the effects of the RGS9-2 complexes on the kinetics of the change in cAMP concentration. The time from baseline to maximal peak response after isoproterenol application and from peak to basal after morphine application were defined as Gas ON and Gai ON phases, respectively.