Changes in Striatal Signaling Induce Remodeling of RGS Complexes Containing G β 5 and R7BP Subunits[∇]

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Neurotransmitter signaling via G protein coupled receptors is crucially controlled by regulators of G protein signaling (RGS) proteins that shape the duration and extent of the cellular response. In the striatum, members of the R7 family of RGS proteins modulate signaling via D2 dopamine and μ -opioid receptors controlling reward processing and locomotor coordination. Recent findings have established that R7 RGS proteins function as macromolecular complexes with two subunits: type 5 G protein β (G β 5) and R7 binding protein (R7BP). In this study, we report that the subunit compositions of these complexes in striatum undergo remodeling upon changes in neuronal activity. We found that under normal conditions two equally abundant striatal R7 RGS proteins, RGS9-2 and RGS7, are unequally coupled to the R7BP subunit, which is present in complex predominantly with RGS9-2 rather than with RGS7. Changes in the neuronal excitability or oxygenation status resulting in extracellular calcium entry, uncouples RGS9-2 from R7BP, triggering its selective degradation. Concurrently, released R7BP binds to mainly intracellular RGS7 and recruits it to the plasma membrane and the postsynaptic density. These observations introduce activity-dependent remodeling of R7 RGS complexes as a new molecular plasticity mechanism in striatal neurons and suggest a general model for achieving rapid posttranslational subunit rearrangement in multisubunit complexes.

Members of the regulator of G protein signaling (RGS) family are ubiquitous negative regulators of signal transmission via G protein-coupled receptors. RGS proteins act to limit the extent and duration of G protein-coupled receptor signaling by accelerating the GTP hydrolysis rate on the α subunits of heterotrimeric G proteins, thus promoting their inactivation (see references 25 and 46 for reviews). The action of RGS proteins is essential for normal functioning of a wide range of fundamental processes including cell division (24), neuronal excitability (47), photoreception (22), angiogenesis (20), vasoconstriction (55), and many others.

R7 RGS subfamily is one of six distinct groups of more than 30 diverse RGS proteins (46, 64). This subfamily is comprised of four proteins: RGS6, RGS7, RGS9, and RGS11 with similar multidomain organizations (46, 64) and predominant neuronal expression patterns (17). Studies in mice indicate that R7 RGS proteins crucially regulate several critical aspects of nervous system function, such as vision (12, 45), motor control (4, 30), and nociception (15, 48, 62), placing a significant emphasis on the elucidation of their mechanisms.

A unique property of R7 RGS proteins is their constitutive association with the type 5 G protein beta (G β 5) subunit (6, 35). Binding to a G protein gamma-like domain in the core of R7 RGS proteins (28), G β 5 is tightly integrated into the structure of the RGS molecule (8). The ability to form complexes with G β 5 was shown to be essential for the folding and stability of R7 RGS proteins (23, 60), and

knockout of G β 5 in mice results in complete abrogation of expression of all four R7 RGS proteins (10). More recent studies revealed that, in addition to G β 5, R7 RGS proteins bind to a two-member family of SNARE-like membrane proteins: the R7 family binding protein (R7BP) (14, 37) and the RGS9 anchor protein (R9AP) (27, 53), which interact with the DEP/R7H domain of the RGS proteins and constitute the third subunit in the complex.

The role of R7BP/R9AP proteins is perhaps best studied for the R7 RGS subfamily member, RGS9. This RGS protein exists in two splice variants exhibiting a very restricted and nonoverlapping expression pattern (17, 63). The short-splice isoform, RGS9-1, is expressed exclusively in photoreceptors (22), where it sets the timing of phototransduction cascade recovery from the light excitation (42). The long-splice isoform is mostly found in the striatum region of the brain (18, 43, 57) and regulates the duration of the G protein signaling through D2 dopamine (30, 44) and μ -opioid receptors (16, 62). Accordingly, knockout of RGS9 in mice not only results in deficits in light adaptation (9) but also affects striatal control of movement and reward (4, 30, 44, 62). We have previously shown that both R9AP and R7BP play crucial roles for targeting and expression of RGS9 splice isoforms. While retina-specific R9AP delivers RGS9-1 to the specific subcellular compartment, the outer segment of photoreceptors (36), R7BP, is indispensable for targeting RGS9-2 to the postsynaptic densities of striatal neurons (2). Furthermore, knockout of either R9AP (29) or R7BP (2) leads to severe downregulation in RGS9 protein levels in the retina and striatum, respectively. It has been proposed that exposure of specific degradation determinants normally shielded by R7BP/R9AP tags RGS9/G β 5 for degradation by cellular cysteine proteases and that the

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balance of RGS9/Gβ5 association with R7BP/R9AP sets its expression levels in vivo (2, 29, 31).

Striatal neurons contain multiple R7 RGS proteins that bind to R7BP; however, only RGS9-2 requires R7BP for its expression (2, 3). In turn, R7BP itself is an unstable protein and is eliminated upon ablation of all R7 RGS proteins (2, 18). Interestingly, knockout of only RGS9 does not affect the stability of R7BP (2), suggesting that multiple striatal R7 RGS/Gβ5 complexes are pivoted by R7BP to allow several possibilities for subunit composition.

The goal of the present study was to investigate the principles and factors that regulate coupling of R7 RGS/Gβ5 complexes to R7BP in striatal neurons. We report that, in the striatum, R7BP mainly exists in complex with two RGS complexes: RGS9-2/Gβ5 and RGS7/Gβ5. However, despite the equimolar amounts of these RGS proteins in striatal neurons, R7BP is coupled preferentially to RGS9-2/Gβ5, whereas the majority of RGS7/Gβ5 is free from R7BP due to its substantially lower binding affinity. As a result, RGS9-2/Gβ5 complex is targeted by R7BP to the plasma membrane, while RGS7/Gβ5 is predominantly found at the intracellular sites. Strikingly, we find that this background complex composition undergoes dramatic remodeling, upon which R7BP recouples to RGS7 to recruit it to the plasma membrane and the postsynaptic density. This remodeling involves selective proteolytic degradation of RGS9-2, during which it uncouples from R7BP. We report that RGS9-2 degradation is controlled by signaling pathways that mediate synaptic activity and oxygen sensing, converging at an elevation of intracellular Ca^{2+} via extracellular Ca^{2+} entry. These findings suggest a novel plasticity mechanism for dynamically regulating subunit composition for major G protein signaling regulators in striatum depending on changes in synaptic activity.

MATERIALS AND METHODS

Antibodies, recombinant proteins, and DNA constructs. Generation of sheep anti-R7BP NT (37) and sheep anti-RGS9-2 CT (37) has been described previously. Rabbit anti-RGS7 (7RC1), rabbit anti-R7BP (TRS), and anti-Gβ5 (SGS) were generous gifts from William Simonds (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health). All custom generated antibodies were tested for the specificity to ensure that (i) they recognize single major band in brain extracts corresponding to size of detection subject, (ii) their immunoreactivity is blocked by peptide containing specific epitope sequence, and (iii) antibodies efficiently performed in immunoprecipitation reactions and the identity of precipitated band was confirmed by mass spectrometry. Recombinant His-tagged and glutathione *S*-transferase (GST)-tagged R7BP were expressed in *Escherichia coli* and affinity purified on Ni-NTA beads (Qiagen) as previously described (3, 37). RGS7 and RGS9-2 were expressed in Sf9 insect cells, together with Gβ5, via baculovirus-mediated delivery, and recombinant complexes were purified by Ni-NTA chromatography utilizing His tag present at the N termini of RGS proteins as described previously (37). Protein concentration was determined by BCA kit (Pierce) and adjusted to reflect the protein purity (ranging between 55 and 90%) determined by densitometry of Coomassie blue-stained gels.

Urea-treated bovine rod outer segment membranes were obtained as described previously (40). The plasmid encoding His₆-Gα_{o1} was a gift from N. O. Artemyev; the plasmid encoding His₆-Gα_{i1} was a gift from N. P. Skiba. Both recombinant proteins were purified from *E. coli* as described previously (32). Gα_i and Gβ_{1γ1} subunits were purified from bovine retinas as described previously (58).

Preparation of brain extracts, immunoprecipitation, and Western blotting. Cellular lysates were prepared by homogenizing brain tissue by sonication in immunoprecipitation buffer composed of phosphate-buffered saline (PBS) (pH 7.4; Fisher) supplemented with an additional 150 mM NaCl, 1% Triton X-100, and Complete protease inhibitors (Roche), followed by 15 min of centrifugation

at 14,000 × *g*. The resulting extract was used for protein concentration determination by BCA assay (Pierce, Rockford, IL). This Triton X-100 extraction procedure yields >90% extraction efficiency of proteins under investigation and was followed for both direct immunoblot and immunoprecipitation experiments. Immunoprecipitation of R7BP was performed using 3 μg of sheep anti-R7BP NT antibody and 10 μl of protein G beads (GE Healthcare, Little Chalfont, United Kingdom) added to the extracts. The mixtures were incubated for 1 h and washed three times with immunoprecipitation buffer, and proteins bound to the beads were eluted with sodium dodecyl sulfate (SDS) sample buffer. Samples were resolved on by SDS-polyacrylamide gel electrophoresis (PAGE), transferred onto polyvinylidene difluoride membrane, and subjected to Western blot analysis using horseradish peroxidase (HRP)-conjugated secondary antibodies and the ECL West Pico (Pierce) detection system. Western blots that were quantified were subjected to analysis of specific bands performed on an Odyssey infrared (IR) imaging system (Li-Cor Biosciences) according to the manufacturer's protocols using IRDye 680- and IRDye 800-labeled secondary antibodies.

Cell culture and transfections. 293FT cells were obtained from Invitrogen and cultured at 37°C and 5% CO₂ in Dulbecco modified Eagle medium supplemented with 100 U of penicillin/ml, 100 μg of streptomycin/ml, 10% fetal bovine serum, 4 mM L-glutamine, and 1 mM sodium pyruvate. Cells were transfected at ~70% confluence using Lipofectamine LTX reagent (Invitrogen) according to the manufacturer's protocol. The ratio of Lipofectamine to DNA used was 6.25 μl: 2.5 μg per 10 cm² of cell surface. A constant level of R7BP-pcDNA3.1 (0.2 μg) vector was cotransfected with an excess of Gβ5-pcDNA3.1 (1.2 μg), variable RGS7-pcDNA3.1, and RGS9-2-pcDNA3.1, balanced with empty pcDNA3.1 vector. The quantity of RGS7-pcDNA3.1 (1 μg) and RGS9-2-pcDNA3.1 (0.2 μg) vector necessary to establish a 1:1 protein expression level was determined by comparison to recombinant protein standard curves. The cells were grown for 36 to 48 h posttransfection prior to collection.

GST pulldown assays. The assays were performed by preparing cellular lysates in Triton X-100-PBS buffer (1× PBS, 150 mM NaCl, 1% Triton X-100, protease inhibitors) and clarified by centrifugation at 14,000 × *g* for 15 min. Concentration of Triton X-100 in lysate was then reduced to 0.2% with binding buffer (1× PBS, 150 mM NaCl, protease inhibitors) prior to the addition of purified recombinant R7BP-GST fusion proteins (175 pmol) attached to 10 μl of glutathione-agarose beads (GE Healthcare). Samples were incubated on rocker for 3 h at 4°C, and the beads were subsequently washed with binding buffer three times. The proteins were eluted in SDS sample buffer, and RGS proteins retained by the beads were detected by Western blotting with specific antibodies.

Surface plasmon resonance spectroscopy. The specific interaction between recombinant R7BP and RGS9-2/Gβ5 and between R7BP and RGS7/Gβ5 was analyzed by using a Biacore processing unit (Biacore 1000) at 23 to 25°C. R7BP was covalently coupled to CM5 chip flow cells (Biacore) by using an amine coupling kit (Biacore) according to the manufacturer's instructions. R7BP (30 μl) was flowed across the flow cell at a concentration of 10 μg/ml in 10 mM ammonium acetate (pH 6.0) at a rate of 5 μl/min, resulting in a net increase of 1,500 to 3,000 resonance units after immobilization. A control flow cell surface was reacted with the amine coupling reagents in the absence of R7BP. RGS protein of several concentrations in HBS buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant p20) was injected over the control and immobilized R7BP flow cell surfaces at a flow rate of 10 μl/min, and the resonance changes were recorded using Biacore 1000 control software v2.1. Changes in surface refraction were recorded for 1 min during protein binding and dissociation. The response from the R7BP surface, which was in the 25%-35% range of the resonance unit response observed in the R7BP cell, was subtracted from that of the control, and the dissociation constants (*K_D*) were determined by using BIAevaluation v3.0.2 software. R7BP surfaces were removed of RGS protein and regenerated by running 100 μl of 10 mM NaOH through the flow cell at 100 μl/min.

Coronal brain slice preparation and pharmacological treatments. The whole brain from 1- to 2-month-old C57BL/6 mice was quickly removed and placed in an ice-cold slicing solution containing 85 mM NaCl, 2.5 mM KCl, 4 mM MgCl₂, 1 mM NaH₂PO₄, 25 mM NaHCO₃, 25 mM glucose, and 75 mM sucrose (pH 7.4; all components were obtained from Sigma, St. Louis, MO). When indicated, the medium was continuously bubbled with 95% O₂-5% CO₂. Coronal slices were prepared 300 μm in thickness by using a Vibratome 3000 Plus sectioning system (Vibratome, St. Louis, MO). Slices were then cut sagittally down the midline, with striatal tissue being isolated from one side and directly frozen in liquid N₂. The contralateral half slice was subsequently placed into artificial cerebrospinal fluid (ACSF; 119 mM NaCl, 2.5 mM KCl, 1 mM NaH₂PO₄, 26.5 mM NaHCO₃, 1.3 mM MgSO₄, 2.5 mM CaCl₂, 11 mM glucose) with the indicated pharmacological agent, brought to 37°C, and continuously bubbled with 95% O₂-5% CO₂. Treatments with 30 mM KCl were performed by concurrently reducing the

FIG. 1. R7BP recouples to RGS7 upon induced degradation of RGS9-2. (A) Culturing striatal slices results in overt RGS9-2 degradation. Coronal sections (300 μ m) were prepared and cut in half down the midline. One side of the section served as control, with striatal tissue isolated and directly frozen in liquid nitrogen. The contralateral section was subsequently cultured for 0, 60, or 180 min in ACSF prior to analysis. Subsequently, the tissue was lysed and subjected to Western blot analysis using anti-RGS9-2 antibodies (left panels) or antibodies against striatally enriched signaling proteins (right panels). Protein bands were quantified by densitometry, and the resulting values were plotted as a function of time. (B) Degradation of RGS9-2 in slices is prevented by the inhibitor of cysteine proteases, E64. 1% Triton X-100 lysates were obtained from striatal slices (300 μ m) cultured for 3 h in the absence or presence of 50 μ M E64 and analyzed for the expression of RGS9-2, RGS7, and R7BP by Western blotting. (C) Western blot analysis of proteins immunoprecipitated by 5 μ g of sheep anti-R7BP from the extracts in panel B. (D) Quantitative analysis of protein band density in panel C. RGS9-2 and RGS7 band densities from three independent experiments were obtained by ImageJ software and normalized against R7BP content. Error bars represent the standard error of the mean (SEM) values. (E) Pull-down binding assay between immobilized recombinant GST-R7BP and native RGS proteins. Extracts obtained from slices either immediately frozen or cultured in the presence of E64 inhibitor were applied to GST-R7BP beads. After a washing step, bound proteins were eluted with the SDS-PAGE sample buffer and detected by Western blotting.

ACSF NaCl concentration to 91.5 mM. (+)-Bicuculline was purchased from Ascent Scientific (Weston-Super-Mare, United Kingdom); nimodipine and phorbol 12-myristate 13-acetate were from Sigma. Striatal tissue was isolated from the slice after 1 h of culture and frozen in liquid N₂ prior to protein extraction and Western blot analysis.

Immunoelectron microscopy. Electron microscopic examination of immunoreactivity for RGS9-2, R7BP, and RGS7 in the striata of wild-type and RGS9-2 KO mice was performed as described previously using either pre- or postembedding immunogold methods (2, 34). Briefly, brains were perfused with 4% paraformaldehyde, 0.05% glutaraldehyde, and 15% (vol/vol) saturated picric acid in 0.1 M phosphate buffer. For pre-embedding immunogold labeling, brain sections (50 to 70 μ m) were cut on a Vibratome and processed for immunohistochemical detection of RGS9-2 and/or R7BP using HRP and silver-enhanced immunogold techniques. For postembedding immunogold labeling, ultrathin sections (70 to 90 nm) from three Lowicryl-embedded blocks slices were cut on an ultramicrotome and processed for immunohistochemical detection of RGS9-2 and/or R7BP. The specificity of RGS7 antibodies (7RC1) for electron microscopy conditions was confirmed by staining the striatal regions of G β 5 knockout mice and observing the marked downregulation of characteristic staining as the levels of RGS7 in these mice are severely reduced (data not shown).

RESULTS

R7BP/RGS complexes in the striatum are remodeled upon induced degradation of RGS9-2. We have previously shown that RGS9-2/G β 5 complexes are proteolytically labile and rapidly degrade when not bound to R7BP. Furthermore, we hypothesized that the concentration of RGS9-2/G β 5 in vivo may be quickly regulated by dynamically changing the RGS9-2/G β 5 association with R7BP. To investigate whether levels of RGS9-2/G β 5 is subject to these posttranslational alterations, we studied modulation of RGS9-2 expression in cultured striatal slices. The data presented in Fig. 1A illustrate that upon culturing a 300- μ m-thick coronal slice through the striatum, the RGS9-2 protein levels rapidly declined, reaching ~30% of initial amount in only 3 h in culture. During this time frame, the levels of other investigated signaling proteins, including the RGS9-2 homolog, RGS7, were unaffected, suggesting a selec-

FIG. 2. RGS9-2 is the primary R7 RGS protein in complex with R7BP in the striatum. (A) Quantification of proteins coimmunoprecipitating with R7BP. Fresh striatal tissue was lysed in 1% Triton extracts and incubated with 5 µg of sheep anti-R7BP antibody. Eluates were loaded onto 4 to 20% PAGE gels adjacent to recombinant protein standards containing 50, 100, 250, and 500 fmol of the indicated RGS proteins. Upon incubation with IRDye-conjugated secondary antibodies images were scanned on Odyssey IR imager. Band densities were quantified by using Odyssey 2.0 software. A calibration curve was plotted from densities of recombinant protein standards. The linear range, typically extending throughout the calibration standard concentrations, was used to determine the RGS protein content in striatal extracts obtained from three separate mice. (B) Quantification of the total levels of R7BP complex components in striatum. Portions (20 µg) of total protein from each of three lysates were loaded next to recombinant protein standards of 20, 50, 100, 150, and 200 fmol for RGS7, RGS9-2, and R7BP or 40, 100, 200, 300, and 400 fmol for Gβ5. Note that the range of protein standards is different from that used in the experiment presented in panel A. Error bars indicate the SEM.

tive nature of this modulation. Inclusion of the cysteine protease inhibitor E64, which was previously shown to be effective for its prevention of RGS9-2/Gβ5 degradation (2), in the culture medium completely blocked its decay, suggesting a proteolytic mechanism for the effect (Fig. 1B).

Because it was previously found that stable expression of RGS9-2/Gβ5 *in vivo* requires its binding to R7BP (2), we next investigated whether degradation of RGS9-2/Gβ5 upon culturing could be explained by the loss of its association with R7BP. In these experiments, R7BP was immunoprecipitated from the striatal slices cultured in the presence of E64 inhibitor, and coprecipitating proteins were analyzed by Western blotting. The data presented in Fig. 1C reveal two key observations. First, the amount of RGS9-2 coprecipitating with R7BP markedly drops after culturing slices for 3 h despite similar levels of RGS9-2 protein present in the total lysates before and after culture in the presence of protease inhibitor. Second, culturing striatal slices substantially increases the amount of RGS7 coprecipitating with R7BP, despite the lack of modulation in its expression level. Indeed, quantitative analysis of data from three independent experiments (Fig. 1D) indicates that while RGS9-2/R7BP complex formation is decreased ~5-fold, the extent of RGS7/R7BP association is increased by a comparable factor.

These observations suggest that R7BP remains competent for binding R7 RGS proteins, and switching of its binding from RGS9-2 to RGS7 is likely induced by the changes affecting RGS proteins during culturing. In order to determine which RGS protein is responsible for the observed remodeling, we conducted a pull-down assay where we studied the ability of recombinant R7BP immobilized on the beads to capture RGS7 and RGS9-2

from striatal slices prepared before and after culture. As evident from Fig. 1E, R7BP was able to efficiently pull down both RGS9-2 and RGS7 from uncultured tissue. However, after culture, R7BP completely lost the ability to retain RGS9-2 while still effectively pulling down RGS7. This suggests that degradation of RGS9-2 in culture and subsequent upregulation of RGS7-R7BP complexes is induced by changes in RGS9-2 protein, leading to its uncoupling from R7BP and subsequent targeting for degradation.

Quantitative analysis of R7 RGS/Gβ5/R7BP complex compositions in the striatum. The observation of R7BP complex remodeling in slices and previous reports that R7BP is capable of forming complexes with all R7 RGS proteins have prompted us to investigate the composition and stoichiometry of R7BP complexes with R7 RGS/Gβ5 in the striatum. We have immunoprecipitated R7BP from striatal lysates and determined the amount of coprecipitating R7 RGS proteins by quantitative Western blotting using recombinant protein standards for each individual R7 RGS protein (Fig. 2A). Plotting the densities of protein standards against their concentration resulted in a linear relationship (data not shown) that was used to determine the concentration of proteins in the lysates from values of their band densities. Of four RGS proteins reported to interact with R7BP, RGS7 and RGS9-2 were found to be the predominant binding partners of R7BP. Furthermore, RGS9-2/R7BP complexes were approximately five times more abundant than RGS7/R7BP. Neither RGS6 nor RGS11 was present in R7BP immunoprecipitate fractions in appreciable quantities, indicating that their association with R7BP and/or abundance in the striatum is substantially lower than that of RGS9-2 and RGS7.

We next measured the absolute amounts of RGS7 and RGS9-2, as well as their binding partners Gβ5 and R7BP in

mouse striatum, using quantitative Western blotting with recombinant protein standards. The data presented in Fig. 2B illustrate that RGS9-2, RGS7, and R7BP are present in near stoichiometric amounts (~ 4 fmol/mg of total striatal protein). At the same time, Gβ5 was twice as abundant (~ 8 fmol/mg), a finding in a close agreement with the requirement of its 1:1 association with both RGS9-2 and RGS7 and the fact that other R7 RGS proteins were found to be considerably less abundant in the striatum (Fig. 2A). These data indicate that, despite the equimolar concentration of RGS9-2/Gβ5 and RGS7/Gβ5 in the striatum, R7BP is coupled predominantly to RGS9-2.

RGS9-2 and RGS7 colocalize in the same neurons of mouse striatum. Given equal amounts of RGS9-2 and RGS7 in the striatum, the disproportionate association of R7BP preferentially with RGS9-2 called for an examination of interaction partner colocalization across striatal neurons. We used double-labeling techniques at the electron microscopic level, combining pre-embedding immunogold with HRP detection, to study the colocalization of RGS7, R7BP, and RGS9-2 in three different double-labeling experiments (Fig. 3). Consistent with previous reports, we found that RGS9-2 is localized in essentially all striatal neurons, where it colocalized with R7BP (2, 30, 44). As reported earlier, we found that RGS9-2/R7BP complexes were predominantly found on the plasma membrane of dendritic spines and shafts. Most of the RGS7 immunoreactivity was found to localize in the cells positive for RGS9-2 and R7BP (Fig. 3). Similarly to RGS9-2 and R7BP, RGS7 antibodies strongly labeled dendritic but not axonal compartments, suggesting its compartmentalization in striatal neurons. These data indicate that RGS9-2, R7BP, and RGS7 extensively overlap in their expression in the same striatal neurons and their subcellular compartments.

Recombinant R7BP has higher affinity for RGS9-2/Gβ5 than for RGS7/Gβ5. In search for the mechanisms behind the preferential complex formation of R7BP with RGS9-2, we compared the binding affinities of R7BP to RGS9-2/Gβ5 and RGS7/Gβ5 using purified recombinant proteins. Association of proteins was monitored by surface plasmon resonance technology using a BIAcore 1000 instrument. Recombinant R7BP was immobilized on the surface of a CM5 chip, which was exposed to either RGS9-2/Gβ5 or RGS7/Gβ5 complexes in solution. Performing protein-protein interaction measurements over a range of analyte concentrations yielded consistent differences in the binding constants (Fig. 4A). The affinity of R7BP for the RGS9-2/Gβ5 ($K_D = 1.1 \pm 0.4$ nM) complex was ~ 10 -fold higher than for RGS7/Gβ5 ($K_D = 12.7 \pm 2.6$ nM). We further sought to determine whether RGS9-2/Gβ5 and RGS7/Gβ5 can simultaneously interact with R7BP using competition binding experiments. We incubated GST-tagged R7BP with a fixed concentration of RGS7/Gβ5 and an increasing concentration gradient of RGS9-2/Gβ5. After pull-down with the glutathione agarose beads, the amounts of retained RGS7 and RGS9-2 were determined. The data in Fig. 4B demonstrate that increasing concentrations of RGS9-2/Gβ5 result in elevation in its binding to R7BP concomitant with the decreased association between RGS7/Gβ5 and R7BP. This result indicates that RGS9-2 and RGS7 compete for R7BP association binding to the same or overlapping determinants and yet with different affinities.

Modulation of R7BP/R7 RGS complex composition in transfected cells. The significantly different affinities of R7BP for RGS7/Gβ5 and RGS9-2/Gβ5 prompted us to analyze how a decrease in individual RGS protein concentration affected the composition of the whole complex. We addressed this issue by modeling the competitive association of R7BP with RGS9-2/Gβ5 and RGS7/Gβ5 in transfected HEK 293FT cells which do not express R7BP, Gβ5, RGS7, or RGS9-2 endogenously (Fig. 5).

Using transient transfection, we delivered a fixed amount of R7BP and Gβ5 message and either a fixed amount of RGS9-2 and variable amounts of RGS7 or a fixed amount of RGS7 and variable amounts of RGS9-2. The amount of Gβ5 construct was kept in sixfold excess to ensure that RGS proteins at all concentrations are not competing for Gβ5. Further, by using quantitative Western blotting with recombinant standards and native striatal extracts, we confirmed that RGS7 and RGS9-2 were present in equimolar concentrations when equal amounts of their expression constructs were delivered into cells (data not shown). Cellular extracts containing variable amounts of RGS7 or RGS9-2 were subjected to immunoprecipitation with anti-R7BP antibodies, and changes in the amounts of each precipitated protein were determined by Western blotting. The data presented in Fig. 5A reveal that RGS9-2 in complex with R7BP is minimally affected by changes in the concentration of RGS7, which can only decrease the abundance of RGS9-2/R7BP complexes by 20% upon reaching equimolar concentration with RGS9-2. In agreement with this estimation, the reciprocal experiment (Fig. 5B) shows that at an equal stoichiometric ratio of RGS proteins, as much as 80% of RGS9-2 is complexed with R7BP. However, unlike the model with variable RGS7, modulation of RGS9-2 concentration appears to markedly affect the extent of RGS7 coupling to R7BP. This indicates that while change in RGS7 expression minimally affects the balance in RGS9-2/R7BP complexes, changes in RGS9-2 levels cause proportionate rebalancing of R7BP coupling to RGS7.

Elimination of RGS9-2 leads to the R7BP-mediated recruitment of RGS7 to the plasma membrane. Modeling of the complex formation in transfected cells suggests that reduction of RGS9-2 in the striatum would entail upregulation in RGS7/R7BP complex formation. In order to investigate whether this is the case, we compared RGS7 coupling to R7BP between the striatal regions of wild-type and RGS9^{-/-} mice in which the expression of RGS9-2 was ablated by gene targeting. The results of the immunoprecipitation experiment presented in Fig. 6 illustrate marked enhancement of RGS7-R7BP association in the absence of RGS9-2 providing further evidence that changes in RGS9-2 expression cause remodeling of R7BP containing complexes.

To begin to analyze the consequences of this remodeling, we examined whether the loss of RGS9 affected the localization of RGS7 in the striatal neurons. Immunogold labeling indicated that in wild-type neurons most of the dendritic RGS7 is found at intracellular sites (Fig. 7A and B), and almost none of it is detected within the postsynaptic density (Fig. 7C). Quantitative analysis of 1,781 gold particles revealed that 1,478 (83%) of them were at the intracellular sites and only 303 (17%) were at the plasma membrane. Since nearly all of the R7BP was previously found associated with the plasma membrane com-

FIG. 3. RGS9-2, RGS7, and R7BP colocalize in the same cell types and subcellular compartments in mouse striata. Electron micrographs show double labeling for RGS9, RGS7, and R7BP in the mouse striata, as revealed using pre-embedding techniques. (A and B) Double-labeling approach showing colocalization for RGS9-2 and RGS7. The peroxidase reaction product (HRP), indicates RGS9 immunoreactivity-filled dendritic shafts (Den) and spines (s), whereas immunoparticles (RGS7 immunoreactivity) were mainly located along the extrasynaptic plasma membrane (e.g., arrows) and at intracellular sites (e.g., crossed arrows). (C and D) Double-labeling approach showing colocalization for RGS9-2 and R7BP. The peroxidase reaction product (HRP) indicates RGS9 immunoreactivity-filled dendritic shafts (Den) and spines (s), whereas immunoparticles (R7BP immunoreactivity) were mainly located along the extrasynaptic plasma membrane (e.g., arrows) and at intracellular sites (e.g., crossed arrows). (E and F) Double-labeling approach showing colocalization for RGS7 and R7BP. The peroxidase reaction product (HRP) indicates RGS7 immunoreactivity-filled dendritic shafts (Den) and spines (s), whereas immunoparticles (R7BP immunoreactivity) were mainly located along the extrasynaptic plasma membrane (e.g., arrows) and at intracellular sites (e.g., crossed arrows). b, axon terminal. Scale bar (A to F), 0.5 μ m.

partment, this distribution is in the close agreement with ~20% fraction of RGS7 associated with R7BP in wild-type striata (Fig. 2). In contrast, in striatal neurons of RGS9 knockout mice, only 22% (374 particles) were found at intracellular sites, and 78% (1,322 particles) were found at the plasma membrane (Fig. 7D and E) and postsynaptic densities (Fig. 7F). No significant change in RGS7 localization was observed in the striatal neurons of R7BP knockout mice (Fig. 7G and H). This result is consistent with the small extent of RGS7 association with R7BP in the presence of RGS9-2 and suggests

that it is the physical association with vacant R7BP rather than changes in the G protein signaling resulting from RGS9 elimination that are responsible for the recruitment of RGS7 to the postsynaptic plasma membrane compartments.

Multiple signaling pathways regulate RGS9-2 degradation in striatal neurons. Since RGS9-2 protein level is the determining factor for remodeling R7 RGS complexes, we investigated signaling mechanisms that are involved in the regulation of RGS9-2 posttranslational stability. The observation that RGS9-2 undergoes spontaneous degradation upon acute cul-

FIG. 4. RGS9-2/G β 5 has higher affinity for R7BP over RGS7/G β 5 and competes for its binding. (A) Surface plasmon resonance analysis of R7BP association with RGS7/G β 5 and RGS9-2/G β 5 performed on a BIAcore 1000 instrument. Purified recombinant R7BP was immobilized on the surface of a CM5 chip and exposed to various concentrations of RGS7/G β 5 (25, 50, 65, 80, and 100 nM) or RGS9-2 (10, 25, 50, 65, and 80 nM). Analyte solutions were injected at a 10 μ l/min rate for 1 min and analyzed for RGS/R7BP association rates, as were the dissociation rates during the first minute after the RGS solution injection was stopped. Each plotted curve is representative of duplicate repetition. The K_D values, calculated using Biacore evaluation software, were 1.1 nM $\pm$ 0.4 nM (standard deviation) for R7BP binding to RGS9-2/G β 5 and 12.7 nM $\pm$ 2.6 nM for R7BP interaction with RGS7/G β 5. (B) RGS7/G β 5 and RGS9-2/G β 5 compete for binding to R7BP. A 16 nM concentration of immobilized GST-R7BP bait was incubated with a fixed concentration of RGS7/G β 5 (55 nM) and various concentrations of RGS9-2/G β 5 (0, 16, 32, 95, 320, 475, and 800 nM). The beads were washed, and bound proteins were analyzed by Western blotting. The right panel represents the quantification of the protein densities normalized to maximal binding in the absence of RGS/G β 5 and plotted as a function of RGS/G β 5 concentration.

turing of striatal slices under standard conditions prompted us to modify the culture conditions in order to minimize RGS9-2 loss. We found that saturating the medium with 95% oxygen was very effective for maintaining RGS9-2 stability, reducing

the rate of its degradation to only 10% per hour (Fig. 8A). Therefore, we used the culturing system with constant oxygenation for subsequent pharmacological manipulations.

We reasoned that increased degradation rate of RGS9-2

FIG. 5. Modeling of competitive association of R7BP with RGS9-2/G β 5 and RGS7/G β 5 complexes in transfected cells. (A) HEK 293FT cells were transfected with equal amounts of R7BP and RGS9-2 expression constructs (0.2 μ g) and various amounts of RGS7 plasmid. Sixfold excess of G β 5 expression construct was used (1.2 μ g) to ensure that its concentration does not limit RGS protein expression. The actual levels of expressed RGS proteins (left panels) were determined by quantitative IR Western blotting. The equality of RGS9-2 and RGS7 protein levels at the maximal concentration of RGS7 construct was verified by comparing band densities to recombinant protein standards and brain extracts (results not shown). Cells were lysed in 1% Triton and incubated with 3 μ g of sheep anti-R7BP antibody. Eluates were subjected to SDS-PAGE separation, followed by Western blot analysis with specific and IR-conjugated secondary antibodies (right panels). Images were scanned on Odyssey IR imager, and band densities were quantified by using Odyssey 2.0 software. The densities of RGS9-2 bands were normalized against those of R7BP and used to define the y axis, with 100% reflecting the maximum band density in the absence of RGS7. The ratios of the actual RGS7 and RGS9-2 band densities present in total lysates before immunoprecipitation (left panels) were used to define the x axis. (B) An inverse experiment was performed with constant RGS7 expression construct and various RGS9-2 amounts. The protein levels in inputs and immunoprecipitation eluates were determined exactly as described for panel A. The experiments shown on both panels are representative out of at least three conducted and yielding similar results.

FIG. 6. Complexes of RGS7 with R7BP are upregulated in the striata of RGS9 knockout mice. Striatal regions were dissected from C57BL/6 wild-type (WT), RGS9 knockout (RGS9^{-/-}), and R7BP knockout (R7BP^{-/-}) mice; lysed in 1% Triton; and used to determine the total protein concentration. Aliquots (20 μ g) of each lysate were separated by SDS-PAGE and analyzed by Western blotting (left panels, "input"). Equal amounts of lysates were subjected to immunoprecipitation with 5 μ g of sheep anti-R7BP antibody. Immunoprecipitated proteins were analyzed by Western blotting. The experiment shown is representative of three conducted that yielded similar results.

protein upon culture could be caused by uncontrollable neuronal activity exacerbated by insufficient oxygenation. Indeed, stimulating neuronal firing by either application of 30 mM KCl, which induces membrane depolarization, or application of 50 μ M bicuculline, which inhibits inhibitory GABA transmission and thus indirectly stimulates synaptic transmission, caused marked RGS9-2 destabilization, essentially abrogating a protective effect of high oxygen concentration (Fig. 8B). Because a rise in the intracellular calcium concentration is the hallmark of increased neuronal activity, we next investigated whether RGS9-2 instability could be mediated by the calcium entry. Preventing extracellular calcium entry by removing it from the medium completely blocked not only depolarization-induced but also residual spontaneous RGS9-2 degradation. Blocking a major class of voltage gated calcium channels (L-type VGCC) by specific inhibitor nimodipine also abolished depolarization-induced RGS9-2 loss, suggesting that the increase in the intracellular calcium entering through plasma membrane calcium channels is the major signaling event that triggers RGS9-2 degradation. Finally, we found that activation of protein kinase C (PKC), a major downstream effector influenced by calcium entry, directly enhances RGS9-2 loss, indicating that PKC plays a critical role in controlling RGS9-2 stability.

DISCUSSION

The main finding of this study is that complexes of neuronal R7 RGS proteins with their subunits R7BP and G β 5 undergo remodeling of their composition in response to changes in neuronal signaling. Striatal neurons express equal amounts of two R7 RGS proteins: RGS9-2 and RGS7, both forming equimolar complexes with G β 5 subunit. The third subunit in the complex, R7BP, is unequally distributed and forms preferential complexes with RGS9-2 owing to the order-of-magnitude higher affinity over RGS7. An intrinsic proteolytic instability of RGS9-2/G β 5 in the absence of R7BP binding further shifts this balance, driving virtually all RGS9-2/G β 5 to be present as a ternary complex with R7BP, while largely leaving RGS7 a dimer with only G β 5. Remarkably, this equilibrium undergoes dramatic rearrangement when R7BP uncouples

from RGS9-2/G β 5 complex, targeting RGS9-2 for degradation and funneling R7BP into complex formation with RGS7/G β 5, generating nascent RGS7/G β 5/R7BP trimer (Fig. 9). R7BP, because of its proteolytically labile nature, requires association with R7 RGS proteins to maintain stable expression (2, 18), which further ensures the high efficiency of this complex remodeling process. Indeed, while completely absent in the striata of G β 5 knockouts lacking both RGS7 and RGS9-2 (2), the levels of R7BP are not altered upon RGS9-2 elimination (2 and Fig. 5) providing further support to the large extent of R7BP switching from RGS9-2 to RGS7.

Changes in subunit composition of the macromolecular complexes are a fundamental mechanism that underscores the plasticity of many biological processes including chromatin remodeling (33), immune cell differentiation (19), proteasome-mediated protein degradation (21), synaptic activity (41, 49, 54), gene transcription (59), and many others. Although the tremendous significance of this organizational flexibility is undoubted, much remains to be learned about their underlying mechanisms. In the present study, we describe a mechanism for rapid posttranslational changes in subunit composition of G protein signaling regulators which could serve as a general model for understanding remodeling principles of many complexes build from proteolytically labile components. In a proposed three-way equilibrium model, a stable protein complex of three unstable subunits (ABC) donates one of the subunits (A) to the alternatively configured stable dimer (DC), while the remaining unstable components (BC) undergo selective degradation. These events allow for the rapid shift in subunit composition from the basal state of ABC to a new ADC state in a condition-dependent manner and could be easily reversed by events that promote stabilization of BC subunits, thus providing a point for regulatory input.

Our results indicate that remodeling of trimeric R7 RGS complexes is triggered by changes that affect the binding of RGS9-2/G β 5 to R7BP. More specifically, we found that it is RGS9-2 that is modified in a way that renders it unable to associate with R7BP, targeting it for degradation. Although the molecular identity of the modification(s) remains unknown at the moment, several changes in neuronal signaling status appear to specifically modulate RGS9-2/R7BP coupling and the resultant RGS9-2 degradation. Our pharmacological data argue for the involvement of at least two major mechanisms: oxygen sensing and synaptic activity along voltage-gated Ca²⁺ channels–PKC axis. The disruption in cellular Ca²⁺ homeostasis appears to be the common element unifying both mechanisms. Indeed, changes in neuronal signaling and excitotoxicity is a well-documented occurrence that accompanies hypoxia/ischemia in the brain (13). One of the major mechanisms contributing to neuronal excitotoxicity associated with increased neuronal excitability under hypoxic stress is calcium dysregulation. Hypoxic stress leads to elevated Ca²⁺ influx, release from intracellular Ca²⁺ stores, and generalized disruption in Ca²⁺ buffering, resulting in constant activation of tightly regulated signaling molecules (i.e., PKC), which can ultimately culminate in neuronal injury and subsequent long-term disabilities for stroke victims (5, 61). However, it is also possible that oxygen sensing and changes in neuronal excitability are separate mechanisms that converge only at the level of modulating RGS9-2/R7BP coupling. In any case, our findings

FIG. 7. Loss of RGS9-2 but not R7BP induces changes in RGS9 localization in striatal neurons. Electron micrographs showing immunoreactivity for RGS7 in wild-type (WT), RGS9 knockout (RGS9 KO), and R7BP knockout (R7BP KO) mice in the striatum, as revealed using pre-embedding (A, B, D, E, G, and H) and postembedding (C and F) immunogold techniques. Using the pre-embedding immunogold method, immunoparticles for RGS7 in wild-type neurons (A and B) were mainly located at intracellular sites (e.g., crossed arrows), associated with intracellular membranes within dendritic shafts (Den) and spines (s) establishing excitatory synapses with axon terminals (b). In less proportion, immunoparticles for RGS7 were also detected along the plasma membrane (e.g., arrows). Using the postembedding immunogold method (C), immunoparticles for RGS7 in wild-type neurons were mainly located at intracellular sites (e.g., crossed arrows) and occasionally along the postsynaptic density of excitatory synapses (e.g., arrowheads). Using the pre-embedding immunogold method, immunoparticles for RGS7 in RGS9 KO neurons (D and E) were mainly located along the plasma membrane (e.g., arrows) of dendritic shafts (Den) and spines (s) establishing excitatory synapses with axon terminals (b). In less proportion, immunoparticles for RGS7 were also detected at intracellular sites (e.g., crossed arrows), associated with intracellular membranes. Using the postembedding immunogold method (F), immunoparticles for RGS7 in the RGS9 KO were mainly located along the postsynaptic density of excitatory synapses (e.g., arrowheads) and occasionally at intracellular sites (e.g., crossed arrows). RGS7 immunoreactivity in R7BP KO neurons (G and H) replicated the pattern observed in wild-type samples. That is, immunoparticles for RGS7 in the R7BP KO were mainly located along the plasma membrane (e.g., arrows) of dendritic shafts (Den) and spines (s), establishing excitatory synapses with axon terminals (b). In less proportion, immunoparticles for RGS7 were also detected at intracellular sites (e.g., crossed arrows), associated with intracellular membranes. Scale bar: A, B, D, E, G, and H, 0.5 μ m; C and F, 0.2 μ m.

strongly suggest that changes in R7 RGS complex composition is another molecular hallmark of changes induced by oxygen deprivation and excessive synaptic activity.

It is interesting to consider the observed degradation-triggered remodeling in the context of the recent finding that R7BP/RGS9-2 complexes are enriched in postsynaptic densities of excitatory striatal synapses (2). A formidable body of evidence implicates dynamic regulation of postsynaptic density components as a central mechanism underlying the plasticity of synaptic communication between neurons (reviewed in reference 52). Studies in mice have shown that both elimination and overexpression of RGS9-2 affect the sensitivity of the μ -opioid

and D2 dopamine receptor signaling and influence movement control and reward processing, two highly adaptable functions of the striatum. Here, we report that fluctuations in RGS9-2 levels result in an unexpected molecular consequence: change in the targeting of RGS7/G β 5/R7BP GAP complex to the postsynaptic density. It is tempting to speculate that these changes in subunit composition and localization of the major G protein signaling regulator in the region underlie activity-dependent modulation of the opioid and dopamine systems in striatal neurons and thus could be viewed as a molecular substrate of synaptic plasticity. Indeed, levels of RGS9-2 expression have been reported to be sensitive to a number of manip-

FIG. 8. Oxygenation status, neuronal activity, and extracellular calcium entry regulates RGS9-2 protein levels. (A) Overt RGS9-2 degradation upon striatal slice culturing can be slowed down by increasing oxygenation. Coronal brain slices were prepared and cut in half down the midline. One side of the section served as control, with striatal tissue isolated and directly frozen in liquid nitrogen. The contralateral section was subsequently cultured for 60 min in ACSF at atmospheric oxygen levels, 5% CO₂ (Low O₂), or ACSF bubbled with 95% O₂ and 5% CO₂ (High O₂) prior to analysis. Subsequently, the tissue was lysed in 1% Triton, and equal amounts of lysates were subjected to Western blot analysis using RGS9-2 specific antibody. Upon incubation with IRDye-conjugated secondary antibodies, images were scanned on an Odyssey IR imager. The band densities were quantified by using Odyssey 2.0 software, and the cultured tissue RGS9-2 percent loss was calculated compared to the uncultured tissue control band density ($n = 8$ to 12). (B) Neuronal depolarization initiates RGS9-2 loss in an extracellular-calcium-dependent fashion. All striatal coronal sections were prepared similarly as in panel A, cultured for 60 min in ACSF with the indicated medium modification or pharmacological treatment, and actively bubbled with 95% O₂–5% CO₂. Western blot analysis and the percent RGS9-2 loss quantitation was performed as in panel A and plotted as cumulative results ($n = 8$ to 12).

ulations that alter opioid and dopamine signaling in the region (44, 51, 56, 62) and are therefore expected to cause remodeling of RGS complexes. In this light, it appears noteworthy that major determinants of various forms of neuronal plasticity, such as Ca²⁺ influx (11) and oxygen deprivation (7), also regulate the homeostasis of R7 RGS complexes. Furthermore, alterations in the activity of the proteolytic machinery have been implicated as the earliest and most rapid mechanism utilized in neurons as a part of the biochemical and structural remodeling that occurs in response to changes in synaptic activity. Both the ubiquitin-proteasome system (38) and the lysosomal autophagy (1) pathways have been shown to play a key role in the rapid synaptic remodeling that is observed with ischemic tolerance. A connection to the lysosomal degradation mechanisms is particularly intriguing because degradation of RGS9-2/Gβ5 dissociated from R7BP was shown to be mediated by cysteine proteases, major proteolytic enzymes of the lysosomes (2).

Finally, we want to discuss the potential consequences of the described R7 RGS complex remodeling on striatal signaling,

FIG. 9. Proposed model for activity-dependent regulation of R7 RGS subunit composition. Under basal conditions, striatal neurons contain RGS9-2/Gβ5/R7BP trimer located at the postsynaptic sites and RGS7/Gβ5 dimer located intracellularly. Several signaling events can trigger RGS9-2 uncoupling from R7BP targeting RGS9-2/Gβ5 complex for degradation. Released R7BP becomes available for RGS7/Gβ5 binding, resulting in the production of RGS7/Gβ5/R7BP trimer, which undergoes targeting to the postsynaptic density. This process is sensitive to oxygenation status and the level of neuronal excitability and is regulated by changes in calcium influx and PKC activation.

the extreme case of which is observed in RGS9 knockout mice, where all of R7BP is recoupled to RGS7. First, relocation of RGS7 to the postsynaptic sites is expected to reduce its regulatory influence on the G proteins located at the intracellular sites. Second, binding of R7BP to RGS7/Gβ5 has been shown to negatively affect the capacity of the RGS7/Gβ5 dimer to dampen Gα_q-mediated signaling (39), suggesting that the remodeling would also lead to an enhancement in Gα_q signaling events. Third, it has been demonstrated that RGS9-2 and RGS7 proteins differ in their substrate selectivity for members of the Gi/o family of proteins, with RGS9 being more potent than RGS7 in regulating the GTPase activity of Gα_i (26). This is expected to lead to the selective reduction in regulation of the Gα_i-mediated signaling pathways at the postsynaptic density. Taken together, the R7 RGS complex remodeling upon a decrease in the RGS9-2 concentration suggests that global

changes in G protein inactivation specificity would occur, rather than a simple decrease in RGS9-2 specific GAP activity in the striatum. These global changes in signaling events may in fact occur in RGS9 knockout mice, where increased behavioral responses to the stimulation of μ -opioid and D2 dopamine receptors (44, 50, 62) might stem from the global remodeling rather than simple loss of RGS9-2's GAP activity. This model and its role in the sensitization of μ -opioid and D2 dopamine receptors observed in RGS9-2 knockout mice therefore requires further investigation. It is clear, however, that changes in multiple signaling pathways beyond that normally controlled by RGS9-2 alone occurs upon its elimination, providing additional complexity to the molecular mechanisms that determine basal ganglial responses to the action of psychostimulants and addictive drugs.

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REFERENCES

- Adhami, F., A. Schloemer, and C. Y. Kuan. 2007. The roles of autophagy in cerebral ischemia. *Autophagy* 3:42–44.
- Anderson, G. R., R. Lujan, A. Semenov, M. Pravetoni, E. N. Posokhova, J. H. Song, V. Uversky, C. K. Chen, K. Wickman, and K. A. Martemyanov. 2007. Expression and localization of RGS9-2/G 5/R7BP complex in vivo is set by dynamic control of its constitutive degradation by cellular cysteine proteases. *J. Neurosci.* 27:14117–14127.
- Anderson, G. R., A. Semenov, J. H. Song, and K. A. Martemyanov. 2007. The membrane anchor R7BP controls the proteolytic stability of the striatal specific RGS protein, RGS9-2. *J. Biol. Chem.* 282:4772–4781.
- Blundell, J., C. V. Hoang, B. Potts, S. J. Gold, and C. M. Powell. 2008. Motor coordination deficits in mice lacking RGS9. *Brain Res.* 1190:78–85.
- Bright, R., and D. Mochly-Rosen. 2005. The role of protein kinase C in cerebral ischemic and reperfusion injury. *Stroke* 36:2781–2790.
- Cabrera, J. L., F. De Freitas, D. K. Satpaev, and V. Z. Slepak. 1998. Identification of the Gβ5-RGS7 protein complex in the retina. *Biochem. Biophys. Res. Commun.* 249:898–902.
- Calabresi, P., D. Centonze, A. Pisani, L. Cupini, and G. Bernardi. 2003. Synaptic plasticity in the ischaemic brain. *Lancet Neurol.* 2:622–629.
- Cheever, M. L., J. T. Snyder, S. Gershbarg, D. P. Siderovski, T. K. Harden, and J. Sondek. 2008. Crystal structure of the multifunctional Gβ5-RGS9 complex. *Nat. Struct. Mol. Biol.* 15:155–162.
- Chen, C. K., M. E. Burns, W. He, T. G. Wensel, D. A. Baylor, and M. I. Simon. 2000. Slowed recovery of rod photoreponse in mice lacking the GTPase accelerating protein RGS9-1. *403:557–560.*
- Chen, C. K., P. Eversole-Cire, H. K. Zhang, V. Mancino, Y. J. Chen, W. He, T. G. Wensel, and M. I. Simon. 2003. Instability of GGL domain-containing RGS proteins in mice lacking the G protein β-subunit Gβ5. *Proc. Natl. Acad. Sci. USA* 100:6604–6609.
- Citri, A., and R. C. Malenka. 2008. Synaptic plasticity: multiple forms, functions, and mechanisms. *Neuropsychopharmacology* 33:18–41.
- Cowan, C. W., W. He, and T. G. Wensel. 2000. RGS proteins: lessons from the RGS9 subfamily. *Prog. Nucleic Acid Res. Mol. Biol.* 65:341–359.
- Dirnagl, U., C. Iadecola, and M. A. Moskowitz. 1999. Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci.* 22:391–397.
- Drenan, R. M., C. A. Doupnik, M. P. Boyle, L. J. Muglia, J. E. Huettner, M. E. Linder, and K. J. Blumer. 2005. Palmitoylation regulates plasma membrane-nuclear shuttling of R7BP, a novel membrane anchor for the RGS7 family. *J. Cell Biol.* 169:623–633.
- Garzon, J., A. Lopez-Fando, and P. Sanchez-Blazquez. 2003. The R7 subfamily of RGS proteins assists tachyphylaxis and acute tolerance at μ -opioid receptors. *Neuropsychopharmacology* 28:1983–1990.
- Garzon, J., M. Rodriguez-Diaz, A. Lopez-Fando, and P. Sanchez-Blazquez. 2001. RGS9 proteins facilitate acute tolerance to μ -opioid effects. *Eur. J. Neurosci.* 13:801–811.
- Gold, S. J., Y. G. Ni, H. G. Dohlman, and E. J. Nestler. 1997. Regulators of G-protein signaling (RGS) proteins: region-specific expression of nine subtypes in rat brain. *J. Neurosci.* 17:8024–8037.
- Grabowska, D., M. Jayaraman, K. M. Kaltenbronn, S. L. Sandiford, Q. Wang, S. Jenkins, V. Z. Slepak, Y. Smith, and K. J. Blumer. 2008. Postnatal induction and localization of R7BP, a membrane-anchoring protein for regulator of G protein signaling 7 family-Gβ5 complexes in brain. *Neuroscience* 151:969–982.
- Grumot, R. J., and S. Gerondakis. 1994. The subunit composition of NF- κ B complexes changes during B-cell development. *Cell Growth Differ.* 5:1321–1331.
- Hamzah, J., M. Jugold, F. Kiessling, P. Rigby, M. Manzur, H. H. Marti, T. Rabie, S. Kaden, H. J. Grone, G. J. Hammerling, B. Arnold, and R. Ganss. 2008. Vascular normalization in Rgs5-deficient tumours promotes immune destruction. *Nature* 453:410–414.
- Hanna, J., and D. Finley. 2007. A proteasome for all occasions. *FEBS Lett.* 581:2854–2861.
- He, W., C. W. Cowan, and T. G. Wensel. 1998. RGS9, a GTPase accelerator for phototransduction. *Neuron* 20:95–102.
- He, W., L. S. Lu, X. Zhang, H. M. El Hodiri, C. K. Chen, K. C. Slep, M. I. Simon, M. Jamrich, and T. G. Wensel. 2000. Modules in the photoreceptor RGS9-1.Gβ5L GTPase-accelerating protein complex control effector coupling, GTPase acceleration, protein folding, and stability. *J. Biol. Chem.* 275:37093–37100.
- Hess, H. A., J. C. Roper, S. W. Grill, and M. R. Koelle. 2004. RGS-7 completes a receptor-independent heterotrimeric G protein cycle to asymmetrically regulate mitotic spindle positioning in *Caenorhabditis elegans*. *Cell* 119:209–218.
- Hollinger, S., and J. R. Hepler. 2002. Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. *Pharmacol. Rev.* 54:527–559.
- Hooks, S. B., G. L. Waldo, J. Corbitt, E. T. Bodor, A. M. Krumins, and T. K. Harden. 2003. RGS6, RGS7, RGS9, and RGS11 stimulate GTPase activity of Gi family G-proteins with differential selectivity and maximal activity. *J. Biol. Chem.* 278:10087–10093.
- Hu, G., and T. G. Wensel. 2002. R9AP, a membrane anchor for the photoreceptor GTPase accelerating protein, RGS9-1. *Proc. Natl. Acad. Sci. USA* 99:9755–9760.
- Jones, M. B., D. P. Siderovski, and S. B. Hooks. 2004. The Gβγ dimer as a novel source of selectivity in G-protein signaling: GGL-ing at convention. *Mol. Interv.* 4:200–214.
- Keresztes, G., K. A. Martemyanov, C. M. Krispel, H. Mutai, P. J. Yoo, S. F. Maison, H. A. Burns, V. Y. Arshavsky, and S. Heller. 2004. Absence of the RGS9Gβ5 GTPase-activating complex in photoreceptors of the R9AP knockout mouse. *J. Biol. Chem.* 279:1581–1584.
- Kovoor, A., P. Seyfarth, J. Ebert, S. Barghshoon, C. K. Chen, S. Schwarz, J. D. Axelrod, B. N. Cheyette, M. I. Simon, H. A. Lester, and J. Schwarz. 2005. D2 dopamine receptors colocalize regulator of G-protein signaling 9-2 (RGS9-2) via the RGS9 DEP domain, and RGS9 knockout mice develop dyskinesias associated with dopamine pathways. *J. Neurosci.* 25:2157–2165.
- Krispel, C. M., D. Chen, N. Melling, Y. J. Chen, K. A. Martemyanov, N. Quillinan, V. Y. Arshavsky, T. G. Wensel, C. K. Chen, and M. E. Burns. 2006. RGS expression rate-limits recovery of rod photoreponses. *Neuron* 51:409–416.
- Lee, E., M. E. Linder, and A. G. Gilman. 1994. Expression of G-protein α subunits in *Escherichia coli*. *Methods Enzymol.* 237:146–164.
- Lessard, J., J. I. Wu, J. A. Ranish, M. Wan, M. M. Winslow, B. T. Staahl, H. Wu, R. Aebersold, I. A. Graef, and G. R. Crabtree. 2007. An essential switch in subunit composition of a chromatin remodeling complex during neural development. *Neuron* 55:201–215.
- Lujan, R., Z. Nusser, J. D. Roberts, R. Shigemoto, and P. Somogyi. 1996. Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. *Eur. J. Neurosci.* 8:1488–1500.
- Makino, E. R., J. W. Handy, T. S. Li, and V. Y. Arshavsky. 1999. The GTPase activating factor for transducin in rod photoreceptors is the complex between RGS9 and type 5 G protein β subunit. *Proc. Natl. Acad. Sci. USA* 96:1947–1952.
- Martemyanov, K. A., P. V. Lishko, N. Calero, G. Keresztes, M. Sokolov, K. J. Strissel, I. B. Leskov, J. A. Hopp, A. V. Kolesnikov, C. K. Chen, J. Lem, S. Heller, M. E. Burns, and V. Y. Arshavsky. 2003. The DEP domain determines subcellular targeting of the GTPase activating protein RGS9 in vivo. *J. Neurosci.* 23:10175–10181.
- Martemyanov, K. A., P. J. Yoo, N. P. Skiba, and V. Y. Arshavsky. 2005. R7BP, a novel neuronal protein interacting with RGS proteins of the R7 family. *J. Biol. Chem.* 280:5133–5136.
- Meller, R., S. J. Thompson, T. A. Lusardi, A. N. Ordenez, M. D. Ashley, V.

- Jessick, W. Wang, D. J. Torrey, D. C. Henshall, P. R. Gafken, J. A. Saugstad, Z. G. Xiong, and R. P. Simon. 2008. Ubiquitin proteasome-mediated synaptic reorganization: a novel mechanism underlying rapid ischemic tolerance. *J. Neurosci.* **28**:50–59.
39. Narayanan, V., S. L. Sandiford, Q. Wang, T. Keren-Raifman, K. Levay, and V. Z. Slepak. 2007. Intramolecular interaction between the DEP domain of RGS7 and the G β 5 subunit. *Biochemistry* **46**:6859–6870.
40. Nekrasova, E. R., D. M. Berman, R. R. Rustandi, H. E. Hamm, A. G. Gilman, and V. Y. Arshavsky. 1997. Activation of transducin guanosine triphosphatase by two proteins of the RGS family. *Biochemistry* **36**:7638–7643.
41. Paoletti, P., and J. Neyton. 2007. NMDA receptor subunits: function and pharmacology. *Curr. Opin. Pharmacol.* **7**:39–47.
42. Pugh, E. N., Jr. 2006. RGS expression level precisely regulates the duration of rod photoresponses. *Neuron* **51**:391–393.
43. Rahman, Z., S. J. Gold, M. N. Potenza, C. W. Cowan, Y. G. Ni, W. He, T. G. Wensel, and E. J. Nestler. 1999. Cloning and characterization of RGS9-2: a striatal-enriched alternatively spliced product of the RGS9 gene. *J. Neurosci.* **19**:2016–2026.
44. Rahman, Z., J. Schwarz, S. J. Gold, V. Zachariou, M. N. Wein, K. H. Choi, A. Kovoar, C. K. Chen, R. J. DiLeone, S. C. Schwarz, D. E. Selley, L. J. Sim-Selley, M. Barrot, R. R. Luedtke, D. Self, R. L. Neve, H. A. Lester, M. I. Simon, and E. J. Nestler. 2003. RGS9 modulates dopamine signaling in the basal ganglia. *Neuron* **38**:941–952.
45. Rao, A., R. Dallman, S. Henderson, and C. K. Chen. 2007. Gbeta5 is required for normal light responses and morphology of retinal ON-bipolar cells. *J. Neurosci.* **27**:14199–14204.
46. Ross, E. M., and T. M. Wilkie. 2000. GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu. Rev. Biochem.* **69**:795–827.
47. Saitoh, O., Y. Kubo, Y. Miyatani, T. Asano, and H. Nakata. 1997. RGS8 accelerates G-protein-mediated modulation of K⁺ currents. *Nature* **390**:525–529.
48. Sanchez-Blazquez, P., M. Rodriguez-Diaz, A. Lopez-Fando, M. Rodriguez-Munoz, and J. Garzon. 2003. The G β 5 subunit that associates with the R7 subfamily of RGS proteins regulates mu-opioid effects. *Neuropharmacology* **45**:82–95.
49. Schmid, A., S. Hallermann, R. J. Kittel, O. Khorramshahi, A. M. Frolich, C. Quentin, T. M. Rasse, S. Mertel, M. Heckmann, and S. J. Sigrist. 2008. Activity-dependent site-specific changes of glutamate receptor composition in vivo. *Nat. Neurosci.* **11**:659–666.
50. Seeman, P., J. Schwarz, J. F. Chen, H. Szechtman, M. Perreault, G. S. McKnight, J. C. Roder, R. Quirion, P. Boksa, L. K. Srivastava, K. Yanai, D. Weinshenker, and T. Sumiyoshi. 2006. Psychosis pathways converge via D2high dopamine receptors. *Synapse* **60**:319–346.
51. Sharifi, J. L., D. L. Brady, and J. I. Koenig. 2004. Estrogen modulates RGS9 expression in the nucleus accumbens. *Neuroreport* **15**:2433–2436.
52. Sheng, M., and C. C. Hoogenraad. 2007. The postsynaptic architecture of excitatory synapses: a more quantitative view. *Annu. Rev. Biochem.* **76**:823–847.
53. Song, J. H., H. Song, T. G. Wensel, M. Sokolov, and K. A. Martemyanov. 2007. Localization and differential interaction of R7 RGS proteins with their membrane anchors R7BP and R9AP in neurons of vertebrate retina. *Mol. Cell Neurosci.* **35**:311–319.
54. Sprengel, R. 2006. Role of AMPA receptors in synaptic plasticity. *Cell Tissue Res.* **326**:447–455.
55. Tang, M., G. Wang, P. Lu, R. H. Karas, M. Aronovitz, S. P. Heximer, K. M. Kaltenbrunn, K. J. Blumer, D. P. Siderovski, Y. Zhu, and M. E. Mendelsohn. 2003. Regulator of G-protein signaling-2 mediates vascular smooth muscle relaxation and blood pressure. *Nat. Med.* **9**:1506–1512.
56. Tekumalla, P. K., F. Calon, Z. Rahman, S. Birdi, A. H. Rajput, O. Hornykiewicz, T. Di Paolo, P. J. Bedard, and E. J. Nestler. 2001. Elevated levels of DeltaFosB and RGS9 in striatum in Parkinson's disease. *Biol. Psychiatry* **50**:813–816.
57. Thomas, E. A., P. E. Danielson, and J. G. Sutcliffe. 1998. RGS9: a regulator of G-protein signalling with specific expression in rat and mouse striatum. *J. Neurosci. Res.* **52**:118–124.
58. Ting, T. D., S. B. Goldin, and Y.-K. Ho. 1993. Purification and characterization of bovine transducin and its subunits. *Methods Neurosci.* **15**:180–195.
59. Verma, S., Y. Xiong, M. U. Mayer, and T. C. Squier. 2007. Remodeling of the bacterial RNA polymerase supramolecular complex in response to environmental conditions. *Biochemistry* **46**:3023–3035.
60. Witherow, D. S., Q. Wang, K. Levay, J. L. Cabrera, J. Chen, G. B. Willars, and V. Z. Slepak. 2000. Complexes of the G protein subunit G β 5 with the regulators of G protein signaling RGS7 and RGS9: characterization in native tissues and in transfected cells. *J. Biol. Chem.* **275**:24872–24880.
61. Yao, H., and G. G. Haddad. 2004. Calcium and pH homeostasis in neurons during hypoxia and ischemia. *Cell Calcium* **36**:247–255.
62. Zachariou, V., D. Georgescu, N. Sanchez, Z. Rahman, R. DiLeone, O. Bertoni, R. L. Neve, L. J. Sim-Selley, D. E. Selley, S. J. Gold, and E. J. Nestler. 2003. Essential role for RGS9 in opiate action. *Proc. Natl. Acad. Sci. USA* **100**:13656–13661.
63. Zhang, K., K. A. Howes, W. He, J. D. Bronson, M. J. Pettenati, C. K. Chen, K. Palczewski, T. G. Wensel, and W. Baehr. 1999. Structure, alternative splicing, and expression of the human RGS9 gene. *Gene* **240**:23–34.
64. Zheng, B., L. De Vries, and M. G. Farquhar. 1999. Divergence of RGS proteins: evidence for the existence of six mammalian RGS subfamilies. *Trends Biochem. Sci.* **24**:411–414.