Published in final edited form as:
Cell Biochem Biophys. 2009 ; 54(1-3): 33–46. doi:10.1007/s12013-009-9052-9.

THE R7 RGS PROTEIN FAMILY: MULTI-SUBUNIT REGULATORS OF NEURONAL G PROTEIN SIGNALING

Garret R. Anderson, **Ekaterina Posokhova**, and **Kirill A. Martemyanov***
From the Department of Pharmacology, University of Minnesota, Minneapolis, MN 55455 USA

Abstract

G protein-coupled receptor (GPCR) signaling pathways mediate the transmission of signals from the extracellular environment to the generation of cellular responses, a process that is critically important for neurons and neurotransmitter action. The ability to promptly respond to rapidly changing stimulation requires timely inactivation of G proteins, a process controlled by a family of specialized proteins known as regulators of G protein signaling (RGS). The R7 group of RGS proteins (R7 RGS) has received special attention due to their pivotal roles in the regulation of a range of crucial neuronal processes such as vision, motor control, reward behavior and nociception in mammals. Four proteins in this group: RGS6, RGS7, RGS9 and RGS11 share a common molecular organization of three modules: (i) the catalytic RGS domain, (ii) a GGL domain that recruits $G\beta_5$, an outlying member of the G protein beta subunit family, and (iii) a DEP/DHEX domain that mediates interactions with the membrane anchor proteins R7BP and R9AP. As heterotrimeric complexes, R7 RGS proteins not only associate with and regulate a number of G protein signaling pathway components, but have also been found to form complexes with proteins that are not traditionally associated with G protein signaling. This review summarizes our current understanding of the biology of the R7 RGS complexes including their structure/functional organization, protein-protein interactions and physiological roles.

INTRODUCTION

The role of RGS proteins in setting the timing of G protein signaling

G protein signaling pathways are ubiquitous systems that mediate the transmission of signals from the extracellular environment to generate cellular responses. In these pathways, propagation of a signal from plasma membrane receptors to effectors is mediated by molecular switches known as heterotrimeric G proteins (1,2). In the prototypical sequence of events, G protein-coupled receptors (GPCRs) are activated by ligand binding, which catalyzes GDP/GTP exchange on many Gα protein molecules. Upon GTP binding, Gα-GTP and Gβγ subunits dissociate from one another, and both proceed to activate or inhibit a variety of downstream signaling molecules (ranging from enzymes that regulate second messenger homeostasis to ion channels and protein kinases) that are collectively referred to as effectors (reviewed in (3,4)). Thus, a cellular response is elicited by modulation of the activity of an effector molecule by G protein subunits. The extent of effector activity regulation, and consequently the magnitude and duration of the response, depends on how long the G proteins stay in the activated state. Processes that inactivate G proteins therefore play critical roles in shaping the kinetics of the response. The first recognized molecular events that contribute to the inactivation of G protein signaling were those that lead to GPCR desensitization, including phosphorylation by receptor kinases, binding of arrestin molecules and internalization via endocytosis (reviewed in (5)).

***Address correspondence to**: Dr. Kirill Martemyanov, Department of Pharmacology, University of Minnesota, 6-120 Jackson Hall, 321 Church St. SE Minneapolis, MN 55455, Phone: (612) 626-5309, Fax: (612) 625-8408, martemyanov@umn.edu.

Currently well accepted, these reactions represent powerful mechanisms for limiting G protein activation during sustained stimulation of GPCRs. Controlling G protein activation can be further modulated by controlling the inactivation of G protein subunits, which occurs when the Gα subunit hydrolyzes GTP and its inactive GDP-bound state re-associates with Gβγ subunits (6). Although Gαsubunits can hydrolyze GTP and self-inactivate, this process is rather slow and does not account for the fast deactivation kinetics observed under physiological conditions (discussed in (7)). Timely inactivation of G proteins is controlled by a specialized family of proteins classified as regulators of G protein signaling (RGSs). Comprising more than 30 members, RGS proteins act to accelerate the rate of GTP hydrolysis of G protein α subunits (8–10). This activity makes RGS proteins key elements that determine the lifetime of the activated G proteins in the cell, thus determining the overall duration of the response to GPCR activation. The importance of RGS proteins in regulating the magnitude of cellular reactions within an organism is underscored by a number of studies with genetic mouse models either deficient in genes encoding individual RGS proteins (11–18) or carrying G proteins insensitive to RGS action (19). These mouse models often suffer from a range of dysfunctions that severely affect most systems in the organism. Furthermore, recent evidence suggests that the activity of RGS proteins may in fact be a rate-limiting step in the termination of G protein-mediated responses in a similar way to that of the visual signal transduction pathway in retinal photoreceptors (20). In this context, understanding the mechanisms that regulate RGS protein function will provide critical insight into how the timing of G protein-mediated cellular reactions is achieved.

Regulation of G protein signaling in the nervous system and the R7 group of the RGS family

Perhaps one of the most impressive features of G protein signaling in neuronal cells is the exquisite timing of signaling events. Neurons heavily rely on GPCR pathways for mediating neurotransmitter action, requiring simultaneous processing of multiple incoming signals in a rapid timeframe and in a constantly changing environment (reviewed in (21)). In many cases, changes in the precise timing of these signaling events lead to a range of grave dysfunctions of the nervous systems (22,23). Thus, it is perhaps not surprising that regulation of neuronal G protein signal termination mediated by RGS proteins has raised considerable interest. Neuronal RGS proteins have been implicated in many neurological conditions such as anxiety, schizophrenia, drug dependence and visual problems (See(23–25) for reviews).

Although the expression of several RGS proteins has been detected in the nervous system, the R7 group of RGS proteins has received special attention due to their pivotal roles in the regulation of a range of crucial neuronal processes such as vision, motor control, reward behavior and nociception in animals from *C. elegans* to humans (10,26). Additionally, R7 RGS proteins are key modulators of the pharmacological effects of drugs involved in the development of tolerance and addiction (27–29). In mammals, the R7 subfamily consists of four highly homologous proteins, RGS6, RGS7, RGS9 and RGS11, all of which are expressed predominantly in the nervous system (30). Despite the important role that R7 RGS proteins play in controlling neuronal G protein signaling, relatively little was known about their operational principles. Over the last few years, significant progress has been achieved in elucidating many exciting principles underlying the function of R7 RGS proteins, essentially making them one of the best understood subfamilies of the RGS family. The purpose of this review is to summarize our understanding of this important protein family and its role in regulating neuronal processes. We hope that the lessons learned from the studies on R7 RGS proteins may lead to better understanding of the general principles underlying G protein signaling in neurons and help spur the progress in studying other members of the RGS protein family with less understood roles.

R7 RGS proteins are multi-domain protein complexes

A major characteristic feature of R7 RGS proteins is their modular organization. These RGS proteins contain four distinct structural domains and form tight stoichiometric complexes with two binding partners. In fact, due to the obligatory nature of the association between three constituent components, R7 RGS proteins are increasingly viewed as heterotrimeric complexes composed of three subunits (Figure 1).

The central element of this complex is formed by the RGS molecule itself that shares a common domain organization across all R7 RGS members. The defining feature of all RGS proteins, the catalytic RGS domain, is located at the C-terminus of the molecule and constitutes the only enzymatically active portion of the complex. The RGS domains of all R7 RGS proteins were shown to be capable of stimulating GTP hydrolysis on Gα protein subunits (31–37,38,39). From an enzymatic perspective, this process could be regarded as the conversion of active Gα-GTP species into inactive Gα-GDP species, accompanied by the release of the inorganic phosphate (40) commonly referred to as GAP (GTPase activating protein). Interestingly, the RGS domains of the R7 RGS proteins act as potent GAPs, even when isolated from the other, non-catalytic domains (see (31–33,37) for examples). However, studies with RGS7 and RGS9 indicate that these other non-catalytic domains contribute to setting the maximal catalytic activity and refining Gα specificity (31–33). *In vitro* enzymatic studies have demonstrated that full-length R7 RGS proteins containing all non-catalytic domains selectively stimulate GTP hydrolysis on α subunits of the Gi/o class of G proteins but not on $G\alpha_{q/11}$, $G\alpha_z$ or $G\alpha_s$ (24, 39).

Crystal structures of isolated RGS homology domains have been solved for RGS7 (41) and RGS9 (42), both alone and, in the case of RGS9, in a complex with activated $G\alpha_t$. Analysis of these structures reveals a high degree of conformity to the all-helical bundle organization observed in a number of other RGS proteins (41,43–45). The loops connecting the bundled helices form direct contacts with the switch region of the activated Gα subunit to stabilize it in the transition state of GTP hydrolysis, thereby providing a mechanism for the GAP activity (42). The RGS domain undergoes very little conformational change upon Gα binding, affecting mainly the α5/6 loop, which contains the catalytically critical Asn residue (42).

Upstream from the RGS domain, R7 RGS proteins carry a second conserved feature, the GGL (G protein gamma-like) domain. This domain is structurally homologous to the conventional γ subunits of G proteins (38). Like all Gγ subunits, the GGL domain binds to its obligatory partner, the Gβ subunit. However, unlike conventional Gγ subunits, this interaction of the GGL domain is incredibly specific, as it is capable of forming a coiled-coil interaction only with $G\beta_5$ (type 5 G protein β subunit), a distant member of the G protein β subunit family (35,46, 47). A recently solved crystal structure of the RGS9-$G\beta_5$ complex reveals that the interaction between GGL and $G\beta_5$ closely follows the same orientation and association mechanisms as those observed in conventional Gβγ dimers (48).

Finally, the N-terminus of R7 RGS proteins is formed by the DEP (Disheveled, Egl-10, Pleckstrin) (49) and DHEX (DEP helical extension) (48) domains. While the DEP domain is found in many signaling proteins (49), the DHEX domain is unique to R7 RGS proteins (10). Both crystal structure (48) and chimeric mutagenesis (50) studies suggest that the DEP and DHEX domains form a single, functional domain in the molecule. Recent studies have revealed that the DEP/DHEX module of R7 RGS proteins is responsible for their interaction with two novel membrane proteins, R9AP (RGS9 anchor protein) and R7BP (R7 family binding protein), which are discussed in detail below.

Increasing evidence suggests that alternative splicing is a powerful mechanism that affects three members of the R7 RGS family: RGS6 (51), RGS9 (52–54) and RGS11(55). Combined

with the modular principle of R7 RGS organization, differential splicing generates variability in domain composition, leading to the loss or gain of functions mediated by those affected domains. An extreme example of the extensive splicing patterns of R7 RGS proteins was recently provided by studies of RGS6. Alternative splicing of this protein generates 36 isoforms containing virtually all possible combinations of non-catalytic domains in addition to the RGS catalytic domain (51). Remarkably, several studied isoforms of RGS6 showed differential distribution patterns across cellular compartments (51,56), suggesting that domain composition may regulate subcellular targeting of RGS6 in cells. The splicing pattern of RGS9 is much less complex, but nonetheless provides the best understood example of functional implications. Two splice variants of RGS9, which differ only in their composition at the C-termini, have been described (52–54). The short splice isoform, RGS9-1, contains only 18 amino acid residues at the C-terminus and is exclusively expressed in photoreceptors. In the long splice isoform, RGS9-2, the short C-terminus is replaced by a longer region of 209 amino acids. RGS9-2 is expressed in the striatum and is not present in photoreceptors (52,57). The ability of the RGS9-1 isoform to recognize its cognate G protein target $G\alpha_t$ is regulated by the effector enzyme of the visual cascade in photoreceptors, PDEγ (58–60), which acts to dramatically enhance the affinity of RGS9-1 for $G\alpha_t$ (61). As PDEγ is absent in the striatum, G protein recognition is enhanced by the additional C-terminal PDEγ-like domain (PGL) domain that is unique to RGS9-2 (62). It is likely that future studies on the role of alternative splicing in R7 RGS proteins will yield additional insights into the fundamental principles regulating these proteins.

In summary, R7 RGS proteins are built from the three constituent modules: (i) the catalytic RGS domain, (ii) the GGL domain that recruits the $G\beta_5$ subunit and (iii) the DEP/DHEX domain that mediates interactions with the membrane proteins R7BP and R9AP. As will be detailed in the following sections, the interplay between these functional domains determines expression level, intracellular localization and ultimately the GAP properties of the R7 family members.

$G\beta_5$, an obligate subunit with an enigmatic functional role

$G\beta_5$ was first discovered as a novel type of Gβ subunit exclusively expressed in the nervous system (63). It was shown to selectively interact with $G\gamma_2$ *in vitro*, although the existence of this interaction *in vivo* has never been demonstrated (63,64). Despite this fact, most subsequent studies focused on analyzing the ability of the $G\beta_{5\gamma2}$ complex to mediate classical Gβγ functions such as interactions with Gα subunits and effectors. It was found that $G\beta_{5\gamma2}$ has an unusual selectivity for its effectors, as it potently regulates the activities of $PLC\beta_2$, N-type calcium channels and GIRK channels, but not $PLC\beta_3$, PI3Kγ or adenylate cyclase II (63,65–69). Likewise, $G\beta_{5\gamma2}$ was shown to interact with GDP-bound Gα subunits (70,71). However the specificity of these interactions is more controversial. While one group reported that $G\beta_{5\gamma2}$ can bind to $G\alpha_q$ but not to $G\alpha_i$ or $G\alpha_o$ (70), another group detected stable interactions with both $G\alpha_i$ and $G\alpha_o$ (71). Although no explanation for these discrepancies exists, it was noticed that the complex of $G\beta_5$ with $G\gamma_2$ is abnormally weak and prone to spontaneous dissociation, leading to loss of $G\beta_5$ activity (72,73). Overall, these findings demonstrate that $G\beta_5$ exhibits some properties that are common to the conventional Gβ subunits, such as interaction with Gα and Gγ subunits as well as with effectors. A recently solved crystal structure supports this idea, as it indicates that most of the critical amino acids that build the protein interaction interface in $G\beta_5$ are conserved (48). However, the physiological function of $G\beta_5$ remained a mystery until the discovery that $G\beta_5$ readily forms complexes with members of the R7 family of RGS proteins instead of Gγ subunits *in vivo* (46,47,74). Unlike the $G\beta_{5\gamma2}$ association, $G\beta_5$·RGS complex formation is very strong and resistant to dissociation in detergent solutions, allowing for its purification by various chromatographic and immunoprecipitation strategies (46,47,64). It should be noted, however, that the debate on

whether $G\beta_5$ can also exist and function in complex with conventional Gγ subunits continues (see (75) for most recent example), as it remains to be established whether $G\beta_5$ can be found outside of the complexes with R7 RGS proteins *in vivo*.

Two splice isoforms of $G\beta_5$ have been described (71). $G\beta_{5S}$, a 39 kDa short splice isoform, is ubiquitously expressed in the retina and brain, where it forms complexes with all R7 RGS proteins, except RGS9-1 (46,64,76). The 44 kDa long splice variant, $G\beta_{5L}$, containing 42 extra amino acids at the N-terminus, is exclusively present in the outer segments of photoreceptors (ROS), where it forms a complex with RGS9-1 (47). The longer N-terminal portion of the photoreceptor $G\beta_{5L}$ isoform has been shown to contribute to a high affinity to RGS9-1, selectively with a $G\alpha_t$-PDEγ complex, as opposed to free, activated $G\alpha_t$. However, the precise role that alternative splicing of $G\beta_5$ plays for RGS9-1 function is not fully understood.

From early studies on the functional significance of R7 RGS·$G\beta_5$ complex formation, it was unequivocally determined that $G\beta_5$ is essential for the stability and expression of all R7 RGS proteins. Co-expression with $G\beta_{5S}$ was shown to be necessary for achieving high expression levels of RGS6 and RGS7 via protecting them from proteolytic degradation (35,74), resulting in the enhancement of RGS activity in regulating GIRK channel kinetics (77). Likewise, experiments with recombinant overexpression in heterologous systems indicate that functionally active proteins can only be obtained when R7 RGS proteins are co-expressed with $G\beta_5$ (32,34). Finally, the ultimate proof of the importance of the interaction between R7 RGS proteins and $G\beta_5$ arose from knockout mouse studies that demonstrated that the genetic ablation of $G\beta_5$ resulted in the loss of all R7 RGS proteins (78). Conversely, deletion of RGS9, the only R7 RGS protein in photoreceptors, results in the degradation of $G\beta_5$. This indicates that, at least in this cell type, $G\beta_5$ exists only in complex with RGS proteins and becomes destabilized in the absence of its interaction with the GGL domain (13). These observations are reminiscent of the reciprocal stabilization seen in conventional Gβγ subunits, which are thought to form inseparable entities (see (79,80) for examples). Overall, most of the accumulated evidence establishes R7 RGS proteins and $G\beta_5$ as obligate subunits of a complex that exists and functions *in vivo* as a single entity.

Delineation of the functional roles that $G\beta_5$ plays as a part of the heterodimeric complex with RGS proteins beyond proteolytic protection has proven to be more difficult. The regulatory effector and Gα binding properties observed for $G\beta_{5\gamma 2}$ have not been found for $G\beta_5$ in complex with R7 RGS proteins. RGS6·$G\beta_5$ and RGS7·$G\beta_5$ were shown to not modulate either PLCβ or adenylate cyclase (39). Similarly, recombinant RGS6·$G\beta_5$, RGS7·$G\beta_5$ and RGS9·$G\beta_5$ were demonstrated to be incapable of interacting with GDP-bound $G\alpha_{i/o/t}$ subunits (33,39,62). The crystal structure of the RGS9·$G\beta_5$ complex sheds some light on the apparent discrepancy between the capability of $G\beta_5$ to interact with Gα subunits and effectors when in complex with $G\gamma_2$ but not when in complex with RGS proteins (48). Analysis of the structure indicates that although the protein interaction interface that mediates association of Gβ subunits with Gα subunits and effectors is conserved in $G\beta_5$, it is inaccessible due to its interactions with the N-terminal DEP domain. The DEP domain is intricately interwoven with the adjacent DHEX domain, with both of the domains forming a single structural domain that caps the protein interaction interface of $G\beta_5$. This cap is connected to the rest of the RGS polypeptide via an unstructured hinge region, which is postulated to bear significant conformational flexibility (48). These observations led to the idea that the complex in the crystal structure was captured in the “closed” conformation, which could be transformed into the “open” state by conformational changes that would disrupt the interactions between the DEP domain and $G\beta_5$ (48,81). Intriguingly, it is speculated that the R7BP and R9AP proteins that bind to the DEP/DHEX domains could impact the equilibrium between “open” and “closed” conformations, thus altering access to the protein-protein interaction interface of $G\beta_5$.

An alternative possibility is that the GGL-$G\beta_5$ module could be employed by RGS complexes to play a role in setting their G protein selectivity, thus regulating the GAP activity of RGS proteins. Indeed, several similar effects of $G\beta_5$ have been reported. Deletion mutagenesis studies on RGS9-1·$G\beta_5$ complexes indicate that the GGL-$G\beta_5$ module acts to non-specifically reduce the affinity of the RGS catalytic domain to its two G protein targets: free activated $G\alpha_t$ and $G\alpha_t$-PDEγ complexes (33). In contrast, the non-catalytic domains of RGS9-1 enhance binding specifically for $G\alpha_t$-PDEγ complexes. In conjunction with the function of $G\beta_5$, this activity is thought to be required for setting the high degree of RGS9-1·$G\beta_5$ discrimination for its physiological substrate, $G\alpha_t$-PDEγ, and for preventing short-circuiting of the cascade due to deactivation of $G\alpha_t$ before it can relay the signal to the effector (32,33). The ability of $G\beta_5$ to affect RGS interactions with Gα was also observed for RGS7, which was shown to bind to activated $G\alpha_o$ more tightly alone than when in complex with $G\beta_5$ (82). Finally, $G\beta_5$, in complex with the GGL domain of RGS9, was found to be important for sustaining the high turnover rate of $G\alpha_t$ on the RGS domain of RGS9 (33). These results suggest that $G\beta_5$ is involved in regulating GAP properties of R7 RGS proteins. However, much of the underlying mechanisms remain to be elucidated.

R7BP and R9AP: Adaptor subunits specifying expression, localization and activity of R7 RGS complexes

The function of many signaling proteins in cells is determined to a great extent by their targeting to specific subcellular compartments. Photoreceptor neurons have served as a convenient model for delineating compartmentalization mechanisms of several signaling molecules, including that of R7 RGS proteins (83–85). In these cells, the visual signal transduction pathway is physically restricted to a specialized compartment, the outer segment, which is separated from the rest of the cellular compartments containing other G protein pathways (86). The outer segment is also the exclusive localization site for RGS9-1, which is tightly bound to the disc membranes (87,88). Biochemical reconstitution studies and experiments with transgenic animals have indicated that the association of RGS9-1·$G\beta_5$ with the disc membranes and its specific targeting to the outer segment is mediated by the DEP domain (88,89). Proteomic screening for the molecules that mediate this function in the photoreceptors resulted in the identification of the membrane anchor protein R9AP (90). Similar to RGS9-1, RGS9-2 also associates with membranes and is specifically targeted to the postsynaptic density site in striatal neurons (91). The absence of R9AP in the brain led to another proteomic search that identified R7BP, an R9AP homologue that binds to RGS9-2 and all other R7 RGS proteins in striatal neurons (76). At the same time, R7BP was also independently discovered as a binding partner of R7 RGS proteins via bioinformatics homology searches using R9AP as bait (92). Although the binding of both R9AP and R7BP to RGS proteins has been shown to be mediated by the DEP domain (50,76), complex formation exhibits clear interaction specificity. Although all four R7 RGS proteins can bind to R7BP, only RGS9 and RGS11 are capable of forming complexes with R9AP (76,92).

At the amino acid sequence level, the similarity between R9AP and R7BP is limited to only 30% (15% identity). However, both proteins share a significant homology and similarity in overall architecture with SNARE proteins (88,93). SNAREs are membrane-associated proteins involved in the vesicular trafficking and exocytosis that underlie synaptic fusion events (for review, see (94,95). Like the SNARE protein syntaxin, R9AP and R7BP are predicted to contain an N-terminal three-helical bundle followed by an extensive coiled-coil domain and a membrane attachment site (Figure 2). This similarity invites speculation that the interaction between DEP domains and SNARE-like proteins may be a common principle underlying the targeting of DEP domain-containing proteins, which include numerous signaling proteins (9, 49). In this context, it is intriguing that in yeast, syntaxin homologues are found among the binding partners of the DEP domain-containing RGS protein, Sst2 (96).

Although both R9AP and R7BP are membrane proteins, the mechanisms of their binding to membranes differ. R9AP is anchored via a single-pass C-terminal transmembrane helix, making it an integral membrane protein (90). In contrast, association of R7BP with the plasma membrane is mediated by two palmitoyl lipids that are post-translationally attached to the C-terminal cysteine residues, acting synergistically with an upstream polybasic stretch of six amino acids (92,97). The labile nature of palmitoylation provides R7BP with flexibility in its localization. In cultured cells, it has been shown that de-palmitoylation of R7BP not only removes it from the plasma membrane but also uncovers a nuclear localization signal, resulting in its translocation into the nucleus (92,97). This mechanism is thought to contribute to the regulation of R7 RGS protein availability at the plasma membrane (92,98). However, the exact functional implications of R7BP shuttling from the plasma membrane to the nucleus are unknown. Furthermore, in native neurons R7BP has been primarily found at the plasma membrane compartments (91,97,99) and its translocation into the nucleus has not been established despite several reports documenting nuclear localization of R7 RGS proteins *in vivo* (56,100,101).

What does appear to be firmly established is the role of R7BP/R9AP-mediated membrane association in the function of R7 RGS proteins. First, the membrane anchors regulate the activity of R7 RGS proteins. Studies have shown that association of RGS9-1·$G\beta_5$ with R9AP causes a dramatic potentiation of the ability of RGS9-1 to activate transducin GTPase (88, 89,102). Under optimal conditions, the degree of this potentiation can be as large as 70-fold (40,89). Similar to R9AP, it was found that co-expression of RGS7·$G\beta_5$ with R7BP in *Xenopus* oocytes enhances the ability of RGS7 to augment M2 receptor-elicited GIRK channel kinetics, presumably due to the stimulation of the catalytic activity of RGS7 (92,98). Because the effects of both R7BP and R9AP require the presence of the elements that mediate their membrane attachment, it is reasonable to assume that stimulatory activity of R7BP/R9AP can be attributed to a large extent to concentrating R7 RGS proteins on the membranes and in close proximity to membrane-bound G proteins. However, direct allosteric mechanisms also appear to contribute to the effects of anchors on R7 RGS proteins, as suggested by the observation that R9AP influences not only the catalytic rate of RGS9-1·$G\beta_5$ GAP activity but also its affinity to activated $G\alpha_t$ (103). Second, R7BP and R9AP play major roles in dictating the subcellular localization of R7 RGS proteins. In addition to translocation of R7 RGS proteins to the plasma membrane, as observed in transfected cells upon co-expression with R7BP/R9AP (90,92,97), membrane anchors target RGS proteins to unique subcellular compartments in neurons. In photoreceptors, R9AP mediates RGS9-1 delivery to the outer segments and excludes it from the axonal terminals (88,104). In striatal neurons, R7BP specifies the targeting of RGS9-2 to the postsynaptic density (91). Interestingly, R7BP/R9AP activity is not universally required for targeting all R7 RGS proteins in all cells, as it was recently shown that targeting of RGS7·$G\beta_5$ in retinal bipolar neurons occurs independently from its association with R7BP (105).

Studies with mouse knockout models revealed that R9AP and R7BP also play an important role in determining the expression levels of R7 RGS·$G\beta_5$ complexes. Knockout of R9AP in mice results in nearly complete elimination of detectable RGS9-1 and RGS11 proteins in the retina (105,106). Similarly, knockout of R7BP leads to severe down-regulation of RGS9-2 protein levels in the striatum (91). At the same time, transcription of the RGS9 and RGS11 genes is unaltered, as evidenced by similar levels of mRNA in both knockout and wild type tissues (91,106). The protein levels of RGS9-1, RGS9-2 and RGS11 are reduced by half in the tissues of heterozygous mice carrying one R9AP- or R7BP-deficient allele, which corresponds to the extent of the reduction in R7BP or R9AP expression, respectively. Conversely, overexpression of R9AP in the photoreceptors and R7BP in the striatum led to an increase in the levels of RGS9-1 (20) and RGS9-2 (91), respectively. Examination of the mechanisms by which R7BP/R9AP confer their effects revealed that RGS9 isoforms, even when in complex

with $G\beta_5$, are proteolytically unstable proteins with an estimated half life in the cell of less than one hour (50). RGS9 isoforms carry instability determinants located within their N-terminal DEP/DHEX domains that target they for degradation by cellular cysteine proteases (91). Binding of R7BP or R9AP to this region is thought to shield these determinants and thus prevent the degradation of RGS9, drastically prolonging its life time. Thus, R9AP and R7BP proteins could be viewed as subunits whose expression levels ultimately set the levels of RGS9- and RGS11-containing complexes in cells. Interestingly, RGS7 (and likely RGS6) does not possess these instability determinants and is therefore resistant to degradation when present in complex only with $G\beta_5$ (35,50). Consistent with this observation, the levels of RGS6 and RGS7 are unaltered in R9AP or R7BP knockout tissues (91,105). These observations suggest that RGS9 and RGS11 likely exist as obligate heterotrimeric complexes with either R9AP or R7BP, while RGS7·$G\beta_5$ and RGS6·$G\beta_5$ dimers with could associate with R7BP conditionally. In summary, current evidence indicates that R7BP and R9AP are integral subunits of R7 RGS proteins and play critical roles in regulating the (i) catalytic activity, (ii) subcellular targeting and (iii) protein expression levels of R7 RGS complexes.

R7 RGS proteins associate with a wide spectrum of cellular proteins

As discussed above, R7 RGS proteins form trimeric complexes with R7BP (or R9AP) and $G\beta_5$ subunits. These interactions are intrinsic to all members of the R7 family and have been demonstrated to play critical roles in their activity. Interactions of R7 RGS complexes with their G protein substrates and the Gα subunits of the heterotrimeric G proteins of the $G_{i/o}$ family in the transition state of GTP hydrolysis are equally well established (31,42,61), (37,38,107). Interestingly, in addition to these well accepted interactions, R7 RGSs have been also reported to bind a number of other proteins, suggesting that these RGS proteins are likely integrated into larger macromolecular complexes in cells. Additional interactions were found for RGS6 and both splice isoforms of RGS9 and RGS7, but not for RGS11 (Table 1). In contrast to the conventional complexes of R7 RGS proteins with R9AP, R7BP and $G\beta_5$, most interactions reported in Table 1 were shown only for some members of the family, and their universality is unknown. Furthermore, for most of these interactions, it is unknown whether the binding occurs directly or is mediated by other proteins. Information about the binding determinants is often missing, and most of these interactions were not considered in the context of constitutive R7 RGS complexes with $G\beta_5$ and R7BP or R9AP. Despite these limitations, analysis of the patterns of these interactions may be productive, as it may suggest not only a potential involvement of R7 RGS proteins in the regulation of discrete cellular processes, but may also provide models of the regulation of RGS protein function. Interaction partners of R7 RGS proteins can be divided into three groups: (i) components of G proteins receptor complexes, (ii) signaling proteins outside of classical GPCR pathways and (iii) proteins that modulate RGS function.

The first consistent theme of R7 RGS proteins is the association with components of GPCR signaling complexes. In brain lysates, RGS9-2 was co-precipitated with the μ-opioid receptor (108,109). Furthermore, targeting of RGS9-2 to membrane compartments required the presence of its DEP domain and co-transfection with μ-opioid (109) or D2 dopamine (110) receptors in transfected cells. Similarly, RGS7 was shown to directly interact with the intracellular loops of the muscarinic M3 receptor through its N-terminus (111). The interactions of mammalian R7 RGS proteins with GPCRs are further supported by the observation that the DEP domain of the yeast RGS protein Sst2 directly interacts with the C-terminal domain of its cognate receptor, Ste2 (112). Hypothetically, the RGS-GPCR pairing can serve as a powerful mechanism that provides the specificity of RGS activity and shapes the kinetics of the response. In this respect, it is important to note the discovery of the polypeptide that contains both GPCR and RGS domains, which allow it to effectively modulate cell proliferation (113). Interestingly, binding partners of R7 RGS proteins also include proteins that are

normally found in complexes with GPCRs. Receptor kinase GRK2, β-arrestin and the GPCR scaffold spinophilin were found to co-immunoprecipitate with RGS9-2 in brain tissue (109, 114). Although it is unclear whether these interactions occur directly or are mediated by μ-opioid receptors, they are thought to contribute to the regulation of receptor internalization and the development of tolerance, both of which are influenced by RGS9-2 (28,109,114).

The second large group of R7 RGS binding partners is composed of the non-conventional interactions of R7 RGS proteins with signaling proteins outside of G protein signaling pathways. For example, a yeast two hybrid screen has revealed interactions between RGS6 and the transcriptional repressor complex DMAP1/Dnmt1 (115), an observation that is consistent with the previously reported localization of RGS6 in the nucleus (56). Nuclear localization has also been reported for other R7 RGSs (100,101,116) and is thought to be mediated by R7BP, which can serve as a membrane-nuclear shuttle in a palmitoylation-dependent fashion (97,98). This raises the possibility that additional interactions of R7 RGS proteins with components of signaling pathways in the nucleus exist. The discovery of these interactions may provide significant insight into the function of these proteins in the nucleus. In the cytoplasm, RGS6 was found to be associated with the microtubule destabilizing protein SCG10. This interaction that was shown to result in the enhancement of neurite outgrowth when studied in transfected cells (117). Similarly, RGS9-2 was reported to be associated with another cytoskeletal protein, α-actinin-2 (118). In transfected cells, this interaction was demonstrated to link RGS9-2 to the regulation of NMDA receptor function (118). Finally, RGS7 was found to bind a component of the synaptic fusion complex, snapin, leading to the hypothesis that R7 RGS proteins can also regulate exocytosis (93,119). More studies will be needed to delineate the exact roles of R7 RGS proteins in mediating these signaling processes and fully validate these novel interactions. Likewise, it remains to be established whether the non-conventional functions of R7 RGS proteins are mediated by G proteins or occur via other, yet undetermined pathways.

The last group of R7 RGS binding partners consists of the proteins that serve to regulate RGS proteins themselves. Although there are only two reported observations in this category, the number of examples is expected to grow substantially as the organization of R7 RGS proteins and their reliance on protein-protein interactions for determining their cellular function are complex. In studies of the established interactions with R7BP/R9AP and $G\beta_5$, association with other cellular proteins was shown to affect catalytic activity and proteolytic stability of R7 RGS proteins. This is a recurring theme for the regulation of this RGS family. Indeed, the binding partner of RGS7, polycystin, was shown to protect it from rapid proteolytic degradation by the ubiquitin proteasome system (120), whereas association with the 14-3-3 protein was shown to inhibit RGS7 activity in a phosphorylation-dependent manner (121).

Physiological roles of R7 RGS proteins: insights from mouse models

Most of what we know about the physiological roles of R7 RGS proteins comes from studies on selective elimination or overexpression of R7 RGS proteins in murine models. Among the four R7 RGS proteins, the function of RGS9 is best understood due to its localized expression and the abundance of mouse genetic models. The functional role of this member can serve as a valuable example of the other R7 RGS family members, the physiological roles of which remain largely unknown.

Targeting of the RGS9 gene produced a line of knockout mice that lack the expression of both splice isoforms: RGS9-1 in the retina and RGS9-2 in the brain (13). Elimination of RGS9-1 in the retina resulted in a substantial delay in the termination of photoreceptor responses to light, a process mediated by the GPCR phototransduction cascade (13). In this pathway, the activated receptor (photoexcited rhodopsin) triggers the activation of the G protein transducin ($G\alpha_t$), which in turn stimulates the activity of the effector enzyme cGMP phosphodiesterase.

This leads to transient membrane hyperpolarization, a major response of the photoreceptor to light (reviewed in (25,122). Following extinction of light excitation, wild type rod photoreceptors quickly return to the resting state, with an average time constant of approximately 200 ms. This rapid recovery requires G protein inactivation in the cascade and is critical for the high temporal resolution of our vision (123). In contrast, rods of mice lacking RGS9-1 show recovery kinetics that are an order of magnitude slower (time constant ~ 2.5 s) (13). This phenotype is thought to result from delayed transducin inactivation, which is mediated by RGS9-1. This suggests that this regulator is the GAP in the phototransduction cascade. Similar recovery deficiencies were also described in cone cells, suggesting that this function of RGS9-1 is conserved in all photoreceptor cells (124). Consistent with its obligatory trimeric organization, the function of RGS9-1 in providing timely transducin deactivation has been shown to depend on its association with R9AP and $G\beta_5$ subunits. Elimination of these subunits in mice results in an identical slow photoreceptor deactivation phenotype (106,125). In line with the observations in mice, mutations disrupting RGS9-1 and R9AP were found to cause the human visual disease bradyopsia, which disrupts the ability of those affected to adapt to changes in luminance and to recognize moving objects (126–128). Conversely, overexpression of RGS9-1·$G\beta_5$·R9AP in mouse rods results in an acceleration of photoresponse inactivation, demonstrating that it serves as a key rate-limiting enzyme in the cascade of recovery reactions that bring photoreceptors to a resting state (20).

The other splice isoform, RGS9-2, was found to be enriched in the striatum, a region commonly associated with reward and motor control functions. It was also found, albeit at much lower levels, in the periaqueductal gray matter, the dorsal horns of the spinal cord and the cortex, which are structures that mediate nociception (28,118,129,130). This expression pattern has prompted several groups to evaluate the contribution of RGS9-2 to specific behaviors controlled by these systems. RGS9 knockout mice had the following phenotypic properties: (i) increased sensitivity to the rewarding properties of cocaine, amphetamine and morphine (27,131,132), (ii) increased sensitivity to the anti-nociceptive action of morphine (109,131) (similar observation were also made with the down-regulation of RGS9-2 expression by antisense oligonucleotides (129), (iii) delayed development of tolerance to the administration of morphine (131), (iv) enhanced severity of withdrawal symptoms following the cessation of morphine administration (131) (v) rapid development of tardive dyskinesia in response to suppression of dopaminergic signaling (110) and (vi) deficits in motor coordination and working memory (133). Conversely, viral-mediated overexpression of RGS9-2 in the rat striatum resulted in the reduction of locomotor activity potentiation in response to cocaine administration (12). Similarly, overexpression of RGS9-2 in a MPTP monkey Parkinson's model has been reported to diminish L-DOPA-induced dyskinesia symptoms (134). Despite the long list, these deficiencies are likely to arise from alterations in specific pathways, as RGS9 knockout mice are quite normal in many behavioral aspects. They exhibit unaltered basal locomotor activities, cognitive function, fear conditioning and pre-pulse inhibition (12,131, 133).

These described phenotypical observations suggest a model in which the function of RGS9-2 in the striatum negatively regulates the sensitivity of the signaling pathways that process reward and nociceptive cues. Indeed, growing pharmacological evidence supports the idea that RGS9-2 moderates signaling via D2 dopamine and μ-opioid receptors, two prominent systems that are thought to critically regulate reward, nociception and locomotor functions (27,110, 131,132,134,135). Moreover, signaling through D2 and μ-opioid receptors appears to be connected to RGS9-2 expression through feedback mechanisms that adjust the level of this negative regulator, thus allowing dynamic modulation of the signaling intensity (12,131,136, 137). Furthermore, the RGS9-2 complex physically associates with D2 and μ-opioid receptors (see previous chapter and Table 1), although it is currently unknown what mediates this interaction.

In contrast to the thorough understanding of the role of RGS9-1in the phototransduction cascade, the mechanistic picture of RGS9-2 activity and the second messengers and effector systems that are involved in this activity are far less clear. Studies that have addressed this issue have found that introduction of the catalytically active portion of the RGS9 protein into the striatal cholinergic interneurons reduced the modulation of N-type voltage gated calcium channels by dopamine, suggesting that ion channels that regulate neuronal excitability are a potential target of RGS9-2 activity (135). This observation is in line with reconstitution studies in *Xenopus* oocytes that demonstrated that full length RGS9-2, both alone and in complex with $G\beta_5$, can powerfully modulate the kinetics of GIRK channel gating (12,77). Studies with RGS9 knockout mice also revealed enhanced D2 dopamine receptor-mediated suppression of NMDA currents in striatal medium spiny neurons lacking RGS9-2. Furthermore, RGS9-2 was found to regulate Ca^{2+}-dependent NMDA inactivation via complex formation with α-actinin-2 in transfected cells (118). Although the mechanisms by which RGS9-2 controls these reactions are unclear, these studies implicate RGS9-2 in the regulation of excitatory glutamatergic transmission and potentially synaptic plasticity. Finally, RGS9-2·$G\beta_5$ was reported to diminish ERK1/2 kinase activation in response to the activation of μ-opioid receptor in transfected cells (109). While these studies outline the range of the effector systems that can be regulated by RGS9-2, much of the underlying mechanisms remain unclear. Among key unanswered questions are whether the effects of RGS9-2 require its GAP activity (as, for example, in the regulation of calcium channels (135)) or if these effects can be explained by direct association with receptors (as, for example, in the regulation of μ-opioid receptor internalization (109)). Equally important is the question whether RGS9-2 is a specific regulator of select receptors or if it can function as a universal regulator of several GPCRs in neurons (discussed in (138)). Finally, since RGS9-2 forms a constitutive complex with $G\beta_5$ and R7BP, elucidating the contribution of these subunits to its activity and selectivity will have a significant impact on our understanding of RGS9-2 function.

Our knowledge of the physiological roles played by other R7 RGS members is substantially more limited. Knockdown studies using antisense oligonucleotides have implicated RGS6, RGS7 and RGS11 in regulating nociception mediated by μ- and δ-opioid receptors and the development of tolerance to morphine administration (29,139). In addition, the expression levels of these R7 RGS proteins have been reported to be modulated in response to changes in signaling via a range of pathways (for examples see (140–143)). Broad expression profiles across the nervous system (30,38,74) and the ability to regulate responses elicited by a variety of GPCRs that are coupled not only to Gi/o (34,38,39,77) but also to Gq (144,145) suggest that R7 RGSs may be critical regulators in a range of signaling pathways. Indeed, the development of the $G\beta_5$ knockout mouse provides a glimpse into the range of dysfunctions that are caused by the elimination of all R7 family members at once (78). Aside from the known defects associated with the loss of RGS9, $G\beta_5$ knockouts exhibit a range of developmental anomalies. Homozygous mice lacking $G\beta_5$ are smaller in size at birth, gain weight at a slower rate, do not gain body weight in the critical period prior to weaning between postnatal days 15 to 20 and exhibit a high pre-weaning mortality rate (up to ~60%) by 21 days of age (Chen et al. 2003). In addition, retinas of $G\beta_5$ knockouts are unable to relay light excitation from rod photoreceptors to downstream ON-bipolar cells, as revealed by the lack of the characteristic b-wave on electroretinograms (146). This deficiency in synaptic transmission is underlined by the failure of ON-bipolar cells to establish synaptic contacts with rod terminals during the critical developmental window (146). In light of these widespread developmental deficiencies, it is interesting to note that the expression of R7BP, a universal subunit of R7 RGS proteins, is tightly and developmentally controlled. R7BP mRNA and protein are largely undetectable at birth and exhibit a rapid and dramatic induction, peaking around the age of weaning (91, 99). Delineating the roles of R7 RGS complexes in regulating the specific pathways that shape developmental processes and the establishment of synaptic connectivity will be an exciting future direction.

Acknowledgments

We thank Mr. Perry Anderson for his help with illustrations. Studies on R7 RGS proteins in our laboratory are supported by the NIH grants EY018139 and DA 021743. Garret Anderson is a recipient of the Ruth L. Kirschstein National Research Service Award DA024944.

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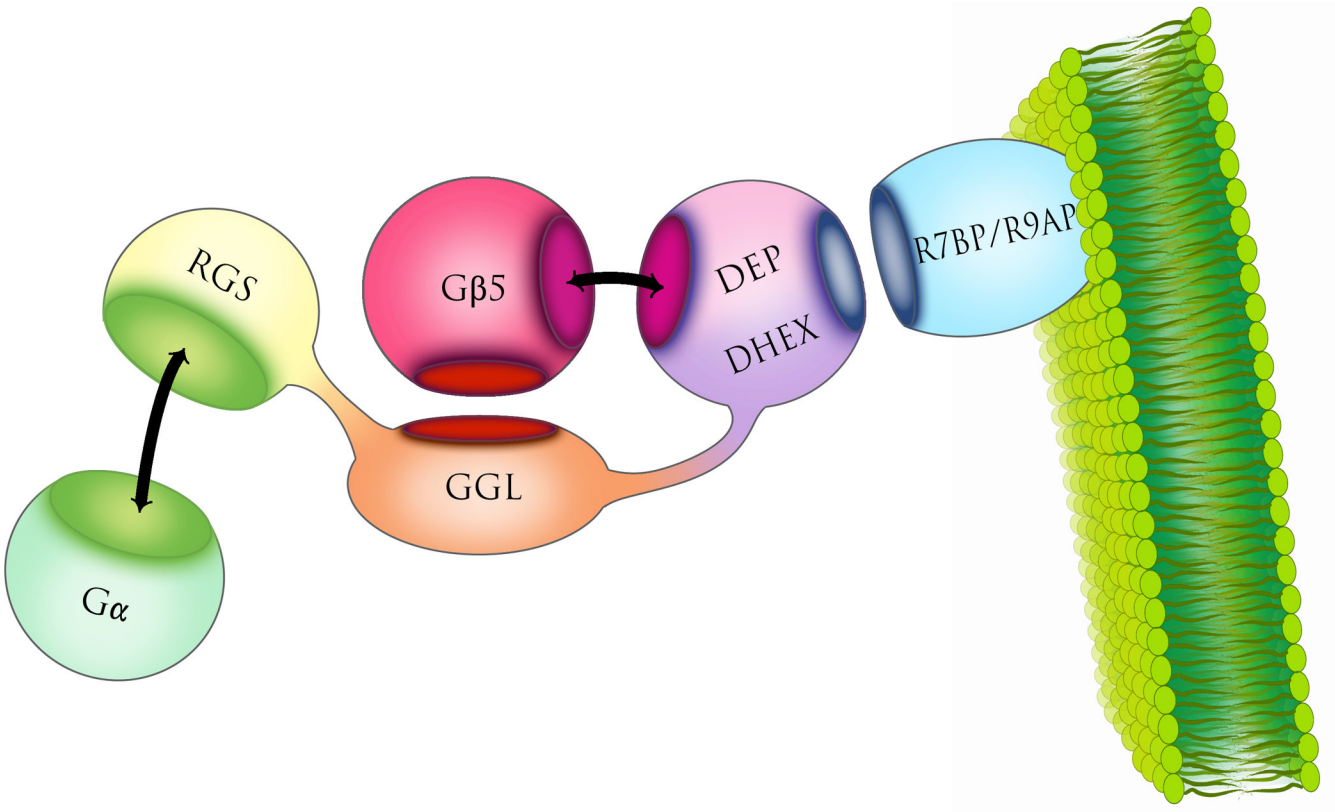

Figure 1. Organization of trimeric complexes between R7 RGS proteins and their subunits: R7BP/R9AP and $G\beta_5$

R7 RGS proteins consist of three functional modules. The N-terminal DEP (Disheveled, EGL-10, Pleckstrin) and DHEX (DEP helical extension) domains mediate binding to the membrane anchors R7BP and R9AP. The central GGL (G Protein gamma-like) domain forms a complex with the $G\beta_5$ (G protein β subunit, type 5). The C-terminal RGS (regulator of G protein signaling) domain mediates transient association with Gα-GTP subunits, during which GTP hydrolysis is stimulated. In addition to the GGL domain, $G\beta_5$ also associates with the DEP/DHEX module.

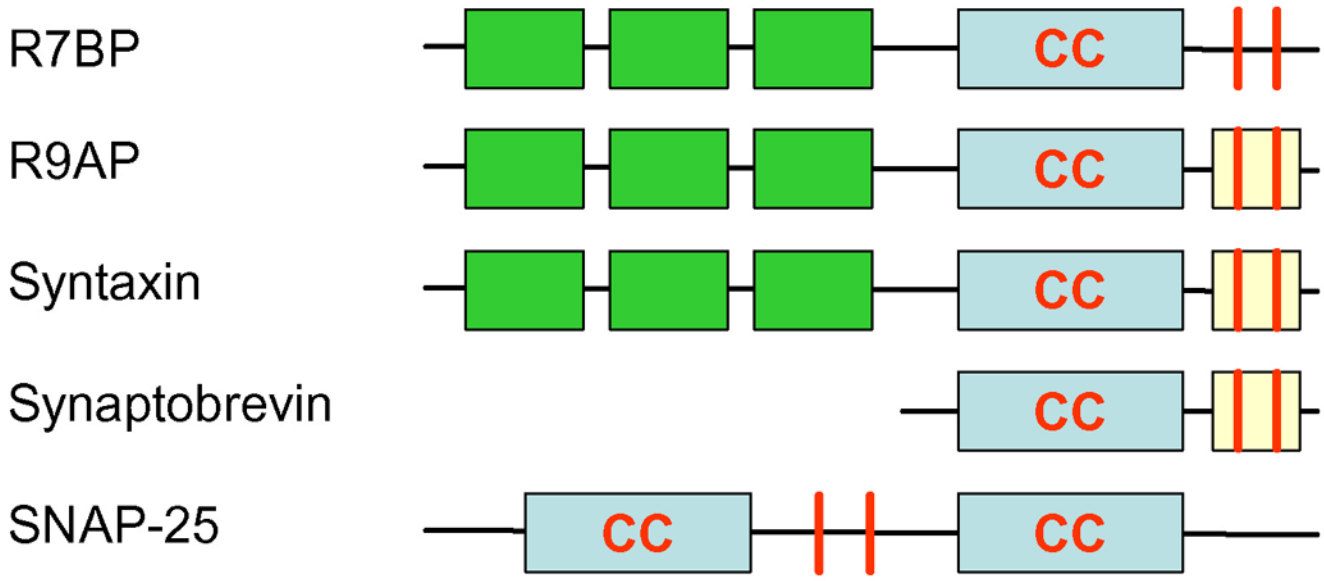

Figure 2. Membrane anchors R7BP and R9AP share structural similarities with SNARE proteins

Schematic representation of R7BP and R9AP domain compositions in comparison with three canonical SNARE proteins. Green boxes represent the alpha helical regions, blue boxes indicate conservative coiled-coil domains that participate in SNARE complex formation, yellow boxes indicate transmembrane regions and red lines indicate sites of membrane attachment.

Table 1

Interactions of R7 RGS proteins outside of the complexes with Gβ5 and membrane anchors R7BP/R9AP.

Interaction partner	R7 RGS	System	Method	Domain	Reference
μ-opioid receptor	RGS9-2	PC12	Co-IP	N/A	106
		periaqueductal gray matter	Co-IP	N/A	105
M3 receptor	RGS7	CHO-K1	Pull-down	DEP	108
β-arrestin	RGS9-2	PC12	Co-IP	N/A	106
α-actinin-2	RGS9-2	HEK293, brain	Y2H, Co-IP	N/A	117
NMDAR, subunit NR1	RGS9-2	HEK293, brain	Co-IP	N/A	117
14-3-3	RGS7	HEK293, brain	Co-IP, pull-down	RGS	120
	RGS9-2	periaqueductal gray matter	Co-IP	N/A	105
DMAP1, DNMT1	RGS6	COS-7, SH-SY5Y, brain	Y2H, Co-IP, pull-down	GGL	112
SCG10	RGS6	COS-7	Y2H, Co-IP	GGL	116
Snapin	RGS7	CHOK1	Co-IP, pull-down	DEP	118
Polycystin	RGS7	HEK293	Co-IP, pull-down	GGL	119
Spinophilin	RGS9-2	striatum	Co-IP	N/A	111
GRK2	RGS9-2	striatum	Co-IP	N/A	111
Guanylyl cyclase	RGS9-1	bovine ROS	Overlay	N/A	147