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INTERNATIONAL UNION OF BASIC AND CLINICAL PHARMACOLOGY REVIEW

The evolution of regulators of G protein signalling proteins as drug targets – 20 years in the making: IUPHAR Review 21

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Regulators of G protein signalling (RGS) proteins are celebrating the 20th anniversary of their discovery. The unveiling of this new family of negative regulators of G protein signalling in the mid-1990s solved a persistent conundrum in the G protein signalling field, in which the rate of deactivation of signalling cascades *in vivo* could not be replicated in exogenous systems. Since then, there has been tremendous advancement in the knowledge of RGS protein structure, function, regulation and their role as novel drug targets. RGS proteins play an important modulatory role through their GTPase-activating protein (GAP) activity at active, GTP-bound Gα subunits of heterotrimeric G proteins. They also possess many non-canonical functions not related to G protein signalling. Here, an update on the status of RGS proteins as drug targets is provided, highlighting advances that have led to the inclusion of RGS proteins in the IUPHAR/BPS Guide to PHARMACOLOGY database of drug targets.

Abbreviations

DEP, Disheveled Egl-10 Pleckstrin; GAP, GTPase-activating protein; GGL, G protein γ-like; PPI, protein–protein interaction; RGS, regulator of G protein signalling; R7BP, R7 binding protein; R9AP, RGS9 associated protein

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Tables of Links

TARGETS	
R4 family	**R7 family**
RGS1	RGS6
RGS2	RGS7
RGS3	RGS9
RGS4	RGS11
RGS5	**R12 family**
RGS8	RGS10
RGS13	RGS12
RGS16	RGS14
RGS18	**RZ family**
RGS21	RGS17
	RGS19
	RGS20

LIGANDS
5nd
CCG-4986
CCG-63802
CCG-63808
CCG-50014
CCG-203769 (RGS4 inhibitor 11b)
YJ34

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

Introduction

G protein-mediated signalling pathways have played a pivotal role in drug discovery and development for many decades. The large family of GPCRs or their downstream effectors are the target of 40% of clinically used drugs and thus represent a multi-billion-dollar industry (Wise *et al.*, 2002). Interestingly, of the more than 300 non-olfactory GPCRs known, only a fraction of them are targeted by drugs. Thus, there is a large untapped area of drug development still available. Moreover, many GPCR drugs are associated with low efficacy and/or side effects. More targeted therapies are therefore required, and as we learn more about the structure and function of GPCRs and their regulators, these goals will be achievable.

All biological signals are tightly regulated and for every on-switch there is usually an off-switch. GPCRs are activated by ligands, transmitting signalling information to Gα subunits of heterotrimeric G proteins by enhancing the exchange of GDP for GTP in the Gα nucleotide binding site, which results in the dissociation of Gα from Gβγ dimers and activation of both G protein components. Deactivation of G proteins does not occur by simple reversal of nucleotide exchange, but rather by an independently regulated GTPase activity, hydrolyzing GTP to GDP. Although Gα proteins possess an intrinsic ability to hydrolyze GTP, this process is very slow and cannot account for the transient nature of intracellular signalling cascades *in vivo*. Hence, additional kinetic mechanisms are required for the physiological timing of signals. One of the most critical of these kinetic mechanisms is mediated through regulator of G protein signalling (RGS) proteins, which have received increasing interest as novel drug targets in the past two decades. As a result, RGS proteins have now been added as the most recent addition to the International Union of Basic and Clinical Pharmacology/British Pharmacology Society (IUPHAR/BPS) Guide to PHARMACOLOGY database of drug targets (www.guidetopharmacology.org) (Alexander *et al.*, 2015; Sjögren *et al.*, 2016a).

RGS proteins all share a common RGS domain that directly interacts with active, GTP-bound Gα subunits of heterotrimeric G proteins. RGS proteins stabilize the transition state for GTP hydrolysis on Gα (Berman *et al.*, 1996a; Tesmer *et al.*, 1997) and thus induce a conformational change in the Gα subunit that accelerates GTP hydrolysis, thereby effectively turning off signalling cascades mediated by GPCRs (Figure 1). To date, there have been many excellent reviews published on the structure and function of RGS proteins as well as on their role in drug discovery. The purpose of this review is not to give a comprehensive summary of the RGS literature, but rather to serve as a guide to current advances and ways of thinking in the field of RGS protein drug discovery. For more extensive reviews on RGS proteins and their potential as therapeutic targets, see for example, Ross and Wilkie, 2000; Zhong and Neubig, 2001; Hollinger and Hepler, 2002; Druey, 2003; Cho *et al.*, 2004; Siderovski and Willard, 2005; Blazer and Neubig, 2009; Gu *et al.*, 2009; Sjögren *et al.*, 2010; Sjögren, 2011; Zhang and Mende, 2011.

RGS proteins – a brief history

The existence and characterization of negative regulators of G protein activity was almost simultaneously demonstrated in *Saccharomyces cerevisiae* (*S. cerevisiae*), *Caenorhabditis elegans* (*C. elegans*) and mammalian cells in key publications in the mid-1990s, and the specific identification of the RGS proteins in each of these systems followed soon after

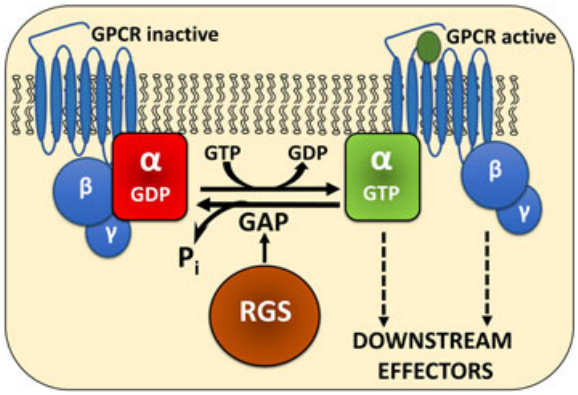

Figure 1

The canonical action of RGS proteins. In its inactive state, Gα is bound to GDP. Upon receptor activation, GDP is exchanged for GTP, Gα dissociates from Gβγ and both can mediate signalling cascades. RGS proteins bind to the transition state of GTP-bound Gα, accelerate GTP hydrolysis and effectively reduce the amplitude and duration of GPCR signalling.

(Siderovski *et al.*, 1994; Dohlman *et al.*, 1995; Wu *et al.*, 1995; De Vries *et al.*, 1996; Druey *et al.*, 1996; Koelle and Horvitz, 1996; Siderovski *et al.*, 1996; Watson *et al.*, 1996; Koelle, 1997). Within the span of a few short years, a new family of G protein regulators was established as a critical piece of the G protein regulation cycle.

As early as 1982, a novel factor regulating pheromone sensitivity and G1 cell cycle arrest was identified in yeast (Chan and Otte, 1982a,b). This factor, Sst2, was subsequently identified as a negative regulator of the G protein Gpa1 in *S. cerevisiae* (Dohlman *et al.*, 1995; 1996) and later demonstrated to be a GTPase-activating protein (GAP) for the yeast G protein Gpa1 (Apanovitch *et al.*, 1998). This feature is the hallmark of all RGS proteins, and this work established Sst2 and Gpa1 as the cognate G protein-RGS pair in yeast.

Around the same time, Koelle and Horvitz (1996) demonstrated that loss-of-function mutations in the *egl-10* gene led to reduced egg-laying behaviour and locomotion behaviour in *C. elegans* (Koelle and Horvitz, 1996). This effect was the opposite of loss-of-function mutations in the *C. elegans* G protein GOA-1, and the authors postulated that the two proteins might function in a common signalling pathway, one with positive and one with negative regulation. They subsequently demonstrated that EGL-10 shows high sequence similarity to the yeast protein Sst2 as well as several mammalian proteins that we now know as RGS proteins, including RGS1 (formally known as BL34 and 1R20), RGS2 (formally known as G0S8) and, most closely related, RGS7 (Koelle and Horvitz, 1996).

Finally, the Gilman lab described the first biochemical function of mammalian RGS proteins, demonstrating that the proteins RGS4 and GAIP (now known as RGS19) could serve as GAPs at certain Gα subtypes *in vitro*, including all members of the $G\alpha_i$ subfamily (Berman *et al.*, 1996b). The following year, in 1997, Doupnik *et al.* demonstrated that heterologous expression of RGS4 in *Xenopus* oocytes could replicate the temporal characteristics of G protein-coupled inward rectifying potassium channel deactivation following GPCR activation observed in endogenous systems, such as atrial myocytes (Doupnik *et al.*, 1997). This demonstrated functionality of mammalian RGS proteins in a biologically relevant setting and established that RGS proteins account for physiological GTPase kinetics.

It is now recognized that RGS proteins make up a large family of proteins containing a common ~120 residue RGS domain, responsible for their GAP activity towards Gα subunits of heterotrimeric G proteins. The 20 classical RGS proteins are divided into four subfamilies (R4, R7, R12 and RZ) based on sequence and domain homology (Figure 2). In addition, several other families of proteins have been identified containing an RGS homology domain. These include GPCR kinases (GRK1–7), ankyrin, AKAPs, Rho-GEFs, and sorting nexin proteins (SNX13, 14 and 25) (Siderovski and Willard, 2005). For the purpose of this review, the focus will be limited to the 20 classical RGS proteins.

To understand the importance of RGS proteins *in vivo*, numerous genetic models have been produced, such as global knockouts, as well as conditional and/or tissue specific knockout or transgenic models. While some of these display a clear phenotype, as will be exemplified in later sections, some RGS protein knockout models have produced little to no effect, most likely due to redundancy where one RGS protein can substitute for another. Early work from the Dohlman lab had identified a point mutation in the yeast Gα protein Gpa1 that made it insensitive to RGS protein action (DiBello *et al.*, 1998). Subsequently, the corresponding mutations were identified in mammalian $G\alpha_o$ (G184S) and $G\alpha_{i1}$ (G183S) (Lan *et al.*, 1998). This glycine to serine mutation prevents binding of all RGS proteins to Gα, thus enabling studies of global disabling of RGS protein GAP activity. Transgenic animal models

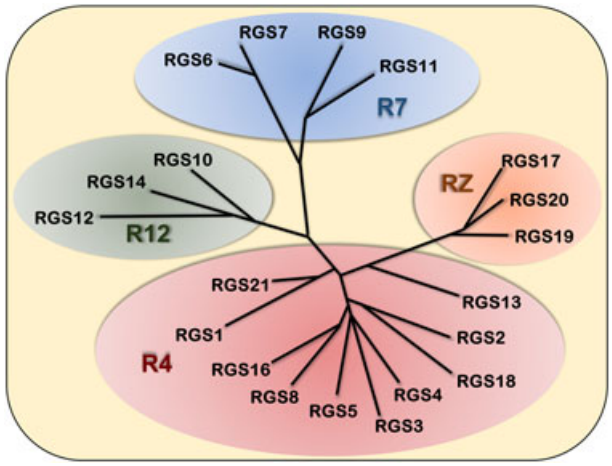

Figure 2

Classification of the 20 canonical human RGS proteins. The classical RGS proteins are divided into four families based on sequence and domain homology. The largest, the R4 family, contains RGS1, 2, 3, 4, 5, 8, 13, 16, 18 and 21. The R7 family consists of RGS6, 7, 9 and 11. The R12 family members are RGS10, 12 and 14. Finally the RZ family consists of RGS17, 19 and 20. This unrooted dendrogram was created using ClustalW alignment of the full-length RGS protein sequences and Dendroscope (Huson and Scornavacca, 2012) was used for visualization.

of these mutated Gα subunits have since been created, and have proven valuable tools to study the effects of global RGS protein action towards specific Gα subunits. In addition, they have provided key insights into the role of individual Gα subtypes. The phenotypes of these mice are extensively discussed in a recent review (Neubig, 2015) and reveal major roles for RGS proteins in regulating key physiological functions.

Why target RGS proteins in drug discovery?

In the past two decades, RGS proteins have received increasing interest as potential drug targets in numerous therapeutic areas, including cardiovascular disease, multiple CNS disorders and several types of cancer (Mittmann *et al.*, 2002; Riddle *et al.*, 2005; Hurst and Hooks, 2009; Sjögren *et al.*, 2010). While GPCR signalling has been a major focus in drug development, traditional GPCR agonists and antagonists are often associated with side effects due to widespread expression of many receptors and lack of receptor selectivity of drugs. Furthermore, many receptors can couple to more than one Gα subunit as well as initiate β-arrestin-mediated signalling pathways, resulting in several different signalling pathways being activated by the same receptor. This raises the possibility that while activation of one pathway may lead to a desired therapeutic effect, another might result in an unwanted side effect. It is therefore clear that drugs or drug combinations that are able to fine-tune cellular responses by selectively modulating a subset of downstream pathways are desirable over a simple receptor on/off switch. At the level of the receptor, great progress is being made in the field of biased signalling, as well as the development of positive and negative modulators of receptor activity (see e.g. Kenakin, 2012; Khoury *et al.*, 2014; Shukla *et al.*, 2014; Bertekap *et al.*, 2015; Bisignano *et al.*, 2015; Burford *et al.*, 2015). There is also significant potential for RGS proteins to serve a similar signalling pathway-specific role at the level of the G protein in order to improve the selectivity and efficacy of GPCR-targeted approaches.

Many RGS proteins have selectivity towards different Gα subtypes and thus can affect one pathway over another. In the early days of RGS proteins, the Gilman lab demonstrated that RGS4 has high affinity for all members of the $G\alpha_{i/o}$ subtypes, while showing lower affinity for $G\alpha_q$ and no activity towards $G\alpha_s$ and $G\alpha_{12}$ (Berman *et al.*, 1996a). RGS2, on the other hand, was demonstrated to be selective for $G\alpha_q$ over all other Gα subtypes tested (Heximer *et al.*, 1997), although later studies demonstrated that RGS2 can also inhibit $G\alpha_i$-mediated signalling *in vivo* (Chakir *et al.*, 2011). Furthermore, all members of the R7 family of RGS proteins (RGS6, 7, 9 and 11) are selective for $G\alpha_{i/o}$ proteins (Anderson *et al.*, 2009) while other RGS proteins are more promiscuous in their selectivity, for example, RGS1, RGS8, RGS13, RGS16 (Johnson and Druey, 2002; Soundararajan *et al.*, 2008). To date, no RGS proteins have been shown to have GAP activity towards $G\alpha_s$, although RGS2 has been demonstrated to associate with $G\alpha_s$ in cells (Roy *et al.*, 2006). These differences in G protein selectivity among RGS proteins could enable signalling pathway-specific regulation of GPCRs in drug development.

A second opportunity for enhanced GPCR selectivity via RGS regulation is based on expression patterns. The tissue distribution of RGS proteins is often more discrete than the G proteins they regulate, and thus, an RGS protein modulator would enable tissue-specific regulation of GPCR signalling. One example is RGS9-2, which is specifically enriched in striatum (Mancuso *et al.*, 2009) [an alternative isoform, RGS9-1, is exclusively expressed in photoreceptor cells in the retina (He *et al.*, 1998)]. This selective distribution matches the critical site of L-DOPA-induced dyskinesia, and the $G\alpha_{i/o}$-protein selectivity of RGS9 GAP activity corresponds to the $G\alpha_{i/o}$ coupling of the dopamine D_2 receptor, which is critical in mediating L-DOPA effects in the striatum (Gold *et al.*, 2007; Blundell *et al.*, 2008). The broad distribution of D_2 receptor expression limits the use of D_2 receptor targeted approaches to regulate striatal signalling pathways. However, the overlapping G protein selectivity and expression of RGS9-2 and D_2 receptors in striatum suggests that RGS9-2 may be a useful complementary target for the treatment of involuntary movements following L-DOPA treatment in Parkinson's disease (PD). In this approach, an RGS modulator would result in selective regulation of GPCR signalling only in locations where the RGS protein is expressed. The effect of a receptor-targeted drug would then be selectively altered in these tissues, enabling the use of lower doses for an effective therapeutic effect (see Blazer and Neubig, 2009 for an expanded discussion on this topic). Thus, dual targeting of RGS proteins and GPCRs can add selectivity to GPCR targeting strategies by virtue of their ability to modulate a subset of downstream pathways and their unique distribution in tissues.

Additional domains and non-canonical functions of RGS proteins

Apart from the canonical GAP activity encoded by the common RGS domain, many RGS proteins also possess additional domains and functions that provide additional potential targets for drug discovery. In many cases, GAP-independent domains and regulatory elements serve to target or regulate the RGS domain GAP functionality, while in some cases these additional domains possess signalling functionality of their own. Numerous non-canonical functions have been demonstrated for RGS proteins; due to space limitations, only a few will be discussed here. For a more comprehensive review see Sethakorn *et al.*, 2010.

Non-canonical functions of RGS proteins are typically mediated through protein–protein interactions (PPI), many of which result from additional domains present in many RGS proteins. A classic example of domain-mediated targeting is found in the R7 family of RGS proteins. The interaction between the R7 family members and binding partners $G\beta_5$ and R7BP/R9AP are mediated by the G protein γ-like (GGL), Disheveled, Egl-10, pleckstrin (DEP) and DEP helical extension domains present in these proteins (Anderson *et al.*, 2009). R7BP (for R7 binding protein) and R9AP (RGS9 associated protein, specifically in photoreceptor cells) are membrane tethered proteins that mediate targeting of R7 family RGS proteins to the plasma membrane, thereby enhancing proximity to the G protein target (Drenan *et al.*, 2005; Grabowska *et al.*, 2008). The functionality of all R7 family members is enhanced in the presence of R7BP (Drenan *et al.*, 2006; Jayaraman *et al.*, 2009). $G\beta_5$ interaction with the

GGL domain of R7 protein regulates protein stability and is described below. Additional interactions between the DEP domain and intracellular regions of GPCRs have also been demonstrated (Sandiford and Slepak, 2009) providing additional mechanisms whereby this family of RGS proteins can regulate GPCR signalling in a non-canonical manner.

Another example of a GAP-independent domain that mediates independent functionality is the $G\alpha_{i/o}$-Loco (GoLoco) motif present in the R12 family members RGS12 and 14 (Kimple *et al.*, 2001; Siderovski and Willard, 2005). Like the RGS domain, the GoLoco motif binds Gα, but this interaction inhibits GTP exchange, thereby preventing G protein activation. It also blocks the association of Gα with Gβγ, potentially leading to prolonged Gβγ signalling. This enables dual regulation of Gα by RGS12 and RGS14 (Traver *et al.*, 2004). RGS12 and RGS14 are two of the largest classical RGS proteins with additional domains apart from the RGS and GoLoco domains. Notably, the Ras binding domain(s) present in these proteins has been demonstrated to integrate GPCR and Ras/MAPK signalling pathways (Shu *et al.*, 2010; Zhao *et al.*, 2013; Brown *et al.*, 2015). Furthermore, through an additional domain, the phosphotyrosine binding (PTB) domain, RGS12 can interact with, and modulate the activity of, N-type calcium channels in a phosphorylation-dependent manner (Schiff *et al.*, 2000). Altogether, these examples shed light on the important role additional protein domains can play in mediating non-canonical functions of multidomain RGS proteins.

The presence of additional domains is not always necessary for an RGS protein to exert GAP-independent PPIs and non-canonical functions. The R4 family member RGS2 is one of the smallest RGS proteins with only a small N- and C-terminus flanking the RGS domain. Despite this, several functions have been attributed to RGS2 that are not related to its GAP activity. Firstly, RGS2 can suppress $G\alpha_s$ signalling through direct interactions with adenylate cyclase I, II and VI (Salim *et al.*, 2003; Roy *et al.*, 2006). Secondly, RGS2 suppresses general protein translation through interaction with eukaryotic initiation factor 2Bε (eIF2Bε) (Nguyen *et al.*, 2009; Chidiac *et al.*, 2014). Thirdly, RGS2 has been shown to interact directly with several GPCRs, including the α_{1A} adrenoceptor (Hague *et al.*, 2005) and the M_1 muscarinic receptor (Bernstein *et al.*, 2004b). Another member of the R4 family, RGS13, can bind directly to the transcription factor CREB and act as a transcriptional repressor (Xie *et al.*, 2008). Like RGS2, RGS13 does not contain any additional protein domains.

Together, these examples of mechanisms of regulation and non-canonical functions described above and elsewhere (Sethakorn *et al.*, 2010) reveal the complexity of RGS protein biology and contribute to their diverse potential as drug targets.

Regulation of function, localization and expression

Mechanisms regulating RGS protein levels and function range from posttranslational modifications, such as phosphorylation and palmitoylation, to control of degradation, transcription and subcellular localization. Correct function of RGS proteins requires rapid spatial and temporal regulation. Post-translational modifications, such as phosphorylation, can either enhance or inhibit RGS protein function in a rapid, cell state-specific manner. Phosphorylation of RGS14 by PKA at Thr^{494}, adjacent to the GoLoco motif, enhances its guanine nucleotide dissociation inhibitory activity towards $G\alpha_i$, while having no effect on GAP activity (Hollinger *et al.*, 2003).In contrast, GPCR ligand-dependent phosphorylation of RGS16 at Ser^{53} has been shown to inhibit GAP activity (Chen *et al.*, 2001), while Src-mediated phosphorylation at Tyr^{168} protects RGS16 from degradation leading to enhanced GAP activity in cells (Derrien and Druey, 2001; Derrien *et al.*, 2003). Another member of the R4 family, RGS2, was demonstrated to be phosphorylated by PKC, which inhibited GAP activity of RGS2 *in vitro* (Cunningham *et al.*, 2001). In contrast, we recently demonstrated that activation of PKC enhances RGS2 protein levels, leading to increased RGS2-mediated suppression of GPCR signalling in HEK-293 cells (Raveh *et al.*, 2014). Although it is not clear whether this effect is due to direct phosphorylation of RGS2 by PKC, it provides a clear example of the importance of the experimental context in which RGS protein function is studied and the complexity of RGS protein biology.

Canonical RGS domain GAP functionality requires localization to the plasma membrane, the site of action of G proteins. For several RGS proteins, palmitoylation of the N-terminus provides this targeting mechanism. Both RGS4 and RGS16 are palmitoylated at their amino-terminal, anchoring them to the plasma membrane and the GPCR-G protein complex (Chen *et al.*, 1999; Druey *et al.*, 1999; Hiol *et al.*, 2003; Bastin *et al.*, 2012). Palmitoylation within the RGS domain of these and other RGS proteins can also modulate GAP activity (Tu *et al.*, 1999; Castro-Fernandez *et al.*, 2002; Hiol *et al.*, 2003; Osterhout *et al.*, 2003; Jones, 2004; Bernstein *et al.*, 2004a; Ni *et al.*, 2006). For the members of the R7 family, as mentioned above, this function is accomplished through PPI-mediated interaction with R7BP. The protein stability of these RGS proteins is also regulated through the formation of obligatory dimers with $G\beta_5$ (Anderson *et al.*, 2009). In the absence of this Gβ subunit, as in $G\beta_5^{-/-}$ mice, all members of the R7 family (RGS6, 7, 9 and 11) are also absent due to robust protein degradation (Chen *et al.*, 2003). While R7BP binding is not necessary for protein stability of all members of the R7 family, the exception is RGS9, which in the absence of R7BP has been shown to be degraded by cysteine proteases (Anderson *et al.*, 2007a,b).

The expression of RGS proteins is not only spatially regulated per cell type and subcellular localization but also temporally regulated by mechanisms that induce or suppress RGS expression in response to specific cues or during pathological conditions. Examples include RGS2 down-regulation in androgen-independent prostate cancer (Cao *et al.*, 2006) and hypertension (Semplicini *et al.*, 2006), the down-regulation of RGS10 and RGS17 in models of chemoresistance in ovarian cancer (Hooks *et al.*, 2010), as well as the up-regulation of RGS17 in lung and prostate cancer (James *et al.*, 2009; Bodle *et al.*, 2013). Importantly, RGS transcript and protein levels may be independently regulated. Xie *et al.* (2009) demonstrated that RGS4 mRNA levels were greatly enhanced in human breast cancer

tumours. In contrast, protein levels of RGS4 from the same tissues were virtually absent, due to enhanced proteasomal degradation of RGS4 protein (Xie *et al.*, 2009). Furthermore, re-expression of RGS4 in invasive cancer cell lines in which RGS4 protein is down-regulated suppresses cancer cell invasion and migration (Xie *et al.*, 2009). Apart from RGS4, several other members of the R4 family, including RGS2 and RGS5 are substrates for the ubiquitin-proteasomal pathway and are rapidly and constitutively degraded (Davydov and Varshavsky, 2000; Lee *et al.*, 2005; Bodenstein *et al.*, 2007; Lee *et al.*, 2011; Sjögren *et al.*, 2015). This mechanism may be a way for physiological systems to very rapidly adapt to new environments. In the study by Xie *et al.* (2009), mentioned above, inhibition of proteasome activity could restore RGS4 protein levels in invasive breast cancer cells and thereby suppress invasion and migration. Altogether, this suggests that stabilizing RGS4 protein could be a promising strategy in the treatment of invasive breast cancer. In contrast, inhibiting RGS4 could also have therapeutic merit. In animal models of PD, several groups found that RGS4 mRNA is increased and contributes to the development of involuntary movement disorders following L-DOPA treatment, an effect that could be blocked by silencing RGS4 by RNAi (Lerner and Kreitzer, 2012; Ko *et al.*, 2014).

The notion that one might seek to inhibit or enhance RGS protein function depending on the therapeutic indication is further highlighted by the R7 family member RGS6 (reviewed in Ahlers *et al.*, 2016). Prolonged alcohol exposure in mice leads to increased levels of both RGS6 mRNA and protein in the ventral tegmental area (VTA), a brain region strongly associated with drug addiction. Furthermore, RGS6$^{-/-}$ mice display a reduction in alcohol seeking behaviour compared to wild-type mice, as well as diminished symptoms of conditioned reward and withdrawal (Stewart *et al.*, 2015). Inhibition of RGS6 has also been implicated as a promising therapeutic strategy in depression and anxiety (Stewart *et al.*, 2014). In contrast, RGS6 protects against dopaminergic neuron loss in the VTA, indicating that enhancing RGS6 could be beneficial in the treatment of PD (Bifsha *et al.*, 2014). This would suggest that an RGS6 modulator would have broad implications in CNS diseases. An RGS6 enhancer could also be beneficial as a novel cancer therapeutic. RGS6 has been proposed as a tumour suppressor in several types of cancer, including bladder, lung and breast cancer (Berman *et al.*, 2004; Gu *et al.*, 2006; Maity *et al.*, 2011; Maity *et al.*, 2013). While the effects of RGS6 in the CNS seem to mainly be mediated through is canonical GAP activity, its action as a tumour suppressor is mediated through non-G protein mechanisms (Maity *et al.*, 2011).

These and other studies not only further demonstrate the potential for RGS proteins as potential targets in drug development for a wide range of therapeutic indications but also highlights the complexity and challenges facing investigators that wish to pursue this avenue. While enhancement of an RGS protein may be beneficial in one disease model, other indications might benefit from an RGS protein inhibitor. Furthermore, the specific function to be targeted – GAP versus non-canonical function – may also differ between therapeutic areas.

Advances in RGS protein drug discovery – from biochemical activity to *in vivo* efficacy

Based on the non-canonical activities described above, successful RGS targeted drug discovery efforts will ultimately have to take into account that RGS proteins are not only GAPs for active, GTP-bound Gα subunits. Nevertheless, the early efforts to target RGS proteins have focused on this feature, which is the common structural element for all RGS protein family members. More recent efforts are starting to elucidate other strategies for targeting non-canonical functions and mechanisms that control expression and localization.

RGS proteins are challenging targets for small molecules. Firstly, because they are intracellular proteins, a potential RGS-modulating drug needs to be both cell permeable as well as stable in the intracellular environment. However, this is not a particularly high obstacle to overcome, and advances have been made in the drug discovery of many other intracellular protein families, such as kinases, phosphatases and nuclear receptors (Rask-Andersen *et al.*, 2011; He *et al.*, 2014; Barnes, 2016; Shang *et al.*, 2016). Indeed, small molecules have recently emerged that are active as RGS inhibitors both in cells and *in vivo* (see below).

The second, and more daunting, challenge for the development of small molecule RGS inhibitors is the task of inhibiting a PPI. The canonical mode of action of RGS proteins is through a transient PPI with active, GTP-bound Gα, a flat surface with an area of more than 2000 Å^2. PPIs are receiving increasing interest in drug discovery and this mechanism, that historically has been considered 'undruggable', is now one of the fastest expanding areas in drug development (Arkin *et al.*, 2014). Thus, while these obstacles are significant, they have not prevented several efforts to identify inhibitors of RGS proteins, with growing success. Early work on identifying RGS protein inhibitors used yeast two-hybrid and biochemical methods to detect peptides that could serve as inhibitors of the RGS-Gα interaction. These studies led to several peptides that effectively blocked RGS protein activity *in vitro* (e.g. YJ34 and 5nd; Jin *et al.*, 2004; Young *et al.*, 2004; Roof *et al.*, 2006; 2008; 2009; Wang *et al.*, 2008).

The first published small molecule RGS inhibitor, CCG-4986, was identified by the group of Richard Neubig, using a novel flow cytometry-based PPI assay (Roman *et al.*, 2007). Subsequent work from the same group used biochemical time-resolved FRET (TR-FRET) (Leifert *et al.*, 2006) to identify CCG-63802, and analogues thereof, as the first reversible RGS protein inhibitor (Blazer *et al.*, 2010). Like CCG-4986, CCG-63802 showed selectivity for RGS4 over other RGS proteins studied. A third series of small molecule RGS4 inhibitors is represented by CCG-50014, which is the first RGS inhibitor that has shown activity in cells (Blazer *et al.*, 2011). A derivative of CCG-50014, CCG-203769, was also demonstrated to have effects *in vivo*. In a mouse model of Parkinson's disease (PD), CCG-203769 was able to reverse raclopride-induced akinesia and bradykinesia. It also potentiated $G\alpha_i$-dependent muscarinic bradycardia (Blazer *et al.*, 2015), thereby replicating a phenotype previously demonstrated in RGS4$^{-/-}$ mice

to be dependent on RGS4 (Cifelli *et al.*, 2008). This shows that RGS protein inhibitors may be used in a clinical setting alone or in conjunction with other therapies.

Although CCG-50014 and CCG-203769 have been shown to be active in biological systems, many early inhibitors identified in biochemical screens failed to move forward due to lack of cellular activity. We attempted to overcome this problem by developing a cell-based high-throughput assay for RGS4 inhibitors. We used the FlpIn-TREx system (Invitrogen™) to develop a cell line with stable expression of the $G\alpha_q$-coupled M_3 muscarinic receptor and doxycycline-inducible RGS4 expression and screened for compounds that could reverse RGS4-mediated suppression of Ca^{2+} signalling (Storaska *et al.*, 2013). This screen identified several novel inhibitors and studies are ongoing to characterize them further.

Apart from RGS4, RGS17 has been a focus for high-throughput screening for small molecule inhibitors. As discussed above, RGS17 is one of several RGS proteins that are up-regulated in different cancers. In both lung and prostate cancer, RGS17 mRNA is significantly increased and this contributes to tumour progression (Bodle *et al.*, 2013). This led the lab of David Roman to develop an Alpha Screen assay to screen for small molecule inhibitors of the RGS17-$G\alpha_o$ interaction (Mackie and Roman, 2011). The hits identified from this screening campaign inhibited this interaction with micro molar potency. Future development of these or other RGS17 inhibitors could serve as novel cancer therapeutics.

Although much of RGS protein drug discovery efforts have been focused on identifying inhibitors of GAP activity, identifying enhancers of RGS protein function may be equally important from a clinical perspective. However, this is a more daunting task, since enhancing a PPI is far more difficult than blocking it. Several RGS proteins are down-regulated during pathological insults so finding ways to increase their expression, and thereby function, is an attractive alternative strategy to achieve this goal. This encouraged us to develop a cell based enzyme complementation assay to screen for small molecule stabilizers of RGS2 (Sjögren *et al.*, 2012; Raveh *et al.*, 2014). As discussed above, RGS2 is one of several RGS proteins that is rapidly degraded through the ubiquitin-proteasomal pathway. Low RGS2 protein levels are associated with hypertension and other cardiovascular pathologies (Heximer *et al.*, 2003; Takimoto *et al.*, 2009; Tsang *et al.*, 2010) and could also be involved in the progression of prostate and breast cancer (Cao *et al.*, 2006; Lyu *et al.*, 2015). In our initial screen, we identified digoxin and other cardiotonic steroids as selective stabilizers of RGS2 protein levels (Sjögren *et al.*, 2012). In subsequent work, we demonstrated that digoxin is protective in a murine model of cardiac injury, an effect that was lost in $RGS2^{-/-}$ mice (Sjögren *et al.*, 2016b). This is the first study demonstrating that pharmacological enhancement of an RGS protein has effects *in vivo* and opens up new avenues for RGS protein drug discovery.

RGS protein drug discovery – what does the future hold?

Although great progress has been made in the field of RGS protein biology, many mechanisms still need to be elucidated. What has become clear is that members of this family are more than just GAPs for G proteins, and the emerging plethora of non-canonical functions may become a more prominent focus in the future. Given the important role of GPCRs in physiology and drug discovery, however, the canonical G protein regulatory role of RGS proteins is likely to remain a focus in future drug development efforts. Early drug discovery efforts focused solely on the inhibition of RGS proteins interacting with Gα subunits, but other functions, as well as dynamic regulation of expression, were ignored. Future efforts may investigate these regulatory mechanisms further, especially for the development of RGS protein enhancers.

The RGS proteins that have been targeted in drug discovery thus far (RGS2, 4 and 17 as described above) all have in common that they are small, containing no additional domains apart from the RGS domain. This makes them 'easier' to work with from an experimental stand point. Although other RGS proteins might also have great therapeutic potential, such as RGS9 in PD drug development, the additional non-canonical functions of additional protein domains present in these RGS proteins make drug discovery efforts less straight-forward. In some cases, targeting GAP activity may not be the primary goal when developing small molecules to target these larger, multi-domain, RGS proteins. Thus, although many RGS proteins could have great therapeutic potential, more studies are required to determine their physiological function and how best to target them. These may depend on a detailed knowledge of the mechanisms of RGS protein regulation that control their expression, post-translational modifications and other mechanisms that have yet to be elucidated. After all, the existence of RGS proteins has only been acknowledged in the last 20 years. What will the next 20 years bring?

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Conflict of interest

The author declares no conflicts of interest.

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