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Review

RGS Proteins in Sympathetic Nervous System Regulation: Focus on Adrenal RGS4

Anastasios Lymperopoulos[1,*], Renee A. Stoicovy[1]

[1]Department of Pharmaceutical Sciences, Laboratory for the Study of Neurohormonal Control of the Circulation, Nova Southeastern University Barry and Judy Silverman College of Pharmacy, Davie/Fort Lauderdale, FL 33328-2018, USA
*Correspondence: al806@nova.edu (Anastasios Lymperopoulos)
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Abstract

The sympathetic nervous system (SNS) consists largely of two different types of components: neurons that release the neurotransmitter norepinephrine (NE, noradrenaline) to modulate homeostasis of the innevrvated effector organ or tissue and adrenal chromaffin cells, which synthesize and secrete the hormone epinephrine (Epi, adrenaline) and some NE into the blood circulation to act at distant organs and tissues that are not directly innervated by the SNS. Like almost every physiological process in the human body, G protein-coupled receptors (GPCRs) tightly modulate both NE release from sympathetic neuronal terminals and catecholamine (CA) secretion from the adrenal medulla. Regulator of G protein Signaling (RGS) proteins, acting as guanosine triphosphatase (GTPase)-activating proteins (GAPs) for the Gα subunits of heterotrimeric guanine nucleotide-binding proteins (G proteins), play a central role in silencing G protein signaling from a plethora of GPCRs. Certain RGS proteins and, in particular, RGS4, have been implicated in regulation of SNS activity and of adrenal chromaffin cell CA secretion. More specifically, recent studies have implicated RGS4 in regulation of NE release from cardiac sympathetic neurons by means of terminating free fatty acid receptor (FFAR)-3 calcium signaling and in regulation of NE and Epi secretion from the adrenal medulla by means of terminating cholinergic calcium signaling in adrenal chromaffin cells. Thus, in this review, we provide an overview of the current literature on the involvement of RGS proteins, with a particular focus on RGS4, in these two processes, i.e., NE release from sympathetic nerve terminals & CA secretion from adrenal chromaffin cells. We also highlight the therapeutic potential of RGS4 pharmacological manipulation for diseases characterized by sympathetic dysfunction or SNS hyperactivity, such as heart failure and hypertension.

Keywords: catecholamine secretion; chromaffin cell; free fatty acid receptor-3; G protein-coupled receptor; norepinephrine release; regulator of G protein signaling-4; signal transduction; sympathetic nervous system

1. Introduction

G protein-coupled receptors (GPCRs) represent the most diverse and populated cell membrane receptor superfamily, with ~350 (non-sensory) members mediating regulation of all aspects of cell physiology by various chemical molecules and hormones [1–4]. The catecholamines (CAs) norepinephrine (NE) and epinephrine (Epi) mediate all the actions of the sympathetic branch of the autonomic nervous system (SNS), as a neurotransmitter released by sympathetic neurons and a hormone secreted by the adrenal medulla, respectively [3]. NE is also secreted alongside Epi from the adrenal gland but to a lesser extent [5]. Both NE and Epi exert their actions in cells through nine different adrenergic receptor (AR) subtypes, all of which are cell membrane-residing GPCRs. In other words, all ARs couple to heterotrimeric guanine nucleotide-binding proteins (G proteins) to transduce their signals inside the cell [6]. These heterotrimeric G proteins consist of four families: $G_{i/o}$, $G_{s/olf}$, $G_{q/11/14/15}$, $G_{12/13}$, based on the 16 different Gα subunits they contain. GPCRs (ARs) do not only mediate the cellular actions of NE and Epi but they also tightly regulate their release/secretion. For example, $G_{q/11}$ protein-coupled muscarinic chloninergic receptors of acetylcholine (mAChRs) are also GPCRs and mediate, together with nicotinic cholinergic receptors, the physiological stimulus of CA secretion from the adrenal medulla, which acetylcholine-induced exocytosis [5]. On the other hand, $G_{i/o}$ protein-coupled α_2ARs inhibit NE release from SNS terminals and CA secretion from adrenal chromaffin cells [7–9].

All GPCRs act as a guanine nucleotide exchange factors (GEFs) for the Gα subunit of a heterotrimeric G protein, in essence separating the Gα subunit from its bound G$\beta\gamma$ dimer thanks to the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) on the Gα subunit. Bound to GDP and the G$\beta\gamma$ dimer, Gα is inactive but capable of surveing the intracellular interface of the cell membrane, via its C-terminal α5 helix for agonist-activated GPCRs to interact with [10]. Upon such interaction, Gα releases GDP and binds the much more abundant GTP in its place [11]. This GTP binding induces large structural rearrangements in the Gα subunit, which dramatically reduce Gα subunit's affinity for both G$\beta\gamma$ and the receptor [11,12]. Thus, GTP-bound Gα dissociates from G$\beta\gamma$ and

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the GPCR and instead interacts with effectors to modulate their activity [13]. Dissociated ("free") G$\beta\gamma$ also modulate activity of various enzymes and ion channels [12], and, thus, the entire heterotrimer is now signaling-competent (active). Termination of G protein signaling is equally important to its activation for every cell and is achieved at both the receptor level, via the process of desensitization, i.e., G protein uncoupling [14], and the G protein level, via GTP hydrolysis to GDP by the Gα subunit that possesses GTPase activity [13]. Upon GTP conversion to GDP, GDP-bound Gα now re-associates with G$\beta\gamma$ and the G protein reurns to its inactive state [15,16]. Despite having GTPase activity of their own though, most Gα subunits utilize co-factors (GTPase activating proteins, GAPs) to get inactivated quickly enough for normal cellular function and homeostasis. These GAPs are called "Regulator of G protein signaling (RGS)" proteins, because they all contain a conserved ~120-amino acid-long domain (the "RGS box"), which dramatically accelerates GTP hydrolysis by stabilizing its transition state [16–22]. Over 37 mammalian RGS proteins exist, with several more containing non-functional "RGS homology" domains [21,22]. Some RGS proteins can interfere with the interaction of active Gα free G$\beta\gamma$ subunits with effectors, while some others can interact directly with GPCRs [15]. Importantly, RGS proteins inactivate not only Gα but also the free G$\beta\gamma$ subunits, since GTP hydrolysis causes Gα-G$\beta\gamma$ reassociation [15]. Several RGS proteins act upon more than one Gα type/G protein family (e.g., RGS4 regulates G$\alpha_{i/o}$ and G$\alpha_{q/11}$) [16]. Importantly, most (if not all) RGS proteins inactivate their G protein substrates in a cell type- and GPCR type-specific manner, with the identity of the receptor that has stimulated the G protein dictating whether that G protein will be inactivated by which RGS protein. For example, RGS4 inactivates angiotensin II (AngII) type 1 receptor (AT_1R)-stimulated Gαq but not gonadotropin-releasing hormone (GnRH) receptor-stimulated Gαq subunits [23,24]. Certain RGS proteins have been implicated in regulation of NE release from SNS neurons and in CA secretion from adrenal glands. In this review, we provide an overview of these (admittedly few) studies documenting these regulatory roles of RGS proteins, particularly of RGS4, and we also underline the potential of targeting these RGS proteins therapeutically for diseases associated with abnormally elevated CA levels.

2. RGS Proteins and NE Release from SNS Terminals

The roles of several RGS proteins, e.g., RGS6, RGS4, RGS2, in parasympathetic regulation of cardiac function, particularly in modulation of acetylcholine (ACh)-dependent bradycardia and slowing of atrial conduction, have been extensively documented [15–20]. However, rather surprisingly, very little is known regarding regulation of sympathetic neuronal function/nerve activity (SNA) and NE release by RGS proteins. Studies on RGS2 knockout mice indicated that these animals display a hypertensive phenotype with central SNS activation, increased urinary NE levels, and impaired nitric oxide-mediated vasodilation [25,26]. However, a later study failed to replicate these findings and reported indistinguishable circulating NE and Epi levels between RGS2 knockout and control wild type mice, as well as no change in mean blood pressure [27]. On the other hand, RGS2 ameliorates AngII-induced hypertension [28] and its levels can be reciprocally regulated by the vascular AT_1R [29–32]. Therefore, it is quite plausible that the effect of the genetic deletion of RGS2 on systemic hypertension is (primarily) mediated by elevated AngII signaling in the vasculature and not so much by elevated SNS activity and NE release. In any case, irrespective of the mechanism(s) involved, it appears that RGS2 plays a minimal (if at all) role in NE release from sympathetic neurons (Fig. 1).

In a recent study from our lab, RGS4 was shown to regulate NE release from sympathetic-like neurons *in vitro* via its actions on the short chain free fatty acid receptor (FFAR)-3, also known as GPR41, a GPCR that mediates signaling by short chain (up to 5 carbon atoms) carboxylic acids, such as propionate and butyrate [33]. FFAR3 is Gi/o protein-coupled GPCR robustly expressed in murine peripheral sympathetic neurons, including cardiac SNS terminals, wherein it regulates SNA/SNS firing by stimulating NE release [34] (Fig. 1). Although both NE and Epi mediate the effects of the sympathetic nervous system on all cells and tissues of the entire body, NE is the actual neurotransmitter synthesized, stored, and released from sympathetic neurons, since SNS neurons lack the enzyme phenyl-ethanolamine-N-methyltransferase (PNMT), which converts NE to Epi [35–37]. FFAR3 promotes neuronal firing and NE synthesis and release in SNS neurons via stimulation of Phospholipase C (PLC)-$\beta_{2/3}$ by Gi/o protein-derived free G$\beta\gamma$ subunits [34] (Fig. 1). Free $G_{\beta\gamma}$-activated PLC$\beta_{2/3}$ activates, in turn, the mitogen activated protein kinases (MAPKs) extracellular signal regulated kinase (ERK)1/2, which phosphorylate synapsin-2β to induce vesicle fusion with the neuronal plasma membrane and thus, NE release from SNS terminals [38] (Fig. 1). RGS4 can bind Gi/o protein-derived free G$\beta\gamma$ subunits directly, as well as PLCβ, thereby inhibiting PLCβ activation independently of its GAP activity [39–41]. This prompted us to speculate that RGS4 may negatively regulate (suppress) FFAR3 signaling towards NE release stimulation. Indeed, this proved to be the case [42] (Fig. 1). More specifically, in rat H9c2 cardiomyocytes, RGS4 dampened propinic acid-activated FFAR3 signaling towards cyclic adenosine monophosphate (cAMP) lowering via Gi/o protein activation, as well as p38 MAPK activation, pro-inflammatory interleukin (IL)-1β and IL-6 production, and pro-fibrotic transforming growth factor (TGF)-β synthesis [42]. Importantly, RGS4 also reduced FFAR3-dependent NE release from sympathetic-like neurons (differentiated Neuro-

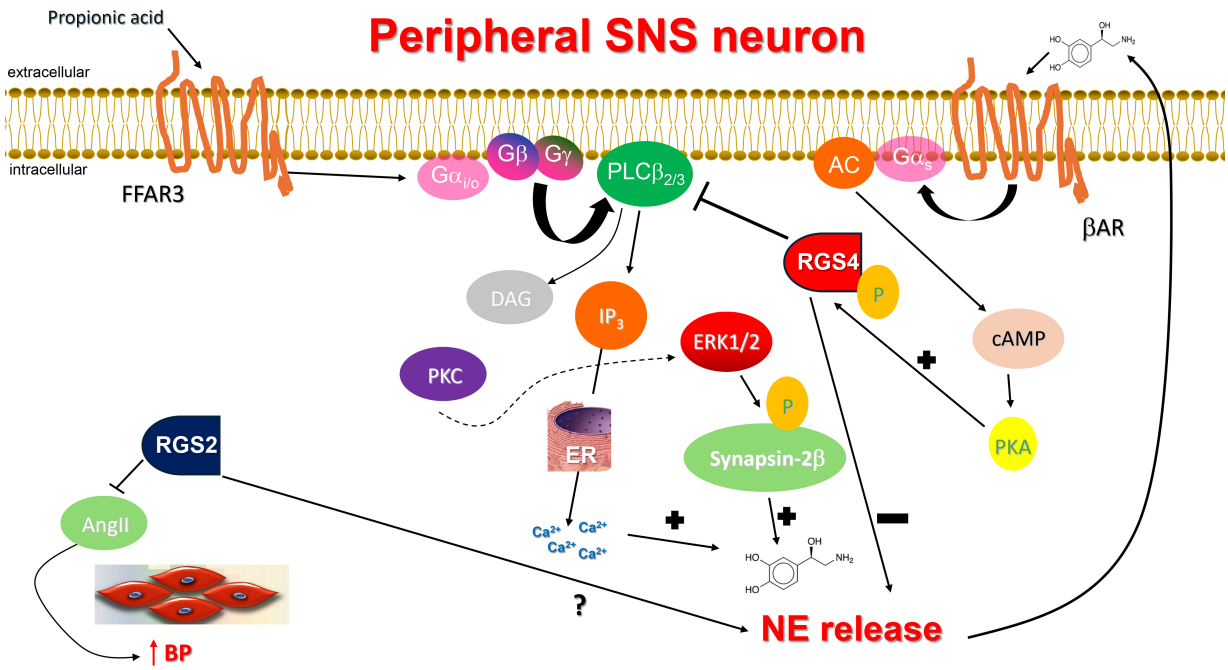

Fig. 1. RGS4 inhibits NE release from sympathetic neurons and & RGS2 reduces AngII secretion in the vasculature. AC, Adenylyl cyclase; AngII, Angiotensin II; AR, Adrenergic receptor; BP, Blood pressure; cAMP, Cyclic 3′, 5′-adenosine monophosphate; DAG, 1′, 2′-Diacylglycerol; ER, Endoplasmic reticulum; ERK1/2, Extracellular signal-regulated kinase-1/2; $G_{\alpha i/o}$, Inhibitory/other alpha subunit of heterotrimeric guanine nucleotide-binding protein; $G_{\alpha s}$, Stimulatory alpha subunit of heterotrimeric guanine nucleotide-binding protein; IP_3, Inositol 1′, 4′, 5′-trisphosphate; PKA, Protein kinase A; PKC, Protein kinase C; PLCβ, Phospholipase C-beta; NE, Norepinephrine; RGS, Regulator of G protein Signaling; SNS, Sympathetic nervous system. "P" enclosed in a dark yellow circle indicates phosphorylation, arrows with "+"indicate stimulation, upward arrows indicate increase, arrows with "–" indicate decrease, "⊣' indicates inhibition. "?" indicates action/effect presently unknown/undefined.

2a cells) co-cultured with H9c2 cardiomyocytes, which prevented cardiomyocyte βAR downregulation [42] (Fig. 1). These findings await confirmation in animal models *in vivo*; however, they provide unquestionable evidence for the important role RGS4 plays in inhibition of NE release from SNS neurons via termination of FFAR3 signaling in sympathetic neurons and, by extension, in SNS activity mitigation (Fig. 1).

Finally, RGS4 can be activated via phosphorylation by protein kinase A (PKA), the kinase activated by cyclic adenosine monophosphate (cAMP) produced by NE-activated βARs [42,43] (Fig. 1), as well as by cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG), activated by natriuretic peptides [43,44], and GPCR kinase (GRK)-2 [43], which phosphorylates and desensitizes several GPCRs, including the βARs [45,46]. Since βAR-stimulated cAMP/PKA promotes NE release from SNS terminals [47], PKA-activated RGS4 acting as GAP for FFAR3 signaling may serve the purpose of closing a negative feedback loop in regulation of NE release from SNS neurons: βARs in conjunction with FFAR3 promote NE release and, in parallel, βARs activate RGS4 via PKA phosphorylation to reduce NE release via FFAR3 signaling termination (Fig. 1).

Notably, the ketone body β-hydroxybutyrate (or 3-hydroxybutyrate) has been shown to act as FFAR3 antagonist, also reducing SNS neuronal firing rate [34]. This means that β-hydroxybutyrate (and possibly other ketones) mimics RGS4's actions on FFAR3 signaling in SNS neurons. Given that sodium/glucose co-transporter (SGLT)-2 inhibitor drugs, such as dapagliflozin and empagliflozin, which exert a multitude of beneficial cardiovascular effects, including SNS activity lowering, and increase ketone body (including β-hydroxybutyrate) production [48–50], it is quite plausible that the sympatholytic effects of SGLT2 inhibitors are mediated in part by FFAR3 signaling attenuation through β-hydroxybutyrate antagonism or even possibly through RGS4 potentiation [51]. The interplay between FFAR3, RGS4, and β-hydroxybutyrate appears to play a central role in SNS activity modulation and thus, future studies are warranted to fully delineate it.

3. RGS Proteins and Adrenal CA Secretion

Certain RGS proteins, i.e., RGS4 and RGS2, play major roles in modulation of adrenal CA secretion. Several $G_{q/11}$-coupled GPCRs, including muscarinic M_1 mAChRs and AT_1Rs, that raise free intracellular Ca^{2+} levels act

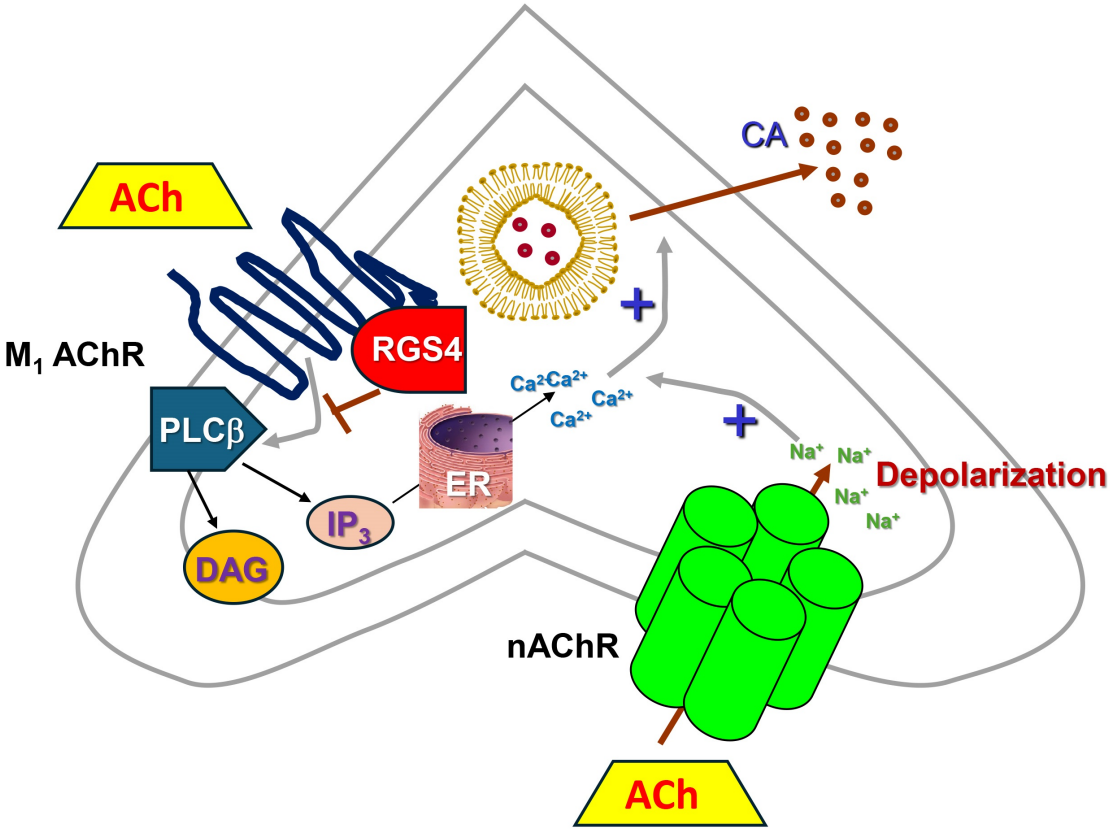

Fig. 2. RGS4 inhibits CA secretion in adrenal chromaffin cells. M_1 AChR, Muscarinic cholinergic receptor type 1; nAChR, Nicotinic cholinergic receptor; Ach, Acetylcholine; CA, Catecholamine (norepinephrine or epinephrine); DAG, 1′, 2′-Diacylglycerol; ER, Endoplasmic reticulum; IP_3, Inositol 1′, 4′, 5′-trisphosphate; PLCβ, Phospholipase C-beta.

as secretagogues for Epi and NE secretion from the chromaffin cells of the adrenal medulla via the classic Ca^{2+}-dependent process of exocytosis/secretion [52–57]. Interestingly, AT_1R-dependent Ca^{2+}-dependent exocytosis leading to CA secretion can also be promoted by the β-arrestins, two GPCR adapter proteins and signal transducers that normally terminate G protein signaling [58,59]. The mechanism appears to be the direct interaction of βarrestin1 with the Ca^{2+} channel TRPC3 (short transient receptor potential channel-3) in adrenal chromaffin cells [60]. RGS4 is highly expressed in the adrenal medulla, where it inhibits cholinergic-induced CA secretion from chromaffin cells by serving as GAP for the $G_{\alpha q/11}$ subunits activated by the M_1 mAChR [57] (Fig. 2). $G_{\alpha q/11}$ subunits activate PLCβ which, in turn, converts PIP_2 to the second messengers Inositol 1′, 4′, 5′-trisphosphate (IP_3) and 1′, 2′-Diacylglycerol (DAG) [61]. IP_3 is highly hydrophilic, so it readily diffuses through the cytoplasm to bind its receptor in the endoplasmic reticulum membrane (IP_3R), which is essentially a Ca^{2+} channel that releases the stored Ca^{2+} into the cytosol [62] (Fig. 2). DAG also contributes to raised free intracellular [Ca^{2+}] courtesy of activating protein kinase C (PKC) [63]. It should be noted here that the physiological stimulus of adrenal CA secretion, acetylcholine released from preganglionic sympathetic splanchnic nerves, primarily induces CA secretion via its nicotinic receptors (nAChRs), which are ligand (acetylcholine)-gated sodium channels [64,65]. However, muscarinic receptors, particularly of the M_1 subtype, play a big part, almost of equal importance with that of the nAChRs, in cholinergic-dependent CA secretion from the adrenal medulla [64,66]. Therefore, RGS4-mediated termination of M_1 mAChR (potentially also of AT_1R [22]) $G_{q/11}$/PLCβ signaling in chromaffin cells serves as a molecular "brake" on adrenal CA secretion (Fig. 2). Indeed, both Epi and NE secretions *in vitro* in response to acute acetylcholine stimulation (for 1 and 5 minutes) were markedly (over 4 times) higher in adrenal slices lacking RGS4 (isolated from RGS4 global knockout mice) compared to control, wild type murine adrenal glands [57]. This finding is very important because, although the mice used in that study [57] were global knockouts, the in ivtro CA secretion experiments were performed in iso-

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lated adrenal glands, i.e., the CA secretion measured could not have been influenced by the lack of RGS4 in other tissues/organs outside the adrenals (e.g., autonomic neurons). Thus, they confirmed the direct regulation of CA secretion by adrenal RGS4.

Of note, RGS4 has been shown to also inhibit AT_1R-stimulated aldosterone synthesis and secretion via downregulation of aldosterone synthase (CYP11B2) expression in adrenocortical cells [67], an effect shared with RGS2 [68]. Interestingly, adrenal RGS2 can be upregulated by angiotensin II (AngII), so RGS2-dependent aldosterone secretion inhibition might serve as a negative feedback mechanism for AngII-induced aldosterone production [68]. However, unlike adrenocortical aldosterone production which has been shown to be blocked by both RGS2 and RGS4, RGS4 remains the only RGS protein reported to date to inhibit CA secretion from the adrenal medulla [57]. Indeed, RGS4 knockout mice exhibit elevated adrenal CA secretion but RGS2 knockout animals have apparently normal circulating Epi (and NE) levels [27], which, given that the adrenal gland is the sole source of circulating Epi, argues against a significant impact of adrenal RGS2 on CA secretion. Thus, adrenal CA secretion inhibition, coupled with its effects on aldosterone secretion from the cortex, place RGS4 front and center in adrenal hormone secretion regulation, uniquely positioned to filter down excess CA and aldosterone production. Thus, strategies to potentiate adrenal RGS4 activity/levels may afford a significant therapeutic benefit in diseases characterized by elevated CA and/or aldosterone levels, such as chronic heart failure, hypertension, and other heart diseases [69,70].

One such strategy is the use of the neurostimulant parachloroamphetamine (PCA), which specifically inhibits the Arg/N-end rule pathway, delaying the degradation of RGS4 [71]. RGS4 is a substrate of the so-called "Arg/N-end rule" pathway, in which proteins with either positively charged residues (the case of RGS4), such as Arg, Lys, and His, or bulky, hydrophobic residues, such as Phe, Trp, Leu, Tyr, and Ile, at their N-termini undergo proteasomal degradation and PCA has been reported to inhibit the Arg/N-end rule pathway slowing down RGS4 degradation *in vitro* and *in vivo* [71]. Indeed, intraperitoneal injection of PCA significantly increased endogenous RGS4 protein levels in the brain, particularly in the frontal cortex and hippocampus, in mice [71]. Thus, PCA or PCA derivatives may be utilized for therapeutic boosting of protein levels/activity specifically of RGS4 in the central nervous system or in the adrenal glands.

4. Conclusions

Considerable progress has been made over the past two decades in delineating the various signaling properties and biological actions of RGS proteins in almost every organ system, including in the central and autonomic nervous systems. As key regulators of GPCR signaling through G proteins, RGS proteins are enticing therapeutic targets based on their physiological and pathophysiological importance in the heart, kidneys, central nervous system, oncology, and other disease areas. On the other hand, there is a plethora of pathological situations where enhanced G protein signaling, accompanied by reduced RGS protein activity, is involved in the pathophysiology of a cardiovascular disease, so augmentation of RGS protein function would be desirable.

It is clear from the discussion above that particularly adrenal RGS4 could be targeted for sympatholytic therapy in the context of heart failure, hypertension, and other diseases accompanied and aggravated by elevated SNS activity. Although it is generally very hard to enhance the activity of an RGS protein pharmacologically, the inhibition of the N-end rule pathway with agents like parachloroamphetamine provides a realistic, paves a viable way forward for the pharmaceutical and, importantly, selective potentiation of RGS4 activity. Regarding the central SNS, the picture is still quite foggy, unfortunately. More studies are urgently needed to address the roles of specific RGS proteins in regulation of SNA and NE release from the central nervous system (CNS). For the time being, RGS4, again, appears to be the RGS protein family member standing out for therapeutic targeting. Coupled with its established role in prevention of atrial fibrillation and of other cardiac arrhythmias, as well as its potential role in mitigating cardiac hypertrophy and heart failure (reviewed in Ref. [33]), it is easily conceivable that pharmacological targeting of RGS4 may be pursued for treatment of various cardiovascular conditions in the near future. On the other hand, given the multifaceted role of RGS4 in neuropsychiatric disorders (e.g., schizophrenia), potentiation of RGS4 in the CNS might turn out to be a "double edged sword". Hence, the need for more studies on RGS4 and the other R4 family RGS proteins is more urgent than it has ever been.

Author Contributions

RS and AL performed literature research and contributed to the writing of the manuscript and the drawing of the figures. AL supervised the project, led the writing of, and edited the manuscript. Both authors read and approved the final manuscript. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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