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A role of RGS proteins in drug addiction

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ABSTRACT

The diverse family of Regulators of G protein signaling (RGS) proteins are widely distributed proteins with multiple functions, including GAP activity for heterotrimeric G protein alpha subunits. Three members of the RGS family, RGS9-2, RGS4 and RGSz, have been shown to play an essential modulatory role in psychostimulant and opiate drug actions. Interestingly, these proteins show distinct structure, distribution pattern and cellular localization. In addition, each of these proteins is differentially regulated by drugs of abuse in particular brain networks and appears to modulate distinct signal transduction events. The striatal enriched RGS9 plays a prominent role in opiate and psychostimulant drug reward; RGS4 appears to modulate opiate dependence via actions in the locus coeruleus, whereas RGSz modulates analgesia via activation of the PKC pathway.

Drug addiction is a chronic disease, triggered by repeated exposure to substances such as opioids and psychostimulants, and it is generally defined as compulsive drug use and loss of control over drug intake [1–5]. Exposure to drugs of abuse affects the function of several brain networks including the mesoaccumbens dopamine pathway, extending from the ventral tegmental area of the midbrain to the nucleus accumbens (ventral striatum) and from there to the prefrontal cortex [6]. The addiction process also involves several nuclei of the amygdala [2,7], the hippocampus [8,9] the locus coeruleus [2,10] the periaqueductal gray [8] and the spinal cord [11,12]. The neurochemical changes that follow exposure to addictive substances involve the dopaminergic, opioidergic and noradrenergic systems. Each of these neurotransmitter systems function by binding and activating cell surface G protein-coupled receptors (GPCRs) expressed in pre-synaptic or post-synaptic cell membranes, resulting in the initiation of cellular signaling events that mediate neuronal responses to these neurotransmitters. GPCRs are a major class of transmembrane receptors that are targeted in greater than half of all pharmaceutics on the market and nearly all drugs of abuse. Many of the adaptive changes that occur during the progression of addiction involve GPCRs and other molecules that regulate the activity of G proteins or their downstream effectors.

GPCRs and the cellular proteins with which they are associated transmit signals across the plasma membrane through conformational changes, enzymatic activity, and formation of multi-protein complexes. The immediate effect of receptor activation by ligand is the activation of heterotrimeric G proteins, which act as molecular switches in diverse cell signaling pathways [13]. When bound to guanine nucleotide diphosphate (GDP), the Gα subunit associates with a Gβγ dimer in an inactive heterotrimer. When bound to guanine nucleotide triphosphate (GTP) the Gα subunit undergoes conformational changes in three switch regions involved in binding to the Gβγ dimer and to effector molecules. The Gα subunit then dissociates from the Gβγ dimer, both of which

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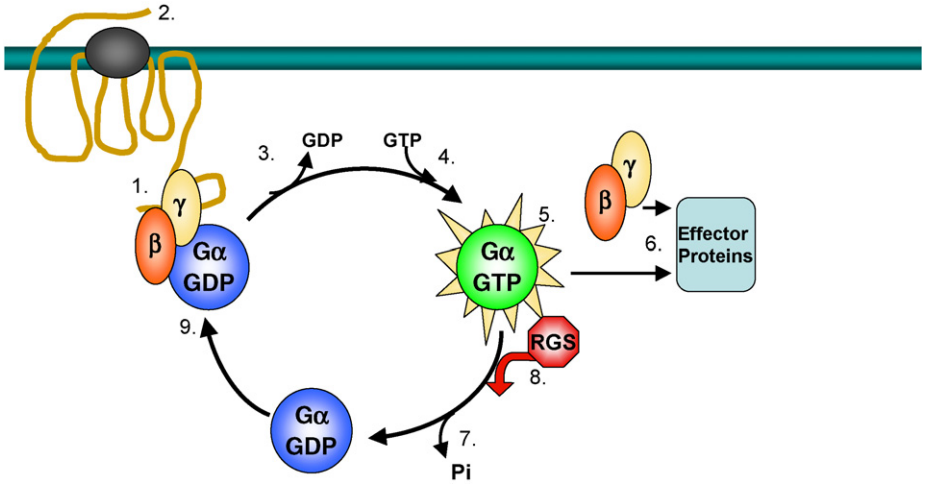

Fig. 1 – G-protein nucleotide binding and activation cycle. In the basal state, the Gα subunit is bound to GDP and forms an inactive heterotrimer with Gβγ dimers. The heterotrimer associates with cytosolic portions of GPCRs to promote the high-affinity state of the receptor (1). Agonist binds receptor, inducing conformational changes in the transmembrane domains of the receptor (2). These conformational changes alter receptor interaction of the G-protein heterotrimer in such a way to promote the dissociation of GDP from the Gα nucleotide binding pocket (3). Once GDP is removed from the binding pocket, GTP, which is present in cytosol at much higher concentrations than GDP, quickly binds the Gα subunit, causing major changes in the conformation of three helical "switch regions" of the Gα subunit (4). The switch regions of Gα contain critical contact points for association with Gβγ dimers. Structural changes in these regions induced by GTP binding cause dissociation of Gβγ dimers from the Gα subunit (5). Dissociation of the heterotrimer unmasks active binding sites on both the Gα subunit and the Gβγ dimer that are responsible for activating effector enzymes that regulate neuronal activity (6). The Gα subunit functions as a GTPase enzyme, hydrolyzing the third phosphate group of GTP to generate GDP in the nucleotide binding pocket (7). While the endogenous Gα GTPase activity is very slow, this rate is accelerated dramatically by RGS proteins, which directly bind GTP-bound Gα subunits and enhance their GTPase activity (8). Once returned to the inactive GDP bound state, Gα subunits reassociate with Gβγ dimers to return to the basal state (9).

are then active to trigger downstream signaling events. Receptors activate specific subtypes of G proteins, each of which mediates neuronal responses to the neurotransmitter signal by regulating second messenger levels or by directly regulating ion channel function. There are four major classes of Gα G protein subunits, which differ in their effects on downstream effectors. Briefly, $G\alpha_s$ proteins activate adenylyl cyclase to increase cellular cAMP concentrations; $G\alpha_{i/o}$ proteins inhibit adenylyl cyclase to oppose $G\alpha_s$ activity, activate G protein regulated inwardly rectifying potassium (GIRK) channels, and inhibit voltage-gated calcium channels; $G\alpha_q$ proteins activate phospholipase C enzymes to produce diacyl glycerol and inositol triphosphate, and inhibit GIRK channels; $G\alpha_{12/13}$ proteins activate the small G protein Rho and some phospholipase subtypes; and Gβγ dimers activate phospholipases and ion channels (reviewed in [10,14–16]). Thus, there is remarkable functional diversity among G protein families [17].

Both the activation and deactivation of G proteins are highly regulated events in cells. Neurotransmitter binding to the receptor initiates conformational changes in the transmembrane domains, particularly TM3 and TM6 [18], that interact with heterotrimeric G proteins. Upon ligand activation, GPCRs stimulate exchange of GDP for GTP, thereby turning G proteins "on". The lifetime of this active state of G proteins is regulated in cells by the rate at which the Gα subunit hydrolyzes GTP, and thereby returns to the GDP bound inactive state. G protein alpha subunits possess weak GTPase catalytic activity, but the basal rates of this activity are much slower than the deactivation kinetics of G proteins in vivo. Accounting for this discrepancy, diverse GTPase accelerating proteins (GAPs) function to enhance the GTPase activity of the G-α subunit [19], thereby turning G proteins "off" (Fig. 1). While GPCRs and their ligands have been extensively investigated as G protein activators, relatively little is known about the more recently discovered proteins that deactivate G proteins. A diverse family of Regulator of G protein signaling (RGS) proteins that act as GAPs for heterotrimeric G protein α subunits has emerged over the last decade [20–22]. By accelerating the return of the activated GTP-bound Gα protein to its basal GDP-bound state, RGS proteins terminate effector activation by both Gα and Gβγ subunits, thus regulating the kinetics and amplitude of signaling.

1. GPCR families in addiction biology

Changes in dopamine receptor signaling in the nucleus accumbens underlie addiction to many abused drugs. To date, more than five dopamine receptor subtypes have been found to be expressed in the mesolimbic dopamine system [23]. Dopamine receptor subtypes are linked to different G proteins, with D_1 and D_5 associated with G_s activity, whereas D_2-like receptors (D_2, D_3, D_4) are thought to act primarily via

$G_{i/o}$ proteins [24,25]. Activation of D_1 and D_5 receptors leads to stimulation of adenylyl cyclase to increase cellular cAMP levels, increased CREB phosphorylation and changes in gene transcription. D_2-like receptor activation leads to inhibition of adenylyl cyclase or no effect on the enzyme activity [25]. Both D_1 and D_2 receptor agonists have reinforcing and locomotor activating properties, but recent evidence primarily attributes aspects of drug reward to D_1 actions, and links D_2-like receptors to relapse and drug seeking [26,27].

Opioids such as morphine, heroin and methadone exert their rewarding and analgesic actions via G protein coupled μ opioid receptors (MOR) [28,29], and, to a lesser extent, δ and κ opioid receptors (DOR and KOR) [28,30], all three of which couple to $G_{i/o}$ type G proteins. In the striatum, μ opioid receptors are primarily expressed in dendritic spines and axon terminals [31]. Activation of $G_{i/o}$ proteins by μ opioid receptors leads to several events, including activation of post-synaptic GIRK channels, inhibition of pre-synaptic voltage gated calcium channels, inhibition of adenylyl cyclase, and reduced protein phosphorylation [32].

Over the last decade, it has become clear that addiction is a result of adaptations in GPCR signaling in the brain. In most cases, there is little or no change in neurotransmitter levels or levels of their cell surface receptors, suggesting that changes take place downstream of agonist–receptor interaction. Some of these events include superactivation of the cAMP system, changes in ERK phosphorylation, changes in the rate of receptor recycling, or altered ion channel function [10,28,33,34]. For example, morphine dependence has been associated with increased activity of the cAMP pathway and increased firing of locus coeruleus neurons [10,35,36]. This contrasts the known immediate response to opioid receptor activation of decreasing cAMP levels via $G_{i/o}$, and thus suggests adaptive changes downstream of receptor activation of the G proteins. In the locus coeruleus, activation of presynaptic α_2-adrenoreceptor subtypes by agonists such as clonidine or lofenexide alleviates some of the physical symptoms of opiate withdrawal by decreasing the firing of locus coeruleus neurons [37]. α_2-Adrenoceptors mediate inhibition of cAMP production, and therefore may function to counteract the increase in cAMP activity during withdrawal. Thus, the long term effects that follow exposure to drugs of abuse include changes in the responsiveness of several pathways activated by G protein coupled receptors throughout the brain. These adaptive responses have been extensively studied in cultured cells and brain slice models. Understanding which of these cellular adaptations occur in particular cell populations and how they affect neural responses in vivo may lead to new approaches in addiction prevention and pain management.

1.1. *RGS proteins: structural and functional diversity*

Regulator of G protein signaling (RGS) proteins have emerged over the past decade as potential mediators of the adaptive changes that take place in the addiction process. As negative regulators of G protein signaling, RGS proteins have been shown to have profound effects on the kinetics and strength of in vivo receptor signaling pathways in the central nervous system, sensory systems, and heart [38]. RGS proteins comprise a functionally and structurally diverse superfamily of proteins with approximately 40 distinct gene products in humans and homologues represented in yeast and plants, suggesting that they serve a fundamental role in eukaryotic cellular signaling [21,38]. RGS proteins are defined by the presence of a conserved RGS domain of approximately 125 amino acids of largely alpha helical structure that forms two sub-domains. The four helical bundle comprised of helices 4, 5, 6, and 7 forms the major contact sites with the switch regions of the Gα subunit and is responsible for accelerated GTPase catalytic activity, the primary mechanism by which RGS proteins inhibit G protein signaling [38]. The RGS domain binds with highest affinity to Gα bound to transition state analogs of GTP hydrolysis ($GDP + AlF_4^-$). This suggests that RGS proteins accelerate GTP hydrolysis not by any catalytic activity of their own, but by stabilizing the transition state and thus enhancing the catalysis by Gα [39].

In addition to its ability to accelerate GTPase activity, a second mechanism of RGS-domain mediated regulation of G protein activity is that of effector antagonism. Because RGS proteins bind to the same site of the G proteins that is required to bind and activate effector molecules, RGS binding to G proteins may inhibit their activity independent of GTPase activity. This has been demonstrated by the ability of RGS proteins to inhibit the activity of G proteins bound to the hydrolysis-resistant GTP analog GTPγS [40] or to inhibit the activity of GTPase deficient Gα mutants [41].

All proteins grouped within the RGS superfamily contain a conserved RGS domain (although not all have been demonstrated to possess functional GAP activity). Many RGS proteins contain functional domains in addition to the RGS domain. These diverse proteins are grouped into eight major subfamilies based on homology within the RGS domain and in many cases subfamilies also share common multi-domain structure [38,42]. The other domains in RGS proteins confer specificity, stability, sub-cellular targeting, and interaction with related cellular signaling components, suggesting that the multi-domain RGS proteins may integrate diverse signaling functions or synergistically regulate a single pathway. For example, the R7 RGS subfamily contains a N-terminal Dishevelled-EGL10-Pleckstrin (DEP) homology domain that mediates binding to membrane associated adaptor proteins and a G-gamma like (GGL) domain that confers protein stability by dimerization with Gβ5 [43]. Members of the R12 subfamily contain ''GoLoco'' motifs that function as Guanine nucleotide dissociation inhibitors, or GDIs, which lower the exchange rate of GDP/GTP and thus inhibit G protein function [44]. Additionally, members of the GEF RGS subfamily contain exchange factor domains for small G proteins that are activated upon the interaction of the RGS domain with active $G\alpha_{12}$ subunits, thereby linking signaling between heterotrimeric and small monomeric G proteins [45]. Through their binding to multiple signaling mediators, RGS proteins may serve as physical scaffolding proteins, bringing together multiple activators, regulators, and effectors of G proteins function. Further, a ''kinetic scaffolding'' mechanism has been proposed in which RGS proteins may serve to regulate the speed of both the activation and deactivation of signaling pathways [46].

2. Post-translational regulation of RGS proteins

A significant body of evidence indicates that RGS proteins are subject to regulation at the post-translational level. Many RGS proteins undergo post-translational modifications. Prominent examples of such modifications include phosphorylation [47–50], palmitoylation [51–53] ubiquitination [54,55], arginylation [56,57] and sumoylation [58]. Given that activation of protein kinases is one of the major intracellular responses to GPCR stimulation, phosphorylation of RGS proteins represents an important feedback mechanism that could regulate the sensitivity of G protein signaling via modulation of RGS protein function. Indeed, phosphorylation of RGS proteins was shown to affect multiple aspects of RGS function in the cell such as its GAP activity [47,59–61] protein stability [48,62] and subcellular localization [49].

Modulation of protein concentration through regulation of proteolytic degradation is another critical function of post-translational modifications that regulate RGS protein function in the cell. Emerging evidence suggests that several RGS proteins are liable to rapid proteolytic degradation mediated by the proteasome system. Two kinds of instability elements directing such degradation have been identified in the RGS proteins so far. Elements found in RGS2, RGS4 and RGS5 consist of de-stabilizing amino acids at the N-terminus of the molecules (N-end rule) which trigger argynilation and promote subsequent ubiquitination, thereby targeting the proteins to the proteasome [56,57,63]. Another instability element, called PEST sequence, is found in the GGL domain of RGS7. The presence of this amino acid motif was shown to be essential for RGS7 ubiquitination followed by rapid proteasomal degradation [54]. Although the mechanisms of degradation targeting by PEST sequences are understood much less than N-end rule dependent targeting, it was proposed that masking the PEST sequences by interacting partners such as polycystin or Gβ5 prevents RGS7 degradation [54,64]. This protection mechanism might control proteolytic stability of the entire R7 family of RGS proteins to which RGS7 belongs, because knockout of their interaction partner Gβ5 in mice leads to dramatic destabilization of these proteins at the post-translational level [65].

Another dimension in post-translational regulation of RGS proteins is uncovered by the association of various RGS proteins with a number of interacting partners. In fact, increasing evidence points to a model in which RGS proteins function as a part of a bigger signaling complex involving receptors, effector enzymes, scaffolding proteins and other signaling proteins (reviewed in [66]). Similarly to covalent post-translational modifications, interactions of RGS proteins with components of such complexes were shown to greatly affect their localization, [67–69] activity [70–72] and stability [54,73]. Despite their clear importance in controlling the function of RGS proteins, very little is known about how post-translational mechanisms are modulated by signaling perturbations (e.g. induced by drugs of abuse), providing an exciting direction for future research.

2.1. *RGS protein expression in the brain*

The lack of selective antibodies for most of the RGS proteins has been a major limitation in determining their regional distribution and regulation. Most of the information on RGS expression patterns in the nervous system is based on in situ hybridization studies. The first evidence of RGS mRNA expression in networks associated with motivation, reward, dependence, or analgesia came from studies by Gold et al. in 1997 [74]. Several members of the RGS family are found in dorsal and ventral striatum. RGS9 message is extremely abundant throughout the striatum and present in both D_1 and D_2 positive neurons. RGS8, RGS7 and RGS10 also show moderate to high expression in the striatum, whereas RGS2 is present in low amounts. The same study shows that RGS10 is densely expressed in the dentate gyrus, whereas RGS7 and RGS8 are present in the pyramidal cell layers of the hippocampus. In the amygdala, RGS4 is the most abundant RGS protein in the bed nucleus of the stria terminalis and the central amygdaloid nucleus. These regions also express low amounts of RGS7, RGS8 and RGS9. Interestingly, RGS8 is the predominant RGS protein in the ventral tegmental area and RGS10 is the predominant RGS protein in the dorsal raphe. RGS4 and RGS7 are abundant in the noradrenergic nuclei of the locus coeruleus [74]. At the spinal cord level, the most interesting expression pattern concerns RGS9 and RGS4 mRNA. RGS9 mRNA is localized in lamina I of the dorsal horn [75], whereas RGS4 message is enriched in the superficial laminae (I–III) of the dorsal horn [76].

Protein expression follows the pattern of mRNA expression for most RGS proteins. Western blot analysis has confirmed high abundance of RGS4 in the cortex, cerebellar enrichment of RGS8, and striatal specific expression of RGS9 [77–79]. RGS2 and RGS10 proteins are present in low to moderate levels throughout the brain [80,81].

2.2. *Regulation of RGS proteins by drugs of abuse*

Some RGS proteins appear to be regulated at the transcriptional level by drugs of abuse, while other RGS proteins known to play an essential role in the addiction process such as RGS9 (see below) show little or no regulation at the transcriptional level (V. Zachariou, unpublished observations). RGS4 and RGS2 appear to be regulated by D_1 and D_2 receptor agonists at the transcriptional level. Agonist-stimulation of D_1 receptor, or blockade of D_2 receptors, induces RGS2 transcription in a fast and transient manner [82], while transcription of the RGS4 gene in striatum is induced only by application of D_2 agonists or by blockade of D_1 receptors. Amphetamine treatment appears to robustly induce RGS2 expression in striatum [83]. Interestingly, this effect is regulated by both D_1 and D_2 receptors: activation of the Gs coupled D_1 receptors or blockade of the Gi coupled D_2 receptors induces RGS2 mRNA. Evidence from cell culture suggests that regulation of RGS2 transcription is mediated via activation of the cAMP pathway [84]. To date, there is no report on RGS2 action in the long-term effects of addictive substances.

In contrast to the rapid induction of RGS2 gene transcription, RGS4 mRNA induction in striatum occurs in a more delayed fashion [81]. Further, stress, corticosteroids, and drugs of abuse have been shown to affect RGS4 mRNA levels in various brain sites [77,85,86]. The role of RGS4 in D_1 receptor function is highlighted by Schwendt et al. [87] in studies showing that amphetamine downregulates RGS4 mRNA in the

caudate putamen and frontal cortex. This decrease in RGS4 message may function to enhance G_i/G_q signaling to counteract D_1 receptor stimulation of G_s.

A study by Bishop and colleagues demonstrates that RGS4 is differentially regulated by psychostimulants and opioids in locus coeruleus versus striatum. This observation is particularly interesting since the noradrenergic nuclei of the locus coeruleus play a critical role in the drug dependence and expression of opiate withdrawal. In particular, acute morphine or amphetamine injection leads to a decrease in RGS4 expression in the locus coeruleus, whereas the same treatment upregulates RGS4 in the nucleus accumbens [86]. Interestingly, in the locus coeruleus, repeated morphine administration leads to increased RGS4 transcription [77]. Consistent with the hypothesis that RGS4 is a negative modulator of MOR function, overexpression of RGS4 reduced electrophysiological responses to morphine in locus coeruleus slices [77]. Further, a study by Garnier et al. [88] suggests involvement of RGS4 in endogenous analgesic responses. RGS4 mRNA expression increases in neuropathic pain models, which is thought to contribute to adaptive changes that follow chronic pain signal processing and lead to the development of hyperalgesia.

Another RGS protein strongly implicated in the regulation of both dopamine and opioid receptor pathways in addiction is RGS9-2, a member of the multi-domain R7 RGS subfamily (RGS6, RGS7, RGS9, RGS11). The R7 family RGS domain selectively deactivates $G_{i/o}$ family of G proteins [89]. RGS9-2 is a product of the alternative splicing of the RGS9 gene [90–92]. The short splice isoform, RGS9-1, contains only 18 unique amino acid residues at the C-terminus and is exclusively expressed in photoreceptors of the retina. In the long splice isoform, RGS9-2, the short C-terminus is replaced by a longer region of 209 amino acids. RGS9-2 is highly expressed in the striatum but is not present in the photoreceptors [92]. The unique C-terminal domain of RGS9-2 has been shown to be critical for the efficient interaction of RGS9-2 with G protein alpha subunits. Mimicking the organization of the γ subunit of the effector enzyme PDE6, the C-terminus of RGS9-2 relieves the structural constraint of RGS9 catalytic domain to require the presence of PDE6 γ for its efficient GAP activity [93]. The enriched expression of RGS9-2 in medium spiny neurons and cholinergic interneurons [90,94] suggest that his RGS protein has a specialized role in striatal function.

All members of R7 RGS family share common structural/functional features (Fig. 2). In addition to the core RGS catalytic domain, a hallmark feature of all RGS proteins, R7 RGS proteins have several non-catalytic domains (DEP, R7H and GGL). One of the most striking structural features of R7 RGS proteins, is the presence of the G protein γ-like (GGL) domain which forms a highly specific coil–coil interaction with the type 5 G protein β subunit (Gβ5) [64,95,96]. As evident from experiments with recombinant proteins [89,97] and most importantly, from a recent study using the Gβ5 knockout mouse [65] association of R7 RGS proteins with Gβ5 is critical for their proper folding and post-translational stability. N-terminal R7 Homology (R7H) and Disheveled, Egl-10, Pleckstrin (DEP) domains together form a binding site for the interaction with R7 Binding Protein (R7BP), another universal partner of all R7 RGS proteins [73]. Association with R7BP was shown to mediate plasma membrane localization of R7 RGS proteins [68–70]. Studies with RGS9-2 also indicate that R7BP is important for mediating its proteolytic stability [73]. Interestingly, in related yeast RGS proteins, the DEP domain was shown to directly interact with GPCRs [98]. Likewise, deletion of the DEP domain of RGS9-2 disrupted its co-localization with D_2 receptors [67]. However, neither the determinants of direct R7 RGS–GPCR interactions nor the role of R7BP in this complex formation have been established.

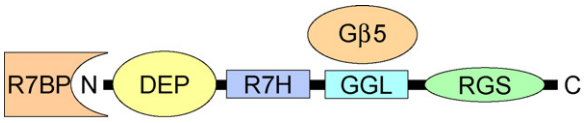

Fig. 2 – Structural organization of the R7 family of RGS proteins. R7 RGS proteins contain four distinct modules: the N-terminal disheveled-EGL10-Pleckstrin homology (DEP) domain of ~110 amino acids; the R7 family homology (R7H) of ~80 amino acids; the G protein gamma subunit-like domain (GGL) of ~80 amino acids; the RGS homology domain (RGS) of ~120 amino acids. The GGL domain is responsible for interaction with Gβ5, the constitutive binding partner of RGS9. The DEP domain mediates binding to membrane anchors R7 family Binding Protein (R7BP) in all R7 RGS protein and RGS9 Anchor Protein (R9AP) in RGS9 and RGS11.

Apart from RGS9-2 the other members of the R7 RGS proteins are expressed in the brain in low to moderate amounts. RGS7 is one of the most abundant proteins in the locus coeruelus, it is not present in the spinal cord, and it is moderately expressed in striatum [74,77]. Interestingly, there is no regulation of RGS7 in the locus coeruleus by opiates, although acute morphine upregulates RGS7 in the striatum [77].

2.3. *Modulation of dopamine responses by RGS9-2*

In striatum, RGS9-2 is upregulated by intermittent cocaine administration, or cocaine self administration [79]. Behavioural characterization of mice lacking RGS9 revealed an important role of this protein in basal ganglia function. Lack of RGS9 delays signaling termination and enhances responses to D_2 agonists. The importance of D_2 sensitivity due to prolonged signaling in the RGS9 knockout mice has been demonstrated by behavioural assays on cocaine reward, locomotor activation and locomotor sensitization [79]. In particular, RGS9 knockout mice condition place preference to lower doses of cocaine than their wild type controls. Additionally, there is an increase in the potency of psychostimulants with respect to locomotor activation. RGS9-2 plays a critical role in behavioural sensitization to cocaine, as knockout of RGS9 increases the rate of sensitization whereas RGS9-2 overexpression in the nucleus accumbens prevents sensitization [79]. Additional evidence of the regulation of D_2 receptor function by RGS9 derives from recordings from striatal slices expressing different forms of RGS9 [94]. These electrophysiological studies reveal that RGS9-2 in striatum modulates D_2 receptor mediated control of calcium channel function in cholinergic

interneurons. In particular, overexpression of RGS9 reduced D_2 receptor modulation of calcium Cav2.2 channels. Expression of the DEP-GGL domain of RGS9-2 antagonizes endogenous RGS9-2 activity and enhances D_2 receptor modulation of calcium currents. This is a D_2 receptor selective action, as modulation of Cav2.2 currents by M_2 muscarinic receptors is not affected by dialysis of cholinergic interneurons with RGS9 constructs. The same study points to the importance of the DEP-GGL domains in modulation of GAP function of the RGS9-2 protein in striatum. Regulation of D_2 receptor function by RGS9-2 has also been shown by Kovoor et al. [67]: recordings from striatal neurons of RGS9 knockout mice reveal that activation of D_2-like receptors leads to abnormal inhibition of glutamate-elicited currents.

2.4. *Modulation of opiate responses by RGS proteins*

The bulk of information on modulation of opiate responses by RGS proteins derives from cell culture studies. RGS2, RGS4, RGS9-2, RGS7, RGS8 and RGSz are among the RGS proteins that are present in brain networks associated with opiate addiction. Data from in vitro studies link three of these proteins (RGS9-2, RGSz and RGS4) with MOR function [99–101,58]. RGSz1 appears to have a role in μ opioid receptor signaling modulation via a mechanism that involves interactions with a PKC interacting protein, PKC-1 [102]. As described above, some of the best characterized signal transduction events associated with opiate addiction include superactivation of the cAMP system, and phosphorylation of MAP kinase [28,32,34,103,104]. The first evidence for the role of RGS proteins in signal transduction events associated with addiction comes from Clark et al. [100] who used PC12 cells stably expressing an RGS insensitive form of $G\alpha_o$ protein to demonstrate that prevention of RGS function leads to prolonged signaling duration, increased cAMP responses and increased phosphorylation of MAP kinase (ERK). Similar findings have been obtained by other groups who knocked down RGS9 [105] using antisense oligonucleotides in CHO cells expressing human MOR. As expected, reduction of RGS9 expression and activity leads to increased cAMP inhibition by opioid agonists in forskolin activated cells. A recent study by Psifogeorgou et al. reveals that RGS9-2 associates with MOR after morphine treatment and prevents internalization of the receptor [106].

In the brain, members of the R7 RGS family appear to be dynamically regulated by acute morphine. The pattern of RGS7 regulation by acute morphine is similar to that of RGS9-2: both proteins are upregulated in striatum 2 h after morphine administration [106]. Unlike RGS9-2, RGS7 levels do not change following chronic exposure to morphine [75].

Analysis of RGS9 knockout animals, revealed an essential role for RGS9 in the rewarding actions of opiates. Functional deletion of the RGS9 gene results in a 10-fold increase in sensitivity in the place conditioning paradigm response to morphine [75]. This phenotype reflects functions mediated by the nucleus accumbens, and can be reversed by overexpression of RGS9-2 specifically in the nucleus accumbens of RGS9 knockout mice. As expected, the increased sensitivity to the actions of morphine in RGS9 knockout mice leads to a more severe dependence: morphine withdrawal intensity in RGS9 knockout mice is much higher than in their wild type littermates. These actions of RGS9 are thought to be mediated via mechanisms that involve compromised opioid and dopamine receptor signaling in striatum. Notably, lack of RGS9 increases analgesic efficacy and delays tolerance but does not affect the duration of the analgesic response [75,106]. Delivery of antisense RNA to the rat periaqueductal gray has also been used to demonstrate a role of RGS9 and RGSz in acute analgesic tolerance to opioid agonists [107].

Evidence for direct association of RGS4 with MOR was recently reported by Georgoussi et al. [101]. The group used GST fusion proteins of the carboxy termini of the MOR, DOR, or the third intrecellular loop of DOR and showed direct association of RGS4 with all these proteins. In the same study, investigators performed cAMP inhibition assays in cells transiently transfected with RGS4, demonstrating that RGS4 is a negative modulator of MOR signaling. Application of KOR or MOR agonists leads to upregulation of RGS4 mRNA in PC12 cells stably overexpressing these receptors via a pertussis toxin-sensitive and naloxone reversible mechanism [108]. It is yet to be determined if the association between MOR and RGS4 observed in vitro also occurs in the CNS and whether long-term opiate exposure affects this association. Recent evidence from knockout mice points to a potent role of RGS4 in opiate dependence [76]. In particular, loss of RGS4 sensitizes the cAMP pathway in locus coeruleus neurons and leads to development of severe dependence to morphine. Constitutive or conditional RGS4 knockout mice show normal analgesic responses to morphine [76,109]. Notably, findings by Grillet et al. [109] show no significant opiate dependence phenotype in RGS4 mice generated by that group.

3. RGS proteins as drug targets

As described, RGS proteins clearly regulate critical signaling events that may contribute to the addiction process and are therefore potential molecular targets in addiction therapeutics. Most RGS proteins display specificity for a subset of G proteins, and in many cases RGS proteins only deactivate G proteins that are coupled to specific receptors [110]. Small molecule inhibitors of RGS protein activity have recently been described [111], and it has been shown that small molecules that bind an allosteric regulatory site on RGS proteins may increase RGS ability to deactivate G proteins [112]. Thus, RGS proteins are attractive targets to selectively manipulate G protein pathways and regulate the potency, selectivity, or duration of action of receptor-targeted drugs or endogenous neurotransmitters.

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