



Review

Regulators of G protein signalling as pharmacological targets for the treatment of neuropathic pain

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ABSTRACT

Neuropathic pain, a specific type of chronic pain resulting from persistent nervous tissue lesions, is a debilitating condition that affects about 7% of the population. This condition remains particularly difficult to treat because of the poor understanding of its underlying mechanisms. Drugs currently used to alleviate this chronic pain syndrome are of limited benefit due to their lack of efficacy and the elevated risk of side effects, especially after a prolonged period of treatment. Although drugs targeting G protein-coupled receptors (GPCR) also have several limitations, such as progressive loss of efficacy due to receptor desensitization or unavoidable side effects due to wide receptor distribution, the identification of several molecular partners that contribute to the fine-tuning of receptor activity has raised new opportunities for the development of alternative therapeutic approaches. Regulators of G protein signalling (RGS) act intracellularly by influencing the coupling process and activity of G proteins, and are amongst the best-characterized physiological modulators of GPCR. Changes in RGS expression have been documented in a range of models of neuropathic pain, or after prolonged treatment with diverse analgesics, and could participate in altered pain processing as well as impaired physiological or pharmacological control of nociceptive signals. The present review summarizes the experimental data that implicates RGS in the development of pain with focus on the pathological mechanisms of neuropathic pain, including the impact of neuropathic lesions on RGS expression and, reciprocally, the influence of modifying RGS on GPCRs involved in the modulation of nociception as well as on the outcome of pain. In this context, we address the question of the relevance of RGS as promising targets in the treatment of neuropathic pain.

1. Introduction

Neuropathic pain refers to a chronic pain syndrome that is induced by a lesion or disease affecting the sensory nervous system and results from alteration(s) of its structure and function. Neuropathic pain is a consequence of maladaptive plastic changes, termed peripheral and central sensitization [1,2], within the synapses and along the nerve fibre pathways of the nociceptive system [3]. These changes affect the properties and activity of cells in the central nervous system (CNS), i.e. neurons and glial cells, leading to painful sensations, even in the absence of a peripheral insult [4]. This condition affects nearly 7% of the general population [5] and frequently remains debilitating. Many patients with neuropathic pain fail to respond adequately to commonly used analgesics, such as non-steroidal anti-inflammatory drugs or opioids. Several therapeutic alternatives have been proposed, including anti-depressants and anti-epileptic drugs, but their use may be limited by issues of

variable efficacy [6] and the incidence of side effects (e.g. sedation, somnolence and anti-cholinergic effects) [7]. In most cases, the effectiveness of currently available medications is unpredictable and their dosage requires individual modification. It is therefore essential to develop new therapeutic strategies.

The difficulty in treating neuropathic pain or in designing new effective treatments resides in the heterogeneity of the mechanisms underlying its development and maintenance, the full scope of which remains only partially understood [5]. Enhanced pain associated with neuropathies is thought to result from molecular and cellular mechanisms directly affecting neurons involved in nociceptive transmission, both in the peripheral nervous system (PNS) and CNS. This sensitization largely relies on alterations of the expression and function of proteins participating in the detection, transmission or modulation of nociceptive signals as well as on the emergence of aberrant causes of nociceptor activation [8]. The enhanced transmission of such signals also results

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from a dysfunction of inhibitory descending pathways and spinal inhibitory interneurons [1,5]. These mechanisms, summarized in Fig. 1, have been reviewed in the recent literature, but here, we focus on the pathophysiological activities that involve G protein coupled receptors (GPCRs). The present review summarizes the experimental data documenting the implication of physiological modulators of G protein signalling in the development of pain and, more precisely, in the pathophysiological mechanisms of neuropathic pain. In this context, we also address the question of the relevance of developing drugs targeting these GPCR signalling partners as innovative treatment strategies for neuropathic pain.

2. GPCRs in pain modulation

The detection of nociceptive stimuli at the peripheral nerve terminal of primary afferent neurons and the transmission of signals to the spinal cord and to the brain essentially involve ligand-dependent or voltage-dependent cation channels such as sodium and calcium channels, ionotropic glutamate receptors, purinergic receptors and transient receptor potential (TRP) ion channels. In addition, a large range of GPCRs participate in the physiological control of nociceptive transmission. As recently reviewed by Stone and Molliver, receptor members belonging to almost all families of GPCRs have been associated with the modulation of pain [9]. Receptors that putatively influence pain are localized on nociceptive neurons or on modulatory interneurons, but are also expressed by glial cells within the dorsal root ganglia and in the spinal cord. Indeed, as in many neuropathological conditions, these glial cells influence excitatory transmission and participate in inflammatory processes, two mechanisms that likely contribute to the development of

chronic pain. In peripheral tissues, inflammation decreases the activation threshold of nociceptors [10,11] through a mechanism involving cyclooxygenase 2 and the subsequent production of diverse prostanoids that exert most of their actions via GPCRs [12]. A strong inflammatory component also supports central sensitization during the development of neuropathic pain. The neuroinflammation driven by glial cells largely relies on the production and release of pro-inflammatory cytokines such as tumour necrosis factor α (TNF α), which directly interacts with membrane receptors influencing intracellular kinases, but also by a variety of other cytokines such as interleukin 1 β (IL-1 β) and chemokines that bind to GPCRs [13].

GPCRs constitute the largest and currently best-characterized family of membrane receptors in eukaryotes. They mediate their signalling through either G_{i/o}, G_s or G_q proteins. GPCRs recognize a large number of molecules, including ions, neurotransmitters and hormones, and thereby participate in cell-to-cell communication throughout the organism. GPCRs represent the targets of many drugs commonly used in the treatment of a wide range of diseases such as cardiovascular diseases, psychosis or allergies, to name but a few (for a review, see [14]). GPCRs are highly expressed in nervous tissues and, disregarding olfactory and photoreceptors, it is estimated that 90% of these receptors are located on neurons and glia of the CNS [15].

In the context of pain, depending on the type of receptors and their cellular localization, GPCRs can reinforce or inhibit nociception. Accordingly, GPCRs associated with pain transmission systems can be sub-divided into two groups: the pro-nociceptive and the anti-nociceptive GPCRs. Unsurprisingly, anti-nociceptive GPCRs constitute the targets of diverse therapeutic agents, acting as agonists of these receptors. Indeed, the effects of several analgesics, such as opioids as well

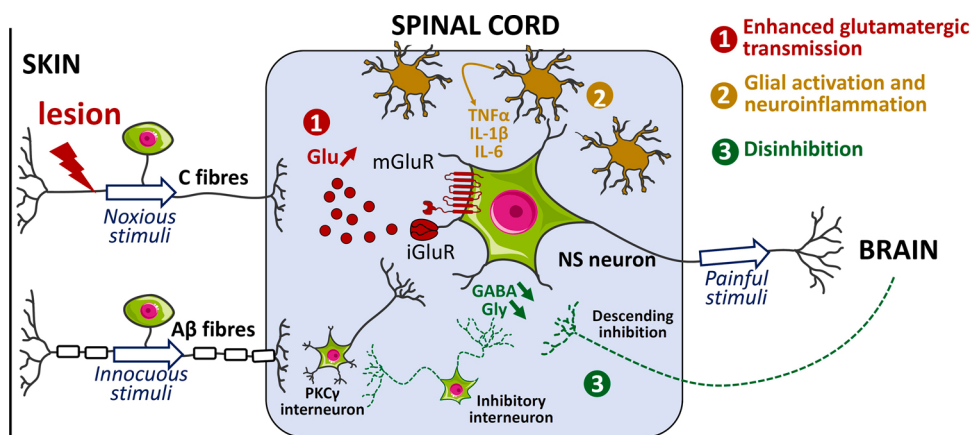


Fig. 1. Principal mechanisms of central sensitization that support chronic pain.

1. Enhanced glutamatergic transmission. Following a persistent injury, activated C fibre nociceptors projecting on spinal nociceptive specific (NS) neurons in laminae I and II of the superficial dorsal horn release a variety of neurotransmitters. Among these, glutamate triggers the activation of both ionotropic (iGluR) and metabotropic (mGluR) subtypes. Through phosphorylation and up regulation of these receptors as well as recruitment of signalling partners, an increased excitability of NS neurons develops that facilitates the transmission of nociceptive signals to the brain.

2. Glial activation and neuroinflammation. Peripheral nerve injury promotes the release of neuromediators, such as ATP and inflammatory cytokines that activate glial cells. Activated microglia and astrocytes release various cytokines, such as TNF α , IL-1 β and IL-6 that contribute to central sensitization. These cytokines also activate the glial cells themselves, supporting an amplification process.

3. Disinhibition. Under normal circumstances, inhibitory interneurons (green) continuously release GABA and glycine (Gly) to decrease the excitability of central projection neurons and modulate nociceptive transmission. However, in the case of injury, this inhibition can be lost. Similarly, descending inhibitory pathways from the brain are attenuated. Finally, disinhibition enables non-nociceptive myelinated A β primary afferents to activate the pathway for the transmission of nociception, turning normally innocuous stimuli into painful stimuli. This occurs, in part, through the disinhibition of excitatory PKC γ -expressing interneurons in lamina II.

as adrenergic- and serotonergic receptor and transporter ligands, directly or indirectly rely on the activation of GPCRs in descending pathways [16]. Agonists of these GPCRs reinforce endogenous inhibitory controls of pain transmission through the modulation of second messengers that can, in turn, activate a series of downstream effectors, such as transcription factors, that are able to change the physiological properties of affected neurons [9,17].

Neuronal GPCRs that mediate the induction of analgesia act through $G_{i/o}$ proteins and reduced cAMP production oppose to the effects of G_s proteins, and thereby decrease neuronal excitability [18]. $G_{i/o}$ -coupled receptors are often present at presynaptic locations, where they exert an inhibitory effect on responses associated with ion channels [19]. Some GPCRs have also been shown to regulate the transcription of diverse genes, such as those encoding inflammatory factors involved in the control of nociception transmission [20]. The family of $G_{i/o}$ -coupled receptors comprises (amongst others) delta, mu and kappa opioid receptors (also abbreviated as DOR, MOR and KOR), cannabinoid receptors (CB_1 and CB_2), muscarinic acetylcholine receptors (M_2 and M_4), gamma-aminobutyric acid B receptors ($GABA_B$), α -2-adrenoreceptors and type 3 metabotropic glutamate receptors (mGluR3), all of which are known to play a role in neuropathic pain. Experimental data has demonstrated that agonists of these receptors exert anti-nociceptive effects at both supraspinal and spinal levels, [16,18,21–24]. Significantly, numerous studies have also documented altered expression and functionality of these receptors in animal models of neuropathic pain. For instance, it is acknowledged that the density of MOR is reduced in the dorsal horn of the spinal cord after peripheral nerve injury, and that the analgesic effect of MOR agonists is decreased in this context [25].

The majority of pro-nociceptive GPCRs are coupled to G_q or G_s proteins [9] which facilitate the transmission of nociceptive signals. A G_s -mediated increase in cAMP and activation of protein kinase A (PKA) is known to increase the activity of nociceptors through phosphorylation of essential signalling proteins. Also, phosphorylation of transcription factors such as the cAMP response element-binding protein (CREB), likely contributes to long-term modifications in neurons and other cells, potentially leading to increased excitability [9]. Similarly, intracellular responses induced by G_q proteins (especially inositol trisphosphate (IP_3)-mediated calcium release from cellular stores and diacylglycerol-mediated activation of protein kinase C (PKC)) are commonly associated with increased neuronal activity. The subsequent elevation of the intracellular calcium concentration $[Ca^{2+}]_i$ facilitates the release of neurotransmitters and promotes the activation of transcription factors, in particular via a calmodulin-dependent mechanism [26]. Furthermore, PKC isozymes such as PKC ϵ are involved in the sensitization of primary afferent nociceptors [27]. GPCRs that are currently considered as pro-nociceptive include (among others), the P2Y purinoreceptor 2 (P2RY $_2$), the histamine 1 receptor (H_1) and type 5 metabotropic glutamate receptors (mGluR5), all of which are coupled to G_q proteins, as well as vasoactive intestinal peptide (VIP) receptors that are coupled to G_s proteins [28–30]. Some of these receptors, such as P2RY $_2$, are expressed by astrocytes and microglia. Although circumstantial, this evidence may support the involvement of these glial cells in neuropathic pain [31].

At variance with treatment strategies for acute pain, which suppress the symptoms of pain, therapeutic approaches for the treatment of neuropathic pain should aim to restore normal nociceptive function. At present, the most commonly prescribed medications for neuropathic pain belong to the family of anticonvulsants, antidepressants and opioids [5]. The anticonvulsants such as gabapentin, pregabalin or valproate inhibit ion channels and decrease the propagation of action potentials in affected axons, whereas antidepressants and opioids influence transmission through mechanisms that depend on GPCR activity. While morphine and its analogues directly activate opioid receptors in descending pain-modulating pathways, antidepressants such as amitriptyline, nortriptyline and duloxetine inhibit the re-uptake of noradrenaline and serotonin, thereby promoting activation of their

cognate receptors in these pathways. Furthermore, other GPCRs have also been considered as promising targets in the development of new treatments for neuropathic pain, such as tachykinin receptors, cannabinoid receptors, purinergic receptors of the P2X family, adenosine receptors and mGluRs [32].

3. Regulators of G protein signalling

The biochemical mechanism for the production of intracellular messengers through GTP hydrolyzing proteins in response to extracellular transmitter-mediated activation of membrane receptors was defined in the early 1970's. Subsequently, the observation that purified $G\alpha$ protein subunits hydrolyze GTP too slowly to match the transient profile of the responses observed *in vivo* led researchers to hypothesize the existence of physiological intracellular activators of G protein GTPase activity. Regulatory proteins capable of influencing G protein GTPase activity were rapidly identified and referred to as regulators of G protein signalling (RGS) (for early seminal reviews on their discovery, see [33–35]). The identification of a multitude of RGS subtypes in mammals established the existence of a large family of these GPCR partners. While sharing the common RGS domain or RGS-like domain that directly binds the $G\alpha$ subunit [36], these proteins differ in the presence of other functional domains that may influence their regulation, localization or interaction with other cell components [37]. In addition to other proteins that participate to the regulation of signalling via GPCR, such as G-protein receptor kinases (GRKs) and axins, more than 20 other RGS have been described in mammals and have been divided into four sub-families (for recent update, see [38]).

The best-characterized function of RGS that is shared by all members of the family is the activity of the 'GTPase activating protein' (GAP) (Fig. 2). RGS reduce the duration of GPCR-mediated signals through the canonical activity that is driven by the common RGS domain. Indeed, RGS accelerate intrinsic GTPase activity and the subsequent hydrolysis of GTP favours the reformation of the inactive $G\alpha\beta\gamma$ heterotrimer. This GAP activity is an essential feature of RGS that has been validated in several studies using purified proteins [39–41]. A second function exerted by RGS is to physically impede the interaction between G protein subunits and their effectors. Such blockade of G protein signalling operates independently of GAP activity and some RGS have therefore, been proposed to antagonize effectors or to sequester G protein subunits [42,43].

RGS should be considered as modulators of G protein signalling rather than as strict inhibitors. For example, RGS modulate (rather than abolish) the calcium signalling associated with G_q -coupled receptors [44,45]. Therefore, in addition to these diverse GAP-dependent and independent activities, RGS can also act as adaptor molecules, integrating G proteins into complex signalling networks. Through this scaffolding function, RGS influence GPCR activation by assembling them with related signalling proteins such as adenylyl cyclase or ion channels [46,47]. Such GAP-independent non-canonical RGS functions are attributed to functional protein-protein interaction domains which are located outside of the RGS domain (for review, see [48]). Interestingly, the level of expression of RGS is thought to be important in determining the mode of regulation [49]. Accumulating data demonstrates that a variety of mechanisms regulate RGS, operating at the transcriptional or translational levels, or influencing their subcellular localization or degradation [50]. While these mechanisms contribute to the modulation of RGS activity in physiological or pathological conditions, it also opens the way for the development of novel therapeutic approaches for a number of diseases or disorders.

4. RGS in neuropathic pain

The first indications of a putative role of RGS in the modulation of nociceptive processing were published in the early 2000's, since RGS had been shown to influence the signalling of anti-nociceptive receptors,

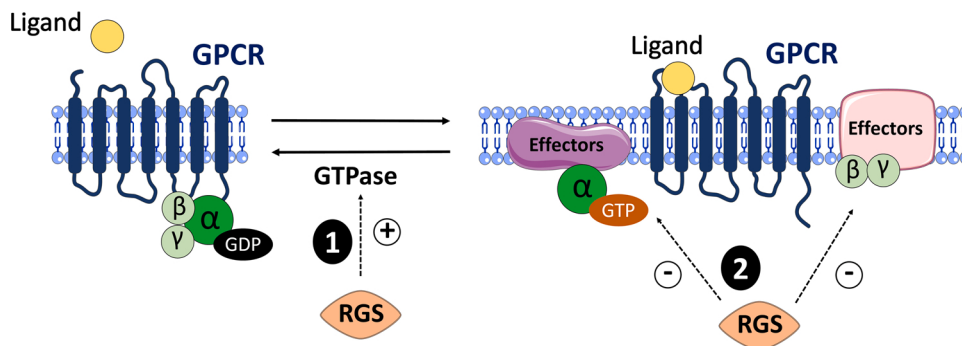


Fig. 2. Functions of RGS on GPCR-mediated signalling. Simplified model highlighting the two main functions that are attributed to RGS in modulating signalling via GPCRs. 1. RGS accelerate GTPase function through their GTPase activating protein (GAP) activity, causing a rapid silencing of the activated G protein. 2. RGS can block the interaction between G protein subunits and their effectors, thereby preventing the activation of downstream signalling cascades. Note that a series of non-canonical functions has also been described for RGS. These functions are not shown in this figure.

in particular of opioid receptors. Cell responses mediated by these receptors were found to be modified upon manipulation of RGS expression [51–54]. Altering RGS expression *in vivo* was found to modulate the physiological control of nociception, as well as opioid-mediated analgesia and opioid tolerance [55–58]. Furthermore, an altered expression of selected RGS was documented in several rodent models of neuropathic/chronic surgical pain [59]. Subsequent data indicated that the regulation of RGS could influence signalling of both anti- and pro-nociceptive GPCRs, highlighting the role of these signalling partners in the mechanisms of pathological pain (Fig. 3).

4.1. Alterations of RGS in the context of neuropathic pain

Shortly after the discovery of RGS, several research groups examined possible changes in the expression of these proteins in models of human diseases, including schizophrenia, Parkinson's disease and chronic heart failure [60–62]. Later, in the context of pathological pain, the expression of several RGS (RGS2, 3, 4, 7 and 9-2) was studied in CNS structures that

are associated with the processing of nociceptive signals. In neuropathic pain, selected RGS appeared to be either negatively or positively altered, depending on the RGS subtype, the experimental model, and the nervous tissue structure that were being analysed (see Table 1). Many studies were restricted to the analysis of RGS mRNA levels, probably due to low endogenous protein levels or the lack of validated tools for the detection of RGS proteins [63;64].

The first experimental evidence for regulation of RGS in experimental models of neuropathic pain was reported by Garnier and colleagues who demonstrated that RGS4 mRNA levels increased significantly at the first synaptic junction in the nociceptive transmission pathway in the lumbar spinal cord of animals with a partial sciatic nerve ligation [59]. This early study raised the hypothesis that an increased expression of RGS could reduce the activity or responsiveness of MOR to endogenous opioids, thereby supporting the development of pain. The relevance of such regulation was further consolidated when examined in a modified model of spared nerve injury, in which only a fraction of the lesioned animals developed neuropathic pain [65]. In this model, where

Endogenous/exogenous analgesic ligand

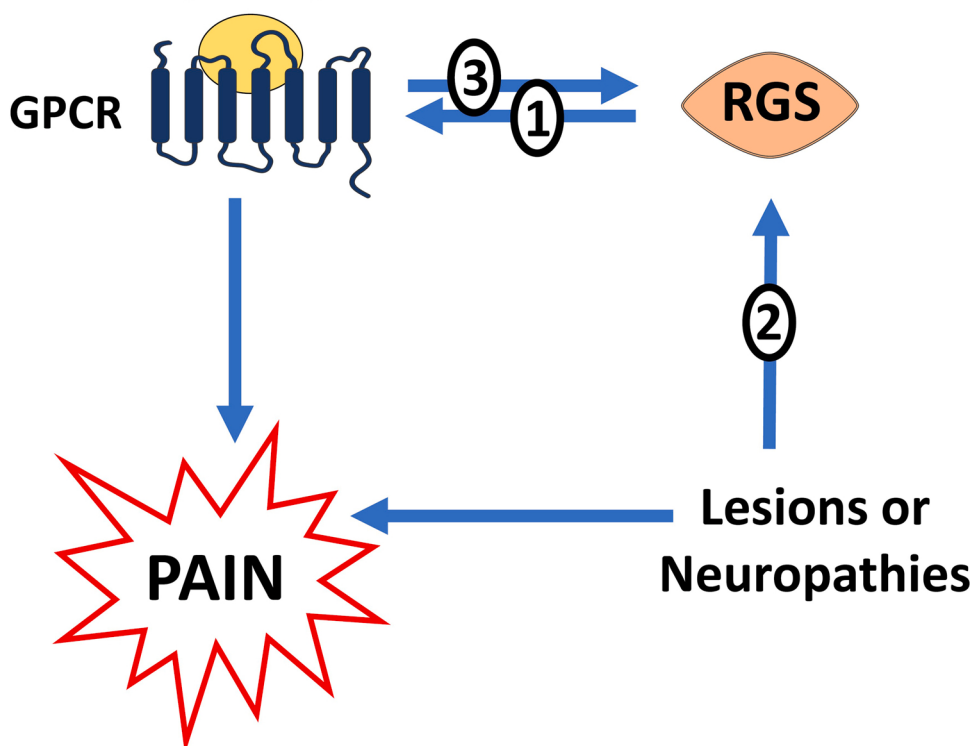


Fig. 3. RGS and neuropathic pain.

RGS exert an influence on the signalling associated with several GPCRs that modulate the transmission of nociceptive stimuli throughout the CNS. Considering the existence of pro- and anti-nociceptive GPCRs, RGS may have a negative or positive impact on endogenous pain modulators as well as on drugs used in the treatment of pain syndromes (1). There is abundant evidence for altered RGS expression in experimental models of neuropathic pain which could contribute to the impaired control of nociception by endogenous or exogenous pain modulators (2). Moreover, the expression of RGS has been shown to be regulated by several drugs acting on GPCRs with possible influence on their analgesic properties (3).

Table 1

Alterations of RGS in experimental models of neuropathic pain.

RGS types	Models of neuropathic pain	Analyses	Results	Time points	Reference
RGS2	Spinal nerve injury	mRNA level in the lumbar spinal cord	Downregulation	d21 post-surgery	[73]
RGS2	Modified spinal nerve injury	mRNA level in DRG and dorsal spinal cord	Unchanged	d7 post-surgery	[62]
RGS3	Spinal nerve transection (axotomy)	mRNA level in DRG	Downregulation	d1, 3, 7 and 14 post-surgery	[63]
RGS3	Spinal nerve transection (rhizotomy)	mRNA level in DRG	Unchanged	d3 post-surgery	[63]
RGS3	Spinal nerve injury	mRNA level in the lumbar spinal cord	Upregulation	d3 and 5 post-surgery	[64]
RGS4	Partial sciatic nerve ligation	mRNA level in dorsal spinal cord	Upregulation	d7 post-surgery	[67]
RGS4	Partial sciatic nerve ligation	mRNA level in the lumbar spinal cord	Upregulation	d7 post-surgery	[56]
RGS4	Spinal nerve injury	mRNA level in the lumbar spinal cord	Downregulation	d7 post-surgery	[64]
RGS4	Spinal nerve transection (axotomy)	mRNA level in DRG	Downregulation	d1, 3, 7 and 14 post-surgery	[63]
RGS4	Spinal nerve transection (rhizotomy)	mRNA level in DRG	Unchanged	d3 post-surgery	[63]
RGS4	Modified spinal nerve injury	mRNA level in ipsilateral DRG and dorsal spinal cord	Upregulation only in animals developing hypersensitivity	d7 post-surgery	[62]
RGS7	Spinal nerve injury	mRNA level in the lumbar spinal cord	Downregulation	From d3 post-surgery and outlasted until d21	[64]
RGS7	Partial sciatic nerve ligation	mRNA level in the lumbar spinal cord	Downregulation	d7 post-surgery	[56]
RGS7	Spinal cord contusion	Protein expression (immunohistochemistry) in the spinal cord	Upregulation in activated microglia and macrophage	d1, 3 and 7 post-surgery	[69]
RGS9	Partial sciatic nerve ligation	mRNA level in the lumbar spinal cord	Downregulation	d7 post-surgery	[56]
RGS10	Partial sciatic nerve ligation	Protein expression (immunoblotting) in the spinal cord	Downregulation	d3 post-surgery	[74]
RGS12	Partial sciatic nerve ligation	mRNA level in the lumbar spinal cord	Downregulation	d7 post-surgery	[56]

Altered expression of several RGS has been documented in a variety of experimental models of neuropathic pain. Up and down regulation of selected RGS have been reported, depending on the RGS subtype, the experimental model, the delay after the lesion and the nervous tissue structure that is analyzed.

the surgical lesion was restricted to a single peripheral branch of the sciatic nerve, up-regulation of RGS4 mRNA was only observed in the ipsilateral spinal cord and dorsal root ganglia of those animals that demonstrated nociceptive hypersensitivity [65].

For several RGS, the modulation appears to be influenced by the nature of the lesion applied in the rodent models. At variance with data that had been obtained using partial nerve lesions, a down-regulation of RGS4 in ipsilateral dorsal root ganglia was observed in a model of complete sciatic nerve transection (axotomy) [66]. Similarly, RGS4 in the lumbar spinal cord was down-regulated in a model of spared nerve injury [67]. This observation is paradoxical as it contradicts earlier reports [59,65] despite sharing common signs of strong inflammation, glial activation and hypersensitivity [68]. This however suggests that distinct molecular or cellular adaptive changes are triggered in these models of neuropathic pain, highlighting the complexity of the disease. Furthermore, it is worth noting that in these models, different RGS regulation profiles have been observed when examined in distinct nervous tissues associated with the processing of nociceptive stimuli. Such observations raise the question of the relevance of targeting RGS4 with inhibitors or activators in the treatment of neuropathic pain (see below). As shown in Table 1, different and sometimes opposite effects have been observed in the brain, spinal cord or in dorsal root ganglia [67,69–71], suggesting that site-specific modulation of RGS should be taken into consideration when targeting RGS.

While these sometimes puzzling data appear difficult to integrate, they might reflect the modulation that operates in different cell types, including neurons and glial cells, or in different CNS structures where diverse GPCRs participate in the processing of nociceptive signals. Furthermore, it is worth mentioning that the regulation profiles of these RGS showed different kinetics, since some RGS were modified at particular time points (RGS2, RGS3 and RGS4) whereas others underwent more persistent modifications (RGS7) [67]. This might suggest a

differential role for these RGS in the onset or maintenance of the pathological mechanisms supporting the development of chronic pain.

Although the regulation of RGS expression has been demonstrated in a variety of models of neuropathic pain and other disorders, little is known about the molecular and cellular mechanisms that are involved. Some studies have examined RGS regulation in a subset of cells within nervous tissues, e.g. the up-regulation of RGS7 in macrophage/activated microglia after spinal cord injury [72]. In the context of nociception, the down-regulation of RGS3 mRNA observed in the dorsal root ganglia of rats undergoing sciatic nerve transection was demonstrated to essentially concern C-fibre primary sensory neurons [66]. Since neuro-inflammation at the level of the dorsal spinal cord is a key feature in the development of chronic pain, it has been proposed that inflammatory mediators may play a role in the regulation of RGS [67,73]. This suggestion was convincingly supported in the model of modified spared nerve injury, in which only those animals that presented with an up-regulation of RGS4 and hyperalgesia also demonstrated an activation of microglia and astrocytes [65]. Inflammatory stimuli, such as exposure to lipopolysaccharide, have been reported to reduce RGS10 expression in microglia [74] and a brief exposure of cultured astrocytes to the cytokines IL-1 β , TNF α and IL-6 has been found to result in decreased expression of RGS2 and increased expression of RGS4 [67]. Considering the role of diverse GPCRs in glial cells in the modulation of synaptic signalling, the regulation of RGS in these cells could participate in the alteration of glial responses observed under neuropathic conditions [75].

4.2. Alterations of RGS induced by pharmacological analgesic treatments

In several tissues, including the heart, the nervous system and also in some cancer cells (e.g. breast, prostate), the regulation of RGS expression has been reported to be triggered by ligands that interact with their

cognate GPCRs, providing evidence for modulation through both positive and negative feedback mechanisms [50]. In the CNS, the expression of RGS encoding genes and the corresponding protein levels have also been shown to be influenced (with distinct mechanisms and time courses) by drugs, such as antipsychotics [76], cocaine [77] or amphetamines [78]. With respect to neuropathic pain, several treatments used to modulate nociceptive transmission appear to regulate levels of RGS. This has been experimentally demonstrated by studying the influence of opioids on selected RGS, particularly RGS4 and RGS9-2. In rodents, the regulation of RGS in response to opioid treatments was first observed in brain structures associated with reward and addiction behaviours (e.g. the locus coeruleus and nucleus accumbens), where RGS4 expression was found to be altered after acute or chronic administration of morphine [79]. With respect to RGS9-2, increased expression in the striatum, periaqueductal grey matter and spinal cord was observed after acute morphine administration whereas down-regulation was observed after chronic administration [80]. This suggested an involvement of this RGS splice variant in both the acute reward effect as well as in the progressive tolerance to analgesia after repeated administration of opiates [57]. Consistent with these observations in rodents, examination of human tissues revealed that RGS4 protein level was increased in the prefrontal cortex of long-term opioid abusers while no change was observed after short-term administration [81]. Together, these data highlighted a putative role for RGS in the development of tolerance to opiates and one may hypothesize that similar altered expression or function of RGS may influence the analgesic properties of opiates after chronic administration.

Regarding other members of the RGS family that are expressed in the CNS, Gold et al. reported an up-regulation of RGS2 mRNA level in the locus coeruleus of rats upon opiate withdrawal, while its level remained unchanged following chronic morphine administration [82]. Similarly, the members of the R7 sub-family of RGS (which consists of the four closely related proteins, RGS6, RGS7, RGS9 and RGS11) were shown to be differentially regulated in discrete regions of the mouse brain after acute and chronic morphine exposures [80]. The authors also pointed out that chronic opioid treatment modulates the expression of the G β 5 subunit, which commonly and specifically heterodimerizes with members of the RGS7 sub-family [80].

As mentioned earlier, opioids are not the only therapeutic agents that are administered for the treatment of neuropathic pain, nor are they the only agents capable of influencing the expression of RGS. Stratinaki and colleagues demonstrated a substantial up-regulation of RGS4 in the nucleus accumbens of human patients that had received monoamine-directed anti-depressants [69]. It is worth noting that in the same nervous tissue, opiates down-regulated the levels of RGS4 [70], suggesting a differential regulation of RGS by distinct classes of drugs that are commonly both used in the treatment of persistent pain.

The modulation of RGS expression by drugs acting on receptors, the signalling of which, is itself influenced by the RGS is highly suggestive of a form of feedback control. Although the up- or down-regulation of GPCRs by prolonged drug administration (antagonists and agonists, respectively) is well documented, the targeting of these receptors with drugs should also take into consideration the putative impact on their RGS partners [56,82]. Therefore, the reduced efficacy of endogenous as well as exogenous pain modulators could depend on alterations of RGS levels [70;71].

5. Influence of RGS on GPCRs controlling nociception

To study the role of RGS in the processing of nociceptive information, a number of different techniques that manipulate RGS expression and function have been developed using both genetic and pharmacological approaches.

5.1. Tools used to manipulate RGS expression and function

Models of RGS knockdown and knockout have permitted the specific control of individual RGS levels to explore their contribution in physiological processes. This approach does not, however, provide any indication of specific RGS-G protein interactions and suffers from a possible redundancy in RGS function that can compensate for RGS down-regulation. To date, diverse knockout animal models for RGS (including conditional knockout of the protein allowing its suppression within discrete regions of the brain) have been developed for RGS2, RGS4, RGS7 and RGS9 [58;69;83-85]. Additionally, the injection of a viral construct into selected areas of the adult rat brain has been used to over-express RGS4 [69,70].

Another elegant approach that has been developed consists of the use of mutated G α proteins (in particular of G α_o) which is insensitive to the GAP activity of RGS [51,86]. This overcomes the issue of RGS redundancy and permits the study of the role of endogenous RGS. Moreover, it provides an indication of the specific role of G α_o -RGS interactions. Knock-in mice with RGS-insensitive G α_o have been used to explore the functional interaction between RGS and opioid-mediated signalling in spinal and supraspinal tissues [87]. This approach still suffers from a major drawback in that it is not possible to discriminate between the roles of individual RGS.

In parallel with these genetic tools, the opportunity to specifically target RGS in order to modulate GPCR signalling has emerged after the development of synthetic modulators of RGS [88,89]. It has been suggested that these modulators are likely to have potential clinical usefulness in potentiating the actions of endogenous or exogenous GPCR ligands [90,91]. Many of these compounds inhibit the GAP activity of RGS, in particular of RGS4, by interfering with the interaction with G α proteins. For instance, CCG 63802 has been shown to inhibit the interaction between G α_o and RGS4 with a micromolar potency [88]. Such inhibition should also impact other G α members, since the compound binds to RGS4 but not to G α_o . Indeed, other more recently developed derivatives that block the interaction between RGS4 and G α_o , have also been shown to alter both G q and G i -dependent signalling [89]. These compounds are thiadiazolidinone derivatives with structures that slightly differ from that of CCG 63802. Amongst these derivatives, CCG 203769 presents a nanomolar potency in inhibiting RGS4. The inhibitor is 8-fold more selective for RGS4 than for RGS19 and up to >5000-fold more selective than for other RGS. This contrasts starkly with the lower selectivity of CCG 63082 for RGS4 (being only 2.2-fold more selective for RGS4 than for RGS19).

While the development of such RGS4 inhibitors has provided a means of manipulating RGS activity, other approaches, such as the prevention of degradation, have also proven to be effective in modulating RGS expression and function. Ubiquitin-mediated proteasomal degradation is responsible for the short half-life of several RGS, but is also involved in various disease states where RGS expression appears to be reduced [92]. Interfering with such degradative processes could stabilize RGS, thereby extending their activity. Some proteasome inhibitors, such as bortezomib, are already being applied in the treatment of certain cancers and might also be useful in the treatment of other disorders by influencing RGS degradation [92]. Similarly, it has been suggested that MG132, a proteasome inhibitor, inhibits breast cancer cell invasion and migration in a RGS4-dependent mechanism [93].

5.2. Influence of RGS on GPCRs involved in nociception

RGS functionally interact with a large variety of GPCRs and their GAP activity principally operates via G α_i and G α_q subunits [64]. In a variety of experimental settings, this interaction has been shown to support the modulation of cell signals specifically involved in nociception. The best documented example of this is the functional interaction between RGS and opioid receptors, which are preferentially coupled to G i/o proteins. Furthermore, the modulation of other

predominantly anti-nociceptive receptors, such as CB₁, has also been studied in the context of pain. Conversely, RGS may influence GPCRs preferentially coupled to G_α_i-protein [45;84;85], some of which are known to facilitate nociceptive transmission, but such modulation has, so far, not been specifically examined in context of pain. The following sections provide a non-exhaustive description of experimental works that underline such interactions.

6. In vitro influence of RGS on GPCR signalling involved in nociception

The initial identification of RGS was rapidly followed by investigations into their influence on opioid receptors. It was hypothesized that these proteins dampened the responses to opioid agonists. Early *in vitro* studies demonstrated that RGS-dependent GAP activity influenced MOR signalling, causing a shift to the right of agonist concentration-response curves [51;56;57;83]. The generation of a RGS-insensitive G_α_o in C6 glioma cells carrying recombinant MOR resulted in an increased potency and efficacy of morphine in inhibiting adenylyl cyclase [83]. Accordingly, it was demonstrated that interfering with RGS activity on G_α_o could turn partial opioid agonists, such as buprenorphine, into full agonists [86]. Similarly, the functional interaction of RGS with DOR and KOR was shown to influence the amplitude of downstream signals or to contribute to defined G-protein coupling specificity [53;94].

The influence of RGS4 on opioid receptor-associated signalling has been abundantly examined in systems co-expressing selected receptors and RGS proteins. Using MOR expressing HEK293 T cells, a decreased agonist-induced inhibition of adenylyl cyclase activity was observed when the cells over-expressed RGS4 [59]. Other studies conducted in transfected cells demonstrated that RGS4 could reduce MAPK phosphorylation following MOR, KOR or DOR activation. Such modulation of morphine-mediated signalling by RGS4 has been shown to rely on the direct interaction between the opioid receptors and the RGS protein [87, 88,94]. It was demonstrated that in MOR expressing C6 cells, RGS4 and RGS8 were more potent than RGS7 in reducing the signalling of MOR agonists [89]. Furthermore, RGS9 has also been shown to modulate MOR-associated responses in *in vitro* studies [56,90].

Apart from opioid receptors, any GPCR that modulates the nociceptive processing may also be regarded as a putative target for the modulatory role of RGS. For instance, through its interaction with metabotropic GABA_B receptors, GABA is known to interfere with the propagation of nociceptive signals, and specific agonists such as baclofen, have been suggested to be promising analgesic drugs [91]. The GABA_B receptor-mediated inhibitory signal is regulated by RGS4 [92, 93]. This interaction may play a role in pathological pain or influence the efficacy of GABA_B receptor ligands. Similarly, RGS4 can negatively modulate the signalling of anti-nociceptive cannabinoid receptors (particularly CB₁). The over-expression of RGS4 in transfected cells has been reported to attenuate the CB₁-induced inhibition of cAMP production and phosphorylation of ERK1/2 [71]. This effect is consistent with the documented GAP activity exerted by RGS4 on both CB₁ and CB₂ [95].

The influence of RGS is not restricted to G_α_i proteins and several studies have revealed their impact on G_α_q-associated pathways [45,96], as is the case for mGluRs. In *Xenopus* oocytes, RGS4 has been demonstrated to block activation of mGluR1a- and mGluR5a-mediated calcium-dependent chloride currents, an effect that has been attributed to the blockade of G_q [84,85]. In hippocampal neurons, RGS4 has been reported to attenuate the mGluR5-dependent inhibition of potassium currents. Such interactions have also been demonstrated *in vivo*, such as in the dorsal striatum where RGS4 over-expression attenuated ERK phosphorylation that had been mediated by mGluR5 activation [85]. The direct interaction between RGS4 and mGluR5 was further demonstrated by co-immunoprecipitation studies conducted on samples of striatal tissue. Since mGluR5 is recognized as a pro-nociceptive receptor

participating in central sensitization [97], the impact of RGS4 on nociceptive processing mediated by this pathway and its relevance to the development of novel analgesic therapies deserves further experimental exploration.

7. Influence of RGS on the modulation of pain-induced behaviour by GPCR

The demonstration of the influence of RGS on the pharmacological responses of GPCRs in several *in vitro* models was rapidly followed by the suggestion that they might also exert an influence on a range of mammalian behaviours influenced by these receptors. Although the present review has focused on the influence of RGS on GPCR-associated modulation of nociception, the alteration of opioid mediated-responses by RGS was first demonstrated in other contexts, such as addiction, reward and tolerance [57]. As detailed below, the main conclusions that can be drawn from a number of studies is that: (1) their effect is dependent on the agonist used, even when acting on the same receptor, (2) individual members of the RGS family influence nociception in distinct manners, (3) these effects appear to be site-specific along the neuraxis.

Although *in vitro* studies have revealed a negative modulation of MOR signalling by RGS4, transgenic mice lacking RGS4 have unexpectedly revealed no alteration of the analgesic response to morphine when compared to the responses of their wild-type littermates using the hot plate assay [70]. However, the responses to fentanyl and methadone, two potent MOR agonists, have been found to be dramatically reduced in the same transgenic mouse strain. These observations were suggested to reflect differences in the impact of RGS4 on G_α_i or G_α_q subunits that are differentially recruited in response to selected agonists. In another independent study, the suppression of RGS4 in total knockout mice has been shown to potentiate the anti-nociceptive (acetic acid stretch assay) and anti-hyperalgesic effect (nitroglycerin-induced thermal hyperalgesia assay) of SNC80, a DOR agonist [98].

Although the consequence of manipulating RGS4 seems to depend on the agonists tested, it also exhibits a site-specific profile, with distinct outcomes resulting from their action in different spinal or supraspinal structures [99]. As expected, conditional RGS4 suppression that was restricted to the nucleus accumbens affected opioid reward associated behaviours [70,100]. Similar observations were drawn for RGS7, of which both systemic or selective striatal suppression increased the sensitivity to morphine reward. However, although total RGS7 knockout mice showed a potentiated analgesic response to morphine, RGS7 suppression restricted to the striatal neurons had no apparent influence on hypersensitivity as measured with the hot plate assay [101]. The development of a mouse strain expressing a RGS-insensitive G_α_o protein has undoubtedly contributed to a better understanding of RGS involvement in numerous pathological contexts involving G_α_o-coupled receptors [102]. Suppression of RGS activity on G_α_o resulted in increased supraspinal antinociception induced by morphine measured by the hot plate test [99]. However, the use of the tail-withdrawal test as an indicator of spinal cord-dependent responses revealed that the insensitivity of the mutated G_α_o to endogenous RGS resulted in a decreased analgesic effect of both morphine and methadone [99]. Such unexpected results were also demonstrated for RGS9, which is largely expressed in the brain. The supraspinal anti-nociceptive effect of morphine appeared to be enhanced in RGS9-knockout mice [57]. However, in a tail immersion test, these knockout mice demonstrated reduced sensitivity to noxious thermal stimuli and electrophysiological recordings performed on spinal slices from these transgenic animals also showed decreased neuronal hyperpolarization in response to morphine [103]. The mechanisms underpinning this differential role of RGS at spinal and supraspinal levels remain unknown.

Other RGS that are commonly detected in the nervous system have also been observed to exert an influence on the anti-nociceptive action of opioids, depending on the agonist in question. For example, RGS9-

knockout mice showed a decreased methadone and fentanyl-mediated analgesia in the hot-plate test, but enhanced morphine-mediated analgesia [56,104]. Similarly, RGS7-knockout mice exhibited enhanced analgesia after morphine administration as compared to their control wild-type littermates, probably through a functional interaction with MOR [101]. Finally, knocking down RGS2 or RGS3 reduced morphine and beta-endorphin mediated analgesia, whereas a lower expression of RGS12 had the opposite effect [56].

Binding partners of RGS have also been found to play a role in the regulation of opioid-mediated analgesia. It has been demonstrated that the R7 binding protein acts as a negative modulator of the analgesic response to morphine. R7-binding protein knockout mice display a higher sensitivity to KOR agonists and enhanced analgesia after morphine administration [105,106]. Similarly, the Gβ5 subunit, known to combine with members of the R7 sub-family of RGS, has been shown to modulate the analgesic effect of morphine. Accordingly, reducing Gβ5 expression, either by blocking its mRNA with antisense oligodeoxynucleotides or by using a knockout model, was associated with an enhanced analgesic response to morphine and an increased responsiveness to MOR and DOR agonists [55,107].

8. Influence of RGS modulation in neuropathic pain models

The impact of modulating RGS expression or activity has been examined on both the development of neuropathic pain and its modulation by diverse pharmacological agents. Inhibition of RGS by the pharmacological compound CCG63802 decreases the hypersensitivity developed in two animal models of neuropathic pain: intrathecal administration resulting in a transiently reduction of lesion-associated thermal hyperalgesia in the widely used model of partial sciatic nerve ligation, as well as reducing tactile hypersensitivity in the spared nerve injury model [65,71].

More recently, the role played by RGS4 in the maintenance of chronic pain symptoms has been explored in mice. It was demonstrated that female RGS4-knockout mice display attenuated mechanical and cold allodynia several days after complete Freund's adjuvant injection while no effect on thermal hyperalgesia was observed [58]. Similarly, the authors showed that in the spared nerve injury neuropathic pain model, female RGS4-knockout mice recovered more quickly from mechanical allodynia than wild-type littermates.

The results described here show the effect of suppressing the expression of RGS4 or reducing its activity on the hypersensitivity developed in different models of neuropathic pain. However, other studies investigated the modulatory role of RGS on the effect of analgesics in these models. It was demonstrated that knocking out RGS9 could attenuate the intensity of thermal hyperalgesia and mechanical allodynia during the first few days after injury using the spared nerve injury model [108]. Similarly, after a nerve lesion, pain relief mediated by anti-depressants is modified in several models of RGS knockout mice. In a model of spared nerve injury, the anti-nociceptive effect of desipramine appeared to be amplified in transgenic mice lacking RGS9 in the nucleus accumbens [109], but was decreased in mice lacking RGS4 [69]. In contrast, in the same lesion model, RGS4 suppression had an opposite influence on the analgesic effect mediated by ketamine, a non-monoamine acting antidepressant [69]. With respect to cannabinoid ligands, it was reported that sciatic nerve lesioned animals displayed a loss of cannabinoid-mediated analgesia but that the spinal inhibition of RGS4 could restore this activity, indicating that RGS4 acts as a negative modulator of cannabinoid-mediated control of nociception in the spinal cord [71]. Altogether, these data provide strong evidence for the importance to develop RGS inhibitors as valuable analgesic tools.

9. Conclusion

9.1. RGS and pain

Based on the abundant literature regarding the influence of RGS on nociceptive transmission in physiological or pathophysiological contexts, including sometimes apparently contradictory results, one cannot strictly define RGS as pro- or anti-nociceptive mediators. Intuitively, inhibiting GAP activity mediated by RGS should reinforce the signalling by GPCRs, such as opioid or cannabinoid receptors, thereby promoting analgesia. However, many GPCRs contribute positively or negatively to the physiological modulation of pain through a variety of mechanisms. In this context, RGS may either reinforce or silence these different types of receptors (Fig. 4). The diversity of RGS functions, their regulation, their tissue distribution along the nociceptive pathways and their specificity towards individual receptors likely explains the complex and sometimes puzzling outcomes observed when examining their impact on nociception, pain and analgesia. Furthermore, one may also anticipate that reinforcing the signal triggered by a given anti-nociceptive GPCR (such as opioid receptors) could eventually favour the development of tolerance, leading to some paradoxical loss of the expected analgesic benefit [110].

The link between RGS and the modulation of nociception has been considered from several perspectives, either by testing drugs and determining the extent of nociception after manipulating RGS expression and/or activity, or by studying RGS function in animal models of neuropathic pain. A wide range of GPCRs contribute to the physiological control of nociceptive transmission and many drugs acting on these receptors have been studied for their analgesic properties. It is therefore not surprising that manipulating RGS can influence the response to these drugs. Moreover, the expression of RGS proteins is altered in models of neuropathic pain and one may reasonably suggest that the regulation of RGS participates in the altered transmission of nociceptive signals in pathological contexts. Hence, current research about RGS should also contribute to the understanding of some of the pathological mechanisms that are responsible for the development or persistence of pain in neuropathic conditions. In the search for disease modifying drugs, targeting RGS in neuropathic pain conditions could help to restore a normal control of nociception.

10. RGS as a drug target

GPCRs constitute the largest family of targets for approved and commercialized drugs. While these membrane proteins are still the subject of intense research, associated signalling partners such as GPCR kinases (GRK) or arrestins have started to receive growing attention. Since their discovery, RGS have emerged as promising therapeutic targets for drug development [111]. They could offer a complementary approach to conventional receptor agonists and antagonists that frequently present limitations in terms of efficacy or undesirable side effects. Nevertheless, because many RGS share several functions and a wide distribution throughout the organism, the selective manipulation of RGS function appears to be extremely challenging. Therefore, the increased understanding of these proteins should help in the move towards this goal. Firstly, the spatiotemporal expression has been shown to differ for many RGS subtypes; not all RGS are expressed in the same tissues nor do they exert the same function in physiological or pathophysiological situations [47,50]. For example, splice variants of RGS9 show a peculiar distribution pattern, as RGS9-1 is found in retinal cells and displays a GAP activity for transducin, a photoreceptor-associated GPCR, while RGS9-2 is predominantly detected in the striatum, where it modulates MOR-associated responses [56,90,112]. Furthermore, RGS differ according to the presence of molecular domains that control their interactions with other proteins with distinct outcomes on GPCR-associated signalling cascades [48]. Developing drugs that could specifically recognize these domains or interfere with their interaction

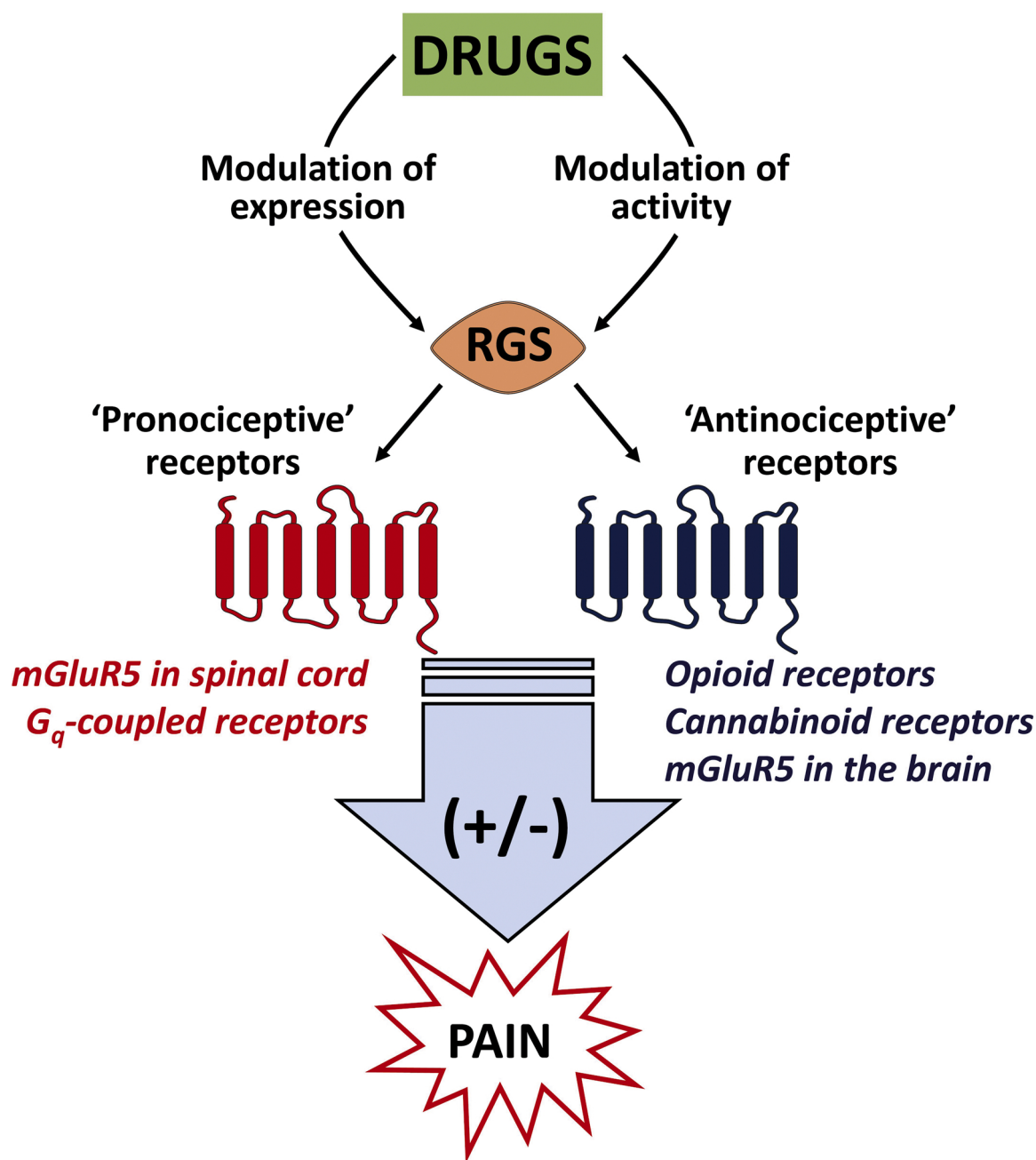


Fig. 4. Opportunity of pain modulation by drugs that influence RGS.

Drugs that influence the expression or activity of RGS along the nociceptive neuraxis should have an influence on the functional response of diverse GPCRs involved in the modulation of nociception. The balance between the activities of receptors that positively and negatively control nociception will eventually define the impact on pain.

characteristics could contribute to their selective action. Finally, an up or down-regulation of individual RGS has been associated with diverse disorders such as schizophrenia, Alzheimer's and Parkinson's diseases, opening the door to a specific action of certain RGS-targeting drugs in these pathological contexts [113]. In the present review, we highlighted their increased or decreased expression in several models of neuropathic pain. While it frequently remains unclear whether this regulation participates in disease progression or is a consequence of it, this should be considered when developing pharmacological strategies involving these proteins. Indeed, in the neuropathic pain model of partial sciatic nerve ligation, the up-regulation of RGS4 alone, amongst 10 RGS examined [59] provides an important rationale for its pharmacological targeting.

For the last 20 years, the multiplicity of cell signalling associated

with a given GPCR has raised the hope of developing biased ligands that could activate a subset of the downstream molecular responses [114]. Such functional selectivity is exemplified in the case of opioid receptors, for which biased ligands that activate G proteins without recruiting β -arrestin were expected to promote analgesia without inducing respiratory depression [115]. The concept presently lacks clinical validation, but the capacity of RGS to bias the response to GPCR ligands certainly holds great promise for future drug development [116,117]. In this respect, analgesic strategies should preferentially combine RGS-targeting drugs with GPCR ligands in order to improve their efficacy as well as their specificity. Obviously, opioid receptor ligands as well as cannabinoids represent promising candidates, but ligands of other receptors should also be considered for RGS modulation, including

G_q-coupled receptors, which are often documented as pro-nociceptive. To date, RGS4 appears to be one of the most appropriate targets for the potentiation of several analgesic systems and the effective treatment of neuropathic pain. Not surprisingly, RGS4 has received prominent attention for the development of selective pharmacological modulators. However, further studies on RGS4, as well as other RGS that are widely expressed in the CNS, deserve future attention. In particular, conditional transgenic models allow the manipulation of the expression of selected RGS in defined CNS structures, in individual cell types or at a specific time after injury should help to clarify their respective roles in the context of neuropathic pain. RGS operate through a variety of molecular mechanisms and the identification of specific inhibitors or activators may require a series of functional assays [118]. Only a few small inhibitors have been developed so far [119], some of which show promising results in models of neuropathic hypersensitivity [65,71]. Nevertheless, considering that RGS expression levels are altered in the context of disease, future research should also consider the possibility of influencing the mechanisms that regulate these proteins [50]. This should also support more long-term benefits for patients suffering from chronic conditions or diseases, such as neuropathic pain.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.phrs.2020.105148>.

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