G Protein-Coupled Receptor Kinases (GRKs) and G Protein-Coupled Receptors (GPCRs): GRK6 as a Potential Drug Target for CNS Disorders

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G protein-coupled receptor kinases (GRKs) specifically interact with G protein-coupled receptors (GPCRs) and play an important role in terminating agonist-induced GPCR signaling. Activated by an agonist-stimulated GPCR, GRKs in turn phosphorylate the GPCR, which leads to recruitment and activation of β-arrestin and subsequent receptor desensitization. Through activation of β-arrestins, GRK can also participate in downstream signaling events. GRK6, one of the GRK isoforms, is expressed abundantly in the brain. In recent years, potential links between GRK6 and central nervous system (CNS) disorders, in particular Parkinson's disease (PD), have been suggested. Specifically, GRK6 appears to play a significant role in dopamine-mediated locomotor activity and dopamine agonist-induced dyskinesia. Therefore, GRK6 should be considered as a potential drug target for CNS disorders.

Keywords: *GPCR, GRK, GRK6, Parkinson's disease, dopamine, L-DOPA, dyskinesia*

Mechanism and regulation of GPCR signaling

GPCRs, such as rhodopsin, prostaglandin E_2 receptors and β adrenergic receptors, are members of the seven transmembrane receptor family. G proteins are heterotrimeric complexes comprised of G_α and $G_{\beta\gamma}$ subunits. Upon agonist binding to a GPCR, the G_α subunit exchanges its bound GDP with GTP, which triggers release of G_α and its subsequent association with effectors. The released $G_{\beta\gamma}$ subunit can also stimulate downstream signaling[1]. GPCR signaling is controlled by receptor desensitization, a process that involves receptor phosphorylation by G protein-could receptor kinases (GRKs) and subsequent β-arrestin binding[2]. β-arrestin binding to the receptor sterically blocks the interaction between GPCR and G proteins, preventing further signaling[3]. β-arrestins also promote endocytosis by functioning as adaptors between endocytotic elements and the receptor[2], promoting internalization of GPCRs through clathrin-coated pits[4]. In addition to receptor desensitization, β-arrestins can coordinate the process of signaling termination through degradation of second messengers[5] such as cAMP[6]. In other instances, arrestins can also act as signaling molecules independent of G-proteins by, for example, binding directly to mitogen activated protein kinases (MAPKs)[3]. Furthermore, upon GPCR activation, β-arrestin1 can translocate into the nucleus, where it facilitates the recruitment of histone acetyltransferase, leading to transcription of genes encoding proteins such as c-fos[7].

Activation of arrestins depends on the phosphorylation of agonist-stimulated GPCRs. Importantly, phosphorylation of GPCRs specifically by GRK enhances the inhibitory effect of arrestins[4]. Furthermore, the pattern of GRK-mediated receptor phosphorylation determines how tightly β-arrestins bind to the activated and phosphorylated receptor[2]. In summary, GPCR activity and signaling are positively and negatively regulated through β-arrestins. Activity of β-arrestins, in turn, depends on the phosphorylation state of the activated GPCR, which is controlled by GRKs.

Overview of G protein-coupled receptor kinases

The seven vertebrate GRKs (GRK1-7) are grouped into three subfamilies: GRK1 (GRK1 and 7), GRK2 (GRK2 and 3), and GRK4 (GRK4, 5 and 6)[8,9]. GRK1 and 7 are exclusively expressed in photoreceptors, where they phosphorylate rhodopsin[10,11]. GRK2 and 3 are broadly distributed in the CNS[12,13], while expression of GRK4 is mostly found in testis[14]. GRK5 is most abundantly expressed in lung, heart, retina, and lingual epithelium, with moderate expression in brain[50,53,54]. The highest expression of GRK6 is found in brain and skeletal muscle, followed by pancreas, and much

lower levels in other organs[15].

All GRKs have in common a multi-domained structure consisting of (1) the N-terminal region, (2) the regulator of G-protein signaling homology domain, (3) a protein kinase domain, and (4) a variable C-terminal domain[16]. In general, the N-terminal region of GRK is believed to recognize the activated form of GPCRs[17]. Lipid modification of the C-terminus, as seen in GRK2, is also involved in this process[18,19]. Binding of a lipid-modified $G_{\beta\gamma}$ subunit to the C-terminus of GRK2 facilitates its translocation from cytoplasm to membrane, where the target GPCR is located. In addition, binding of lipids to the C-terminal pleckstrin homology domain can directly regulate GRK2 activity[20]. In effect, these two mechanisms can synergistically enhance the activity of GRK2[21,22]. Increases in activity of GRKs by lipid binding have been observed for all members of the GRK4 subfamily[23]. In addition to lipid modifications, kinase activity of GRKs can be regulated by other kinases[24]. For example, GRK5 activity can be inhibited by autophosphorylation promoted by calmodulin (CaM)[25-27] or by protein kinase C (PKC)-mediated phosphorylation[28]. Interestingly, GRK2 activity is inhibited by CaM, but this effect is reversed by PKC[29,30]. Other kinases such as Src and MAPK have also been found to have regulatory effects on GRKs[31,32]. In another case, Raf kinase inhibitor protein (RKIP), upon its phosphorylation by PKC, releases from its normal target of Raf1 and binds to GRK2, inhibiting its activity. This change in RKIP function from Raf-1 inhibition to GRK2 inhibition results in enhanced receptor signaling[33]. Taken together, these findings indicate that the activity of GRKs, and therefore subsequent changes in GPCR-mediated signaling, are regulated by many different mechanisms. The effects of CaM and PKC, for example, suggest a model of coordinated regulation of GPCRs[16]. Therefore, any alterations in GRK activity may have a critical impact on cellular functions.

Molecular properties of GRK6

The GRK of most relevance to CNS disorders is GRK6, which is the GRK with highest expression in the brain. Alternative splicing of the C-terminal end of GRK6 yields three variants: GRK6A, B and C, with the sizes of 576, 589, and 560 amino acid residues, respectively[34]. Only GRK6A has the palmitoylation site within the C-terminal domain, which allows membrane localization[35]. The C-terminal region of GRK6B contains consensus phosphorylation sites for PKC and cAMP/cGMP-dependent protein kinases that may contribute to phosphorylation-dependent membrane association. GRK6C, on the other hand, has a truncated C terminus and lacks both the palmitoylation and phosphorylation sites; therefore, it is suspected to have poor membrane association[34,36]. The existence of a fourth variant, GRK6D, has also been reported. This variant, however, lacks a functional catalytic domain and is speculated to act as an inhibitor of other GRK6 isoforms[36].

Palmitoylation of GRK6A has important consequences. Palmitoylated GRK6 is membrane-associated, localized in close proximity to target GPCRs and thus has increased activity[37,38]. However, palmitoylation is not the only mechanism leading to membrane localization for GRK6 variants. When overexpressed in COS-7 cells, the three variants of GRK6 (A, B and C) are membrane-associated despite the lack of putative C-terminal palmitoylation sites in both GRK6B and C[39]. Furthermore, the GRK6C isoform has the highest catalytic activity, suggesting that the C-terminal extensions found in variants A and B, but not in variant C, result in decreased activity[39]. According to the crystal structure, there are two sets of complementary interactions that bring GRK6 into close proximity of a GPCR. First, two regions of GRK6, the α helix region in the N-terminus and the C-terminal tail, directly bind to GPCRs. Second, GRK6 associates with membrane through the phospholipid-binding surface[40]. It has been suggested that the complementary receptor binding provides the free energy needed to induce and stabilize the active form of GRK6[40,41].

While the molecular mechanisms of interactions between GRK6 and GPCRs are becoming better understood, studies attempting to identify the native receptor substrate of GRK6 have yielded inconclusive results. Nevertheless, some candidate receptors have emerged. For example, despite the negative results of earlier studies[42,43], several, more recent studies verified the M3 muscarinic acetylcholine receptor as a target of GRK6[44-46]. Other receptors identified as potential targets of GRK6 are the β2 adrenergic receptor[47] and the insulin-like growth factor 1 receptor[48]. In the CNS, one study demonstrated that the D_2-like dopamine receptor was a target of GRK6[49]. However, more studies are needed to verify the identity of receptor substrates for GRK6 *in vivo*.

GRK6 involvement in PD and animal models of PD

As mentioned earlier, GRK6 is highly expressed in the brain[15]. It reaches its highest expression in the striatum[13,50],

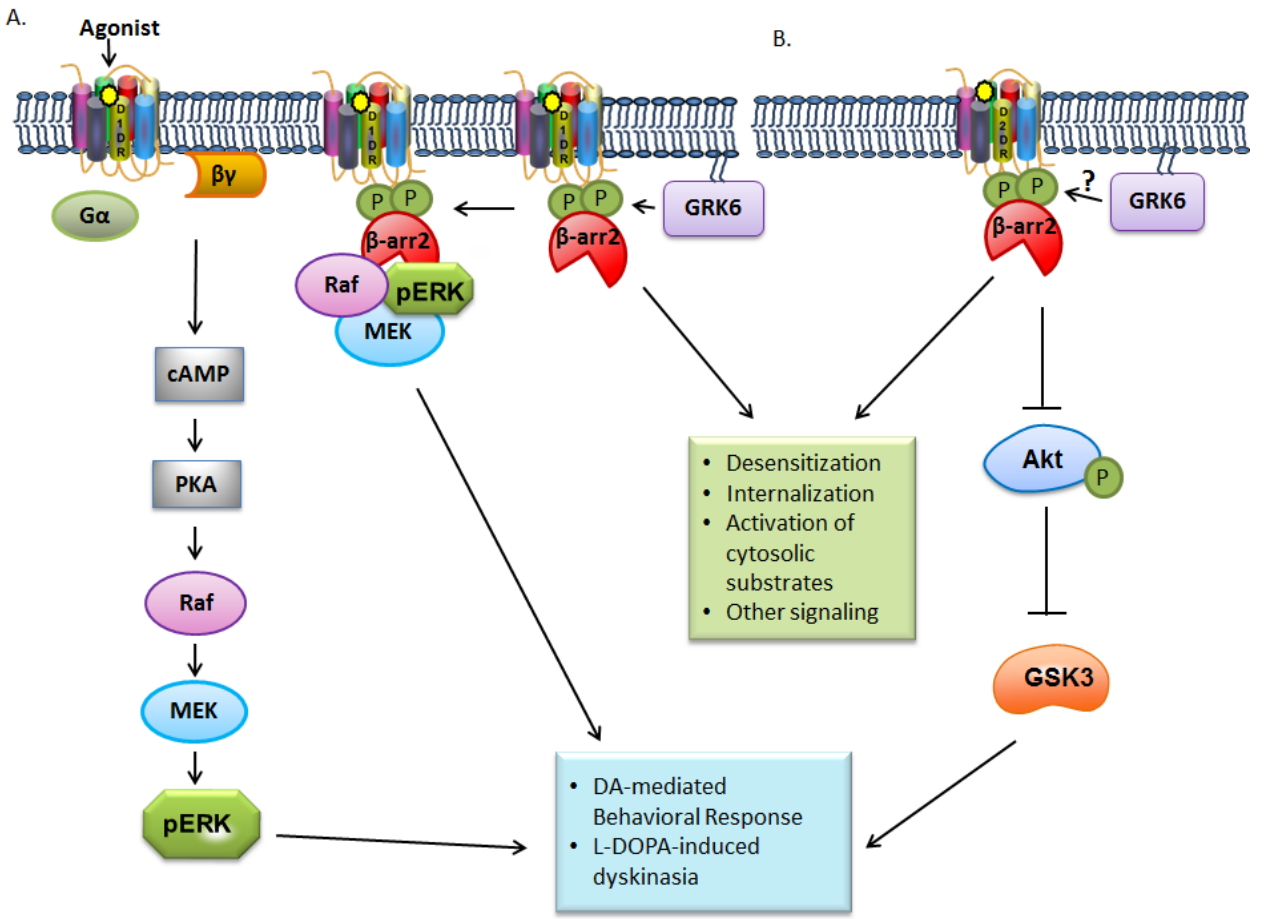

Figure 1: *Signal transduction mediated by dopamine receptors that may lead to Parkinson's disease-related motor dysfunction.* (A) D1 receptor-mediated signal transduction. G protein-mediated signal transduction (left). Activation of trimeric G protein results in increased level of cAMP and activation of PKA, which leads to activation of MAPK cascade and increased Erk phosphorylation. β-arrestin-dependent activation of MAPK (middle). β-arrestin binds to agonist-activated, GRK-phosphorylated receptor, and acts as signal transducer independent of G proteins. Increased activation of ERK by β-arrestin in this manner may contribute to L-DOPA-induced dyskinasia. β-arrestin binding to the activated receptor also leads to receptor internalization (right), followed by receptor desensitization or activation of other signaling cascades. (B) D2 receptor- mediated β-arrestin dependent signal transduction. β-arrestin inactivates Akt through dephosphorylation, and Akt inactivates GSK3 through phosphorylation. Therefore, β-arrestin contributes to dopamine (DA)-mediated signal activation as well as receptor desensitization and internalization. Increased activity of GSK3 has been linked to L-DOPA-induced dyskinasia.

and changes in GRK6 expression levels have been observed in human clinical conditions as well as in animal models of neurological disorders. Therefore, it has been speculated that alterations in GRK6 activity may contribute to pathophysiology of CNS disorders. One of the CNS disorders in which changes in GRK6 have been reported is Parkinson's disease (PD). For example, in postmortem human brain samples, increased GRK6 mRNA levels were detected among the PD patients who also exhibited dementia compared to PD patients without dementia or the control group[50].

PD is a neurological disorder characterized by bradykinesia (slowness of movement), rigidity, and tremor. PD symptoms are the result of a loss of the striatal innervation by dopaminergic neurons in the substantia nigra pars compacta. Currently, L-3,4-dihydroxyphenylalanine (L-DOPA), a precursor of dopamine, is the most effective pharmacological therapy for the treatment of motor symptoms of PD[51]. Unfortunately, after 4-6 years of L-DOPA treatment, about 40% of PD patients develop increased involuntary movements known as dyskinesias. After prolonged L-DOPA therapy, almost 90% of patients develop dyskinesia[52]. The loss of striatal dopamine in PD can be modeled in rodents and non-human primates by injection of dopaminergic neurotoxins. In rats, the neurotoxin 6-hydroxydopamine (6-OHDA) is directly injected into the nigrostriatal pathway or the striatum, whereas dopamine depletion in

non-human primates is achieved by systemic injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Like in humans, dopamine-depleted animals develop dyskinesias following repeated treatment with L-DOPA.

There are a series of studies investigating the role of GRK6 in animal models of PD that were aimed at gaining valuable insight into the molecular mechanisms underlying the altered dopamine signaling in this disorder and, in particular, L-DOPA-induced dyskinesia. Biochemical studies in both MPTP-lesioned monkeys and 6-OHDA-lesioned rats revealed increased GRK6 expression levels in the dorsal and ventral striatum[53,54]. Behavioral studies have also provided insights into the connection between GRK6 and motor function. A study by Ahmed et al. (2010)[55] demonstrated that L-DOPA- or dopamine receptor agonist-induced rotation in 6-OHDA-lesioned rats produces a progression of adverse involuntary movements (AIMs). Similar results were seen in MPTP-lesioned monkeys. It was shown that overexpression of GRK6 alleviated AIMs, and down-regulation exacerbated them. However, in another study, 6-OHDA-lesioned GRK6 kockout (KO) mice showed significantly less dyskinesia as measured by AIM score compared to lesioned wild-type (WT) mice[49]. Therefore, while the former study demonstrated that an increase in GRK6 availability alleviated dyskinesias, the latter study showed GRK6 depletion suppressed dyskinesia. Although these results seem conflicting, it should be noted that there is a caveat in using knockout animals. For example, since GRK2 is also expressed in striatum[54], although at lower levels than GRK6, there may be a compensatory expression of GRK2 or other GRK isoforms in GRK6 KO animals. If that is the case, decreased dyskinesias in GRK6 KO animals may be a result of other GRK isoforms being upregulated. Further studies are needed to address these possibilities.

To further understand the role of GRK6 in dopamine receptor-mediated locomotor activity, Gainetdinov et al. (2003)[56], using GRK6 KO mice, took a pharmacological approach in the GRK6 KO mice. This study showed that GRK6 KO mice had an increased locomotor response to the D_2 agonist quinpirole. *In vitro*, GRK6-mediated desensitization of D_2-like, but not D_1-like dopamine receptors. In mouse striatum, one possible signaling mechanism downstream of the D_2 dopamine receptor in which GRK6 plays a role involves Akt and glycogen synthase kinase 3 (GSK3)[57]. Activation of β-arrestin 2 via D_2 receptor stimulation leads to dephosphorylation and inactivation of Akt which, through phosphorylation, has an inhibitory effect on GSK3. In support of GRK6's involvement in this signaling cascade, GRK6 KO mice had significantly higher basal levels of pGSK3β compared to WT mice[49].Interestingly, there are also some data suggesting that GRK6 may exert its effect on dyskinesia via the MAPK signaling cascade. For example, after continuing L-DOPA treatment on 6-OHDA-lesioned mice, expression of phosphorylated extracellular signal-regulated kinase 2 (pErk2), one member of the MAPK family, was significantly increased compared to sham-operated control. There were no changes in pErk expression, however, between controls and 6-OHDA-lesioned GRK6-KO mice[49]. In a separate study, Westin et al. (2007)[58] showed that there was a positive correlation between the severity of L-DOPA-induced dyskinesia and the amount of pErk expression in 6-OHDA-lesioned rat striatum. In addition, in those animals, application of a D_1 antagonist reduced the development of dyskinesia as well as the amount of pErk. Together, these results suggest that dyskinesia induced by the action of L-DOPA on D_1 receptors may involve GRK6 through its activation of the MAPK cascade.

GRK6 as a drug target

Development of drugs for CNS disorders has been less successful than in other areas of disorders because of limited knowledge of the underlying pathophysiology, limitations in current preclinical models of CNS disorders, and a lack of good biomarkers[59]. For treatment of PD, there are many available drugs that include, for example, L-DOPA, D_2 dopamine receptor agonists, catechol-O-methyltransferase inhibitors, and monoamine oxidase inhibitors. Despite the fact that L-DOPA treatment loses efficacy with time and produces adverse side effects, it remains the most widely used therapy today due to the limited efficacy of other drugs[60]. Currently, amantadine, a non-competitive N-methyl-D-aspartate receptor antagonist is the only drug used to alleviate the symptoms of L-DOPA-induced dyskinesia, but prolonged use of amantadine leads to the development of adverse side effects such as dizziness, confusion and hallucinations[51]. Another, non-pharmacological approach to symptomatic treatment of PD that can reduce L-DOPA induced dyskinesia is deep brain stimulation (DBS) of the subthalamic nucleus. DBS, however, involves neurosurgeries that are expensive, come with potential risks, and require regular maintenance of the stimulator[51]. Because of the limitations associated with all the current therapies, there is a need for continuing efforts to develop novel therapies aimed at new potential drug targets. As an

alternative to drugs that directly activate or inhibit GPCRs, there is the potential for therapeutic value in regulators of GPCR activities[1]. Although lagging behind, continuing efforts have been made for development of drugs targeting CNS protein kinases such as GSK3 for Alzheimer's disease and neuropsychiatric disorders, PKC for bipolar disorder, and mammalian target of rapamycin for autism[61]. Some interesting properties of GRK-mediated GPCR regulation make GRK an attractive drug target. Probably one of the most intriguing discoveries is the ability of GRK-produced phosphorylation patterns to stimulate distinct downstream signaling through β-arrestin in a GRK variant-specific manner[62]. The mechanism of this process was unlocked in a study by Nobles et al. (2011)[63]. In this experiment, the authors demonstrated that, although both GRK2 and GRK6 participated in the phosphorylation of the β_2 adrenoreceptor, the functional outcome was different depending on the distinct phosphorylation patterns that were produced by each kinase. Furthermore, binding of different ligands to GPCRs could determine which GRK would be activated.

Conclusions and future directions

As discussed in the previous sections, several lines of evidence suggest that GRK6 has a critical role in PD. Its tissue-specific expression and intricate network of regulatory functions make GRK6 a good drug target for PD treatment. The future of successful drug development may lie in the discovery of selective ligands for GRK6, which may enable precisely targeted treatment for CNS disorders and/or alleviate undesired side effects when used in combination with other GPCR ligands. By manipulating the activity of GRK6, it is possible to selectively target and fine-tune the cellular signaling events that contribute to CNS disease phenotypes downstream of GPCRs.

One of the most prominent drug-discovery techniques, high-throughput screening (HTS), is a promising route to identifying possible GRK6-targeted drugs. HTS of compound libraries has evolved into a successful approach for discovering novel drugs for both CNS disorders and other indications. Examples of successful HTS efforts include the discovery of drugs that target tyrosine kinase for cancer treatment (e.g. Gefitinib, Lapatinib), proteases for HIV (Tipranavir) and GPCRs for hypertension (Ambrisentan)[64]. It should be noted, however, that screening for drugs that target protein kinases is in its infancy, and there are undoubtedly many challenges in the process. Some of the difficulties include assay design, finding compounds with sufficient affinity and selectivity for the target as well as appropriate chemical properties for CNS penetrance[61]. Another inherent challenge is that most protein kinase inhibitors identified by HTS are ATP derivatives that compete for the binding site with endogenous ATP[65]. Therefore, it will require careful planning and optimization of the assay conditions before screening attempts should be made. With utilization of proper conditions and technique, the HTS approach may lead to the discovery of compounds that selectively act on GRK6 and could be used to further define the role of GRK6 in PD.

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