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G protein-coupled receptor kinases as regulators of dopamine receptor functions

Eugenia V. Gurevich[a,*], **Raul R. Gainetdinov**[b,c], and **Vsevolod V. Gurevich**[a]

[a]Department of Pharmacology, Vanderbilt University, Nashville, TN 37221, USA

[b]Institute of Translational Biomedicine, St. Petersburg State University, St. Petersburg, 199034, Russia

[c]Skolkovo Institute of Science and Technology, Skolkovo, 143025, Moscow, Russia

Abstract

Actions of the neurotransmitter dopamine in the brain are mediated by dopamine receptors that belong to the superfamily of G protein-coupled receptors (GPCRs). Mammals have five dopamine receptor subtypes, D1 through D5. D1 and D5 couple to $G_{s/olf}$ and activate adenylyl cyclase, whereas D2, D3, and D4 couple to $G_{i/o}$ and inhibit it. Most GPCRs upon activation by an agonist are phosphorylated by GPCR kinases (GRKs). The GRK phosphorylation makes receptors high-affinity binding partners for arrestin proteins. Arrestin binding to active phosphorylated receptors stops further G protein activation and promotes receptor internalization, recycling or degradation, thereby regulating their signaling and trafficking. Four non-visual GRKs are expressed in striatal neurons. Here we describe known effects of individual GRKs on dopamine receptors in cell culture and in the two *in vivo* models of dopamine-mediated signaling: behavioral response to psychostimulants and L-DOPA-induced dyskinesia. Dyskinesia, associated with dopamine super-sensitivity of striatal neurons, is a debilitating side effect of L-DOPA therapy in Parkinson's disease. *In vivo*, GRK subtypes show greater receptor specificity than *in vitro* or in cultured cells. Overexpression, knockdown, and knockout of individual GRKs, particularly GRK2 and GRK6, have differential effects on signaling of dopamine receptor subtypes in the brain. Furthermore, deletion of GRK isoforms in select striatal neuronal types differentially affects psychostimulant-induced behaviors. In addition, anti-dyskinetic effect of GRK3 does not require its kinase activity: it is mediated by the binding of its RGS-like domain to $G_{\alpha q/11}$, which suppresses $G_{q/11}$ signaling. The data demonstrate that the dopamine signaling in defined neuronal types *in vivo* is regulated by specific and finely orchestrated actions of GRK isoforms.

Keywords

G protein-coupled receptor kinase; Dopamine receptor; Psychostimulants; L-DOPA; Dyskinesia; Parkinson's disease

*Corresponding author at: 2200 Pierce Ave., PRB417C, Department of Pharmacology, Vanderbilt University, Nashville, TN 37221, USA. Eugenia.Gurevich@vanderbilt.edu (E.V. Gurevich).

Conflict of interest

The authors declare no conflict of interest.

1. Introduction

The dopaminergic system of the brain is critical for the control of the brain functions, such as motor behavior, reward mechanisms and cognition, to name just a few. The highest density of the dopaminergic innervation and, correspondingly, the highest concentration of dopamine (DA) receptors are found in the striatum, the subcortical structure that plays an essential role in the movement control, as well as in motivation and reward.

The dopaminergic system is targeted by multiple therapeutically active drugs as well as drugs of abuse. The latter include psychostimulants such as amphetamine and cocaine that increase effective concentration of DA in the striatum by either blocking the DA transporter or inducing transporter-mediated DA release [1,2]. Excessive DA acting via DA receptors alters the signaling transduction pathways, which contributes to drug-induced molecular and behavioral effects [3–7]. Additionally, deregulation of the signaling via DA receptors contributes to motor deficits in Parkinson's disease (PD), a common age-related neurodegenerative disorder caused by degeneration of dopaminergic neurons in the substantia nigra that supply DA to the striatum [reviewed in Ref. [8]]. The symptomatic therapy of PD, which includes the use the DA precursor levolopa (or L-DOPA) to restore DA to the striatum, as well as DA agonists, although effective, with time causes significant side effects associated with abnormal signaling via DA receptors [9]. Since DA receptors play critical roles in many functions in normal and diseased brain and are targeted by numerous drugs, the mechanisms that regulate their signaling are of interest and may represent promising therapeutic targets for brain disorders.

DA receptors belong to the superfamily of G protein-coupled receptors (GPCR) and, similar to many other members of the GPCR superfamily, DA receptors were identified by the similarity between their sequences and their overall topology to β2-adrenergic receptor and rhodopsin [10]. The DA receptors were first cloned in 1988-91, beginning with the most abundant receptor in the brain, D2 [11], which has two known splice variants [12,13]. The other four DA receptor subtypes are D1 [14–16], D3 [17], D4 [18], and D5 [19]. The five DA receptor subtypes present in mammals are classified into two subfamilies, D1-like (D1 and D5) and D2-like (D2, D3, and D4) [20,21]. The D1-like receptors generally couple to G proteins of the G_s subfamily (G_s and G_{olf}) that stimulate the adenylyl cyclases and cAMP production [22], whereas the D2-like receptors couple to members of the inhibitory G_i family members (G_i and G_o) [22]. In the striatum, the action of the D1 receptor is primarily mediated by highly expressed G_{olf} isoform [23–25]. Similarly, the action of the D2 receptor in the striatum seems to be mostly mediated via coupling with highly abundant G_o, to which the D2 receptor has higher affinity, although it is capable of interacting with Gi as well [26–28]. Out of five DA receptor subtypes, striatal neurons express four (D1, D2, D3, and D5), with the D1 and D2 receptors being expressed at the highest levels (Fig. 1A) [8].

The signaling parameters of most G protein-coupled receptors (GPCRs), including the D1-D5 dopamine (DA) receptors, are fine-tuned to a significant extent by a conserved desensitization mechanism that is orchestrated by GPCR kinases (GRKs) (Fig. 1B) [29]. Ligand-activated DA receptors are phosphorylated by one or several GRKs. They are then bound by multifunctional scaffolding arrestin proteins that specifically recognize active

phosphorylated GPCRs [30]. These proteins block (“arrest”) further activation of G proteins by competing for overlapping binding sites on the receptor [31]. Importantly, when an arrestin binds to a receptor, it also initiates GPCR internalization and other events ultimately leading to the receptor recycling or downregulation [32]. This mechanism controls the intensity of GPCR signaling to ensure that GPCR appropriately modulates G protein activation, thereby preventing G protein over-activation. Furthermore, when arrestin binds to a receptor, it initiates an additional wave of G protein-independent signaling by scaffolding multiple signaling partner proteins [33]. The number of signaling pathways regulated via arrestin-dependent scaffolding continues to grow [33] and includes such critical signaling pathways as MAPK kinases and Akt [5,34,35].

The GPCR-mediated signaling is strictly controlled by this desensitization process, and the rate and extent of GPCR desensitization depend on the functional activities of GRKs [36–46]. Because most GPCRs must be phosphorylated by GRKs to allow high-affinity binding with arrestins [32], the termination of G protein activation by arrestin biding is dependent on the GRK activity. Furthermore, GRKs also control arrestin-dependent signaling by modulating the formation of arrestin-dependent signaling complexes that in most cases require arrestin binding to the receptor [47,48].

There are seven GRKs in mammals classified into 3 subfamilies. Four of these, including GRK2 and GRK3 belonging to the GRK2 subfamily (Fig. 1C), and GRK5, and GRK6 belonging to the GRK4 subfamily, are generally ubiquitous, and these proteins are responsible for regulating hundreds of known GPCR subtypes [49]. The striatal neurons express GRKs 2, 3, 5, and 6, albeit the expression levels and the cellular and subcellular distribution of these GRK isoforms vary substantially (Fig. 1A) [50–54]. It has been reported that phosphorylation of a receptor by different GRKs triggers differential downstream events leading to the receptor internalization or initiation of arrestin-mediated signaling [47,48]. It was hypothesized that each GRK isoform preferentially phosphorylates a limited number of distinct residues, and this specific set of phosphorylated residues (termed “barcode”) recognized by arrestins upon their binding to the phosphorylated receptor induces unique arrestin conformation and, consequently, specific signaling outcomes [55]. This idea is known as the “barcode hypothesis” [56,57]. So far, no data exists to demonstrate whether or how the DA receptor subtypes are “barcoded” by GRK-dependent phosphorylation. However, sheer abundance of potential phosphorylation sites in DA receptors suggests a possibility of such scenario.

Therefore, it is necessary to explore which GRK isoforms are capable of phosphorylating specific DA receptor subtypes *in vitro* and whether such phosphorylation brings about distinct functional consequences. Furthermore, the functional significance of this regulation should be demonstrated in the *in vivo* studies. While these topics are related, they are not identical. In living tissues, the activity of GRKs depends not only on their biochemical specificity but also on their level of expression and subcellular distribution. Therefore, demonstrating their biochemical activity in the *in vitro* experiments is necessary but not sufficient to show that a specific GRK plays a role in regulating a particular DA receptor *in vivo*.

2. Regulation of dopamine receptors by GRKs in heterologous expression systems

2.1. GRK-mediated phosphorylation of dopamine receptor subtypes

Even when using *in vitro* experimental conditions, which allow the simplest biochemical analysis, determination of the specificity of GRK-mediated DA receptor phosphorylation has proven to be non-trivial. The first such study focused on the ability of GRK2, GRK3, and GRK5 to regulate the heterologously expressed rat D1 DA receptor (called D1A at the time of its discovery to distinguish it from another G_s-coupled DA subtype, D1B; later renamed D5) in HEK293 cells [58]. Notably, co-expression of all of these GRKs induced the D1 receptor phosphorylation to a similar extent by preferentially phosphorylating serine residues of the receptor. However, the functional effects of this phosphorylation on cAMP production were found to be quite different. All of the GRKs shifted the DA dose-response curve to the right toward higher DA concentrations. However, while GRK2/3 did not affect the maximum response, GRK5 reduced it by ~40% [58]. Another study focused on the sites on the D1 receptor that were not targeted by GRKs but instead targeted by cAMP-activated protein kinase A (PKA). The authors analyzed the effects of removing these sites by mutagenesis on the rate of desensitization of the D1-mediated activation of adenylyl cyclase [59]. Among the four known potential PKA phosphorylation sites, Thr268 was identified as the most critical. Importantly, removing the PKA sites slowed desensitization to dopamine, but did not affect the rate of receptor internalization [59]. It was therefore concluded that PKA phosphorylation affects the D1 coupling to G proteins but does not promote its endocytosis, which is likely controlled by GRK phosphorylation and subsequent arrestin binding [59].

One interesting model that has been proposed to describe the role of GRK-mediated phosphorylation of the D1 receptor was based on a detailed mutagenesis study that included the progressive truncation of the C-terminus and the elimination of putative phosphorylation sites in the third cytoplasmic loop (i3) [60]. Most importantly, this investigation revealed that while the full deletion of the C-terminus, including all of its Ser/Thr residues, completely blocked D1 phosphorylation, it did not prevent desensitization, arrestin recruitment, or the endocytosis of the receptor. The elimination of putative phosphorylation sites from i3 reduced phosphorylation and significantly impaired desensitization and arrestin recruitment to the receptor [60]. These observations indicate that the phosphorylation of the D1 C-terminus and i3 likely results in the unmasking of the arrestin binding site. Thus, in the D1 receptor lacking the C-terminus, unmasking is not necessary, and arrestin can bind to the receptor even when it has not been phosphorylated [60]. Interestingly, a similar mechanism was previously proposed to describe the regulation of the M2 muscarinic [61] and δ-opioid [62] receptors.

While both the D2 and the D3 receptors couple to members of the Gi subfamily of G proteins, It has been shown that D2 receptors undergo rapid GRK phosphorylation and arrestin binding, which leads to their internalization, while D3 receptors are relatively resistant to this regulation [40]. In these in vitro studies, GRK2, GRK3, and both non-visual arrestins seem to play critical roles during phosphorylation and internalization of D2

receptors [40]. Interestingly, exchanging the i2 and i3 intracellular loops between the D2 and D3 receptors reversed this phenotype, indicating that these elements determine the susceptibility of the receptors to GRK/arrestin-mediated regulation [40].

Another unconventional GRK-mediated regulatory process was found for the D3 dopamine receptor, which acts in part as a presynaptic autoreceptor in dopaminergic cell bodies and terminals [47]. It has been reported that the phosphorylation of the D3 receptor by GRK2/3 disrupts the interaction between the D3 receptor and filamin, which appeared to control the localization of D3 dopamine receptors in filamin-rich lipid rafts enriched with cognate G proteins. Notably, arrestin-3 (a.k.a. β – arrestin-2) also seems to be localized near D3 receptor-expressing sites because of its direct interaction with filamin [47]. Thus, it seems likely that phosphorylation of D3 receptors by GRK2/3 desensitizes the D3 receptors by breaking their association with filamin and thereby preventing its localization to the G protein-rich subcellular compartments. The interaction between GRK-phosphorylated D3 receptors and arrestin might play a relatively secondary role in the regulation of these receptors [47].

2.2. Selectivity of GRK isoforms for active dopamine receptors

It is commonly accepted that GRKs are able to selectively phosphorylate active GPCRs (reviewed in [49]). This mode of activity has been strongly supported by multiple studies, including those that focused on determining the molecular mechanism of rhodopsin kinase (GRK1) activation [63]. These studies have convincingly demonstrated that GRK1 physically binds to light-activated rhodopsin and that this interaction increases its enzymatic activity [63]. Importantly, the D1 dopamine receptor was the first GPCR, for which this model was shown not to be universal [64]. It has been demonstrated that the α isoform of GRK4 (but not the other three splice variants of this GRK) constitutively phosphorylates the D1 dopamine receptor at two sites (Thr428 and Ser431) in its C-terminus. This phosphorylation reduces the responsiveness of the D1 receptor and facilitates its endocytosis from the plasma membrane. Both of these effects were fully abolished by the elimination of these phosphorylation sites in the T428V + S431A mutant [64]. It is therefore likely that at least some GPCRs are directly regulated by the complement of GRKs that is expressed in the cell [64]. Two splice variants of GRK4, GRK4α and GRK4γ, can phosphorylate another dopamine receptor subtype, D3, only in an activation-dependent manner [65]. Eliminating GRK4 using RNAi prevented ERK1/2 activation and a mitogenic response to dopamine [65], indicating that these responses are likely mediated by arrestin proteins that associate with phosphorylated D3 dopamine receptors.

It has been shown that GRK2 and GRK3, but not other GRKs, are able to phosphorylate the agonist-activated D2 receptor by targeting eight Ser/Thr residues in its i3 loop [66]. Interestingly, the mutational elimination of all of these GRK targets prevented D2 phosphorylation but did not affect the receptor’s association with arrestin-3 or its internalization [66]. Thus, similar to the D1 dopamine receptor [60], the phosphorylation of the D2 receptor by GRKs is not required to form an arrestin-3 binding site, but it does allow arrestin access to a site that is created by the activation of the receptor [66]. Intriguingly, unphosphorylated D2 receptors are generally degraded following internalization, whereas

phosphorylated wild type D2 receptors are recycled. Thus, in the particular case of the regulation of the D2 dopamine receptor, phosphorylation by GRKs appears to be critical for its post-endocytic trafficking [66]. Another study that focused on the regulation of D2 receptors also concluded that agonist-dependent GRK phosphorylation of the D2 receptor and its arrestin-dependent internalization played more important role in receptor resensitization than in its desensitization [67]. The authors found that phosphorylated D2 internalized in complex with arrestin recycles, and therefore resensitizes, whereas unphosphorylated D2 internalized via arrestin-independent mechanism does not recycle and resensitize [67].

Recently, the results of a systematic analysis of the phosphorylation of six different GPCRs, including two dopamine receptor subtypes, by four ubiquitously expressed receptor kinases, including GRK2, GRK3, GRK5, and GRK6, were published. This investigation demonstrated a lack of a universal rule to describe the dependence of receptor phosphorylation on the receptor activation state [68]. In most cases, GRK2/3 were specifically selective for active GPCRs, whereas GRK5 and GRK6, which belong to the GRK4 subfamily [49], phosphorylated both active and inactive receptors [68]. The D1 dopamine receptor demonstrated a higher dependence on activation than other GPCRs, but GRK4α similarly phosphorylated both the active and inactive form of the D1 receptor [64]. The other members of the GRK4 subfamily, GRK5 and GRK6, like GRK2 and GRK3, preferentially phosphorylated agonist-activated D1 receptors [68]. However, the regulation of the D2 receptor was found to be somewhat different: it was phosphorylated in an activation-dependent manner by GRK2 and GRK3, but it was not phosphorylated at all by GRK5 and GRK6 [68], which is in agreement with the previous report [66].

It has been shown that three classes of proteins preferentially bind ligand-activated GPCRs. These include G proteins [69,70], GRKs [63,71,72], and arrestins [73,74]. A growing body of evidence indicates that all three classes of proteins engage the cavity between the GPCR helices that opens on the cytoplasmic side upon receptor activation [75,76]. Apparently, G proteins [77], arrestins [78], and GRKs [79,80] are able to insert an amphipathic α-helix into this cavity. The recent observation that certain synthetic ligands can bias signaling towards either G proteins or arrestins (see [81] and references therein) suggests that the binding of various agonists to the same GPCR can produce very different conformational changes that could lead to preferential engagement with G proteins or arrestins. The fact that there are significant structural differences between G_s-associated β2-adrenergic receptors [76] and arrestin-1-associated rhodopsin [78] supports this model, although additional structural studies on GPCR complexes that include G proteins and arrestins are needed to determine whether these differences reflect differences in the receptors or in their interaction partners.

3. DA receptor regulation by GRKs during psychostimulant drug activity

Investigations into the roles played by GRKs in the regulation of dopamine receptors are facilitated by the opportunity to analyze the behaviors that are induced by drugs that target DA receptors. Experiments in living animals bypass the obvious problems that are associated with inducing the heterologous expression of receptors and GRKs in cells and allow analyses aimed at determining the specificity of GRK regulation based on cell-specific

expression and/or subcellular localization rather than only their biochemical specificity. These *in vivo* studies have convincingly demonstrated that GRKs play important roles in modulating DA-dependent behaviors and indicated that GRK could be novel therapeutic targets in various pathological conditions related to abnormal dopaminergic transmission.

3.1. Psychostimulants and DA neurotransmission

Various psychostimulatory drugs, including cocaine and amphetamines, produce multiple physiological and psychological effects in humans, such as increased psychomotor stimulation, euphoria and exhilaration, reduced fatigue and appetite, increased blood pressure, heart rate and respiration, and improved performance during cognitive and motor tasks [82]. In mice and rats, the systemic administration of psychostimulants induces a sharp elevation in locomotor activity. Psychostimulatory drugs are known to elevate the extracellular concentration of monoamines (e.g., DA, serotonin and norepinephrine) by blocking their respective plasma membrane transporters (e.g., DAT, SERT and NET) and/or inducing transporter-mediated outward transport. The activity that is primarily responsible for their physiological and behavioral effects is thought to be their effect on the mesolimbic dopaminergic system. Psychostimulants are known to elevate the DA concentration in the nucleus accumbens, a key brain structure in the reward system that receives dopaminergic projections from neurons that are located in the ventral tegmental area [83,84]. The dramatic increases in extracellular dopamine levels that are induced by psychostimulants result in the enhanced stimulation of DA receptors, which manifests as psychomotor agitation that is reflected in rodents as elevated locomotor activity.

These dopaminergic projections transmit dopaminergic signaling largely to medium-spiny GABAergic neurons in the striatal area, including the nucleus accumbens (Fig. 2A). Based on the anatomical structures that these output striatal neurons project to, they are classified as striatonigral (projecting to the substantia nigra reticulata) or striatopallidal (projecting to the external globus pallidus) neurons. The D1-like and D2-like DA receptors are the main subtypes that are expressed by the output neurons in the striatum and nucleus accumbens. In the caudate-putamen region (Figs. 1 and 2A), the D1 and D2 subtypes are generally segregated: D1 receptors are expressed predominantly in striatonigral neurons, while D2 receptors are expressed primarily in the striatopallidal neurons, and there is little overlap in the expression of these subtypes in striatal neurons [85–92]. It has been shown that in the nucleus accumbens, the concentrations of D1 and D2 receptors are lower than their concentrations in the caudate-putamen, and the number of cells with co-expression of these receptors is somewhat higher [91–93]. The third DA receptor subtype that is expressed in the nucleus accumbens is the D3 receptor, which is particularly abundant in its shell region. These receptors are localized both presynaptically and postsynaptically, where they generally co-express with D1 receptors [94,95]. Large cholinergic interneurons, which exert a powerful influence over striatal functions in general, and striatal output neurons [96–98] express both D2 and D5 receptors [91,99–101]. As the major receptor subtypes that are expressed in the nucleus accumbens and striatum, D1 and D2 dopamine receptors play a critical role in mediating the psychomotor effects of psychostimulants [102,103]. Because GRKs are known to effectively regulate dopamine receptors *in vitro* (reviewed in [104,105]), several studies have focused on evaluating the roles of particular GRKs in regulating

dopamine receptor responsiveness *in vivo* using simple assessments of behavioral responses to psychostimulants, such as cocaine and amphetamine, in various strains of mutant mice that lack individual GRKs.

3.2. GRK2-mediated regulation and behavioral effects of psychostimulants

GRK2 is ubiquitously expressed in multiple brain areas, including major dopaminergic areas [106] (Figs. 1 and 2A), and it has been shown to regulate D1, D2, and D3 dopamine receptors in cultured cells [40,58,107–110]. Alterations in striatal GRK2 levels following chronic exposure to cocaine have also been reported [111]. Mice with global GRK2 deficiency have been developed, but the homozygous mice in this line were unfortunately not viable because of embryonic lethality that was caused by cardiac hypoplasia [112]. Thus, only the surviving heterozygous GRK2+/− mice were initially used to investigate the role of GRK2 in the regulation of dopamine receptors in vivo [104]. These studies revealed that the locomotor effects of the psychostimulatory drug amphetamine and the D1/D2 dopamine agonist apomorphine, were not altered in heterozygous GRK2+/− mice. However, when the mice were treated with cocaine in a narrow range of doses, they exhibited enhanced locomotor activity, indicating that partial GRK2 deficiency is sufficient to cause changes in the functions of dopamine receptors [104]. The fact that such enhancement was observed only at certain doses suggests that the regulation of dopamine receptors by GRK2 might be a complex and intricate process and that it might be different in various cellular groups. Recently, mice were developed in which GRK2 was deleted in a cell-specific manner in targeted groups of striatal neurons. The results of these studies demonstrated the strictly organized role of GRK2 during the control of dopamine receptors and the related activities of psychostimulants. Several strains of mice with cell-specific deficiencies in GRK2 have been developed using the Cre-lox-mediated conditional transgenic deletion of this kinase under the control of cell-specific promoters, and these lines have been analyzed to determine their responses to psychostimulants [113,114].

The following mice were used in these studies: mice lacking GRK2 selectively in cholinergic interneurons (ChATcreGrk2f/f mice) (Fig. 2B), D1 dopamine-expressing striatal direct pathway output neurons (D1RcreGrk2f/f mice) (Fig. 2C), DAT-expressing dopaminergic neurons (DATcreGrk2f/f mice) (Fig. 2D), D2 dopamine receptor-expressing presynaptic dopaminergic neurons and striatal indirect pathway output neurons (D2RcreGrk2f/f mice) (Fig. 2E), or adenosine A2A receptor-expressing striatal indirect pathway output neurons (the same neurons that express the D2 DA receptor) (A2AcreGrk2f/f mice) (Fig. 2F). These mutant mice were tested to determine basal and cocaine-induced locomotor activity [113,114]. Interestingly, previous studies have shown that mice that globally lack specific GRKs throughout the body display a very modest phenotype in unchallenged animals. These data suggest that lacking these GRKs has little effect on dopamine-related functions under basal non-stimulated conditions [44,46]. Accordingly, no overt alterations were observed in spontaneous locomotor activity in non-treated mice that lacked GRK2 in either cholinergic [113] or the D1R-expressing neurons [114]. At the same time, selectively deleting GRK2 in the D2 dopamine receptor-expressing neurons and DAT-expressing dopaminergic neurons, but not in postsynaptic A2AR-expressing striatopallidal MSNs, significantly increased basal locomotor activity, suggesting

that the specific loss of GRK2 in dopaminergic neurons was responsible for the hyperactivity phenotype that was observed in D2RcreGrk2f/f mice.

The cocaine-induced locomotor activity remained intact after GRK2 was deleted in the A2AR-expressing striatopallidal MSNs or cholinergic neurons [113], but deleting GRK2 in the D1R- and D2R-expressing neurons resulted in potent effects on acute cocaine-induced locomotion. Mice lacking GRK2 in the D1 DA receptor-containing neurons showed strongly increased hyperlocomotor responses to cocaine, while mice lacking GRK2 in the D2 DA receptor-containing neurons showed the opposite: less hyperactivity following the administration of cocaine. Mice lacking GRK2 selectively in the dopaminergic neurons were also less sensitive to the hyperlocomotor effects of cocaine, indicating that the reduced sensitivity of D2RcreGrk2f/f mice to cocaine was likely caused by the disruption in the regulation of D2 DA autoreceptors that resulted from the loss of GRK2 functions in the dopaminergic neurons.

Locomotor sensitization to the effects of chronic exposure to cocaine was also investigated in all of these mutants. Among the tested strains, only the mice with GRK2 deficiency in the D2R-expressing neurons demonstrated a reduced sensitization response. These results may indicate that the altered regulation of D2 receptors that was caused by lacking GRK2 may have been observed not only in striatal cells but also in other brain areas, such as the medial prefrontal cortex [115], and these non-striatal D2 receptors might be required for the full expression of behavioral sensitization to chronic exposure to cocaine. Furthermore, the role of GRK2 in regulating presynaptic D2 DA autoreceptors has been supported in studies that examined Fast Scan Cyclic Voltammetry measurements in extracellular DA dynamics. Selectively inducing GRK2 deficiency in the D2R-expressing neurons resulted in a significant reduction in evoked striatal DA release, which directly demonstrates that GRK2 deficiency in the D2R-expressing neurons leads to overactivity of presynaptic D2 autoreceptors and that this altered activity likely causes the persistent suppression of DA release amplitudes that was observed in striatal DA terminals. It is likely that enhanced D2 autoreceptor activity may be the result of the impaired GRK2-mediated desensitization of these presynaptic receptors. Importantly, while GRK2 is known to be expressed at a higher level in striatal cholinergic interneurons than in striatal output neurons [53], the selective knockout of GRK2 in cholinergic neurons did not change the effects of acute or chronic exposure to cocaine much [113], indicating a minimal role for GRK2 in regulating dopaminergic responses in these neurons.

3.3. Roles of other GRKs in the effects of psychostimulants

Similar direct assessments of the potential roles of GRK3, GRK4 and GRK5 (Fig. 1C) in regulating DA receptors in mice, in which these GRKs have been globally deleted revealed no alterations in responses to psychostimulants and some other dopaminergic drugs, such as cocaine, amphetamines and apomorphine, indicating limited roles for these GRK isoforms in the regulation of DA receptors [46]. However, the knockout of GRK6 resulted in strongly enhanced striatal D2 DA receptor activity [44], In contrast to GRK2, GRK6 seems to be predominantly implicated in the regulation of postsynaptic D2 DA receptors. GRK6 was found to be one of the most prominently expressed GRKs in the striatum and other

dopaminergic brain areas [116]. In fact, GRK6 was found to be robustly expressed in DA-receptive striatal GABAergic medium spiny neurons, in addition to large cholinergic interneurons in the striatum [44] (Fig. 1A). Mice lacking GRK6 showed significantly enhanced responses to dopaminergic psychostimulatory drugs, such as cocaine and amphetamines, in addition to direct DA agonists [44,117]. In biochemical studies, the increased coupling of striatal D2-like DA receptors with G proteins and a higher proportion of high-affinity D2 receptors were observed in striatal tissues that were obtained from GRK6-KO mice [44,118]. These data suggest that postsynaptic D2 DA receptors are direct physiological targets of GRK6-related processes. Taken together, these observations indicate that multiple GRKs regulate DA receptors *in vivo* and that the regulation of D2 DA receptors by GRK2 and GRK6 seems to be functionally the most important in striatal functions. These two GPCR kinases demonstrated the following defined neuronal specificity in their regulatory roles: GRK2 and GRK6 regulate distinct D2 DA receptor populations, i.e., with the D2 receptors that are localized presynaptically on dopaminergic neurons and terminals being regulated by GRK2 and those on postsynaptic striatal output neurons being regulated by GRK6. The role of GRK2 in the regulation of D1 DA receptors also seems to be functionally important. Thus, GRKs play important roles in the functional regulation of dopamine receptors and the central dopaminergic system in general, and these GRKs (particularly GRK2 and GRK6) should therefore be further explored as potential novel therapeutic targets for DA-related disorders.

4. Role of GRKs in the regulation of DA receptor signaling in parkinson's disease

4.1. Dopamine receptors and signaling in models of parkinson's disease and L-DOPA-induced dyskinesia

The major dopaminergic structure in the brain, the striatum, receives dense dopaminergic innervation, and medium-spiny GABAergic striatal output neurons express high levels of DA receptors. The DA released by midbrain dopaminergic neurons cooperates with frontostriatal glutamatergic input to regulate striatal output via medium-spiny GABAergic neurons, and this process plays an essential role in controlling movement. The degeneration of dopaminergic neurons and the resulting loss of DA in the striatum in Parkinson's disease (PD) causes deregulation of the striatal circuits and motor deficits. In the striatum, DA acts via DA receptors that belong to the superfamily of G protein-coupled receptors. Out of 5 DA receptors, 4 are expressed in the striatum with the D1 and D2 receptor subtypes expressed at the highest level by the striatal medium spiny neurons (Fig. 1A).

PD is a debilitating neurodegenerative disorder caused by the progressive degeneration of nigral dopaminergic neurons that project DA terminals to the striatum. When damage was induced in DA neurons (often unilateral) in rodents using the DA neuron-specific neurotoxin 6-hydroxydopamine (6-OHDA), it caused a deficit in DA and motor abnormalities, and this protocol therefore serves as one of the best available neurotoxic models of PD. Most of the information that is currently available on the signaling alterations that occur in striatal neurons in PD has been gained in studies that have used this model. Treatment of non-human primates with another dopaminergic neurotoxin, 1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine (MPTP), faithfully reproduces essentially all of the major PD symptoms, and this approach is therefore considered the “golden standard” of animal models of PD. However, while it theoretically presents the best possible validity, this model is less practical than hemiparkinsonian rodents for molecular studies. The loss of striatal DA innervation in PD causes complex rearrangements in DA receptor-related intracellular signaling: several pathways in the striatum show strikingly exaggerated responses when stimulated with dopaminergic drugs [87,119–121] because of the supersensitivity of D1 [122,123] and D2 [124,125] DA receptors. The mechanisms that are involved in these aberrant receptor responses during DA depletion remain poorly understood.

The best symptomatic antiparkinsonian drug is the immediate DA precursor L-3,4-dihydroxyphenylalanine (L-DOPA), which is the most effective drug that initially reverses primary motor deficits in PD patients. However, long-term treatment with L-DOPA results in a loss in effectiveness and significant motor abnormalities, including L-DOPA-induced dyskinesia (LID), or abnormal involuntary movements [126–128]. The pathophysiology of LID continues to be investigated in part because of the extreme complexity of the mechanisms that are involved in the intracellular signaling alterations caused by the loss of DA innervation and chronic L-DOPA treatment. In unilaterally 6-OHDA-lesioned rodents, treatment with L-DOPA induces rotations, with the frequency of rotations increasing following repeated administration (commonly defined as behavioral sensitization to L-DOPA) and abnormal involuntary movements (AIMs) following chronic drug treatment. It has been shown that chronic treatment with L-DOPA, in paradigms that ameliorate movement deficits, suppresses the supersensitivity of the ERK pathway that is caused by loss of DA [119,129–132]. At the same time, chronic L-DOPA treatment can augment the lesion-induced supersensitivity of certain pathways and/or further deregulate their activity [119,132,133] in parallel with causing a progressive increase in the frequency of L-DOPA-induced rotations [50,94,119,134] and the development of AIMs [50,135–138]. It has been shown that exaggerated signaling via the striatal D1 [122,133,137], D2 [125], and D3 [94,119,139] DA receptors is involved in the development of LID in rodents and primates.

Because both the antiparkinsonian and the dyskinetic effects of L-DOPA are mediated by DA receptors, the molecular intracellular signaling mechanisms that are involved in these effects are likely interconnected. The exaggerated signaling via DA receptors observed in LID suggests that normalizing excessive signaling may be beneficial. However, the critical challenge is to suppress the abnormal signaling responsible for LID while preserving sufficient dopaminergic activity to support the antiparkinsonian activity of the drug [125,139]. Thus, DA receptor-related intracellular signaling mechanisms, under both normal and pathological conditions, must be explored to enable the selective targeting of the mechanisms that are specifically responsible for LID.

4.2. Effect of DA deficiency and L-DOPA treatment on the expression of GRKs

Among the five GRK isoforms that are known to be expressed in the brain [53,106,129,140,141], striatal neurons express four: GRK2, GRK3, GRK5, and GRK6 (Fig. 1A,C). In the rat striatum, GRK6 is expressed at the highest level, followed by GRK2 [51].

In most striatal subdivisions of the monkey and human brain, GRK5 is the most highly expressed, followed by GRK2 [52,129].

A simple potential mechanism that explains how the loss of DA and/or L-DOPA treatment may alter signaling via GPCRs is the modulation of the availability or activity of GRKs (Fig. 3A,B). In rats with unilateral DA lesions, the concentrations of the GRK6 and GRK3 isoforms are reduced in the dopamine-depleted motor striatum, but L-DOPA fails to alter their expression [50,134]. Although the degree of reduction in GRK6 is not large (25–40%), it could still be highly functionally significant because of the overactivity of DA receptors that is created by the high concentration of DA produced from L-DOPA. Furthermore, heterozygous GRK6 knockout mice that express approximately 50% of normal GRK6 levels display the same supersensitive phenotype as full GRK6-KO mice [44], suggesting that a normal concentration of GRK6 is critically important for the normal functions of DA receptors, particularly under conditions involving dopaminergic overactivity. GRK3 is the only other GRK isoform that is consistently reduced across striatal regions in hemiparkinsonian rats [51,54]. In contrast, GRK2, one of the major GRK isoforms in the rat striatum and the closest relative of GRK3, was not altered by the loss of DA but was up-regulated following chronic L-DOPA treatment [51,54].

In parkinsonian monkeys, the loss of DA innervation following treatment with MPTP leads to the up-regulation of several GRKs [129], suggesting that the tempering of dopaminergic signaling that accompanies early L-DOPA administration may ensure a therapeutic response to the drug. However, chronic L-DOPA treatment suppressed the expression of GRKs [129]. Notably, elevated membrane expression and reduced internalization of D1 receptors was observed in the striatum of dyskinetic monkeys [142], suggesting that LID is associated with deficits in D1 receptor desensitization, internalization and trafficking. Furthermore, a small decrease in the concentrations of GRK2 and GRK5 in striatal regions was observed in human tissues obtained from PD patients [141], indicating the possible loss of GRK function following long-term use of L-DOPA.

Taken together, these data suggest that the reduced availability of GRK caused by either the loss of DA innervation or chronic treatment with L-DOPA might contribute to exaggerated dopaminergic signaling in dyskinetic subjects (Fig. 3B). Alternatively, the defective desensitization that causes DA receptor supersensitivity could result from lowered GRK availability in specific compartments (even without changes in total GRK levels) that is insufficient to meet the demand imposed by the DA surges during the course of chronic L-DOPA therapy. Thus, it seems plausible that increasing the capacity of the DA receptor desensitization machinery in the parkinsonian striatum may be an effective strategy to ameliorate LID.

4.3. GRK-mediated regulation of L-DOPA effects

Recent studies have focused on evaluating the effect of enhancing the capacity of the GRK-mediated desensitization system on the development of LID. This was achieved by virally overexpressing one of the most prominent striatal GRKs that controls DA receptor functions, GRK6 [44,104], in the DA-depleted striatum in hemiparkinsonian rats using lentivirus-mediated gene transfer [50]. It has been shown that lentivirus-mediated overexpression of

GRK6 in the denervated striatum of hemiparkinsonian rats significantly suppressed L-DOPA-induced contralateral rotations and ameliorated AIMs [50] (Fig. 3C). Consistent with these results, experiments that were performed using a lentivirus-mediated miRNA knockdown approach revealed that reducing the availability of GRK6 promoted rotational behavior and increased AIM scores [134]. These results are consistent with observations in GRK6-KO mice, in which the effects of L-DOPA treatment and dopaminergic stimulation were generally increased [44,143].

Two splice variants of GRK6, GRK6A and GRK6B, caused by alternative exon usage and differing in the C-terminus have been described in the rodent [144]. The mRNA transcript of a shorter variant, GRK6C, has also been isolated [145–147], but the corresponding protein has not been detected in the brain. GRK6A variant is the most studied and possesses C-terminal palimitoylation residues as well as amphipathic helix, the structures characteristic of the GRK4 subfamily, mediating semi-permanent membrane association of these kinases [49,145,146]. GRK6B lacks these structural elements, and it remains unclear whether or how it is recruited to the membrane to be able to interact with the receptors [145]. Importantly, although both GRK6A and GRK6B seem to be reduced by DA depletion, GRK6A is most affected by the lesion. Inducing the miRNA-mediated knockdown of GRK6 further exacerbated the decrease in the GRK6 expression that was observed in the lesioned hemisphere and aggravated the behavioral consequences of DA deficiency and chronic L-DOPA treatment. These findings support a role for deficient GRK6 functionality in the development of LID. Notably, it has been observed that lesions reduced the GRK6 concentration by approximately 40% and that lentiviral knockdown further reduced the level of expression by 36% to 40%, whereas overexpression approximately doubled the concentration of GRK6. Thus, even modest modulations of the GRK6 concentration seem to have a critical impact on dopaminergic signaling and dopamine-dependent behavior, as previously indicated in studies involving GRK6 heterozygous mice [44]. These data reveal important functional roles for GRK6, especially in the signaling mechanisms that underlie the development of LID (Fig. 3B).

In other experiments, the effect of up-regulating GRK6 on the manifestations of LID weas explored in parkinsonian monkeys. The monkeys were bilaterally lesioned with MPTP and then chronically treated with L-DOPA until they developed LID. Then, they were given injections of a lentivirus encoding human GRK6 into the putamen. Upon recovery, the animals were tested for LID and for the antiparkinsonian effects of L-DOPA. It was found that restoring GRK6 levels in the striatum led to the strong amelioration of LID while essentially completely preserving the therapeutic antiparkinsonian activity of L-DOPA. In fact, the therapeutic effects of L-DOPA were even extended in duration in animals that overexpressed GRK6 compared to their duration in control monkeys. Furthermore, in GRK6-expressing monkeys, even half of the L-DOPA dose was as therapeutically effective as the full dose in control animals, and it did not cause any LID. In contrast, following the administration of a halved dose of L-DOPA, the control animals also showed reduced LID, albeit to a lesser extent than was observed in the GRK6-expressing monkeys, but that was at the expense of a greatly reduced therapeutic antiparkinsonian effect [50]. Thus, promoting GPCR desensitization in the DA-depleted striatum via the viral overexpression of GRK6 attenuated LID in both primate and rodent models without significantly compromising the

antiparkinsonian activity of L-DOPA. In fact, GRK6 overexpression prolonged the duration of antiparkinsonian effect, especially in animals administered the lower L-DOPA dose, without promoting LID. Collectively, these data from rodents and monkeys strongly indicate that strategies aimed at increasing the availability or function of GRK6 may help to control LID without sacrificing the antiparkinsonian benefits of L-DOPA.

In addition to a kinase domain, GRKs are known to have a RGS homology (RH) domain [148]. Notably, in all GRKs, the kinase domain is inserted within the structure of the RH domain (Fig. 1C). The regulator of G protein signaling (RGS) family of proteins characterized by the presence of RH domain are thought to be critical regulators of GPCR signaling and to play important roles in a variety of physiological and pathological processes [149,150]. RGS proteins acts as GTPase accelerating proteins (GAPs) for Gα subunit of G proteins enhancing its intrinsic GTPase activity and increasing the rate of GTP hydrolysis, thereby promoting G protein deactivation [149–151]. While it was originally identified *in silico* [148], the RH domain of GRKs was later shown to be functionally active in cultured cells [152–157]. The RGS proteins of several subfamilies interact with Gα of either the G_i family (G_i, G_o) inhibiting adenylyl cyclase, such as, for example, striatum-enriched RGS9, a member of the R7 subfamily [150]. Others interact with members of the G_q (G_q, G_{11}) (activating phospholipase C) or G_{12} families (Rho activation, stress fiber formation) [22,158], and some members of the R4 subfamily can interact with G proteins of both G_i and G_q classes [150]. It is important to note that interaction of RGS proteins with individual members of G proteins classes have rarely been tested, and the information is limited to the most common representatives of each G protein class.

The GRK RH domain interacts with Gα of all members of the Gq family (G_q, G_{11}, G_{14}) except $G_{15/16}$ [G_{15} is murine and G_{16} is the human orthologue [158]] [153]. Of the four members of the G_q family, G_q and G_{11} are expressed in the central nervous system almost ubiquitously [159] and show high functional redundancy [160]. It is unknown whether G_{14} is expressed in the striatum. It is important to note that the RH of GRKs possesses almost no GAP activity, in contrast to “conventional” RGS proteins such as most members of the R4 or R7 subfamilies. Instead, RH of GRKs sequesters active Gα thereby reducing signaling via $G_{q/11}$-coupled GPCRs in cultured cells [152–156,161,162]. However, this activity has not been demonstrated under *in vivo* conditions. Thus, GRK2/3 likely suppresses GPCR signaling through two independent mechanisms: via receptor phosphorylation and by scavenging active $G_{\alpha q/11}$.

Because GRK3 is known to be down-regulated in the denervated striatum in hemiparkinsonian rats, the effect of overexpressing GRK3 on L-DOPA-induced rotations and AIMs was investigated, and potent inhibitory activity was observed [54]. If GRK3 activity were mediated by DA receptor phosphorylation, it might result from enhanced DA receptor desensitization or potentiated arrestin-mediated signaling following arrestin recruitment to phosphorylated receptors [47,48]. However, unlike GRK6, which is known to act via DA receptor phosphorylation, the effect of GRK3 has been shown to be phosphorylation-independent, because kinase-dead mutant GRK3 was shown to be fully active [54]. In contrast, the over-expression of a GRK3 construct containing an inactivated RH domain but with all other domains functionally intact (GRK3-RHD) did not alter L-

DOPA-induced rotations or LID, indicating that a functional RH domain is selectively responsible for the anti-LID activity of GRK3. Furthermore, overexpressing the construct containing isolated RH domain that lacked other domains recapitulated the effects of full-length GRK3, further demonstrating the critical role of the RH domain in GRK3 activity in the DA system and its anti-LID effect [54] (Fig. 3C). In fact, the predominant phosphorylation-mediated activity of GRK6 on DA receptors might result from the fact that the GRK6 RH domain contains a shorter α5 helix than is present in the RH domain in GRK2 and GRK3, which are capable of binding $G_{\alpha q/11}$ [151,153]. Thus, GRK6 lacks structural elements that are required for $G_{\alpha q/11}$ binding [152,155,156].

4.4. Receptor specificity in GRK-mediated control of LID

Previous work with knockout mice has demonstrated that the behavioral supersensitivity to psychostimulants and dopaminergic agents that is caused by GRK6 deficiency results from the altered regulation of the D2 but not the D1 DA receptor [44]. However, protracted DA deficiency and the subsequent development of LID during the course of chronic L-DOPA treatment cause multiple dramatic changes in striatal signaling pathways [123,163]. Although both receptor subtypes are involved in LID development, the role of the D1 receptor seems to be the most prominent [122,142,164]. Deleting the D1, but not the D2 receptor, abrogated both L-DOPA-induced rotations and AIMs [165], indicating that only the D1 receptor is indispensable for LID and L-DOPA responses in general. Thus, it might be expected that the anti-dyskinetic activity of GRK6 involves D1 receptors or both D1 and D2 DA receptor subtypes. In fact, GRK6 overexpression reduced the LID that was caused by both selective D1 and D2 agonists in parkinsonian monkeys, indicating that signaling via both receptor subtypes was involved. It should be noted that the effect of GRK6 overexpression is qualitatively different from those in previous similar studies of RGS9-2 [125], in which alterations were mediated by only D2 receptors and not D1 receptors [149]. Inducing GRK6 overexpression in the denervated striatum of hemiparkinsonian rats promoted the internalization of D1 receptors and suppressed the L-DOPA-induced up-regulation of prodynorphin and D3 dopamine receptors, effects that are believed to be related to enhanced D1 receptor signaling [94]. When similar manipulations were performed in dyskinetic monkeys, they also reduced prodynorphin expression. While no increase in D2 receptor internalization was observed, GRK6 overexpression reduced the up-regulation of pre-proenkephalin mRNA, which is normally expressed in D2 receptor-containing neurons [87,88,166].

It should be mentioned that multiple studies that have used selective D1 and D2 agonists have demonstrated functional cooperation between these receptor systems. In several experimental paradigms, a ***synergism*** between the D1 and D2 receptors (a phenomenon in which the concomitant administration of a D1 and a D2 agonist produced a much more noticeable effect than administration of either drug alone) has been documented. This synergism, in addition to ***cross-priming effects*** (a phenomenon in which the response to an agonist of one subtype of DA receptor is enhanced by pre-treatment with an agonist of another subtype of DA receptor) between D1 and D2 DA receptors, strongly complicates our understanding of their relative roles and our approaches to pharmacological interventions (reviewed in Refs. [8,9]). The mechanisms that underlie these phenomena remain unknown,

but they likely involve indirect glutamate transmission [167–169]. While it is likely that GRK6 overexpression alters D1 receptor desensitization, as shown by alterations in D1 receptor trafficking, it is possible that the behavioral changes caused by GRK6 following administration of the D2 selective agonist were the result of indirect circuitry mechanisms, because no change was observed in D2 receptor trafficking [50]. Further experiments using neuron-selective manipulations of GRK6 expression and/or activity are necessary to clarify this issue.

In animals that were treated with selective D1 or D2/D3 agonists, GRK6 overexpression not only suppressed LID but also shortened the overall duration of antiparkinsonian activity. These effects were likely caused by faster receptor desensitization, which resulted from increased GRK6 availability. In GRK6-expressing monkeys, however, L-DOPA-induced antiparkinsonian effects lasted longer than their duration in control animals. It is known that GPCRs must be activated before they can be desensitized by GRK-mediated phosphorylation [170]. It is possible that these initial signaling events are sufficient for the antiparkinsonian effect but not for LID. While GRK6 is able to phosphorylate many GPCRs, even in an inactive state, it has shown a high preference for active D1 receptors over inactive D1 receptors [68]. Because the effects of L-DOPA likely arise from its simultaneous activity at both D1 and D2 receptors, the up-regulation of GRK6 might shift the balance in favor of D2 receptors, because they generally do not desensitize as readily as D1 receptors [40,58]. In fact, in monkeys, GRK6 had only a modest effect on the duration of D2-mediated effects, whereas it substantially shortened the effects of a D1 agonist. This rebalancing of D1 and D2 receptor-mediated functions and activities and the corresponding alterations in the activity of the direct and indirect pathways might contribute to extended antiparkinsonian benefits of administering L-DOPA following GRK6 overexpression.

Work with GRK6 knockout mice has demonstrated that GRK6 is the key isoform that regulates striatal signaling via DA receptors [44]. More recent studies have supported this conclusion, although they have emphasized that GRK6 plays roles in regulating both D1 receptors and D2 receptors [50]. In regard to GRK3, the situation is quite different. The canonical belief is that neither D1 nor D2 signaling involves $G_{q/11}$. However, several lines of evidence have suggested that D1R can couple with G_q as an alternative to $G_{s/olf}$ [93,171–183]. Recent in vivo studies have demonstrated that the striatal D1 receptors that are located on striatonigral neurons and the D5 receptors located on cholinergic interneurons couple with G_q in native tissues [184,185]. Furthermore, potential D1R/D2R heterodimers were reported to couple preferentially with G_q [90,93]. However, it should be noted that dimer formation requires D1/D2 receptor co-expression within the same neuron, and the currently available evidence indicates that only a small proportion (5–7%) of neurons in the dorsal striatum co-express both receptor subtypes [91–93,178,186–188]. In addition, the existence of D1/D2R dimers in native striatal tissues has recently been questioned [189]. Thus, further studies are necessary to understand the nature of GRK3-related $G_{q/11}$ signaling in LID. Because RH can directly bind to active $G_{\alpha q/11}$, this activity does not necessarily require GRK3 to bind to a DA or any other particular receptor. GRK3 could sequester, via its RH domain, the active $G_{\alpha q/11}$ that is generated by the activation of a number of non-DA $G_{q/11}$-coupled receptors expressed in striatal neurons. For example, active $G_{\alpha q/11}$ can be generated by Group I mGluRs [190–193], 5-HT2A receptors [194] and M1 muscarinic receptors

[195,196]. Several studies show that drugs that target these receptors can exert antiparkinsonian effects and/or modulate L-DOPA-induced behaviors and LID [197,198] [196,199].

4.5. GRK-dependent signaling mechanisms in the parkinsonian brain

One attractive hypothesis suggests that multiple signaling abnormalities in the DA-depleted striatum might result from inadequate desensitization of DA receptors, which is then even further deregulated by chronic L-DOPA treatment. Abnormal receptor desensitization may in part result from the reduced expression of GRK isoforms in DA-depleted striatum and the fact that GRK levels are not normalized by L-DOPA [134]. As discussed above, GRK6 overexpression directly affects desensitization of DA receptors via a conventional phosphorylation mechanism, whereas GRK3 likely acts via its RH domain to facilitate the inactivation of active $G_{\alpha q/11}$, like many other RGS proteins. Thus, it is possible that the elevated expression of GRKs improves motor functions by rescuing deficient desensitization of DA receptors and, as a result, normalizing abnormal signaling via multiple pathways.

Several signaling pathways that are not activated by DA (or L-DOPA) in normal animals can be strongly activated by L-DOPA or other dopaminergic agonists under conditions involving DA deficiency. Following DA depletion, the supersensitivity of the MAP kinase ERK pathway to dopaminergic stimulation has been documented by several laboratories, and elevated ERK activity has been linked to the development of LID [87,119,120,129,132,200,201]. However, it has been shown that chronic treatment with L-DOPA desensitizes the ERK response, suggesting that high ERK activity is associated with a priming effect rather than with the development of LID *per se* [87,119,129,132]. Because priming is an important component of LID pathophysiology, strategies aimed at reducing ERK hyperactivity may have an anti-LID effect. Thus, the successful amelioration of LID was achieved by controlling the activity of the neuron-specific ERK1/2 activator Ras-GRF1 [202]. It has also been demonstrated that overexpressing GRK6 in the DA-depleted striata significantly suppressed ERK1/2 activation in response to L-DOPA. We also found, in agreement with previous reports, that the effect of GRK6 was most apparent in drug-naïve animals but that it diminished with chronic L-DOPA treatment, which is known to reduce the degree of ERK supersensitivity to L-DOPA [119,129–132].

While there is evidence showing that the p38 pathway may be involved in neuronal death in PD, the role of this pathway in the development of LID has not been previously appreciated [203]. Elevated basal p38 kinase activity was observed in lesioned striatum. Similar to ERK1/2, these cells were rendered supersensitive to L-DOPA challenge. In the DA-depleted striatal tissues obtained from saline-treated animals, similarly elevated levels of phosphop38 and total p38 were detected. In L-DOPA-treated animals, the ratio of phospho-p38 to total p38 was elevated to a comparable degree in saline- and L-DOPA-challenged rats, suggesting that following chronic L-DOPA treatment the increased activity of the p38 pathway was not affected by acute challenge with L-DOPA. Thus, elevated p38 activity may be related not only to peak-dose LID, as is the case for ERK1/2, but it may also be important for the dyskinetic state itself. Importantly, in contrast to ERK regulation, chronic L-DOPA treatment did not desensitize the p38 response to L-DOPA challenge. Overexpressing GRK6

also reduced the level of p38 supersensitivity across all experimental groups and reduced its basal activity in L-DOPA-treated rats.

It has been shown that DA negatively regulates Akt activity via D2 receptors and arrestin-3 (a.k.a. beta-arrestin-2) mediated mechanisms [4,5,204]. Furthermore, it has been shown that the loss of DA in the striatum causes a sustained elevation in the activity of the Akt pathway in response to dopaminergic stimulation that was not sensitive to chronic L-DOPA treatment [119]. The hyperphosphorylation of Akt (at the major activating residue Thr^{308}) that resulted from L-DOPA challenge in both drug-naïve and L-DOPA-pretreated rats was ameliorated by overexpression of GRK6, as was the case for both ERK1/2 and p38 activation. Taken together, these data demonstrate that overexpressing GRK6 in the lesioned striatum likely facilitates the desensitization of DA receptors and partially normalizes signaling via at least three intracellular pathways, including ERK, p38, and Akt, all of which became supersensitive to dopaminergic stimulation upon DA depletion.

DA stimulation in DA-depleted striatal tissues enhanced ERK activity, and this effect is believed to be related to supersensitivity of the D1 DA receptor [87,120,205,206]. It is likely that the overexpression of GRK6 reduced ERK activation by L-DOPA by facilitating D1 receptor desensitization, which normalized D1 intracellular trafficking and regulation [50]. While sensitization of the p38 or Akt pathways has not yet been linked to a specific DA receptor subtype, it is reasonable to conclude that they were also normalized by GRK6 as a result of improved receptor desensitization. While GRKs are known to possess broad receptor specificity (for review see [145]), GRK6 has been shown to preferentially regulate the D2 receptor *in vivo* and to therefore affect Akt signaling [44]. In addition, under DA-depleted conditions, GRK6 can effectively modulate the signaling and trafficking of supersensitive D1 receptors [50]. It should also be noted that there is currently little information regarding the mechanisms by which GRK6 regulates other GPCRs that are expressed by striatal neurons and might be also involved in movement control and/or LID.

Improved GRK-dependent desensitization of DA receptors may also reduce the accumulation of the transcription factor ΔFosB in the lesioned striatum of rats that overexpress GRK6. The accumulation of ΔFosB is known to contribute to the development of LID [207–211], and a lower level of ΔFosB in GRK6-overexpressing rats is therefore consistent with the relatively less-pronounced manifestations of behavioral sensitization and LID observed in these animals. It has been shown that ΔFosB concentrations in lesioned striatum are more dramatically increased in the nuclear fraction, and this accumulation is reduced by GRK6 overexpression. The decreased priming effect of every consecutive L-DOPA dose during the course of chronic treatment may eventually result in the reduced expression of genes that are associated with LID. Interestingly, In GRK6-overexpressing rats, dynorphin and enkephalin mRNA were up-regulated to a lesser degree and the increase in the striatal D3 DA receptor concentration was also less evident [50]. These responses are considered to be biochemical markers of LID [8,9], but their roles in the development of LID have not been identified.

Importantly, no significant alterations in the MAP kinase or Akt signaling pathways were found, but the diminished accumulation of ΔFosB was observed when full-length GRK3 or

the isolated GRK3 RH domain was overexpressed [54]. The effect of GRK3 on the accumulation of ΔFosB was phosphorylation-independent and was mediated via the RH domain of GRK3 [54], which is capable of interacting with $G_{\alpha q/11}$ [153]. It is possible that G_q-mediated signaling could potentially affect ΔFosB accumulation. This could occur via the modulation of either protein kinase C activity [212] or calcium/calmodulin-dependent protein kinase II (CaMKII) activity [213].

4.6. Conclusions

Taken together, these studies, which range from exploring in vitro activity to in vivo responses to psychostimulant and antiparkinsonian drugs in normal and genetically modified animals, convincingly demonstrate that certain GRK isoforms play important roles in regulating DA receptor signaling. In particular, GRK2, GRK3 and GRK6 seem to be the most critical for regulating both the D1-like and the D2-like DA receptors via a number of mechanisms, as shown in cell culture and in experiments using *in vivo* genetic manipulations in animals. Better understanding of the complicated mechanisms involved in these regulatory processes is crucial for determining the pathological mechanisms involved in dopamine-related conditions and disorders. In particular, psychostimulant addiction remains on the rise worldwide, and there is no viable strategy for treating addiction or preventing relapse in psychostimulant drug users. It is likely that GRKs, which are the key regulators of DA receptor signaling, play an essential role in the neuronal adaptations that underlie the transition from persistent drug use to addiction. GRKs could impact addiction processes by affecting, for example, ΔFosB accumulation, which is believed to be involved in cocaine-induced neuroplasticity [214], or by affecting the recruitment of arrestin to receptors and arrestin-dependent signaling, which is known to play an important role in psychostimulant activities [3–5].

Understanding the regulation of GRK-dependent signaling is particularly important in PD and LID, which remains an unmet medical need in the management of PD. In recent years, biochemical investigations have revealed several signaling alterations that are associated with the development of LID (reviewed in Ref. [8,9,136,215]). Among these, the less-pronounced sensitization of the ERK pathway and the reduced accumulation of ΔFosB, as means to ameliorate LID, have received the most attention [202,207,208,211]. However, many other signaling pathways that operate in the striatum have never been investigated with regard to their potential connections with PD or LID. One such example is the involvement of $G_{q/11}$-mediated signaling in LID, which is related to alterations in GRK3 functions that have never been previously explored. Thus, alterations in several other GRK-related signaling pathways may potentially be implicated in LID, and strategies aimed at normalizing these intracellular pathways may achieve sustained anti-LID benefits. Managing signaling at the level of DA receptors by targeting GRKs may be a highly effective strategy for normalizing multiple pathways at the same time instead of separately targeting each pathway.

Finally, GPCRs, including DA receptors, are known to use two general modes of signaling: G protein-dependent and arrestin-dependent [216]. The role of GRKs in regulating arrestin-dependent signaling remains essentially unknown. It would be interesting to determine

whether certain GRK subtypes specifically contribute to one or another mode of signaling or whether the process of GRK-mediated phosphorylation is equally important for both events. Increasing our understanding of such GRK-mediated mechanisms may suggest new approaches for targeting DA-related disorders.

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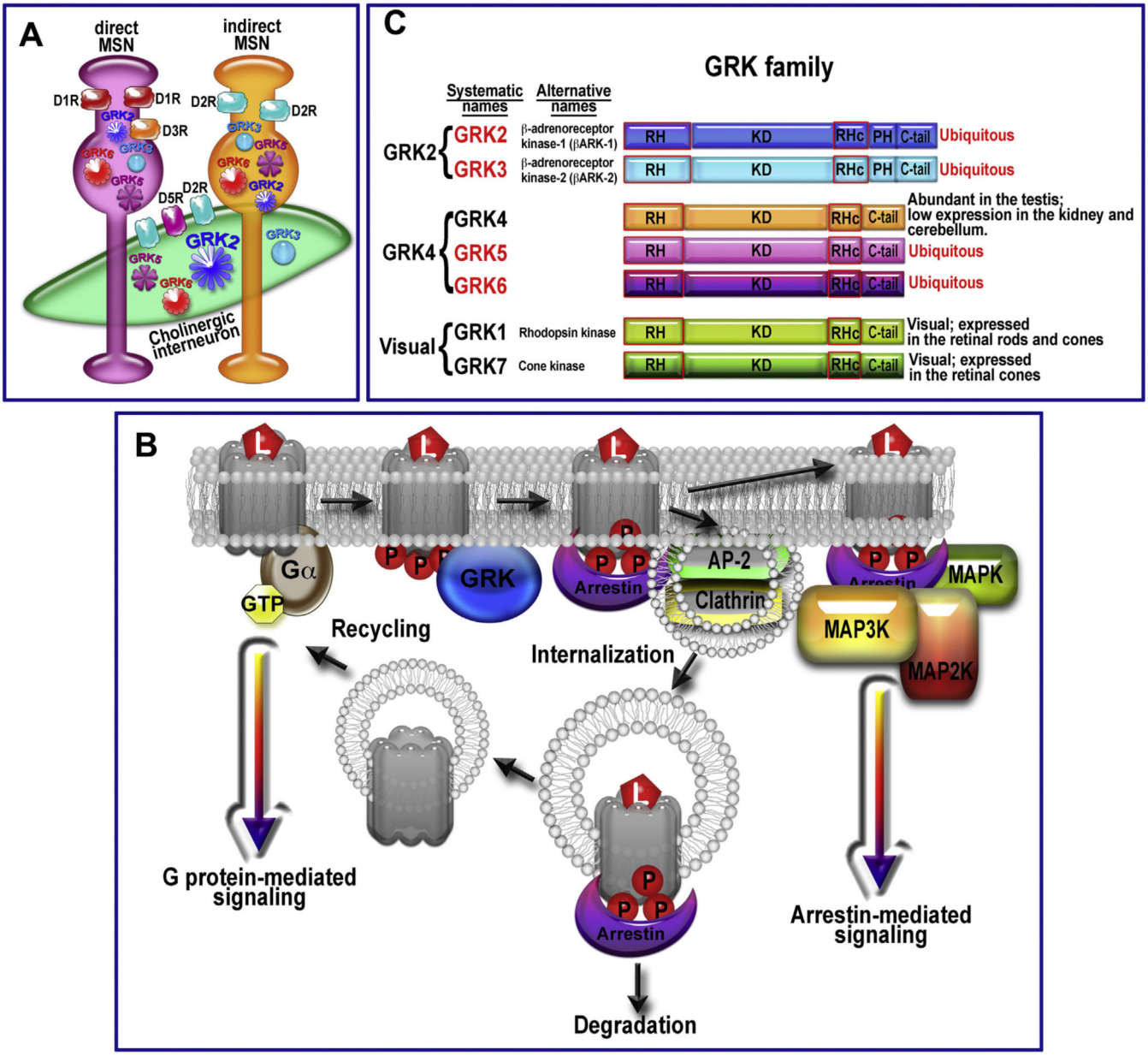

Fig. 1.

GRK isoforms regulating the signaling of the dopamine receptors in the striatum. (**A**) Striatal medium spiny neurons (MSN) express D1, D2, and D3 dopamine receptors. The D1 receptor is selectively expressed by the direct pathway neurons, whereas the D2 receptor is found in the indirect pathway neurons. The D3 receptor co-expresses with the D1 receptor in the direct pathway neurons, at least, in the rodent (in the human, the D3 receptor expression is much higher and it extensively co-localizes with the D2 receptor). Cholinergic interneurons express the D2 and D5 receptors. MSN express four GRK isoforms, 2, 3, 4, and 5, as do cholinergic interneurons. The latter are enriched with GRKs 2 and 3, as compared to MSN, whereas the levels of GRKs 5 and 6 do not differ between MSN and interneurons. (B) Schematic representation of the homologous desensitization mechanism. Liganded receptor

is phosphorylated by a GRK followed by arrestin biding. Arrestin binding precludes further G protein activation and initiates receptor internalization via coated pits by virtue of arrestin interaction with clathrin and AP-2 (adapter protein-2), main components of the coated pit. Following a sorting process, internalized receptor is recycled back to the plasma membrane or sent to lysosomes to be degraded. Arrestin binding to the receptor also promotes the formation of the multi-protein signaling complexes where arrestin acts as a scaffold capable of activating multiple signaling pathways. Here the mitogen activated protein kinase (MAPK) pathway is shown as an example. Arrestin binds the upstream MAPK kinase (MAP3 K), MAPK kinase (MAP2 K) and MAPK promoting their sequential activation due to proximity. (C) The GRK isoforms are classified into three subfamilies: GRK2, GRK4 and visual. The visual subfamily comprising GRKs1 and 7 is largely restricted to the retinal photoreceptors. GRKs are multi-domain proteins with unusual domain structure: their kinase domain (KD) is inserted inside the RGS homology domain (RH), so that the last two helices of RH (RHc) are positioned after KD. GRKs 2 and 3 possess pleckstrin homology domain (PH) that other isoforms lack.

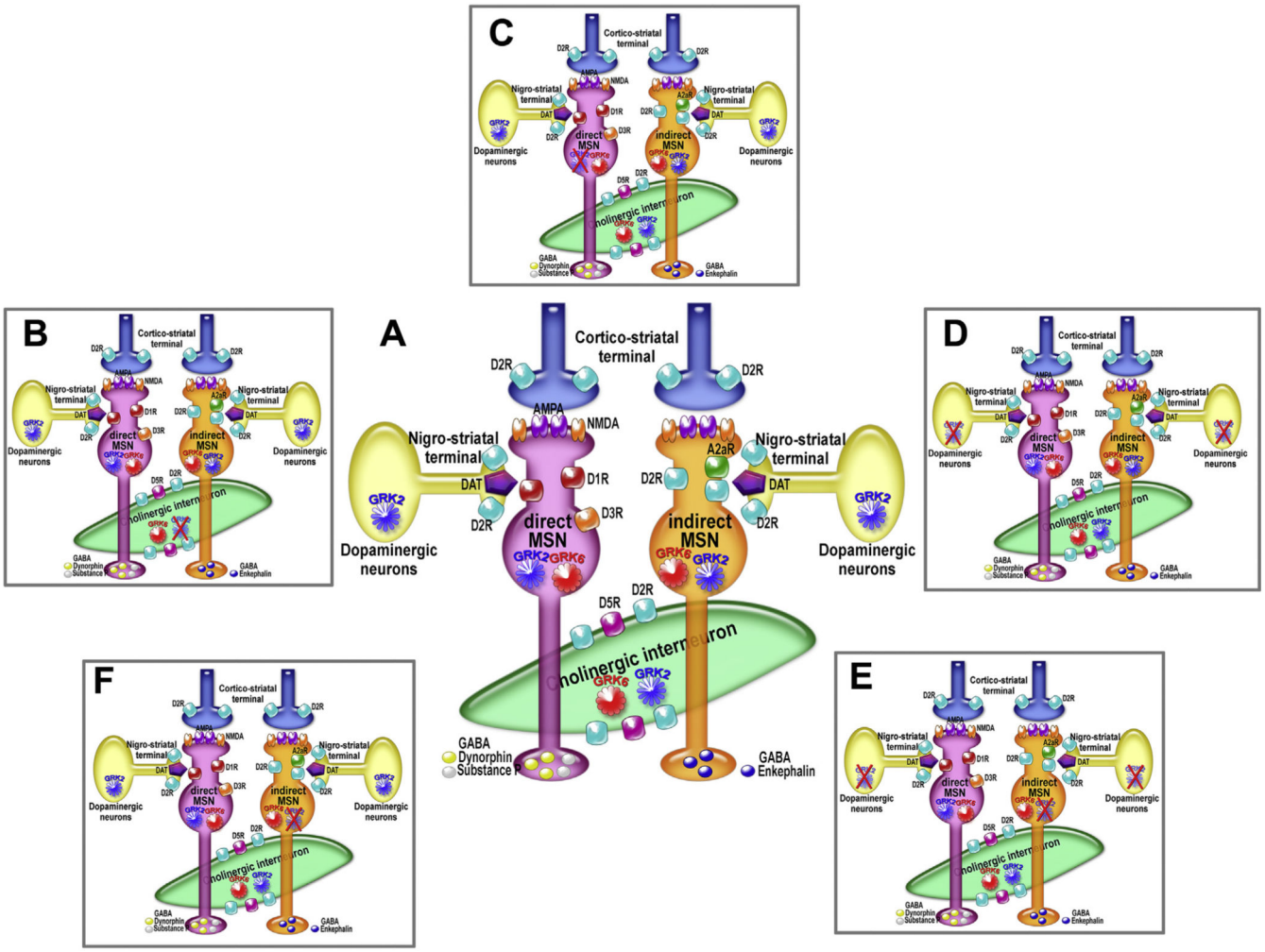

Fig. 2.

Creation of knockout mouse lines lacking GRK isoforms in defined cellular populations in the basal ganglia (A) In the striatum, the D1 and D2 dopamine receptors are largely segregated on the direct (D1-expressing) and indirect (D2-expresing) pathway medium spiny output neurons. D2 receptors are also expressed postsynaptically on cholinergic interneurons and presynaptically – on the nigrostriatal dopaminergic terminals and cortico-striatal glutamatergic terminals. The indirect pathway neurons selectively express the A2 adenosine receptors. GRK2 and GRK6 isoforms are ubiquitous and expressed in medium spiny neurons as well as in cholinergic interneurons. (B) Mice lacking GRK2 selectively in cholinergic interneurons were created using mice with floxed GRK2 gene bred with the strain expressing Cre recombinase under choline acetyltransferate (ChaT) promoter. (C) The use of the strain with Cre expressed under the D1 receptor promoter resulted in the elimination of GRK2 selectively in the direct pathway neurons. (D) The use of the strain with Cre expressed under the dopamine transporter (DAT) receptor promoter lead to the elimination of GRK2 selectively in the dopaminergic neurons of the substantia nigra. (E) When the D2 receptor promoter was used to control Cre expression, GRK2 was knocked out both in dopaminergic nigral neurons and indirect pathway striatal neurons. (F) To knockout

GRK2 selectively in the indirect pathway striatal neurons, the promoter of A2a adenosine receptor was used for Cre expression.

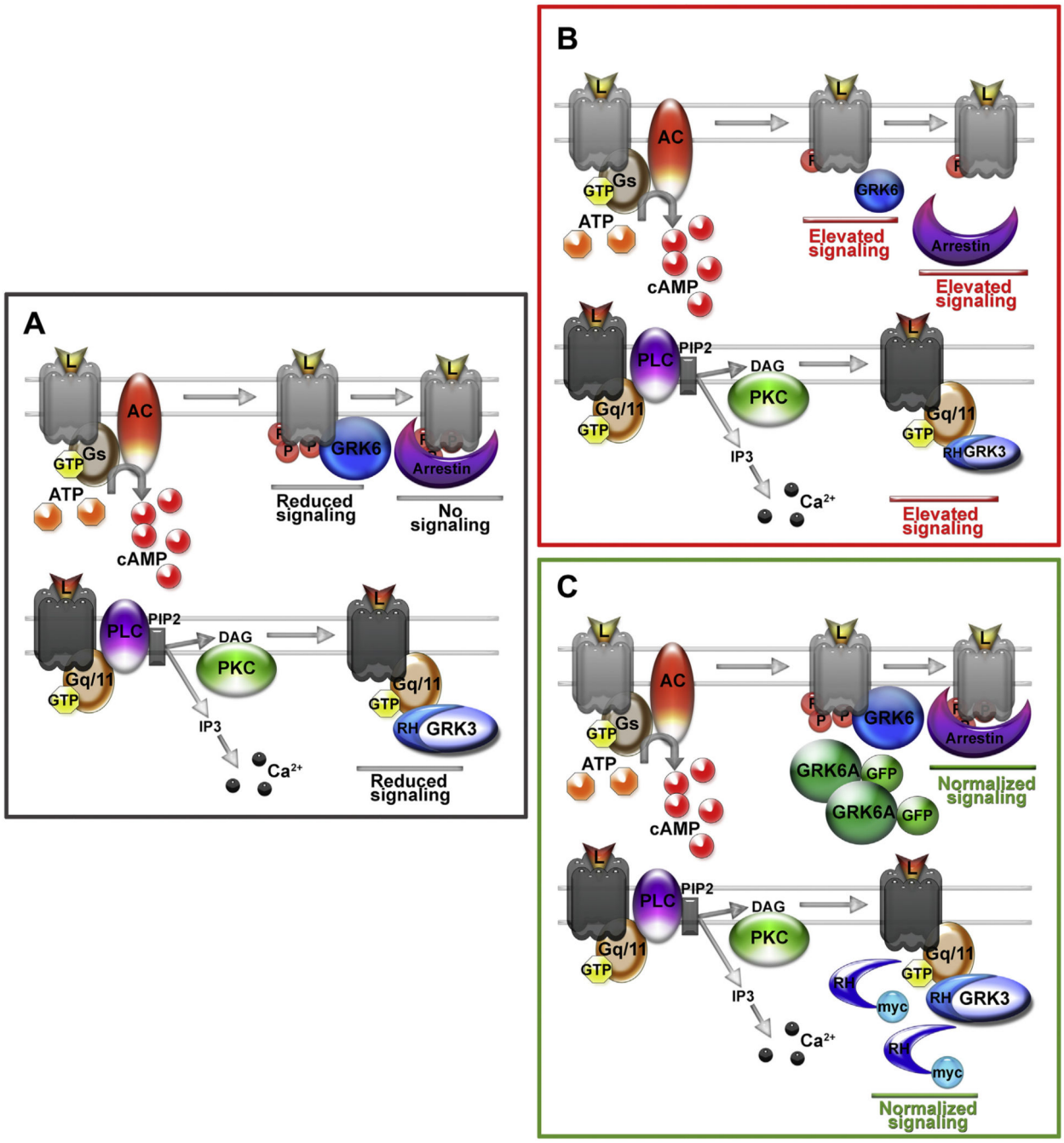

Fig. 3.

GRK isoforms play a critical role in L-DOPA-induced dyskinesia. (A) In the normal striatum, GRK6 regulates signaling of dopamine receptor via phosphorylation-dependent desensitization mechanisms that involves phosphorylation of activated receptor followed by high-affinity binding of arrestins, which abort the signaling. In contrast, GRK3 acts in a phosphorylation-independent manner by sequestering active $G_{\alpha q/11}$ via interaction with its RGS homology domain (RH). (B) In the dyskinetic subjects, the level of GRKs is reduced or insufficient to meet the demand. Therefore, the signaling is enhances as result of deficient

desensitization. (C) Lentiviral overexpression of GRK6 (GRK6A splice variant tagged with GFP) or isolated GRK3 RH domain (tagged with myc) rescue the dopamine receptor desensitization thereby alleviating dyskinesia.