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Lentiviral Overexpression of GRK6 Alleviates L-Dopa–Induced Dyskinesia in Experimental Parkinson's Disease

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Abstract

Parkinson's disease is caused primarily by degeneration of brain dopaminergic neurons in the substantia nigra and the consequent deficit of dopamine in the striatum. Dopamine replacement therapy with the dopamine precursor L-dopa is the mainstay of current treatment. After several years, however, the patients develop L-dopa–induced dyskinesia, or abnormal involuntary movements, thought to be due to excessive signaling via dopamine receptors. G protein–coupled receptor kinases (GRKs) control desensitization of dopamine receptors. We found that dyskinesia is attenuated by lentivirus-mediated overexpression of GRK6 in the striatum in rodent and primate models of Parkinson's disease. Conversely, reduction of GRK6 concentration by microRNA delivered with lentiviral vector exacerbated dyskinesia in parkinsonian rats. GRK6 suppressed dyskinesia in monkeys without compromising the anti-parkinsonian effects of L-dopa and even prolonged the antiparkinsonian effect of a lower dose of L-dopa. Our finding that increased availability of GRK6 ameliorates dyskinesia and increases duration of the antiparkinsonian action of L-dopa suggests a promising approach for controlling both dyskinesia and motor fluctuations in Parkinson's disease.

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SUPPLEMENTARY MATERIAL
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Materials and Methods
Fig. S1. The GFP-tagged GRK6 is functional and has the subcellular localization of the endogenous GRK6.
Fig. S2. Antibodies to GRK6 selectively recognize GRK6A or GRK6B splicing variants.
Fig. S3. The lentivirus carrying two chained miRNAs targets both GRK6A and GRK6B splice variants.
Fig. S4. Infection of the rat striatum with the miRNA lentivirus induces the GRK6 knockdown.
References

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder primarily caused by the degeneration of nigral dopaminergic neurons that provide dopamine to the striatum. The best symptomatic therapeutic agent is the dopamine precursor L-dopa. Long-term treatment leads to L-dopa–induced dyskinesia (LID) or involuntary aimless movements (1). Loss of dopamine in PD causes complex alterations in cellular signaling: Numerous pathways in the dopamine-depleted striatum show exaggerated responses to stimulation by dopaminergic drugs. The signaling is further distorted by chronic L-dopa treatment (2–4). Super-sensitivity of the D1 (5) and D2 (6,7) dopamine receptors is thought to be among the molecular mechanisms underlying LID. Because both the dyskinetic and antiparkinsonian actions of L-dopa are mediated by signaling through dopamine receptors, the molecular mechanisms of these effects are likely intertwined. Previous attempts to dissociate the detrimental and beneficial effects of the drug with pharmacological or molecular tools that inhibit the former while preserving the latter have been only moderately successful (7,8). Underlying molecular mechanisms must be identified and selectively targeted to effectively manage LID.

A conserved desensitization mechanism terminates signaling by G protein–coupled receptors (GPCRs). The first rate-limiting step in this process is activation-dependent receptor phosphorylation by GPCR kinases (GRKs). Binding of arrestins (regulatory proteins) to phosphorylated receptors blocks further G protein activation and initiates receptor internalization (9,10). GPCR signaling is strictly controlled by this process, and the rate and extent of desensitization depend on the availability of GRKs (11–14). In rats with dopamine depletion in one hemisphere, the concentration of GRKs in the dopamine-depleted motor striatum is reduced, and L-dopa fails to alter the GRK expression (15). In parkinsonian monkeys, loss of dopamine leads to the up-regulation of several GRKs (2), which may temper dopaminergic signaling on initial L-dopa administration and ensure a therapeutic response to the drug. However, chronic L-dopa treatment suppresses the GRK expression (2). Previously, we demonstrated elevated membrane expression and reduced internalization of D1 receptors in the striatum of dyskinetic monkeys (16), suggesting that LID is associated with deficits in D1 receptor desensitization and trafficking. Reduced GRK availability likely contributes to the exaggerated dopaminergic signaling in the dyskinetic brain. Collectively, these results suggest that increasing the capacity of the desensitization machinery in the parkinsonian striatum may ameliorate LID.

Five GRK isoforms are expressed in the brain (2,17–19). GRK6 has been implicated in the regulation of dopaminergic signaling in the striatum (13). Here, we demonstrate that overexpression of GRK6 in the striatum facilitates receptor desensitization and ameliorates LID while preserving or even enhancing the antiparkinsonian effects of L-dopa.

RESULTS

Overexpression of GRK6 ameliorates behavioral sensitization to L-dopa in 6-hydroxydopamine–lesioned rats

We constructed lentiviruses encoding green fluorescent protein (GFP) (control) or rat GRK6 [GRK6A splice variant, which is the major messenger RNA (mRNA) variant in the brain] (20) tagged with GFP for easy detection (fig. S1A). Using an in vitro rhodopsin phosphorylation assay, we ascertained that GFP-tagged rat and human GRK6 is functional (fig. S1B). By subcellular fractionation, we found that the exogenous GRK6-GFP was enriched in synaptic membranes, similarly to the endogenous GRK6 (fig. S1C).

First, we tested whether overexpression of GRK6 in the dopamine-depleted striatum would suppress contralateral rotations in response to dopamine agonists in 6-hydroxydopamine (6-

OHDA)–hemilesioned rats. We measured the rotation frequency induced by apomorphine in rats injected with either GFP (control) or GRK6 virus into the striatum on the lesioned side. Both the control and GRK6 groups had similarly extensive lesions (Fig. 1A). Fewer than 3% of the tyrosine hydroxylase (TH)–positive terminals remained in the lesioned caudate putamen (CPu) (Fig. 1B). The GRK6-GFP lentivirus induced GRK6 expression throughout the CPu (Fig. 1C). GRK6 expression was detected in medium spiny neurons as determined by double immunohistochemistry for GFP and the marker of these neurons, DARPP-32 (21) (Fig. 1D). Western blots (Fig. 1D) demonstrated the presence of the transgenic GRK6-GFP in the infected striatum. We used rabbit polyclonal antibody to label both endogenous and overexpressed GRK6A (Fig. 1F). The antibody specificity was demonstrated in a separate experiment (fig. S2). Quantification of the transgenic GRK6A demonstrated that the gene transfer increased the total amount of GRK6A in the lesioned striatum by a factor of ~2 (Fig. 1G).

Drug-naïve rats overexpressing GRK6 displayed significantly less frequent apomorphine-induced rotations than control animals (Fig. 2A). Rodents with unilateral 6-OHDA lesion respond to repeated administration of L-dopa with a progressive increase in the rotation frequency (3,15,22). Overexpression of GRK6 significantly reduced the rotation frequency after repeated L-dopa treatment as compared to the control group [$P = 0.0014$ by two-way repeated-measures analysis of variance (ANOVA)] (Fig. 2B), although it did not prevent the increase in the rotation frequency from session to session.

GRK6 suppresses the sensitized rotational response to L-dopa and abnormal involuntary movements in 6-OHDA–lesioned rats

Because antidyskinetic therapy is required for already dyskinetic PD patients, we tested whether overexpression of GRK6 would influence preexisting sensitization or dyskinesia. We treated 6-OHDA–lesioned rats with L-dopa for 5 days before injection of GRK6 or control viruses (Fig. 2C). The preinjection rotation frequencies were the same in the control and GRK6 groups, with both groups showing marked behavioral sensitization. However, after the injection, rotation frequencies in the GRK6 group were reduced ($P = 0.0247$) (Fig. 1C).

We also studied the effect of GRK6 overexpression on a rodent analog of dyskinesia, abnormal involuntary movements (AIMs) (23,24). Repeated administration of L-dopa to 6-OHDA–lesioned rats leads to a progressive increase in the AIM score (Fig. 2D). The score was markedly reduced in animals expressing GRK6 (Fig. 2D). Thus, the increased availability of GRK6 alleviates already established dyskinesia.

GRK6 knockdown augments the sensitized rotational response to L-dopa and AIMs in 6-OHDA–lesioned rats

To evaluate the role of endogenous GRK6, we tested whether knockdown of GRK6 with lentivirus-delivered microRNA (miRNA) would influence the behavioral effects of L-dopa. We have constructed a lentivirus carrying two miRNA sequences directed against different regions of GRK6 and co-cistronic GFP to label infected cells (fig. S3A). A lentivirus encoding nonsense miRNA and GFP served as control.

6-OHDA–lesioned rats were treated with L-dopa for 5 days before virus injection. The preinjection rotation frequencies were the same in the control and knockdown groups, but after the virus injection, the rotation frequencies in the GRK6 knockdown group were significantly increased ($P = 0.0057$ by two-way repeated-measures ANOVA) (Fig. 2E). The effect was most evident on the last testing days because there was a significant increase in the sensitization slope in the knockdown group ($P = 0.001$). Similarly, GRK6 knockdown increased the frequency of AIMs and exacerbated the progressive increase in AIM score (Fig. 2F).

Postmortem examination of the infected striatum revealed miRNA expression in DARPP-32–positive medium spiny striatal neurons (fig. S4A). The expression of GRK6A was significantly decreased in the lesioned as compared to the intact striatum (fig. S4, B and C), in agreement with our previous report (15). We found that the decrease was largely due to the GRK6A variant, whereas the change in GRK6B was much smaller, albeit significant (fig. S4, D and E). The GRK6 concentration was significantly decreased by the GRK6 miRNA as compared to the control lentivirus (fig. S4, B to E). Because the miRNA sequences were directed against the regions common to GRK6A and GRK8B isoforms (fig. S3B), both isoforms were down-regulated to a similar extent (38.5% GRK6A and 36% GRK6B) (fig. S4, C and E). Thus, decreased availability of GRK6 exacerbates dyskinesia.

GRK6 promotes internalization of D1 receptors and attenuates the signaling effects of L-dopa in 6-OHDA rats

We examined the subcellular localization of three GPCRs, D1 and D2 dopamine receptors and mGluR5 glutamate receptor, after acute L-dopa challenge in rats expressing GRK6 or GFP (Fig. 3). Although D1 immunostaining was restricted to the plasma membrane in the GFP group (Fig. 3, top right panel) (16,25), it was clearly intracellular in GRK6 animals (Fig. 3, top left panel), indicating greater D1 receptor internalization. Conversely, as a substantial proportion of the D2 receptor was already internalized in GFP-expressing animals (Fig. 3, right middle panel) (16), GRK6 did not further promote the D2 receptor internalization (Fig. 3, left middle panel). For comparison, we examined internalization of the mGluR5 receptor in both D1-and D2-expressing medium spiny neurons and found no differences in mGluR5 internalization between the GFP- and GRK6-expressing animals (Fig. 3, lower panels).

Next, we tested whether overexpression of GRK6 in the lesioned striatum blunted dopaminergic signaling after L-dopa administration. The amount of dynorphin, which is largely coexpressed with D1 receptors (26,27), is reduced by the loss of dopamine but significantly up-regulated by subsequent chronic L-dopa treatment (3,22). Similarly, D3 receptors are reduced by dopamine depletion but up-regulated by chronic L-dopa in the CPu (3,22). GRK6 expression suppressed L-dopa–induced up-regulation of prodynorphin mRNA (Fig. 4, A and B) and D3 receptor binding (Fig. 4, C and D) in the CPu. Collectively, these data suggest that expressed GRK6 normalizes the D1 signaling by promoting receptor desensitization.

Enkephalin, which is usually coexpressed with D2 receptors (26,27), is up-regulated in the dopamine-depleted striatum (3,28). GRK6 significantly reduced the expression of preproenkephalin mRNA in the striatum in both saline- and L-dopa–treated lesioned rats (Fig. 4, E and F). Thus, GRK6 also normalizes the signaling in D2 receptor–bearing indirect pathway neurons.

GRK6 alleviates LID in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine–lesioned macaques

Next, we investigated the effectiveness of GRK6 in the gold standard model of LID, L-dopa–treated, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)–lesioned macaque monkeys (29). L-Dopa–treated dyskinetic macaques received GFP (n = 6) or GRK6-GFP (n = 6) virus in the motor striatum. Upon completion of the behavioral experiments, all monkeys were tested to evaluate the lesion and GRK6 expression. Both groups had similar extensive dopamine depletion, as evidenced by the marked decrease in the TH immunoreactivity and dopamine transporter (DAT) binding (Fig. 5, A and B). GRK6-GFP was readily detectable in the motor putamen by immunohistochemistry (Fig. 5C). Using Western blotting, we further confirmed the presence of GRK6-GFP at the level targeted during surgery [anterior commissural, 0 mm (AC0)], whereas there was no expression in the adjacent nontargeted area (AC + 3 mm) (Fig. 5D).

Before GRK6 administration, parkinsonian disability scores in both OFF (before L-dopa administration) and ON (after L-dopa administration) states (Fig. 6A), LID scores in the ON state (Fig. 6B), and the time course of L-dopa–induced locomotor activity (Fig. 6C) were indistinguishable between the two groups. Starting at 6 weeks after surgery, when behavioral experiments resumed, the antiparkinsonian efficacy of L-dopa was comparable in GRK6 and GFP animals (Fig. 6, A and D). There was an overall positive effect of GRK6 on the parkinsonian disability score, suggesting that GRK6 animals benefited longer from L-dopa than controls (Fig. 6, A and D). In agreement with our rodent data, monkeys expressing GRK6 had significantly less intense LID (Fig. 6, B and E). There was also a significant decrease in locomotor activity during the ON state in GRK6 animals in comparison to the GFP group (Fig. 6, C and F). Thus, GRK6 expression in the macaque motor striatum diminished LID intensity without interfering with the antiparkinsonian action of L-dopa.

As in the rat, prodynorphin mRNA expression detected by in situ hybridization was reduced in the GRK6-GFP group in comparison to the GFP group (Fig. 5E), with the latter showing the enhanced prodynorphin expression typical for dyskinetic animals in comparison with non–L-dopa–treated MPTP monkeys (Fig. 5E) (30). Collectively, these data indicate that expressed GRK6 improves LID by normalizing D1 receptor signaling.

GRK6 prolongs the antiparkinsonian action of a lower L-dopa dose

One strategy for controlling LID severity is to decrease the L-dopa dosage. In the L-dopa–treated, MPTP-lesioned macaque monkey, a dose corresponding to 50% of the optimal yields shorter antiparkinsonian effect. Control monkeys displayed this phenomenon (Fig. 7, A and C), with the corresponding decrease in LID severity and duration (Fig. 7, B and D). In contrast, in GRK6-expressing monkeys treated with the 50% L-dopa dose, the duration of the antiparkinsonian effect was comparable to that of the 100% dose (Fig. 7, A and C) but without LID (Fig. 7, B and D). The overall locomotor activity of control animals treated with the 50% dose of L-dopa was reduced by 50%, reflecting shorter duration of L-dopa effect. The GRK6-expressing monkeys showed a less pronounced decrease in locomotor activity due to longer L-dopa effect (Fig. 7E).

GRK6 reduces LID induced by selective D1 and D2 agonists

Because L-dopa is a D1 or D2 indirect agonist, we investigated how GRK6 expression modifies the effects of D1 and D2 agonists. GRK6 monkeys displayed a shortening of the D1 agonist–mediated anti-parkinsonian action compared to controls ($P < 0.05$) (Fig. 7, F and H), which was accompanied by a significant reduction in LID severity and duration ($P < 0.05$) (Fig. 7, G and I). We also detected a significant difference in the antiparkinsonian action of a D2 agonist between the two groups ($P < 0.05$) (Fig. 7, F and H), with GRK6 monkeys displaying lower LID intensity than controls ($P < 0.05$) (Fig. 7, G and I). Thus, the anti-LID effect of GRK6 is mediated by reduced super-sensitivity of both D1 and D2 receptors.

DISCUSSION

Loss of dopamine in PD causes multiple changes in the dopamine-mediated signaling (2,3, 26). The initial dysregulation of signaling pathways is further aggravated by chronic L-dopa treatment, eventually leading to dyskinesia and other motor complications. Although the exact molecular mechanisms of LID remain to be elucidated, exaggerated signaling of the striatal D1 (4,5,23), D2 (7), and D3 (3,8,22) receptors has been implicated in LID in rodents and primates, suggesting that normalization of this excessive signaling may be beneficial. The challenge is to reduce the signaling in a way that alleviates LID while preserving the antiparkinsonian activity of the drug, which is also mediated by dopamine receptors.

Our data demonstrate that promoting GPCR desensitization in the dopamine-depleted striatum via virus-mediated overexpression of GRK6 attenuates LID in both primate and rodent models. GRK6 suppresses LID in dyskinetic monkeys without compromising the antiparkinsonian effects of L-dopa. GRK6 prolongs the antiparkinsonian effect, especially at the lower L-dopa dose. The duration of the anti-parkinsonian effect of the half-dose in GRK6-expressing animals was even slightly longer than that of the full L-dopa dose in controls. The additional time afforded by GRK6 was LID-free. In the rodent model, GRK6 consistently reduced the rotation frequency and the appearance of AIMs. The inhibition of the rotations and AIMs in rats by GRK6 parallels its potent antidyskinetic activity in the primate model of PD, suggesting an overlap between the molecular mechanisms underlying LID in primates and dyskinetic behaviors in rodents. Collectively, these data demonstrate that increased availability of GRK6 helps to control LID without sacrificing the antiparkinsonian benefits of L-dopa.

The rat knockdown studies demonstrated that reduced availability of GRK6 promoted rotational behavior and increased AIM scores, in agreement with our finding (15) that dopamine depletion reduces the expression of GRK6 and L-dopa treatment does not reverse this reduction. Here, we showed that GRK6A splice variant is most affected by the lesion. The loss of GRK6 in the lesioned hemisphere suggests a link between lower GRK6 availability and dyskinesia. MiRNA-mediated GRK6 knockdown exacerbated the decrease in the GRK6 expression in the lesioned hemisphere and aggravated the behavioral consequences of dopamine depletion and L-dopa treatment, supporting the role of low GRK6 in dyskinesia. Conversely, via overexpression of GRK6A, the splice variant most affected by the lesion, we significantly ameliorated dyskinetic behavior. The lesion reduced the GRK6 concentration by ~40%, and lentiviral knockdown further reduced it by 36 to 40%, whereas overexpression doubled GRK6 concentration. These numbers are in good agreement with the work by Gainetdinov *et al*. (13), who found in GRK6 hemizygous mice (with ~50% reduction in the GRK6 concentration) a behavioral phenotype close to that of knockout animals. Thus, even a modest modulation of GRK6 concentration seems to have critical impact on dopaminergic signaling and dopamine-dependent behavior. These data underscore an important functional role of GRK6 in signaling mechanisms underlying dyskinesia.

GRK6 likely alters dopamine-dependent behavior by facilitating desensitization of dopamine receptors. Previous work with mice has demonstrated that behavioral supersensitivity to psychostimulants caused by GRK6 knockout is due to modified signaling through the D2 but not the D1 receptor (13). However, dopamine depletion and subsequent development of LID in the course of L-dopa treatment precipitates multiple marked changes in the striatal signaling pathways (29,31). Although both receptor subtypes are involved in LID, the D1 receptor seems to play a particularly important role (5,16,25). Thus, we expected that in the dyskinetic brain, GRK6 might act on both major dopamine receptor subtypes, which proved to be the case. GRK6 reduced LID caused by selective D1 and D2 agonists, indicating that desensitization of both receptor subtypes was facilitated. In this respect, the effect of GRK6 is qualitatively different from our previous results with RGS9-2 (7), which affected D2 receptors coupled to its target $G\alpha_{i/o}$ but not D1 receptors coupled to $G\alpha_s$ (32). In hemiparkinsonian rats, GRK6 promoted D1 receptor internalization and suppressed the L-dopa–induced up-regulation of prodynorphin and D3 receptor attributed to the enhanced D1 receptor signaling (22). Similarly, GRK6 reduced the prodynorphin expression in dyskinetic monkeys. Although we did not detect any increase in D2 receptor internalization, GRK6 reduced the up-regulation of preproenkephalin mRNA expressed in D2 receptor–bearing neurons (26–28). Thus, the pattern of behavioral and molecular effects is consistent with the conclusion that GRK6 alleviated LID by facilitating the desensitization of both major dopamine receptor subtypes.

In animals treated with selective D1 or D2/D3 agonists, GRK6 not only suppressed LID but also shortened the overall duration of their effects, including the antiparkinsonian activity,

which is consistent with faster receptor desensitization due to increased GRK6 availability. Conversely, in GRK6-expressing animals, L-dopa–induced antiparkinsonian effect lasted longer than in control monkeys. Because of the high selectivity of GRKs for active receptors (33), we expected the anti-LID effect of GRK6 to be coupled with the preservation of the anti-parkinsonian activity. The receptor must be activated, allowing the signal to go through, before it is desensitized by GRK-mediated phosphorylation. Apparently, this initial signaling is sufficient for the antiparkinsonian effect but not for LID. Unique effects of L-dopa might arise from its simultaneous action at both D1 and D2 receptors. The presence of GRK6 is likely to shift the balance in favor of D2-like receptors because they do not desensitize as readily as D1 receptors (34,35). This conclusion is consistent with our finding that in monkeys GRK6 had only a modest effect on the duration of D2-mediated effects, whereas it substantially shortened that of the D1 agonist (Fig. 7). Such rebalancing of the activity of D1 and D2 receptors and, consequently, of the direct and indirect pathways might contribute to extended antiparkinsonian benefits.

Our results demonstrate that a targeted enhancement of GPCR desensitization machinery substantially relieves dyskinesia in two animal models. This amelioration of LID is combined with a longer duration of the antiparkinsonian benefits of L-dopa, offering the hope of achieving the elusive goal of controlling both LID and motor fluctuations. These results pave the way for the development of treatments for dyskinesia in Parkinson's patients based on judicious manipulation of receptor signaling. Our data identify the receptor desensitization machinery as a therapeutic target in numerous disorders associated with aberrant signaling via GPCRs, including schizophrenia and drug abuse.

MATERIALS AND METHODS

Virus construction and preparation

The full-length coding sequence of the rat or human GRK6A C-terminally tagged with GFP or GFP alone (control) was cloned into the lentiviral vector pLenti6/V5-DEST, and the virus was produced using ViraPower system (Invitrogen) (fig. S1A). MiRNA sequences targeting rat GRK6 were selected with Invitrogen Block-iT RNAi Designer software (fig. S3), and miRNA-encoding viruses were produced with Block-iT HiPerform Lentiviral Pol II RNAi expression system (Invitrogen).

Rat experiments

Adult Sprague-Dawley rats (Charles River) were used. The animals were housed at the Vanderbilt University animal facility in a 12:12 light-dark cycle with free access to food and water. All procedures followed the National Institutes of Health guidelines and were approved by the Vanderbilt University Institutional Animal Care and Use Committee. The 6-OHDA lesion was performed as described (3,15). The viruses were injected either at the time of the 6-OHDA lesion or after the behavioral pretesting. The animals were tested for rotations in an automated rotometer (AccuScan Instruments) as described (3,15). In the experiments with AIMs, rats were assessed for AIMs on a 0 to 4 AIM rating scale (24).

Monkey experiments

All experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/ EEC) for the care of laboratory animals in an American Association for Accreditation of Laboratory Animal Care–accredited facility. Veterinarians skilled in the healthcare and maintenance of nonhuman primates supervised animal care. Experiments followed published procedures (5,7,8,31). After parkinsonian syndrome stabilized, all 12 MPTP-treated monkeys were treated with Modopar for 6 months to develop dyskinesia. The improved Horsley-Clarke stereotactic technique was used as

described (2,5,7,8,36,37). Either GRK6-GFP ($n = 6$) or GFP ($n = 6$) lentivirus was injected into the dorsolateral putamen as described (7). The response of monkeys to L-dopa was assessed as described (7,8,31,36).

Data analysis

The rotation data were analyzed by two-way repeated-measures ANOVA, with Group (GFP versus GRK6) as the between-group factor and Session as the repeated-measures factor. If the significant effect of Group was observed, the data for individual sessions were compared by unpaired Student's *t* test. The AIM scores were compared for each session with the Mann-Whitney nonparametric test. Neurochemical data were analyzed by repeated-measures ANOVA, with Hemisphere (intact versus lesioned) as the within-group factor and Group (GRK6 versus GFP) as the between-group factor, or by Mann-Whitney test where appropriate. The value of $P < 0.05$ was considered significant. Detailed description of rat experiments is given in Supplementary Material. Monkey behavioral data were analyzed by two-way repeated-measures ANOVA, with Group (GRK6 versus GFP) as the between-group factor and Session (before and after surgery) as the within-group factor. Additional analysis was performed with Wilcoxon matched-pairs signed-ranks test or Mann-Whitney test where appropriate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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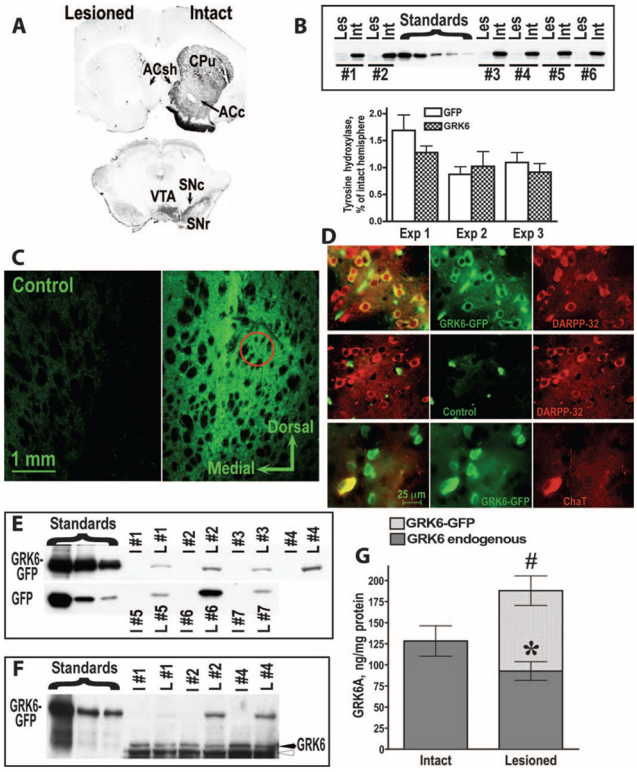

Fig. 1.

Lentivirus-induced GRK6 expression in the rat dopamine-depleted striatum. (**A**) Loss of dopaminergic innervation in the striatum and dopaminergic neurons in the substantia nigra was detected by immunohistochemistry for TH. ACsh, shell of the accumbens; ACc, core of the accumbens; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; VTA, ventral tegmental area. (**B**) Quantification of the Western blots for TH in the lesioned (Les) and intact (Int) striatum in animals expressing GFP or GRK6. The inset shows a representative Western blot for TH. Numbers refer to individual animals. $n = 8$ in each group displayed in lower panel. (**C**) Photomicrograph of the intact striatum (left panel) and the striatum infected with the GRK6-GFP lentivirus (right panel) immunostained for GFP. (**D**) Expression of GRK6-GFP in striatal neurons. The upper row of images demonstrates the expression of GRK6 in medium spiny neurons by double staining for GFP and DARPP-32. The circle in (C) (right panel) indicates the approximate position where these photographs were taken. The middle row of images shows photomicrographs taken from similar positions in the control uninfected hemisphere [left image in (C)]. The lower row of images demonstrates the expression of GRK6 in cholinergic interneurons by double staining for GFP and choline acetyltransferase (ChAT). (**E**) Detection of the GRK6-GFP expression by Western blot. The expression of the GRK6 transgene in four animals in the intact and lesioned injected hemisphere detected with antibody to GFP is shown. The lower row shows expression of GFP in control animals. (**F**) Detection of the endogenous GRK6 and GRK6-GFP transgene expression by Western blot. Black arrowhead, GRK6A; white arrowhead, nonspecific band. (**G**) Quantification of the Western blot data for the expression of GRK6-GFP and endogenous GRK6A in the intact and lesioned hemisphere. Serial dilutions of purified human GRK6A were used as standards (15). The expression of endogenous GRK6A and total GRK6A (endogenous plus transgenic) in the intact and lesioned (infected) hemisphere was compared by paired *t* test. $^{*}P < 0.05$ to the intact striatum for endogenous GRK6A; $^{\#}P < 0.05$ to the intact striatum, total GRK6A.

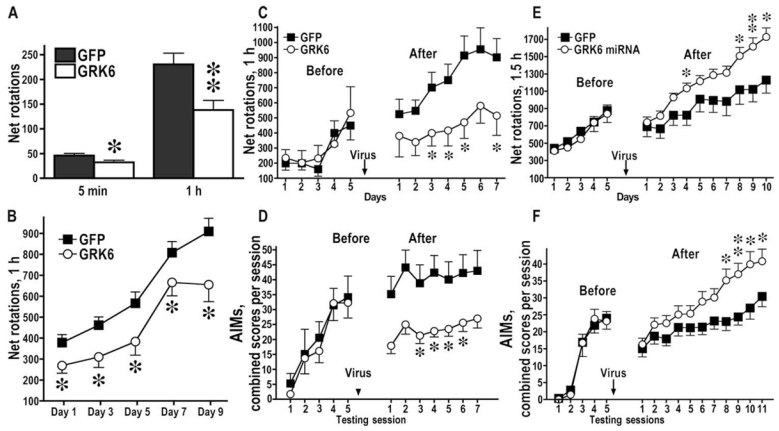

Fig. 2.

The expression of GRK6 in the lesioned striatum inhibits, and knockdown of GRK6 exacerbates, rotations and AIMs in the hemiparkinsonian rat. Data are shown as means ± SEM. (**A**) Overall frequency (for 1 hour) and peak frequency (5 min) of apomorphine-induced net contralateral rotations (contralateral - ipsilateral) in rats expressing GRK6 as compared to animals expressing GFP (control). **P* < 0.05, ***P* < 0.01. (**B**) L-Dopa–induced rotations on repeated L-dopa treatment in rats expressing GRK6. **P* < 0.05, between the GRK6 and control GFP groups, post hoc Student's *t* test. (**C**) L-Dopa–induced rotations in rats expressing GRK6 presensitized with L-dopa for 5 days before virus injection. **P* < 0.05, between the GRK6 and control GFP groups, post hoc unpaired Student's *t* test. (**D**) Combined AIM scores per session in rats expressing GRK6 or GFP pretreated with L-dopa before the virus injection. **P* < 0.05, between the GRK6 and GFP groups, Mann-Whitney test. (**E**) L-Dopa–induced rotation frequencies in rats injected with the GRK6 miRNA virus as compared to control (GFP). The animals were presensitized with L-dopa for 5 days before the virus injection. **P* < 0.05, between the GRK6 miRNA and GFP groups, post hoc unpaired Student's *t* test. (**F**) Combined AIM scores per session in rats with GRK6 knockdown as compared to control. Both groups were pretreated with L-dopa before the virus injection. **P* < 0.05, between the GRK6 miRNA and GFP groups, Mann-Whitney test.

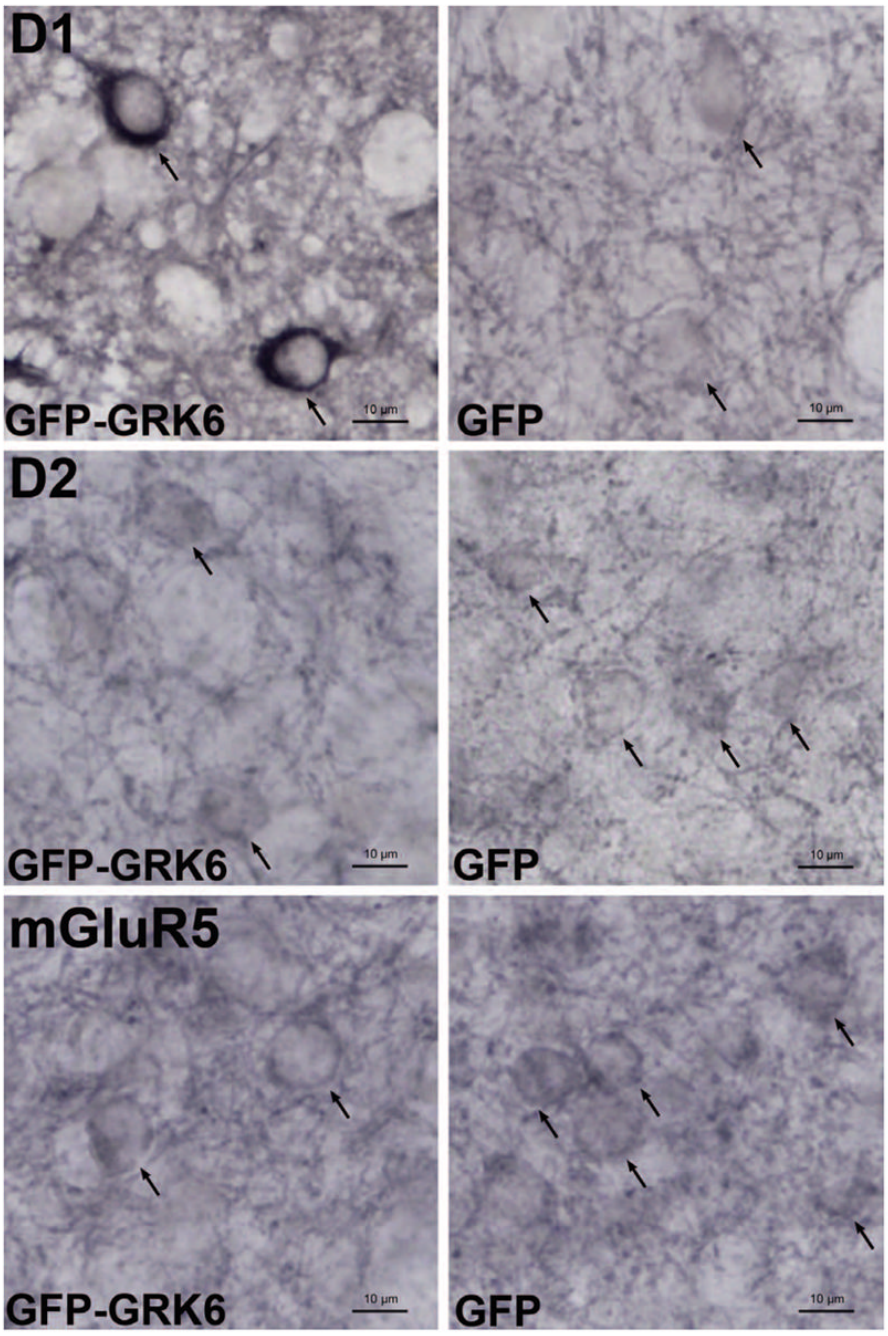

Fig. 3.

GRK6 promotes D1 dopamine receptor internalization in the lesioned striatum but does not affect D2 dopamine and mGluR5 glutamate receptor localization. In an area delineated by GFP immunohistochemistry, medium spiny neurons immunopositive for the D1 receptor showed plasma membrane and neuropil D1 receptor localization in GFP animals (top right) and prominent cytoplasmic localization in GRK6-expressing animals (top left; arrows). This effect was specific for the D1 receptor, as two other GPCRs, D2 receptor (middle) expressed in different medium spiny neurons and mGluR5 (lower) expressed in both D1- and D2-bearing neurons, remained cytoplasmic (D2, arrows) and both membranous and cytoplasmic (mGluR5, arrows) in both groups.

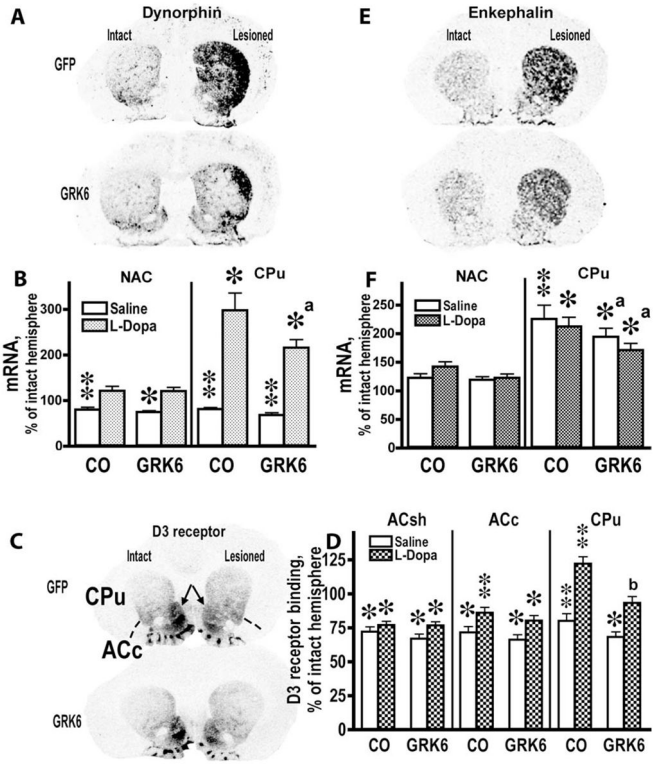

Fig. 4.

The expression of GRK6 in the lesioned striatum inhibits D1 receptor– and D2 receptor–mediated signaling. Rats lesioned with 6-OHDA and treated with L-dopa for 10 days expressed either GFP (control) or GRK6 in the lesioned striatum. (**A**) Representative autoradiograms showing the expression of prodynorphin mRNA. (**B**) Quantification of the prodynorphin in situ hybridization. Data are shown for the lesioned hemisphere as percentages of the intact hemisphere values (means ± SEM). **P* < 0.001, ***P* < 0.01, as compared to the intact hemisphere by repeated-measures ANOVA; [a]*P* < 0.05, as compared to the L-dopa–treated GFP-expressing group (CO), Mann-Whitney test. (**C**) Representative autoradiograms showing the D3 receptor binding in the lesioned striatum. Note the reduction of the D3 receptor concentration in the shell (arrows) and core (approximate borders indicated by dashed lines) of the nucleus accumbens. Also note the up-regulation of the D3 receptor in the lesioned striatum caused by L-dopa and the reduction of the D3 receptor in the CPu of GRK6-expressing rats. (**D**) Quantification of the D3 receptor binding data (means ± SEM). **P* < 0.001, ***P* < 0.01, as compared to the intact hemisphere by repeated-measures ANOVA; [b]*P* < 0.01, as compared to the L-dopa–treated GFP-expressing group, Mann-Whitney test. (**E**) Representative autoradiograms showing the expression of preproenkephalin mRNA in the lesioned striatum. (**F**) Quantification of the preproenkephalin in situ hybridization data (mean ± SEM). **P* < 0.001, ***P* < 0.01, as compared to the intact hemisphere by repeated-measures ANOVA; [a]*P* < 0.05, as compared to the GFP-expressing group, Mann-Whitney test.

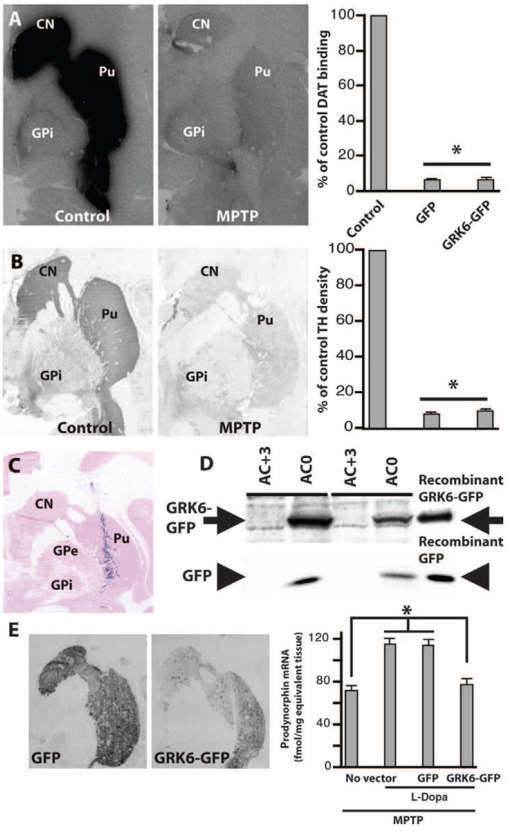

Fig. 5.

Extent of dopaminergic lesion and lentivirus-mediated expression of GRK6 in the motor striatum of MPTP-lesioned monkeys. (**A** and **B**) Representative examples and quantification (right panel) of DAT binding (A) and TH immunohistochemistry (B) in the motor striatum show marked dopamine denervation in both the caudate nucleus (CN) and the putamen (Pu) (37) with a faint signal around the globus pallidus (GPi), as reported (38). Both GFP and GRK6-GFP groups showed lesions of comparable extent [DAT: $F(2,17) = 3828$, $P < 0.0001$; TH: $F(2,17) = 3331$, $P < 0.0001$; $^*P < 0.001$ versus control animals, one-way ANOVA followed by Bonferroni; means ± SEM]. (**C**) Representative GFP immunostaining at the most caudal level targeted in the striatum [globus pallidus pars externalis (GPe)] with a needle track. (**D**) Detection of the GRK6-GFP expression by Western blot. Samples of the monkey putamen were collected at the most rostral injection site (AC0) and 3 mm further rostrally to the injection site (AC+3). Total protein (5 μg) was loaded per lane, and GRK6-GFP and GFP were detected with mouse antibody to GFP (Clontech). Expression of the GRK6 and GFP transgenes in two GRK6-GFP–injected and two GFP-injected monkeys is shown. Recombinant proteins were loaded for comparison (right side of the blot). (**E**) Prodynorphin mRNA in situ detection in non–L-dopa–treated MPTP, L-dopa–treated MPTP, (L-dopa–treated MPTP) GFP, and (L-dopa–treated MPTP) GRK6-GFP monkeys. Quantitative analysis (right panel) showed that prodynorphin mRNA is reduced in the GRK6-GFP group to a level comparable to that of the non–L-dopa–treated situation [$F(3,23) = 14.01$, $P < 0.0001$; $^*P < 0.01$ versus non–L-dopa–treated MPTP and (L-dopa–treated MPTP) GRK6-GFP monkeys, one-way ANOVA followed by Bonferroni correction; means ± SEM].

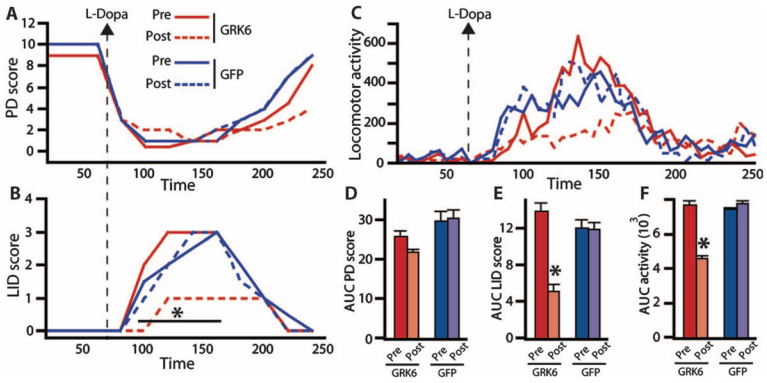

Fig. 6.

Virus-mediated GRK6 expression in the macaque motor striatum decreases LID intensity. (**A** and **B**) Only the median scores are shown, without ranges for readability. The dashed vertical line indicates the administration of L-dopa (L-dopa–carbidopa, 4:1; see Materials and Methods). (A) GRK6 expression had no impact on parkinsonian disability (PD) score at any time point. (**D**) Analysis of the area under the curve (AUC) of PD scores revealed a trend for a positive effect (mean ± SEM). The data for pretesting and posttesting sessions were analyzed separately by two-way repeated-measures ANOVA, with Group (GRK6 versus GFP) as the between-group factor and Session (before and after surgery) as the repeated-measures factor [Group effect: $F(1,21) = 6.85$, $P = 0.027$; Session effect: $F(1,21) = 5.69$, $P = 0.04$; Interaction: $F(1,21) = 13.83$, $P = 0.004$]. The significant interaction suggests that the GRK6 animals benefit longer from L-dopa than the GFP animals. (B) GRK6 expression reduced L-dopa–induced LID from 100 min until 160 min in comparison with the presurgery situation (median scores; $*P < 0.05$, Wilcoxon matched-pairs signed-ranks test). (**E**) The overall positive effect on LID severity is further exemplified by the AUC data [means ± SEM; Group effect: $F(1,21) = 11.09$, $P = 0.008$; Session effect: $F(1,21) = 30.23$, $P = 0.0003$; Interaction: $F(1,21) = 11.09$, $P = 0.008$], which show a significant difference in LID severity in GRK6 animals after surgery compared to their scores before surgery and to the GFP animals. $*P < 0.05$ versus all others. (**C** and **F**) Consequently, locomotor activity was lower in GRK6 animals [Group effect: $F(1,21) = 31.82$, $P = 0.0003$; Session effect: $F(1,21) = 157.41$, $P < 0.0001$; Interaction: $F(1,21) = 206.75$, $P < 0.0001$]. $*P < 0.05$ versus all others.

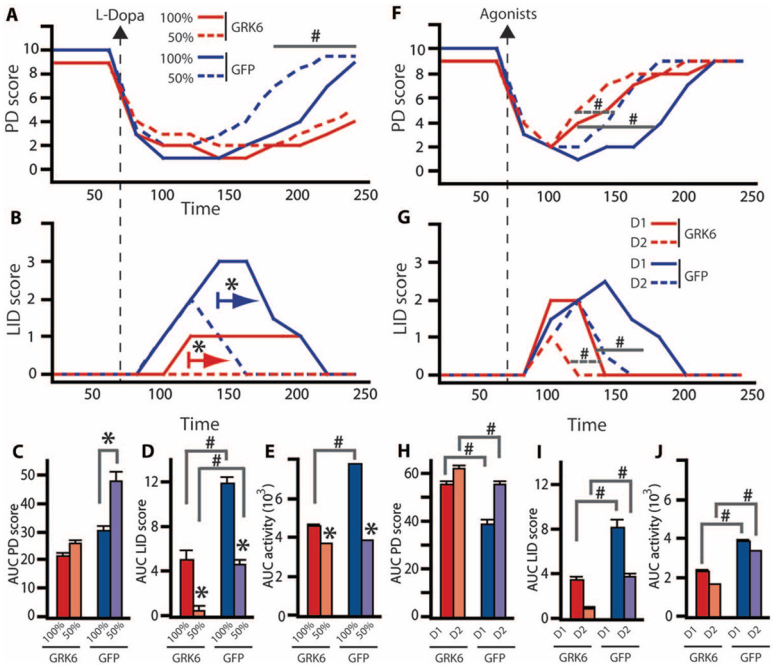

Fig. 7.

Virus-mediated GRK6 expression in the macaque motor striatum prolongs L-dopa action and reduces the dyskinesia elicited by D1 and D2 agonists. (**A**) Parkinsonian scores as function of time after administration of full or 50% L-dopa dose in GFP- or GRK6-expressing animals (#, scores not statistically different from baseline from 180 min onward in the GFP group treated with 50% dose). (**C**) AUC data of PD scores. The GFP group displayed worse PD scores with the 50% dose as compared to the full dose (**P* < 0.05, Wilcoxon matched-pairs signed-ranks test), whereas the GRK6 group did not. (**B**) Dyskinesia scores as function of time after administration of full or 50% L-dopa dose in GFP- or GRK6-expressing animals. Dyskinesia is reduced with 50% dose in GFP from 140 min onward (blue arrow; **P* < 0.05 to 100% dose, Wilcoxon matched-pairs signed-ranks test) and GRK6 from 120 min (red arrow). (**D**) Analysis of the AUC of LID scores shows significant reduction in LID in both groups (**P* < 0.05, between 100 and 50% doses, Wilcoxon matched-pairs signed-ranks test). GRK6 animals display less severe LID than their GFP counterparts ($^{\#}P < 0.05$, Mann-Whitney test). (**E**) GRK6 animals show lower AUC of activity counts than the GFP group ($^{\#}P < 0.05$) with the full dose but the same AUC at 50% dose. Both groups show a significant reduction in activity counts at the 50% dose in comparison with their respective 100% dose (**P* < 0.05). (**F**) GRK6 animals displayed reduced duration of antiparkinsonian effects of D1-and D2-selective agonists, with PD scores worsening from 120 min until 180 min (solid gray line) and from 120 min until 140 min (dashed gray line), respectively (median scores; $^{\#}P < 0.05$, Mann-Whitney test). (**H**) AUC analysis shows shorter effects of D1 and D2 agonists in GRK6 than in GFP animals [$^{\#}P = 0.004$ (D1 agonist) and 0.03 (D2 agonist), Mann-Whitney test]. (**G**) GRK6 animals displayed a reduced duration of D1 agonist– and D2 agonist–induced dyskinesia from 140 min until 160 min (solid gray line) and around 120 min (dashed gray line), respectively (median scores; $^{\#}P < 0.05$, Mann-Whitney test). (**I**) AUC analysis shows reduced duration of D1 agonist– and D2 agonist–induced dyskinesia in GRK6 animals [$^{\#}P = 0.004$ (D1 agonist) and 0.004 (D2 agonist), Mann-Whitney test]. (**J**) AUC of activity counts is reduced in GRK6 animals as compared to GFP animals after both D1 and D2 agonist administration ($^{\#}P < 0.05$, unpaired *t* test).