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Selective Deletion of GRK2 Alters Psychostimulant-Induced Behaviors and Dopamine Neurotransmission

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GRK2 is a G protein-coupled receptor kinase (GRK) that is broadly expressed and is known to regulate diverse types of receptors. GRK2 null animals exhibit embryonic lethality due to a severe developmental heart defect, which has precluded the study of this kinase in the adult brain. To elucidate the specific role of GRK2 in the brain dopamine (DA) system, we used a conditional gene knockout approach to selectively delete GRK2 in DA D1 receptor (D1R)-, DA D2 receptor (D2R)-, adenosine 2A receptor (A2AR)-, or DA transporter (DAT)-expressing neurons. Here we show that select GRK2-deficient mice display hyperactivity, hyposensitivity, or hypersensitivity to the psychomotor effects of cocaine, altered striatal signaling, and DA release and uptake. Mice with GRK2 deficiency in D2R-expressing neurons also exhibited increased D2 autoreceptor activity. These findings reveal a cell-type-specific role for GRK2 in the regulation of normal motor behavior, sensitivity to psychostimulants, dopamine neurotransmission, and D2 autoreceptor function.
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INTRODUCTION

Neuronal G protein-coupled receptors (GPCRs) represent a large class of proteins that orchestrate a diverse array of physiological processes critical for maintaining normal brain function. Central to their regulation are a family of G protein-coupled receptor kinases (GRKs) that are actively engaged following agonist-mediated receptor activation and function in part to dampen intracellular signaling via homologous desensitization. Seven GRK family members have been identified to date, four of which (GRKs 2, 3, 5, and 6) are highly expressed and in diverse cellular populations in the brain (Premont and Gainetdinov, 2007). The physiological functions of GRKs 3–6 have been previously examined using isoform-specific KO mice to reveal critical roles for these kinases in the regulation of muscarinic, dopamine (DA), and opioid receptor function in adult animals (Gainetdinov *et al*, 2003; Gainetdinov *et al*, 1999; McLaughlin *et al*, 2004). In contrast, germline deletion of GRK2 in mice results in hypoplasia of the ventricular myocardium and embryonic lethality (Jaber *et al*, 1996). This has consequently hampered efforts to understand the physiological importance of GRK2 function in the adult brain, leaving relatively little known.

DA is a catecholamine neurotransmitter involved in a diverse array of functions in mammals, including the control of movement, addiction, emotion, and cognition. Consistent with its vital role as a neuromodulator, dopaminergic dysfunction has been implicated in various pathological states, such as Parkinson's disease, Huntington's disease, Tourette's syndrome, and attention deficit and hyperactivity disorder (Beaulieu and Gainetdinov, 2011). Two distinct classes of DA-responsive GPCRs, D1-like (D1R and D5R) and D2-like (D2R, D3R, and D4R), are expressed within the basal ganglia and signal through opposing G-protein-dependent pathways following activation by DA (Bateup *et al*, 2008). Previous work from our lab also suggests that striatal D2 receptors signal in a G-protein-independent manner via the scaffolding protein βarrestin-2 to modulate striatal protein kinase B (Akt) and GSK3β signaling (Beaulieu *et al*, 2005; Beaulieu *et al*, 2007). Genetic and/or pharmacological disruption of downstream effectors engaged by DA receptors (eg, DA- and cAMP-regulated phosphoprotein of molecular weight 32 kDa (DARPP-32) and GSK3β) robustly alters motor behavior and sensitivity to antipsychotic and psychostimulant drugs (Bateup *et al*, 2010; Beaulieu *et al*, 2004; Urs *et al*, 2012). Thus, a more comprehensive understanding of proteins involved in DA receptor signaling may yield valuable mechanistic insights

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regarding several important clinically relevant drugs and provide novel therapeutic targets.

Several studies in heterologous cells have demonstrated that GRK2 can profoundly regulate the trafficking and agonist-induced signaling of D1 and D2 receptors (Ito *et al*, 1999; Iwata *et al*, 1999; Namkung *et al*, 2009a; Namkung *et al*, 2009b; Sedaghat and Tiberi, 2011; Tiberi *et al*, 1996). However, evidence for this regulation *in vivo* or even in systems with more physiologically relevant levels of GRK2 (eg, cultured neurons) remains scarce. Studies utilizing pharmacological interventions that perturb the DA system, such as the psychostimulant cocaine, or potent neurotoxins, such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and 6-hydroxydopamine, have found region-specific alterations in GRK2 levels (Ahmed *et al*, 2008a; Bezard *et al*, 2005; Schroeder *et al*, 2009). Additionally, it was found that fragile X mental retardation protein deficiency in mice causes a coincident increase in GRK2 levels in the frontal cortex and D1R hyperphosphorylation (Wang *et al*, 2008). Although these studies provide a potential link between GRK2 and the DA system, the physiological consequences of altered GRK2 levels to DA-dependent behaviors remain largely unknown.

To examine the possibility that GRK2 regulates DA-dependent behaviors, we generated mice with the conditional deletion of GRK2 in either DA-receptive neurons (D1R-, D2R-, and A2AR-expressing) or in midbrain DA neurons (DA transporter (DAT)-expressing neurons) and utilized behavioral and electrophysiological approaches to determine the impact of GRK2 deletion. Our results reveal cell-type-specific effects of GRK2 deletion on motor behavior, sensitivity to cocaine-induced locomotion, cocaine-induced striatal signaling, and DA uptake inhibition, DA release and uptake, and D2 autoreceptor function.

MATERIALS AND METHODS

Generation of Experimental Animals

All animals studies were conducted with an approved protocol from the Duke University Institutional Animal Care and Use Committee in accordance with the National Institutes of Health guidelines. Floxed *Grk2* mice were obtained from Dr Gerald Dorn II (Washington University, St Louis, Missouri) (Matkovich *et al*, 2006) and Rosa26-floxed-stop-EYFP reporter mice were obtained from Dr Brigid Hogan (Duke University, Durham, NC) (Srinivas *et al*, 2001). The $D1R^{cre}$ (founder line EY262), $D2R^{cre}$ (founder line ER44), and $A2AR^{cre}$ (founder line KG139) bacterial artificial chromosome (BAC) transgenic mouse lines were purchased from GENSAT (The Rockefeller University). The DAT^{cre} knock-in mouse line was obtained from Dr Xiaoxi Zhuang (University of Chicago, Chicago, IL) and has been previously published (Zhuang *et al*, 2005). Experimental animals were obtained by crossing homozygous floxed *Grk2* (129/C57BL/6J) mice to homozygous floxed mice that were also hemizygous (from BAC transgenic lines) or heterozygous (for the knock-in mouse line) for cre. These breedings yielded homozygous floxed *Grk2* mice that were also cre-positive (KO animals) and floxed *Grk2* littermates (control animals). A summary of the mouse lines generated can be found in Supplementary Table S1. Drug and experiment-naive adult mice (2–6 months of age) and of mixed sex were used for all studies. Animals were placed on a 12-h light/dark cycle in a temperature- and humidity-controlled environment with *ad libitum* access to food and water.

Drugs

Cocaine hydrocholoride, quinpirole hydrochloride, sulpiride, and apomorphine hydrochloride hemihydride were purchased from Sigma-Aldrich, and SKF81297 was purchased from Tocris Biosciences. All drugs were dissolved in isotonic, sterile saline and injected as described at a volume of 10 ml/kg body weight.

Locomotor Activity and Cocaine Sensitization

Locomotor activity was measured at 5-min intervals in an automated Omnitech Digiscan open-field ($20 \times 20\,cm^2$) apparatus. Animals were tested during the light phase of the cycle and habituated to the room before testing. For the acute cocaine experiments, mice were habituated in the open field for 30 min, removed, injected with the indicated dose of cocaine, and immediately placed back into the apparatus. Sensitization experiments were performed as previously described (Gainetdinov *et al*, 2003). Briefly, on day 1 of the protocol, mice were injected with the indicated dose of cocaine and placed into the open field. On days 2–5, mice were injected with the same dose of cocaine and placed back into their home cage. After a 1-day reprieve from treatment, mice were challenged on day 7 with the same dose of cocaine and monitored in the open field for 90 min postinjection.

Conditioned Place Preference

Mice were tested using a three-compartment apparatus (Med Associates) as previously described (Daigle and Caron, 2012).

Fast-Scan Cyclic Voltammetry (FSCV)

Mice received one injection daily of either vehicle or cocaine (20 mg/kg, i.p.) for 5 consecutive days. Approximately 40 h after the last injection, mice were killed for slice voltammetry experiments. Note that while this is the same day that mice in the behavioral sensitization experiments received cocaine challenge and locomotor activity assessment, no injections were given to mice in the voltammetry study on day 7. Rather, all drugs were bath applied on brain slices as described in detail below. After killing, multiple coronal slices (400 μm) containing the NAc and CPu were prepared from each animal with a vibrating tissue slicer while immersed in oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl (126), KCl (2.5), NaH_2PO_4 (1.2), $CaCl_2$ (2.4), $MgCl_2$ (1.2), $NaHCO_3$ (25), glucose (11), and L-ascorbic acid (0.4), and pH was adjusted to 7.4. Once sliced, tissue was transferred to the testing chambers containing bath aCSF (32 °C), which flowed at 1 ml/min. A cylindrical carbon fiber microelectrode (100–200 mM length, 7 mM radius) and a bipolar stimulating electrode was placed into the dorsolateral CPu, and DA was evoked by

a single, rectangular, electrical pulse (750 μA, 4 ms) applied every 5 min. Extracellular DA was monitored at the carbon fiber electrode every 100 ms using FSCV by applying a triangular waveform (−0.4 to +1.2 to −0.4 V *vs* Ag/AgCl, 400 V/s) (Kennedy *et al*, 1992). Once the extracellular DA response was stable (ie, did not exceed 10 % variation in peak height for three successive stimulations), a single experiment was performed on each slice containing the CPu as described below.

After obtaining stable DA to single-pulse stimulation, multiple concentrations of cocaine (0.3, 1.0, 3.0, 10.0, and 30.0 μM) were bath applied cumulatively to the slice while continuing single-pulse stimulations every 5 min. Each concentration of cocaine was allowed to stabilize before adding the next concentration (typically 45 min at each concentration, or eight stimulations). For a separate set of multiple-pulse experiments without cocaine, stimulation parameters were adjusted from the single pulse to include five-pulse stimulations across frequencies that mimic both tonic and phasic firing of DA neurons, including 5, 10, 20, 25, and 100 Hz frequencies. Following the five-pulse series of stimulations, the D2R antagonist sulpiride (10 μM) was then added to the bath, and DA evoked by single-pulse stimulation was allowed to stabilize to the drug before repeating the multiple-pulse protocol. Immediately following the completion of each experiment, recording electrodes were calibrated by recording their response (in electrical current; nA) to a known concentration of DA in aCSF (3 mM) using a flow-injection system. This value was then used to convert electrical current to DA concentration. Evoked levels of DA were modeled using Michaelis–Menten kinetics, as a balance between release and uptake (Wightman *et al*, 1988). Michaelis–Menten modeling provides parameters that describe the amount of DA released following stimulation (ie, the peak height of the signal) and the maximal rate of DA uptake (V_{max}). We followed standard voltammetric modeling procedures by setting the apparent K_m parameter to 160 nM for each animal based on well-established research on the affinity of DA for the DAT (Wu *et al*, 2001), while baseline V_{max} values were allowed to vary as the baseline measure of rate of DA uptake. All modeled data were required to exceed a goodness-of-fit $\geqslant R^2 = 0.98$, and all V_{max} values fell within an acceptable range of physiological rates of uptake described in previous work. In experiments that incorporated the use of cocaine, the time constant tau (measured as 2/3 return to baseline from peak height) and peak height of the signal was calculated for each concentration of cocaine as a percentage of baseline tau or peak height in order to account for changes in cocaine-induce DA uptake inhibition and release. All voltammetry data were collected and modeled using the Demon Voltammetry and Analysis Software (Yorgason *et al*, 2011).

Immunohistochemistry

Staining of free-floating brain sections was performed as previously described (Daigle *et al*, 2011). Briefly, mice were anesthetized with chloral hydrate (500 mg/kg i.p.) and perfused with ice-cold PBS followed by 10% formalin solution (Sigma Aldrich). Brains were postfixed overnight in 10% formalin and sectioned (50 μm) on a vibratome. Following permeabilization and blocking, sections were incubated overnight at 4 °C in one or more of these antibodies: GRK2 (1 : 500; Santa Cruz Biotechnology), rabbit anti-GFP (1 : 5000; abcam), chicken anti-GFP (1 : 500; abcam), DAT (DA transporter) (1 : 300; Millipore), and met-Enkephalin (1 : 250; Millipore). Primary antibody was detected with Alexa dye-conjugated secondary antibodies (Life Technologies). Images (1024 × 1024 pixels) were acquired on either a Zeiss LSM-510 confocal microscope or an Olympus FluoView FV1000 confocal microscope.

Western Blotting Analyses

Determination of phosphoprotein levels in dorsal striatal extracts was performed as previously described (Daigle *et al*, 2011). Blots were probed with antibodies directed against pT308 Akt (1 : 100), Akt (1 : 1500), pS9 GSK3 (1 : 100), or GSK3 (1 : 500) (all from Cell Signaling Technology), pT34 DARPP-32 (1 : 300; Phosphosolutions), or DARPP-32 (1 : 1000; BD Transduction Laboratories). Primary antibodies and direct fluorescent signal from secondary antibodies were detected using an Odyssey Infrared imaging system (LI-COR Biosciences). Phosphoprotein levels were normalized to the corresponding level of total protein and then normalized to the average for the wild-type group ($Grk2^{f/f}$) for each separate experiment.

Tyramide Signal Amplification (TSA) Immunohistochemistry

Free-floating brain sections were incubated for 5 min in PBS containing 0.5% $NaBH_2$, 10% MeOH, and 3% H_20_2. Sections were then washed in PBS followed by incubation for 15 min in PBS containing 1.2% Triton X-100, blocked in PBS containing 5% normal goat serum, 2% bovine serum albumin, and 0.2% Triton X-100 for 1 h, and then incubated overnight at 4 °C in the indicated antibody. Sections were incubated the following day in either an Alexa-dye-conjugated (Invitrogen) or an HRP-conjugated secondary antibody. The latter was detected by brief incubation with an Alexa488-labelled tyramide reagent (Invitrogen).

Data and Statistical Analyses

All statistical analyses were performed using the GraphPad Prism 4.0 software (GraphPad Software). The results presented are means with the SEMs. An unpaired Student's *t*-test, one-way ANOVA, or two-way repeated measures (RM) ANOVA was used for the different comparisons as indicated in the text and figure legends.

RESULTS

Selective GRK2 Deletion Alters Spontaneous Locomotion and Causes a Bidirectional Change in Sensitivity to Cocaine

To determine whether GRK2 regulates DA receptor function *in vivo* and, in turn, DA-dependent behaviors, we generated conditional knockout (KO) mice in which GRK2 was selectively deleted from D1R- and D2R-expressing neurons in the murine brain. This was accomplished by crossing

BAC transgenic mice in which Cre recombinase was driven under the D1R and D2R promoter elements (*D1R*^{cre} and *D2R*^{cre} mice, respectively) to mice with a floxed *Grk2* locus (*Grk2*$^{f/f}$) to yield conditional KO mice (*D1R*^{cre}*Grk2*$^{f/f}$ and *D2R*^{cre}*Grk2*$^{f/f}$) and littermate controls (*Grk2*$^{f/f}$). As D2Rs are expressed in cholinergic neurons within the striatum, striatopallidal neurons, and midbrain DA projection neurons, we expected GRK2 deletion in all of these subpopulations in *D2R*^{cre}*Grk2*$^{f/f}$ mice. To confirm Cre-mediated recombination in the intended cellular populations, *D1R*^{cre} and *D2R*^{cre} mice were also crossed to Rosa26-EYFP (R26-EYFP) reporter mice, and the brains were fixed and processed for immunostaining against EYFP (anti-GFP) and met-Enkephalin (anti-Enk), a marker of striatopallidal MSNs (Supplementary Figures S1 and S2). We detected Cre-mediated recombination in the expected brain regions (including strong anti-GFP staining in striatonigral MSNs and cortical layer VI neurons for the *D1R*^{cre} line and in striatopallidal MSNs for the *D2R*^{cre} line), consistent with the known expression patterns for endogenous D1 and D2 DA receptors (Supplementary Figures S1A, 1B, S2A, and 2B). In addition, co-labeling of EYFP with Enk was observed in brain sections from *D2R*^{cre}R26-EYFP but not *D1R*^{cre}R26-EYFP mice (Supplementary Figures S1A, S1B S2A, and S2B). Deletion of GRK2 from D1R- and D2R-expressing neurons was also verified using an isoform-specific GRK2 antibody (Ahmed *et al*, 2008b) and an antibody directed against GFP. Here we observed, in part, deletions of GRK2 within the cortex in the D1R line (Supplementary Figure S1C) and within striatal cholinergic neurons (Supplementary Figure S2C) and DA neurons in the D2R line (data not shown). Collectively, these results confirm that *D1R*^{cre}*Grk2*$^{f/f}$ and *D2R*^{cre}*Grk2*$^{f/f}$ mice exhibit selective deletion of GRK2 in genetically defined D1R- and D2R-expressing cellular populations, respectively.

To investigate the role of GRK2 in DA-dependent behaviors, we first analyzed spontaneous locomotion and the effects of cocaine in mice with the conditional deletion of GRK2 from D1R-expressing neurons. Although no difference in baseline locomotor activity between genotypes was observed in drug-naive mice, the acute administration of cocaine to *D1R*^{cre}*Grk2*$^{f/f}$ mice resulted in significantly enhanced psychomotor activation relative to control animals (interaction, $F_{(23,897)} = 2.71$, $p < 0.0001$; genotype, $F_{(1,897)} = 9.39$, $p = 0.0039$; Figure 1a and b). Analysis of the sum of the distance travelled 90 min postinjection of 10 or 20 mg/kg cocaine (i.p.) revealed a 2.5- and a 1.6-fold increase, respectively, in the activity of *D1R*^{cre}*Grk2*$^{f/f}$ mice compared with control animals (Figure 1c). To determine whether GRK2 deficiency in D1R-expressing neurons altered sensitization to cocaine, we next evaluated the locomotor activity of *D1R*^{cre}*Grk2*$^{f/f}$ mice following repeated, daily injections of cocaine (20 mg/kg, i.p.). As expected, *D1R*^{cre}*Grk2*$^{f/f}$ mice were hypersensitive to the initial drug exposure, displaying increased activity on day 1 of the treatment regimen (interaction, $F_{(23,506)} = 2.19$, $p = 0.0012$; genotype, $F_{(1,506)} = 11.05$, $p = 0.0031$; Figure 1d, left and $p = 0.0039$; Figure 1e). However, repeated cocaine injections produced robust sensitization in both genotypes, with no significant difference in activity observed between groups on day 7 (Figure 1d, right and Figure 1e). These data suggest that one function of GRK2 in D1R-expressing neurons may be to negatively regulate psychomotor activation induced by acute cocaine treatment.

In direct contrast to the D1R conditional KO line, *D2R*^{cre}*Grk2*$^{f/f}$ mice exhibited increased spontaneous locomotor

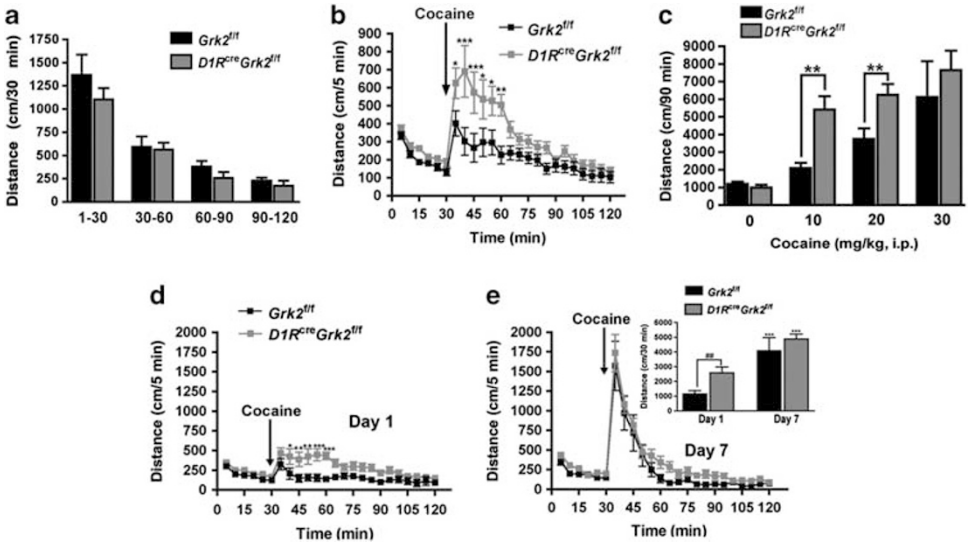

Figure 1 Deletion of GRK2 from D1R-expressing neurons promotes hypersensitivity to cocaine. (a) Spontaneous locomotor activity of control and *D1R*^{cre}*Grk2*$^{f/f}$ mice ($n = 9$ mice per group) analyzed in 30-min intervals over a 2-h period. (b) Locomotor activity of mice (*Grk2*$^{f/f}$ mice, $n = 20$; *D1R*^{cre}*Grk2*$^{f/f}$ mice, $n = 21$) administered cocaine (20 mg/kg, i.p.). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, comparison of drug effect between genotypes by two-way RM ANOVA. (c) Cocaine dose-response for *D1R*^{cre}*Grk2*$^{f/f}$ ($n = 6$–21) and *Grk2*$^{f/f}$ ($n = 6$–20) mice. $^{**}p < 0.01$, comparison relative to control mice by unpaired Student's *t*-test. (d) Cocaine sensitization in *D1R*^{cre}*Grk2*$^{f/f}$ and *Grk2*$^{f/f}$ mice ($n = 8$–12 mice for each group). Locomotor activity of cocaine-treated mice on days 1 and 7 are shown. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ for comparison of the drug effect between genotypes by two-way RM ANOVA. (e) Cumulative distance traveled by cocaine-treated mice in the first 30 min postinjection of cocaine. $^{\#\#}p < 0.01$ for comparison of the drug effect between genotypes on day 1 and $^{***}p < 0.001$ for comparison of the drug effect on day 1 *vs* day 7 by unpaired Student's *t*-test.

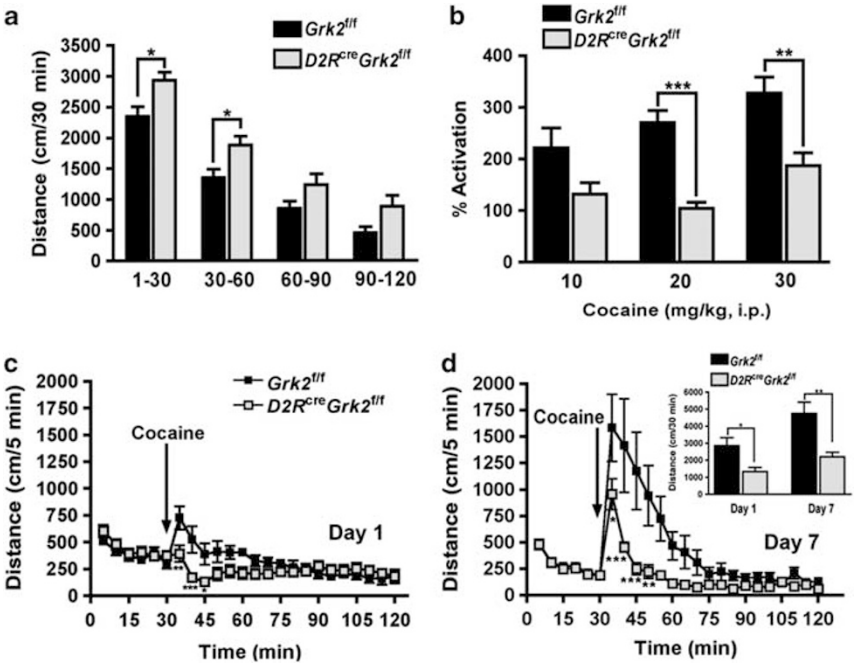

Figure 2 Deletion of GRK2 from D2R-expressing neurons enhances spontaneous locomotion and decreases sensitivity to cocaine. (a) Spontaneous locomotion of *Grk2*$^{f/f}$ ($n=19$) and *D2R*^{cre}*Grk2*$^{f/f}$ ($n=17$) mice analyzed in 30-min intervals over a 2-h period. *$p<0.05$, comparison between genotypes by two-way RM ANOVA. (b) Cocaine-induced psychomotor activation of *Grk2*$^{f/f}$ ($n=8$–16) and *D2R*^{cre}*Grk2*$^{f/f}$ ($n=8$–15) mice expressed as a percentage of activation (or fold increase) relative to vehicle-treated animals of the same genotype. **$p<0.01$ and ***$p<0.001$, comparison of the drug effect between genotypes by unpaired Student's *t*-test. (c) Cocaine sensitization in *D2R*^{cre}*Grk2*$^{f/f}$ and *Grk2*$^{f/f}$ mice ($n=7$–8 mice for each group). Locomotor activity in cocaine-treated mice was analyzed on days 1 and 7. *$p<0.05$, **$p<0.01$, ***$p<0.001$, comparison of the drug effect between genotypes by two-way RM ANOVA. (d) Cumulative distance traveled by cocaine-treated mice in the first 30 min postinjection of cocaine. *$p<0.05$ and **$p<0.01$, comparison of the drug effect between genotypes on days 1 and 7 by unpaired Student's *t*-test. (e) Apomorphine-induced vertical activity in control and *D2R*^{cre}*Grk2*$^{f/f}$ mice ($n=10$–12 mice for each group). *$p<0.05$, comparison of the drug effect between genotypes by unpaired Student's *t*-test.

activity (genotype, $F_{(1,102)}=9.14$, $p=0.0047$; Figure 2a) and reduced responsiveness to acute cocaine (20 mg/kg, i.p.) treatment (Figure 2b). Analysis of the fold increase in locomotor activity induced by cocaine (fold activation) revealed a robust reduction in responsiveness of *D2R*^{cre}*Grk2*$^{f/f}$ mice to multiple independent doses of the stimulant relative to control animals ($p<0.0001$ and $p=0.0033$, 20 and 30 mg/kg doses, respectively; Figure 2b). A cocaine sensitization paradigm was used to determine whether *D2R*^{cre}*Grk2*$^{f/f}$ mice could mount a response to a sensitizing regimen of cocaine (20 mg/kg, i.p.). This analysis revealed that *D2R*^{cre}*Grk2*$^{f/f}$ mice were significantly less active following cocaine treatment on both days 1 and 7 relative to control animals (day 1: interaction, $F_{(23,299)}=4.02$, $p<0.0001$ and day 7: interaction, $F_{(23,299)}=3.24$, $p<0.0001$; genotype, $F_{(1,299)}=8.78$, $p=0.011$; Figure 2c and d). Interestingly, peak activity (35 min time point) on day 1 *vs* day 7 for conditional KO mice was increased (393 ± 77 *vs* 960 ± 145 cm), indicating some degree of sensitization to the locomotor response. However, analysis of the total distance travelled 30 min postinjection revealed a significant reduction in activity of *D2R*^{cre}*Grk2*$^{f/f}$ mice relative to control animals on both days (day 1, $p=0.0167$; and day 7, $p=0.0036$; Figure 2d). These results strongly suggest that GRK2 in D2R-expressing neurons has a role during spontaneous locomotion and is required for the locomotor-stimulating effects of cocaine.

To determine whether the rewarding properties of cocaine were altered by the selective deletion of GRK2, we used a conditioned place preference paradigm. Independent cohorts of *D1R*^{cre}*Grk2*$^{f/f}$ and *D2R*^{cre}*Grk2*$^{f/f}$ mice were conditioned to two different doses of cocaine (10 and 20 mg/kg, i.p.), and their preference for the drug-paired chamber was analyzed. Robust conditioned responses were observed, and no significant differences were identified between genotypes, suggesting that GRK2 in D1R- and D2R-expressing neurons does not have a role in associative learning and cocaine reward (Supplementary Figure S3A and B).

The phenotypes observed in *D1R*^{cre}*Grk2*$^{f/f}$ and *D2R*^{cre}*Grk2*$^{f/f}$ mice are not due to the BAC transgenes, because both *D1R*^{cre} and *D2R*^{cre} hemizygous mice showed no spontaneous locomotor phenotype (Supplementary Figures S1D, 1F S2D, and F) and exhibited normal locomotor activity responses to cocaine (Supplementary Figures S1E, S1F S2E, and S2F). These results argue against any contribution of BAC transgene expression or integration site to DA-related phenotypes in these animals and are consistent with other published observations using these BAC transgenic animals (Bateup *et al*, 2010; Guzman *et al*, 2011).

Enhanced DARPP-32 Signaling in the Striatum of Cocaine-Treated *D1R*^{cre}*Grk2*$^{f/f}$ Mice

To determine whether the hypersensitivity phenotype of *D1R*^{cre}*Grk2*$^{f/f}$ mice was accompanied by alterations in striatal signaling, we next analyzed phospho-DARPP-32 levels because (1) cocaine has been shown to increase the phosphorylation of DARPP-32 at threonine 34 (T34) selectively in striatonigral neurons (Bateup *et al*, 2008) and

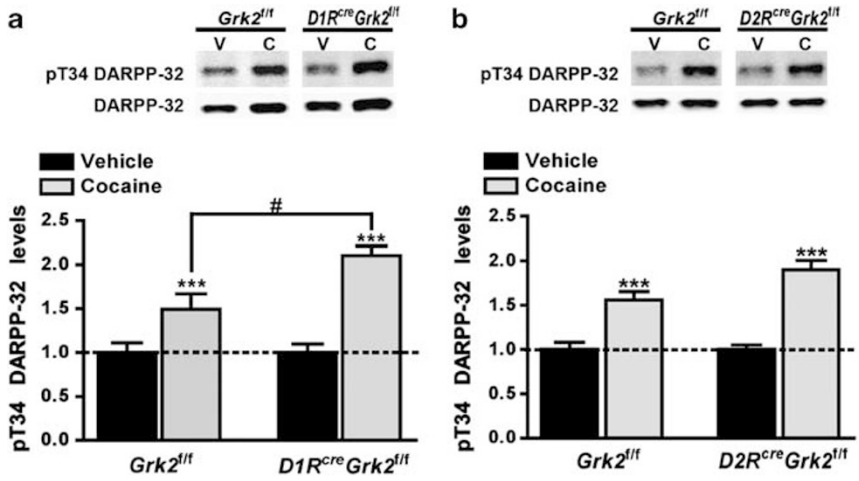

Figure 3 Cocaine-induced DARPP-32 phosphorylation is enhanced in the striatum of *D1R*^{cre}*Grk2*$^{f/f}$ mice. (a and b) Mice were injected with either vehicle or cocaine (20 mg/kg, i.p.) and killed 15 min postinjection. pT34 DARPP-32 and total DARPP-32 levels were measured in extracts prepared from the dorsal striatum. Representative western blots (upper panels; V = vehicle and C = cocaine) and the corresponding densitometric analyses are shown. $n = 4$–8 mice for each treatment group. pT34 DARPP-32 levels were normalized to the levels of DARPP-32 in the respective striatal extracts. ***$p < 0.001$, vehicle *vs* cocaine treatment within genotypes and $^{\#}p < 0.05$ for comparison between genotypes of the drug effect by two-way ANOVA.

(2) because DARPP-32 regulates basal and cocaine-induced locomotion (Bateup *et al*, 2010). Here we observed that acute cocaine administration promoted a robust increase in striatal pT34 DARPP-32 levels in both genotypes (interaction, $F_{(1,17)} = 34.47$, $p < 0.0001$); however, the effect was significantly enhanced in *D1R*^{cre}*Grk2*$^{f/f}$ mice relative to control animals ($210 \pm 11\%$ *vs* $149 \pm 17\%$ of control, respectively; interaction, $F_{(1,17)} = 5.06$, $p = 0.03$; Figure 3a). In contrast, a similar magnitude response to cocaine was observed in *D2R*^{cre}*Grk2*$^{f/f}$ mice (Figure 3b). Evaluation of basal pT34 DARPP-32 levels in drug-naive *D1R*^{cre}*Grk2*$^{f/f}$ and *D2R*^{cre}*Grk2*$^{f/f}$ mice revealed no significant differences relative to matched controls (data not shown). These results suggest that GRK2 deletion in D1R-expressing neurons selectively enhances striatal D1R signaling.

To determine whether D2R-mediated Akt and GSK3 signaling in striatopallidal neurons was disrupted by selective GRK2 deletion, we analyzed the levels of phosphorylated threonine 308 (T308) Akt and serine 9 (S9) GSK3 in the striatum of both conditional KO lines. This analysis revealed no significant differences in the basal levels of striatal pT308 Akt and pS9 GSK3 in *D1R*^{cre}*Grk2*$^{f/f}$ and *D2R*^{cre}*Grk2*$^{f/f}$ mice relative to matched control animals (Supplementary Figure S4A–D). Furthermore, we observed no differences between genotypes in striatal Akt and GSK3 signaling following acute cocaine exposure (data not shown). These results suggest that GRK2 deficiency in D1R- or D2R-expressing neurons does not alter βarrestin-2-dependent D2R signaling in striatopallidal neurons, and appear to be consistent with the observation that GRK2 phosphorylation is not required for the association of D2R with βarrestin-2 (Namkung *et al*, 2009a).

D1R^{cre}*Grk2*$^{f/f}$ Mice Exhibit Enhanced DA Release, Uptake, and Sensitivity to Cocaine

To investigate whether GRK2 deficiency in D1R- or D2R-expressing neurons caused perturbations to the DA system, we measured evoked DA release and uptake using FSCV in dorsal striatal slices prepared from the conditional KO mice. We observed that, in slices from drug-naive *D1R*^{cre}*Grk2*$^{f/f}$ mice, electrically evoked DA release (either to single or multiple stimuli) was robustly increased ($\sim$2-fold at each frequency) relative to that measured in slices from control animals (genotype, $F_{(1,30)} = 24.08$, $p = 0.0027$; Figure 4a and b). Additionally, as shown in Figure 4c, we found that the maximal rate of DA uptake (V_{max}) was dramatically increased in slices from *D1R*^{cre}*Grk2*$^{f/f}$ mice ($p = 0.038$). This robust increase in V_{max} levels was similar to what we frequently observe in cocaine-treated wild-type animals (data not shown) and may be due to an increase in cell-surface DAT levels as has been previously described for cocaine (Brown *et al*, 2001; Daws *et al*, 2002).

To determine whether *D1R*^{cre}*Grk2*$^{f/f}$ mice were also hypersensitive to cocaine at the neurochemical level, we measured evoked DA release in the presence of cocaine (10 μM) in slices from both genotypes. This analysis revealed a significant increase in evoked DA levels in striatal slices prepared from *D1R*^{cre}*Grk2*$^{f/f}$ mice ($p = 0.03$; Figure 4d). Interestingly, we did not observe a difference in the rate of DA uptake inhibition (τ) between genotypes (data not shown), indicating that GRK2 deficiency in D1R-expressing neurons does not alter the effectiveness of cocaine at blocking DA reuptake.

As D2 autoreceptors present on DA neurons have been shown to tightly regulate striatal DA release (Bello *et al*, 2011), we next investigated whether the neurochemical phenotype observed in *D1R*^{cre}*Grk2*$^{f/f}$ mice was due to altered D2 autoreceptor activity. Evoked DA release in striatal slices was measured in the presence of the D2R antagonist sulpiride (10 μM) and expressed as a percentage of the baseline value (ie, release at one pulse; Figure 4e). Here we observed that sulpiride did not affect DA release in sections prepared from either genotype. To further evaluate D2 autoreceptor function in this mouse line, we measured

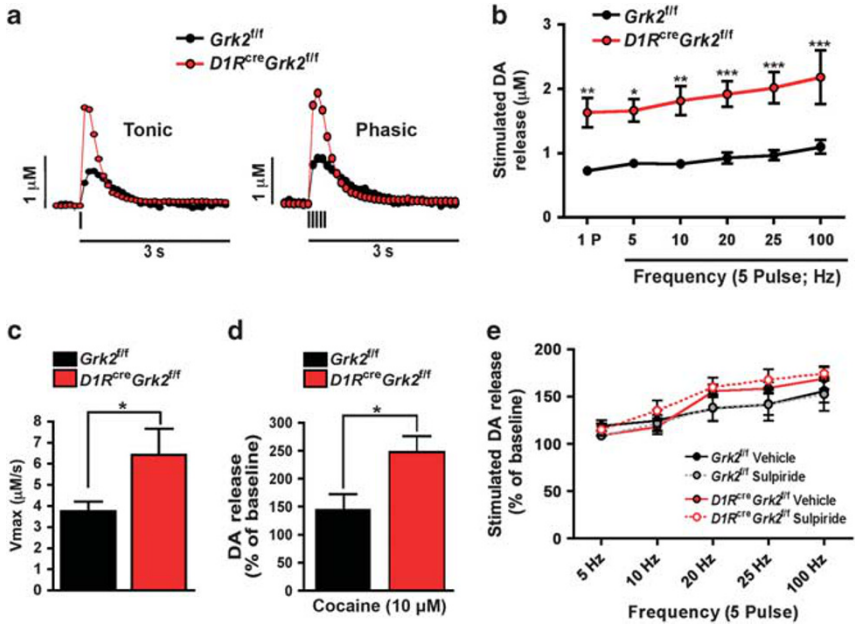

Figure 4 Enhanced baseline and cocaine-induced DA release in $D1R^{cre}Grk2^{f/f}$ mice. (a) Representative DA traces (no drug applied) from electrical stimulations that mimic tonic (one pulse) and phasic (five pulses at 25 Hz) firing of DA neurons in slices prepared from drug-naive $Grk2^{f/f}$ (black) and $D1R^{cre}Grk2^{f/f}$ mice (red). (b) Frequency–DA response curves for slices from control and $D1R^{cre}Grk2^{f/f}$ mice ($n=3$–5 mice for each group). $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$, $Grk2^{f/f}$ *vs* $D1R^{cre}Grk2^{f/f}$ by two-way RM ANOVA. (c) Maximal rates of DAT uptake (V_{max}) in slices prepared from drug-naive $Grk2^{f/f}$ ($n=11$) and $D1R^{cre}Grk2^{f/f}$ ($n=8$) mice. $^{*}p<0.05$, $Grk2^{f/f}$ *vs* $D1R^{cre}Grk2^{f/f}$ by unpaired Student's *t*-test. (d) Cocaine response (expressed as percentage of predrug baseline) demonstrates the relationship between bath-applied cocaine (10 μM) and electrically stimulated DA release. Slices were prepared from drug-naive $Grk2^{f/f}$ ($n=5$) and $D1R^{cre}Grk2^{f/f}$ ($n=4$) mice. $^{*}p<0.05$, $Grk2^{f/f}$ *vs* $D1R^{cre}Grk2^{f/f}$ by unpaired Student's *t*-test. (e) Frequency–response curves (expressed as percentage of one-pulse stimulation) before (solid lines) and after (dotted lines) bath application of sulpiride (10 μM). $n=4$–6 mice for each group.

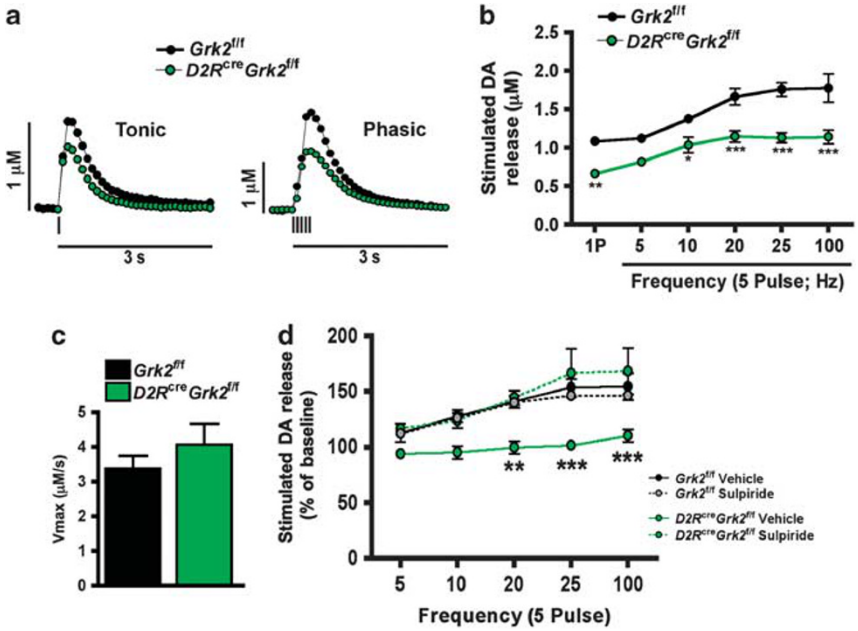

Figure 5 Depressed baseline DA release and increased D2 autoreceptor activity in $D2R^{cre}Grk2^{f/f}$ mice. (a) Representative DA traces (no drug applied) from electrical stimulations that mimic tonic and phasic firing of DA neurons in slices prepared from drug-naive $Grk2^{f/f}$ (black) and $D2R^{cre}Grk2^{f/f}$ mice (green). (b) Frequency–DA response curves for control and conditional KO mice ($n=4$–5 mice for each group). $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$, $Grk2^{f/f}$ *vs* $D2R^{cre}Grk2^{f/f}$ mice by two-way RM ANOVA. (c) Maximal rates of DAT uptake in drug-naive $Grk2^{f/f}$ ($n=6$) and $D2R^{cre}Grk2^{f/f}$ ($n=10$) mice. (d) Frequency–response curves before (solid lines) and after (dotted lines) bath application of sulpiride (10 μM). $n=4$–7 mice for each group. $^{**}p<0.01$ and $^{***}p<0.001$, vehicle *vs* sulpiride-treated $D2R^{cre}Grk2^{f/f}$ mice by two-way RM ANOVA.

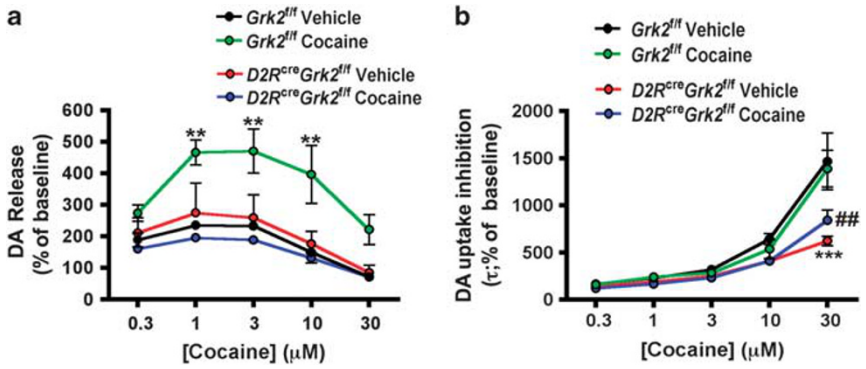

Figure 6 Reduced cocaine-induced sensitization of DA release and inhibition of DA uptake in $D2R^{cre}Grk2^{f/f}$ mice. (a and b) Cocaine concentration–response curves in slices from $Grk2^{f/f}$ ($n=3$) and $D2R^{cre}Grk2^{f/f}$ mice treated acutely (vehicle group) or exposed repeatedly to cocaine (cocaine group) demonstrate the relationship between bath-applied cocaine (10 μM) and electrically stimulated DA release (a) or DA uptake inhibition (b). $^{**}p<0.01$, vehicle *vs* cocaine-treated $Grk2^{f/f}$ mice in panel (a). $^{***}p<0.001$, comparison of acute cocaine treatment groups (black *vs* red in panel (b) by two-way RM ANOVA. $^{\#\#}p<0.01$, cocaine-sensitized $Grk2^{f/f}$ mice *vs* cocaine-sensitized $D2R^{cre}Grk2^{f/f}$ mice (green *vs* blue in panel (b) by two-way RM ANOVA.

evoked DA release in the presence of quinpirole, a direct D2R agonist. This analysis revealed no significant differences in quinpirole-induced inhibition of DA release between genotypes, regardless of treatment history (Supplementary Figure S5). Collectively, these results strongly suggest that GRK2 deficiency in D1R-expressing neurons does not alter D2 autoreceptor function.

Reduced DA Release and Increased D2 Autoreceptor Activity in $D2R^{cre}Grk2^{f/f}$ Mice

Conversely, measurement of DA release evoked by a single or multiple stimuli in slices prepared from drug-naive $D2R^{cre}Grk2^{f/f}$ mice revealed a robust decrease in baseline DA release relative to that measured in control slices (genotype, $F_{(1,35)}=39.35$, $p=0.0004$; Figure 5a and b). The depression of baseline DA levels was not due to alterations in the rate of DA uptake, because measured V_{max} levels were similar in slices between $D2R^{cre}Grk2^{f/f}$ and control animals (Figure 5c). To determine whether altered D2 autoreceptor receptor activity in $D2R^{cre}Grk2^{f/f}$ mice may underlie the hypodopaminergia phenotype, we bath applied sulpiride to slices prepared from drug-naive mice of both genotypes following electrical stimulation. As expected, stimulated DA release in $D2R^{cre}Grk2^{f/f}$ slices was reduced relative to controls (solid lines, black *vs* green; interaction, $F_{(4,40)}=4.95$, $p=0.0025$; genotype, $F_{(1,40)}=22.08$, $p=0.0008$; Figure 5d). But interestingly, while sulpiride treatment had no effect in control slices, D2 autoreceptor antagonism relieved the blunted DA release observed in KO slices (dashed *vs* solid green lines; interaction, $F_{(4,32)}=2.74$, $p=0.04$; genotype, $F_{(1,32)}=28.98$, $p=0.0007$; Figure 5d). These findings suggest that under basal conditions, GRK2 regulates D2 autoreceptor function in this cell population, and the absence of this kinase leads directly to increased receptor activity and dampened evoked DA release.

$D2R^{cre}Grk2^{f/f}$ Mice Exhibit Reduced Neurochemical Sensitivity to Cocaine

We next examined whether the deficiency of GRK2 in D2R-expressing neurons altered sensitivity to cocaine at the neurochemical level. For these studies, mice were injected with either vehicle or cocaine (20 mg/kg, i.p.) once daily for 5 consecutive days, and on day 7 (approximately 40 h after the last injection) striatal slices were prepared from cocaine-sensitized mice (green and blue groups; Figure 6) and vehicle-treated control animals (black and red groups; Figure 6). Cocaine was then bath applied to slices from both treatment groups, and evoked DA release was measured. As expected, repeated drug administration to control mice resulted in a robust dose-dependent enhancement in cocaine-induced DA release and overflow (black *vs* green lines; genotype, $F_{(1,16)}=15.42$, $p=0.017$; Figure 6a and b). In contrast, cocaine at all concentrations tested did not augment evoked DA release in sections from sensitized-$D2R^{cre}Grk2^{f/f}$ mice (red *vs* blue lines; Figure 6a). Additionally, we analyzed the relationship between increasing concentrations of bath-applied cocaine and rates of DA uptake. These measurements revealed that cocaine was less effective at inhibiting DA uptake in $D2R^{cre}Grk2^{f/f}$ mice regardless of vehicle or cocaine treatment (vehicle groups: interaction, $F_{(4,28)}=6.01$, $p=0.0013$; genotype, $F_{(1,28)}=8.31$, $p=0.02$ and cocaine groups: interaction, $F_{(4,20)}=5.28$, $p=0.0046$; Figure 6b). These findings strongly suggest that GRK2 in D2R-expressing neurons is required for cocaine-induced sensitization of DA release and cocaine-mediated inhibition of DA uptake in the striatum.

$DAT^{cre}Grk2^{f/f}$ Mice are Hyperactive and Less Sensitive to Acute Cocaine Treatment

Because $D2R^{cre}Grk2^{f/f}$ mice have GRK2 deleted in both postsynaptic DA-receptive neurons (ie, striatopallidal MSNs) and presynaptic DA neurons, we next sought to discern the role of GRK2 in these two distinct cell types using a genetic approach. We generated two independent lines of conditional KO mice in which GRK2 was selectively deleted from either presynaptic DA neurons or postsynaptic A2AR-expressing striatopallidal MSNs. This was accomplished by crossing $Grk2^{f/f}$ mice to either DAT^{cre} or $A2AR^{cre}$ mouse lines to yield conditional KO mice ($DAT^{cre}Grk2^{f/f}$ and $A2AR^{cre}Grk2^{f/f}$) and control littermates. Immunostaining with cell-type-specific markers (DAT and DARPP-32)

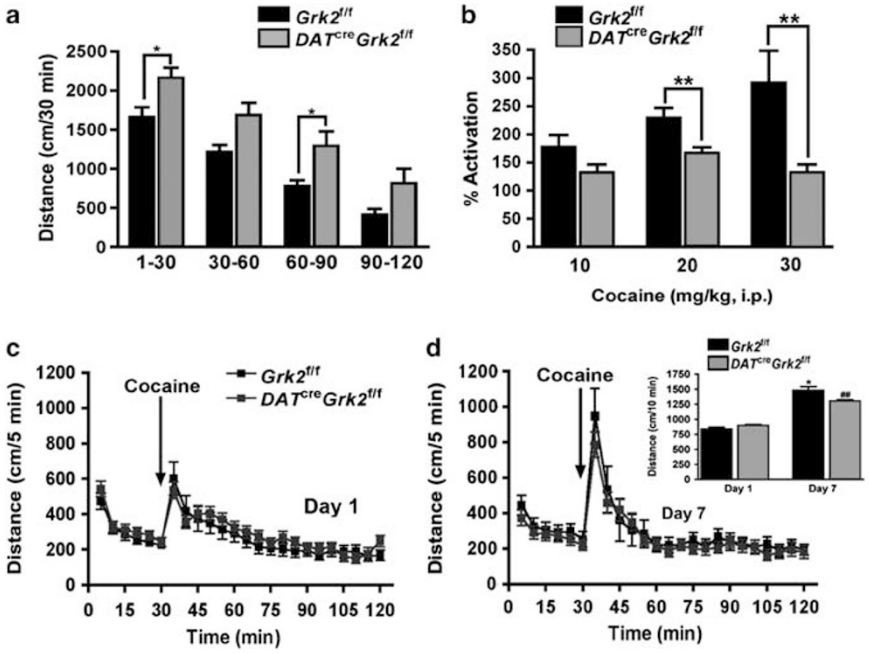

Figure 7 *DAT*^{cre}*Grk2*$^{f/f}$ mice exhibit enhanced spontaneous locomotion and reduced sensitivity to cocaine. (a) Spontaneous locomotion of *Grk2*$^{f/f}$ and *DAT*^{cre}*Grk2*$^{f/f}$ ($n = 20$ mice per group) mice was analyzed in 30-min intervals over a 2-h period. $^{*}p < 0.05$, comparison between genotypes by two-way RM ANOVA. (b) The change in locomotor activity (expressed as a percentage) of *Grk2*$^{f/f}$ ($n = 6$–20) and *DAT*^{cre}*Grk2*$^{f/f}$ ($n = 8$–20) mice following acute cocaine treatment. $^{*}p < 0.05$ and $^{**}p < 0.01$, comparison of the drug effect between genotypes by unpaired Student's *t*-test. (c) Cocaine sensitization in *DAT*^{cre}*Grk2*$^{f/f}$ and *Grk2*$^{f/f}$ mice ($n = 19$–21 mice for each group). Locomotor activity in cocaine-treated mice on days 1 and 7 are shown. (d) Cumulative distance traveled by cocaine-treated mice in the first 10 min postinjection of cocaine. $^{*}p < 0.05$ and $^{\#\#}p < 0.01$, comparison of the drug effect between days 1 and 7 in each respective genotype by unpaired Student's *t*-test.

confirmed Cre-mediated recombination in the intended cell populations (Supplementary Figures S6A and S7). Interestingly, we did not observe GRK2 expression in A2AR-expressing MSNs within the dorsal striatum or a notable loss of GRK2 staining in the striatum of *A2AR*^{cre}*Grk2*$^{f/f}$ KO mice (Supplementary Figure S7A and data not shown). These results strongly suggest that GRK2 is not expressed at measurable levels in MSNs and are consistent with the recent observation that GRK2 is most highly expressed in cholinergic interneurons within the striatum (Bychkov *et al*, 2012; Daigle and Caron, 2012).

Similar to the *D2R*^{cre}*Grk2*$^{f/f}$ line, *DAT*^{cre}*Grk2*$^{f/f}$ mice were hyperactive over a 2-h period relative to control animals (genotype, $F_{(1,114)} = 9.20$, $p = 0.0043$; Figure 7a). In addition to the marked hyperactivity, *DAT*^{cre}*Grk2*$^{f/f}$ mice also exhibited reduced sensitivity to acute cocaine administration. Analysis of the fold activation induced by cocaine over a 90-min period revealed a striking reduction in responsiveness of *DAT*^{cre}*Grk2*$^{f/f}$ mice to two doses of the stimulant relative to control animals ($p = 0.0045$ and $p = 0.0097$, 20 and 30 mg/kg doses, respectively; Figure 7b). In contrast, analysis of locomotor activity following repeated cocaine injections revealed no significant differences between genotypes on days 1 or 7 (Figure 7c and d). Additionally, no difference in cocaine reward was observed in the conditioned place preference paradigm (Supplementary Figure S3C). To confirm that mice heterozygous for the *DAT*^{cre} allele alone do not have altered locomotor behavior, we performed open field studies on *DAT*^{cre} heterozygous animals and wild-type littermates (Supplementary Figure S6). We observed no differences in the total distance travelled by drug-naive or cocaine-treated animals, indicating that the insertion of the IRES-Cre at the endogenous DAT locus does not significantly alter locomotor behavior (Supplementary Figure S6B–D). Taken together, these results indicate that GRK2 in DA neurons has a critical role in the regulation of spontaneous locomotion and acute cocaine-induced psychomotor activation.

A2AR^{cre}*Grk2*$^{f/f}$ Mice Exhibit Normal Locomotor Behavior and Sensitivity to Cocaine

Although we were unable to detect appreciable GRK2 expression in A2AR-expressing striatal MSNs (Supplementary Figures S7 and S8), we could not exclude the possibility that an extremely low, yet functionally relevant level of GRK2 was present in this cell population. We therefore performed the same battery of behavioral experiments in *A2AR*^{cre}*Grk2*$^{f/f}$ mice as with the other conditional KO lines (Figure 8). However, analysis of spontaneous locomotor activity revealed no significant differences between genotypes (Figure 8a and c). Further studies with cocaine also revealed no significant differences between genotypes in sensitivity to either an acute cocaine exposure or to a sensitizing regimen of cocaine (Figure 8b–d). Additionally, cocaine-induced conditioned place preference was unaltered in *A2AR*^{cre}*Grk2*$^{f/f}$ mice (Supplementary Figure S3D). These results strongly suggest that the hyperactivity and hyposensitivity to cocaine phenotypes observed in D2R^{cre}Grk2$^{f/f}$ mice are not due to a deficiency of GRK2 within postsynaptic striatopallidal MSNs.

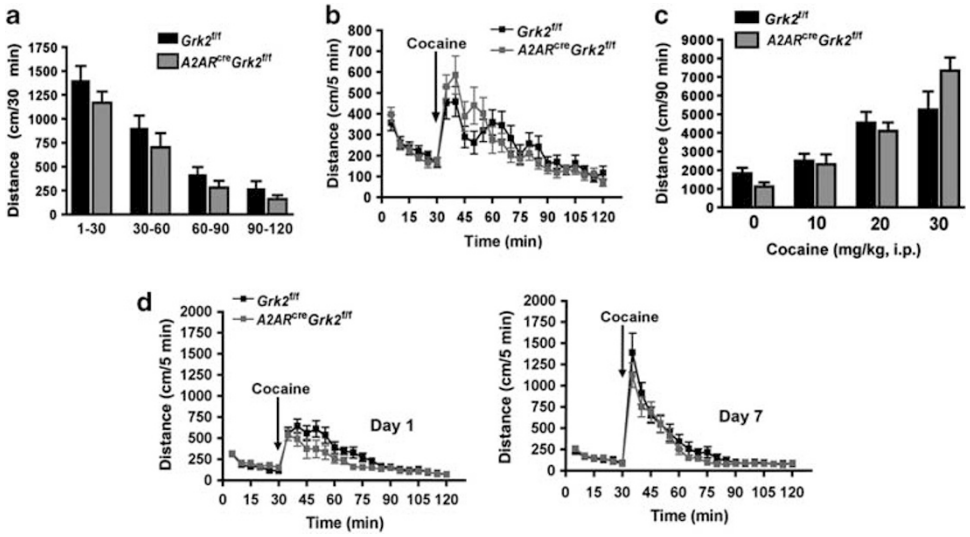

Figure 8 Deletion of GRK2 from A2AR-expressing neurons does not alter basal locomotor activity or sensitivity to cocaine. (a and b) Spontaneous activity (analyzed in 30-min intervals over a 2-h period) or cocaine-induced (20 mg/kg, i.p.) locomotion of $Grk2^{f/f}$ and $A2AR^{cre}Grk2^{f/f}$ mice (vehicle, $n = 13$–16 mice per group; cocaine, $n = 15$ mice per group). (c) The sum of distance travelled by $Grk2^{f/f}$ and $A2AR^{cre}Grk2^{f/f}$ mice postinjection of vehicle or cocaine ($n = 8$–16 mice per group). (d) Cocaine sensitization in $Grk2^{f/f}$ and $A2AR^{cre}Grk2^{f/f}$ mice ($n = 13$–16 mice for each group). Locomotor activity in cocaine-treated (20 mg/kg, i.p.) mice on days 1 and 7 are shown.

DISCUSSION

Although previous studies have implicated GRK2 as an essential mediator of DA receptors *in vitro*, no investigations to date have examined whether the deficiency of this kinase in the mature brain alters DA receptor function or animal behaviors classically associated with the actions of DA. Here we used a genetic strategy to dissect GRK2 function within the mammalian brain and present evidence in support of a cell-type-specific role for GRK2 in the control of spontaneous locomotion, DA neurotransmission, D1R signaling, D2 autoreceptor function, and the behavioral response to psychostimulants. Collectively, our behavioral, biochemical, and electrophysiological results (largely summarized in Supplementary Table S2) provide novel mechanistic insight into DA receptor function *in vivo* and the actions of psychostimulant drugs in the murine brain. Although our current efforts were arguably DA-centric, it is plausible that GRK2 deficiency alters the function of additional GPCRs within the studied neuronal populations. This possibility should be examined in greater detail in future studies.

Earlier work with GRK5 and GRK6 germline KO mice revealed a very modest phenotype in unchallenged animals, suggesting that these kinases have little impact on diverse physiological processes under basal conditions (Gainetdinov *et al*, 2003; Gainetdinov *et al*, 1999). Similar to these findings, we observed no overt behavioral phenotype in drug-naive mice lacking GRK2 in either cholinergic (Daigle and Caron, 2012) or D1R-expressing neurons (present study). In contrast, the selective deletion of GRK2 in D2R-expressing neurons resulted in increased spontaneous locomotor activity, decreased apomorphine-induced vertical activity, and impaired habituation to a novel environment (data not shown). Basal hyperactivity was also observed in mice lacking GRK2 in DA neurons, but interestingly, not in mice with GRK2 deficiency in postsynaptic A2AR-expressing striatopallidal MSNs. The similar impairment in baseline locomotion between conditional KO mice (ie, $D2R^{cre}Grk2^{f/f}$ mice *vs* $DAT^{cre}Grk2^{f/f}$ mice) strongly suggests that the hyperactivity phenotype of $D2R^{cre}Grk2^{f/f}$ mice is caused by the loss of GRK2 in DA neurons.

Psychostimulant drugs such as cocaine promote robust increases in extracellular DA levels, resulting in enhanced DA receptor signaling and elevated locomotor activity. Because D1 and D2 receptors have a vital role in mediating the locomotor-stimulating effects of cocaine (Chausmer and Katz, 2001; Xu *et al*, 1994), and because GRK2 can regulate these receptors *in vitro* (Ito *et al*, 1999; Iwata *et al*, 1999; Namkung *et al*, 2009a; 2009b; Sedaghat and Tiberi, 2011; Tiberi *et al*, 1996), we hypothesized that GRK2 may regulate behavioral sensitivity to cocaine. Here we report that the deletion of GRK2 in D1R- and D2R-expressing neurons causes opposite effects on acute cocaine-induced locomotion, resulting in hypersensitive or hyposensitive mice, respectively. Interestingly, while all cocaine-mediated effects remained intact following deletion of GRK2 from A2AR-expressing striatopallidal MSNs (present study) or cholinergic neurons (Daigle and Caron, 2012), mice with GRK2 deficiency in DA neurons were also markedly less sensitive to the acute effects of cocaine. Collectively, these results strongly suggest that the reduced sensitivity of $D2R^{cre}Grk2^{f/f}$ mice to cocaine is primarily due to the loss of GRK2 function in DA neurons.

Sensitization to the locomotor-stimulating effects of cocaine was also dramatically reduced by the deficiency of GRK2 in D2R-expressing neurons, but unlike for spontaneous and acute cocaine-induced locomotion, this behavioral impairment was not recapitulated in $DAT^{cre}Grk2^{f/f}$ or in $A2AR^{cre}Grk2^{f/f}$ mice. These results suggest that GRK2 function in D2R-expressing neurons outside of the striatum may be required for the full expression of behavioral

sensitization to cocaine. Although further studies are still needed to pinpoint a contributing neuronal population, one promising candidate brain region is the medial prefrontal cortex (mPFC). Beyer and Steketee (2000, 2002) reported that quinpirole infusion into the mPFC blocks acute cocaine-induced locomotor activity and sensitization, suggesting that cortical D2R activation negatively regulates the actions of cocaine. Their behavioral results essentially phenocopy what we observe in $D2R^{cre}Grk2^{f/f}$ mice, raising the possibility that increased activity of cortical D2Rs may also contribute to the reduction in the cocaine-induced effects in these conditional KO animals.

Deletion of GRK2 from D1R-expressing neurons caused a robust increase in striatal DA release and uptake in drug-naive mice. Particularly intriguing was the dramatic increase in V_{max} levels in these mice, because the rate of uptake here was similar to that observed in control animals exposed to cocaine (data not shown). One explanation for these effects is that the loss of GRK2 in this neuronal population causes an increase in the membrane-associated levels of DAT in the striatum. This perturbation could presumably also enhance DA release, because DA recycling into synaptic vesicles should also be augmented in response to a protracted increase in surface DAT levels. The initial increase in cell-surface DAT levels may be caused by elevated glutamate levels in the VTA that result from increased activity of D1Rs located on afferent glutamatergic terminals originating from forebrain regions (Kalivas and Duffy, 1995; Lu *et al*, 1997; Sesack and Pickel, 1992). Enhanced glutamate release directly onto DA neurons may increase neuronal firing frequency, which would thereby result in enhanced striatal DA release. If this were the case, cell-surface DAT levels may increase to compensate for the chronic elevation of extracellular DA levels. We also cannot exclude the possibility that GRK2 deletion altered the activity of additional GPCRs in this neuronal population, and these potential perturbations contributed to the profound neurochemical phenotype. In addition to these baseline alterations, enhanced DA release in response to acute cocaine exposure was also observed in striatal slices from $D1R^{cre}Grk2^{f/f}$ mice. This observation strongly suggests that locomotor supersensitivity to cocaine may be due to elevated drug-induced DA release in the striatum of $D1R^{cre}Grk2^{f/f}$ mice.

In contrast, the elimination of GRK2 from D2R-expressing neurons caused a significant reduction in striatal DA release under basal conditions. Remarkably, we found that this depression could be completely reversed by application of sulpiride, a classical D2R antagonist. These results demonstrate that GRK2 deficiency in D2R-expressing neurons leads to overactivity of presynaptic D2 autoreceptors, which thereby results in the persistent suppression of DA release from striatal DA terminals. Enhanced D2 autoreceptor activity may be due to impaired GRK2-mediated desensitization of these presynaptic receptors. Importantly, the absence of GRK6 has also been reported to enhance striatal D2R activity (Gainetdinov *et al*, 2003), but in contrast to the present findings, this work implicated postsynaptic D2Rs as the principal physiological target of GRK6. Taken together, these data provide the first evidence that the regulation of presynaptic autoreceptors by GRKs occurs in a similar manner to the regulation of postsynaptic receptors, albeit by different GRKs. Furthermore, while it is now evident that multiple GRKs can regulate DA receptors *in vivo*, GRK2- and GRK6-mediated regulation exhibit neuronal specificity and regulate distinct populations of striatal D2Rs (presynaptic *vs* postsynaptic).

The reduced behavioral sensitivity of $D2R^{cre}Grk2^{f/f}$ mice to cocaine was paralleled by changes at the neurochemical level. Here we observed that repeated cocaine treatment of $D2R^{cre}Grk2^{f/f}$ mice was less effective at promoting the expected sensitized increase in striatal DA release. Additionally, we uncovered a dramatic impairment in cocaine-mediated uptake inhibition in the striatum of both drug-naive and drug-sensitized animals. Therefore, in addition to the regulation of D2 autoreceptors, GRK2 deficiency may also impact the sensitivity of DAT for cocaine and/or the availability of releasable DA. Detailed investigation will be required in future work to establish the entire collection of presynaptic targets affected by GRK2 deletion.

Mice with the genetic deletion of D2 autoreceptors exhibit hyperactivity when placed in a novel environment and are hypersensitive to the acute locomotor-stimulating effects of cocaine (Anzalone *et al*, 2012; Bello *et al*, 2011). Our results are partially consistent with these earlier findings in that the reverse perturbation, increased D2 autoreceptor activity, causes reduced locomotor sensitivity to cocaine. However, both $D2R^{cre}Grk2^{f/f}$ mice and D2 autoreceptor-deficient mice exhibited hyperactivity when exposed to a novel environment. This seemingly paradoxical phenotype in baseline locomotor activity between $D2R^{cre}Grk2^{f/f}$ mice and D2 autoreceptor-deficient mice is likely due to the constitutive deletion of GRK2 from D2R-expressing cells, which could lead to multiple aberrant physiological processes in diverse brain regions (eg, potential cortical D2R hyperactivity in the mPFC). It is also important to note that both chronic hyperdopaminergia and hypodopaminergia cause a remarkable array of adaptive changes at the molecular level, including alterations in DA synthesis, storage, and DA receptor levels and functioning, as well as distinct behavioral impairments (Giros *et al*, 1996; Jones *et al*, 1999; Salahpour *et al*, 2008; Sotnikova *et al*, 2005). Therefore, the lack of an opposite behavioral outcome in this context (ie, spontaneous locomotion) may also be due to divergent compensatory mechanisms that exist between genetic models and result from alterations in extracellular DA levels.

The mystery of GRK2 function in the adult brain is beginning to become unraveled. This pursuit has been greatly aided by genetic methods that improve on the traditional gene KO approach and allow for the cell-type-specific deletion of proteins in the mature brain. Here we provide evidence that GRK2 deficiency in select neuronal populations in adult animals profoundly alters cocaine-dependent behaviors and striatal DA neurotransmission. Intriguingly, we found that the deletion of GRK2 in D1R- and D2R-expressing neurons differentially affects locomotor sensitivity to cocaine and striatal DA dynamics. D2 autoreceptor function was also significantly impaired in the absence of GRK2, suggesting that this receptor is robustly regulated by GRK2 under physiological conditions. Taken together, it appears that GRK2 has a vital role in the central DA system and thus may represent a novel therapeutic target for DA-related brain disorders.

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