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RGS9 Modulates Dopamine Signaling in the Basal Ganglia

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Summary

Regulators of G protein signaling (RGS) modulate heterotrimeric G proteins in part by serving as GTPase-activating proteins for Gα subunits. We examined a role for RGS9-2, an RGS subtype highly enriched in striatum, in modulating dopamine D2 receptor function. Viral-mediated overexpression of RGS9-2 in rat nucleus accumbens (ventral striatum) reduced locomotor responses to cocaine (an indirect dopamine agonist) and to D2 but not to D1 receptor agonists. Conversely, RGS9 knockout mice showed heightened locomotor and rewarding responses to cocaine and related psychostimulants. In vitro expression of RGS9-2 in *Xenopus* oocytes accelerated the off-kinetics of D2 receptor-induced GIRK currents, consistent with the in vivo data. Finally, chronic cocaine exposure increased RGS9-2 levels in nucleus accumbens. Together, these data demonstrate a functional interaction between RGS9-2 and D2 receptor signaling and the behavioral actions of psychostimulants and suggest that psychostimulant induction of RGS9-2 represents a compensatory adaptation that diminishes drug responsiveness.

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Introduction

RGS proteins constitute a large family of proteins that potently modulate the functioning of heterotrimeric G proteins in part by stimulating the GTPase activity of the G protein α subunits (reviewed in Dohlman and Thorner, 1997; Berman and Gilman, 1998; De Vries et al., 2000). RGS proteins can thus dampen or help terminate G protein-mediated signaling. In addition, RGS proteins have been shown to alter the kinetics of G protein signaling (Zerangue and Jan, 1998) and may even function as effector molecules in certain signaling networks (De Vries and Farquhar, 1999). As a result, RGS proteins exert complex effects on G protein signaling. While RGS proteins have been shown to negatively modulate G protein-coupled neurotransmitter responses in cultured neurons (Saugstad et al., 1998), the net effect of RGS proteins in an intact neural system remains largely unexplored.

RGS proteins differ in their size, composition, and tissue distribution, but all members contain a conserved, approximately 120 amino acid sequence, referred to as the RGS domain. Since their initial description, the RGS family of proteins has grown rapidly and includes homologs throughout the animal and fungal kingdoms (Ross and Wilkie, 2000). To date, at least 25 mammalian gene products containing the core RGS domain have been identified. Numerous RGS genes are expressed in the brain with highly region-specific expression patterns (Gold et al., 1997).

The expression pattern for one RGS family member, RGS9, is particularly striking. The RGS9 gene gives rise to two products, RGS9-1 and RGS9-2, via alternative splicing (He et al., 1998; Rahman et al., 1999; Zhang et al., 1999). Each splice variant displays highly specific and nonoverlapping tissue distribution. RGS9-1 is expressed exclusively in retina, while RGS9-2 is highly enriched in striatal regions of brain, which include dorsal striatum (caudoputamen), ventral striatum (nucleus accumbens), and olfactory tubercle, with very low levels of expression seen throughout the rest of brain or in peripheral tissues. The striatal-enriched expression of RGS9-2 and its localization in medium spiny projection neurons (Thomas et al., 1998) suggests that it has a role in dopamine-mediated behavior. One feature of striatal neurons is their rich innervation by dopamine and high levels of expression of dopamine receptors (Aizman et al., 2000; Graybiel, 2000). D2 receptors in the nucleus accumbens are particularly important in mediating the locomotor-activating and rewarding effects of psychostimulant drugs of abuse, such as cocaine and amphetamine (Self and Nestler, 1998; Wise, 1998; Koob, 1999).

The goal of the present study was to explore a functional relationship between RGS9-2 and D2 receptors and psychostimulant action in the nucleus accumbens. We describe several experiments undertaken to under-

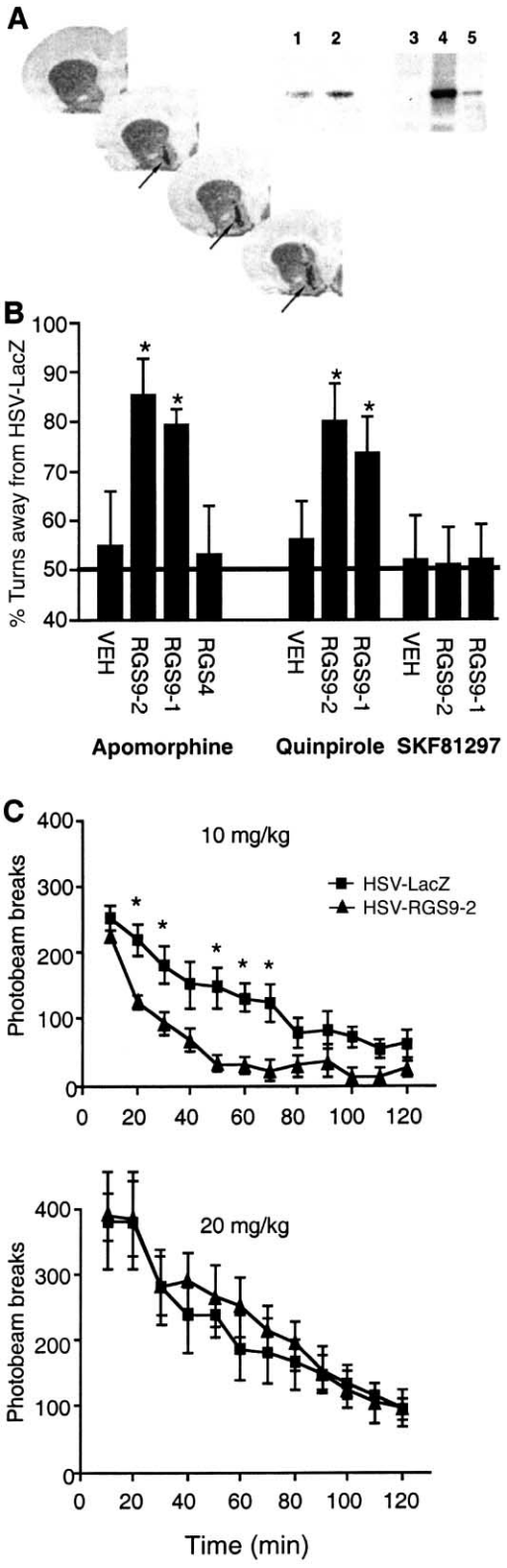

Figure 1. Effect of HSV-Mediated Overexpression of RGS9-2 on Behavioral Responses to Dopaminergic Agonists

(A) Sections of rat brain show highly localized overexpression of RGS9-2 mRNA in the medial aspect of the nucleus accumbens by in situ hybridization in a representative animal 3 days after injection of HSV-RGS9-2 into this brain region (arrows indicate overexpressed RGS9-2 mRNA). Inset shows Western blots labeled for RGS9-2. Lanes 1 and 2, nucleus accumbens from an HSV-RGS9-2-injected animal (lane 2) and the contralateral side injected with HSV-LacZ (lane 1). Levels of RGS9-2 on the HSV-RGS9-2-injected side were 82% ± 5% higher than on the contralateral control side (mean % of control ± SEM, n = 4, $p < 0.05$ by t test). Lanes 3–5, PC12 cells infected with HSV-RGS9-2 (lane 4) or HSV-LacZ (lane 3); lane 5 shows protein extract from normal striatum as a positive control.

(B) (Left) HSV-RGS9-2, HSV-RGS9-1, HSV-RGS4, or sucrose vehicle (VEH) was unilaterally injected in the medial nucleus accumbens of rats; HSV-LacZ was injected on the other side as a control. On days 3 and 4 after surgery, at which time transgene expression is maximal, animals were challenged with apomorphine (1 mg/kg sc) and circling behavior was measured. Data were calculated as % of total turns away from HSV-LacZ during a 40 min test period (n = 6–12). No rotational bias would be indicated as 50% on this scale. Asterisk, significantly different from VEH ($p < 0.01$ by χ^2 test). (Right) Animals received HSV-RGS9-2, HSV-RGS9-1, or VEH injections on one side of the nucleus accumbens, and HSV-LacZ on the other side, and then challenged with the D2-selective agonist quinpirole (1 mg/kg i.p.) or the D1-selective agonist SKF81297 (3 mg/kg s.c.) (n = 8). Quinpirole and SKF81297 produced equivalent degrees of general locomotor activity (~300 total rotations in 40 min; not shown). Asterisk, significantly different from VEH ($p < 0.01$ by χ^2 test).

(C) Animals received bilateral injections of HSV-RGS9-2 or HSV-LacZ, and 3 and 4 days later cocaine (10 or 20 mg/kg i.p.)-induced locomotor activity was measured. Each point represents the mean ± SEM number of ambulatory counts recorded in 10 min bins over a 2 hr test period. Asterisk, significantly different from HSV-LacZ ($p < 0.05$ by t test).

stand RGS9-2 function in vivo using both overexpression of the protein by viral-mediated gene transfer in rats and deletion of the protein in RGS9 knockout mice. Results of these experiments support an inhibitory influence of RGS9-2 on D2 receptor signaling and on the behavioral effects of psychostimulants and suggest a role for RGS9-2 in aspects of psychostimulant addiction.

Results

Inhibition of Dopamine D2 Receptor-Mediated Locomotor Responses upon Overexpression of RGS9-2

As a first step in testing the hypothesis that RGS9-2 modulates dopaminergic neurotransmission, we overexpressed the protein specifically in the nucleus accumbens (Figure 1A) using a herpes simplex virus (HSV) system demonstrated to be effective for in vivo gene transfer (Carlezon et al., 1997, 1998). This treatment was found to result in a ~80% increase in RGS9-2 levels compared to basal levels of the protein (Figure 1A). As shown previously for other HSV-expressed proteins, we found that RGS9-2 overexpression was transient (maximal 3–4 days after virus injection) and not associated with detectable toxicity. The consequence of RGS9-2 overexpression on behavioral responses to dopamine was assessed in a circling model, which has been used for more than 30 years as a standard test of dopaminergic function (Ungersted and Arbuthnott, 1970). For example, unilateral lesions of midbrain dopamine neurons that innervate the striatum, followed by administration of apomorphine (a nonselective dopamine receptor agonist), causes dramatic circling behavior of the animal away from the side of the lesion. This is interpreted as

being due to supersensitivity of dopamine receptors on the denervated side. In contrast, apomorphine does not induce circling in a normal animal.

In the present study, HSV-RGS9-2 was injected on one side of the nucleus accumbens, and HSV-LacZ was injected on the other. Apomorphine challenge induced circling in the animals with a strong directional bias: animals rotated in a direction toward the side of RGS9-2 overexpression (Figure 1B). These results suggest an imbalance in dopaminergic neurotransmission, with the side overexpressing RGS9-2 being less responsive than the other. Overexpression of RGS9-1 induced a similar degree of circling, whereas animals injected with vehicle showed minimal circling and no rotational bias. Similarly, little circling and no rotational bias was observed when RGS4, another RGS protein prevalent in striatum (Gold et al., 1997), was overexpressed unilaterally in nucleus accumbens (Figure 1B).

To determine whether the effect of RGS9-2 on responses to apomorphine was specific to a particular dopamine receptor subtype, we tested the effect of D1-selective and D2-selective dopamine receptor agonists on circling behavior of animals unilaterally injected with HSV-RGS9-2 into nucleus accumbens, with HSV-LacZ again injected on the other side. Animals administered quinpirole, a D2-selective agonist, showed similar rotational bias as seen for apomorphine (Figure 1B), although the total amount of circling was not as great as for apomorphine. In contrast, animals administered SKF81297, a D1-selective agonist, showed no rotational bias, although the animals displayed a general increase in locomotor activity that was comparable to that seen with quinpirole. The findings indicate that the effect of RGS9-2 on dopaminergic neurotransmission was selective for the D2 receptor. Quinpirole and SKF81297 had similar effects in animals injected with HSV-RGS9-1, indicating that the unique C-terminal tail of RGS9-2 is not required for these behavioral effects.

Inhibition of Cocaine-Induced Locomotor Activity upon Overexpression of RGS9-2

Psychostimulants, such as cocaine, induce locomotor activity via actions on D2 and D1 dopamine receptors, an action mediated in large part via the nucleus accumbens. It was, therefore, of interest to study the effect of RGS9-2 overexpression in this brain region on locomotor responses to cocaine. As shown in Figure 1C, bilateral overexpression of RGS9-2 in nucleus accumbens had no effect on baseline locomotor activity but significantly attenuated the ability of a moderate dose of cocaine (10 mg/kg i.p.) to stimulate locomotion in these animals compared to control animals. This effect of RGS9-2 could be overcome by a higher dose of cocaine (20 mg/kg), which induced similar levels of locomotor activity in animals injected with HSV-RGS9-2 or HSV-LacZ.

Cellular Localization of RGS9-2 in Nucleus Accumbens

The nucleus accumbens, like the striatum as a whole, contains two major subtypes of medium spiny projection neurons, which together comprise 90%–95% of the neurons in this region. These neuronal subtypes can be differentiated by the neuropeptide they express; one subtype expresses enkephalin, whereas the other expresses substance P and dynorphin (Aizman et al., 2000; Graybiel, 2000). While the precise functional roles played by these two subtypes of neurons remain incompletely understood, there is substantial evidence that both contribute to the locomotor-activating and rewarding actions of psychostimulants (Graybiel, 2000; Self and Nestler, 1998). We used a double-labeling in situ hybridization protocol to study the distribution of RGS9-2 between these two subtypes of nucleus accumbens neurons. As shown in Figure 2, RGS9-2 is present in a majority of both enkephalin+ and substance P+ neurons. In general, enkephalin+ neurons express high levels of D2 receptors, whereas dynorphin/substance P+ neurons express high levels of D1 receptors (Aizman et al., 2000; Graybiel, 2000). Consistent with these patterns of expression, double-labeling immunohistochemistry confirmed that a majority of D2+ neurons also express RGS9-2 (not shown). Together, these anatomical data indicate that while RGS9-2 is broadly expressed in nucleus accumbens and dorsal striatum, it is heavily colocalized in neurons that express D2 receptors. This complements the behavioral-pharmacological data to further indicate an association between RGS9-2 and signaling via dopamine D2 receptors.

Enhanced Locomotor Responses to Psychostimulants in RGS9 Knockout Mice

To complement our studies of RGS9-2 overexpression in rats, we characterized locomotor responses to psychostimulants in RGS9 knockout mice. Consistent with the overexpression data in rats, we found that mice with a null mutation in the RGS9 gene show augmented locomotor activation in response to amphetamine (Figure 3A). This enhanced responsiveness was apparent at lower doses of amphetamine (1–2 mg/kg), while a higher dose (5 mg/kg) elicited similar responses in the mutants and wild-type littermates. There was no difference between mutant and wild-type mice in baseline locomotor activity throughout a 30 min habituation period (not shown) or in response to saline injections (Figure 3A). Figures 3B and 3C show, respectively, that RGS9 knockout mice also exhibit increased locomotor responses to cocaine as well as to the direct dopamine receptor agonist apomorphine. This enhanced behavioral responsiveness to dopamine agonists in the RGS9 knockouts is consistent with enhanced biochemical responsiveness as well. The highly selective D2 agonist quinelorane produced a 52% $\pm$ 4% ($n = 4$; $p < 0.025$ by t test) greater inhibition of forskolin-stimulated adenylyl cyclase activity in striatal extracts from RGS9 knockout mice compared to their wild-type littermate controls.

Repeated exposure to psychostimulants causes progressively greater locomotor activity, a phenomenon called locomotor sensitization. To study locomotor sensitization in RGS9 mutant mice, we repeatedly administered cocaine at a low dose (7.5 mg/kg). As shown in Figure 3D, this dose of cocaine, upon initial exposure, caused relatively small increases in locomotor activity that were indistinguishable between RGS9 knockout mice and their wild-type littermates. Repeated cocaine administration produced significant locomotor sensitization in both wild-type and RGS9 mutant mice (locomo-

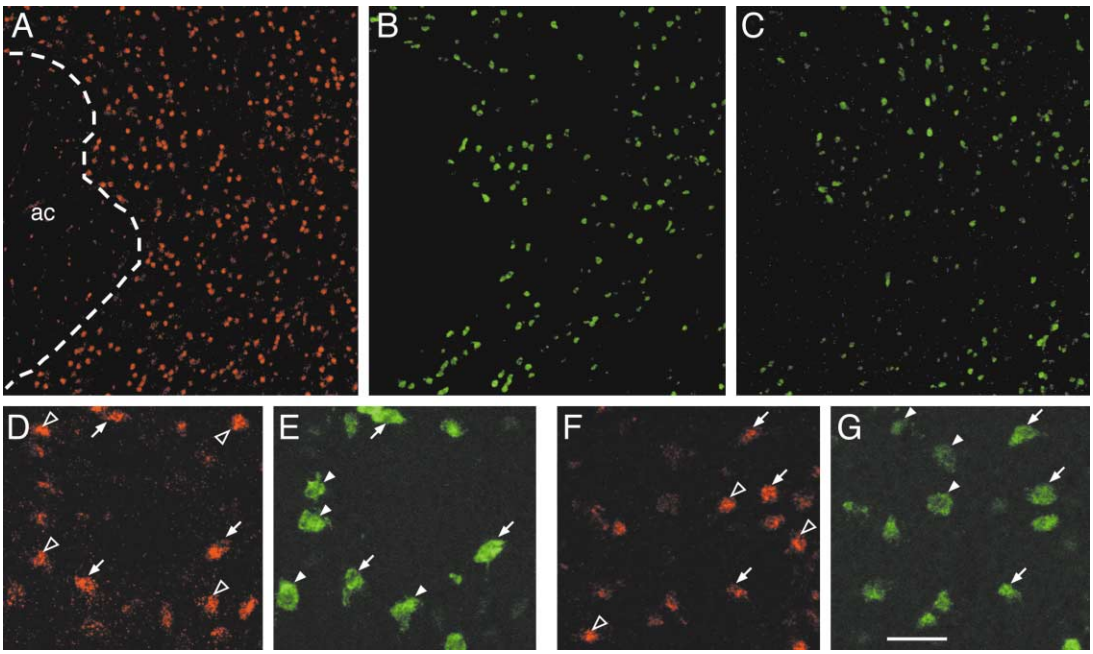

Figure 2. Cellular Localization of RGS9 in Nucleus Accumbens

Double-labeling flourescent in situ hybridization of rat striatum demonstrates RGS9-2 expression in both enkephalin+ and substance P+ neurons.

(A–C) Representative low-power photomicrographs of fluorescent in situ hybridization to RGS9 (A), enkephalin (B), and substance P (C) mRNA via biotin-labeled (Cy3-RGSS9; red) and digoxigenin-labeled (FITC-enkephalin and substance P; green) riboprobes in coronal sections of nucleus accumbens.

(D–G) Representative high-power, confocal photomicrographs of sections double-labeled for RGS9 and enkephalin (D and E) or for RGS9 and substance P (F and G). Cells single-labeled for (1) RGS9 are indicated by open arrowheads and for (2) enkephalin or substance P by solid arrowheads. Cells double-labeled for RGS9 and either enkephalin or substance P are indicated by arrows; for clarity not all cells are highlighted. RGS9-2 was roughly equally distributed between these two cell types: 65.3% ± 1.2% of enkephalin+ cells are positive for RGS9-2; 65.4% ± 3.1% of substance P+ cells are positive for RGS9-2 (from analysis of ~250 cells/rat, n = 3 rats). Scale bars equal 190 μm in (A)–(C) and 33 μm in (D)–(G).

tor activity was greater on day 5 compared to day 1), but the degree of sensitization was markedly potentiated in the RGS9 knockouts.

Enhanced Rewarding Effects of Cocaine in RGS9 Knockout Mice

To determine whether the rewarding effects of cocaine are also altered in RGS9 mutant mice, we used the conditioned place preference test, a classical conditioning paradigm in which animals learn to prefer an environment associated with drug exposure (Wise, 1998). Both wild-type and RGS9 knockout mice showed no baseline preference for either side of the test chambers prior to cocaine administration, and both developed significant place conditioning after training with cocaine (Figure 3E). Notably, the RSG9 mutants showed more than double the degree of place conditioning compared to their wild-type littermates to a moderate dose of cocaine (10 mg/kg), with no apparent difference observed at a higher dose (15 mg/kg). Mice heterozygote for the RGS9 deletion showed intermediate place conditioning to cocaine (not shown). These results indicate greater sensitivity to the rewarding effects of cocaine, as seen for locomotor effects of the drug, in the absence of the RGS9 gene.

Normal Dopamine Receptor Levels in RGS9 Null Mice

Given the influence of RGS9-2 on dopamine signaling in the nucleus accumbens, it was important to determine whether mice lacking the protein show normal levels of D1 and D2 dopamine receptors or whether levels of the receptors are abnormal in the mutant mice due to developmental compensations. Therefore, direct radioligand binding studies were performed to compare the expression levels of D1-like and D2-like dopamine receptors in the striatum of wild-type and RGS9-2 null mice. The D1-like selective radioligand, ^{3}H SCH 23390, was used to quantify D1 receptors; the D2-like selective radioligand, ^{125}I-IABN, was used to quantify D2-like receptors (Luedtke et al., 2000). These binding studies showed that there was no difference in the level of expression of D1 or D2 receptors between wild-type and knockout mice (Table 1).

Increased RGS9 Expression in Brain by Chronic Cocaine Administration

To further characterize a possible role for RGS9-2 in mediating responses to psychostimulants, we examined the ability of cocaine to alter levels of RGS9-2 expression in striatum. Levels of RGS9 immunoreactivity were analyzed by Western blotting, using a polyclonal anti-RGS9 antibody, which detects a protein band (76 kDa) that corresponds to the calculated size of RGS9-2, is striatal-enriched, and is absent in striatal extracts from RGS9 null mice. Chronic administration of cocaine for 7 days produced a small, but statistically significant, increase in levels of RGS9-2 immunoreactivity in the nucleus ac-

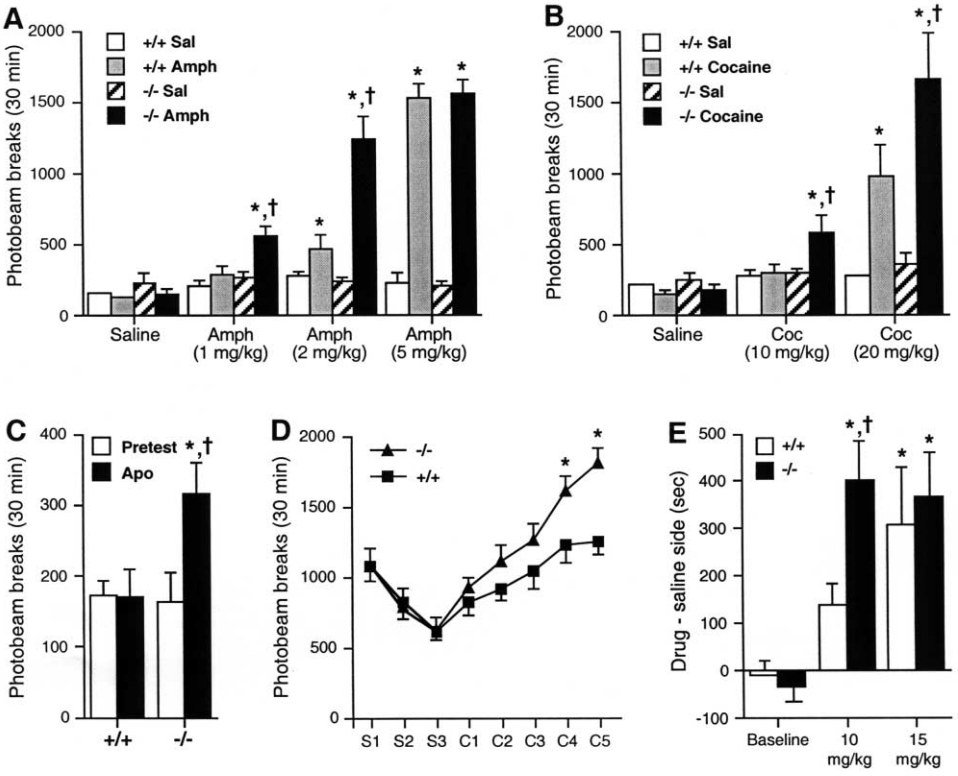

Figure 3. Behavioral Responses of RGS9 Knockout Mice to Psychomotor Stimulants

(A–C) Total locomotor activity scores of RGS9 knockout mice (−/−) and their wild-type littermates (+/+) over 30 min after saline (Sal) or single doses of (A) amphetamine (Amph; 1, 2, or 5 mg/kg i.p.), (B) cocaine (Coc; 10 or 20 mg/kg i.p.), or (C) apomorphine (Apo; 1 mg/kg i.p.). Data are expressed as mean ± SEM photocell disruptions (+/+, n = 6–12 mice in each group). Asterisk, significantly different from saline (A and B) or baseline (C); dagger, significantly different from wild-type ($p < 0.05$, ANOVA followed by Fisher test).

(D) RGS9 knockout mice and their wild-type littermates were given daily injections of saline for 3 days (S1–S3) followed by daily injections of cocaine (7.5 mg/kg) for 5 days (C1–C5). Locomotor activity was measured for 10 min after each injection. Data are expressed as mean ± SEM photocell disruptions over the 10 min test period. Wild-type (n = 11) and mutant (n = 13) mice showed small increases in locomotor activity after the first dose of cocaine, with no difference apparent between the two genotypes. Both groups of mice also showed significantly greater locomotor responses to the fifth dose of cocaine compared to the first dose (i.e., sensitization) ($p < 0.05$, ANOVA and t test), although the knockouts showed significantly greater levels of sensitization ($p < 0.05$, ANOVA and t test).

(E) Place conditioning to cocaine (10 or 15 mg/kg) in RGS9 knockout mice and their wild-type littermates. Data are expressed as the mean ± SEM time spent on drug-paired side minus that on saline-paired side, which provides a measure of place conditioning. Neither genotype showed a significant baseline preference for either side of the apparatus before conditioning, but both developed a significant place preference after cocaine training ($p < 0.05$ ANOVA and Fisher). In addition, RGS9 knockouts showed significantly greater place conditioning to cocaine at the 10 mg/kg dose than wild-type mice ($p < 0.05$ by ANOVA and Fisher test), a difference not seen at the 20 mg/kg dose.

Table 1. Comparison of D1-like and D2-like Dopamine Receptor Binding in the Striatum of Wild-Type and RGS9 Knockout Mice

Ligand	Kd (nM)	Bmax (fmol/mg protein)
^{3}H-SCH23390		
RGS9-2 (+/+)	0.79 ± 0.10	629 ± 104
RGS9-2 (−/−)	0.88 ± 0.08	616 ± 132
^{125}I-IABN		
RGS9-2 (+/+)	0.012 ± 0.001	173 ± 35
RGS9-2 (−/−)	0.014 ± 0.001	185 ± 12

Radioligand binding techniques were used to determine the dissociation constant (Kd) and the maximum density (Bmax) of dopamine receptors in RGS9 knockout mice (−/−) and their wild-type littermates using a radioligand selective for either D1-like (^{3}H-SCH 23390) or D2-like (^{125}I-IABN) dopamine receptors. Data are expressed as means ± SEM (n = 3–5 animals/group). Equivalent results were obtained in two additional experiments on separate groups of animals.

cumbens as well as in the dorsal striatum (Figure 4A). This effect required repeated cocaine administration, since no change was observed after acute exposure to the drug (97% ± 7% and 99% ± 6% of control in nucleus accumbens and dorsal striatum, respectively; n = 7). In addition, chronic cocaine administration did not alter levels of RGS9-2 mRNA in either the nucleus accumbens or dorsal striatum as determined by in situ hybridization (not shown).

We next examined RGS9-2 levels in rats after cocaine self-administration. Interestingly, chronic self-administration of cocaine causes an even greater induction of RGS9-2 levels in striatum than observed after investigator-administered drug (Figure 4B). This greater effect was not due to the increased dose or duration of cocaine exposure in the self-administering animals, since investigator-administered cocaine of a dose and duration that mirrors self-administration led to the same smaller increase in RGS9-2 levels (not shown). As with investigator-administered cocaine, self-administration of cocaine

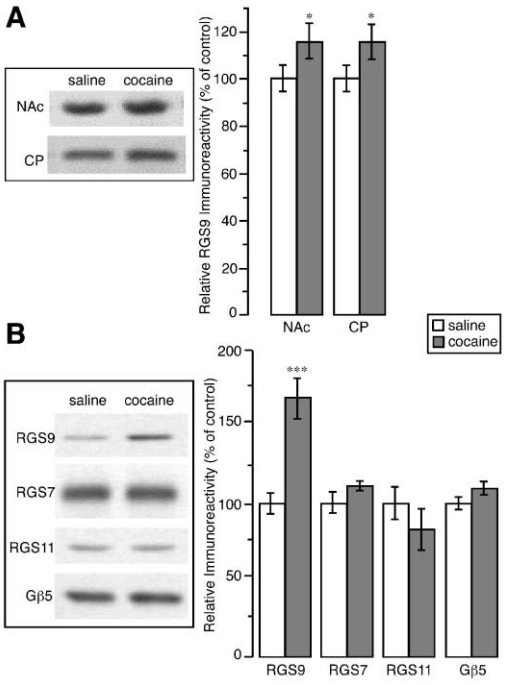

Figure 4. Regulation of RGS9-2 Immunoreactivity by Chronic Cocaine Administration

(A) Rats were treated daily with saline or cocaine (20 mg/kg i.p.) for 7 days; the following day levels of RGS9-2 were measured in extracts of nucleus accumbens (NAc) and dorsal striatum (caudate-putamen or CP) by Western blotting. Data are expressed as mean ± SEM of saline-treated animals (n = 15). The inset shows representative lanes of resulting blots. Asterisk, significantly different from saline ($p < 0.05$ by t test corrected for multiple comparisons).

(B) Rats were allowed to self-administer cocaine or saline daily for 4 hr for a period of 15 days and were used the day after the last self-administration session. Striatal dissections were then subjected to Western blotting for RGS9-2, RGS7, RGS11, or Gβ5. Data are expressed as mean ± SEM of saline-administering animals (n = 12). The inset shows representative lanes of resulting blots. Asterisk, significantly different from saline ($p < 0.05$ by t test corrected for multiple comparisons).

failed to alter RGS9-2 mRNA levels (not shown). Cocaine regulation of RGS9-2 showed some specificity for this protein, since no effect was observed on two related RGS proteins expressed in striatum, RGS7 or RGS11, or on the G protein subunit, Gβ5 (Figure 4B). This subunit is of particular interest, because it has been reported to uniquely associate with RGS9-1 in retina (Kovoor et al., 2000; Martemyanov and Arshavsky, 2002; Nair et al., 2002), although a specific association with RGS9-2 has yet to be established.

Effect of RGS9-2 on Kinetics of Dopamine D2 Receptor Signaling

We used dopamine-evoked currents, mediated by inwardly rectifying K^+ channels (GIRKs), recorded from *Xenopus* oocytes coexpressing functional Kir3.1/Kir3.2 channel heteromultimers along with the dopamine D2 receptor to study the direct influence of RGS9-2 on D2 receptor signaling. Regulation of GIRK channels is one mechanism by which D2 receptors produce physiological responses in striatal neurons, and the Kir3.1/Kir3.2 channel subtypes are widely expressed in brain including striatum (Karschin et al., 1996). When D2 receptors and GIRK channels are heterologously expressed in oocytes, activation of the receptors by dopamine causes a time- and dose-dependent activation of an inward current (Figures 5A and 5B). Coexpression of RGS9-2 significantly accelerated both the on- and off-kinetics of D2 responses but had no effect on the amplitude of the current. This is similar to the effect of RGS9-2 and other RGS proteins on several G protein-coupled receptors (Kovoor et al., 2000). To examine the effect of Gβ5 on RGS9-2 function, we repeated the above experiment after reducing the amount of RGS9-2 cRNA injected per oocyte (from 10 ng to 5 ng). At this level of RGS9-2 cRNA, the deactivation rate was not significantly different from control. However, we found that coexpression of Gβ5 with RGS9-2 significantly accelerated the speed of GIRK deactivation when compared to RGS9-2 alone (Figure 5C, $p < 0.05$). Expression of Gβ5 in the absence of RGS9-2 had no effect on GIRK kinetics (not shown), indicating the Gβ5 functions to enhance the action of RGS9-2 on dopamine receptor-elicited GIRK kinetics. Thus, RGS9-2 and Gβ5 appear to modulate dopamine receptor-GIRK signaling in a manner that is equivalent to the way they affect m2 muscarinic acetylcholine receptor coupling to GIRK channels (Kovoor et al., 2000). The functional activity of RGS9-2 when expressed alone is not due to endogenous Gβ5 expressed by *Xenopus* oocytes, which contain no detectable Gβ5 immunoreactivity (Kovoor et al., 2000).

Note that the deactivation rates for the control group and the group expressing RGS9-2 in Figure 5B are faster than the deactivation rates for the corresponding groups in Figure 5C. This is because the data for the two figures were generated at different times from oocytes obtained from different donors. We have observed that the GIRK deactivation rates measured in oocytes from one donor can be significantly different from the rates measured in an identical experiment with oocytes from another donor. Therefore, comparisons between different experimental groups are always made using oocytes from the same donor. RGS proteins consistently accelerate the deactivation kinetics of receptor-GIRK coupling across donors, although the mean deactivation rates of the RGS-expressing oocyte groups may be significantly different from donor to donor.

Discussion

The highly enriched expression of RGS9-2 in striatum and the prevalence of dopamine neuromodulation in this structure suggest that RGS9-2 could serve specialized functions for striatal neurons and modulate signaling through dopamine receptors. We have tested this hypothesis in vivo, using several well-established behavioral paradigms for dopamine function. Our results indicate that overexpression of RGS9-2 diminishes sensitivity to the behavioral effects of dopamine agonists, while loss of RGS9 has the opposite effect. These actions of RGS9-2 seem most related to the D2 class of dopamine receptor, which is not surprising since the D2 receptor signals via Gi are known to be influenced by

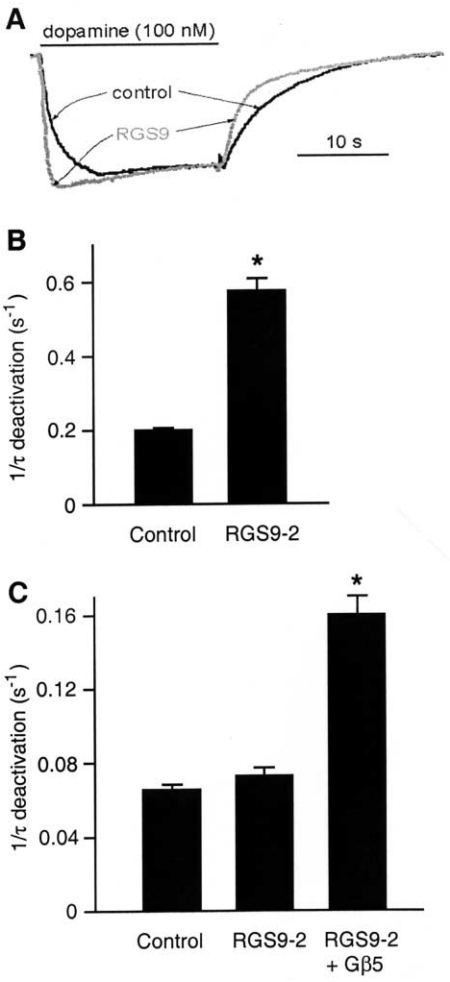

Figure 5. Effect of RGS9-2 on Kinetics of Dopamine D2 Receptor Signaling in *Xenopus* Oocytes

(A and B) Control (Con) oocytes were injected with 1 ng of cRNA for the dopamine D2 receptor, and 0.02 ng of cRNA each for GIRK1 and GIRK2 channels. Another group of oocytes (RGS) was in addition injected with 10 ng of cRNA for RGS9-2.

(A) Representative traces of dopamine-evoked GIRK currents. The solid horizontal line above the traces represents dopamine application. The traces were normalized to the amplitude of the response just prior to dopamine wash off.

(B) Comparison of the deactivation ($1/\tau_{deactivation}$) rate constants derived from the exponential fits of the deactivation phase of the dopamine-evoked GIRK currents in the two oocyte groups. Data are expressed as means ± SEM from 4–7 oocytes from the same oocyte donor. Similar results were repeated in oocytes from two other donors. The mean steady-state current amplitudes in nA ± SEM for the oocyte groups were as follows: control, 708 ± 135; RGS9-2 alone, 1206 ± 157*; asterisk, significantly different from Con (by $p < 0.01$ by t test).

(C) Control (Con) oocytes were prepared as described for (A) and (B). The other oocyte groups were injected in addition with RGS9-2 cRNA (5 ng) alone or RGS9-2 cRNA (5 ng) and Gβ5 cRNA (10 ng). The figure shows a comparison of the deactivation ($1/\tau_{deactivation}$) rate constants derived from the exponential fits of the deactivation phases of the dopamine-evoked GIRK currents in the three oocyte groups. Data are expressed as means ± SEM from 4–6 oocytes from the same oocyte donor; similar results were repeated in oocytes from a second donor. The mean steady-state current amplitudes in nA ± SEM for the different oocyte groups were as follows: Con, 452 ± 73; RGS, 187 ± 54; RGS + Gβ5, 88 ± 13. Asterisk, significantly different from Con and RGS groups ($p < 0.01$ by t test).

RGS proteins, while the D1 receptor signals via Gs are known to be insensitive to most RGS proteins. Importantly, loss of RGS9-2 increases an animal's sensitivity to the behavioral effects of cocaine, whereas overexpression of RGS9-2 has the opposite effect. Moreover, chronic administration of cocaine increases levels of RGS9-2 in striatum. Together, these results suggest a role for RGS9-2 in mediating some of the functional changes in dopamine signaling induced in striatum after chronic cocaine exposure.

Circling behavior or rotation in rodents has been used for more than 30 years as a model system in which to study dopaminergic function in the basal ganglia. A functional imbalance in the dopaminergic system between the two sides of the brain, followed by administration of a dopamine agonist, results in circling: it is interpreted that the animal turns away from the side of higher dopaminergic activity. For example, upon unilateral lesioning of dopamine neurons with 6-hydroxydopamine (a dopaminergic neurotoxin), animals turn away from the lesioned side when challenged with apomorphine, a D1/D2 mixed receptor agonist (Ungersted and Arbuthnott, 1970). This circling is thought to be caused by greater sensitivity of striatal neurons on the lesioned side to dopamine stimulation, although the mechanism underlying this functional supersensitivity has remained unknown (Schwarting and Huston, 1996).

In the present study, we used this established and highly reproducible behavioral response to dopaminergic agents to test the hypothesis that RGS9-2 may alter dopaminergic neurotransmission in striatum. Unilateral injections of HSV-RGS9-2 into the nucleus accumbens caused circling behavior to apomorphine. This is a striking finding, because apomorphine does not induce circling in a normal animal, and only dramatic manipulations (such as unilateral lesions of the dopamine system) have previously been shown to induce circling behavior. These results establish a robust effect of RGS9-2 overexpression on the balance of dopaminergic sensitivity on the two sides of the brain. More specifically, the results suggest that RGS9-2 overexpression decreases sensitivity to dopamine receptor activation. This effect of RGS9-2 was specific for dopamine D2 receptors, since it was seen after administration of a D2-selective agonist, but not a D1-selective agonist. Moreover, the effect of RGS9-2 was not a nonspecific effect of overexpressing an RGS protein per se, since overexpression of RGS4, which is also highly expressed in nucleus accumbens, had no effect on circling behavior. It will be interesting to examine in future studies what feature of RGS9-2 is required to elicit this effect (for example, its RGS domain or any of several other domains [GGL, DEP] contained within this RGS subtype [Ross and Wilkie, 2000]).

Further evidence for a functional connection between RGS9-2 and D2 receptors was the finding that the two proteins show prominent coexpression within striatum. Thus, RGS9-2 was present in a large fraction of D2 receptor-containing neurons. We also directly studied interactions between RGS9-2 and D2 receptors in *Xenopus* oocytes, where expressed GIRK channels respond to Gi activation; this assay has been used widely to study Gi-coupled pathways (reviewed in Zerangue and Jan, 1998; Kovoor and Lester, 2002). Coexpression of

RGS9-2 accelerated both the on- and off-kinetics of D2-mediated channel activation but did not alter the amplitude of the current. One of these effects, the acceleration of deactivation kinetics, is consistent with the classical concept that RGS proteins stimulate the GTPase activity of the Gαi subunits through which D2 receptors signal (Dohlman and Thorner, 1997; Berman and Gilman, 1998; De Vries et al., 2000). To some extent, the increased rate constant for activation also derives from the fact that equilibrium is approached more rapidly if channels close more quickly. However, that steady-state amplitudes show little or no decrease in the presence of RGS9-2 is a well-known paradox of RGS protein action (Doupnik et al., 1997; Zerangue and Jan, 1998; Kovoor et al., 2000; Kovoor and Lester, 2002; Zhang et al., 2002). According to present concepts, there is probably a "precoupled" macromolecular complex or "scaffold" comprising the D2 receptor, the Gαβγ trimer, RGS9-2, and an effector (such as a GIRK channel), involving direct contacts among several component proteins, with the result that RGS9-2 shapes both kinetic and steady-state aspects of signaling through the D2 receptor (Ross and Wilkie, 2000; Kovoor and Lester, 2002; Zhang et al., 2002; Peleg et al., 2002). Importantly, for the subsecond agonist pulses that characterize synaptic transmission, the expected overall result is to decrease the overall current, which would dampen average levels of D2 receptor signaling (Doupnik et al., 1997).

Consistent with the hypothesis that RGS9-2 negatively modulates dopamine signaling are our findings that RGS9 knockout mice show heightened behavioral responses to amphetamine and to cocaine. The heightened locomotor responses were observed for both acute and repeated drug exposure. RGS9 mutants also showed increased sensitivity to the rewarding effects of cocaine as measured by place conditioning, which involves repeated drug exposures. Furthermore, we provided direct evidence for enhanced dopamine receptor signaling in the RGS9 knockouts: the mutants showed greater locomotor responses to apomorphine, as well as greater D2-receptor-mediated inhibition of adenylyl cyclase in striatal extracts.

The finding that a protein highly enriched in striatal regions can influence behavioral responses to psychostimulants is not surprising, since these regions are known to play a central role in mediating both the locomotor activating and rewarding responses of the drugs. The dorsal striatum contributes to the locomotor effects of the drugs, whereas the nucleus accumbens is involved in both the locomotor and rewarding effects (Self and Nestler, 1998; Wise, 1998). These drugs act by increasing dopaminergic transmission in striatal regions, either by inhibiting dopamine reuptake (cocaine) or stimulating dopamine release (amphetamine). The precise contribution of D1 and D2 receptor subtypes in mediating these responses remains unclear, although both receptors are clearly involved (Self and Nestler, 1998; Wise, 1998; Self et al., 1998). Our results would suggest that RGS9-2 selectively modulates responses mediated via the D2 receptor.

We also found that chronic, but not acute, administration of cocaine increases levels of RGS9-2 immunoreactivity in the nucleus accumbens and dorsal striatum. Importantly, this increase was observed with chronic self-administration of the drug. This effect was specific for RGS9-2, since no change was observed in levels of Gβ5 or of RGS subtypes (RGS7, RGS11), which belong to the same subfamily of RGS proteins as RGS9-2. Increased levels of RGS9-2, based on our in vivo behavioral data, would be expected to diminish D2 signaling. Hence, the upregulation of RGS9-2 by cocaine could represent a homeostatic response of D2 receptor-containing striatal neurons to excessive dopaminergic transmission. This adaptation would therefore be expected to diminish the animal's subsequent responsiveness to cocaine. Reduced responsiveness or "tolerance" to the behavioral effects of cocaine is seen in human addicts and is thought to contribute to escalating patterns of cocaine use, particularly during binges of drug administration. Our data, therefore, implicate upregulation of RGS9-2 as one mechanism responsible for this effect. It is interesting that the cocaine-induced increase in RGS9-2 immunoreactivity was not associated with an increase in RGS9-2 mRNA expression. This observation raises the possibility that RGS9-2 may be regulated posttranslationally, although this requires further investigation.

G protein-coupled receptors undergo a complex process of desensitization upon prolonged exposure to an agonist. The best-established mechanism underlying this process involves phosphorylation of the receptors by G protein receptor kinases and the subsequent interaction of the receptors with arrestins (reviewed in Lefkowitz, 1998). Indeed, recent work has shown that mice that lack arrestin-2 show reduced desensitization to the cellular effects of opiates (which act on Gi-coupled receptors) and reduced tolerance to the analgesic effects of opiates after repeated exposure (Bohn et al., 1999, 2000). The in vitro actions of RGS proteins suggest that they, too, may contribute to receptor desensitization (reviewed in Dohlman and Thorner, 1997; Nestler and Aghajanian, 1997; Berman and Gilman, 1998; Potenza et al., 1999; Gold et al., 2003). Results of the present study provide the first in vivo evidence consistent with this possibility. It will be interesting in subsequent studies to evaluate a role for RGS proteins in other in vivo systems of receptor desensitization and tolerance; for example, as seen with chronic opiate exposure. Results of the present study also raise the possibility that drugs targeting RGS9-2 function might be useful in the treatment of psychostimulant and possibly other drug addictions.

The results presented here further demonstrate that behavioral plasticity to drugs of abuse can be linked to specific proteins that modulate cellular responses to the drugs (Nestler, 2001). Thus, RGS9-2 levels are increased in striatum by chronic exposure to cocaine, and modulating RGS9-2 levels in animals alters their behavioral responses to cocaine. Together, these results establish a functional interaction between RGS9-2 and drugs of abuse and contribute toward a more complete understanding of the molecular and cellular mechanisms by which repeated cocaine exposure causes a state of addiction.

Experimental Procedures

All animal procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by our

Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (initial weight ~250 g; Charles River, Kingston RI) and RGS9 mutant mice (Chen et al., 2000) and their wild-type littermates (10–14 weeks old; see below) were used in these studies. All mice were bred from heterozygous crossings (mixed 129-B6 background); male and female homozygous null mice and their wild-type littermates were used. There was no apparent gender difference in subsequent behavioral analyses. Mice were housed by litter and not by genotype. The animal colony was climate controlled and kept on a 12 hr light/dark cycle. Animals were group housed; food and water was made available ad libitum.

Generation and Use of HSV Constructs

HSV vectors containing RGS9-2, RGS9-1, RGS4, and LacZ were prepared as described (Neve et al., 1997). The average titer of the purified virus stocks was $>10^8$ infectious units/μl. Rats received unilateral microinjections (2 μl) of HSV vectors delivered over 10 min into rat nucleus accumbens by stereotaxic surgery; sucrose vehicle was microinjected into the contralateral side. Half of the animals received HSV injections on the right side and half on the left side. Each HSV vector caused roughly comparable expression of the encoded transgene as assessed by in situ hybridization. As demonstrated in several previous studies (Carlezon et al., 1997, 1998; Neve et al., 1997; Kelz et al., 1999), HSV injections were not associated with detectable toxicity: Nissl staining was indistinguishable on the side that received the HSV vector versus sucrose vehicle.

Intracranial surgery was performed, using a 26 gauge hamilton syringe, exactly as described (Carlezon et al., 1997). The following coordinates were used for nucleus accumbens: anteroposterior, −1.8 mm posterior to bregma; lateral/medial, +2.5 mm from midline; and dorsal/ventral, −6.8 mm below dura; the injection syringe was angled at 10° from the midline. The syringe was left in place for 5 min before injection.

Rotation Behavior in Rats

Animals were injected unilaterally with an HSV vector into the nucleus accumbens. HSV-LacZ was injected on the other side of the brain as a control. Three and four days after surgery, when transgene expression is maximal, animals were given apomorphine (1 mg/kg s.c.), quinpirole (1 mg/kg i.p.), or SKF-81297 (3 mg/kg s.c.) and placed in a large (16 in diameter) round chamber for a period of 40 min after drug administration. All rotations were recorded on videotape and the net rotation asymmetry was calculated. At the time of scoring the behavior, the researcher was blind to the orientation (side of brain) of the HSV injections. All animals were pretested with apomorphine (1 mg/kg) for 3 days (each with a day interval between); animals that showed no directional bias and a robust locomotor response were used for the HSV injections; more than 80% of all pretested animals were used for subsequent experiments. Data were calculated as percentage of total turns away from HSV-LacZ and expressed as mean percentages; data from days 3 and 4, which were indistinguishable, were combined. After testing, rats were perfused with paraformaldehyde, and the brains analyzed by Nissl to confirm the placement of the injection needle and the lack of toxicity. Some sections were examined for expression of RGS9-2 by in situ hybridization (Rahman et al., 1999) or β-galactosidase by immunohistochemistry (Carlezon et al., 1997).

Locomotor Activity Assays

For rats, spontaneous locomotor activity was monitored in a circular corridor (10 cm wide and 60 cm in diameter with walls 30 cm high; Med Associates Inc., St Albans, VT). Four photoelectric cells located equidistantly around the walls of the circles detected an animal's horizontal ambulatory activity by way of beam interruptions. Data were recorded via a PC equipped with a customized software (Med Associates). Separate groups of animals were tested with 10 mg/kg ($n = 6$ per group) and 20 mg/kg ($n = 12$ per group) cocaine. Animals were randomized into treatment groups (HSV-LacZ and HSV-RGS9-2) and habituated to the locomotor apparatus for 2 hr. On the next day, animals received HSV vectors in the nucleus accumbens shell on a stereotaxic frame. Following 2 days of recovery, animals were tested with cocaine on locomotor activity for 2 hr. Data were analyzed by two-way ANOVA (HSV × time) with Bonferroni post hoc test.

For mice, locomotor activity was determined in an automated system in which the activity chambers were plastic cages (12 × 18 × 33 cm) with 10 pairs of photocell beams dividing the chamber into 11 rectangular fields, as described previously (Hiroi et al., 1997). Mice were tested at the same time each day by an experimenter who did not know the genotype of the mice. For acute experiments, animals were habituated to the chambers for 30 min, after which time they received i.p. injections of saline or of varying doses of amphetamine, cocaine, or apomorphine, and locomotor activity was assessed for an additional 30 min. For chronic experiments, animals were placed in the chambers immediately after an i.p. saline injection on the first 3 days (S1–S3). Horizontal activity was then measured for 10 min. On days 4–8 (C1–C5), animals were given cocaine (7.5 mg/kg i.p.) and activity was measured for 10 min. The short time periods used for rats and mice have been shown in previous studies to avoid the potentially confounding effects of stereotypy in measures of ambulatory locomotor activity (Carlezon et al., 1997; Kelz et al., 1999; Hiroi et al., 1997).

Conditioned Place Preference Assay

The conditioned place preference assay used in this study has been described elsewhere (Carlezon et al., 1997; Kelz et al., 1999; Hiroi et al., 1997). During the first session, mice were allowed to explore the three chambers (two large chambers with a small chamber in between) of the place conditioning apparatus freely for 20 min. Neither mutant mice nor their wild-type littermates showed a bias to either of the two large chambers. The following three sessions were used for conditioning: mice were alternatively confined to either of the two large chambers immediately after administration of cocaine (10 mg/kg i.p.) or saline for 20 min. On the fifth session, the mice were placed in the central chamber and were allowed to move freely among the three chambers for 20 min. The time they spent in the cocaine-paired chamber minus that in the saline-paired chamber provides a measure of place conditioning.

Cocaine Self-Administration

Cocaine self-administration was carried out according to published procedures (Self et al., 1998). Briefly, male Sprague-Dawley rats weighing 300–325 g were randomized into two groups (saline and cocaine) and trained to respond for saline or cocaine infusions in 15 daily 4 hr test sessions (5–6 days/week). Animals were killed 16–20 hr after the last self-administration session and the brains were rapidly dissected in ice-cold artificial CSF.

In Situ Hybridization and Immunohistochemistry

Isotopic in situ hybridization for localization of HSV-RGS9-2 overexpression in brain was performed exactly as described (Gold et al., 1997). For double-labeling flourescent in situ hybridization, rat brains were rapidly frozen on dry ice and stored at −80°C. Coronal 14 μm sections through striatum (~1.6 to 0.9 mm rostral to bregma) were cryosectioned and thaw-mounted onto Superfrost Plus glass slides (Fisher, Pittsburgh, PA). Sections were pretreated by fixation in ice-cold 4% paraformaldehyde (20 min), dehydrated in increasing concentrations of ethanol, and allowed to air dry. Sections were rehydrated, acetylated for 5 min, dehydrated, and air-dried as before, hybridized coverslipped in buffer containing 50% formamide, 5× SSC, 5× Denhardts, 250 μg/ml yeast RNA, 0.5 mg/ml salmon testes DNA, 200–300 ng/ml RNA probe overnight at 65°C, in a humidified chamber. Slides were washed to a stringency of 0.2× SSC at 65°C and then blocked with 5% normal rabbit IgG and 1% blocking reagent (Roche). Digoxigenin-labeled probes were detected first using a rabbit anti-digoxigenin antibody coupled to horseradish peroxidase (1:200: DAKO). The signal was amplified and detected using tyramide signal amplification; TSA-direct by deposition of Flourescein (Perkin-Elmer). The HRP conjugated to the anti-digoxigenin antibody was quenched by H_2O_2 treatment (3%, 15 min), and the biotin-labeled probes were detected with 1:1000 streptavidin conjugated to HRP (Perkin-Elmer) followed by amplification with TSA-direct deposition of Cy3 (Perkin-Elmer). The slides were then dehydrated and mounted in DPX (Fluka). Biotinylated and digoxigenin-labeled antisense riboprobes complementary to RGS9-2, enkepha-

lin, or substance P were transcribed in vitro using digoxigenin and biotin in vitro transcription kits (Roche). All three transcripts localized by FISH in these experiments are striatally abundant mRNAs with relatively far lower mRNA concentrations in overlying neocortex. This regional labeling footprint was utilized to assess background labeling. In all experiments, the digoxigenin-labeled riboprobes had very low background signal. The biotinylated-labeled riboprobes had consistently higher background signal, but specific signal was easily distinguished by subtracting out the background as measured in neocortex. As an additional tool for assessing background labeling, all experiments included control conditions, where sections were mock-hybridized with no probe and then processed through the streptavidin-HRP-TSA amplification. To assess the degree of TSA-mediated crossamplification, single-labeling controls for both haptens were included in each experiment. No significant differences in the numbers of RGS9 or peptide mRNA positive cells per unit area were found between the double- and single-labeled conditions, indicating insignificant amounts of crossamplification. As an additional control for crossamplification between the two hapten-labeled riboprobes, double-labeling for substance P and enkephalin mRNAs was conducted with both possible hapten-labeled riboprobe combinations. In either case, $<10\%$ of substance P+ cells were colocalized with enkephalin+ cells. Taken together, these control experiments suggest that crossamplification could not account for an appreciable number of false positives for a given riboprobe. Colocalization was assessed using a Zeiss LSM510 confocal microscope. Background labeling for both the Cy3 and flourescein signal was established in the overlying neocortex for each section. Then, single- and double-labeled cells were counted by an observer blind to the experimental conditions, with an average of 125 cells sampled per section and two sections sampled per rat.

Double-labeling immunohistochemistry of mouse striatum was sequentially performed with anti-D1 receptor polyclonal rabbit antiserum, anti-D2 receptor polyclonal rabbit antiserum (Research & Diagnostics Antibodies, San Francisco, CA), and RGS9 polyclonal goat antiserum (Santa Cruz Biotechnologies, CA) by use of standard procedures (Carlezon et al., 1997). Primary receptor antibodies were labeled with Alexa 488-conjugated anti-rabbit IgG made in sheep and RGS9 antibody with Cy5-conjugated anti-goat IgG made in donkey.

Brain Dissections and Western Blotting

Nucleus accumbens and dorsal striatum were dissected as punches with a 12 gauge syringe needle from 1 mm thick coronal sections of rat brain according to published procedures (Kelz et al., 1999). Rats that had been forcibly given cocaine (20 mg/kg i.p. daily for 7 or 15 days) or those that self-administered cocaine (for 15 days) were used in this experiment. Brain samples were solubilized in 1% SDS, and levels of RGS9-2 immunoreactivity in the extracts were measured by Western blotting exactly as described (Rahman et al., 1999; Tekumalla et al., 2001). The following antibodies were used: a sheep affinity-purified polyclonal anti-RGS9 antibody (Makino et al., 1999), a rabbit affinity-purified polyclonal RGS7 antibody (Khawaja et al., 1999), a rabbit anti-RGS11 peptide antibody (a gift from Andrej Krummins), and a rabbit polyclonal antibody (SGS) against the C terminus of Gβ5 (Zhang et al., 1996). Equal loading and transfer of proteins was confirmed by Ponceau staining and by immunolabeling the same blots for α-tubulin, which showed no cocaine-induced change.

Oocyte Expression Assay

Published methods were used for these studies (Kovoor et al., 2000). cRNA was synthesized in vitro from plasmids containing the cDNA and appropriate promoters for cRNA transcription. Plasmids were linearized prior to cRNA synthesis and mMESSAGE MACHINE kits (Ambion) were used to generate capped cRNA. The dopamine D2 receptor (long form) was provided by Marc Caron, Duke University. The cDNA for the GIRK subunits, rat Kir3.1 (Dascal et al., 1993) and mouse Kir3.2 (Kofuji et al., 1996), and mouse RGS9-2 were generated in our laboratory. *Xenopus* oocyte preparation was previously described (Quick and Lester, 1994). cRNA was injected into oocytes at a volume of 50 nl/oocyte using a Drummond microinjector. Oocytes were maintained in a saline buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM HEPES [pH 7.5]) solution supplemented with sodiumpyruvate (2.5 mM) and gentamycin (50 μg/ml). All oocytes were injected with 1 ng of cRNA for the D2 receptor, and 0.02 ng of cRNA each for GIRK1 and GIRK2. Dopamine (1 μM) was used as the dopamine receptor agonist. A valve system controlled by the data acquisition software pCLAMP 6 (Axon Instruments) was used to control solution changes and to minimize washing and washout times. Two-electrode voltage clamp recordings of the oocytes were performed 36–72 hr after cRNA injection. Membrane potential was clamped at -80 mV using a Geneclamp 500 amplifier (Axon Instruments) and pCLAMP 6 software. Electrodes were filled with 3 M KCl and had resistances of 0.5–1.5 MΩ. To reveal inward currents through the inwardly rectifying GIRK channels, recordings were performed in oocyte saline buffer with elevated (16 mM) KCl concentration (other components: 82 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM HEPES [pH 7.5]).

Dopamine Receptor Binding and Adenylyl Cyclase Studies

Published methods were used for radioligand binding studies (Luedtke et al., 2000). The D1-like selective radioligand, ^{3}H SCH 23390 (PerkinElmer Life Sciences, Boston, MA), was used to quantify D1 receptors, while the D2-like selective radioligand, ^{125}I-IABN, was used to quantify D2-like receptors. Nonspecific binding was defined by including (+)-butaclamol (2.5 μM) in the binding assays. D1 binding assays were performed in the presence of 100 nM ketanserin, a serotonin 5HT2 receptor antagonist. Protein was quantified using a BCA protein reagent kit (Pierce, Rockford, IL) with bovine serum albumin as the standard. Estimates of Kd and Bmax values were obtained using unweighted linear regression analysis of transformed data.

For measures of adenylyl cyclase activity, membranes were prepared from striatum of RGS9 mutant mice and their wild-type littermates according to standard methods and resuspended in 50 mM Tris-HCl, 100 mM NaCl, 3 mM $MgCl_2$, and 0.2 mM EGTA. Aliquots (containing 3–5 μg protein) were incubated with a maximal concentration of quinelorane (10 μM) for 15 min at 30°C in the above buffer containing 50 μM ATP, [α-^{32}P]ATP (1.5 μCi), 0.2 mM DTT, 0.01% (wt/vol) BSA, 50 μM cAMP, 50 μM GTP, 3 mM papaverine, 5 mM phosphocreatine, and 20 units/ml creatine phosphokinase in a final volume of 100 μl. The reaction was terminated by boiling in a water bath for 2 min. [^{32}P]cAMP was isolated by the method of Salomon (1979).

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