

# Activator of G Protein Signaling 3: A Gatekeeper of Cocaine Sensitization and Drug Seeking

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## Summary

**Chronic cocaine administration reduces G protein signaling efficacy. Here, we report that the expression of AGS3, which binds to  $G_{i\alpha}GDP$  and inhibits GDP dissociation, was upregulated in the prefrontal cortex (PFC) during late withdrawal from repeated cocaine administration. Increased AGS3 was mimicked in the PFC of drug-naïve rats by microinjecting a peptide containing the  $G_{i\alpha}$  binding domain (GPR) of AGS3 fused to the cell permeability domain of HIV-Tat. Infusion of Tat-GPR mimicked the phenotype of chronic cocaine-treated rats by manifesting sensitized locomotor behavior and drug seeking and by increasing glutamate transmission in nucleus accumbens. By preventing cocaine withdrawal-induced AGS3 expression with antisense oligonucleotides, signaling through  $G_{i\alpha}$  was normalized, and both cocaine-induced relapse to drug seeking and locomotor sensitization were prevented. When antisense oligonucleotide infusion was discontinued, drug seeking and sensitization were restored. It is proposed that AGS3 gates the expression of cocaine-induced plasticity by regulating G protein signaling in the PFC.**

## Introduction

Enduring molecular changes associated with chronic cocaine administration and withdrawal are believed to mediate addiction and relapse to drug taking (Berke and Hyman, 2000; Nestler, 2001). Neuroimaging experiments with nonhuman primates (Morgan et al., 2002) and human addicts (Goldstein and Volkow, 2002) suggest that reduced signaling through  $G_{i\alpha}$ -coupled receptors may be an important neuroadaptation in cocaine addiction. Also, *in vivo* studies in rodents demonstrate that reducing  $G_{i\alpha}$  with pertussis toxin alters the expression of cocaine-induced sensitization and self administration (Self et al., 1994; Steketee and Kalivas, 1991). More direct evidence of reduced  $G_{i\alpha}$  function has been observed following repeated cocaine administration at the level of protein expression (Nestler et al., 1990; Striplin and Kalivas, 1993),  $G_{i\alpha}$ -mediated signaling events (Xi et al., 2003, 2002; Zhang et al., 2000), and membrane

electrophysiology (Henry and White, 1995; Shoji et al., 1997). While the accumulating evidence poses reduced  $G_{i\alpha}$ -coupled cell signaling as important in cocaine addiction, specific cellular changes that might cause this deficit are not known and may involve accessory proteins that influence the transfer of signal from receptor to G protein or directly regulate the activation state of G proteins (Blumer and Lanier, 2003; Ribas et al., 2002).

Two families of G protein regulators are important to the specificity and kinetics of the G protein signaling cascade. One family of G protein modulators, the regulators of G protein signaling (RGS), regulates signaling through  $G_{i\alpha}$  by stimulating GTPase activity or blocking effector activation by G proteins (De Vries et al., 2000). Some members of the RGS family are regulated by cocaine. The mRNAs encoding RGS2, 3, 4, and 5 are upregulated in the striatum by acute psychostimulant administration (Bishop et al., 2002; Burchett et al., 1999), and the protein content of RGS9 is increased by chronic cocaine (Rahman et al., 2003). In this report, we investigate a potential role in the cellular and behavioral consequences of chronic cocaine administration for a member of the other family of G protein regulators, the activator of G protein signaling (AGS) family.

The AGS family consists of three structurally and functionally distinct members that were identified in a yeast-based functional screen for mammalian cDNAs that activated G protein signaling in the absence of a receptor (Takesono et al., 1999). AGS1, a ras-related protein also known as RASD1, promotes nucleotide exchange by  $G_i/Go$  and thereby stimulates G protein activation. AGS2 interacts with  $G_{\beta\gamma}$  and is absent in brain (Blumer and Lanier, 2003). AGS3 is enriched in neurons (Blumer et al., 2002), contains no structural homology with AGS1 or AGS2 (Blumer and Lanier, 2003), and is the first known G protein dissociation inhibitor for heterotrimeric G proteins (Takesono et al., 1999).

AGS3 consists of seven tetratricopeptide repeats in the amino terminus, which is followed by an  $\sim 100$  amino acid linker region connecting four  $\sim 25$  amino acid G protein regulator (GPR) motifs in the carboxyl domain. The GPR repeats selectively stabilize up to four  $G_{i\alpha}1-3$  subunits in the inactive GDP conformation, thereby inhibiting  $G_{i\alpha}$  binding to  $G_{\beta\gamma}$  (Bernard et al., 2001; Peterson et al., 2002; Takesono et al., 1999). In heterologous systems,  $G_{i\alpha}$  complexed with a GPR motif is not recognized by either receptor or effector (Peterson et al., 2000). Thus, overexpression of AGS3 may selectively regulate receptor signaling through  $G_{i\alpha}$  without affecting signaling through receptors that couple to other G proteins (Bernard et al., 2001; Peterson et al., 2002; Takesono et al., 1999).

To begin investigating if the regulation of  $G_{i\alpha}$  by AGS3 is relevant to cocaine addiction, immunoblots were obtained following withdrawal from repeated administration of cocaine, and AGS3 expression was upregulated in the prefrontal cortex (PFC) and core compartment of the nucleus accumbens. Given the inhibitory role of AGS3 upon  $G_{i\alpha}$  activation and reduced signaling through  $G_{i\alpha}$  during cocaine withdrawal, alterations in

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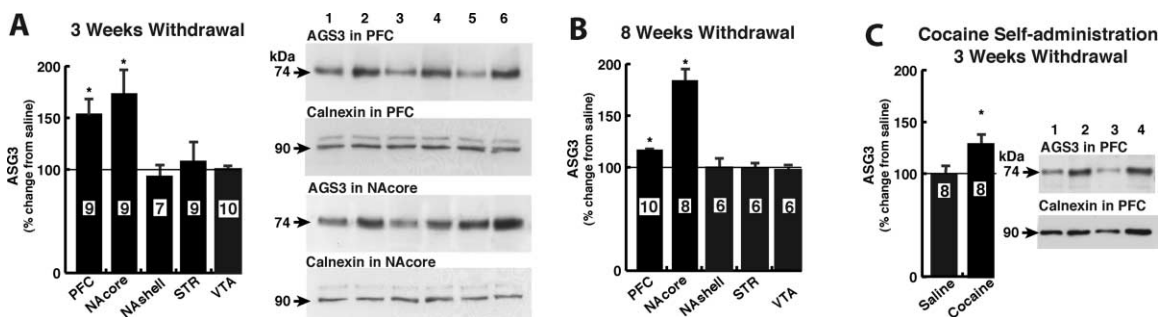


Figure 1. AGS3 Was Upregulated during Withdrawal from Repeated Cocaine Administration

(A and B) After 3 and 8 weeks of withdrawal, AGS3 was upregulated in the prefrontal cortex (PFC) and nucleus accumbens core (NAcore) but not in the shell of the nucleus accumbens (NAshell), the dorsal striatum (STR), or the ventral tegmental area (VTA). (C) AGS3 was elevated in the PFC at 3 weeks after discontinuing cocaine self administration compared with yoked saline control subjects. Representative blots of AGS3 (A and C) are shown as well as blots probed for calnexin, an integral membrane protein of the endoplasmic reticulum, to demonstrate even loading across the gel. Odd numbers, repeated saline group; even numbers, repeated cocaine group. Data were normalized to percent change from saline control values within each blot. \* $p < 0.05$ , comparing withdrawal from repeated saline to withdrawal from repeated cocaine using a two-tailed unpaired Student's  $t$  test.

AGS3 levels in the PFC and accumbens core are consistent with the fact that the glutamatergic projection from the PFC to the accumbens core is necessary for the expression of addiction-related behaviors (Li et al., 1999a; McFarland and Kalivas, 2001; Pierce et al., 1998). Also, cocaine addiction is associated with reduced PFC function, including deficits in adaptive decision making and impulse control (Bonson et al., 2002; Goldstein and Volkow, 2002). Accordingly, experiments were conducted to determine if the upregulation of AGS3 in the PFC may mediate the behavioral and neurochemical phenotype observed during withdrawal from repeated cocaine administration. AGS3 levels in the PFC were pharmacologically regulated using antisense oligonucleotides and a Tat-GPR fusion peptide, and effects were measured on receptor coupling to  $G_{i\alpha}$ , glutamate release, locomotor activity, and the reinstatement of cocaine seeking.

## Results

### Increased AGS3 Expression during Withdrawal

Immunoblots were obtained in tissue collected from the brain regions that are commonly associated with the effects of abused drugs (Everitt and Wolf, 2002). Increased content of AGS3 was observed in the PFC and the core region of the nucleus accumbens following 3 weeks of withdrawal from repeated cocaine but not in the shell of the accumbens, ventral tegmental area, or

striatum (Figure 1A). By 8 weeks of withdrawal, the levels of AGS3 remained elevated in the accumbens core, and while significantly elevated above control in the PFC, the levels were reduced compared to 3 weeks of withdrawal (Figure 1B). AGS3 expression was not different between repeated cocaine and saline treatment groups at earlier withdrawal time points (1 and 7 days) or after acute cocaine treatment in any brain region examined (Tables 1 and 2). Finally, animals were trained to self administer cocaine for 2 weeks, and after 3 weeks of withdrawal, the level of AGS3 in the PFC was elevated compared with yoked saline control subjects (Figure 1C).

### Elevating AGS3 Caused a Cocaine Sensitization-like Phenotype

$G_{i\alpha}$  inhibition by AGS3 can be mimicked by a consensus peptide derived from the four GPR domains contained within AGS3, and a single point mutation in the GPR domain (mGPR) abolishes  $G_{i\alpha}$  binding (Bernard et al., 2001; Peterson et al., 2002). The GPR peptides were rendered cell permeable by fusing the protein transduction domain from HIV-Tat to the amino terminus of the GPR consensus peptide (Schwarze et al., 1999). Like the GPR domain of AGS3, Tat-GPR peptides inhibited [ $^{35}$ S]GTP $\gamma$ S binding to purified  $G_{i\alpha}$ , while Tat-mGPR was without effect (Figure 2A). Also, high-affinity agonist binding to  $\alpha_{2AD}$  adrenergic receptors in DDT-MF2 cell membrane extracts was inhibited by Tat-GPR but not

Table 1. Mesocorticolimbic AGS3 Expression following Withdrawal from Repeated Cocaine

	24 Hrs Withdrawal		1 Week Withdrawal	
	Sal (O.D.)	Coc (O.D.)	Sal (O.D.)	Coc (O.D.)
PFC	100 $\pm$ 5.5 (5)	104.7 $\pm$ 8.7 (5)	100 $\pm$ 7.3 (5)	101.9 $\pm$ 7.4 (6)
NAc	100 $\pm$ 9.2 (6)	116.2 $\pm$ 10.6 (6)	100 $\pm$ 8.6 (6)	130.9 $\pm$ 17.8 (5)
NAs	100 $\pm$ 5 (6)	93.5 $\pm$ 5 (6)	100 $\pm$ 17.4 (6)	121.2 $\pm$ 18.4 (5)
STR	100 $\pm$ 9.5 (4)	113.7 $\pm$ 14.1 (5)	100 $\pm$ 4.3 (6)	99.6 $\pm$ 9 (6)
VTA	100 $\pm$ 9.2 (6)	108.3 $\pm$ 9.2 (6)	100 $\pm$ 8.3 (6)	80.9 $\pm$ 12.3 (6)

Data are expressed as percent expression in optical density values from animals treated with acute saline at equivalent withdrawal periods  $\pm$  SEM (n). NAc, nucleus accumbens core; NAs, nucleus accumbens shell; PFC, prefrontal cortex; STR, striatum; VTA, ventral tegmental area; Sal, saline; Coc, cocaine; ND, not determined.

Table 2. Mesocorticolimbic AGS3 Expression following Withdrawal from Acute Cocaine

	1 Week Withdrawal		3 Weeks Withdrawal	
	Sal (O.D.)	Coc (O.D.)	Sal (O.D.)	Coc (O.D.)
PFC	100 ± 5.5 (4)	108.5 ± 4.2 (4)	100 ± 19.3 (6)	90.9 ± 15.9 (6)
NAc	100 ± 19.2 (4)	116.3 ± 12.5 (4)	100 ± 12.4 (6)	94.0 ± 7.5 (6)
NAs	100 ± 4.2 (4)	109.9 ± 2.6 (4)	100 ± 1.6 (6)	97.6 ± 4.9 (6)
STR	100 ± 16.5 (4)	100.7 ± 1.7 (4)	100 ± 20.8 (6)	103.2 ± 16.7 (6)
VTA	100 ± 16.1 (4)	107.2 ± 37.7 (3)	ND	ND

Data are expressed as percent expression in optical density values from animals treated with acute saline at equivalent withdrawal periods ± SEM (n). NAc, nucleus accumbens core; NAs, nucleus accumbens shell; PFC, prefrontal cortex; STR, striatum; VTA, ventral tegmental area; Sal, saline; Coc, cocaine; ND, not determined.

by Tat-mGPR (Figure 2B). Thus, Tat-GPR effectively mimicked the ability of the AGS3 GPR domain to inhibit  $G_{i\alpha}$ . It should be noted that in the brain LGN, Rap1Gap, RGS12, and RGS14 contain at least one GPR domain each (Blumer and Lanier, 2003), and increasing intracellular content of the four GPR domains contained in AGS3 by Tat-GPR could also be mimicking the action of these proteins.

A Tat-fluorescein conjugate was microinjected into the PFC, and in vivo transduction of cells was examined 30 min following microinjection. Transduction of both neurons and glia was found at the site of microinjection (Figures 3A and 3B). Nissl staining of adjacent sections revealed an abundance of healthy-appearing neurons and no apparent neurotoxicity outside the mechanical damage resulting from cannula implantation (Figure 3C). Immunoblotting for Tat-GPR at different times after microinjection into the PFC revealed maximum Tat-GPR content at 30 min that gradually diminished to undetectable levels by 120 min (Figure 3D).

The behavioral effect of transducing PFC cells with Tat-GPR was examined in drug-naive animals using photobeam breaks to estimate locomotor activity. Thirty minutes after microinjecting Tat-peptide, cocaine (15 mg/kg, i.p.) was administered, and the motor stimulant

response was markedly enhanced between 10 and 60 min after cocaine injection in Tat-GPR-pretreated animals, compared to subjects microinjected with Tat-mGPR (Figures 4A and 4B). In contrast, there was no difference in behavioral activation produced by injecting

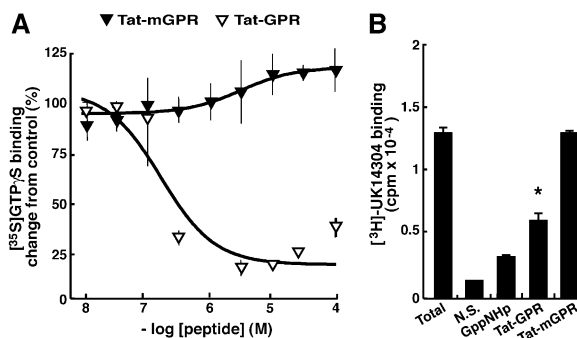


Figure 2. Tat-GPR Prevents  $G_{i\alpha}$  Signaling  
(A)  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding to 100 nM purified  $G_{i\alpha}$  was blocked by increasing concentrations of Tat-GPR (100  $\mu\text{M}$ ), while Tat-mGPR (100  $\mu\text{M}$ ) was ineffective. (B) High-affinity (4 nM)  $[^3\text{H}]\text{-UK14304}$  binding in DDT1-MF2 membranes stably expressing the  $\alpha_{2A/D}$  adrenergic receptor was inhibited by Tat-GPR but not by Tat-mGPR. N.S., nonspecific binding; GppNHp, guanylyl imidodiphosphate is a non-hydrolyzable form of GTP that produced maximal reduction in  $[^3\text{H}]\text{-UK14304}$  binding. Data depict mean ± SEM, n = 2–5, run in duplicate. \*p < 0.01, using two-tailed unpaired Student's t test comparing Tat-GPR with Tat-mGPR

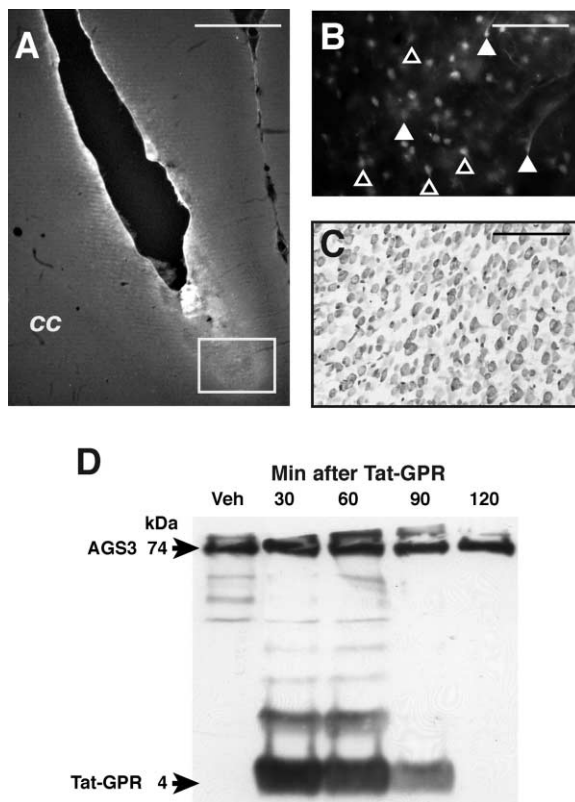
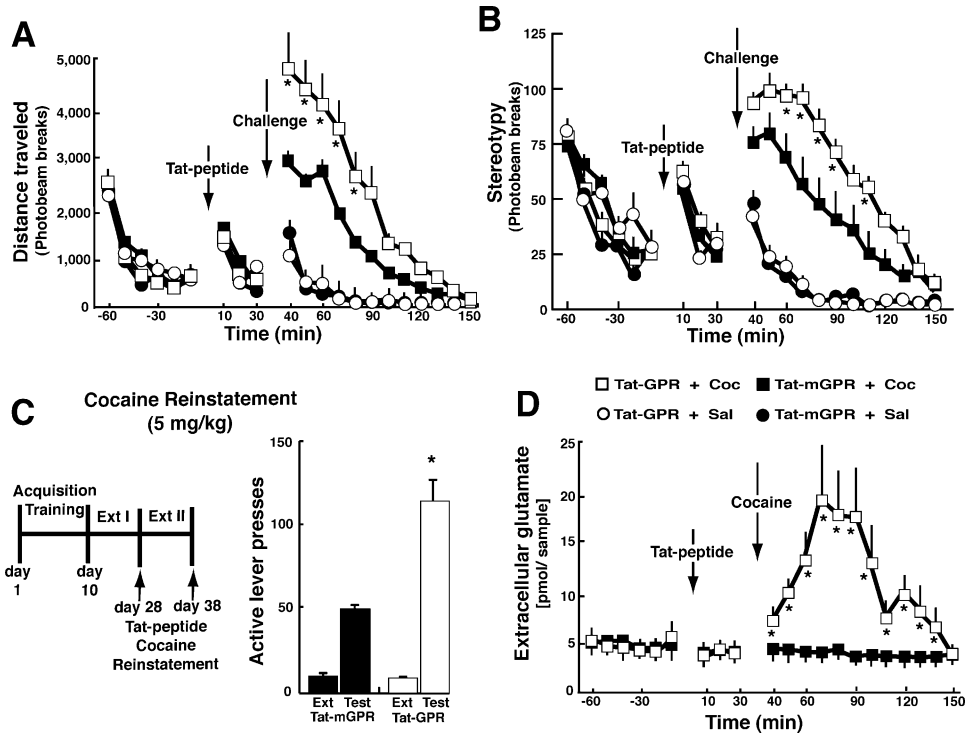


Figure 3. Tat-Peptides Were Efficiently Transduced and Exhibited No Apparent Toxicity  
(A) Epifluorescent micrograph illustrates Tat-fluorescein-containing cells at the tip of the microinjection cannulae 30 min after microinjection. Cannulae placements are within area Cg3 (Paxinos and Watson, 1998) near the prelimbic/infralimbic border of the PFC. Scale bar, 1 mm; cc, corpus callosum. (B) Tat-fluorescein fills the cell bodies of pyramidal neurons and extends into the dendrites (white triangle). Astroglia were also transduced (black triangle). Scale bar, 100  $\mu\text{m}$ ; cc, corpus callosum. (C) Nissl staining revealed an abundance of healthy-appearing neurons at the infusion site. (D) A pan GPR antibody illustrates that after microinjection into the PFC, Tat-GPR (4 kDa) is cleared within 120 min. Crossreactivity of the GPR antibody to AGS3 (74 kDa) demonstrated even gel loading. Veh, vehicle administration.



**Figure 4.** Tat-GPR in the PFC Produces a Sensitization-like Behavioral and Neurochemical Phenotype

(A and B) Microinjection of Tat-GPR into the PFC produced a sensitized motor response to cocaine (15 mg/kg, i.p.) in drug-naive subjects. Animals were habituated to the test chamber (time, -60-0), microinjected with either Tat-GPR or Tat-mGPR (time, 0) and injected with cocaine or saline (time, 30). Data points depict mean distance traveled or number of stereotypies  $\pm$  SEM,  $n = 8$ . (C) Microinjection of Tat-GPR into the PFC augmented the reinstatement of drug seeking (active lever pressing) by a cocaine priming injection (5 mg/kg, i.p.). Two reinstatement trials were conducted in animals extinguished to criterion (Ext I and Ext II), and Tat-GPR or Tat-mGPR were administered 30 min prior to the cocaine priming injection in random order. Data are shown as mean  $\pm$  SEM; active lever presses,  $n = 9$ . Ext, number of active lever presses on the day of extinction training prior to the reinstatement trial. (D) Cocaine-induced (15 mg/kg, i.p.) increase in extracellular glutamate in the accumbens core was augmented by pretreatment of the PFC with Tat-GPR. Data points depict mean  $\pm$  SEM pmol/dialysis sample,  $n = 8$ . Tat-peptide, either Tat-GPR or Tat-mGPR. Challenge, either cocaine (15 mg/kg, i.p.) or saline (1 ml/kg, i.p.). \* $p < 0.05$ , using a two-way ANOVA with repeated measures over time using a least significant difference test for posthoc comparisons of Tat-GPR to Tat-mGPR (A, B, and D) or a two-tailed paired Student's *t* test (C).

saline between the Tat-GPR and Tat-mGPR-pretreated groups (Figures 4A and 4B). Both distance traveled (estimate of locomotion, Figure 4A) and number of stereotypies (repeated breaking of the same photocell beam to estimate stereotyped behavior, Figure 4B) were augmented by Tat-GPR, indicating that the increase in motor activity probably did not involve switching between competing behaviors but rather that inhibition of  $G_{i\alpha}$  by Tat-GPR imparts increased sensitivity to the motor-stimulant properties of cocaine. In the experiment shown in Figures 4A and 4B, subjects were microinjected with Tat-GPR or Tat-mGPR at 1 week intervals in a random design such that each animal received all four treatments. Thus, in accord with the apparent short half-life of the Tat-GPR fusion peptide (Figure 3D), the capacity of Tat-GPR pretreatment to produce a sensitization-like augmentation in the behavioral stimulant effect of cocaine was reversible.

In addition to motor stimulation, the capacity of Tat-GPR to potentiate cocaine-primed reinstatement of drug seeking was examined (Figure 4C). The reinstatement of drug-seeking behavior is a widely employed animal model of relapse (Shalev et al., 2002). Animals were trained to self administer intravenous cocaine and fol-

lowing extinction training were administered an injection of cocaine at a dose (5 mg/kg, i.p.) that produces submaximal reinstatement of drug seeking (Baker et al., 2003). Two separate reinstatement trials were conducted, and animals were microinjected with either Tat-GPR or Tat-mGPR in counterbalanced order 30 min prior to injecting cocaine (Figure 4C shows protocol). The number of active lever presses elicited by cocaine was significantly augmented in the group pretreated with Tat-GPR (Figure 4C).

Animals that develop behavioral sensitization or have been trained to self administer cocaine demonstrate an elevation in extracellular glutamate in the core compartment of the nucleus accumbens following a subsequent injection of cocaine and/or exposure to an environment that was paired with cocaine administration, and the enhanced glutamate release arises primarily from prefrontal cortical glutamatergic afferents (Hotsenpiller et al., 2001; Li et al., 1999a; McFarland et al., 2003; Pierce et al., 1996, 1998). Corresponding with Tat-GPR inducing a behavioral sensitization-like phenotype, cocaine produced an increase in extracellular glutamate in the accumbens core of drug-naive animals following Tat-GPR pretreatment of the PFC (Figure 4D). Similar to previous

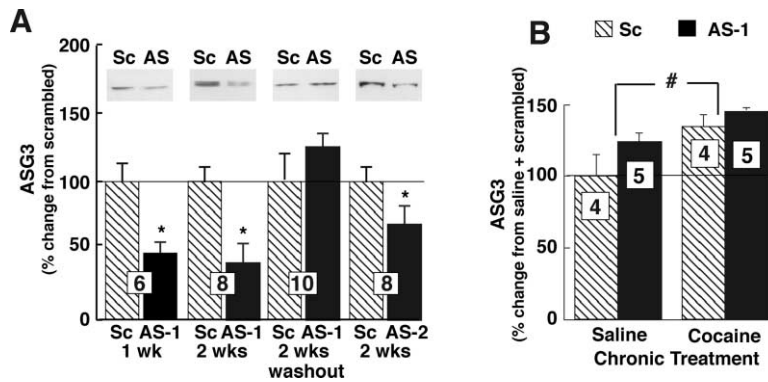


Figure 5. Regulating AGS3 Expression with Antisense Oligonucleotides (A) AGS3 was downregulated following either 1 or 2 weeks of continuous infusion of antisense into the PFC and returned 2 weeks after antisense infusion was terminated. (B) Animals were injected with daily saline or cocaine for 1 week, oligonucleotide was bilaterally infused for 2 weeks, pumps were removed, and 2 weeks later, levels of AGS3 in PFC were measured. AS-1, antisense flanking the AGS3 initiation codon; Sc, a scrambled form of AS-1 that does not bind any known gene; AS-2, antisense directed entirely within the AGS3 open reading frame. Data points are depicted as mean  $\pm$  SEM percent change in optical density from scrambled (A) or chronic saline + scrambled (B). n is indicated in each bar. \* $p < 0.05$ , comparing AS-1 or AS-2 with SC using a two-tailed paired Student's t test. # $p < 0.05$ , comparing chronic saline with chronic cocaine; a two-way ANOVA revealed a significant effect of chronic treatment but no effect of oligonucleotide or interaction between chronic drug and oligonucleotide.

reports in drug-naïve animals (McFarland et al., 2003; Smith et al., 1995), extracellular glutamate in the accumbens core was not altered by an acute injection of cocaine (15 mg/kg, i.p.) in animals pretreated with Tat-mGPR (Figure 4D).

#### Antisense Oligonucleotides Reversibly Lower Levels of AGS3

An antisense oligonucleotide strategy was used during withdrawal to reversibly inhibit the increase in AGS3 in the PFC. Two antisense constructs, one that flanked the AGS3 initiation codon (AS-1) or one directed within the AGS3 open reading frame (AS-2), were continuously infused bilaterally into the PFC using subcutaneously implanted osmotic minipumps. To determine a concentration of AS that would restore the cocaine withdrawal-elevated AGS3 content to approximate control levels (e.g.,  $\sim 40\%$  reduction, see Figure 1A), AS-1 or AS-2 was continuously infused into one side of the PFC for 1–2 weeks. A scrambled oligonucleotide sequence was infused into the opposite hemisphere as a control. An antisense infusion rate of 504 pmol/24 hr was found to reduce AGS3 expression by  $\sim 35\%$ – $60\%$  (Figure 5A). Additional immunoblotting revealed that 2 weeks of AS-1 infusion did not affect expression of proteins in the PFC that are functionally related to signaling through  $G_{i\alpha}$  including  $G_{i\alpha 1,3}$  ( $111\% \pm 9.5\%$ ;  $n = 8$ ; mean optical density  $\pm$  SEM change from scrambled oligonucleotide) or  $G_{i\beta}$  ( $110\% \pm 11\%$ ;  $n = 8$ ). In some animals, the osmotic minipumps were removed after 2 weeks to determine if the reduction in AGS3 by AS-1 was reversible. Two weeks after discontinuing oligonucleotide infusion, AGS3 content was equivalent between the AS-1 and scrambled control group (Figure 5A). Moreover, animals were administered daily saline or cocaine for 7 days and 1 week later implanted with bilateral minipumps to deliver AS-1 or scrambled oligonucleotide into the PFC for 2 weeks. Two weeks following pump removal, the level of AGS3 in the PFC of both oligonucleotide groups was elevated in the animals treated with chronic cocaine compared with chronic saline (Figure 5B), indicating that cocaine-induced elevations in AGS3 were restored after discontinuing AS-1 infusion.

Neurotoxicity is produced by relatively high concentrations of phosphorothioate-substituted oligonucleo-

tides (Braasch and Corey, 2002). Due to using low concentrations and having only the five terminal bases phosphorothioate substituted, after 2 weeks of infusion, no evidence of neurotoxicity was found outside of the mechanical disruption produced by implantation of the infusion cannula (Figure 6). Nissl staining revealed an abundance of healthy-appearing cells (Figures 6B, 6E, and 6H), and staining for glial proliferation with glial fibrillary acidic protein (GFAP) showed equivalent staining between the three oligonucleotide infusion groups (Figures 6C, 6F, and 6I).

#### Elevated AGS3 Contributes to Reduced $G_{i\alpha}$ Signaling Produced by Repeated Cocaine

The effect of increased AGS3 expression on G protein signaling after 3 weeks of withdrawal from repeated exposure to cocaine was assayed via agonist-induced guanosine 5'-[ $\gamma$ - $^{35}S$ ]thiotriphosphate ( $[^{35}S]GTP\gamma S$ ) binding in ex vivo PFC membranes with ligands selective for  $G_{i\alpha}$ -coupled group II metabotropic glutamate receptors (mGluR2/3) and D2/3 dopamine receptors, or  $G_{s\alpha}$ -coupled D1 dopamine receptors. At 1 week after the last daily cocaine or saline injection, either scrambled oligonucleotide or AS-1 was infused into the PFC for 2 weeks. Following infusion of scrambled oligonucleotide,  $[^{35}S]GTP\gamma S$  binding induced by stimulating mGluR2/3 or dopamine D2/3 receptors with 2R,4R-4-aminopyrrolidine-2,4-dicarboxylate (APDC) or quinpirole, respectively, was significantly decreased after withdrawal from cocaine, compared with control subjects (e.g., compare Sc + Coc with Sc + Sal in Figures 7A and 7B). Agonist-induced  $[^{35}S]GTP\gamma S$  binding was restored in rats withdrawn from repeated cocaine to levels equivalent to control by infusing AS-1 (e.g., no significant difference existed between AS + Coc, Sc + Sal, and AS + Sal groups). In contrast, AS-1 in repeated saline animals did not significantly alter the level of quinpirole-stimulated  $[^{35}S]GTP\gamma S$  binding (this treatment group was not examined with APDC treatment, Figure 7B). Consistent with the selective binding of AGS3 to  $G_{i\alpha}$  (Bernard et al., 2001),  $[^{35}S]GTP\gamma S$  binding induced by stimulating the  $G_{s\alpha}$ -coupled dopamine D1 receptors was equivalent in all groups (Figure 7C). The restoration of quinpirole and APDC-induced  $[^{35}S]GTP\gamma S$  binding by AS-1 was not associated with alterations in PFC content

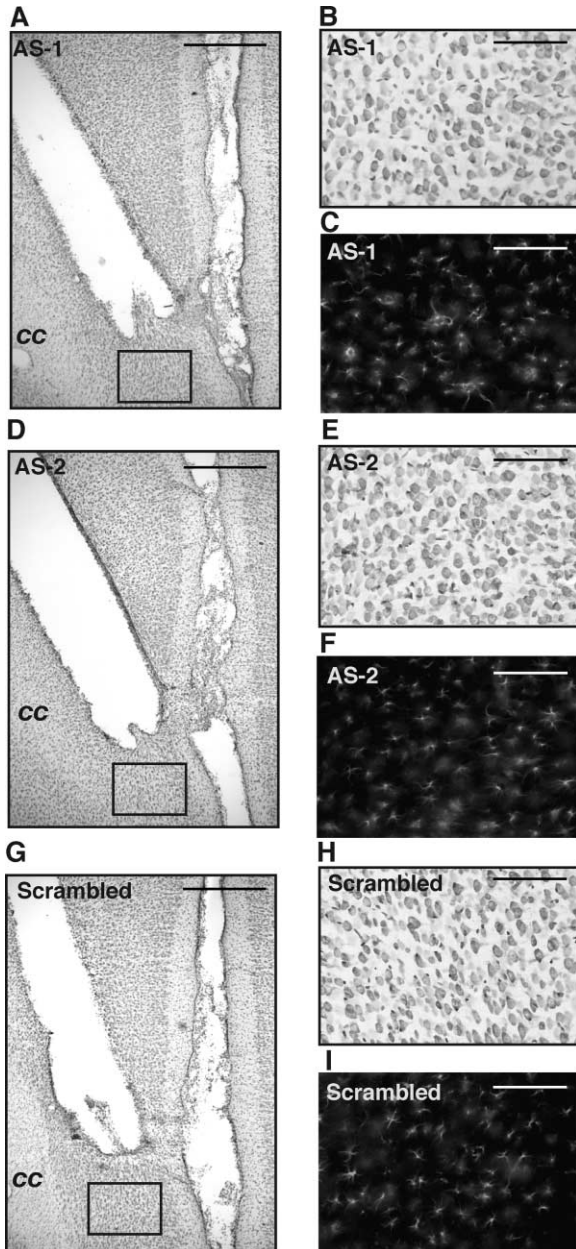


Figure 6. Lack of Neurotoxicity after 2 Weeks of Oligonucleotide Infusion into the PFC

(A, D, and G) Cannulae placements were near the prelimbic/infralimbic border of the PFC (Paxinos and Watson, 1998). Scale bar, 1 mm; cc, corpus callosum. (B, E, and H) Visual inspection of Nissl staining for chromatolysis and microglial reactivity directly below the infusion site revealed an abundance of healthy-appearing neurons. (C, F, and I) No difference was detected between treatments when glial fibrillary acidic protein (GFAP) immunoreactivity was utilized to assess activated or proliferating astroglia. Increased GFAP expression was apparent after all oligonucleotides within 100  $\mu$ m of the indwelling cannula. Scale bar, 100  $\mu$ m.

of D2 (AS-1 =  $96.8 \pm 6.2$ ,  $n = 8$ , data normalized to percent change from Sc) or mGluR2/3 (monomer, AS-1 =  $100.1 \pm 1.3$ ,  $n = 8$ ; dimer, AS-1 =  $92.4 \pm 10.3$ ,  $n = 8$ ) (Figures 7A and 7B).

### Decreasing AGS3 Reversibly Inhibits Behavioral Sensitization

Repeated exposure to cocaine produces an enduring augmentation (sensitization) in the motor stimulant effects of a subsequent cocaine injection (Everitt and Wolf, 2002). Some lesion studies (Pierce et al., 1998; Tzschenke and Schmidt, 1999), but not all (Li et al., 1999b), demonstrate that the glutamatergic PFC projection to the core of the nucleus accumbens is necessary for the expression of behavioral sensitization following repeated cocaine administration. Experiments were conducted to determine if the upregulation of AGS3 in the PFC during withdrawal from repeated treatment with cocaine contributes to the expression of cocaine-sensitized motor behavior.

Rats were repeatedly administered intraperitoneal cocaine for 1 week, and following 1 week of withdrawal, osmotic minipumps were implanted to deliver AS-1 or scrambled oligonucleotide into the PFC (Figure 8A). After 2 weeks of oligonucleotide infusion, all animals were administered cocaine (15 mg/kg, i.p.) to test for the presence of a sensitized motor response. The pumps were removed, and 2 weeks later, all animals were again tested for the presence of behavioral sensitization to cocaine. The first injection of cocaine (i.e., on day 1 before oligonucleotide infusion) elicited an equivalent motor response in both groups (Figure 8B). Following 3 weeks of withdrawal from daily cocaine and 2 weeks of oligonucleotide infusion, the motor response to cocaine in the scrambled group was significantly augmented compared to day 1 (Figures 8B and 8C). In contrast, infusion of AS-1 inhibited the expression of a sensitized motor response (day 28; Figures 8B and 8C). Two weeks following pump removal, both oligonucleotide groups showed significant and equivalent behavioral sensitization in response to an injection of cocaine (Figure 8D). The sensitized response in the scrambled group was equivalent between days 28 and 42, while the response on day 42 was significantly increased over day 28 in the AS-1 group. Thus, in accord with the protein expression studies (Figure 5), the inhibition of sensitized motor activity by AS-1 was reversible.

In contrast to the marked effect of AS-1 on the expression of the sensitized motor response to cocaine, 2 weeks of AS-1 infusion did not affect the motor stimulant response to an acute injection of cocaine (15 mg/kg, i.p.) in drug-naïve rats (Figure 8E). Similarly, the exploratory response to novelty (habituation to the photocell chamber) or the locomotor response to an acute injection of saline was not affected by AS-1 infusion (Figure 8E).

### Decreasing AGS3 Reversibly Inhibits Cocaine Reinstatement of Drug Seeking

Pharmacological inactivation of the PFC prevents the expression of drug-seeking behavior induced by a cocaine injection or an environmental stressor (Capriles et al., 2003; McFarland and Kalivas, 2001). Also, the release of glutamate from the PFC into the accumbens core is required for cocaine-induced reinstatement of drug seeking (McFarland et al., 2003). Based on the critical role of the PFC in drug-seeking behavior, experiments were conducted to evaluate the hypothesis that the

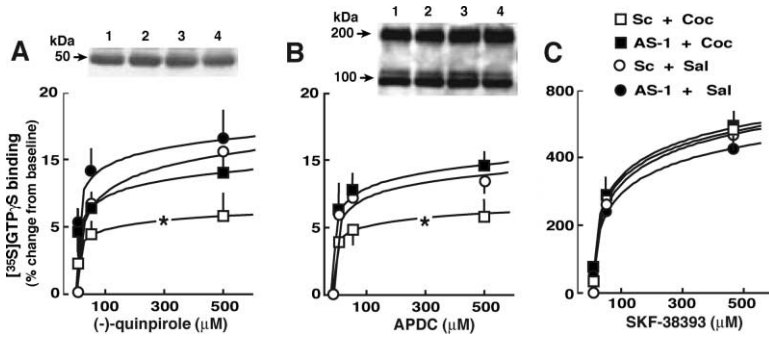


Figure 7. Elevation of AGS3 Parallels Decreased Signaling through  $G_i\alpha$ -Coupled Receptors  
(A) Three weeks of withdrawal from 1 week of daily cocaine administration decreased (-)-quinpirole stimulated  $G_i\alpha$  signaling at D2/3 receptors in the PFC. This effect was reversed by AS-1. Representative immunoblot showing lack of effect of AS-1 on D2 levels in PFC. (B) APDC stimulation of mGluR2/3 receptors and representative immunoblot of mGluR2/3 monomer and dimer. The AS-1 + Sal group was not examined. (C) Signaling through  $G_s\alpha$ -coupled D1 dopamine receptors was equivalent for all treatment groups. Data in each panel are shown as mean  $\pm$  SEM and were analyzed using a three-way ANOVA with repeated measures over drug concentration. For mGluR2/3 and D2/3 receptors, there was a significant effect of nucleotide and drug concentration but no interaction between oligonucleotide and concentration. No significant effect of factor or interaction between factors was found for D1 receptors. Sc, scrambled; Coc, repeated cocaine; Sal, repeated saline. \* $p < 0.01$ , comparing Sc + Coc with all other treatment groups using posthoc three-way repeated-measures ANOVAs over drug concentration.

upregulation of AGS3 in the PFC during withdrawal from cocaine self administration mediates the reinstatement of cocaine-primed drug seeking.

Rats were trained to self administer cocaine by associating a lever press with a cocaine infusion. Lever pressing for cocaine was extinguished by replacing cocaine with saline, and during extinction training, AS-1, AS-2, or scrambled oligonucleotides were bilaterally infused into the PFC for 2 weeks. No difference between oligonucleotide treatment groups was measured in the number of days required to achieve the extinction criterion (see Experimental Procedures). After 14 days of oligonucleotide infusion, rats were administered a single injection of cocaine (10 mg/kg, i.p.) to reinstate drug seeking (lever pressing for saline). Following this reinstatement trial, oligonucleotide infusion was terminated by removing the osmotic minipumps, and the propensity to reinstate drug seeking was reassessed 2 weeks later (Figure 9A).

Drug seeking was elicited by a cocaine injection on day

28 in the group infused with scrambled oligonucleotide (Figure 9C). In contrast, drug seeking was prevented by infusion of either of the two antisense oligonucleotides (Figure 9C). However, a second cocaine injection administered to the same animals 2 weeks after discontinuing oligonucleotide infusion (day 42) elicited reliable reinstatement to a cocaine injection in all oligonucleotide treatment groups (Figure 9D). The reduction in AGS3 by AS-1 did not affect food-induced reinstatement in rats trained to self administer food pellets instead of cocaine, indicating that AS-1 did not produce motor or learning deficits that prevented rats from responding to the cocaine-priming injection (Figure 9B).

### Histology

The site of oligonucleotide infusion (Figure 6) or Tat-GPR microinjection (Figure 3A) was primarily in the ventral prelimbic and dorsal infralimbic cortex (Paxinos and Watson, 1998). The dialysis probes were implanted into the core of the nucleus accumbens with  $<15\%$  of the

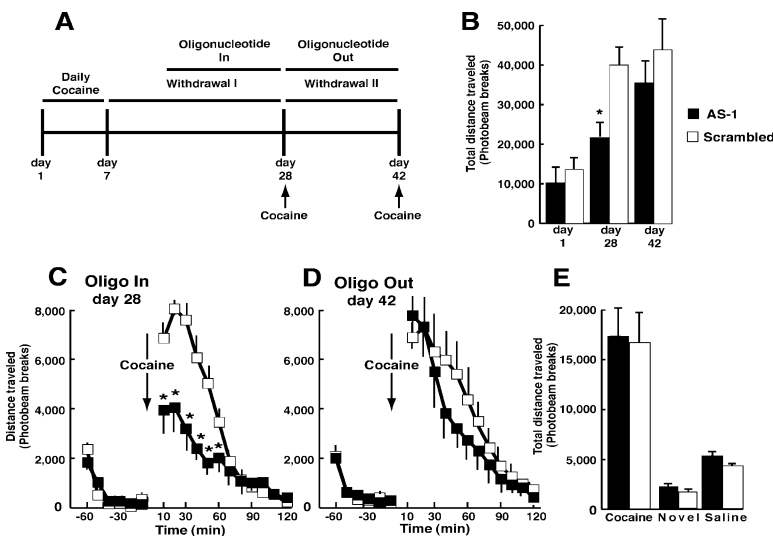


Figure 8. Reduction of AGS3 in the PFC by Antisense Oligonucleotide Inhibits Locomotor Sensitization

(A) Timeline summarizing the behavioral protocol. (B) The cumulative motor response to the cocaine at days 1, 28, and 42. (C) The motor response to cocaine injection on day 28 in the presence of oligonucleotide. (D) The motor response to cocaine on day 42, 2 weeks after removing the osmotic minipumps delivering oligonucleotide into the PFC. (E) Two weeks of oligonucleotide infusion did not affect the drug-naïve animals to a novel environment (open-field photocell chamber) or an injection of saline (1 ml/kg, i.p.) or cocaine (15 mg/kg, i.p.). The data are shown as cumulative distance traveled over 60 min after injection or exposure to a novel environment.  $n = 8$  in all treatment groups. Data in (B)–(D) were analyzed together using a three-way ANOVA with repeated measures over time. A significant effect was found over treatment

and time as well as a significant three-way interaction between treatment, oligonucleotide, and time. Data in (E) were analyzed using an unpaired Student's  $t$  test, and no differences were found. \* $p < 0.05$ , using a least significant difference test for posthoc comparisons between AS-1 and scrambled on day 28. + $p < 0.05$ , comparing day 28 with day 42 in the AS-1 group. # $p < 0.05$ , comparing day 28 with day 1 in the AS-1 group.

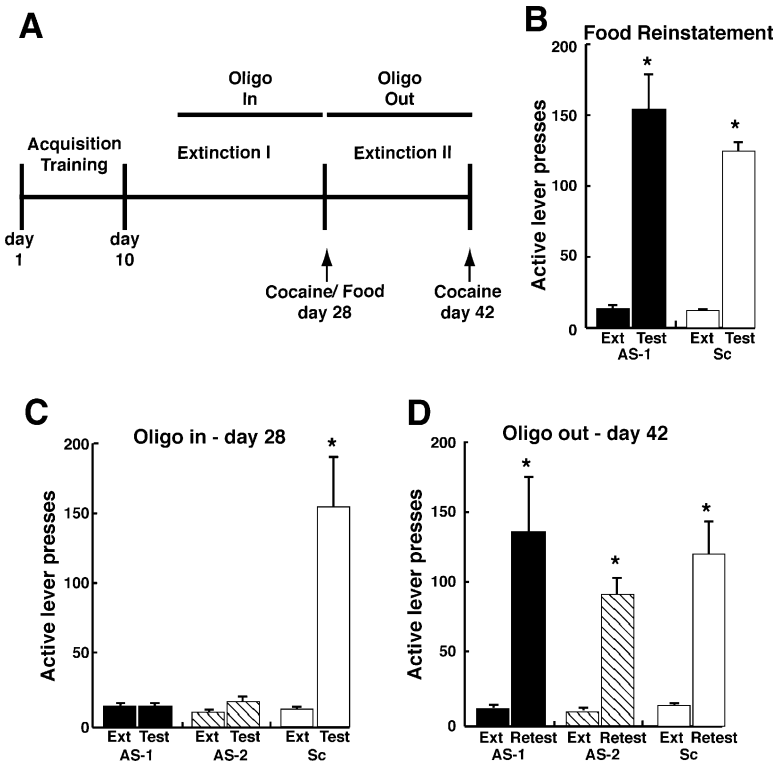


Figure 9. Prefrontal Cortical AGS3 Regulates Cocaine-Seeking Behavior

(A) Timeline outlining the experimental protocol. (B) Oligonucleotide infusion did not impair food seeking. (C) Bilaterally reducing AGS3 blocked the reinstatement of drug-seeking behavior, while infusion with scrambled oligonucleotide had no effect. (D) Reinstatement of drug seeking was initiated by a cocaine injection 2 weeks after discontinuing oligonucleotide infusion in all groups. Data points depict mean active lever presses  $\pm$  SEM,  $n = 8$  in all groups. Ext, total active lever presses made during the extinction trial the day prior to the cocaine-induced reinstatement trial; Test, total active lever presses made during cocaine reinstatement; Retest, total active lever presses following a second extinction period and a second cocaine reinstatement; Sc, scrambled oligonucleotide. \* $p < 0.05$ , using two-way ANOVA with repeated measures over time using a least significant difference test for posthoc comparisons.

active membrane dorsal or ventral to the accumbens core, in the striatum or ventral limb of the accumbens shell, respectively (e.g., see Figure 4 in McFarland et al. [2003] for similar probe placements).

## Discussion

This study demonstrated that AGS3 expression was induced in the PFC during withdrawal from repeated cocaine. When the increased PFC expression of AGS3 was mimicked by microinjecting Tat-GPR, drug-naive animals demonstrated a sensitization-like phenotype by manifesting augmented motor activity and drug seeking as well as increased extracellular glutamate in the core compartment of the nucleus accumbens in response to an acute injection of cocaine. When the elevation in AGS3 was inhibited by infusing antisense oligonucleotides into the PFC, the cocaine-induced reduction in  $G_{i\alpha}$  coupling to mGluR2/3 and D2/3 receptors was reversed. Antisense oligonucleotide infusion also reversed the behavioral consequences of repeated cocaine, including reinstatement of drug seeking and locomotor sensitization. Taken together, these data indicate that upregulation of AGS3 in the PFC following withdrawal from repeated cocaine administration contributes to cocaine-induced behavioral and neurochemical plasticity.

### AGS3, $G_{i\alpha}$ Regulation, and Addiction

AGS3 complexes  $G_{i\alpha}GDP$  and thereby reduces signaling through  $G_{i\alpha}$ -coupled receptors in heterologous cells and in brain tissue (Figure 7). AGS3 can scaffold up to four GDP bound  $G_{i\alpha}$  subunits via GPR repeats, which interferes with signal transduction from G protein-coupled receptors to signaling pathways by disrupting the

$G_{i\alpha}$ - $G\beta\gamma$  interaction and/or by altering the subcellular localization of  $G_{i\alpha}$ -related signaling complexes (Blumer et al., 2002). It is presently unclear whether AGS3 actively promotes G protein subunit dissociation while maintaining  $G_{i\alpha}$  in the GDP bound state, catches a transiently nucleotide-free conformation of  $G_{i\alpha}$  that is then stabilized by the binding of GDP, and/or binds  $G_{i\alpha}GDP$  during the G protein activation-deactivation cycle. Regardless of the precise molecular interactions, inhibition of  $G_{i\alpha}$  by AGS3 in the PFC represents a unique mode of cocaine-induced plasticity. Reduced signaling through  $G_{i\alpha}$ -coupled receptors has been previously described within the addiction literature (Hummel and Unterwald, 2003; Nestler et al., 1990; Self et al., 1994; Shoji et al., 1997; Steketee and Kalivas, 1991; Xi et al., 2003, 2002; Zhang et al., 2000), and the present data identify AGS3 as a link between  $G_{i\alpha}$  signaling and cocaine-induced behavioral and neurochemical plasticity. Preventing the expression of cocaine-induced behavioral sensitization and the reinstatement of drug seeking by normalizing the expression of AGS3 and thereby restoring  $G_{i\alpha}$  signaling is consistent with a role in addiction for elevated AGS3 and the correspondingly reduced signaling through  $G_{i\alpha}$ . However, it should be noted that elevated AGS3 was not measured after 1 week of withdrawal, yet behavioral sensitization and cocaine-primed reinstatement are both present at that withdrawal time, indicating additional mechanisms.

Exactly how compromised  $G_{i\alpha}$  coupling may contribute to other cellular adaptations associated with withdrawal from repeated cocaine administration is not clear. However, decreased  $G_{i\alpha}$  signaling may promote signaling through other G proteins. The mutually antagonistic effects of  $G_{i\alpha}$  and  $G_{s\alpha}$  upon adenylyl cyclase are illustra-

tive of this possibility, since  $G_s\alpha$  signaling through adenylyl cyclase, cAMP-dependent protein kinase (PKA), and proteins downstream from PKA such as  $\Delta$ -fosB is elevated in the nucleus accumbens after repeated cocaine administration (Bibb et al., 2001; Nestler et al., 2001). Moreover,  $G_s\alpha$ -coupled D1 dopamine receptors have been shown to increase membrane excitability in a PKA-dependent manner in pyramidal neurons of the PFC (Gonzalez-Islas and Hablitz, 2003), and D1 dopamine receptor activation in the PFC is hypothesized to underlie the sustained network activity that allows for focused behavior (Durstewitz and Seamans, 2002) such as compulsive drug use (Berke and Hyman, 2000). Also, membrane potential in PFC pyramidal cells is bistable (Steriade et al., 1993), and stimulation of D1 receptors promotes and prolongs the more depolarized "up" state, from which the neuron can more easily emit action potentials in response to excitatory input (Lewis and O'Donnell, 2000). Consistent with pyramidal neurons being more easily stimulated, the membrane potential of pyramidal cells is no longer biphasic and rests closer to the up state after withdrawal from repeated cocaine (Trantham et al., 2002). Also, the reinstatement of drug seeking is associated with increased glutamate release into the accumbens core from prefrontal cortical glutamatergic afferents (McFarland et al., 2003) and can be blocked by dopamine antagonist administration into the PFC (Capriles et al., 2003; McFarland and Kalivas, 2001). These data suggest a hypothesis that high levels of AGS3 may alter dopaminergic signaling in the PFC such that D1-stimulated  $G_s\alpha$  signaling is preferentially activated, which shifts pyramidal cells toward sustained activity, allowing for more focused behaviors. This mechanism is consistent with the critical role of dopamine transmission at D1 receptors in the PFC in cocaine-induced drug seeking and conditioned place preference (Capriles et al., 2003; Sanchez et al., 2003), as well as a role for increased glutamate transmission in the projection from the PFC to the accumbens core in the reinstatement of drug seeking (Capriles et al., 2003; McFarland and Kalivas, 2001; McFarland et al., 2003; Park et al., 2002). Enhanced sequestering of  $G_i\alpha$  by the upregulation of AGS3 may also affect other signaling systems by increasing free  $G\beta\gamma$ . For example, increased  $G\beta\gamma$  could impinge upon information processing within the PFC by opening the G protein-coupled inwardly rectifying potassium (GIRK) channels (Lei et al., 2000), and deletion of the gene encoding the Kir3.2 or Kir3.3 subunits of GIRK channels reduces intravenous cocaine self administration (Morgan et al., 2003).

### Prefrontal Cortex and Addiction

No effective pharmacotherapy exists for the treatment of cocaine addiction, in part because the molecular basis of relapse to drug taking has yet to be elucidated (National Institute on Drug Abuse, 2000). The most successful treatment strategies rely on learning cognitive-behavioral coping skills that strengthen one's ability to self monitor and enable responding with greater self control when presented with a situation that would typically lead to relapse (Carroll, 1998). Appropriate functioning of the PFC is integral to self monitoring (Blake-more et al., 1998), and when this region is damaged,

decision making is impaired (Anderson et al., 1999). Accordingly, dysfunction of this brain region is implicated in psychostimulant addiction and may underlie relapse (Berke and Hyman, 2000; Goldstein and Volkow, 2002; Jentsch and Taylor, 1999; McFarland and Kalivas, 2001). The present study poses increased AGS3 in the PFC as a potential pathological change contributing to drug seeking and behavioral sensitization. The precise molecular mechanisms were not identified whereby the reduced  $G_i\alpha$  signaling resulting from enhanced AGS3 expression contributes to changes in prefrontal glutamatergic output to the nucleus accumbens and the associated cocaine-induced behavioral plasticity. However, it is clear that AGS3 levels in the PFC have a profound influence on the expression of behaviors that are strongly associated with cocaine addiction. Inasmuch as the PFC is playing a critical role in regulating the expression of motor sensitization and relapse to drug seeking, the data in this study demonstrate that the level of AGS3 expression within the PFC is a molecular determinant of this gatekeeper function.

### Experimental Procedures

#### Subjects

All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted by the NIH, and with the approval of an institutional animal care and use committee. Male Sprague-Dawley rats (Harlan, Indianapolis, IN; or Charles River, Wilmington, MA, for self-administration and microdialysis studies) were individually housed in an AAALAC-approved facility. Animals were maintained on a 12 hr light-dark cycle (lights on at 7 a.m. or lights off at 7 a.m. for self-administration studies) with ad libitum access to food and water unless otherwise stated. Upon arrival, subjects were weighed and handled daily for 1 week. All experimentation was conducted during the light cycle (or dark cycle for self administration). Every effort was employed to reduce animal discomfort during sacrifice through either rapid decapitation (for immunoblotting) or overdosing with pentobarbital (100 mg/kg, i.p.) for histological preparation of brain slices.

#### Surgery

##### Osmotic Minipumps

Animals were anesthetized with pentobarbital (50 mg/kg, i.p.) and bilateral 7 mm 28-gauge stainless steel microinjection cannulae (Plastics 1, Roanoke, VA) lowered into the PFC (from Bregma: AP 3.0, ML 2.0, DV -3.7 with manipulator arms set to a 20° angle) (Paxinos and Watson, 1998). Cannulae were fitted with thick-wall PE 60 tubing attached to osmotic minipumps (Durect, Cupertino, CA). These pumps deliver 0.5  $\mu$ L per hour for 14 days, which equals 504 pmol oligonucleotide per hemisphere within a 24 hr period when filled with a 42  $\mu$ M solution. After 2 weeks, pumps were removed and tubes sealed after anesthetizing the animals briefly with Brevital (45 mg/kg, i.p.).

##### Self Administration

Rats were anesthetized as described above and fitted with unilateral jugular catheters that were externalized on the animals' backs to attach to an infusion apparatus (McFarland and Kalivas, 2001).

##### Microinjection and Microdialysis Cannulae

Animals were anesthetized as described above and 14 mm, 26-gauge stainless steel microinjection guide cannulae (Small Parts, Roanoke, VA) implanted into the PFC (Paxinos and Watson, 1998) at AP 3.0 mm, ML 2.0 mm, DV -2.7 mm from Bregma with manipulator arms set to a 20° angle from vertical. Dialysis guide cannulae (20-gauge, 14 mm, Small Parts, Roanoke, VA) were implanted over the nucleus accumbens (AP 1.2 mm, ML 1.6 mm, DV -4.7 mm from Bregma using a 6° angle from vertical) (Paxinos and Watson, 1998).

## Behavioral Assays

### *Noncontingent Cocaine Administration and Behavioral Sensitization*

Cocaine HCl was a gift from the National Institute on Drug Abuse. Daily cocaine or saline injections (1.0 ml/kg, i.p.) were made for 1 week (Pierce et al., 1996). On the first and last days of daily cocaine administration, 15 mg/kg, i.p., was administered and locomotor activity monitored in a photocell apparatus (Omnitech, Columbus, OH). Locomotor activity was quantified as distance traveled (determined by consecutive breaking of adjacent photobeams). Stereotypy was estimated as consecutive breaking of the same photocell beam. Animals were adapted to the photocell box for 60 min prior to injection of cocaine or saline. On the intervening days (days 2–6 of daily injection), rats were administered 30 mg/kg, i.p., of cocaine. A single investigator injected all rats at the same time each day during the light cycle. If animals were used for immunoblotting, they were killed at various times after discontinuing daily cocaine or saline administration. If they were used for behavioral sensitization studies, the tests for behavioral sensitization were conducted in the same photocell box following administration of 15 mg/kg, i.p., cocaine.

To examine the effect of reducing AGS3 expression by antisense oligonucleotide infusion into the PFC, after 1 week of withdrawal from repeated cocaine, animals were fitted with indwelling microinjection cannulae attached to osmotic pumps as described above, and either AS-1, AS-2, or the scrambled oligonucleotide was infused bilaterally. Two weeks after beginning oligonucleotide infusion (day 28 of the experiment, see Figure 4A), the expression of behavioral sensitization was assessed by injecting cocaine (15 mg/kg, i.p.). The next day, animals were anesthetized briefly with Brevital (45 mg/kg, i.p.), the osmotic minipumps removed, and the tubing sealed. Animals were again challenged with cocaine (15 mg/kg, i.p.) 2 weeks after terminating oligonucleotide infusion to assess the expression of sensitization (day 42 of the experiment, see Figure 8A).

In the Tat-GPR experiment, microinjection cannulae were implanted over the PFC (see above), and 1 week later, the animals were habituated to the photocell apparatus with a sham (vehicle only) injection. On the day of the experiment, animals were habituated to the photocell chamber for 1 hr, obdurator removed, and 1  $\mu$ l Tat-peptide infused over 90 s with 33-gauge stainless steel microinjection needles for a total of 4.5  $\mu$ g Tat-peptide per hemisphere. After 2 additional minutes, needles were removed, obdurator replaced, and animals returned to the photocell chamber. Thirty minutes later, animals were challenged with either cocaine (15 mg/kg, i.p.) or an identical volume of 0.9% saline (1 ml/kg, i.p.). Two days of sham experiments followed, except that no microinjection occurred. Five days later, an additional experiment was conducted, and this process was repeated until all animals received all treatments in a counterbalanced design (e.g., Tat-GPR + cocaine; Tat-GPR + saline; Tat-mGPR + cocaine; and Tat-mGPR + saline).

### *Cocaine Self Administration and Reinstatement*

Animals were trained to self administer cocaine (0.25 mg/infusion) (McFarland and Kalivas, 2001). Some rats served as yoked saline controls and received an infusion of saline (0.9%) in the same temporal pattern as that of cocaine self administered by a paired rat. After reaching criterion (<10% deviation in the number of active lever presses over the 2 hr daily trial), daily extinction trials were begun, in which cocaine was replaced with saline. One week after beginning extinction, rats were given food ad libitum for 5 days prior to being implanted in the PFC with microinjection cannulae attached to osmotic minipumps to continuously infuse oligonucleotide (see above). Five days after beginning oligonucleotide infusion, extinction training was reinstated. All subjects had achieved extinction criterion by 14 days after reinstating extinction training (<10% of the average number of active lever presses made during the last 3 days of cocaine self administration), and the animals' propensity to reinstate drug-seeking behavior was assessed by administering cocaine (5 or 10 mg/kg, i.p.) and monitoring active lever presses for 2 hr. During reinstatement testing, active lever presses resulted in saline delivery. The following day, the osmotic minipumps were removed as described above, and the rats received daily extinction trials for 14 additional days, by which time all rats had achieved extinction criterion. After this second extinction period, all subjects were given a cocaine injection (10 mg/kg, i.p.) and reinstatement assessed as described above.

### *Food Self Administration and Reinstatement*

To control for nonspecific behavioral effects, a separate group of animals was trained to self administer food (McFarland and Kalivas, 2001) and implanted with osmotic minipumps during extinction training as described above. Rats were trained to lever press on an FR-1 schedule of reinforcement (each reinforcement consisted of a single 45 mg Noyes food pellet) in daily 2 hr sessions. Once stable responding was achieved, the schedule of reinforcement was increased to FR-2 and then to FR-5. Subjects remained in maintenance until lever press responding stabilized at less than 10% variation across 3 consecutive days (ranging from 12 to 15 days of training). Subjects then entered behavioral extinction, in which lever press responding no longer resulted in food delivery, and were implanted with osmotic minipumps during extinction training as described above. Rats were tested for their propensity to reinstate responding for noncontingent food delivery 14 days after implanting the osmotic minipumps, and all subjects tested had met extinction criteria (3 consecutive days of less than 10% maintenance rate of lever pressing). Rats received two pellets immediately upon initiation and an additional 10 pellets for the first 20 min of the reinstatement session (one pellet at 2 min intervals). Lever presses never resulted in food delivery.

### *Microdialysis*

Concentric probes were constructed and dialysis conducted in the accumbens core (McFarland et al., 2003) following pretreatment of the PFC with either Tat-GPR or Tat-mGPR and systemic cocaine (15 mg/kg). Dialysis probes were inserted the night prior to the experiment. The next morning, microdialysis samples were obtained every 10 min (dialysis buffer flow rate = 2.0  $\mu$ l/min). After collecting samples for 2 hr, rats were microinjected with Tat peptide as described above and three more samples collected. Rats were then injected with cocaine (15 mg/kg, i.p.) or saline and samples collected for 2 more hours. The concentration of glutamate in the dialysis samples was measured using HPLC with fluorometric detection (McFarland et al., 2003).

### *Immunoblotting*

After various withdrawal times (1, 7, 21, or 56 days after discontinuing repeated cocaine or saline; 7 or 21 days after acute injection), animals were decapitated, and brain nuclei were excised and subjected to blotting procedures (Bowers and Kalivas, 2003). Proteins of interest were immunolabeled using specific antisera (AGS3 and pan-GPR 1:3000 [Bernard et al., 2001]; G $\beta$  1:3000, Gi $\alpha_{1,3}$  1:1000, mGluR2/3 1:3000, Upstate, Lake Placid, NY), DR2 receptor (1:1000, Santa Cruz, CA), and activity of the secondary HRP conjugate (1:7000, Upstate, Lake Placid, NY) determined using enhanced chemiluminescence reagents and hyperfilm (Amersham Biosciences, Piscataway, NJ). Immunolabeled bands were identified by their molecular weight and quantified by densitometry (NIH Image 1.62).

### *Antisense Design and Delivery*

BLASTn searches (Altschul et al., 1994) (nonredundant, expect of 10,000 and word sizes of seven) were conducted to design potential DNA antisense and scrambled constructs that hybridize to AGS3 mRNA specifically or to no known gene, respectively. These constructs were synthesized with 5' phosphorothioate bonds (indicated by an "m") at each end and purified using reversed phase HPLC by IDT (Coralville, IA). The scrambled sequence was 5'-T<sup>m</sup>A<sup>m</sup>C<sup>m</sup>G<sup>m</sup>GCT ACGACCGACCG<sup>m</sup>T<sup>m</sup>C<sup>m</sup>A<sup>m</sup>A<sup>m</sup>G<sup>m</sup>-3', AS-1 was 5'-C<sup>m</sup>C<sup>m</sup>A<sup>m</sup>G<sup>m</sup>A<sup>m</sup>CAG GAGGCCTCCAT<sup>m</sup>C<sup>m</sup>T<sup>m</sup>G<sup>m</sup>A<sup>m</sup>G<sup>m</sup>-3', and AS-2 was 5'-C<sup>m</sup>G<sup>m</sup>C<sup>m</sup>T<sup>m</sup>C<sup>m</sup>ACC TTCCAGTGCCAG<sup>m</sup>C<sup>m</sup>T<sup>m</sup>C<sup>m</sup>A<sup>m</sup>-3'. These constructs were placed into osmotic minipumps at a concentration of 42  $\mu$ M in saline.

### *Tat-Peptide Construction*

Tat-GPR GRKKRRQRRRPTMGEDFFDLLAKSQRKRDQDQV LAK, Tat-mGPR (Tat-GPRQ22A), which contains A in place of Q at residue 34 of the Tat-GR peptide, and Tat-fluorescein were synthesized and purified by LSU Health Sciences Center Core Labs (New Orleans, LA) and peptide mass and purity verified by matrix-assisted laser desorption ionization time of flight mass spectrometry. Peptides were dissolved in 136 mM NaCl, 3 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>,

2 mM NaH<sub>2</sub>PO<sub>4</sub>•H<sub>2</sub>O, and 1% glycerol for a final concentration of 4.5 μg/μl.

#### [<sup>35</sup>S]GTPγS Binding for Rat Brain

One week after the repeated cocaine treatment regimen, AS-1 and the scrambled construct were delivered into opposite hemispheres of the PFC for 2 weeks, animals were rapidly decapitated, and the PFC was excised. Membrane preparation and binding were modified from Schaffhauser et al. (2000). Similarly treated hemispheres were pooled from two rats whose locomotor response to repeated cocaine was similar and homogenized on ice in 320 mM sucrose, 20 mM HEPES, 10 mM EDTA, 0.002 μM Brij 30 (Aldrich, Milwaukee, WI), and two tablets protease inhibitor cocktail (Roche, Mannheim) (pH 7.4) with a tissumizer (Teckmar, Cincinnati, OH) and spun down at 52,000 RCF for 20 min at <4°C. The resulting pellet was resuspended twice in 20 mM HEPES, 10 mM EDTA, 0.002 μM Brij 30, and two tablets protease inhibitor (pH 7.4) before being suspended in binding buffer (50 mM Tris, 2 mM MgCl<sub>2</sub>, 1 mM EGTA [pH 7.4]). Protein concentrations were determined utilizing the Bio-Rad DC assay immediately prior to conducting the binding assay. Protein (30 μg) from the membrane preparation, 1 U/ml adenosine deaminase (Sigma, St. Louis, MO), 1 μM GDP, and binding buffer (for a final volume of 1 ml) were incubated for 15 min at 37°C with shaking prior to the addition of ligand (Tocris, Ellisville, MO) and 0.5 μM [<sup>35</sup>S]-GTPγS (Amersham, Piscataway, NJ). Basal binding was determined in the absence of agonist and nonspecific binding measured in the presence of 10 μM unlabeled GTPγS (Sigma, St. Louis, MO). After 45 min, the reaction was terminated by filtration under vacuum through prewet GF/B glass fiber filters (Whatman, Maidstone, UK), followed by three washes with cold 50 mM Tris buffer (pH 7.4). Filters were extracted overnight with 10 ml of Cytoscient ES scintillation fluid (ICN, Aurora, OH) and radioactivity measured by liquid scintillation spectrophotometry (Beckman Coulter, Fullerton, CA). Each sample was run in triplicate and averaged. After background was subtracted, each sample was normalized as percent change from baseline for statistical analysis.

#### [<sup>35</sup>S]GTPγS Binding for GPR-Peptides

GTPγS binding assays were conducted as described (Ross and Higashijima, 1994). Giα1 was purified in the GDP bound state from Sf9 insect cells infected with recombinant virus and was kindly provided by Dr. Stephen G. Graber (West Virginia University School of Medicine, Morgantown, WV). G proteins (100 nM) were preincubated for 20 min at 24°C in the presence and absence of GPR peptides in 50 mM HEPES (pH 7.4), 1 mM EDTA, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 50 μM adenosine triphosphate, and 10 μg/ml bovine serum albumin. Binding assays were initiated by addition of 0.5 mM GTPγS (4.0 × 10<sup>4</sup> dpm/pmol, Dupont/NEN, Boston, MA), and incubations (total volume = 50 μl) continued 30 min at 24°C. Both preincubations and GTPγS binding assays were conducted in binding buffer containing 2 mM MgCl<sub>2</sub>. Reactions were terminated by rapid filtration through nitrocellulose filters (Schleicher and Shuell, Keene, NH) with 4 × 4 ml washes of stop buffer (50 mM Tris, 2 mM MgCl<sub>2</sub>, 1 mM EDTA [pH 7.4], 4°C). Radioactivity bound to the filters was determined by liquid scintillation counting using 8 ml Ecoscint (National Diagnostics, Manville, NJ). Nonspecific binding was defined by 100 μM GTPγS (Sigma, St. Louis, MO).

#### High-Affinity Agonist Binding

Membranes from DDT-MF2 cells expressing α<sub>2A/D</sub> receptors were used to measure high-affinity agonist binding with [<sup>3</sup>H]-UK14304 (Dupont/NEN, Boston, MA). Membranes were prepared by washing confluent cells twice (137 mM NaCl, 2.6 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 4°C) that were then collected by scraping and centrifuged at 220 × g. The pellet was homogenized with a 1 ml tuberculin syringe (5 mM Tris, 5 mM EDTA, 5 mM EGTA [pH 7.4], 4°C), centrifuged at 12,000 RCF in a microcentrifuge, and the pellet washed (50 mM Tris, 0.6 mM EDTA, 5 mM MgCl<sub>2</sub> [pH 7.4], 4°C). High-affinity agonist binding was measured with 50 μg membrane preincubated with GPR peptides for 30 min at 25°C. Binding assays were initiated by addition of 4 nM [<sup>3</sup>H]-UK14304, and incubations (total volume, 50 μl) continued for 30 min at 24°C. Nonspecific binding was determined in the presence of 10 μM rauwolscine (Carl-Roth, Germany).

Low-affinity agonist binding was determined in the presence of 1 mM GppNhp (Roche, Indianapolis, IN). Binding reaction mixtures were incubated at 25°C for 30 min and terminated by rapid filtration through glass fiber filters (Schleicher and Shuell, Keene, NH) with 4 × 4 ml washes of stop buffer (100 mM Tris [pH 7.4], 4°C). Radioactivity bound to the filters was determined by liquid scintillation.

#### Histology and Immunohistochemistry

Histological evaluation of cannula tract placement was conducted in all animals not used for immunoblotting. Subjects were administered an overdose of pentobarbital (100 mg/kg, i.p.) and the brains fixed via intracardiac infusion of formalin. Coronal sections (100 μm thick) were obtained of the cannulae tracts and stained with cresyl violet. For immunocytochemistry, the brains were perfused with phosphate-buffered saline followed by 4% paraformaldehyde. Alternate sections through the cannulae tracts were processed for Nissl staining or GFAP immunoreactivity (Bowers and Kalivas, 2003). Tat-fluorescein transduction of cells in the PFC was evaluated with direct fluorescence, and chromatolysis was assessed with a Nikon Optiphot-II in brightfield.

#### Statistical Analysis

Statistical analysis of optical density measures obtained through immunoblotting of AGS3 during withdrawal was determined by an unpaired Student's t test using StatView (Cary, NC). The effect of antisense was determined using a paired Student's t test, since opposite sides of the same brain were being compared. Analysis of [<sup>35</sup>S]-GTPγS binding studies was conducted with three-way ANOVA with repeated measures over dose followed by a two-way ANOVA comparing treatment groups. A two-way ANOVA with repeated measures over time, followed by least significant difference posthoc comparisons was utilized to analyze behavioral and neurochemical data (Milliken and Johnson, 1984). The absence of ordering effects in Figure 7A was determined using a three-way ANOVA.

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