Activator of G protein signaling 3 regulates opiate activation of protein kinase A signaling and relapse of heroin-seeking behavior

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The nucleus accumbens (NAc) is central to heroin addiction. Activation of opiate receptors in the NAc dissociates $G_{i/o}$ into α and $\beta\gamma$ subunits. $G\alpha_i$ inhibits cAMP production, but $\beta\gamma$ regulates several molecular pathways, including protein kinase A (PKA). We show in NAc/striatal neurons that opiates paradoxically activate PKA signaling by means of $\beta\gamma$ dimers. Activation requires $G\alpha_{i3}$ and an activator of G protein signaling 3 (AGS3). AGS3 competes with $\beta\gamma$ for binding to $G\alpha_{i3}$–GDP and enhances the action of unbound $\beta\gamma$. AGS3 and $G\alpha_{i3}$ knockdown prevents opiate activation of PKA signaling. In rats self-administering heroin, AGS3 antisense in the NAc core, but not shell, eliminates reinstatement of heroin-seeking behavior, a model of human relapse. Thus, $G\alpha_{i3}/\beta\gamma/AGS3$ appears to mediate μ opiate receptor activation of PKA signaling as well as heroin-seeking behavior.

μ opiate receptor | nucleus accumbens core | addiction | $\beta\gamma$ subunits | $G\alpha_{i3}$

Heroin addiction, a worldwide socioeconomic and public health problem, is difficult for physicians to treat, often because addicts frequently relapse when they try to stop using heroin during abstinence. Heroin activates opiate receptors (1). However, we do not understand the postsynaptic cellular mechanisms produced by μ opiate receptor (MOR) activation that contribute to craving for and relapse to heroin. Sharma *et al.* (2) provided pioneering evidence in NG108-15 cells that δ opiate receptor (DOR) activation initially decreases cAMP levels, followed by restoration of cAMP to normal levels during continued exposure to morphine; cAMP increases further in response to opiate receptor blockade. Many investigators have confirmed an opiate-induced increase of cAMP/protein kinase A (PKA) signaling that is exaggerated upon opiate withdrawal (3, 4). *In vivo*, increased cAMP/PKA activity may be associated with opiate-induced tolerance and dependence and is thought to contribute to the reinforcing properties of opiates (5).

Postsynaptic $G_{i/o}$-coupled opiate receptors are expressed on GABAergic medium spiny neurons in the nucleus accumbens (NAc), a brain region thought to account, in part, for craving, reward, and reinforcement of addicting drugs (6, 7). MOR and DOR activation dissociates heterotrimeric $G_{i/o}$ proteins into free α and $\beta\gamma$ subunits. $G\alpha_i$ released from $G_{i/o}\beta\gamma$ inhibits several isoforms of adenylyl cyclase (AC) (8) and appears to account for opiate inhibition of cAMP production. By contrast, $\beta\gamma$ dimers, which are simultaneously released during receptor activation, can stimulate AC2 and AC4 (9–13). However, the precise molecular mechanisms that facilitate this paradoxical $G_{i/o}$-coupled opiate receptor-induced stimulation of cAMP/PKA signaling are not clearly understood.

Recent evidence suggests that an activator of G protein signaling 3 (AGS3) regulates G protein signaling in the NAc (14). By competing with $\beta\gamma$ for binding to $G\alpha_{i3}$, AGS3 appears to stabilize a specific $G\alpha_{i3}$–GDP complex formed before the reassociation of free $G\alpha_{i3}$ and $\beta\gamma$ subunits (15). The bound form of $G\alpha_{i3}$ does not inhibit AC. Both AGS3 and MOR are highly expressed in the NAc (6, 16). This expression suggests that AGS3 could play a role in MOR-induced G protein signaling and heroin addiction. Here, we identify $G\alpha_{i3}$ and AGS3 in primary NAc/striatal cultures as molecular components preferentially associated with the MOR. $G\alpha_{i3}$ and AGS3 appear to be required to enhance the action of free $\beta\gamma$ dimers, which mediate MOR stimulation of PKA signaling. Relapse, or reinstatement of heroin addiction, is a major limitation for effective treatment of human heroin addiction. In rats trained to self-administer heroin, we find that selective expression of AGS3 antisense in the NAc core prevents reinstatement of heroin-seeking behavior, a valid model of human heroin craving and relapse. By contrast, AGS3 antisense in the NAc shell is without effect. These findings suggest that AGS3 may play a critical role in MOR stimulation of PKA signaling in NAc neurons and in the development or maintenance of heroin addiction.

Methods

Preparation and Analysis of Cells. Cells were prepared from NAc/striatum of newborn (postnatal day 0) male Sprague–Dawley rat pups, plated at 5×10^4 cells per slide, and grown in Neurobasal A medium (GIBCO) supplemented with B27 supplement (GIBCO) and glutamate. One-half of the medium was changed 1 day after plating and weekly thereafter. Cells were treated on day 10. Immunostaining and images were obtained as described in ref. 17. For quantification of PKA Cα translocation, random fields on each slide were selected, and cells scored for PKA Cα staining in Golgi, nucleus, cytoplasm, and neurites. For all experiments, at least five fields were scored for each experiment for a total number of at least 40 cells per slide. Data shown were obtained by two independent observers who were blind to the experimental condition. Staining for glutamic acid decarboxylase indicates that >90% of cells are GABAergic neurons (data not shown).

Total RNA was isolated by using TRIzol (Invitrogen) and treated with DNase. First-strand cDNA was synthesized by using 1 μg of RNA in a final volume of 40 μl containing 0.5 μg of oligo(dT), 500 μM each dNTP, and 20 units of Superscript reverse transcriptase (GIBCO). Ten microliters of this reaction was taken for PCR using AGS3-specific primers. PCR was performed at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 25 cycles. PCR products were analyzed on a 1.2% agarose gel.

Herpes simplex virus (HSV) vectors expressing antisense RNA were created by cloning antisense oligonucleotides into HSVLacZ vector under control of mouse U6 polymerase III promoter. The sequences for AGS3 antisense correspond to nucleotides 306–330 of AGS3. The sequences of antisense for $G\alpha_i$, $G\beta$, or $G\gamma$ were designed as described in ref. 18. Ad5AGS3 antisense (AS) under the control of a CMV promoter was created by replacing the E1A gene of the adenovirus. HSV (multiplicity of infection 1) and adenovirus (multiplicity of infection 100) were used to infect primary NAc/striatal neurons.

To assay cAMP-response element (CRE)-luciferase, rat NAc/striatal neurons were plated at 2.5×10^4 cells per 24-well plate and grown for 10 days. Cell transfection, drug treatment, and luciferase assay were carried out as described in ref. 10.

Preparation of Membrane and Solubilized MOR. Confluent primary NAc/striatal cultures in 100-mm dishes were washed with PBS and homogenized with a Brinkmann Polytron homogenizer in 1 ml of buffer (20 mM Tris, pH 7.4/1 mM EGTA/1 mM $MgCl_2$/250 mM sucrose). The homogenate was centrifuged at $120 \times g$ for 30 min. The resulting supernatant was centrifuged at $27,000 \times g$ for 30 min. Crude membrane pellet was resuspended in buffer A, which contains 50 mM Hepes (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 1 mM EGTA, 2 mM DTT, and a protease inhibitor mixture (Roche Molecular Biochemicals). Membranes were centrifuged at $27,000 \times g$ for 30 min, resuspended in fresh buffer A, incubated with 100 nM [D-Ala^2,N-MePhe^4,Gly^5-ol]enkephalin (DAMGO) for 10 min at 30°C, and then chilled on ice for 10 min. 3-[(3-Cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate was added to the membrane suspension to obtain a final concentration of 10 mM. The preparation was incubated on ice with moderate agitation for 30 min and subsequently centrifuged for 1 h at $50,000 \times g$. The supernatant fraction was saved for immunoprecipitation.

Immunoprecipitation and Western Blotting. Solubilized membranes, prepared from cells stimulated with 100 nM DAMGO, were immunoprecipitated with anti-MOR antibodies. Protein complexes were captured by adding Protein A/G-Sepharose beads (Santa Cruz Biotechnology) and continuing the incubation for 30 min at 4°C. The mixture was then microcentrifuged at 4°C, and pellets were washed and resuspended in 2× Laemmli loading buffer. Resuspended samples were boiled for 5 min, and supernatant was loaded on 10% SDS/10% PAGE. Membrane transfer and protein detection were carried out as described in ref. 17.

Animal Surgery and Behavioral Assay. Sprague–Dawley male rats were anesthetized, implanted with jugular catheters and bilateral intracranial guide cannulas, and trained as described in refs. 19 and 20. After animals met maintenance criterion (active lever presses do not vary by >10% across three consecutive days), extinction and reinstatement were conducted as described (19, 20), except that a heroin prime was used. Before reinstatement, animals received a microinfusion of Ad5AGS3 AS or Ad5LacZ. Ad5LacZ was used to confirm viral expression and to control for the effect of viral expression on reinstatement. On days 4–6 after infusion, the subjects were returned to extinction conditions. Reinstatement testing was conducted on day 7 after infusion. Each rat received a priming injection of heroin (0.25 mg/kg s.c.) before placement in the self-administration chamber for a 3-h extinction session. After behavioral testing, rats were anesthetized, and brains were fixed and sectioned as described in ref. 19 to verify infusion sites. There was no evidence of toxicity on histological examination of the NAc from experimental animals (data not shown).
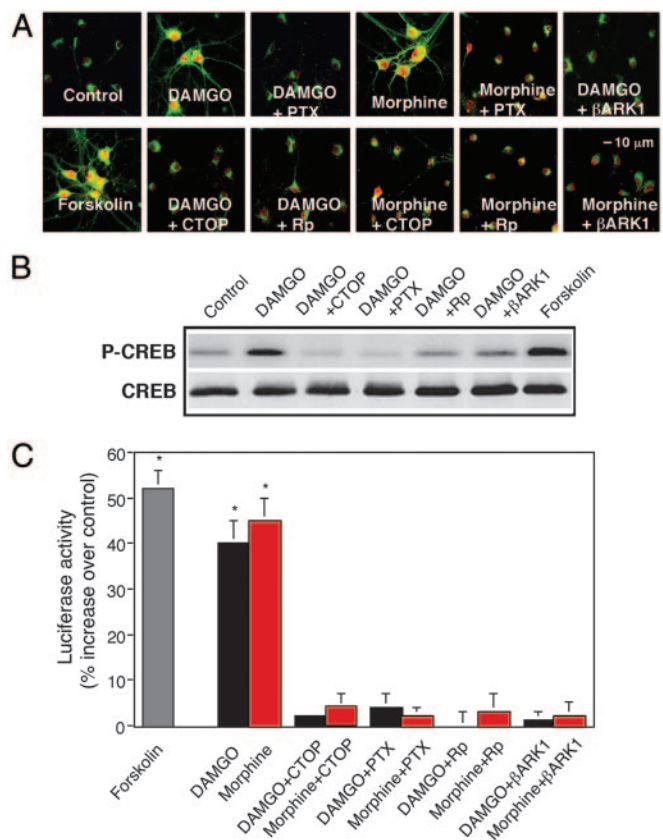

Fig. 1. MOR promotes PKA activation in primary NAc/striatal neurons. (*A*) Confocal analysis of PKA Cα translocation. Cells were incubated with or without 100 nM DAMGO, 100 nM morphine, or 1 μM forskolin for 10 min. Where indicated, cells were preincubated for 30 min with or without 100 nM D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH$_2$ (CTOP) or 20 μM R_P-adenosine 3′,5′-cyclic monophosphorothioate (Rp), overnight with pertussis toxin (PTX) (50 ng/ml) or Ad5βARK1. (*B*) Western blot analysis of CREB phosphorylation (P-CREB) at serine-133. (*C*) Gene activation measured by CRE-luciferase activity. Cells were incubated with DAMGO or morphine as described above. Luciferase was assayed 5 h after drug treatment. Data are the mean ± SEM of at least three experiments. *, $P < 0.01$, compared with control (one-way ANOVA and Dunnett's test).

Results

Activation of MOR Induces PKA Signaling. DAMGO (100 nM) or morphine (100 nM) each causes activation and translocation of PKA Cα from Golgi to the nucleus, cytoplasm, and neurites in primary NAc/striatal neurons (Fig. 1*A*). CTOP, a MOR antagonist, or R_P-adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMPS), a PKA inhibitor, blocks DAMGO and morphine-induced PKA translocation (Fig. 1*A*). By contrast, activation of nonaddictive $G_{i/o}$-coupled adrenergic receptor α2b or muscarinic receptor M4 by UK 14,304 or carbachol, respectively, has no effect (data not shown). As a positive control, forskolin, a direct and potent activator of AC, causes PKA activation and translocation, like DAMGO and morphine (Fig. 1*A*).

PKA Cα translocated into the nucleus phosphorylates CRE-binding protein (CREB) leading to increased CRE-mediated gene expression. DAMGO increases CREB phosphorylation in 10 min (Fig. 1*B*) and CRE-mediated luciferase activity 5 h later (Fig. 1*C*); these effects also are blocked by CTOP or Rp-cAMPS. As a positive control, forskolin produced a similar activation (Fig. 1 *B* and *C*). These data suggest that increases in cAMP-dependent signaling and CRE-mediated gene expression are MOR- and PKA-dependent.
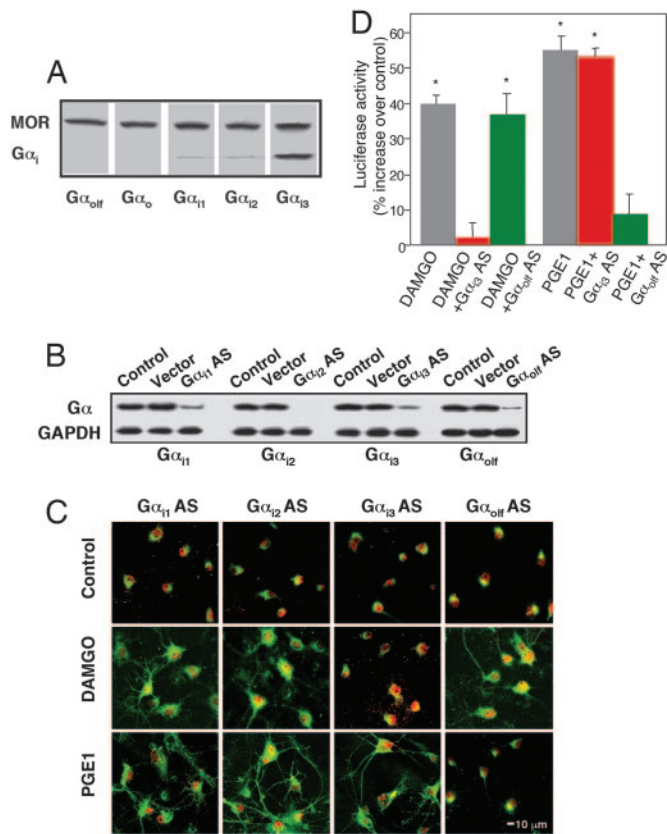
Fig. 2. Gα$_{i3}$ AS blocks PKA activation. (*A*) Coimmunoprecipitation of MOR with Gα$_{i3}$ subunits. MOR was immunoprecipitated with anti-MOR antibodies and probed with anti-Gα$_{olf}$, -Gα$_{o}$, -Gα$_{i1}$, -Gα$_{i2}$, or -Gα$_{i3}$ antibodies. (*B*) Gα$_{i}$ expression was determined by Western blot analysis in cells transfected with or without HSVGα$_{i1}$ AS, Gα$_{i2}$ AS, Gα$_{i3}$ AS, Gα$_{olf}$ AS, or vector control overnight. GAPDH was assayed as control for protein loading. (*C* and *D*) Gα$_{i3}$ AS inhibition of Cα translocation (*C*) and CRE-luciferase expression (*D*). PGE1, prostaglandin E1. Data are the mean ± SEM of at least three experiments. *, *P* < 0.01, compared with control.

MOR Is Preferentially Coupled with Gα$_{i3}$. Activation of G$_{i/o}$-coupled receptors is thought to inhibit cAMP production by means of Gα$_{i}$, but MOR activation also can paradoxically stimulate cAMP/PKA signaling (12, 21). We asked whether MOR couples with a specific G protein to facilitate AC stimulation. Solubilized rat NAc/striatal membranes were immunoprecipitated with anti-MOR antibodies and Western-blotted with anti-Gα$_{o}$, -Gα$_{i1}$, -Gα$_{i2}$, -Gα$_{i3}$, or -Gα$_{olf}$ plus MOR (Fig. 2*A*). We found that only Gα$_{i3}$ coimmunoprecipitates predominantly with MOR (Fig. 2*A*), suggesting that MOR interacts preferentially with Gα$_{i3}$. This finding was confirmed functionally by using AS directed against Gα$_{i1}$, Gα$_{i2}$, Gα$_{i3}$, or Gα$_{olf}$ to knock down expression of each of these proteins in NAc/striatal neurons (Fig. 2*B*). Knockdown of Gα$_{i3}$ specifically blocks DAMGO-induced PKA Cα transloca-tion (Fig. 2*C*) and CRE-mediated luciferase activity (Fig. 2*D*). In contrast, AS for Gα$_{i1}$, Gα$_{i2}$, or Gα$_{olf}$ was without effect (Fig. 2*C*). In control studies, AS directed against Gα$_{olf}$, but not Gα$_{i3}$, blocks Gα$_{s}$/Gα$_{olf}$-coupled prostaglandin E1-induced PKA sig-naling (Fig. 2 *C* and *D*). Antisense for Gα$_{i1}$ or Gα$_{i2}$ does not change DAMGO-induced PKA Cα translocation (Fig. 2*C*) or CRE-mediated gene expression (data not shown), confirming the preferential requirement of Gα$_{i3}$.

AGS3 Enhances MOR-Induced PKA Signaling. AGS3 also appears to interact preferentially with Gα$_{i3}$, which is released upon MOR activation. When AGS3 binds to Gα$_{i3}$, a stable AGS3–Gα$_{i3}$–
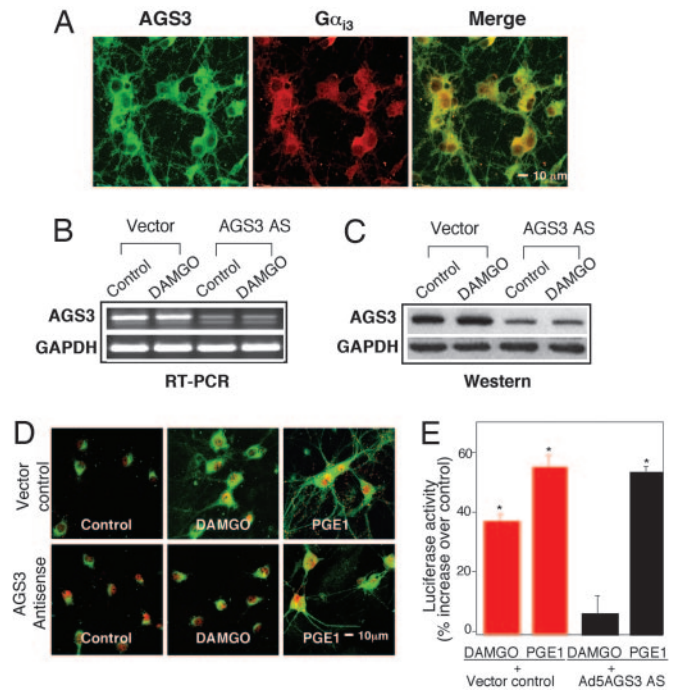
Fig. 3. AGS3 AS blocks PKA signaling. (*A*) Confocal analysis of AGS3 expres-sion and colocalization with Gα$_{i3}$ subunits. Shown are AGS3 (green), Gα$_{i3}$ (red), merge (yellow). (*B* and *C*) AGS3 expression detected by RT-PCR (*B*) and Western blot analysis (*C*). Cells were transfected with or without Ad5AGS3 AS or vector control overnight, treated with or without DAMGO for 10 min, and then lysed for RT-PCR or Western blot analysis. (*D* and *E*) AGS3 AS inhibition of Cα translocation (*D*) and CRE-luciferase expression (*E*). Cells were transfected with Ad5AGS3 AS or vector control (*D*) and HSVCRE-luciferase (*E*) overnight. Data are the mean ± SEM of at least three experiments. *, *P* < 0.01, compared with control.

GDP complex is formed that maintains inactivation of Gα$_{i3}$, thereby limiting Gα$_{i3}$ recycling and its potential inhibition of AC (15). AGS3 and MOR are expressed in the NAc (6, 16). Therefore, AGS3 could modulate MOR signaling through Gα$_{i3}$. By using immunocytochemistry, we determined that AGS3 and Gα$_{i3}$ are expressed and colocalized in membranes, cytoplasm, and Golgi in primary NAc/striatal neurons (Fig. 3*A*). AS directed against AGS3 knocks down AGS3 mRNA and protein by >80% (Fig. 3 *B* and *C*) and prevents DAMGO-induced PKA Cα translocation (Fig. 3*D*) and CRE-mediated luciferase activity (Fig. 3*E*). By contrast, AGS3 AS is without effect on prosta-glandin E1-induced PKA translocation and gene expression, because this activation depends on Gα$_{olf}$ or Gα$_{s}$, not Gα$_{i3}$ (Fig. 3 *D* and *E*). These findings are consistent with the possibility that, after MOR-induced release of Gα$_{i3}$ and βγ subunits, AGS3 competes with βγ binding to a Gα$_{i3}$–GDP complex (22), thereby enhancing MOR-dependent stimulation of AC by βγ. This possibility was confirmed in NAc/striatal neurons preincubated with PTX to inhibit Gα$_{i/o}$ dissociation into α$_{i3}$ and βγ, or the Ad5βARK1 dominant-negative minigene to inhibit the action of βγ (23). We find that PTX or Ad5βARK1 each blocks DAMGO- or morphine-induced PKA Cα translocation (Fig. 1*A*), CREB phosphorylation (Fig. 1*B*), and CRE-mediated luciferase expression (Fig. 1*C*).

MOR Activation Stimulates AC2 and AC4. Several lines of evidence indicate that βγ dimers stimulate AC2, AC4, and perhaps AC7 (9–13). Western blot analysis shows that AC2, AC4, and AC7 are expressed in primary NAc/striatal neurons; AS against AC2, AC4, or AC7 knocks down the expression of each isoform,

Fig. 4. AC2 and AC4 AS block PKA activation. (*A*) AC2, AC4, and AC7 expression. Cells were transfected with or without HSVAC2 AS, AC4 AS, or AC7 AS overnight and then lysed. Lysates (20 μg) were Western-blotted with antibodies specific for AC2, AC4, or AC7, respectively. (*B* and *C*) AC2 AS and AC4 AS inhibition of Cα translocation (*B*) and CRE-luciferase expression (*C*). Cells were transfected with HSVAC2 AS, AC4 AS, AC7 AS, or AC3 AS overnight. Data are the mean ± SEM of at least three experiments. *, $P < 0.01$, compared with control.

respectively (Fig. 4*A*). DAMGO-induced PKA Cα translocation (Fig. 4*B*) and CRE-luciferase expression (Fig. 4*C*) are inhibited by AC2 and AC4 antisense. Antisense for AC3 and AC7 have no effect on DAMGO-induced PKA signaling (Fig. 4 *B* and *C* and data not shown). These findings suggest that AC2 and AC4, which are known to be expressed in the NAc (24, 25), appear to be required for βγ-mediated stimulation of cAMP/PKA signaling upon MOR activation.

AGS3 Is Required for Heroin-Seeking Behavior. MOR activation of PKA signaling in NAc/striatal neurons requires AGS3. The NAc core has been implicated in heroin-induced reinstatement of drug-seeking behavior (25). Heroin self-administration was established in rats (26). We asked whether heroin-induced reinstatement would be regulated by AGS3 in the NAc. Expression of AGS3 antisense in the core of the NAc abolishes heroin-seeking behavior but is without effect when expressed in the NAc shell (Fig. 5). Moreover, AGS3 AS in the NAc did not affect heroin reinforcement as measured by heroin self-administration (data not shown). Therefore, AGS3 appears to be required in the NAc core primarily for the reinstatement of heroin-seeking behavior, a model of human relapse.

Discussion

In this paper, we identify an important MOR-activated pathway in NAc/striatal neurons that stimulates cAMP/PKA signal transduction, followed by activation of CRE-mediated gene expression. AGS3 appears to be a key component of this

Fig. 5. Ad5AGS3 AS eliminates heroin-seeking behavior in rats. AS for AGS3 (Ad5AGS3 AS, 2 μl per side, 10^{12} plaque-forming units/ml) was infused into the core or the shell of the NAc. AGS3 AS in the core of the NAc prevented reinstatement of lever pressing elicited by a priming injection of heroin. Data represent mean active lever presses ± SEM, $n = 8$ in all groups. *, $P < 0.01$, compared with extinction responding (EXT) (two-way ANOVA, Tukey's posttest).

pathway, because it is required for MOR-induced PKA signaling in primary NAc/striatal neurons. AGS3 in the core of the NAc is also required for reinstatement of heroin-seeking behavior. Our major findings are that: (*i*) $Gα_{i3}$ and AGS3 associate preferentially with the MOR in NAc/striatal neurons, which appears to provide specificity for MOR-induced release of βγ dimers from $G_{i/o}$; (*ii*) $Gα_{i3}$, AGS3, and βγ dimers are required for MOR stimulation of cAMP/PKA signaling and CRE-mediated gene expression in NAc/striatal neurons; and (*iii*) AGS3 in the core of the NAc is required for heroin-seeking behavior during opiate withdrawal. MOR agonists paradoxically induce PKA Cα activation and translocation to the nucleus and CREB phosphorylation within 10 min, followed by CRE-mediated gene expression hours later (Fig. 1). These events appear to be determined first by the predominant association of the MOR with $Gα_{i3}$ (Fig. 2) and subsequently, after $Gα_{i3}$ and βγ subunits are dissociated upon MOR activation, by the binding interaction of $Gα_{i3}$ with AGS3 (Figs. 2 and 3). AGS3 is thought to bind transiently to the GDP-bound conformation of $Gα_{i3}$, maintaining attenuation of $Gα_{i3}$ inhibition of AC (15). Simultaneously, by competing with βγ for binding to $Gα_{i3}$ (22), AGS3 delays the reassociation of free βγ dimers with $Gα_{i3}$ and would be expected to prolong βγ stimulation of cAMP production unencumbered by $Gα_i$ inhibition (27). Thus, interventions to prevent AGS3 from binding to the $Gα_{i3}$–GDP complex would be expected to hasten reassociation of free βγ dimers with $Gα_{i3}$, thereby reducing βγ stimulation of AC. Consistent with this possibility, AS knockdown of AGS3 selectively prevents MOR-dependent increases in cAMP/PKA signaling (Fig. 3); whereas AS knockdown of AGS1 and AGS2 is without effect (data not shown).

The NAc core is thought to contribute to addictive behaviors (28). Inactivation of the NAc core, but not the shell, inhibits heroin self-administration (26) and heroin-induced reinstatement of drug-seeking behavior (K.M., unpublished data). Further, self-administration of heroin is associated with up-regulation of gene transcripts involved in neuronal function within the NAc core but down-regulation of these same tran-

scripts within the NAc shell (29). Notably, inactivation of the NAc core (but not shell) (19, 30) or interference with prefrontal glutamatergic projections to the NAc core (20) also prevents cocaine-induced reinstatement of drug-seeking behavior (14). In that study, AGS3 AS in the prefrontal cortex prevented relapse of cocaine-seeking behavior. In this study, AGS3 AS in the NAc core, but not the shell, abolishes reinstatement of heroin-seeking behavior during withdrawal, a valid model of relapse in human heroin addiction (Fig. 5). It is of interest that AGS3 AS in the NAc did not block heroin reinforcement as measured by heroin self-administration (data not shown). This result argues for a specific effect of AGS3 AS on reinstatement and that MOR receptor blockade is not involved in this action. Our findings are consistent with current concepts about the special importance of the core of the NAc in addictive behavior (25, 27) and suggest that AGS3 in the core may regulate craving and relapse in heroin addiction.

The specific requirement for AGS3 in MOR-activated cAMP/PKA signaling appears to depend on the preferential association of $G\alpha_{i3}$ with the MOR (Fig. 2A). Thus, knockdown of $G\alpha_{i3}$ eliminates MOR-induced PKA signaling and CRE gene expression; knockdown of other $G\alpha_i$ subunits is without effect (Fig. 2C and data not shown). We propose that in the absence of $G\alpha_{i3}$, when $\beta\gamma$ dimers would be rapidly degraded (31), a $G\alpha_{i3}\beta\gamma$ complex is not available to dissociate upon MOR activation so that free $\beta\gamma$ is not formed and cannot stimulate AC2 or AC4 (12). Thus, $G\alpha_{i3}$ as well as AGS3 appears to be required for $\beta\gamma$ action. Our finding that the MOR preferentially interacts with $G\alpha_{i3}$ (Fig. 2A) is consistent with other reports that $G\alpha_{i3}$ is associated with the MOR and the DOR (32). However, adrenergic receptor α2b and muscarinic receptor M4, which are coupled primarily to $G\alpha_{i2}$, only inhibit cAMP production in the same cells (data not shown) and do not activate PKA signaling (10, 33, 34). This result suggests that the preferential association of $G\alpha_{i3}$ with the MOR appears to be required for AGS3 regulation of $\beta\gamma$-stimulated cAMP/PKA signaling. However, we have not proven that AGS3/$\beta\gamma$ regulation of PKA signaling, which we show in NAc/striatal neurons *in vitro*, is responsible in the NAc for AGS3/$\beta\gamma$ regulation of heroin-seeking behavior *in vivo*. Other actions of $\beta\gamma$ subunits, such as activation of G protein-activated inwardly rectifying potassium channels, could also be involved (35).

A prevailing concept is that short-term activation of MOR and DOR only inhibits AC activity. Several laboratories, however, find that increased cAMP production can be detected within minutes after exposure to opiate agonists (10, 12, 21, 36, 37). Our work suggests that unbound $\beta\gamma$ dimers could be responsible for these recent observations. Moreover, our studies also suggest the possibility that $\beta\gamma$ dimers may play a more general role in cellular mechanisms involved in addiction to other drugs. Dopamine D2, δ-opioid, and cannabinoid CB1 receptors also appear to be coupled preferentially to $G\alpha_{i3}$ (38, 39). We find that $\beta\gamma$ dimers are required for dopamine, opiate, cannabinoid, and ethanol stimulation of cAMP/PKA signaling and for voluntary alcohol consumption (10, 17). Thus, $G\alpha_{i3}$, $\beta\gamma$, and AGS3 appear to be components of a regulatory mechanism shared in common by those $G_{i/o}$-coupled receptors involved in addiction. It is well established that $\beta\gamma$ dimers have diverse effects in cells, including stimulation of PKA signaling by means of AC2 and AC4 (11, 40), which could be involved in addiction (3).

Uncontrolled craving and relapse is perhaps the most important clinical problem that limits effective medical treatment of addiction. Current concepts suggest that reinstatement during withdrawal is a valid model of human drug-seeking behavior (craving). One of our most important findings during heroin withdrawal is that AS directed against AGS3 in the core of the NAc, but not the shell, abolishes reinstatement in heroin-addicted rats. This finding is consistent with recent findings that AGS3 appears to be required for cocaine-induced behavioral sensitization and reinstatement (14). We propose that, by competing with $\beta\gamma$ for binding to the $G\alpha_{i3}$–GDP complex (22), AGS3 enhances the action of $\beta\gamma$ dimers, and that $G\alpha_{i3}$, AGS3, and $\beta\gamma$ dimers appear to be components of a signaling pathway that underlies addictive behavior. Our results suggest that molecular mechanisms that differentially regulate the action of $G\alpha_{i3}$, AGS3, and $\beta\gamma$ dimers could underlie drug-seeking behavior (craving) and mediate relapse in heroin addicts. These observations open an avenue to develop treatments for heroin addiction directed against relapse.

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