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Up-Regulation of AGS3 during Morphine Withdrawal Promotes cAMP Superactivation via Adenylyl Cyclase 5 and 7 in Rat Nucleus Accumbens/Striatal Neurons

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ABSTRACT

Effective medical treatment of opiate addiction is limited by a high relapse rate in abstinent addicts. Opiate withdrawal causes cAMP superactivation, but the underlying molecular mechanisms are not clear. Recent evidence implicates an activator of G-protein signaling 3 (AGS3) in opiate addiction. We found previously that during a 10-min activation of opioid receptors, AGS3 binds $G\alpha_i$-GDP to promote free $G\beta\gamma$ stimulation of adenylyl cyclase (AC) 2 and 4, and/or inactivate $G\alpha_i$ inhibitory function, thereby transiently enhancing cAMP-dependent protein kinase A (PKA) activity. In contrast, we report here that in nucleus accumbens/striatal neurons, morphine withdrawal induces cAMP superactivation, which requires up-regulation of AGS3. cAMP increases as a function of withdrawal time, by approximately 20% at 10 min and 75% at 5 h. However, cAMP superactivation does not require $G\beta\gamma$. Instead, adenosine A2A receptor activation of $G\alpha_{s/olf}$ seems to initiate cAMP superactivation and promote AGS3 up-regulation. Elevated AGS3 binds to $G\alpha_i$ to prevent its inhibition on AC activation. Moreover, withdrawal-induced increases in cAMP/PKA activate phospholipase C and ε protein kinase C to further stimulate AC5 and AC7, causing cAMP superactivation. Our findings identify a critical role for AC 5 and 7 and A2A receptors for up-regulation of AGS3 in morphine withdrawal-induced cAMP superactivation.

Opiate addiction is a worldwide public health problem, but effective treatment is limited by high rates of relapse during abstinence (O'Brien, 2005). Sharma et al. (1975) developed model neuronal cell systems expressing opiate receptors to identify molecular mechanisms associated with exposure to and withdrawal from opiates. They discovered that activation of opioid receptors in NG108-15 cells produced an initial reduction in cAMP followed by compensatory up-regulation during continued exposure to morphine. It is noteworthy that cAMP increased even further after opiate receptor blockade with antagonists or by morphine withdrawal, a phenomenon described as cAMP overshoot, superactivation, supersensitization, or heterologous sensitization of adenylyl cyclase (AC) (Watts, 2002). Increased cAMP activates cAMP-dependent protein kinase A (PKA) and cAMP response element (CRE)-dependent gene transcription, thought to regulate the development of tolerance and dependence (Chao and Nestler, 2004). Opiates exert their actions by binding to the δ-opioid receptor (DOR), the μ-opioid receptor (MOR), and the κ-opioid receptor. Morphine, an opiate agonist, acts primarily on the MOR to promote opiate seeking behavior (Narita et al., 2001).

Opiate receptors belong to a superfamily of pertussis toxin (PTX)-sensitive G-protein coupled receptors. Activation of MOR releases $G\alpha_i$ and $G\beta\gamma$. $G\alpha_i$ inhibits adenylyl cyclase to decrease cAMP production. On the other hand, $G\beta\gamma$ can stimulate AC and also activate several other down-stream signaling molecules, including phospholipase C (PLC), potassium channels, and so forth. (Gautam et al., 1998). Emerging evidence suggests that cAMP superactivation consists of enhanced $G\alpha_s$-receptor coupling, G-protein dissociation, and $G\alpha_s$-adenylyl cyclase interaction (Watts and Neve, 2005; Chakrabarti and Gintzler, 2007). However, it is unclear how

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ABBREVIATIONS: AC, adenylyl cyclase; PKA, cAMP-dependent protein kinase A; CRE, cAMP response element; MOR, μ-opioid receptor; DOR, δ-opioid receptor; PTX, pertussis toxin; PLC, phospholipase C; AGS3, activator of G-protein signaling 3; NAc, nucleus accumbens; Rp-cAMPS, adenosine-3′,5′-cyclic monophosphorothioate, *R*p-isomer; GF109203X, bisindolylmaleimide I; siRNA, small interfering RNA; PBS, phosphate-buffered saline; CTOP, H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; PKC, protein kinase C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANOVA, analysis of variance; A2A, adenosine A2A receptor; Et-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphorylcholine (edelfosine); KW6002, 7-methyl-3,7-dihydro-1*H*-purine-2,6-dione (istradefylline).

G-protein dissociation could occur in the absence of morphine during withdrawal and how $G\alpha_s$ can be involved in $G\alpha_i$-coupled MOR signaling. Recent evidence suggests that signaling via G-proteins can be regulated by receptor-independent activators (Takesono et al., 1999). A family of such regulatory accessory proteins includes an activator of G-protein signaling 3 (AGS3) (Blumer et al., 2007). AGS3 binds to $G\alpha_i$-GDP (De Vries et al., 2000), enhances unbound free $G\beta\gamma$ stimulation of AC2 and AC4 (Yao et al., 2005), and/or diminishes $G\alpha_i$-GTP inhibition of AC (Takesono et al., 1999; Kimple et al., 2002). We have shown that knockdown of AGS3 or expressing a $G\beta\gamma$ inhibitor blocks morphine-induced cAMP/PKA signaling in primary nucleus accumbens/striatal neurons (Yao et al., 2005). Inhibition of AGS3 expression in rat nucleus accumbens (NAc) core or rat prefrontal cortex also eliminates withdrawal-induced relapse of heroin-, cocaine-, and ethanol-seeking behavior (Bowers et al., 2004, 2008; Yao et al., 2005). Moreover, withdrawal from cocaine or ethanol increases AGS3 expression in rat prefrontal cortex and the core of nucleus accumbens (Bowers et al., 2004, 2008). These findings suggest that up-regulation of AGS3 may be a decisive molecular mechanism underlying opiate withdrawal-induced cAMP superactivation and relapse.

Materials and Methods

Materials. All reagents were purchased from Sigma (St. Louis, MO), except where indicated. Rp-cAMPS was obtained from BioLog (La Jolla, CA). Bisindolylmaleimide I (GF109203X) was purchased from Calbiochem (San Diego, CA). cAMP assay kit and [γ-^{32}P]ATP (6000 Ci/mmol) were purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Complete protease inhibitor tablets were purchased from Roche Molecular Biochemicals (Indianapolis, IN).

Cell Culture and Transfection. Primary nucleus accumbens (NAc)/striatal neuron preparations and culture were carried out as described previously (Yao et al., 2002). In brief, NAc/striatum cells from Sprague-Dawley rat newborn brain (postnatal day 0) were plated at 5×10^4 cells/cm^2 and grown in Neurobasal A medium (Invitrogen, Carlsbad, CA) supplemented with 1× B27 supplement (Invitrogen). Half the medium was changed 1 day after plating and weekly thereafter. Experiments were carried out on day 10. For siRNA transfection, cells were incubated with 100 pmol of siRNA for AC2, AC4, AC5, or AC7 (Santa Cruz Biotechnology, Santa Cruz, CA) plus 5 μl of Lipofactamine 2000 (Invitrogen). Transfection was performed as described by the manufacturer. For adenovirus transfection, cells were incubated with Ad5βARK1 (Yao et al., 2002) or Ad5AGS3AS (Yao et al., 2005) at a multiplicity of infection of 100.

cAMP Assay. NAc/striatal neurons were treated with 100 nM morphine or not treated for 18 h. To withdraw, cells were completely washed three times with culture medium and continued incubation for the indicated time in the absence of morphine. Cells were then harvested, suspended in 1 ml of Opti-MEM I (Invitrogen) containing 1 unit/ml adenosine deaminase and 10 μM rolipram, and incubated at 37°C for 10 min. cAMP was assayed using HitHunter cAMP XS Assay kit (GE Healthcare) as described by the manufacturer. Data are mean ± S.E.M. obtained from three to six independent experiments, with each treatment carried out in triplicate.

Immunochemistry and Microscopy. Cells were fixed with 4% formaldehyde in 160 mM sucrose/PBS at room temperature for 15 min, then with 4% formaldehyde plus 0.5% Triton X-100 for another 15 min, rinsed three times with PBS, incubated at room temperature with blocking buffer (1% normal goat serum in PBS and 0.1% Triton X-100) for 3 to 4 h and overnight at 4°C in PBS containing 0.1% Triton X-100, 2 mg/ml fatty acid-free bovine serum albumin (Dohrman et al., 1996), and primary antibody specific for PKA Cα (BD Biosciences, San Jose, CA). The cells were then washed three times with PBS, incubated for 1 h at room temperature with goat anti-mouse IgM (Cappel, Aurora, OH) (diluted 1:1000) and anti-NeuN (Millipore Bioscience Research Reagents, Temecula, CA), washed three times with PBS, and coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Cells were imaged using a Carl Zeiss LSM PASCAL (Jena, Germany) laser scanning microscope equipped with an argon laser attached to an Axiovert 200 M microscope. Images were collected as z-series. Collected data were processed using NIH Image (http://rsb.info.nih.gov/nih-image/) and Photoshop software (Adobe Systems, Mountain View, CA). All images shown were obtained under 40× magnification from individual middle sections of the projected z-series. To view PKA Cα translocation, random fields on each slide were selected, and cells were examined for PKA Cα staining in the Golgi, nucleus, cytoplasm, and neurites.

Cell Fractionation. Cells in 100-mm dishes (2×10^6 cells/dish) were washed with ice-cold PBS and lysed in 1 ml of lysis buffer containing 20 mM Tris, pH 7.5, 2 mM EDTA, 10 mM EGTA, 0.25 M sucrose, and one tablet of protease inhibitor/10 ml. Cells were homogenized by 10 passes through a 26-gauge needle and centrifuged at 3000 rpm for 5 min at 4°C. The supernatant was centrifuged for 8 min at 70,000 rpm at 4°C in a TLA 120 rotor (Beckman Coulter, Fullerton, CA) to separate the membrane pellet from the cytosol. The supernatant was saved as the cytosolic fraction. The remaining pellets were suspended in 1 ml of lysis buffer containing 1% Triton X-100, titrated, and incubated on ice for 20 min. This suspension was centrifuged as described above, and the Triton-soluble material was collected as the original particulate fraction. Each experiment was repeated three times with each treatment in triplicates.

Immunoprecipitation and Western Blot. Cells in 100-mm dishes (2×10^6 cells/dish) were washed with ice-cold PBS, harvested in 1 ml of lysis buffer, and lysed on ice for 20 min. The lysates were centrifuged at 14,000 rpm at 4°C in an Eppendorf centrifuge and the supernatant was subject to immunoprecipitation. Five micrograms of pan PKC polyclonal immunoglobulin G antibody or AGS3 polyclonal immunoglobulin G antibody was incubated with 50 μl of protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Antibody-bound beads were then washed twice with PBS and blocked with 3% bovine serum albumin for 2 h at 4°C. Protein samples were precleared with protein A/G beads for 30 min at 4°C, incubated with the antibody-bound beads overnight at 4°C, and subsequently washed four times with PBS. Bound material was eluted with SDS sample buffer, run on a 10% SDS/polyacrylamide gel electrophoresis, transferred, and probed for AGS3 (EMD Chemicals, Gibbstown, NJ), PKA Cα (BD Biosciences), εPKC, or $G\alpha_{i3}$ (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibody was horseradish peroxidase-linked goat anti-mouse or anti-rat (1:1000) (PerkinElmer Life and Analytical Sciences, Waltham, MA). Proteins were detected using chemiluminescence substrate (PerkinElmer Life and Analytical Sciences).

PKC Activity Assay. Cells in 100-mm dishes (2×10^6 cells/dish) were washed with ice-cold PBS, harvested in 1 ml of lysis buffer, and lysed on ice for 20 min. The lysates were centrifuged at 14,000 rpm at 4°C in an Eppendorf centrifuge. The supernatant was immunoprecipitated for PKC as described above. To assay PKC activity, immunoprecipitates were incubated at 30°C for 20 min with 10 μM ATP, 0.5 μCi of [γ-^{32}P]ATP, and a peptide substrate mixture from SignaTECT PKC Assay System (Promega, Madison, WI). PKC activity was detected as described by the manufacturer. The values are represented as the mean ± S.E.M. of three independent experiments with each treatment in triplicates.

Results

Morphine Withdrawal Induces AGS3 Up-Regulation. Recent studies suggest that increased AGS3 expression seems to be required for cocaine- and ethanol-induced rein-

statement (Bowers et al., 2004, 2008). We therefore sought to determine whether morphine withdrawal increases AGS3 expression. NAc/striatal neurons were exposed to 100 nM morphine for 18 h. To withdraw, cells were washed three times with growth medium and cultured for the indicated times in the absence of morphine. Western blot analysis shows that such long-term exposure to morphine did not alter AGS3 expression (Fig. 1, C and D). However, morphine withdrawal increased AGS3 expression, starting at 2 h and reaching a maximum at 3 h (Fig. 1, A and B). Because exposure to morphine can stimulate PKA signaling, we sought to determine whether PKA also plays a role in morphine withdrawal-induced AGS3 expression. Figure 1, C and D, shows that the PKA antagonist Rp-cAMPS prevented AGS3 up-regulation measured 5 h after morphine withdrawal, suggesting that the increase in AGS3 expression is PKA-dependent. In contrast, Rp-cAMPS had no effect on basal AGS3 expression (data not shown).

Morphine Withdrawal Increases cAMP and Activates PKA. Because PKA seems to be required for AGS3 up-regulation during morphine withdrawal, we sought to determine whether morphine withdrawal increases cAMP in NAc/striatal neurons. Withdrawal from morphine induced cAMP production as a function of time, increasing by approximately 20% at 10 min and reaching 75% at 5 h (Fig. 2A). Increasing morphine exposure time from 18 to 24 or 48 h, or increasing morphine concentrations from 100 nM to 300 nM or 1 μM, did not further increase morphine withdrawal-induced cAMP production (data not shown).

The NAc/striatum expresses DOR and MOR. To determine which receptor is involved in cAMP superactivation, cells were pretreated with opioid receptor antagonists for 30 min and continued incubation with 100 nM morphine for 18 h, followed by 5-h withdrawal. Withdrawal-induced cAMP superactivation was attenuated by the DOR antagonist naltrindole (100 nM) and largely prevented by 0.1 μM CTOP, an MOR antagonist. Higher concentrations of CTOP (0.3 and 1 μM) were not more effective. In contrast, cAMP superactivation was completely blocked by the nonselective opioid receptor antagonist naltrexone (1 μM) (Fig. 2B). In addition, pretreatment with PTX also blocked the cAMP increase (Fig. 2B), suggesting that $G\alpha_{i/o}$ is required for cAMP superactivation. In control studies, opioid antagonists or PTX did not affect basal cAMP levels (data not shown).

Increased cAMP activates PKA by binding to its regulatory subunit and releasing the catalytic subunit (Cα), which translocates from the Golgi to other cell compartments to phosphorylate its substrates (Yao et al., 2006). Confocal microscopy shows that 10 min after morphine withdrawal, PKA Cα was translocated from the Golgi to the cytoplasm and the nucleus, similar to the effect of a 10-min treatment with the AC activator, forskolin (Fig. 2C). Western blots confirmed Cα translocation from the membrane to the cytosolic fraction (Fig. 2D). This finding is consistent with an opiate withdrawal-induced increase in cAMP at 10 min, suggesting that a 20% increase in cAMP is sufficient to activate PKA. By contrast, long-term exposure to morphine did not cause PKA Cα translocation (Fig. 2, C and D).

Adenosine A2A Receptors Promote cAMP Superactivation via AC5 and AC7. Increasing evidence suggests that cAMP superactivation involves receptor-$G\alpha_s$ coupling and enhanced $G\alpha_s$ sensitization (Chakrabarti and Gintzler, 2007). The NAc/striatum is enriched in $G\alpha_{s/olf}$-coupled adenosine A2A receptors (A2A), which stimulate cAMP production in response to released adenosine (Yao et al., 2006) and modulate the neuronal responses to heroin (Yao et al., 2006). Thus, we first determined whether A2A is involved in the initiation of withdrawal-induced cAMP superactivation by eliminating adenosine or blocking A2A before withdrawal. Indeed, adenosine deaminase, which degrades adenosine, or

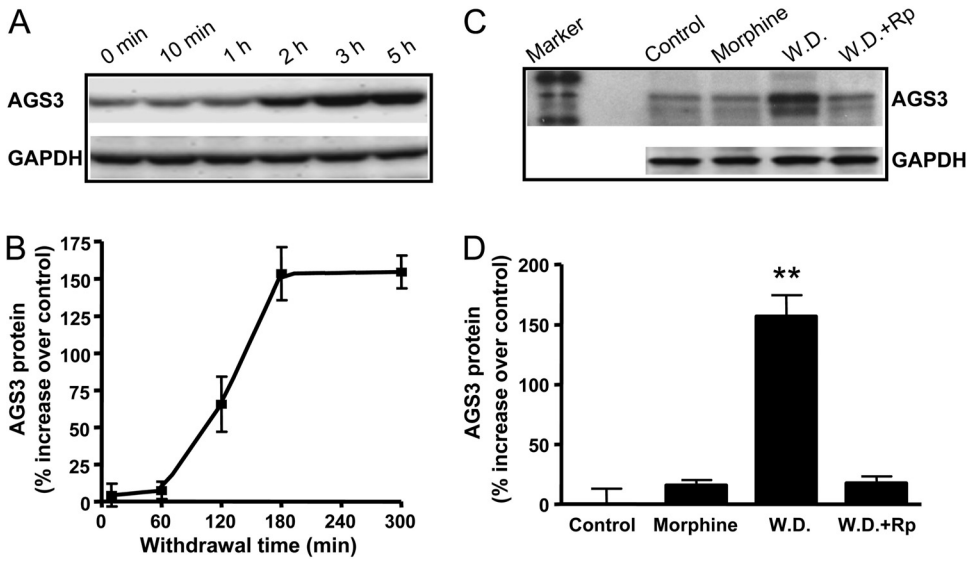

Fig. 1. Morphine withdrawal increases AGS3 expression. A, representative Western blot showing AGS3 expression as a function of time. NAc/striatal neurons were treated with morphine (100 nM) for 18 h. To withdraw, cells were washed with growth medium for three times and continue cultured for the indicated time. Cells were then lysed for Western analysis of AGS3 expression. B, relative intensity of individual bands from A was quantitated using http://rsbweb.nih.gov/ij/ software. Blots were normalized to GAPDH. Data are the mean ± S.E.M. of three independent experiments. C, representative Western blot showing PKA-dependent AGS3 up-regulation. Morphine withdrawal (W.D.) was performed as above, and cells were then incubated for 5 h with or without Rp-cAMPS (Rp, 20 μM). D, relative intensity of individual bands from C was quantitated using ImageJ software. Blots were normalized to GAPDH. Data are the mean ± S.E.M. of three independent experiments. **, $p < 0.01$ compared with control (one-way ANOVA and Dunnett's test).

the A2A antagonist KW-6002 inhibited morphine withdrawal-induced cAMP superactivation, respectively (Table 1). Then, we determined whether A2A plays a role in the sustained cAMP superactivation by treating cells with adenosine deaminase or KW-6002 at 3 h after withdrawal. We found that A2A was not involved in sustaining cAMP superactivation (Table 2), suggesting that A2A is required only for the initial stimulation of cAMP superactivation. Next, we determined which ACs are involved in morphine withdrawal-induced cAMP superactivation by knocking down each AC isoform with a sequence-specific siRNA. Each siRNA suppressed its corresponding AC expression by more than 80% but had no significant effect on basal cAMP levels (data not shown). However, siRNA for AC5 or AC7 alone significantly attenuated morphine withdrawal-induced cAMP superactivation; when the siRNA were added together, morphine withdrawal-induced cAMP superactivation was completely blocked. It is noteworthy that siRNA for AC2, AC4, and other ACs had no effect (Table 1, or data not shown). Activation of A2A releases $G\alpha_{s/olf}$, which should stimulate all AC isoforms (Tang and Gilman, 1991). The selective requirement for AC5 and AC7 in opiate-withdrawal induced superactivation suggests that other signaling molecules are likely to be involved.

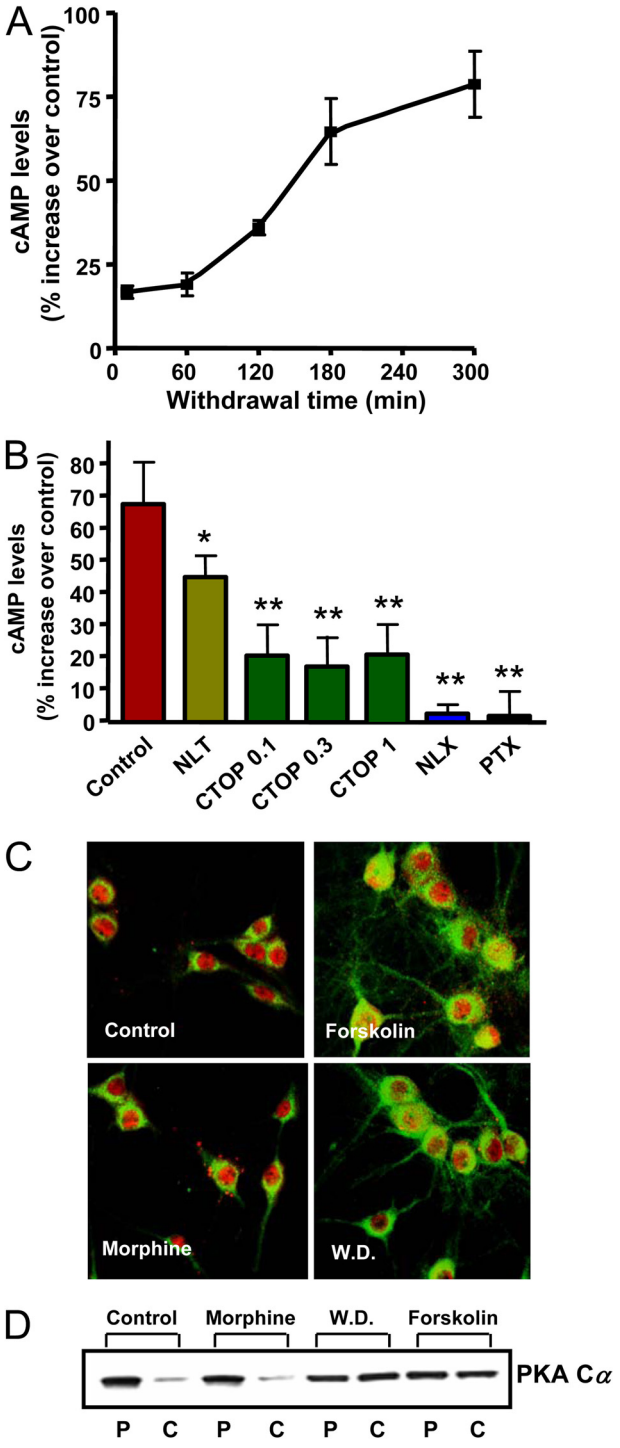

Fig. 2. Morphine withdrawal induces cAMP/PKA signaling. A, cAMP increases as a function of time. NAc/striatal neuron culture and morphine withdrawal (W.D.) were conducted as Fig. 1A. Cells were harvested at the indicated time, and cAMP was determined as described under *Materials and Methods*. Data are the mean ± S.E.M. of four independent experiments with each treatment carried out in triplicate. B, the role of MOR and DOR in cAMP superactivation. Cells were preincubated with the DOR antagonist naltrindole (NLT, 100 nM), the MOR antagonist CTOP (0.1 μM, CTOP 0.1; 0.3 μM, CTOP 0.3; or 1 μM, CTOP 1), or the nonselective opioid receptor antagonist naltrexone (NLX, 1 μM) for 30 min, or PTX (50 ng/ml) overnight, respectively. Cells were then treated with 100 nM morphine for 18 h. cAMP was assayed 5 h after withdrawal. Data are the mean ± S.E.M. of six independent experiments with each treatment carried out in triplicates. C, representative confocal microscopic image showing PKA Cα translocation from the Golgi to the cytoplasm determined 10 min after withdrawal (W.D.). Forskolin (1 μM for 10 min) was used as a positive control. Green indicates Cα, and red indicates nucleus stained with NeuN. D, representative Western blot showing PKA Cα translocation from particulate fraction (P) to cytosolic fraction (C) determined 10 min after withdrawal. *, $p < 0.05$; **, $p < 0.01$ compared with control (one-way ANOVA and Dunnett's test).

εPKC Is Required for cAMP Superactivation and AGS3 Expression. It has been reported that protein kinase C (PKC) stimulates AC2, AC5, and AC7 in neuronal and non-neuronal cell lines and is implicated in morphine tolerance (Narita et al., 1994). We next sought to determine whether cAMP superactivation requires PKC. GF109203X, a PKC inhibitor, attenuated cAMP superactivation (Fig. 3A). This observation led us to determine whether morphine withdrawal activates PKC. We found that morphine withdrawal increased PKC activity by approximately 125%; this increase in PKC activity was blocked by the PKA inhibitors, Rp-cAMPS, or the PLC inhibitor Et-18-OCH_3 (edelfosine), respectively (Fig. 3B), consistent with evidence for cross-talk between PKA and PKC signaling (Yao et al., 2008). We next identified a specific PKC isoform activated during morphine withdrawal. Figure 3C shows that the εPKC-specific peptide inhibitor εV1-2 blocked PKC activation, but there was no effect of the δPKC peptide inhibitor δV1-1, or the classic PKC peptide inhibitor βC2-4. These findings suggest that morphine withdrawal seems to activate εPKC. Indeed, Western blots show that during morphine withdrawal, activated εPKC translocates from membrane to cytoplasm as a function of time, starting at 2 h and peaking at 5 h (Fig. 3, D and E). Because AGS3 up-regulation requires PKA (Fig. 1, C and D), and cAMP superactivation requires εPKC (Fig. 3A), AC5, and AC7 (Table 1), we next sought to determine whether inhibition of PKC, AC5, or AC7 prevents AGS3 up-regulation. Figure 3F shows that inhibition of εPKC either by GF109203X or by εV1-2 reduced AGS3 up-regulation and that knockdown of AC5 and AC7 prevented AGS3 up-regulation. These findings suggest that PKA and εPKC seem to interact with each other to mediate AGS3 up-regulation and cAMP superactivation during morphine withdrawal.

Up-Regulation of AGS3 Contributes to cAMP Superactivation. To determine the role of AGS3 in the cAMP superactivation, we transfected cells with adenovirus carrying AGS3 antisense before long-term morphine exposure. We found that AGS3 antisense largely prevented morphine withdrawal-induced cAMP superactivation. In addition, the role of AGS3 in the superactivation was confirmed by treating cells with AGS3 inhibitor peptide Tat-GPR (Bowers et al., 2004) (Fig. 3A). Increased AGS3 expression is thought to drive the formation of AGS3-$G\alpha_i$-GDP, thus reducing the amount of $G\alpha_i$ available to inhibit AC (Takesono et al., 1999; Kimple et al., 2002). To test this possibility, we sought to determine whether $G\alpha_{i3}$ is coimmunoprecipitated with AGS3 using AGS3 antibody. Withdrawal from morphine promoted the formation of AGS3-$G\alpha_i$ (Fig. 4, A and B). This increase was blocked by the PKC inhibitor GF109203X or the PKA inhibitor Rp-cAMPS, respectively. Moreover, increased formation of AGS3-$G\alpha_i$ is not due to a concomitantly increased expression of $G\alpha_{i3}$, because withdrawal did not affect $G\alpha_{i3}$ expression (Fig. 4, C and D).

Discussion

The major findings in this study are that morphine withdrawal induces up-regulation of AGS3, which in turn seems to regulate morphine withdrawal-induced cAMP superactivation. Elevated AGS3 binds to $G\alpha_i$, preventing inhibitory $G\alpha_i$ from restraining cAMP signaling. Thus, inactivation of $G\alpha_i$ signaling facilitates activation of PKA. Increased PKA, known to increase PKC activity via PLC (Yao et al., 2008), promotes PKC stimulation of AC5 and AC7 activity, boosting cAMP production further, contributing to superactivation. We know that AGS3 binding to $G\alpha_i$-GDP liberates free $G\beta\gamma$ and have reported that unbound $G\beta\gamma$ subunits stimulate AC2 and AC4 (Yao et al., 2005). However, during morphine withdrawal, inhibition of $G\beta\gamma$ function did not inhibit withdrawal-induced cAMP superactivation. Thus, in contrast to short-term morphine-induced increases in cAMP via $G\beta\gamma$ subunits, morphine withdrawal-induced cAMP superactivation does not seem to require $G\beta\gamma$ function but does require up-regulation of AGS3 as a central molecular event. Overall, the mechanism of action of morphine withdrawal-induced cAMP superactivation requires up-regulation of AGS3, A2A receptors, AC5, AC7, PKA, and εPKC. A schematic presentation of these molecular mechanisms is presented in Fig. 5.

TABLE 1

The role of adenosine A2A receptors and adenylyl cyclases in withdrawal-induced cAMP superactivation

NAc/striatal neurons were transiently transfected with or without siRNAs for AC2, AC4, AC5, AC7, AC5 + AC7, or scramble control, respectively. Twenty-four hours after transfection, cells were treated with 100 nM morphine for 18 h followed by 5-h withdrawal (W.D.) in the presence or absence of adenosine deaminase (ADA, 1 unit/ml) or the A2A antagonist KW-6002 (100 nM). Data are the mean ± S.E.M. of at least three experiments with each treatment carried out in triplicates.

Treatment	cAMP Increase
	%
W.D.	78 ± 9
W.D. + ADA	2 ± 5*
W.D. + KW-6002	1 ± 3*
W.D. + Control SiRNA	71 ± 5
W.D. + AC2 SiRNA	69 ± 12
W.D. + AC4 SiRNA	72 ± 10
W.D. + AC5 SiRNA	24 ± 4*
W.D. + AC7 SiRNA	49 ± 7*
W.D. + (AC5 + AC7) SiRNA	11 ± 4*

* $P < 0.05$ compared with withdrawal control (one-way ANOVA and Dunnett's test).

TABLE 2

The role of adenosine A2A receptors in cAMP superactivation 3 h after withdrawal

NAc/striatal neurons were treated with 100 nM morphine for 18 h. Three hours after withdrawal (W.D.), cells were then treated with or without adenosine deaminase (ADA, 1 unit/ml) or the A2A antagonist KW-6002 (100 nM) for 2 h. Data are the mean ± S.E.M. of at least three experiments with each treatment carried out in triplicates.

Treatment	cAMP Increase
	%
W.D.	75 ± 7
W.D.+ADA	79 ± 11
W.D.+KW-6002	70 ± 6

cAMP/PKA. Sharma et al. (1975) provided pioneering evidence in NG108-15 cells that DOR activation initially decreases cAMP levels, but during continued exposure to morphine cAMP is restored to normal levels (tolerance); cAMP levels are increased further (superactivation) upon opiate receptor blockade or opiate withdrawal (Avidor-Reiss et al., 1995; Watts, 2002). Up-regulation of the cAMP pathway occurs in vivo during long-term exposure to opioids (Nestler and Aghajanian, 1997), and cAMP/PKA signaling is thought to mediate many neural responses to drugs in models of addiction (Nestler, 2001). Increased cAMP activates PKA- and CRE-dependent gene transcription, thought to mediate both tolerance and dependence (Chao and Nestler, 2004). In this study, we used NAc/striatal neurons to first confirm that morphine withdrawal induces cAMP superactivation, and then we investigated the molecular mechanisms underlying this event. We found that morphine withdrawal for 10 min enhanced basal cAMP levels by approximately 20%. cAMP levels increase further 1 to 3 h after withdrawal, reaching a maximum at 5 h. cAMP superactivation seems to involve both DOR and MOR, because the DOR antagonist naltrindole and the MOR antagonist CTOP both attenuate morphine withdrawal-induced increases in cAMP. This is consistent with the previous finding that both DOR and MOR mediate the rewarding effects of morphine (Shippenberg et al., 2008). It is noteworthy that a 20% increase in cAMP seems to be sufficient to activate PKA as well as increase AGS3 expression.

We previously showed in NAc/striatal neurons that short-term activation of MOR paradoxically activates cAMP/PKA signaling via free $G\beta\gamma$ subunit stimulation of AC2 and AC4 (Yao et al., 2005). However, cAMP superactivation after withdrawal from morphine is independent of $G\beta\gamma$ function because the dominant-negative $G\beta\gamma$ inhibitor βARK1 has no effect. Our finding is not in concert with the observation that AC5 superactivation induced by long-term morphine requires $G\beta\gamma$ in MOR-transfected COS-7 cells (Avidor-Reiss et al., 1996). This discrepancy may be due to the fact that NAc/striatal neurons and COS-7 cells express different types and amounts of G-proteins and adenylyl cyclases. Nonetheless, AC5 and AC7 seem to be required for superactivation. Thus, siRNA for AC5 or AC7, but not that for AC2 or AC4, prevents cAMP superactivation.

AGS3. AGS3 was identified as a receptor-independent activator of heterotrimeric G-protein signaling pathways (Takesono et al., 1999). AGS3 seems to be required for cAMP/

PKA activation caused both by short-term exposure to morphine and by withdrawal after long-term exposure to morphine. Thus, knockdown of AGS3 expression completely blocks the increase in cAMP/PKA signaling in both conditions. AGS3 selectively interacts with the GDP-bound conformation of α-subunits of the $G\alpha_{i/o}$ family, enabling free $G\beta\gamma$ to stimulate AC2 and AC4 (Yao et al., 2005). However, G-protein dissociation is not expected to occur in the absence of morphine. Therefore, free $G\beta\gamma$ released from $G\alpha_{i/o}$ stimulating AC cannot explain cAMP superactivation during withdrawal from long-term exposure. Indeed, we found that cAMP superactivation, which requires AC5 and AC7, did not require $G\beta\gamma$ function. AGS3 also binds to and inactivates $G\alpha_{i/o}$, preventing $G\alpha_{i/o}$ from restraining AC activation (Takesono et al., 1999; Kimple et al., 2002; Peterson et al., 2002). Because superactivation requires AGS3, it is possible that

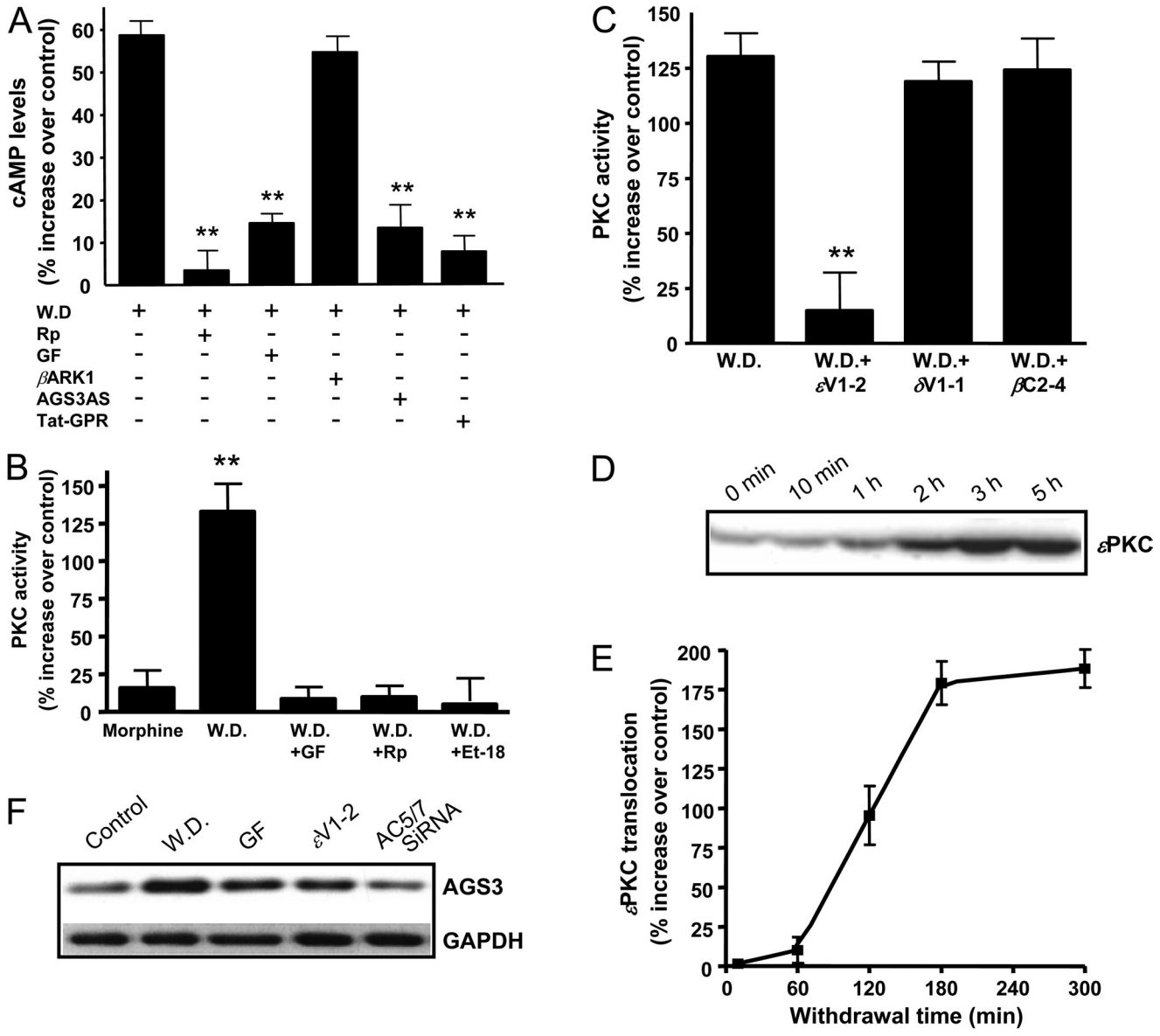

Fig. 3. PKA, PKC, and AGS3 each contribute to cAMP superactivation. A, cAMP assay. NAc/striatal neurons were transfected with or without Ad5βARK1 or Ad5AGS3AS (AGS3 antisense). Twenty-four hours later, cells were treated with 100 nM morphine for 18 h and followed by 5-h withdrawal (W.D.) in the presence or absence of the AGS3 peptide inhibitor Tat-GPR (1 μM), the PKA inhibitor Rp-cAMPS (Rp, 20 μM), or the PKC inhibitor GF109203X (GF, 1 μM). Data are the mean ± S.E.M. of four independent experiments, with each treatment carried out in triplicates. B, withdrawal increases PKC activity. During withdrawal, cells were incubated with or without the PKC inhibitor GF109203X (GF, 1 μM), the PKA inhibitor Rp-cAMPS (Rp, 20 μM), or the PLC inhibitor Et-18-OCH_3 (Et-18, 1 μM) for 5 h and lysed for assay of PKC activity. Data are the mean ± S.E.M. of three independent experiments with each treatment carried out in triplicates. C, withdrawal activates εPKC. Cells were incubated with or without εPKC-specific peptide inhibitor εV1-2 (1 μM), δPKC-specific inhibitor δV1-1 (1 μM), or conventional PKC-specific inhibitor βC2-4 (1 μM) during 5-h withdrawal, respectively. PKC activity was assayed as above. Data are the mean ± S.E.M. of three independent experiments with each treatment carried out in triplicates. D, representative Western blot showing εPKC translocation from the membrane to the cytoplasm as a function of time. After withdrawal, cells were lysed at the indicated time and lysates were subject to fractionation. Cytosolic fractions were analyzed for εPKC translocation by Western blot. E, relative intensity of individual bands from D was quantitated using ImageJ software. Data are the mean ± S.E.M. of three independent experiments. F, representative Western blot showing AGS3 expression in cells transfected with or without siRNAs for AC5 plus AC7 (AC5/7) for 24 h, treated with 100 nM morphine for 18 h, and followed by 5 h withdrawal in the presence or absence of the PKC inhibitor GF109203X (GF, 1 μM) or εPKC inhibitor εV1-2 (1 μM). **, $p < 0.01$, compared with control (one-way ANOVA and Dunnett's test).

up-regulation of AGS3 selectively diminishes signaling through $G\alpha_i$. Indeed, we found that up-regulation of AGS3 may contribute to morphine withdrawal-induced cAMP superactivation apparently by binding with the $G\alpha_{i/o}$-GDP complex, thereby preventing free $G\alpha_{i/o}$ from inhibiting AC.

cAMP activates PKA and causes its translocation from the Golgi to the nucleus, where PKA phosphorylates the cAMP response element binding protein to activate CRE-mediated gene transcription (Chao and Nestler, 2004). It is possible that up-regulation of AGS3 could occur as a result of increased gene transcription. It is noteworthy that Rp-cAMPS, a PKA inhibitor, prevented up-regulation of AGS3, suggesting the possibility that the AGS3 gene may contain CREs regulated by cAMP/PKA, although this is unknown. Despite this limitation, however, it seems that up-regulation of AGS3 by drugs of abuse seems to be another common determinant in addiction biology, possibly related to drug-seeking behavior and locomotor sensitization. Thus, AGS3 antisense prevents cocaine-induced behavioral sensitization and cocaine-, heroin-, and ethanol-seeking behavior (Bowers et al., 2003, 2008; Yao et al., 2005).

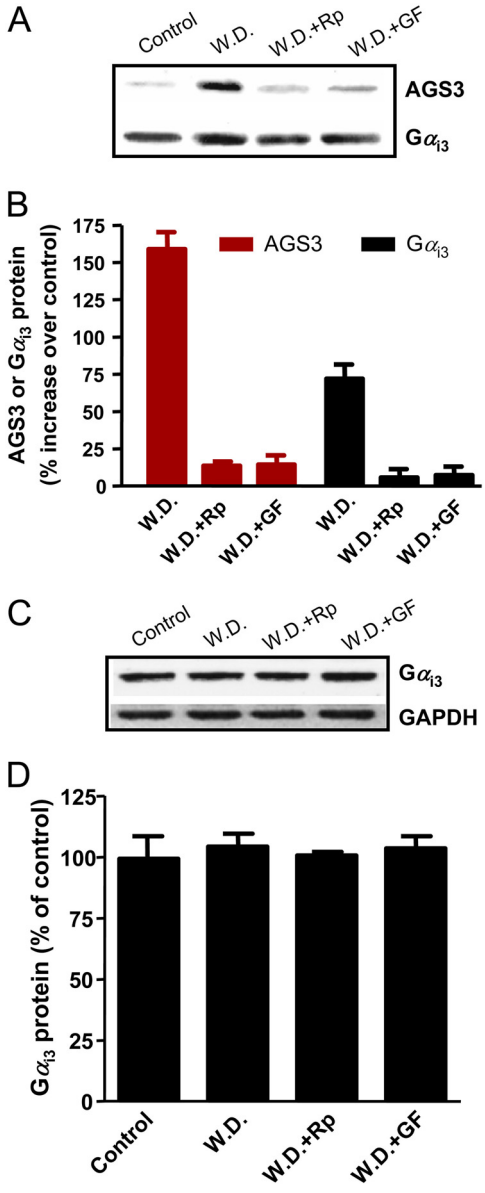

Fig. 4. $G\alpha_i$ but not $G\beta\gamma$ is involved in withdrawal-induced cAMP/PKA signaling. A, representative Western blot showing that the increased AGS3 binds to $G\alpha_i$. NAc/striatal neurons were incubated with or without the PKC inhibitor GF109203X (GF, 1 μM) or the PKA inhibitor Rp-cAMPS (Rp, 20 μM) during 5-h withdrawal (W.D.). Cells were then lysed and immunoprecipitated with AGS3 antibody. Membranes were blotted with antibodies for $G\alpha_{i3}$ or AGS3, respectively. B, relative intensity of individual bands from A was quantitated using ImageJ software. Data are the mean ± S.E.M. of three independent experiments. C, representative Western blot showing $G\alpha_{i3}$ expression 5 h after withdrawal. D, relative intensity of individual bands from B was quantitated using ImageJ software. Blots were normalized to GAPDH. Data are the mean ± S.E.M. of three independent experiments.

Adenosine A2A Receptors. Adenosine has been implicated in responses to several addictive agents such as ethanol (Arolfo et al., 2004), opiates (Brundege and Williams, 2002), and cannabinoids (Soria et al., 2004). The striatum is enriched in postsynaptic adenosine A2A receptors that couple to the stimulatory G-protein $G\alpha_s$ in most cells and primarily $G\alpha_{olf}$ in brain (Kull et al., 2000). Long-term morphine exposure can increase extracellular adenosine in striatum by up-regulating adenosine transporter binding sites (Kaplan and Leite-Morris, 1997), and adenosine seems to act on A2A. We found that A2A is required for initiating but not sustaining morphine withdrawal-induced cAMP superactivation. The sustained cAMP superactivation seems to require AGS3 up-regulation because treatment with adenosine deaminase or KW-6002 at 3 h after withdrawal, when AGS3 has been up-regulated, did not inhibit cAMP superactivation. This finding is consistent with a role for A2A in contribution to morphine-seeking behaviors. Thus, adenosine receptor antagonists attenuate development of morphine sensitization in mice (Weisberg and Kaplan, 1999) and inhibit morphine self-administration (Sahraei et al., 1999) and heroin-induced reinstatement in rats (Yao et al., 2006).

Adenylyl Cyclases. Nine different AC isoforms have been identified that are regulated by different effectors, such as $G\alpha$ and $G\beta\gamma$ subunits and protein kinases. AC2, AC4, and AC7 are conditionally stimulated by $G\beta\gamma$ subunits; AC2, AC5, and AC7 are activated by PKC (Watts and Neve, 2005). We previously reported that AC2 and AC4 are stimulated by $G\beta\gamma$ and involved in short-term neural responses to morphine (Yao et al., 2005). By contrast, we show here that AC5 and AC7, not AC2 and AC4, are required for morphine with-

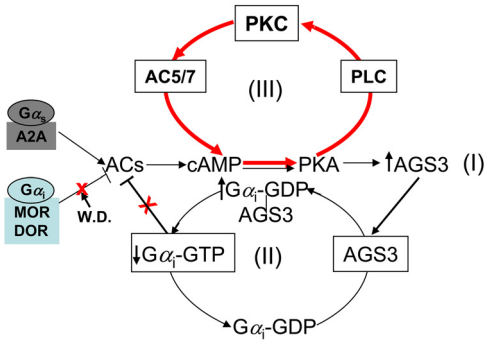

Fig. 5. Schematic representation of AGS3 up-regulation and cAMP superactivation. I, withdrawal removes the inhibitory effect of morphine and enables adenosine to increase the basal cAMP levels. This increase in cAMP is sufficient to activate PKA and up-regulate AGS3 expression. II, increased AGS3 binds to $G\alpha_i$-GDP and inactivate the $G\alpha_i$ signaling, further enhancing cAMP levels. III, unrestrained enhancement of cAMP/PKA signaling activates the PLC/PKC system to stimulate AC5 and AC7, ultimately leading to cAMP superactivation. Red arrows indicate cAMP superactivation pathway.

drawal-induced cAMP superactivation. Thus, opiate withdrawal seems to require specific AC isoform (Avidor-Reiss et al., 1997). AC5 is highly enriched in the striatum (Defer et al., 2000). AC5 knockouts show a dramatic reduction in morphine-induced physical dependence and withdrawal symptoms (Kim et al., 2006). PKC has been implicated in the mechanism of action of addictive drugs (Olive and Messing, 2004). PKC phosphorylates AC5 and enhances basal and forskolin- and $G\alpha_s$-stimulated cAMP accumulation (Kawabe et al., 1994). Furthermore, PKC can be activated by PKA via a cross-talk mechanism (Yao et al., 2008). Consistent with these reports, we found that morphine withdrawal activates and translocates εPKC. It is noteworthy that cAMP superactivation is blocked by PKC inhibitors and attenuated by siRNA for AC5 or AC7, suggesting that activation of εPKC may enhance AC5 and AC7 activity contributing to cAMP superactivation during withdrawal.

Figure 5 schematically illustrates a putative model of AGS3 up-regulation and cAMP superactivation. During long-term morphine exposure, both MOR/DOR and A2A modulate basal cAMP levels. Morphine decreases cAMP via $G\alpha_i$, whereas adenosine increases cAMP via $G\alpha_{s/olf}$. Withdrawal removes the inhibitory effect of morphine and enables A2A-stimulated increases in cAMP to rise above basal levels. This increase in cAMP is sufficient to activate PKA and increase AGS3 expression. Increased AGS3 binds $G\alpha_i$-GDP and inactivates $G\alpha_i$ signaling, further increasing cAMP production. Unrestrained enhancement of cAMP/PKA signaling activates the PLC/PKC system to stimulate AC5 and AC7 and boost cAMP production, ultimately leading to cAMP superactivation. Taken together, up-regulation of AGS3 seems to play a central role in the cooperation of these diverse pathways, facilitating morphine withdrawal-induced cAMP superactivation and, perhaps, underlying some drug-seeking behaviors associated with opiate withdrawal and relapse. It is possible that agents that interfere with AGS3 function might be potential therapies for opiate and other drug addictions.

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References

Arolfo MP, Yao L, Gordon AS, Diamond I, and Janak PH (2004) Ethanol operant self-administration in rats is regulated by adenosine A2 receptors. *Alcohol Clin Exp Res* **28:**1308–1316.

Avidor-Reiss T, Bayewitch M, Levy R, Matus-Leibovitch N, Nevo I, and Vogel Z (1995) Adenylylcyclase supersensitization in mu-opioid receptor-transfected Chinese hamster ovary cells following chronic opioid treatment. *J Biol Chem* **270:** 29732–29738.

Avidor-Reiss T, Nevo I, Levy R, Pfeuffer T, and Vogel Z (1996). Chronic opioid treatment induces adenylyl cyclase V superactivation. Involvement of Gbetagamma. *J Biol Chem* **271:**21309–21315.

Avidor-Reiss T, Nevo I, Saya D, Bayewitch M, and Vogel Z (1997) Opiate-induced adenylyl cyclase superactivation is isozyme-specific. *J Biol Chem* **272:**5040–5047.

Blumer JB, Smrcka AV, and Lanier SM (2007) Mechanistic pathways and biological roles for receptor-independent activators of G-protein signaling. *Pharmacol Ther* **113:**488–506.

Bowers MS, Hopf FW, Chou JK, Guillory AM, Chang SJ, Janak PH, Bonci A, and Diamond I (2008) Nucleus accumbens AGS3 expression drives ethanol seeking through G betagamma. *Proc Natl Acad Sci U S A* **105:**12533–12538.

Bowers MS, Lake RW, McFarland K, Peterson YK, Lanier SM, Lapish CC, and Kalivas PW (2003) AGS3: a G-protein regulator of addiction-associated behaviors. *Ann N Y Acad Sci* **1003:**356–357.

Bowers MS, McFarland K, Lake RW, Peterson YK, Lapish CC, Gregory ML, Lanier SM, and Kalivas PW (2004) Activator of G protein signaling 3: a gatekeeper of cocaine sensitization and drug seeking. *Neuron* **42:**269–281.

Brundege JM and Williams JT (2002) Increase in adenosine sensitivity in the nucleus accumbens following chronic morphine treatment. *J Neurophysiol* **87:** 1369–1375.

Chakrabarti S and Gintzler AR (2007) Phosphorylation of Galphas influences its association with the micro-opioid receptor and is modulated by long-term morphine exposure. *Molecular pharmacology* **72:**753–760.

Chao J and Nestler EJ (2004) Molecular neurobiology of drug addiction. *Annu Rev Med* **55:**113–132.

De Vries L, Fischer T, Tronchère H, Brothers GM, Strockbine B, Siderovski DP, and Farquhar MG (2000) Activator of G protein signaling 3 is a guanine dissociation inhibitor for Galpha i subunits. *Proc Natl Acad Sci U S A* **97:**14364–14369.

Defer N, Best-Belpomme M, and Hanoune J (2000) Tissue specificity and physiological relevance of various isoforms of adenylyl cyclase. *Am J Physiol Renal Physiol* **279:**F400–F416.

Dohrman DP, Diamond I, and Gordon AS (1996) Ethanol causes translocation of cAMP-dependent protein kinase catalytic subunit to the nucleus. *Proc Natl Acad Sci U S A* **93:**10217–10221.

Gautam N, Downes GB, Yan K, and Kisselev O (1998) The G-protein betagamma complex. *Cell Signal* **10:**447–455.

Kaplan GB and Leite-Morris KA (1997) Up-regulation of adenosine transporter-binding sites in striatum and hypothalamus of opiate tolerant mice. *Brain Research* **763:**215–220.

Kawabe J, Iwami G, Ebina T, Ohno S, Katada T, Ueda Y, Homcy CJ, and Ishikawa Y (1994) Differential activation of adenylyl cyclase by protein kinase C isoenzymes. *J Biol Chem* **269:**16554–16558.

Kim KS, Lee KW, Lee KW, Im JY, Yoo JY, Kim SW, Lee JK, Nestler EJ, and Han PL (2006) Adenylyl cyclase type 5 (AC5) is an essential mediator of morphine action. *Proc Natl Acad Sci U S A* **103:**3908–3913.

Kimple RJ, Kimple ME, Betts L, Sondek J, and Siderovski DP (2002) Structural determinants for GoLoco-induced inhibition of nucleotide release by Galpha subunits. *Nature* **416:**878–881.

Kull B, Svenningsson P, and Fredholm BB (2000) Adenosine A(2A) receptors are colocalized with and activate g(olf) in rat striatum. *Mol Pharmacol* **58:**771–777.

Narita M, Funada M, and Suzuki T (2001) Regulations of opioid dependence by opioid receptor types. *Pharmacol Ther* **89:**1–15.

Narita M, Makimura M, Feng Y, Hoskins B, and Ho IK (1994) Influence of chronic morphine treatment on protein kinase C activity: comparison with butorphanol and implication for opioid tolerance. *Brain Res* **650:**175–179.

Nestler EJ (2001) Molecular basis of long-term plasticity underlying addiction. *Nat Rev Neurosci* **2:**119–128.

Nestler EJ and Aghajanian GK (1997) Molecular and cellular basis of addiction. *Science* **278:**58–63.

O'Brien CP (2005) Anticraving medications for relapse prevention: a possible new class of psychoactive medications. *Am J Psychiatry* **162:**1423–1431.

Olive MF and Messing RO (2004) Protein kinase C isozymes and addiction. *Mol Neurobiol* **29:**139–154.

Peterson YK, Hazard S 3rd, Graber SG, and Lanier SM (2002) Identification of structural features in the G-protein regulatory motif required for regulation of heterotrimeric G-proteins. *J Biol Chem* **277:**6767–6770.

Sahraei H, Motamedi F, Khoshbaten A, and Zarrindast MR (1999) Adenosine A(2) receptors inhibit morphine self-administration in rats. *Eur J Pharmacol* **383:**107–113.

Sharma SK, Klee WA, and Nirenberg M (1975) Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc Natl Acad Sci U S A* **72:** 3092–3096.

Shippenberg TS, LeFevour A, and Chefer VI (2008) Targeting endogenous mu- and delta-opioid receptor systems for the treatment of drug addiction. *CNS Neurol Disord Drug Targets* **7:**442–453.

Soria G, Castañé A, Berrendero F, Ledent C, Parmentier M, Maldonado R, and Valverde O (2004) Adenosine A2A receptors are involved in physical dependence and place conditioning induced by THC. *Eur J Neurosci* **20:**2203–2213.

Takesono A, Cismowski MJ, Ribas C, Bernard M, Chung P, Hazard S 3rd, Duzic E, and Lanier SM (1999) Receptor-independent activators of heterotrimeric G-protein signaling pathways. *J Biol Chem* **274:**33202–33205.

Tang WJ and Gilman AG (1991) Type-specific regulation of adenylyl cyclase by G protein beta gamma subunits. *Science* **254:**1500–1503.

Watts VJ (2002) Molecular mechanisms for heterologous sensitization of adenylate cyclase. *J Pharmacol Exp Ther* **302:**1–7.

Watts VJ and Neve KA (2005) Sensitization of adenylate cyclase by Galpha i/o-coupled receptors. *Pharmacol Ther* **106:**405–421.

Weisberg SP and Kaplan GB (1999) Adenosine receptor antagonists inhibit the development of morphine sensitization in the C57BL/6 mouse. *Neuroscience Letters* **264:**89–92.

Yao L, Arolfo MP, Dohrman DP, Jiang Z, Fan P, Fuchs S, Janak PH, Gordon AS, and Diamond I (2002) betagamma Dimers mediate synergy of dopamine D2 and adenosine A2 receptor-stimulated PKA signaling and regulate ethanol consumption. *Cell* **109:**733–743.

Yao L, Fan P, Jiang Z, Gordon A, Mochly-Rosen D, and Diamond I (2008) Dopamine and ethanol cause translocation of epsilonPKC associated with epsilonRACK: cross-talk between cAMP-dependent protein kinase A and protein kinase C signaling pathways. *Mol Pharmacol* **73:**1105–1112.

Yao L, McFarland K, Fan P, Jiang Z, Inoue Y, and Diamond I (2005) Activator of G protein signaling 3 regulates opiate activation of protein kinase A signaling and relapse of heroin-seeking behavior. *Proc Natl Acad Sci U S A* **102:**8746–8751.

Yao L, McFarland K, Fan P, Jiang Z, Ueda T, and Diamond I (2006) Adenosine A2a blockade prevents synergy between mu-opiate and cannabinoid CB1 receptors and eliminates heroin-seeking behavior in addicted rats. *Proc Natl Acad Sci U S A* **103:**7877–7882.

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