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Amphetamine Self-Administration Attenuates Dopamine D2 Autoreceptor Function

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Dopamine D2 autoreceptors located on the midbrain dopaminergic neurons modulate dopamine (DA) neuron firing, DA release, and DA synthesis through a negative-feedback mechanism. Dysfunctional D2 autoreceptors following repeated drug exposure could lead to aberrant DA activity in the ventral tegmental area (VTA) and projection areas such as nucleus accumbens (NAcc), promoting drug-seeking and -taking behavior. Therefore, it is important to understand molecular mechanisms underlying drug-induced changes in D2 autoreceptors. Here, we reported that 5 days of amphetamine (AMPH) self-administration reduced the ability of D2 autoreceptors to inhibit DA release in the NAcc as determined by voltammetry. Using the antibody-capture [^{35}S]GTPγS scintillation proximity assay, we demonstrated for the first time that midbrain D2/D3 receptors were preferentially coupled to Gαi2, whereas striatal D2/D3 receptors were coupled equally to Gαi2 and Gαo for signaling. Importantly, AMPH abolished the interaction between Gαi2 and D2/D3 receptors in the midbrain while leaving striatal D2/D3 receptors unchanged. The disruption of the coupling between D2/D3 receptors and Gαi2 by AMPH is at least partially explained by the enhanced RGS2 (regulator of G-protein signaling 2) activity resulting from an increased RGS2 trafficking to the membrane. AMPH had no effects on the midbrain expression and trafficking of other RGS proteins such as RGS4 and RGS8. Our data suggest that midbrain D2/D3 receptors are more susceptible to AMPH-induced alterations. Reduced D2 autoreceptor function could lead to enhanced DA signaling and ultimately addiction-related behavior. RGS2 may be a potential non-dopaminergic target for pharmacological intervention of dysfunctional DA transmission and drug addiction.
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INTRODUCTION

Amphetamine (AMPH) addiction is a serious public health concern. However, the mechanisms underlying AMPH addiction remain elusive. The mesolimbic dopaminergic system, originating from dopamine (DA) neurons of the ventral tegmental area (VTA) and projecting to the nucleus accumbens (NAcc), has a critical role in drug addiction (Wise, 1996). The ability of AMPH to act on DA neurons to elicit an increase in extracellular DA levels in the NAcc has been profoundly implicated in the rewarding and reinforcing properties of AMPH. Although DA activity in the NAcc is necessary and sufficient to drive drug-seeking behavior (Leyton *et al*, 2005), the amount of DA released in the NAcc is tightly controlled by VTA DA neuron activity (Sombers *et al*, 2009; Yun *et al*, 2004). Compared with studies on DA-related changes in DA neuron terminals located in the NAcc, there are notably fewer reports that have evaluated alterations of DA activity in the cell bodies located in VTA or substantia nigra (SN) following repeated exposure to psychostimulants.

A key modulator of DA neuron activity is the D2 autoreceptor, which operates through a negative-feedback mechanism. D2 autoreceptors are located in the somatodendritic compartments of DA neurons of the VTA and SN, and presynaptically on their nerve terminals in the NAcc and dorsal striatum. The function of D2 autoreceptors is to inhibit neuron firing, DA synthesis, and DA release. In the VTA, D3 receptors are also modestly present, but D4 receptors seem to be absent (Diaz *et al*, 2000; Mengod *et al*, 1992; Mercuri *et al*, 1997; Sesack *et al*, 1994). Given the role of D2 autoreceptors in the regulation of neuron excitability and DA release, impaired autoreceptor function would lead to aberrant DA activity in the VTA and thereby produce altered DA transmission in the NAcc. Moreover, newly emerging evidence from human studies indicate that functional expression of midbrain D2/D3 autoreceptors is inversely correlated with impulsivity for

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drugs and the amount of DA release elicited by AMPH (Buckholtz *et al*, 2010). This notion was also supported by findings from D2 autoreceptor conditional knockout mice, in which knockout mice display supersensitivity to cocaine-induced locomotor activity and conditioned place preference (Bello *et al*, 2011). Thus, it is important to understand functional adaptations of D2 autoreceptors in response to repeated drug exposure, which may be a mechanism underlying susceptibility to drug addiction.

D2 receptors are also located postsynaptically on DA neurons in the striatum. Reduced postsynaptic D2 receptor availability is often associated with long-term psychostimulant exposure in drug addicts and non-human primates (Nader *et al*, 2006; Shumay *et al*, 2012; Volkow *et al*, 1990; Volkow *et al*, 1999) and has been considered to be a neurochemical endophenotype for drug dependence and relapse. Thus, it appears that both D2 autoreceptors and postsynaptic D2 receptors contribute to drug addiction. There are no reports on whether there is a differential time-dependent drug effect on alterations of D2 autoreceptor and postsynaptic D2 receptor function; therefore, our aim was to evaluate whether D2 autoreceptors were more vulnerable to AMPH-induced changes than postsynaptic D2 receptors or vice versa.

D2 receptors are capable of coupling to and activating all subtypes of Gαi/o proteins to exert cellular effects on heterologous systems albeit with different efficiency based on the cell type (Ghahremani *et al*, 1999; Nickolls and Strange, 2004). However, there are no reports on the specific coupling between D2 receptor and subtypes of Gαi/o proteins in the brain. Furthermore, termination of D2 receptor signaling is accelerated by the regulator of G-protein signaling (RGS) proteins. There are more than 20 different subtypes of RGS proteins, and most are promiscuous in their Gα subunit binding. Identification of RGS proteins that are specific for D2 receptor function will improve our understanding of D2 receptor signaling and may lead to a future non-dopaminergic strategy for interference with DA transmission and drug abuse.

As there are no selective pharmacological ligands that can differentiate D2 receptors from D3 receptors, results from experiments using quinpirole, a D2/D3 agonist, were applied to both D2 and D3 receptors. Here, we demonstrated that short-term AMPH self-administration reduced the ability of D2/D3 autoreceptors to regulate DA release and synthesis. Moreover, we showed for the first time that midbrain D2/D3 receptors were preferentially coupled to Gαi2, whereas striatal D2/D3 receptors were coupled equally to Gαi2 and Gαo for signaling. Importantly, AMPH self-administration abolished the interaction between Gαi/o proteins and D2/D3 receptors in the midbrain while leaving striatal D2/D3 receptors unaffected. The disruption of the coupling between D2/D3 receptors and Gαi2 is at least partially explained by the enhanced RGS2 activity resulting from an increased RGS2 trafficking to the membrane.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (375–400 *g*; Harlan Laboratories, Frederick, Maryland) were used for all experiments. Rats were maintained on a 12 : 12 h reverse light/dark cycle (0300 hours lights off; 1500 hours lights on) with food and water *ad libitum*. All animals were maintained according to the National Institutes of Health guidelines in Association for Assessment and Accreditation of Laboratory Animal Care accredited facilities. The experimental protocol was approved by the Institutional Animal Care and Use Committee at Wake Forest School of Medicine.

Self-Administration

Rats were anesthetized and implanted with chronic indwelling jugular catheters as previously described (Liu *et al*, 2007). Animals were singly housed, and all sessions took place during the active/dark cycle (0900 hours–1500 hours). After a 2-day recovery period, animals underwent a training paradigm within which animals were given access on a fixed-ratio one (FR1) schedule to an AMPH-paired lever, which, upon responding, initiated an intravenous delivery of 0.187 mg/kg per AMPH infusion in 100 μl saline over 4 s. This dose was chosen because it is the most reinforcing dose as measured by the peak of the progressive ratio dose–response curve for AMPH (Richardson and Roberts, 1996). After each AMPH delivery, the lever was retracted and a stimulus light was illuminated for a 20-second timeout period. Acquisition occurred when an animal responded for 20 injections for two consecutive days and a stable pattern of infusion intervals was present. Following training, rats were allowed to self-administer AMPH for 6 h daily with a maximum of 40 injections for five consecutive days. Rats were killed ~18 h after the final self-administration session. The half-life of AMPH clearance is ~60 min for intravenous self-administration (Hutchaleelaha *et al*, 1994). Previous work has shown that animals maintain an approximate blood level of 1 mg/kg AMPH at any given time during a 6-h self-administration session, independent of dose (Yokel and Pickens, 1974). This is because animals titrate their responding in order to achieve a preferred drug level, that is, animals respond less when the dose is high and more when the dose is low. In our study, the animals administered 7.48 mg/kg (0.187 mg/kg/injection × 40 injection/day = 7.48 mg/kg) over a 6-h period. Thus, the 18-h withdrawal from the last session of AMPH self-administration is a sufficient time period to ensure the clearance of 1 mg/kg AMPH blood level that was present at the end of the self-administration session.

Control and AMPH self-administering animals were housed in the same room under the same light cycle (lights on 1500 hours, off 0300 hours) for the same period of time. Experiments were conducted between 0800 hours and 1500 hours daily. Control animals did not have sham surgery. Using the same treatment paradigm, we have repeatedly shown previously that there are no neurochemical differences in DA-related measurements such as DA uptake and release between animals with and without sham surgery for catheter implantation (Calipari *et al*, 2013a; Calipari *et al*, 2013b; Ferris *et al*, 2012).

Fast-Scan Cyclic Voltammetry for Measuring the Function of D2/D3 Autoreceptor and DA Transporter in the NAcc

Ex vivo fast-scan cyclic voltammetry (FSCV) was used to characterize D2/D3 autoreceptor function, DA transporter

(DAT) activity, and DA release in the NAcc. Voltammetry experiments were conducted during the dark phase of the light cycle 18 h after commencement of the final AMPH self-administration session. A vibrating tissue slicer was used to prepare 400-μm-thick coronal brain sections containing the NAcc, which were immersed in oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl (126), KCl (2.5), NaH_2PO_4 (1.2), $CaCl_2$ (2.4), $MgCl_2$ (1.2), $NaHCO_3$ (25), glucose (11), L-ascorbic acid (0.4) and pH was adjusted to 7.4. Then the slice was transferred to the testing chambers containing aCSF at 32 °C with a 1 ml/min flow rate. A carbon fiber microelectrode (100–200 μM length, 7 μM radius) and a bipolar stimulating electrode were placed into the core of the NAcc. DA release was evoked by a single electrical pulse (300 μA, 4 ms, monophasic) applied to the tissue every 5 min. The extracellular DA level was recorded by applying a triangular waveform (− 0.4 to + 1.2 to − 0.4 V *vs* Ag/AgCl, 400 μV/s). Once the extracellular DA level was stabilized, the amount of evoked DA release and a maximal rate of uptake (V_{max}) were assessed. Subsequently, quinpirole, a D2/D3 receptor agonist, was applied cumulatively to the brain slice to determine a dose–response curve (0.01–1 μM) for the quinpirole effects on DA release. Data were modeled using Michaelis–Menten kinetics to determine DA released and V_{max}. The quinpirole concentration–response curves were assessed by determining the peak height of DA release at each dose and expressed as a percentage of the pre-drug basal DA release. Recording electrodes were calibrated by recording responses (in electrical current; nA) to a known concentration of DA (3 μM) using a flow-injection system. This was used to convert an electrical current to a DA concentration. Demon Voltammetry and Analysis software (Yorgason *et al*, 2011) was used to analyze FSCV data.

The Antibody-Capture [^{35}S]GTPγS Scintillation Proximity Assay for Determining the Coupling of D2/D3 Receptors and the Subtypes of Gαi/o Proteins in the Midbrain and Striatum

Eighteen hours after the last session of AMPH self-administration, tissues from the midbrain (including VTA and SN) or the whole striatum (ventral and dorsal striatum) of AMPH self-administering rats and controls were dissected using a rat brain matrix. Tissues from the VTA and SN of each animal were pooled so that we could have enough tissues to perform D2 receptor binding for each animal. The membrane was prepared as described (DeLapp *et al*, 1999). Briefly, tissue was homogenized in homogenization buffer containing10 mM NaHepes, pH 7.4, 1 mM NaEGTA, a complete protease inhibitor cocktail (Sigma Aldrich), 1 mM dithiothreitol (DTT), and 10% sucrose. The homogenates were centrifuged at 1000 *g* for 10 min at 4 °C. The supernatants were centrifuged at 11 000 *g* for 20 min. The resulting pellets were homogenized in 40 volumes of 10 mM NaHepes, pH 7.4, 1 mM MgCl2, 1 mM EGTA, and 1 mM DTT, and were centrifuged at 27 000 *g* for 20 min. The resulting pellets were suspended in the same buffer at a protein concentration of 1.5 mg/ml and aliquots were frozen and stored at − 80 °C.

Stimulation of [^{35}S]GTPγS binding to the membranes was performed using an antibody-capture [^{35}S]GTPγS scintillation proximity assay on 96-well plates (Eldeeb *et al*, 2013). The membranes were incubated with 500 pM [^{35}S]GTPγS (PerkinElmer, Billerica, MA, USA), 10 μM GDP and D2/D3 receptor agonist quinpirole (Quin, 10 μM) or antagonist (raclopride, 1 μM) in the assay buffer (20 mM NaHepes, pH 7.4, 100 mM NaCl, 5 mM MgCl2, and 1 mM DTT) for 1 h at 30 °C. Membranes were then placed on ice (4 °C), lysed with 3% IGEPAL CA-630 for 30 min, incubated with primary antibodies anti-Gαi1, anti-Gαi2, anti-Gαi3 or anti-Gαo (Santa Cruz) for 1 h. Anti-IgG-coated scintillation proximity assay beads coated with the secondary anti-rabbit or anti-mouse antibodies (PerkinElmer) were added for 30 min, and the plates were then centrifuged at 1000 *g* for 5 min. The radioactivity was detected on a Top-Count microplate scintillation counter (PerkinElmer). The non-specific binding was determined in the presence of unlabeled 10 μM GTPγS. Basal values were in the absence of the agonist quinpirole.

Analyses of Midbrain RGS mRNA Levels by Quantitative Real-Time PCR

qPCR was performed to analyze the mRNA levels of RGS2, RGS4, and RGS8 in the midbrain. Total RNA was isolated from tissues using the RNeasy isolation kit (Qiagen, Valencia, CA, USA). Purity and quantity of RNA were measured on the NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). Total RNA was converted to single-stranded cDNA using a high-capacity cDNA reverse transcription kit (Invitrogen, Carlsbad, CA, USA). Oligonucleotide primers for qPCR were designed in the following using NCBI Primer-BLAST. RGS2 (*Rgs2*; 5′-CACCACAGAAACTGTCCTCAA-3′ and 5′-CCACTTGTAGCCTCTTGGATATT-3′), RGS4 (*Rgs4*; 5′-ACCGTCGTTTCCTCAAGTCTCGAT-3′ and 5′-TTTCGGTTCTCTGCCTCTGTGTGA-3′), RGS8 (*Rgs8*; 5′-ACGAGGTGGGCAGATTCCTTTGAT-3′ and 5′-TAGCTTTGCAGTCGACCTGGTCTT-3′), β-actin (*Actb*; 5′-TGAGAGGGAAATCGTGCGTGACAT-3′ and 5′-ACCGCTCATTGCCGATAGTGATGA-3′), and GAPDH (*Gapdh*; 5′-TGATGCTGGTGCTGAGTATGTCGT-3 and 5′-TTCTCGTGGTTCACACCCATCACA-3′). Primers were obtained from Integrated DNA technologies (Coralville, Johnson County, IA, USA).

qPCR was performed using Fast SYBR Green Master Mix (Invitrogen) in a 96-well format using an ABI 7500 Fast real-time PCR System (Applied Biosystems, Forster City, CA, USA). Samples containing no cDNA template and no reverse transcriptase were run as controls for contamination and amplification of genomic DNA, respectively. All samples were run in triplicate. For each gene, qPRC reactions for control and AMPH self-administration groups were run concurrently on the same 96-well plate. Levels of mRNA expression were normalized to the average of the two housekeeping genes: actin and GAPDH.

Fractionation of The Midbrain Homogenates

Tissue dissected from the midbrain was processed by centrifugation to separate the cytosolic and the membrane fractions as described (Carrasco *et al*, 2003). Briefly, tissue was homogenized in 50 mM TrisCl buffer (pH 7.4) containing 150 mM NaCl, 10% sucrose, and a cocktail of protease and phosphatase inhibitors (Sigma Aldrich). A portion of

the homogenates was saved, lysed in RIPA buffer, and used as the total crude homogenate. The remaining homogenates were centrifuged at 20 000 *g* for 1 h. The supernatant was collected and stored at −80°C for further analyses of the cytosol-associated protein levels. The pellets were collected and resuspended by sonication in 20 mM Tris buffer (pH 8, containing 1 mM EDTA, 100 mM NaCl, 1% sodium deoxycholate, and 1 mM DTT and a cocktail of protease and phosphotase inhibitors) and lysed for 1 h at 4 °C. The lysate was centrifuged at 100 000 *g* for 60 min and the supernatants were collected for analyses of membrane-associated protein levels. Protein concentrations were measured using a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL, USA).

Western Blot Analyses on Samples Prepared as Crude Homogenates, Cytosol Fractions, or Membrane Fractions from the Midbrain and the Striatum

Quantitation of subtypes of Gαi/o proteins, total RGS proteins, tyrosine hydroxylase (TH), phosphorylated TH at serine 31 and 40, was performed by western blot for the lysed crude homogenates. Translocation of RGS proteins was determined in the fractionated cytosolic and membrane portions. Samples containing 5–20 μg protein were submitted to 10% or 12% SDS-polyacrylaminde gel electrophoresis. Gels were transferred electrophoretically onto nitrocellulose membranes. After incubation with the blocking buffer containing 5% non-fat milk or 3% BSA in phosphate-buffered saline, the membranes were probed overnight at 4 °C with various primary antibodies. The following primary antibodies were used: mouse anti-RGS2 (Sigma Aldrich), rabbit anti-RGS4 (Cell Signaling), mouse anti-RGS8 (Santa Cruz), mouse anti-TH (Santa Cruz), rabbit anti-TH ser 31(Cell signaling), and rabbit anti-TH ser 40 (Cell Signaling). Following primary antibody incubation, the membranes were incubated with appropriate secondary antibodies conjugated with peroxidase. The membranes were incubated with the enhanced chemiluminescence substrate solution (Pierce Chemical) and then exposed to an X-ray film. Films were analyzed densitometrically using the Image J (NIH). Actin was used as a loading control. The band density was normalized to its respective actin level for quantitation of Gαi/o proteins and RGS proteins. The expression of pTH was normalized by the expression of the total TH.

Statistics

Graph Pad Prism (version 6, La Jolla, CA, USA) was used for statistic analyses. Data for AMPH self-administration, quinpirole dose–response curve, and subtypes of Gαi/o activation were analyzed by a one- or two-way analysis of variance (ANOVA). The *post hoc* Bonferroni test was used to compare group differences if necessary. A two-tailed Student's *t*-test was performed to compare differences in the evoked DA levels, V_{max}, phosphorylation of TH, membrane and cytosolic localization of RGS proteins between control and AMPH self-administering rats.

RESULTS

Escalation of AMPH Self-Administration over Sessions

Rats self-administered AMPH (0.187 mg/kg/infusion) for five consecutive days in a daily 6-h session with a maximum of 40 total injections. The linear regression analysis revealed that rats showed an escalation in the rate of intake across sessions ($\beta = 0.5195 \pm 0.1546$, $P < 0.01$), indicating that rats gradually take less time to achieve 40 injections of AMPH across 5 days of the self-administration session (Figure 1).

AMPH Self-Administration Produced an Increase in Evoked DA Release *Ex Vivo*

In order to determine the effect of AMPH self-administration on presynaptic DA transmission, we investigated the amount of evoked DA release and DAT function by voltammetry. The DA level measured upon each pulse ([DA]p) was significantly greater in AMPH self-administering rats compared with controls (a two-tailed Student's *t*-test, $t_{10} = 3.187$, $P < 0.01$) as indicated in Figure 2a and b. However, AMPH self-administration did not alter the maximal rate of DA uptake as the values for V_{max} (μM/s) were comparable in both the AMPH self-administration (2.244 ± 0.1164) and control (2.356 ± 0.1581) groups (Figure 2c).

AMPH Self-Administration Resulted in Subsensitivity of D2/D3 Autoreceptors in Inhibition of Evoked DA Release

To determine the ability of D2 autoreceptors to inhibit evoked DA release, increasing concentrations of the D2/D3 receptor agonist quinpirole (0.01–1 μM) were added to the NAcc-containing slices to establish a dose–response curve. The peak in DA release was established at each concentration and expressed as the percent of control (Figure 2d). The IC50 for quinpirole was 22 nM in control rats and 463 nM in AMPH rats. A two-way ANOVA revealed a significant main effect of the quinpirole dose ($F_{5,65} = 71.95$, $P < 0.001$), and the drug treatment ($F_{1,65} = 51.69$, $P < 0.01$)

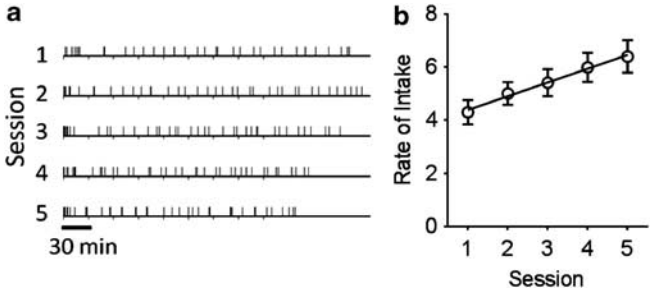

Figure 1 Amphetamine (AMPH) self-administration results in an escalation of rate of intake over sessions. Rat received AMPH (0.187 mg/kg/infusion) on a fixed-ratio one schedule of reinforcement for five consecutive days with a maximum of 40 infusions per day. Session 1 is counted as the first session that occurs following acquisition. (a) Representative operant behavior from a rat undergoing AMPH self-administration. Tick marks represent AMPH infusions delivered. (b) The rate of AMPH intake (infusion/hr/day) from AMPH self-administering rats was averaged. Rats escalate their rate of intake over days ($N = 6$). Data are reported as mean ± SEM.

on DA release. There was also an interaction (dose × drug treatment) effect ($F_{5,65} = 4.021$, $P < 0.05$). *Post hoc* Bonferroni analysis indicated that quinpirole was less able to inhibit DA release at the 0.03, 0.1, and 0.3 μM concentrations for AMPH self-administering rats as compared with controls, suggesting subsensitivity of D2/D3 autoreceptor function (Figure 2d).

AMPH Self-Administration Reduced D2/D3 Autoreceptor Function by Decreasing Phosphorylation of TH in the Midbrain

To measure the ability of D2/D3 autoreceptor to regulate DA synthesis, we examined the level of TH phosphorylation in lysed crude homogenates. Ser31 and Ser40 of TH are believed to be directly involved in DA synthesis because increases in the phosphorylation of TH at these two sites enhance the catalytic activity of TH (Colby *et al*, 1989; Dunkley *et al*, 2004). Compared with control animals, AMPH self-administration did not change the expression of TH, but significantly reduced the level of TH phosphorylation at serine 31 ($N = 5$–6, two-tailed unpaired *t*-test, $t_9 = 3.054$, $P < 0.05$) and serine 40 ($t_9 = 2.427$, $P < 0.05$) as shown in Figure 3.

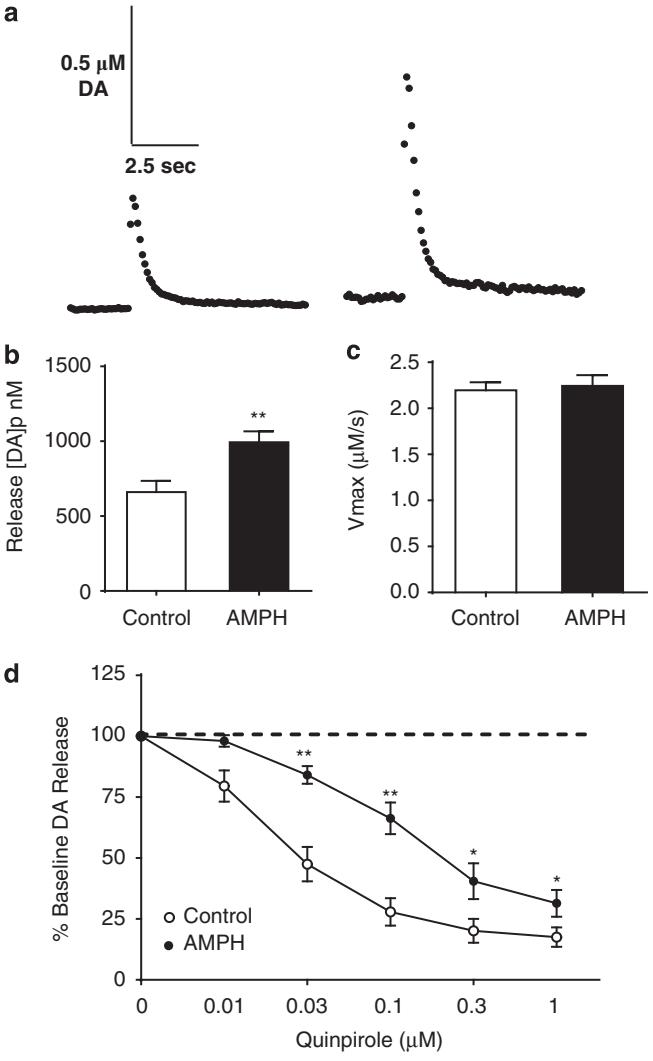

Figure 2 Amphetamine (AMPH) self-administration results in increased evoked dopamine (DA) release and subsensitive D2-like autoreceptors. (a) Representative DA traces showing evoked DA release over time as measured by voltammetry. (b) Evoked DA release was enhanced following AMPH self-administration in the nucleus accumbens core compared with controls. (c) The maximal rate of DA uptake (V_{max}) via the DA transporter (DAT) remains unchanged following AMPH self-administration. (d) The ability of D2/D3 autoreceptors in inhibition of evoked DA release from the nucleus accumbens core was dose-dependently decreased following AMPH self-administration as compared with controls. Data are expressed as mean ± SEM ($N = 6$–7). *$P < 0.05$; **$P < 0.01$ *vs* controls.

AMPH Self-Administration Abolished D2/D3 Autoreceptor Coupling to Gαi/o Proteins in the Midbrain

To determine the potential mechanism for the subsensitivity of D2/D3 autoreceptor following AMPH self-administration, we examined the activation of Gαi/o proteins by D2/D3 agonist quinpirole (10 μM). Using the antibody-capture [^{35}S]GTPγS scintillation proximity assay, we found that midbrain D2/D3 receptors were primarily coupled to the subtype Gαi2 for receptor activation and signaling. A two-way ANOVA revealed a significant main effect on activation of subtypes of Gαi/o proteins ($F_{3,32} = 10.52$, $P < 0.001$) and an interaction effect between drug treatment and G-protein activation ($F_{3,32} = 6.861$, $P < 0.01$). Bonferroni *post hoc* analysis indicated a significant difference in Gαi2 activation between AMPH self-administration and control groups. There was a significant increase in [^{35}S]GTPγS binding to the subtype Gαi2 protein upon stimulation of D2/D3 receptor by quinpirole ($N = 5$–6, $P < 0.01$), whereas

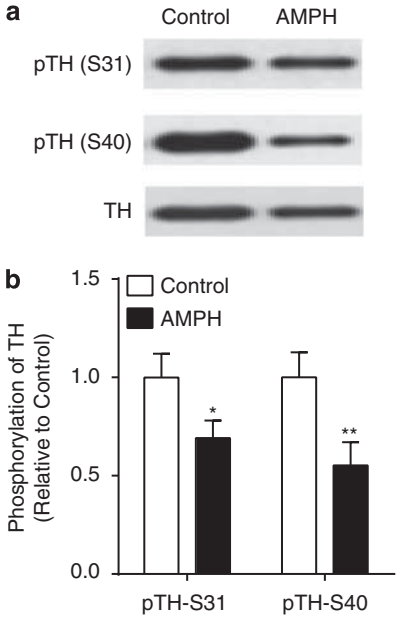

Figure 3 AMPH self-administration reduced phosphorylation of TH (pTH) in the midbrain. (a) Representative western blots of midbrain samples from a control and an AMPH self-administration rat. Samples were blotted for pTH at Ser 31 and Ser 40, and total TH. (b) Summary of the expression of the pTH at Ser 31 and 40. pTH was normalized to its own total TH and was calculated as a ratio relative to the value of control animals. Data are expressed as mean ± SEM ($N = 5$–6), *$P < 0.05$; **$P < 0.01$ *vs* controls.

no stimulation of other subtypes of Gαi/o was observed in control rats (Figure 4a). Following 5 days of AMPH self-administration, activation of Gαi2 by quinpirole was completely abolished when compared with the controls. As AMPH self-administration did not alter the expression of any subtypes of Gαi/o proteins (Supplementary Figure S1) or the membrane expression of D2/D3 receptors (Supplementary Figure S2A), we concluded that the difference in quinpirole-stimulated [^{35}S]GTPγ binding between the control and AMPH self-administration group was due to an impairment in the activation of Gαi/o proteins.

AMPH Self-Administration had no Effect on D2/D3 Receptor Coupling to Gαi/o Proteins in the Striatum

In contrast to the abolished quinpirole effect on midbrain D2/D3 autoreceptor-stimulated Gαi/o activation in AMPH self-administering rats, AMPH had no effect on the striatal D2/D3 receptor coupling to Gαi/o proteins. A two-way ANOVA indicated that there was no significant main effect on drug treatment. However, there was a significant main effect on the activation of subtypes of G proteins ($F_{3,32} = 25.88$, $P < 0.01$), suggesting that the activation of Gαi/o proteins in the striatum was subtype-dependent. Unlike midbrain D2/D3 receptors that were primarily coupled to Gαi/2, striatal D2/D3 receptors seemed equally coupled to both Gαi2 and Gαo for receptor activation (Figure 4b). No significant activation of Gαi1 or Gαi3 was observed in both control and AMPH groups. Furthermore, AMPH self-administration did not alter the availability of D2/D3 receptor as indicated by [^{3}H]raclopride binding on the purified striatal membranes (Supplementary Figure S2B).

AMPH Self-Administration Altered RGS2 mRNA Levels but not Protein Levels in the Midbrain

To determine whether AMPH self-administration had any effect on RGS mRNA induction, we performed qPCR to examine the mRNA levels of RGS proteins present in the midbrain. Compared with the control animals, 5 days of AMPH self-administration significantly enhanced the mRNA levels of RGS2 (*t*-test, $t_8 = 3.073$, $P < 0.05$) and RGS4 (*t*-test, $t_8 = 2.508$, $P < 0.05$), but had no effect on RGS8 (Figure 5a). However, the total protein levels of RGS2, RGS4, or RGS8 in the midbrain did not differ between control and AMPH self-administering animals (Figure 5b). Interestingly, AMPH self-administration significantly reduced striatal RGS2 protein levels when compared with controls shown in Figure 5c (*t*-test, $t_{10} = 2.886$, $P < 0.05$), although there was no change in

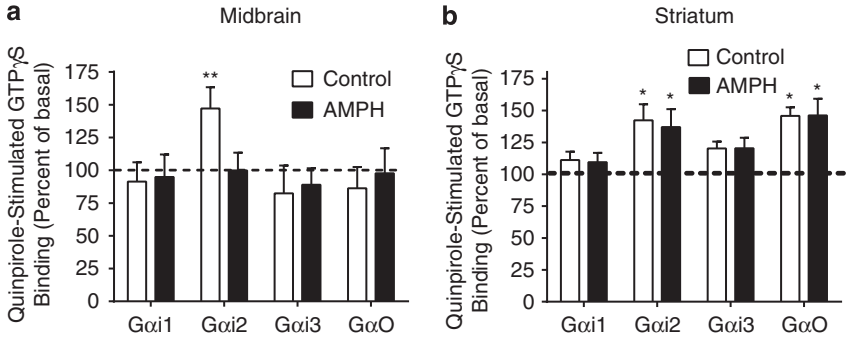

Figure 4 AMPH self-administration had a differential effect on D2/D3 receptor coupling to Gαi/o proteins in the midbrain and striatum. (a) Midbrain or (b) striatal membranes from control and amphetamine-treated rats were assayed for individual G-protein activation by quinpirole (10 μM) using a antibody-capture [35S]GTPγS scintillation proximity assay as described in the Materials and Methods. Data were reported as percent increase over basal (no quinpirole) ($N = 4$–6). In the midbrain, D2/D3 receptors were primarily coupled to Gαi2, which was abolished by AMPH self-administration (a). In contrast to the midbrain, striatal D2/D3 receptors were primarily coupled to Gα2 and Gαo, and AMPH self-administration did not have any effects on D2/D3 receptors and Gαi/o proteins (b). Data are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$ compared with the basal level.

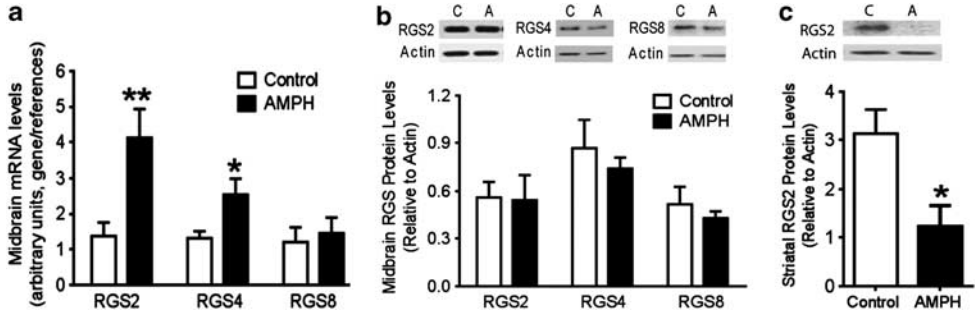

Figure 5 Differential effects of AMPH self-administration on the expression of RGS genes and proteins. (a) AMPH self-administration induced a significant increase in levels of RGS2 and RGS4 mRNA in the midbrain when compared with the control treatment ($N = 5$). However, AMPH self-administration did not alter the mRNA expression of RGS8. Data were normalized by the average mRNA expression of actin and GAPDH. (b) AMPH self-administration did not cause any changes in the total protein expression of RGS2, RGS4, and RGS8 proteins in the midbrain. Data were normalized by actin ($N = 5$). (c) AMPH self-administration significantly reduced RGS2 protein expression in the striatum when compared with controls ($N = 6$). Data are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$ *vs* controls.

striatal D2/D3 receptor function (Figure 4b) or expression (Supplementary Figure S2B).

AMPH Self-Administration Selectively Enhanced a Membrane Translocation of RGS2 Protein from the Cytosol in the Midbrain

We further examined whether AMPH self-administration caused any changes in RGS2 protein translocation in the midbrain. We performed tissue fractionation to separate the cytosolic and membrane fractions. Interestingly, AMPH self-administration selectively promoted RGS2 protein translocation to the membrane (Figure 6a, *t*-test, $t_6 = 8.089$, $P < 0.01$), and reduced the cytosolic RGS2 protein localization relative to controls (Figure 6a, *t*-test, $t_6 = 3.587$, $P < 0.05$). Furthermore, AMPH self-administration did not have any effect on redistribution of RGS4 and RGS8 proteins (Figure 6b and c). In addition, we determined whether RGS2 proteins were expressed in dopaminergic neurons by immunocytochemistry. TH was indeed colocalized with RGS2 protein in the dopaminergic neurons of the VTA (Supplementary Figure S3).

DISCUSSION

Our study demonstrated a profound impact of short-term AMPH exposure on midbrain DA transmission. Five days of AMPH self-administration reduced the function and signaling of midbrain D2/D3 receptors without any effect on striatal D2/D3 receptors. The subsensitivity of midbrain D2/D3 receptors likely resulted from AMPH-induced loss of coupling between Gαi2 protein and D2/D3 receptors. Moreover, AMPH self-administration induced a pronounced translocation of RGS2 protein to the membrane, which may also contribute to the reduced D2/D3 receptor function by rapidly turning off Gαi/o protein signaling. Using the antibody-capture [^{35}S]GTPγS scintillation proximity assay, we showed for the first time that midbrain D2/D3 receptors were primarily coupled to Gαi2, whereas the striatal D2/D3 receptors were equally coupled to Gαi2 and Gαo for activation.

Escalation of AMPH Intake Rate Paralleled Reduced D2/D3 Autoreceptor Function and Enhanced DA Release

We used extended access AMPH self-administration to model the escalation of intake that is often seen in human drug abusers. Although the total amount of AMPH intake was limited, rats still escalated in the rate of intake over sessions. In this experimental paradigm, AMPH self-administration reduced the ability of D2/D3 autoreceptors to inhibit DA release measured by voltammetry, and reduced the ability of D2/D3 autoreceptors to synthesize DA as demonstrated by a decreased level of phosphorylated TH. Our results are consistent with a few other reports. For instance, prior AMPH exposure or repeated systemic injections of AMPH to rats markedly increases neuron firing in the VTA, which is due to the reduced ability of D2 autoreceptors to suppress VTA DA neuron firing (Kamata and Rebec, 1984; Nimitvilai and Brodie, 2010; Seutin *et al*, 1991; White and Wang, 1984a, 1984b). Here, we provide another line of evidence using contingent AMPH self-administration paradigm to support the notion that D2/D3 autoreceptors are less able to inhibit DA release following AMPH self-administration. Our data further indicate that the reduced D2/D3 autoreceptor function increases the probability of DA release. A persistent increase in synaptic DA levels could alter DA transmission in the NAcc and ultimately, drug addiction-related behavior.

Limited data suggest that D2/D3 autoreceptor function or expression may be a crucial factor contributing to drug reward. For instance, microinjection of quinpirole into the VTA, but not the SN, of rats dose-dependently decreases cocaine-induced reinstatement (Xue *et al*, 2011). Furthermore, the availability of midbrain D2/D3 autoreceptors is inversely correlated with drug reward and impulsivity (Bello *et al*, 2011; Buckholtz *et al*, 2010). In agreement with the literature, our study clearly demonstrates that 5 days of AMPH self-administration compromises midbrain D2/D3 receptor function and signaling. These data collectively indicate that reduced midbrain D2/D3 autoreceptor function or expression may be a common neurochemical phenotype that predicts susceptibility to drug addiction.

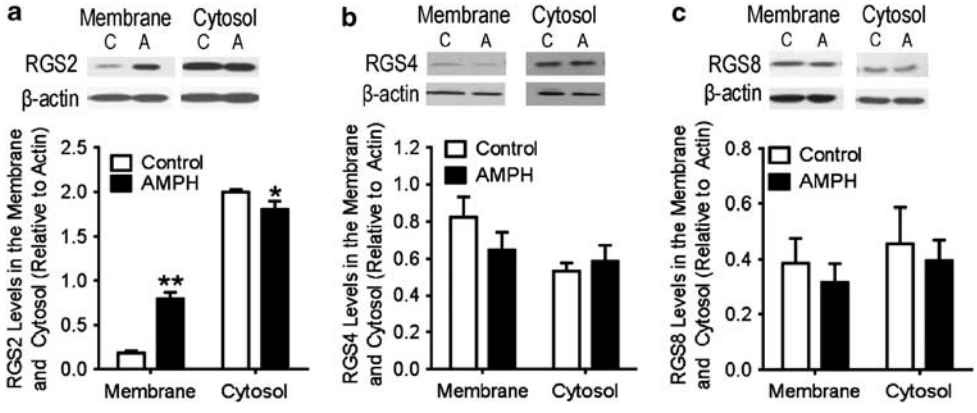

Figure 6 AMPH self-administration induced differential trafficking of RGS2, RGS4, and RGS8 proteins in the midbrain. Top panels: representative western blots of the midbrain RGS2, RGS4, and RGS8 protein expression in the membrane and the cytosol of a control (c) and an AMPH (a) self-administering rat. Bottom panels: a summary of the membrane and the cytosol expression of RGS2 (a), RGS4 (b), and RGS8 (c) in the midbrain. AMPH self-administration resulted in an increase in the membrane insertion and a decrease in the cytosolic translocation of RGS2 proteins without any effects on RGS4 or RGS8 protein trafficking ($N = 4$–5). Data were normalized by actin, and expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$ *vs* controls.

AMPH Self-Administration Abolished Midbrain D2/D3 Receptor-Activated G-Protein Signaling Without any Effect on Postsynaptic D2/D3 Receptor signaling

The D2 receptor is capable of coupling to various subtypes of Gαi/o proteins in heterologous expression systems (Ghahremani *et al*, 1999; Nickolls and Strange, 2004). There are no reports on whether D2 receptors are preferentially coupled to specific subtypes of Gαi/o proteins in specific brain regions. Here, we demonstrate that midbrain D2/D3 receptors are primarily coupled to Gαi2 for receptor activation. However, striatal D2/D3 receptors have a different profile of coupling to Gαi/o proteins, showing a robust response to quinpirole activation of Gαi2 and Gαo and a lesser activation of Gαi3. It has long been speculated that D2 autoreceptors and postsynaptic D2 receptors may have a different signaling pathway because of their differential spatial distribution *in vivo*. This is the first demonstration of the differential G-protein coupling to D2 autoreceptors and postsynaptic D2 receptors in animal brain tissues. We are aware that the striatum contains presynaptic D2 receptors located at dopamine neuron nerve terminals and postsynaptic D2 receptors primarily residing in GABAergic neurons. Functionally, D2 autoreceptors can be distinguished from postsynaptic D2 receptors by using FSCV to measure D2 autoreceptor-mediated DA release as demonstrated in quinpirole dose-dependent inhibition of DA release in Figure 2d. Anatomically and biochemically, it is difficult to dissect out the D2 autoreceptors from postsynaptic D2 receptors in the striatum. However, it has been shown that striatal D2 receptors are predominantly postsynaptic D2 receptors. Mice with conditional knockout of D2 autoreceptors (both somatodendritic and presynaptic D2 receptor deletion in DA neurons) showed 80–100% binding to [^{3}H]spiperone (a membrane permeable D2 receptor ligand) in the striatum when compared with their corresponding wild-type controls (Anzalone *et al*, 2012; Bello *et al*, 2011), suggesting that $>80\%$ receptors are postsynaptic and $<20\%$ receptors in the striatum are presynaptic D2 receptors. Because of the predominant expression of postsynaptic D2 receptors relative to presynaptic D2 receptors in the striatum, activation of Gαi/o proteins by quinpirole (Figure 4b) is reasonably considered to result from stimulation of postsynaptic D2 receptors. Future studies using conditional D2 autoreceptor knockout mice would provide a more accurate contribution of postsynaptic D2 receptors in activation of G protein by quinpirole in the striatum.

Importantly, we show that AMPH disrupts D2 receptor-mediated activation of Gαi/o proteins in the midbrain without any effect on the coupling between postsynaptic D2 receptors and Gαi/o proteins in the striatum. Our observation of no changes in striatal D2 receptor function and signaling is in contrast to reported alterations in striatal D2 receptor density and/or signaling following lengthy treatments with psychostimulants *in vivo*. For example, repeated and intermittent AMPH treatment for 14 days to rats reduces the surface availability of D2/D3 receptors and abolishes D2 receptor agonist-induced inhibition of forskolin-enhanced adenylyl cyclase activity in the NAcc as compared with saline controls (Chen *et al*, 1999). Repeated cocaine exposure for 14 days leads to a reduced binding affinity of D2 receptor ligands to striatal D2/D3 receptors (Maggos *et al*, 1998; Tsukada *et al*, 1996). Although the routes of drug treatment (contingent *vs* non-contingent AMPH) may contribute to the discrepancies between our data and that from the literature, another likely confounding factor is the length of AMPH treatment. It seems that longer drug exposure to psychostimulants is required to induce alterations in striatal D2/D3 receptors as compared with midbrain D2/D3 receptors. Changes in midbrain D2/D3 autoreceptors may precede changes in postsynaptic D2/D3 receptors during the addiction process. The subsensitivity of D2 autoreceptor may be the first step in a cascade of events, which could influence continued drug-taking and -seeking behavior, and could be a potential mechanism that furthers the addiction process.

AMPH Self-Administration Selectively Promoted Membrane Translocation of RGS2

Changes in RGS protein levels have a functional significance *in vivo*. For example, overexpression of RGS9 in the NAcc attenuates acute and chronic cocaine-induced locomotor activity, which parallels the reduced locomotor stimulation by D2 receptor, but not D1 receptor, agonists (Rahman *et al*, 2003). Furthermore, lentiviral RGS4 overexpression in the dorsal striatum suppresses acute AMPH-induced locomotor stimulation and weakens the AMPH-induced phosphor-ERK level, which may be mediated by glutamate receptor 5 (Schwendt *et al*, 2012). These data suggest that RGS proteins can modulate the effect of psychostimulants through specific interactions with certain receptors in a brain region-dependent manner. To date, there is no report on changes in the midbrain RGS mRNA and protein levels by psychostimulants. In the current study, we found a significant increase in the midbrain RGS2 and RGS4 mRNA levels 18 h following 5 days of AMPH self-administration. Reports on RGS2 mRNA levels following non-contingent AMPH treatment have not been consistent with no change (Burchett *et al*, 1998) and an increase (Seeman *et al*, 2007) owing to different lengths of AMPH treatment and withdrawal. These data suggest that AMPH modulates RGS2 mRNA levels in a brain region-dependent manner. Although there was an increase in RGS2 and RGS4 mRNA levels after AMPH self-administration in our study, their protein levels did not differ from control animals. It is possible that the changes in RGS protein levels are transient or it may take a longer length of withdrawal to see changes in RGS proteins. It has been shown that RGS mRNA levels are affected by the length of drug withdrawal from repeated psychostimulant exposure (Park *et al*, 2011; Piechota *et al*, 2012); thus future studies are warranted to determine the time course for changes of RGS proteins following AMPH self-administration.

Our data demonstrate for the first time that AMPH selectively enhances midbrain RGS2 function by promoting RGS2 translocation from the cytosol to the membrane without any effect on RGS4 and RGS8. The enhanced RGS2 membrane translocation may underlie subsensitivity of D2 autoreceptor following AMPH self-administration. It has been demonstrated previously that RGS2 mRNA is expressed in dopaminergic neurons as shown by quantitative gene expression analysis in single cells (Labouebe *et al*,

2007). We further confirmed that RGS2 protein exists in the midbrain dopaminergic neurons by immunocytochemistry. Given that repeated treatment with psychostimulants leads to persistent stimulation of DA receptors by DA, an upregulation of an RGS protein would lead to desensitization of receptor function and signaling, whereas a downregulation of an RGS protein may result in receptor sensitization. Therefore, the enhanced RGS2 trafficking to the membrane by AMPH self-administration may facilitate the termination of D2/D3 receptor-activated Gαi/o signaling, dampening D2/D3 receptor function in the midbrain. Future studies should focus on whether there is a direct interaction between RGS2 and D2 receptors *in vivo*. It is worth noting that striatal RGS2 protein was significantly reduced by 5 days of AMPH self-administration. Because the striatal D2 receptor function and expression remained unchanged after AMPH self-administration, a decrease in striatal RGS2 proteins suggests that RGS2 may not directly regulate D2 receptor function in this brain region. Instead, RGS2 may have specificity for *N*-methyl-D-aspartate receptor (NMDA) in the striatum because acute treatment of the NMDA receptor antagonist MK-801 reduces striatal RGS2 protein expression (Taymans *et al*, 2005). Therefore, it seems that AMPH self-administration has a differential effect on RGS2 protein expression or translocation in a brain region-dependent manner.

To summarize, this study demonstrates that AMPH self-administration dampens midbrain D2/D3 receptor function and signaling. D2/D3 autoreceptors are more susceptible to changes by AMPH compared with striatal postsynaptic D2 receptors. The rapid desensitization of autoreceptors by short-term AMPH self-administration suggests that the inhibitory feedback mechanism by D2/D3 autoreceptor is rapidly turned off, whereas postsynaptic responses to DA are still fully functional, producing a magnification of responses to AMPH. The sustained dysfunction of D2/D3 autoreceptors could ultimately lead to long-lasting changes in postsynaptic D2/D3 receptors and thus addiction-related behavior. AMPH-induced selective membrane insertion of RGS2 protein in the midbrain indicates that RGS2 could be a potential pharmacological target for intervention of DA transmission and thus treatment of drug abuse.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)