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Repeated cocaine administration changes the function and subcellular distribution of adenosine A₁ receptor in the rat nucleus accumbens

Shigenobu Toda,* Luis F. Alguacil*† and Peter W. Kalivas*

*Physiology and Neuroscience, Medical University of South Carolina, Charleston, South Carolina, USA
†Dpto. Farmacología, Tecnología y Desarrollo Farmacéutico, University San Pablo CEU, Madrid, Spain

Abstract

Adenosine A₁ receptor (A₁) protein and mRNA is increased in the nucleus accumbens following repeated cocaine treatment. In spite of this protein up-regulation, A₁ agonist-stimulated [³⁵S]GTPγS binding was attenuated in accumbens homogenates of rats withdrawn for 3 weeks from 1 week of daily cocaine injections. Cellular subfractionation revealed that the discrepancy between total A₁ protein and G protein coupling resulted from a smaller proportion of receptors in the plasma membrane. The decrease in functional receptor in the plasma membrane was further indicated by diminished formation of heteromeric receptor complex consisting of A₁ and dopamine D₁A receptors. To explore the functional significance of the altered distribution of A₁ receptors, at 3 weeks after discontinuing repeated cocaine or saline, animals were injected with cocaine and 45 min later the subcellular distribution of A₁ receptors quantified. Whereas a cocaine challenge in repeated saline-treated animals induced a marked increase in membrane localization of the A₁ receptor, the relative distribution of receptors in repeated cocaine rats was not affected by acute cocaine. These data suggest that the sorting and recycling of A₁ receptors is dysregulated in the nucleus accumbens as the consequence of repeated cocaine administration.

Keywords: adenosine A₁ receptor, cocaine, dopamine D₁A receptor, receptor sorting.


Repeated cocaine or amphetamine administration causes a variety of enduring behavioral changes that are characteristic of addiction, including drug craving, relapse and paranoia (Dackis and O’Brien 2001). The search for the cellular underpinnings of these behavioral changes in animal models of addiction has resulted in many potential candidate proteins whose levels or phosphorylation states are altered after extended withdrawal from repeated cocaine or amphetamine administration (Nestler 2001). The search for neural substrates of addiction is focused within brain circuits that mediate the pharmacological effects of the psychostimulants, notably in dopamine terminal fields such as the nucleus accumbens. Within the nucleus accumbens, changes by repeated cocaine or amphetamine have been found in the level of many G-protein coupled receptors, including GABA, glutamate and opioid receptors (Turchan et al. 1999; Sharpe et al. 2000; Xi et al. 2002, 2003). In addition, a recent study using accumbens tissue from animals withdrawn for 3 weeks from repeated cocaine administration identified an induction of adenosine 1 (A₁) receptor protein and mRNA (Toda et al. 2002).

The increase in A₁ receptors in the accumbens by withdrawal from repeated cocaine is interesting as A₁ receptors form a heteromeric receptor complex with dopamine D₁A receptors (Férré et al. 1994; Ginés et al. 2000). Many studies show that the electrophysiological effects of D₁ receptor stimulation are enhanced by withdrawal from cocaine, while binding to D₁ receptors is not altered (Kleven et al. 1990; Henry and White 1991), indicating enhanced signaling distal to D₁ ligand binding. Also, many of the enduring neuroadaptations elicited by repeated cocaine administration arise from cocaine indirectly stimulating D₁ receptors.

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Address correspondence and reprint requests to Shigenobu Toda, MD, PhD, Department of Physiology and Neuroscience, Medical University of South Carolina, 173 Ashley Avenue, BSB-408, Charleston, SC 29425, USA. E-mail: todas@musc.edu

Abbreviations used: A₁, adenosine A₁ receptor; CPA, N-cyclopentyladenosine; D₁, dopamine D₁ receptor; ER, endoplasmic reticulum; GPCR, G protein coupled receptor; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.
receptors (Nestler 2001). The formation of A1-D1A heteromeric receptor complex causes D1A receptors to favor the low-affinity agonist binding state by disassociating Gsα from D1A (Ferre et al. 1998; Torvinen et al. 2002). Thus, increasing A1 receptors by withdrawal from repeated cocaine would be predicted to reduce signaling by D1 receptor stimulation. The fact that, after repeated cocaine, the elevation in A1 protein is associated with enhanced D1 function indicates that increased total A1 protein may not be commensurate with increased functional A1 receptors. A1 receptors are Gsα coupled and the possibility of a dissociation between A1 protein content and function is consistent with the effect of withdrawal from repeated cocaine to reduced signaling through other Gαi receptors, including GABAβB, and group II metabotropic glutamate receptors (Zhang et al. 2000; Xi et al. 2002, 2003).

In order to examine the functional status of the elevated A1 protein, at 3 weeks after discontinuing repeated cocaine administration, the present study examined A1 receptor coupling to Gαi, the formation of A1-D1A heteromeric receptor complex and the relative distribution of A1 protein to the plasma membrane. In addition, repeated cocaine pretreatment sensitizes the behavioral response to a subsequent cocaine injection (Vanderschuren and Kalivas 2000), and it was determined if an acute injection of cocaine changed the subcellular distribution of A1 receptors commensurate with the altered behavioral response.

**Materials and methods**

**Animal housing**

Male Sprague-Dawley rats weighting 250–350 g (Harlan, Indianapolis, IN, USA) were housed in groups of two, with food and water available *ad libitum*. A 12-h light/dark cycle was used with the lights on at 07.00 hours. All saline or cocaine injections were performed during the light cycle. All experiments were conducted according to specifications of the National Institute of Health guide for the Care and Use of Laboratory Animals.

**Repeated cocaine or saline treatment and tissue preparation**

All rats were acclimatized to the housing facility for 1 week prior to beginning cocaine injections using a regimen shown previously to produce behavioral sensitization (Pierce et al. 1996). Rats were treated with either daily saline or cocaine in their home cages (× 7 days; 15 mg/kg, i.p., on the first and last day, 30 mg/kg, i.p., on the intervening 5 days). After a 3-week withdrawal period, the animals were decapitated without any challenge of saline or cocaine or decapitated 45 min after an acute injection of saline (1.0 mL/kg, i.p.) or cocaine (30 mg/kg, i.p.) for subcellular fractionation. The nucleus accumbens was dissected and homogenized immediately for subcellular fractionation or frozen on dry ice and stored at −80°C.

**Immunoblotting**

Total or fractionated proteins (10–20 μg; determined using DC assay; Bio-Rad, Hercules, CA, USA) from each animal were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The following antibodies were obtained from commercial sources; polyclonal adenosine A1 receptor (diluted 1:500, Sigma, St Louis, MO, USA), polyclonal adenosine A1 receptor (diluted 1:500, Affinity BioReagents, Golden, CO, USA), polyclonal actin (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal dopamine D1A receptor (1:200, Santa Cruz Biotechnology), polyclonal calnexin (1:80 000, Stressgen, San Diego, CA, USA), and monoclonal PSD-95 (1:1000, Sigma). Immunolabeling was detected using ECL system (Amersham, Piscataway, NJ, USA). Levels of immunoreactivity were quantified by measuring the density of each band using NIH image 1.62.

**[^1S]GTPγS binding assay**

Tissue was homogenized in the buffer containing 320 mM sucrose, 20 mM HEPES and 10 mM EDTA, pH 7.4. The homogenate was centrifuged three times at 48 000 g at 4°C for 15 min and resuspended in assay binding buffer (50 mM Tris–HCl, 3 mM MgCl₂ and 1 mM EGTA, pH 7.4). The binding assay was performed according to the method described elsewhere with some minor modifications (Xi et al. 2002). Briefly, one mL of assay buffer was combined with 30 μg of proteins, 150 μM GDP, 0.1 U of adenosine deaminase (EC 3.5.4.4., Sigma), 0.1 nM[^35]S]GTPγS (1 mCi/mL, Amersham) and various concentration of adenosine A1 receptor agonist N-cyclopentyladenosine (CPA) (Sigma). Basal binding was measured in the absence of agonist, and non-specific binding was measured in the presence of 10 μM unlabeled GTPγS (Sigma). The reaction mixture was incubated for 30 min at 30°C, and the reaction was terminated by filtration under vacuum through GF/B glass fiber filters. The radioactivity was measured by liquid scintillation spectrophotometry.

**Co-immunoprecipitation**

Total protein was homogenized in PBS containing 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS, pH 7.4 (Ginés et al. 2000). Homogenates were pre-cleared by overnight incubation with protein-A immunoabsorbent (Sigma) at 4°C. After centrifugation at 10 000 g for 5 min, the supernatants were transferred to the tube containing anti-A1 antibody covalently coupled to protein-A sepharose beads (Sigma), and shaken for 4 h at 4°C. Then, the collected resin was washed with bovine serum albumin (BSA) lysis buffer three times. Finally, immuno-complexes treated with 2 × SDS buffer for 2 min at 37°C were resolved by SDS–PAGE in 4–15% gradient gel (Bio-Rad).

**Subcellular fractionation**

Subcellular fractionation was performed as described previously with minor modifications (Huttner et al. 1993; Dunah and Standaert 2001). Briefly, fresh brain tissues were homogenized in cold buffer containing 0.32 M sucrose and 1 mM EDTA, pH 7.4. Homogenates were cleared three times at 1000 g for 10 min to remove nuclei and large debris (i.e. the P1 nuclear fraction). The resulting supernatants were concentrated twice at 10 000 g for 15 min to obtain a crude synaptosome fraction, and subsequently were lysed hypo-osmotically and centrifuged at 25 000 g for 20 min to pellet synaptosomal membrane fractions (LP1). The resulting supernatants were then centrifuged at
165 000 g for 2 h to obtain synaptic vesicle-enriched fraction (LP2). Concurrently, the supernatant above the crude synapticomatal fractions were centrifuged at 165 000 g for 2 h to obtain a light membrane/Golgi apparatus (golgi)/endoplasmic reticulum (ER)-enriched fraction (P3). The integrity of the P3 and LP1 fractions was verified by immunoblotting for calnexin (P3) and PSD-95 (LP1).

Data analysis
Dose–response curves were analyzed using Prism (GraphPad Software Inc., San Diego, CA, USA). A two-way ANOVA with repeated measures over dose was used to evaluate the data of $[^{35}S]GTP_\gamma S$ binding. Immunoblotting data were analyzed using a two-tailed Student's $t$-test or two-way ANOVA. Data from subfractionated samples were normalized first to calnexin (P3) or PSD-95 (LP1), then as per cent of control treatment. The co-immunoprecipitation data were evaluated as the ratio of $D_{1A}$ to $A_1$ in each sample.

Results

$A_1$-stimulated $[^{35}S]GTP_\gamma S$ binding is reduced
Rats were injected with daily cocaine or saline for 7 days, and 21 days later G protein coupling of $A_1$ in the nucleus accumbens was examined between saline- and cocaine-treated animals. $[^{35}S]GTP_\gamma S$ binding was stimulated using the selective $A_1$ agonist CPA. Figure 1 shows that the $B_{max}$ of $[^{35}S]GTP_\gamma S$ binding was significantly reduced in the accumbens of cocaine-compared with saline-treated animals without a significant change of $K_d$ (saline = 2.48 ± 0.81 nM; cocaine = 1.76 ± 0.25 nM). These data indicate that the previously reported increase in $A_1$ protein after repeated cocaine is not functionally inserted into the plasma membrane (Toda et al. 2002).

Relative subcellular location of $A_1$ receptors is altered
Figure 2(a) illustrates the relative location of $A_1$ and $D_{1A}$ receptors in the ER/golgi (P3) and plasma membrane (LP1) fractions in accumbens homogenates of untreated animals. Figure 2(b) shows representative immunoblots and quantitative comparisons of $A_1$ in P3 and LP1 fractions. S, chronic saline-treated animals; C: chronic cocaine-treated animals. Quantification of $A_1$ protein in P3 and LP1 fractions was normalized using calnexin and PSD-95, respectively, as internal control. Data are shown as mean ± SEM per cent change from the saline group. *$p < 0.05$, comparing saline with cocaine using a two-tailed Student's $t$-test.

Fig. 1 Adenosine $A_1$ receptor shows altered function in the nucleus accumbens at 3 weeks after discontinuing daily cocaine injections. $[^{35}S]GTP_\gamma S$ binding assay was stimulated using a selective $A_1$ agonist CPA. A two-way ANOVA with repeated measures over dose revealed a significant effect of chronic cocaine treatment [$F_{5,18} = 95.5$, $p < 0.001$]. Data are shown as mean ± SEM.

Fig. 2 Adenosine $A_1$ receptor shows altered subcellular distribution in the nucleus accumbens at 3 weeks after discontinuing daily chronic cocaine injections. (a) Representative subcellular distribution of $A_1$, $D_{1A}$ and marker proteins. Equal amounts of protein (10 µg) were loaded in each lane and calnexin and PSD-95 were used as internal controls for P3 and LP1 fractions, respectively. (b) Representative immunoblots and quantitative comparisons of $A_1$ in P3 and LP1 fractions. S, chronic saline-treated animals; C: chronic cocaine-treated animals. Quantification of $A_1$ protein in P3 and LP1 fractions was normalized using calnexin and PSD-95, respectively, as internal control. Data are shown as mean ± SEM per cent change from the saline group. *$p < 0.05$, comparing saline with cocaine using a two-tailed Student's $t$-test.
postsynaptic scaffolding protein PSD-95 was relatively enriched in LP1. Figures 2 (b and c) show that the relative amounts of A1 protein in P3 and LP1 in accumbens tissue obtained 21 days after discontinuing repeated saline (S) and cocaine (C). A1 protein content was relatively enriched in the P3 compared with LP1 fraction of cocaine versus saline-treated animals.

To further examine a functional change in A1 receptors, the formation of A1-D1A heteromeric receptor complex was measured with co-immunoprecipitation using anti-A1 antibody (Fig. 3). Consistent with previous reports using membrane fraction of co-transfected cell homogenates (Gínez et al. 2000), we confirmed that D1A and A1 receptors are co-immunoprecipitated in accumbens homogenates that were not subfractionated. Following withdrawal from repeated cocaine, the co-immunoprecipitation of D1A and A1 receptors was reduced, indicating a reduction in heteromeric receptor complex formation. This reduction was associated with an increase in total A1 receptor (Toda et al. 2002), and no change in D1A receptors (saline = 1.00 ± 0.068, n = 6; cocaine = 0.97 ± 0.168, n = 5; also, see Kleven et al. 1990).

Acute cocaine administration induces rapid A1 cycling
According to the data above, we speculated that the retention of A1 receptors in the ER/golgi of cocaine-withdrawn subjects may function as a receptor reserve that would cycle to the membrane compartment in response to an acute cocaine injection. However, Fig. 4 shows that in chronic cocaine-withdrawn subjects the amount of A1 protein in both the P3 and LP1 fractions was equivalent between the acute saline and cocaine (30 mg/kg, i.p.) injection groups. In contrast, an injection of cocaine at 3 weeks after discontinuing repeated saline administration produced a significant increase in A1 protein in both P3 and LP1 fractions without significantly altering total protein content. To further validate these findings, the LP1 fraction was evaluated using a second A1 antibody and all groups were normalized to the group receiving repeated saline and an acute saline challenge injection (SS). Not only did this additional experiment verify the elevation by acute cocaine administration in control animals, but consistent with the data shown in Fig. 2(b), in repeated cocaine animals the levels of A1 proteins in LP1 fraction were significantly decreased compared with repeated saline animals, regardless of whether the animals were injected with acute cocaine or saline (Fig. 4b).

Discussion
The present study showed that in spite of increased synthesis of A1 protein (Toda et al. 2002), there is a functional attenuation of A1 receptor stimulation in the nucleus accumbens of animals withdrawn from repeated cocaine treatment. This was evidenced by a reduction in A1 receptor stimulated G protein coupling, as well as decreased formation of A1-D1A heteromeric receptor complex. The reduced function may be due to retention of A1 in the ER/golgi as relatively greater amounts of A1 protein were found in the P3 compared with LP1 fraction of cocaine versus saline-treated animals.

To further examine a functional change in A1 receptors, the formation of A1-D1A heteromeric receptor complex was measured with co-immunoprecipitation using anti-A1 antibody (Fig. 3). Consistent with previous reports using membrane fraction of co-transfected cell homogenates (Gínez et al. 2000), we confirmed that D1A and A1 receptors are co-immunoprecipitated in accumbens homogenates that were not subfractionated. Following withdrawal from repeated cocaine, the co-immunoprecipitation of D1A and A1 receptors was reduced, indicating a reduction in heteromeric receptor complex formation. This reduction was associated with an increase in total A1 receptor (Toda et al. 2002), and no change in D1A receptors (saline = 1.00 ± 0.068, n = 6; cocaine = 0.97 ± 0.168, n = 5; also, see Kleven et al. 1990).

Acute cocaine and A1 receptor sorting and resensitization
This is the first report to demonstrate an effect by psychostimulant administration on the sorting and/or recycling of a G protein coupled receptor (GPCR) to the
plasma membrane in vivo. The data also indicate that this rapid sorting of A1 includes a route through the ER/golgi. The source of additional A1 receptor to the P3 and LP1 fractions is unknown. However, GPCRs are generally recycled through early endosome directly to the plasma membrane, therefore the endosomal compartment is a likely candidate (Ferguson 2001). In addition, the non-significant trend towards an increase in total protein indicates that de novo GPCR synthesis may contribute to the net increase on the cell surface. Consistent with this possibility, similar to the cocaine-induced elevation in A1 in the ER/golgi compartment, several reports show agonist-induced increase in GPCRs in the ER/golgi as a result of increased protein synthesis (Dumartin et al. 1998; Bernard et al. 1999; Csaba et al. 2001; Liste et al. 2002). Finally, a role by altered degradation cannot be ruled out as a contributing factor in the apparent cocaine-induced redistribution of A1 receptor.

Repeated cocaine and A1 receptor sorting and resensitization

The mechanisms mediating ER/golgi retention of A1 receptors and the attenuated recycling/resensitization of A1 protein in the accumbens of animals withdrawn from repeated cocaine administration cannot be discerned from the present data. However, these two cocaine-induced adaptations might be related. One possibility is an alteration in the basal tone provided by the endogenous ligand adenosine following repeated cocaine administration. Enhanced adenosine uptake has been reported in the accumbens following repeated cocaine treatment (Manzoni et al. 1998), and could affect A1 trafficking. Another possibility is that, generally, GPCRs are phosphorylated when internalized and dephosphorylated when sorted back to the plasma membrane (Tsao and von Zastrow 2001), and repeated cocaine has been shown to increase the phosphorylation state of other Giα-coupled GPCRs, including GABAB and mGluR2/3 receptors (Xi et al. 2002, 2003).

Functional significance of altered A1 receptor sorting and resensitization

The fact that alterations in A1 subcellular localization occur after 3 weeks of withdrawal from repeated cocaine poses the possibility that this neuroadaptation may play a role in the enduring behavioral effects induced by repeated cocaine, including motor sensitization. In general, Giα signaling in the nucleus accumbens is reduced following repeated cocaine, due to a reduction in Giα (Nestler et al. 1990; Striplin and Kalivas 1993), an increase in the Giα binding protein AGS3 (Bowers et al. submitted) and a phosphorylation-induced receptor desensitization (Xi et al. 2002, 2003). The present data are the first to suggest that sorting of receptor out of the plasma membrane may also be a contributing factor. Presynaptic A1 receptor activation reduces the synaptic release of glutamate and dopamine, and enhanced cocaine-
induced release of both transmitters is observed in the accumbens of behaviorally sensitized animals after withdrawal from repeated cocaine (Heidbreder et al. 1996; Pierce et al. 1996). Thus, the functional down-regulation of A1 receptors could contribute to the sensitized release of dopamine and glutamate. Also potentially important to augmented transmitter release is the inability of an acute cocaine injection to redistribute A1 protein to the cell surface. Thus, this potential compensatory mechanism for reducing transmitter release and thereby limiting the motor stimulant response to cocaine is not available after withdrawal from repeated cocaine.

The present study demonstrated, for the first time, the in vivo presence of A1-D1A heteromeric receptor complexes. In addition to the loss of A1 receptors in the plasma membrane compartment, the reduction in A1-D1A heteromeric receptor complex may contribute to the well-established enduring increase in D1 signaling produced by withdrawal from repeated cocaine (Henry and White 1991; Nestler 2001). When D1A receptors are dimerized with A1 receptors, the binding affinity for dopamine is reduced (Ferre S., Popoli P., Gimenez-Llort L., Finman U.-B., Martinez E., Scotti de Carolis A. and Fuxe K. (1994) Postsynaptic antagonistic interaction between adenosine A1 and dopamine D1 receptors. Neuroreport 6, 73–76.


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