A Region-Specific Increase in $G\alpha_q$ and $G\alpha_{11}$ Proteins in Brains of Rats during Cocaine Withdrawal

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ABSTRACT
Serotonin 2A (5-HT$_{2A}$) receptor-mediated increases in plasma hormone levels become supersensitive after 42 h of withdrawal from cocaine treatment. The present study investigated which components of the 5-HT$_{2A}$ receptor signaling system are associated with this supersensitivity. Rats were injected daily for 14 days with either saline or cocaine (15 mg/kg i.p.) twice a day or were injected using a “binge” protocol (three injections per day, 1 h apart). Rats were sacrificed 2 or 7 days after the last cocaine injection, and the levels of membrane and cytosol-associated 5-HT$_{2A}$ receptors, $G\alpha_q$, $G\alpha_{11}$, regulators of G protein signaling (RGS4), and RGS7 proteins were assayed in the hypothalamic paraventricular nucleus, amygdala, and frontal cortex using Western blot analysis. Two days of withdrawal from cocaine, administered twice a day or using a binge protocol, produced an increase in membrane-associated $G\alpha_q$ and $G\alpha_{11}$ proteins in the paraventricular nucleus and the amygdala (but not in the frontal cortex). This effect was reversible after 7 days of withdrawal. The protein levels of the 5-HT$_{2A}$ receptor, $G\alpha_q$ protein, and RGS4 or RGS7 proteins were not altered by cocaine withdrawal in any of the above-mentioned brain regions. These findings suggest that the supersensitivity of the 5-HT$_{2A}$ receptors, during withdrawal from chronic cocaine, is associated with an increase in membrane-associated $G\alpha_q$ and $G\alpha_{11}$ proteins and not with changes in the expression of 5-HT$_{2A}$ receptors.

Serotonergic mechanisms play an important role in cocaine addiction (Levy et al., 1992; Baumann and Rothman, 1996). Cocaine binds with high affinity to serotonin (5-HT) uptake sites on nerve terminals, inhibiting serotonin reuptake (Ritz et al., 1990). Consequently, acute exposure to cocaine alters serotonin neurotransmission, increasing the concentration of serotonin in the synapse (Parsons et al., 1996). Furthermore, cocaine reduces the activity of serotonin neurons in the dorsal raphe nucleus (Cunningham and Lakoski, 1988), presumably as a consequence of increasing the stimulation of somatodendritic 5-HT$_{2A}$ autoreceptors in the cell body regions. Human cocaine abusers have a withdrawal-induced deficit in serotonergic nerve terminals in the hypothalamus (Buydens-Branchey et al., 1999) that could be associated with mild to severe depression and anxiety (Gawin, 1991).

Behavioral and neuroendocrine studies have demonstrated that chronic exposure to cocaine produces supersensitivity of 5-HT$_{2A}$ receptor function (Levy et al., 1992; Baumann and Rothman, 1996). Behavioral studies have shown that withdrawal (48 h) from intermittent cocaine administration (15 mg/kg i.p. twice a day for 7 days) enhances the head shake response elicited by DOI, a 5-HT$_{2A}$/2C receptor agonist (Baumann and Rothman, 1996). After 8 days of withdrawal, the response is not significantly different from rats that receive saline (Baumann and Rothman, 1996). Additionally, withdrawal from intermittent cocaine (5 days) has been reported to enhance the basal locomotor activity of rats, an effect that is inhibited by ketanserin, a 5-HT$_2$ receptor antagonist with modest $\alpha_1$-adrenergic antagonist activity, but not by prazosin, an $\alpha_1$-adrenergic antagonist (Filip et al., 2001). Using neuroendocrine responses to DOI, we also found increased sensitivity of 5-HT$_{2A}$ receptors after 42 h withdrawal from repeated cocaine (15 mg/kg i.p., twice a day for 7 days) (Levy et al., 1992). Similar results for prolactin have been described after 2 days of cocaine withdrawal, an effect that disappears after 8 days of withdrawal (Baumann and Rothman, 1996). Additionally, an increase in DOI-induced dopamine release in the nucleus accumbens was observed, using in vivo microdialysis after 7 days of cocaine withdrawal, in rats treated twice a day with cocaine for 14 days (30 mg/kg s.c.) (Yan et

ABREVIATIONS: 5-HT, 5-hydroxytryptamine; DOI, (±)-1-(2,5 dimethoxy-4-iodophenyl)-2-amino-propane HCl; IOD, integrated optical density; ANOVA, analysis of variance; ACTH, adrenocorticotropic hormone.
al., 2000). This effect was inhibited by ketanserin (Yan et al., 2000).

The mechanisms underlying the supersensitivity of 5-HT2A receptors during cocaine withdrawal are not understood. However, autoradiographic examination of 5-HT2A receptors in different brain areas of the rat, including frontal cortex, nucleus accumbens, caudate putamen, and thalamus revealed no changes between saline- and cocaine-treated rats (15 mg/kg i.p., three times a day for 14 days) (Perret et al., 1998). Similar results were reported in ligand binding studies using [1H]ketanserin in the hippocampus, choroid plexus, and frontal cortex of cocaine-treated rats (20 mg/kg i.p. twice a day for 14 days) (Johnson et al., 1993). Together, these findings suggest that the supersensitivity of 5-HT2A receptors induced by withdrawal from chronic intermittent cocaine is not associated with changes in the density of the 5-HT2A receptor. It is possible that this supersensitivity of 5-HT2A receptors is related to other downstream components of the 5-HT2A signaling cascade.

5-HT2A receptors are coupled via Goq and G11 proteins to the phosphoinositide signaling cascades (Rotth et al., 1998a). Also, regulators of G protein signaling, specifically regulators of G protein signaling (RGS4) and RGS7 proteins, are involved in 5-HT2A receptor signaling (Huang et al., 1997). These proteins enhance the intrinsic GTPase activity of Goq and G11 proteins, stimulating GTP hydrolysis (Huang et al., 1997). In the present study, we examined the protein levels of 5-HT2A receptors, Go11, Goq, G11, RGS4, and RGS7 proteins in membranes and cytosol of the hypothalamic paraventricular nucleus, amygdala, and frontal cortex of cocaine- and saline-treated rats. These three regions were selected because of their prominent role in stress, anxiety, and neuroendocrine function (Van de Kar and Blair, 1999; Carrasco and Van de Kar, 2003). The hypothalamic paraventricular nucleus plays a central role in mediating neuroendocrine responses to serotonergic activation (Bagdy, 1996). This nucleus receives serotonergic projections from the raphe nuclei, which also send collaterals to other limbic structures, such as amygdala (Petrov et al., 1994). The amygdala is a limbic structure with interconnections to the cortex and nucleus accumbens (Davis et al., 1994). Previous reports have shown that injection of cocaine produces a dramatic decrease in the 5-hydroxyindolacetic acid/5-HT ratio in the amygdala, cortex, and hypothalamus of mice (Hadfield, 1995). This effect is dose-dependent and is more prominent in the hypothalamus, amygdala, and cortex than in all the other brain areas studied, including the septum, hippocampus, and olfactory bulbs (Hadfield, 1995).

Materials and Methods

Animal Treatment. Male Sprague-Dawley rats (225–275 g) (Harlan, Indianapolis, IN) were housed two per cage in an environment controlled for lighting, temperature, and humidity. Food and water were available ad libitum. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as approved by the Loyola University Institutional Animal Care and Use Committee. The rats were allowed to acclimate to their environment for at least 4 days before the start of the treatment period. Eight to 12 rats were randomly assigned to four treatment groups: 1) saline twice a day (8:30 AM and 4:30 PM) for 14 days; 2) cocaine-HCl (15 mg/kg i.p., 8:30 AM and 4:30 PM) for 14 days; 3) saline three times a day (4:00, 5:00, and 6:00 PM) for 14 days; and 4) cocaine-HCl (15 mg/kg i.p.) three times a day (4:00, 5:00, and 6:00 PM) for 14 days. Rats were killed 2 or 7 days after the last injection. The brains were immediately removed and the frontal cortex was dissected and frozen in liquid nitrogen. The remainder of the brain was frozen and stored at −70°C.

Tissue Preparation. Rat brains were placed in a cryostat at −10°C, and coronal sections were cut to obtain a 700-μm-thick section containing the paraventricular nucleus and rostral amygdala and a 1200-μm-thick section containing the caudal amygdala. The paraventricular nucleus and amygdala were microdissected from these frozen sections with the aid of a dissecting stereomicroscope. Plasma membranes of frontal cortex, amygdala, and paraventricular nucleus of the hypothalamus were prepared as described previously (Serres et al., 2000). All procedures were conducted at 4°C. Briefly, the frontal cortex, hypothalamic paraventricular nucleus, and amygdala were homogenized in 50 mM Tris buffer (pH 7.4) containing 150 mM NaCl, 10% sucrose, and 0.5 mM phenylmethylsulfonyl fluoride and additional protease inhibitors purchased as a cocktail (containing 4:2-aminomethyl benzenesulfonyl fluoride, pepstatin A, trans epoxysuccinyl-l-leucyl-amido(4-guanidino)butane, bestatin, leupeptin, 10,000 units of Sigma-Aldrich (St. Louis, MO)). After centrifugation at 20,000g for 60 min, the supernatant was collected and stored at −70°C for further analyses of cytosol-associated protein levels. The pellets were collected and resuspended by sonication in a 20 mM Tris buffer (pH 8, containing 1 mM EDTA, 100 mM NaCl, 1% sodium cholate, and 1 mM dithiothreitol) plus the protease inhibitory cocktail (1.5 μl cocktail/30 mg of tissue). The resuspended pellets were incubated while shaking for 1 h at 4°C and then centrifuged at 100,000g for 60 min. The supernatant was collected for the Western blot analyses of membrane-associated protein levels. Protein concentration was measured using a bichinonic acid protein assay kit (Pierce Chemical, Rockford, IL). The membrane proteins were stored at −70°C for Western blot analyses.

Western Blot Analysis. Quantification of 5-HT2A receptors, Goq, G11, Goq, G11, RGS4, and RGS7 proteins was performed by Western blot of cytosol and membrane-associated proteins. Samples containing 2 to 4 μg of protein were submitted to SDS-polyacrylamide gel electrophoresis containing 0.1% SDS, 12.5% acrylamide/bisacrylamide (30:0.2), 4.6 M urea, and 275 mM Tris, pH 8.7. Gels were transferred electrophoretically by semidyed blot to nitrocellulose membranes. After incubation with a blocking buffer (phosphate-buffered saline containing 0.2% casein, 0.1% Tween 20), the nitrocellulose membranes were probed overnight at 4°C with polyclonal antiserum. Immunodetection was performed with either rat anti-5-HT2A receptor (1:500 dilution; BD PharMingen, San Diego, CA), anti-Gq11 (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz Biotechnology, Inc.), anti-Gq11 (1:500; Santa Cruz Biotechnology, Inc.), anti-Gq11 (1:6000; Santa Cruz Biotechnology, Inc.), anti-RGS4 (1:500, N-16; Santa Cruz Biotechnology, Inc.), and anti-RGS7 (1:1000; generously donated by Drs. Philip Jones and Kathleen Young, Wyeth Discover Research, Princeton, NJ). After incubation with the 5-HT2A receptor antibody, the membranes were incubated at room temperature with a peroxidase-labeled anti-mouse secondary antibody (1:10,000; Jackson Immunoresearch Laboratories, West Grove, PA). The overnight incubation with Gq11, Gq11, Gq11, and RGS7 antibodies was followed by incubation with peroxidase-labeled anti-rabbit antibody (1:20,000, 1 h at room temperature; Santa Cruz Biotechnology, Inc.). Incubation with RGS4 antibody was followed by incubation at room temperature for 1 h with an anti-goat antibody (1:2000, IgG1; Sigma-Aldrich) and a goat peroxidase-antiperoxidase solution (1:10,000; Cappel ICN Pharmaceuticals, Aurora, OH). Finally, the membranes were incubated with the enhanced chemiluminescence substrate solution (Amersham Biosciences Inc., Piscataway, NJ) and then exposed to Kodak X-ray film. Films were analyzed densitometrically using the Scion Corporation (Frederick, MD) Image program. Protein loading for each lane was verified using an anti-actin antibody (1:20,000; Santa Cruz Biotechnology, Inc.). Negative con-
trols included either omission of primary antibody or addition of preimmune rabbit immunoglobulins.

**Film Analysis.** Films were analyzed densitometrically using Scion Image software (Scion Corporation). The gray scale density readings were calibrated using a transmission step-wedge standard. The integrated optical density (IOD) of each band was calculated as the sum of optical densities of all the pixels within the area of the band outlined. An adjacent area was used to calculate the background optical density of the film. The IOD for the film background was subtracted from the IOD for each band. The resulting IOD for each protein band was then divided by the amount of protein loaded on the corresponding lane, and each sample was expressed as IOD per microgram of protein. Each sample was measured on three independent gels. All samples were standardized to controls and normalized to its respective actin level.

**Statistics.** All data are expressed as the mean ± S.E.M., where n indicates the number of rats per group. A three-way (cocaine × withdrawal time × injection frequency) analysis of variance (ANOVA) was used to analyze the Western blot data, considering p < 0.05 statistically significant. Group means were compared by Newman-Keuls multiple range test (Steel and Torrie, 1960). GB-STAT software (Dynamic Microsystems, Inc., Silver Spring, MD) was used for all statistical analyses.

## Results

### Western Blot Analysis

**Gα11 Proteins.** Gα11 protein was detected as a single band at approx. 40 kDa (Fig. 1). The three-way ANOVA revealed that cocaine [F(1,86) = 17.17634, p < 0.0002] and withdrawal time [F(1,86) = 11.22402, p < 0.0001] have a significant main effect on the level of membrane-associated Gα11 protein in the hypothalamic paraventricular nucleus (Figs. 1, 2, and 4). The protocol of injection did not significantly affect the levels of membrane-associated Gα11 proteins [F(1,86) = 0.11522, p > 0.73]. The interaction between cocaine treatment and withdrawal time was significant [F(1,86) = 9.44, p < 0.03], but no significant interaction was observed between cocaine treatment and injection protocol [F(1,86) = 0.05079, p > 0.82], or withdrawal time and injection protocol [F(1,86) = 4.22, p > 0.05].

In rats injected twice a day with cocaine, the level of membrane-associated Gα11 protein was 60% higher (p < 0.01) than in the saline group (Fig. 4). An increase of approx. 40% (p < 0.05) was observed in the levels of membrane-associated Gα11 protein in rats injected with cocaine using a binge protocol (Fig. 4). The level of cytosol-associated Gα11 proteins was not affected by cocaine treatment [F(1,78) = 0.17975, p > 0.67], withdrawal time [F(1,78) = 0.88253, p > 0.35], or protocol of injection [F(1,84) = 0.02592, p > 0.8725] (Fig. 3).

In the amygdala, the level of membrane-associated Gα11 protein was elevated 2 days after withdrawal from cocaine (Figs. 2 and 5). The three-way ANOVA indicated a significant main effect of cocaine [F(1,104) = 15.06632, p < 0.0002] and the withdrawal time [F(1,104) = 4.27547, p < 0.05]; but no significant interaction was observed between the twice a day versus the binge protocol [F(1,104) = 0.3645, p > 0.80]. The interaction between cocaine treatment and withdrawal time was significant [F(1,104) = 11.1878, p < 0.05], but no significant interaction was observed between cocaine treatment and injection protocol [F(1,104) = 0.51317, p > 0.45], or withdrawal time and injection protocol [F(1,104) = 0.04123, p > 0.80]. The levels of membrane-associated Gα11 proteins were increased in rats injected twice a day with cocaine when measured after 2 days of withdrawal (p < 0.01) (Fig. 2). However, after 7 days of withdrawal, Gα11 proteins returned to control levels (Fig. 5). A similar and reversible increase in membrane-associated Gα11 proteins was detected in rats injected using a binge protocol (p < 0.001) (Fig. 5). No differences were found in the levels of membrane-associated Gα11 proteins between rats injected with cocaine twice a day and rats that received cocaine using a binge protocol (p > 0.05). The levels of cytosol-associated Gα11 proteins were not affected by cocaine treatment [F(1,84) = 1.14955, p > 0.28], withdrawal time [F(1,84) = 0.07533, p > 0.78] or protocol of injection [F(1,84) = 0.10804, p > 0.74] (Fig. 5).

In the frontal cortex, cocaine did not produce any change in the levels of membrane-associated Gα11 proteins between rats injected with cocaine twice a day and rats that received cocaine using a binge protocol (p > 0.05). The levels of Gα11 protein in the paraventricular nucleus of rats injected twice a day with saline or cocaine for 14 days and after 2 days of withdrawal. An increase in the levels of membrane-associated Gα11 proteins in the paraventricular nucleus of cocaine-treated rats was found without changes in the levels of actin, which was used as a control of protein loading; 2.4 μg of protein was loaded per lane.

**Gαq Proteins.** Gαq protein was detected in the hypothalamic paraventricular nucleus, amygdala, and frontal cortex as a single band at approx. 42 kDa (Fig. 2). The levels of membrane-associated Gαq protein in the paraventricular nucleus were also affected by cocaine [F(1,78) = 32.9463, p < 0.0001] and withdrawal time [F(1,78) = 23.66472, p < 0.0001], but were not affected by the injection protocol [F(1,78) = 0.61814, p > 0.43]. Both cocaine injected twice a day (p < 0.01) and cocaine injected using a binge protocol (p < 0.01) increased the levels of membrane-associated Gαq by approx. 50% (Figs. 2 and 4). After 7 days of withdrawal, the levels of Gαq were not significantly different (p > 0.05) in cocaine-treated rats compared with controls (Fig. 4). The level of cytosol-associated Gαq protein was not affected by cocaine treatment [F(1,78) = 0.49812, p > 0.48], withdrawal time [F(1,78) = 0.20112, p > 0.65], or protocol of injection [F(1,78) = 0.25489, p > 0.61] (Fig. 5).

In the amygdala, the three-way ANOVA indicated a significant main effect of cocaine [F(1,93) = 20.70083, p < 0.0001]
and the withdrawal time [$F_{(1,93)} = 26.63021, p < 0.0001$], and the injection protocol [$F_{(1,93)} = 4.70565, p < 0.0326$] for the membrane-associated $G_{\alpha_1}$ protein. The level of membrane-associated $G_{\alpha_1}$ proteins was increased by approximately 80% over the saline control ($p < 0.01$) in rats injected twice a day with cocaine and withdrawn for 2 days (Figs. 2 and 5). This effect of cocaine was transient. After 7 days of withdrawal, the levels of $G_{\alpha_1}$ were not different in cocaine-treated rats compared with control rats (Fig. 5). Also, the level of membrane-associated $G_{\alpha_1}$ was increased in rats injected with cocaine using a binge protocol ($p < 0.05$) (Fig. 5). After 2 days of withdrawal, the levels of $G_{\alpha_1}$ were significantly higher in the rats injected with cocaine twice a day than in the rats injected using the binge protocol ($p < 0.01$). Levels of cytosol-

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Fig. 2. Representative Western blots of membrane-associated $G_{\alpha_{11}}, G_{\alpha_4}, G_{\alpha_2}, 5$-HT$_2A$, RGS4, and RGS7 proteins in the paraventricular nucleus, the amygdala, and frontal cortex of rats injected twice a day with saline (S) or cocaine (C) for 14 days and after 2 days of withdrawal. An increase in the levels of membrane associated $G_{\alpha_{11}}$ and $G_{\alpha_4}$ proteins in the paraventricular nucleus and the amygdala of cocaine treated rats was detected.

Fig. 3. Representative Western blots of cytosol-associated $G_{\alpha_{11}}, G_{\alpha_4}, G_{\alpha_2}, 5$-HT$_2A$, RGS4, and RGS7 proteins in the paraventricular nucleus, the amygdala, and frontal cortex of rats injected twice a day with saline (S) or cocaine (C) for 14 days and after 2 days of withdrawal. No changes were detected in the levels of any of the cytosol-associated proteins studied.
associated $G_{\alpha}$ proteins were not affected by cocaine treatment [$F_{(1,90)} = 0.8505, p > 0.45$], withdrawal time [$F_{(1,90)} = 1.00249, p > 0.31$], or protocol of injection [$F_{(1,90)} = 0.24681, p > 0.6205$] (Fig. 5).

In the frontal cortex, no effect of cocaine was detected on the levels of membrane-associated $G_{\alpha}$ protein [$F_{(1,94)} = 1.74212, p > 0.19$] or the levels of cytosol-associated $G_{\alpha}$ protein [$F_{(1,94)} = 0.76994, p > 0.38$] (Figs. 2, 3, and 6).

$G_{\alpha}$ Protein. $G_{\alpha}$ is a 40-kDa protein that has not been reported to associate with the 5-HT$_{2A}$ receptor signaling cascade. The determination of $G_{\alpha}$ protein was used as a negative control to gain valuable information related the specificity of effects of cocaine on G proteins. The ANOVA for the levels of membrane and cytosol-associated $G_{\alpha}$ protein in the paraventricular nucleus showed no significant main effect of cocaine treatment [$F_{(1,76)} = 0.24112, p > 0.62$, and $F_{(1,78)} = 0.89069, p > 0.34$, for membrane and cytosol, respectively], no significant main effect of withdrawal time [$F_{(1,76)} = 1.51877, p > 0.22$ and $F_{(1,76)} = 0.07612, p > 0.78$, for membrane and cytosol, respectively], and no significant effect of the protocol of injection [$F_{(1,76)} = 2.50595, p > 0.11$ and $F_{(1,78)} = 0.95208, p > 0.33$, for membrane and cytosol, respectively] (Figs. 2–4). In the amygdala, the statistical analysis for the levels of membrane and cytosol-associated $G_{\alpha}$ proteins showed no significant main effects of cocaine treatment [$F_{(1,90)} = 3.11896, p > 0.08$ and $F_{(1,97)} = 3.90838, p > 0.05$, for membrane and cytosol, respectively], withdrawal time [$F_{(1,90)} = 2.19543, p > 0.14$ and $F_{(1,97)} = 0.99243, p > 0.75$, for membrane and cytosol, respectively] and protocol of injection [$F_{(1,90)} = 0.15504, p > 0.21$ and $F_{(1,97)} = 2.97737, p > 0.08$, for membrane and cytosol, respectively] (Figs. 2, 3, and 5).

Additionally, cocaine did not affect the levels of membrane and cytosol-associated $G_{\alpha}$ protein in the frontal cortex [$F_{(1,92)} = 0.51117, p > 0.47$ and $F_{(1,90)} = 0.87196, p > 0.35$, membrane and cytosol, respectively] (Figs. 2, 3, and 6). Furthermore, no significant effects of withdrawal time [$F_{(1,92)} = 0.03475, p > 0.85$ and $F_{(1,90)} = 0.23258, p > 0.63$, membrane and cytosol, respectively] or injection protocol [$F_{(1,92)} = 0.42745, p > 0.51$ and $F_{(1,90)} = 0.71749, p > 0.39$, membrane and cytosol, respectively] were observed for the levels of $G_{\alpha}$ proteins (Figs. 2, 3, and 6).

5-HT$_{2A}$ Receptor. The 5-HT$_{2A}$ receptor antibody recognized two bands (approx. at 55–58 kDa), both of which were included in the quantification of the protein levels (Fig. 2). Cocaine did not produce a significant main effect on the levels of membrane-associated 5-HT$_{2A}$ receptors in the paraventricular nucleus [$F_{(1,73)} = 0.11501, p > 0.73$], amygdala [$F_{(1,97)} = 3.90838, p > 0.05$], or frontal cortex [$F_{(1,90)} = 1.65965, p > 0.20$] (Figs. 2, 4, 5, and 6). Similarly, no main effect of cocaine was found on the levels of cytosol-associated...
5-HT$_{2A}$ receptors in the paraventricular nucleus [F($1,76$) = 0.83247, p > 0.36], amygdala [F($1,86$) = 0.6883, p > 0.40], or frontal cortex [F($1,80$) = 0.0004, p > 0.98] (Figs. 3–6).

**RGS4 and RGS7 Proteins.** RGS4 and RGS7 proteins were detected each as a single band at 35 and 56 kDa, respectively. No significant effect of cocaine was observed on the levels of membrane-associated RGS4 or RGS7 proteins in the paraventricular nucleus [F($1,70$) = 0.04613, p > 0.83 and F($1,73$) = 0.01464, p > 0.90, for RGS4 and RGS7, respectively], amygdala [F($1,87$) = 2.0139, p > 0.15 and F($1,81$) = 0.23344, p > 0.163, for RGS4 and RGS7, respectively] or frontal cortex [F($1,83$) = 0.01972, p > 0.88 and F($1,82$) = 0.84628, p > 0.36, for RGS4 and RGS7, respectively] (Figs. 2, 4, 5, and 6).

The cytosol-associated levels of RGS4 were not affected by cocaine treatment in the paraventricular nucleus [F($1,73$) = 0.01464, p > 0.904], amygdala [F($1,88$) = 0.009994, p > 0.75], or frontal cortex [F($1,83$) = 0.05357, p > 0.81] (Fig. 3). Similarly, no main effect of cocaine was detected for cytosol-associated RGS7 proteins in the hypothalamic paraventricular nucleus [F($1,77$) = 4.05986, p > 0.05], amygdala [F($1,86$) = 0.08454, p > 0.77], or frontal cortex [F($1,88$) = 0.17454, p > 0.67] (Fig. 3).

**Discussion**

A cocaine-induced supersensitivity of the 5-HT$_{2A}$ receptor might be expected to be accompanied by increased levels of the 5-HT$_{2A}$ receptor and/or $G_{oq}$ and $G_{a11}$ proteins and/or reduced expression of RGS4 or RGS7 proteins. The main findings of this article are 1) exposure to cocaine produces a transient and a region-specific increase in the levels of membrane-associated $G_{oq}$ and $G_{a11}$ proteins in the paraventricular nucleus and the amygdala, but not in the frontal cortex; 2) no changes were detected in the cytosol-associated $G_{oq}$ and $G_{a11}$ proteins in the paraventricular nucleus, amygdala, or frontal cortex during withdrawal from cocaine; and 3) exposure to cocaine does not produce changes in the levels of membrane or cytosol-associated 5-HT$_{2A}$ receptors, RGS4, RGS7, and $G_{oq}$ proteins.

Exposure of rats to cocaine for 7 days, followed by 2 days of withdrawal, leads to supersensitivity of 5-HT$_{2A}$-receptor-mediated phenomena, such as head shake behaviors and hormone responses induced by the 5-HT$_{2A/C}$ receptor agonist DOI (Levy et al., 1994; Baumann and Rothman, 1998). Interestingly, in both the behavioral and the neuroendocrine studies, there was no increase in the maximal responses to DOI but instead a leftward-shift in the dose-response curves. These observations suggest alterations in the coupling state or postreceptor signal transduction mechanisms, rather than increased density of 5-HT$_{2A}$ receptors. Our results support this conclusion, because we detected no changes in the protein levels of the 5-HT$_{2A}$ receptor in the hypothalamic paraventricular nucleus, the amygdala, or frontal cortex. Consistent with our results, no changes in 5-HT$_{2A}$ receptors have been described in frontal cortex, nucleus accumbens, caudate putamen, or thalamus of cocaine-treated rats (Johnson et al., 1993; Perret et al., 1998).

To our knowledge, no studies examined the levels of $G_{oq}$ or $G_{a11}$ proteins in the brain of cocaine-treated rats. Studies regarding the effect of cocaine on other G proteins have been reported (Przewlocka et al., 1994; Self et al., 1994). These studies include chronic and acute effects of cocaine on $G_{oq}$, $G_{a11}$, and $G_{o}$ proteins in the hippocampus, ventral tegmental area, nucleus accumbens, and locus coeruleus (Przewlocka et al., 1994; Self et al., 1994). In human cocaine overdose victims, a down-regulation of the $G_{o1}$ subunit was found in the ventral tegmental area (Tang et al., 2003). We found a specific and transient increase in $G_{oq}$ and $G_{a11}$ proteins in the hypothalamic paraventricular nucleus and amygdala during withdrawal from cocaine. The specificity of this increase was evident when no changes were detected in the protein levels of $G_{oq}$ protein, which is associated with the signaling cascade of the 5-HT$_{1A}$ receptor in the hypothalamic paraventricular nucleus (Serres et al., 2000). Interestingly, our results showed that the cocaine-induced increases in levels of membrane-associated $G_{oq}$ and $G_{a11}$ proteins in the paraventricular nucleus were not affected by the injection protocol. In the amygdala, the levels of $G_{oq}$ protein were 40% lower when the rats were injected using a binge protocol than when they received cocaine injections twice a day. We used a binge regimen of cocaine administration in this study to mimic the pattern frequently seen in human cocaine abusers (Gawin, 1991). This is an important neuroendocrine consideration, because human cocaine abusers often engage in binges lasting several hours (Zhou et al., 2003a), and it is reported that the frequency of drug administration, among other variables, may be of critical importance in the activity of the hypothalamic-pituitary-adrenal axis (Torres and Rivier, 1992).
Although the increase in the levels of $\alpha_q$ and $\alpha_{11}$ proteins might explain the cocaine-induced supersensitivity of the 5-HT$_{2A}$ receptor, additional receptors are coupled to $\alpha_q$ and $\alpha_{11}$ proteins and need to be explored. 5-HT$_{2C}$ and metabotropic glutamate receptors are also coupled to $\alpha_q$ and $\alpha_{11}$ proteins (Li et al., 2003). Cocaine-induced alterations in both 5-HT$_{2C}$ and metabotropic glutamate receptors have been reported (Cornish and Kalivas, 2001; Rocha et al., 2002; Xi et al., 2002). Interestingly, 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors have similar molecular structures and similar affinities for most of their agonists (Barnes and Sharp, 1999) and are coupled to $\alpha_{q}$/$\alpha_{11}$ proteins and subsequently activate phospholipase C to trigger signaling pathways (Roth et al., 1998b). 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors are also reported to participate in the control of brain dopamine neurotransmission (Rocha et al., 2002). Similar to 5-HT$_{2A}$ receptors, 5-HT$_{2C}$ receptors are widely distributed in brain regions, including the hypothalamus, amygdala, and frontal cortex (Li et al., 2003). Currently, there is no evidence of alterations in hypothalamic 5-HT$_{2C}$ receptors in cocaine-treated rats. On the other hand, evidence supports a role for metabotropic glutamate receptors in mediating behavioral and neuroendocrine effects of cocaine (Swanson et al., 2001; Xi et al., 2002). However, most of these data have been obtained in the ventral tegmental area, nucleus accumbens and striatum (Cornish and Kalivas, 2001; Swanson et al., 2001; Xi et al., 2002).

To our knowledge, there is no evidence of cocaine-induced alterations in metabotropic glutamate receptor signaling in the hypothalamic paraventricular nucleus or the amygdala.

RGS proteins are a family of more than 20 proteins that stimulate the intrinsic GTP-hydrolyzing activities of G$_\alpha$-protein subunits and thereby promote the conversion of G$_\alpha$-protein from an active monomeric to an inactive trimeric state (Hollinger and Hepler, 2002). At least 10 RGS proteins have been identified in the brain (Taymans et al., 2002). Three of these, RGS2, RGS4, and RGS7 proteins, modulate $\alpha_q$ and $\alpha_{11}$ protein signaling (Hollinger and Hepler, 2002). Supersensitization of 5-HT$_{2A}$ receptor signaling could be induced by a decrease in RGS4 and/or RGS7 protein levels. We did not find changes in the membrane or cytosol levels of RGS4 or RGS7 proteins during withdrawal from cocaine. Although, no studies reported cocaine-induced changes in the level of RGS7 protein, some changes have been reported for RGS4 proteins in other areas of the brain (Bishop et al., 2002; Yuferov et al., 2003). The locus coeruleus exhibited a biphasic response, with decreased RGS4 mRNA levels after acute administration and increased levels of RGS4 mRNA after chronic administration (Bishop et al., 2002). In human cocaine overdose abusers, up-regulation of RGS3 and down-regulation of RGS12 mRNAs in the ventral tegmental area has been reported (Tang et al., 2003).

One feature common to many drugs of abuse is the withdrawal syndrome that results from termination of drug administration. We observed that the increase in the levels of $\alpha_q$ and $\alpha_{11}$ proteins that occurred 2 days after withdrawal from cocaine was reversed after 7 days of withdrawal. Interestingly, this transient increase in the protein levels of $\alpha_q$ and $\alpha_{11}$ corresponds with previous neuroendocrine data indicating supersensitivity of DOI-induced ACTH, corticosterone, and prolactin secretion in cocaine-treated rats (Levy et al., 1992; Baumann and Rothman, 1996). In these studies, the supersensitivity in DOI-induced hormone release is present after 2 days of withdrawal from chronic cocaine-treated rats (Levy et al., 1992; Baumann and Rothman, 1996). This effect disappears after 8 days of withdrawal (Baumann and Rothman, 1996). Similarly, behavioral studies showed a supersensitized DOI-induced increase of head shakes, a specific 5-HT$_{2A}$ receptor-induced behavior, after 2 days of withdrawal from cocaine (Baumann and Rothman, 1996). This behavioral effect disappeared after 8 days of withdrawal from cocaine (Baumann and Rothman, 1996). Although further studies are required, the similarities between our findings on $\alpha_q$ and $\alpha_{11}$ protein levels with previous neuroendocrine and behavioral data are suggestive of a specific effect of cocaine on 5-HT$_{2A}$ receptor signaling in the hypothalamic-pituitary-adrenal axis and the limbic system.

There is limited information concerning the effects of withdrawal from chronic cocaine on the hypothalamic-pituitary-adrenal axis (Zhou et al., 2002, 2003a,b). A recent report showed that rats injected with cocaine (15 mg/kg i.p., 14 days, using binge protocol) showed a significant attenuation of ACTH and corticosterone responses 30 min after the last cocaine injection (Zhou et al., 2002, 2003a,b). However, 24 h after the final administration of binge cocaine, a significant elevation of the plasma levels of ACTH and corticosterone was found (Zhou et al., 2002, 2003a,b). Interestingly, increased mRNA expression encoding CRF$_1$ receptors is increased in the anterior pituitary during cocaine withdrawal and this effect is not found on the 10th day of cocaine withdrawal (Mantsch et al., 2003). A possible explanation for this phenomenon could involve upstream elements that regulate CRF and ACTH secretion in the hypothalamus and pituitary, respectively. 5-HT$_{2A}$ receptors activate the release of hypothalamic CRF and thus ACTH release (Van de Kar et al., 2001; Zhang et al., 2002). Because an increase in the activity of the hypothalamic-pituitary-adrenal axis has been reported in human cocaine users (Vescovi et al., 1992) and rats (Zhou et al., 2003a,b) we can speculate that the increased levels of $\alpha_q$ and $\alpha_{11}$ proteins in the hypothalamic paraventricular nucleus and amygdala could be responsible for this effect.

In summary, the mechanisms by which drugs of abuse, and specifically cocaine, mediate their varied effects are incompletely understood. Cocaine-induced supersensitivity of some behavioral and neuroendocrine effects associated with 5-HT$_{2A}$ receptors seems to be mediated by increased levels of $\alpha_q$ and $\alpha_{11}$ proteins.

References


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