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## COCAINE-MEDIATED SUPERSENSITIVITY OF 5-HT<sub>2A</sub> RECEPTORS IN HYPOTHALAMIC PARAVENTRICULAR NUCLEUS IS A WITHDRAWAL-INDUCED PHENOMENON

Gonzalo A. Carrasco, Louis D. Van de Kar, Nicole R. Sullivan, Michelle Landry, Francisca Garcia, Nancy A. Muma, and George Battaglia\*

Department of Pharmacology, Loyola University of Chicago, 2160 South First Avenue, Maywood, IL 60153

### Abstract

We previously reported that treatment and withdrawal from cocaine increases: (1) serotonin 2A (5-HT<sub>2A</sub>) receptor-mediated neuroendocrine responses, and (2) G<sub>i</sub> and G<sub>11</sub> G-protein levels in the hypothalamic paraventricular nucleus (PVN) at 48 h post-treatment. This study investigates changes in the initial 24 h of withdrawal to discern whether 5-HT<sub>2A</sub> receptor supersensitivity is due to cocaine treatment or is induced during the withdrawal period. We report here increases in 5-HT<sub>2A</sub> receptor-mediated neuroendocrine responses only 12 or 24 h post-treatment, but not during the initial 4 h withdrawal period. Levels of membrane- or cytosol-associated G<sub>i</sub> or G<sub>11</sub> proteins in PVN are not altered during the first 24 h of withdrawal. However, the density of <sup>125</sup>I-DOI-labeled high-affinity 5-HT<sub>2A</sub> receptors in PVN increased 35% in rats withdrawn from cocaine for 24 h. These findings demonstrate that cocaine-induced increases in 5-HT<sub>2A</sub> receptor function in PVN represents a withdrawal-induced phenomena that: (1) is likely attributed to increased G-protein coupled/high-affinity conformational state of the 5-HT<sub>2A</sub> receptor, and (2) occurs in the absence of changes in the levels of associated G proteins during the first 24 h.

### Keywords

serotonin; hypothalamus; prolactin; corticosterone; G proteins; rats

### INTRODUCTION

The activity of the hypothalamicâpituitaryâadrenal (HPA) axis before, during and after cocaine exposure appears to be one of the important determinants of whether or not individuals will engage in cocaine-seeking behavior (Sinha et al., 2006; Fox et al., 2005; Mantsch et al., 2003; Sinha et al., 2000; Sinha et al., 2003). While chronic cocaine administration is associated with neuroadaptive changes that lead to attenuated responses to cocaine in humans and rats (Levy et al., 1993; Mendelson et al., 1998), withdrawal from cocaine is associated with

\*Correspondence: George Battaglia, PhD, Department of Pharmacology, Loyola University of Chicago, Stritch School of Medicine 2160 South First Avenue Maywood, IL 60153, Phone: 708-216-3264 Fax: 708-216-6596 Email: gbattag@lumc.edu  
Dr. Louis D. Van de Kar, a good friend, colleague and internationally recognized scientist passed away on September 4, 2004. His contribution and memory will endure through all of those whose lives he enriched.

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activation of the HPA axis (Peltier et al., 2001;Zhou et al., 2003). This enhanced response of the HPA axis during withdrawal from chronic cocaine has been reported 24 h to 48 h after the last cocaine injection in rats (Peltier et al., 2001;Zhou et al., 2003). Similar results have been reported in human cocaine addicts (Vescovi et al., 1992;Mendelson et al., 1998).

We and others have previously reported unique neuroadaptive changes in the serotonin 2A (5-HT<sub>2A</sub>) receptor signaling pathway in the hypothalamic paraventricular nucleus (PVN) after 2 days of withdrawal from cocaine. These include: (1) increases in 5-HT<sub>2A</sub> receptor-mediated neuroendocrine responses, suggesting supersensitivity of postsynaptic 5-HT<sub>2A</sub> receptors in PVN (Levy et al., 1992;Baumann et al., 1993;Baumann and Rothman, 1998); and (2) increases in G<sub>i</sub> and G<sub>11</sub> G-proteins in the membrane fraction of PVN (Carrasco et al., 2003;Carrasco et al., 2004b). Since agonists and antagonists of 5-HT<sub>2A</sub> receptors produces desensitization of 5-HT<sub>2A</sub> receptors (Gray and Roth, 2001;Hanley and Hensler, 2002;Damjanoska et al., 2004), the increases in 5-HT<sub>2A</sub> receptor signaling during withdrawal from cocaine represents a unique adaptive response of this system.

The objectives of the present study were to determine if changes in neuroendocrine responses to (α)DOI (a 5-HT<sub>2A/2C</sub> receptor agonist) and the levels of G<sub>11</sub> and G<sub>i</sub> G-proteins in the PVN are produced during the cocaine treatment period or develop during withdrawal. G<sub>i</sub> and G<sub>11</sub> proteins were examined because these are the G-proteins that mediate 5-HT<sub>2A</sub> receptor function. As 5-HT<sub>2A</sub> receptors in PVN are important for the regulation of mood, impulse control and responses to stressors (Kroeze et al., 2002;Aghajanian and Marek, 2000), withdrawal-induced increases in 5-HT<sub>2A</sub> receptor function in this limbic region may be clinically relevant with respect to drug relapse.

## MATERIALS AND METHODS

### Drugs

(α)Cocaine-HCl was obtained from the National Institute on Drug Abuse (Bethesda, MD). A fresh cocaine solution (15mg/ml) was prepared in 0.9% saline prior to each dosing. (α)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl [(α)DOI] was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in 0.9% saline to the concentration of 0.5 mg/kg/ml. All solutions were made fresh before administration and injected at a volume of 1 ml/kg.

### Treatment

Male Sprague-Dawley rats (225±275 g) were purchased from Harlan (Indianapolis, IN). The rats were housed two per cage in a temperature-, humidity-, and light-controlled room (12 hr light/dark cycle, lights on 7:00 AM±19:00 PM). 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 Loyola University Institutional Animal Care and Use Committee (IACUC).

### Experimental Protocols

After arrival, the rats were allowed to acclimate to their environment for at least 4 days prior to the start of the treatment period. Eight to twelve rats were randomly assigned to each group. Cage-mates were assigned to the same treatment group. Rats were injected with either 0.9% saline (1ml/kg, i.p., 8:30 h and 15:30 h) or cocaine (15mg/kg, i.p., 8:30 h and 15:30 h) for 5 days. At different time points after the last saline or (α)cocaine injection (0.5, 1, 2, 4, 12, and 24 hrs), rats were sacrificed by decapitation. The rats were challenged with either saline (1ml/kg) or (α)DOI (0.5 mg/kg) 30 min prior to sacrifice. The trunk blood was collected in centrifuge tubes containing a 0.5 ml solution of 0.3 M EDTA (pH 7.4). After centrifugation, the plasma

was stored at  $-80^{\circ}\text{C}$  for radioimmunoassays of plasma hormone concentrations. The brains were immediately removed and frozen in dry ice and stored at  $-80^{\circ}\text{C}$ .

### Radioimmunoassay

Plasma prolactin and corticosterone concentrations were determined by radioimmunoassays as previously described in detail (Li et al., 1993; Li et al., 1997).

### Tissue preparation

Rat brains were placed in a cryostat at  $-12^{\circ}\text{C}$ , and coronal sections were cut to obtain a 700  $\mu\text{m}$  thick section containing the PVN. The PVN was microdissected from these frozen sections with the aid of a dissecting stereomicroscope.

### Western blots

Plasma membranes and cytosol fractions of the PVN were prepared as previously reported (Carrasco et al., 2003). Expression of membrane- and cytosol-associated  $\text{G}_i^q$ ,  $\text{G}_i^{11}$  and  $\text{G}_i^z$  proteins was measured by Western blot as previously described (Carrasco et al., 2004b). Negative controls included either omission of primary antibody or addition of pre-immune rabbit immunoglobulins.

### Western Blot film analysis

Films were analyzed densitometrically with values calculated from the integrated optical density (IOD) of each band using Scion Image software (Scion Corporation, Frederick, MD, USA), as previously described (Carrasco et al., 2004a). Each sample was measured on three independent gels. All samples were standardized to controls and normalized to their respective actin levels.

### Autoradiography of $^{125}\text{I}$ -DOI binding

Only rats injected with saline before sacrifice were used for receptor autoradiography. The density of 5-HT<sub>2A</sub> receptors in PVN was examined by autoradiographic labeling with  $^{125}\text{I}$ -DOI (2,200 Ci/mmol) in slide mounted sections as previously described (Appel et al., 1990). Nonspecific binding was determined in the presence of 100nM spiperone to block  $^{125}\text{I}$ -DOI binding to 5-HT<sub>2A</sub> receptors. A set of  $^{125}\text{I}$  microscale standards (Amersham Biosciences, Piscataway NJ) were included with samples to equate film optical density readings to fmol/mg of tissue equivalent based on the specific activity of the radioligand. All slides were apposed to Kodak Bio-Max MR film for 6 days at  $4^{\circ}\text{C}$ . Images were scanned and analyzed using MCID Elite 7.0 software (Imaging Research, St-Catharines, Ontario, Canada). Specific  $^{125}\text{I}$ -DOI binding was determined by subtracting the nonspecific binding from the total binding. Data for each rat are the mean of five adjacent sections.

### Statistics

All data are expressed as the mean  $\pm$  S.E.M., where  $n$  indicates the number of rats per group. Hormone data were analyzed by three-way analyses of variance (ANOVA). Immunoblot data was analyzed by two-way ANOVA. Group means were compared by a Newman-Keuls multiple-range test (Steel and Torrie, 1960). Autoradiography data was analyzed by an unpaired Student's  $t$ -test. GB-STAT software (Dynamic Microsystems, Inc., Silver Spring, MD, USA) was used for all statistical analyses.

## RESULTS

### Cocaine withdrawal associated increases in ( $\hat{a}$ )DOI-induced hormone levels

Our previous experiments did not discern whether changes in the activity of 5-HT<sub>2A</sub> receptors in the PVN after treatment and withdrawal from cocaine were a cocaine-treatment effect or a withdrawal-induced phenomena. Therefore, we investigated the neuroendocrine responses to ( $\hat{a}$ )DOI in rats injected for 5 days with either saline or cocaine and withdrawn for 0.5 to 24 h. Increased sensitivity of 5-HT<sub>2A</sub> receptors that stimulate the secretion of corticosterone and prolactin was observed only following 12 or 24 h withdrawal from repeated cocaine treatment (Fig. 1A and 1B). No supersensitivity occurred within the first 1 h of cocaine withdrawal. As shown in Fig. 1, basal corticosterone and prolactin levels were similar ( $p > 0.05$ ) in saline and cocaine treated rats at all post-treatment withdrawal times. ( $\hat{a}$ )DOI significantly increased the plasma levels of corticosterone and prolactin in saline- or cocaine-treated rats at all post-treatment times (Fig. 1). However, in cocaine-treated rats, the ( $\hat{a}$ )DOI stimulated increase in corticosterone and prolactin was significantly greater only at 12 and 24 h withdrawal times, indicating that 5-HT<sub>2A</sub> receptor supersensitivity is a result of changes occurring specifically during the withdrawal period.

For plasma corticosterone, the three-way ANOVA indicated a significant main effect of treatment ( $F_{(1,140)} = 26.25, p < 0.0001$ ) and a significant main effect of ( $\hat{a}$ )DOI challenge ( $F_{(1,140)} = 922.23, p < 0.0001$ ), but not a main effect of withdrawal time ( $F_{(1,140)} = 3.53, p < 0.88$ ). Significant interactions between treatment and the ( $\hat{a}$ )DOI challenge ( $F_{(1,140)} = 12.25, p < 0.0006$ ), and between treatment, withdrawal time and ( $\hat{a}$ )DOI challenge ( $F_{(5,140)} = 2.39, p < 0.0407$ ) were found. The *post hoc* Newman-Keuls test indicated no differences in the ( $\hat{a}$ ) DOI-induced plasma levels of corticosterone between cocaine- and saline-treated rats at any of the times during the first four hours of withdrawal. However, ( $\hat{a}$ )DOI-induced plasma corticosterone levels in cocaine-treated rats were significantly ( $p < 0.01$ ) higher than in saline-treated controls at 12 and 24 h of withdrawal from cocaine (Fig. 1A). The magnitude of increase in ( $\hat{a}$ )DOI-induced corticosterone responses were similar (45% increase) at 12 and 24 h of withdrawal from cocaine (Fig. 1A).

For plasma prolactin, the three-way ANOVA indicated a significant main effect of withdrawal time ( $F_{(5,158)} = 12.02, p < 0.0001$ ) and ( $\hat{a}$ )DOI challenge ( $F_{(1,158)} = 924.52, p < 0.0001$ ) but not a main effect of treatment ( $F_{(1,158)} = 4.79, p < 0.73$ ). There was a significant interaction between treatment and withdrawal time ( $F_{(5,158)} = 20.12, p < 0.00001$ ), and between withdrawal time and ( $\hat{a}$ )DOI challenge ( $F_{(5,158)} = 12.94, p < 0.0001$ ). There was also a significant interaction between cocaine treatment, withdrawal time and ( $\hat{a}$ )DOI challenge ( $F_{(5,158)} = 18.03, p < 0.0001$ ). At 30 and 60 min of withdrawal the plasma levels of prolactin are lower in cocaine-treated rats than in saline-treated rats ( $p < 0.01$  and  $p < 0.05$ , respectively) (Fig. 1B). The *post hoc* Newman-Keuls test indicated that ( $\hat{a}$ )DOI-induced plasma prolactin levels in cocaine-treated rats are significantly ( $p < 0.01$ ) higher than the ( $\hat{a}$ )DOI-induced increases in saline treated rats at 12 or 24 h of withdrawal (Fig. 1B). The magnitude of increases in ( $\hat{a}$ )DOI-induced prolactin levels were similar (65% increase) at 12 and 24 h of withdrawal from cocaine.

### Membrane levels of G $\hat{I}$ proteins

G $\hat{I}$ <sub>q</sub> protein was detected in PVN at approximately 42–43 kDa (Fig. 2A). The levels of membrane-associated G $\hat{I}$ <sub>q</sub> in the PVN were not affected by cocaine treatment ( $F_{(1,71)} = 0.70536, p > 0.41$ ) or withdrawal period ( $F_{(5,71)} = 0.2532, p > 0.94$ ) (Fig. 2B). Neither cocaine treatment nor withdrawal time had a significant ( $p > 0.05$ ) effect on the levels of cytosol-associated G $\hat{I}$ <sub>q</sub> in PVN (Fig. 2A).

$G\hat{I}_{11}$  protein was detected in PVN at approximately 40 kDa (Fig. 2B). There was no significant main effect of cocaine treatment on the levels of membrane-associated  $G\hat{I}_{11}$  protein in the PVN ( $F_{(1,71)} = 0.3035, p < 0.5837$ ). Similarly, there was no significant main effect of withdrawal time on the levels of membrane-associated  $G\hat{I}_{11}$  protein in the PVN ( $F_{(5,71)} = 0.2434, p < 0.9415$ ). Neither cocaine treatment nor withdrawal time had a significant ( $p > 0.05$ ) effect on the levels of cytosol-associated  $G\hat{I}_{11}$  in PVN (Fig. 2B).

$G\hat{I}_z$  is a 40 kDa protein that has not been reported to be associated with the 5-HT<sub>2A</sub> receptor signaling cascade. The determination of  $G\hat{I}_z$  protein was used to verify the specificity of the effects of cocaine treatment on G-proteins associated with 5-HT<sub>2</sub> receptor signal transduction. There was no significant main effect of cocaine on the levels of membrane-associated  $G\hat{I}_z$  in PVN ( $F_{(1,71)} = 0.1726, p < 0.6792$ ) (Fig. 2C). There was no significant effect of withdrawal time on the levels of membrane-associated  $G\hat{I}_z$  in PVN ( $F_{(5,71)} = 0.09458, p < 0.9927$ ). The levels of cytosol-associated  $G\hat{I}_z$  in PVN were not affected ( $p > 0.05$ ) by cocaine treatment or withdrawal time (Fig. 2C).

### Autoradiography of 5-HT<sub>2A</sub> Receptors

Figure 3A shows representative brightfield autoradiograms of the distribution of <sup>125</sup>I-DOI binding in coronal sections of rats at 24 h withdrawal from five days treatment with either saline or cocaine. The insert is a magnified view of the paraventricular nucleus of the hypothalamus in the respective sections. As shown in Fig. 3B, the density of <sup>125</sup>I-DOI-labeled 5-HT<sub>2A</sub> receptors in PVN was significantly ( $p < 0.05$ ) increased (+35%) in rats at 24 hours withdrawal from cocaine.

### Discussion

The present findings demonstrate that 5-HT<sub>2A</sub> receptor supersensitivity in PVN is not present immediately following cocaine treatment but develops during the early withdrawal period. More specifically, cocaine-treated rats required at least 4 h of withdrawal to develop increases in (̂)DOI-induced secretion of corticosterone and prolactin, since changes were first observed at 12 h post-treatment. The supersensitivity of 5-HT<sub>2A</sub> receptors in the PVN is associated with increases in the density of the G-protein coupled/high-affinity conformational state of 5-HT<sub>2A</sub> receptors at 24h withdrawal but not with increased levels of membrane- or cytosol-associated  $G\hat{I}_q$  and  $G\hat{I}_{11}$  proteins, as these were not altered during the first 24 h of withdrawal.

It is well known that chronic treatment with agonists or antagonists of 5-HT<sub>2A</sub> receptors typically produces desensitization of 5-HT<sub>2A</sub> receptors (Roth et al., 1998; Gray and Roth, 2001; Hanley and Hensler, 2002; Damjanoska et al., 2004). In contrast, withdrawal from cocaine produces supersensitivity of 5-HT<sub>2A</sub> receptors (Carrasco et al., 2003; Carrasco et al., 2004b). In the present study, the (̂)DOI-evoked release of corticosterone and prolactin was greater in rats undergoing cocaine withdrawal, a result that is consistent with reports in the literature (Baumann et al., 1993; Baumann and Rothman, 1998; Levy et al., 1992). We have previously reported that the neuroendocrine effects of (̂)DOI, are mediated exclusively by activation of 5-HT<sub>2A</sub> receptors (but not 5-HT<sub>2C</sub> receptors) in the PVN (Zhang et al., 2002). Consequently, the potentiated responses to (̂)DOI of two different hormones (prolactin and corticosterone) that are mediated by distinct signaling pathways suggests supersensitivity mediated at the level of 5-HT<sub>2A</sub> receptor signal transduction.

In contrast to the increased 5-HT<sub>2A</sub> receptor-mediated prolactin response at 12 and 24 h post-treatment, the prolactin response to (̂)DOI was blunted after 0.5 and 1 h of withdrawal from cocaine. Increased dopamine levels, due to cocaine blockade of dopamine re-uptake and D<sub>2</sub> dopaminergic inhibition of prolactin release, could explain this blunted response to (̂)DOI at 0.5 and 1 h post-treatment, when cocaine is most likely to still be present (Samson et al.,

2003). As no increases in 5-HT<sub>2A</sub> receptor-mediated corticosterone levels were observed at 0.5 and 1 h post-treatment times, these data provide further evidence that 5-HT<sub>2A</sub> receptor supersensitivity is a result of changes occurring during the withdrawal period.

Behavioral and neuroendocrine studies in rats withdrawn from cocaine indicate no increase in the maximal responses to (â)DOI, but do reveal a left-ward-shift in the dose-response curves (Baumann et al., 1993;Baumann and Rothman, 1998;Levy et al., 1992). These data suggest that 5-HT<sub>2A</sub> receptor supersensitivity could be the result of alterations in receptor-G-protein coupling or alterations in G-protein activation of signal transduction mechanisms. Our present results support this interpretation, as we detected an increase in <sup>125</sup>I-DOI-labeled 5-HT<sub>2A</sub> receptors in rats withdrawn from cocaine for 24 h in the absence of increases in the levels of membrane-associated GÎ<sub>q</sub> and GÎ<sub>11</sub> proteins in the PVN. Importantly, we have reported increases in the levels of membrane-associated GÎ<sub>q</sub> and GÎ<sub>11</sub> proteins in the PVN that require at least 48 h of withdrawal (Carrasco et al., 2003;Carrasco et al., 2004b), suggesting longer withdrawal times may evoke additional mechanism(s) of supersensitivity of 5-HT<sub>2A</sub> receptor-mediated responses.

In summary, our results demonstrate that supersensitivity of 5-HT<sub>2A</sub> receptors in the PVN is a result of changes occurring specifically during the withdrawal period that requires at least 4 h to develop. Although, the mechanisms by which cocaine withdrawal mediates 5-HT<sub>2A</sub> receptor supersensitivity in PVN are not completely understood, our present and published data indicates that they could involve: (1) increased density of 5-HT<sub>2A</sub> receptors in a âhigh-affinityâ state at least at 24 h of withdrawal; and (2) increased levels of membrane-associated GÎ<sub>q</sub> and GÎ<sub>11</sub> proteins at 48 h of withdrawal. As 5-HT<sub>2A</sub> receptors in PVN are important for the regulation of mood, impulse control and responses to stressors (Kroeze et al., 2002;Aghajanian and Marek, 2000), withdrawal-induced increases in 5-HT<sub>2A</sub> receptor function in this limbic region may be clinically relevant with respect to drug relapse.

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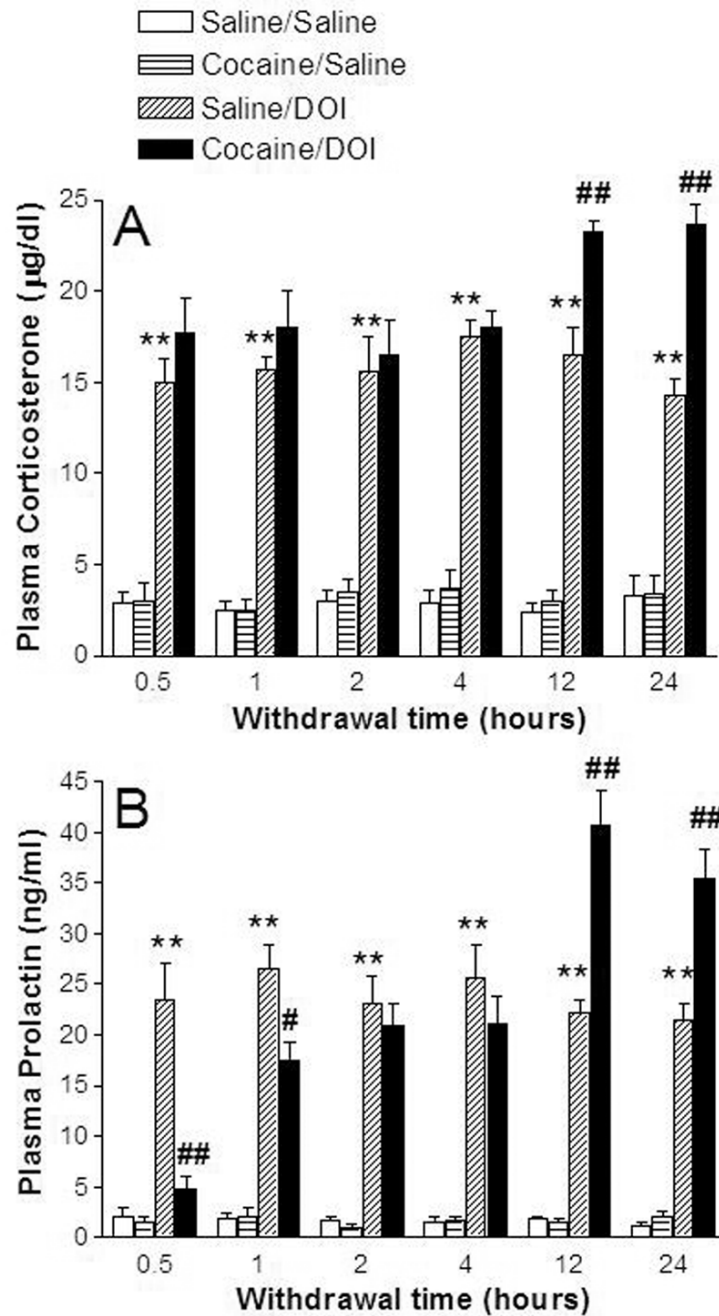
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## Abbreviations

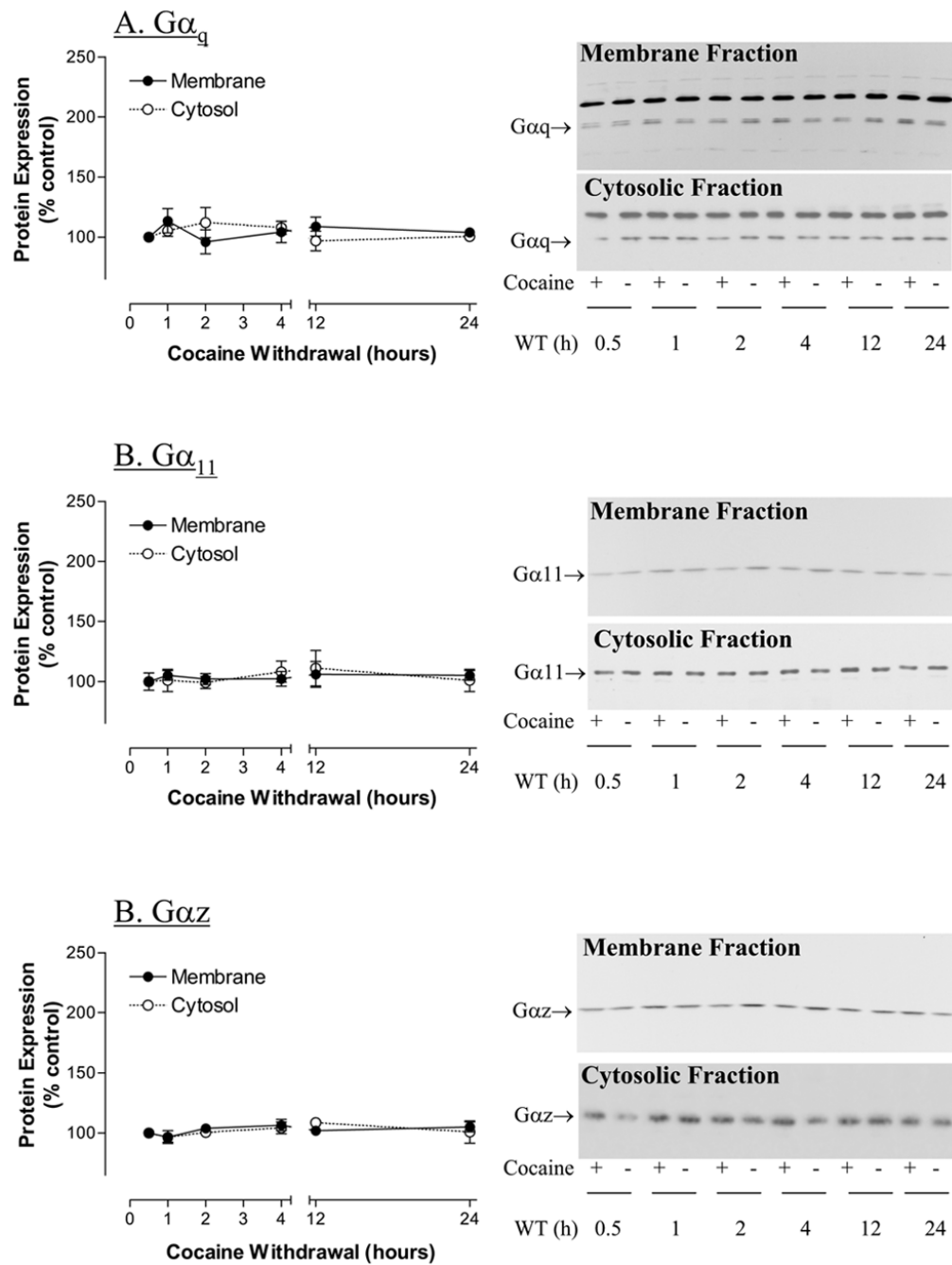
- (HPA) axis**    Â  
HypothalamicâPituitaryâAdrenal
- (PVN)**        Â  
Hypothalamic Paraventricular Nucleus
- (5-HT)**        Â  
5-hydroxytryptamine
- (5-HT<sub>2A</sub> receptors)**  
Serotonin 2A receptors
- HCL (DOI)**    Â  
(â)-1-(2,5 dimethoxy-4-iodophenyl)-2-amino-propane





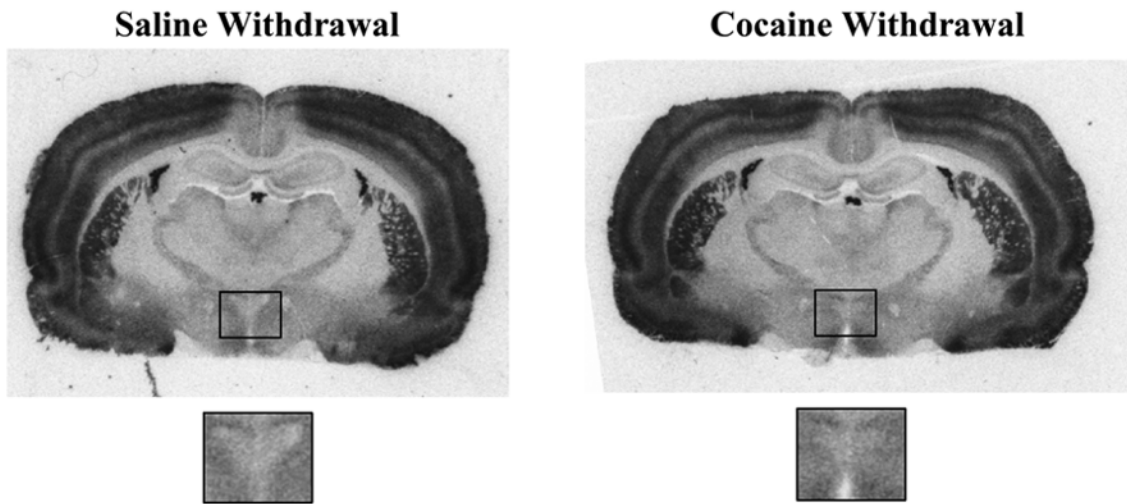
**Figure 1.**

Time-dependent supersensitivity of the ( $\Delta$ )DOI-induced neuroendocrine responses in cocaine-treated rats at various post-treatment withdrawal times. The data represent the plasma levels of corticosterone (A) and prolactin (B) in rats treated chronically with saline (1ml/kg, i.p., bid) or cocaine (15mg/kg, i.p., bid) and challenged with saline (1 ml/kg) or ( $\Delta$ )DOI (0.5 mg/kg) (mean  $\pm$  SEM of 8-12 rats per group). \*\* $p < 0.01$ , significant effect of ( $\Delta$ )DOI compared with the respective saline challenge group. ## $p < 0.01$ , significant effect of ( $\Delta$ )DOI challenge in cocaine-treated rats compared with ( $\Delta$ )DOI challenge in saline-treated rats (three-way ANOVA and Newman-Keuls multiple range test).

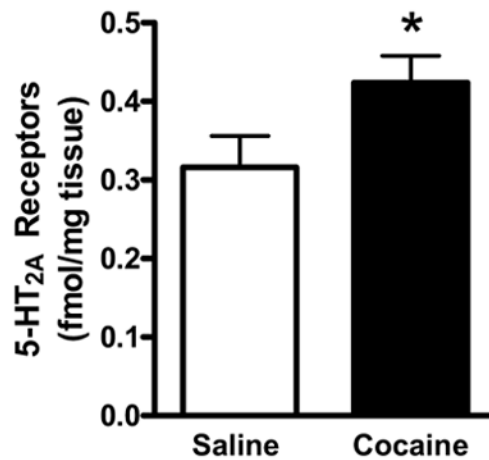
**Figure 2.**

The effect of withdrawal time on the levels of membrane- and cytosol-associated protein levels of (A)  $G\alpha_q$  proteins; (B)  $G\alpha_{11}$  proteins; and (C)  $G\alpha_z$  proteins in hypothalamic paraventricular nucleus of rats treated with saline or cocaine (15 mg/kg, ip, 5 days). WT= withdrawal time in hours (h).  $\beta$ -Actin was used as control of protein loading (data not shown). The data represent mean IOD (Integrated Optical Density)  $\pm$  SEM as % control (n=6 $\times$ 8) measured in triplicate.

### A. Representative Autoradiogram of total $^{125}\text{I}$ -DOI binding after 24 h withdrawal



### B. Density of $^{125}\text{I}$ -DOI labeled $5\text{-HT}_{2A}$ receptors in PVN at 24 h withdrawal



**Figure 3.**

$^{125}\text{I}$ -DOI-labeled  $5\text{-HT}_{2A}$  receptors in hypothalamic paraventricular nucleus of rats withdrawn from cocaine for 24 h. (A) Representative autoradiogram of  $^{125}\text{I}$ -DOI binding performed in coronal sections from rats withdrawn for 24 h from saline or cocaine treatment. The box on this coronal section indicates the region of the paraventricular nucleus of the hypothalamus. Higher power images of the paraventricular nucleus of the hypothalamus are shown for a representative saline- and cocaine-treated rats. (B) Quantification of  $^{125}\text{I}$ -DOI labeled  $5\text{-HT}_{2A}$  receptors in PVN of rats withdrawn from either saline or cocaine for 24 h.  $^{125}\text{I}$ -DOI-labeled  $5\text{-HT}_{2A}$  receptors were significantly increased in rats withdrawn from cocaine for 24 h. The data represent mean  $\pm$  SEM of 5 rats per group. \*  $p < 0.05$  between cocaine- and saline-treated rats (unpaired Student-t test).