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SHORT-TERM COCAINE TREATMENT CAUSES NEUROADAPTIVE CHANGES IN $G\alpha_q$ AND $G\alpha_{11}$ PROTEINS IN RATS UNDERGOING WITHDRAWAL[1]

Gonzalo A. Carrasco, Katerina J. Damjanoska, Deborah N. D'Souza, Yahong Zhang, Francisca Garcia, George Battaglia, Nancy A. Muma, and Louis D. Van de Kar.

Center for Serotonin Disorders Research and Department of Pharmacology Loyola University of Chicago, Stritch School of Medicine, 2160 South First Avenue, Maywood, IL 60153

Correspondence: Louis D. Van de Kar, PhD

Department of Pharmacology Phone:708-216-3263

Loyola University of Chicago, Stritch School of Medicine Fax: 708-216-6596

2160 South First Avenue E-mail:lvandek@lumc.edu

Maywood, IL 60153 http://www.luhs.org/SerotoninResearch

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ABSTRACT

One of the characteristics of drug dependence is that a drug has to be administered repeatedly before withdrawal effects can be observed. We have previously shown that withdrawal after 14 days of cocaine treatment produces a supersensitivity of hypothalamic serotonin 2A (5-HT_{2A}) receptors, which is accompanied by increases in the levels of $G\alpha_q$ and $G\alpha_{11}$ proteins. Unfortunately, the exact duration of cocaine treatment necessary to induce alterations in G protein levels during cocaine withdrawal is unknown. The present study investigated the minimum cocaine treatment period required to produce changes in protein levels of membrane- and cytosol-associated $G\alpha_q$ and $G\alpha_{11}$ proteins in the hypothalamic paraventricular nucleus, amygdala and frontal cortex. Rats were injected with cocaine (15 mg/kg, i.p., b.i.d.) for 0, 1, 3, 5 and 7 days and tested following 2 days of withdrawal. The levels of $G\alpha_q$ and $G\alpha_{11}$ proteins were increased in the paraventricular nucleus and the amygdala but not in the frontal cortex. Although, 1 and 3 days of cocaine treatment were sufficient to maximally elevate the protein levels of $G\alpha_{11}$ and $G\alpha_q$ proteins in the amygdala, 5 days of treatment were required to maximally increase the levels of $G\alpha_{11}$ and $G\alpha_q$ proteins in the paraventricular nucleus. The data suggest that the amygdala shows a faster neuroadaptation to the effects of cocaine than the hypothalamic paraventricular nucleus. These findings provide insight into the relative importance of individual components of 5-HT_{2A} receptor signal transduction system in regulating the overall sensitivity of this signaling in cocaine treated rats.

INTRODUCTION

Cocaine produces a variety of actions in neuronal function (Levy et al., 1994a). Cocaine binds with high affinity to neurotransmitter uptake sites on monoaminergic (serotonergic, dopaminergic, and noradrenergic) neurons in the brain and peripheral tissues, blocking the reuptake of serotonin, dopamine, and norepinephrine into the pre-synaptic neuron (Levy et al, 1994a).

The influence of cocaine on serotonergic neurotransmission has received increasing attention in recent years. The cocaine-induced reuptake blockade leads to an increased concentration of 5-HT in the synapse and thus to stimulation of post-synaptic 5-HT receptors (Hanson et al., 1987). Furthermore, cocaine reduces the activity of 5-HT neurons in the dorsal raphe (Cunningham and Lakoski, 1988), presumably as a consequence of increased stimulation of somatodendritic 5-HT_{1A} autoreceptors in the dorsal raphe nucleus (Pan et al., 1989).

5-HT_{2A} and 5-HT_{2C} receptors, which activate the dopamine mesoaccumbens pathway, play an important role in the behavioral effects of cocaine (McMahon et al., 2001). In addition, 5-HT_{2A} receptors are expressed by neurons in the hypothalamic paraventricular nucleus (Zhang et al., 2002). Activation of 5-HT_{2A} receptors in the hypothalamic paraventricular nucleus increases the secretion of ACTH, corticosterone, oxytocin and prolactin (Van de Kar et al., 2001). Using neuroendocrine responses to the 5-$HT_{2A/2C}$ receptor agonist DOI, we found increased sensitivity of 5-HT_{2A} receptors that stimulate the secretion of ACTH, corticosterone and prolactin following 42 hours withdrawal from repeated cocaine treatment (15 mg/kg, i.p., twice a day for 7 days) (Levy et al., 1992). The mechanism of this cocaine-induced supersensitivity of 5-HT_{2A} receptors in the paraventricular nucleus might be an altered expression of proteins associated with the 5-HT_{2A} receptor signaling pathway, including $G\alpha_q$ and $G\alpha_{11}$ proteins, and regulators of G protein signaling proteins, such as RGS4 and RGS7

proteins. Our previous study (Carrasco et al., 2003) showed that withdrawal (2 days) from chronic cocaine treatment (14 days, 15mg/kg twice a day or using a binge protocol) produces a transient and region-specific increase in the levels of membrane-associated $G\alpha_q$ and $G\alpha_{11}$ proteins in the hypothalamic paraventricular nucleus and the amygdala, but not in the frontal cortex (Carrasco et al, 2003). Exposure to chronic cocaine does not produce changes in the levels of membrane- or cytosol-associated 5-HT_{2A} receptors, RGS4 or RGS7 proteins in frontal cortex, amygdala or paraventricular nucleus (Carrasco et al, 2003). These results support the conclusion that withdrawal from chronic cocaine treatment increases 5-HT_{2A} receptor function by altering post-receptor signal transduction mechanisms, rather than increasing 5-HT_{2A} receptor density.

In this paper, we focused on the minimum number of days of cocaine treatment required to change the levels of 5-HT_{2A} receptor signaling proteins in the hypothalamic paraventricular nucleus, amygdala and frontal cortex. These regions were selected because of their prominent role in stress, anxiety, neuroendocrine function and addiction (Carrasco and Van de Kar, 2003;Yun and Fields, 2003). The hypothalamic paraventricular nucleus plays a central role in mediating neuroendocrine responses to serotonergic activation (Bagdy, 1996). The hypothalamic paraventricular nucleus receives serotonergic projections from the raphe nuclei, which also send collaterals to other limbic structures, notably the amygdala (Liposits et al., 1987;Petrov et al., 1994). The amygdala is a limbic structure with interconnections to the cortex and the nucleus accumbens and plays a central role in the reinforcing effects of drugs of abuse (Yun and Fields, 2003).

Various drugs of abuse require repeated administration before withdrawal effects can be observed (Levy et al, 1994a). Since the immediate effect of cocaine is to reduce serotonergic firing rate (Cunningham and Lakoski, 1988), there is a good likelihood that the reduction in the

5-HT release in the hypothalamus will gradually lead to supersensitive postsynaptic 5-HT_{2A} receptors. However, the minimum duration of cocaine exposure required to induce the adaptative mechanisms leading to an increase in the levels of $G\alpha_q$ and $G\alpha_{11}$ proteins in the hypothalamic paraventricular nucleus and amygdala during withdrawal from cocaine has not been determined. In the present study we evaluated the minimum number of days of cocaine treatment that will produce an increase in the levels of $G\alpha_q$ and $G\alpha_{11}$ proteins in rats withdrawn from cocaine.

METHODS

Animal Treatment:

Male Sprague-Dawley rats (225-275 g) (Harlan Laboratories, Indianapolis, IN, USA) were housed 2 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 (IACUC). 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. Rats received injections of cocaine (15mg/kg, i.p., 8:30 h and 15:30 h) for 1, 3, 5 and 7 days. A control group of rats was injected with 0.9% saline (1ml/kg, i.p., 8:30 h and 15:30 h) for 7 days. All the rats were killed 2 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 in dry ice and all tissues were stored at -80^0C.

Tissue preparation

Rat brains were placed in a cryostat at -10^0C, and coronal sections were cut to obtain a 700µm thick section containing the paraventricular nucleus and rostral amygdala and a 1200µm 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 previously described (Carrasco et al, 2003). 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 phenylmethanesulfonyl fluoride (PMSF) and additional

protease inhibitors purchased as a cocktail (containing: 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, trans-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane, bestatin, leupeptin, and aprotinin) from Sigma Chemical Co (St. Louis, Mo., USA; 1.5 µl/30 mg tissue). After centrifugation at 20,000 x g for 60 min, the supernatant was collected and stored at –80°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 1mM dithiothreitol, plus the protease inhibitory cocktail (1.5 µl cocktail/30 mg tissue). The resuspended pellets were incubated while shaking for 1 hour at 4°C and then centrifuged at 100,000 x g for 60 min. The supernatant was collected for the Western blot analyses of membrane-associated protein levels. Protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). The membrane proteins were stored at –80 °C for Western blot analyses.

Western Blot Analysis

Samples containing 2 µg (hypothalamic paraventricular nucleus), 3 µg (amygdala) and 4 µg (frontal cortex) of protein were separated by sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) 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 semi-dry 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 antisera. Immunodetection was performed with either anti-$G\alpha_{11}$ (1:500, Santa Cruz Biotechnology, CA, USA), anti-$G\alpha_q$ (1:500, Santa Cruz Biotechnology, CA, USA), or anti-$G\alpha_z$ (1:6,000, Santa Cruz Biotechnology, CA, USA). The overnight incubation with $G\alpha_q$, $G\alpha_{11}$ and $G\alpha_z$ antibodies was followed by incubation with peroxidase-labelled anti-rabbit antibody (1:20,000, one hour at room temperature, Santa Cruz

Biotechnology, CA, USA). Finally, the membranes were incubated with the ECL chemiluminescence substrate solution (Amersham, Arlington Heights, IL, USA) and then exposed to Kodak X-ray film. Protein loading for each lane was verified using an anti-actin antibody (1:20,000, Santa Cruz Biotechnology, CA, USA). Negative controls included either omission of primary antibody or addition of pre-immune rabbit immunoglobulins.

Film analysis

Films were analyzed densitometrically using Scion Image software (Scion Corporation, Frederick, MD, USA). 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 the 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. 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 one-way analysis of variance (ANOVA) was used to analyze the Western blot data, considering $p < 0.05$ as 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, USA) was used for all statistical analyses.

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RESULTS

Western Blot Analysis

$G\alpha_{11}$ protein

$G\alpha_{11}$ protein was detected as a single band at approximately 40 kDa (Fig.1A). The level of membrane-associated $G\alpha_{11}$ protein in the hypothalamic paraventricular nucleus was elevated in rats treated with cocaine for 1 to 7 days and withdrawn for 2 days. The ANOVA revealed that cocaine treatment ($F_{(4,49)}$= 39.42315, $p< 0.0001$) had a significant main effect on the level of membrane-associated $G\alpha_{11}$ protein. The increase in the levels of $G\alpha_{11}$ protein ranged from 24.5% over the control levels ($p<0.01$) in rats that received cocaine treatment for one day to 95% over the control levels ($p<0.01$) in rats that received cocaine treatment for five days (Fig.1A). Rats that received 7 days of cocaine treatment showed an increase of 69% over the control levels ($p<0.01$) (Fig.1A). The levels of membrane-associated $G\alpha_{11}$ protein were significantly higher in rats which received 5 days of cocaine treatment than those which received 1, 3 or 7 days of cocaine treatment ($p<0.01$) (Fig.1A). Cocaine treatment did not have a significant effect on the levels of cytosol-associated $G\alpha_{11}$ protein in the hypothalamic paraventricular nucleus ($F_{(4,49)}$= 0.25323, $p> 0.9$) (Fig.1A).

Cocaine treatment had a significant main effect on the levels of membrane-associated $G\alpha_{11}$ protein in the amygdala ($F_{(4,49)}$= 26.19591, $p<0.0001$) (Fig.1B). The peak in the levels of membrane-associated $G\alpha_{11}$ protein in the amygdala was found after 1 day of cocaine treatment (107% over control levels, $p<0.01$). The levels of membrane-associated $G\alpha_{11}$ protein in the amygdala stayed elevated during the first 5 days of cocaine treatment ($p<0.01$) (Fig.1B). No significant differences were observed for the levels of membrane-associated $G\alpha_{11}$ protein among 1 to 5 days of cocaine treatment ($p>0.05$). After 7 days of cocaine treatment, the levels

of membrane-associated $G\alpha_{11}$ protein (76% over control levels) were significantly lower than after 1, 3 or 5 days of cocaine treatment ($p<0.05$). No significant effect of cocaine was found on the levels of cytosol-associated $G\alpha_{11}$ in the amygdala ($F_{(4,49)}= 0.79477$, $p> 0.5$) (Fig.1B).

In the frontal cortex, cocaine treatment did not produce a significant change in the levels of membrane-associated ($F_{(4,49)}= 0.55749$, $p> 0.69$) or cytosol-associated ($F_{(4,49)}= 0.3706$, $p> 0.8283$) $G\alpha_{11}$ protein (Fig.1C) across the different treatment durations.

$G\alpha_q$ protein

$G\alpha_q$ proteins were detected in the hypothalamic paraventricular nucleus, amygdala and frontal cortex as a single band at approximately 42 kDa (Fig.2). The levels of membrane-associated $G\alpha_q$ protein in the paraventricular nucleus were also affected by cocaine treatment ($F_{(4,49)}= 23.63318$, $p< 0.0001$). Cocaine treatment gradually increased the levels of membrane-associated $G\alpha_q$ (Fig.2A): 60% over control levels after 1 day and 86% over control levels after 3 days of cocaine treatment (Fig.2A). The peak levels of membrane-associated $G\alpha_q$ protein in the paraventricular nucleus were detected after 5 days of cocaine treatment (105% over control levels, $p<0.001$). The levels of membrane-associated $G\alpha_q$ protein were significantly higher in rats which received 5 days of cocaine treatment than those which received 1, 3 or 7 days of cocaine treatment ($p<0.05$) (Fig.2A). After 7 days of cocaine treatment, the levels of membrane-associated $G\alpha_q$ protein were 60% over control levels ($p<0.01$)(Fig.2A). The levels of cytosol-associated $G\alpha_q$ protein were also significantly altered by cocaine ($F_{(4,49)}= 13.76312$, $p< 0.0001$). However, whereas the levels of cytosol-associated $G\alpha_q$ protein were not significantly altered ($p>0.05$) during the first 5 days of cocaine treatment (Fig.2A), a 30% reduction ($p<0.01$) was detected after 7 days of cocaine treatment.

In the amygdala, the ANOVA indicated a significant main effect of cocaine treatment ($F_{(4,49)}$= 28.35248, p< 0.0001) on the levels of membrane-associated $G\alpha_q$ protein. In rats that received cocaine for 1 day, the levels of membrane-associated $G\alpha_q$ proteins were increased by approximately 74% over the saline control (p< 0.01) (Fig.2B). In rats that received cocaine for 3 days, the levels of membrane-associated $G\alpha_q$ protein were even higher (103% over control levels, p<0.01). The levels of membrane-associated $G\alpha_q$ protein in rats which received 3 days of cocaine treatment were higher than those which received 1, 5 or 7 days of cocaine treatment (p<0.05) (Fig.2B). After 5 and 7 days of cocaine treatment the levels of membrane-associated $G\alpha_q$ protein were deduced to a 57% and 43% over control levels, respectively. Levels of cytosol-associated $G\alpha_q$ proteins in the amygdala were also affected by cocaine treatment ($F_{(4,49)}$= 15.18487, p<0.001). The levels of cytosol-associated $G\alpha_q$ proteins were increased by 25% over the control levels in rats that received cocaine for 1 day (p<0.005) and 55% in rats treated with cocaine for 3 days (p<0.001)(Fig.2B). The levels of cytosol-associated $G\alpha_q$ stayed elevated after 5 and 7 days of cocaine treatment by 50% and 45% higher over control levels (p<0.01) (Fig.2B). In rats treated with cocaine for 7 days, the levels of cytosol-associated $G\alpha_q$ proteins were still 43% higher (p<0.01) than control levels (Fig.2B).

In the frontal cortex, cocaine treatment did not alter the levels of membrane-associated $G\alpha_q$ protein ($F_{(4,49)}$= 1.43207, p> 0.24) or the levels of cytosol-associated $G\alpha_q$ protein ($F_{(4,49)}$= 0.55094, p> 0.69) (Fig.2C).

$G\alpha_z$ protein

$G\alpha_z$ is a 40 kDa protein that has not been reported to associate with the 5-HT_{2A} receptor signaling cascade. The determination of $G\alpha_z$ protein was used to verify the specificity of the

effects of cocaine treatment on G proteins. Since $G\alpha_z$ proteins are not involved in 5-HT_{2A} receptor signaling during withdrawal from chronic cocaine treatment, changes in the levels of membrane and cytosol-associated $G\alpha_z$ proteins were also evaluated as negative controls. The ANOVA for the levels of membrane and cytosol-associated $G\alpha_z$ protein in the paraventricular nucleus showed no significant main effect of cocaine treatment ($F_{(4,49)}$= 0.60125, p> 0.66 and $F_{(4,49)}$= 0.28564, p> 0.88, for membrane and cytosol, respectively; Fig.3A). In the amygdala, the statistical analysis for the levels of membrane and cytosol-associated $G\alpha_z$ proteins showed no significant main effects of cocaine treatment ($F_{(4,49)}$= 0.55889, p> 0.69 and $F_{(4,49)}$= 0.2732, p> 0.89, for membrane and cytosol, respectively; Fig.3B). Additionally, cocaine treatment did not affect the levels of membrane or cytosol-associated $G\alpha_z$ protein in the frontal cortex ($F_{(4,49)}$= 1.21265, p> 0.31 and $F_{(4,49)}$= 1.24992, p> 0.30, for membrane and cytosol, respectively; Fig.3C).

DISCUSSION

Chronic cocaine has substantial effects on 5-HT_{2A} receptor function (Levy et al, 1994a). Cocaine administration (15 mg/kg, i.p, twice a day for seven days) enhances the head shake response elicited by a specific 5-$HT_{2A/2C}$ receptor agonist (DOI) (Baumann et al., 1993). We have also previously reported that DOI-mediated increases in plasma levels of prolactin, corticosterone and ACTH become supersensitive following an identical cocaine paradigm (Levy et al, 1992). Interestingly, in both the behavioral and the neuroendocrine studies, there was no increase in the maximal responses to DOI but instead a left-ward-shift in the dose-response curve (Baumann and Rothman, 1998;Levy et al, 1994a), suggesting alterations in the coupling state or post-receptor signal transduction mechanisms. As no differences in the density of 5-HT_{2A} receptors have been found in the frontal cortex, amygdala, nucleus accumbens, caudate putamen and thalamus of cocaine-treated rats, when measured by quantitative receptor autoradiography and binding assays (Perret et al., 1998), the increased sensitivity must be due to changes in the efficiency of 5-HT_{2A} receptor transduction mechanisms. 5-HT_{2A} receptors are coupled via $G\alpha_q/\alpha_{11}$ and $G\beta\gamma$ proteins to phospolipase C and A_2 signaling cascades (Roth et al., 1998).

The present results indicate that the increase in the levels of $G\alpha_q$ and $G\alpha_{11}$ proteins in the amygdala peaks during withdrawal from 1-3 days of cocaine treatment. In the hypothalamic paraventricular nucleus, the increase in the levels of $G\alpha_q$ and $G\alpha_{11}$ proteins is more gradual and peaks after withdrawal from 5 days of cocaine treatment. Thus, cocaine induced a more rapid increase in the membrane-associated levels of $G\alpha_q$ and $G\alpha_{11}$ proteins in the amygdala than in the hypothalamic paraventricular nucleus, suggesting that the amygdala shows a faster neuroadaptation to the effects of cocaine. While the neuroadaptations occurring in both of these brain regions may not be related, there are three lines of evidence supporting a

role of the amygdala in the function of the hypothalamic paraventricular nucleus. First, injection of ketanserin, a 5-HT_2 receptor antagonist into the amygdala inhibited the effect of photic stress on the release of ACTH and corticosterone (Feldman et al., 1998). Secondly, as mentioned earlier, activation of 5-HT_{2A} receptors expressed by neurons in the hypothalamic paraventricular nucleus increases the secretion of ACTH, corticosterone, oxytocin and prolactin (Van de Kar et al, 2001). We recently reported (Zhang et al, 2002) that when MDL100,907, a 5-HT_{2A} receptor antagonist, is injected directly into the hypothalamic paraventricular nucleus, it completely blocked the prolactin and oxytocin responses to DOI, whereas, the same dose of MDL 100,907 produced an incomplete inhibition of the of ACTH response to DOI, suggesting a role of extra-hypothalamic structures in the regulation of ACTH release. Lastly, 5-HT_{2A} receptors are found in oxytocin and corticotrophin releasing factor-immunoreactive cells in the hypothalamic paraventricular nucleus, whereas in the amygdala, 5-HT_{2A} receptors are found in corticotrophin releasing factor-immunoreactive cells (Zhang et al., 2001;Gray et al., 2003). 5-HT_{2A} receptors are not found on enkephalin neurons, which are part of an inhibitory local circuit in the amygdala, further emphasizing their excitatory role in this brain area (Gray et al, 2003). Interestingly, a number of studies suggest that amygdaloid corticotrophin releasing factor plays an important role in the expression of cocaine-induced behavior (Yun and Fields, 2003).

We further speculate that serotonin release in the amygdala activates 5-HT_{2A} receptors (located on corticotrophin releasing factor expressing neurons) and induces corticotrophin releasing factor secretion from terminals in the brainstem that mediate anxiety and stress-like responses. Thus, 5-HT_{2A} receptors could activate corticotrophin releasing factor neurons in the amygdala, which excite dorsal raphe neurons to induce ACTH release via their projection to hypothalamic neurons (Commons et al., 2003). Also, direct projections from the central

amygdaloid nucleus to the hypothalamic paraventricular nucleus could mediate this effect (Gray et al., 1989). In this way, the activation of 5-HT_{2A} receptors in the amygdala could be one mechanism by which neuroendocrine (hypothalamic corticotrophin releasing factor) and autonomic/behavioral (amygdaloid corticotrophin releasing factor) responses are integrated during responses to stressful or aversive stimuli (Gray et al, 2003). Thus, it is possible, that in rats withdrawn from cocaine, an exacerbation of the 5-HT_{2A} receptor signaling in the amygdala, produced by an increase in the membrane-associated levels of $G\alpha_q$ and $G\alpha_{11}$, could lead to a supersensitivity of the 5-HT_{2A} receptor signaling in the paraventricular nucleus.

The increase in $G_{\alpha q}$ and $G_{\alpha 11}$ protein levels during cocaine withdrawal may be due to three different phenomena: 1) an increased expression of the G_α proteins specifically at the cell membrane; 2) a general increase in G_α protein levels in both membrane and cytosolic cellular compartments; 3) a translocation of the G proteins from the cytoplasmic to the membrane portion. Since no reductions in the levels of cytosol-associated $G\alpha_q$ and $G\alpha_{11}$ proteins were found, it is not likely that the supersensitivity of 5-HT_{2A} receptor signaling in cocaine-treated rats involves a re-distribution or translocation of $G\alpha_q$ and $G\alpha_{11}$ proteins from the cytosol to the plasma membrane in the amygdaloid-hypothalamic neuronal circuits. The significant increase in the cytosol-associated levels of $G\alpha_q$ proteins in the amygdala could involve increase in the mRNA levels encoding $G\alpha_q$ and $G\alpha_{11}$ proteins or decreased rate of degradation of these proteins in this brain region. Altough, $G\alpha_q$ and $G\alpha_{11}$ proteins are targets for N-myrisyoylation but not ADP-ribosylation (Chen and Manning, 2001), there is no evidence that treatment with cocaine leads to changes in the post-translational modifications of $G\alpha_q$ or $G\alpha_{11}$ proteins.

Additionally, it is possible that other proteins involved in the function of the 5-HT_{2A} receptor and $G\alpha_q$ and $G\alpha_{11}$ proteins could be modified by cocaine treatment. These include

two families of G protein regulators important to the specificity and kinetics of the G protein signaling: the Regulators of G protein Signaling (RGS) proteins and the activator of G protein signaling (AGS) family. In a previous report (Carrasco et al., 2003), we did not find changes in the levels of membrane and cytosol associated-levels of RGS4 and RGS7 proteins, two RGS proteins associated with the $G\alpha_q$ and $G\alpha_{11}$ protein signaling (Hollinger and Hepler, 2002), in the hypothalamic paraventricular nucleus, amygdala or frontal cortex. However, others RGS proteins are up-regulated by cocaine (Bishop et al., 2002;Rahman et al., 2003). RGS4 shows a biphasic response in the locus coeruleus, with decreased RGS4 mRNA levels after acute administration and increased levels of RGS4 mRNA after chronic administration (Bishop et al, 2002). Also, the protein content of RGS9 is increased by chronic cocaine in the nucleus accumbens (Rahman et al, 2003). 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). On the other hand, AGS3 one member of the AGS proteins is upregulated in the prefrontal cortex during withdrawal from cocaine (Bowers et al., 2004). AGS3 binds to $G\alpha_i$ proteins and inhibits GDP dissociation (Bowers et al, 2004). To the best of our knowledge there is no current evidence of AGS proteins associated to the $G\alpha_q$ and $G\alpha_{11}$ protein signaling.

The activity of the hypothalamic–pituitary–adrenal axis prior to, at the time of, and subsequent to cocaine exposure appears to be an important determinant of whether or not individuals will engage in cocaine-seeking behavior (Mantsch et al., 2003). Whereas acute administration of cocaine stimulates the hypothalamic–pituitary–adrenal axis in rats (Levy et al., 1991;Rivier and Vale, 1987), chronic cocaine administration is associated with neuroadpative changes that lead to attenuated responses to cocaine in rats and humans (Levy et al., 1993;Zhou et al., 2003). Rats chronically treated with cocaine showed a deficit in serotonergic nerve terminal function as seen from a reduced ACTH response to serotonin

releasing drugs p-cloroamphetamine and fenfluramine (Levy et al., 1994b;Van de Kar et al., 1995). On the other hand, withdrawal from cocaine is associated with activation of the hypothalamic–pituitary–adrenal axis in rats (Peltier et al., 2001;Zhou et al, 2003). This enhanced response of the hypothalamic-pituitary-adrenal axis during withdrawal from chronic cocaine has been reported 24 h to 48 h after the last cocaine injection (Peltier et al, 2001;Zhou et al, 2003). Similar results have been reported in human cocaine addicts (Vescovi et al., 1992;Baumann et al., 1995). These observations suggest that following development of adaptation or tolerance to chronic cocaine that produces deficits in the serotonergic activity, and during the withdrawal period, there is an increase in the activity of the hypothalamic-pituitary-adrenal axis. This increased activity would be associated to the supersensitivity of the 5-HT_{2A} receptors and can be measured as increased levels of 5-HT_{2A} receptor-mediated release of ACTH, corticosterone and prolactin. This supersensitivity of postsynaptic 5-HT_{2A} receptors in the hypothalamic-pituitary-adrenal axis may be mediated through increased levels of membrane-associated of $G\alpha_q$ or $G\alpha_{11}$ proteins in the hypothalamic paraventricular and/or amygdala. However, at this point in time we cannot conclusively show whether the changes observed in $G\alpha_q$ or $G\alpha_{11}$ proteins are due to cocaine treatment or the withdrawal period.

In summary, our results reveal unique neuroadaptative mechanisms in the hypothalamic paraventricular nucleus and the amygdala but not frontal cortex of rats withdrawn from cocaine. The mechanisms by which cocaine mediates its region-specific effects are incompletely understood, although some behavioral and neuroendocrine effects associated with 5-HT_{2A} receptor regulation appear to involve increased levels of membrane-associated $G\alpha_q$ and $G\alpha_{11}$ proteins. These findings provide insight into the relative importance of individual components of the 5-HT_{2A} receptor signal transduction system in regulating the overall sensitivity of this signaling transduction pathway.

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Footnotes

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LEGENDS OF FIGURES

Figure 1.- Representative Western blots and densitometric analysis of the membrane and cytosol-associated $G\alpha_{11}$ proteins in the hypothalamic paraventricular nucleus (A), amygdala (B) and frontal cortex (C) of rats injected twice a day with cocaine for 0, 1, 3, 5 and 7 days and then withdrawn for 2 days. Actin was used as a control of protein loading. The data represent the mean Integrated Optical Density (IOD)± SEM as % saline control (n=6-8) measured in triplicate (**p< 0.01, significant effect of cocaine compared with rats injected with saline for 7 days; $^{\#\#}$ indicates significant difference (p< 0.01) from rats injected with cocaine for 1, 3 or 7 days; $^{\&\&}$ indicates significant difference (p< 0.01) from rats injected with cocaine for 7 days).

Figure 2.- Representative Western blots and densitometric analysis of the membrane and cytosol-associated $G\alpha_{q}$ in the hypothalamic paraventricular nucleus (A), amygdala (B) and frontal cortex (C) of rats injected twice a day with cocaine for 0, 1, 3, 5 and 7 days and then withdrawn for 2 days. Actin was used as a control of protein loading. The data represent the mean Integrated Optical Density (IOD)± SEM as % saline control (n=6-8) measured in triplicate (** indicates significant difference (p< 0.01) from rats injected with saline for 7 days; $^{\$}$ indicates significant difference (p< 0.05) from rats injected with cocaine for 1, 3 and 7 days; $^{@@}$ indicates significant difference (p< 0.01) from rats injected with cocaine for 1, 3 and 5 days; $^{\&}$ indicates significant difference (p< 0.05) from rats injected with cocaine for 1, 5 and 7 days; $^{\#\#}$ indicates significant difference (p< 0.01) from rats injected with saline for 7 days; $^{\#}$ indicates significant difference (p< 0.05) from rats injected with saline for 7 days; $^{\%}$ indicates significant difference (p< 0.05) from rats injected with cocaine for 1 day).

Figure 3.- Representative Western blots and densitometric analysis of the membrane and cytosol-associated $G\alpha_z$ proteins in the hypothalamic paraventricular nucleus (A), amygdala (B) and frontal cortex (C) of rats injected twice a day with cocaine for 0, 1, 3, 5 and 7 days and then withdrawn for 2 days. Actin was used as a control of protein loading. The densitometric data represent the mean Integrated Optical Density (IOD)± SEM as % saline control (n=6-8) measured in triplicate.

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$G\alpha_{11}$ Protein

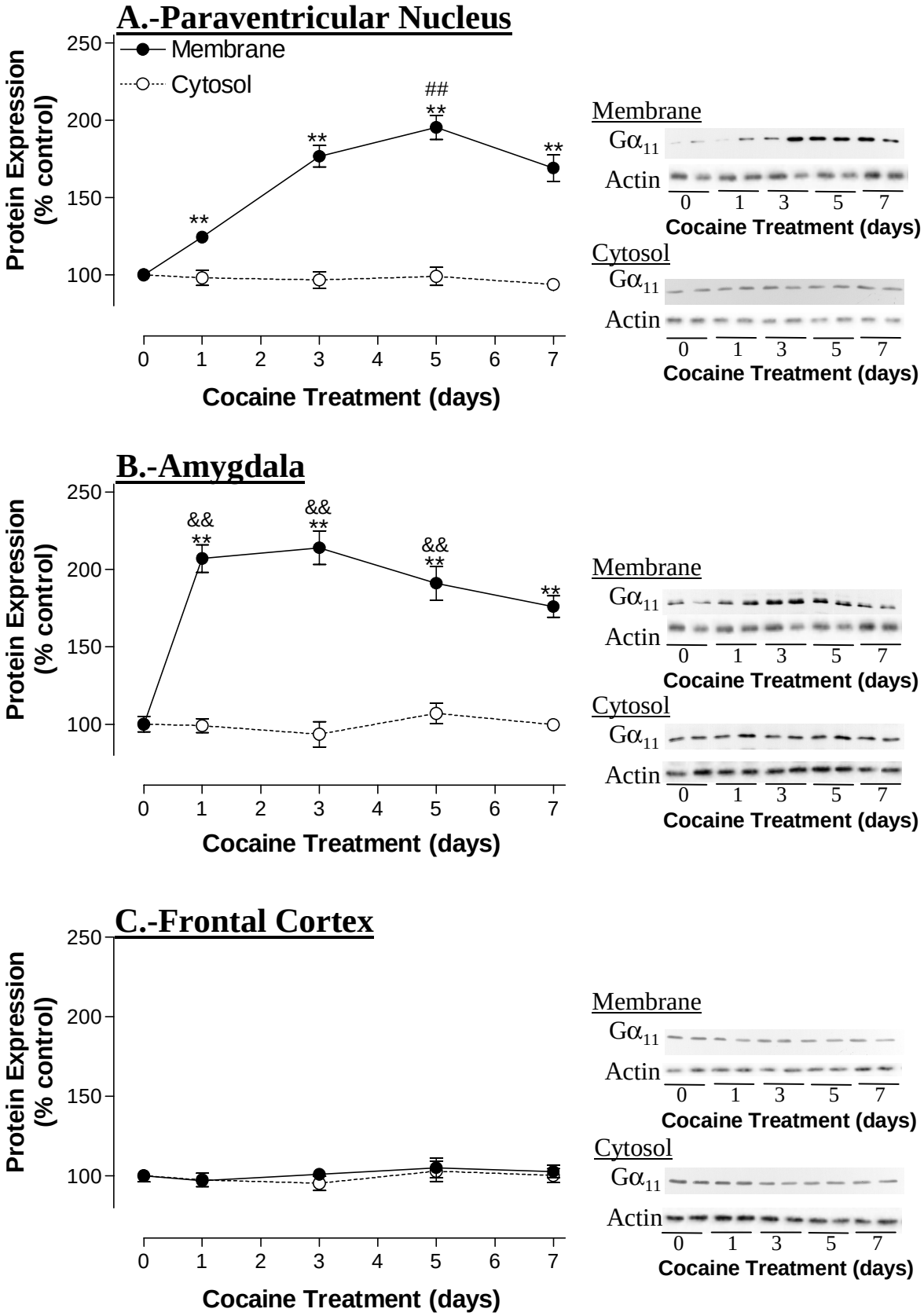

Figure 1

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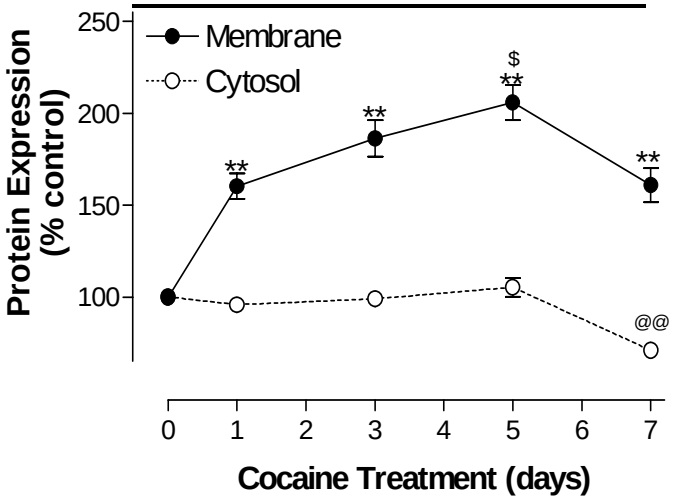
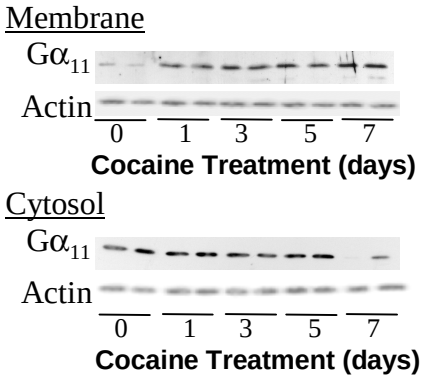
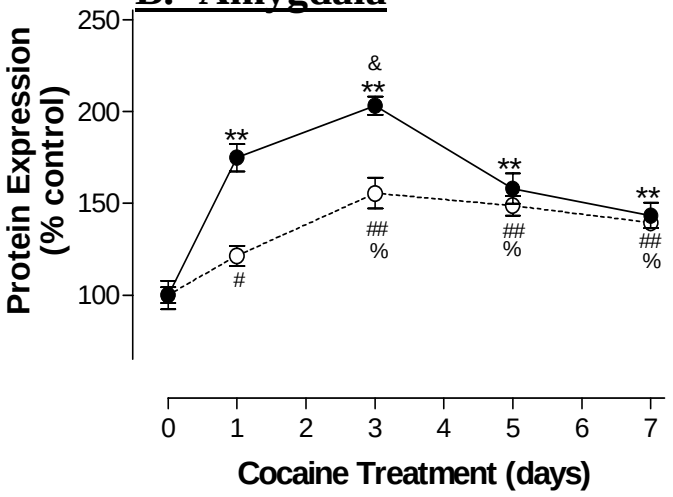
$G\alpha_q$ Protein

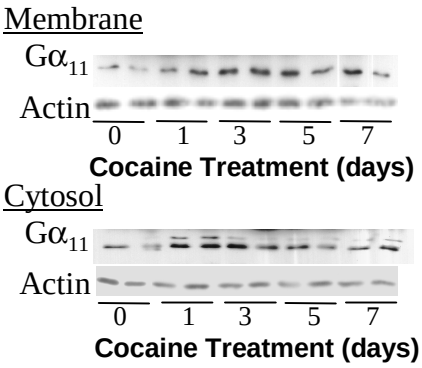
A.- Paraventricular Nucleus



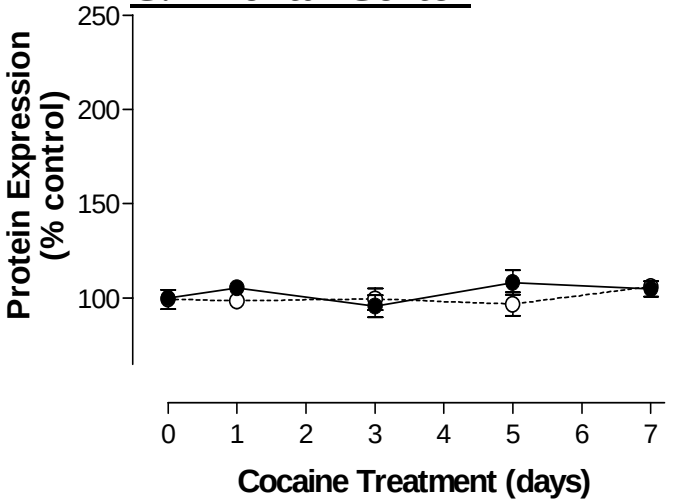


B.- Amygdala





C.- Frontal Cortex



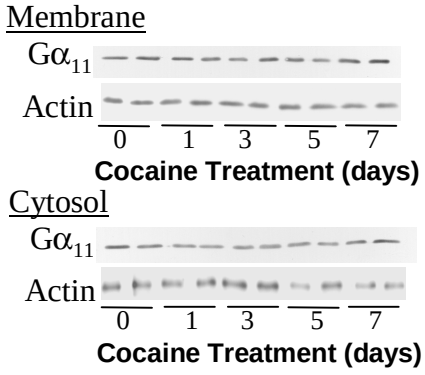


Figure 2

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$G\alpha_z$ Protein

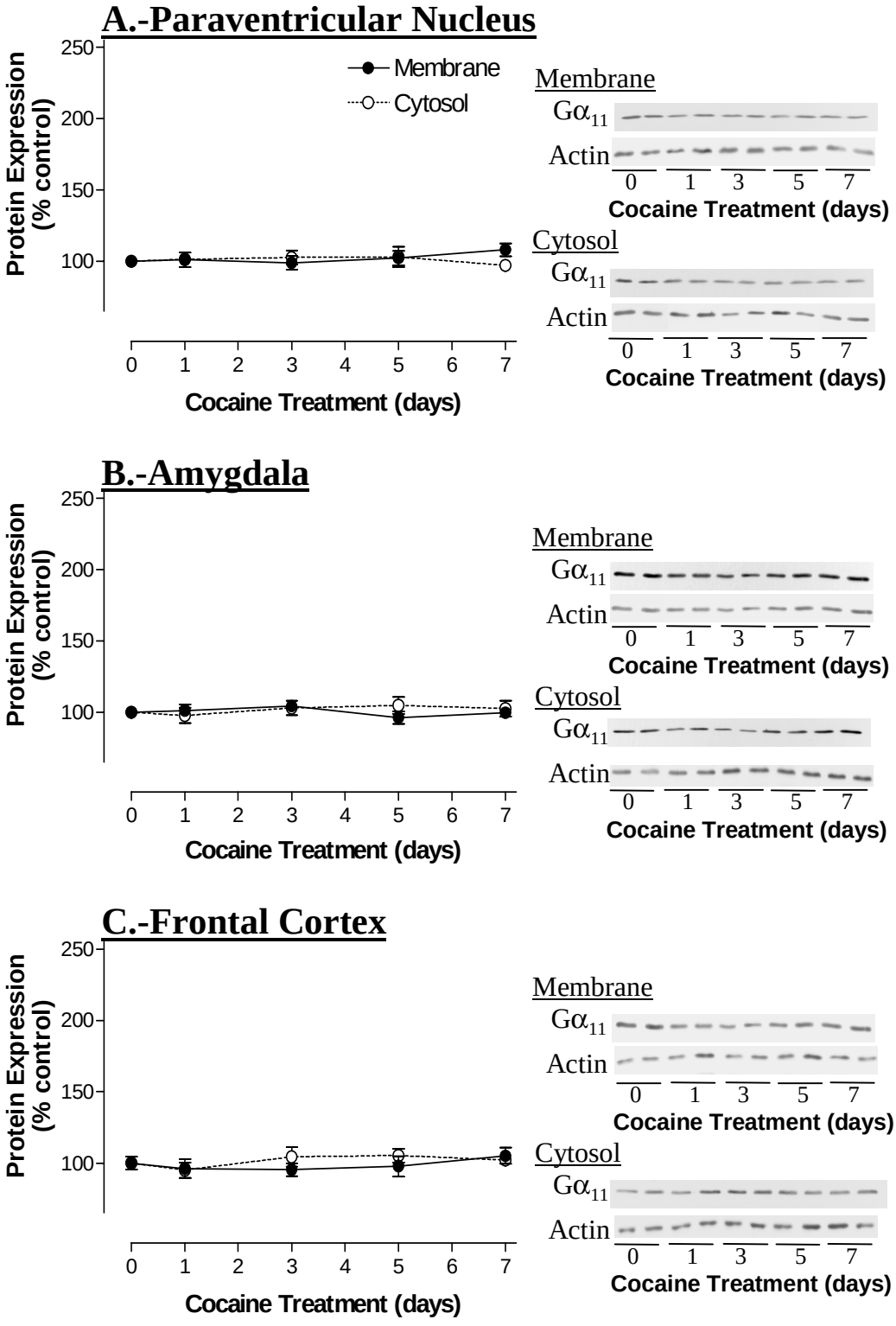

Figure 3