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The effects of cocaine on CART expression in the rat nucleus accumbens: A possible role for corticosterone

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Abstract

CART (Cocaine- and Amphetamine-Regulated Transcript) was initially described as an mRNA which had increased expression in the rat striatum following administration of acute cocaine or amphetamine but not saline. However, not all subsequent studies confirmed this. The present study aimed to repeat experiments with conflicting results and to reexamine and extend the original finding of acute regulation of nucleus accumbens CART mRNA by cocaine. Acute administration of cocaine failed to produce any change in levels of CART mRNA or peptide. Chronic administration of cocaine, as well as unilateral 6-hydroxydopamine lesions, also failed to alter CART mRNA levels in the accumbens. However, binge administration of cocaine, which also caused some seizures, did cause a significant increase in CART message. Given the involvement of corticosteroids with both stress and the effects of psychostimulants, we examined the possible effects of corticosteroids. We acutely administered ascending doses of corticosterone and found an increase in CART message. Similar effects were seen on CART peptides after acute corticosterone administration, and acute metyrapone administration was found to reduce CART peptide levels in the accumbens. This suggests that CART mRNA may be regulated by cocaine under certain conditions, such as binge administration, and this may at least partly involve corticosterone.

Keywords: CART; Cocaine; Corticosterone; Dopamine; Nucleus accumbens

1. Introduction

Douglass et al. (1995) identified the CART (Cocaine- and Amphetamine-Regulated Transcript) transcript via PCR (polymerase chain reaction) differential display as a striatal transcript that was up-regulated by cocaine and amphetamine but not by saline. Analysis of the sequence of the transcript demonstrated that the deduced polypeptide sequence contained the same sequence as a partial peptide isolated by Spiess et al. (1981). In situ hybridization showed that, within the striatum, the mRNA was restricted to the nucleus accumbens. Early anatomical work showed that CART peptides were also expressed in the accumbens and that, in the monkey accumbens, CART-containing neurons were in synaptic contact with dopaminergic nerve terminals (Smith et al., 1997a,b, 1999). Recently it has also been demonstrated that populations of these neurons express dopamine D2 and D3 receptors (Beaudry et al., 2004). Further work has demonstrated that CART is involved in a number of behavioral and physiological processes from drug reward to feeding (for review see Hunter and Kuhar, 2003).

The initial findings, discussed above, suggested that CART was of particular interest as a potential element in the mechanism of psychostimulant action. Indeed, subsequent investigation has shown that intra-ventral tegmental area infusion produces an increase in locomotor activity and a conditioned place preference (Kimmel et al., 2000), behaviors associated with psychostimulants (Wise and Bozarth, 1987). Others have shown that i.c.v. CART increased the levels of dopamine metabolites in the

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accumbens shell (Shieh, 2003; Yang et al., 2004). These findings suggested that CART may be an endogenous psychostimulant. However, analysis of the interaction of intra-ventral tegmental area CART peptide with concurrent cocaine administration showed that CART's effects were sub-additive; that is, CART actually reduced the locomotor stimulant effects of cocaine (Jaworski et al., 2002). Similarly, injection of CART peptide into the accumbens blunted the effects of systemic cocaine (Jaworski et al., 2003). This finding suggests that, contrary to the earlier hypothesis that CART was an endogenous psychostimulant, it may actually be part of the homeostatic mechanisms which serve to control hyper-activation of the mesolimbic dopamine system. Vrang et al. (2002), using in situ hybridization, could not confirm that CART was regulated by amphetamine in either the accumbens or the hypothalamus at the doses used by Douglass et al. (1995). Marie-Claire et al. (2003) has also shown that acute treatment with cocaine, morphine, methylenedioxymethamphetamine (MDMA) or tetrahydrocannabinol (THC) did not alter accumbal CART mRNA. Nonetheless, studies using higher, binge doses of cocaine such as those by Fagergren and Hurd (1999) and Brenz Verca et al. (2001) did show increases in CART expression, although these treatments can be highly stressful to the animals.

To test whether CART is regulated by cocaine and to further explore and define the interrelationship of CART and dopamine at the level of the nucleus accumbens, the present studies were performed. Some of the data presented here has appeared previously in abstract form (Hunter and Kuhar, 2004; Vicentic et al., 2003).

2. Materials and methods

2.1. Animals and drug treatments

Male Sprague–Dawley rats of 250–300 g were housed on a 7:00 to 19:00 light–dark cycle and fed and watered ad libitum. All animal care and experimentation was performed in accordance within the guidelines set forth in the National Institutes of Health guide for the care and use of laboratory animals. With the exception of the chronic cocaine study, all experiments were performed so as to terminate between 14:00 and 16:00. All experimental handling of animals was done by the first author alone with the exception of the last two experiments.

Cocaine was provided by the National Institute for Drug Abuse and dissolved in 0.9% saline. For the acute cocaine study, animals were given either 1 ml/kg 0.9% saline or 20 mg/kg cocaine i.p. and sacrificed 2 h later; their brains were processed for in situ or RIA as described above. Rats in the binge study, following the method used by Brenz Verca et al. (2001), were given 4 doses of 30 mg/kg cocaine or 1 ml/kg 0.9% saline at 2 h intervals and sacrificed 2 h after the last dose was administered. Several animals died following this treatment and were excluded from analysis. Chronic cocaine treatment consisted of 1 ml/kg 0.9% saline or 20 mg/kg cocaine, i.p., once per day for 14 days; animals were sacrificed 22 h after the last dose of cocaine.

Rats in the 6-hydroxydopamine study were treated as described by Robertson et al. (1992). Briefly, rats were treated with desipramine, 20 mg/kg in 0.9% saline 30 min prior to surgery. Rats were then anesthetized using isoflurane gas and placed in a stereotaxic apparatus. Lesions were made by injection of 12 ìg 6-hydroxydopamine in 4 ìl saline with 0.05% ascorbic acid. Injections were made over an interval of 10 min to the coordinates AP 4.0, ML 1.3, and DV 1.6 from interaural zero according to the atlas of Swanson (1998). Animals were allowed to recover for 2 or 14 days, then sacrificed and their brains frozen at −80 °C to be processed for CART in situ hybridization and [^{125}I] RTI-121 (Research Triangle Institute drug number) autoradiography.

For the corticosterone experiments, corticosterone was obtained from Sigma-Aldrich (St. Louis, MO) and metyrapone from Biomol Research Laboratories (Plymouth Meeting, PA). Corticosterone was dissolved in 90% ethanol, warmed up to 50 °C and then diluted to 40% with physiological 0.9% saline solution. metyrapone was dissolved in water.

Rats treated with corticosterone received i.p. injections of either 1 ml/kg 0.9% saline, 0.1, 1 or 10 mg/kg corticosterone for the acute dose–response or 10 mg/kg for the acute peptide study. 1 ml/kg 0.9% saline or 30 mg/kg metyrapone was injected i.p. for the acute metyrapone study. Animals were sacrificed 2 h after corticosterone or metyrapone treatment.

2.2. In situ hybridization

The general protocol, with some modifications, for in situ mRNA hybridization follows that of Dagerlind et al. (1992), as utilized for detection of CART message by Broberger (Broberger, 1999; Broberger et al., 1999). Briefly, male Sprague–Dawley rats of 250–300 g were sacrificed by decapitation and their brains quickly dissected out and fast frozen. The rat brains were cut in 14 ìm thick sections on a cryostat (Leica Instruments GmbH, Nussloch, Germany), thaw-mounted on Probe On Plus slides (Fisher Scientific, Pittsburgh, PA) and processed as previously described (Dagerlind et al., 1992). Sections were kept at −80 °C until processed for in situ. The probe for rat CART mRNA nt223-270 (Douglass et al., 1995) was synthesized by the Emory Microchemical Facility. The CART probe was labeled with [^{35}S]-dATP (NEN, Boston, MA) as described previously at the 3′ end using terminal deoxynucleotidyl transferase (Amersham Pharmacia Biotech, Piscataway, NJ) to a specific activity of 5×10^9 cpm/ug. The probe was then purified with a Quiaquick nucleotide removal kit (Quiagen, Valencia, CA).

Sections were processed by passing through one 5 min 4% paraformaldehyde wash, two 5 min phosphate buffered saline (PBS) washes, dipped in deionized water, 10 min in 0.1 M triethanolamine with 5ml/l acetic anhydride, 3 min in 2× salt–sodium citrate buffer (SSC), then washed in ascending concentrations of ethanol, followed by a 5 min wash in chloroform before a final wash in 95% ethanol. Sections were then air dried and incubated for 2 h with 150 µl hybridization buffer without probe, followed by a wash in 2× SSC for 3 min, followed by 95% ethanol. Sections were air dried and incubated 16 h at 42°C with 1.5 ng of the CART probe diluted in 50% deionized formamide (GibcoBRL, Gaithersburg, MD), 4× SSC, 1× Denhardt's solution (Roche), 0.02M $NaPO_4$ (pH 7.0), 1% *N*-lauroylsarcosine (Sigma, St. Louis, MO), 10% dextran sulfate (Sigma), 500 mg/l denatured salmon testis DNA (Sigma) and 200

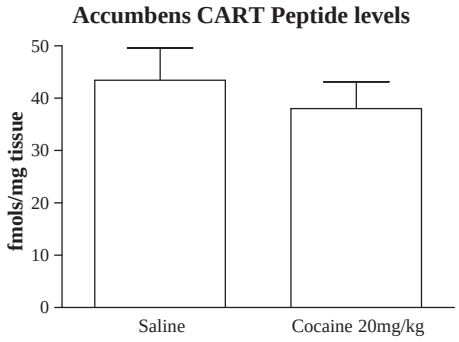

Fig. 1. Levels of CART peptide immunoreactivity in the nucleus accumbens after acute cocaine administration as measured by RIA (two tailed *t*-test, $n=4$, $P>0.05$).

mM dithiothreital (Sigma). After hybridization, sections were washed 4× in 1× SSC for 15 min at 55 °C and left in SSC to cool to room temperature for 1 h, then washed for 3 min in 50% ethanol and 0.3 M ammonium acetate, 3 min in 85% ethanol and 0.3 M ammonium acetate and 3 min in 100% ethanol. Slides were then air dried and exposed to Kodak MR autoradiography films for 10 days and analyzed for optical density using MCID (Imaging Research, St. Catharine's, OT, Canada). Measurements were made bilaterally in the rostral pole +2.7 mm from bregma and the shell and core of the accumbens +1.6 mm from bregma; for the present analysis these were averaged to provide a single overall measure for the nucleus for each animal. More caudal regions were not examined, as CART has a primarily rostral distribution in the accumbens.

2.3. Radioimmunoassay

The concentration of CART peptide was determined in the nucleus accumbens by radioimmunoassay (RIA). The procedure was carried out generally as previously described (Murphy et al., 2000), but with a commercially available [^{125}I]-RIA kit (Phoenix Pharmaceuticals, Belmont, MA). The RIA kit was validated before use with tissue extracts and dose–response curves, and increasing concentrations of authentic rat CART 55-102 standard (Peptide International, Louisville, Kentucky) added to tissue extracts were parallel to the standard curve. The assay sensitivity was 10 pg/tube; intra-assay variability was 5%. In order to determine the efficiency of the procedure, internal standards (80 pg/tube) of authentic CART 55-102 were added and recovered to a level of 57%, and the values deviated less than 5%. All values are corrected for the loss of CART peptide using the recovery factor. Samples were purified by Sep Pak C18-E columns (Phoenix Pharmaceuticals, Belmont, MA). The eluates were freeze-dried overnight using a lyophilizer and later were dissolved in RIA buffer. Extraction of CART peptide from tissues was carried out as previously described by Murphy et al. (2000). Briefly, 0.5 M acetic acid was added to each tissue sample, which was then transferred to boiling water bath for 15 min. After cooling on ice, samples were homogenized and centrifuged at 13,000 g for 15 min. The supernatant was collected and the pellet re-extracted with acetic acid. The supernatant was divided into two tubes. One tube was used for RIA analysis of CART peptides and the other for quantification of soluble protein. The supernatants were further purified on Sep Pak C18-E columns. Following extraction, the eluates were freeze-dried overnight using a lyophilizer, and samples were dissolved in RIA buffer for assay.

2.4. Dopamine transporter autoradiography

Dopamine transporter autoradiography using the method of Boja et al. (1995) was used on serial sections from the 6-hydroxydopamine lesion study to confirm the extent of the lesion. Slides were incubated in phosphate buffered saline containing 10 mM NaI and 15 pM [^{125}I] RTI-121 for 1 h at room temperature. Sections were then washed 2× for 20 min in the same assay buffer at 0 °C without ligand, followed by a dip in deionized water. Sections were dried with a stream of cool dry air and exposed to Kodak Biomax MR film for 3 days.

2.5. Statistics

Two tailed *t*-tests for two group comparisons and one way analysis of variance (ANOVA) with Bonferroni post hoc analysis for multiple groups was used to analyze the data using Graph Pad Prism 3.0 (Graph Pad Software, San Diego, CA).

3. Results

In an attempt to replicate the findings of Douglass et al. (1995), we gave acute i.p. injections of 20 mg/kg cocaine to adult male Sprague–Dawley rats and euthanized the animals 1 h later. This treatment produced no measurable change in nucleus accumbens CART message as measured by in situ hybridization, which is in agreement with the findings of Marie-Claire et al. (2004) and Vrang et al. (2002) (two tailed *t*-test, $n=6$, $P>0.05$, data not shown). Similarly, the same treatment produced no change in CART peptide levels in the accumbens as measured by RIA (two tailed *t*-test, $n=4$, $P>0.5$, see Fig. 1).

However, binge cocaine administration (four injections of 30 mg/kg cocaine, once every 2 h) produced a 34% increase (±16%, two tailed *t*-test, cocaine $n=3$, saline $n=5$, $P<0.05$) in CART mRNA in the accumbens (Fig. 2). However, the binge paradigm also produced seizures in all the animals and death in two of the animals soon after the fourth dose; the deceased animals were excluded from this analysis.

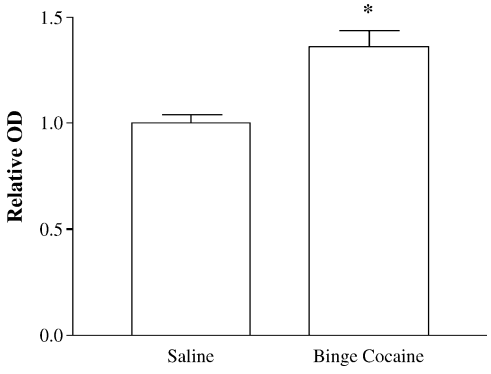

Fig. 2. The effect of binge cocaine administration (4× 30 mg/kg cocaine one dose every 2 h) on nucleus accumbens CART mRNA as measured by in situ hybridization. Results are mean±S.E.M. in relative optical density normalized to saline controls (two tailed *t*-test, saline $n=5$, cocaine $n=3$, *$=P<0.05$ binge cocaine versus saline).

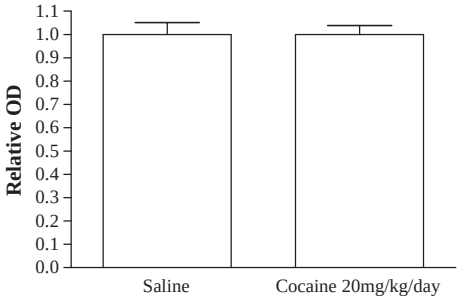

Fig. 3. Levels of CART mRNA after 14 days chronic cocaine as measured by in situ hybridization. Results are mean±S.E.M. in relative optical density normalized to saline controls (two tailed *t*-test, $n=6$, $P>0.9$).

Chronic administration of 20 mg/kg cocaine once per day for 14 days also failed to result in detectable alterations in CART mRNA levels as measured by in situ hybridization (two tailed *t*-test, $n=6$, $P>0.9$) (see Fig. 3). Unilateral 6-hydroxydopamine lesion of the dopaminergic projections in the median forebrain bundle produced a complete ablation of dopamine transporter binding as measured by [^{125}I] RTI-121 autoradiography (see Fig. 4A). On the contrary, CART mRNA levels in serial sections from the same brain did not change (two tailed *t*-test, $n=5$, $P>0.05$, see Fig. 4B).

We examined the effects of corticosterone administration because CART mRNA is regulated by this hormone in the hypothalamus (Balkan et al., 2001), and it was evident that binge administration caused significant stress to the rats. Corticosterone administration produced a significant increase of 38±6% in CART mRNA in the accumbens at the 10 mg/kg dose, as can be seen in Fig. 5, but not at lower doses (one way ANOVA, $P<0.002$, $n=5$, $F=7.612$, Bonferroni's post hoc:10 mg/kg corticosterone versus saline, $P<0.01$). We also examined peptide levels after acute corticosterone administration and found an 80±9% increase in peptide levels in the accumbens (two tailed *t*-test: saline $n=4$, corticosterone $n=9$, $P<0.01$, see Fig. 6). Acute treatment with 30 mg/kg metyrapone i.p., which blocks the synthesis of corticosterone, produced a 23±8% decrease in CART 55-102 peptide levels 2 h after administration (two tailed *t*-test: $n=5$, $P<0.005$, see Fig. 7). Thus, it appears that corticosterone acutely influences the levels of both CART mRNA and peptide levels in the nucleus accumbens.

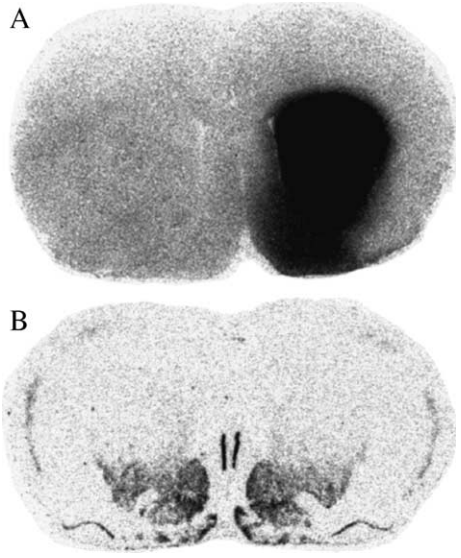

Fig. 4. A) Autoradiogram of [^{125}I] RTI-121 binding in the rat striatum 14 days after a 6-hydroxydopamine lesion, demonstrating a complete absence of dopamine transporter binding on the lesioned side (left). B) A serial section from the same brain processed for CART in situ hybridization; no change is evident between the lesioned and unlesioned sides (detailed results not shown, but two tailed *t*-test, $n=6$, $P>0.05$).

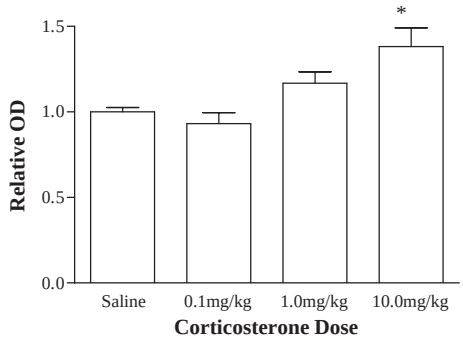

Fig. 5. Corticosterone dose–response effects on nucleus accumbens CART mRNA as measured by in situ hybridization. Shown are the effects of ascending doses of corticosterone on CART mRNA levels, reported as relative optical density normalized to saline controls (one way ANOVA $n=5$, $P<0.002$, $F=7.612$; *=Bonferroni post hoc $P<0.01$ for 10 mg/kg dose versus saline).

4. Discussion

The results presented here support the findings of Vrang et al. (2002) and Marie-Claire et al. (2003) with regard to the absence of effect of acute psychostimulant administration on CART mRNA. We show in this study that the peptide levels do not change as well. Further, chronic cocaine administration or removal of dopamine input to the striatum (which mediates cocaine's effects) also failed to produce a detectable effect on CART message. However, binge administration did produce an increase in mRNA levels, but this finding was confounded in our hands by the production of seizures in all and death in some of the subjects, making it impossible to conclude if the change in message levels were the result of increased dopamine levels, a side effect of the seizures

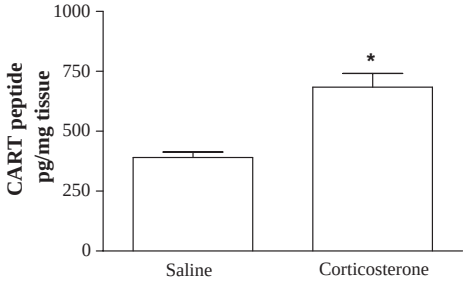

Fig. 6. Effects of acute (10 mg/kg) corticosterone on nucleus accumbens CART peptide immunoreactivity as measured by RIA (two tailed *t*-test, saline $n=4$, corticosterone $n=9$, *=$P<0.01$ corticosterone versus saline).

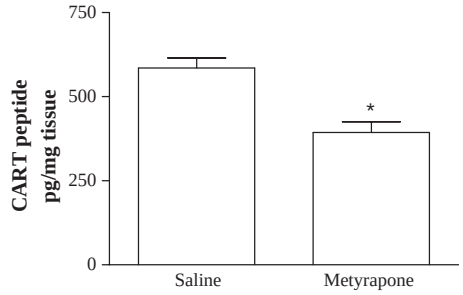

Fig. 7. Effects of acute (30 mg/kg) metyrapone on nucleus accumbens CART peptide immunoreactivity as measured by RIA (two tailed t-test, $n=5$, $*=P<0.005$ saline versus metyrapone).

or some other non-specific consequence of the high doses of cocaine used.

A number of explanations can be offered for the disparity of results with regard to dopaminergic regulation of CART mRNA levels in the accumbens in different laboratories. Vrang et al. (2002) proposed that errors in dissection may have biased the initial finding by Douglass et al. (1995). Alternatively, the disparity of effects seen here and elsewhere could be explained, at least in part, by a non-dopaminergic mechanism. It has been shown that glucocorticoids increase CART message in the rat hypothalamus (Balkan et al., 2001; Vrang et al., 2003). We have demonstrated here that corticosterone administration increases CART mRNA and peptide levels in the accumbens and that peptide levels are reduced by acute administration of metyrapone, an inhibitor of the 11β-hydroxylation in corticosterone synthesis (Haleem et al., 1988; Haynes, 1990). The hypothesis that glucocorticoids may be confounding some of the studies of psychostimulant interactions with CART could explain the finding that binge cocaine administration increases CART mRNA in the accumbens, whereas in lower dose acute studies there is often no observable change, as the increase in corticosterone produced by cocaine administration is dose-dependent (Levy et al., 1991; Mello and Mendelson, 1997). It is also plausible to posit that variations in animal handling between individual experimenters may contribute to substantially higher corticosterone levels in some animals treated with psychostimulants than in others. This could produce detectable changes in CART levels in combination with lower doses of psychostimulants than in animals whose handling stress was minimized. Differences in the time of day during which the experiments are performed could also have the same impact due to diurnal fluctuations in corticosterone levels. In fact, we have recently shown that CART peptide levels in the nucleus accumbens and blood also show diurnal variation that appears to be at least partially corticosterone-dependent (Vicentic et al., 2004). The corticosterone data presented here lend support to the possibility that some of the regulatory effects of psychostimulants on CART in the nucleus accumbens are mediated by corticosterone.

We did not attempt to show that metyrapone blocked the binge-induced increase in CART because of various confounds. There was a high mortality rate in the treated group and use of metyrapone to blunt the stress response could induce an even higher mortality rate. Also, metyrapone alters dopamine release (Rouge-Pont et al., 1995) and the metabolism of cocaine (Kloss et al., 1983). We did, however, produce the novel observation that metyrapone reduced CART in the nucleus accumbens.

A number of investigators have shown (for review see Goeders, 2002) that corticosterone in the nucleus accumbens is necessary for the acquisition of psychostimulant self-administration in animals, a model of human drug abuse. The mechanisms for this effect are not completely understood, and it is possible that CART is a downstream mediator of this process. It has been found that CART is regulated selectively by dopamine D1 and D3 receptors under certain conditions (Beaudry et al., 2004), so an interaction between CART expression and dopamine receptors seems to be present even if drugs of abuse lack a prominent regulatory effect, i.e., causes a change in levels of mRNA or peptide. Moreover, it is possible that CART-containing neurons may respond to drugs of abuse without showing detectable changes in levels of CART mRNA or peptides.

Acknowledgements

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