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Cocaine Administration Increases the Fraction of CART Cells in the Rat Nucleus Accumbens that Co-immunostain for c-Fos

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Abstract

In order to further test whether or not psychostimulant drugs activate CART peptide-containing cells in the nucleus accumbens, we examined the fraction of CART positive cells that co-immunostained for c-Fos after administration of saline or cocaine (10 and 25 mg/kg i.p.). There was about a 45% increase in the fraction of cells that stained for both CART and c-Fos after administration of cocaine, but there was no change in the fraction after administration of saline. Moreover, the increase was not found 24 hours after injection and is therefore reversible. These results support the notion that psychostimulant drugs activate CART cells in the nucleus accumbens, even under conditions where it is difficult to show a change in CART levels.

Introduction

CART mRNA was discovered as a transcript whose levels increased after acute administration of cocaine or amphetamine (Douglass et al., 1995). Evidence for translation of the mRNA was that part of its coding sequence corresponded to the amino acid sequence of a previously identified peptide from ovine hypothalamus (Speiss et al., 1981). The increase in the level of the transcript suggested that the product of this gene was a substance related to the action of psychostimulant drugs, and the increased level further suggested an increased need or release of the gene product. Since that discovery, many publications have shown that the product of the gene is at least a pair of peptides (rat CART 62–102, CART 55–102) that function as peptide neurotransmitters. In the ventral striatum, CART peptides are co transmitters with GABA in some of the medium spiny neurons. In the nervous system, the peptides are localized in widespread but discrete groups of neurons that suggest many functions of the peptides. The general importance of CART peptides in a variety of physiologic functions, including food intake, stress, drug reward, endocrine control, and other processes has been demonstrated (Dominguez et al., 2004; Murphy 2005; Larsen et al., 2002; Hurd et al., 1999; Hunter and Kuhar 2003; Thim et al., 1998; Kuhar et al., 2005; also see the August issue of Peptides for reviews).

Several studies have not confirmed the up regulation of CART mRNA under the original conditions of a single, acute injection of drug (Vrang et al., 2002; Marie-Claire et al., 2003; Hunter et al., 2005; Jones and Kuhar 2006). Rather, binge or repeated dosing has more reliably resulted in increases of CART mRNA and peptide in the ventral striatum (Fagergren and Hurd,

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1999; Brenz Verca et al., 2001; Hunter et al., 2005), and corticosterone may play a role in this increase (Hunter et al., 2005; Hunter et al., 2007). Nevertheless, these disparate results have lead to controversy over whether or not administration of psychostimulant drugs activate CART-containing neurons in the nucleus accumbens. An appropriate issue has been that CART neurons could be activated without a significant change in levels of CART peptide or mRNA, and that searching for changes in levels is not a reliable approach for determining neuronal activity.

In this study, in order to additionally test if psychostimulants activate CART-containing neurons, we measured c-Fos levels, another possible indicator of activation, in accumbal CART neurons after acute administration of cocaine. This approach would not require changes in CART levels to suggest changes in the activity of CART neurons. It is known that cocaine administration can increase Fos levels in brain (Marie-Claire et al., 2003; Graybiel et al., 1990; Gutstein et al 1998; Erdtmann-Vourliotis et al., 1999), but the colocalization of CART and Fos in the nucleus accumbens has not yet been reported. In this study we test if cocaine administration, in physiologically active doses, results in an increase in c-Fos in CART-containing neurons in the rat nucleus accumbens.

Materials and Methods

Animals

Thirty six adult, male, Sprague-Dawley rats (Charles River, Wilmington MA) were injected with either cocaine (10 or 25mg/kg, i.p.) or saline (vehicle) and anesthetized with chloral hydrate either thirty minutes, one hour or 24 hours after injection. Within minutes of loss of tail pinch reflex, all rats were transcardially perfused with 100 ml of cold, oxygenated Ringer's solution. This was followed by perfusion with 500 ml of fixative containing 4.0% paraformaldehyde and 0.1% glutaraldehyde, dissolved in phosphate buffer (PB, 0.1M, pH 7.4). Brains were removed from the skull and stored in 30% sucrose solution in PB at 4°C until brains were saturated. The brains were then cut into 60μm-thick coronal sections on a freezing microtome. Sections were put in a 1.0% sodium borohydride solution dissolved in phosphate buffered saline (PBS) for 20 min and rinsed with PBS before being processed for immunocytochemistry. The anesthesia and euthanasia procedures were carried out according to the National Institutes of Health Guidelines and a protocol describing these experiments has been approved by the Institutional Animal Care and Use Committee of Emory University.

Antisera

Commercially available antibodies for c-Fos raised in mouse (Calbiochem, San Diego CA), and custom antibodies for CART peptide raised in rabbit (C4 in Koylu et al., 1997) were used. Secondary antibodies raised in donkey coupled to either CY3 or FITC (Jackson Laboratories, West Grove, PA) were used to visualize the localization of the proteins of interest.

Immunofluorescent Localization of CART and c-Fos

The tissue sections were preincubated in a solution containing 5% normal donkey serum (NDS), 1.0% bovine serum albumin (BSA), and 0.3% Triton X-100 in PBS for one hour. They were then incubated overnight with primary antisera for CART and/or c-Fos diluted to 0.5–1.0 μg/ml in a solution containing 1.0% NDS, 1.0% BSA, 0.3% Triton X-100 in PBS. Next, the sections were rinsed in PBS and transferred for one hour to a secondary antibody solution containing FITC- (for CART) and CY3-(for c-Fos) conjugated secondary antibodies diluted 1:100 in the primary antibody diluent solution. The tissue sections were then washed in PBS and incubated in cupric sulfate solution for 30 minutes to enhance visualization. Sections were then mounted on gelatin-coated slides, dried, and a coverslip was applied with Vectashield mounting agent (Vector, Burlingame, CA) and sealed using clear fingernail polish. Specimens

were observed throughout the rostrocaudal length (2.7 to 0.7 mm according to Paxinos and Watson,1986) of the nucleus accumbens using a Zeiss LSM 410 confocal microscope. Accumbal neurons that displayed CART-IR were scanned on the confocal microscope for the presence of double labeling. The total number of CART positive cells examined under each condition are given in Table 1. Due to the limitations of darkfield, oil-immersion confocal microscopy, where it is difficult to precisely define the border between core and shell, no distinction made between the accumbens core and shell.

Results

Animals were prepared and tissues processed as described in Methods. CART positive cells and cFos positive cells were examined in multiple sections from each animal as described in Methods. The total number of Immunofluorescent cells that stained for CART are given in Table 1 for each experimental condition. CART cells were rated as either co-staining or not co-staining with c-Fos. Examples of such accumbal cells are shown in Figure 1.

Figure 2 shows the quantitative analysis of the data. A two way analysis of variance revealed a significant interaction, a significant effect of time and a significant effect of dose on the fraction of CART cells staining for c-Fos, all at the $P<0.0001$ level. Subsequent analysis of the data at each time point with a one way ANOVA revealed significant differences at 30 min [$F(2,6)= 122.8$, $P<0.0001$] and 1 hr [$F(2,6)=169.3$, $P<0.0001$], but not at 24 hours [$F(2,6)=0.0586$, $P=0.94$]. Tukey's post hoc analysis revealed that, at 30 min, the fractions at both cocaine doses were significantly greater than that at saline ($P<0.001$), but were not significantly different from each other. The same results were found for fractions measured at 1 hour after cocaine. However, 24 hours after cocaine, there were no significant differences among the fractions for the different doses of cocaine (Figure 2).

Discussion

Several studies support the notion that psychostimulants, which increase extracellular dopamine levels, can activate CART-containing neurons in the nucleus accumbens through dopaminergic mechanisms. One is that CART neurons in the accumbens receive nerve terminals that contain tyrosine hydroxylase (Smith et al., 1999). Another study showed that accumbal CART neurons express protein or mRNAs for dopamine receptors (Hubert and Kuhar 2005; Beaudry et al., 2004), and that the neurons are affected by some dopamine agonists and antagonists (Hunter et al., 2006; Beaudry et al., 2004). Moreover, various publications indicate a CART-dopamine interaction in the nucleus accumbens (Dominguez et al., 2004; Philpot and Smith, 2006; Jaworski and Jones,2006). However, it has not been clear that administration of psychostimulants such as cocaine results in an activation of CART neurons in the nucleus accumbens. Part of the problem is that most studies used the *levels* of CART mRNA or peptide in the ventral striatum as an indicator of changed activity. However, the activity of the neurons could change without a measurable change in CART levels, provided the changes in activity were small or if the rate of synthesis of the peptide could keep up with demand. Therefore, we measured the effect of cocaine administration on the fraction of CART neurons that contain c-Fos, a marker for increased activity and possibly neuroplasticity, in the nucleus accumbens.

It has long been known that measurement of neurotransmitter levels alone are not adequate for an accurate determination of neuronal activity, but measurement of turnover rates of neurotransmitters is more sensitive (Iversen and Glowinski, 1966; Brodie et al., 1966). Turnover measurements reflect release and/or synthesis rates that have been found to change and reflect activity even when levels do not. Similarly, protein levels may not change while changes in turnover rates are significant (for example, Kimmel et al., 2003). However, methods

for measuring turnover rates of most peptides have not been developed. Therefore, other approaches are useful, and we utilize induction of c-Fos levels as a rough indicator of activity.

The results of this study were straightforward. Injection of cocaine, at 10 or 25 mg/kg i.p., for 30 min or 1 hr caused an increase in the fraction of CART cells that stained for c-Fos, strongly suggesting that cocaine increases the activity of accumbal CART cells. C-Fos is also associated with plasticity (McClung et al., 2004). Twenty-four hrs after cocaine injection, there were no significant differences in the fractions indicating that the increase in c-Fos in CART cells was reversible. In several other studies, CART levels have been measured in the accumbens after cocaine, and no changes in levels after acute cocaine were found (Vrang et al., 2002; Marie-Claire et al., 2003; Hunter et al., 2005; Jones and Kuhar 2006). This makes it unlikely that the overall number of CART positive cells changed in the accumbens after drug administration.

The doses of cocaine and the time points were selected because of the data showing increases in c-Fos under roughly these conditions (Marie-Claire et al., 2003; Graybiel et al., 1990; Gutstein et al 1998; Erdtmann-Vourliotis et al., 1999). Now we show that the increase in c-Fos occurs at least partly in CART positive cells. Also, it is known that cocaine can increase locomotor activity under these conditions (For example, see Jaworski et al., 2003a: 2007). This strongly supports that idea the cocaine activates CART cells in the nucleus accumbens and implicates CART in drug abuse, and other studies with CART implicate CART in drug abuse as well (Jaworski and Jones, 2006; Jaworski et al 2006; Hurd et al., 1999; Freeman et al., 2007; Dayas et al., 2007; Moffett et al., 2006; Salinas et al., 2006; Couceyro et al., 2005; Kim et al., 2003; Kim et al., 2007; Yoon et al., 2007).

The fraction of cells staining with both CART and cFos after either 10 or 25 mg/kg were the same at 30 min and 1 hour. This suggests that the effects of cocaine on CART were maximal at these times and doses. A major hypothesis is that CART peptides in the accumbens act in a corrective or homeostatic mechanism such that the effects of high levels of dopamine, including those caused by psychostimulants, are opposed by CART peptide in accumbal neurons (Jaworski et al 2003a,b, 2006, 2007). Evidence for this has been that injection of CART into the nucleus accumbens has no effect on locomotor activity, but coinjection of CART with cocaine or dopamine results in a decrease in the cocaine- or dopamine-induced locomotor activity (Jaworski et al 2003a,b, 2006, 2007). Other neurotransmitters could also be involved in such a mechanism that tends to limit and control accumbal output.

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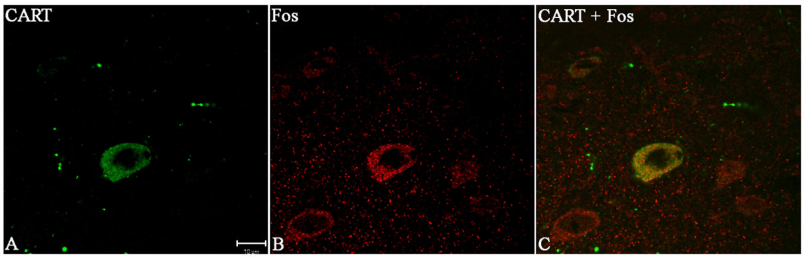

Figure 1.
Examples of immunostaining for c-Fos and CART peptide in neurons of the nucleus accumbens. In A, a CART positive cell is stained green and a c-Fos positive cell is stained red in B. In C, the neuron co-staining for both c-Fos and CART is yellow which is expected. All three frames show the same tissue field.

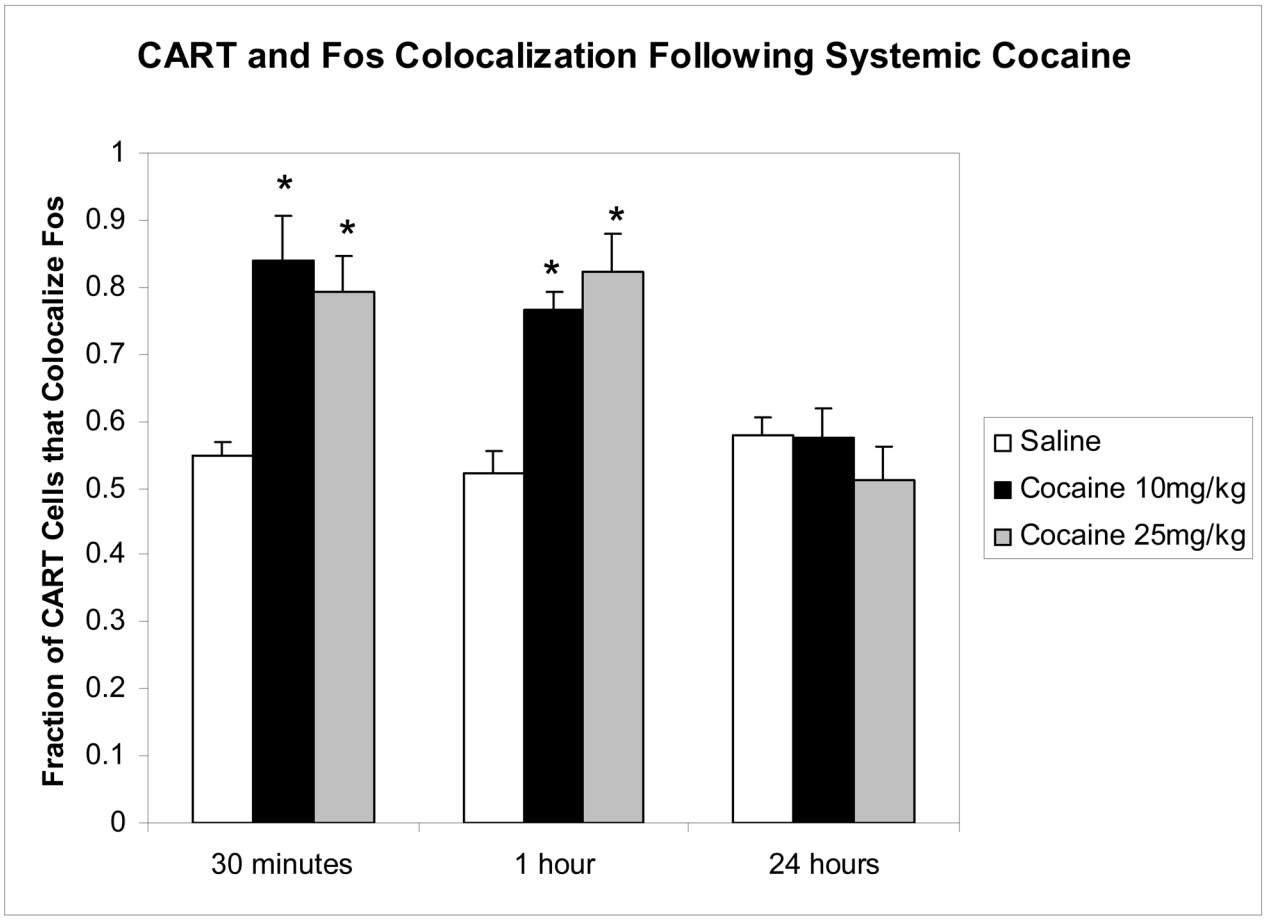

Figure 2.

Effect of cocaine administration on the fraction of CART positive cells staining for c-Fos in the nucleus accumbens. Tissues were prepared and examined as described in Methods. Administration of either 10 or 25 mg/kg of cocaine i.p., resulted in a significant increase in the fraction of CART positive cells that stained for cFos in the nucleus accumbens (2 way ANOVA, with a $P<0.0001$ effect in interaction, drug, and time). Asterisks indicate a significant difference ($P<0.05$) between values from cocaine treated animals and saline treated animals at the same time point. At 24 hours after cocaine injection, there was no difference among the groups. N = 3 animals for each bar. Data are mean +/− SEM for each bar.

Table 1

Total number of CART-positive cells examined under each condition. The number of Fos-positive CART cells is divided by the total number of CART-positive cells to determine the proportion of cells activated by cocaine, shown graphically in Figure 2.

	Saline	Cocaine, 10mg/kg	Cocaine, 25mg/kg
30 min	232	254	273
1hr	189	222	239
24hrs	252	218	246