Fos but not Cart (cocaine and amphetamine regulated transcript) is overexpressed by several drugs of abuse: a comparative study using real-time quantitative polymerase chain reaction in rat brain.

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Modulation of Cocaine and Amphetamine Regulated Transcript (*CART*) and *c-fos* expression by several drugs of abuse : a comparative study using real-time quantitative RT-PCR in rat brain

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ABSTRACT

It has been reported that cocaine and amphetamine-regulated transcript (CART) peptides can increase locomotor activity and produce a conditioned place preference. To establish whether or not CART can be consider as a valuable marker of addiction we performed a comparative study of the expression of *CART* gene by several drugs of abuse. This was achieved using real-time quantitative PCR in four rat brain structures: prefrontal cortex, caudate putamen, nucleus accumbens and hippocampus. As expected, a significant induction of the immediate early gene *c-fos* was observed after acute administration of morphine, cocaine, 3, 4-methylenedioxymethamphetamine and Δ^9-Tetrahydrocannabinol. On the contrary none of these drugs was able to produce a significant change in *CART* mRNA levels demonstrating that the expression of this gene is not modulated by the four drugs of abuse in these brain structures.

133 words

Keywords : CART, c-fos, quantitative RT-PCR, rat, gene expression, drug of abuse

Cocaine- and amphetamine- regulated transcript (CART) was initially isolated by PCR differential display as an up-regulated mRNA in the rat striatum following acute psychostimulant-drug administration [4]. *CART* mRNA and CART peptides are expressed throughout the mammalian brain as shown by immunohistochemical and *in situ* hybridization studies [2, 4, 13, 14]. In the rat, alternative splicing produce two CART peptides (116 and 129 amino acids) [4], which are tissue-specifically processed into several fragments. It has been shown that the C-terminal region, CART(55-102), is sufficient to obtain a biological activity [16].

Neuro-anatomical and behavioral studies have confirmed the implication of CART in the control of appetite in the last few years (review in [10, 15]). On the other hand, CART peptides and CART mRNA, were found in brain structures associated with drug-induced reward and reinforcement such as the nucleus accumbens [4]. All drugs of abuse induce an increase of dopamine in this region [3], although these drugs have different mechanisms of action (review in [18]). Some of them target dopamine and/or serotonine transporters like cocaine and 3, 4-methylenedioxymethamphetamine (MDMA). Others interact with specific receptors like morphine or Δ^9-Tetrahydrocannabinol (Δ^9-THC). Nevertheless, all drugs of abuse induce hyperlocomotion and activate the reward pathway which includes dopaminergic neurons in the ventral-tegmental area (VTA) and their direct or relayed projections to nucleus accumbens (N. Acc), amygdala, prefrontal cortex (PFC) and other forebrain regions. Interistingly, Kimmel et al, have recently shown that injection of CART(55-102) into the VTA of rats induced locomotor activity and a slight conditioned place preference [11, 12] suggesting that two of the most important behavorial responses of all drugs of abuse could be induced by CART itself: Based on these results it was interesting to to test the possible role of CART as a common effector of drugs of abuse such as morphine, cocaine, MDMA and Δ^9-THC.

This was investigated by using real time quantitative RT-PCR to measure the expression of the CART gene. The immediate early gene c-*fos* which is a marker of neuronal activation is overexpressed by these drugs in rat brain [7, 8, 17, 19] and was used here as a positive control. Moreover, we have examined the levels of *CART* mRNA in different structures implicated in memory, locomotion and reward to test the specificity of *CART* induction by the four drugs of abuse.

Male Sprague-Dawley rats (250-300 g) were used (Charles-Rivers, France). Experiments were performed according to the European Communities Concil Directive (86/609/EEC). Animals were housed under controlled conditions (12h alterning light/dark cycle and 21±1°C) and had free access to food and water. Morphine (Francopia, France), cocaine (Sigma, France) and MDMA (Lipomed, Switzerland) were dissolved in 0.9% saline and Δ^9–THC (Sigma, France) in 80% saline, 10% EtOH, 10% cremophor (Sigma, France). The rats received a single 0.1 ml/100g intraperitoneal (i. p.) injection of morphine (20 mg/kg; n = 4), cocaine (20 mg/kg; n = 4), MDMA (10mg/kg; n = 5) or Δ^9–THC (5 mg/kg; n =3). Controlled rats received 0.9% saline or vehicle i.p. Immediately after the injection, the rats were placed in their home cages and grouped according to the substance administered. One hour after the injection, the animals were killed by decapitation and the four brain structures were rapidly dissected : prefrontal cortex (PFC), caudate putamen (CPu), nucleus accumbens (N. Acc) and hippocampus. The tissues were frozen in isopentane at –50°C and stored at –80°C until use.

The theoretical and practical aspects of real-time quantitative RT-PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) have been described elsewhere [1]. Briefly, total RNA extracted from frozen tissues is reverse-transcribed before real-time PCR amplification. Quantitative values are obtained from the threshold cycle (Ct) number at which the increase in the signal associated with exponential growth of PCR products begins to be detected using PE Biosystems analysis software, according to manufacturer

recomandations. The precise amount of total RNA added to each reaction mix (based on optical density) and its quality (lack of extensive degradation) are both difficult to assess. We therefore also quantified transcripts of the *RPLP0* gene encoding the rat acidic ribosomal phosphoprotein P0 as the endogenous RNA control, and each sample was normalized on the basis of its *RPLP0* content.

As described, [1] differences in target gene expression (*c-fos* or *CART*) relative to the *RPLP0* gene, termed N_{target} were determined by the formula $N_{target} = 2^{\Delta Ctsample}$, where ΔCt value of the sample was determined by substracting the average Ct value of the target gene from the average Ct value of the *RPLP0* gene. The N_{target} values of the samples (drug-treated and controls) were then subsequently normalized to a calibrator consisting of a naïve rat brain tissue sample. The nucleotide sequences of the specific primers used are shown in table I. PCR was performed using the SYBR® Green PCR Core Reagents kit (Perkin Elmer Applied Biosystems). The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 50 cycles at 95°C for 15s and 65°C for 1 min

The quantification were performed in duplicate on a pool of structures from 6 animals for each point, the results represents the mean value ± SEM of 4 independent experiments. One-way analysis of variance (ANOVA), followed by Student's t test, was used for the statistical evaluation. The level of significance was chosen as $P<0.05$.

As compared to the controls, acute treatment with morphine, cocaine, Δ^9–THC and MDMA led to a significant increase in *c-fos* mRNA in the CPu (1.4; 6.84; 2.52 and 5.44 fold respectively). Similarly all the tested drugs induced a marked increase in the mRNA level of this gene in the hippocampus (1.48; 2; 1.62 and 2.18 fold respectively). However, the modulation of *c-fos* mRNA was significantly induced in the PFC by three of the tested drugs : cocaine, Δ^9–THC and MDMA (2.11; 2.26 and 4.2 fold respectively). These three drugs also

induced a significant increase of this mRNA in the N. Acc (2.26; 2.11 and 3.9 fold respectively).

These results are in accordance with those of Erdtmann-Vourliotis et al. (1999) [5] who showed by *in situ* hybridization that drugs with low addictive potential like Δ^9–THC and MDMA induced a larger increase in *c-fos* mRNA than morphine or cocaine. Thus the quantitative RT-PCR protocol used in the present study appears to be a satisfactory method to quantify the modulation of genes after drug treatments.

Therefore this method was used to evaluate the possible modulation of *CART* mRNA level following treatments by the four drugs chosen (Fig. 2). Surprisingly, one injection of cocaine did not lead to a significant increase of *CART* in any of the structures studied: N. Acc, CPu, PFC and hippocampus (Fig. 2). Moreover, none of the four drugs appear to significantly modulate *CART* mRNA in any of the selected cerebral structures (Fig. 2). However, there is a slight but not significant increase in *CART* mRNA in the N. Acc of the rats treated with MDMA. In this study we could not detect a significant modulation of *CART* in any of the two major components of the striatum (N. Acc and CPu). Furthermore, we did not see any regulation of *CART* mRNA with repeated fifteen daily doses of cocaine (data not shown). Moreover, in the case of the amphetamine-derived compound (MDMA) no significant modulation of this gene was observed.

Our study showed that the modulation of *c-fos* by different drugs of abuse can be assessed by real-time quantitative RT-PCR. The results obtained are consistent in range and localization with those obtained previously [5]. However, although we used strictly the same protocol of cocaine injection as Douglass et al [4] we did not find any regulation of *CART* by this drug and by any of the drugs tested. These authors reported a 4-5 fold increase in *CART* mRNA in rat striatum using Northern blot analysis. Here, we could detect as little as 1.6 fold increase in *c-fos* mRNA. Thus, the absence of regulation seen here is clearly not a limitation

of the method used. It can be proposed that the increase in *CART* mRNA may be localized in a subset of neurons or a certain area of the striatum. However, Vrang and al [20] reported recently that in similar conditions as those used here they could not detect any modulation of *CART* by amphetamine using *in situ* hybridization. Moreover, except for the original report on amphetamine and cocaine only two other publications examined the effects of cocaine on *CART* expression. One of them reported a modest increase of *CART* mRNA in the N. Acc of female rats after binge injections of cocaine [6]. The other one reported that cocaine had no effect on the expression of CART in the N. Acc [9]. Taken together these results and those obtained in this study with extension to other drugs of abuse raise the question of the relevance of the modulation of *CART* by drugs of abuse including psychostimulants.

CART peptides have been discovered in 1995 as psychostimulants regulated peptides but their implication in drug addiction appears unlikely. This does not impede the interest of CART peptides in feeding modulation as shown by several groups.

LEGENDS

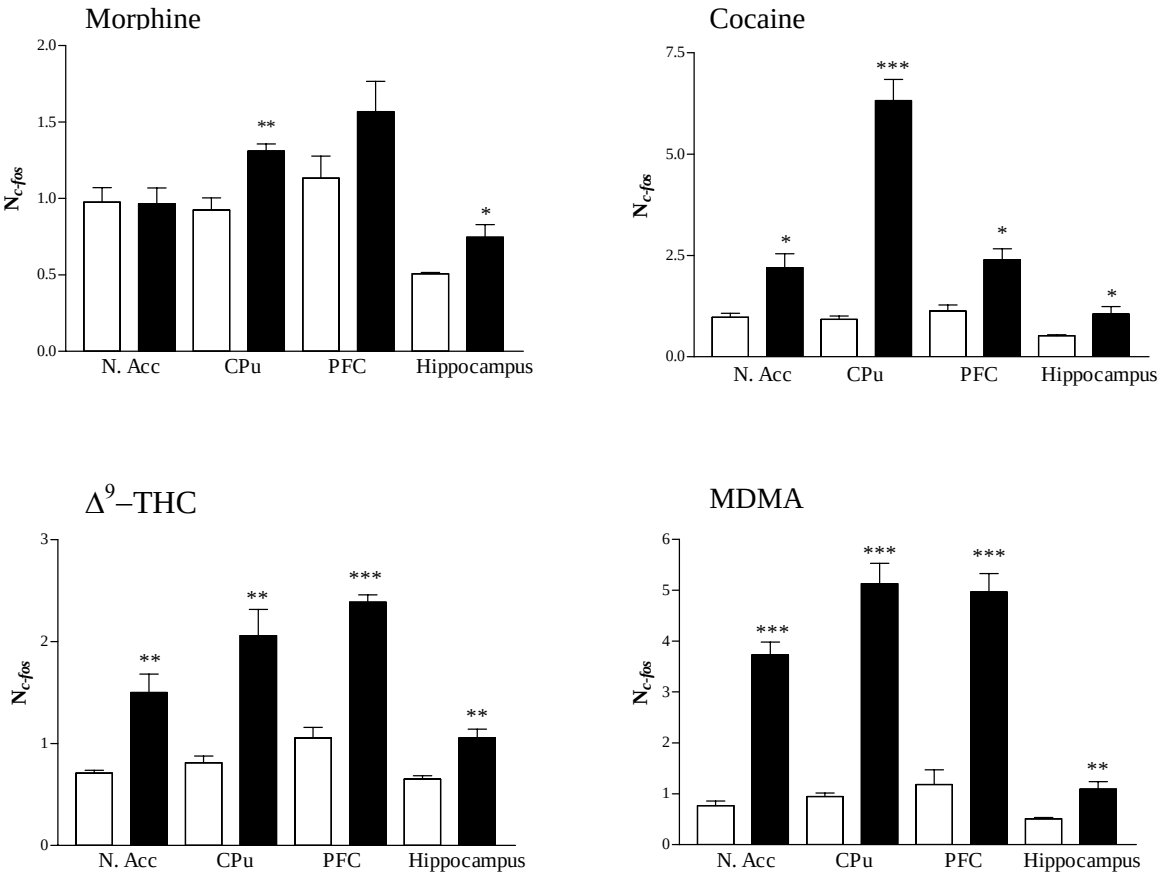
Figure 1 : Real-time quantitative RT-PCR analysis of the effect of acute injection of morphine (20 mg/kg), cocaine (20 mg/kg), Δ^9–THC (10 mg/kg) and MDMA (10 mg/kg) on *c-fos* mRNA level in the different brain structures tested (N. Acc, Cpu, PFC and hippocampus). ☐ saline, ■ drug. Data are the means ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 by the Student's *t*-test (n = 4)

Figure 2 : Real-time quantitative RT-PCR analysis of the effect of acute injection of morphine (20 mg/kg), cocaine (20 mg/kg), Δ^9–THC (10 mg/kg) and MDMA (10 mg/kg) on *CART* mRNA level in the different brain structures tested (N. Acc, Cpu, PFC and hippocampus). ☐ saline, ■ drug. Data are the means ± SEM (n = 4)

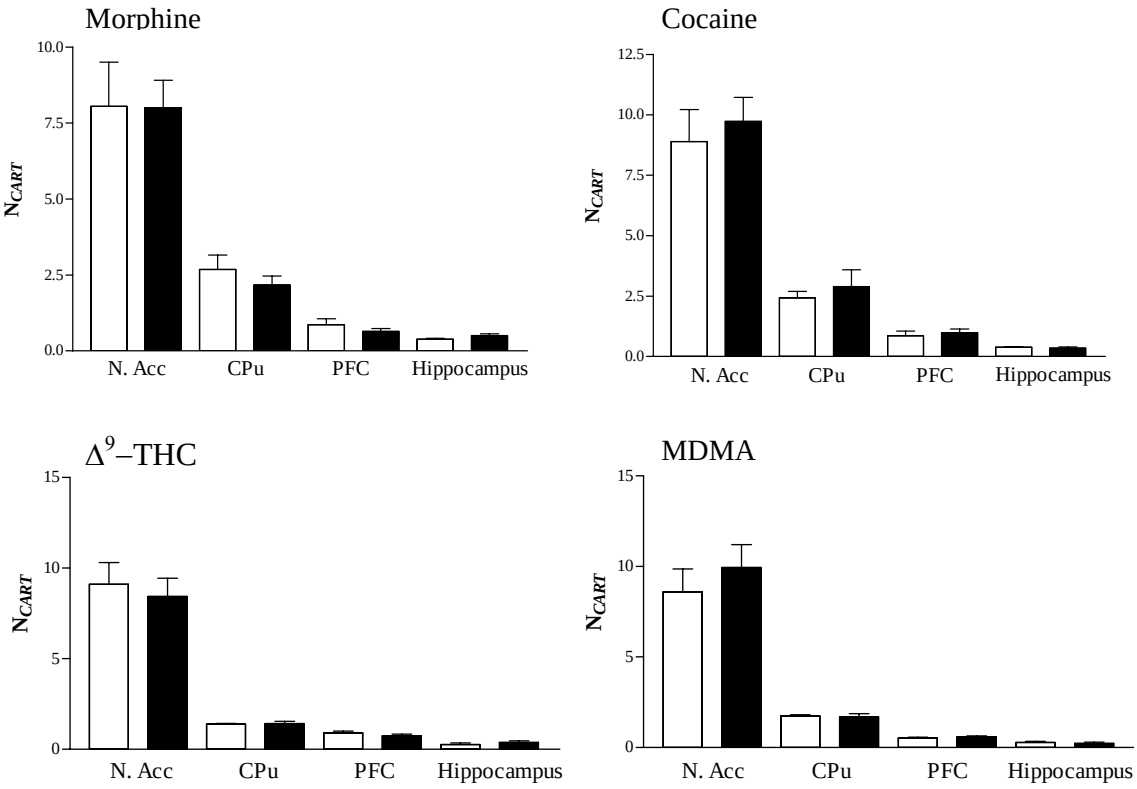
Genes	Oligonucleotide	Sequence	Amplicon size
RPLP0	Forward primer	5'-GGCGACCTGGAAGTCCAACT-3'	149 bp
	Reverse primer	5'-CCATCAGCACCACAGCCTTC-3'	
CART	Forward primer	5'-GCCAAGTCCCCATGTGTGAC-3'	129 bp
	Reverse primer	5'-CACCCCTTCACAAGCACTTCA-3'	
c-fos	Forward primer	5'-GGCAAAGTAGAGCAGCTATCTCCT-3'	106 bp
	Reverse primer	5'-TCAGCTCCCTCCTCCGATTC-3'	

Table I : Primers sequences used. Primers were chosen with the assistance of Oligo 6™ software. *CART* primers were chosen in the region of the cDNA corresponding to the C-terminal part of the protein which shows physiological activity. c-*fos* and *RPLP0* primers were chosen on different exons or at exon boundaries in order to avoid amplification of putative contaminating genomic DNA.

c-*fos*



CART



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