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CART expression in the rat nucleus accumbens is regulated by adenylyl cyclase and the cyclic-AMP/PKA second messenger system.

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Abbreviations: CART, Cocaine-amphetamine Regulated Transcript; NAc, Nucleus Accumbens; DA, Dopamine; CREB, cyclic-AMP Response Element Binding Protein; PKA, Protein Kinase A; AC, Adenylyl Cyclase; cAMP, cyclic adenosine monophosphate; ANOVA, analysis of variance; OD, Optical Density; ip, intraperitoneal

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ABSTRACT

Cocaine-amphetamine regulated transcript (CART), a neuropeptide involved in the brain's reward/reinforcement circuit, modulates the effects of psychostimulants, including cocaine. The CART gene has been characterized and binding sites for multiple transcription factors have been identified within the promoter region, including the cyclic AMP-response element (CRE), which serves as a binding site for cyclic-AMP responsive binding protein (CREB). CART gene expression appears to be regulated via cAMP/PKA/CREB-mediated signaling in cell culture. Therefore, the goal of these studies was to examine the involvement of cAMP/PKA/CREB-mediated signaling in CART mRNA and peptide expression in-vivo in the rat nucleus accumbens. Intra-accumbal injections of forskolin, an adenylyl cyclase activator, stimulated the phosphorylation of CREB and increased both CART mRNA and peptide levels, an effect attenuated by inhibition of PKA with H89 and Rp-cAMPS. In addition, Rp-cAMPS alone decreased CART mRNA as compared to saline-injected controls, suggesting that CART expression may be tonically regulated by PKA. Under certain conditions, cocaine increases CART mRNA levels; thus, we examined the effects of cocaine on forskolin-induced CART mRNA expression in the rat NAc. Cocaine plus forskolin significantly increased CART mRNA over either of the drugs administered independently, suggesting that under conditions of heightened cAMP signaling, cocaine may impact CART gene expression. These results suggest that CART expression in-vivo in the rat nucleus accumbens is regulated by adenylyl cyclase and cAMP/PKA-mediating signaling, and likely, through the activation of CREB.

INTRODUCTION

CART neuropeptides are involved in numerous physiological processes, including feeding behavior (Lambert *et al.*, 1998), response to fear and stress (Vicentic *et al.*, 2004), endocrine regulation (Kuriyama *et al.*, 2004), and the reward/reinforcing effects of psychostimulants (Douglass *et al.*, 1995; Brenz-verca *et al.*, 2001; Jaworski *et al.*, 2003; Couceyro *et al.*, 2005). Expressed throughout the CNS, CART peptides are partly located in the ventral tegmental area (VTA) and nucleus accumbens (NAc), regions of the brain included in the mesolimbic dopamine (DA) pathway and associated with reward and reinforcement (Dallvechia-Adams *et al.*, 2002). Interestingly, CART modulates mesolimbic dopaminergic activity (Yang *et al.*, 2004) and attenuates the locomotor effects of cocaine (Jaworski *et al.*, 2003, Kim *et al.*, 2003) and feeding (Yang *et al.*, 2005), suggesting a complex relationship between CART, mesolimbic DA, and the brain's reward/reinforcement pathways.

Due to the unique anatomical distribution of CART peptides and the nature and variety of the stimuli that influence changes in CART levels, CART expression must be highly regulated. CART levels in the NAc and other brain regions follow a diurnal rhythm (Vicentic *et al.*, 2004), which may influence the diurnal variation in cocaine sensitization and reward. In addition, CART expression appears to be under hormonal regulation as CART levels are affected by glucocorticoids (Vicentic *et al.*, 2004; Hunter *et al.*, 2005), which, similar to CART, follow diurnal rhythms and are involved in the modulation of rewarding behaviors, including food and drug intake (Goeders, 2002). The mechanisms underlying the regulation of the CART gene are not fully understood and thus, are the focus of the current study. Several genes associated with reward and

reinforcement are regulated via cAMP response binding protein (CREB). The CART promoter contains a cAMP response element (CRE) binding site (Dominguez *et al.*, 2002), therefore, it was hypothesized that the CART gene may be regulated by CREB. Indeed, *in-vitro* studies suggest that CREB, a transcription factor activated via phosphorylation by protein kinase A (PKA), is involved in the regulation of the CART gene. For instance, activation of adenylyl cyclase (AC) increases CART mRNA levels in a PKA-dependent manner in cell culture (Lakatos *et al.*, 2002). Moreover, mutations of the CRE binding site on the CART promoter decreases promoter activity (Barrett *et al.*, 2002; Dominguez and Kuhar, 2004) and thus, likely decreases CART mRNA levels.

Both mesolimbic DA and CART peptides are involved in processes associated with reward and reinforcement, including feeding, stress, and psychostimulants (Dallvechia-Adams *et al.*, 2002). For instance, cocaine targets the DA system, activating G-proteins, cAMP signal transduction pathways and ultimately, pCREB. Thus, it is feasible that cocaine, which increases mesolimbic DA, may indirectly affect CART expression through the stimulation of some DA receptors and the downstream activation of CREB. Indeed, under binge dosing regimes, cocaine reliably increases CART expression (Fagergren and Hurd, 1999; Brenz-verca *et al.*, 2001; Hunter *et al.*, 2005). In addition, pCREB which, in part, is activated via the stimulation of DA receptors, has been implicated in the rewarding effects of food intake and drugs of abuse, including opiates and cocaine (Carlezon *et al.*, 1998; Nestler, 2001). Cocaine increases the phosphorylation of CREB in the NAc (Walters *et al.*, 2003) which up-regulates several genes associated with reward and reinforcement (Carlezon *et al.*, 2005). Interestingly, cocaine overdose victims

demonstrate increased levels of pCREB and CART expression, suggesting a possible relationship between the transcription factor and neuropeptide (Tang *et al.*, 2003).

Therefore, we hypothesize that *in-vivo* regulation of the CART gene is, in part, mediated by cAMP signaling and CREB. The activation of DA receptors subsequently activates G proteins and affects cAMP signaling, which in turn, increases pCREB levels and modulates CART expression. Using intra-accumbal administration of forskolin, we examined the role of the AC/cAMP/PKA/CREB pathway (Figure 1) on CART mRNA and peptide levels *in-vivo* in the rat NAc. Because i) a potential role for cocaine in CART gene regulation has been suggested and, ii) pCREB expression is increased in cocaine overdose victims, we examined the effect of cocaine on forskolin-induced (cAMP-mediated) CART mRNA levels. Understanding the regulation of CART peptide and its role in addiction will not only allow us to better understand the function of neuropeptides, but will also contribute to our understanding of the addiction process.

MATERIALS AND METHODS

Drugs. Ketaset (Ketamine HCL) was supplied by Fort Dodge Animal Health (Fort Dodge Iowa) and Domitor (medetomidine HCL) was from Pfizer Animal Health (Exton, PA). Cocaine HCL was a gift from the National Institute on Drug Abuse (NIDA; National Institute of Health, Bethesda, Maryland). N-lauroylsarcosine, dextran sulfate, and denatured salmon testis DNA were purchased from Sigma (St. Louis, Mo). 1,9-dideoxyforskolin, *Coleus forskohlii* (Forskolin), 7-Deacetyl-7-[O-(N-methylpiperazino)-g-butyryl]-, Dihydrochloride (INAF), N-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinoline-sulfonamide hydrochloride (H89), and adenosine-3', 5'-cyclic monophosphorothioate, Rp-isomer (Rp-cAMPS) were from Calbiochem (La Jolla, CA). Terminal deoxynucleotide transferase (tdt) was purchased from Amersham Biotech (Piscataway, NJ). Deionized formamide was from GibcoBRL (Gaithersburg, MD) and Denhardt's solution was from Roche Applied Science (Indianapolis, IN). Radiolabeled S^{35} was obtained from PerkinElmer Life and Analytical Sciences, Boston, MA. CREB and pCREB antibodies were from Cell Signaling Technology Inc. (Beverly, MA) and C4 CART antibody was purchased from Phoenix Pharmaceuticals Inc. (Belmont, CA).

Animals. Male Sprague-Dawley rats (Charles River Laboratories Inc., Wilmington, MA) weighing ~300g were used in all experiments. Animals were group-housed prior to surgery and individually housed thereafter. Animals were allowed access to food and water ad libitum and maintained on a 12h light/dark cycle. All experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Bilateral guide cannula surgery. Cannula surgeries were performed according to Jaworski *et al.* (2003) with modifications. Rats were anesthetized with a mixture of ketamine HCL (75 mg/kg ip) and medetomidine HCL (0.5 mg/kg ip). A bilateral stainless steel guide cannula assembly (22 gauge with a center to center distance of 3.0 mm; Plastics One, Roanoke, VA) was implanted directly over the NAc in both hemispheres via aseptic stereotaxic surgery. Stereotaxic coordinates for the guide cannula (relative to bregma) were A/P +1.7mm, M/L ± 1.5mm, D/V -5.7mm (Paxinos and Watson, 1998). Guide cannulas were anchored to the skull using dental acrylic and three stainless steel screws driven into the skull. A dummy cannula was inserted to prevent blockage and a dust cap was screwed onto the top of the assembly. The rats were allowed to recover for 7-10 days. Cannulas were successfully implanted into the NAc approximately 98% of the time, consequently minimizing the number of animals eliminated from analysis.

Intra-accumbal infusions. Stainless steel injector cannulas (28 gauge, Plastics One), which extend 2 mm beyond the tips of the guide cannulas, and thus were centered over the shell/core junction of the NAc, were used for all drug infusions. While there could be differences in CART regulation between the shell and core of the NAc, the shell/core junction was chosen as the target site for injections because, i) this is a novel experiment and CART expression in the whole NAc was examined, and ii) injections of CART peptide into the shell/core junction of the NAc attenuated the rewarding effects of cocaine (Jaworski *et al.*, 2003). The injector cannulas were attached to 10µl Hamilton syringes (Hamilton Co., Reno NV) via polyethylene-10 tubing. Rats were placed in a polyethylene box (12 x 36 x 48 cm) and all infusions were conducted on freely moving

rats. Left and right hemispheres were simultaneously infused with 0.5µl volume/side over 30 seconds using an infusion pump (PHD 2000, Harvard Apparatus, Cambridge, MA). Infusions were conducted so that each rat received drug in one hemisphere and a vehicle in the other, thus permitting each rat to serve as its own control. Injector cannulas were left in place for an additional 30 seconds to permit drug diffusion and prevent backflow. To control for possible hemispheric differences, drug infusions were alternated between hemispheres so that some animals received drug in the left and others received drug in the right hemisphere. The location of cannula injection sites was determined histologically and only animals with injector sites in the desired anatomical location, on or near the accumbal shell/core junction, were used for analysis (Figure 2).

In-situ-hybridization. *In-situ* hybridization was preformed as previously described (Hunter *et al.*, 2005). Following infusions, rats were decapitated; the brain removed and 14-µm-thick sections encompassing the injection site were cut on a cryostat and slide mounted. Sections were post-fixed in 4% paraformaldehyde followed by consecutive washes in 2X SSC, triethanolamine/0.5% acetic anhydride (0.1M), H_20, 70%, 95%, and 100% ethanol, chloroform (5%), 95% and 70% ethanol, and then air dried. Slides were incubated at 37°C in pre-hybridization buffer (50% deionized formamide, 4X SSC, 1X Denhardt's solution, 0.02M NaPO4 (pH 7.0), 1% N-lauroylsarcosine), 10% dextran sulfate) in a humidifying chamber for 2 hrs. An oligodeoxynucleotide probe complementary to rat CART mRNA (nucleotides 223-270) was synthesized by Emory Microchemical Facility (Emory University, Atlanta, GA). The probe was labeled on the 3' end with ^{35}S-dATP (NEN, Boston, MA) to a specific activity of 5 X 10^9 cpm/µl using

terminal deoxynucleotide transferase (Amersham Biotech, Piscataway, NJ) and then purified with a QIAquick Nucleotide removal kit (Qiagen Inc, Valencia, CA). Hybridization solution (pre-hybridization buffer plus 500 mg/l denatured salmon testis DNA and 200mM dithiothreitol) containing the CART probe (~5 X 10^5 cpms/slide) was applied to each section. Slides were hybridized overnight in a humidifying chamber at 42°. Post-hybridization washing consisted of successive washes in 2X SSC, 50% ethanol/0.3M ammonium acetate, 85% ethanol/0.3M ammonium acetate, 100% ethanol, and H_20. Sections were air dried and exposed to Kodak BioMax MR autoradiography film for either 10-12 days.

Autoradiogram image analysis. Levels of CART mRNA in the NAc were quantified by capturing the autoradiograms with a Photometrics CoolSNAP camera (Photometrics, Roper Scientific Inc, Tucson, AZ) with the illuminating light adjusted so that the optical densities (OD) of the probe signal fell within the linear portion of a standard curve generated from C^{14} microscales (American Radiolabeled Chemicals Inc., St Louis, MO). Analysis was conducted using MCID Basic imaging software (Imaging Research Inc, Ontario, Canada). Relative OD of the autoradioactive regions corresponding to the NAc of each hemisphere were measured using an outline with a consistent area (60 x 80 pixels) centered over the NAc shell/core junction. Magnification was held constant throughout the analysis. Measurements were taken through the injection site and without knowledge of treatment in brain slices at 2.2, 1.7, 1.6, and 1.2mM from bregma (Figure 2), which represents a major portion of the NAc.

Western immunoblot analysis. Western blot analysis for CART peptide (Vicentic *et al.*, 2004) and CREB/pCREB (Walters *et al.*, 2003) expression was carried out as previously described with modifications. Briefly, following infusions, rats were decapitated and the NAc was dissected out and frozen. Total protein was extracted in 50µl lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 5 nM acidic acid, 1 µg/ml leupeptin and 1 mM PMSF. 15-20µg total protein was loaded in 1 x sample buffer (62.5 mM Tris-HCl (pH 6.8), 2 % (w/v) SDS, 10 % glycerol and 0.01 % (w/v) bromophenol blue) onto 16% Novex® pre-cast SDS-Tris-Glycine gel (Invitrogen, Carlsbad, CA). Proteins were transferred at 30V at 4°C overnight. Membranes were blocked with 5% nonfat milk in 1 x TBS-T (Tris-Buffered Saline, 0.1% Tween-20; pH 7.6) for 1 hour, and then incubated with primary antibodies, either polyclonal antiserum to C4 peptide (CART nucleotides 61-102; 1:5000) or polyclonal antiserum to pCREB (1:1000) at 4° overnight. Chemiluminescent signal was detected by using horseradish peroxidase (HRP)-conjugated chicken anti-rabbit (1:1000) secondary antibody, and an enhanced chemiluminescence kit (ECL; Amersham, Arlington Heights, IL). For CREB detection, pCREB membranes were stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford IL) and re-probed with a polyclonal antiserum against CREB (1:1000). Quantitative analysis was conducted using Scion Image (Scion Corporation, Frederick, Maryland) and measuring the relative OD of each band.

Statistical analyses: MCID, Scion Image, and GraphPad Prism (GraphPad Software Inc, San Diego, CA) were utilized for data analysis. Data are presented as mean ± SEM. Statistical analyses were carried out by either a students t test or a one-way ANOVA followed by Tukey's post hoc test and $p<0.05$ was considered statistically significant. Comparisons made and specific analyses conducted for each experiment are detailed in the figure legends.

RESULTS

Centrally administered forskolin increased CART mRNA levels in the rat NAc. Optimal dose (Figure 3a) and time course (Figure 3b) for intra-accumbal forskolin administration were determined. Forskolin (0.5, 1.0, or 2.5 μg) was infused into either the left or right accumbens, and the vehicle (saline) was injected into the opposite hemisphere and rats were sacrificed at different times (30, 60, 90, 120 min) following treatment. Brains were removed and processed for *in-situ* hybridization to examine CART mRNA levels. Forskolin increased CART mRNA expression in the treated hemisphere in a dose-dependent manner (Figure 3a). Doses of 1.0 and 2.5μg significantly increased CART mRNA levels whereas 0.5μg had no effect. Because the difference between these doses was negligible, a dose of 1.0μg was used for further experiments with the exception of the cocaine experiment (Figure7). Time course analysis indicated that 60 min post infusion was the optimal time to sacrifice the animals (Figure 3b). Prior to 60 min there was little effect and time periods longer than 60 min (90 and 120 min) demonstrated a decreased CART mRNA levels, suggesting a transient effect. A representative autoradiogram of forskolin-induced CART mRNA expression in the left accumbens is shown in Figure 4a. As expected, CART mRNA levels in brain slices taken at the level of the hippocampus (approximately -2.8 to -3.3mM from bregma), a region of the brain distant from the injection site which expresses CART mRNA and peptide (Koylu *et al*, 1998), were unaffected by intra-accumbal forskolin injection (data not shown) indicating a local effect. To confirm the effect of forskolin on CART mRNA levels, a second experiment was conducted, infusing forskolin into one hemisphere and the vehicle or an inactive forskolin analog, 7-Deacetyl-7-[O-(N-methylpiperazino)-g-butyryl]-, Dihydro-

chloride (INAF), into the other and sacrificing the animals 60 min following treatment. Forskolin (1.0μg) significantly increased CART mRNA levels in the NAc approximately two-fold over saline and INAF (Figure 4b). Finally, as a positive control for forskolin-induced pCREB activity, preprodynorphin (PPD) mRNA levels, which are known to increase in response to forskolin and are regulated by pCREB (Simpson and McGinity, 1994), were measured with *in-situ* hybridization using an oligonucleotide targeting rat PPD mRNA. As expected, intra-accumbal forskolin injections increased PPD mRNA levels in a PKA-dependent manner (data not shown).

Inhibition of PKA activity attenuated forskolin-induced increase in CART mRNA. Forskolin-induced stimulation of adenylyl cyclase increases cAMP levels, activates PKA, and increases pCREB levels (Simpson and McGinity, 1994). Phosphorylation of CREB by PKA is a necessary step in the activation of CREB, permitting it to regulate transcriptional activity. Consequently, inhibition of PKA activity should block forskolin's cAMP-mediated activation of CREB and subsequently, should attenuate forskolin-induced CART mRNA expression. H89 or Rp-cAMPS, PKA inhibitors with different mechanisms of action, were injected into one hemisphere 20 min prior to the administration of forskolin (1.0μg) into both hemispheres. The doses chosen for intra-accumbal administration of H89 (2μg) and Rp-cAMPS (2μg) were based on previous studies (Sutton *et al.*, 2000; Cervo *et al.*, 1997). Both H89 and Rp-cAMPS attenuated forskolin-induced CART mRNA expression by approximately 30% and 40%, respectively (Figure 5), indicating the involvement of PKA in CART gene regulation. Interestingly, hemispheres treated with Rp-cAMPS alone demonstrated a significant

decrease in CART mRNA expression compared to control levels (Figure 5), suggesting CART gene regulation may be under the tonic regulation of PKA. Representative autoradiograms of forskolin-induced (and PKA attenuated) CART mRNA expression in the rat NAc are shown in Figure 6.

Cocaine potentiated forskolin-induced CART mRNA expression in the rat NAc. Interestingly, both pCREB and CART have been linked to the rewarding effects of cocaine (Carlezon *et al.*, 1998; Jaworski *et al.*, 2003; McClung and Nestler, 2003; Couceyro *et al.*, 2005; Hunter *et al.*, 2005). Consequently, it was hypothesized that if pCREB modulates the effects of cocaine *and* regulates the expression of the CART gene, then cocaine may potentiate forskolin-induced CART mRNA expression. Indeed, acute systemic administration of cocaine (20 mg/kg; ip) immediately following intra-accumbal infusions of forskolin (0.5μg) significantly increased CART mRNA expression in the rat NAc compared to forskolin or cocaine alone (Figure 7). A dose of 0.5μg forskolin (rather than a near maximal dose of 1.0μg) was used because it produces a minimal effect on CART expression and thus should show clear potentiation with cocaine. Consistent with other reports (Vrang *et al.*, 2002; Marie-Claire *et al.*, 2003; Hunter *et al.*, 2005) a single dose of cocaine failed to increase CART expression. However, in combination with forskolin, CART mRNA levels increased by approximately 42% compare to forskolin alone suggesting that, under conditions of enhanced cAMP signaling, cocaine may influence CART levels.

Intra-accumbal forskolin administration increased CART peptide levels in the rat NAc. Because the CART peptide, not the mRNA, is functionally active, it is important to understand not only the regulation of mRNA, but also the regulation of CART peptide. Therefore, we measured CART peptide levels in the NAc following intra-accumbal forskolin administration. Western immunoblot analysis showed that intra-accumbal forskolin administration significantly increased CART peptide levels in the rat NAc (Figure 8). Accumbi treated with forskolin had CART peptide levels 2.5 fold greater than those of saline (control) treated accumbi, suggesting that changes in CART mRNA expression lead to changes in CART peptide levels. Inhibition of PKA with H89 attenuated forskolin-induced CART peptide expression. Therefore, it appears that, similar to CART mRNA, CART peptide levels in the rat NAc are regulated, at least in part, by AC/cAMP/PKA-mediated signaling.

Intra-accumbal forskolin administration increased pCREB levels. We hypothesized that cAMP/PKA-mediated phosphorylation and activation of CREB is a key mediator in the regulation of CART expression in the rat NAc. This hypothesis was examined by using forskolin to activate, and PKA inhibitors to block, the cAMP/PKA second messenger system. To confirm that forskolin is indeed stimulating the phosphorylation of CREB in our model, accumbal tissue from forskolin-treated animals was subjected to western blot analysis with antibodies targeting pCREB and CREB. Consistent with other reports (Simpson and McGinity, 1994), central forskolin administration significantly increased pCREB levels while simultaneously decreasing the amount of CREB protein in the rat NAc (Figure 9). Inhibition of PKA with H89 attenuated pCREB expression. Although this finding does not directly implicate pCREB in CART gene regulation, it

provides evidence indicating that intra-accumbal forskolin injections increase the phosphorylation of CREB and hence, may be involved in regulating CART mRNA and peptide levels.

DISCUSSION

CART neuropeptides have been implicated in the reward/reinforcement and addictive properties of psychostimulants, including cocaine and amphetamine (Jaworski *et al.*, 2003; Kim *et al.*, 2003 Kuhar *et al.*, 2005; Couceyro *et al.*, 2005). The regulatory pathways involved in CART expression in the brain have yet to be elucidated. Our hypothesis states that pCREB ultimately regulates expression of the CART gene via AC/cAMP/PKA-mediated signaling (Figure 1). Consequently, manipulations of this pathway should affect CART mRNA and peptide levels. Accordingly, various stimulators and inhibitors of the AC/cAMP/PKA pathway were centrally administered and CART mRNA and peptide, as well as pCREB, levels in the rat NAc were measured. As predicted by the model, forskolin increased CART mRNA levels, an effect attenuated by the inhibition of PKA. Interestingly, Rp-cAMPS alone reduced mRNA levels compared to controls indicating a tonic regulation by PKA on CART gene expression. Forskolin significantly increased CART peptide levels in a manner consistent with the observed increase in CART mRNA levels. Finally, cocaine potentiated forskolin-induced CART mRNA expression in the accumbens suggesting that, under conditions of enhanced cAMP signaling, cocaine may affect CART-containing neurons. Forskolin increases CART mRNA expression in cell culture (Dominguez *et al.*, 2002; Lakatos *et al.*, 2002; Barrett *et al.*, 2002) and mutations of the CRE binding site in the CART promoter decrease CART promoter activity (Dominguez and Kuhar, 2004). To our knowledge, these findings are the first to provide evidence demonstrating CART gene regulation via AC/cAMP/PKA-mediated signaling *in-vivo* in the rat NAc, likely via the transcriptional activity of pCREB.

Inhibition of PKA attenuated forskolin-induced CART expression. Inhibition of forskolin-induced CART mRNA expression appears greater with Rp-cAMPS than with H89. Reasons for this difference are unclear; however, differences in the drugs mechanism of action could provide some insight. H89 inhibits the phosphorylation process by blocking ATP binding sites on PKA. Thus, like most ATP-site inhibitors, H89 may have effects on other protein kinase pathways and ATP receptors within the cell. Rp-cAMPS, on the other hand, is highly specific for PKA, working a step earlier in the activation process by inhibiting the dissociation of the catalytic and regulatory units, thus preventing any possible physiological actions by the regulatory units. Indeed, numerous differences between the inhibitory actions of H89 and Rp-cAMPS have been reported (Biolog Life Sci Institute. Tech Info No. 1003). Regardless of mechanism, the present results indicate that both PKA inhibitors significantly attenuated forskolin induced CART mRNA expression, providing strong evidence for the involvement of PKA in the regulation of the CART gene.

Interestingly, in addition to attenuating forskolin-induced CART expression, Rp-cAMPS alone significantly reduced CART mRNA levels compared to controls, suggesting that the expression of CART may be, in part, tonically regulated by PKA. Although treatment with forskolin stimulated CART expression, CART mRNA was also detected in control samples (vehicles and INAF; Figure 4) indicating basal expression of CART in the NAc. Consequently, perhaps CART expression is under the tonic regulation of PKA. Receptor-mediated signal transduction pathways are often under a continuous basal level of signaling independent of receptor stimulation. Protein kinases and phosphatases, for example, may regulate basal level expression of multiple proteins by

spontaneously activating (phosphorylation) and de-activating (de-phosphorylation) targeted proteins. The steady state level of expression in un-stimulated cells, therefore, is the consequence of a balance between positive and negative regulators of a given pathway. Receptor stimulation produces an increase in expression over basal levels. The finding that inhibition of PKA decreases CART expression compared to basal levels suggests that CART expression in the NAc may be tonically regulated by PKA. Consequently, other secondary signaling pathways that ultimately converge on PKA and CREB may also have a role in CART gene regulation. CREB is activated by multiple cell surface receptors and intracellular signaling cascades, including neurotrophin, glutamate, NMDA, and g-protein coupled receptors, as well as L-type Ca^{2+} channels (Carlezon *et al.*, 2005). Ca^{2+} mediates multiple signaling pathways that result in the phosphorylation of CREB (Carrion *et al.*, 1999) and the subsequent expression of proteins associated with addiction including dynorphin, fos, CRF, and BDNF (Carlezon *et al.*, 2005). Therefore, it is possible that Ca^{2+}-mediated signaling may participate in CART gene regulation. Interestingly, several of the biological processes that CART is associated with, including feeding, anxiety/fear, and psychostimulant addiction have also been linked to increases in pCREB, supporting the contention that CREB is associated with CART.

Although CREB is regulated by multiple signaling pathways and in turn, regulates several genes associated with reward and reinforcement, the present study focused on a single CREB-regulatory pathway (ie. cAMP/PKA) because in the NAc, this pathway has been closely linked to the addictive properties of drugs of abuse. Adenylyl cyclase proteins are plasma membrane-bound and coupled upstream to g-proteins. DA, which activates multiple g-protein coupled receptors ultimately affecting cAMP signaling and pCREB-mediated gene transcription,

modulates the effects of many drugs of abuse, including cocaine (Ikegami and Duvauchelle, 2004). Therefore, because, 1) the rewarding effects of cocaine are partially modulated by pCREB (Carlezon *et al.*, 1998; McClung and Nestler, 2003), and 2) cocaine increases CART mRNA levels (Fagergren and Hurd, 1999; Brenz Verca *et al*, 2001; Hunter *et al.*, 2005), it is plausible to suggest that DA receptors may regulate CART expression. Indeed, CART expression in the NAc may be partially regulated via DA D3 receptors (Beaudry *et al.*, 2004; Hunter *et al.*, in press). Moreover, dopaminergic nerve terminals in the NAc synapse on CART-containing neurons (Koylu *et al.*, 1998; Smith *et al.*, 1999) and CART and DA receptor mRNAs are co-localized (Beaudry *et al.*, 2004), providing the proximity required for neurotransmitter signaling. Finally, CART levels are affected by glucocorticoids (Vicentic *et al.*, 2004; Hunter *et al.*, 2005), which appear to modulate the dopaminergic system (Czyrak *et al.*, 2003). These studies suggest that dopamine may play a role in regulating CART gene expression. This is relevant because drugs of abuse, including cocaine, ultimately lead to the stimulation of DA receptors, thus, cocaine may indirectly impact the CART gene.

Studies of the relationship between cocaine and CART have not been without controversy. Douglass *et al.* (1995) reported that cocaine increased CART mRNA levels. Although this finding has been difficult to replicate (Vrang *et al.*, 2002; Marie-Claire *et al*, 2003), it appears that a binge-dosing regime, rather than acute administration of cocaine, more reliably increases CART mRNA levels (Fagergren and Hurd, 1999; Brenz Verca *et al*, 2001; Hunter *et al.*, 2005). The effect of acute cocaine on forskolin-induced CART gene expression was examined. Cocaine significantly potentiated forskolin-induced CART mRNA expression in the rat NAc compared to either of the drugs administered

independently. Therefore, cocaine appears to impact the regulation of the CART gene under conditions of enhanced cAMP signaling, which, interestingly, is increased in the VTA of human cocaine overdose victims (Tang *et al.*, 2003) and increased cAMP/CREB expression in the addiction process has been associated with the development of dependence and tolerance to several common drugs of abuse (Nestler, 2004). Thus, it is plausible to suggest that perhaps CART levels are regulated by cocaine in a chronic abuser, whose basal cAMP signaling is enhanced due to constant cocaine use. Moreover, both manipulations of the cAMP pathway (Knapp *et al.*, 2001; Carlezon *et al.*, 1998) and central administration of CART peptide (Jaworski *et al.*, 2003; Kim *et al.*, 2003) modulate some of the behavioral effects of cocaine. These findings are compatible with the view that CART peptides are involved in the rewarding properties of cocaine. Interestingly, as CART peptides appear to oppose some of cocaine's effects, the stimulatory effect of cocaine on CART expression suggests the existence of an endogenous feedback mechanism meant to decrease the behavioral effects of cocaine (i.e. cocaine increases CART which in turn attenuates the effects of cocaine).

CART peptides are involved in the modulation of the brain's reward circuitry, appearing to oppose the reinforcing properties of several processes, including feeding and drug addiction. Therefore, because CART may serve as a therapeutic target for obesity and drug addiction, understanding its regulation is important. The present results demonstrate the *in-vivo* regulation of the CART gene. CART mRNA and peptide levels in the rat NAc were elevated following intra-accumbal activation of adenylyl cyclase, an effect attenuated by PKA inhibitors, suggesting that CART gene expression in the rat NAc is regulated, in part, via cAMP signaling and, most likely, through pCREB. Cocaine potentiated

forskolin-induced CART expression suggesting that cocaine may participate in the regulation of the CART gene, likely via its effects on DA receptors and cAMP/PKA-mediated signaling. These results provide a basis for future studies on the mechanisms underlying the *in-vivo* regulation of CART expression and its complex relationship with cocaine, feeding, anxiety/fear, and other processes associated with the brain's reward and reinforcement pathways.

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FIGURE LEGENDS

Figure 1. Schematic of experimental hypothesis. Briefly, we hypothesize that; **(1)** forskolin-induced stimulation of AC at the plasma membrane will increase CART mRNA and peptide levels in the rat NAc via cAMP/PKA/CREB-mediated signaling. **(2)** AC converts ATP to cAMP, which in turn activates cytoplasmic PKA **(3)** by releasing the catalytic units (C) from the regulatory units (R). **(4)** The catalytic units of PKA then cross the nuclear membrane and phosphorylate (P) inactive CREB, which is associated with the cAMP response element (CRE) site of the CART promoter, to form transcriptionally active pCREB. **(5)** With the help of co-activators, including CREB-binding protein (CBP), pCREB acts on the promoter to drive the expression of the CART gene resulting in increased levels of CART mRNA and peptide.

Figure 2. Anatomical location of microinjection sites in the NAc. Brain images (Paxinos and Watson, 1998) corresponding to the NAc are overlapped and injection sites represented by black dots. Not all sites are visible due to overlap. The majority of injector sites are located +1.6-1.7 mm from bregma as desired, and only 3 animals were eliminated from analysis due to improper cannula placement. Quantitative analysis was done using an outline with a consistent area (60 x 80 pixels) centered over the accumbens shell (NAcS) and core (NAcC) and measuring relative OD in the NAc on brain slices comprising the injection site (2.2, 1.7, 1.6, and 1.2 mm from bregma).

Figure 3. Dose-response (A) and time-course (B) for forskolin-induced CART mRNA expression in the rat NAc. **(A)** Forskolin (0.5, 1.0, or 2.5μg in 0.5 μl volume)

was injected into one hemisphere and the vehicle (saline) into the contra-lateral hemisphere and animals (n = 3) were sacrificed 60 min following infusion. **(B)** Forskolin (2.5μg) was injected into one hemisphere, saline into the other and animals were sacrificed at 30, 60, 90, or 120 min following infusion. For each animal, data from accumbal slices taken within the injection site (8-12 slices per animal) were averaged prior to analysis. Quantitative analysis was conducted by measuring the relative OD of the radioactive signal at the level of the NAc (bregma 1.2-2.2). Data is expressed as the mean ± SEM and significance was tested with a one-way ANOVA and Tukey's post hoc test. Overall F and P values are [$F(3, 11) = 425.4$, $p < 0.001$] for panel A and [$F(4, 14) = 30.71$, $p < 0.001$] for panel B. Significant differences from vehicle control are represented with asterisks (*; $p < 0.001$). Differences from 0.5μg in panel A, and from 60 min in panel B, are represented with daggers (†; $p < 0.01$).

Figure 4. Effect of forskolin on CART mRNA expression in the rat NAc. Forskolin (1.0μg; vehicle A, saline) was injected into the NAc of one hemisphere and either saline or INAF (1.0μg; vehicle B, DMSO/saline; 1:1) was injected into the contra-lateral hemisphere and animals (n = 8) were sacrificed 60 min following treatment. A second set of animals received vehicle A in one hemisphere and vehicle B in the other and no significant difference was detected between vehicles. **(A)** Representative autoradiogram showing significantly increased CART mRNA levels in the forskolin-treated left hemisphere (LH) compared to the INAF-treated right hemisphere (RH). **(B)** For each animal, data from accumbal slices taken within the injection site (8-12 slices per animal) were averaged prior to analysis**.** Quantitative analysis was conducted by measuring the

relative OD of the radioactive signal at the level of the NAc (Bregma 1.2-2.2). Data is expressed as the mean ± SEM and significance was tested with a one-way ANOVA and Tukey's post hoc test. Overall F and P values are [$F(3, 31) = 18.45$, $p < 0.001$]. Significant differences from vehicle A (control) are represented with asterisks (*; $p < 0.001$) and differences from INAF are represented with daggers (†; $p < 0.001$).

Figure 5. Inhibition of PKA decreased CART mRNA levels and attenuated forskolin-induced CART expression in the rat NAc. In one group of animals (n = 8), either H89 (2μg) or Rp-cAMPS (2μg) was injected into the NAc of one hemisphere and the vehicle (DMSO/saline; 1:1) into the other; Rp-cAMPS inhibited CART mRNA expression compared to controls (4th bar from left). A second group of animals was administered H89 or Rp-cAMPS into one hemisphere 20 min prior to the administration of forskolin (1.0μg) into both hemispheres; inhibition of PKA attenuated forskolin-induced CART mRNA expression (right two bars). For each animal, data from accumbal slices taken within the injection site (8-12 slices per animal) were averaged prior to statistical analysis**.** Quantitative analysis was conducted by measuring the relative OD of the radioactive signal at the level of the NAc (bregma 1.2-2.2). Data is expressed as the mean ± SEM and significance was tested with a one-way ANOVA and Tukey's post hoc test. Overall F and P values are [$F(5, 47) = 35.24$, $p < 0.001$]. Significant differences from control are represented with asterisks (*; $p < 0.001$) and differences from forskolin are represented with daggers (†; $p < 0.01$).

Figure 6. Representative autoradiograms showing the effects of PKA inhibition on forskolin-induced CART mRNA expression in the rat NAc. (**A)** Injection of Rp-cAMPS into the left hemisphere (LH) decreased CART mRNA expression compared to the vehicle-injected (control) right hemisphere (RH). **(B)** H89 was injected into the left hemisphere 20 min prior to the administration of forskolin into both hemispheres. Inhibition of PKA attenuated forskolin-induced CART expression.

Figure 7. Cocaine potentiated forskolin-induced CART mRNA expression in the rat NAc. Animals (n = 8) received; **a)** an acute dose of cocaine (20mg/kg; ip), **b)** intra-accumbal forskolin injections (0.5μg), or **c)** acute cocaine immediately following intra-accumbal infusions of forskolin. Animals were sacrificed 60 min following forskolin administration. Cocaine alone had no effect on CART mRNA levels compared to controls; however, in combination with forskolin, it significantly potentiated CART mRNA expression over forskolin alone. For each animal, data from accumbal slices taken within the injection site (8-12 slices per animal) were averaged prior to statistical analysis**.** Quantitative analysis was conducted by measuring the relative OD of the radioactive signal at the level of the NAc (bregma 1.2-2.2). Data is expressed as the mean ± SEM and significance was tested with a one-way ANOVA and Tukey's post hoc test. Overall F and P values are [$F(3, 31) = 143.5$, $p < 0.001$]. Significant differences from control (and cocaine) are represented with asterisks (*; $p < 0.001$) and differences from forskolin treated animals are represented with daggers (†; $p < 0.01$).

Figure 8. Forskolin increased CART peptide levels in the rat NAc. Western immunoblot analysis for CART peptide following intra-accumbal administration of forskolin (1.0μg) revealed increased CART peptide levels. Authentic rat C4 peptide (CART 55-102; Peptide International, Louisville, Ky) was used as positive control. Immunoblots are representative of the accumbi from either the left or right hemisphere of a single animal. Quantitative results are representative of 3 experiments using accumbal tissue from 3 different animals from each treatment group. Data is expressed as the mean ± SEM (n=3) and significance was tested with a one-way ANOVA and Tukey's post hoc test. Overall F and P values are [$F(3, 8) = 79.1$, $p < 0.001$]. Significant differences from saline-injected controls are represented with asterisks (*; $p < 0.01$) and differences from forskolin alone are represented with daggers (†; $p < 0.01$).

Figure 9. Forskolin induced pCREB expression in the rat NAc. Western immunoblot analysis for pCREB and CREB expression following intra-accumbal administration of forskolin revealed increased pCREB and decreased CREB protein levels. Animals were treated as follows; one group was sham infused, a second group received forskolin (1 or 2μg) in one hemisphere and either saline or INAF (1μg) in the contra-lateral hemisphere, and a third group received H89 (2μg) in one hemisphere followed by forskolin (1μg) in both hemispheres. For each animal the NAc was dissected out and separated into left and right hemispheres for analysis. Immunoblots were probed for pCREB, stripped, and re-probed for CREB. A strong band of approximately 43 kDa representing immunoreactive pCREB was detected in forskolin treated accumbi. H89, a PKA inhibitor attenuated forskolin-induced pCREB expression. Strong bands of CREB immunoreactivety were

detected in control samples whereas forskolin treated accumbi showed a trend towards decreased CREB levels. Immunoreactive bands shown are representative of the accumbi from either the left or right hemisphere of a single animal. Quantitative results are representative of 3 experiments using accumbal tissue from 3 different animals from each treatment group. Data is expressed as the mean ± SEM (n=3) and significance was tested with a one-way ANOVA and Tukey's post hoc test. Overall F and P values are [$F(5, 12) = 633.8$, $p < 0.001$] for pCREB and [$F(5, 12) = 35.78$, $p < 0.001$] for CREB. Significant differences from saline-injected controls (and INAF) are represented with asterisks (*; $p < 0.01$) and differences from forskolin (2μg) are represented with daggers (†; $p < 0.01$).

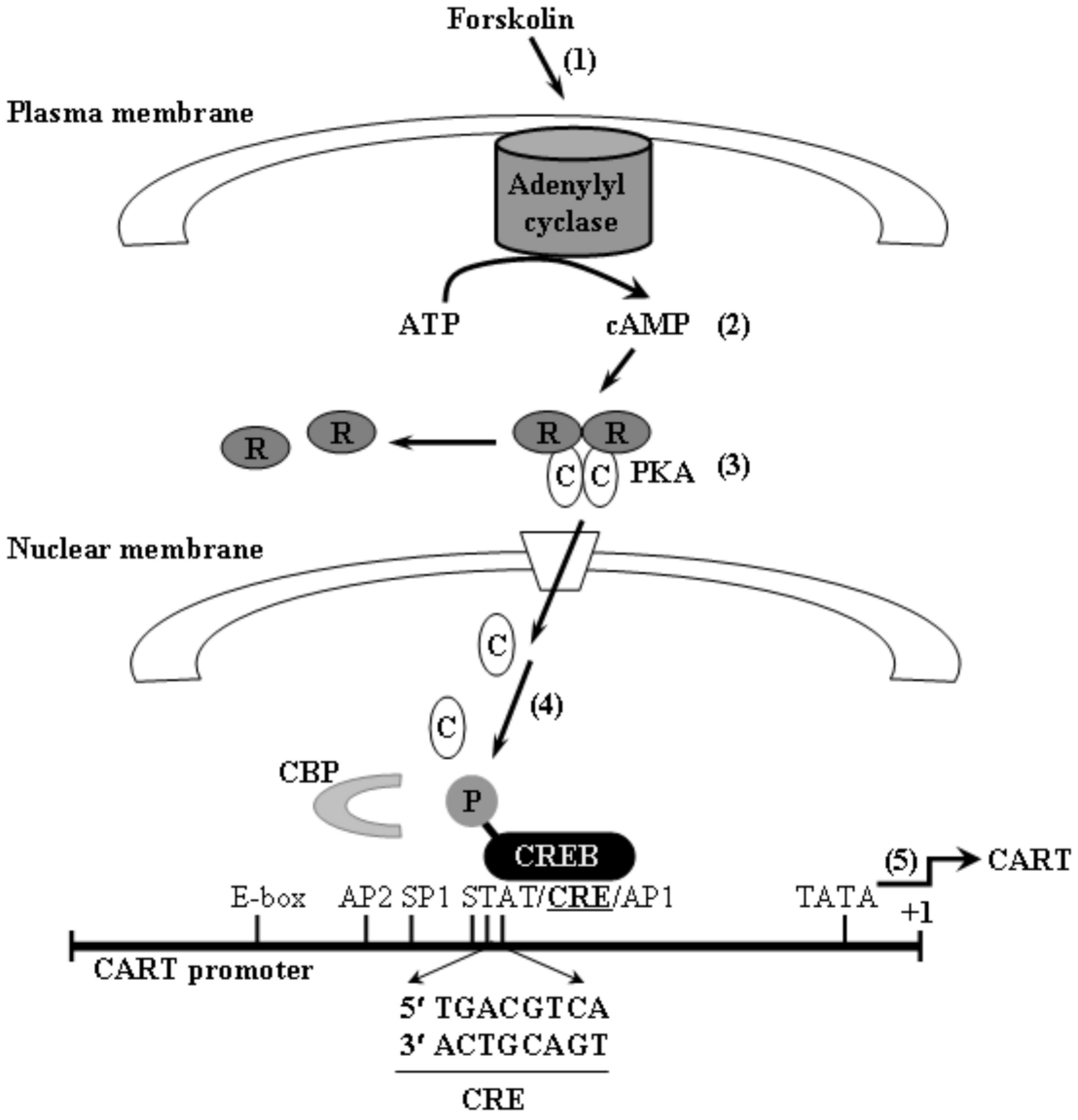
Forskolin
(1)
Plasma membrane
Adenylyl cyclase
ATP
cAMP
(2)
R
R
R
R
C
C
PKA
(3)
Nuclear membrane
C
C
(4)
CBP
P
CREB
(5)
CART
E-box
AP2
SP1
STAT/CRE/AP1
TATA
+1
CART promoter
5′ TGACGTCA
3′ ACTGCAGT
CRE

Figure 1

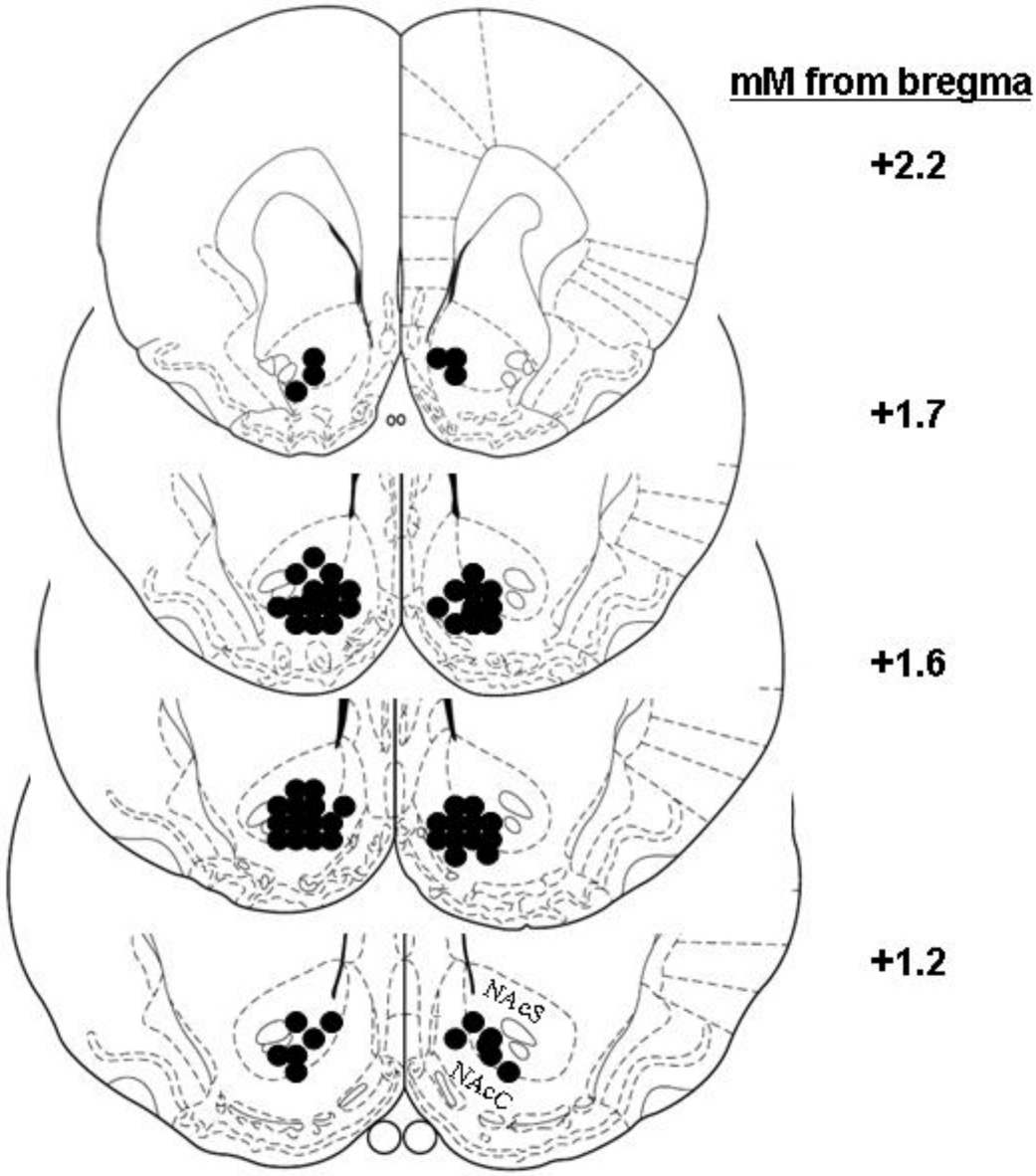

Figure 2

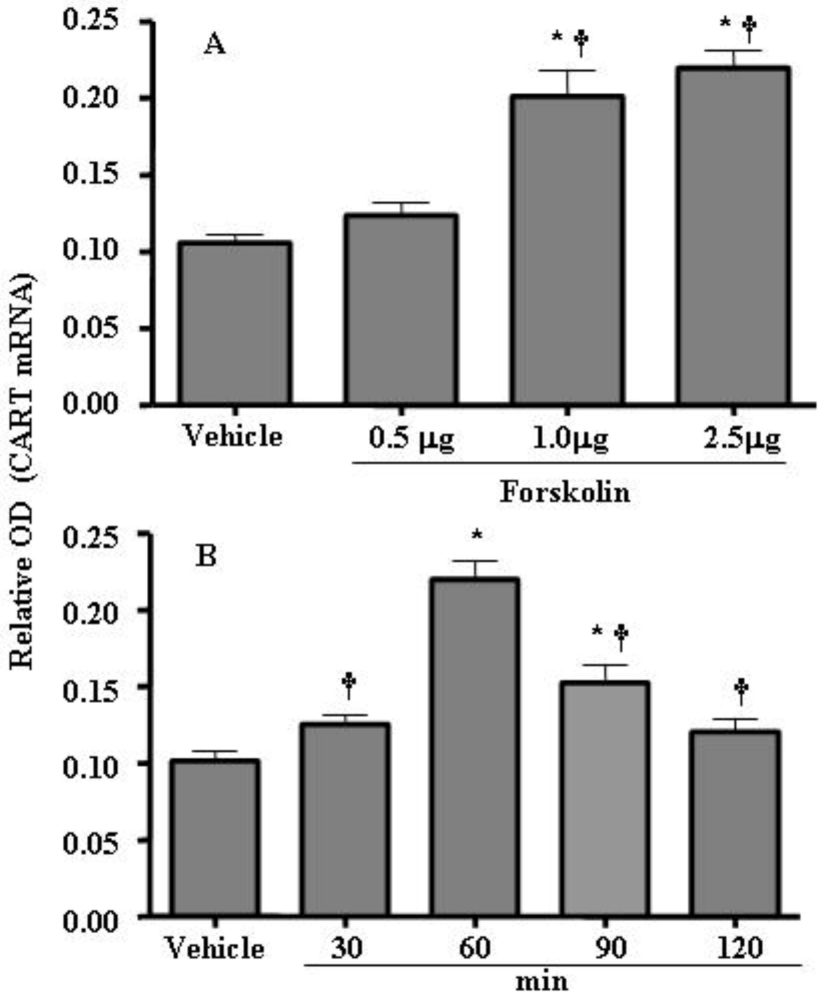
A
B
Relative OD (CART mRNA)
0.25
0.20
0.15
0.10
0.05
0.00
Vehicle
0.5 µg
1.0µg
2.5µg
Forskolin
30
60
90
120
min
*
†

Figure 3

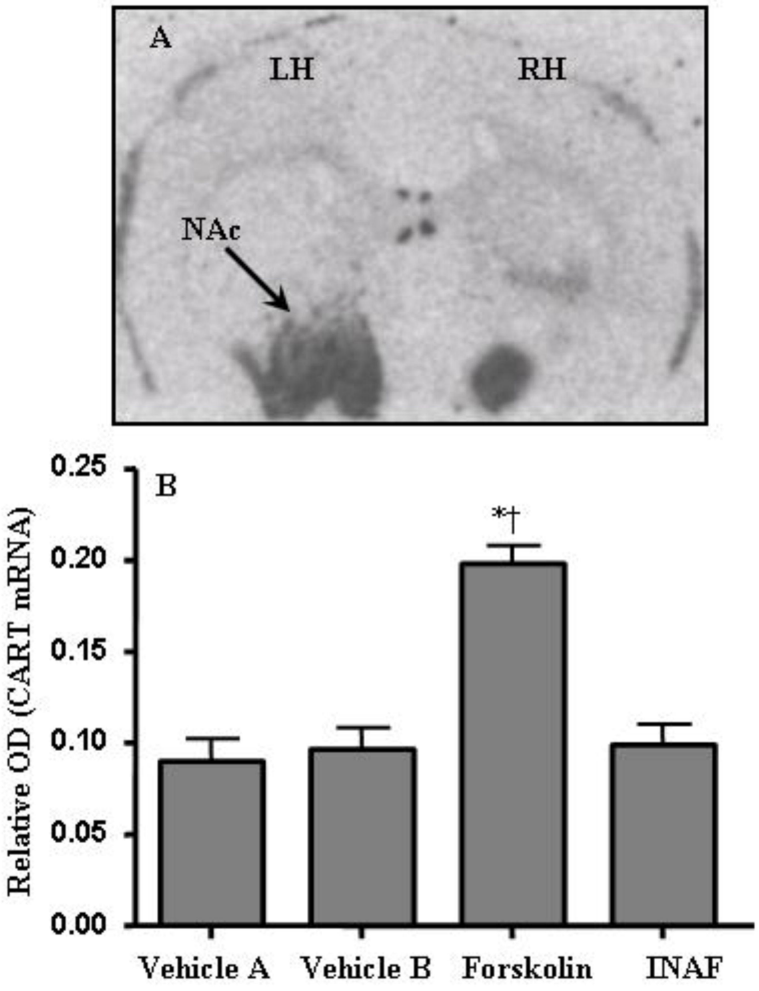
A
LH
RH
NAc
B
*†
Relative OD (CART mRNA)
0.25
0.20
0.15
0.10
0.05
0.00
Vehicle A
Vehicle B
Forskolin
INAF

Figure 4

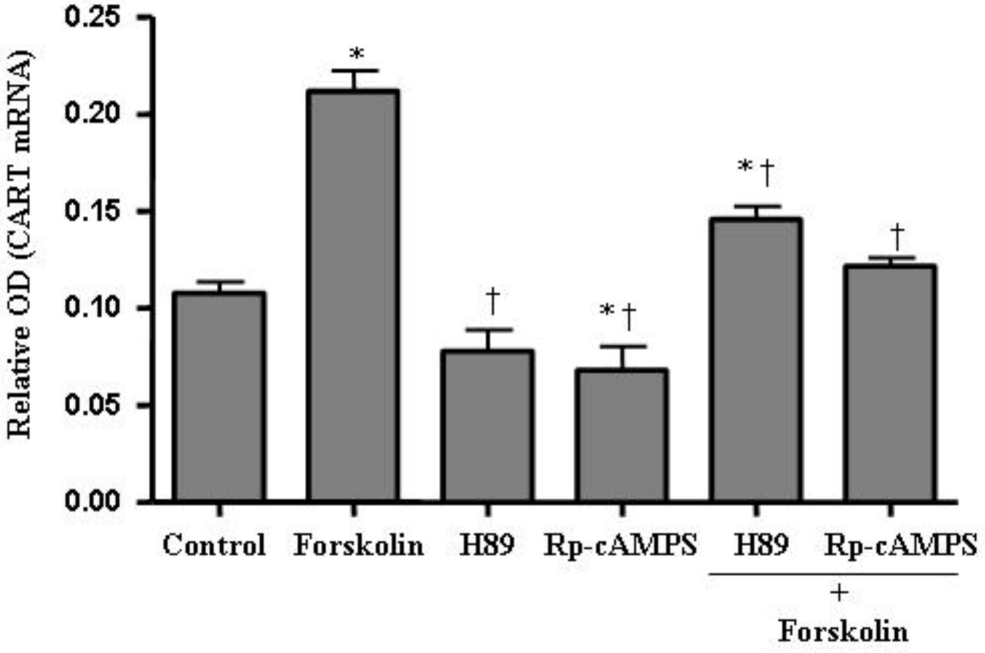
Relative OD (CART mRNA)
0.25
0.20
0.15
0.10
0.05
0.00
*
†
*†
*†
†
Control
Forskolin
H89
Rp-cAMPS
H89
Rp-cAMPS
+
Forskolin

Figure 5

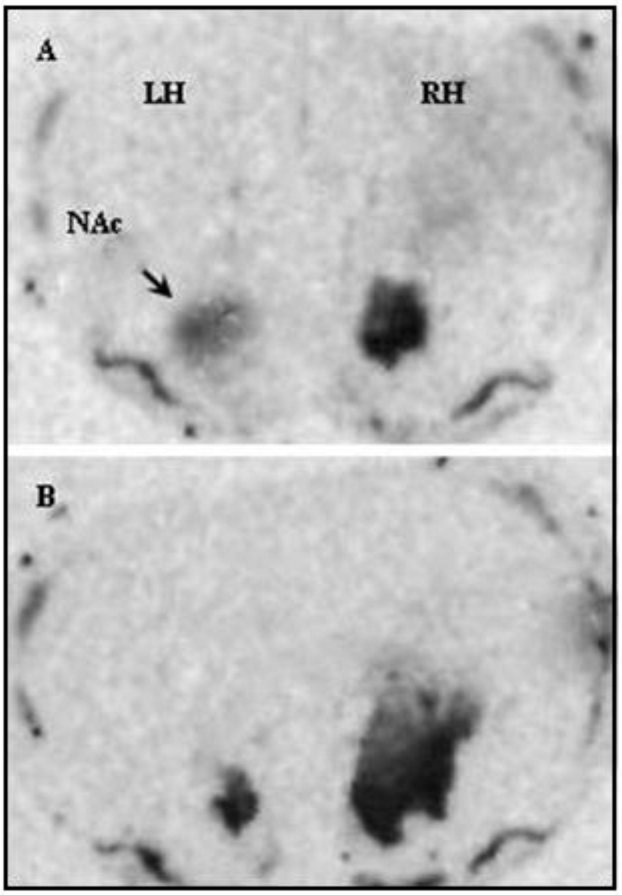

Figure 6

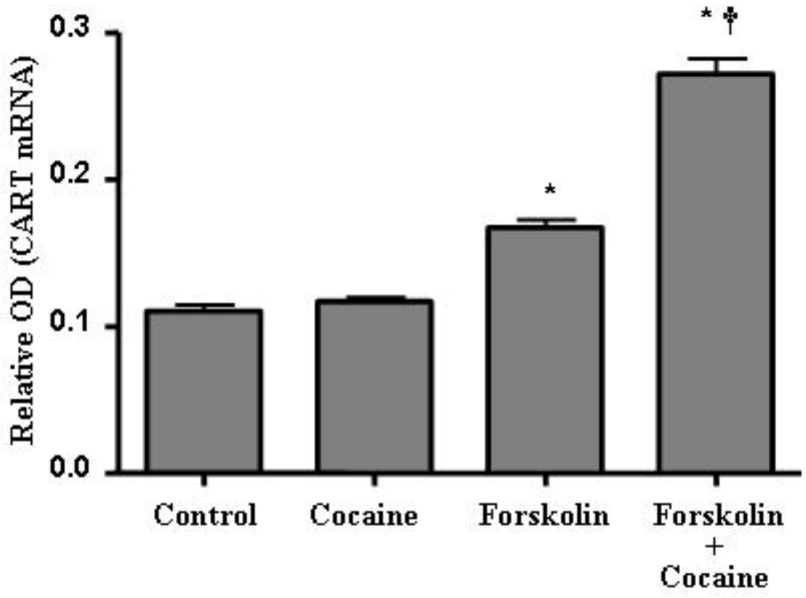
Relative OD (CART mRNA)
0.3
0.2
0.1
0.0
*
* †
Control
Cocaine
Forskolin
Forskolin + Cocaine

Figure 7

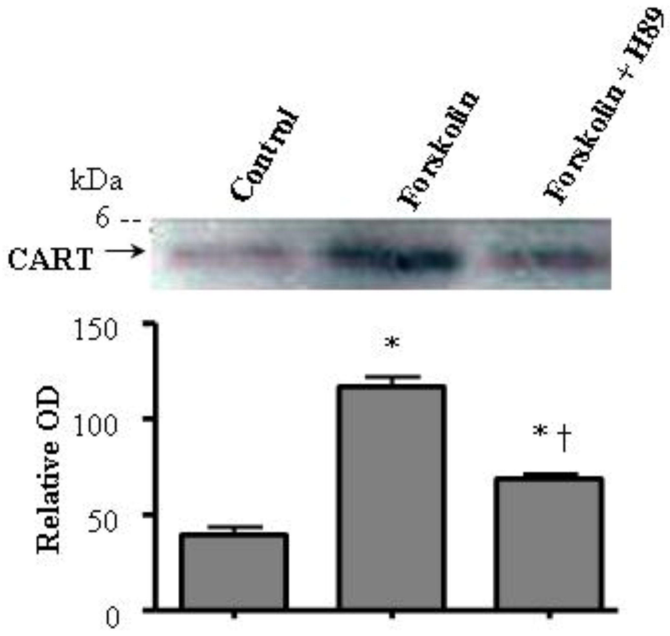
Control
Forskolin
Forskolin + H89
kDa
6 --
CART
150
100
50
0
Relative OD
*
* †

Figure 8

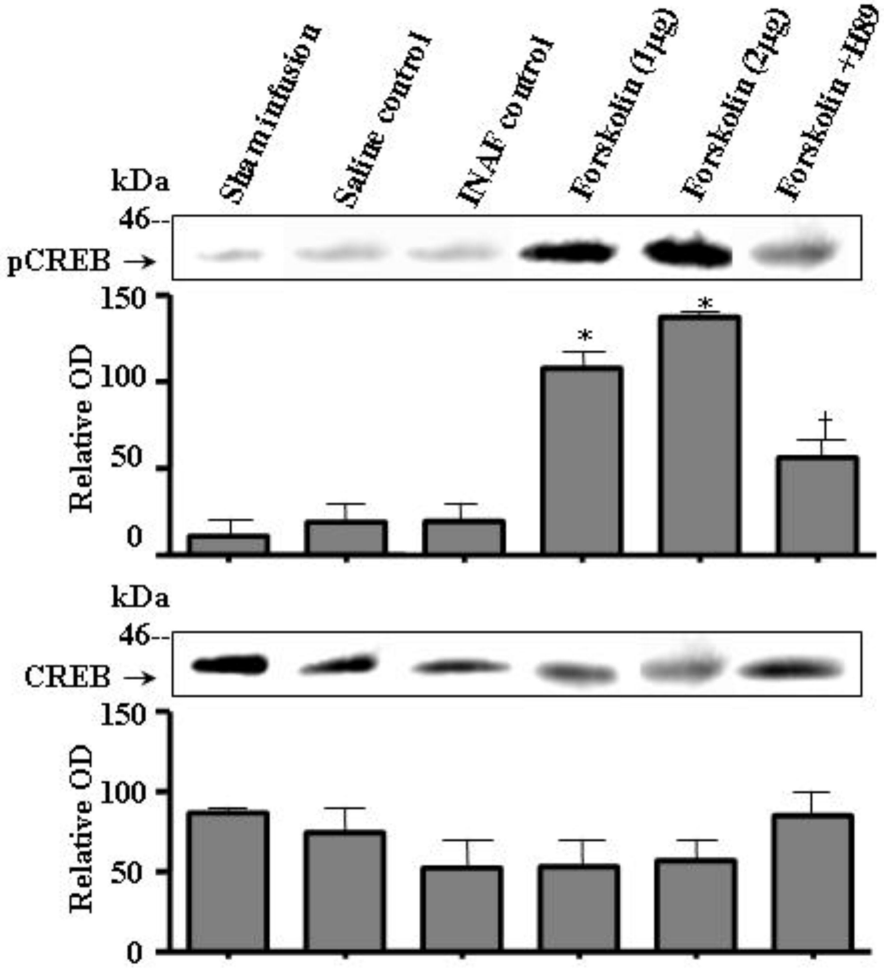
Sham infusion
Saline control
INAF control
Forskolin (1μg)
Forskolin (2μg)
Forskolin +H89
kDa
46--
pCREB →
150
100
50
0
Relative OD
*
*
†
kDa
46--
CREB →
150
100
50
0
Relative OD

Figure 9