Behavioral Neuroscience
2007, Vol. 121, No. 1, 212–217

0735-7044/07/$12.00 DOI: 10.1037/0735-7044.121.1.212

BRIEF COMMUNICATIONS

Differential Activation of cAMP Response Element Binding Protein in Discrete Nucleus Accumbens Subregions During Early and Late Cocaine Sensitization

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The present study examined the differential cocaine-induced activation of the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) throughout discrete zones of analysis of the nucleus accumbens (NAc) in rats. CREB-dependent gene transcription, which may underlie long-lasting drug-induced changes in behavior and the subjective effects of cocaine, varies depending on the stage of drug exposure or withdrawal and the cell population involved. Using immunohistochemistry, the authors analyzed changes in CREB phosphorylation in the NAc after 5 days of cocaine, a short or long drug-free period, and a subsequent challenge injection. The NAc shell was separated into 5 zones of analysis previously defined by neurochemistry and connectivity. Repeated cocaine resulted in CREB phosphorylation in all analyzed subregions of the NAc excluding the most ventrolateral region of the shell 2 weeks after cessation of repeated cocaine, but rats challenged after 2 drug-free days yielded a more localized activation of CREB in the 3 most dorsomedial zones of the shell. The temporal and anatomical determinants of cocaine-induced CREB activity may indicate functional differences among NAc shell subregions and suggest the involvement of CREB in early and late cocaine effects.

Keywords: CREB, psychostimulants, immunohistochemistry, behavioral sensitization

The neuroadaptations associated with cocaine sensitization occur in mesolimbic structures, particularly in the ventral tegmental area (VTA; McFarland & Kalivas, 2001; Vezina & Stewart, 1989, 1990), nucleus accumbens (NAc; Nestler, 2001; Robinson & Kolb, 1999), and medial prefrontal cortex (mPFC; Sorg, Davidson, Kalivas, & Prasad, 1997). One way to study these cellular changes in response to psychostimulants is to make a survey of changes in transcription factor expression. Transcription factor proteins modulate the expression of other genes involved in activation, differentiation, and plasticity. Expression of several transcription factors is altered in response to repeated psychostimulant exposure and has been causally linked to the subjective effects of these drugs. One such transcription factor is the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB). Administration of drugs of abuse cause CREB activation, via phosphorylation (Yamamoto, Gonzalez, Biggs, & Montminy, 1988), in the NAc (Walters, Kuo, & Blendy, 2003) and dorsal striatum (Kano, Suzuki, Shibuya, Kiuchi, & Hagiwara, 1995). Introduction of herpes simples (HSV)-CREB virus into the NAc reduces the rewarding effects of cocaine (Carlezon et al., 1998), and upregulation of CREB in the NAc by cocaine and other drugs mediates tolerance to the reinforcing effects of the drugs and may mediate a state of aversion or dysphoria during drug withdrawal (Barrot et al., 2002).

Repeated cocaine administration in rats leads to sensitization, as evidenced by an increased locomotor response to an acute (challenge) dose after a drug-free period. This sensitization has been shown to facilitate acquisition of a conditioned place preference or drug self-administration behavior (Horger, Shelton, & Schenk, 1990; Piazza, Deminiere, LeMoal, & Simon, 1989). Therefore, changes in locomotor sensitization may reflect changes in mechanisms that are responsible for the subjective effects of cocaine (Carlezon & Nestler, 2002).

Transcription factor expression changes within the NAc that correspond to the development of sensitization depend on both the period of time lapsed since repeated cocaine exposure, as well as on the cell population examined (Brenhouse & Stellar, 2006; Todtenkopf, Mihalakopoulos, & Stellar, 2002). Because CREB has been shown to mediate the behavioral response to cocaine, the pattern of CREB activation during early and late withdrawal from cocaine could further explain the differential role of discrete NAc subregions in the circuitry mediating psychostimulant sensitization. In the present study, changes in phosphorylated CREB immunoreactivity (pCREB-ir) in response to repeated cocaine exposure were analyzed in the NAc after 2 or 14 drug-free days. For anatomical analysis, the shell of the NAc was subdivided into its five functionally distinct segments: the vertex (NAc_{VERT}), arch (NAc_{ARCH}), cone (NAc_{CONE}), intermediate zone (NAc_{INT}), and

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We express thanks to Christine Konradi for her generous insight and suggestions on pCREB immunohistochemistry. We would also like to thank Veronica Dobrovitsky, Linnea Freeman, and Danielle Zito for their technical assistance.

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ventrolateral zone (NAc_{LAT}), as previously reported (Brenhouse & Stellar, 2006; Todtenkopf, Mihalakopoulos, & Stellar, 2002; Todtenkopf & Stellar, 2000). The localization of pCREB-ir across these regions at different time points after repeated cocaine exposure should provide further insight into the complex anatomical and molecular associations underlying cocaine-induced neuroplasticity in the NAc.

Method

Subjects

All procedures were conducted according to National Institutes of Health guidelines (U.S. National Institutes of Health, 1986) and were approved by the Institutional Animal Care and Use Committee at Northeastern University. Adult male Sprague–Dawley rats ($N = 30$; 275–325 g; Taconic Farms, Germantown, NY) were allowed free access to food and water and were housed individually in plastic cages on a 12-hr reversed light–dark cycle (lights on at 19:00) at an ambient temperature of 22–24 °C with a controlled relative humidity of 55%.

Behavioral Testing

The basic comparison was between two groups of rats treated twice daily with cocaine (Research Biochemicals International, Natick, MA) and then challenged with cocaine either 2 days or 14 days after the end of treatment without intervening treatment or handling. All cocaine injections were doses of 15 mg/kg ip that had been prepared in sterile 0.9% isotonic saline. Twice-daily repeated injections were separated by approximately 6 hr for 5 consecutive days. As a control, saline was substituted in the treatment phase of the experiment. Thus, there were four treatment conditions: repeated saline with a cocaine challenge after 2 treatment-free days (SC2; $n = 6$), repeated cocaine with cocaine challenge after 2 treatment-free days (CC2; $n = 6$), repeated saline with cocaine challenge after 14 treatment-free days (SC14; $n = 6$), and repeated cocaine with cocaine challenge after 14 treatment-free days (CC14; $n = 6$). A separate negative control group (SS; $n = 6$) comprised rats exposed only to saline.

All testing was done in a separate, dimly lit room with a white-noise generator (San Diego Instruments, San Diego, CA) that masks any external sounds. On the day prior to the first day of treatment, rats were habituated for 1 hr to one of four Plexiglas activity monitor chambers (43.2 cm × 43.2 cm × 30.5 cm; Med-Associates, St. Albans, VT). All injections were performed in each of the rats' assigned activity chamber, and locomotor behavior was measured after the first injections on the first and last day of the repeated treatment. Behavior was recorded for an initial 20 min to establish baseline after which the rats were given an injection of either saline or cocaine intraperitoneally. Behavior was then recorded for an additional 40 min. After the 2- or 14-day drug-free period, we tested all rats using the same procedure described above, with each rat receiving either a challenge injection of cocaine (15 mg/kg ip) or saline (1 ml/kg ip). Total distance (in meters) that each rat traveled in 40 min was recorded. A greater than 20% increase in locomotion from Day 1 of treatment to the challenge day was used as the criterion for locomotor sensitization.

pCREB and Tyrosine Hydroxylase (TH) Immunohistochemical Staining

Exactly 1 hr (Jang, Lee, Lee, Suh, & Song, 2002) following administration of the challenge injection, rats were anesthetized with a ketamine–xylazine mixture and were intracardially perfused with 300 ml of ice-cold 4% paraformaldehyde solution in 0.1 M phosphate buffered saline (PBS, pH 7.4). The exsanguination step in which saline is introduced first was eliminated to minimize postdeath phosphorylation (Konradi, Cole, Heckers, & Hyman, 1994). The brains were then removed and postfixed in the same solution for 2 hr and then in 20% glycerol with 50 mM sodium fluoride (NaF) and 2 mM sodium orthovanadate (to inhibit phosphatases) until sunk. Coronal sections (40 μm) were taken using a vibrating microtome. Sections were stored in a cryoprotectant solution (30% ethylene glycol, 1% polyvinylpyrolidone-40, 30% sucrose, 50 mM NaF, 2 mM orthovanadate in 0.1 M PBS) at −20 °C until immunohistochemical processing.

Sections were washed six times for 10 min in dilution media (0.9% w/v sodium chloride [NaCl], 0.7% w/v trisma, 0.05% triton-X, 50 mM NaF, 2 mM orthovanadate in distilled water) and then endogenous peroxidases were inhibited with a 20-min bath in 0.1M sodium periodate. Sections were then again washed three times in dilution media and then blocked for 1 hr in dilution media containing 3% normal goat serum (NGS) and 2% w/v bovine serum albumin (BSA). Blocked sections were incubated with the primary antibody (in a solvent of PBS containing 1% NGS, 1% BSA, and 0.4% triton-X) containing polyclonal rabbit anti-pCREB-1 (ser-133) gamma globulin [IgG] (1:200; Santa Cruz Biochemicals, Santa Cruz, CA) for 48 hr at 4 °C. Primary deletions were made in order to verify antibody specificity; between three and five randomly selected sections per run were treated identically to the rest of the sections, except that they were incubated in the primary antibody solution with the primary antibody omitted. After six washes, sections were incubated for 1 hr at room temperature with biotinylated secondary antibody (1:200 polyclonal goat anti-rabbit IgG; Vector Laboratories, Burlingame, CA), washed again six times, and incubated for 75 min with an avidin-biotin-peroxidase complex (1:100, Vector Laboratories). After three 10-min washes, sections were reacted for 7 min with Vector VIP peroxidase substrate, which yields a dark purple reaction product. To visualize the different subregions of the NAc shell (Todtenkopf, Mihalakopoulos, & Stellar, 2002; Todtenkopf & Stellar, 2000), we subsequently processed the tissue for TH. The sections were washed three times for 15 min and incubated overnight with a primary polyclonal antiserum (rabbit anti-TH, 1:5000; Chemicon, Temecula, CA). Sections were then processed as above but instead were reacted for 6 min with 0.05% 3,3′-diaminobenzidine tetrahydrochloride with 0.01% hydrogen peroxide (DAB kit, Vector Laboratories), washed in distilled water, mounted, dehydrated through a graded series of ethanol and xylenes, and coverslipped using Cytoseal 60 mounting medium (VWR Scientific, West Chester, PA).

Microscopic Analysis

Slides from each rat were coded and analyzed by an experimenter unaware of the experimental conditions. Each subregion was identified at low power (2×) on a Nikon Eclipse E600 light

microscope (Microscope Video Instruments, Avon, MA) equipped with a DEI-750 CE camera (Optronics, Goleta, CA) connected to an image analysis software package (BioQuant NOVA 98, R & M Biometrics, Nashville, TN) running on a Pentium (Intel, Santa Clara, CA) PC computer. For each subject, we used a mouse-driven cursor to outline representative sections of the subregions of the shell and the core as evident from the TH-ir staining (Todtenkopf & Stellar, 2000; Todtenkopf, Mihalakopoulos, & Stellar, 2002). Area measurements for each of the subregions mentioned above were obtained, and then under higher magnification (10×), the number of CREB-positive nuclei was quantified for each outlined subregion. Before they were counted, the images were thresholded at a standard red-green-blue-scale level empirically determined by observers unaware of the treatment conditions so as to allow detection of nuclei stained with moderate to high intensity, while suppressing lightly stained nuclei.

Statistical Analysis

Data representing the amount of pCREB-ir in each subregion were expressed as an average density of immunoreactive nuclei per square millimeter of tissue. This was done by dividing the number of immunoreactive nuclei quantified by the total area of the subregion. The individual densities obtained across multiple sections and then across all rats in each treatment group for each subregion were then averaged. To study the effects of pretreatment and withdrawal time on pCREB following cocaine challenge, we performed two-way analyses of variance (ANOVAs) and Tukey–Kramer multiple comparisons to compare the subregions of CC rats with the subsections of SC rats at each challenge time point. To study the effect of cocaine on cocaine-exposed rats compared with control SS rats, we also conducted one-way ANOVA with Tukey–Kramer multiple comparisons within each subregion for all four treatment groups: SS, SC (collapsed across challenge time-point because SC2 and SC14 were not different from one another), CC2, and CC14. Activity levels were analyzed using meters traveled in 40 min as a measure of locomotion. Two of 12 cocaine-treated rats did not display behavioral sensitization and were processed for immunohistochemistry, but were not included in their respective groups for analysis of CREB. All rats were included in the analysis of behavioral data, including those that did not become sensitized. We performed two-way ANOVAs on locomotion scores using day as a repeated measure, with Bonferroni posttests to determine differences between groups on each test day.

Results

As presented in Figure 1 histologically and Figure 2 graphically, CREB phosphorylation to a cocaine challenge after a cocaine treatment was augmented by the elapsed time between challenge on Day 2 and challenge on Day 14 after treatment, but only in discrete zones of analysis of the NAc. Although the two-way ANOVA revealed no significant interaction between treatment and withdrawal time in the NAc_{VERT}, NAc_{ARCH}, or NAc_{CONE}, significant interactions between treatment and withdrawal time were found in the NAc_{INT}, $F(1, 16) = 23.3$, $p < .01$, and in the core,

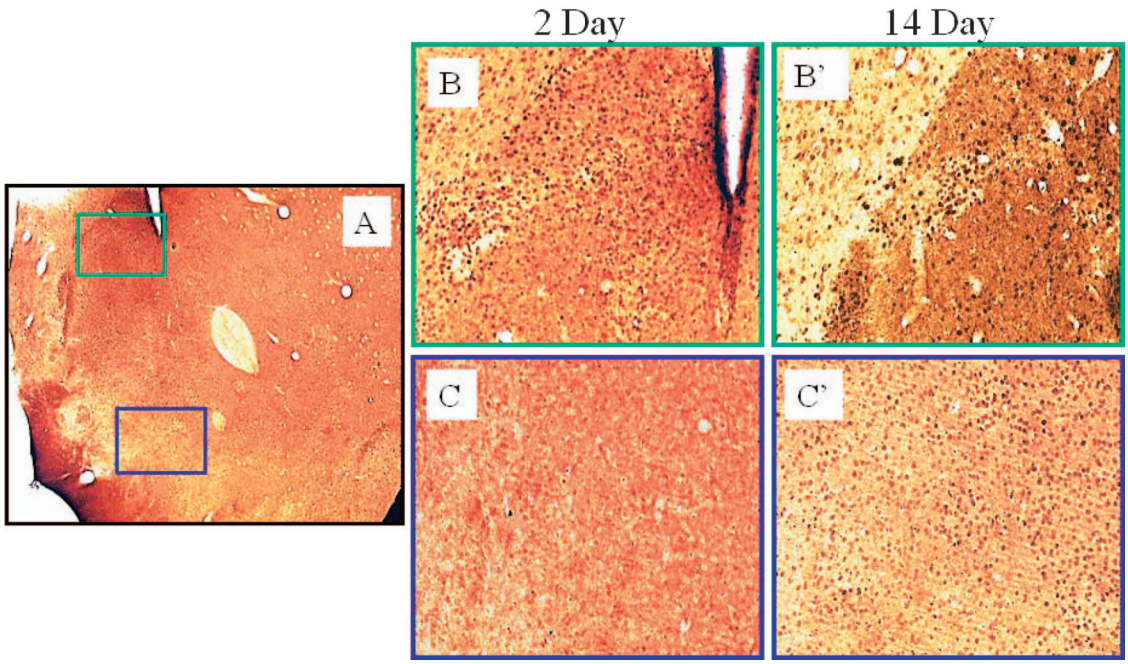

Figure 1. A: Representative low power (2×) photomicrograph shows phosphorylated cyclic adenosine monophosphate (cAMP) response element binding protein (pCREB) immunoreactivity (ir; dark dots) and tyrosine hydroxylase (TH)-ir (brown staining pattern) in the nucleus accumbens. B and B′: 10× magnification of the dorsomedial shell (area corresponds to green box in A) after a challenge 2 days (B) or 14 days (B′) after repeated cocaine treatment. C and C′: 10× magnification of the intermediate zone (area corresponds to blue box in A) after a challenge 2 days (C) or 14 days (C′) after repeated cocaine treatment.

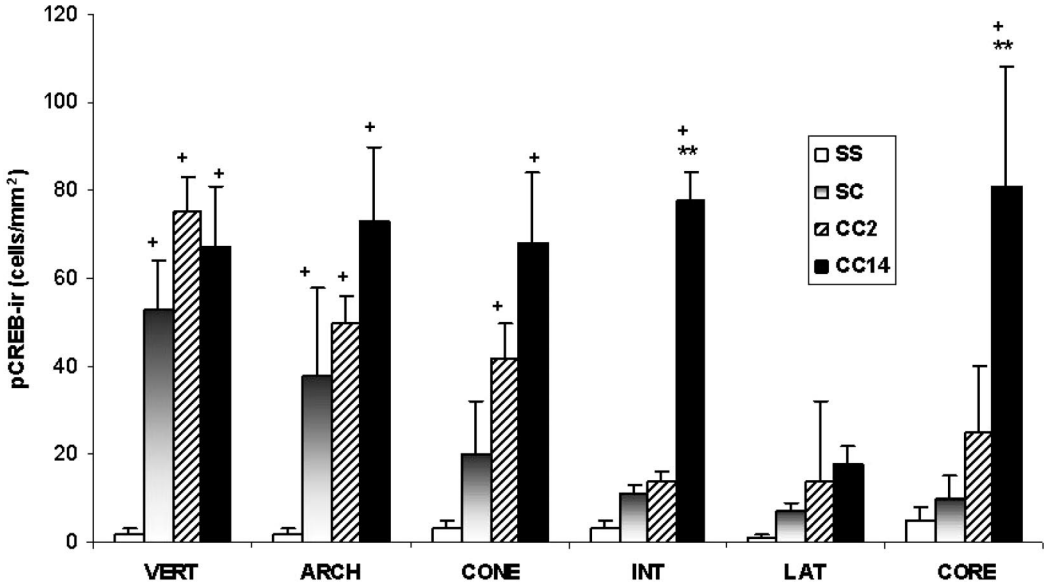

Figure 2. Density of phosphorylated cyclic adenosine monophosphate (cAMP) response element binding protein (pCREB) immunoreactive (ir) cells in animals challenged either 2 or 14 days after repeated cocaine or saline across six regions of analysis in the nucleus accumbens. SS = repeated saline with saline challenge 2 days following repeated treatment. SC = repeated saline with cocaine challenge, collapsed across challenge time point; CC2 = repeated cocaine with cocaine challenge 2 days following repeated treatment; CC14 = repeated cocaine with cocaine challenge 14 days following repeated treatment; VERT = vertex; ARCH = arch; CONE = cone; INT = intermediate zone; LAT = ventrolateral zone; CORE = core. Error bars = standard error. $^{\dagger}p < .01$ difference from SS group. $^{**}p < .01$ difference from CC2 group.

$F(1, 16) = 11.0$, $p < .01$. Rats pretreated with cocaine ($p < .01$)—but not those pretreated with saline—and challenged after 14 days of withdrawal displayed higher levels of pCREB than those challenged after 2 days of withdrawal in both these subregions. Figure 2 also shows that pCREB was increased by the basic cocaine challenge even under saline pretreatment conditions in the dorsal medial shell of the NAc and that without any cocaine administration, pCREB levels were low in all NAc areas. Specifically, SC rats displayed significantly higher pCREB density than SS controls in the NAc_{VERT} ($p < .001$) and NAc_{ARCH} ($p < .001$), but in no other subregion. Additionally, CC2 rats displayed increased pCREB-ir in the NAc_{VERT} ($p < .001$), NAc_{ARCH} ($p < .001$), and NAc_{CONE} ($p < .001$) compared with SS controls. However, cocaine administration 14 days after repeated treatment yielded additional pCREB-ir in the NAc_{INT} and core only in rats with previous cocaine exposure (Figures 1 and 2).

As expected, the subjects displayed locomotor sensitization after 2 and 14 days of withdrawal. The behavioral activity of SC2 and SC14 groups was not statistically different from one another, so the results for these two groups were combined for analysis. A main effect of treatment group, $F(1, 74) = 15.7$, $p < .01$, and treatment day, $F(3, 74) = 2.9$, $p < .05$, on locomotion was found. By Day 5 of repeated cocaine, rats receiving cocaine began to show increased locomotion compared with the locomotion that they had exhibited on Day 1, although this increase only approached significance ($p = .06$). Significant increases over the activity exhibited on Day 1 were not seen until Challenge Day 2 ($p < .05$), and locomotor activity remained elevated through Challenge Day 14 ($p < .05$). On cocaine challenge after both 2 ($p < .05$) and 14 ($p < .01$) treatment-free days, rats that had received previous cocaine treatment displayed significantly more locomotion than those that had no previous experience with cocaine.

Discussion

In this study, early and late cocaine challenges after sensitization to cocaine resulted in differential CREB activation (pCREB) in distinct subregions of the NAc. More specifically, repeated cocaine administration resulted in substantial CREB phosphorylation following a cocaine challenge in all subregions of the NAc shell excluding the NAc_{LAT}. This widespread increase was evident only after 14 drug-free days. Rats challenged after only 2 drug-free days following repeated exposure yielded a pattern of pCREB that was localized to the dorsomedial zones of the shell only (comprising the NAc_{VERT}, NAc_{ARCH}, and NAc_{CONE}), and levels were no different from those found in vehicle controls in the NAc_{INT}, NAc_{LAT}, and core subregions. Thus the NAc_{INT} and NAc core subregions stand out as different in the rats challenged at 14, but not at 2, days following cocaine treatment.

Recent data show electrolytic NAc_{INT} lesions attenuate behavioral sensitization to a challenge injection after 14, but not after 2, days of withdrawal following repeated cocaine injections, indicating a role for this subregion in the long-term expression of sensitization (Brenhouse, Montalto, & Stellar, 2006). Further supporting evidence of a role for the NAc_{INT} in postcocaine reaction has been provided in prior studies that found *c-fos* expression—which requires CREB activation—increased after 14 days of withdrawal

from repeated cocaine exposure (Brenhouse & Stellar, 2006; Todtenkopf, Mihalakopoulos, & Stellar, 2002). It is interesting that increased *c-fos* was not found in the dorsomedial shell after repeated cocaine exposure, whereas CREB activation was found in the current study. It is possible that with repeated activation of CREB (such as after each cocaine exposure), *c-fos* transcription is no longer seen, since *c-fos* has been shown to downregulate after repeated activation (Moratalla, Elibol, Vallejo, & Graybiel, 1996). Together, these studies suggest differential involvement of the dorsomedial shell and the NAc_{INT} in sensitization.

We note that we obtained pCREB levels 1 hr after cocaine challenge to allow the analysis of cocaine-induced locomotor behavior. Recently, peak accumbal CREB phosphorylation has been found to occur 20 min after cocaine challenge (Mattson et al., 2005). Given that dephosphorylation may occur at different rates across the accumbens subregions, future work with the 20-min time point would be useful. We also note that other studies support functional differences between NAc shell subregions in behavioral sensitization and further suggest that the NAc shell is not a homogeneous region. Previous work in our laboratory has shown that the dorsomedial shell plays a role in the development and earlier phase of cocaine sensitization because both electrolytic (Todtenkopf, Carreiras, Melloni, & Stellar, 2002) and excitotoxic (Walsh et al., 2004) lesions of this area attenuate sensitization only if the lesions exist prior to repeated cocaine treatment (Todtenkopf, Stellar, & Melloni, 2002). It is important to note that dorsomedial shell lesions have the greatest attenuating effect on locomotor sensitization during repeated exposure and after a short drug-free period (Todtenkopf, Carreiras, et al., 2002).

The connectivity of the dorsomedial shell and NAc_{INT} may shed some light onto their functional roles. Efferents from the NAc_{INT} include the ventrolateral periaqueductal gray matter, the lateral VTA, and the substantia nigra pars compacta, whereas afferents include the basal amygdala and the lateral VTA (Wright, Beijer, & Groenewegen, 1996). The involvement of the basal amygdala in the expression of sensitized responses to cocaine and cocaine-related cues (e.g., Knapp, Printseva, Cottam, & Kornetsky, 2002) supports the importance of the NAc_{INT} in late-phase sensitization that we found in this study. It is also possible that the increase in pCREB in the dorsomedial shell during early sensitization is due to a cocaine-induced increase in dopamine transmission. This regulation is likely provided by glutamatergic terminals in the shell, which originate in the infralimbic and prelimbic cortices as well as in the paraventricular thalamic nucleus (PV; Groenewegen & Berendse, 1994; Moga, Weis, & Moore, 1995) and terminate most densely in the dorsomedial shell regions (Wright & Groenewegen, 1995). Lesions of the prelimbic region of the mPFC attenuate the development of cocaine sensitization (Tzschentke & Schmidt, 1998), whereas a lesion of the PV that preceded the repeated cocaine treatment produces an augmented locomotor response on the 1st day of cocaine treatment but not after 5 days of repeated treatment (Young & Deutch, 1998). Glutamatergic inputs from the PV may therefore facilitate the CREB activation in the dorsomedial zones upon acute cocaine administration (as seen in the SC rats). In contrast, NAc_{INT} neurons are known to exhibit relatively low neurotensin levels (Zahm, Williams, Krause, Welch, & Grosu, 1998) and a possible lower level of regulation of the dopamine response during sensitization (see Todtenkopf & Stellar, 2000, for review).

Like the NAc_{INT}, the NAc core also displayed increased pCREB after 14 days of withdrawal compared with the level after 2 days of withdrawal following repeated cocaine treatment. This is cohesive with reports that significant neuroplasticity is seen in the core of sensitized animals 2 weeks following repeated cocaine exposure (Li, Acerbo, & Robinson, 2004). This phenomenon is also seen in the core of self-administering animals that have been allowed extended drug access and are sensitized to the locomotor-activating effects of cocaine (Ferrario et al., 2005). In these reports, no changes were seen in the NAc shell; however, because the shell was analyzed as a homogeneous region, differences in the NAc_{INT} may have been dissipated.

It appears that CREB is phosphorylated within the dorsomedial NAc in response to each cocaine exposure and that this pattern of cocaine-induced activation does not change until a considerable drug-free period has lapsed. Recent studies in our laboratory have found a similar pattern of expression for Δ*fosB*, lasting as long as 14 drug-free days after repeated cocaine administration (Brenhouse & Stellar, 2006). McClung and Nestler (2003) reported that gene expression in the NAc switches from dependence on CREB early after repeated cocaine exposure to a largely Δ*fosB*-mediated gene expression profile after longer cocaine-treatment periods. Therefore, if withdrawal time also affects gene expression, it is possible that cocaine-induced gene expression in dorsomedial shell neurons is mediated first by CREB and later by Δ*fosB* as time progresses after cocaine treatment.

The anatomical expression profiles of Δ*fosB* and CREB may identify functional differences in the NAc_{INT} and the dorsomedial shell during phases of response to repeated cocaine exposure. Other researchers have proposed that during sensitization to drugs of abuse, a switch occurs from drug "liking" to drug "wanting" via two distinct neural incentive systems (Robinson & Berridge, 1993). These temporal changes in the anatomical distribution of transcription factor activities may provide insight about the early "reward stamp" of drug use (i.e., liking) involving the dorsomedial shell, as well as the involvement of the NAc_{INT} during the persistent motivational, or wanting, stages of drug abuse. CREB activation has been attributed to aversive states (Barrot et al., 2002), and late-phase sensitization may reflect an aversive craving effect, driven by a circuit involving the NAc_{INT}.

Further study of the differential activation of transcription factors in distinct zones of analysis within the NAc, including within the NAc shell itself, seems likely to contribute to our understanding of the changing behavioral reactions as cocaine is repeatedly administered.

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Received May 24, 2006
Revision received September 27, 2006
Accepted October 6, 2006 ■