pression, confirming that other mechanisms (for example, c-*myc* overexpression, receptor mutation, and so forth) must also contribute to TGF-β resistance.

As breast carcinomas and melanomas become metastatic, they secrete large amounts of TGF-β (*25, 27*). This may enhance tumor cell invasion through effects on extracellular matrix (*27, 33*). Thus, TGF-β resistance may be an essential adaptation to the metastatic phenotype. In accord with this notion, the extent of TGF-β resistance correlates with metastatic progression (*28, 30*), and targeted deletion of an essential component of the TGF-β signaling cascade, *Smad3*, promotes the formation of metastatic tumors (*1*). Although TGF-β resistance can be achieved through multiple routes, increased expression of MDM2 is sufficient to confer this phenotype.

Previous work indicated that MDM2 may contribute to transformation through mechanisms that are independent of effects on p53. For example, in some human breast carcinomas and lymphomas, p53 mutation and MDM2 overexpression occur together (*31, 32*). Recently, alternatively spliced forms of MDM2 were identified in bladder and ovarian carcinomas (*34*). These alternative forms lack the p53-binding domain but still transform NIH-3T3 cells. We have demonstrated that MDM2 can overcome growth inhibition by TGF-β through effects on the RB/E2F pathway. These results provide a potential mechanism underlying p53-independent oncogenic activities of MDM2. Thus, in tumors, MDM2 may antagonize both the Rb and p53 pathways, functioning in many respects as a cellular version of SV40 large T antigen.

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8. The cDNA expression vector (HygroMaRXII; P. Sun, G. J. Hannon, D. Beach, unpublished data) was designed based on Molony murine leukemia virus (MoMLV). We included a recognition site (loxP) for Cre recombinase in a 3′ long terminal repeat (LTR) and a bacterial replicon and a bacterial selectable marker within the retroviral genome. These modifications allow easy and efficient recovery of cDNAs by Cre-mediated excision of integrated proviruses from the genome. The recovered circular plasmids contained a single LTR, and thus could be directly used to produce recombinant viruses for further studies.
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# Regulation of Cocaine Reward by CREB


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Cocaine regulates the transcription factor CREB (adenosine 3′,5′-monophosphate response element binding protein) in rat nucleus accumbens, a brain region that is important for addiction. Overexpression of CREB in this region decreases the rewarding effects of cocaine and makes low doses of the drug aversive. Conversely, overexpression of a dominant-negative mutant CREB increases the rewarding effects of cocaine. Altered transcription of dynorphin likely contributes to these effects: Its expression is increased by overexpression of CREB and decreased by overexpression of mutant CREB. Moreover, blockade of κ opioid receptors (on which dynorphin acts) antagonizes the negative effect of CREB on cocaine reward. These results identify an intracellular cascade—culminating in gene expression—through which exposure to cocaine modifies subsequent responsiveness to the drug.


Cocaine causes complex molecular adaptations in brain reward systems, some of which affect its addictive qualities (*1*). For example, chronic cocaine use increases formation of adenosine 3′,5′-monophosphate (cAMP) and activity of cAMP-dependent protein kinase (PKA) in the nucleus accumbens (*2*), a neural substrate for the rewarding actions of cocaine (*3, 4*). Stimulation of PKA in the nucleus accumbens counteracts the rewarding properties of cocaine (*5*), which suggests a neural mechanism of drug tolerance. Increased PKA activity would be expected to lead to increased phosphorylation of CREB, which mediates many of the effects of cAMP and PKA on gene expression (*6, 7*). However, direct evidence for a role of CREB in cocaine actions has been lacking. To address this issue, we selectively induced CREB overexpression in the nucleus accumbens with microinjections of a herpes simplex virus vector (HSV-CREB) and measured alterations in the rewarding properties of cocaine with place conditioning (*8*). We performed the same experiments in other rats after overexpression of a dominant negative mutant CREB (mCREB) (*8*), which contains a single point


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mutation (Ala for Ser at residue 133) that prevents its phosphorylation and transactivation (*6*).

In our place conditioning protocol, control rats given intraperitoneal (ip) injections of cocaine at 5.0 mg/kg or more spend significantly more time in environments previously associated with the drug, whereas cocaine at 1.25 mg/kg or less does not reliably affect preferences. The effect of cocaine at 1.25 mg/kg was not altered by bilateral microinjections (*9*) of vehicle (10% sucrose) or of HSV-LacZ (expressing β-galactosidase, a control protein) (*10*) into the nucleus accumbens shell (Fig. 1A), an especially sensitive substrate of drug reward (*4, 11*). However, this threshold dose of cocaine established conditioned place preferences in rats microinjected with HSV-mCREB. The rewarding effect was "inversed" to place avoidance in rats given HSV- CREB, which suggests that this dose of cocaine was made aversive by increased quantities of CREB in this region. Qualitatively similar differences were observed between animals given HSV-CREB and HSV-mCREB into the core subregion of the nucleus accumbens, although the effects—particularly the rewarding effect of HSV-mCREB—were less reliable and not statistically significant (Fig. 1B). The effects of gene transfer were transient: when cocaine was administered a week (rather than 3 days) after HSV treatment, cocaine was devoid of rewarding or aversive effects (Fig. 1C). This finding is consistent with our previous observations (*12*) that the behavioral consequences of HSV viral vectors are transient and reversible and have a time course that parallels that of transgene expression (see below).

Dose-response analyses suggested that HSV-mCREB and HSV-CREB were producing, respectively, approximately parallel leftward (more rewarding) and rightward (less rewarding) shifts in the effects of cocaine (Fig. 1D). At a high dose of cocaine (5.0 mg/kg), rats given HSV-mCREB and those given vehicle displayed equivalent place preferences, which is consistent with previous observations that there is an upper limit to the magnitude of place preferences that can be observed in this model (*8*). In rats given HSV-CREB, cocaine at 5.0 mg/kg was less aversive than at 1.25 mg/kg; this suggests a rightward shift in the effects of cocaine and that higher concentrations of cocaine can counteract the aversive consequences of increased amounts of CREB.

Histological examination confirmed viral-mediated gene expression. Vector microinjections intended for the nucleus accumbens shell were aimed at the ventromedial region of the nucleus accumbens, whereas those intended for the nucleus accumbens core were aimed more laterally (Fig. 2A). In rats given HSV-LacZ (Fig. 2B), expression of β-galactosidase (*13*) peaked between days 3 and 4, was restricted to an area of the nucleus accumbens of ~1.5 mm in diameter, and was accompanied by minimal damage (for example, gliosis) (Fig. 2C) that was indistinguishable from that caused by microinjection of vehicle. On day 3, about 2000 β-galactosidase-labeled cells were visible in the area of the injection. In rats given HSV-CREB, moderate numbers of highly CREB-immunoreactive cells (*13*) were observed at the injection site (Fig. 2, D and E); however, the number of neurons overexpressing CREB is likely underrepresented because the immunohistochemical conditions used minimized detection of endogenous CREB. CREB immunoreactivity did not increase in rats given HSV-LacZ, confirming that increased CREB expression is not a nonspecific reaction to surgery or viral infection. Although there has been concern about potential toxicity of viral vectors (*12*), there was little evidence of gliosis found with Nissl staining (as in Fig. 2C). Moreover, there was no detectable toxicity on the dopamine-containing terminals in the nucleus accumbens (Fig. 2F), the proximate neural substrate of the rewarding actions of cocaine (*3, 4*). Viral-mediated expression of mCREB was immunohistochemically indistinguishable from that of CREB (Fig. 2G), as expected because the antibody used cannot distinguish between CREB and mCREB. Expression of LacZ, CREB, and mCREB transgenes in the nucleus accumbens dissipated by day 7, consistent with previous in vitro and in vivo studies (*10, 12*).

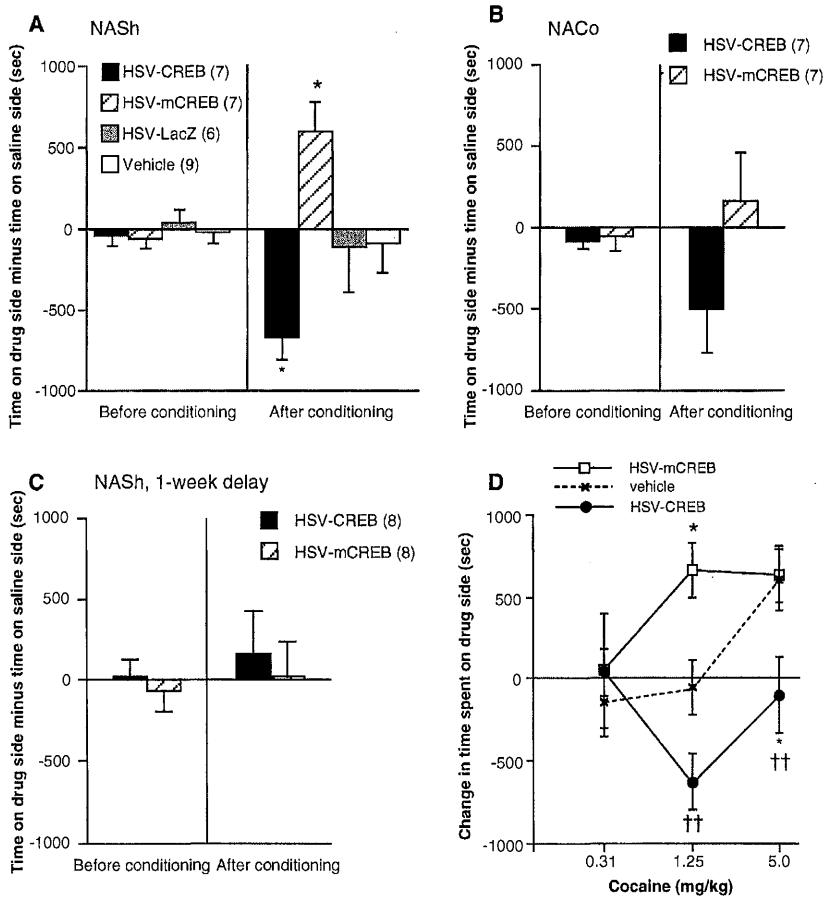


**Fig. 1.** Sensitivity to cocaine after gene transfer. (**A**) Rats spent significantly less time in cocaine-associated environments after microinjections of HSV-CREB into the nucleus accumbens shell but significantly more time after similar microinjections of HSV-mCREB (mean ± SEM) (treatment × days interaction: $F_{3,25} = 4.16$, $P < 0.02$). (**B**) Effects were not statistically reliable with nucleus accumbens core microinjections (treatment × days interaction: $F_{1,12} = 2.70$, not significant). (**C**) Differences between groups did not persist when place conditioning occurred on day 7 or 8 rather than on day 3 or 4 after gene transfer (treatment × days interaction: $F_{1,14} = 0.16$, not significant). (**D**) Dose dependency of changes in effects of cocaine expressed as change (before minus after) in time spent in the cocaine-associated environment. Effects of cocaine depended on vector treatment and dose (treatment × dose interaction: $F_{4,67} = 2.77$, $P < 0.05$). In rats given vehicle microinjections, cocaine was rewarding at 5.0 mg/kg only. In rats given HSV-mCREB, cocaine was maximally rewarding at 1.25 mg/kg. In rats given HSV-CREB, cocaine was maximally aversive at 1.25 mg/kg, whereas higher doses occasionally established place preferences. Groups consisted of 7 to 11 rats; *$P < 0.05$ compared with vehicle, ††$P < 0.01$ compared with HSV-mCREB (Fisher's $t$ test). NASh, nucleus accumbens shell; NACo, nucleus accumbens core.

Although the effects of CREB on nucleus accumbens neurons (and hence on cocaine reward) are likely mediated via many targets, we focused on the effects of HSV-CREB and HSV-mCREB on expression of dynorphin, an endogenous ligand of κ opioid receptors (*14*). The dynorphin gene is known to be CREB regulated in vitro (*7*), and repeated cocaine administration increases its expression in the nucleus accumbens and dorsal striatum (*15*). Microinjections of a κ opioid agonist into the nucleus accumbens establish place aversions (*16*) that are qualitatively similar to those observed in this study with cocaine in animals given HSV-CREB (Fig. 1A). Northern blot analysis (*17*) 3 days after treatment with HSV vectors revealed a 42% increase in dynorphin mRNA in rats overexpressing CREB and a 33% decrease in dynorphin mRNA in rats overexpressing mCREB (Fig. 3). These results show that CREB regulates dynorphin expression in the nucleus accumbens in vivo.

To determine whether increased dynorphin expression is involved in cocaine aversions caused by HSV-CREB, we blocked brain κ receptors with intracerebroventricular microinjection of the irreversible κ receptor antagonist norBNI (*18*). Treatment with norBNI before cocaine place conditioning blocked the aversive effects associated with a 1.25-mg/kg dose of cocaine in animals given HSV-CREB into the nucleus accumbens shell but did not have a significant effect in rats given microinjections of vehicle or HSV-mCREB (Fig. 4). The fact that only the aversive properties of cocaine are altered by norBNI suggests that microinjections of HSV-CREB into the nucleus accumbens shell enhance the aversive aspects of cocaine by promoting dynorphin actions at κ opioid receptors.

Our results indicate that κ opioid receptors are involved in cocaine valence (reward versus aversion) and suggest that CREB-mediated transcription in the nucleus accumbens shell serves as a "drug reward rheostat" in part via effects on dynorphin expression (*15, 19*). Moreover, they suggest a sequence of intracellular events, initiated by drug admin-

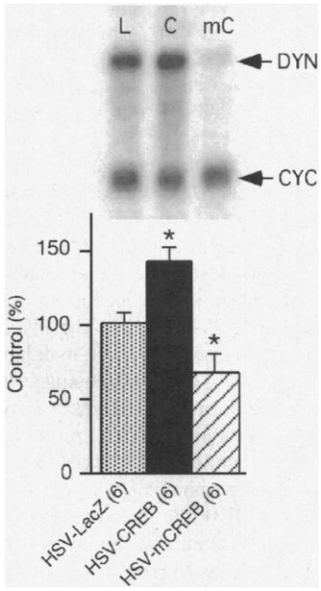


**Fig. 3.** Northern blot of dynorphin (DYN) mRNA in nucleus accumbens shell after gene transfer. L, LacZ; C, CREB; mC, mCREB. Dynorphin mRNA concentrations were significantly increased by HSV-CREB and significantly decreased by HSV-mCREB ($F_{2,15} = 13.4$, $P < 0.001$). Data are expressed as percentage (mean ± SEM) of HSV-LacZ and are corrected for cyclophilin (CYC) mRNA content. *$P < 0.05$ compared with HSV-LacZ (Fisher's $t$ test).

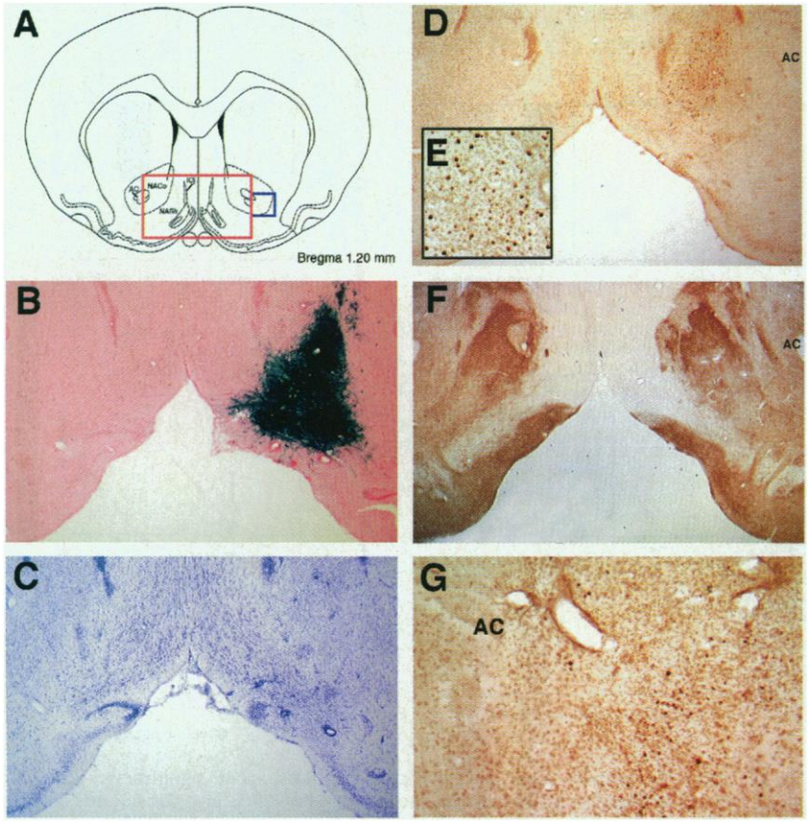


**Fig. 2.** Histological examination of nucleus accumbens after gene transfer. (**A**) Schematic of nucleus accumbens (9). Red box shows field of view in (B), (C), (D), and (F); blue box shows field of view in (G). (**B**) Expression of β-galactosidase 3 days after unilateral microinjection of HSV-LacZ (×25) (*13*). Brain slices were reacted in sodium phosphate buffer solution (pH 7.4) containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (0.2 mg/ml; American Bioanalytical). (**C**) An adjacent, Nissl-stained slice from the same brain. (**D**) Expression of CREB 3 days after microinjection of HSV-CREB into the right nucleus accumbens shell (*13*). (**E**) Higher magnification (×100) of the injection site in (D), showing nuclear localization of CREB expression. (**F**) Tyrosine hydroxylase expression (*13*) in a slice adjacent to that in (D). (**G**) Expression of mCREB 3 days after injection of HSV-mCREB into the right nucleus accumbens core (×100), using the same antibody to CREB as in (D). AC, anterior commissure; NASh, nucleus accumbens shell; NACo, nucleus accumbens core; ICj, Islands of Calleja.

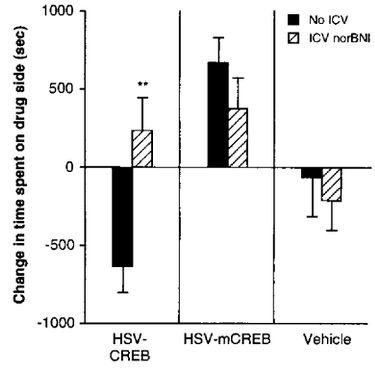


**Fig. 4.** Effects of norBNI (5.0 μg, intracerebroventricularly) on cocaine (1.25 mg/kg, ip) place conditioning in rats given gene transfer, expressed as change (before minus after) in time spent in the cocaine-associated environment. The effects of norBNI on place conditioning depended on HSV vector treatment (vector × intracerebroventricular treatment interaction: $F_{2,45} = 4.77$, $P < 0.02$). Aversive effects of cocaine were blocked by norBNI in rats given HSV-CREB but were not significantly altered in rats given HSV-mCREB or vehicle. Groups consisted of 7 to 10 rats; **$P < 0.01$ compared with HSV-CREB/no intracerebroventricular (ICV) treatment (Fisher's $t$ test).

istration and culminating in altered gene transcription, through which previous exposure to cocaine can influence the subsequent subjective qualities of the drug. Repeated exposure to cocaine causes an up-regulation of dynorphin expression through stimulation of dopamine D1-type receptors and the cAMP pathway (*2, 7, 15*). Upon subsequent exposure to cocaine, augmented release of dynorphin could inhibit local dopamine release through actions at κ opioid receptors on terminals of mesolimbic dopaminergic neurons that innervate the nucleus accumbens (*19, 20*). Diminished release of dopamine in the nucleus accumbens may be aversive, or it may unmask other actions of cocaine that oppose drug reward (*3, 21*).

With repeated use of cocaine in humans, rewarding effects of the drug reportedly diminish and are overshadowed by unpleasant side effects including anxiety and irritability (*22*). Our data provide evidence that cocaine-induced increases in CREB and dynorphin in the forebrain could contribute to these changes. Indeed, cocaine users exhibit increased expression of dynorphin mRNA in the nucleus accumbens (*23*). Up-regulation of CREB-mediated transcription in the nucleus accumbens may counteract positive feedback-type adaptations that tend to intensify drug reward [for example, see (*12, 24*)]. Sensitization to the reward-related properties of psychostimulants also contributes importantly to addictive behavior (*25*). Individual variability in the balance and time course of positive and negative feedback-type changes in brain biochemistry may ultimately influence vulnerability to addiction and relapse.

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9. Bilateral microinjections (2.0 μl) of HSV vectors were delivered over 10 min into the nucleus accumbens shell (relative to bregma: AP = +1.7, lat = ±2.3, DV = 6.8 mm below dura) or core (AP = +1.7, lat = ±3.9, DV = 6.5 mm below dura) [G. Paxinos and C. Watson, *The Rat Brain in Stereotaxic Coordinates* (Academic, Sidney, 1986)] of anesthetized rats (325 to 375 g) as described in (*12*). The injection syringe was angled at 10° from the midline.
10. cDNAs for CREB and mCREB (obtained from M. E. Greenberg, Harvard University) and LacZ were inserted into the HSV amplicon HSV-PrpUC and were packaged into virus using the helper 5*dl*1.2 [R. L. Neve, J. R. Howe, S. Hong, R. G. Kalb, *Neuroscience* **79**, 435 (1997); F. Lim *et al.*, *Biotechniques* **20**, 460 (1996)]. Average titer of the purified virus stocks was $4.0 \times 10^7$ infectious units per milliliter.
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# A Family of cAMP-Binding Proteins That Directly Activate Rap1


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cAMP (3′,5′ cyclic adenosine monophosphate) is a second messenger that in eukaryotic cells induces physiological responses ranging from growth, differentiation, and gene expression to secretion and neurotransmission. Most of these effects have been attributed to the binding of cAMP to cAMP-dependent protein kinase A (PKA). Here, a family of cAMP-binding proteins that are differentially distributed in the mammalian brain and body organs and that exhibit both cAMP-binding and guanine nucleotide exchange factor (GEF) domains is reported. These cAMP-regulated GEFs (cAMP-GEFs) bind cAMP and selectively activate the Ras superfamily guanine nucleotide binding protein Rap1A in a cAMP-dependent but PKA-independent manner. Our findings suggest the need to reformulate concepts of cAMP-mediated signaling to include direct coupling to Ras superfamily signaling.


Since the discovery that cAMP activates the phosphorylating enzyme PKA (*1*), the cAMP messenger system has been shown to involve the sequential activation (or inhibition) of cAMP production by heteromeric guanine nucleotide–binding proteins (G proteins), subsequent binding of cAMP to PKA, and consequent phosphorylation of PKA substrates (*1*). PKA is considered to be the essential effector molecule mediating many of the wide range of physiological effects initiated by receptors coupled to generation of cAMP (*1*, *2*). cAMP has also been implicated in neuronal functions, including neurotransmitter-initiated signaling and the neuroplasticity underlying development and memory (*3*, *4*), but PKA has not been clearly linked to all of these neuronal functions (*5*). We initi-