Brief Communications

Altered Sensitivity to Rewarding and Aversive Drugs in Mice with Inducible Disruption of cAMP Response Element-Binding Protein Function within the Nucleus Accumbens

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The transcription factor cAMP response element-binding protein (CREB) within the nucleus accumbens (NAc) plays an important role in regulating mood. In rodents, increased CREB activity within the NAc produces depression-like signs including anhedonia, whereas disruption of CREB activity by expression of a dominant-negative CREB (mCREB, which acts as a CREB antagonist) has antidepressant-like effects. We examined how disruption of CREB activity affects brain reward processes using intracranial self-stimulation (ICSS) and inducible bitransgenic mice with enriched expression of mCREB in forebrain regions including the NAc. Mutant mice or littermate controls were prepared with lateral hypothalamic stimulating electrodes, and trained in the ICSS procedure to determine the frequency at which the stimulation becomes rewarding (threshold). Inducible expression of mCREB did not affect baseline sensitivity to brain stimulation itself. However, mCREB-expressing mice were more sensitive to the rewarding (threshold-lowering) effects of cocaine. Interestingly, mCREB mice were insensitive to the depressive-like (threshold-elevating) effects of the κ-opioid receptor agonist U50,488. These behavioral differences were accompanied by decreased mRNA expression of G-protein receptor kinase-3 (GRK3), a protein involved in opioid receptor desensitization, within the NAc of mCREB mice. Disruption of CREB or GRK3 activity within the NAc specifically by viral-mediated gene transfer enhanced the rewarding impact of brain stimulation in rats, establishing the contribution of functional changes within this region. Together with previous findings, these studies raise the possibility that disruption of CREB in the NAc influences motivation by simultaneously facilitating reward and reducing depressive-like states such as anhedonia and dysphoria.

Key words: **aversion; cocaine; dysphoria; GRK3; κ-opioid receptors; ICSS; reward**

Introduction

The nucleus accumbens (NAc) is implicated in disorders including addiction and depression (Nestler and Carlezon, 2006). Drugs of abuse (Turgeon et al., 1997) and stress (Pliakas et al., 2001) have profound effects on the NAc, including enhanced activation of the transcription factor cAMP response element-binding protein (CREB) (Carlezon et al., 2005). Elevated CREB function in the NAc triggers increased immobility in the forced swim test (Pliakas et al., 2001), an effect opposite to that induced by antidepressants. It also reduces the rewarding effects of drugs of abuse and food, reflecting depressive-like signs such as anhedonia (Carlezon et al., 1998; Barrot et al., 2002; Nestler and Carlezon, 2006). In contrast, disruption of CREB function by expression of a dominant-negative CREB (mCREB) in the NAc produces antidepressant-like effects and increased reward (Pliakas et al., 2001; Barrot et al., 2002). Mice lacking key CREB isoforms display fewer signs of opiate withdrawal, and are more sensitive to cocaine and morphine reward (Walters et al., 2005). The depressive-like behaviors that accompany elevated CREB function in the NAc are likely related to CREB induction of dynorphin (Carlezon et al., 1998), an endogenous peptide that acts at κ-opioid receptors (KORs). Indeed, KOR agonists produce depressive-like effects (anhedonia, dysphoria) in rodents, whereas KOR antagonists have antidepressant-like effects (Bals-Kubik et al., 1993; Pliakas et al., 2001; Newton et al., 2002; Mague et al., 2003; Carlezon et al., 2006).

The present studies were designed to further characterize how CREB function affects motivation. We examined the effects of cocaine (a rewarding drug) and the KOR agonist U50,488 (a drug that induces depressive-like signs) in bitransgenic mice that inducibly express mCREB. In these mutants, mCREB expression is enriched in forebrain (NAc, striatum, cortex), reaches maximal levels 6–8 weeks after discontinuation of doxycycline (DOX; which suppresses transgene expression), and reduces expression of CREB-regulated genes including prodynorphin (Sakai et al., 2002; McClung and Nestler, 2003). Drug effects were examined

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using intracranial self-stimulation (ICSS), a procedure that is sensitive to treatments with rewarding or depressive effects in humans and impervious to satiation and novelty-related anxiety states, which can complicate studies involving other types of rewards (food, sex, drugs of abuse) (Carlezon and Chartoff, 2007). Because these studies revealed a novel interaction between mCREB and G-protein receptor kinase-3 [GRK3, a protein involved in opioid receptor desensitization (McLaughlin et al., 2004)], we used ICSS and viral-mediated gene transfer in rats to evaluate how NAc-restricted alterations in the function of CREB and GSK3 each affect reward.

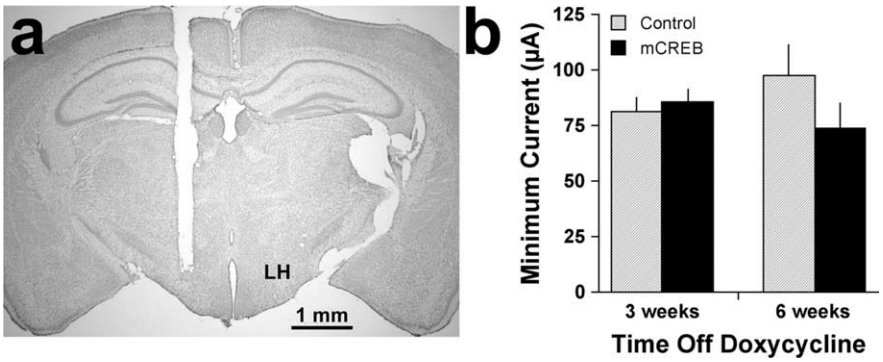

Figure 1. Effect of inducible expression of mCREB on brain stimulation reward. ***a***, Stimulating electrodes were located in the MFB. ***b***, There were no differences between mCREB transgenic mice and controls in the minimal current (mean ± SEM) required for reliable ICSS at 3 weeks ($n = 19–21$) or 6 weeks ($n = 7–10$) after DOX discontinuation.

Materials and Methods

Animals. Inducible bitransgenic mice expressing mCREB (A6 line) were generated using a tetracycline-regulated gene expression system (Sakai et al., 2002). Male mice carrying the NSE-tTA and TetOp-mCREB transgenes (mCREB group), or the NSE-tTA transgene only (control group), were raised on water containing DOX (100 μg/ml; Sigma). The experimental and control mice were littermates, and had been backcrossed to C57BL/6 for at least 12 generations. Most testing occurred at 6–8 weeks after DOX discontinuation, when transgene expression is maximal (McClung and Nestler, 2003). Male Sprague Dawley rats (Charles River Laboratories) were used for viral gene transfer studies. Animals were housed individually with *ad libitum* access to food and water, and maintained on a 12 h light (7:00 A.M. to 7:00 P.M.) cycle. Procedures were conducted in accordance with the 1996 National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* and McLean Hospital policies.

ICSS. Mice (28–35 g) or rats (325–350 g) were prepared with monopolar electrodes aimed at the medial forebrain bundle (MFB) as described previously (Gilliss et al., 2002; Todtenkopf et al., 2006). Rats were simultaneously implanted with bilateral guide cannulae aimed at the NAc shell, to enable gene transfer, as described previously (Todtenkopf et al., 2006).

One week later, animals were trained to respond for brain stimulation (Gilliss et al., 2002; Todtenkopf et al., 2006). The stimulation current was adjusted to the lowest value that would sustain reliable responding (>40 rewards per min) for 3 consecutive days. This was considered the "minimal current," reflecting sensitivity to the rewarding effects of the stimulation. For studies involving mice, two separate cohorts of mutants and controls were studied to ensure that sensitivity to the brain stimulation and the drugs was evaluated at a time coinciding with peak mCREB expression. One cohort was tested for minimal current sensitivity 3 weeks after DOX before undergoing additional training (see below) to enable drug testing 6 weeks after DOX. The other was tested for minimal current sensitivity 6 weeks after DOX, but did not receive further training or testing. Rats were assigned such that minimum currents were equivalent among groups (~170 μA).

Once the minimal current was identified for each animal, it was held constant. Animals were then adapted to brief tests over a series of 15 stimulation frequencies. Each series (or rate–frequency "curve") comprised 1 min test trials at each frequency: a 5 s "priming" phase of noncontingent stimulation, a 50 s test phase, and a 5 s time-out. The stimulation frequency was then lowered by 10% (0.05 $\log_{10}$ units), and another trial was started. After responding had been evaluated at each of the 15 frequencies, the procedure was repeated such that each animal was given 6 "curves" per day (90 min of training). During training, the range of frequencies was adjusted so that the highest 6–7 frequencies would sustain responding. ICSS thresholds (the frequency at which the stimulation becomes rewarding) were expressed as theta-0 (see Carlezon and Chartoff, 2007). Testing (drug treatments for mice, viral-mediated gene transfer for rats) started when mean thresholds varied by <10% over 3 consecutive days.

Drug testing in mice. Cocaine HCl and U50,488 (U50) (Sigma) were dissolved in 0.9% saline and injected intraperitoneally in a volume of 10 ml/kg. Three rate–frequency curves were determined immediately before drug treatment; the second and third curves were averaged to obtain the baseline (threshold and maximal response rate) parameters. Each mouse then received an intraperitoneal injection of drug or vehicle, and four (cocaine study) or six (U50 study) more 15 min rate–frequency curves were obtained. Mice received doses of cocaine (0.625–10.0 mg/kg) or U50 (0.03–5.5 mg/kg) twice; doses were given in ascending and then descending order. Pilot studies demonstrated that higher doses of these drugs disrupt responding. Each drug treatment followed a test with vehicle on the preceding day to ensure that the mouse had recovered from prior treatments and to minimize conditioned drug effects. Group differences were analyzed using ANOVAs; significant effects were analyzed further using *post hoc* tests (simple main effects tests, Newman–Keuls *t* tests). Electrode placements were confirmed by histology.

Viral-mediated gene transfer in rats. Herpes simplex virus (HSV) vectors encoding mCREB, wild-type GRK3 (wtGRK3) [tagged with green fluorescent protein (GFP)], or dominant-negative GRK3 (dnGRK3) (tagged with GFP) were produced and microinjected as described previously (Todtenkopf et al., 2006). The control condition comprised rats treated with vectors encoding GFP or β-galactosidase (which did not differ). Rats were tested for 30 min on days 3 and 5 (when transgene expression is elevated) and day 7 (when transgene expression is waning) (see Pliakas et al., 2001). The mean threshold for each day was calculated and compared with the baseline threshold. Effects of gene transfer on ICSS thresholds were evaluated with a two-way (treatment × day) ANOVA with repeated measures, and significant effects were analyzed further using simple main effects tests and Newman–Keuls *t* tests.

Quantitative PCR. Levels of mRNA for key G-protein signaling proteins were measured by quantitative PCR in mCREB and control mice: GRK3; regulator of G-protein signaling-9 (RGS9); G-protein β subunit-5 (Gβ5); and G-protein inhibitory subunit-2 (Gαi2). The mice had not been used in behavioral studies. Total RNA was harvested from bilateral 15 gauge punches of fresh NAc tissue as described previously (Tsankova et al., 2006). Each PCR ($n = 4$ mice per group) was repeated in triplicate by real-time PCR (Applied Biosystems). Data were normalized to GAPDH (glyceraldehyde-6-phosphate dehydrogenase). Fold differences of mRNA levels over control values were calculated and group differences were analyzed using Student's *t* tests for each gene.

Results

Mice responded at high rates for stimulation delivered through MFB electrodes (Fig. 1*a*). There were no group differences in sensitivity to the rewarding effects of MFB stimulation (minimum current) at 3 weeks (in mice that would eventually be tested with drug) or 6 weeks (in mice not tested with drug) (Fig. 1*b*). The nominal difference at 6 weeks is largely attributable to one con-

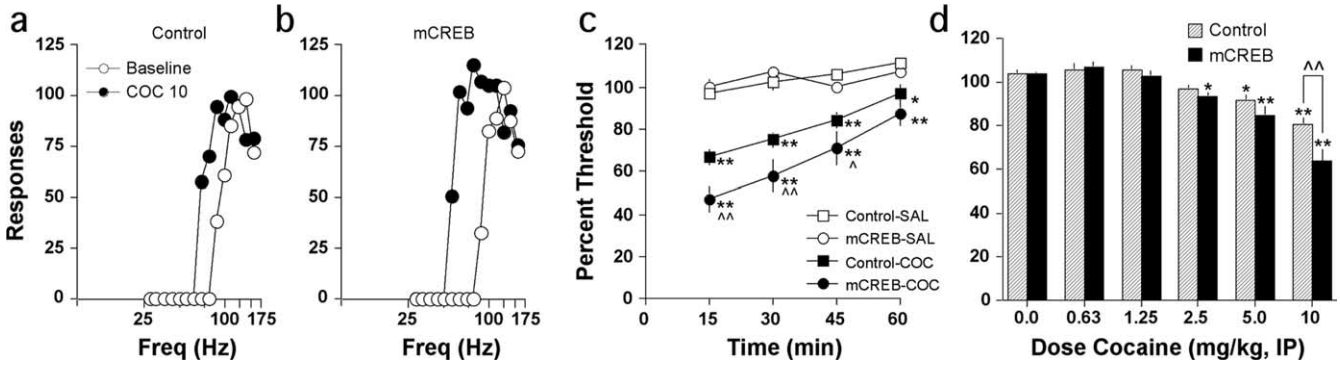

Figure 2. Effect of inducible expression of mCREB on sensitivity to cocaine (COC) in the ICSS test 6 weeks after DOX discontinuation. ***a***, ***b***, Rate of responding as a function of stimulation frequency during preinjection baseline, and after COC (10 mg/kg, i.p.). COC induced parallel leftward shifts and reductions in ICSS thresholds in control (***a***) and mCREB (***b***) mice, but the effects were larger in the mutants. Data are from representative mice. ***c***, Time course of COC effects (10 mg/kg, i.p.); drug effects were evident over the 1 h test period. ***d***, When data are expressed as mean (±SEM) percentage of pretreatment threshold for this test period, COC had significant threshold-lowering effects at a lower dose in mCREB mice (2.5 mg/kg) than in controls (5.0 mg/kg). Furthermore, the threshold-lowering effects of 10 mg/kg COC were significantly larger in mCREB mice. $^{*}p < 0.05$, $^{**}p < 0.01$ for within-group comparisons with saline (SAL; 0.0 dose) treatment, $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ for between-genotype comparisons, $n = 9$ mice/group.

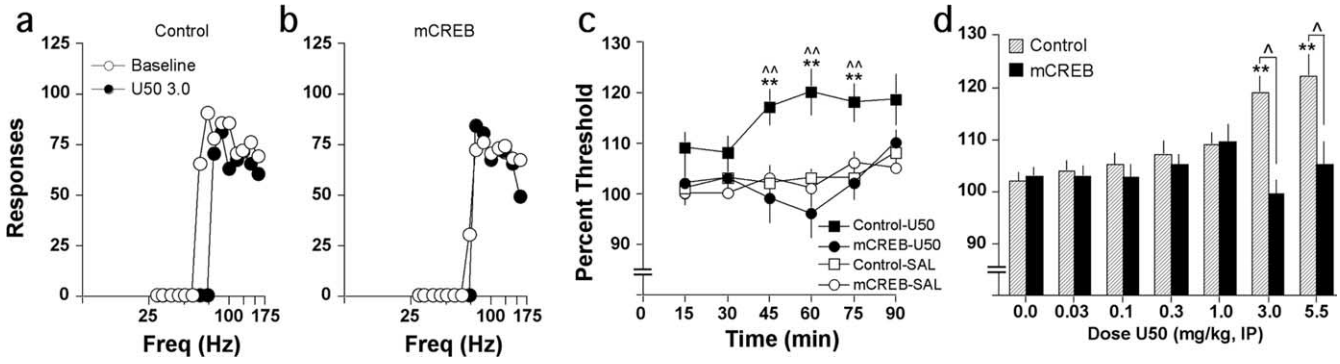

Figure 3. Effect of inducible expression of mCREB on sensitivity to U50 in the ICSS test 6 weeks after DOX discontinuation. ***a***, ***b***, Rate of responding as a function of stimulation frequency during preinjection baseline, and after U50 (3.0 mg/kg, i.p.). U50 induced parallel rightward shifts and increases in ICSS thresholds in control mice (***a***), but had negligible effects in mCREB mice (***b***). Data are from representative mice. ***c***, Time course of U50 effects (3.0 mg/kg, i.p.); drug effects were evident from 30 to 75 min of the 90 min test period. ***d***, When data are expressed as mean (±SEM) percentage of pretreatment threshold for the 30–75 min period, U50 had significant threshold-elevating effects in control mice (3.0 and 5.5 mg/kg) without affecting mCREB mice. $^{*}p < 0.05$, $^{**}p < 0.01$ for within-group comparisons with saline (SAL; 0.0 dose) treatment; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ for between-genotype comparisons, $n = 10$–12 mice/group.

trol mouse that had a particularly high minimum current (185 μA) but fulfilled criteria for inclusion.

Cocaine decreased mean ICSS thresholds in both groups of mice (Fig. 2), causing parallel leftward shifts in ICSS rate–frequency functions (Fig. 2*a*,*b*). However, cocaine effects were significantly larger in mCREB-expressing mice. Time course analysis of the most effective dose of cocaine (10 mg/kg) indicated that the effects of the drug were detectable for the entire 1 h of testing (Fig. 2*c*) (genotype × treatment × time interaction: $F_{(3,48)} = 3.16$, $p < 0.05$); subsequent analyses included all data from this period. Repeated testing did not alter the effect of cocaine on thresholds or response rates (data not shown), so data for each dose were compiled into single means. The effects of cocaine depended on an interaction between genotype and dose ($F_{(5,80)} = 2.34$, $p < 0.05$) (Fig. 2*d*). Cocaine lowered ICSS thresholds in control mice at 5.0 mg/kg (Newman–Keuls tests: $p < 0.05$) and 10 mg/kg ($p < 0.01$), whereas it lowered thresholds in mCREB transgenic mice at 2.5 mg/kg ($p < 0.05$), 5.0 mg/kg ($p < 0.01$), and 10 mg/kg ($p < 0.01$). In addition, the threshold-lowering effects of 10 mg/kg cocaine were significantly larger in the mCREB mice ($p < 0.01$). Cocaine had no effects on maximum response rates (data not shown).

U50 increased ICSS thresholds in control mice (Fig. 3), causing parallel rightward shifts in the ICSS rate–frequency functions (Fig. 3*a*,*b*). However, U50 had minimal effects in mCREB mice. The highest dose of U50 (5.5 mg/kg) affected response rates in a small number of mice: it transiently eliminated responding in a control mouse, and it transiently induced large (>200%) increases in rates in two mCREB mice. These data were not included in the analyses. Time course analysis of the highest dose of U50 without effects on response rates (3.0 mg/kg) indicated that the effects of the drug became detectable 30–45 min after administration and lasted ~45 min (Fig. 3*c*) (genotype × treatment × time interaction: $F_{(5,100)} = 3.33$, $p < 0.01$). Accordingly, subsequent analyses included only data collected during the 30–75 min period of testing. Since repeated testing did not alter the effect of U50 on thresholds or response rates (data not shown), data for each dose were compiled into single means. The effects of U50 depended on an interaction between genotype and dose ($F_{(6,120)} = 4.57$, $p < 0.01$) (Fig. 3*d*). U50 elevated ICSS thresholds in control mice at 3.0 mg/kg and 5.5 mg/kg (p values <0.01), whereas it had no effect in mCREB mice. The threshold-elevating effects of U50 were significantly larger in control mice at 3.0 mg/kg and 5.5 mg/kg (p values <0.01). U50 also affected maximum response rates (main effect of dose: $F_{(6,120)} = 2.40$, $p < 0.05$) (data not shown). Simple main effects tests indicated that 5.5 mg/kg U50 decreased response rates (to ~85% baseline) in control mice ($p < 0.05$).

Inducible expression of mCREB in NAc decreases expression of CREB-dependent genes (Sakai et al., 2002; McClung and Nestler, 2003). We quantified mRNA levels of several proteins that regulate G-protein signaling and are associated with altered drug sensitivity, but have not been previously examined in these mutants. GRK3 gene expression was repressed in NAc by mCREB ($t_{(4)} = 5.28$, $p < 0.01$) (Table 1). None of the other genes examined were altered. To examine the consequences of altered function of CREB or GRK3 specifically within the NAc, we used HSV vectors to transiently express mCREB, wtGRK3, or dnGRK3 in this area. Dominant-negative activity of dnGRK3 (containing a lysine–arginine point mutation at residue 220) was confirmed as described previously (Benovic and Gomez, 1993): phosphorylation of rhodopsin, a GRK3 substrate, was attenuated at dnGRK3:wtGRK3 ratios of 3:1 or higher ($F_{(4,8)} = 27.2$, $p < 0.01$; Newman–Keuls tests, p values <0.01; data not shown). The effects of gene transfer on ICSS depended on main effects of treatment ($F_{(3,40)} = 6.13$, $p < 0.01$) and day ($F_{(2,80)} = 8.83$, $p < 0.01$) (Fig. 4). Simple main effect tests on individual test days indicated that expression of mCREB or dnGRK3 decreased ICSS thresholds on days 3 and 5 ($p < 0.05$), but these effects had waned by day 7. Overexpression of wtGRK3 had no effect, implying maximal function under baseline conditions.

Table 1. Effect of inducible expression of mCREB on mRNA levels (mean ± SEM) of select G-protein-related genes in the NAc

Genotype	GRK3	RGS9	Gβ5	Gαi2
Control	1.00 (±0.09)	1.00 (±0.18)	1.00 (±0.09)	1.00 (±0.16)
mCREB	0.46 (±0.08)**	1.07 (±0.13)	1.17 (±0.09)	0.92 (±0.02)

**$p < 0.01$, $n = 4$ per group.

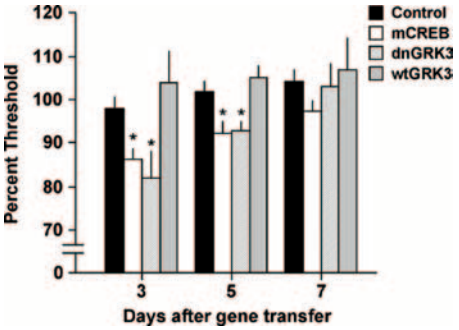

Figure 4. Effects of HSV-mediated transgene expression in the NAc shell on ICSS. Expression of mCREB and dnGRK3 reduced mean (±SEM) ICSS thresholds in 30 min test on days 3 and 5. Data are expressed as percentage pre-gene transfer baselines. *$p < 0.05$, $n = 9$–14 rats/group.

Discussion

We show that mCREB-expressing mice are more sensitive to cocaine and less sensitive to U50,488, a prototypical representative of a class of drugs (KOR agonists) that produces anhedonia and dysphoria in laboratory animals (Bals-Kubik et al., 1993; Carlezon et al., 2006) and depressive effects in humans (Wadenberg, 2003). Cocaine induced larger decreases in ICSS thresholds in mCREB mice, indicating that it has greater reward-facilitating actions in mice with reduced CREB function. In contrast, the threshold-elevating effects of U50 were substantially reduced in mCREB mice, if evident at all, indicating that this drug has fewer depressive-like effects in these mutants. These results are broadly consistent with those from studies in which viral vectors were used to enhance or disrupt CREB function in NAc: elevated CREB expression reduced the rewarding effects of cocaine and induced depression-like effects, whereas mCREB expression increased cocaine reward and had antidepressant-like effects (Carlezon et al., 1998; Pliakas et al., 2001). Indeed, mCREB expression is enriched in forebrain areas (including NAc) of these mice (Sakai et al., 2002). We also show that, in NAc, mCREB decreases mRNA levels for GRK3, a protein that regulates opioid receptor internalization (McLaughlin et al., 2004). Finally, we show that disruption of CREB or GRK3 function specifically in the NAc is sufficient to increase sensitivity to brain stimulation reward. When considered with previous findings, these studies raise the possibility that disruption of CREB in the NAc influences motivation by simultaneously facilitating reward and reducing depressive-like states such as anhedonia and dysphoria, rather than by simply increasing sensitivity to all stimuli.

ICSS has several advantages for these types of studies (Carlezon and Chartoff, 2007): it is a highly trained behavior that is sensitive to treatments that induce rewarding or depressive states in humans, and it enables "real-time" analysis of treatment effects (e.g., while the animal is under the influence of a drug). As such, it is a powerful complement to tests such as place preference, which evaluates the conditioned effects of rewarding or aversive treatments. Virtually all drugs of abuse decrease ICSS thresholds, suggesting that their rewarding effects summate with the rewarding effects of MFB stimulation. In contrast, treatments with depressive effects in humans (including drug withdrawal, KOR agonists) increase ICSS thresholds, suggesting that they detract from the rewarding effects of the stimulation. One advantage of the "curve-shift" variant of the ICSS paradigm is that it distinguishes effects on motivation from effects on response capabilities, particularly when the shifts in rate–frequency functions are parallel as in our studies. Thus despite the propensity of high doses of U50 to decrease response rates, the fact that the drug increases ICSS thresholds is evidence that it decreases the rewarding effects of the stimulation.

The mechanisms by which mCREB transgenic mice have altered sensitivity to cocaine and U50 are not understood. Previous work suggests that CREB induction of dynorphin within the NAc contributes to depressive-like behaviors. For example, KOR agonists induce conditioned place aversions (Bals-Kubik et al., 1993), increase immobility in the forced swim test (Mague et al., 2003), and increase ICSS thresholds (Carlezon et al., 2006). These results are consistent with the idea that stimulation of KORs induces complex mood states that involve dysphoria, behavioral despair, and anhedonia. Indeed, KOR agonists are "depressogenic" in humans (Wadenberg, 2003). The mCREB mice express less dynorphin in the NAc, and exhibit other reductions in CREB-dependent transcription, which could contribute to the behavioral phenotype seen here and in other studies (Newton et al., 2002; Sakai et al., 2002; McClung and Nestler, 2003). However, the A6 mutants also express mCREB outside the NAc (dorsal striatum, cortex), and disrupted CREB function in these areas might affect behavior. We found that viral-mediated expression of mCREB in the NAc of rats was sufficient to induce increased sensitivity to reward. When considered with the fact that the A6 mutants were not more sensitive to the rewarding effects of MFB stimulation per se, this finding is consistent with evidence that CREB disruption outside the NAc has opposite effects on reward function (Carlezon et al., 2005). Similarly, the finding that reduced GRK3 function in the NAc has motivational consequences

supports the idea that lower levels of GRK3 expression in the mCREB mutant mice contributes to altered sensitivity to cocaine and U50. One consequence of decreased GRK3 expression in NAc might be reduced internalization of μ- and δ-opioid receptors, which could augment the ability of brain stimulation to induce endogenous opioid activation of these receptor subtypes, thereby enhancing reward. Indeed, naltrexone blockade of opioid receptors decreases the rewarding effects of MFB stimulation (West and Wise, 1988), suggesting an important role for endogenous opioids. As was the case with viral-mediated expression of mCREB, expression of dnGRK3 in the rat NAc increased sensitivity to the rewarding effects of MFB stimulation even though sensitivity to the stimulation itself was not altered in the A6 mice. Again, these results suggest that the consequences of CREB disruption are brain region specific (Carlezon et al., 2005). Although it is of interest to determine whether NAc expression of mCREB or dnGRK3 affects sensitivity to cocaine and U50 in rats, such work is complicated by the baseline shifts in ICSS thresholds caused by these viral-mediated manipulations. Baseline shifts make it imperative to test each drug over a complete range of doses, as we did in the mice, which is not currently possible within the time frame of HSV-mediated transgene expression.

Other molecular alterations in the mCREB mice may contribute to their phenotype. CREB regulates the firing rate of NAc medium spiny neurons: elevated CREB function increases the activity of NAc neurons and opposes the effects of cocaine (Dong et al., 2006). Our observation that disruption of CREB function induces the opposite effect (enhanced sensitivity to cocaine) is consistent with these electrophysiological data. Decreased sensitivity to U50 in these same mice suggests that the depressive-like effects of this drug involve increased activity of NAc medium spiny neurons, which are inhibited by mCREB expression (Dong et al., 2006). Enhanced inhibitory tone in NAc neurons might reduce the influence of U50-induced stimulation of KORs, which regulate dopaminergic inputs to this region (Svingos et al., 1999; Carlezon et al., 2006).

We have proposed that CREB in the NAc acts as a "hedonic rheostat," in part by regulation of dynorphin function (Nestler and Carlezon, 2006). According to this model, elevated CREB function in NAc dampens the sensitivity of reward circuits and contributes to depressive-like effects, whereas low CREB activity sensitizes reward circuits and induces antidepressant-like effects. The recent observation that CREB activity serves as a rheostat of NAc electrical excitability, with high CREB activity mediating NAc activation and low CREB activity mediating NAc inhibition (Dong et al., 2006), is consistent with NAc CREB functioning as a behavioral rheostat. The present data support this hypothesis and identify an additional gene (GRK3) that contributes to plasticity within motivational systems.

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