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ANTISENSE-INDUCED REDUCTION IN NUCLEUS ACCUMBENS CYCLIC AMP RESPONSE ELEMENT BINDING PROTEIN ATTENUATES COCAINE REINFORCEMENT

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Abstract—Repeated cocaine exposure up-regulates cyclic AMP signaling and increases the transcriptional activity of cyclic AMP response element binding protein (CREB) in the nucleus accumbens. To study the possibility that nucleus accumbens CREB activity regulates self-administration behavior, we tested the effects of a single, bilateral infusion of CREB antisense oligonucleotide into nucleus accumbens core and shell sub-regions on cocaine self-administration in rats. Nucleus accumbens core infusions of CREB antisense reduced CREB and the CREB-regulated immediate early gene brain-derived neurotrophic factor by 31 and 27%, respectively, but failed to alter levels of the homologous CREB family proteins cyclic AMP response element modulator and activating transcription factor 1, and had no effect on CREB levels in adjacent nucleus accumbens shell tissue. Similar infusions of CREB antisense in either core or shell produced a transient downward shift in cocaine self-administration dose-response curves on a fixed ratio 5 (five responses/injection) reinforcement schedule, indicating a reduction in cocaine reinforcement that fully recovered 3 days after treatment. CREB antisense also increased the threshold dose of cocaine required for reinstating cocaine self-administration, indicating that nucleus accumbens CREB levels regulate the incentive properties of cocaine. When access to cocaine was less restricted on a fixed ratio 1 schedule, infusion of CREB antisense in the core, but not shell, caused a transient (1–2 days) reduction in stabilized cocaine self-administration, but had no effect on responding maintained by sucrose pellets, indicating that basal CREB levels in the nucleus accumbens core regulate drug intake. None of these effects were produced by nucleus accumbens infusions of complementary sense oligonucleotide. These results suggest a necessary role for nucleus accumbens CREB activity in cocaine reinforcement, and, by converse analogy, up-regulation in CREB activity after chronic cocaine use could contribute to addiction-related increases in cocaine self-administration.

Key words: addiction, reinstatement, reward, self-administration, cyclic AMP response element binding protein, brain-derived neurotrophic factor.

Repeated psychostimulant use produces numerous cellular and physiological changes, but relatively few are known to contribute to addiction-related changes in self-administration behavior (Koob and Le Moal, 2001; Nestler, 2001; Self, 2004; Kalivas et al., 2005). Acute treatment with psychostimulants activates the transcription factor CREB (cyclic AMP response element binding protein) through dopamine- and glutamate-dependent pathways in the dynorphin-containing subpopulation of striatal neurons (Montminy, 1997; Shaywitz and Greenberg, 1999; Andersson et al., 2001; Lonze and Ginty, 2002; Walters et al., 2003). In addition, up-regulation in cyclic AMP (cAMP) signaling pathways following chronic drug use could lead to persistent activation of CREB-regulated gene transcription (Nestler et al., 1990; Terwilliger et al., 1991; Striplin and Kalivas, 1993; Unterwald et al., 1996; Freeman et al., 2001). Although such up-regulation in CREB activity following chronic cocaine exposure has not been reported, repeated exposure to amphetamine induces prolonged increases in striatal CREB phosphorylation that coincide with behavioral sensitization (Cole et al., 1995; Simpson et al., 1995; Turgeon et al., 1997). Furthermore, repeated treatment increases the degree of cAMP response element (CRE)-mediated gene transcription induced by amphetamine, but only in nucleus accumbens (NAc) neurons, and also recruits additional cell types in the NAc that otherwise do not show CREB activity with acute amphetamine treatment (Shaw-Lutchman et al., 2003).

Neuroadaptations in the dopamine- and glutamate-responsive cells of the NAc are thought to play a significant role in addiction-related changes in drug intake, reinforcing efficacy, and the propensity for relapse in withdrawal (Nestler, 2001; Self, 2004; Kalivas et al., 2005). We previously reported that sustained activation of cAMP-dependent protein kinase in the NAc increases cocaine intake consistent with addiction-like changes in drug self-administration (Self et al., 1994, 1998). While these tolerance-like effects could involve local cytoplasmic phosphorylation events leading to dopamine receptor desensitization or down-regulation, persistent up-regulation in cAMP signaling also could invoke downstream effects on CREB-regulated gene expression (Nestler, 2001; Self, 2004).

In this regard, viral-mediated overexpression of CREB in the NAc shell region reduces sensitivity to cocaine re-

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Abbreviations: ATF-1, activating transcription factor 1; BDNF, brain-derived neurotrophic factor; cAMP, cyclic AMP; CRE, cyclic AMP response element; CREB, cyclic AMP response element binding protein; CREM, cyclic AMP response element modulator; FR, fixed ratio; NAc, nucleus accumbens; NGS, normal goat serum; PBS, phosphate-buffered saline; PKA, protein kinase A; TO, time out.

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ward in a conditioned place preference paradigm, whereas, expression of dominant negative CREB in the NAc, or knockout of CREB α and Δ isoforms, increases sensitivity to cocaine (Carlezon et al., 1998; Pliakas et al., 2001; Walters and Blendy, 2001). These studies suggest that NAc CREB activity also could regulate drug-taking and -seeking behaviors associated with cocaine self-administration.

Previous studies suggest that within the NAc, the shell subregion may have a predominant role in regulating drug taking (reward), whereas the NAc core may have a predominant role in regulating drug seeking (incentive motivation), although substantial overlap exists (Ito et al., 2000; Alderson et al., 2001; Di Ciano and Everitt, 2001; Hutcheson et al., 2001; Rodd-Henricks et al., 2002). In this study, we used an antisense oligonucleotide directed against CREB mRNA to test the effects of down-regulating endogenous CREB levels in NAc core and shell subregions on various aspects of cocaine self-administration, including cocaine reinforcement, the ability of cocaine priming to reinstate self-administration behavior, and regulation of cocaine intake with relatively unrestricted access.

EXPERIMENTAL PROCEDURES

Experimental subjects

Animals were maintained under ethical standards to minimize pain and distress according to the National Institutes of Health (USA) *Guide for the Care and Use of Laboratory Animals*. Serial testing was implemented when possible to reduce the number of animals used. Outbred male Sprague–Dawley rats, initially weighing 300–325 g on arrival (Charles River, Kingston, NY, USA), were housed individually in a climate-controlled environment (21 °C) on a 12-h light/dark cycle (lights on at 7:00 AM).

CREB sense and antisense oligos

Sense and antisense treatments involved a single infusion of fully phosphorothioate-modified, 20 base oligo sequences: CREB antisense, 5′TGGTCATCTAGTCACCGGTG3′; CREB sense, 5′CACCGGTGACTAGATGACCA3′ (Midland Certified Reagent Co., Midland, TX, USA). The antisense sequence was directed at the translation start site of the CREB message and was chosen based on its published efficacy at reducing CREB in the striatum (Konradi et al., 1994). Prior to brain infusion, the oligos were ethanol precipitated, washed three times with 70% ethanol, and resuspended in sterile phosphate-buffered saline (PBS, pH 7.4). The concentration was determined by absorbance at a wavelength of 260 nm and adjusted to 10 μg/μl accordingly.

CREB immunoreactivity in brains slices and NAc homogenates

CREB immunohistochemistry. Under sodium pentobarbital anesthesia (60 mg/kg, i.p.), drug naïve rats were given unilateral stereotaxic infusions of CREB antisense (or sense) oligo (10 μg/1.0 μl), while the contralateral side (balanced left and right) received infusion of the PBS vehicle. NAc infusions were delivered through 26 gauge Hamilton microsyringes (Hamilton, Reno, NV, USA) over a 3 min period in the NAc core at +1.7 mm anterior to bregma, 1.5 mm lateral to midline, and −6.7 mm ventral to dura with level skull according to Paxinos and Watson (1998). Eighteen hours after the infusion, rats were anesthetized with chloral hydrate and transcardially perfused with ice-cold 1× PBS followed by 4% para-formaldehyde. Rat brains were cryoprotected overnight in 20% glycerol, and frozen coronal brain slices (40 μm) were sectioned, washed with 1% H_2O_2/PBS for 30 min, and blocked with 3% normal goat serum (NGS)/PBS containing 0.3% Triton X-100 for 1 h at 23 °C. Free floating sections were incubated overnight with rabbit primary anti-CREB (1:500, Upstate Biotechnology, Lake Placid, NY, USA), followed by 1 h in biotinylated goat anti-rabbit IgG (1:200; DAKO Corporation, Carpinteria, CA, USA) secondary in 1% NGS/PBS and 1 h with avidin–biotin–peroxidase complex (1:50; DAKO Corporation). Peroxidase activity was labeled for 10 min with a 3,3-diaminobenzidine kit (Vector Laboratories, Inc., Burlingame, CA, USA), and sections were mounted on slides.

Immunoblot analysis. Eighteen hours following unilateral CREB antisense oligo and PBS infusions (as described above), animals were removed from their home cages and immediately decapitated in a separate room; the brains were rapidly dissected and chilled in ice-cold artificial CSF (126 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 10 mM D-glucose, pH 7.4). Medial NAc core samples were obtained with a 16 gauge punch from chilled coronal brain slices (~+1.2–2.2 mm anterior to bregma; Paxinos and Watson, 1998) and immediately frozen and stored at −80 °C. Half-moon-shaped samples of the remaining adjacent ventromedial shell tissue were obtained with a 14 gauge punch. Tissue samples were homogenized by sonication and protein concentrations were determined (Lowry et al., 1951). Twenty microgram protein samples were subjected to SDS–polyacrylamide gel electrophoresis in (7.5% acrylamide–0.12% bisacrylamide), followed by electrophoretic transfer to PVDF (polyvinylidene fluoride) membranes. CREB, CREM (cyclic AMP response element modulator), and ATF-1 (activating transcription factor-1) were sequentially immunolabeled with rabbit anti-CREB (1:2000; Upstate Biotechnology) or anti-CREM (1:1000), and mouse anti-ATF-1 (1:500) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) overnight at 4 °C in blocking buffer consisting of 5% non-fat dried milk powder in PBST (10 mM $NaPO_4$, 0.9% NaCl, 0.1% Tween 20, pH 7.4). Blots were stripped after anti-CREB and anti-CREM labeling with Restore (Pierce, Rockford, IL, USA). BDNF (brain-derived neurotrophic factor) was isolated on 12% acrylamide–0.12% bisacrylamide gels using a Tris/Tricine/SDS electrophoresis buffer (Bio-Rad, Hercules, CA, USA), and membranes were immunolabeled with anti-BDNF (1:3000) from Santa Cruz Biotechnology as described above. After incubation with the primary antibody, blots were washed with blocking buffer and incubated for 1 h at 23 °C with peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (1:25,000; Chemicon, Temecula, CA, USA). Immunoreactivity of labeled protein was visualized with enhanced chemiluminescence for peroxidase labeling (NEN, Boston, MA, USA). Immunoreactivity was quantified by densitometric analysis using NIH image 1.57 (National Institutes of Health, Bethesda, MD, USA). Immunoreactivity under the conditions was linear over a four-fold concentration range for each protein.

Cocaine and sucrose self-administration

To facilitate acquisition of cocaine self-administration, animals initially were maintained on a restricted diet of laboratory chow at 85% of their original body weight, and trained to press a lever for 45 mg sucrose pellets on a fixed ratio 1 reinforcement schedule (FR1) until acquisition criteria were achieved (100 pellets self-administered for three consecutive days). Animals then were fed *ad libitum* for at least 1 day before surgery.