Neuroscience 137 (2006) 373–383

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

K.-H. CHOI, K. WHISLER, D. L. GRAHAM AND D. W. SELF*

Department of Psychiatry, Seay Center for Basic and Applied Research in Psychiatric Illness, University of Texas Southwestern Medical Center, Dallas, TX 75390-9070, USA

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. © 2005 Published by Elsevier Ltd on behalf of IBRO.

*Corresponding author. Tel: +1-214-648-1237; fax: +1-214-648-4947.
E-mail address: david.self@utsouthwestern.edu (D. W. Self).
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.

0306-4522/06$30.00+0.00 © 2005 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2005.10.049

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-

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.

Surgical catheterization/cannulation procedures. Under sodium pentobarbital anesthesia (60 mg/kg, i.p.), a chronic indwelling catheter composed of SILASTIC tubing (Green Rubber, Woburn, MA, USA) and treated with tridodecylmethyl ammonium chloride (TDMAC) heparin (Polysciences Inc., Warrington, PA, USA), was surgically placed in the animals' jugular vein according to published procedures (Self et al., 1998). The catheter was secured with Mersilene surgical mesh (General Medical, New Haven, CT, USA) at the jugular vein, and passed s.c. to exit the animals' back through a 22 gauge cannula (Plastics One, Roanoke, VA, USA) imbedded in dental cement on a Marlex surgical mesh (Bard Inc., Cranston, RI, USA). In addition, bilateral 26 gauge guide cannulae (Plastics One) were stereotaxically implanted in the NAc core or shell subregions in the same surgical procedure. Stereotaxic coordinates were +1.7 mm anterior to bregma, ±1.5 mm (core) or ±0.8 mm (shell) lateral, and −5.7 mm ventral to dura. Dummy cannulae, cut to extend 1 mm beyond guide cannulae, were left in place throughout the experiment. Animals received a prophylactic injection of penicillin (60,000 IU/0.2 ml, i.m.) and antibiotic ointment was applied daily to the catheter exit wound. Catheters were flushed daily with 0.2 ml of heparinized (20 U/ml), bacteriostatic saline containing gentamycin sulfate (0.33 mg/ml).

Apparatus. Operant chambers (Med Associates Inc., St. Albans, VT, USA) for cocaine and sucrose pellet self-administration were contextually different from the animals' home cage, and located in a different room. Each chamber was equipped with an infusion pump assembly consisting of a Razel Model A pump (Stamford, CT, USA) and 10 ml glass syringe connected to a fluid swivel (Instech, Plymouth Meeting, PA, USA) by Teflon tubing. Tygon® tubing enclosed by a metal spring connected the swivel to the animal's catheter exit port and was secured to Teflon threads on the catheter assembly. Each operant chamber contained two levers, and either one (FR1) or five (FR5) 20 g lever press responses on the active lever delivered an i.v. injection of sterile-filtered cocaine (NIDA, Research Triangle Park, NC, USA) dissolved in 0.9% saline. During each injection, a cue light above the active lever was illuminated, and the house light was extinguished. Each injection was followed by an additional "time-out" (TO) period in which the cue light was extinguished; lever press responses during the entire injection-TO period were recorded but had no programmed consequences. Responses on the inactive lever were recorded but had no scheduled consequence.

Effects of CREB sense and antisense on cocaine self-administration dose-response curves. After a minimum 6 day recovery period, animals were placed in operant test chambers during their light cycle and allowed to acquire cocaine self-administration (500 μg/kg/0.1 ml in a 5 s injection) on a FR1:TO 15 s (FR1:TO 15) reinforcement schedule in daily 2-h self-administration test sessions for 5–6 days per week. After a minimum of 15 test sessions to acquire self-administration, the response requirement was gradually raised to five lever presses/injection over several days, and training continued until cocaine intake stabilized (number of total injections varied <10% from the mean of three consecutive sessions). Following stabilization on the FR5 schedule, animals were trained in daily within-session dose-response tests where five ascending injection doses were available in sequential 30 min components (the FR5 schedule facilitates stability in this dose-response procedure). The unit dose/injection was increased in each 30 min component by prolonging the injection time (volume) for 0, 2.5, 5, 10, and 20 s to produce unit injection doses of 0, 125, 250, 500, 1000 μg/kg cocaine in volumes of 0, 0.05, 0.1, 0.2, 0.4 ml, respectively. Each infusion accompanied by a cue light was followed by a 20 s time out period. The sequential 30 min components were separated by a 2 min TO period (lights off), and two non-contingent priming injections of cocaine at the dose available for self-administration (concurrent with cue light) were given 20 s apart at the onset of each 30 min component. The number of self-injections at each dose was measured, and animals were trained until both the threshold dose required for reinstating self-administration (priming dose eliciting the first self-injection), and the dose producing peak self-administration rates, remained constant for at least three consecutive sessions.

After demonstrating stability in dose-response testing, animals were given a single 16–18 h pretreatment via intra-NAc infusion of CREB antisense or CREB sense oligo (10 μg/side) through 33 gauge bilateral infusion cannulae (Plastics One) in 1.0 μl/side over a 3 min period. The effects of CREB antisense or sense on both the threshold dose of cocaine required to reinstate self-administration behavior, and the dose-response curve following initiation of cocaine self-administration, were determined for at least 3 days after the oligo treatment. Some rats that were tested with one oligonucleotide (sense or antisense) subsequently were tested with the alternate oligo (counterbalanced for order) following recovery of stability and at least 2 weeks of further cocaine self-administration in the dose-response procedure. One of six rats (core) developed catheter failure before testing with the alternate oligo; in the shell, only two of six rats (antisense) and two of five rats (sense) were tested with the alternate oligo following recovery of stabilized baseline dose-response responding.

Effects of CREB sense and antisense on stabilized unrestricted cocaine intake. Animals initially were trained to self-administer cocaine (500 μg/kg/injection) as described above on a FR1:TO 15 schedule of reinforcement in daily 2-h self-administration test sessions for 5–6 days per week. After a minimum of 15 test sessions and achievement of stabilized cocaine self-administration on the FR1 schedule, animals were given the NAc oligo infusions as described above, and the effects of CREB antisense or CREB sense oligo on stabilized cocaine intake were followed for at least 6 days after the NAc infusion. Most rats were tested with the alternate oligo (counterbalanced for order) following at least 2 weeks and recovery of stabilized baseline cocaine self-administration. Two of 10 rats (core) and one of eight rats (shell) developed catheter failures before testing with the alternate oligo.

Effects of CREB sense and antisense on stabilized sucrose pellet self-administration. Bilateral NAc guide cannulae were implanted in the NAc core of drug-naïve rats, and the rats were trained to self-administer sucrose pellets on a FR1 reinforcement schedule as described above. Following acquisition, the reinforcement schedule was changed to FR1:TO 2 min for 2 h/day (maximum of 60 pellets available), where a 2 min TO period with lights off followed each sucrose pellet delivery. Animals were trained on this schedule until total non-reinforced responding during the TO periods stabilized (number of TO responses varied <15% from the mean of three consecutive sessions). Stabilized animals received NAc infusions of CREB sense or CREB antisense 16–18 h prior to testing as described above. All animals subsequently were tested with the alternate oligo (counterbalanced for order) following demonstration of stabilized TO responding and at least 2 weeks of sucrose pellet self-administration training on the FR1:TO 2 min schedule.

Verification of NAc infusion sites. Following successful completion of self-administration experiments, animals were anesthetized with chloral hydrate (300 mg/kg, i.p.) and bilateral 0.5 μl Cresyl Violet infusions were delivered through the guide cannulae as described above. Immediately following the infusions, animals were decapitated, brains dissected and 0.8 mm thick coronal slices through the forebrain were collected and analyzed under a dissecting microscope for location of infusion sites according to stereotaxic coordinates of Paxinos and Watson (1998).

Data analysis. Immunoblots from CREB antisense-infused tissue were compared with contralateral PBS-infused tissue by Student's paired *t*-tests. Cocaine self-administration data (number

of self-injections) in CREB sense and antisense-infused animals were analyzed by multivariate ANOVA (region×treatment) with repeated measures on test session and cocaine dose; responding in sucrose self-administration utilized repeated measures on both treatment and test session. Significant interactions were followed by one-factor ANOVA across daily test sessions (within treatment), or across treatments at a given dose followed by Tukey's post hoc comparisons.

RESULTS

Effects of CREB antisense on CREB and BDNF immunoreactivity in the NAc

NAc infusions of CREB antisense oligo reduced endogenous CREB immunoreactivity in both coronal brain sections and immunoblots labeled with anti-CREB when compared with PBS-infused tissue. Fig. 1A shows that CREB antisense reduced the intensity of CREB labeling primarily in the vicinity of the infusion site within the medial NAc core, without altering CREB-labeled cells in the adjacent NAc shell region (results are representative of four CREB antisense-infused animals). Higher magnification shows that CREB antisense generally reduced the intensity of staining within CREB-positive cells, without reducing the overall number of CREB-positive cells (except in the immediate vicinity of the infusion site). In contrast, infusion of complementary CREB sense oligo had no effect on the intensity of anti-CREB staining compared with the contralateral side infused with PBS (Fig. 1B), consistent with a previous study using immunoblot procedures (Widnell et al., 1996). There was no evidence for extensive cell damage or gliosis in CREB sense- or antisense-infused tissue in consecutive Nissl-stained brain slices.

NAc infusions of CREB antisense reduced CREB protein levels by 31±9.6% (T_{12}=2.929; P=0.013) in core

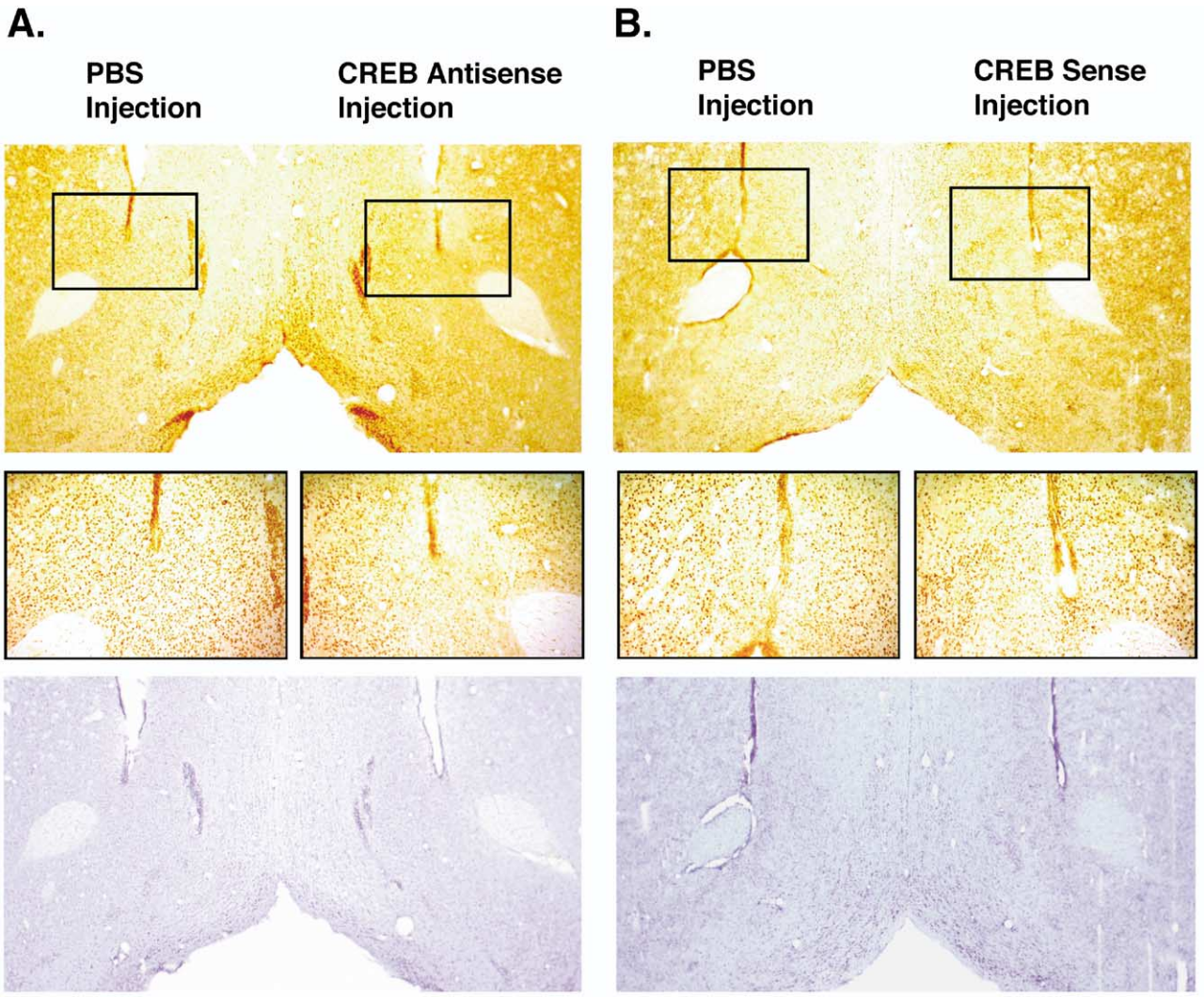

Fig. 1. A single unilateral infusion of CREB antisense (A), but not sense (B), oligonucleotide (10 μg/1.0 ul/side) in the NAc core subregion reduces endogenous CREB immunoreactivity when compared with contralateral infusions of the PBS vehicle. The middle panels (magnified 100×) show that CREB antisense decreases the intensity of CREB labeling in the medial core region primarily around the injections site, but not in adjacent medial shell tissue. Nissl-stained sections show no evidence of overt tissue damage induced by CREB antisense and sense oligos (lower panels). Oligos were infused into the NAc 18 h earlier.

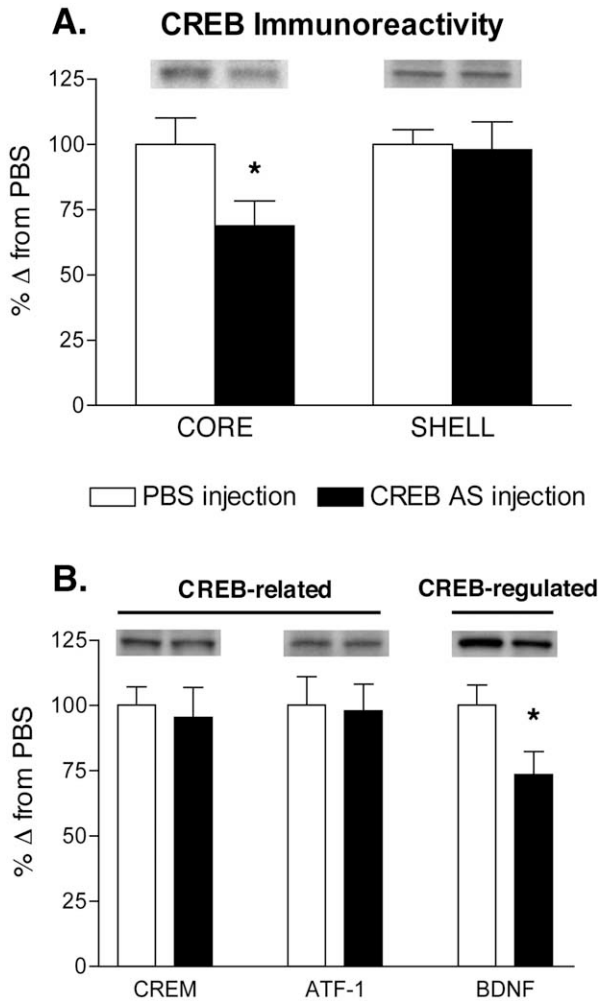

Fig. 2. (A) NAc core infusion of CREB antisense reduces CREB protein levels by 31% in homogenates of core tissue, but not in adjacent shell tissue ($N=13$). (B) CREB antisense has no effect on homologous CREB family members with CRE binding activity (CREM and ATF-1), but reduces levels of the CREB-regulated immediate early gene BDNF in the core. Asterisk indicates protein levels differ from contralateral infusions of PBS by paired t test ($P<0.05$).

tissue punches surrounding the infusion site when compared with contralateral PBS infusions (Fig. 2A), but did not alter levels of ATF-1 and CREM, two CREB family members with >65% homology in primary structure and similar CRE binding activity (Shaywitz and Greenberg, 1999). When CREB was normalized to CREM as an internal standard, CREB levels were reduced by 27±5.3% ($T_{12}=3.138$; $P=0.009$). The reduction in CREB levels was entirely limited to the subregion receiving the infusion, since CREB levels in homogenates of adjacent NAc shell tissue were not altered. Together with immunohistochemical labeling in brain slices, these results indicate that the CREB antisense effects remained highly localized within the subregion receiving the infusion. In addition, Fig. 2B shows that NAc infusions of CREB antisense also reduced levels of the CREB-regulated immediate early gene BDNF by 27% ($T_{10}=3.013$; $P=0.013$). These data indicate that CREB antisense selectively reduced CREB-regulated gene expression in the NAc.

Effects of CREB antisense on cocaine reinforcement and reinstatement of self-administration

A within-session dose-response procedure was used to test the effects of bilateral NAc infusions of CREB antisense and sense on cocaine self-administration dose-response curves. Fig. 3A shows that NAc infusions of CREB antisense produced transient downward shifts in the inverted U-shaped dose-response, resulting in significant oligo treatment×test session ($F_{2,36}=6.588$, $P=0.004$), oligo treatment×dose ($F_{4,72}=5.419$, $P=0.001$), and dose×test session ($F_{8,144}=2.327$, $P=0.022$) interactions in test sessions that spanned baseline (day prior to treatment), 1 day (16–18 h) and 3 days post-infusion. There were no significant interactions with NAc subregion or main effect of subregion reflecting similar flattening of dose-response curves following both core and shell infusions. Subsequent analyses of interactive effects found that CREB antisense reduced cocaine self-administration at the dose producing peak self-administration rates (250 μg/kg/injection) when compared with sense-infused controls 1 day after treatment ($F_{3,18}=9.333$, $P=0.001$). In addition, core, but not shell, infusions of CREB antisense reduced cocaine self-administration at the 500 μg/kg/injection dose on the descending limb of the curve when compared with pre-infusion baselines ($F_{1,20}=5.633$, $P=0.028$), but not at the 1000 μg/kg dose, possibly due to the relatively lower number of self-injections (floor effect). The downward shift in self-administration dose-response curves suggests that intra-NAc infusions of CREB antisense weakened the reinforcing efficacy of cocaine. Self-administration dose-response baselines were similar for both oligo treatments in core and shell subregions, and dose-response curves generally recovered from antisense effects by 3 days post-infusion (Fig. 3A).

The ascending dose-response procedure also determined the threshold dose for cocaine-primed reinstatement of cocaine self-administration, since two non-contingent priming injections of the available dose were given at the onset of each 30 min component of dose-response testing. NAc infusions of CREB antisense increased the threshold dose required to reinstate the first self-injection (Fig. 3B), resulting in a significant oligo treatment×test session interaction ($F_{2,36}=7.674$, $P=0.002$), but no difference between core and shell infusions was found, similar to effects on cocaine reinforcement. Subsequent analysis of oligo treatment effects found that antisense-induced increases in dose thresholds achieved significance when infused in the shell, but not core, subregion compared with sense-infused controls on the day after treatment ($F_{3,18}=4.320$, $P=0.018$), and baseline thresholds recovered fully by 3 days after the infusion. These data suggest that intra-NAc infusions of CREB antisense reduced sensitivity to the incentive properties of the cocaine priming injections (i.e. their ability to trigger a cocaine-seeking response). In contrast, NAc infusions of CREB sense failed to alter either the self-administration dose-response curves or the dose

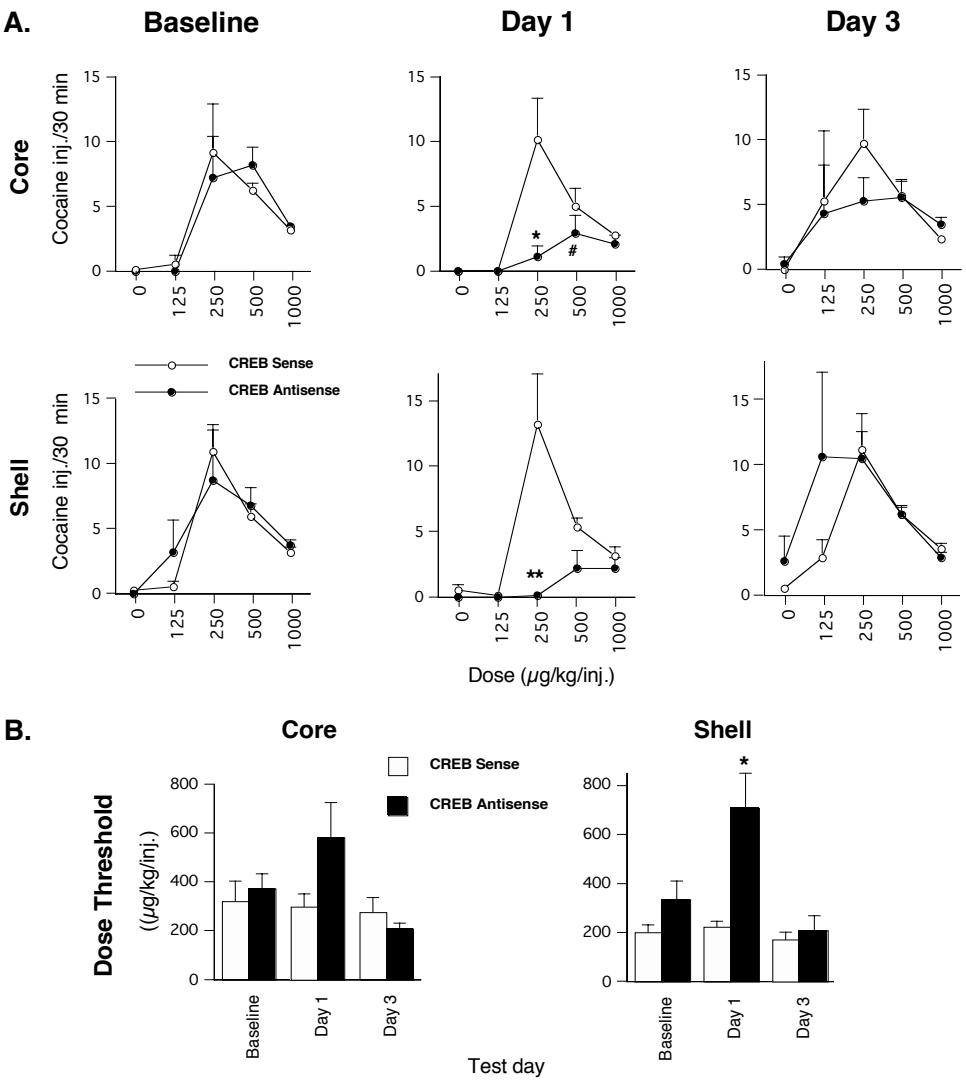

Fig. 3. (A) NAc core and shell infusions of CREB antisense (N=6/region) produce transient downward shifts in cocaine self-administration dose-response curves compared with control infusions of CREB sense (N=5–6/region) in a within-session ascending dose-response procedure (FR5:TO 20 s reinforcement schedule). (B) NAc infusions of CREB antisense increase the threshold dose of cocaine needed to reinstate self-administration behavior when administered as two non-contingent priming injections of the dose available for self-administration. The effects of CREB antisense on both dose-response curves and dose thresholds recover after 3 days post-infusion. Asterisks indicate * $P<0.05$ and ** $P<0.01$ compared with sense-infused controls, or # $P<0.05$ compared with pre-infusion baselines, by Tukey's post hoc tests.

thresholds for reinstatement when compared with pre-infusion baselines.

Effects of CREB antisense on cocaine intake with unrestricted access (FR1)

To determine whether NAc CREB levels regulate stabilized levels of cocaine intake when access to cocaine is relatively unrestricted, we tested the effects of NAc core and shell infusions of CREB antisense in animals self-administering cocaine on a FR1 reinforcement schedule in daily 2 h test sessions. Bilateral CREB antisense infusions reduced cocaine intake in self-administering animals 16–18 h following the NAc infusions in the core, but not shell subregion (Fig. 4). Response patterns from a representative animal show that core infusions of CREB antisense lengthened the post-injection pause between successive self-injections (Fig. 4A), an effect similar to increasing the unit dose of cocaine per injection. Analysis of self-administration data collected from baseline to 3 days post-infusion found a significant subregion×test session interaction ($F_{3,90}$=3.907, P=0.011), and a main effect of test session ($F_{3,90}$=8.349, $P<0.001$), reflecting the differential effect of CREB antisense and sense infusions in the core and shell over the course of testing (Fig. 4B). Within-region ANOVA found that NAc core infusions of CREB antisense, but not sense-infused controls, decreased stabilized cocaine intake when compared with pre-infusion self-administration baselines ($F_{3,27}$=8.347, $P<0.001$). These results indicate that NAc core infusions of CREB antisense prolonged the rate-limiting effects of cocaine resulting in fewer self-injec-

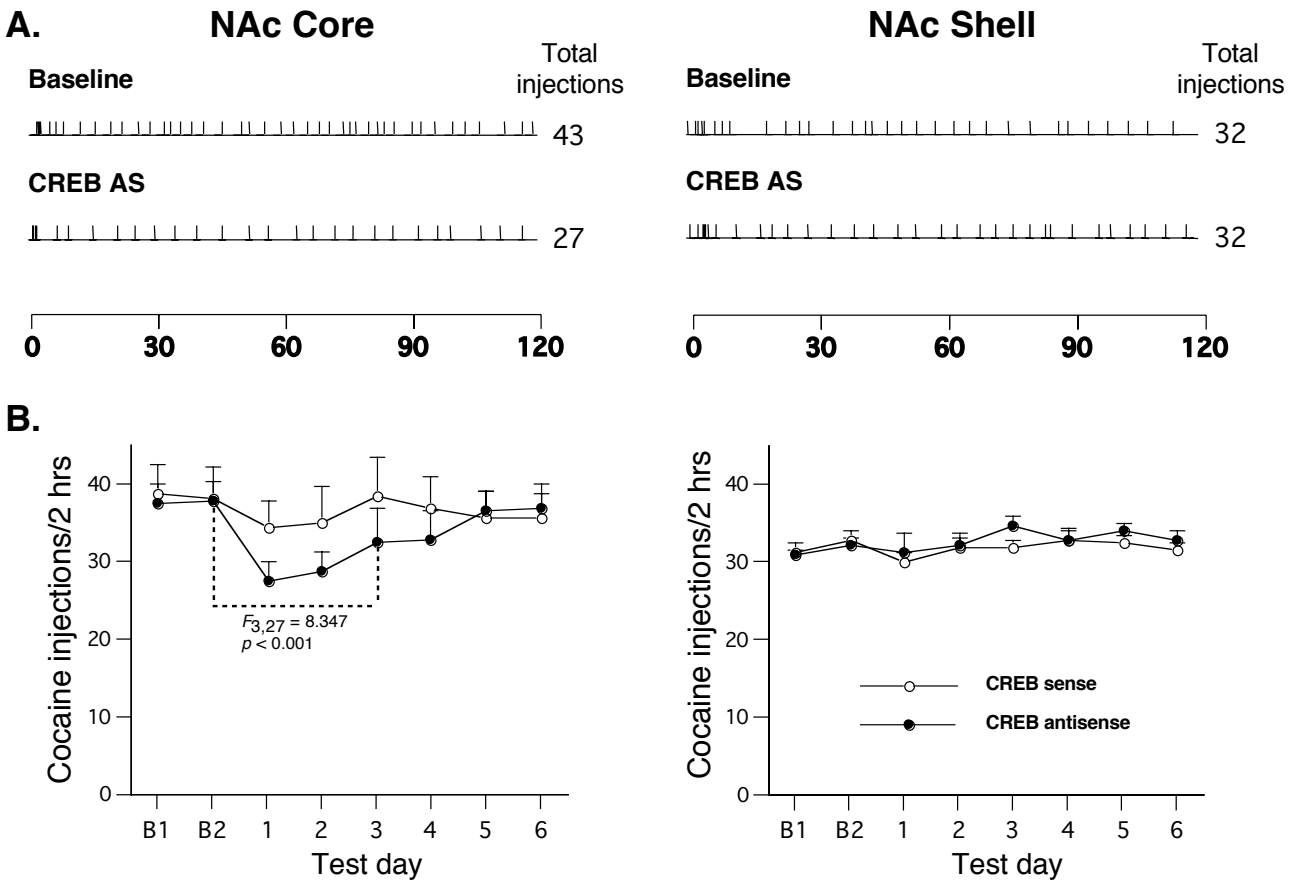

Fig. 4. NAc core, but not shell, infusion of CREB antisense oligo (AS) decreases cocaine intake in animals self-administering cocaine (500 μg/kg/injection) with relatively unrestricted access (FR1:TO 15 s reinforcement schedule). (A) Individual self-administration response records from representative animals show that CREB antisense decreases cocaine intake by prolonging the time interval between successive self-injections in 2 h test sessions conducted 1 day (16–18 h) following CREB antisense treatment. (B) NAc core infusion of CREB antisense ($N=10$) decreases cocaine intake by an average of 27% from pre-infusion self-administration baselines; baselines fully recover over consecutive daily test sessions. Similar NAc core infusion of CREB sense ($N=8$) fails to reduce cocaine intake, and neither oligo alters cocaine intake when infused in the NAc shell subregion ($N=8$/treatment).

tions over the course of testing. The antisense-induced reduction in cocaine intake was maximal the first day after the core infusion, but self-administration baselines recovered completely 5 days after the infusions.

Effects of CREB antisense on responding for sucrose pellets

To control for possible generalized effects on response rates with NAc core infusions of CREB antisense, we infused both oligos into the NAc core in animals trained to self-administer sucrose pellets on a FR1:TO 2 min reinforcement schedule. In this schedule, well-trained animals typically respond at high rates near the end of each TO period in anticipation of reward availability. Neither CREB antisense nor sense infusions altered high rates of anticipatory responding in the TO periods when compared with pre-infusion baseline response rates, and the number of sucrose pellets consumed remained near the maximum allowed in a 2 h session (Fig. 5). These data indicate that reductions in cocaine intake under conditions of relatively unrestricted access (FR1) with NAc core infusions of CREB antisense did not result from generalized rate-reducing effects.

Localization of NAc core and shell infusions sites

Fig. 6 shows the localization of bilateral NAc infusion sites in either core ($n=24$) or shell ($n=18$) for rats used in the analysis of behavioral data. NAc shell sites were clustered in the ventral medial shell region, whereas core sites were clustered in the medial core, typical of previously published studies (Self et al., 1998; Sutton et al., 2003).

DISCUSSION

A single NAc infusion of CREB antisense *in vivo*, but not sense, reduced the intensity of anti-CREB-labeled cells in the vicinity of the infusion site in rat brain slices. NAc core infusions of CREB antisense produced a 31% reduction in CREB protein levels without altering CREB levels in the adjacent medial shell subregion, indicating that antisense-induced reductions in CREB did not spread substantially beyond the infused subregion. Given that α and Δ isoforms are the predominant CREB proteins expressed in brain,

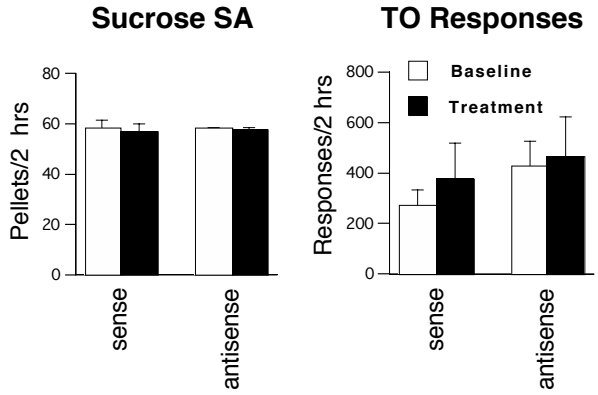

Fig. 5. NAc core infusion of CREB antisense and sense oligo fails to alter sucrose pellet self-administration when compared with pre-infusion baseline response rates ($N=8$/treatment). The left panel shows that animals self-administer near maximal sucrose pellets available in 2 h on a FR1:TO 2 min reinforcement schedule. The right panel shows that oligo treatment also fails to alter high rates of anticipatory responding in the 2 min TO periods that precede availability of each sucrose pellet.

and CREB antisense targets a homologous translational start site for both isoforms (Shaywitz and Greenberg, 1999), a reduction in CREB immunoreactivity probably reflects a reduction in both isoforms. The ability of CREB antisense to partially "knock down" CREB levels with subregional specificity and in adult animals has certain advantages over traditional knockout approaches, since $CREB^{\alpha\Delta}$ knockout mice show developmental or compensatory up-regulation of the β CREB isoform in the brain (Hummler et al., 1994; Blendy et al., 1996). Moreover, $CREB^{\alpha\Delta}$ knockout mice also show compensatory up-regulation in both activator and repressor isoforms of CREM (Shaywitz and Greenberg, 1999). In contrast, CREB antisense infusions selectively reduced total CREB levels without altering CREM, and had no effect on ATF-1, another CREB-related transcription factor that activates CRE-regulated transcription. Further evidence for reduced CREB function is indicated by the ability of CREB antisense to decrease the CREB-regulated immediate early gene, BDNF, and a previous study found that a single NAc infusion of this CREB antisense oligo also reduces cAMP-dependent protein kinase A (PKA) catalytic subunit levels, without altering numerous other signaling proteins (Widnell et al., 1996). Therefore, the effects of CREB antisense on cocaine self-administration behavior are likely attributable to a transient and selective down-regulation in CREB-regulated gene transcription, rather than a generalized impairment in CRE-regulated gene transcription.

In animals trained to self-administer cocaine, NAc infusions of CREB antisense (but not sense) produced downward shifts in self-administration dose-response curves in both core and shell subregions. Thus, even a 31% reduction in basal CREB levels limited to the NAc core subregion substantially reduced cocaine reinforcement. Similar effects with shell infusions of CREB antisense suggest that CREB levels in both subregions maintain sensitivity to cocaine reinforcement. It is unlikely that shell infusions spread to the core, since there was no evidence for diffusion in the converse direction, and shell infusions of CREB antisense failed to recapitulate the reduction in cocaine intake produced by core infusions under relatively unrestricted access conditions. Furthermore, the failure of shell infusions to affect unrestricted cocaine intake, and the finding that core infusions failed to alter even higher response rates in animals trained to self-administer sucrose pellets, suggest that downward shifts in cocaine self-administration dose-response curves reflect reduced motivation for cocaine rather than overt deficits in performance capability.

Antisense-induced reduction in NAc CREB levels also increased the threshold dose of cocaine needed to reinstate self-administration behavior when cocaine was administered as response-independent priming injections. Thus, in addition to regulation of cocaine reinforcement, basal levels of CREB in the NAc apparently maintain sensitivity to the incentive properties of cocaine, and lower CREB levels would reduce the propensity for cocaine-induced relapse to cocaine self-administration. The transient effects of CREB antisense on cocaine reinforcement, cocaine intake, and cocaine-induced reinstatement all diminished 3 days after the antisense infusions, consistent with a previous study showing full recovery of CREB levels 3 days following a single NAc infusion of an identical CREB antisense oligo (Widnell et al., 1996). This feature, and the absence of prominent gliosis or scarring in antisense- (or sense-) infused tissue, together with normal levels of CREM, ATF-1 and several other signaling proteins as discussed above, indicate that changes in these cocaine-regulated behaviors were not caused by cell damage or other potential neurotoxicity with oligonucleotide infusions.

The descending limb of the self-administration dose-response curve reflects the fact that higher injection doses of cocaine have an inhibitory "feedback" effect on further cocaine self-administration behavior. Core infusions of CREB antisense decreased cocaine intake at an intermediate injection dose on the descending limb (500 μg/kg/injection) by prolonging the time interval between successive self-injections, but animals maintained regular temporal patterns of self-administration (see Fig. 4A). This effect

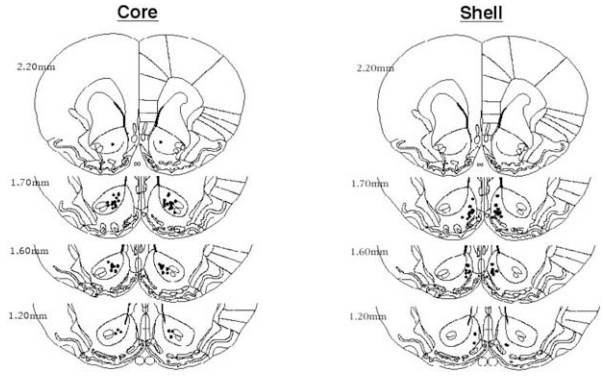

Fig. 6. Localization of NAc CREB oligo infusion sites in the core ($N=24$) and shell ($N=18$) subregions in cannulated animals used in behavioral tests.

is identical to increasing the injection dose of cocaine, resulting in fewer self-injections over a given period of time. Therefore, while a reduction in CREB levels in the NAc core attenuated the reinforcing efficacy of cocaine, it also enhanced, or prolonged, cocaine's inhibitory effects on further self-administration. Conversely, blockade of cocaine effects under these conditions (as produced by dopamine antagonist treatment) typically increases cocaine intake as animals self-administer cocaine with shorter inter-injection intervals (Koob and Goeders, 1989).

The ability of core and shell infusions of CREB antisense to attenuate cocaine-induced reinstatement agrees with studies showing that dopamine agonists reinstate cocaine-seeking behavior in both core and shell subregions (Bachtell et al., 2005), and other studies suggest a role for dopaminergic and glutamatergic neurotransmission in both NAc subregions in cocaine-seeking behavior (Di Ciano and Everitt, 2001; Cornish and Kalivas, 2000; Ito et al., 2000; Sutton et al., 2003). In addition, both cocaine reinforcement and regulation of cocaine intake are sensitive to dopamine receptor blockade in the NAc, although core and shell subregions were not compared in these studies (McGregor and Roberts, 1993; Caine et al., 1995). Our results suggest that basal levels of CREB-regulated gene expression in both core and shell subregions are necessary to maintain sensitivity to cocaine reinforcement and cocaine-induced reinstatement of cocaine self-administration, while CREB-regulated genes in the NAc core may play a greater role in regulating unrestricted cocaine intake.

The mechanism whereby CREB antisense regulates cocaine reinforcement, reinstatement of cocaine self-administration, and cocaine intake is unknown, but could involve down-regulation of PKA catalytic subunits or BDNF levels. In this regard, the effects of CREB antisense on cocaine intake were somewhat similar to the effects of the PKA inhibitor, Rp-cAMPS, when infused into the NAc (Self et al., 1998). However, NAc infusions of the PKA inhibitor enhanced the ability of cocaine to elicit cocaine-seeking responses, whereas CREB antisense reduced this ability, suggesting that changes in other CREB-regulated genes underlie the effect on reinstatement. A reduction in BDNF levels could contribute to this effect, since BDNF infusions in the NAc facilitate cocaine effects on conditioned reward (Horger et al., 1999). Ultimately, the effects of CREB on these behaviors could involve alterations in dopaminergic and glutamatergic input to NAc neurons.

The ability of CREB antisense to attenuate cocaine reinforcement and cocaine-induced reinstatement in self-administering animals differs from the increased sensitivity to low doses of cocaine in CREB$^{\alpha\Delta}$ knockout mice and with dominant negative CREB expression in rats in conditioned place preferences tests (Carlezon et al., 1998; Walters and Blendy, 2001). A primary reason for this discrepancy may involve differences between instrumental and Pavlovian conditioning procedures, including voluntary versus involuntary drug administration, or the fact that conditioned place preference is conducted in initially drug naïve animals, whereas our studies were conducted in cocaine-experienced and potentially sensitized animals. These differences underscore the notion that conditioned place preference and self-administration represent fundamentally distinct behaviors, and caution against extrapolating results from one procedure to the other.

There are other biological differences between CREB antisense in rats and CREB$^{\alpha\Delta}$ knockout mice including 1) regional versus generalized targeting, 2) partial versus complete knockdown of CREB, and 3) differential biological responses with antisense (reduced PKA and BDNF) and CREB$^{\alpha\Delta}$ knockout mice (increased CREB$^{\beta}$ and CREM) as discussed above. Regarding the latter, CREB$^{\alpha\Delta}$ knockout fails to abolish cocaine-induced CREB phosphorylation in the NAc (although it is substantially reduced from wild type controls), and cocaine-induced increases in dynorphin expression, a CREB-regulated gene, are greater in CREB$^{\alpha\Delta}$ knockouts (Walters et al., 2003). Moreover, cocaine-induced reinstatement of an established cocaine place preference is not altered in CREB$^{\alpha\Delta}$ knockout mice, whereas stress-induce reinstatement is abolished (Kreibich and Blendy, 2004). When compared with dominant negative approaches, it is important to note that dominant negative CREB occludes all CRE binding activity, including regulation by other CREB family members (CREB, CREM, ATF-1, etc.), although viral-mediated expression methods provide good regional control and neuronal specificity. In contrast, CREB antisense is targeted specifically to CREB isoforms, but its effects are not restricted to neuronal tissue. Further investigation is needed to determine whether behavioral and/or biological methodology can account for opposite effects on cocaine responses with these varied approaches.

Chronic exposure to abused drugs and alcohol upregulates cAMP signaling proteins and can produce prolonged CREB activation in the NAc (see introduction). By converse analogy to our results with CREB antisense, such increases in CREB function in NAc neurons could produce vertical shifts in the self-administration dose-response curve, indicating an overall increase in cocaine reinforcement, and may contribute to escalating cocaine intake during self-administration, whereas increased sensitivity to the incentive properties of cocaine would contribute to craving and relapse in withdrawal. Such reciprocal changes in cocaine-taking and -seeking behaviors are hallmarks of cocaine-addicted phenotypes in animal models (Ahmed and Koob, 1998; Piazza et al., 2000; Sutton et al., 2000). Given that a relatively minor 1/3 reduction in CREB levels in core or shell subregions substantially alters these aspects of cocaine self-administration, CREB and its downstream target genes in the NAc may play a prominent role in contributing to these important behavioral changes in cocaine addiction.

Acknowledgments—This work is supported by the United States Public Health Service grants DA 10460, DA 08227, and by the Wesley Gilliland Professorship in Biomedical Research (UTSW).

REFERENCES

Ahmed SH, Koob GF (1998) Transition from moderate to excessive drug intake: change in hedonic set point. Science 282:298–300.

Alderson HL, Parkinson JA, Robbins TW, Everitt BJ (2001) The effects of excitotoxic lesions of the nucleus accumbens core or shell regions on intravenous heroin self-administration in rats. Psychopharmacology 153:455–463.

Andersson M, Konradi C, Cenci MA (2001) cAMP response element-binding protein is required for dopamine-dependent gene expression in the intact but not the dopamine-denervated striatum. J Neurosci 21:9930–9943.

Bachtell RK, Whisler K, Karanian DA, Self DW (2005) Effects of intra-nucleus accumbens shell administration of dopamine agonists and antagonists on cocaine-taking and -seeking behavior. Psychopharmacology 183(1):41–53.

Blendy JA, Kaestner KH, Schmid W, Gass P, Schutz G (1996) Targeting of the CREB gene leads to up-regulation of a novel CREB mRNA isoform. EMBO J 15:1098–1106.

Caine SB, Heinrichs SC, Coffin VL, Koob GF (1995) Effects of the dopamine D-1 antagonist SCH 23390 microinjected into the accumbens, amygdala or striatum on cocaine self-administration in the rat. Brain Res 692:47–56.

Carlezon WA, Thome J, Olson VG, Lane-Ladd SB, Brodkin ES, Hiroi N, Duman RS, Neve RL, Nestler EJ (1998) Regulation of cocaine reward by CREB. Science 282:2272–2275.

Cole RL, Konradi C, Douglass J, Hyman S (1995) Neuronal adaptation to amphetamine and dopamine: molecular mechanisms of prodynorphin gene regulation in rat striatum. Neuron 14:813–823.

Cornish JL, Kalivas PW (2000) Glutamate transmission in the nucleus accumbens mediates relapse in cocaine addiction. J Neurosci 20:RC89 (1–5).

Di Ciano P, Everitt BJ (2001) Dissociable effects of antagonism of NMDA and AMPA/KA receptors in the nucleus accumbens core and shell on cocaine-seeking behavior. Neuropsychopharmacology 25: 341–360.

Freeman WM, Nader MA, Nader SH, Robertson DJ, Gioia L, Mitchell SM, Daunais JB, Porrino LJ, Friedman DP, Vrana KE (2001) Chronic cocaine-mediated changes in non-human primate nucleus accumbens gene expression. J Neurochem 77:542–549.

Horger BA, Iyasere CA, Berhow MT, Messer CJ, Nestler EJ, Taylor JR (1999) Enhancement of locomotor activity and conditioned reward to cocaine by brain-derived neurotrophic factor. J Neurosci 19: 4110–4122.

Hummler E, Cole TJ, Blendy JA, Ganss R, Aguzzi A, Schmid W, Beermann F, Schutz G (1994) Targeted mutation of the CREB gene: compensation within the CREB/ATF family of transcription factors. Proc Natl Acad Sci U S A 91:5647–5651.

Hutcheson DM, Parkinson JA, Robbins TW, Everitt BJ (2001) The effects of nucleus accumbens core and shell lesions on intravenous heroin self-administration and the acquisition of drug-seeking behaviour under a second-order schedule of heroin reinforcement. Psychopharmacology 153:464–472.

Ito R, Dalley JW, Howes SR, Robbins TW, Everitt BJ (2000) Dissociation in conditioned dopamine release in the nucleus accumbens core and shell in response to cocaine cues and during cocaine-seeking behavior in rats. J Neurosci 20:7489–7495.

Kalivas PW, Volkow N, Seamans J (2005) Unmanageable motivation in addiction: a pathology in prefrontal-accumbens glutamate transmission. Neuron 45:647–650.

Konradi C, Cole RL, Heckers S, Hyman SE (1994) Amphetamine regulates gene expression in rat striatum via transcription factor CREB. J Neurosci 14:5623–5634.

Koob GF, Goeders NE (1989) Neuroanatomical substrates of drug self-administration. In: The neuropharmacological basis of reward (Leibman JM, Cooper SJ, eds), pp 214–263. Oxford: Clarendon Press.

Koob GF, Le Moal M (2001) Drug addiction, dysregulation of reward, and allostasis. Neuropsychopharmacology 24:97–129.

Kreibich AS, Blendy JA (2004) cAMP response element-binding protein is required for stress but not cocaine-induced reinstatement. J Neurosci 24:6686–6692.

Lonze BE, Ginty DD (2002) Function and regulation of CREB family transcription factors in the nervous system. Neuron 35:605–623.

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275.

McGregor A, Roberts DCS (1993) Dopaminergic antagonism within the nucleus accumbens or the amygdala produces differential effects on intravenous cocaine self-administration under fixed and progressive ratio schedules of reinforcement. Brain Res 624:245–252.

Montminy M (1997) Transcriptional regulation by cyclic AMP. Annu Rev Biochem 66:807–822.

Nestler EJ (2001) Molecular basis of long-term plasticity underlying addiction. Nat Rev Neurosci 2:119–128.

Nestler EJ, Terwilliger RZ, Walker JR, Sevarino KA, Duman RS (1990) Chronic cocaine treatment decreases levels of the G-protein subunits G_{ia} and G_{oa} in discrete regions of the rat brain. J Neurochem 55:1079–1082.

Paxinos G, Watson GC (1998) The rat brain in stereotaxic coordinates, 4th edition. New York: Academic Press.

Piazza PV, Deroche-Gamonent V, Rouge-Pont F, Le Moal M (2000) Vertical shifts in self-administration dose-response functions predict a drug-vulnerable phenotype predisposed to addiction. J Neurosci 20:4226–4232.

Pliakas AM, Carlson RR, Neve RL, Konradi C, Nestler EJ, Carlezon WA (2001) Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens. J Neurosci 21:7397–7403.

Rodd-Henricks ZA, McKinzie DL, Li TK, Murphy JM, McBride WJ (2002) Cocaine is self-administered into the shell but not the core of the nucleus accumbens of Wistar rats. J Pharmacol Exp Ther 303:1216–1226.

Self DW (2004) Regulation of drug-taking and -seeking behaviors by neuroadaptations in the mesolimbic dopamine system. Neuropharmacology 47:242–255.

Self DW, Genova LM, Hope BT, Barnhart WJ, Spencer JJ, Nestler EJ (1998) Involvement of cAMP-dependent protein kinase in the nucleus accumbens in cocaine self-administration and relapse of cocaine-seeking behavior. J Neurosci 18:1848–1859.

Self DW, Terwilliger RZ, Nestler EJ, Stein L (1994) Inactivation of G_i and Go proteins in nucleus accumbens reduces both cocaine and heroin reinforcement. J Neurosci 14:6239–6247.

Shaw-Lutchman TZ, Impey S, Storm D, Nestler EJ (2003) Regulation of CRE-mediated transcription in mouse brain by amphetamine. Synapse 48:10–17.

Shaywitz AJ, Greenberg ME (1999) CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem 68:821–861.

Simpson JN, Wang JQ, McGinty JF (1995) Repeated amphetamine administration induces a prolonged augmentation of phosphorylated cyclase response element-binding protein and Fos-related antigen immunoreactivity in rat striatum. Neuroscience 69:441–457.

Striplin CD, Kalivas PW (1993) Robustness of G protein changes in cocaine sensitization shown with immunoblotting. Synapse 14: 10–15.

Sutton MA, Karanian DA, Self DW (2000) Factors that determine a propensity for cocaine-seeking behavior during abstinence in rats. Neuropsychopharmacology 22:626–641.

Sutton MA, Schmidt EF, Choi K-H, Schad CA, Whisler K, Simmons D, Karanian DA, Monteggia LM, Neve RL, Self DW (2003) Extinction-induced up-regulation in AMPA receptors reduces cocaine-seeking behaviour. Nature 421:70–75.

Terwilliger RZ, Beitner-Johnson D, Sevarino KA, Crain SM, Nestler EJ (1991) A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. Brain Res 548:100–110.

Turgeon SM, Pollack AE, Fink JS (1997) Enhanced CREB phosphorylation and changes in c-Fos and FRA expression in striatum accompany amphetamine sensitization. Brain Res 749:120–126.

Unterwald EM, Fillmore J, Kreek MJ (1996) Chronic repeated cocaine administration increases dopamine D1 receptor-mediated signal transduction. Eur J Pharmacol 318:31–35.

Walters CL, Blendy JA (2001) Different requirements for cAMP response element binding protein in positive and negative reinforcing properties of drugs of abuse. J Neurosci 21:9438–9444.

Walters CL, Kuo YC, Blendy JA (2003) Differential distribution of CREB in the mesolimbic dopamine reward pathway. J Neurochem 87:1237–1244.

Widnell KL, Self DW, Lane SB, Russell DS, Vaidya VA, Miserendino MJ, Rubin CS, Duman RS, Nestler EJ (1996) Regulation of CREB expression: in vivo evidence for a functional role in morphine action in the nucleus accumbens. J Pharmacol Exp Ther 276:306–315.

(Accepted 17 October 2005)
(Available online 15 December 2005)