Regulation of CREB Expression: \textit{In Vivo} Evidence for a Functional Role in Morphine Action in the Nucleus Accumbens$^1$

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ABSTRACT

Previous work has shown that chronic opiate administration regulates protein components of the cAMP signaling pathway, specifically in the nucleus accumbens (NAC), a brain region implicated in the reinforcing properties of opiates, and that such adaptations may contribute to changes in reinforcement mechanisms that characterize opiate addiction. In the present study, we examined a possible role for the transcription factor CREB (cyclic AMP response element-binding protein) in mediating these long-term effects of opiates in the NAC. Chronic, but not acute, opiate administration was found to decrease levels of CREB immunoreactivity in the NAC, an effect not seen in other brain regions studied. The functional significance of this CREB down-regulation was then investigated by the use of an antisense oligonucleotide strategy that produces a specific and sustained decrease in CREB levels in the NAC, without detectable toxicity. It was found that the antisense oligonucleotide-induced reduction in CREB levels mimicked the effect of morphine on certain, but not all, cAMP pathway proteins in this brain region, whereas a large number of other signal transduction proteins tested were unaffected by this treatment. Our results support a role for CREB in autoregulation of the cAMP pathway in the nervous system, as well as in mediating some of the effects of morphine on this signaling pathway in the NAC.

The NAC is an important substrate for the acute reinforcing properties of opiates and possibly also for the long-term motivational changes associated with opiate addiction (Koob, 1992; Kuhar et al., 1991; Self and Nestler, 1995; Wise, 1990). We have shown that chronic opiate treatment increases levels of adenyl cyclase and cAMP-dependent protein kinase in the NAC, but not in several other brain regions, such as the striatum and frontal cortex, that are generally not implicated in drug reinforcement mechanisms (Nestler, 1992; Terwilliger et al., 1991). Chronic opiate administration also decreases levels of Gi$_\text{a}$ specifically in the NAC, but does not alter levels of other G protein subunits, including Go$_\text{o}$ and Gi$_\text{d}$; this reduction in Gi$_\text{a}$ would further increase the functional activity of the cAMP pathway in this brain region (Terwilliger et al., 1991). Recent work has provided direct evidence that opiate regulation of Gi$_\text{a}$ and the cAMP pathway plays a role in drug reinforcement and addiction: intra-NAC administration of agents that inhibit Gi$_\text{a}$ or activate cAMP-dependent protein kinase increases heroin self-administration, whereas agents that produce the opposite effect decrease drug self-administration (Self et al., 1994; Self and Nestler, 1995). Similar evidence implicates Gi$_\text{a}$ and the cAMP pathway in drug regulation of locomotor activity (Cunningham and Kelley, 1993; Miserendino and Nestler, 1996; Striplin and Kalivas, 1993), an effect that is also mediated by the NAC.

These opiate-induced adaptations in G proteins and the cAMP pathway involve changes in protein levels, raising the possibility that they are mediated at least in part by alterations in gene expression (Nestler et al., 1993). The transcription factor CREB mediates many of the effects of the cAMP signaling pathway on gene expression and thus may be involved in morphine regulation of Gi$_\text{a}$ and cAMP pathway proteins in the NAC. CREB is a 43-kDa protein that binds to specific DNA sequences called CREs, which are found in the promoters of many neurally expressed genes (e.g., see Hyman et al., 1988; Lewis et al., 1987; Montminy et al., 1986; Tsukada et al., 1987). We show that chronic exposure to

ABBREVIATIONS: CRE, cyclic AMP response element; CREB, cyclic AMP response element-binding protein; NAC, nucleus accumbens; NIDA, National Institute on Drug Abuse; PBS, phosphate-buffered saline.
morphine leads to a down-regulation of CREB immunoreactivity in the NAc, an effect not seen in the striatum and frontal cortex.

To assess the functional consequences of CREB down-regulation in the NAc, we used an antisense oligonucleotide strategy to specifically decrease CREB levels in this brain region. Antisense oligonucleotides result in down-regulation of specific target proteins by either (1) forming a physical barrier to translation initiation and/or mRNA processing or (2) providing a target for RNase H, an endogenous enzyme that cleaves RNA at the site of an RNA/DNA heteroduplex (Bischofberger and Shea, 1992; Milligan et al., 1993). Concern over antisense oligonucleotide stability and sequence nonspecific effects has limited the effective use of this potentially powerful technique (Stein and Cheng, 1993). Recently, we established a series of criteria to ensure the specificity of an antisense effect in the brain (Russell et al., 1996). In the current study, we demonstrate that antisense oligonucleotides targeted to CREB mRNA specifically down-regulate CREB and selectively down-regulate Gae and cAMP-dependent protein kinase catalytic subunit in the NAc. These data support a scheme whereby the morphine-induced down-regulation of CREB mediates some of the long-term adaptations in signaling proteins seen in the NAc. The results also delineate an experimental protocol by which a specific transcription factor, and other signaling proteins not readily affected by traditional pharmacological agents, can be studied within a functional context in the brain in vivo.

Methods

Animals and in vivo treatments. Outbred male Sprague-Dawley rats (CAMM, Wayne, NJ) were used in all studies (initial weight: ~170 g for morphine studies, ~270 g for antisense studies). All animals were housed in groups of two or three per cage and maintained on a controlled 12-hr light/dark cycle, with free access to food and water. Chronic morphine treatment involved the subcutaneous implantation of morphine pellets (containing 75 mg of morphine base; NIDA) daily for 5 days, with rats killed on day 6. This treatment results in profound states of tolerance, dependence and withdrawal (see Guitart et al., 1992). Control rats were implanted with equivalent placebo pellets (NIDA). In some experiments, rats were treated concomitantly with morphine plus naltrexone under conditions that have been shown to completely block the development of tolerance and dependence based on behavioral, electrophysiological and biochemical criteria (see Nestler et al., 1989). Acute morphine treatment involved the subcutaneous injection of morphine sulfate (10 mg/kg in 0.9% saline; NIDA), with rats used 2 hr later, a time point of maximal biochemical effects of morphine (Guitart et al., 1992). Acute cocaine treatment involved the intraperitoneal injection of cocaine HCl (20 mg/kg in 0.9% saline; NIDA), with rats used 2 hr later, a time point at which c-Fos is maximally induced (Hope et al., 1994; Moratalla et al., 1993). Control rats received equivalent saline injections.

Antisense treatment involved intra-NAc injections or infusions of the following oligonucleotide sequences: CREB antisense, 5’TGGTG-CATCTAGTCACCAGGTG3’; CREB sense, 5’CAGCGGCTAGACTAGAC- CAAA3’; and CREB scrambled, 5’GACGCTAGCTAGCTGTTGTTG3’ (Midland Certified Reagent Co., Midland, TX). The antisense sequence was directed at the translation start site of the CREB message and was chosen based on its published efficacy in the striatum (Konradi et al., 1994). In initial experiments, fully phosphorothioate-modified oligonucleotides were used. In subsequent studies involving chronic infusion, we used oligonucleotides that were phosphorothioate-modified only on the terminal base pairs since chronic infusion of totally phosphorothioate-modified oligonucleotides produced localized tissue damage (see Results). Before administration to the animals, the oligonucleotides were ethanol precipitated, washed 3 times with 70% ethanol, and resuspended in sterile PBS (Sigma Chemical Co.). The concentration of oligonucleotide was determined by optical density. Rats were anesthetized with an intraperitoneal injection (3.3 ml/kg) of equithesin (8.1 mg/ml sodium pentobarbitol, 43 mg/ml chloral hydrate, 21 mg/ml MgSO4, 10% by vol propylene glycol and 10% by vol ethanol) and placed in a Kopf stereotaxic instrument. Stereotaxic coordinates with the top of the skull level were +1.7 mm anterior to bregma, + or − 1.5 mm lateral, and −6.7 mm ventral to dura (Paxinos and Watson, 1982). Rats were injected unilaterally (half on the right, half on the left) with antisense (or sense) oligonucleotide (20 μg/1 μl/side) and the contralateral side received the PBS vehicle (sham). Injections into the NAc were delivered over 2 min through 33-gauge bilateral injection cannulae (Plastics One, Roanoke, VA), and the rats were killed 18 hr after a single injection. The dose of oligonucleotide (20 μg) was based on a published report demonstrating a maximal reduction in CREB in the striatum (Konradi et al., 1994) and on preliminary studies in which the effectiveness of this dose in reducing CREB was more consistent than a 50-μg dose. For chronic treatments, two different paradigms were used. In one paradigm, 26-gauge bilateral guide cannulae (Plastics One) were chronically implanted into the NAc 5.7 mm ventral to dura. After 5–7 days of recovery, the antisense (or sense) oligonucleotide (20 μg/1 μl/side) and vehicle (1 μl/side) were injected as above into freely moving animals through 33-gauge bilateral injection cannulae (Plastics One) that extended 1 mm beyond the guide cannulae. The rats were given 5 daily injections and killed 18 hr after the final injection. In the other chronic paradigm, osmotic minipumps (Alzet 2001; ALZA, Palo Alto, CA) were used to deliver continuous infusions of oligonucleotide (20 μg/day) and vehicle into the NAc at a rate of 0.5 μl/hr. These animals were surgically implanted with 28-gauge bilateral osmotic minipump cannulae 6.7 mm ventral to dura. Before implantation, the minipumps were filled, and a 2.5-cm length of polyethylene tubing (PE-80; Clay Adams, NJ) was connected to the minipump flow moderator and sealed with LocTite glue (Bearing Distributors, CT). The minipumps were primed by submersion in sterile saline at 37°C overnight. Immediately after cannulae implantation, two minipumps, one containing oligonucleotide and one containing vehicle, were attached to the bilateral minipump cannulae via the PE tubing and sealed with LocTite. The minipumps and tubing were implanted subcutaneously between the animal’s scalp. In these chronic minipump experiments, rats were killed 5 days after implantation. In some experiments, pumps containing antisense oligonucleotide were switched after 5 days with pumps containing the PBS vehicle, and the rats were killed 5 days later.

In all experiments, levels of immunoreactivity of a protein on the oligonucleotide-injected side were compared with the immunoreactivity on the contralateral, sham-injected side. This enabled a within-animal comparison, as opposed to a between-animal comparison, which reduced variability in the data. For each experiment, an equal number of animals received oligonucleotide on the left and right sides, with no differences observed between the two groups. To take into account the observed interanimal variability of protein levels for statistical purposes, the percent difference in immunoreactivity between each sham-injected side and the average sham value per group of animals tested was calculated. Due to interanimal variability in levels of all proteins tested, the S.E.M. for the sham group is larger than the corresponding S.E.M. for the antisense or sense group. This larger error further demonstrates the utility of the within-animal comparison, so that variability in endogenous levels of a protein do not mask an antisense effect. In all experiments, treated vs. sham samples were compared using an unpaired Student’s two-tailed t test.

In some experiments, the integrity of the brain tissue around the site of infusion was studied by standard histological techniques. Rats

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were perfused with saline followed by paraformaldehyde, and 40-μm-thick coronal sections were obtained from fixed brains through the NAc. The sections were subjected to Cresyl violet staining. The slides were coverslipped and observed under light microscopy. Photographs were obtained under the same exposure conditions, and prints were developed together.

Isolation of brain regions. Brains were removed rapidly from decapitated rats and cooled immediately in ice-cold buffer containing 126 mM NaCl, 5 mM KCl, 1.25 mM NaH2PO4, 25 mM NaHCO3, 2 mM CaCl2, 2 mM MgCl2 and 10 mM d-glucose, pH 7.4. The NAc and striatum were excised from 1-mm-thick coronal brain slices using a 12-gauge needle as described (Terwilliger et al., 1991). Frontal cortex was isolated by gross dissection. In some experiments, a ~0.5-mm-thick ring of tissue surrounding NAc was dissected around the NAc punch. For morphine experiments, bilateral punches of NAc or striatum were pooled.

Preparation of brain extracts and immunoblot analysis. Brain samples were Dounce homogenized or sonicated (1–2 mg wet weight/0.1 ml) for 5 sec (sonication resulted in a higher total protein yield of equivalent purity) in EMSA buffer containing 20 mM HEPES, 0.4 M NaCl, 20% glycerol, 5 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 10 μg/ml leupeptin, 0.1 mM p-aminoazobenamidene, 1 μg/ml pepstatin, 1% nonidet P-40, 1 mM phenylmethylsulfonyl fluoride and 5 mM dithiothreitol. Brain homogenates were then incubated on ice for 25 min and centrifuged at 15,000 rpm for 25 min at 4°C in a Tomy MTX-150 centrifuge, and supernatants were reserved for immunoblotting. Aliquots of brain extracts (50 μg for CREB and PKC analyses, 10 μg for all other protein analyses) were subjected to SDS-polyacrylamide gel electrophoresis (Widnell et al., 1994). After electrophoretic transfer to nitrocellulose, proteins were immunolabeled with the following antibodies. Anti-CREB antiserum (1:20,000), raised against a TrpE-CREB fusion protein, was provided by David Ginty and Michael Greenberg (Harvard Medical School, Boston, MA). The anti-CREB antiserum is specific for CREB and does not recognize other family members (Ginty et al., 1993, Widnell et al., 1994). Anti-CAMP-dependent protein kinase catalytic subunit anti-serum (1:1000) was provided by Charles Rubin (Albert Einstein Medical School, Bronx, NY). Anti-M peptide (anti-Fos-related antigen) antiserum (1:4000) was provided by Michael Iadarola (National Institute of Dental Research, National Institutes of Health, Bethesda, MD). This antiserum recognizes c-Fos and several Fos-like proteins; all of the bands detected on immunoblots between 30 and 60 kDa are specific based on their ability to be completely and selectively blocked by preabsorption of the antibody with purified M-peptide (Hope et al., 1994). Anti-G protein anti-sera (Gαi, Gαs, Gαq, Gβ12, Gγ12, 1:4000) were purchased from NEN (Boston, MA). Anti-Ca2+/calmodulin kinase IIα or IIβ anti-sera (1:3500), anti-phospholipase Cδ-1 anti-serum (1:400), anti-phospholipase Cγ-1 anti-serum (1:1000) and anti-phosphatidylinositol-3-kinase anti-serum (1:2000) were purchased from UBI. Anti-protein kinase Cβ or Cγ anti-sera (1:1500) were purchased from GIBCO. The blots were incubated with a 1:4000 dilution of goat anti-rabbit antibody (for CREB, protein kinase A, FRA, and G protein blots) or with goat anti-mouse antibody (for Ca2+/calmodulin kinase I or II, phospholipase Cδ-1 or Cγ-1, phosphatidylinositol-3-kinase and protein kinase Cβ or Cγ blots) conjugated to horseradish peroxidase (Vector Laboratories), developed with the enhanced chemiluminescence system of Amersham and exposed to Hyperfilm-ECL (Amersham) for 5 sec to 2 min. Bands on the autoradiogram were analyzed using a Macintosh-based Image Analysis 1.52 system. All blots were stained with Amido black to confirm equal loading of protein.

Results

Regulation of CREB protein expression by chronic morphine administration in the NAc. Immunoblot analysis revealed significant levels of CREB immunoreactivity in the NAc of control rats (fig. 1). Reaction with the identified 43-kDa band corresponding to CREB is specific, based on the finding that it is selectively and completely blocked when the anti-CREB antiserum is preabsorbed with Trp-CREB (see Methods). As shown in figure 1, we observed a 48% decrease in CREB levels in the NAc compared with control after chronic morphine treatment. In contrast to the NAc, striatum and frontal cortex showed no change in levels of CREB immunoreactivity after chronic morphine treatment. The morphine effect in the NAc was blocked by concomitant administration of naltrexone, an opioid receptor antagonist, indicating that it is mediated via opioid receptors (~5 ± 10% change from control ± S.E.M., n = 6). Furthermore, acute (2 hr) morphine treatment did not regulate levels of CREB immunoreactivity in the NAc (9 ± 16% change from control ± S.E.M., n = 6).

Development of a procedure to reduce CREB protein expression by administration of CREB antisense oligonucleotide in the NAc. To investigate the functional role of the morphine-induced down-regulation of CREB in the NAc, preliminary studies were performed using antisense oligonucleotides directed against CREB. Initial studies involved a single injection of a 20-bp phosphorothioate-modified antisense oligonucleotide directed against CREB. Treatment involved a single injection of a 20-bp phosphorothioate-modified antisense oligonucleotide directed against CREB. In contrast to the NAc, striatum and frontal cortex showed no change in levels of CREB immunoreactivity after chronic morphine treatment. The morphine effect in the NAc was blocked by concomitant administration of naltrexone, an opioid receptor antagonist, indicating that it is mediated via opioid receptors (~5 ± 10% change from control ± S.E.M., n = 6). Furthermore, acute (2 hr) morphine treatment did not regulate levels of CREB immunoreactivity in the NAc (9 ± 16% change from control ± S.E.M., n = 6).

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figure 2B, 1 day after administration of CREB sense oligonucleotide, levels of CREB immunoreactivity were not altered compared with the sham side. Moreover, CREB immunoreactivity returned to basal levels 3 days after a single injection of CREB antisense oligonucleotide (data not shown). No evidence of tissue damage was seen 1 day after the antisense treatment, consistent with previous reports (Konradi et al., 1994).

To more closely parallel the chronic morphine-induced down-regulation of CREB, we altered the method of antisense oligonucleotide administration to one involving repeated daily injections (20 μg in 1 μl) or constant infusions (20 μg/day at a rate of 0.5 μl/hr) via Alzet minipumps for a period of 5 days into the NAc. Both techniques resulted in decreases in CREB immunoreactivity in the NAc, with a constant infusion appearing to down-regulate CREB levels slightly more than repeated injections (data not shown). However, in >50% of the rats studied, we observed tissue changes (hemorrhagic or other nonspecific alterations in tissue quality) not seen after a single injection; this was more obvious with the repeated injections. Because the oligonucleotide with phosphorothioate-modifications on all base pairs was apparently toxic on chronic administration, we switched to an oligonucleotide with phosphorothioate modifications on the two end nucleotides only and used the continuous infusion with minipumps in subsequent studies.

As shown in figure 2A, a 5-day infusion of partially modified CREB antisense oligonucleotide resulted in a similar degree of down-regulation as the 1-day injection, with a 40% decrease in CREB immunoreactivity observed on the antisense oligonucleotide-treated side compared with the sham side. In contrast, chronic administration of CREB sense oligonucleotide failed to alter levels of CREB immunoreactivity compared with the sham side (fig. 2B). Similarly, infusion of a partially phosphorothioate-modified CREB scrambled oligonucleotide (consisting of a randomly shuffled sequence with the same GC and phosphorothioate content as the CREB antisense oligonucleotide) for 5 days had no effect on CREB immunoreactivity in the NAc (4 ± 8% change from sham, n = 5).

In addition to the lack of effect of sense and scrambled oligonucleotides, we used several other criteria (see Russell et al., 1996) to demonstrate the specificity of the partially modified antisense oligonucleotide treatment. First, there appeared to be little diffusion of CREB oligonucleotides outside of the NAc, as a 5-day infusion of CREB antisense oligonucleotide into the NAc did not affect levels of CREB immunoreactivity in a 0.5-mm-thick ring of tissue surrounding the NAc (5 ± 8% change from sham, n = 6). Second, there was no evidence of tissue damage at the time of brain dissection. Third, the various antibody banding patterns on immunoblotting, including even nonspecific bands, were unaffected by the antisense, sense or sham infusions relative to brain extracts from naive rats (data not shown). Fourth, the banding pattern of Coomassie blue-stained gels of NAc extracts from rats infused for 5 days with partially phosphorothioate-modified CREB antisense or sense oligonucleotide was identical to tissue extracts from sham or untreated rats. In contrast, NAc extracts from rats infused for 5 days with totally phosphorothioate-modified CREB antisense oligonucleotide demonstrated a markedly different banding pattern (data not shown). Fifth, Cresyl violet stains of rat brain sections through the NAc demonstrated no detectable tissue alterations on the side infused for 5 days with partially phosphorothioate-modified antisense oligonucleotide compared with the contralateral sham-treated side (fig. 3). The Cresyl violet stain also confirmed the anatomic localization of the implanted cannulae in the NAc. Finally, the CREB antisense oligonucleotide-induced down-regulation of CREB was reversible, in that levels of CREB immunoreactivity returned to basal levels 5 days after replacement of the Alzet minipump containing CREB antisense oligonucleotide with a minipump containing the PBS vehicle only (6 ± 15% change from sham, n = 8). Based on these findings, subsequent studies with CREB antisense oligonucleotides used the single injection paradigm (with fully modified oligonucleotides) or the 5-day continuous infusion paradigm (with partially modified oligonucleotides).

**Regulation of Gs immunoreactivity by administration of CREB antisense oligonucleotide in the NAc.** Selective morphine regulation of CREB levels in the NAc, but not in brain areas where morphine-induced alterations in components of the cAMP signaling pathway have not been observed, suggested a possible role for CREB in mediating morphine regulation of these intracellular messenger proteins. Given the observed down-regulation of Gs in the
NAc by morphine, we examined the effect of CREB down-regulation on this G protein subunit in this brain region. As shown in figure 4A, 1-day treatment of CREB antisense oligonucleotide did not alter levels of $G_{\alpha_{1/2}}$ immunoreactivity on the antisense-treated side compared with the sham side. However, a 5-day continuous infusion of CREB antisense oligonucleotide resulted in a significant 21% decrease in $G_{\alpha_{1/2}}$ immunoreactivity on the CREB antisense oligonucleotide-treated side compared with the sham side (fig. 4A).

Several lines of evidence indicate that this down-regulation of $G_{\alpha_{1/2}}$ is specific, as demonstrated for the reduction in CREB itself. Infusion of CREB sense oligonucleotide had no effect on $G_{\alpha_{1/2}}$ immunoreactivity after a single injection or a 5-day infusion (fig. 4B). Similarly, infusion of CREB scrambled oligonucleotide had no effect on levels of $G_{\alpha_{1/2}}$ immunoreactivity (2 ± 9% change from sham, n = 8). The CREB antisense oligonucleotide-induced down-regulation of $G_{\alpha_{1/2}}$ was reversible, in that levels of $G_{\alpha_{1/2}}$ immunoreactivity returned to basal levels 5 days after replacement of the Alzet minipump containing CREB antisense oligonucleotide with a minipump containing PBS only (−3 ± 5% change from sham, n = 7). Finally, a 5-day infusion of CREB antisense oligonucleotide into the NAc had no effect on $G_{\alpha_{1/2}}$ immunoreactivity in a 0.5-mm ring of tissue surrounding the NAc (−4 ± 3% change from sham, n = 6).

**Regulation of cAMP-dependent protein kinase catalytic subunit immunoreactivity by administration of CREB antisense oligonucleotide in the NAc.** We next examined the levels of immunoreactivity of cAMP-dependent protein kinase catalytic subunit, also known to be regulated by morphine in the NAc, after antisense oligonucleotide administration in this brain region. The antibody used is unable to distinguish between the two known forms of catalytic subunit, $C \alpha$ and $C \beta$. As shown in figure 5A, 1-day of CREB antisense oligonucleotide treatment resulted in a 25% decrease in cAMP-dependent protein kinase catalytic subunit immunoreactivity compared to the sham side. A five day infusion of CREB antisense oligonucleotide resulted in a similar degree of down-regulation of catalytic subunit immunoreactivity, with a 27% decrease observed (fig. 5A). This down-regulation of catalytic subunit was specific for the CREB antisense oligonucleotide. As shown in figure 5B, injections of CREB sense oligonucleotide had no effect on cAMP-dependent protein kinase catalytic subunit immunoreactivity after a single injection or a 5-day infusion. Similarly, a 5-day infusion of CREB scrambled oligonucleotide had no effect on catalytic subunit immunoreactivity (4 ± 15% change from sham, n = 7). The CREB antisense oligonucleotide-induced down-regulation of catalytic subunit was reversible in that levels of catalytic subunit immunoreactivity returned to basal levels 5 days after replacement of the Alzet minipump containing CREB antisense oligonucleotide with a minipump containing the PBS vehicle (1 ± 12% change from sham, n = 8). Finally, a 5-day infusion of CREB antisense oligonucleotide into the NAc had no effect on catalytic sub...

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**Fig. 3.** Cresyl violet stains of brain sections illustrating lack of toxicity of CREB antisense oligonucleotide infusions in the rat NAc. Rats received 5 days of infusion of saline vehicle into one NAc (A) and of CREB antisense oligonucleotide into the centralateral NAc (B) and were then perfused with saline and paraformaldehyde. Then, 40-μm-thick coronal sections of brain, at the level of the NAc, were analyzed by Cresyl violet staining. Low-power (25x) (shown) and high-power (not shown) analyses revealed no detectable evidence of toxicity: no changes in cellular patterns or numbers and no evidence of an infusion artifact. The photographs shown in the figure are representative of results obtained from 3 rats.

**Fig. 4.** Effect of CREB antisense and sense oligonucleotide administration on $G_{\alpha_{1/2}}$ immunoreactivity in the rat NAc. NAc from rats after administration of CREB antisense (AS) or sense (S) oligonucleotide for 1 or 5 days were analyzed for $G_{\alpha_{1/2}}$ immunoreactivity by immunoblotting. Representative autoradiograms and the bar graphs summarize CREB AS (A) and S (B) regulation of $G_{\alpha_{1/2}}$ immunoreactivity at 1 and 5 days of treatment. Data are expressed as percent change from sham ± S.E.M. (1-day CREB antisense, n = 12; 5-day CREB antisense, n = 21; 1-day CREB sense, n = 4; 5-day CREB sense, n = 6). *P < .001 by unpaired Student’s t test.
unit immunoreactivity in a 0.5-mm ring of tissue surrounding the NAc (−2 ± 3% change from sham, n = 6).

Lack of regulation of other signal transduction proteins by administration of CREB antisense oligonucleotide in the NAc. We investigated the effect of the 5-day CREB antisense oligonucleotide treatment on several other signal transduction proteins in the NAc. The results of these experiments are shown in figure 6. In contrast to our findings for Gα1/2 and the catalytic subunit of cAMP-dependent protein kinase, there was no detectable effect of antisense oligonucleotide to CREB on two other G protein subunits, Gαs or Gβ. There also was no observed effect on calcium/calmodulin-dependent protein kinase IIα or β, protein kinase Cβ or γ, phospholipase Cγ or δ or phosphatidylinositol-3-kinase, nor were there alterations in the levels of these various proteins after 5 days of CREB sense oligonucleotide infusion (table 1).

**Functional correlates of CREB regulation by administration of CREB antisense oligonucleotide in the NAc.** Konradi *et al.* (1994) demonstrated that a single injection of CREB antisense oligonucleotide into the striatum completely abolished, 24 hr later, the ability of an acute exposure to amphetamine to induce c-Fos (whose induction is believed to be mediated by CREB; Morgan and Curran, 1991; Sheng and Greenberg, 1990) in this brain region. We carried out an analogous experiment with our 5-day treatment paradigm, with one NAc infused with CREB antisense oligonucleotide and one infused with CREB sense oligonucleotide. Representative autoradiograms and the bar graphs summarize CREB AS (A) and S (B) regulation of PK-C immunoreactivity at 1 and 5 days of treatment. Data are expressed as percent change from sham ± S.E.M. (within-animal comparison), as described in Methods.

**Table 1**

Lack of effect of CREB sense oligonucleotide infusions on several signal transduction proteins in the rat NAc

<table>
<thead>
<tr>
<th>Protein</th>
<th>Treatment</th>
<th>Change from control</th>
<th>S.E.M.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gαs</td>
<td>Sham</td>
<td>0</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Sense</td>
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<td>12 (4)</td>
<td></td>
</tr>
<tr>
<td>Gβ</td>
<td>Sham</td>
<td>0</td>
<td>9 (6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sense</td>
<td>−1</td>
<td>5 (6)</td>
<td></td>
</tr>
<tr>
<td>Ca2⁺/calmodulin kinase</td>
<td>Sham</td>
<td>0</td>
<td>7 (6)</td>
<td></td>
</tr>
<tr>
<td>IIα</td>
<td>Sense</td>
<td>4</td>
<td>7 (6)</td>
<td></td>
</tr>
<tr>
<td>Ca2⁺/calmodulin kinase</td>
<td>Sham</td>
<td>9</td>
<td>5 (5)</td>
<td></td>
</tr>
<tr>
<td>IIβ</td>
<td>Sense</td>
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<td>14 (5)</td>
<td></td>
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<tr>
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<td></td>
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<td>Sham</td>
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<td>9 (5)</td>
<td></td>
</tr>
<tr>
<td>Phospholipase</td>
<td>Sham</td>
<td>9</td>
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<tr>
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<td>Sham</td>
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<tr>
<td>kinase</td>
<td>Sense</td>
<td>8</td>
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Data are expressed as percent change from sham (within-animal comparison) ± S.E.M. The S.E.M. for sham animals was calculated based on an average sham value (determined by a between-animal comparison), as described in Methods.
morphine administration reduces levels of CREB immunoreactivity and motivational aspects of opiate dependence. We be regulated in neuronal cells (Brecht et al., 1994).

Activity in the NAc, a brain region implicated in opiate reinforcement and motivational aspects of opiate dependence, has been thought to be constitutive and not subject to general transcription on induction of c-Fos in the rat NAc by acute cocaine administration. Cocaine (20 mg/kg i.p.) or saline was administered to rats after 5 days of infusion of CREB antisense (AS) oligonucleotide into one NAc and sham (S) oligonucleotide into the central lateral NAc. Levels of c-Fos and Fos-like proteins were analyzed by immunoblotting. Acute administration of cocaine results in the induction of c-Fos (58 kDa), as well as several Fos-like proteins in the 45- and 40-kDa range, on the sense-treated side. In contrast, cocaine induction of c-Fos, but not of the Fos-like proteins, is attenuated on the antisense-treated side. The autoradiograms shown in the figure are representative of results obtained from 4 saline-treated and 4 cocaine-treated animals.

**Fig. 7.** Effect of CREB antisense and sense oligonucleotide administration on induction of c-Fos in the rat NAc by acute cocaine administration. Cocaine (20 mg/kg i.p.) or saline was administered to rats after 5 days of infusion of CREB antisense (AS) oligonucleotide into one NAc and sham (S) oligonucleotide into the central lateral NAc. Levels of c-Fos and Fos-like proteins were analyzed by immunoblotting. Acute administration of cocaine results in the induction of c-Fos (58 kDa), as well as several Fos-like proteins in the 45- and 40-kDa range, on the sense-treated side. In contrast, cocaine induction of c-Fos, but not of the Fos-like proteins, is attenuated on the antisense-treated side. The autoradiograms shown in the figure are representative of results obtained from 4 saline-treated and 4 cocaine-treated animals.

The results of the present study demonstrate that chronic morphine administration reduces levels of CREB immunoreactivity in the NAc, a brain region implicated in opiate reinforcement and motivational aspects of opiate dependence. We demonstrated previously that chronic morphine treatment reduces levels of $G_{i/o}$ and increases levels of adenylyl cyclase and cAMP-dependent protein kinase in the NAc and provided direct evidence that these adaptations can modulate alterations in drug reinforcement mechanisms, and possibly drug craving in addicted subjects, after long-term exposure to opiates (see introductory paragraphs). In contrast, we show here that chronic morphine administration does not alter CREB levels in the striatum and frontal cortex, two brain regions generally not implicated in opiate reinforcement and where morphine regulation of G proteins and the cAMP pathway has not been detected. The functional consequences of the morphine-induced down-regulation of CREB in the NAc were then examined in the present study by use of a novel antisense oligonucleotide treatment paradigm, which achieves a specific and sustained (yet fully reversible) reduction in CREB levels in this brain region. Results of the antisense oligonucleotide experiments support the hypothesis that the morphine-induced down-regulation in CREB in the NAc could mediate some of the effects of morphine on intracellular messenger proteins in this brain region.

It has proven difficult in vivo to determine the role of a given transcription factor in the regulation of any given target gene, particularly in chronic treatment paradigms. Such work has depended to a large extent on the use of mutant mice in which the transcription factor is either mutated or disrupted completely (Hummler et al., 1994; Struthers et al., 1991). However, interpretation of these studies is complicated by the developmental compensations common in such mutant animals. Therefore, we turned our attention to the use of an antisense oligonucleotide strategy to specifically decrease levels of CREB in a discrete brain region of an adult animal. Antisense technology has been used increasingly to dissect signal transduction pathways (see Albert and Morris, 1994; Russell et al., 1995). This approach has been used successfully to assign function to specific receptor, G protein and protein kinase subtypes in several experimental systems (see Albert and Morris, 1994; Dean and McKay, 1994; Shih and Malbon, 1994; Standifer et al., 1994; Zhang and Creese, 1993). However, this is a technology that, although powerful, is still viewed with much skepticism (see Albert and Morris, 1994). Antisense oligonucleotides are believed to work by one of two mechanisms: either by forming a physical barrier to translation initiation and/or mRNA processing (e.g., splicing, polyadenylation) or by providing a target for RNase H, an endogenous enzyme that cleaves RNA at the site of an RNA/DNA heteroduplex (Bischofberger and Shea, 1992; Milligan et al., 1993).

There are a number of requirements for the usefulness of an oligonucleotide: its stability in a given tissue, its uptake by the target cell and a lack of sequence-nonspecific effects (Stein and Cheng, 1993). Oligonucleotides substituted with a methyl phosphonate or phosphorothioate backbone are commonly used because they have been shown to be more resistant to nuclease (abundant in the brain) than their unmodified counterparts (Agrawal et al., 1991; Whitesell et al., 1993; Woolf et al., 1990). Another advantage in substituting a phosphorothioate (but not methyl phosphonate) backbone is that the resulting DNA/RNA heteroduplex has been shown to act as a substrate for RNase H (Agrawal et al., 1990), thereby providing an additional mechanism for down-regulation of a target protein. However, phosphorothioate modi-
fifications have also been shown to result in sequence-nonspecific effects, including cellular toxicity (Stein and Cheng, 1993; Woolf et al., 1990). An alternative is to infuse an oligonucleotide modified only on the two terminal base pairs, since it has been reported that such chimeric oligonucleotides may confer partial nuclease resistance and continue to act as substrates for RNase H (Agrawal et al., 1990; Stein et al., 1988).

In the present study, we confirmed the utility of such partially modified oligonucleotides in producing sequence-specific effects with no detectable toxicity. We demonstrate the ability of an antisense oligonucleotide to the transcription factor CREB to specifically down-regulate CREB protein levels selectively in the NAc in vivo. This down-regulation occurs to a similar degree after a single injection and a continuous 5-day infusion of antisense oligonucleotide, with a resulting 40–45% down-regulation of CREB protein. The partial reduction in CREB may be due to the fact that despite the continuous administration, the oligonucleotide may be degraded in vivo; the partially modified phosphorothioate oligonucleotide may not have the same degree of nuclease resistance as the fully modified oligonucleotide. The antisense-induced down-regulation of CREB protein is specific in that sense oligonucleotide had no effect on CREB protein levels compared with sham after either a single injection or a 5-day infusion. A 5-day infusion of a CREB scrambled oligonucleotide also did not regulate CREB levels. Furthermore, 5-day intra-NAc infusion of FosB antisense oligonucleotide, which attenuated expression of FosB, failed to alter levels of CREB immunoreactivity. Evidence for a lack of toxicity of the antisense oligonucleotide treatment was provided by the recovery of levels of CREB immunoreactivity on the antisense-treated side to levels of CREB immunoreactivity on the sham side both 3 days after a single injection of fully modified antisense oligonucleotide and 5 days after substitution of the partially modified antisense oligonucleotide with PBS during continuous infusion. Furthermore, banding patterns of immunoblots and of Coomassie blue-stained gels of NAc extracts from rats treated with partially phosphorothioate-modified antisense or sense oligonucleotides were indistinguishable from the patterns seen with tissue from untreated rats. Finally, Cresyl violet stains of tissue sections revealed no evidence of toxicity resulting from the oligonucleotide treatments or the infusions per se.

Single injections or continuous infusions of CREB antisense oligonucleotide in the NAc resulted in a similar degree of down-regulation of CREB protein levels compared with chronic morphine administration (compare figs. 1 and 2). Interestingly, the 5-day infusion, but not the 1-day injection, paradigm of CREB antisense oligonucleotide treatment produced a similar down-regulation of Gαi1,2 as that produced by chronic morphine (see Terttivuori et al., 1991). This finding suggests that CREB levels must be down-regulated for an extended period of time to exert their effect on Gαi1,2 (see fig. 4A). As found with CREB itself, the down-regulation of Gαi1,2 is specifically produced by CREB antisense oligonucleotide and not by CREB sense or CREB scrambled oligonucleotides. Moreover, CREB antisense oligonucleotide failed to regulate levels of Gαs or Gβ in the NAc. It is particularly interesting that Gαs is not regulated, as it is also not regulated by chronic morphine in this brain region. Furthermore, Gαs has a high degree of sequence homology to Gαi and thus would be especially susceptible to independent effects of the CREB antisense oligonucleotide on G protein message (Jones and Reed, 1987). These results demonstrate that the antisense-induced down-regulation of CREB can mimic the morphine-induced reduction in Gαs.

The mechanism by which changes in CREB levels lead to changes in Gαs levels remains unknown. The inability of the 1-day CREB antisense oligonucleotide treatment to affect Gαs levels suggests that the effect may be indirect. On the other hand, activation of the cAMP pathway has been shown to up-regulate Gαs2 gene transcription in cultured cells (Kinane et al., 1993), consistent with a direct effect of CREB on Gαi expression. The degree of down-regulation of Gαi1,2, a 20% decrease from control in response to 5 days of treatment with either morphine or CREB antisense oligonucleotide, is likely to be functionally significant, since small changes in levels of G proteins have significant functional consequences. For example, 10–15% inhibition of Gαs and Gαι, (via pertussis toxin administration) decreases by ~40–50% the ability of neurotransmitters to produce their electrophysiological effects on dorsal raphe neurons, whereas a 50% inhibition completely abolishes the electrophysiological responses (Innis et al., 1988). In addition, pertussis toxin-mediated inhibition of Gαι (and Gαι,1) of these magnitudes in the NAc results in significant changes in heroin and cocaine self-administration (Self et al., 1994) and locomotor activity (Striplin and Kalivas, 1993).

CREB antisense oligonucleotide administration was also found to down-regulate levels of cAMP-dependent protein kinase catalytic subunit in the NAc. This antisense oligonucleotide effect on the protein kinase, like that on CREB and Gαι, is specific and was not observed after sense or scrambled oligonucleotide administration. However, the observed down-regulation of catalytic subunit immunoreactivity is opposite that observed after chronic morphine treatment (see Terttivuori et al., 1991), suggesting that the morphine-induced down-regulation of CREB does not directly contribute to the observed morphine up-regulation of this protein kinase. Rather, it appears that cAMP-dependent protein kinase is up-regulated by morphine in the NAc via a CREB-independent pathway. The observation that cAMP-dependent protein kinase catalytic subunit is similarly down-regulated after a single injection or 5-day infusion of CREB antisense oligonucleotide is consistent with a direct effect of CREB levels on cAMP-dependent protein kinase expression. This view is consistent with the observation that activation of the cAMP pathway produces a small increase in catalytic subunit expression in some cultured cells in vitro (Tasken et al., 1993). However, the transcriptional mechanisms that control the expression of the α and β isoforms of catalytic subunit remain incompletely understood (see McKnight, 1991).

The catalytic subunit of cAMP-dependent protein kinase is known to activate the transcriptional regulatory properties of CREB by phosphorylating the protein on serine-133 (Alberts et al., 1994; Gonzalez and Montminy, 1988; Yamamoto et al., 1988). We therefore examined the effect of CREB antisense treatment on other protein kinases known to phosphorylate and regulate CREB. Calcium/calmodulin-dependent protein kinases are known to phosphorylate the serine-133 site,
which is also a consensus phosphorylation site for protein kinase C (Enslen et al., 1994; Gonzalez et al., 1989; Sheng et al., 1991). However, CREB antisense infusion did not regulate calcium/calcmodulin kinases or protein kinase C subtypes. Since NGF can lead to CREB phosphorylation and activation (Ginty et al., 1994), we also examined the effect of CREB antisense oligonucleotide on two targets of the NGF signaling cascade; no effect was observed on phosphatidylinositol-3 kinase or phospholipase-C subtypes.

We also obtained functional evidence for the efficacy of CREB antisense oligonucleotide treatments. Five-day infusion of CREB antisense oligonucleotide into the NAc decreased the ability of acute cocaine to induce c-Fos in this brain region. This is similar to the effect elicited by a single injection of CREB antisense oligonucleotide into the striatum, which was found to attenuate c-Fos induction in response to acute amphetamine (Konradi, et al., 1994). Furthermore, we have found in preliminary studies that single bilateral injections of CREB antisense oligonucleotide into the NAc produces a transient decrease in cocaine self-administration. This suggests that CREB functions to modulate the reinforcing effects of cocaine in the NAc. The antisense oligonucleotide-induced reduction in cocaine self-administration is consistent with our current finding that the 1-day CREB antisense oligonucleotide treatment decreases levels of CAMP-dependent protein kinase and our previous finding that injection of inhibitors of CAMP-dependent protein kinase into the NAc also decreases cocaine self-administration.

It is unlikely that CREB represents the only transcription factor mediating the long-term adaptations to opiates or other drugs of abuse in the NAc. Certain forms of immediate early gene transcription factors appear to be induced in the NAc uniquely after chronic exposure to a variety of drugs of abuse (Hope, et al., 1994). However, this study demonstrates that CREB regulation affects specific downstream proteins in the NAc and that CREB may be involved in morphine-regulation of certain signaling proteins in this brain region. We propose a model in which morphine, through either direct or indirect actions on NAc neurons, down-regulates CREB expression (fig. 8). The CREB antisense oligonucleotide infusion experiments demonstrate that down-regulation of CREB protein levels is sufficient to down-regulate G$_{\text{ia1/2}}$, and could mediate morphine regulation of this protein. Further support for this possibility will require future studies in which down-regulation of CREB is also shown to be necessary for morphine regulation of G$_{\text{ia1/2}}$. Based on evidence from a neural cell line, in which sustained activation of the CAMP pathway leads to down-regulation of CREB message and protein (Widnell et al., 1994), it is possible that the morphine-induced increase in CAMP-dependent protein kinase, produced by a distinct mechanism, mediates the observed CREB down-regulation in the NAc. This possibility may now be tested directly by infusing inhibitors of CAMP-dependent protein kinase catalytic subunit, or even antisense oligonucleotides to catalytic subunit mRNA, concomitantly with chronic morphine to see whether an attenuation of protein kinase catalytic activity can block morphine's effects on CREB expression.

In the present study, we provide direct evidence that a morphine-induced adaptation in a particular target protein in vivo, an adaptation of known functional significance, results from regulation of a specific transcription factor within a discrete brain region. Further studies along these lines promise to establish the precise molecular steps by which chronic exposure to morphine, and other drugs of abuse, produce long-lasting changes in the brain that underlie addiction. Moreover, our results further demonstrate the functional importance of the transcription factor CREB in the regulation of neural plasticity.

References


