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Inducible and Brain Region-Specific CREB Transgenic Mice

NORIO SAKAI, JOHANNES THOME, SAMUEL S. NEWTON, JINGSHAN CHEN, MAX B. KELZ, CATHY STEFFEN, ERIC J. NESTLER, and RONALD S. DUMAN

Division of Molecular Psychiatry, Departments of Psychiatry and Pharmacology, Yale University School of Medicine and Connecticut Mental Health Center, New Haven, Connecticut (N.S., J.T., S.S.N., J.C., M.B.K., R.S.D.); and Department of Psychiatry, the University of Texas Southwestern Medical Center, Dallas, Texas (C.S., E.J.N.)

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ABSTRACT

To investigate the role of cAMP response element-binding protein (CREB) in the adaptive responses to psychotropic drugs, we have developed inducible, brain region-specific CREB transgenic mice using the tetracycline-regulated gene expression system. The tetracycline transactivator (tTA) was placed under the control of 1.8-kilobase neuron-specific enolase (NSE) promoter for this purpose. Different patterns of CREB overexpression were found in striatum, nucleus accumbens, and cingulate cortex in different lines of bitransgenic mice, and CREB expression was blocked by addition of doxycycline, an analog of tetracycline. Overexpression of CREB influenced the expression of other members of the CREB/ATF family of transcription factors, consistent with previous reports. In addition, psychostimulant induction of dynorphin, a neuropeptide regulated by drugs of abuse, was up-regulated in striatum. Finally, there was a significant reduction in cocaine-induced locomotor activity in the CREB bitransgenic mice. These results are consistent with a role for CREB in mediating adaptive changes that occur in response to drugs of abuse.

A variety of neurotransmitter and endocrine signals which elevate intracellular cAMP levels activate gene transcription via a cAMP response element (CRE) found in the promoter region of target genes (Ziff, 1990). The cAMP response element binding protein (CREB), a substrate of cAMP-dependent protein kinase A, is able to bind to the CRE and *trans*-activate gene expression (Hoeffler et al., 1988; Yamamoto et al., 1988; Gonzalez and Montminy, 1989). In addition to CREB, several other CRE-binding proteins make up the CREB/activation transcription factor (ATF) family (Hai et al., 1989; Maekawa et al., 1989; Hai and Curran, 1991). Binding to a CRE is dependent on dimerization at the basic/leucine zipper domain in the carboxyl terminus of CREB/ATF proteins (Hai et al., 1989; Hai and Curran, 1991). CREB can form functional homodimers, or heterodimers with other CREB/ATF family proteins. After phosphorylation of CREB at the Ser^{133} residue and binding to a CRE site, the CREB binding protein, a coactivator of CREB, binds to the phosphorylated CREB. This leads to activation of a transcription factor/RNA polymerase II complex that directly *trans*-activates target gene expression. Ser^{133} can also be phosphorylated by a number of protein kinases other than protein kinase A, including calcium calmodulin-dependent protein kinase, protein kinase C, and ribosomal S-6 kinase. This suggests that CREB plays an important role in integrating intracellular cAMP and calcium signaling as well as responses to neurotrophic factors (Dash et al., 1991; Matthews et al., 1994).

CREB has been shown to play a critical role in different types of neuronal function, including memory formation and circadian rhythm (Ginty et al., 1993; Milner et al., 1998; Obrietan et al., 1998). Furthermore, region-specific regulation of CREB has been implicated in the long-term neuronal plasticity that underlies the actions of psychotropic drugs (Duman et al., 1997, 2000; Nestler and Aghajanian, 1997). For example, chronic opiate or psychostimulant administration up-regulates the phosphorylation or expression of CREB in specific brain regions, such as the locus ceruleus or nucleus accumbens (Guitart et al., 1992; Widnell et al., 1994; Cole et al., 1995; Widnell et al., 1996; Lane-Ladd et al., 1997; Nestler, 1997; Nestler and Aghajanian, 1997). A role for CREB in the behavioral actions of drugs of abuse is also supported by studies of CREB$\alpha\Delta$ mutant mice or viral mediated gene transfer of CREB (Maldonado et al., 1996; Carlezon et al.,

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ABBREVIATIONS: CRE, cAMP responsive element; CREB, cAMP response element binding protein; ATF, activation transcription factor; tTA, tetracycline transactivator; TetOp, tetracycline-operated promoter; NSE, neuron-specific enolase; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; BSA, bovine serum albumin; pCREB, phospho-cAMP response element binding protein; RPA, RNase protection analysis; RT, reverse transcriptase; bp, base pair(s); CREM, cAMP response element modulator; ICER, inducible cAMP early repressor; DRE, downstream regulatory element.

1998). Another series of studies demonstrates that administration of antidepressants, but not drugs of abuse, increases the phosphorylation and expression of CREB in the hippocampus and cerebral cortex but not in nucleus accumbens or locus ceruleus (Nibuya et al., 1996; Thome et al., 2000). These findings demonstrate that CREB is differentially regulated by psychotropic drugs and may play a role in the cellular and behavioral responses to these agents.

To further elucidate the function of CREB, we have developed inducible, brain region-specific CREB transgenic mice. Conventional transgenic mice, which constitutively overexpress a transgene from early development, can often exhibit compensatory or developmental adaptations that make it difficult to interpret the actions of transgene overexpression. In addition, expression of a transgene throughout the brain, as well as in peripheral tissues, can lead to effects that indirectly influence the function of specific populations of neurons. The use of inducible and region-specific transgenic mice circumvents these problems. We have used the tetracycline-regulated system, in which the expression of CREB is placed under the control of the tetracycline-operated promoter (TetOp). TetOp-CREB is regulated by the tetracycline transactivator (tTA), which is under the control of the neuron-specific enolase (NSE) promoter (Chen et al., 1998; Kelz et al., 1999). Addition of tetracycline induces a conformational change in tTA and thereby blocks its ability to bind TetOp and *trans*-activate CREB expression. This is referred to as the tetracycline-off system (Fig. 1).

In this study, we have characterized the expression of CREB in several lines of inducible and region-specific transgenic mice, and we have examined the effects of doxycycline, an analog of tetracycline, on the expression of CREB in vivo. In addition, we report the regulation of CREB/ATF1 family members, as well as possible target genes, in the CREB transgenic mice. Moreover, we investigate the behavioral consequences of CREB overexpression.

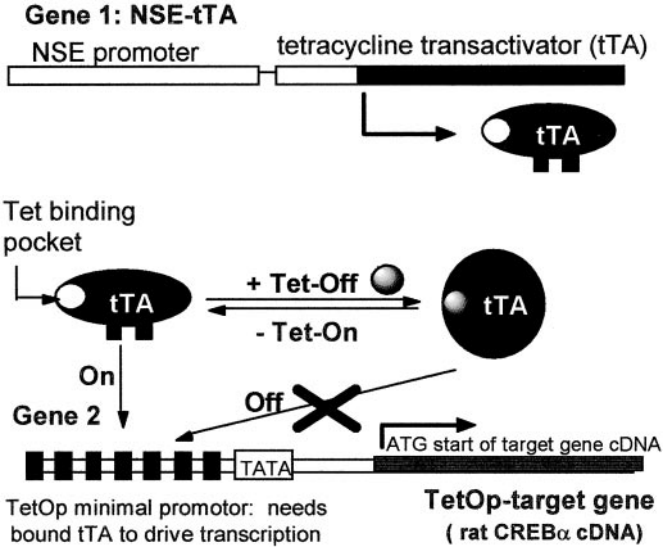

Fig. 1. Schematic diagram of the tetracycline-regulated gene expression system. Gene 1 encodes tTA under the control of NSE promoter. Gene 2 encodes a rat CREBα cDNA under the control of TetOp. Transgenic mice with gene 1 or 2 are developed independently, and these two lines are crossed with each other to obtain NSE-tTA $\times$ TetOp-CREB bitransgenic mice. In NSE-tTA $\times$ TetOp-CREB bitransgenic mice, tTA binds to TetOp and activates the transcription of CREB. In this way, CREB is expressed under the control of NSE promoter in a region-specific manner. In the presence of tetracycline or its derivative, tetracycline binds to tTA and cause a conformational change that blocks its binding to TetOp and CREB expression is turned off.

Materials and Methods

Development of Transgenic Mice and Drug Treatments. The methods for generating inducible and brain region-specific CREB transgenic mice are described in our previous reports (Chen et al., 1998; Kelz et al., 1999). The principle of the Tet-regulated system is summarized in Fig. 1. In brief, plasmids that can drive tTA under the control of 1.8-kilobase NSE promoter (designated pNSE-tTA) and an another that can drive expression of rat CREBα under the control of the TetOp (designated pTetOp-CREB) were constructed. Linearized DNA fragments from pNSE-tTA or pTetOp-CREB, which contain the promoter, open reading frame, simian virus 40 intron, and poly(A)$^+$ signal, were microinjected into pronuclei of oocytes from SJL $\times$ C57BL6 mice. Transgenic mice with a single transgene (either NSE-tTA or TetOP-CREB) were generated independently. These two lines of mice were then crossed to develop NSE-tTA $\times$ TetOp-CREB bitransgenic mice. Genotyping of generated transgenic mice was performed by PCR with specific primer sets for the NSE promoter or rat CREBα cDNA using genomic DNA isolated from mouse tail as template.

To turn off CREB overexpression, the NSE-tTA $\times$ TetOp-CREB bitransgenic mice were given water containing doxycycline (50 μg/ml; Sigma, St. Louis, MO), a derivative of tetracycline, and 5% sucrose. Amphetamine (15 mg/kg), cocaine (10 mg/kg), or saline was administrated i.p. to examine the regulation of dynorphin expression. Animals were killed 3 h after injection. All transgenic mice used in this study were strictly maintained according to the guidelines from the National Institutes of Health and institutional animal care.

Immunohistochemistry. Immunohistochemistry was conducted as described previously (Hiroi and Graybiel, 1996; Hiroi et al., 1997). In brief, mice were perfused with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) after anesthesia with sodium pentobarbital. Brains were removed and further fixed with 4% paraformaldehyde in 0.1 M PBS for 16 h at 4°C. The brains were stored in 20% (v/v) glycerol in 0.1 M PBS for 2 days at 4°C. Coronal brain slices of 20 to 25 μm thickness were cut with the use of a microtome. For CREB immunohistochemistry, slices were blocked with 5% BSA in 0.01 M PBS for 30 min at room temperature and then incubated with a polyclonal CREB antibody (1:500 dilution; Upstate Biotechnology Incorporated, Lake Placid, NY) for 2 to 3 days at 4°C. CREB immunoreactivity was detected using standard avidin-biotin complex-diaminobenzidine method as described previously (Hiroi and Graybiel, 1996; Hiroi et al., 1997).

Immunoblotting of CREB and Phospho-CREB. Dissected striatum and cerebellum from NSE-tTA $\times$ TetOp-CREB bitransgenic (NSE-tTA+/TetOp-CREB+, +/+) mice or NSE-tTA monotransgenic (NSE-tTA+/TetOp-CREB$-$, +/$-$) mice were homogenized and sonicated in immunoprecipitation assay buffer (10 mM Tris-HCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, 20 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4) and centrifuged at 19,000*g* for 15 min at 4°C. Supernatants were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting using a polyclonal CREB antibody (1:500 dilution; Upstate Biotechnology Incorporated) as described previously (Takahashi et al., 1999).

For phospho-CREB immunoblotting (pCREB), forskolin-stimulated striatal slices were prepared. Briefly, mouse brains were quickly removed and immediately immersed in ice-cold HEPES-buffered saline (136.7 mM NaCl, 5 mM KCl, 0.1 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM $MgCl_2$, 16.6 mM glucose, 23.8 mM sucrose, and 9.84 mM HEPES, pH 7.4) agitated with O_2-CO_2 (95:5). After 1 min incubation in ice-cold HEPES buffered saline, brains were trimmed to obtain striatum blocks. Striatum

slices of 200 μm thickness were cut using a vibratome (Technical Products International, Inc., St. Louis, MO) and then slices were preincubated in HEPES-buffered saline agitated with O_2-CO_2 (95:5) at 37°C for 45 min. After the preincubation, slices were treated with 200 μM forskolin (Sigma) for 15 min to activate the cAMP system and induce CREB phosphorylation. Thereafter, treated slices were homogenized in electrophoretic mobility shift assay buffer (20 mM HEPES, 0.4 M NaCl, 20% glycerol, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 20 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.9), containing phosphatase inhibitors (1 mM Na_3VO_4, 1 mM NaF, and 100 nM calyculin A), and incubated for 20 min on ice. After centrifugation at 19,000*g* for 15 min, supernatants were subjected to SDS-polyacrylamide gel electrophoresis, and phospho-CREB immunoblotting was performed using a polyclonal phospho-CREB antibody (1:500 dilution, New England Biolabs, Beverly, MA), as described previously (Takahashi et al., 1999). To compare the precise size of the phospho-CREB band, a lysate of forskolin-treated CATH.a cells was used as positive control. CREB immunoblotting was carried out simultaneously to examine the level of total CREB immunoreactivity.

Phospho-CREB Immunohistochemistry. Immunohistochemistry for pCREB was performed using a slide-based protocol. Cryocut sections (14 μm thick) were briefly fixed in 4% paraformaldehyde followed by rinses in PBS. This was followed by a 5-min treatment with 1% hydrogen peroxide and two 5-min rinses in PBS. Sections were blocked to prevent nonspecific binding of antibody by a 30-min treatment in a blocking solution containing 2.5% BSA.

Sections were incubated overnight at 4°C in antibody solution (0.25% Triton X-100, 1% BSA in PBS) containing anti–phospho-CREB antibody (Upstate Biotechnology) at 1:500 dilution. Antibody-treated sections were given three 5-min washes with PBS to remove unbound primary antibody and were incubated in peroxidase-conjugated goat anti-rabbit IgG at 1:400 dilution for 1 h at room temperature. Unbound secondary antibody was removed by three 5-min rinses in PBS. Sections were then incubated in a preformed avidin and biotinylated horseradish peroxidase macromolecular complex (ABC reagent; Vector Labs, Burlingame, CA) for 1 h. After this incubation, sections were rinsed three times in PBS and then stained with the 3,3′-diaminobenzidine staining kit (Vector Labs) according to manufacturer's directions. Slides were air dried, dehydrated in an alcohol series, and coverslipped with distyrene/plasticizer/xylene mountant.

RNase Protection and in Situ Hybridization Analysis. Total RNA was extracted from various regions of mouse brain using the RNAqueous kit (Ambion, Austin, TX) according to the manufacturer's protocol. RPA was performed with the RPAII kit (Ambion) according to the manufacturer's protocol and using total RNA extracts from mouse brain as templates.

To generate the CREB-specific riboprobes that distinguish the three major CREB isoforms (CREBα, CREBΔ, and CREBβ; Ruppert et al., 1992; Blendy et al., 1996), reverse transcriptase (RT)-polymerase chain reaction was first carried out to obtain partial mouse CREB cDNA. A sense primer that recognizes exon 2 (designated primer 6) of the mouse CREB gene and an antisense primer that recognizes exon 7 (designated primer 7) were designed for RT-PCR. The sequences of primers 6 and 7 were 5′-TAAATGACCATGGAATCTGGAGCA-3′ and 5′-AGTTACACTATCCACAGACTCCTG-3′, respectively. RT-PCR was performed using the Access RT-PCR system (Promega, Madison, WI), according to the manufacturer's recommended method. Total striatal RNA was used as template for RT-PCR, followed by agarose gel separation and ethidium bromide staining to visualize the products. A DNA band of 318 bp, which corresponds to the CREBΔ isoform, was isolated using a gel extraction kit (QIAGEN, Valencia, CA), subcloned into pGEM-T Easy vector (Promega), and verified by sequencing. The resulting plasmid was designated pCREBΔ 6–7. A riboprobe was generated by linearizing pCREBΔ 6–7 with *Spe*I and ^{32}P-labeled using SP6 RNA polymerase (Roche Applied Science, Indianapolis, IN). Protected fragments were loaded onto an 8% acrylamide-Tris/borate/EDTA gel and the separated bands were detected by autoradiography. Measurement and quantification of protected band density was carried out using the Macintosh version of the NIH image analysis program (version 1.52; http://rsb.info.nih.gov/nih-image/).

For CREM (CRE modulator)-specific RPA analysis, RT-PCR was also performed, as described above, to obtain partial cDNA encoding the CREMτ isoform (Foulkes et al., 1992). The sequences of the sense and antisense primers were 5′-GAAACAGTTGAATCACAGCAGGAT-3′ and 5′-TGATTGAATAACCGATGGATGTGG-3′, respectively. RT-PCR products were subcloned (designated as pCREMτ). This riboprobe can distinguish between CREMα and -β, repressor isoforms of CREM, as well as CREMτ, an activator isoform of CREM, by the size of protected fragments. CREMα and -β are recognized as a single band of 105 bp and CREMτ is a protected fragment of 231 bp.

To generate a probe for RPA of ATF1, RT-PCR was performed using mouse ATF1 specific primers (Lee et al., 1992). The sense and antisense primers were 5′-ATAGGCTCCTCACAGAAAGCTCAC-3′ and 5′-TAATGTCTGCAGTGCCTGCACTCC-3′, respectively. The resulting subcloned plasmid was designated pATF1. RPA using the pATF1 riboprobe recognizes an ATF1 mRNA band of 258 bp. Probes used for RPA of inducible cAMP early repressor (ICER) was carried out as described previously (Fitzgerald et al., 1996a).

For RPA and in situ hybridization of dynorphin, a riboprobe was generated from a plasmid encoding the major exon of rat prodynorphin gene, which was provided by Dr. C. R. Gerfen (see Carlezon et al., 1998). Experimental procedures for in situ hybridization of dynorphin were as described previously (Nibuya et al., 1996; Takahashi et al., 1999). After ISH, sections were dipped in nuclear track emulsion (NTB2; Eastman Kodak, Rochester, NY) after diluting emulsion (1:1) in 600 mM ammonium acetate at 42°C. Slides were developed after 1 week exposure in emulsion, counter-stained in cresyl violet (10–15 min), dehydrated in an alcohol series, cleared in Histochoice (15 s; Amresco, Solon, OH), and coverslipped with distyrene/plasticizer/xylene mountant.

Analysis of Cocaine-Induced Locomotor Activity in Bitransgenic Mice. Locomotor sensitization was conducted in mice as described previously (Hiroi et al., 1997). Subjects were habituated to the test apparatus over 5 consecutive days and then received cocaine (10 mg/kg, i.p.) or saline injections for 5 consecutive days. Horizontal locomotor activity was quantified by automated beam crossing for 60 min each day.

Results

Expression Pattern of CREB in NSE-tTA × TetOp-CREB Bitransgenic Mice. We have developed six lines of TetOp-CREB mice, each of which was crossed with NSE-tTA lines A and B generated by our group (Chen et al., 1998). Previous studies demonstrate that these lines induce overexpression of a reporter gene (*luciferase*) and another target gene (Δ*FosB*) under the TetOp promoter to varying degrees in striatum, nucleus accumbens, cortex, and hippocampus (Chen et al., 1998). Immunohistochemical analysis demonstrated significant levels of CREB overexpression in three lines of bitransgenic mice: TetOp-CREB 6 line × NSE-tTA B line (CREB6-B line); TetOp-CREB 6 line × NSE-tTA A line (CREB6-A line); and TetOp-CREB 3 line × NSE-tTA A line (CREB3-A line).

In the 6B line (NSE-tTA+/TetOp-CREB+, +/+), CREB overexpression predominated in the dorsal and medial portion of striatum compared with CREB staining in mice with only NSE-tTA (NSE-tTA+/TetOp-CREB−, +/−) (Fig. 2). Higher magnification (400×) demonstrates that CREB overexpression is restricted to the nucleus of cells. CREB was also overexpressed in nucleus accumbens, primarily in the core

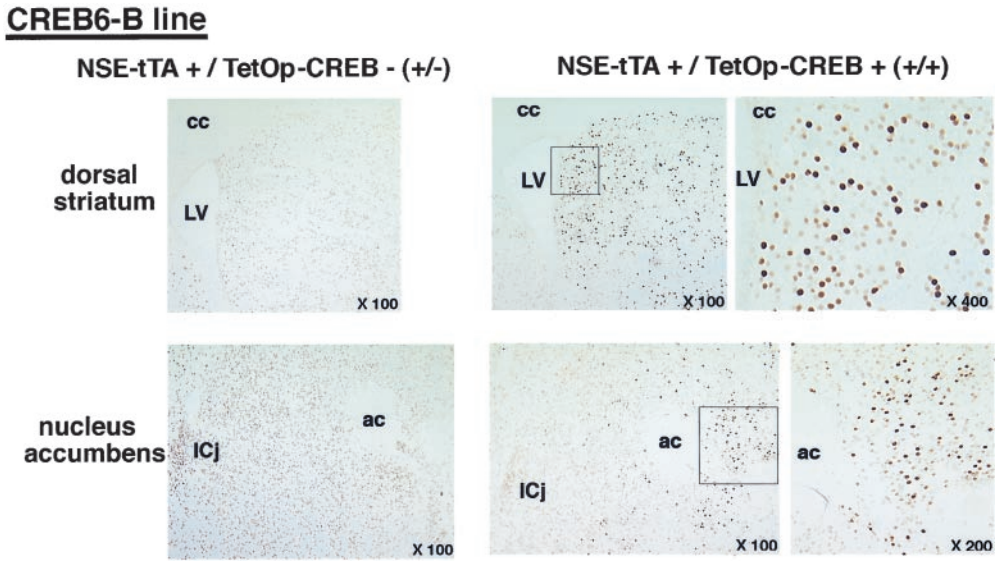

Fig. 2. Pattern of CREB overexpression in striatum and nucleus accumbens of CREB6-B bitransgenic mice. CREB expression was determined by immunohistochemistry in mice expressing one transgene (NSE-tTA+/TetOp-CREB−, +/−) and bitransgenic mice (NSE-tTA+/TetOp-CREB+, +/+) mice. In bitransgenic mice, intense staining of CREB is observed in many cells of the dorsal and medial regions of striatum (upper trace) and in the core of nucleus accumbens (lower trace). Weak nuclear staining of endogenous CREB is seen throughout the striatum and nucleus accumbens of bitransgenic mice, as well as in mice expressing only NSE-tTA. Higher magnification demonstrates that CREB is localized in the nucleus. Results are representative of the analysis of at least three animals in each group. LV, lateral ventricle; cc, corpus callosum; ac, anterior commissure; Icj, islands of Calleja.

subdivision (Fig. 2, lower). In the CREB6-A line, CREB was overexpressed in striatum and nucleus accumbens, similar to the expression pattern in the CREB6-B line (summarized in Table 1). In addition, CREB was sparsely, but significantly, overexpressed in deep and superficial layers of cingulate cortex (Fig. 3). CREB overexpression in deep layers of parietal cortex was also observed (data not shown). No CREB overexpression was observed in hippocampus in either the CREB6-A or -B lines (Table 1). However, low levels of CREB overexpression were observed in hippocampus of the CREB3-A line (Fig. 3, lower trace). CREB was sparsely but clearly overexpressed in CA1 pyramidal cells and granular cells of dentate gyrus (Fig. 3, lower trace). CREB overexpression was also observed in the dorsal striatum and nucleus accumbens but at lower levels than in the CREB6-A or CREB6-B lines (summarized in Table 1). There was no expression of CREB observed in monotransgenic TetOp-CREB mice (i.e., without the NSE-tTA), demonstrating that there is no leak of the TetOp-CREB transgene (not shown).

Overexpression of CREB was further examined by immu-

TABLE 1
Pattern of CREB overexpression in three different lines of NSE-tTA × TetOp-CREB bigenic mice
Level of CREB overexpression is indicated by the number of + symbols. Overexpression of CREB in cerebellum was confirmed by Western blotting (see text).

Line (NSE-tTA × TetOp-CREB)	Striatum	Nucleus Accumbens	Cortex	Hippocampus	Cerebellum
CREB6-A	+++	++	+	−	−
CREB6-B	++++	+++	−	−	+
CREB3-A	++	+	−	+	−

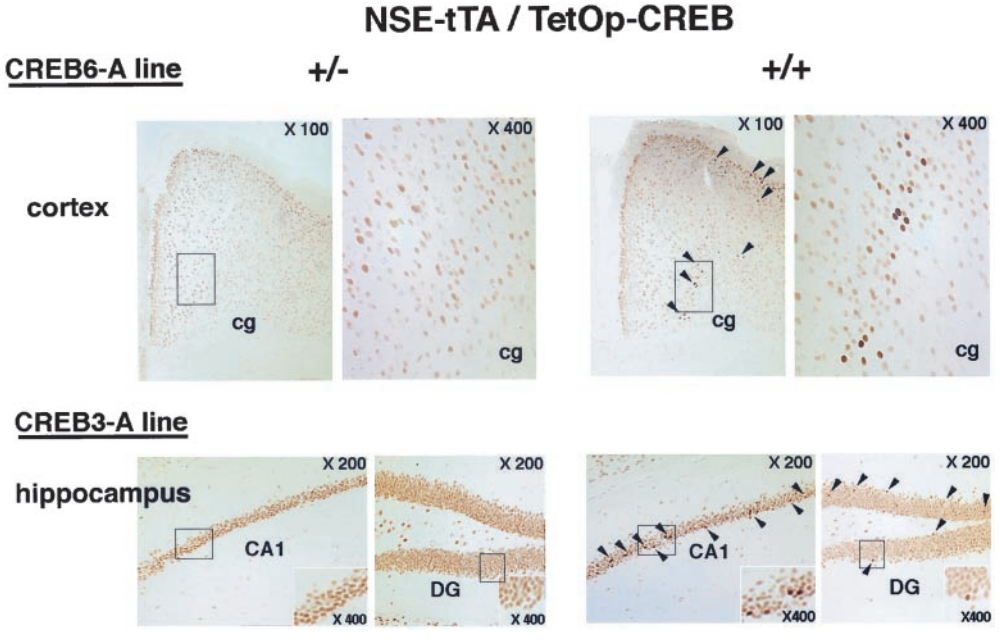

Fig. 3. Pattern of CREB overexpression in CREB6-A and CREB3-A bitransgenic mice. In CREB6-A bitransgenic mice, CREB is overexpressed in deep layers of cingulate cortex (+/+, upper trace). Arrowheads show CREB-overexpressing cells. A small number of cells also exhibit dense CREB staining in the superficial layer of cortex. Higher magnification demonstrate that this staining is localized to the nucleus. In CREB3-A bitransgenic mice (+/+), there is sparse but distinct CREB overexpression in CA1 pyramidal and dentate gyrus granule cells of hippocampus (lower trace). Results are representative of the analysis of at least three animals in each group. cg, cingulum; DG, dentate gyrus.

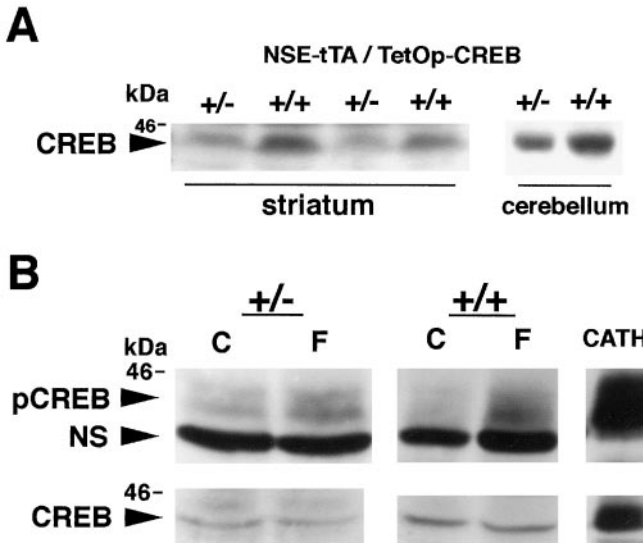

Fig. 4. Immunoblot analysis of CREB overexpression in CREB6-B bitransgenic mice. A, in both striatum and cerebellum, CREB immunoreactive bands of approximately 43 kDa were detected; the intensity of this band was greater in CREB6-B bitransgenic mice relative to the +/− control mice. B, forskolin-stimulated phosphorylation of CREB (pCREB) was enhanced in striatum of CREB6-B bitransgenic mice. Striatal slices were freshly prepared and maintained at 37°C in HEPES-buffered saline as described under *Materials and Methods*. Slices were treated with 200 μM forskolin (F) for 15 min and then subjected to pCREB immunoblot analysis. Samples from forskolin-stimulated CATH.a cells, which express high levels of CREB, were used as a positive control (right lane). The pCREB immunoreactive bands were detected as a doublet just above an intense nonspecific band (NS). The size of the pCREB bands in the bitransgenic mice is the same as those in the forskolin-treated CATH.a cells. In both the +/+ and +/− slices, levels of pCREB staining are greater after incubation with forskolin, but the intensity of the bands is greater in the CREB-overexpressing +/+ slices. Immunoblot analysis of CREB demonstrates similar amounts of CREB regardless of the forskolin incubation, but higher levels of CREB in the bitransgenic mice as expected. Results are representative of three separate experiments.

noblotting of brain regions taken from CREB6-B mice. A CREB immunoreactive band of 43 kDa was overexpressed in NSE-tTA × TetOp-CREB bitransgenic (+/+) mice. CREB overexpression was found in striatum, as well as in cerebellum of the 6B line (Fig. 4A). No additional CREB immunoreactive bands were detected in +/+ mice compared with the genetic controls (+/−) (data not shown).

To determine whether CREB phosphorylation was enhanced in CREB overexpressing bitransgenic mice, phospho-CREB immunoreactivity in striatal slices of CREB6-B +/+ and +/− mice was examined. In preliminary time course experiments, the induction of phospho-CREB by forskolin was found to peak after 15 min and then gradually decrease with longer periods of incubation. Therefore, a 15-min time point was chosen for further studies. Stimulation with forskolin increased phospho-CREB immunoreactivity relative to control, and this effect was greater in the CREB6-B +/+ mice relative to the +/− mice (Fig. 4B). Two phospho-CREB immunoreactive bands, which were just above an intense nonspecific band, were observed in response to forskolin. The phospho-CREB bands in the bitransgenic mice comigrate with the phospho-CREB bands prepared from forskolin-treated CATH.a cells (Fig. 4B), which are also detected as a doublet upon shorter exposure (data not shown). The expression of CREB is relatively high in the CATH.a cells, and we have used them routinely as markers for CREB and phospho-

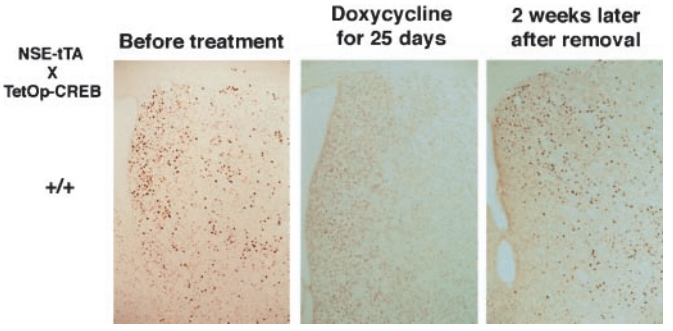

Fig. 5. Influence of doxycycline, a derivative of tetracycline, on the overexpression of CREB in striatum of CREB6-B bitransgenic mice. Immunohistochemistry of CREB was performed to verify the overexpression of CREB. Before the treatment with doxycycline, CREB overexpression was seen in striatum near lateral ventricle, as seen in Fig. 2 (left). After the treatment with doxycycline (50 μg in water) for 25 days, CREB overexpression was almost completely abolished and only staining of endogenous CREB was detected (middle). Overexpression of CREB was once again observed 2 weeks after the removal of doxycycline (right). Data are representative of two separate experiments.

CREB (Widnell et al., 1994, 1996). The relatively lower level of CREB and phospho-CREB immunoreactivity in the mouse brain compared with CATH.a cells is consistent with the low levels observed in our previous studies (Widnell et al., 1994, 1996). Figure 4B also demonstrates that levels of total CREB immunoreactivity are higher in the +/+ mice relative to the +/− mice.

The influence of doxycycline, a derivative of tetracycline, on the expression of CREB in the bitransgenic mice was examined next. This was necessary to confirm that the tTA-TetOp system was functioning correctly in vivo. Application of doxycycline (50 μg/ml in water) for 25 days completely blocked CREB overexpression in the 6-B line (Fig. 5). Removal of doxycycline for 2 weeks allowed for a nearly complete recovery of CREB overexpression (Fig. 5, right). These findings demonstrate that the expression of CREB can be turned off and then back on upon addition and removal of doxycycline.

Regulation of CREB, ATF, and CREM Isoforms in the Bitransgenic Mice. Studies were conducted to determine whether overexpression of CREB alters levels of endogenous CREB or CREB-related transcription factors, including CREM and ATF1. Expression of three major isoforms of CREB (i.e., α, β, and Δ) (Ruppert et al., 1992; Blendy et al., 1996), were analyzed by RPA in NSE-tTA × TetOp-CREB bitransgenic mice. A riboprobe that can distinguish the CREB isoforms by the size of protected fragments was prepared by RT-PCR (Fig. 6A). The relative levels of the three isoforms are similar to what has been reported (Blendy et al., 1996). In wild-type animals, levels of CREBΔ are highest, with approximately equal amounts of CREBα and CREBβ. In striatum from either CREB6-A or -B bitransgenic (+/+) mice, CREBα mRNA, the isoform used to develop the transgenic lines, is significantly increased as expected (Fig. 6, B and C). In addition, levels of CREBβ and Δ isoforms are significantly up-regulated. The induction of the α, as well as β and Δ, isoforms in the CREB6-A line was reversed by addition of doxycycline to the drinking water (Fig. 6C).

Transcription factors in CREB/ATF family modulate transcription via CRE sites, which are found in the promoter region of their target genes by forming homo- or heterodimers with one another (Hai et al., 1989; Ziff, 1990; Hai

and Curran, 1991). Within the CREB/ATF family of transcription factors, CREB, CREM, and ATF1 are known to preferentially form heterodimers with one another in vitro, indicating that these three transcription factors function as partners for regulation of CRE-mediated transcription in vivo (Hai and Curran, 1991). To examine the influence of CREB overexpression on these related proteins, RPA analysis of CREM and ATF1 was performed.

A riboprobe was generated for CREMτ, an activator of CRE-mediated *trans*-activation that can discriminate between this isoform and CREMα and β, two repressors of CRE *trans*-activation, by the size of the protected fragments (Fig. 7A). Both repressor and activator types of CREM (CREMα, β, and τ, respectively) were down-regulated by approximately 20% in striatum and cerebellum of CREB6-B bitransgenic mice (Fig. 7). Down-regulation of CREM was not observed in CREB6-A bitransgenic mice (data not shown). This may be related to the greater induction of CREB in the CREB6-B line relative to the -A line, as demonstrated in Fig. 6B.

We also examined the regulation of ICER, another repressor isoform of CREM. ICER is generated by use of a different promoter in the *CREM* gene than that used for generating CREMα, -β, and -τ (Molina et al., 1993). Basal levels of ICER mRNA were not significantly influenced in striatum of CREB6-B bitransgenic mice (Fig. 7). Because ICER is rapidly induced by activation of the CREB, induction of this isoform in response to amphetamine was also examined in control and CREB-6B bitransgenic mice. Induction of ICER in response to amphetamine was observed in both single (NSE-tTA) and bitransgenic (NSE-tTA × TetOp-CREB) mice, but there was no significant difference between these two groups [273 ± 22.6 and 228 ± 14.6% of saline, for single and bitransgenic CREB6-B mice, respectively (mean ± S.E.M., $n = 10$ per group)]. There was also no significant difference in levels of mRNA for ATF1, another member of the CREB/ATF family, in striatum or cerebellum of the single CREB6-B bitransgenic line (Fig. 7).

Regulation of Dynorphin Expression in NSE-tTA × TetOp-CREB Bitransgenic Mice. Previous reports demonstrate that chronic administration of a psychostimulant (e.g., cocaine or amphetamine) up-regulates the cAMP pathway, which leads to activation of CRE/CREB-mediated transcription in nucleus accumbens and dorsal striatum (Terwilliger et al., 1991; Konradi et al., 1994; Cole et al., 1995; Hyman, 1996; Unterwald et al., 1996; Nestler, 1997; Nestler and Aghajanian, 1997; Turgeon et al., 1997). In addition, dynorphin has been shown to be a target of CREB-mediated *trans*-activation in these regions after chronic cocaine or amphetamine treatment (Hurd et al., 1992; Daunais et al., 1993; Spangler et al., 1993; Cole et al., 1995; Carlezon et al., 1998).

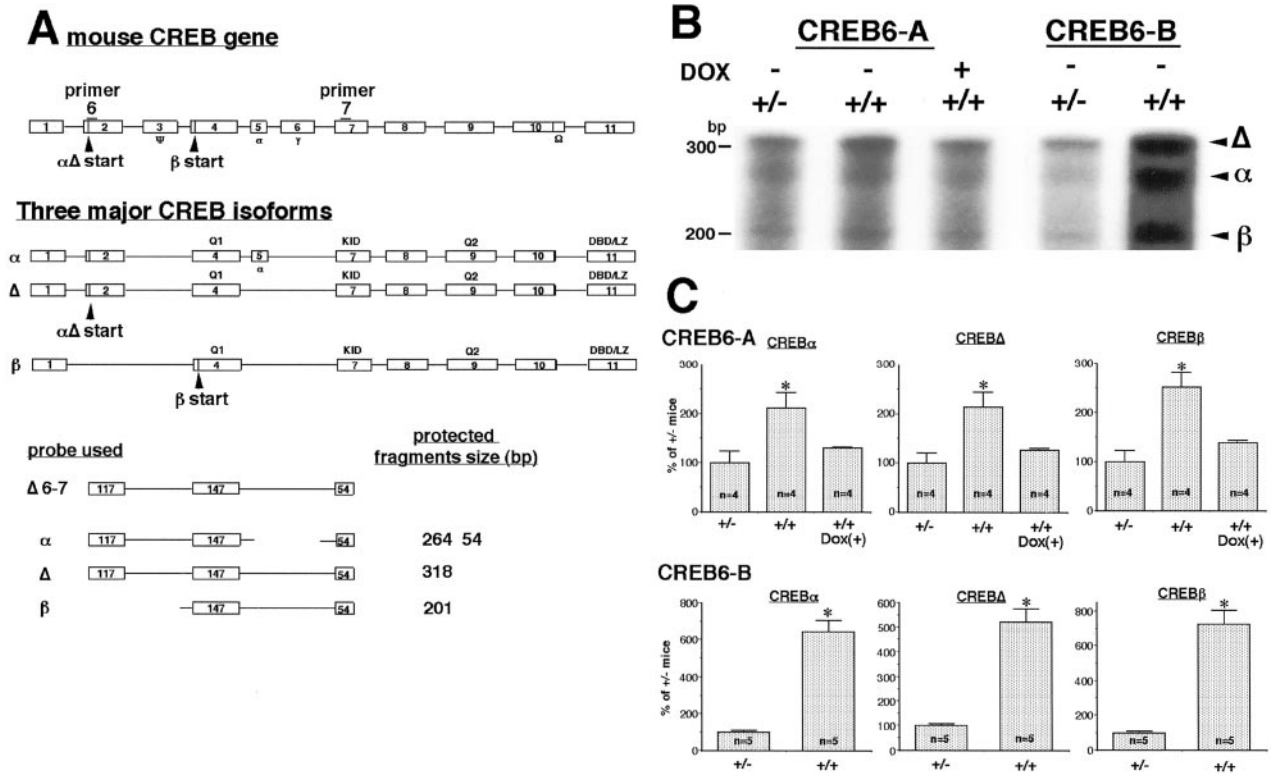

Fig. 6. Analysis of CREBα, -β, and -Δ isoform expression in bitransgenic mice. A, strategy for RNase protection analysis (RPA) of CREBα, -β, and -Δ isoforms. The mouse CREB gene consists of 11 exons (Ruppert et al., 1992). All 11 exons are used for the transcription of the CREBα isoform, whereas CREBΔ lacks exon 5 and CREBβ lacks exons 2 and 5 compared with CREBα. For the translation of CREBα and CREBΔ isoforms, the initiation site in exon 2 is used, whereas the initiation site in exon 4 is used for CREBβ. RT-PCR was carried out to get a partial CREB cDNA that was used to generate probes for RPA. A sense primer (primer 6) and an antisense primer (primer 7) were designed to recognize exon 2 and exon 7, respectively. A partial cDNA of CREBΔ isoform (pCREBΔ 6–7) was obtained and used for making a riboprobe. This probe recognizes CREBα mRNA as two protected fragments of 264 bp and 54 bp, CREBΔ as a fragment of 318 bp, and CREBβ as a fragment of 201 bp. B, analysis of CREBα, -β, and -Δ isoforms by RPA in striatum of CREB6-A and -B line mice. In both NSE-tTA × TetOp-CREB bitransgenic mice (+/+) of CREB6-A and -B lines, all three major CREB isoforms (CREBα, -β, and -Δ) were up-regulated compared with the expression levels in NSE-tTA monotransgenic (+/−) mice or doxycycline-treated NSE-tTA × TetOp-CREB bitransgenic mice [+/+, Dox(+)]. A greater up-regulation of the three CREB isoforms was observed in the CREB6-B line. Results are expressed as mean percentage of control +/− mice ± S. E. M. ($n = 4$ or 5 as indicated). ∗, $P < 0.01$, compared with control (+/−) mice (Student's t test).

The promoter region of the rat dynorphin gene has three predicted CRE sites, and promoter activity is increased by stimulation of receptors that activate the cAMP pathway in vitro (Collins-Hicok et al., 1994). These findings led us to examine the regulation of dynorphin in the dorsal striatum and nucleus accumbens of NSE-tTA × TetOp-CREB bitransgenic mice.

Expression of dynorphin was determined under two conditions. In the first paradigm, CREB6-B bitransgenic mice were raised in the absence of doxycycline (CREB on) throughout development. This would be similar to a constitutive transgenic line of mice in which there is no control over CREB overexpression. In the second paradigm, mice were raised on doxycycline (CREB off) until they were weaned and then withdrawn from doxycycline for 6 weeks before analysis (CREB on). In both cases, monotransgenic mice (+/−) were used as a control. Using the first paradigm, in which CREB overexpression is on throughout development, we found that basal dynorphin expression was depressed in striatum of CREB6-B bitransgenic mice relative to the +/− control mice (Fig. 8). In NSE-tTA × TetOp-CREB bitransgenic (+/+) mice, dynorphin mRNA was decreased by 40 to 60%, determined by either RPA or in situ hybridization, compared with control +/− mice (Fig. 8A). Basal expression of dynorphin was also significantly reduced in the striatum of CREB6-A bitransgenic mice (data not shown). Induction of dynorphin mRNA in response to a relatively high dose of amphetamine (15 mg/kg, i.p.) was also examined. Levels of dynorphin mRNA were determined 3 h after amphetamine administration. In both CREB6-B +/+ and +/− mice, amphetamine increased dynorphin expression, but the induction relative to the basal state was greater in the CREB6-B +/+ mice than in the control +/− mice (Fig. 8B).

The results obtained with the second paradigm, in which CREB expression is not induced until after weaning, resulted in a somewhat different profile. For these studies, expression of dynorphin was visualized by examination of grains over individual cells. This allowed us to look specifically in regions in which there is a high level of CREB overexpression, the medial lateral nucleus accumbens (see Fig. 2). In the naive CREB overexpressing mice, there was an increase in dynorphin expression in this region of the nucleus accumbens (Fig. 9, A and B). Moreover, administration of amphetamine resulted in a greater induction of dynorphin in the CREB overexpressing mice relative to the monotransgenic control mice (Fig. 9, C and D). Levels of pCREB were also determined

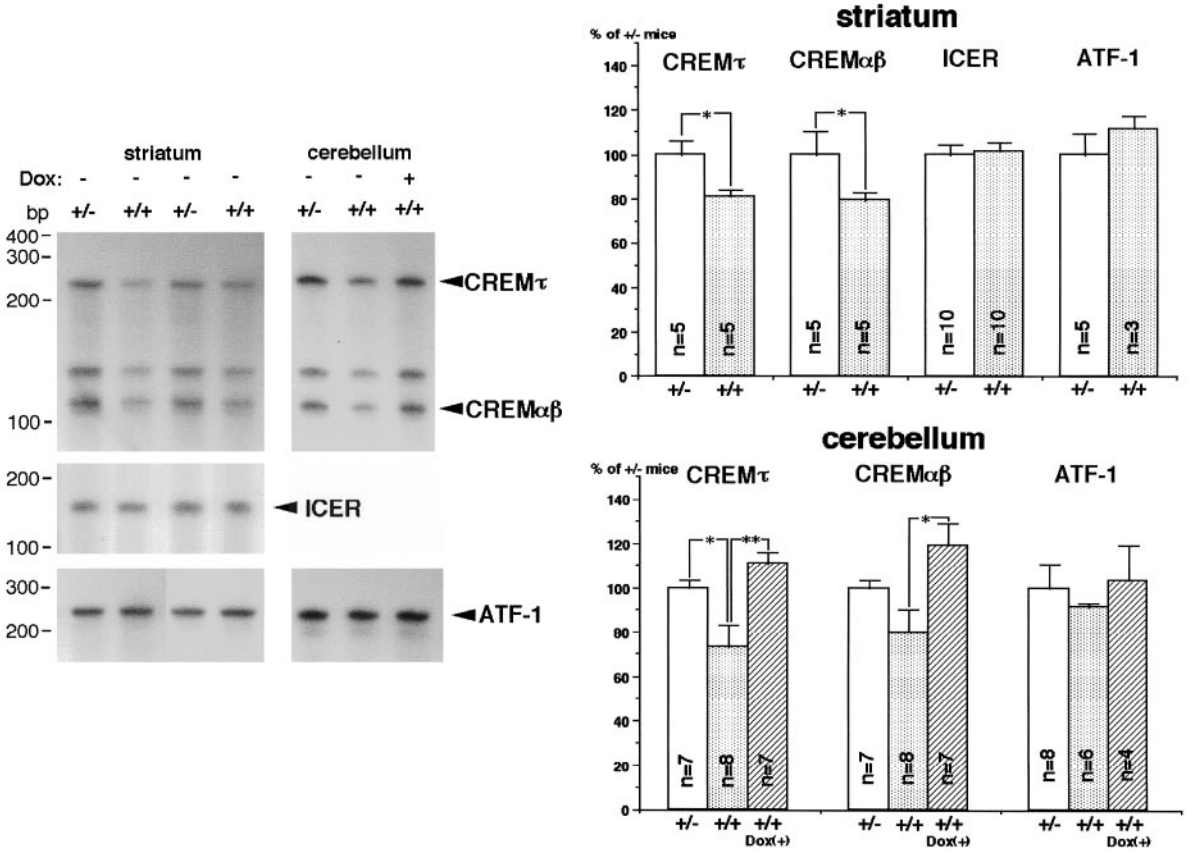

Fig. 7. Analysis of CREM ($\alpha\beta$, τ, and ICER) and ATF1 expression in CREB6-B bitransgenic mice. Left, representative RPA autoradiograms of CREM, ICER, and ATF1 protected fragments. As described under *Materials and Methods*, protected fragments of 231 bp (corresponding to CREMτ) and 105 bp (corresponding to CREM$\alpha\beta$) are activator and repressors of CRE sites, respectively. The α and β isoforms are very similar and cannot be distinguished with the probe used for these studies. Bands of approximately 130 bp were unexpected and may correspond to another unknown isoform of CREM (top). RPA for ICER demonstrates a single major band that corresponds to ICERγ (middle) (Fitzgerald et al., 1996a). Protected fragments for ATF1 are of 258 bp (bottom). In both striatum and cerebellum of NSE-tTA × TetOp-CREB bitransgenic (+/+) mice, CREMτ and -$\alpha\beta$ are down-regulated, compared with the expression level in NSE-tTA monotransgenic (+/−) mice or doxycycline-treated bitransgenic mice [+/+, Dox(+)]. Expression levels of ICER and ATF1 were unchanged in striatum and cerebellum of CREB6-B bitransgenic mice. Results are expressed as mean percentage of control +/− mice ± S.E.M. (n = 3 to 10 as indicated). *, $P < 0.05$; **, $P < 0.01$, compared with +/− mice or +/+, Dox(+) mice (Student's t test).

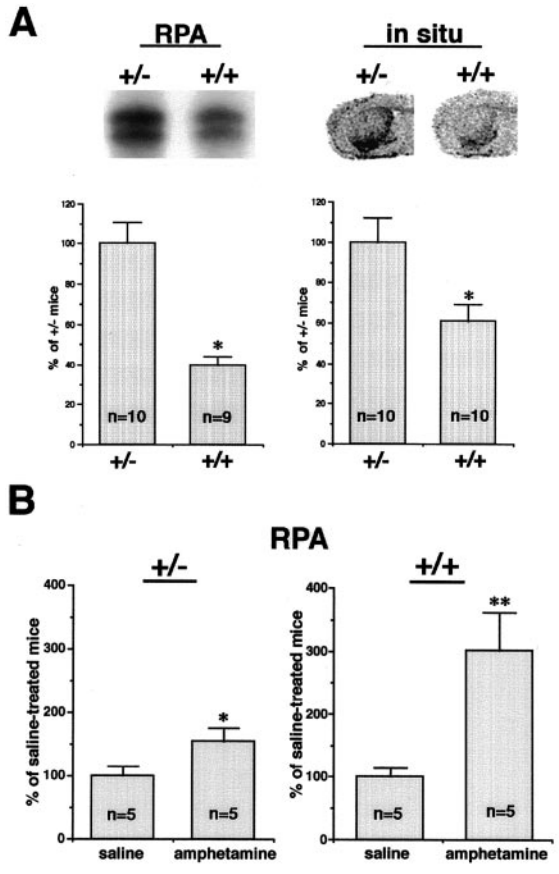

Fig. 8. Regulation of dynorphin expression in bitransgenic mice expressing CREB since birth. Both monotransgenic (NSE-tTA+/TetOp-CREB−) (+/−) and bitransgenic ((NSE-tTA +/TetOp-CREB+) (+/+) mice were raised in the absence of doxycycline from the time of birth. A, the expression of dynorphin mRNA in striatum was examined by RPA and in situ hybridization. A representative autoradiogram of the RPA shows two major protected dynorphin fragments of approximately 150 bp. In situ hybridization analysis demonstrates high levels of dynorphin mRNA in striatum. Levels of dynorphin expression in +/+ mice are reduced by 60 and 40% when determined by RPA and in situ hybridization, respectively, compared with the control +/− mice. Results are expressed as mean percentage of control +/− mice ± S.E.M. (n = 9 or 10 as indicated). *, $P <$ 0.01, compared with the density in +/− mice (Student's t test). B, regulation of dynorphin by a high dose of amphetamine. Amphetamine (15 mg/kg, i.p.) or saline was administered and animals were killed 3 h later. Amphetamine administration increased dynorphin expression in both lines of mice but the effect was more prominent in the CREB bitransgenic animals. Results are expressed as mean percentage of control +/− mice ± S.E.M. (n = 5). *, $P < 0.1$; **, $P < 0.05$, compared with the level in saline-treated mice (Student's t test).

in adjacent sections by immunohistochemistry. Amphetamine-induction of pCREB was significantly elevated in the CREB overexpressing mice relative to the monotransgenic control mice expressing endogenous levels of CREB (Fig. 9, E and F). This demonstrates that increased expression of CREB results in increased levels of pCREB and increased expression of dynorphin. There was also a tendency for levels of pCREB to be increased in the vehicle treated CREB overexpressing mice (data not shown).

Regulation of Cocaine-Induced Locomotor Activity in CREB Bitransgenic Mice. Because of the high level of CREB overexpression in the dorsal striatum and nucleus accumbens and the regulation of dynorphin expression by cocaine, we examined cocaine-induced locomotor activity in the CREB bitransgenic mice. The responses to a single dose of cocaine, as well as to repeated administration of cocaine, which is known to produce locomotor activation, were examined. For these studies, bitransgenic mice maintained on doxycycline in the drinking water (CREB off) were compared with bitransgenic littermates withdrawn from doxycycline at weaning (CREB on). This paradigm was chosen for the behavioral studies because all animals have exactly the same genotype. First, locomotor activity after administration of saline was determined on 5 consecutive days. This allows for analysis of baseline locomotor activity and provides a period of time when the animals can habituate to the test chambers. There was no significant difference between the CREB-on and CREB-off bitransgenic mice when they were first exposed to the chamber (day 1) or upon subsequent exposures (Fig. 10). Administration of cocaine (10 mg/kg) increased locomotor activity on day 1 and this effect was similar in both the CREB-on and -off groups. Repeated cocaine administration on subsequent days resulted in a greater increase in locomotor activity as expected in both groups. However, this effect was significantly lower in the CREB-on group relative to the CREB-off animals (Fig. 10). These findings are consistent with previous reports demonstrating that viral-mediated CREB expression in the nucleus accumbens decreases behavioral responses to cocaine (Carlezon et al., 1998).

Discussion

Characterization of NSE-tTA × TetOp-CREB Bitransgenic Mice. In the present study, we characterized three lines of NSE-tTA × TetOp-CREB bitransgenic mice (CREB6-A, CREB6-B, and CREB3-A lines). In all three lines, CREB was overexpressed in dorsal striatum and nucleus accumbens (Table 1). In the CREB6-A line, CREB overexpression was also observed in deep layers of cortex, whereas in the CREB3-A line, CREB was overexpressed, albeit sparsely, in hippocampal CA1 pyramidal and dentate gyrus granule cell layers (Figs. 2 and 3, Table 1). In the CREB6-B line, immunoblotting also demonstrated overexpression of CREB in striatum and in cerebellum (Fig. 4A). Analysis of CREB mRNA and protein revealed that levels of expression in dorsal striatum and nucleus accumbens are more prominent in CREB6-B than in CREB6-A mice. These patterns of CREB expression in A and B NSE-tTA lines are similar to those seen in TetOp-luciferase reporter mice crossed with these lines (Chen et al., 1998). This indicates that the expression pattern of the TetOp-driven target gene is determined largely by the expression pattern of NSE-tTA. The results also demonstrate that CREB overexpression can be turned off and on by addition or removal, respectively, of doxycycline, an analog of tetracycline. It is notable that a relatively low dose of doxycycline (50 μg/ml in water) can inhibit CREB overexpression. The low dose used could also explain the relatively short washout period required for CREB expression after the removal of doxycycline. These findings demonstrate that the tTA-TetOP system functions correctly in the CREB overexpressing bitransgenic mice.

High-power magnification of brain sections from bitransgenic mice demonstrates that CREB is localized to the cell nucleus, as would be expected for this transcription factor (Fig. 2 and 3). In addition, activation of the cAMP pathway results in increased CREB phosphorylation; this effect is greater in the CREB bitransgenic mice (Fig. 4B). These find-

ings demonstrate that CREB is localized to the appropriate cellular compartment and that it can be regulated by activation of the cAMP cascade. However, it is important to point out that overexpression of transgenic CREB may not result in up-regulation of CRE-mediated gene expression, because endogenous CREB is already expressed in most cells. The functional outcomes of CREB overexpression must be examined by analysis of target genes that are regulated by CREB, as well as by analysis of the behavioral phenotype of the bitransgenic CREB mice. In addition, it will be important to develop additional lines of mice to further examine the function of endogenous CREB. This could include transgenic lines that overexpress a form of CREB that is an active or a dominant negative form of CREB. With regard to the latter, we have made a bitransgenic line that expresses a dominant negative phosphorylation mutant and we are currently characterizing the neurochemical and behavioral phenotype of these animals.

Regulation of CREB/ATF-Like Transcription Factors in CREB Bitransgenic Mice. Within the CREB/ATF family of transcription factors, CREB, CREM, and ATF1 are known to heterodimerize with one another in vitro (Hai and Curran, 1991). In addition, these transcription factors are regulated by the cAMP pathway and share sequence homology (Brindle and Montominy, 1992; Lee and Masson, 1993). Based on homology and sequence conservation, the *CREB* and *CREM* genes are thought to have been formed by duplication of an ancestral gene (Ruppert et al., 1992). Previous reports have revealed that the CREBβ isoform and CREM are up-regulated in mice that have a null mutation of CREBα and -Δ isoforms (Hummler et al., 1994; Blendy et al., 1996). These results indicate that null mutation of CREBα/CREBΔ results in compensatory expression of other members of this transcription factor family. Here we studied what the compensatory responses of CREB-related proteins (CREB, CREM, ICER, and ATF1 isoforms) might be in response to overexpression of CREBα in specific brain regions, which has not yet been examined. The results demonstrate that CREBΔ and CREBβ isoforms are up-regulated in CREBα-overexpressing mice. Levels of ATF1 and ICER were not altered significantly in the bitransgenic mice.

The up-regulation of CREBΔ and CREBβ is consistent with previous reports that activation of the cAMP pathway in most cell types results in up-regulation of *CREB* gene expres-

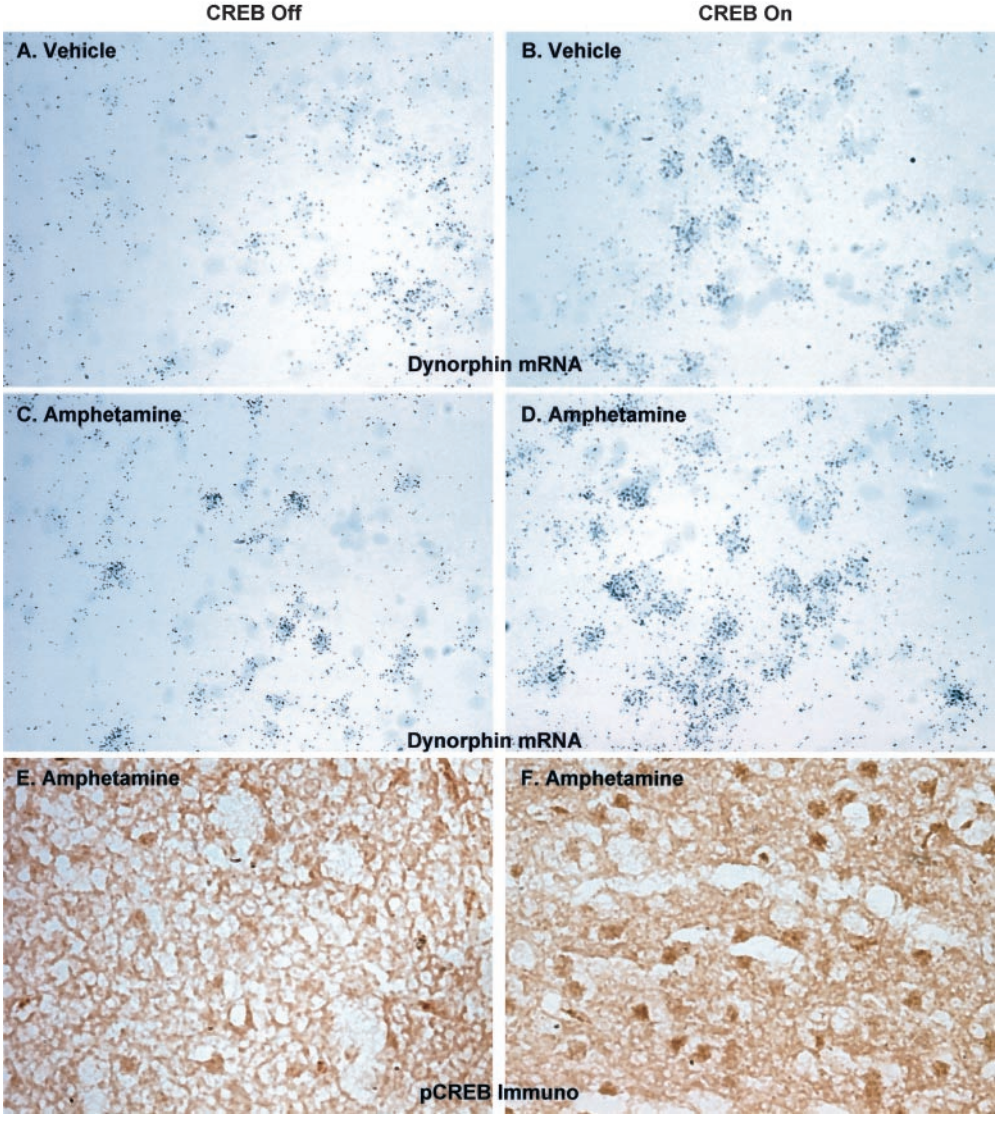

Fig. 9. Regulation of dynorphin expression in bitransgenic mice expressing CREB after weaning. Both monotransgenic (NSE-tTA+/TetOp-CREB−) (CREB Off) and bitransgenic (NSE-tTA+/TetOp-CREB+) (CREB On) mice were raised on doxycycline until weaning. Six weeks later, the mice were treated with vehicle (A, B) or amphetamine (15 mg/kg) (C-F) as indicated. A to D, dynorphin mRNA was determined by in situ hybridization. The sections were then dipped in emulsion and counterstained with cresyl violet. E and F, levels of pCREB were determined by immunohistochemistry using a phosphospecific antibody. Four separate animals were examined in duplicate for each condition and representative figures are shown for each.

sion (Meyer et al., 1993; Walker et al., 1995; Coven et al., 1998). Sequence analysis demonstrates that the *CREB* promoter contains three CRE elements that mediate the up-regulation of *CREB* gene expression in response to stimulation of the cAMP system (Coven et al., 1998). A similar mechanism could underlie the up-regulation of CREBΔ and CREBβ observed in CREB-overexpressing mice. These findings seem to contradict the up-regulation of CREBβ in the CREBα/Δ null mutant mice (Blendy et al., 1996). However, increased CREBβ expression in the latter study was reported to result from alternative splicing and increased mRNA stability, not increased *CREB* gene expression.

The mechanisms underlying the down-regulation of CREM (CREMα, β, and τ isoforms) in CREB-overexpressing bitransgenic mice seem to involve another mechanism. There are no CRE elements in the promoter of the CREM gene that control the expression of these isoforms (Molina et al., 1993). However, previous studies have reported that CREMτ expression during spermatogenesis is controlled by post-transcriptional mechanisms, including alternative splicing and increased mRNA stability (Foulkes et al., 1993). It is possible that down-regulation of the CREM isoforms observed in the present study results from similar mechanisms. There is a CRE in an intronic promoter in the *CREM* gene that controls the induction of ICER (Molina et al., 1993). ICER contains only the DNA binding domain and acts as a repressor of CRE-mediated gene expression. It is surprising that expression of ICER is not up-regulated in the CREB-overexpressing mice. However, ICER has been shown to rapidly counter-regulate its own induction (Molina et al., 1993), and it is possible that a similar mechanism occurs in the CREB-overexpressing mice. The induction of ICER in response to amphetamine, although not different between the single and bitransgenic CREB mice, demonstrates the relatively normal responsiveness of this inducible isoform in the transgenic animals.

Regulation of Dynorphin in CREB Bitransgenic Mice. In bitransgenic mice in which CREB is overexpressed throughout development, we found that dynorphin expression was reduced relative to the littermate monotransgenic control mice. In contrast, in bitransgenic mice in which CREB overexpression is turned on only after weaning, there is an increase in basal dynorphin expression and the induction of dynorphin by amphetamine is increased relative to littermate control mice expressing normal levels of CREB. The repression of basal dynorphin expression could represent a compensatory response to long-term overexpression of CREB. For example, overexpression of CREB in cultured cells is reported to decrease dynorphin promoter activity (Collins-Hicok et al., 1994). One possible explanation is that in the absence of phosphorylation, the overexpressed CREB could compete with endogenous, phosphorylated CREB for the CRE sites and could thereby act as a repressor of dynorphin gene expression. CREB could also compete for other activator proteins (e.g., ATF-2, AP-1) and thereby repress dynorphin expression. Yet another possibility is that overexpressed CREB could interact with other regulatory elements in the prodynorphin gene, such as a downstream regulatory element (DRE) and its binding protein (DRE-antagonist modulator), which represses basal prodynorphin transcription (Carrion et al., 1998, 1999).

Activation of the cAMP pathways is reported to increase dynorphin promoter activity in cultured cells (Collins-Hicok et al., 1994). Studies conducted in primary striatal cultures confirm the induction of dynorphin gene expression by activation of the cAMP pathway and demonstrate that this effect is mediated by three CRE elements in the prodynorphin promoter (Cole et al., 1995). Administration of amphetamine or cocaine is reported to increase the expression of dynorphin in the striatum and nucleus accumbens (Hurd et al., 1992; Daunais et al., 1993; Spangler et al., 1993; Cole et al., 1995; Carlezon et al., 1998). In this study, we demonstrate that administration of amphetamine increases dynorphin expression and that this effect is greater in the CREB-overexpressing mice. Moreover, the induction of dynorphin expression was observed in an area of the nucleus accumbens in which the expression of CREB, as well as pCREB, is greatest in the CREB bitransgenic mice. Taken together, the results indi-

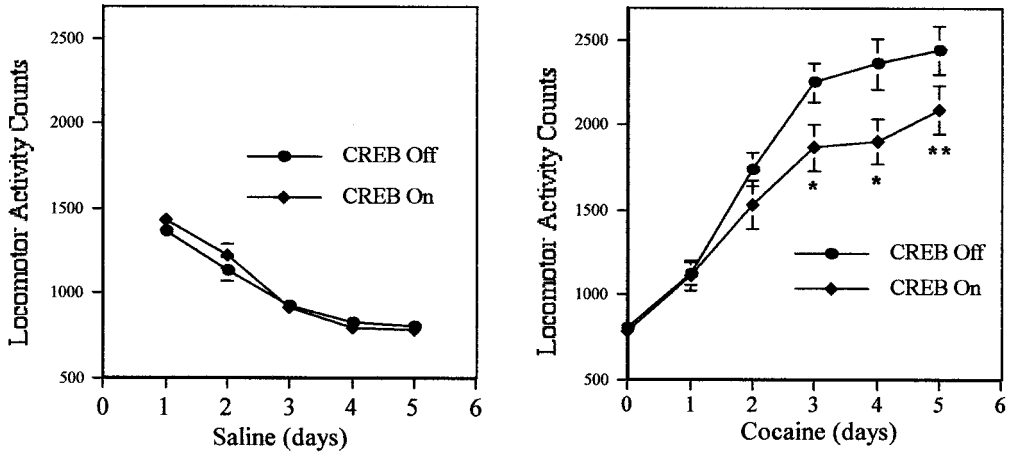

Fig. 10. Analysis of cocaine-induced locomotor sensitization in CREB bitransgenic mice. CREB bitransgenic mice were maintained either in the presence (CREB Off) or absence (CREB On) of doxycycline in the drinking water. The mice were then tested for baseline locomotor activity for 5 days, which also allowed the animals to become habituated to the activity chambers. There was no significant difference between the CREB-on and -off groups upon either the initial or subsequent exposures to the activity chamber. Animals were then administered cocaine (10 mg/kg, i.p.) each day for 5 days. The initial response to cocaine was identical in both the CREB-on and -off groups. However, the enhanced response to cocaine on each consecutive day was lower in the CREB-on group relative to the CREB-off group. The results are expressed as mean activity counts ± S.E.M. (n = 25 per group). *, $P < 0.05$ compared with CREB off (analysis of variance and Fisher's post hoc test. **, $P < 0.07$.

cate that overexpression of CREB in bitransgenic mice increases psychostimulant-induced expression of dynorphin. Moreover, the repression of basal levels of dynorphin expression after long-term expression of CREB, and then psychostimulant induction is consistent with studies of the dynorphin promoter in vitro (Collins-Hicok et al., 1994).

Cocaine-Induced Locomotor Activity Is Decreased in CREB-Overexpressing Mice. The dorsal striatum and nucleus accumbens are targets of the mesolimbic dopamine system and are known to play a prominent role in mediating behavioral responses to drugs of abuse (Nestler and Aghajanian, 1997). Moreover, adaptations of CREB and its target genes, such as dynorphin, in these regions have been implicated in the chronic actions of cocaine and other drugs of abuse (Guitart et al., 1992; Hurd et al., 1992; Spangler et al., 1993; Widnell et al., 1994; Cole et al., 1995; Nestler and Aghajanian, 1997; Carlezon et al., 1998). The results of the present study are consistent with these reports and demonstrate that behavioral responses to cocaine are altered in CREB bitransgenic mice. We found that repeated cocaine treatment for three days or longer caused significantly less locomotor activation in bitransgenic mice relative to monotransgenic littermate control mice. In contrast, there was no difference in the response to the first day of cocaine administration, or in response to saline administration over 5 days of exposure to the test chambers. These finding indicate that CREB overexpression in bitransgenic mice alters the response to repeated cocaine administration, but not to acute cocaine or basal levels of locomotor activity.

The mechanism underlying the down-regulation of cocaine-induced locomotor activation in the CREB bitransgenic mice could involve expression of dynorphin. Dynorphin acts at κ-opioid receptors on dopaminergic terminals in striatum to decrease dopamine release and thereby serve as a feedback inhibitor of dopamine transmission (see Carlezon et al., 1998). Repeated cocaine administration results in a greater induction of dynorphin, which could lead to a greater feedback inhibition of dopamine transmission. Thus, the reduced locomotor response could result from a greater up-regulation of dynorphin in response to repeated cocaine treatment in the CREB bitransgenic mice. This hypothesis must be further tested by analysis of dynorphin peptide expression in the CREB bitransgenic mice. The reason for the delay in the response is not clear, but it may be related to the requirement for up-regulation of other components of the cAMP cascade (e.g., adenylyl cyclase or cAMP-dependent protein kinase) that are necessary for full activation of the overexpressed CREB.

Conclusions. The results outlined in this article represent the initial characterization of inducible CREB overexpressing mice, and additional studies will be needed to further characterize the biochemical and behavioral phenotype of these animals. Studies are currently underway to identify other gene targets regulated by CREB, including glutamate and dopamine receptor subtypes, other neuropeptides, and neurotrophic factors. The behavioral phenotype of these mice will be further characterized in other models of drug abuse (e.g., place preference and self-administration), as well as in behavioral models of depression and anxiety. The results presented here indicate that the CREB over-expressing bitransgenic mice will be beneficial and unique tools to investigate the mechanisms underlying neural plasticity in response to a variety of behavioral and pharmacological stimuli.

Acknowledgments

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References

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

Brindle PK and Montominy MR (1992) The CREB family of transcription activators. *Curr Opin Genet Dev* **2:**199–204.

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

Carrion AM, Link WA, Ledo F, Mellstrom B, and Naranjo JR (1999) DREAM is a Ca^{2+}-regulated transcriptional repressor. *Nature (Lond)* **398:**80–84.

Carrion AM, Mellstrom B, and Naranjo JR (1998) Protein kinase A-dependent derepression of human prodynorphin gene via differential binding to an intragenic silencer element. *Mol Cell Biol* **18:**6921–6929.

Chen J, Kelz MB, Zeng G, Sakai N, Steffen C, Shockett PE, Picciotto MR, Duman RS, and Nestler EJ (1998) Transgenic animals with inducible, targeted gene expression in brain. *Mol Pharmacol* **54:**495–503.

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

Collins-Hicok J, Lin L, Spiro C, Laybourn PJ, Tschumper R, Rapacz B, and McMurray CT (1994) Induction of the rat prodynorphin gene through Gs-coupled receptors may involve phosphorylation-dependent derepression and activation. *Mol Cell Biol* **14:**2837–2848.

Coven E, Ni Y, Widnell KL, Chen J, Walker WH, Habener JF, and Nestler EJ (1998) Cell type-specific regulation of CREB gene expression: mutational analysis of CREB promoter activity. *J Neurochem* **71:**1865–1874.

Dash PK, Karl KA, Colicos MA, Prywes R, and Kandel ER (1991) cAMP response element-binding protein is activated by Ca^{2+}/calmodulin- as well as cAMP-dependent protein kinase. *Proc Natl Acad Sci USA* **88:**5061–5065.

Daunais JB, Roberts DC, and McGinty JF (1993) Cocaine self-administration increases preprodynorphin, but not c-fos, mRNA in rat striatum. *Neuroreport* **4:**543–546.

Duman RS, Heninger GR, and Nestler EJ (1997) A molecular and cellular theory of depression. *Arch Gen Psychiatry* **54:**597–606.

Duman RS, Malberg J, Nakagawa S, and D'Sa C (2000) Neuronal plasticity and survival in mood disorders. *Biol Psychiatry* **48:**732–739.

Fitzgerald LR, Vaidya VA, Terwillinger RZ, and Duman RS (1996a) Electroconvulsive seizure increase the expression of CREM (cyclic AMP response element modulator) and ICER (inducible cyclic AMP early repressor) in rat brain. *J Neurochem* **66:**429–432.

Foulkes NS, Mellstrom B, Benusiglio E, and Sassone-Corsi P (1992) Developmental switch of CREM function during spermatogenesis: from antagonist to activator. *Nature (Lond)* **355:**80–84.

Foulkes NS, Schlotter F, Pevet P, and Sassone-Corsi P (1993) Pituitary hormone FSH directs the CREM functional switch during spermatogenesis. *Nature (Lond)* **362:**264–267.

Ginty DD, Kornhauser JM, Thompson MA, Bading H, Mayo KE, Takahashi JS, and Greenberg ME (1993) Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. *Science (Wash DC)* **260:**238–241.

Gonzalez GA and Montminy MR (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59:**675–680.

Guitart X, Thompson MA, Mirante CK, Greenburg ME, and Nestler EJ (1992) Regulation of CREB phosphorylation by acute and chronic morphine in the rat locus coeruleus. *J Neurochem* **58:**1168–1171.

Hai T and Curran T (1991) Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc Natl Acad Sci* **88:**3720–3724.

Hai T, Liu F, Coukus WJ, and Green MR (1989) Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Gene & Development* **3:**2083–2090.

Hiroi N, Brown JR, Haile CN, Ye H, Greenberg ME, and Nestler EJ (1997) FosB mutant mice: Loss of chronic cocaine induction of Fos-related proteins and heightened sensitivity to cocaine's psychomotor and rewarding effects. *Proc Natl Acad Sci* **94:**10397–10402.

Hiroi N and Graybiel AM (1996) Atypical and typical neuroleptic treatments induce distinct programs of transcription factor expression in the striatum. *J Comp Neurol* **374:**70–83.

Hoeffler JP, Meyer TE, Yun Y, Jameson JL, and Habener JF (1988) Cyclic AMP-responsive DNA-binding proteins: structure based on a cloned placental cDNA. *Science (Wash DC)* **242:**1430–1433.

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

Hurd YL, Brown EE, Finlay JM, Fibiger HC, and Gerfen CR (1992) Cocaine self-administration differentially alters mRNA expression of striatal peptides. *Mol Brain Res* **13:**165–170.

Hyman SE (1996) Addiction to cocaine and amphetamine. *Neuron* **16:**901–904.

Kelz MB, Chen J, Carlezon WA, Whisler K, Gilden L, Beckmann AM, Steffen C,

Zhang YJ, Marotti L, Self DW, et al. (1999) Expression of transcription factor ΔFosB in the brain controls sensitivity to cocaine. *Nature (Lond)* **401:**272–276.
Konradi C, Cole LR, Heckers S, and Hyman SE (1994) Amphetamine regulate gene expression in rat striatum via transcription factor CREB. *J Neurosci* **14:**5623–5634.
Lane-Ladd SB, Pineda J, Boundy VA, Pfeuffer T, Krupinski J, Aghajanian GK, and Nestler EJ (1997) CREB (cAMP response element-binding protein) in the locus coeruleus: biochemical, physiological, and behavioral evidence for a role in opiate dependence. *J Neurosci* **17:**7890–7901.
Lee KA and Masson N (1993) Transcriptional regulation by CREB and its relatives. *Biochim Biophys Acta* **1174:**221–233.
Lee M-R, Chung C-H, Liou M-L, Wu M, Li W-F, Hsueh Y-P, and Lai M-Z (1992) Isolation and characterization of nuclear proteins that bind to T cell receptor Vb decamer motif. *J Immuno* **148:**1906–1912.
Maekawa T, Sakura H, Kanei-Ishii C, Sudo T, Yoshimura T, Fujisawa J, Yoshida M, and Ishii S (1989) Leucine zipper structure of the protein CRE-BP1 binding to the cyclic AMP response element in brain. *EMBO (Eur Mol Biol Organ)J* **8:**2023–2028.
Maldonado R, Blendy JA, Tzavara E, Gass P, Roques BP, Hanoune J, and Schutz G (1996) Reduction of morphine abstinence in mice with a mutation in the gene encoding CREB. *Science (Wash DC)* **273:**657–659.
Matthews RP, Guthrie CR, Wailes LM, X. Z, Means AR, McKnight GS (1994) Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. *Mol Cell Biol* **14:**6107–6116.
Meyer TE, Waeber G, Lin J, Beckmann W, and Habener JF (1993) The promoter of the gene encoding 3′ 5′-cyclic adenosine monophosphate (cAMP) response element binding protein contains cAMP response elements: evidence for positive autoregulation of gene transcription. *Endocrinol* **132:**770–780.
Milner B, Squire LR, and Kandel ER (1998) Cognitive neuroscience and the study of memory. *Neuron* **20:**445–468.
Molina CA, Foulkes NS, Lalli E, and Sassone-Corsi P (1993) Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor. *Cell* **75:**875–886.
Nestler EJ (1997) Molecular mechanism of opiate and cocaine addiction. *Curr Opin Neurobiol* **7:**713–719.
Nestler EJ and Aghajanian GK (1997) Molecular and cellular basis of addiction. *Science (Wash DC)* **278:**58–63.
Nibuya M, Nestler EJ, and Duman RS (1996) Chronic antidepressant administration increase the expression of cAMP response element binding protein (CREB) in rat hippocampus. *J Neurosci* **16:**2365–2372.
Obrietan K, Impey S, and Storm DR (1998) Light and circadian rhythmicity regulate MAP kinase activation in the suprachiasmatic nuclei. *Nat Neurosci* **1:**693–700.
Ruppert S, Cole TJ, Boshart M, Schmid E, and Schutz G (1992) Multiple mRNA isoforms of the transcription activator protein CREB: generation by alternative splicing and specific expression in primary spermatocytes. *EMBO (Eur Mol Biol Organ)J* **11:**1503–1512.
Spangler R, Unterwald EM, and Kreek MJ (1993) 'Binge' cocaine administration induces a sustained increase of prodynorphin mRNA in rat caudate-putamen. *Brain Res Mol Brain Res* **19:**323–327.
Takahashi M, Terwillinger R, Lane C, Mezes PS, Conti M, and Duman RS (1999) Chronic antidepressant administration increases the expression of cAMP-specific phosphodiesterase 4A and 4B isoforms. *J Neurosci* **19:**610–618.
Terwilliger RZ, Beitner JD, Sevarino KA, Crain SM, and Nestler EJ (1991) A general role for adaptation in G-protein and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal junction. *Brain Res* **548:**100–110.
Thome J, Impey S, Storm D, and Duman RS (2000) cAMP-response element-mediated gene transcription is upregulated by chronic antidepressant treatment. *J Neurosci* **20:**4030-4036.
Turgeon SM, Pollack AE, and 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, and Kreek MJ (1996) Chronic repeated cocaine administration increase dopamine D1 receptor-mediated signal transduction. *Eur J Pharmacol* **318:**31–35.
Walker WH, Fucci L, and Habener JF (1995) Expression of the gene encoding transcription factor cyclic adenosine 3′,5′ monophosphate (cAMP) response element-binding protein (CREB): regulation by follicle-stimulating hormone-induced cAMP signaling in primary rat Sertoli cells. *Endocrinology* **136:**3534–3545.
Widnell KL, Russell D, and Nestler EJ (1994) Regulation of cAMP response element binding protein in the locus coeruleus in vivo and in a locus coeruleus-like (CATH.a) cell line in vitro. *Proc Natl Acad Sci* **91:**10947–10951.
Widnell KL, Self DW, Lane SB, Russell DS, Vaidya V, Miserendino MJD, Rubin CS, Duman RS, and Nestler EJ (1996) Regulation of CREB expression: in vivo evidence for functional role in morphine action in the nucleus accumbens. *J Pharmacol Exp Ther* **276:**306–315.
Yamamoto KK, Gonzalez GA, Biggs WHI, and Montminy MR (1988) Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. *Nature (Lond)* **334:**494–498.
Ziff EB (1990) Transcription factors: a new family gathers at the cAMP response site. *Trends Genet* **6:**69–72.

Address correspondence to: Ronald S. Duman, Ph.D., Abraham Ribicoff Research Facilities, Yale University School of Medicine, 34 Park Street, New Haven, CT 06508. E-mail: ronald.duman@yale.edu