Chronic Antidepressant Administration Increases the Expression of cAMP Response Element Binding Protein (CREB) in Rat Hippocampus

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The present study demonstrates that chronic, but not acute, administration of several different classes of antidepressants, including serotonin- and norepinephrine-selective reuptake inhibitors, increases the expression of cAMP response element binding protein (CREB) mRNA in rat hippocampus. In contrast, chronic administration of several nonantidepressant psychotropic drugs did not influence expression of CREB mRNA, demonstrating the pharmacological specificity of this effect. *In situ* hybridization analysis demonstrates that antidepressant administration increases expression of CREB mRNA in CA1 and CA3 pyramidal and dentate gyrus granule cell layers of the hippocampus. In addition, levels of CRE immunoreactivity and of CRE binding activity were increased by chronic antidepressant administration, which indicates that expression and function of CREB protein are increased along with its mRNA. Chronic administration of the phosphodiesterase (PDE) inhibitors rolipran or papaverine also increased expression of CREB mRNA in hippocampus, demonstrating a role for the cAMP cascade. Moreover, coadministration of rolipram with imipramine resulted in a more rapid induction of CREB than with either treatment alone. Increased expression and function of CREB suggest that specific target genes may be regulated by these treatments. We have found that levels of brain-derived neurotrophic factor (BDNF) and trkB mRNA are also increased by administration of antidepressants or PDE inhibitors. These findings indicate that upregulation of CREB is a common action of chronic antidepressant treatments that may lead to regulation of specific target genes, such as BDNF and trkB, and to the long-term effects of these treatments on brain function.

Key words: cAMP; desipramine; imipramine; fluoxetine; sertraline; ECS; tranylcypromine; rolipram; brain-derived neurotrophic factor

Preclinical and clinical studies support the hypothesis that the brain pathways that contain serotonin (5-HT) and norepinephrine (NE) play a role in the therapeutic action of antidepressant treatments, and possibly in the manifestation of certain symptoms of depression (Heninger and Charney, 1987; Maes and Meltzer, 1995; Schatzberg and Schildkraut, 1995). However, these studies have failed to identify a common action of antidepressants at the level of these neurotransmitters or their receptors. This is not surprising given that the different classes of antidepressants currently available, including selective and nonselective 5-HT and NE reuptake blockers, have different effects on levels of these two monoamines. Moreover, elevation of 5-HT and NE levels in response to acute drug exposure is not consistent with the time course for the therapeutic action of antidepressant treatments (i.e., several weeks), suggesting that their mechanism of action involves neuronal adaptations to these acute effects (Heninger and Charney, 1987; Sulser, 1989; Hudson et al., 1993; Duman et al., 1994).

Another explanation to account for the action of different types of antidepressants is that there is a common, intracellular adaptation beyond the levels of 5-HT and NE and their receptors (Sulser, 1989; Hudson et al., 1993; Duman et al., 1994). In this

case, regulation of monoamine levels and receptors may represent initial effects that subsequently lead to regulation of postreceptor pathways and genes that ultimately mediate the therapeutic action of antidepressants. One candidate postreceptor target that could be regulated by both 5-HT and NE and thereby mediate long-term adaptations in response to diverse antidepressant treatments is the nuclear transcription factor cAMP response element binding protein (CREB). The function of CREB is increased by activation of the cAMP cascade and phosphorylation of CREB by cAMP-dependent protein kinase (PKA) (Meyer and Haebner, 1993; Ghosh and Greenberg, 1995). In this way, CREB could be activated by 5-HT and NE receptors that directly stimulate cAMP production (e.g., $5\text{-HT}_{4,6,7}$ or β-adrenergic receptors). In addition, the function of CREB may be regulated by other 5-HT or NE receptors (e.g., $5\text{-HT}_{2A,C}$ and α_1-adrenergic receptors) that lead to stimulation of Ca^{2+}-dependent protein kinases (Meyer and Haebner, 1993; Ghosh and Greenburg, 1995).

A role for the cAMP cascade in the long-term actions of antidepressant treatments is supported by previous studies demonstrating that chronic antidepressant treatments regulate levels of adenylyl cyclase and PKA in the brain (Menkes et al., 1983; Nestler et al., 1989; Perez et al., 1989; Ozawa and Rasenick, 1991). To examine a role for CREB, the influence of chronic administration of several different classes of antidepressants on expression of CREB in hippocampus was examined. Hippocampus has been implicated in both the treatment and pathophysiology of depression and other stress-related psychiatric disorders (Heninger and Charney, 1987; Jacobson and Sapolsky, 1991; Young et al., 1991; Bremner et al., 1995; Duman et al., 1995). The results demonstrate that chronic administration of

several different types of antidepressants, but not nonantidepressant psychotropic drugs, increases the expression of CREB in rat hippocampus.

Increased expression of CREB would be expected to alter the expression of specific target genes. Among the many potential target genes regulated by CREB are those for brain-derived neurotrophic factor (BDNF) and its receptor, trkB (Condorelli et al., 1994; Ghosh et al., 1994; Duman et al., 1995). We have reported that chronic antidepressant administration increases levels of BDNF and trkB in rat hippocampus (Nibuya et al., 1995). Moreover, several lines of behavioral and neurochemical evidence support a role for BDNF in the treatment, and possibly even the pathophysiology, of depression (Suiciak et al., 1994a; Duman et al., 1995; Nibuya et al., 1995; Smith et al., 1995). In the present study, we demonstrate that the regional distribution and time course for induction of CREB correspond to that for BDNF and trkB. In addition, we demonstrate that sustained elevation of cAMP, via chronic administration of a phosphodiesterase (PDE) inhibitor, increases the expression of CREB, as well as BDNF and trkB, and facilitates the action of antidepressants on expression of these proteins.

MATERIALS AND METHODS

Animals and treatment paradigms. Male Sprague–Dawley rats (150–200 gm, CAMM, Wayne, NJ) were group housed and maintained on a 12 hr light/dark cycle with food and water freely available. All treatments were according to standard protocols as described previously (Nibuya et al., 1995). Rats were administered (once daily) electroconvulsive seizure (ECS) via earclip electrodes (50 mA, 0.3 sec) or received sham treatment (handled identically as those that received ECS, but without electrical stimulation). For drug treatments, groups of rats were administered tranylcypromine (7.5 mg/kg for 7 d then 10 mg/kg for 14 d), imipramine (15 mg/kg), desipramine (15 mg/kg), sertraline (10 mg/kg), fluoxetine (5 mg/kg), haloperidol (2 mg/kg), cocaine (15 mg/kg), rolipram (2 or 10 mg/kg, as indicated), papaverine (10 mg/kg), or vehicle (0.9% saline for all treatments, except rolipram, for which the vehicle was 1% dimethyl-sulfoxide, injected in a volume of 0.25 ml) once daily for 21 d via intraperitoneal injection and killed 18 hr after the last treatment. For morphine treatment, rats were administered morphine pellets (75 mg pellets, s.c.) once daily for 5 d or received the same sham treatment (sham surgery without pellet implantation).

Brains were removed and frozen immediately for *in situ* hybridization, or sections of hippocampus were dissected for RNA extraction and Northern blot analysis, or gel-shift analysis, as described below. All animal use procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Yale Animal Care and Use Committee. The drugs used for these studies were obtained from the following sources: tranylcypromine, imipramine, desipramine, papaverine, and haloperidol were purchased from Sigma (St. Louis, MO); fluoxetine was provided by Lilly (Indianapolis, IN); sertraline was provided by Pfizer (Groton, CT); rolipram was provided by Schering (Germany); and morphine and cocaine were obtained from the National Institute on Drug Abuse (Baltimore, MD).

Northern blot analysis. Total RNA was isolated from sections of hippocampus by the guanidine isothiocyanate/cesium chloride centrifugation method, and levels of CREB, BDNF, and trkB mRNA were determined by Northern blot analysis (Nibuya et al., 1995). CREB (S. E. Hyman, Harvard Medical School), BDNF (Regeneron, Tarrytown, NY), and trkB (The National Cancer Institute, Frederick, MD) cDNA clones were used to generate ^{32}P-labeled riboprobes. Briefly, 20 μg of total RNA was electrophoresed on a 1% agarose gel, and the RNA was transferred to nitrocellulose filters. The resulting filters were then incubated with the ^{32}P-labeled CREB, BDNF, or trkB riboprobes for 18 hr at 65°C and washed in 2× SSC (SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% SDS at 65°C for 30 min, and then twice in 0.2× SSC, 0.1% SDS at 65°C for 60 min. Levels of total RNA for each lane were determined by reprobing the nitrocellulose filters with a ^{32}P-labeled cyclophilin cDNA probe that was radiolabeled using a random prime kit (Stratagene, La Jolla, CA). The radiolabeled mRNA bands were visualized by autoradiography and quantified with a GSXL laser densitometer. The levels of

CREB, BDNF, or trkB mRNA were normalized to cyclophilin mRNA levels to account for differences in the amount of RNA per lane.

In situ hybridization analysis. Analysis of CREB, BDNF, and trkB mRNA by *in situ* hybridization was conducted as described previously by this laboratory (Nibuya et al., 1995). Coronal brain sections were hybridized with ^{35}S-labeled CREB, BDNF, or trkB riboprobes (10^6 cpm/section) for 18 hr at 55°C in buffer containing 50% formamide, 0.6 M NaCl, 10 mM Tris, 1× Denhardt's solution, 2 mM EDTA, 10 mM dithiothreitol, 10% dextran sulfate, 50 μg/ml salmon sperm DNA, and 250 mg/ml tRNA. The sections were washed in 2× SSC at 25°C and then treated with 20 μg/ml RNase A for 45 min in 0.5 M NaCl, 10 mM Tris, and 1 mM EDTA. The sections were then washed twice in 0.2× SSC at 55°C, 30 min per wash. The sections were dried, exposed to Hyperfilm (Amersham, Arlington Heights, IL), and subsequently counterstained with cresyl violet to allow alignment with the autoradiogram. The specificity of the hybridization was confirmed by demonstrating that ^{35}S-labeled sense CREB, BDNF, or trkB riboprobes did not yield any significant hybridization (data not shown).

Gel-shift analysis. Extracts of hippocampus from control or antidepressant-treated rats were prepared for CRE gel-shift analysis as described previously (Widnell et al., 1994). The somatostatin consensus CRE (GATCGGC**T**GACGTCAGAG) was used for gel-shift analysis. Double-stranded DNA was labeled using a fill-in reaction with [^{32}P]dGTP and [^{32}P]dTTP and Klenow DNA polymerase to a specific activity of ~8 × 10^8 cpm/μg DNA. Labeled DNA was incubated with ~20 μg of extract protein for 20 min at 20°C. The samples were then electrophoresed (150 V) for 2 hr on a nondenaturing 6% acrylamide: 0.16% *N,N'*-methylenebisacrylamide gel containing 25 mM Tris-borate buffer, pH 8.3, 1 mM EDTA, and 1.6% glycerol. The resulting gels were dried and subjected to autoradiography. Levels of CRE binding were quantified by densitometry. The specificity of the CRE binding was determined by competition with different concentrations (3–100 ng) of cold CRE, mutated CRE (GATCGGCTGACGTC**T**GAG: mutated nucleotide is in bold), or human metalliothionein AP-1 (TCGACGGGACTCAGCGCGC) oligonucleotides: only unlabeled CRE oligonucleotide effectively competed with labeled CRE binding (data not shown). For supershift analysis, 1 μl of anti-CREB (Upstate Biochemical, Lake Placid, NY) or anti-CREM (Santa Cruz Biotechnology, Santa Cruz, CA) antibody was incubated with cell extracts for 18 hr at 4°C before the addition of labeled probe.

Data analysis. Levels of CREB, BDNF, and trkB mRNA were determined by outlining the band on Northern blots or the regions of interest on *in situ* hybridization sections, which were then quantified on a Macintosh-based National Institutes of Health Image analysis program, version 1.52; an equivalent area on each Northern blot or brain section was outlined for the various samples. ^{14}C step standards were used to verify the linearity of densitometry. For *in situ* hybridization sections, the following regions were analyzed: dentate gyrus granule cell layer, CA3 and CA1 pyramidal cell layers, and piriform cortex pyramidal cell layer. For each animal, both sides of three individual brain sections were analyzed, for a total of six determinations, and the mean was determined. The results from several animals were then subjected to statistical analysis. Experiments containing groups of three rats or more (see treatment paradigms above) were subjected to ANOVA, with a significance level of <0.05, and Fisher's post-hoc test. Experiments containing two groups were subjected to Student's *t* test, with significance determined at the <0.05 level.

RESULTS

Chronic (21 d) administration of several different types of antidepressant drugs significantly increased levels of CREB mRNA in rat hippocampus (Figs. 1, 2). The antidepressant drugs tested included desipramine (a selective NE reuptake inhibitor), imipramine (a nonselective 5-HT and NE reuptake inhibitor), tranylcypromine (a monoamine oxidase inhibitor), and sertraline and fluoxetine (two 5-HT selective reuptake inhibitors). Chronic administration (10 d) of ECS, a treatment for which the relevant acute action is unknown, also increased levels of CREB mRNA in hippocampus. In contrast, chronic administration of several nonantidepressant drugs did not influence expression of CREB mRNA in this brain region, demonstrating the pharmacological specificity of this effect for antidepressant treatments (Fig. 1): the drugs tested included morphine (an opiate agonist), cocaine (a
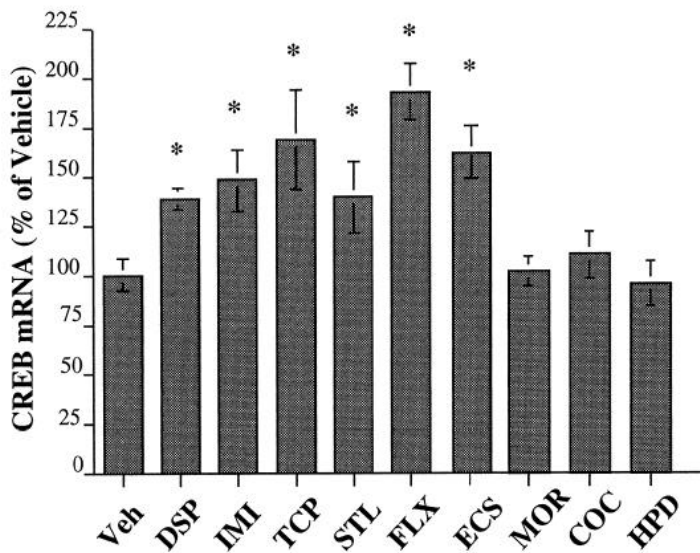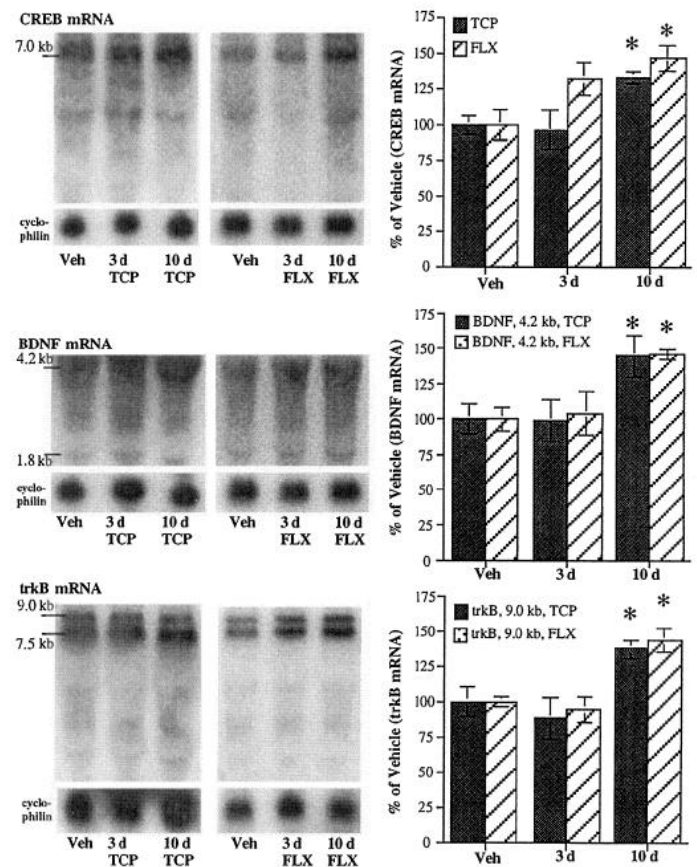
Figure 1. Chronic antidepressant treatment increases levels of CREB mRNA in hippocampus. Desipramine (*DSP*), imipramine (*IMI*), tranyl-cypromine (*TCP*), sertraline (*STL*), fluoxetine (*FLX*), electroconvulsive seizure (*ECS*), morphine (*MOR*), cocaine (*COC*), and haloperidol (*HDP*) were administered chronically, and CREB and cyclophilin mRNA levels in hippocampus were determined by Northern blot analysis. Levels of CREB mRNA were quantitated by densitometry and normalized to cyclophilin mRNA levels. The results are expressed as percent of control and are the mean ± SEM of 5–6 rats for each treatment group. *$p < 0.05$ compared with vehicle control (ANOVA and Fisher's test).

Figure 2. Time course for antidepressant regulation of CREB, BDNF, and trkB mRNA in hippocampus. Fluoxetine (*FLX*) or tranylcypromine (*TCP*) was administered for the times indicated, and levels of CREB, BDNF, and trkB mRNA were determined by Northern blot analysis. Levels of CREB mRNA were quantitated by densitometry and normalized to cyclophilin mRNA levels. Fluoxetine and tranylcypromine administration also increased levels of the 1.8 kb BDNF mRNA band (144 ± 7 and 150 ± 4% of vehicle, respectively) and the 7.5 kb trkB mRNA band (147 ± 8 and 152 ± 6% of control, respectively). Both the 4.4 and the 1.8 kb BDNF transcripts encode full-length BDNF; the 9.0 kb trkB transcript encodes a full-length trkB receptor, and the 7.5 kb trkB mRNA encodes truncated receptor lacking the tyrosine kinase domain. The results are expressed as percent of control and are the mean ± SEM of 4 (*TCP*) or 6 (*FLX*) rats. *$p < 0.05$ compared with vehicle control (ANOVA and Fisher's test).

monoamine reuptake inhibitor and psychostimulant), and halo-peridol (a dopamine D_2 receptor antagonist and antipsychotic). Chronic antidepressant administration did not influence levels of mRNA for one of the PKA catalytic subunits, PKA-Cα, demonstrating that there is not a general increase in expression of cAMP cascade proteins in response to antidepressant treatment (data not shown).

Increased expression of CREB mRNA was observed after 10 d, but not 3 d, of fluoxetine or tranylcypromine administration, demonstrating that upregulation of CREB is dependent on repeated antidepressant treatment (Fig. 2). In addition, acute administration of desipramine, imipramine, sertraline, or ECS did not significantly increase levels of CREB mRNA [102 ± 8, 115 ± 5, 110 ± 4, and 115 ± 9% of control, respectively (mean ± SEM); $n = 6$].

We have reported that chronic administration of several antidepressants (i.e., sertraline, tranylcypromine, desipramine, or ECS), but not nonantidepressants (i.e., morphine, cocaine, or haloperidol), increases the expression of BDNF and trkB mRNA in hippocampus (Nibuya et al., 1995). Moreover, expression of BDNF and trkB is reported to be increased by activation of the cAMP cascade (Condorelli et al., 1994; Ghosh et al., 1994), suggesting that the induction of CREB could contribute to increased expression of BDNF and trkB in response to chronic antidepressant treatments. This possibility is supported by the temporal properties of BDNF and trkB regulation: the time course for induction of CREB was similar to that for induction of BDNF and trkB mRNA in response to these treatments (Fig. 2).
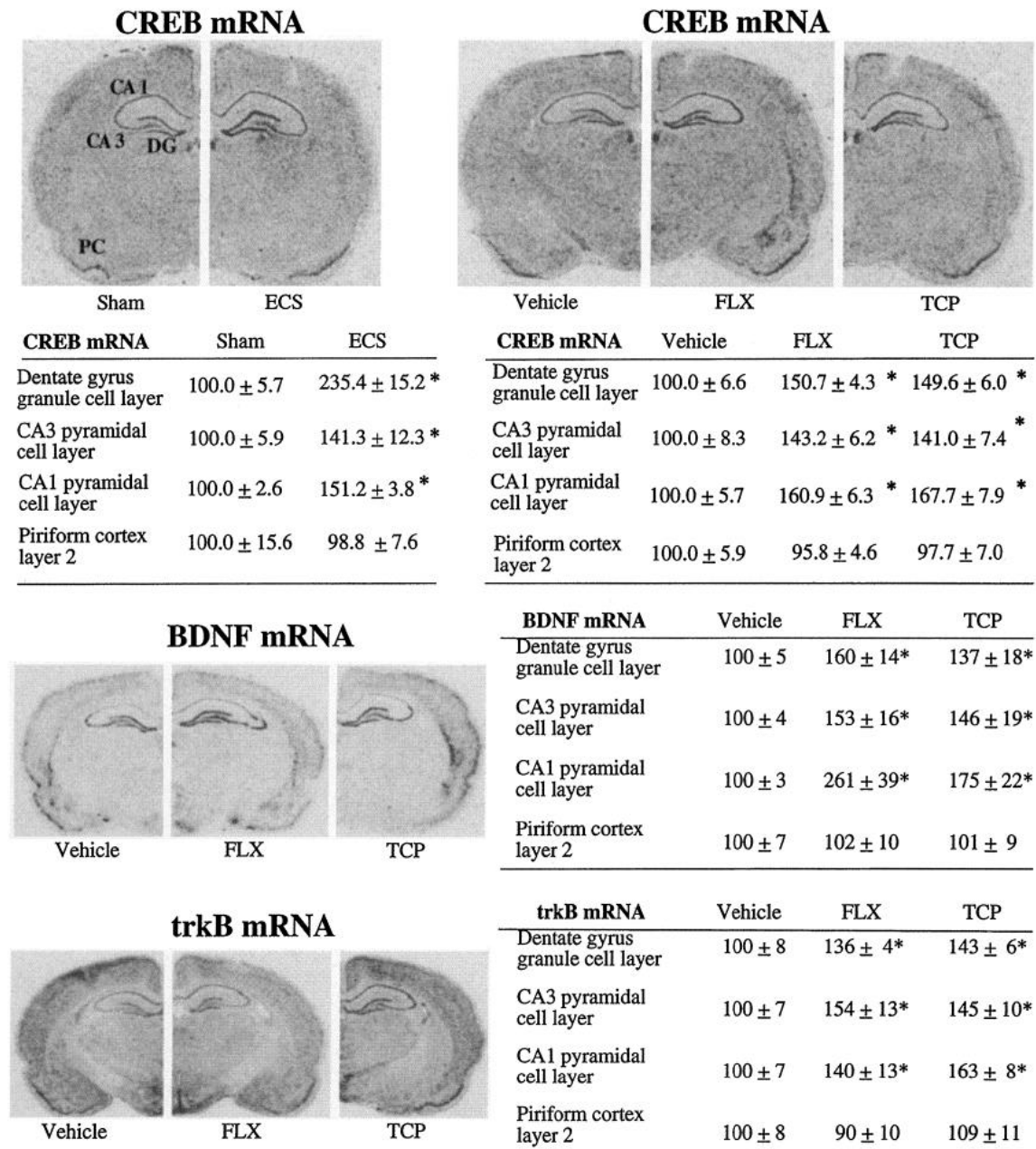
The regional distribution of CREB mRNA expression was examined further by *in situ* hybridization analysis (Fig. 3). Chronic ECS, fluoxetine, or tranylcypromine administration significantly increased levels of CREB mRNA in the dentate gyrus granule cell layer, and CA3 and CA1 pyramidal cell layers in hippocampus

(Fig. 3). We have reported previously that chronic ECS increases or prolongs the expression of BDNF and trkB mRNA in these same cell layers of hippocampus (Nibuya et al., 1995). Here we demonstrate that chronic fluoxetine or tranylcypromine administration also increases the expression of BDNF and trkB mRNA in these three hippocampal cell layers (Fig. 3). In contrast, levels of CREB, BDNF, and trkB mRNA were not significantly influenced in the pyramidal cell layer of piriform cortex (Fig. 3). These findings are consistent with the hypothesis that the induction of CREB is related to the upregulation of BDNF and trkB.

Chronic ECS or fluoxetine administration significantly increased levels of CRE binding activity in extracts of hippocampus, determined by gel-shift analysis (Fig. 4). The levels of CRE binding were 134 ± 5 and 127 ± 4% of control for fluoxetine and ECS treatments, respectively (mean ± SEM, $n = 5$, $p < 0.05$, Student's t test). In contrast, acute ECS or fluoxetine treatment did not significantly influence levels of CRE binding [102 ± 6 and 94 ± 3% of control, respectively (mean ± SEM); $n = 5$]. The

CREB mRNA

Sham   ECS

CREB mRNA	Sham	ECS
Dentate gyrus granule cell layer	100.0 ± 5.7	235.4 ± 15.2 *
CA3 pyramidal cell layer	100.0 ± 5.9	141.3 ± 12.3 *
CA1 pyramidal cell layer	100.0 ± 2.6	151.2 ± 3.8 *
Piriform cortex layer 2	100.0 ± 15.6	98.8 ± 7.6

CREB mRNA

Vehicle   FLX   TCP

CREB mRNA	Vehicle	FLX	TCP
Dentate gyrus granule cell layer	100.0 ± 6.6	150.7 ± 4.3 *	149.6 ± 6.0 *
CA3 pyramidal cell layer	100.0 ± 8.3	143.2 ± 6.2 *	141.0 ± 7.4 *
CA1 pyramidal cell layer	100.0 ± 5.7	160.9 ± 6.3 *	167.7 ± 7.9 *
Piriform cortex layer 2	100.0 ± 5.9	95.8 ± 4.6	97.7 ± 7.0

BDNF mRNA

Vehicle   FLX   TCP

BDNF mRNA	Vehicle	FLX	TCP
Dentate gyrus granule cell layer	100 ± 5	160 ± 14*	137 ± 18*
CA3 pyramidal cell layer	100 ± 4	153 ± 16*	146 ± 19*
CA1 pyramidal cell layer	100 ± 3	261 ± 39*	175 ± 22*
Piriform cortex layer 2	100 ± 7	102 ± 10	101 ± 9

trkB mRNA

Vehicle   FLX   TCP

trkB mRNA	Vehicle	FLX	TCP
Dentate gyrus granule cell layer	100 ± 8	136 ± 4*	143 ± 6*
CA3 pyramidal cell layer	100 ± 7	154 ± 13*	145 ± 10*
CA1 pyramidal cell layer	100 ± 7	140 ± 13*	163 ± 8*
Piriform cortex layer 2	100 ± 8	90 ± 10	109 ± 11

Figure 3. In situ hybridization analysis of CREB, BDNF, and trkB mRNA: regulation by antidepressant treatment. ECS (10 d), fluoxetine (21 d), or tranylcypromine (21 d) was administered, and brain sections were subjected to *in situ* hybridization analysis using ^{35}S-labeled CREB, BDNF, and trkB riboprobes. Representative autoradiograms for each of the conditions are shown. The dentate gyrus granule cell layer (*DG*), CA3 pyramidal cell layer (*CA3*), CA1 pyramidal cell layer (*CA1*), and piriform cortex (*PC*) are indicated. Levels of mRNA were quantitated by densitometry of *in situ* hybridization autoradiograms. Results are expressed as percent of control and are the mean ± SEM of 3 (*ECS*) or 6 (*FLX* and *TCP*) separate animals. *$p < 0.05$ compared with control (Student's *t* test for ECS; ANOVA and Fisher's test for TCP and FLX).

presence of CREB in the CRE binding complex was determined by gel-supershift analysis. Preincubation of hippocampal extracts with anti-CREB antibody completely disrupted the CRE band in both sham- and ECS-treated samples (Fig. 4). In contrast, preincubation with antibody directed against CREM, another transcription factor that specifically binds to the CRE, did not influence levels of the CREB/CRE complex (Fig. 4). The CREM antibody does effectively disrupt a faster migrating CRE binding complex containing CREM isoforms, demonstrating that it is active and specific (Fitzgerald et al., 1995). Competition experiments were also conducted to demonstrate the specificity of our gel-shift assay. The CRE band was completely blocked by addition

of unlabeled CRE oligonucleotide, but was not significantly influenced by a mutated CRE (which differed from the consensus CRE by a single base pair) or by an AP-1 oligonucleotide (data not shown). The influence of antidepressant administration on levels of CREB protein in hippocampus was determined by immunohistochemistry. Chronic administration of fluoxetine or ECS also increased levels of CREB immunoreactivity in the dentate gyrus granule cell layer, and CA3 and CA1 pyramidal cell layers of hippocampus. This is attributable to an increase in the level of CREB immunoreactivity per cell rather than to an increase in the number of CREB-immunoreactive cells. Upregulation of CREB immunoreactivity provides further evidence that upregulation of
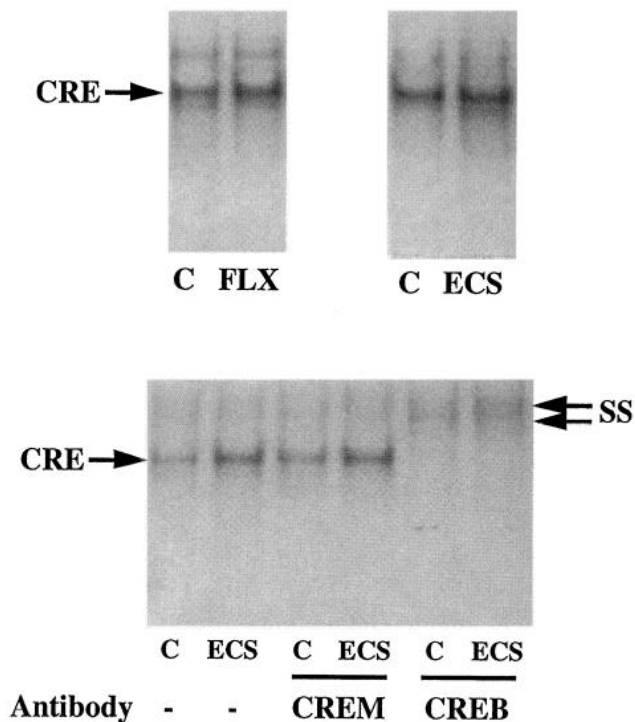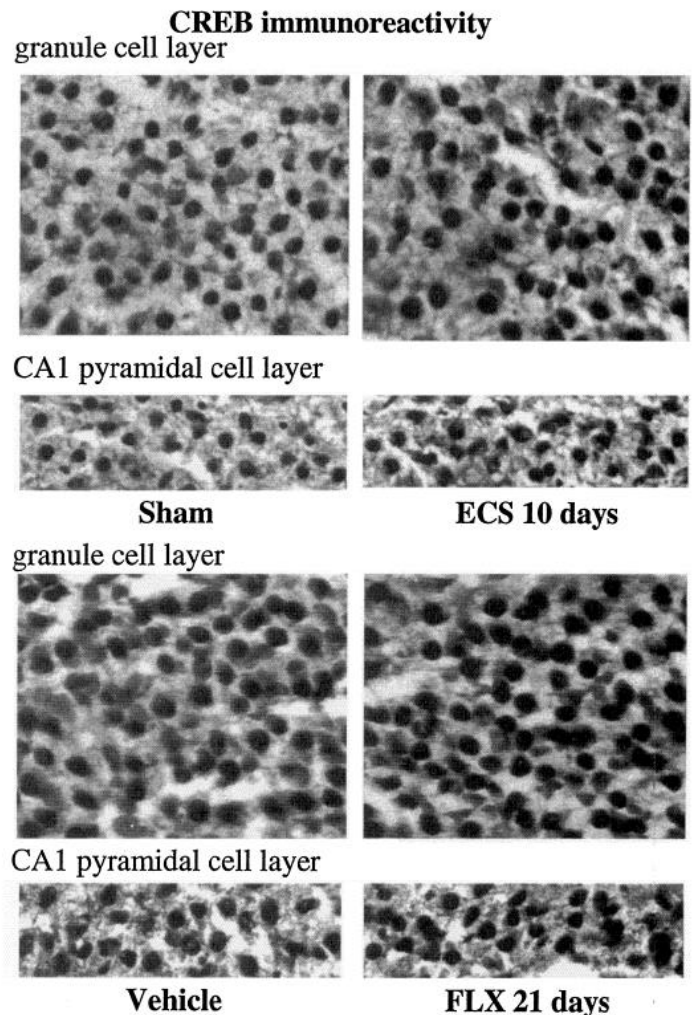
Figure 4. Chronic antidepressant administration increases levels of CRE binding activity in hippocampus. Fluoxetine (*FLX*) or *ECS* was administered chronically, and levels of CRE binding were determined by gel-shift analysis. The presence of CREB in the CRE binding complex was demonstrated by gel-supershift analysis. Preincubation of hippocampal extracts with anti-CREB, but not anti-CREM, antibody disrupted the CRE-binding complex and resulted in the formation of a supershifted band (*SS*). Representative autoradiograms are shown for each.

CREB immunoreactivity

Figure 5. Chronic antidepressant treatment increases levels of CREB immunoreactivity in hippocampus. Fluoxetine (*FLX*; 21 d) or *ECS* (10 d) was administered, and brain sections were subjected to immunohistochemical analysis using an antibody specific for CREB. Representative autoradiograms are shown; equivalent results were obtained from 4 rats examined in each treatment group.

CREB mRNA is accompanied by an increase in levels of CREB protein (Fig. 5).

To examine the role of the cAMP system in regulation of CREB expression, the influence of chronic administration of a PDE inhibitor (i.e., rolipram or papaverine) on levels of CREB mRNA was determined. Chronic administration of rolipram or papaverine significantly increased levels of CREB mRNA in hippocampus (Fig. 6). Upregulation of CREB by these treatments was dependent on chronic administration, because acute administration of these agents did not significantly influence expression of CREB mRNA [100 ± 5 and $104 \pm 6\%$ of control (mean $\pm$ SEM); $n = 6$]. These treatments also increased levels of BDNF and trkB mRNA in hippocampus, further supporting a role for cAMP and CREB in the induction of these mRNAs in response to antidepressant treatment (Fig. 6). We also examined the influence of coadministration of rolipram and imipramine for 7 d on levels of CREB and BDNF mRNA. At this time point, there was no significant effect of either treatment alone, but coadministration of the two drugs resulted in significant upregulation of CREB and BDNF mRNA (Fig. 6). Preliminary results indicate that coadministration of imipramine and rolipram also results in a more rapid upregulation of trkB (data not shown).

DISCUSSION

The results of the present study demonstrate that chronic antidepressant administration increases the expression of CREB mRNA in rat hippocampus. Upregulation of CREB mRNA was observed after chronic, but not acute, administration, consistent with the time course for the therapeutic action of antidepressants. More-

over, levels of CREB mRNA were increased by chronic administration of several different classes of antidepressants with different acute actions, including both 5-HT- and NE-selective reuptake inhibitors. This suggests that upregulation of CREB may be a common target of 5-HT and NE neurotransmitter systems and antidepressant treatments. In contrast, this effect was not observed after chronic administration of several nonantidepressant psychotropic drugs (i.e., cocaine, morphine, and haloperidol), demonstrating that upregulation of CREB in hippocampus is specific to antidepressants. The lack of regulation by cocaine, which also blocks the reuptake of 5-HT and NE, could be explained by additional effects of this drug that oppose the expression of CREB, or by additional actions of antidepressants that are required for induction of CREB. Alternatively, the rapid metabolism of cocaine relative to antidepressants would result in drug levels that are pulsatile, whereas regulation of CREB may require sustained drug levels and sustained blockade of 5-HT and/or NE reuptake that occurs with antidepressants. In either case, opiates and psychostimulants are reported to regulate the expression and/or phosphorylation of CREB in other brain regions, indicating that CREB may subserve some of the transcriptional actions
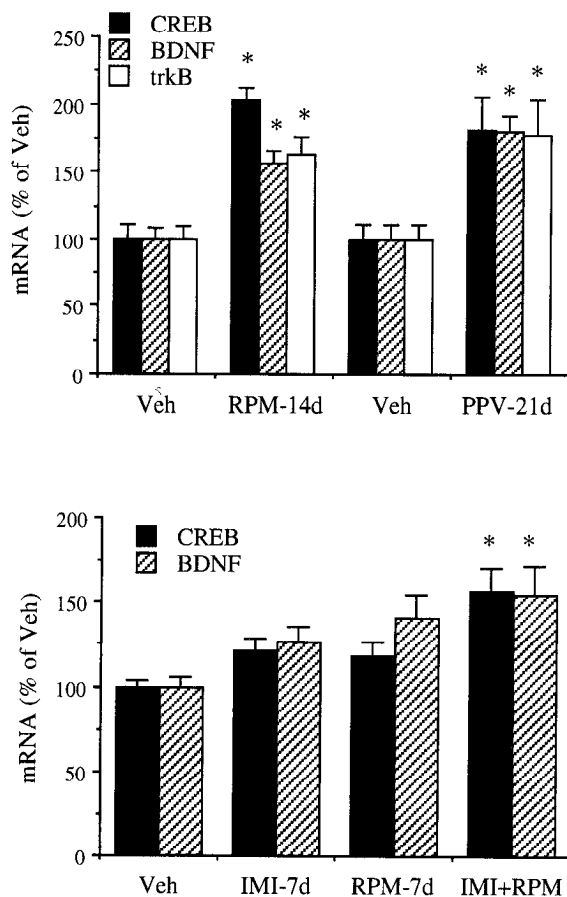
Figure 6. Influence of PDE inhibitors on expression of CREB, BDNF, and trkB mRNA in hippocampus. *Top,* The influence of chronic rolipram (*RPM;* 2 mg/kg for 14 d) or papaverine (*PPV;* 10 mg/kg for 21 d) administration on levels of CREB, BDNF, and trkB mRNA was determined by Northern blot analysis. *Bottom,* Rats were administered imipramine (*IMI;* 15 mg/kg) and *RPM* (10 mg/kg) either alone or in combination for 7 d, and levels of CREB and BDNF mRNA were determined. The results are expressed as percent of vehicle and are the mean ± SEM of 6 separate rats. *$p < 0.05$ compared with vehicle control (*top,* Student's *t* test; *bottom,* ANOVA and Fisher's test).

of these nonantidepressant drugs, but in a region-specific manner (Konradi et al., 1994; Widnell et al., 1994; Cole et al., 1995).

Levels of CRE binding in extracts of hippocampus were also increased by chronic administration of the antidepressants fluoxetine and ECS. The presence of CREB in the CRE binding complex of both sham- and ECS-treated hippocampal extracts was confirmed by demonstrating that preincubation with anti-CREB, but not anti-CREM, antisera disrupted the binding complex. Increased levels of CRE binding indicate that the amount of CREB protein is increased by these treatments, because binding to the symmetrical CRE site (used in this study) is not regulated by the phosphorylation state of CREB (Meyer and Haebner, 1993; Ghosh and Greenburg, 1995). This conclusion is also supported by immunohistochemical studies, which demonstrated that chronic fluoxetine or ECS administration increases levels of CREB immunoreactivity in the same cell layers of hippocampus as those demonstrating an increase in CREB mRNA (i.e., dentate gyrus granule cell layer and the CA3 and CA1 pyramidal cell layers). Taken together, the results indicate that the induction of CREB mRNA is accompanied by a corresponding increase in the expression and function of CREB protein.

Although CREB was originally thought to be constitutively expressed and its function controlled primarily by its phosphorylation (Meyer and Haebner, 1993; Ghosh and Greenberg, 1995), recent studies demonstrate that CREB expression is also subject to regulation (Meyer et al., 1993; Widnell et al., 1994, 1995). The finding that chronic administration of a PDE inhibitor (i.e., rolipram or papaverine) also increases levels of CREB mRNA indicates that the cAMP cascade plays a role in regulation of CREB expression. Indeed, the 5′-flanking region of the human CREB gene contains a CRE that is regulated by cAMP (Ruppert et al., 1992; Meyer et al., 1993). Expression of CREB in sertoli cells is also reported to be increased by activation of the cAMP system (Waeber et al., 1991; Ruppert et al., 1992), and we have found that stimulation of the cAMP system increases expression of CREB in C6 glioma cells (our unpublished observations). Expression of CREB is reported to be decreased by activation of the cAMP system in CATH.a cells (Widnell et al., 1994), indicating that regulation of CREB may be cell type-specific (Widnell et al., 1994). Antidepressant regulation of CREB may also be cell-type- or brain region-specific. For example, chronic antidepressant treatment is reported to decrease levels of PKA, indicating that the function of the cAMP cascade and CREB is reduced in this brain region (Melia et al., 1992). In addition, acute exposure of cultured PC12 cells, an adrenal chromaffin cell line, to certain antidepressants is reported to inhibit the activity of CREB (Schwaninger et al., 1995). In this study, antidepressants inhibited the depolarization-induced activation of CREB, an effect that was demonstrated to result from acute blockade of Ca^{2+} channels. This inhibition of CREB was observed after acute exposure of PC12 cells to antidepressants and has not been demonstrated *in vivo,* where other actions of the antidepressant drugs would be expected to predominate over blockade of Ca^{2+} channels. The results of the present study demonstrate that in the hippocampus the predominant response to administration of a PDE inhibitor, as well as to antidepressant treatments, is upregulation of CREB expression. Antidepressant regulation of CREB (see Fig. 7) could be mediated either by activation of the cAMP cascade (e.g., via $5\text{-}HT_{4,6,7}$ and β-adrenergic receptors) or by Ca^{2+}-activated protein kinases (e.g., $5\text{-}HT_2$ and α_1-adrenergic receptors), which can also activate CREB (Meyer et al., 1991; Ghosh and Greenburg, 1995).

Increased expression of CREB would be expected to lead to regulation of specific target genes, two of which may be BDNF and trkB. In primary cultures of brain, activation of cAMP- or Ca^{2+}-dependent protein kinases increases the expression of BDNF and trkB (Condorelli et al., 1994; Ghosh et al., 1994). In C6 glioma cells, we have found that activation of the cAMP cascade increases expression of BDNF and trkB mRNA (our unpublished observations). The results of the present study, as well as a previous report from this laboratory (Nibuya et al., 1995), demonstrate that the time course and regional distribution observed for antidepressant regulation of CREB correspond to those for regulation of BDNF and trkB mRNA. In addition, chronic administration of the PDE inhibitors increased levels of mRNA for BDNF and trkB. Although it is extremely difficult to demonstrate directly that expression of a gene *in vivo* is mediated by a specific transcription factor, the results of the present study are consistent with the possibility that induction of BDNF and trkB are mediated by CREB. This possibility is supported by studies demonstrating that local infusion of CREB antisense, but not sense, oligonucleotide decreases basal and ECS-induced BDNF mRNA levels in hippocampus (Nibuya et al., 1994; Duman
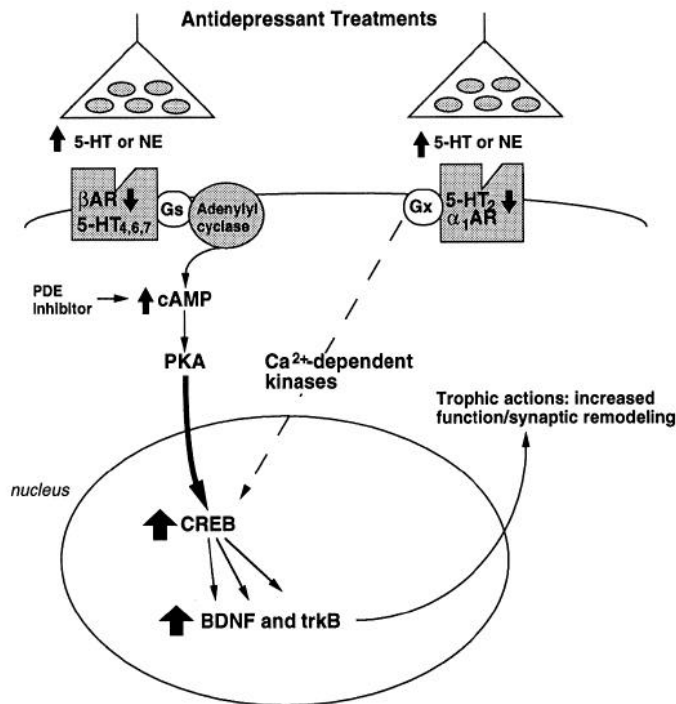
Figure 7. Model of the action of chronic antidepressant treatments on 5-HT and NE receptor-coupled signal transduction pathways and CREB. The results of this study support the hypothesis that CREB is a common target of different types of antidepressant treatments and the 5-HT and NE neurotransmitter systems. CREB could be activated by monoamine receptors that couple to the cAMP–PKA cascade (e.g., $5\text{-}HT_{4,6,7}$ and βAR) or via receptors that lead to activation of Ca^{2+}-dependent kinases (e.g., $5\text{-}HT_2$ and α_1AR). Although chronic treatments may lead to downregulation of 5-HT and NE receptors, the results of the present study demonstrate that the expression and function of CREB are increased, indicating that the functional output of these systems is upregulated, not downregulated, by chronic antidepressant administration. This conclusion is supported further by results demonstrating that levels of BDNF and trkB, two potential targets of CREB, are increased by chronic antidepressant treatment. Upregulation of BDNF and trkB could have autocrine or paracrine actions on hippocampal neurons or neurons innervating this brain region, such as 5-HT neurons. This includes increased neuronal survival, function, and remodeling of synaptic or cellular architecture.

et al., 1995). It is important to point out that regulation of CREB probably leads to regulation of other target genes, which may also play a role in the action of antidepressant treatments. Moreover, it is possible that chronic antidepressant treatments regulate the expression or function of transcription factors in addition to CREB.

The results demonstrate that coadministration of a PDE inhibitor shortens the time required for upregulation of CREB and BDNF expression by imipramine. This interaction could occur if the PDE inhibitor enhances the cAMP response to receptors activated by 5-HT or NE. Enhancement of the cAMP response could be particularly advantageous if the 5-HT or NE receptors coupled to cAMP are downregulated in response to elevated levels of monoamines, as reported for the β-adrenergic receptor (Heninger and Charney, 1987; Duman et al., 1994) and the $5\text{-}HT_7$ receptor (Sleight et al., 1995). Clinical studies are required to determine whether coadministration of a PDE inhibitor is capable of shortening the time required for the therapeutic action of antidepressant treatments.

Increased expression of CREB and induction of BDNF and trkB in response to chronic antidepressant treatments could in-

crease the survival and/or function of neurons, as reported for direct application of BDNF (Ghosh et al., 1994; Lindsay et al., 1994; Lindvall et al., 1994). This could include autocrine actions on hippocampal neurons and paracrine actions on neurons that innervate this brain region. Autocrine actions could be of particular importance for cases of depression associated with severe, chronic stress or other types of neuronal insult that decrease BDNF and lead to atrophy and, in severe cases, reduced survival of hippocampal neurons (Sapolsky et al., 1985, 1990; McEwen and Gould, 1990; Watanabe et al., 1992; Stein-Behrens et al., 1994; Smith et al., 1995). Upregulation of BDNF expression in response to antidepressant treatments could thereby enhance the function and survival of hippocampal neurons. Paracrine actions of BDNF could include increased function of 5-HT and NE neurons that innervate hippocampus, because BDNF is reported to increase the survival and sprouting of 5-HT neurons (Siuciak et al., 1994b; Mamounas et al., 1995; Sklair-Tavron and Nestler, 1995). This could be important, for example, if in certain cases depression is associated with a reduced capacity to synthesize 5-HT. Taken together, the results indicate that 5-HT and BDNF stimulate the expression of each other, and thereby form a positive regulatory loop that may be critical for the function and survival of 5-HT neurons and neurons innervated by 5-HT.

The results demonstrate that chronic administration of several different types of antidepressant drugs, as well as ECS, increases the expression and function of CREB, suggesting that this transcription factor is a common intracellular target of antidepressants. The results also support the hypothesis that the 5-HT- and NE receptor-coupled intracellular cascades that lead to regulation of CREB are activated, not downregulated, by chronic antidepressant treatments (Fig. 7). This suggests that although levels of certain 5-HT and NE receptors (e.g., β-adrenergic, $5\text{-}HT_{2A}$, and $5\text{-}HT_7$ receptors) may be reduced by chronic antidepressant treatments, there is an increase in the functional output of these receptor-coupled intracellular pathways. This is not surprising because the density and function of these receptors are reduced, but not completely eliminated, by chronic antidepressant treatments (Heninger and Charney, 1987). Activation of the remaining 5-HT and NE receptors may be sufficient to stimulate the intracellular signal transduction pathways. This possibility is supported further by the finding that BDNF and trkB, targets of the cAMP–CREB cascade, are also increased by chronic antidepressant treatments. Future studies will test this hypothesis further by examining additional antidepressant and nonantidepressant treatments. Moreover, our studies will attempt to elucidate the cellular consequences of the upregulated cAMP–CREB cascade and of increased expression of BDNF and trkB.

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