

Altered Regulation of CREB by Chronic Antidepressant Administration in the Brain of Transgenic Mice with Impaired Glucocorticoid Receptor Function

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Various effects of antidepressant drugs on gene transcription have been described and altered gene expression has been proposed as being a common biological basis underlying depressive illness. One target for the common action of antidepressants is a modifying effect on the regulation of postreceptor pathways and genes related to the cAMP cascade. Recent studies have demonstrated that long-term antidepressant treatment resulted in sustained activation of the cyclic adenosine 3',5'-monophosphate system and in increased expression of the transcription factor cAMP response element binding protein (CREB). A transgenic animal model of depression with impaired glucocorticoid receptor function was used to investigate the effect of chronic

antidepressant treatments on CREB expression in different brain areas. Wild-type and transgenic mice received one administration of saline, desipramine, or fluoxetine, daily for 21 days. The effects of antidepressants on CREB mRNA were analyzed using a sensitive RNase protection assay. Antidepressant treatment resulted in a neuroanatomically and animal specific expression pattern of CREB. Our findings suggest that life-long central glucocorticoid receptor dysfunction results in an altered sensitivity with respect to the effects of antidepressants on the expression of CREB. [Neuropsychopharmacology 26:605-614, 2002] © 2002 American College of Neuropsychopharmacology. Published by Elsevier Science Inc.

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While the regulation of monoamine levels and their receptors or the restoration of hypothalamus-pituitary-adrenal (HPA) system feedback may represent initial effects of antidepressant drugs, their therapeutic action could be related to subsequent action on genes involved in postreceptor mechanisms. One such postreceptor target that can be influenced by either 5HT or NE as well as by drugs that affect these systems is the nuclear transcription factor cAMP response element binding protein (CREB) (Montminy, et al. 1990; Meyer and Habener 1993; Nibuya et al. 1996; Duman et al. 1997, 1999; Thome et al. 2000). A role for the cAMP cascade in the long-term ac-

tion of antidepressant treatment is supported by research showing that chronic administration of antidepressants regulates brain levels of adenylyl cyclase (Ozawa and Rasenick 1991) and PKA (Nestler et al. 1989) and that the activity of CREB is enhanced following its phosphorylation by PKA (Meyer and Habener 1993; Ghosh and Greenberg 1995). Furthermore, viral mediated over-expression of CREB in two animal models of depression produced an antidepressant effect and resulted in improved behavioral performance in both models (Chen et al. 2001).

Based on the hypothesis that the apparent lack of sensitivity to corticosteroids observed in major depression is causally linked to the pathogenesis of depression (Holsboer and Barden 1996; Holsboer 2000; Pariante and Miller 2001) as well as to the therapeutic effectiveness of antidepressant drugs (Barden 1996), a transgenic animal model of neuroendocrine changes associated with depression was developed (Pepin et al. 1992a). Insertion into the mouse genome of a transgene expressing antisense RNA complementary to a fragment of the glucocorticoid receptor (GR) cDNA produced an animal with a defect in the neuronal glucocorticoid receptors that mediate the effects of high "stress" levels of glucocorticoids on the negative feedback of the HPA system. These transgenic mice displayed impaired endogenous GR function and were characterized by dysfunctional glucocorticoid inhibitory feedback and disturbed ACTH and corticosterone secretory responses (Barden et al. 1997; Karanth et al. 1997; Dijkstra et al. 1998). Long-term treatment of the transgenic mice with antidepressant drugs caused an increase in GR gene expression and produced significant normalization of the HPA system hyperactivity through restoration of functional glucocorticoid inhibitory feedback (Pepin et al. 1992b; Barden et al. 1995; Barden et al. 1997).

Because the second messenger cyclic AMP plays a central role in signaling within the HPA axis, long-term adaptations in cAMP regulated gene expression are critical to chronic HPA dysfunction (Hatalski and Baram 1997). Given that depressed patients display reduced cAMP protein kinase activity (Shelton et al. 1996) and that the cAMP/PKA cascade is involved in mediating the action of antidepressant drugs, GR dysfunction and disruption of proper cAMP/PKA signaling both observed in depression are highly interdependent. The use of this transgenic animal model allowed study of the effectiveness of chronic antidepressant treatment in restoring long-term defective glucocorticoid feedback inhibition and dysregulation of the HPA system by affecting gene expression crucial to the cAMP-signaling pathway.

Consequently, we tested the hypothesis that upregulation of CREB is an important factor in the therapeutic action of antidepressant drugs. This role for CREB, that provides a common intracellular adaptation for the action of diverse types of antidepressants, was tested in various

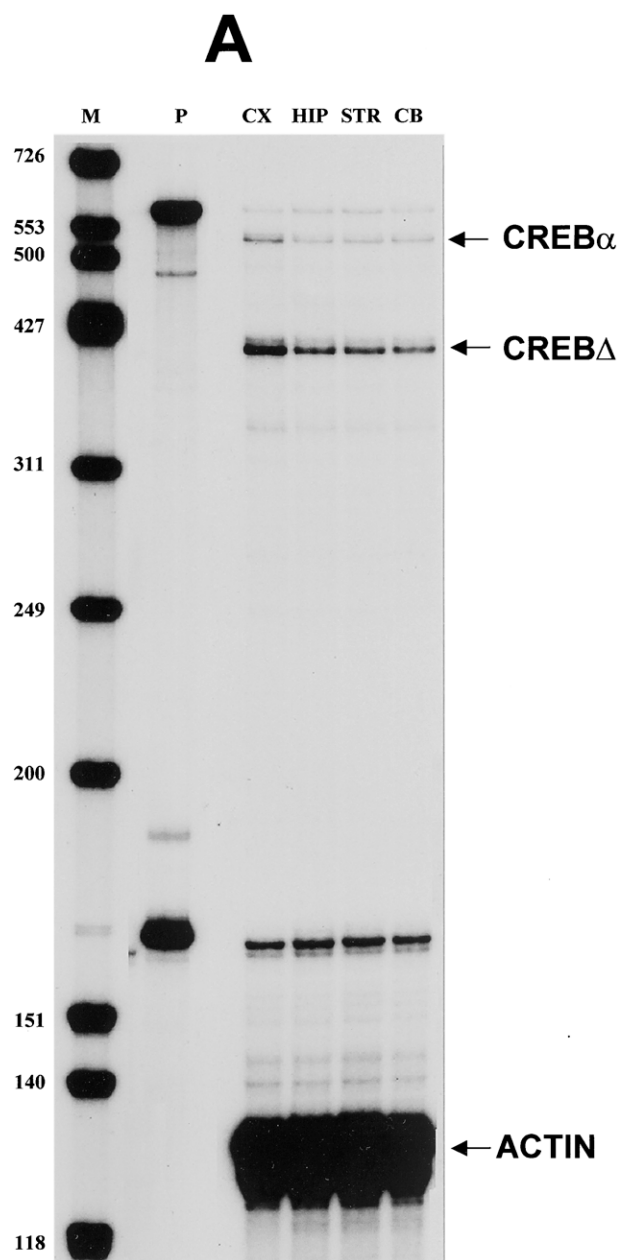


Figure 1. Panel A: Representative autoradiography of an RNase Protection Assay indicating the distribution of CREB- α and CREB- Δ in different brain areas. 10 μ g of total RNA was used in each lane with M: molecular standard; P: Probe; CX: cortex; HIP: hippocampus; STR: striatum; and CB: cerebellum. The lane marked as probe indicates an aliquot (8000cpm) of the hybridization solution containing the antisense cRNA probes to CREB- α , CREB- Δ , and β -actin. The X-ray film was exposed for 16 h at -70°C with an intensifying screen. In all areas analyzed, CREB- Δ expression was 4–5 times higher than CREB- α , with both isoforms expressed most in the cortex. Panel B: Basal mRNA expression of CREB- α and CREB- Δ in the hippocampus and cortex of transgenic and wild-type mice. Levels of CREB- α and CREB- Δ mRNA were determined by RNase Protection Assay. No difference was observed in β -actin expression. The results are expressed as the mean \pm S.E.M. of 6–8 mice in each experimental group.

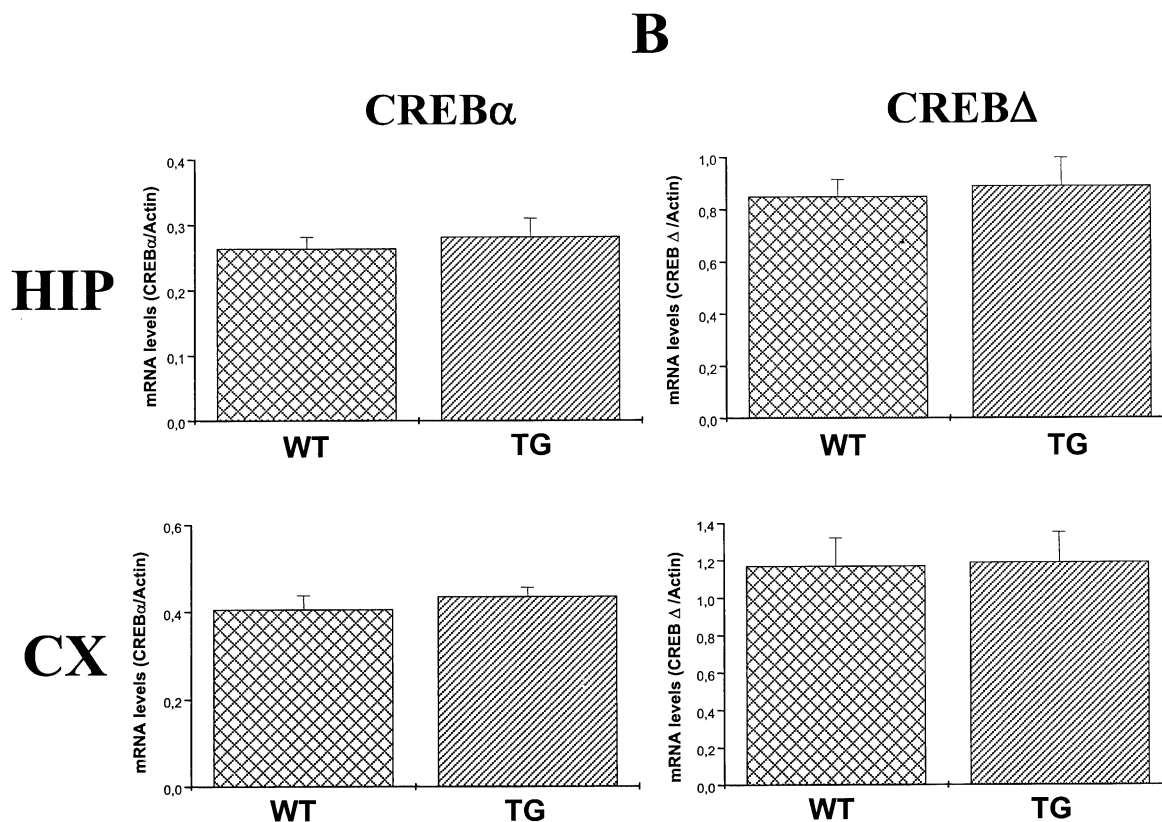


Figure 1. (Continued)

brain structures using both normal and transgenic mice known to be sensitive to antidepressant action.

METHODS

Animals

Adult transgenic (TG) mice (line 5.4) bred and raised in our colony and non-transgenic wild-type (WT) mice (B6C3F1) (Charles River Laboratories, Como) were used in this study. Transgenic mice were maintained as homozygotes. They were constructed using oocytes from B6C3F1 females. All transgenic mice were checked for transgene presence before entering an experiment and all mice were found to be homozygous. Animals were housed in pairs in polycarbonate cages (28 × 17 × 12 cm) with ad libitum access to food and tap water throughout the study, and maintained under a 12:12 light-dark cycle in an ambient temperature of 21 ± 3°C, relative humidity uncontrolled.

Treatment Protocol

TG and WT mice received daily i.p. injections with desipramine (10 mg/kg BW), fluoxetine (10 mg/kg BW) or vehicle for a period of 21 days. Six experimental groups were used with six animals in each group.

RNA Preparation

The tissue from different brain structures was homogenized in 4 M guanidinium isothiocyanate (containing 25 mM sodium citrate pH 7.5, 0.5% sarcosyl and 0.1% 2-mercaptoethanol) and total RNA was isolated by phenol-chloroform extraction (Chomczynski and Sacchi 1987). Quantification was carried out by absorption at 260 nm and RNA was reprecipitated in ethanol for RNase protection assay. In order to verify that equal amounts of total RNA were used in the RNase protection assay, parallel samples were loaded on agarose/formaldehyde gel, run (35V for 16 h) and stained with ethidium bromide (not shown).

CREB Probe Preparation

The cDNA (530 bp EcoRI-StuI fragment) for mouse CREB was subcloned into pBluescript sk- (Stratagene), in order to obtain a template for the *in vitro* transcription of cRNA probes to be employed in the RNase protection assay. cRNA probes were generated using T7 RNA polymerase and ³²P-CTP was used as the radiolabeled nucleotide. The cRNA probe for CREB had 570 bases and after digestion generated two major protected fragments one of 530 bases, CREB- α , and one of 403 bases, CREB- Δ . pTRI-actine (Ambion) containing a portion of β -actine cDNA was utilized as internal standard. The probe for actin was gener-

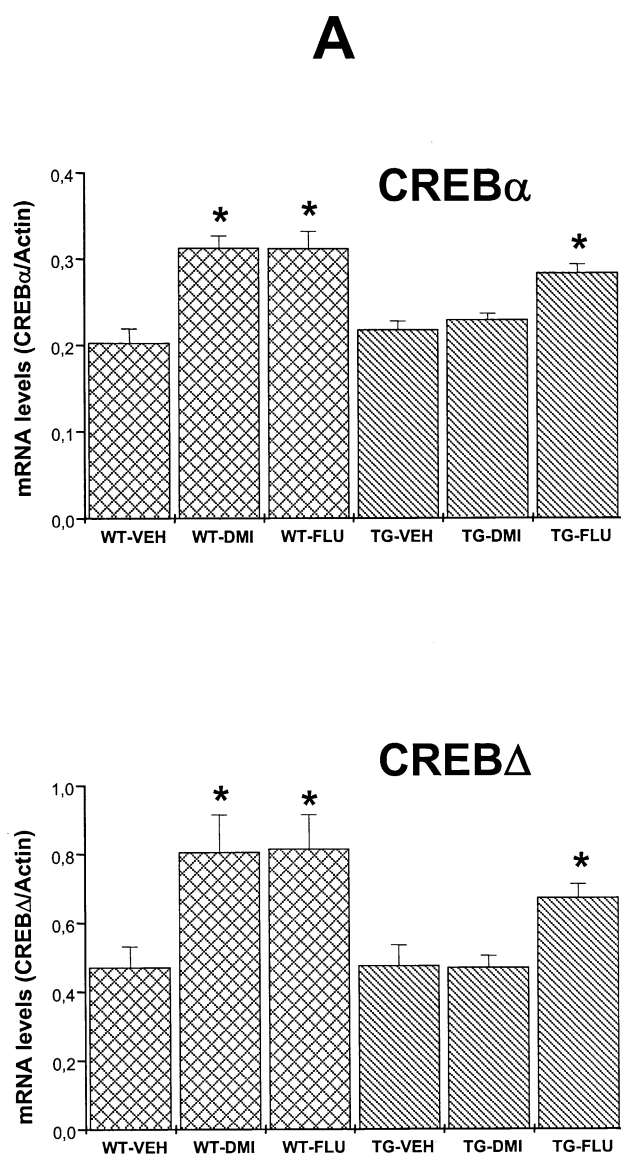


Figure 2. Panel A: mRNA expression of CREB- α and CREB- Δ in the hippocampus of transgenic and wild-type mice after treatment with antidepressant drugs. Levels of CREB- α and CREB- Δ mRNA were determined by RNase Protection Assay. The results, normalized for actin, are expressed as the mean \pm S.E.M. of 6–8 mice in each experimental group. * $p < .05$ compared with WT controls. Panel B: Representative RNase protection assay of CREB transcripts in the hippocampus of transgenic and wild-type mice after treatment with antidepressant drugs.

ated using T3 RNA polymerase, had 160 bases, and generated a protected fragment of 125 bases.

RNase Protection Assay

The RNase protection assay was performed on a 10 μ g sample of total RNA. Briefly, after ethanol-precipitation, total RNA obtained from different tissues was dis-

solved in 20 μ l of hybridization solution (80% formamide, 40 mM PIPES pH 6.4, 400 mM sodium acetate pH 6.4 and 1 mM EDTA) containing 150,000 cpm of each 32 P-labeled cRNA probe (specific activity $> 10^8$ cpm/ μ g). After being heated at 85°C for 10 min, the cRNA probes were allowed to hybridize to the endogenous RNAs at 45°C overnight. At the end of the hybridization, the solution was diluted with 200 μ l of RNase digestion buffer (300 mM NaCl, 10 mM Tris HCl pH 7.4, 5 mM EDTA pH 7.4) containing a 1/200 dilution of a RNase cocktail (1 g/l RNase A and 20 U/l RNase T1) and incubated for 30 min at 30°C. Proteinase K (10 μ g) and SDS (10 μ l of 20% stock solution) were then added to the sample and the mixture was incubated at 37°C for an additional 15 min. At the end of the incubation the sample was extracted with phenol/chloroform and ethanol precipitated. The pellet, containing the RNA:RNA hybrids was dried and resuspended in loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 2 mM EDTA), boiled at 95°C for 5 min and separated on a 5% polyacrylamide gel under denaturing conditions (7M urea). The protected fragments were visualized by autoradiography and their size determined by the use of 32 P-end-labeled (T4 polynucleotide kinase) DNA phiX174-digested Hinf-I fragments.

RNA Calculation

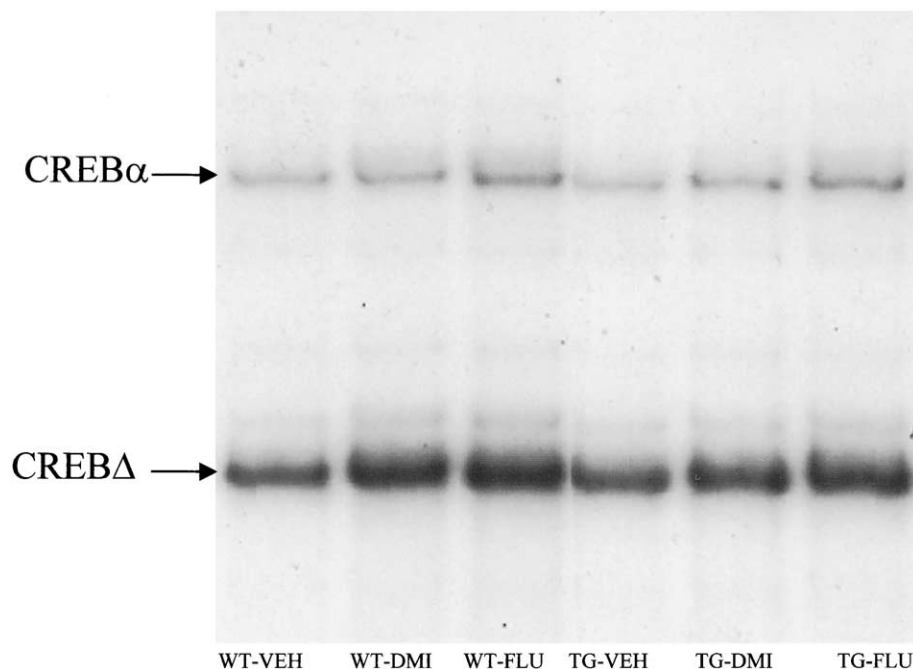
The levels of mRNA were calculated by measuring the peak densitometric area of the autoradiography analyzed with an image analyzer (GS 690 BIORAD). In order to ensure that the autoradiographic bands were in the linear range of intensity, different exposure times were used.

Statistical Analysis

Statistical evaluation of the changes in mRNA levels was performed using an analysis of variance (ANOVA). Significant changes were determined by Dunnett t -test.

RESULTS

CREB is ubiquitously expressed in the CNS of mice and the highest levels of CREB were found in the cortex, the hippocampus, striatum, and cerebellum. Basal expression of CREB mRNA in both hippocampus and cortex did not differ between WT and TG mice (Figure 1, Panels A and B). While no difference was observed in the hippocampus and cortex of TG and WT mice in the expression of the two isoforms of CREB, CREB- α and CREB- Δ , the level of expression of the two isoforms did differ in the cortex and hippocampus as well as in the striatum and cerebellum (Figure 1, Panel B). In all areas analyzed, and irrespective of the type of animal, CREB- Δ expression was 4–5 times higher than CREB- α .

B**Figure 2.** (Continued)

Following chronic administration of antidepressant drugs brain area specific upregulation of CREB- α and CREB- Δ mRNA was displayed. Chronic treatment (21 days) with desipramine (a selective NE reuptake inhibitor) and fluoxetine (a 5-HT selective reuptake inhibitor) increased the expression of CREB- α and CREB- Δ mRNA in the hippocampus of wild type mice (Figure 2, Panels A and B). In transgenic mice, CREB expression in the hippocampus was enhanced only by fluoxetine but not by desipramine. Acute treatment with desipramine or fluoxetine did not affect CREB gene expression in the hippocampus (data not shown).

Chronic treatment with desipramine significantly enhanced levels of CREB- α and CREB- Δ in the cortex of TG-mice but not in wild type mice (Figure 3, Panels A and B).

To determine the area-specific effects of antidepressant drugs on CREB gene expression, other distinct regions of the brain of WT and TG mice were analyzed after long-term treatment with desipramine and fluoxetine. No differences between WT and TG mice were found in the basic expression of CREB- α and CREB- Δ in the cerebellum, striatum and hypothalamus. Furthermore, CREB expression in these brain regions was not affected by chronic antidepressant treatment (Table 1).

DISCUSSION

Depression is an heterogeneous illness that can result from the dysfunction of one or several neurotransmitter

or metabolic systems resulting from a combination of genetic and environmental factors. The cAMP cascade is one of many intracellular pathways regulated by serotonin and NE that could be important for antidepressant action and the present experiments focused on the significance of the cAMP-signaling pathway in a model of a chronically disturbed HPA system that can be normalized by antidepressant drug treatment. The goal of the study was 2-fold: (1) to test whether defective glucocorticoid feedback inhibition of the HPA system leads to changes in the expression of cAMP response element binding protein; and (2) to determine whether or not antidepressant drug treatment affects CREB gene expression *in vivo*.

Three main conclusions arose from our experiments: First, chronic disruption of GR-gene expression leading to defective regulation of the HPA system did not alter basal expression of CREB mRNA in distinct brain regions. Second, the antidepressant drugs desipramine and fluoxetine differentially affected CREB gene expression in WT as compared with TG mice. Chronic antidepressant treatment resulted in brain region-specific upregulation of CREB gene expression according to the type of animal. Third, life-long central glucocorticoid receptor dysfunction resulted in altered sensitivity of CREB expression to antidepressant drugs.

Altered glucocorticoid receptor signaling could be a crucial mechanism in the pathophysiology underlying depression (Holsboer and Barden 1996; Holsboer 2000; Pariante and Miller 2001) and selective reduction of glu-

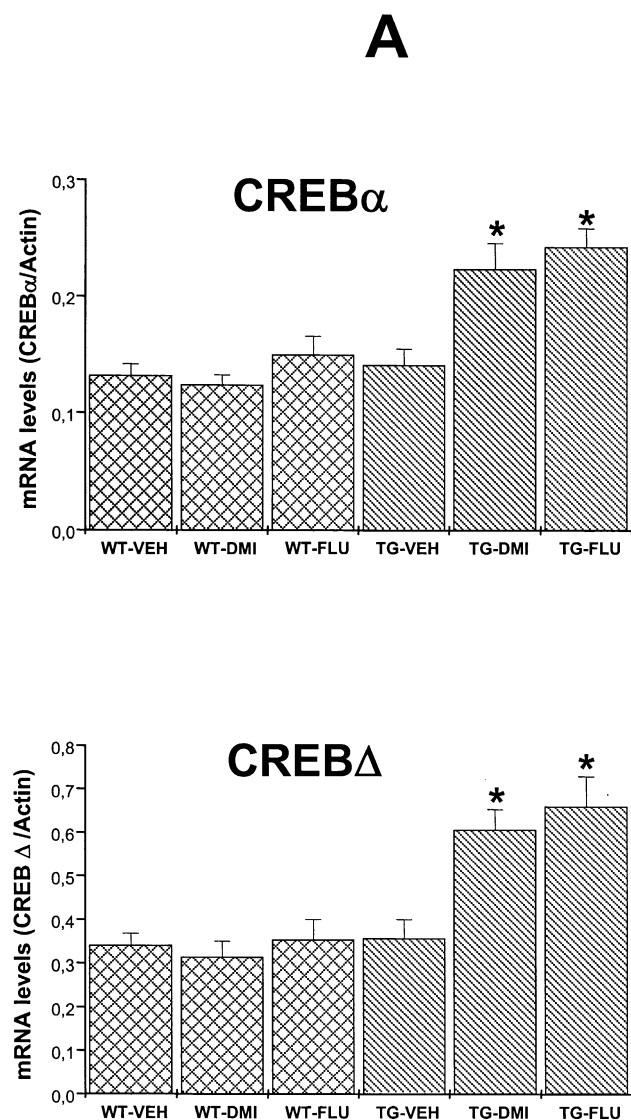


Figure 3. Panel A: mRNA expression of CREB- α and CREB- Δ in the cortex of transgenic and wild-type mice after treatment with antidepressant drugs. Levels of CREB- α and CREB- Δ mRNA were determined by RNase Protection Assay. The results, normalized for actin, are expressed as the mean \pm S.E.M. of 6–8 mice in each experimental group. * p < .05 compared with WT controls (ANOVA). Panel B: Representative RNase protection assay of CREB transcripts in the hippocampus of transgenic and wild-type mice after treatment with antidepressant drugs.

cocorticoid receptor activity in transgenic mice lead to changes in the HPA system similar to those observed in patients suffering from major depression. (Barden et al. 1997; Karanth et al. 1997). Furthermore, modifications in the cAMP cascade play an important role in the pathogenesis of major depression and in the regulation of the HPA axis. cAMP-dependent transcription factors, including CREB, a nuclear protein substrate that binds to a cAMP response element (CRE) present in the

promoter region of a variety of genes, are central to a promising new hypothesis linking alterations in cAMP activation to depression and the action of antidepressant drugs. These transcription factors, in response to cAMP, can also modulate the expression of genes involved in the HPA axis. The first part of the present study, therefore, focused on the question of whether transgenic mice with altered HPA regulation and impaired GR function display altered basal expression of CREB. No such differences were found and GR dysfunction did not affect neither the absolute nor the relative (vs. WT) amounts of CREB mRNA in the cortex and hippocampus of transgenic mice. High, non-basal, levels of ACTH and a blunted circadian hormonal rhythm characteristic of these transgenic mice were not sufficient to modify basal CREB expression.

CREB and related CRE-binding proteins are normally present in their non-phosphorylated form and their activity can be regulated through phosphorylation. The amount of P-CREB is probably more important for transcriptional activation than the absolute amount of CREB (Gonzalez and Montminy 1989; Sassone-Corsi 1995; Lamas and Sassone-Corsi 1997). Consequently, differences in basal levels of CREB gene expression may not be a constitutive feature of central HPA axis dysfunction. In addition, the complexity of the regulation of CREB becomes evident when the elevated numbers of signal transduction pathways that converge onto this transcription factor are considered (Bito et al. 1996). CREB is phosphorylated by many kinases other than PKA, including CAMKIV (Ca⁺⁺-calmodulin kinase IV), PKC (protein kinase Ca⁺⁺ dependent), CKI and CKII (casein kinases I and II) (Sheng et al. 1991; Shaywitz and Greenberg 1999). Also, an abundance of factors as diverse as peptide hormones, neurotransmitter systems, neuro-protective agents, and growth factors, such as BDNF and NGF regulate CREB gene expression and activation. Unmodified basal levels of CREB mRNA may, then, represent the net result of a series of redundant and compensatory processes.

The complexity of the regulation of CREB is further illustrated by a differential-splicing pattern of CREB among CNS regions suggesting a region-specific functional regulation of various CREB-isoforms (Pietruck et al. 1999). While no difference was observed in basal CREB levels, a difference was observed in the expression of the two isoforms of CREB. Expression levels of CREB- Δ were consistently four to five times higher than those of CREB- α . These data are in line with earlier studies (Pietruck et al. 1999) that demonstrated a differential expression pattern of CREB isoforms in the central nervous system and where the CREB- Δ isoform was most abundant. A third isoform, CREB- β , has only been demonstrated in transgenic mice lacking both the α and Δ isoforms, and was therefore not considered in this study (Blendy et al. 1996).

B

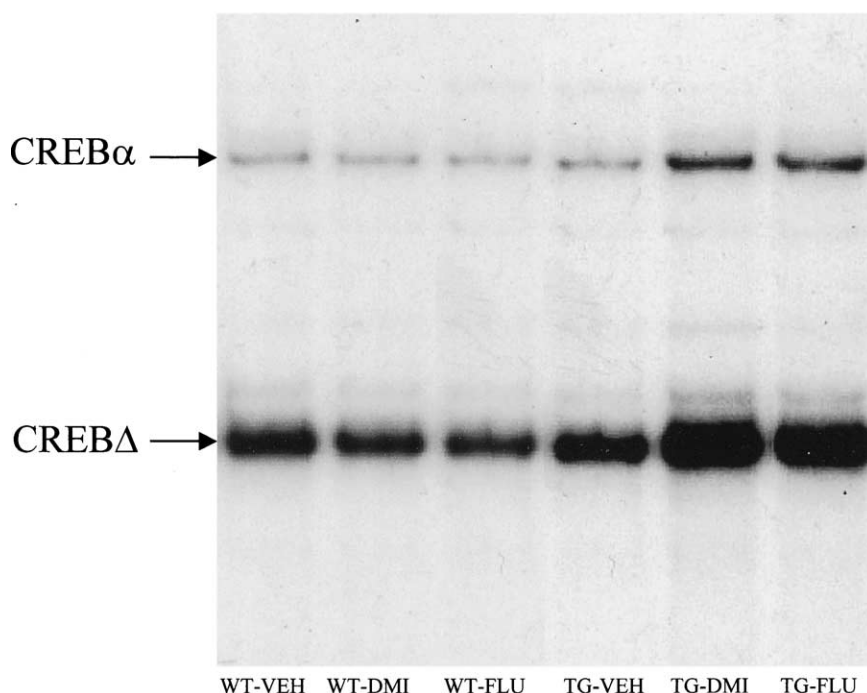


Figure 3. (Continued)

The second part of this study examined the influence of long-term antidepressant treatment on CREB expression. Desipramine and fluoxetine differentially affected CREB gene expression in WT as compared with TG mice. Chronic treatment with desipramine and fluoxetine enhanced the expression of CREB mRNA in the hippocampus of healthy non-transgenic mice. The fact that both drugs affect CREB expression in a similar way cannot be explained by their primary action on cell membrane receptors since fluoxetine is a typical selective serotonin reuptake inhibitor and desipramine is a less

selective norepinephrine reuptake inhibitor. Prolonged administration of various types of antidepressants also reduces the number of biogenic amine receptors and the mechanism underlying antidepressant-induced CREB upregulation is more likely to involve the activation of the intracellular signaling cascade downstream to early stimulation of the cAMP system.

In contrast, in the hippocampus of transgenic mice, the expression of CREB was enhanced only by chronic fluoxetine, but not by desipramine. The molecular mechanisms underlying this altered sensitivity in the expression of CREB in the hippocampus of transgenic mice are not yet known but evidence is accumulating, indicating that different classes of antidepressant drugs activate cAMP signal transduction at different points of the cascade (Frechilla et al. 1998; Takahashi et al. 1999; Popoli et al. 2000).

CREB activation is regulated by phosphorylation and there is substantial interaction between GR related signaling and the phosphorylation of CREB. GR prevents CREB phosphorylation by direct linking to the protein and hindering its binding to CRE. In addition, glucocorticoids can directly affect CREB function by interfering with the phosphorylation of CREB (Legradi et al. 1997). A significant difference in GR number and function in various cerebral areas characterize the transgenic mice used in this study, in the absence of significant elevations in peripheral corticosterone. This suggests that

Table 1. The Effect of Chronic Treatment with Fluoxetine and Desipramine on the Expression of CREB in WT and TG Mice

Tissue Type	WT		TG	
	FLU	DMI	FLU	DMI
Hippocampus	↑	↑	↑	—
Cortex	—	—	↑	↑
Striatum	—	—	—	—
Cerebellum	—	—	—	—
Hypothalamus	—	—	—	—

↑ = Significant induction of CREB gene expression after chronic exposure to fluoxetine (FLU) or desipramine (DMI) with respect to vehicle treated WT or TG mice.

— = No induction of CREB gene expression after chronic exposure to fluoxetine (FLU) or desipramine (DMI) with respect to vehicle treated WT or TG mice.

CREB and, presumably, other transcription factors, such as AP1, activated in these specific brain regions during antidepressant treatment may be influenced either directly by GR number and function or indirectly by the local availability of corticosterone.

Our results not only indicate a differential effect of chronic exposure to two different classes of antidepressant drugs on CREB expression but also imply a region-specific effect. While the expression of CREB in the hippocampus of WT mice was enhanced by chronic administration of both desipramine and fluoxetine, CREB mRNA levels in the cortex were not significantly affected by either antidepressant drug. Support for a selective region-specific effect of antidepressants on CREB expression is provided by a study using post-mortem tissue from patients suffering from major depression that showed CREB expression to be enhanced in the temporal, but not occipital, cortex after treatment with antidepressants (Dowlatabadi et al. 1998).

One explanation for the different expression patterns of CREB displayed by the cortex and hippocampus may be the upregulation of two cAMP specific phosphodiesterase isoforms, PDE4A and PDE4B, that were upregulated in the cortex but not in the hippocampus of rats after chronic antidepressant treatment (Fujimaki et al. 2000; Thome et al. 2000). Phosphodiesterases play a crucial role in converting cAMP to 5'-AMP, and inhibit the transduction pathway leading to the activation of CREB. As a consequence they diminish the responsiveness of CREB gene expression to antidepressant drugs, which seems to be mediated by an upregulation of the cAMP-signaling pathway. In contrast to the lack of effect of antidepressants on the regulation of CREB in the cortex of WT mice, both fluoxetine and desipramine significantly enhanced the expression of CREB in the cortex of transgenic mice. Enhanced responsiveness in the cortex of TG mice may, at least in part, be mediated by the expression and activity of phosphodiesterases. Induction of phosphodiesterases in the cortex is mediated by the cAMP/PKA related signaling pathway (Duman et al. 1999; Fujimaki et al. 2000). Given that substantial interactions exist between cAMP-PKA pathways and glucocorticoid signaling, it is thus possible that the impairment in GR function, characteristic of the transgenic mice used in this study, causes an altered sensitivity in cAMP-PKA induced gene transcription of specific phosphodiesterases. Moreover, an important role for phosphodiesterases in the mechanism of action of antidepressant drugs is suggested by preclinical and clinical studies that show phosphodiesterase inhibitors have antidepressant-like effects on behavior (O'Donnell 1993).

The reason for regional differences between fluoxetine and desipramine is not easy to explain. Both the hippocampus and the cortex receive elaborate projections from the NE and 5-HT systems. However, alternate signaling pathways or different susceptibilities to autoreg-

ulation of these systems may cause, for example, more rapid inhibition of the 5-HT system in response to fluoxetine (Blier and de Montigny 1994). Selective sensitivity to fluoxetine in the cortex of transgenic mice corresponds to other studies where fluoxetine, but not desipramine, significantly increased β -galactosidase and the phosphorylation of CREB in rats (Thome et al. 2000).

In summary, these results support the hypotheses that expression of CREB may be an intracellular target of two different types of antidepressants in two brain areas known to be important sites for depression. The findings further indicate that, while in normal healthy animals, the effects of antidepressants on CREB gene expression depend on the specific brain structure, in transgenic mice CREB expression was more sensitive to the type of antidepressant and less dependent on the cerebral region.

A particular benefit of our model was that it allowed the study of long-term effects of an over-active HPA-system on the regulation of cAMP-mediated gene transcription *in vivo*. We tested the hypothesis that disruption of GR gene expression, resulting in defective glucocorticoid feedback inhibition and dysregulation of HPA system activity result in abnormal CREB gene expression. Furthermore, the hypothesis was tested that the antidepressant drugs desipramine and fluoxetine, not only normalize HPA system function, but also affect CREB gene expression. Changes in CREB gene expression could explain both the time necessary for antidepressants to be therapeutically effective as well as their long-term effects. Given, however, that fluoxetine enhanced the expression of CREB both in cortex and hippocampus of transgenic mice while desipramine affected CREB expression only in the cortex, the signaling pathways activated by the two antidepressants leading to changes in CREB expression may not be the same. Conceptually, this suggests that altered neuroendocrine functioning may lead to a neuroanatomically selective vulnerability of the cAMP mediated signal transduction pathway and, as a consequence, may induce an enhanced sensitivity of cAMP mediated gene transcription to antidepressant treatment. These findings also suggest that HPA axis-mediated vulnerability and life-long central glucocorticoid receptor dysfunction can lead to differential effects of antidepressants on intracellular signal transduction pathways and the regulation of specific target genes including an altered sensitivity of CREB to the effects of antidepressants.

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