Selective Phosphorylation of Nuclear CREB by Fluoxetine is Linked to Activation of CaM Kinase IV and MAP Kinase Cascades

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Regulation of gene expression is purported as a major component in the long-term action of antidepressants. The transcription factor cAMP-response element-binding protein (CREB) is activated by chronic antidepressant treatments, although a number of studies reported different effects on CREB, depending on drug types used and brain areas investigated. Furthermore, little is known as to what signaling cascades are responsible for CREB activation, although cAMP-protein kinase A (PKA) cascade was suggested to be a central player. We investigated how different drugs (fluoxetine (FLX), desipramine (DMI), reboxetine (RBX)) affect CREB expression and phosphorylation of Ser^{133} in the hippocampus and prefrontal/frontal cortex (PFCX). Acute treatments did not induce changes in these mechanisms. Chronic FLX increased nuclear phospho-CREB (pCREB) far more markedly than pronoradrenergic drugs, particularly in PFCX. We investigated the function of the main signaling cascades that were shown to phosphorylate and regulate CREB. PKA did not seem to account for the selective increase of pCREB induced by FLX. All drug treatments markedly increased the enzymatic activity of nuclear Ca^{2+}/calmodulin (CaM) kinase IV (CaMKIV), a major neuronal CREB kinase, in PFCX. Activation of this kinase was due to increased phosphorylation of the activatory residue Thr^{196}, with no major changes in the expression levels of α- and β-CaM kinase kinase, enzymes that phosphorylate CaMKIV. Again in PFCX, FLX selectively increased the expression level of MAP kinases Erk1/2, without affecting their phosphorylation. Our results show that FLX exerts a more marked effect on CREB phosphorylation and suggest that CaMKIV and MAP kinase cascades are involved in this effect.
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INTRODUCTION

Regulation of gene expression represents a major component in long-term plastic changes of CNS (Kandel, 2001; Lonze and Ginty, 2002; West *et al*, 2002). A number of transcription factors have been characterized that regulate gene expression by binding to specific domains in the promoter region of genes and stimulating mRNA transcription. The best characterized among families of transcription factors is that including cAMP-response element-binding protein (CREB). There is ample evidence that CREB regulates the expression of genes involved in neuroplasticity, cell survival, and cognition (Mabuchi *et al*, 2001; Reppert

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and Weaver, 2001; Kandel, 2001; West *et al*, 2002; Lonze and Ginty, 2002; Kida *et al*, 2002; Pittinger *et al*, 2002).

In recent years several lines of investigation showed that expression, phosphorylation, and transcriptional activity of CREB are modulated by psychotropic drugs, particularly by chronic antidepressant treatments (Schwaninger *et al*, 1995; Nibuya *et al*, 1996; Frechilla *et al*, 1998; Thome *et al*, 2000; Chen *et al*, 2001; Manier *et al*, 2002; Nestler *et al*, 2002). These studies were complemented by studies of postmortem brain showing that CREB protein level is higher in patients treated with antidepressants at the time of death compared to untreated patients, and that CREB level and CRE/binding activity are significantly lower in the brain of suicides (Dowlatshahi *et al*, 1998; Dwivedi *et al*, 2003). Although there is general agreement that chronic antidepressants stimulate CREB function and affect neuroplasticity, some authors reported a decrease in CREB expression and/or function following antidepressant treatments (Schwaninger *et al*, 1995; Nibuya *et al*, 1996; Frechilla *et al*, 1998; Thome *et al*, 2000; Chen *et al*, 2001; Manier *et al*, 2002). Interestingly, a number of studies suggested that

different effects on CREB may be dependent on brain areas examined and be selective for drug type (Nibuya *et al*, 1996; Thome *et al*, 2000; Manier *et al*, 2002).

Very little is known about the mechanism whereby psychotropic drugs affect CREB function and regulation of gene expression, although it was proposed that the effects of antidepressants on CREB are mainly due to an upregulation of the cAMP-protein kinase A (PKA) cascade by these drugs (Duman *et al*, 1999; Nestler *et al*, 2002; Manji *et al*, 2003). However, there is an increasing body of evidence showing that several kinase cascades, acting individually or in concert in response to various kinds of stimuli, regulate CREB function in the CNS (Kandel, 2001; Lonze and Ginty, 2002; West *et al*, 2002). Interestingly, it was shown that calcium/ calmodulin (CaM)-dependent kinases, particularly CaM kinase IV (CaMKIV) and mitogen-activated protein kinases (MAPK) have a primary role in the phosphorylation of CREB and in the regulation of activity-dependent neuronal gene expression (Ghosh *et al*, 1994; Kasahara *et al*, 2001; Bito *et al*, 1996). Activation of these signaling cascades and in turn their action on gene expression in response to chronic antide-pressants have been little or not investigated thus far. Furthermore, a critical analysis of the available literature (Tardito *et al*, 2004) showed that the upregulation of cAMP-PKA cascade in response to chronic antidepressants has been clearly demonstrated in the microtubule compartment and at the level of G protein–adenylyl cyclase coupling in the plasma membrane, but not in the nuclear compartment, where Ser[133] phosphorylation and activation of CREB occurs (Chen and Rasenick, 1995; Nestler *et al*, 1989; Perez *et al*, 1989; Popoli *et al*, 2000; Donati and Rasenick, 2003; for a detailed discussion of these aspects see Tardito *et al*, 2004). Therefore, it would be quite interesting to assess what signaling cascades are involved in CREB regulation by antidepressants.

In this work, we asked this question first by investigating how different drugs affect the expression and phosphoryla-tion of CREB, following acute and chronic treatment with three different and representative antidepressants (fluoxetine, desipramine, reboxetine; FLX, DMI, RBX). Furthermore, we studied the signaling pathways involved by assaying expression and activation of different kinases.

MATERIALS AND METHODS

Animal Treatment and Preparation of Homogenate and Nuclear Fraction

Experiments complied with guidelines for use of experi-mental animals of European Community Council Directive 86/609/EEC. Groups of 12 male Sprague–Dawley rats (170– 200 g) were anesthetized and subcutaneously implanted with osmotic minipumps Alzet 2ML2 (release 5 μl/h, capacity 2 ml) (Charles River, Italy), containing either vehicle (5% ethanol) or FLX (a selective 5-HT reuptake inhibitor), DMI (a tricyclic drug mainly inhibiting NA release) or RBX (a selective NA reuptake inhibitor). The drug dosage was 10 mg/kg pro die. Acute treatment was carried out injecting rats (250–270 g) intraperitoneally. Animals were killed after 3 h for acute and after 14 days for chronic treatment; hippocampus (HI) and the whole frontal lobe, referred to as prefrontal/frontal cortex (PFCX), were quickly excised on ice and homogenized 1 : 10 (w/v) by a loose-fitting Potter in homogenization buffer (HB), 0.28 M sucrose, 10 mM HEPES pH 7.4, 0.1 mM EGTA, 20 mM NaF, 5 mM Na_2PO_4, 1 mM Na_2VO_4, and 2 μl/ml of protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). Total homogenates were centrifuged 5 min at 1000 *g*, and the resulting pellets, enriched in nuclei (P1), were resuspended in lysis buffer (LB), 120 mM NaCl, 20 mM HEPES pH 7.4, 0.1 mM EGTA, 0.1 mM DTT, protease and phosphatase inhibitors as in HB.

Real-Time RT-PCR

PCR was carried out using a LightCycler rapid thermal cycler System (Roche Diagnostics, Mannheim, Germany), and the detection was performed by measuring binding of the fluorescent dye SYBR Green I to double-strand DNA. The PCR reaction was set up into microcapillary tubes in a volume of 20 μl with 2 μl of cDNA and 2 μl of 1 × DNA Master SYBR Green I (Roche Diagnostics). In Table 1, an overview of primer and PCR conditions used in this study is reported for CREB and *β*-actin genes. The PCR program also included an initial denaturation step (30 s) followed by 45 cycles. At the end of each cycle, the fluorescence emitted by SYBR Green was measured. After compilation of the cycling process, samples were subjected to a temperature ramp (from 70 to 95°C at 2°C/s) with continuous fluorescence monitoring for melting curve analysis. For each PCR product, a single narrow peak was obtained by melting curve analysis at the specific temperature. Each sample was assayed in duplicate and the analysis was performed for CREB normalized to the *β*-actin gene with the Light Cycler Relative Quantification Software. This new software measures kinetic PCR quantitation at each cycle, during the log-linear phase of a PCR reaction. Data obtained were analyzed with unpaired *t*-test calculated with SPSS software version 10.0.

Table 1 Primer and PCR Conditions Used

Gene	Primer sequence	Primer conc.	Product size (bp)	Annealing temp. (°C)/time (s)	Extension temp. (°C)/time (s)	Acquisition temp.
β-actin	F: GGGAAATCGTGCGTGACATT R: CGGATGTCAACGTCACACTT	2 μ (10 μM)	252	58/8	72/12	88°C
CREB1	F: TACCCAGGGAGGAGCAATAC R: GAGGCAGCTTGAACAACAAC	0.3 μ (10 μM)	183	57/15	72/10	86.5°C

CaMKIV Immunoprecipitation

CaMKIV was immunoprecipitated from 150 μg of total homogenate or P1 proteins using a polyclonal antibody, as described previously (Kasahara et al, 1999). The samples were incubated with antibody in 50 mM Tris pH 7.5, 500 mM NaCl, 10 mM EDTA, 4 mM EGTA, 1 mM Na_3VO_4, 20 mM $Na_2P_2O_4$, 5 mM NaF, 1 mM DTT, 0.5% Triton X-100, 100 nM calyculin A, protease inhibitor cocktail, protein A–sepharose (Sigma-Aldrich) for 4 h at 4°C. The beads were washed with 50 mM HEPES pH 7.5, 1 mM EDTA, 1 mM DTT, 1 mM Na_3VO_4, 20 mM $Na_2P_2O_4$, 5 mM NaF, 100 nM calyculin A, and centrifuged. Immunoprecipitates were used for assay of CaMKIV activity.

Assay of CaMKIV Activity

Immunoprecipitated CaMKIV enzymatic activity was measured by assaying phosphate incorporation in the selective substrate peptide-γ (Primm, Milan, Italy) (Kasahara et al, 1999). The reactions were carried out in standard phosphorylation buffer (50 mM HEPES, 10 mM Mg acetate, 100 μM calyculin A, 1 mM Na_3VO_4, 100 μM peptide-γ, 0.5 mM $CaCl_2$, 0.3 μM CaM (Biomol, Plymouth Meeting, PA, USA), and 100 μM $[\gamma\text{-}^{32}P]ATP$ (200–500 cpm/pmol, Amersham Biosciences, Italy) for 10 min at 30°C and stopped by ice-cold TCA (final concentration 5%). After centrifugation, 25 μl of supernatant were spotted on phosphocellulose P81 paper (Whatman, Maidstone, UK). Filters were washed in 75 mM phosphoric acid, dried, and counted for liquid scintillation. Blanks were incubated in the absence of peptide.

Assay of PKA Basal and cAMP-Stimulated Activity

PKA enzymatic activity was assayed using PKA Assay Kit (Upstate Biotechnology, Lake Placid, NY, USA) following the manufacturer's instructions. Protein/sample (10 and 20 μg) were used for stimulated and basal activity, respectively.

Western Analysis

Western analysis was carried out as described previously (Celano et al, 2003), by incubating PVDF membranes, containing electrophoresed proteins from either total homogenates or P1 nuclear fractions, with monoclonal antibodies for α-CaMKIV (Transduction Laboratories, Lexington) 1:1000, CREB and phospho-Ser[133] CREB 1:1000, polyclonal for p44/42 and monoclonal for phospho-p44/42 MAPK (Thr[202]/Tyr[204]) 1:1000 (Cell Signaling, Beverly, MA), monoclonal for β-actin 1:5000 (Sigma-Aldrich), polyclonal for phospho-Thr[196] α-CaMKIV 1:500, polyclonal for CaMK kinase (CaMKK) β and CaMKK α 1:300, and polyclonal for ribosomal S6 kinases (Rsk)2 phosphorylated in Ser[227] 1:500 (courtesy of Andre' Hanauer, INSERM, Strasbourg). Following incubation with peroxidase-coupled secondary antibodies, protein bands were detected by using ECL (Amersham). Standard curves were obtained by loading increasing amounts of samples on gels as described previously (Verona et al, 2000). All protein bands used were within the linear range of standard curves

and normalized for actin level in the same membrane. Standardization and quantitation was as reported previously, except that Quantity One software (BioRad Laboratories, Italy) was used. Phospho-CREB (pCREB) and pCaMKIV results were further analyzed by normalizing each phosphoprotein value by dividing for respective total protein mean value.

For preparation of anti-CaMKK antibodies, the following peptides conjugated to keyhole lympet hemocyanine were synthesized and served as the immunogen: CGEGGK-SPELPGVQEDEAAS, corresponding to residues 486–505 of rat CaMKK α (Tokumitsu et al, 1995); SEPKEARQRRQPPG-PRASPC, corresponding to residues 528–547 of rat CaMKK β (Kitani et al, 1997). The injection of peptides into rabbits and preparation of the antisera were performed according to the methods previously reported (Kasahara et al, 1999). In short, the antisera were purified as pellets of an ammonium sulfate cut and dissolved in phosphate-buffered saline containing 0.02% sodium azide.

Statistical Analysis

All data from assays of kinase activities and from Western blotting were analyzed by using one-way ANOVA followed by Newman–Keuls post hoc test.

RESULTS

FLX and Pronoradrenergic (PNA) Antidepressants Selectively Affect CREB Expression and Phosphorylation

In order to assess the effect of different classes of antidepressant drugs on CREB, we measured the expression of mRNA, protein expression level, and phosphorylation state of Ser[133] in HI and PFCX of rats acutely or chronically treated with FLX, DMI, and RBX. All mRNA values were normalized for β-actin mRNA in the same samples. No changes in any of the molecules and effectors reported below were observed following acute treatment with the drugs used (not shown).

Following chronic treatments, a general increase in mRNA for CREB was observed with all drugs. In HI, mRNA was significantly increased after treatment with FLX and RBX (Figure 1a). In PFCX, a trend toward the upregulation of CREB mRNA was observed with all the drugs tested, although a significant increase was seen only with RBX (Figure 1a).

The protein expression levels and Ser[133] phosphorylation of CREB were measured by Western analysis in nuclear-enriched fractions. As shown in Figure 1b, the results were somewhat different from CREB mRNA measurements. In HI, a slight, significant increase in CREB protein level was observed only after chronic treatment with DMI, but not with other drugs. In PFCX, both DMI and RBX induced a significant increase in CREB levels. In both areas FLX had no effect on CREB protein level (Figure 1b). Since transcriptional activity of CREB mainly depends on its phosphorylation in Ser[133], this was measured in the same nuclear fractions by using an antibody directed against pCREB. As illustrated in Figure 1c, most antidepressant treatments led to an increase of pCREB in the nuclear fraction. However, in HI this was significant only for FLX.
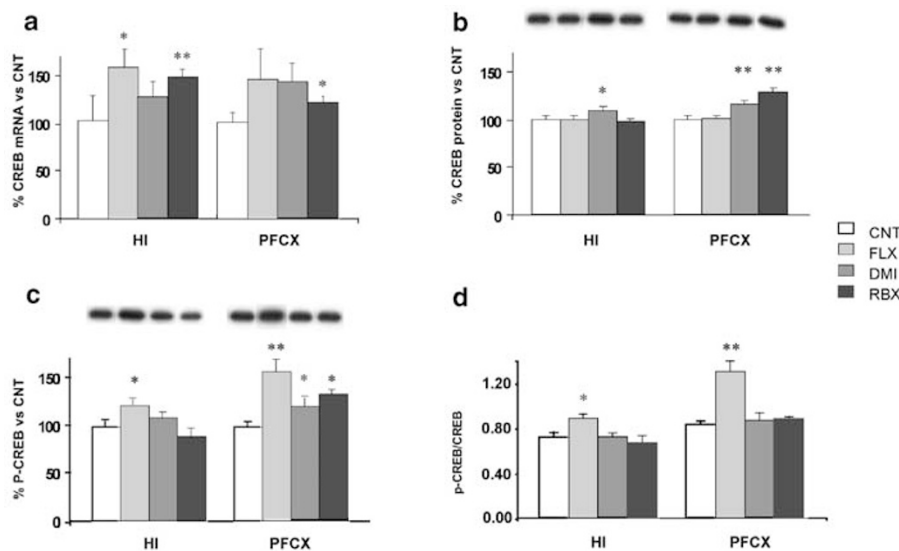
Figure 1 Effects of chronic antidepressants on CREB expression and phosphorylation. (a) CREB mRNA expression was measured in HI and PFCX from rats chronically treated with vehicle (CNT), FLX, DMI, and RBX, by real-time RT-PCR. All mRNA values were normalized for β-actin mRNA in the same sample. A general increase in CREB mRNA was observed with all drugs, significant in HI with FLX and RBX, and in PFCX with RBX. (b) Western analysis of the nuclear fractions showed a slight but significant increase of CREB protein level in HI after chronic treatment with DMI. In PFCX, both DMI and RBX significantly increased CREB protein level. (c) CREB phosphorylation on Ser[133] was measured in nuclear fraction by using a monoclonal antibody. In HI, a significant increase in pCREB was observed only with FLX. In PFCX, although all drugs significantly increased pCREB, FLX induced a more marked increase. (d) pCREB signal was normalized on respective total CREB signal in order to assess the actual fraction of phosphorylated protein; the ratio pCREB/CREB was significantly increased only by FLX. Data shown as mean $\pm$ SEM of the control. $*p < 0.05$; $**p < 0.01$.

In PFCX, although all drugs induced a significant increase, FLX induced a more marked increase in pCREB compared to PNA drugs. These data suggested a more marked effect of FLX on Ser[133] phosphorylation. Indeed, in PFCX the percent increase of pCREB for PNA drugs was quite similar to the increase of total CREB protein (compare Figure 1b and c). To assess the actual change in the fraction of total protein that is phosphorylated (and thus activated), for each drug we normalized pCREB signal on respective total CREB signal (see Materials and methods). The result of this procedure, shown in Figure 1d, suggested that in both brain areas FLX led to a selective increase in the phosphorylation of this transcription factor. Overall, these results suggested that while DMI increased total CREB level in both areas and RBX in PFCX, only FLX actually increased the ratio of pCREB/total CREB, without affecting the protein expression level.

PNA Antidepressants Increase Nuclear PKA Activity in HI, but not in PFCX

Next, the effect of different drugs on signaling cascades regulating CREB phosphorylation and transcriptional activation was investigated. Our aim was to study whether selective activation of different kinase cascades was responsible for selective phosphorylation of CREB by chronic FLX treatment. First, the basal and cAMP-stimulated PKA enzymatic activity was measured by peptide substrate assay in both homogenates and nuclear fractions from HI and PFCX of drug-treated and control rats. It is known that, in order to phosphorylate CREB, the catalytic subunit of PKA must translocate to the nucleus (Hagiwara

et al, 1992, 1993). We hypothesized that, if such transloca-tion was induced by drug treatments, we should observe an increase of PKA activity in the nuclear fraction (henceforth referred to as the nuclei) compared to homogenate, particularly in PFCX (Nestler *et al*, 1989).

As shown in Figure 2a, in homogenate from HI only RBX lead to a significant increase in the basal PKA enzymatic activity, with no significant modifications in cAMP-stimulated PKA activity. In homogenate from PFCX (Figure 2a), PKA basal activity was increased by all drugs (particularly RBX), whereas only a slight increase of cAMP-stimulated activity was observed after chronic FLX (Figure 2a). In the nuclei from HI (Figure 2b), chronic DMI significantly increased basal PKA activity, whereas both DMI and RBX increased stimulated PKA activity. In the nuclei from PFCX, again RBX lead to a significant increase of both basal and stimulated PKA activity; the latter was also increased by chronic DMI. Interestingly, FLX was the only drug that did not increase PKA activity in the nuclei from both HI and PFCX (Figure 2b). Furthermore in PFCX nuclei, we found little or no increase of PKA activity induced by DMI and RBX as compared to homogenate. These results suggested that, although PKA may be involved in nuclear pCREB changes observed with DMI and RBX, the marked pCREB increase observed after chronic FLX (Figure 1d) cannot be due to an increase of nuclear PKA activity.

FLX and PNA Antidepressants Markedly Activate CaMKIV in PFCX

There is compelling evidence that nuclear CaMKIV is crucial for the rapid activity-dependent phosphorylation of
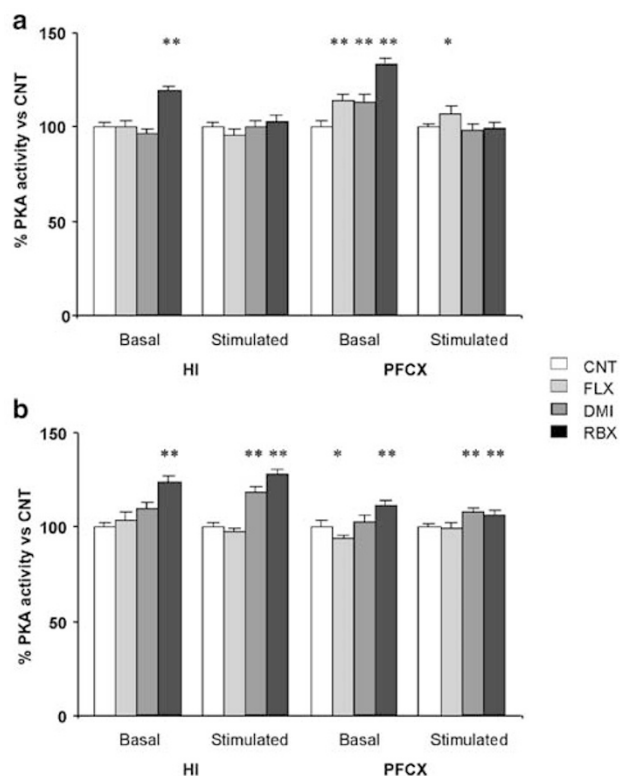
Figure 2 PNA antidepressants increase nuclear PKA activity in HI, but not in PFCX. (a) Total homogenates. In HI, a significant increase of PKA basal activity was observed only after treatment with RBX. In PFCX all drugs increased the basal PKA activity, whereas only FLX induced a slight increase in cAMP-stimulated activity. (b) Nuclear-enriched fractions. In HI, the basal activity of PKA was significantly increased by RBX, whereas both DMI and RBX increased cAMP-stimulated activity. In PFCX, RBX increased both basal and stimulated activity of PKA, DMI increased stimulated activity of the enzyme, and FLX led to a slight decrease of basal PKA activity. Data shown as % mean ± SEM of the control. *$p < 0.05$; **$p < 0.01$. Abbreviations as in Figure 1.

CREB at Ser[133] in neurons (Ghosh *et al*, 1994; Bito *et al*, 1996; West *et al*, 2002). However, the effect of antidepressant treatments on CaMKIV has not been investigated so far. This kinase was immunoprecipitated from the homogenate and nuclei as described previously, and its enzymatic activity measured by an assay using selective peptide substrate (Kasahara *et al*, 2001). In homogenate from HI-only DMI induced an increase in kinase activity, whereas all drugs increased the activity in PFCX (Figure 3a). In HI, no changes were found in the nuclei but all drugs tested induced a marked and significant increase in CaMKIV activity in PFCX (Figure 3b).

The modifications observed in the kinase enzymatic activity could be ascribed to changes in the protein levels of the kinase itself and/or to the phosphorylation of Thr[196] by CaMKK, a mechanism responsible for the activation of this enzyme (Soderling, 1999; West *et al*, 2002). With the exception of HI from FLX-treated animals, no significant change was observed in the protein level of CaMKIV in both HI and PFCX (Figure 3c). Conversely, a marked and significant increase was found in the Thr[196] phosphorylation of CaMKIV in PFCX (Figure 3d), a finding that is fully consistent with the general activation of the kinase induced
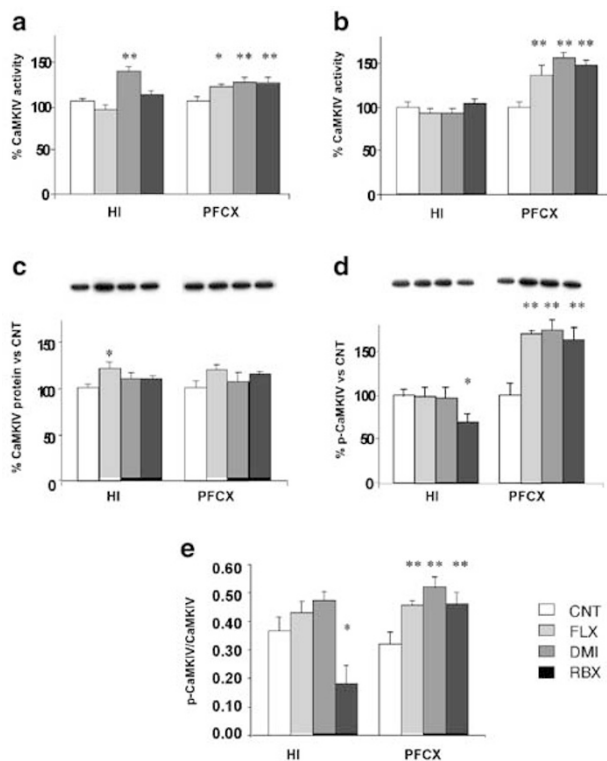

Figure 3 Chronic antidepressants increase nuclear CaMKIV activity in PFCX. (a) CaMKIV enzymatic activity in total homogenates. Enzymatic activity was measured by assaying phosphate incorporation in peptide substrate following immunoprecipitation of CaMKIV. Only DMI increased the kinase activity in HI, whereas a slight but significant increase was observed with all drugs in PFCX. (b) CaMKIV enzymatic activity in nuclear fractions. No changes were detected in HI, but all drugs tested induced a marked increase of CaMKIV activity in PFCX. (c) Total CaMKIV protein levels in nuclear fractions. A slight increase, statistically significant only in HI, was observed after chronic treatment with FLX. In PFCX, although there was a trend toward an increase of CaMKIV protein levels, no significant changes were detected. Representative immunoreactive bands are shown. (d) Thr[196] phosphorylation of CaMKIV in nuclear fractions. A marked increase after treatment with all drugs was observed in PFCX; pCaMKIV significantly decreased with RBX in HI. Data shown as % mean ± SEM of the control. *$p < 0.05$; **$p < 0.01$. Representative immunoreactive bands are shown. (e) pCaMKIV signal was normalized on respective total CaMKIV signal in order to assess the actual fraction of phosphorylated protein; the ratio p-CaMKIV/CaMKIV was significantly increased by all drugs in PFCX, and significantly reduced in HI by RBX. Data shown as mean ± SEM of the control. *$p < 0.05$; **$p < 0.01$.

here by all these drugs. Interestingly, as shown above, we found a more marked activation of CREB phosphorylation in this same area (Figure 1c). As we did for CREB (Figure 1d), we normalized pCaMKIV signal on respective total CaMKIV signal in order to assess the actual change in the fraction of total protein that is phosphorylated (and thus activated). The results, shown in Figure 3d and e, suggested that all drugs significantly increased CaMKIV phosphorylation in the nuclei from PFCX. In contrast, at present we have no explanation for the marked decrease in pCaMKIV with RBX in HI, although this does not seem to affect the kinase activity (Figure 3b).

As the drugs increased CaMKIV activity by altering its phosphorylation state and without affecting its level, we investigated the expression level of α- and β-CaMKK, the

enzymes known to phosphorylate (and activate CaMKIV) in the CaM kinase cascade (Soderling, 1999; West *et al*, 2002). We did not find changes in the levels of the two CaMKK isoforms (Figure 4), with the exception of a small (11%) but significant increase of β-CaMKK in PFCX after chronic FLX treatment. However, these results suggested that other

factors (ie phosphatases) could be responsible for the modifications found in CaMKIV activity.

FLX Selectively Increases the Levels of Erk1/2 in PFCX

The MAPK cascades are among the major pathways leading to the phosphorylation of CREB and modulation of transcriptional activity, mainly in response to growth factors, cytokines, and stress-induced signaling (Ginty *et al*, 1994; Reusch *et al*, 1994). Although increased production of the neurotrophin BDNF and stimulation of its receptor TrkB are purported as major components in the mechanism of action of antidepressants, the effect of these drugs on MAPK pathways have not been investigated. Recently, however, it was shown that lithium and valproate, two medications largely used for the treatment of manic-depressive illness, stimulate the Erk-MAPK pathway (Einat *et al*, 2003). We investigated the expression levels and the phosphorylation state (an index of activation) of Erk1/2 in the homogenate and nuclei of HI and PFCX from rats chronically treated with the three drugs as above. In homogenates, FLX markedly and significantly increased the protein level of both Erk1/2 in PFCX, but not in HI, whereas other drugs showed minor effects (Figure 5a). No significant changes were observed in the level of pErk1/2 (Figure 5b). In the nuclear fraction, no significant changes in expression were found, with a trend for a reduction of both Erk1/2 in PFCX with PNA drugs (Figure 5c). Furthermore, a general trend toward a decrease of Erk1 phosphorylation and an increase of Erk2 phosphorylation was found, with no apparent drug selectivity (Figure 5d). Overall, the most interesting finding in this pathway was the
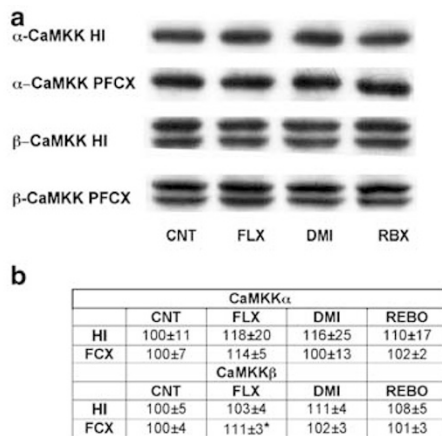
Figure 4 Protein expression levels of α- and β-CaMKK following chronic antidepressants. Protein expression levels of α- and β-CaMKK, the enzymes known to phosphorylate and thus activate CaMKIV, were assessed in total homogenates from HI and PFCX of treated animals. (a) Representative immunoreactive bands of α- and β-CaMKK in HI and PFCX. (b) No significant changes were observed for the two kinase isoforms, except for a slight (but significant) increase of β-CaMKK in PFCX after FLX treatment. Data shown as % mean $\pm$ SEM of the control. *$p < 0.05$.
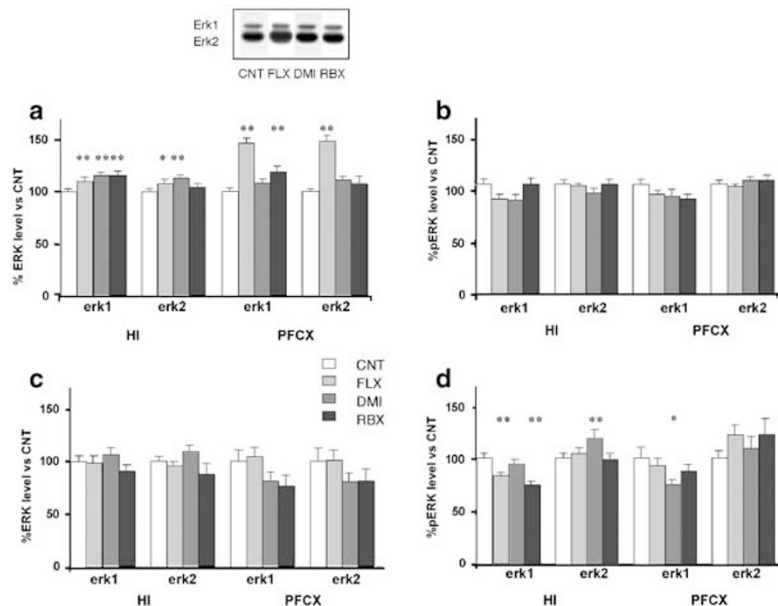
Figure 5 Chronic antidepressants increase Erk1/2 total levels in PFCX. The expression level and phosphorylation state of Erk1/2 were investigated in total homogenates and nuclear fractions of HI and PFCX of the control and treated rats (A and B: total homogenates; B and C: nuclear fractions). (a) Total Erk1/2 levels. In HI slight changes were observed after chronic antidepressants, whereas FLX induced a marked increase of both ERKs in PFCX. Inset: representative immunoblot of Erk1/2 in total homogenate from PFCX in vehicle- (CNT) and drug-treated rats (FLX, DMI, RBX). (b) Phosphorylation of Erk1/2. No significant modification was observed in the homogenates. (c) Total Erk1/2 levels. In nuclear fractions no significant changes were detected in the total levels of both Erk1/2 in both HI and PFCX. (d) Phosphorylation of Erk1/2. A general trend toward a reduction was observed in the phosphorylation of Erk1 in both HI and PFCX, with no apparent drug selectivity. Data shown as % mean $\pm$ SEM of the control. *$p < 0.05$; **$p < 0.01$.

selective increase of Erk1/2 expression induced by FLX; again, this was restricted to PFCX, the area where FLX maximally stimulates CREB phosphorylation.

CREB is not a direct substrate of Erk kinases. Activation of Erk1/2 was shown to induce, in turn, other families of downstream kinases, which carry out the final step of CREB phosphorylation in the nucleus (De Cesare *et al*, 1999; West *et al*, 2002; Lonze and Ginty, 2002). The best known of these is the family of Rsk; Rsk2 was identified as the main kinase responsible for CREB phosphorylation in response to NGF or EGF stimulation; loss of Rsk2 activity impairs CREB phosphorylation and transcriptional induction of *fos* by EGF (Xing *et al*, 1996; De Cesare *et al*, 1998). We investigated whether upregulation of Erk1/2 induced by FLX in PFCX affects the activation of Rsk2, by using Western analysis with an antibody directed against the N-terminal domain containing phospho-Ser[227] of Rsk2, which mediates substrate phosphorylation (Merienne *et al*, 2000). We found no changes in the phosphorylation of Rsk2 (not shown).

DISCUSSION

The main results of this work may be summarized as follows:

1. Chronic treatment with a proserotonergic (PST) antidepressant (FLX) and two PNA antidepressants (DMI and RBX) differently affected the expression and phosphorylation of CREB. Most effects were observed in PFCX, where DMI and RBX increased total CREB protein, while FLX selectively and markedly increased pCREB.
2. Upon assay of both basal and cAMP-stimulated activity of PKA, this kinase did not appear to be responsible for the selective increase of nuclear pCREB induced by FLX, because we found no consistent increase of kinase activity in the nuclear fraction of rats treated with this drug.
3. Both PST and PNA antidepressants markedly activated nuclear CaMKIV in PFCX, by increasing its Thr[196] phosphorylation presumably induced by CaMKK. The expression levels of both CaMKIV and α- and β-CaMKK were unchanged, except for a slight increase in β-CaMKK in PFCX. These results represent the first evidence for involvement of CaMKIV cascade in antidepressant mechanisms.
4. FLX selectively increased the expression level of MAP kinases Erk1/2 in PFCX, without affecting Erk1/2 phosphorylation state. This result represents the first finding for involvement of Erk1/2 in antidepressant mechanisms.

Selective Action of Antidepressants on CREB

Overall, our results would suggest area- and drug-selective effects for different antidepressants. Previous studies suggested selectivity in the action of antidepressants on CREB phosphorylation and CRE-mediated gene expression, showing that FLX is more effective than DMI in some brain areas (Frechilla *et al*, 1998; Thome *et al*, 2000). Further-

more, a recent study found a decrease of pCREB in the frontal cortex from rats chronically treated with DMI or RBX (Manier *et al*, 2002). Although in this work a PST drug was not tested, those findings in principle would not disagree with the concept that chronic FLX is more effective than PNA drugs on CREB phosphorylation. Our present results are in line with this concept, and strenghten the idea that different classes of antidepressants have distinct effects on transcriptional mechanisms.

Selective and Nonselective Action of Antidepressants on Signaling Pathways Regulating CREB

If different antidepressants exert distinct effects on CREB function, this could be accounted for by a different degree of activation of selected signaling pathways regulating CREB. We examined the three main pathways that were previously shown to regulate neuronal CREB function and gene expression in response to a wide variety of stimuli, namely, cAMP-PKA, CaMK, and MAPK-Erk pathways (De Cesare *et al*, 1999; West *et al*, 2002; Lonze and Ginty, 2002). We sought to understand what is different in the action of PST and PNA drugs on the various pathways.

First, we assayed PKA activity in both the homogenate and nuclear fraction of drug-treated rats, because it has been suggested that antidepressant-induced pCREB increase is mainly due to the activation of adenylyl cyclase linked to G-protein-coupled receptors and consequent upregulation of cAMP-PKA cascade (Duman *et al*, 1999; Manji *et al*, 2003). We speculated that if PKA was activated to a greater extent by FLX in the nuclear fraction, this could account for the selective increase induced by this drug in pCREB. We found that this is not the case and that actually RBX and DMI, but not FLX, increase the activity of nuclear PKA. Furthermore, in PFCX nuclear fraction the kinase was less activated compared to PFCX homogenate, ruling out a primary role for PKA in drug-induced phosphorylation of CREB in this area.

Next, we investigated the CaM kinase cascade. Early studies showed that depolarization and activation of neurotransmitter receptors activate the expression of immediate-early genes in a calcium-dependent manner (Morgan and Curran, 1986; Sheng *et al*, 1990; Bading and Greenberg, 1991), and that CREB, besides functioning as a cAMP-inducible transcription factor, also works as a calcium-inducible factor (Sheng *et al*, 1991; Dash *et al*, 1991). Three calcium/CaM-dependent kinases have been shown to phosphorylate CREB in response to calcium and synaptic activity, CaMK I, II, and IV (Bading *et al*, 1993). CaMKIV has emerged as the major CREB kinase in activity-dependent neuronal gene expression (West *et al*, 2002; Lonze and Ginty, 2002). CaMKIV has a pronounced nuclear localization, its kinetics of activation correlates with that of CREB phosphorylation, and cotransfection of cotistutively active kinase drives CRE/CREB-dependent gene expression and inhibition of its function inhibits depolarization-induced CREB phosphorylation (Ghosh *et al*, 1994; Enslen *et al*, 1994; Nakamura *et al*, 1995; Bito *et al*, 1996). Furthermore, *in vitro* studies investigating the regulation of CREB phosphorylation in response to either electrical stimulation of neurons or induction of long-term potentiation (LTP, a paradigm of synaptic plasticity) showed that

CREB phosphorylation in both experimental paradigms was mainly blocked by CaMK inhibitors and not by the inhibition of other kinases (including PKA) (Bito et al, 1996; Kasahara et al, 2001). Therefore, it appears that CaMKIV has a primary role in neuronal activity-dependent phosphorylation of CREB (West et al, 2002; Lonze and Ginty, 2002).

The marked activation of nuclear CaMKIV in PFCX, induced by all drugs we tested, strongly suggests that activation of this kinase is a common effect of different antidepressants. However, additional mechanisms must be activated by FLX with respect to DMI and RBX, because in our hands only the former drug efficiently increased CREB phosphorylation (see below). Our results also clearly show that activation of CaMKIV is due to increased phosphorylation of the regulatory site Thr^{196} by CaMKK and not by changes in the kinase expression. As reported above, we also found no major changes in the expression of α- and β-CaMKK, which are known to phosphorylate and activate CaMKIV in response to stimulation or elevation of calcium fluxes (Soderling, 1999). Therefore, it is possible that the sustained activation of nuclear CaMKIV we observed is due to a decreased function of one or more phosphatases that have been found to interact with the kinase (Westphal et al, 1998; Kasahara et al, 1999; Takeuchi et al, 2001). Decreased dephosphorylation rate of CaMKIV could also explain why this kinase, normally transiently activated in neurons (West et al, 2002), shows a sustained activation following antidepressant treatments. Future experiments will address these issues.

The third kinase cascade we investigated is the MAPK-Erk1/2 cascade. Several lines of evidence showed recently that neuronal activity-dependent phosphorylation of CREB Ser^{133} is induced by sequential activation of CaMK and MAPK cascades (West et al, 2002). A few minutes after neuronal stimulation calcium/CaM-dependent activation of CaMK cascade induces rapid phosphorylation of CREB. Several minutes later MAPK cascade is activated and this event is necessary in order to induce sustained CREB phosphorylation. If MAPK cascade is not activated, CREB phosphorylation is transient and transcription is not activated (West et al, 2002). Our finding that selective induction of CREB phosphorylation by FLX is accompanied by both activation of CaMKIV (all drugs) and selective upregulation of Erk1/2 (FLX only) may suggest a combined action of these two pathways in the chronic action of FLX, although these data are not sufficient to restrict the induction of pCREB to these two pathways. It could be envisaged that additional pathways are involved in this mechanism. Furthermore, it is not clear how Erk1/2 upregulation may affect CREB phosphorylation, because these kinases do not phosphorylate CREB directly. As reported above, Rsk2, the best known CREB kinase activated by Erk1/2, was unchanged after treatment with all drugs we tested. Other downstream nuclear kinases could be involved, such as mitogen- and stress-activated protein kinase 1/2 (Msk1/2), recently identified as CREB kinases activated by either Erk1/2 or SAPK2/p38 (Deak et al, 1998).

We are currently investigating the action of antidepressants on these several signaling pathways in cultured neuroblastoma cells, a model that will allow a better molecular dissection of these effects. However, our results with drug-treated animals suggest that CaMK and MAPK/Erk cascades play a major role in CREB phosphorylation induced by chronic antidepressants. Contrary to previous speculations, the cAMP-PKA cascade does not seem to have a primary role in this effect of antidepressants. Rather, this cascade was clearly shown to be activated at different neuronal compartments, such as microtubules (Perez et al, 2000). Of course, additional experiments with different drugs, such as different SSRI (selective serotonin reuptake inhibitors) as well as MAO (monoamine oxidase) inhibitors will be necessary to extend these observations. We believe that this work may help to dissect common and distinct effects of antidepressants and contribute to the identification of new targets for faster and more efficient treatments.

REFERENCES

Bading H, Ginty DD, Greenberg ME (1993). Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. Science 260: 181–186.

Bading H, Greenberg ME (1991). Stimulation of protein tyrosine phosphorylation by NMDA receptor activation. Science 253: 912–914.

Bito H, Deisseroth K, Tsien RW (1996). CREB phosphorylation and dephosphorylation: a Ca(2+)- and stimulus duration-dependent switch for hippocampal gene expression. Cell 87: 1203–1214.

Celano E, Tiraboschi E, Consogno E, D'Urso G, Mbakop MP, Gennarelli M et al (2003). Selective regulation of presynaptic calcium/calmodulin-dependent protein kinase II by psychotropic drugs. Biol Psychiatry 53: 442–449.

Chen AC, Shirayama Y, Shin K, Neve RL, Duman RS (2001). Expression of the cAMP response element binding protein (CREB) in hippocampus produces an antidepressant effect. Biol Psychiatry 49: 753–762.

Chen J, Rasenick MM (1995). Chronic antidepressant treatment facilitates G protein activation of adenylyl cyclase without altering G protein content. J Pharmacol Exp Ther 275: 509–517.

Dash PK, Karl KA, Colicos MA, Prywes R, 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.

Deak M, Clifton AD, Lucocq LM, Alessi DR (1998). Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. EMBO J 17: 4426–4441.

De Cesare D, Fimia GM, Sassone-Corsi P (1999). Signaling routes to CREM and CREB: plasticity in transcriptional activation. Trends Biochem Sci 24: 281–285.

De Cesare D, Jacquot S, Hanauer A, Sassone-Corsi P (1998). Rsk-2 activity is necessary for epidermal growth factor-induced phosphorylation of CREB protein and transcription of c-fos gene. Proc Natl Acad Sci USA 95: 12202–12207.

Donati RJ, Rasenick MM (2003). G protein signaling and the molecular basis of antidepressant action. Life Sci 73: 1–17.

Dowlatshahi D, MacQueen GM, Wang JF, Young LT (1998). Increased temporal cortex CREB concentrations and antidepressant treatment in major depression. *Lancet* **352**: 1754–1755.

Duman RS, Malberg J, Thome J (1999). Neural plasticity to stress and antidepressant treatment. *Biol Psychiatry* **46**: 1181–1191.

Dwivedi Y, Rao JS, Rizavi HS, Kotowski J, Conley RR, Roberts RC et al (2003). Abnormal expression and functional characteristics of cyclic adenosine monophosphate response element binding protein in postmortem brain of suicide subjects. *Arch Gen Psychiatry* **60**: 273–282.

Einat H, Yuan P, Gould TD, Li J, Du J, Zhang L et al (2003). The role of the extracellular signal-regulated kinase signaling pathway in mood modulation. *J Neurosci* **23**: 7311–7316.

Enslen H, Sun P, Brickey D, Soderling SH, Klamo E, Soderling TR (1994). Characterization of Ca^{2+}/calmodulin-dependent protein kinase IV. Role in transcriptional regulation. *J Biol Chem* **269**: 15520–15527.

Frechilla D, Otano A, Del Rio J (1998). Effect of chronic antidepressant treatment on transcription factor binding activity in rat hippocampus and frontal cortex. *Prog Neuropsychopharmacol Biol Psychiatry* **22**: 787–802.

Ghosh A, Ginty DD, Bading H, Greenberg ME (1994). Calcium regulation of gene expression in neuronal cells. *J Neurobiol* **25**: 294–303.

Ginty DD, Bonni A, Greenberg ME (1994). Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. *Cell* **77**: 713–725.

Hagiwara M, Alberts A, Brindle P, Meinkoth J, Feramisco J, Deng T et al (1992). Transcriptional attenuation following cAMP induction requires PP-1-mediated dephosphorylation of CREB. *Cell* **70**: 105–113.

Hagiwara M, Brindle P, Harootunian A, Armstrong R, Rivier J, Vale W et al (1993). Coupling of hormonal stimulation and transcription via the cyclic AMP-responsive factor CREB is rate limited by nuclear entry of protein kinase A. *Mol Cell Biol* **13**: 4852–4859.

Kandel ER (2001). The molecular biology of memory storage: a dialogue between genes and synapses. *Science* **294**: 1030–1038.

Kasahara J, Fukunaga K, Miyamoto E (1999). Differential effects of a calcineurin inhibitor on glutamate-induced phosphorylation of Ca^{2+}/calmodulin-dependent protein kinases in cultured hippocampal neurons. *J Biol Chem* **274**: 9061–9067.

Kasahara J, Fukunaga K, Miyamoto E (2001). Activation of calcium/calmodulin-dependent protein kinase IV in long term potentiation in the rat hippocampal CA1 region. *J Biol Chem* **276**: 24044–24050.

Kida S, Josselyn SAV, de Ortiz SP, Kogan JH, Chevere I, Masushige S et al (2002). CREB required for the stability of new and reactivated fear memories. *Nat Neurosci* **5**: 348–355.

Kitani T, Okuno S, Fujisawa H (1997). Molecular cloning of Ca^{2+}/calmodulin-dependent protein kinase kinase beta. *J Biochem* **122**: 243–250.

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

Mabuchi T, Kitagawa K, Kuwabara K, Takasawa K, Ohtsuki T, Xia Z et al (2001). Phosphorylation of cAMP response element-binding protein in hippocampal neurons as a protective response after exposure to glutamate *in vitro* and ischemia *in vivo*. *J Neurosci* **21**: 9204–9213.

Manier DH, Shelton RC, Sulser F (2002). Noradrenergic antidepressants: does chronic treatment increase or decrease nuclear CREB-P? *J Neural Transm* **109**: 91–99.

Manji HK, Quiroz JA, Sporn J, Payne JL, Denicoff KA, Gray N et al (2003). Enhancing neuronal plasticity and cellular resilience to develop novel, improved therapeutics for difficult-to-treat depression. *Biol Psychiatry* **53**: 707–742.

Merienne K, Jacquot S, Zeniou M, Pannetier S, Sassone-Corsi P, Hanauer A (2000). Activation of RSK by UV-light: phosphorylation dynamics and involvement of the MAPK pathway. *Oncogene* **19**: 4221–4229.

Morgan JI, Curran T (1986). Role of ion flux in the control of c-fos expression. *Nature* **322**: 552–555.

Nakamura Y, Okuno S, Sato F, Fujisawa H (1995). An immunohistochemical study of Ca^{2+}/calmodulin-dependent protein kinase IV in the rat central nervous system: light and electron microscopic observations. *Neuroscience* **68**: 181–194.

Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM (2002). Neurobiology of depression. *Neuron* **34**: 13–25.

Nestler EJ, Terwilliger RZ, Duman RS (1989). Chronic antidepressant administration alters the subcellular distribution of cyclic AMP-dependent protein kinase in rat frontal cortex. *J Neurochem* **53**: 1644–1647.

Nibuya M, Nestler EJ, Duman RS (1996). Chronic antidepressant administration increases the expression of cAMP response element binding protein (CREB) in rat hippocampus. *J Neurosci* **16**: 2365–2372.

Perez J, Tardito D, Mori S, Racagni G, Smeraldi E, Zanardi R (2000). Abnormalities of cAMP signaling in affective disorders: implication for pathophysiology and treatment. *Bipolar Disorders* **2**: 27–36.

Perez J, Tinelli D, Brunello N, Racagni G (1989). cAMP-dependent phosphorylation of soluble and crude microtubule fractions of rat cerebral cortex after prolonged desmethylimipramine treatment. *Eur J Pharmacol* **172**: 305–316.

Pittinger C, Huang YY, Paletzki RF, Bourtchouladze R, Scanlin H, Vronskaya S et al (2002). Reversible inhibition of CREB/ATF transcription factors in region CA1 of the dorsal hippocampus disrupts hippocampus-dependent spatial memory. *Neuron* **34**: 447–462.

Popoli M, Brunello N, Perez J, Racagni G (2000). Second messenger-regulated protein kinases in the brain: their functional role and the action of antidepressant drugs. *J Neurochem* **74**: 21–33.

Reppert SM, Weaver DR (2001). Molecular analysis of mammalian circadian rhythms. *Annu Rev Physiol* **63**: 647–676.

Reusch JE, Hsieh P, Klemm D, Hoeffler J, Draznin B (1994). Insulin inhibits dephosphorylation of adenosine 3′,5′-monophosphate response element-binding protein/activating transcription factor-1: effect on nuclear phosphoserine phosphatase-2a. *Endocrinology* **135**: 2418–2422.

Schwaninger M, Schofl C, Blume R, Rossig L, Knepel W (1995). Inhibition by antidepressant drugs of cyclic AMP response element-binding protein/cyclic AMP response element-directed gene transcription. *Mol Pharmacol* **47**: 1112–1118.

Sheng M, McFadden G, Greenberg ME (1990). Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. *Neuron* **4**: 571–582.

Sheng M, Thompson MA, Greeberg ME (1991). CREB: a Ca^{2+}-regulated transcription factor phosphorylated by calmodulin-dependent kinase. *Science* **252**: 1427–1430.

Soderling TR (1999). The Ca^{2+}/calmodulin-dependent protein kinase cascade. *TIBS* **24**: 232–236.

Takeuchi F, Ishida A, Kameshita I, Kitani T, Okuno S, Fujisawa H (2001). Identification and characterization of CaMKP-N, nuclear calmodulin-dependent protein kinase phosphatase. *J Biochem* **130**: 833–840.

Tardito D, Tiraboschi E, Perez J, Racagni G, Popoli M (2004). Signaling pathways regulating gene expression, neuroplasticity and neurotrophic mechanisms in the action of antidepressants. A critical overview. *Psychopharmacol Bull* **38**: in press.

Thome J, Sakai N, Shin K, Steffen C, Zhang YJ, Impey S et al (2000). cAMP response element-mediated gene transcription is

upregulated by chronic antidepressant treatment. *J Neurosci* **20**: 4030–4036.

Tokumitsu H, Enslen H, Soderling TR (1995). Characterization of a Ca^{2+}/calmodulin-dependent protein kinase cascade. Molecular cloning and expression of calcium/calmodulin-dependent protein kinase kinase. *J Biol Chem* **270**: 19320–19324.

Verona M, Zanotti S, Schafer T, Racagni G, Popoli M (2000). Changes of synaptotagmin interaction with t-SNARE proteins *in vitro* after calcium/calmodulin-dependent phosphorylation. *J Neurochem* **74**: 209–221.

West AE, Griffith EC, Greenberg ME (2002). Regulation of transcription factors by neuronal activity. *Nat Rev* **3**: 921–931.

Westphal RS, Anderson KA, Means AR, Wadzinski BE (1998). A signaling complex of Ca^{2+}-calmodulin-dependent protein kinase IV and protein phosphatase 2A. *Science* **280**: 1258–1261.

Xing J, Ginty DD, Greenberg ME (1996). Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* **273**: 959–963.