Neuropsychopharmacology (2005) 30, 360–371

www.neuropsychopharmacology.org

Electroconvulsive Seizures Increase the Expression of MAP Kinase Phosphatases in Limbic Regions of Rat Brain

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The mitogen-activated protein (MAP) kinase cascades regulate a variety of cellular activities, including cell growth, proliferation, and apoptosis, and are reported to play a role in the actions of antidepressant treatment. There are a number of different classes of protein phosphatases that could influence the MAP kinase cascade. One of these, the MAP kinase phosphatase (MKP) family, is known to play a key role in dephosphorylation of activated MAP kinase. In the present study, we analyzed the expression of the MKP1, MKP2, and MKP3 isoforms in rat brain after electroconvulsive seizure (ECS), considered the most effective treatment for depression. *In situ* hybridization analysis demonstrates that ECS differentially regulates the expression of the MKP isoforms. Expression of MKP1 mRNA is robustly increased by acute ECS in the major cell layers of the hippocampus, including the dentate gyrus granule cell layer and the CA1 and CA3 pyramidal cell layers. In contrast, MKP2 is induced mainly in the dentate gyrus and MKP3 is preferentially increased in the CA1 and CA3 cell layers. In the prefrontal cortex, all three MKP isoforms are upregulated by acute ECS administration. Chronic ECS resulted in a similar pattern of induction for each of the MKP subtypes, demonstrating that there is little or no desensitization of the response to repeated ECS. The induction of MKP expression serves as negative feedback control for the MAP kinase cascades. Upregulation of MKP expression could dampen the actions of ECS, indicating that blockade of the MKPs could enhance the actions of antidepressant treatment.
Neuropsychopharmacology (2005) **30,** 360–371, advance online publication, 20 October 2004; doi:10.1038/sj.npp.1300588

Keywords: mitogen-activated protein kinase phosphatase; extracellular signal-regulated kinase; antidepressant; mood disorder; fluoxetine; desipramine

INTRODUCTION

Depression is one of the most debilitating and common psychiatric diseases, reported to have a lifetime expectancy of approximately 17%. The pathogenesis of this disease is still unknown. However, recent studies have identified adaptations of intracellular signaling proteins and target genes in response to antidepressant treatment. One gene target of these signaling cascades is brain-derived neurotrophic factor (BDNF). Acute or chronic stress decreases levels of BDNF expression in the dentate gyrus and pyramidal cell layer of the hippocampus in rats (Smith *et al*, 1995). In contrast, electroconvulsive seizure (ECS) or chemical antidepressant administration increases BDNF expression in limbic structures (Nibuya *et al*, 1995). Postmortem studies also demonstrate that BDNF levels are increased in the hippocampus of humans who were taking an antidepressant at the time of death (Chen *et al*, 2001). A role for BDNF in the action of antidepressants is further supported by behavioral studies, demonstrating that infusion of BDNF into the midbrain (Siuciak *et al*, 1997) or hippocampus (Shirayama *et al*, 2002) produces an antidepressant-like response in behavioral models of depression and antidepressant actions, including the forced swim and learned helplessness paradigms.

BDNF influences cellular functions via activation of the tyrosine kinase receptor TrkB. TrkB receptors can activate a number of intracellular signaling cascades, including the Ras/extracellular signal-regulated kinase (ERK) pathway, the phosphatidylinositol-3-OH kinase/Akt kinase pathway, and phospholipase C-γ pathway. Among these pathways, studies to date have demonstrated a role for the ERK pathway in the actions of antidepressant treatment. Coadministration into the hippocampus of an inhibitor of mitogen-activated protein (MAP) kinase kinase or ERK kinase (MEK), which suppresses activation of ERK, blocks the antidepressant effect of BDNF (Shirayama *et al*, 2002). A post-mortem study has also found that levels of ERK activity and expression are decreased in the hippocampus and cerebral cortex of depressed suicide patients (Dwivedi *et al*, 2001). These results suggest that activation of the ERK

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Received 23 December 2003; revised 15 July 2004; accepted 31 August 2004
Online publication: 9 September 2004 at http://www.acnp.org/citations/Npp09090403574/default.pdf

kinase cascade plays a key role in the treatment and possibly pathophysiology of depression.

Activation of ERK requires its phosphorylation by MEK, a dual specific kinase, on both threonine and tyrosine residues (Chang and Karin, 2001; Theodosiou and Ashworth, 2002). The functional control of ERK is complex, as both the duration and magnitude of activation are important for determining the physiological and functional outcome for a given neuronal system. Dephosphorylation of ERK is thought to play a vital role in the control of kinase activity and there are a number of protein phosphatases that dephosphorylate either the threonine or tyrosine, or both residues (Theodosiou and Ashworth, 2002). A protein serine/threonine phosphatase, protein phosphatase 2A (PP2A), is known to be involved in dephosphorylating the phosphothreonine of ERK. Protein tyrosine phosphatases (PTPs) dephosphorylate the regulatory tyrosine residue, which is also sufficient to inactivate ERK.

Dual specificity phosphatases, MAP kinase phosphatases (MKPs), however, are known to inactivate ERK by dephosphorylating both phosphothreonine and phosphotyrosine. Since MKPs are more specific to MAP kinases than the other two kinds of phosphatases, they have been recognized as key players for inactivating the MAP kinases (Theodosiou and Ashworth, 2002). These findings, together with studies demonstrating that antidepressant treatment upregulates BDNF and the MAP kinase cascade, suggest that negative feedback inhibition of ERK signaling by MKP subtypes could inhibit the responses to antidepressant treatment. There are a few previous investigations of the role of MKPs in the pathophysiology and treatment of depression. One study has demonstrated that the expression of hVH-5 (also referred to as DUSP8) is increased by chronic fluoxetine administration in rat hippocampus (Thiriet *et al*, 1998). A post-mortem study has reported that levels of MKP2 are increased in the prefrontal cortex and hippocampus of depressed suicide subjects, and that levels of ERK are decreased in these same regions (Dwivedi *et al*, 2001). There is also an association study of DUSP6, the human homologue of MKP3, in unipolar and bipolar patients but there were no positive findings (Toyota *et al*, 2000).

To further investigate the possible role of MKPs in opposing the actions of antidepressants, the current study examines the relationship between the three MKP subtypes MKP1, MKP2, and MKP3, and antidepressant treatment including ECS and chemical antidepressants.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (Charles River, Wilmington, MA) weighing 225–250 g were housed under a 12-h light/12-h dark cycle (light on at 0700, light off at 1900) and at constant temperature (25°C) and humidity, and allowed free access to food and water. A total of 154 rats were used for the studies described below. Animal use procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Yale University Animal Care and Use Committee.

Antidepressant Treatment Protocols

Rats received ECS (60 mA, 0.3 s) via earclip electrodes or received sham treatment (handled identically as those that received ECS, but without electrical stimulation). The influence of acute and chronic ECS on levels of MKP1, MKP2, and MKP3 mRNA were determined using the following treatment paradigms. Sham animals received sham treatment once daily for 10 days. Animals in the acute ECS group received sham treatment once daily for 9 days followed by a single ECS on the 10th day. Chronic ECS animals received 10 daily ECS treatments. For *in situ* hybridization analysis, rats were killed 1 or 6 h after the last treatment ($n = 5$ or 6 per group). For Western blot analysis, rats were killed 2 or 6 h after the same paradigm ($n = 5$–8 for each group).

For the time-course analysis of ERK phosphorylation, the animals were decapitated 2, 5, 30, 60, 120, 360 min, and 24 h after a single ESC and compared to sham-treated animals ($n = 6$ or 8 for each time point or sham group).

For antidepressant drug treatments, groups of rats were administered desipramine (10 mg/kg/day) or fluoxetine (5 mg/kg/day) in the drinking water for 21 days (drugs are readily soluble in water). All animals consumed approximately the same amount of the drug/drinking water solution. Preliminary studies demonstrate that drug levels are between 100 and 150 ng/ml of plasma, which is in the range of drug levels achieved in patients. The control animals received distilled water only. Fluid intake was measured three times per week and drinking bottles were replenished with fresh drug solution. There were no differences in fluid intake among the treatment groups. On the 21st day of treatment rats were killed ($n = 6$ per group, with separate control groups for each drug).

Synthesis of Oligonucleotide Probes

The antisense oligonucleotide probes complementary to the MKP1 and MKP3 cDNA were produced according to the method of Boschert *et al* (1998). The MKP2 oligonucleotide probe was designed based on a unique sequence. The β-actin probe was also made according to a prior report (Denovan-Wright *et al*, 1998). The sequences used for oligonucleotide probes are: MKP1 (accession number: X84004, 874-913), 5′-AGCATCCTTGATGGAGTCTATAA AGTCAATCGCCTCGTTG-3′; MKP2 (accession number: U23438, 1143-1182), 5′-GCTGGGGGAGGTGGTGATGGG GCTGTGCAGGTACGGCAGG-3′; MKP3 (accession number: X94185, 626-667), 5′-GCAGTGCAGGGCGAACTCGGCCTG GAACTTACTGAAGCCACC-3′; and β-actin (accession number: V01217, 1244-1288), 5′-GCCGGAGCCGTTGTC GACGACGAGCGCAGCGATATCGTCATCCAT-3′.

A search of the Genbank database using the BLAST program revealed no significant homologous sequence with these oligonucleotide probes.

In Situ Hybridization

In situ hybridization analysis was conducted using ^{35}S-labeled oligonucleotide probes as previously described with some minor modifications (Kodama *et al*, 1998). Coronal sections from sham- and ECS-treated animals, two per

animal, were processed at the same time. Sections were chosen based on the rostral–caudal location of the slice, so that all sections were at approximately the same level of the prefrontal cortex or dorsal hippocampus. Briefly, coronal sections (14 μm) were overlaid with 100 μl of hybridization buffer, which contained 50% deionized formamide, 4 × SSC, 1 × Denhardt's solution, 10% dextran sulfate, 250 μg/ml salmon sperm DNA, 100 μg/ml yeast tRNA, 10 mM dithiothreitol and 10^6 c.p.m. of ^{35}S-labeled oligonucleotide probe. The oligonucleotide probe was radiolabeled at the 3′-end with [α-[^{35}S]thio]dATP (specific activity 1250 Ci/mmol, Du Pont-NEN) using terminal deoxyribonucleotidyl transferase (Roche).

In preliminary experiments designed to assess the sequence specificity of the oligonucleotide probes, a 50-fold excess of unlabeled oligonucleotides (MKP1, MKP2, or MKP3) added to the *in situ* hybridization buffer completely abolished the hybridization signal to the corresponding labeled probe (data not shown). The quantitation of the mRNA signals on X-ray films was analyzed using NIH Image analysis software (Version 1.62). The average optical densities of the left and right sides of the medial and lateral frontal cortex, parietal cortex, CA1 and CA3 pyramidal cell layers, and dentate gyrus granule cell layer of the hippocampus were quantified. Background signals were determined by analysis of an area on the slide without brain tissue and the specific signals were calculated by the subtraction of this background value. These densities were converted into kBq/g tissue using a standard curve (log fit) generated from the ^{14}C standards.

RNA Dot Blot Analysis

Rat brains were rapidly removed and the prefrontal cortex and hippocampus dissected over wet ice, and then stored at −80°C. Total RNA was isolated using RNAqueous kit (Ambion). After being denatured at 80°C for 10 min, 2 μl of 40 ng/μl total RNA was blotted on the nylon membrane (Nytran SuperCharge, Schleicher and Schnell). The membranes were dried for 30 min at room temperature and UV crosslinked. They were prehybridized in hybridization buffer (ULTRAhyb-Oligo, Ambion) at 42°C for 60 min and then added 10^6 c.p.m. of ^{33}P-labeled oligonucleotide probes per 1 ml of hybridization buffer for overnight hybridization. The membranes were washed with 0.1 × SSC, 0.5% sodium dodecyl sulfate (SDS) at 42°C twice, wrapped by plastic film, and exposed to a phosphoimage screen (Cyclone storage-phosphor screen, Packard) for 1 week. The signal intensity obtained from the phosphoimage screen was corrected by the β-actin intensity from a separate membrane using the same amount of RNA. In preliminary experiments designed to assess the sequence specificity of the oligonucleotide probes, a 50-fold excess of unlabeled oligonucleotides (MKP1, MKP2, or MKP3) was added to the RNA blot hybridization buffer and this completely abolished the hybridization signal to the corresponding labeled probe (data not shown).

Western Blot Analysis

Western blot analysis was conducted according to standard procedures with some minor modifications (Sakai *et al*, 2002). Briefly, each brain sample was sonicated in ice-cold buffer (20 mM Tris (pH 7.4), 1% SDS, 3 μg/ml leupeptine, 5 μg/ml aprotinin, 3 μg/ml pepstatin, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM phenylmethylsulfonyl fluoride). Sample homogenates (10 μg) for MKP1 and MKP2, or 30 μg for MKP3 were mixed with an equal volume of the sample buffer. Samples were subjected to SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred onto nitrocellulose membranes. After rinsing, the membranes were incubated overnight at 4°C with primary antibodies (Santa Cruz Biotechnology, MKP1 (V-15) sc-1199, 1:5000; MKP3 (C-20) sc-8599, 1:250; BD Transduction Laboratories, MKP2 610850, 1:5000; Cell Signaling, ERK (9102), 1:1000; phospho-ERK (9101), 1:1000). The ERK and phospho-ERK antibodies recognize both the 44 and 42 kDa forms of ERK. After washing, the blots were incubated with a second antibody (Vector Laboratories, peroxidase anti-rabbit IgG, 1:5000 for MKP1; anti-mouse IgG 1:5000 for MKP2; anti-goat IgG, 1:5000 for MKP3; anti-rabbit IgG for ERK and phospho-ERK, 5000) for 60 min at room temperature. Finally, the membranes were rinsed in Tween Tris-buffered saline buffer for 15 min four times, incubated with enhanced chemiluminescence (ECL) reagent (Amersham) for 60 s, and then exposed to ECL-Hyperfilm (Amersham). After development, the bands detected by ECL were analyzed quantitatively.

Immunohistochemistry

Immunohistochemical analysis of ERK and phospho-ERK was conducted according to standard procedures (Shirayama *et al*, 2002). Briefly, slide mounted sections were incubated in rabbit anti-ERK or phospho-ERK antibodies (Cell Signaling, 9102 and 9101, respectively, both 1:100), 3% normal horse serum, 0.3% Triton X-100, phosphate-buffered saline (PBS) overnight at 4°C and incubated for 1 h with secondary antibody (Vector Laboratories, biotinylated horse anti-rabbit 1:200) in 3% normal horse serum, 0.3% Triton X-100, PBS followed by amplification with an avidin–bioin complex (Vector Laboratories), and cells were visualized with DAB (Vector Laboratories).

Statistical Analysis

The differences among groups were analyzed statistically using a one-way ANOVA followed by the Scheffe's *post hoc* test. The level of statistical significance was set at $p < 0.05$.

RESULTS

ECS Increases MKP1 mRNA Expression in Limbic Brain Regions

The distribution of MKP1 mRNA in the rat brain in the naive state and 1 h after acute ECS, as detected by *in situ* hybridization, is shown in Figure 1a. Analysis was conducted on coronal sections of rat brain taken at rostral and midbrain levels. These regions were chosen because they contain several limbic structures that have been implicated in the etiology and treatment of mood disorders, including subregions of the frontal cortex and hippocampus (Duman *et al*, 2000; Nestler *et al*, 2002). MKP1 mRNA levels

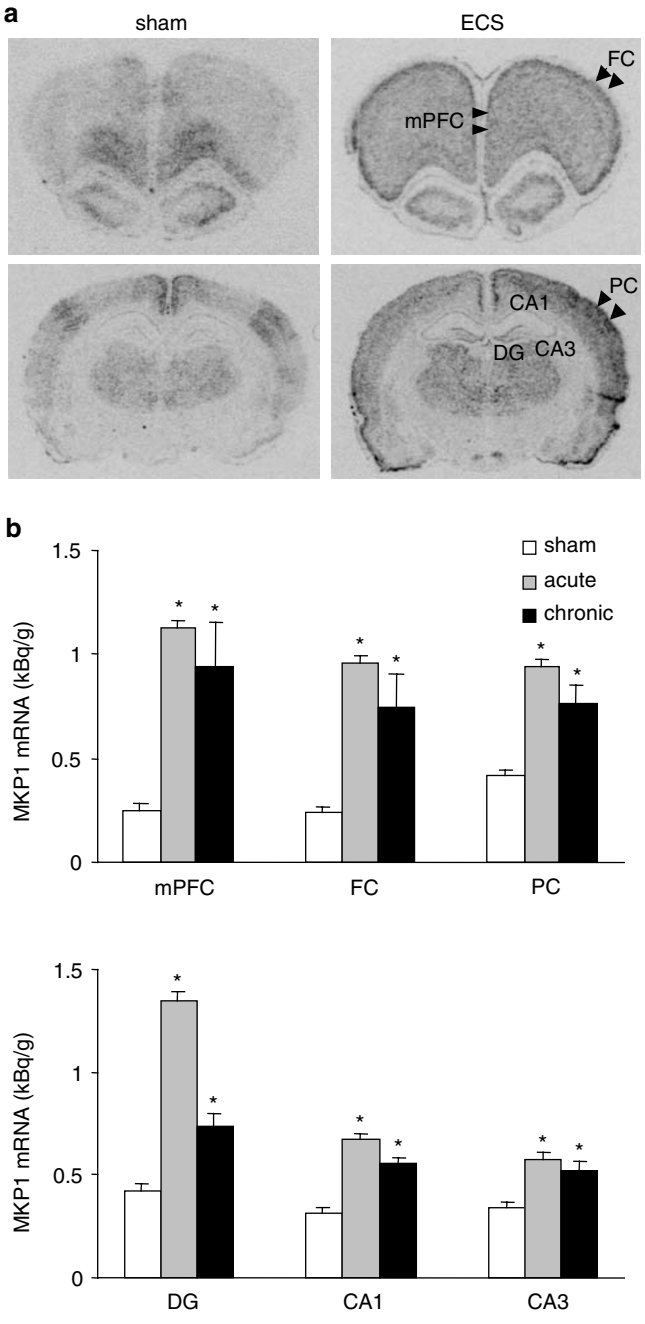

Figure 1 ECS administration increases levels of MKP1 mRNA. Panel (a) shows representative *in situ* hybridization analysis of MKP1 mRNA expression at 1 h after sham (left) or acute ECS (right) administration. Analysis was conducted on coronal sections of rat brain taken at rostral (frontal cortex) and midbrain (hippocampus) levels. Panel (b) shows the quantitative analysis of the effects of acute and chronic ECS on the MKP1 mRNA expression ($n = 5$ or 6 in each group). The values are expressed as the mean ± SEM. The results were subjected to statistical analysis (one-way ANOVA followed by Scheffe's *post hoc* test) with significance set at $^*p < 0.05$. mPFC, medial prefrontal cortex; FC, lateral frontal cortex; PC, parietal cortex; DG, dentate gyrus of the hippocampus, CA1, CA1 pyramidal cell layer; CA3, CA3 pyramidal cell layer.

after acute ECS were distributed most densely in the cerebral cortex and more moderately in the hippocampus, thalamus, and hypothalamus. Analysis of the *in situ* hybridization sections from animals receiving acute or chronic ECS treatment is shown in Figure 1b. There were significant differences in levels of MKP1 mRNA between the three groups for each brain region examined, determined by one-way ANOVA, as follows: medial prefrontal cortex ($F_{(2,14)} = 16.54$, $p = 0.0002$); frontal cortex ($F_{(2,14)} = 20.98$, $p < 0.0001$); parietal cortex ($F_{(2,14)} = 27.49$, $p < 0.0001$); dentate gyrus ($F_{(2,14)} = 110.68$, $p < 0.0001$); CA1 ($F_{(2,14)} = 45.74$, $p < 0.0001$); and CA3 ($F_{(2,14)} = 12.88$, $p = 0.0007$). Differences between two groups were analyzed by the Scheffe's *post hoc* test. MKP1 mRNA levels 1 h after either acute or chronic ECS were significantly greater than those measured after sham treatment in all of the brain areas examined.

Levels of MKP1 mRNA were also analyzed at a later time point (6 h) after either acute or chronic ECS administration. At the 6 h time point, levels of MKP1 mRNA were no longer significantly increased in any of the hippocampal subfields or in the parietal cortex (data not shown), indicating that the induction of MKP1 is transient and rapidly reversible.

ECS Increases MKP2 mRNA Expression in Limbic Brain Regions

In situ hybridization analysis of MKP2 mRNA is shown in Figure 2a. MKP2 mRNA levels after acute ECS treatment is expressed most densely in the dentate gyrus and more moderately in the prefrontal cortex. The results of acute and chronic ECS treatment on MKP2 mRNA levels are shown in Figure 2b. There were significant differences among the MKP2 mRNA levels of the three groups for each brain region examined, determined by one-way ANOVA, as follows: medial prefrontal cortex ($F_{(2,14)} = 16.44$ $p = 0.0002$); frontal cortex ($F2_{(2,14)} = 7.51$, $p < 0.0061$); dentate gyrus ($F_{(2,14)} = 111.49$, $p < 0.0001$); and CA3 ($F2_{(2,14)} = 13.82$, $p = 0.0005$). Differences between two groups were analyzed by the Scheffe's *post hoc* test. MKP2 mRNA levels 1 h after either acute or chronic ECS were significantly greater than those measured after sham treatment in each of these brain areas. In contrast, there was no difference in levels of MKP2 in either the parietal cortex or CA1 pyramidal cell layer of the hippocampus.

Levels of MKP2 mRNA were also analyzed at a later time point (6 h) after either acute or chronic ECS administration. At this time point, levels of MKP2 mRNA were no longer significantly increased in any of the hippocampal subfields where an increase was observed at the 1 h time point (data not shown), indicating that the induction of MKP2, like MKP1, is transient and rapidly reversible.

ECS Increases MKP3 mRNA Expression in Limbic Brain Regions

The distribution of MKP3 mRNA in the rat brain is shown in Figure 3a. MKP3 mRNA expression after acute ECS treatment was distributed most densely in CA1 and CA3 pyramidal cell layers of the hippocampus and prefrontal cortex and more moderately in the parietal cortex. In dentate gyrus, the MKP3 mRNA expression was very low, near background levels. The quantitative analysis of acute and chronic ECS treatment on MKP3 mRNA is shown in Figure 3b. There were significant differences in levels of MKP3 mRNA between the three groups for each brain

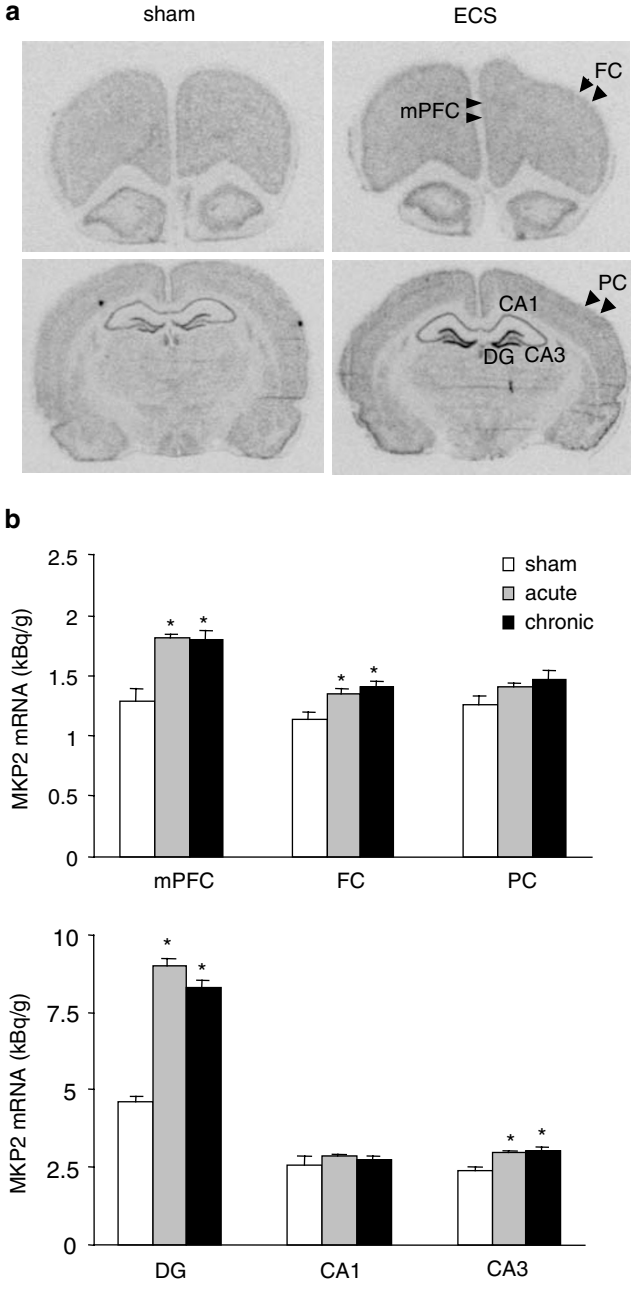

Figure 2 ECS administration increases levels of MKP2 mRNA. Panel (a) shows representative *in situ* hybridization analysis of MKP2 mRNA expression at 1 h after sham (left) or acute ECS (right) administration. Panel (b) shows the quantitative analysis of the effects of acute and chronic ECS on the MKP2 mRNA expression ($n = 5$ or 6 in each group). The values are expressed as the mean ± SEM. The results were subjected to statistical analysis (one-way ANOVA followed by Scheffe's *post hoc* test) with significance set at $^*p < 0.05$. mPFC, medial prefrontal cortex; FC, lateral frontal cortex; PC, parietal cortex; DG, dentate gyrus of the hippocampus, CA1, CA1 pyramidal cell layer; CA3, CA3 pyramidal cell layer.

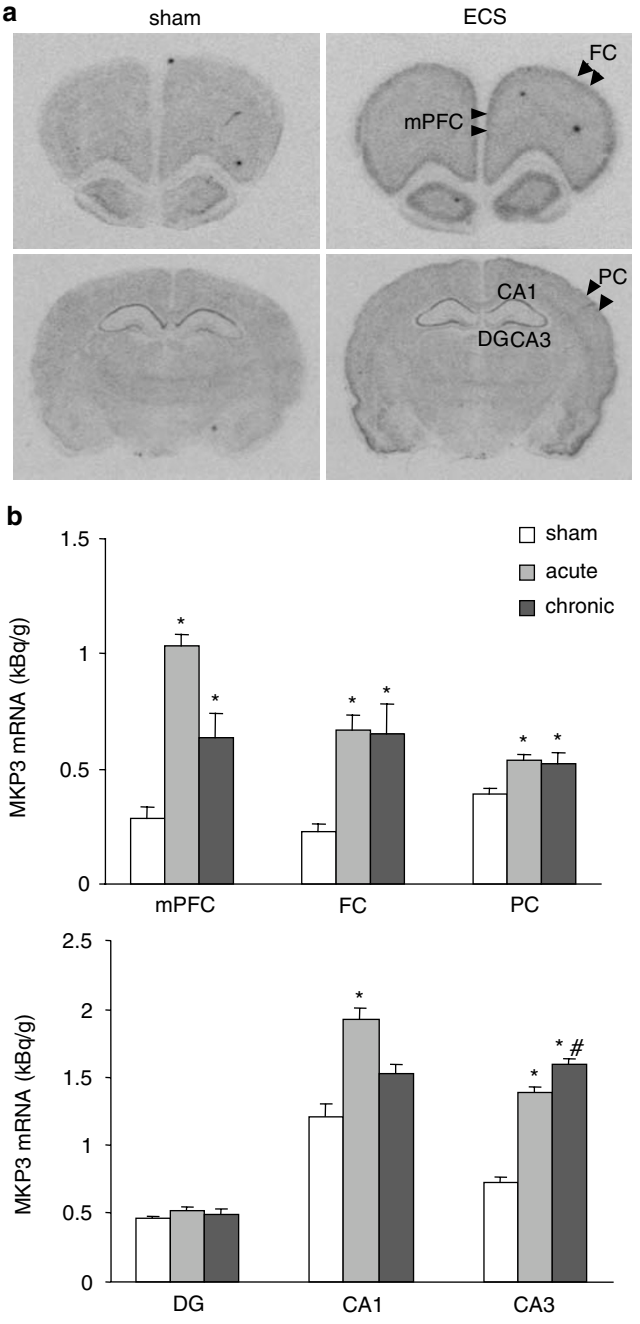

Figure 3 ECS administration increases levels of MKP3 mRNA. Panel (a) shows representative *in situ* hybridization analysis of MKP3 mRNA expression at 1 h after sham (left) or acute ECS (right) administration. Panel (b) shows the quantitative analysis of the effects of acute and chronic ECS on the MKP3 mRNA expression ($n = 5$ or 6 in each group). The values are expressed as the mean ± SEM. The results were subjected to statistical analysis (one-way ANOVA followed by Scheffe's *post hoc* test) with significance set at $^*p < 0.05$. mPFC, medial prefrontal cortex; FC, lateral frontal cortex; PC, parietal cortex; DG, dentate gyrus of the hippocampus, CA1, CA1 pyramidal cell layer; CA3, CA3 pyramidal cell layer.

region examined, determined by one-way ANOVA, as follows: medial prefrontal cortex ($F_{(2,14)} = 31.15$, $p < 0.0001$); frontal cortex ($F_{(2,14)} = 10.93$, $p = 0.0014$); parietal cortex ($F_{(2,14)} = 5.93$, $p = 0.0136$); CA1 ($F_{(2,14)} = 16.17$, $p = 0.0002$); and CA3 ($F_{(2,14)} = 137.68$, $p < 0.0001$). Differences between two groups were analyzed by the Scheffe's *post hoc* test. MKP3 mRNA levels 1 h after acute ECS were significantly greater than those measured after sham treatment in each of these brain areas. Chronic ECS treatment increased MKP3 mRNA in the medial prefrontal

cortex, frontal cortex, parietal cortex, and CA3 compared with sham treatment.

Levels of MKP3 mRNA were also analyzed at a later time point (6 h) after either acute or chronic ECS administration. At this time point, levels of MKP mRNA were no longer significantly increased in any of the hippocampal subfields (data not shown), indicating that the induction of MKP3, like MKP1 and MKP2, is transient and rapidly reversible.

Effect of ECS on MKP1, MKP2, and MKP3 Immunoreactivity

Representative Western blots of MKP1, MKP2, and MKP3 2 h after chronic ECS are shown in Figure 4. MKP1 and MKP2 immunoreactive bands were detected at 38 and 43 kDa, respectively. MKP3 immunoreactive bands were observed at 42 and 44 kDa double bands as reported previously (Camps *et al*, 1998). MKP3 protein levels in both the hippocampus and prefrontal cortex were significantly increased in response to chronic ECS administration (Figure 4) (Student's *t*-test, $p<0.05$ compared to sham). There were no significant differences in levels of MKP1 or MKP2 in the hippocampus or prefrontal cortex. Western blot analysis also demonstrated that only MKP3 immunoreactivity was upregulated in response to acute ECS administration (not shown).

Temporal Profile of ERK Phosphorylation after Acute ECS

The effects of acute ECS on the phosphorylation of ERK (phospho-ERK), determined by Western blot analysis with a phospho-ERK-specific antibody, are shown Figure 5a. As previous reports have indicated, a robust induction of phospho-ERK was seen 2 min after acute ECS (Bhat *et al*, 1998). Levels of phospho-ERK2, the more abundant form of ERK in the hippocampus and prefrontal cortex were quantified and are shown in the accompanying bar graphs. Levels of phospho-ERK2 peaked at 2 min in the hippocampus rising by 346% ($F_{(7,48)}=11.49$, $p<0.0001$) and in the prefrontal cortex by 609% ($F_{(7,40)}=9.85$, $p=0.0026$). Levels of phospho-ERK quickly returned to baseline within 30 min. In the hippocampus, there was a second phase of ERK phosphorylation that was smaller than the first one. The peak time point of the second phase was 6 h after acute ECS and the intensity was 148% of control. In contrast, there was no second phase of increase in the prefrontal cortex. Similar trends were observed for phospho-ERK1. The levels of total ERK immunoreactivity were not changed at any of the time points examined.

Immunohistochemical analysis of phospho-ERK 2 min after sham and ECS are shown in Figure 5c–h. Basal levels of phospho-ERK in the hippocampus are low. A single ECS induces ERK phosphorylation in the granule cells of the dentate gyrus and proximal dendrites in the molecular layer, as well as in the CA3 pyramidal cell layer and the stratum lucidum that includes the mossy fiber axons and CA3 apical dendrites. This is consistent with a previous report on the induction of phospho-ERK (Bhat *et al*, 1998). Total ERK immunoreactivity was not influence by ECS administration (Figure 5g and h).

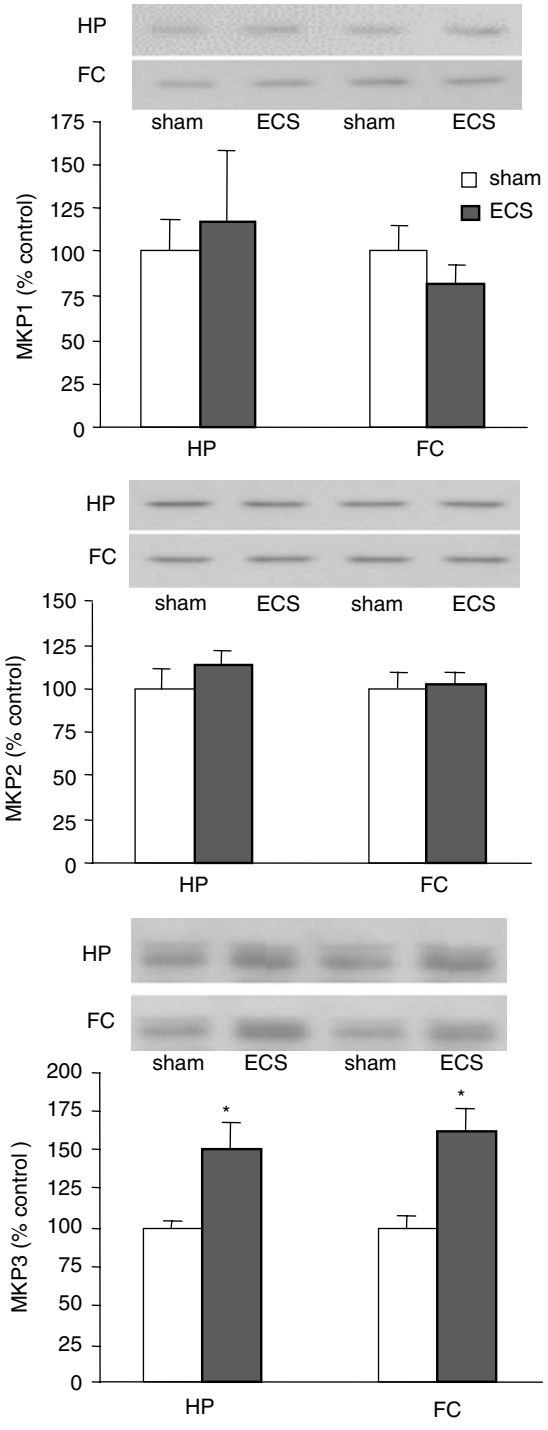

Figure 4 Effects of chronic ECS administration on levels of MKP1, MKP2, and MKP3 immunoreactivity. Representation images MKP1, MKP2, and MKP3 Western blot analysis of the hippocampus and prefrontal cortex. The time point after the last ECS administration was 2 h. The quantitative analysis of the results is presented in the accompanying bar graphs. The values are expressed as the percent of control and are the mean ± SEM ($n=5$, 6 or 8 in each group). *$p<0.05$ compared to corresponding sham treatment (Student's *t*-test). HP, hippocampus; PFC, prefrontal cortex.

Effect of Chemical Antidepressants on MKP1, MKP2, and MKP3 mRNA

The effects of chemical antidepressant treatment on the expression of MKP1, MKP2, and MKP3 mRNA were

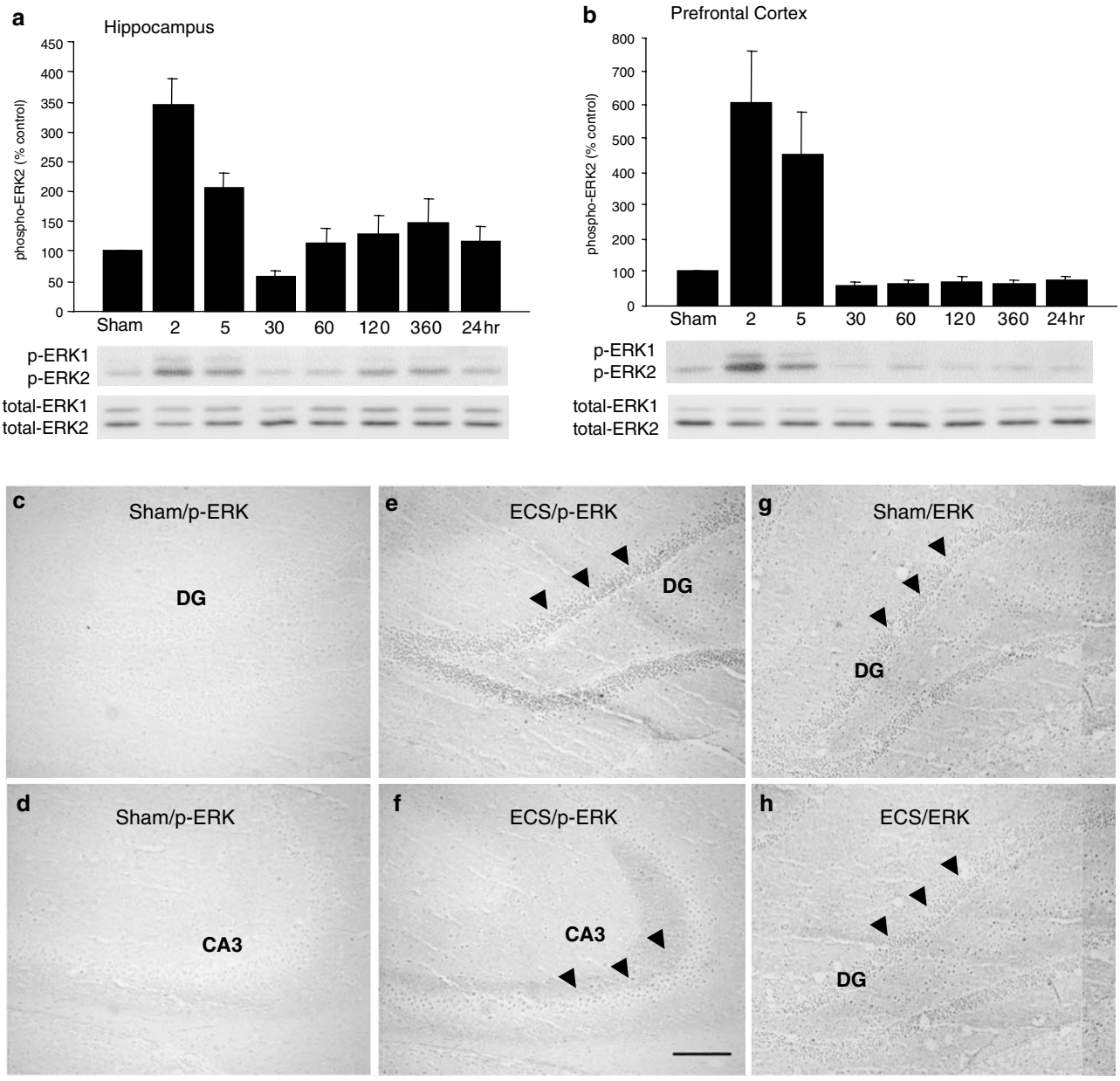

Figure 5 ECS administration increases levels of phospho-ERK. Panels a and b show the time course of ERK phosphorylation, determined by Western blot, after acute ECS in the hippocampus and prefrontal cortex. Rats were decapitated 2, 5, 30, 60, 120, 360 min, and 24 h after a single ECS and compared to sham ($n = 6$ or 8 in each group). Representative images and quantitative results of Western blot analysis are shown. The values are expressed as the mean ± SEM. For comparison, levels of total ERK immunoreactivity were also determined. Panels c–h demonstrate phospho-ERK and total ERK immunohistochemistry after acute ECS in the hippocampus. Rats were killed 2 min after a sham (c, d, and g) or ECS (e, f, and h) treatment as indicated. Levels of phospho-ERK are shown in the dentate gyrus (DG) (c, e) or CA3 (d, f) and levels of total ERK in DG (g, h). Scale bar = 200 μm

determined by RNA dot blot as described in Materials and methods (Figure 6). The RNA dot blot was chosen because it is very sensitive and quantitative, and allows for more rapid analysis of multiple samples. We used two different classes of chemical antidepressants, a selective norepinephrine (desipramine) and a selective serotonin (fluoxetine) reuptake inhibitor. In the hippocampus, there were no significant differences in levels of the three MKP subtypes between the three groups. In the prefrontal cortex, MKP1 mRNA was different among the three groups ($F_{(2,15)} = 4.175$, $p = 0.0362$). Scheffe's *post hoc* test shows that MKP1 mRNA in the fluoxetine-treated group was decreased by 18.6% compared with the control group. MKP3 mRNA was also different among the three groups ($F_{(2,15)} = 4.194$, $p = 0.0358$). Scheffe's *post hoc* test shows that MKP3 mRNA in the fluoxetine-treated group was decreased by 19.3% compared with the control group.

The influence of chemical antidepressant administration on levels of MKP1 and MKP3 mRNA was also examined by *in situ* hybridization analysis for comparison with ECS (Figures 1 and 3). There were no significant effects of fluoxetine administration on levels of MKP1 or MKP3

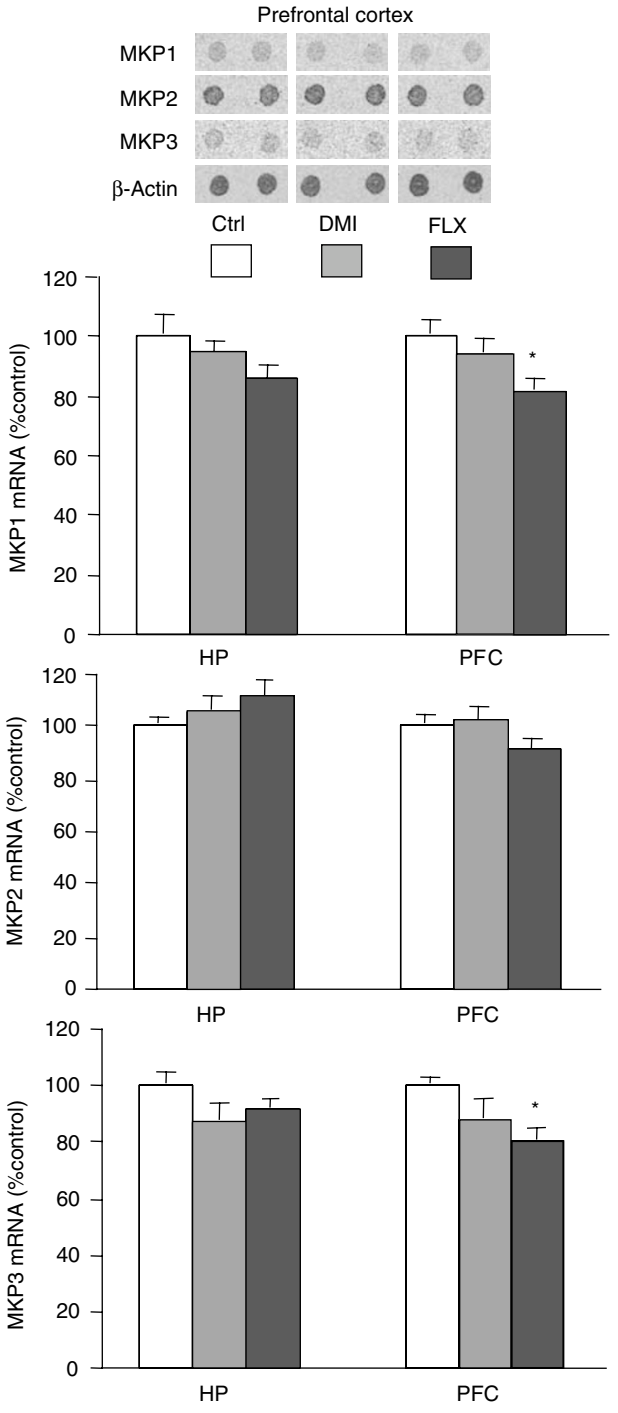

Figure 6 Influence of chemical antidepressant treatment on levels of MKP1, MKP2, and MKP3 mRNA. Rats were administered desipramine (10 mg/kg/day) or fluoxetine (5 mg/kg/day) for 21 days ($n=6$ in each group). Total RNA was isolated from the frontal cortex or hippocampus and analyzed for levels of MKP1, MKP2, or MKP3 mRNA by RNA dot blot analysis. The signal intensity from RNA dot blot analysis was standardized to levels of β-actin. The values are expressed as the percent of control and are the mean $\pm$ SEM. The level of statistical significance was determined by one-way ANOVA followed by Scheffe's *post hoc* test. $^{*}p<0.05$.

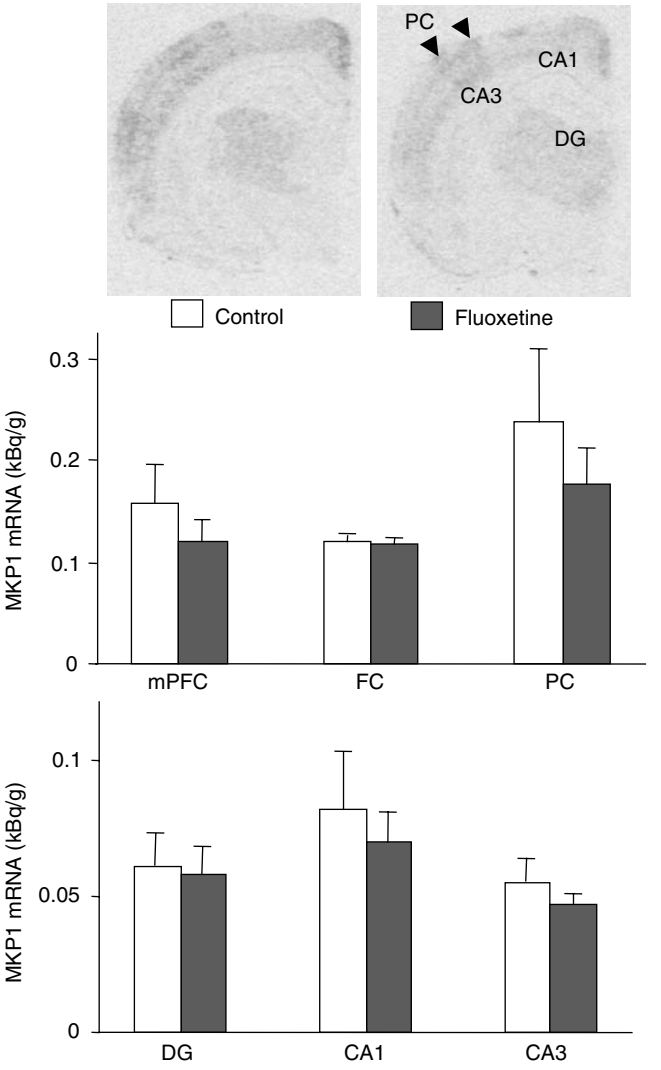

Figure 7 Influence of chronic fluoxetine administration on levels of MKP1 mRNA. Rats were administered fluoxetine (5 mg/kg/day) for 21 days ($n=6$ in each group) and MKP1 mRNA was determined by *in situ* hybridization analysis. The upper panel shows a representative coronal section taken at the level of the hippocampus from control and fluoxetine administered animals. The lower panels show the quantitative analysis of the effects of chronic fluoxetine administration on levels of MKP1 mRNA expression. The values are expressed as the mean $\pm$ SEM. The results were subjected to statistical analysis (Student's *t*-test). mPFC, medial prefrontal cortex; FC, lateral frontal cortex; PC, parietal cortex; DG, dentate gyrus of the hippocampus; CA1, CA1 pyramidal cell layer; CA3, CA3 pyramidal cell layer.

mRNA in subregions of the frontal cortex or the hippocampus (Figures 7 and 8). There were trends for a decrease in levels of MKP1 and MKP3 mRNA in the prefrontal and parietal cortex that are consistent with the effects observed with the RNA blot analysis, but because of the greater variability there was no significant effect.

DISCUSSION

In this study, we describe the influence of antidepressant treatments, including ECS and chemical antidepressants, on the expression of three MKPs, protein phosphatases that control the MAP kinase cascade. Each subtype of MKP mRNA in the hippocampus is induced in a particular spatial pattern in response to acute or chronic ECS administration.

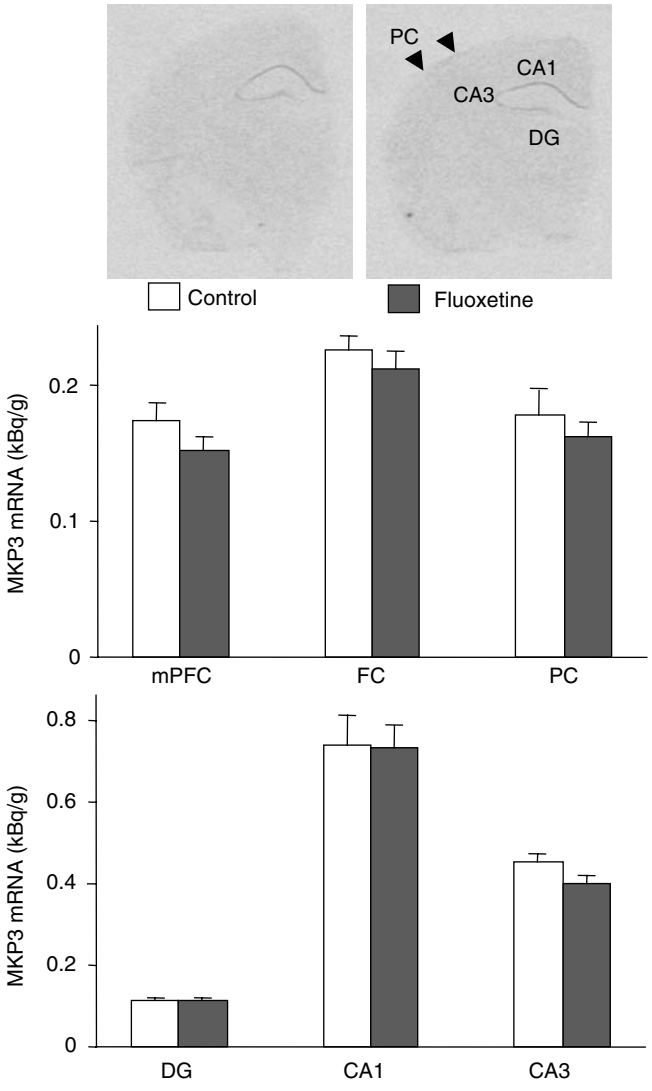

Figure 8 Influence of chronic fluoxetine administration on levels of MKP3 mRNA. Rats were administered fluoxetine (5 mg/kg/day) for 21 days ($n = 6$ in each group) and MKP3 mRNA was determined by *in situ* hybridization analysis. The upper panel shows a representative coronal section taken at the level of the hippocampus from control and fluoxetine administered animals. The lower panels show the quantitative analysis of the effects of chronic fluoxetine administration on levels of MKP3 mRNA expression. The values are expressed as the mean ± SEM. The results were subjected to statistical analysis (Student's *t*-test). mPFC, medial prefrontal cortex; FC, lateral frontal cortex; PC, parietal cortex; DG, dentate gyrus of hippocampus; CA1, CA1 pyramidal cell layer; CA3, CA3 pyramidal cell layer.

MKP1 was induced robustly in all subregions of the hippocampus, while MKP2 was induced primarily in the dentate gyrus granule cell layer and MKP3 was preferentially expressed in the CA1 and CA3 pyramidal cell layers. The sustained induction of MKP expression indicates that there is no desensitization of the response to repeated ECS administration. This indicates that there may be continued negative feedback inhibition of ERK signaling and the antidepressant effects of ECS.

The influence of ECS administration on levels of MKP immunoreactivity was also determined by Western blot analysis. Levels of MKP3 in both the prefrontal cortex and hippocampus were significantly increased by chronic ECS administration, but there was no effect on levels of MKP1 or MKP2. The lack of effect on MKP1 and MKP2 could be due to transient elevation of mRNA that does not lead to corresponding increases in protein levels. Alternatively, there could be subregion or subcellular increases in levels of these MKP subtypes that are not observed when analyzing homogenates of dissected brain regions. Attempts to examine the expression of all three MKP subtypes by immunohistochemistry were unsuccessful with the antibodies currently available, and improved reagents will be required for further analysis at the cellular level. In any case, the current results indicate that MKP3 may be the most important isoform for negative feedback control of the responses to ECS.

In all, 10 different MKP subtypes have been identified to date and have been classified into three subgroups on the basis of a MAP kinase-docking site and substrate specificity (Theodosiou and Ashworth, 2002). MKP1 and MKP2 are in subgroup I that can dephosphorylate a wide range of MAP kinases, including c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), p38, and ERK. MKP3 belongs to subgroup II that is highly specific for ERK. Members of subgroup III can dephosphorylate JNK/SAPK and p38, but not ERK. This indicates that the MKPs play a role in negative feedback control of specific activated MAP kinase cascades.

In turn, the individual MAP kinases are reported to activate the expression of specific MKPs. For example, ERK can induce the expression of MKP1, 2, and 3, while JNK/SAPK can induce MKP1 (Bokemeyer *et al*, 1996; Muda *et al*, 1996; Brondello *et al*, 1997; Hafen, 1998; Sgambato *et al*, 1998). These findings suggest that induction of a particular MKP may be an indication that a particular MAP kinase is activated. Assuming that this is the case, the spatial pattern of MKP expression in the hippocampus in response to ECS administration may reflect a particular spatial pattern of activation of different groups of MAP kinases. On the other hand, the induced pattern after acute ECS in the prefrontal cortex is similar among all three kinds of MKPs. This suggests that in the prefrontal cortex, different classes of MAP kinases may be activated simultaneously.

The results of the current study also demonstrate that ECS induces a highly robust activation of ERK in the hippocampus and prefrontal cortex, which is consistent with previous studies (Stratton *et al*, 1991; Baraban *et al*, 1993; Kang *et al*, 1994; Bhat *et al*, 1998). We also demonstrate that there is a late as well as early phase of phospho-ERK induction in the hippocampus that has not been reported. Immunohistochemical analysis demonstrates that the induction of ERK phosphorylation occurs in the cell bodies and fibers in the dentate gyrus and CA3 pyramidal cell layers. Other kinds of MAP kinases, including JNK/SAPK and p38, are also activated by ECS in the hippocampus (Brecht *et al*, 1999; Oh *et al*, 1999). A detailed characterization of the spatial pattern of activation of other MAP kinase cascades has not been determined.

The early phase of ERK phosphorylation after ECS begins almost immediately, peaks at approximately 2 min, and lasts for less than 30 min. This rapid phosphorylation and dephosphorylation of ERK occurs prior to the induction

of MKP expression and may thereby also involve other classes of protein phosphatases that can influence the ERK cascade (Camps *et al*, 2000). First, the protein serine/threonine phosphatase, PP2A can dephosphorylate the phosphothreonine residue of MAP kinase. PP2A inactivates not only ERK but also MAP kinase kinase or MEK. Second, PTPs dephosphorylate the regulatory tyrosine residue, which is sufficient to inactivate MAP kinase. Among the many kinds of PTPs previous studies have found that PTP-SL, HePTP, and striatal enriched phosphatase (STEP) are associated with the MAP kinase pathway and could also contribute to dephosphorylation of ERK after ECS. STEP has recently been found to participate in rapid dephosphorylation of ERK that is activated via NMDA-mediated influx of calcium (Paul *et al*, 2003).

It is also important to keep in mind that although the MKPs are not induced until after the early phase of ERK phosphorylation, it is possible that the MKPs are expressed at basal levels that are sufficient to contribute to the dephosphorylation of ERK during the early phase as well. This possibility is supported by the results of the Western blot studies that demonstrate that all three MKPs can be detected in the sham-treated animals. The presence of MKP3 is particularly notable because this subtype has been reported to be one of the key regulators of ERK dephosphorylation (Zhou *et al*, 2002). This is due in part to the presence of an ERK kinase interaction domain in MKP3. In addition, phosphorylation of tyrosine residues on ERK inhibits the dephosphorylation of threonine residues by phosphatases such as PP2A. This indicates that MKP3, or other tyrosine phosphatases such as STEP, must dephosphorylate ERK before PP2A can influence ERK (Zhou *et al*, 2002). This further highlights the potential significance of the induction of MKP3 protein as well as mRNA expression in both the prefrontal cortex and hippocampus.

The increased expression of MKP1, MKP2, and MKP3 at the 1 h time point also suggests that the induced levels of these phosphatases could be more relevant to the second peak of ERK phosphorylation that is observed in the hippocampus. However, if this is the case it is surprising that there is a second peak of ERK phosphorylation when levels of the MKPs are induced. There are several possible reasons why this may occur. First, there could be an even greater increase in phospho-ERK, similar to that observed during the early phase, but the increase is blunted because of increased MKP expression. This could explain the lack of a second peak in the prefrontal cortex. Second, it is also possible that this inhibitory feedback mechanism would decrease the response to subsequent stimuli that would stimulate ERK phosphorylation. Further studies of the mechanisms underlying the second phase of ERK phosphorylation as well as the role of MKP in modulating this response will be needed to address this question.

Previous studies demonstrate that the temporal activation of ERK is critical for the appropriate functional response. For example, transient activation of ERK plays a pivotal role in cell proliferation, whereas sustained activation can induce cell cycle arrest and differentiation of PC12 cells (Marshall, 1995). Furthermore, chronic activation and nuclear retention of ERK may be critical factors in triggering proapoptotic signals and neuronal cell death. Sustained activation of ERK brought about by protein phosphatase inhibition induces neuronal cell death in hippocampal slices (Runden *et al*, 1998). These findings highlight the importance of the duration as well as the magnitude of ERK activation in determining cellular responses (Marshall, 1995). The multiple classes and subclasses of phosphatases likely play a key role in the regulation of ERK signaling with each phosphatase possibly playing a different role in determining cell function and even cell fate.

In this study, we found that chronic as well as acute ECS treatment significantly increased MKP expression in subregions of the frontal cortex and hippocampus. Chronic administration of ECS as well as chemical antidepressants is required for a therapeutic response. This has led to the hypothesis that long-term adaptations, including regulation of gene expression, are required for the actions of antidepressant treatment (Duman *et al*, 2000; Nestler *et al*, 2002). Stimulation of the ERK cascade can lead to the activation of several transcription factors, including the cAMP/calcium-responsive element binding protein, Elk-1, and c-Myc. In a previous report, chronic ECS administration increased the phosphorylation of ERK to a level comparable to that after a single ECS (Stratton *et al*, 1991). Sustained activation of the ERK cascade with repeated ECS treatment indicates that this cascade may contribute to the long-term adaptations required for antidepressant effects.

Even though ECS rapidly activates ERK signaling, it may take time for the functional as well as therapeutic consequences of this system to accumulate and to be expressed. The influence of ECS and other antidepressants on adult neurogenesis and neuronal morphology may be particularly time-dependent and require prolonged treatment for full functional expression (Duman *et al*, 2000; Nestler *et al*, 2002). With regard to the regulation of MKP expression, the interpretation of the results presented is that the induction of MKP could act as a negative feedback on ERK signaling and thereby blunt the effects of ECS. This is based on studies demonstrating that the antidepressant effects of BDNF are blocked by infusion of a MEK inhibitor that blocks the phosphorylation and activation of ERK (Shirayama *et al*, 2002). The fact that there is no tolerance to the induction of MKP expression indicates that the dephosphorylation and inactivation of ERK would continue throughout the course of the treatment regimen. These findings suggest that blockade of MKP expression or function could enhance activation of ERK signaling and thereby provide a mechanism for increasing the effects of antidepressant treatment.

We have also investigated the effect of chemical antidepressant treatment on MKP mRNA expression. Two different types of antidepressants, a selective serotonin reuptake inhibitor and a selective norepinephrine reuptake inhibitor, were tested. It has been reported that chronic chemical antidepressant administration increases ERK phosphorylation in the rat hippocampus (Schultz *et al*, 2001). Studies using primary hippocampal and cerebral cortical cultures have directly shown that serotonin and norepinephrine increase the phosphorylation of ERK via specific receptor subtypes for each monoamine (Errico *et al*, 2001; Tolbert *et al*, 2003). Recently, it has also been reported that lithium and valproate, commonly used mood

stabilizers for the treatment of manic-depressive illness, increase phospho-ERK in the rat hippocampus and frontal cortex (Einat *et al*, 2003). Our present data indicate that chronic fluoxetine administration decreases MKP1 and MKP3 mRNA in the frontal cortex. Analysis of the expression of MKP1 and MKP3 mRNA in subregions of the frontal cortex and hippocampus by *in situ* hybridization analysis did not reveal any significant effects. Although there was a trend for a decrease in the prefrontal and parietal cortices, the lack of significance could be due to the greater variability of the *in situ* hybridization results compared to the dot blot. Even though the effects are small, it is possible that decreased expression of MKP1 and MKP3 could lead to greater activation of ERK in the prefrontal cortex. Alternatively, even if we conclude that decreased expression of MKP1 and MKP3 do not play a role in the actions of chemical antidepressants, this does not preclude the possibility that blockade of MKPs could produce an antidepressant response or enhance the response to an antidepressant. This possibility must be directly tested using one of several knockdown strategies, such as MKP null mutant mice, dominant-negative MKPs, or RNAi followed by behavioral studies. These approaches are currently under development to address this question.

The potential behavioral consequences of altered MKP expression are highlighted by a previous study demonstrating that inhibition of ERK blocks the antidepressant effects of BDNF in animal models of depression (Shirayama *et al*, 2002). More recent reports of ERK pathway inhibitors administered systemically have been mixed. One study found that blockade of the ERK pathway produces an antidepressant-like response, although this effect was accompanied by increased locomotor activity that complicates the interpretation of these results (Einat *et al*, 2003). Another report from our laboratory has found that inhibition of the ERK pathway results in prodepressant effects that are time- and dose-dependent, and that inhibitors produce a consistent blockade of chemical antidepressants in the forced swim, learned helplessness, and tail suspension paradigms (Duman *et al*, 2003). These differential effects may be due to dose and time differences, as well as the level of stress exposure. Post-mortem analysis demonstrates that levels of ERK activity and expression are decreased in suicide, depressed patients, and that levels of MKP2 are increased (Dwivedi *et al*, 2001). Levels of MKP1 and MKP3 were not examined in this study. These results are consistent with the hypothesis that decreases in ERK function, resulting in part from increased MKP levels, are correlated with depression.

Although additional studies are required, the reported depressive effects of ERK pathway inhibitors (Shirayama *et al*, 2002; Duman *et al*, 2003) and decreased ERK expression in suicide, depressed patients (Dwivedi *et al*, 2001), together with the results of the current study, indicate that increased MKP expression and function would oppose the actions of antidepressant treatment. These findings also suggest that decreased expression or blockade of specific MKP subtypes, most notably MKP3 because of elevated protein levels for this isoform as well as the critical role of MKP3 in ERK dephosphorylation, could produce an antidepressant response. At the present time, there are no selective inhibitors that could be used to test this hypothesis. We are currently investigating alternative approaches, including analysis of mutant mice or the use of inhibitory RNA technology to directly test the hypothesis that blockade of MKP3, or MKP1 and MKP2, could produce an antidepressant response in behavioral models. Additional post-mortem studies will be required to verify the regulation of the ERK signaling cascade and MKP expression in depressed patients.

ACKNOWLEDGEMENTS

This work is supported by USPHS Grants MH45481 and two PO1 MH25642, a Veterans Administration National Center Grant for PTSD, and by the Connecticut Mental Health Center.

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