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Regulation of cAMP phosphodiesterase mRNAs expression in rat brain by acute and chronic fluoxetine treatment. An in situ hybridization study

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Abstract

Changes in brain cyclic AMP (cAMP) have been suggested to underlie the clinical action of antidepressant treatments. Also, a regionally-selective regulation of cAMP-specific phosphodiesterases (PDEs) has been demonstrated for some antidepressants. To further investigate the effects of antidepressant treatments on PDEs, we examined the expression of different cAMP-specific PDEs in the brain of rats treated (1 and 14 days) with fluoxetine 3 mg/kg day. The mRNAs coding for PDE4A, PDE4B, PDE4D, and the five known PDE4D splice variants were analyzed by in situ hybridization on 45 brain structures of acute and chronic fluoxetine-treated rats. We also examined the binding sites for the putative antidepressant drug [^{3}H]rolipram, a PDE4-selective inhibitor. In some brain areas single fluoxetine administration increased the density of the mRNA of all PDE4 isozymes, except PDE4D and PDE4D5. Chronic fluoxetine treatment increased PDE4A mRNA levels and decreased those for PDE4B, PDE4D and PDE4D1 mRNAs in some brain regions. The study was complemented with the analysis of the expression of the transcripts of BDNF. Chronic fluoxetine treatment down-regulated the expression of BDNF. These results show that the expression of PDE4 isozymes is modulated by a clinically relevant fluoxetine dose. The significance of these changes in PDE4 expression to the antidepressant effect of fluoxetine is discussed.

Keywords: Antidepressants; In situ hybridization; Fluoxetine; Rolipram; PDE4; PDE4D splice variants; BDNF

1. Introduction

Selective serotonin reuptake inhibitors (SSRIs) are extensively used in the treatment of major depression. They exert their therapeutic effect enhancing brain serotonergic activity by preventing serotonin (5-HT) reuptake and thereby increasing its concentration in the synaptic cleft. But all antidepressant treatments require an administration of at least two weeks before inducing a clinically significant improvement. The mechanisms underlying in this delay in the therapeutic effect of SSRIs remain unknown.

Cyclic AMP (cAMP) plays an important role in signal transduction processes (Houslay, 1998). A role of the cAMP pathway has been suggested in several CNS diseases such as depression (Duman et al., 1997) and Alzheimer's disease (Cowburn et al., 1996; Bonkale et al., 1999). The hydrolysis of cAMP is regulated by a family of cyclic nucleotide phosphodiesterases (PDEs). Twelve members of this family have been identified until now on the basis of their substrate specificities, kinetic properties, allosteric regulators, inhibitor sensitivities and amino acid sequences (Conti and Jin, 1999; Soderling and Beavo, 2000; Houslay, 2001). Families 4, 7 and 8 specifically hydrolyze cAMP. The PDE4 family has four members (PDE4A through PDE4D) (Houslay, 1998), the PDE7 family, two (PDE7A, PDE7B) (Conti

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and Jin, 1999) and the PDE8 two (PDE8A, PDE8B) (Fisher et al., 1998; Hayashi et al., 1998) encoded by different genes.

Selective inhibitors of cAMP-specific PDEs have been proposed for the treatment of CNS disorders, such as depression (O'Donnell and Frith, 1999), multiple sclerosis (Genain et al., 1995), ischaemia-reperfusion injury (Kato et al., 1995), Alzheimer's (McGeer and McGeer, 1995) and Parkinson's diseases (Hulley et al., 1995). Rolipram, a PDE4 selective inhibitor, has antidepressant properties (Wachtel, 1983), yet its side effects prevented a widespread clinical use (Fleischhacker et al., 1992).

The long-term treatment with antidepressant drugs results in an increase of the levels of cAMP and of the expression of CREB (cyclic adenosine monophosphate response element-binding protein) in specific brain regions (Nibuya et al., 1996; Duman et al., 1997). These treatments also increased the expression of the brain-derived neurotrophic factor (BDNF), which has been suggested to play a role in the therapeutic action of antidepressant drugs (Duman et al., 1997; Siuciak et al., 1997).

The main objective of the present study was to examine the putative involvement of PDE4s in the mechanism of action of antidepressant drugs. For this, we analyzed the effects of single and repeated fluoxetine treatment on the regulation of the expression of different cAMP-specific PDE4s in a large number of brain structures. The study was complemented by the analysis of the expression of BDNF and [^{3}H]rolipram binding on the same brain structures.

2. Materials and methods

2.1. Animals and treatments

Adult male Wistar rats (200–300 g) were purchased from Iffa Credo (Lyon, France). Animals were acclimated to standard laboratory conditions (14-h light/10-h dark cycle) with free access to rat chow and water. Each rat was used only once for experimentation, and all the procedures conformed to the European Communities Council directive of November 24, 1986 (86/609/EEC). Animals were conscious and freely moving at all times throughout the experimental procedure. Rats were randomly assigned to receive vehicle or fluoxetine. In the case of acute treatments, rats were administered i.p. with fluoxetine (3 mg/kg) or vehicle and killed by decapitation 2 h later. For the chronic treatments, rats under light ether anesthesia were implanted s.c. with Alzet 2002 minipumps filled to deliver vehicle (water/DMSO 50%/50%) or fluoxetine dissolved in vehicle (3 mg/kg day) for 14 days. Animals were killed by decapitation with the minipumps on board. These were removed to check that they were empty by visual inspection. The use of dimethyl sulfoxide (DMSO) to dissolve fluoxetine was necessary due to the small volume of the minipumps. Given the weight gain of the animals, the doses used correspond to the 7th day of treatment. The animals were killed by decapitation without using anesthesia to prevent potential interferences on the variables measured. Brains were quickly removed from the skull, frozen on dry ice and kept at −20°C. Tissue sections, 14-μm thick, were cut on a microtome–cryostat (Microm HM500 OM, Waldorf, Germany), thaw-mounted onto APTS (3-aminopropyltriethoxysilane, Sigma, St. Louis, MO) and kept at −20°C until used.

2.2. In situ hybridization histochemistry

The oligonucleotides used were complementary to the following base sequences (Acc. no. inside the brackets): PDE4A 3649–3693 (L27057), PDE4B 2639–2687 (U95748), PDE4D, bases 1586–1630 (U09456); PDE4D1, bases 180–219 (U09455); PDE4D2, bases 117–163 (U09456); PDE4D3, bases 1–45 (U09457); PDE4D4, bases 360–404 (AF031373); PDE4D5, bases 251–295 (AF012073). They were synthesized by Amersham Pharmacia Biotech (Little Chalfont, UK). The oligonucleotide used for BDNF was the same as described previously (Rocamora et al., 1992).

The oligonucleotides were labeled at their 3′-end by using [α-^{32}P]dATP (3000 Ci/mmol, New England Nuclear, Boston, MA) and terminal deoxynucleotidyltransferase (TdT, Roche Molecular Biochemicals, Mannheim, Germany) to specific activities of 0.7–1.2×10^4 Ci/mmol. Labeled probes were purified by QIAquick Nucleotide Removal Kit (QIAGEN, Hilden, Germany).

Prior to hybridization frozen tissues were brought to room temperature, air dried and fixed for 20 min in 4% paraformaldehyde in phosphate-buffered saline (PBS: 2.6 mM KCl, 1.4 mM KH_2PO_4, 136 mM NaCl, 8 mM Na_2HPO_4), washed once in 3×PBS, twice in 1×PBS, 5 min each, and incubated in a freshly prepared solution of predigested pronase at a final concentration of 24 U/ml in 50 mM Tris–HCl pH 7.5, 5 mM EDTA for 2 min at room temperature. Proteolytic activity was stopped by immersion for 30 s in 2 mg/ml glycine in PBS. Tissues were rinsed in PBS and dehydrated in a graded series of ethanol 100%. For hybridization, labeled probes were diluted to a final concentration of approximately 10^4 cpm/μl in a solution containing 50% formamide, 4×SSC, 1×Denhardt's solution, 1% sarkosyl, 10% dextran sulfate, 20 mM phosphate buffer pH 7, 250 μg/ml yeast tRNA, and 500 μg/ml salmon sperm DNA. Tissues were covered with 100 μl of the hybridization solution and overlaid with Nescofilm coverslips to prevent evaporation. Tissues were incubated in humid boxes overnight at 42°C and then washed 4 times (45 min

each) in 600 mM NaCl, 20 mM Tris–HCl, pH 7.5, 1 mM EDTA at 60°C. Hybridized sections were exposed to Hyperfilm ß-max (Amersham) for 3–5 weeks at −70°C with intensifying screens.

2.3. Autoradiography

Autoradiography with [^{3}H]-rolipram was performed as previously described (Kaulen et al., 1989; Perez-Torres et al., 2000). [^{3}H]Rolipram (85 Ci/mmol) was purchased from Amersham (Little Chalfont, UK). (±)-Rolipram was a generous gift from Almirall Prodesfarma (Barcelona, Spain). Tissue sections were preincubated for 15 min at room temperature in 150 mM phosphate buffer pH 7.4, containing 2 mM $MgCl_2$ and 100 μM dithiothreitol. They were then incubated for 1 h at 0°C in the same buffer with [^{3}H]rolipram 2 nM. Adjacent sections were incubated in the presence of 1 μM (±)-rolipram to determine the non-specific binding. After incubation, the sections were washed twice for 5 min each in the same buffer at 0°C, dipped in distilled water at 0°C and rapidly dried under a cold air stream (4°C). Autoradiograms were generated by apposing the labeled tissue sections to a Hyperfilm ^{3}H (Amersham) together with plastic ^{3}H-standards (^{3}H-Microscales, Amersham) in X-ray cassettes for 4 weeks at 4°C.

To analyze the degree of blockade of the 5-HT transporter by acute and chronic fluoxetine treatments, we carried out an additional autoradiographic study using [^{3}H]citalopram as a ligand. Tissue sections were preincubated for 15 min at room temperature in a buffer containing 120 mM NaCl, 5 mM KCl, and 50 mM Tris–HCl (pH 7.4). Additional experiments were conducted using a much longer (4 h) preincubation time. The reason to use two different preincubation times was the following. Differences in [^{3}H]citalopram binding between control and treated rats could theoretically be due to the presence of the drug in brain or (less likely) to fluoxetine-induced changes in the density of the 5-HT transporter. To examine the second possibility, a long preincubation time was additionally used to wash away the drug remaining in the tissue sections. Thereafter, sections were incubated for 1 h at room temperature in the same buffer with 1 nM [^{3}H]citalopram (79.5 Ci/mmol, NEN, Boston, USA). Adjacent sections were incubated in the presence of 1 μM imipramine (RBI, Natick, USA), to determine non-specific binding. Sections were washed 2×10 min in the same buffer at 4°C, dipped in distilled water at 4°C and dried rapidly under a cold air stream (4°C). Autoradiograms were generated by apposing the labeled tissue sections to a ^{3}H-Hyperfilm (Amersham) together with plastic ^{3}H-standards (^{3}H-Microscales, Amersham) in X-ray cassettes for 6 weeks at 4°C. Single and repeated treatment with fluoxetine elicited a dramatic reduction of the binding of [^{3}H]citalopram in brain compared to controls, as assessed by autoradiography using standard experimental conditions (15 min preincubation time). The use of a much longer preincubation time (4 h) resulted in a recovery of the autoradiographic signal to values similar to those in controls. The binding of [^{3}H]citalopram in controls to the 5-HT transporter was unaffected by the length of the preincubation period (data not shown). These observations support the notion that the dose of fluoxetine used (3 mg/kg, single or continuously for 14 days) elicited a significant blockade of the 5-HT transporter.

2.4. Data analyses

Quantitative image analysis was performed with the MCID-4 computerized image analysis system (St Catharines, Ontario, Canada) on 45 brain regions.

Quantitative measures were analyzed using two-way ANOVA (SigmaStat, Jandel Co, Chicago, IL) analyses for each PDE isozyme, BDNF and [^{3}H]rolipram binding using the regions in which the effect of fluoxetine treatment (single or chronic) differed ±10% from control values. These regions were analyzed considering the effect of fluoxetine treatment (vehicle and treated), the anatomical region, and treatment-region interaction. The latter assesses whether the possible effect of fluoxetine treatment is different for the different regions considered. Since the anatomical region factor was statistically significant for all variables examined (PDE4s, BDNF and [^{3}H]rolipram binding), we only give the p values corresponding to the treatment factor and/or the treatment×region interaction, both being representative of a significant effect of the treatments. Significance level has been set at $p<0.05$ (two-sided). Post hoc analysis (Tukey's test) was performed with a significance set at $p<0.05$ when ANOVA indicated that the treatment factor and/or treatment×region interaction was significant ($p<0.05$). In the Figs 2–5, asterisks (∗) were placed only when ANOVA and Tukey's post test indicated a significant treatment×region interaction ($p<0.05$).

3. Results

We have examined the effects of the acute and chronic treatment with the selective serotonin reuptake inhibitor fluoxetine on the expression of the four members of the PDE4 family (PDE4A, PDE4B, PDE4C and PDE4D, including its five mRNA splice variants), and BDNF mRNAs, by in situ hybridization histochemistry. We have also analyzed the effects of fluoxetine treatments on the density of [^{3}H]rolipram binding sites by autoradiography. Figure 1 shows representative autoradiograms of rat coronal sections of a similar level from control (A1–J1), acute (A2–J2), and chronic (A3–J3) fluoxetine treatment from [^{3}H]rolipram binding sites (A1–A3) and in situ hybridization for mRNAs coding for PDE4A

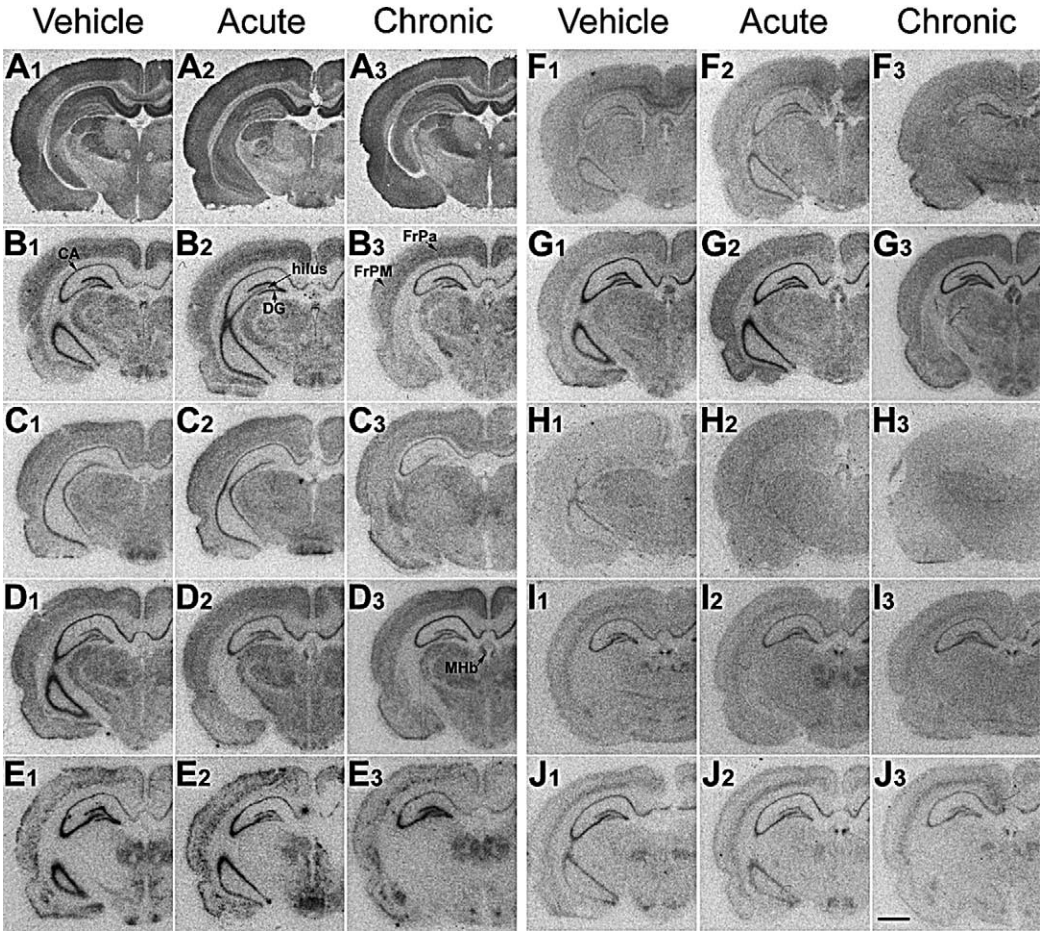

Fig. 1. Rat coronal brain sections from control (A1–J1), acute (A2—J2), and chronic (A3–J3) fluoxetine treated. Rats were treated as described in Material and Methods. Film autoradiogram pictures from [^{3}H]rolipram incubation (A1–A3), and in situ hybridization for mRNAs coding for PDE4A (B1–B3), PDE4B (C1–C3), PDE4D (D1–D3), BDNF (E1–E3), and PDE4D splice variants PDE4D1 (F1–F3), PDE4D2 (G1–G3), PDE4D3 (H1–H3), PDE4D4 (I1–I3), PDE4D5 (J1–J3). Frontoparietal cortex (FrPa, FrPm), anterior cingulate cortex (ACg), Ammon's horn (CA), dentate gyrus (DG), hilus, medial habenular nucleus (MHb). Bar=2 mm.

(B1–B3), PDE4B (C1–C3), PDE4D (D1–D3), BDNF (E1–E3), and PDE4D splice variants PDE4D1 (F1–F3), PDE4D2 (G1–G3), PDE4D3 (H1–H3), PDE4D4 (I1–I3), PDE4D5 (J1–J3). The results of the optical density measurements obtained are summarized in Figs 2 to 5.

Due to the fact that PDE4C mRNA could be detected exclusively in the anterior part of the main olfactory bulb, as we have previously described (Perez-Torres et al., 2000), this PDE4 isozyme was not included in the present work.

3.1. Regulation of PDE4 isoforms and BDNF mRNAs expression by antidepressant treatments

The density of [^{3}H]rolipram binding sites increased after single fluoxetine treatment in the frontal cortex, frontoparietal cortex, anterior cingulate cortex, caudate-putamen, parabigeminal nucleus, inferior olive and in white matter tracts such as corpus callosum. In contrast, an important reduction in the area postrema (Fig. 2A) could be observed. Two-way ANOVA revealed a significant effect of the treatment ($F_{1,54}$=6.32; p=0.015; Tukey's post hoc test $p<0.05$). Conversely, long-term fluoxetine treatment decreased [^{3}H]rolipram binding sites in these same regions except in the parabigeminal nucleus, inferior olive and area postrema (Fig. 4A). However, an increase in [^{3}H]rolipram binding sites was found in the hilus, medial habenular nucleus, medial geniculate nucleus, and pontine nuclei (region×treatment interaction effect; $F_{8,54}$=2.83; p=0.01, Tukey's post hoc test $p<0.05$) where no change in binding was found following the single injection of fluoxetine, while levels in the dorsal part of the medial geniculate nucleus were increased by both treatments.

In the case of PDE4A, acute fluoxetine treatment increased its mRNA levels in only three structures: parabigeminal nucleus, vestibular nuclei and cerebellum (treatment effect; $F_{1,18}$=8.14; p=0.01; Tukey's post hoc test $p<0.05$) (Fig. 2B). Chronic fluoxetine treatment increased PDE4A mRNA levels in dentate gyrus, hilus and cerebellum; but they were significantly reduced (treatment×region interaction; $F_{5,36}$=3.01; p=0.022) in

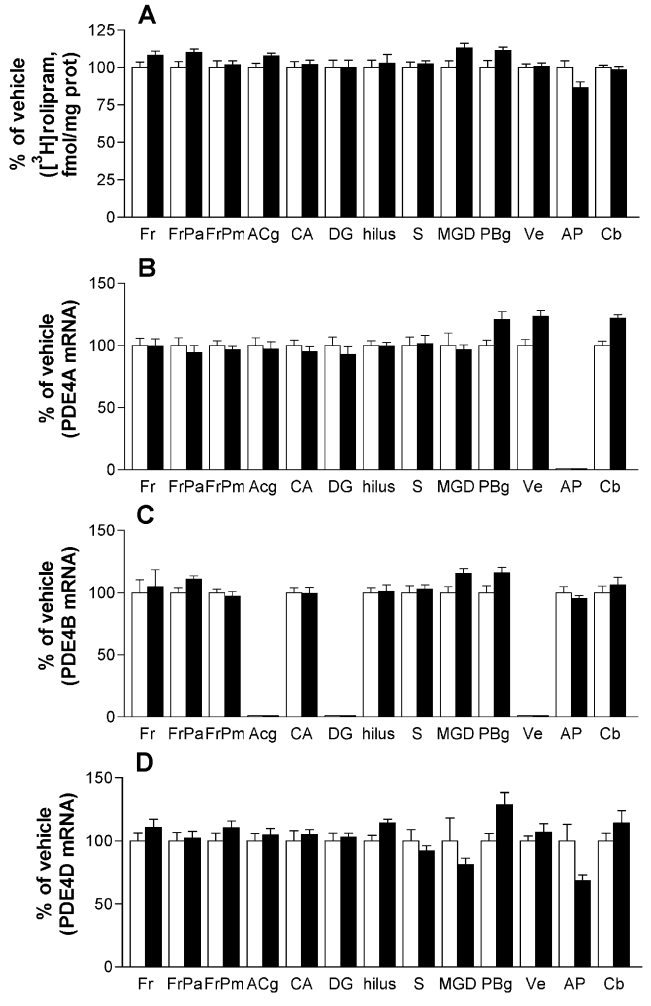

Fig. 2. Effects of acute fluoxetine treatment on the binding of [^{3}H]rolipram (A) and the expression of PDE4A (B), PDE4B (C), PDE4D (D) mRNAs in rat brain. Binding density and mRNA expression levels were determined by receptor autoradiography and in situ hybridization histochemistry analyses as those shown in Fig. 1. The results are expressed as mean ±SEM percent of control; n=4 per group. Two-way ANOVA analysis revealed a significant effect of the treatment or treatment×region interaction for [^{3}H]rolipram binding, all PDEs except PDE4D (see Results for more detailed statistical analysis). Asterisks (∗) indicate a significant treatment×region interaction, $*p<0.05$ compared with control (Two-way ANOVA and Tukey's post hoc test). Open bars represent control values and filled bars represent values from treated rats. Abbreviations: frontal cortex (Fr), frontoparietal cortex (FrPa, FrPm), anterior cingulate cortex (ACg), Ammon's horn (CA), dentate gyrus (DG), hilus, subiculum (S), dorsal part of medial geniculate nucleus (MGD), parabigeminal nucleus (PBg), pontine nuclei (Pn), vestibular nuclei (Ve), area postrema (AP), cerebellum (Cb).

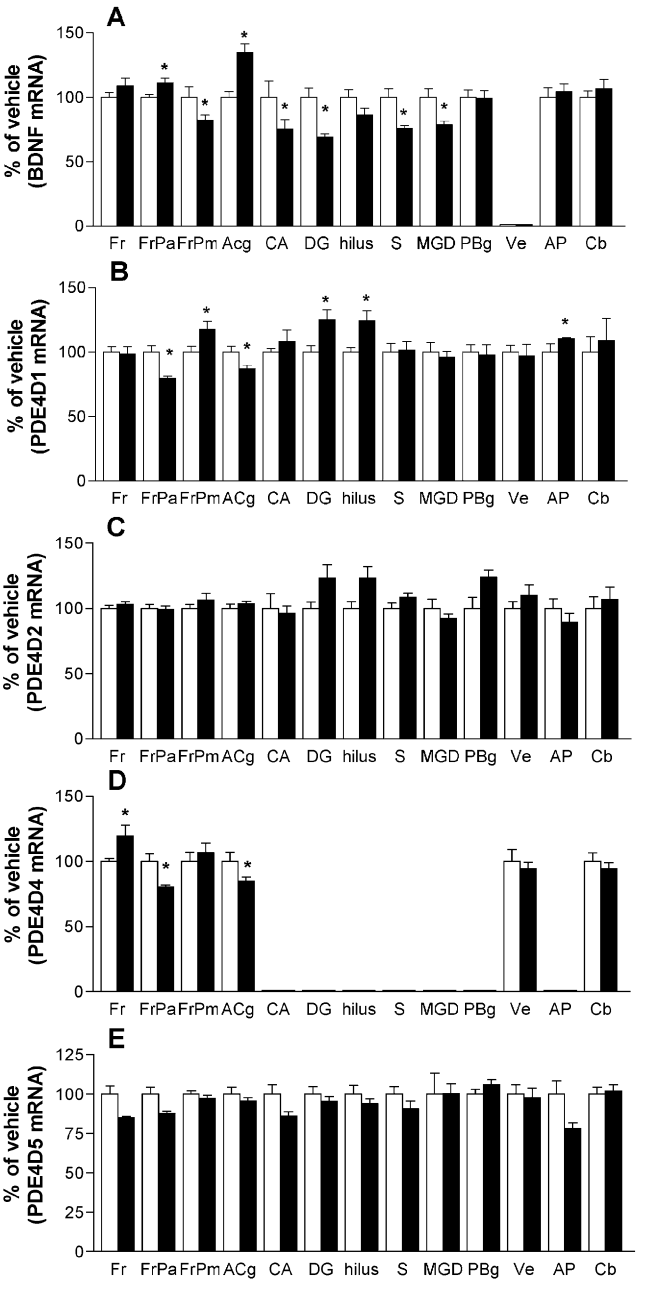

Fig. 3. Bar chart showing the effect of acute fluoxetine treatment on the expression of mRNAs coding for BDNF (A), PDE4D1 (B), PDE4D2 (C), PDE4D4 (D), and PDE4D5 (E). The results are expressed as mean ±SEM percent of control; n=4 per group. Two-way ANOVA analysis showed a significant effect of the treatment or treatment×region interaction for PDE4D splicing isoforms. Asterisks (∗) indicate a significant treatment×region interaction, $*p<0.05$ compared with control (Two-way ANOVA and Tukey's post hoc test). Levels of PDE4D3 mRNA were not significantly altered by the acute treatment (data not shown). Open bars represent control values and filled bars represent values from treated rats. Abbreviations are as in Fig. 2.

cingular and frontoparietal cortices, and medial septal nucleus. Multiple comparison Tukey's post hoc test showed a significant interaction of the treatment in cortical and hipocampal regions ($p<0.05$) (Fig. 4B).

Acute fluoxetine treatment increased PDE4B mRNA levels in seven brain structures: frontoparietal cortex, caudate-putamen, medial habenular nucleus, dorsal part of the medial geniculate nucleus, parabigeminal nucleus, inferior olive and white matter tracts such as corpus callosum (treatment effect $F_{1,42}$=21.84; p=0.0003; Tukey's post hoc analysis $p<0.05$) (Fig. 2C). Conversely, a reduction in PDE4B mRNA expression was measured

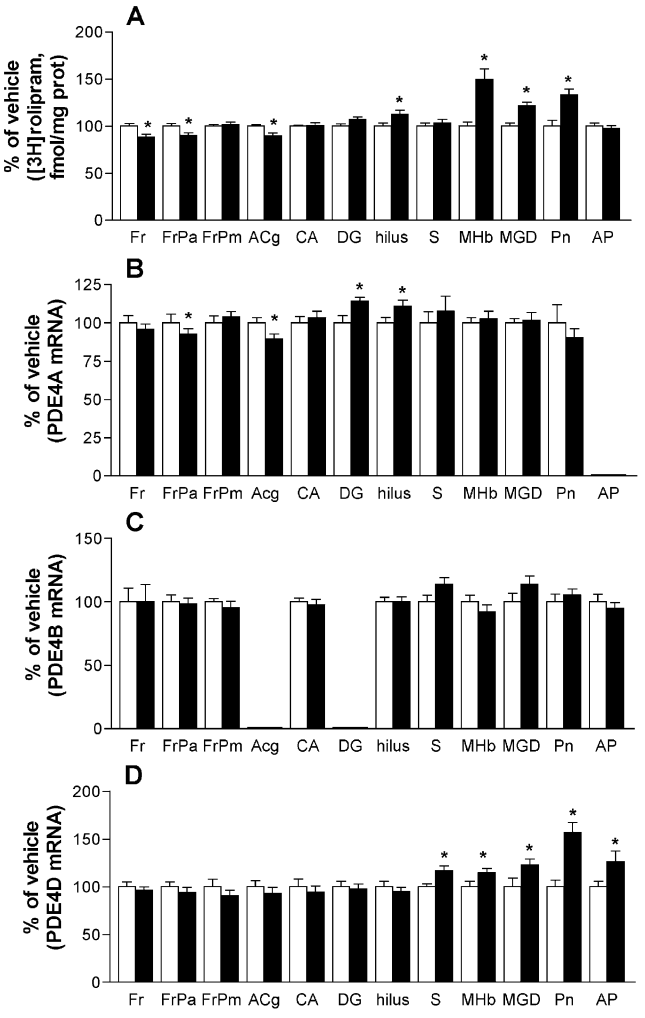

Fig. 4. Influence of chronic fluoxetine treatment on the binding of [^{3}H]rolipram (A) and the expression of PDE4A (B), PDE4B (C), and PDE4D (D) mRNAs. Binding density and mRNA expression levels were determined by receptor autoradiography and in situ hybridization histochemistry analyses as those shown in Fig. 1 and quantified by densitometry as described in Materials and Methods. The results are expressed as mean ±SEM percent of control; n=4 per group. Two-way ANOVA analysis revealed a significant effect of the treatment or treatment×region interaction for [^{3}H]rolipram binding, all PDEs (see Results for more detailed statistical analysis). Asterisks (∗) indicate a significant treatment×region interaction, $*p<0.05$ compared with control (Two-way ANOVA and Tukey's post hoc test). Open bars represent control values and filled bars represent values from treated rats. Abbreviations are as in Fig. 2.

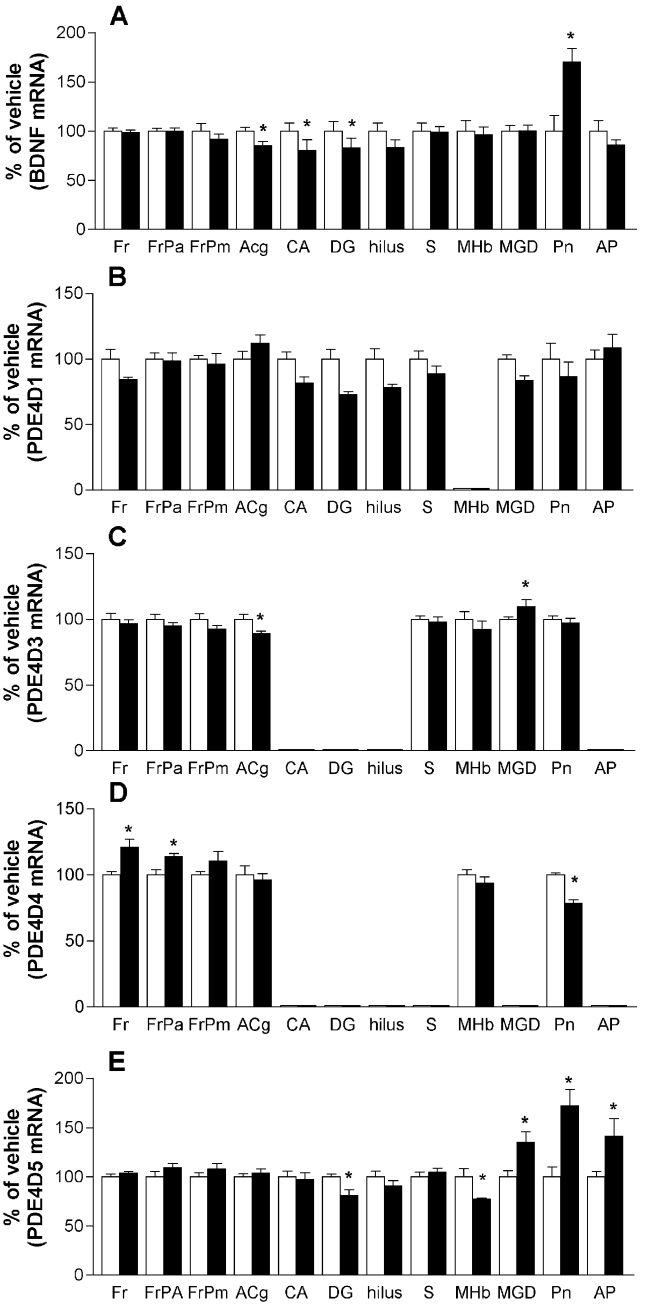

Fig. 5. Bar chart showing the effect of chronic fluoxetine treatment on the expression of mRNAs coding for BDNF (A), PDE4D1 (B), PDE4D3 (C), PDE4D4 (D), and PDE4D5 (E). The results are expressed as mean ±SEM percent of control; n=4 per group. Two-way ANOVA analysis showed a significant effect of the treatment or treatment×region interaction for PDE4D splicing isoforms. Asterisks (∗) indicate a significant treatment×region interaction, $*p<0.05$ compared with control (Two-way ANOVA and Tukey's post hoc test). Levels of PDE4D2 mRNA were not significantly altered by the chronic treatment (data not shown). Open bars represent control values and filled bars represent values from treated rats. Abbreviations are as in Fig. 2.

after chronic fluoxetine treatment in caudate-putamen, inferior olive, cerebellum, pineal gland and corpus callosum and a significant increase (treatment effect; $F_{1,42}$=5.96; p=0.019; Tukey's post hoc test $p<0.05$) (Fig. 4C) in subiculum and medial geniculate nucleus.

PDE4D mRNA transcripts were increased after single fluoxetine treatment in frontal cortex, hilus, medial habenular nucleus and parabigeminal nucleus (treatment effect; $F_{1,24}$=7.07; p=0.014; Tukey's post hoc test $p<0.05$). These mRNA levels were decreased in the dor-

sal part of the medial geniculate nucleus and in the area postrema (Fig. 2D). However, these latest changes did not attain statistical significance (treatment effect; $F_{1,12}$=2.3; p=0.15). In contrast, chronic fluoxetine treatment significantly increased PDE4D mRNA expression in subiculum, medial habenular nucleus, medial geniculate nucleus, pontine nuclei, and area postrema (treatment×region interaction; $F_{6,42}$=5.16; p=0.0005). Multiple comparison Tukey's post hoc test revealed a significant difference ($p<0.05$) between control and treatment group in these regions (Fig. 4D).

Acute fluoxetine treatment resulted in a reduction of BDNF transcripts in the Ammon's horn, dentate gyrus, subiculum and dorsal part of medial geniculate nucleus and in the posterior part of frontoparietal cortex but in an increase in frontoparietal and anterior cingulate cortices and inferior olive (treatment×region interaction; $F_{6,42}$=3.42; p=0.0077; Tukey's post hoc test $p<0.05$) (Fig. 3A). After 14-day treatment, BDNF mRNA levels decreased in the Ammon's horn, dentate gyrus, anterior cingulate cortex, parabigeminal nucleus and inferior olive. In contrast, high BDNF mRNA levels were detected in the pontine nuclei. Two-way ANOVA and Tukey's post hoc test revealed a significant treatment×region interaction in these regions. ($F_{5,36}$=6.17; p=0.00032; Tukey's post hoc test $p<0.05$) (Fig. 5A).

3.2. Regulation of PDE4D splice variants mRNA by antidepressant treatments

We extended our study to the expression of the five PDE4D splice variants in the same brain structures. The hybridization conditions and differential anatomical localization in the rat brain has been previously described (Miro et al., 2002). Each PDE4D splice variant was differentially affected by acute and chronic fluoxetine treatments. A single fluoxetine dose increased PDE4D1 mRNA transcript levels in the posterior part of frontoparietal cortex, lateral septal nucleus, dentate gyrus, hilus and area postrema whereas they were decreased in the anterior cingulate cortex and anterior part of frontoparietal cortex (treatment effect; $F_{1,36}$=6.02; p=0.019 and treatment×region interaction; $F_{5,36}$=5.3; p=0.0009) (Fig. 3B). Multiple comparison Tukey's post hoc analysis showed a significant effect of the treatment ($p<0.05$). Long-term fluoxetine treatment reduced PDE4D1 mRNA levels in frontal cortex, Ammon's horn, dentate gyrus, hilus, dorsal part of medial geniculate nucleus and parabigeminal nucleus (treatment effect; $F_{1,30}$=17.16; p=0.00026; Tukey's post hoc test $p<0.05$) (Fig. 5B).

The mRNA coding for PDE4D2 increased after acute but not chronic treatment. A significant effect of a single fluoxetine administration was observed in the dentate gyrus, hilus, medial habenular nucleus and parabigeminal nucleus (treatment effect; $F_{1,24}$=17.14; p=0.0004; Tukey's post hoc test $p<0.05$) (Fig. 3C). The effects did not reach statistical significance in the chronically-treated group. Conversely, the chronic (but not acute) fluoxetine treatment reduced the expression of PDE4D3 mRNA splice variant in the anterior cingulate cortex and increased it in the dorsal part of medial geniculate nucleus (treatment×region interaction; $F_{1,12}$=5.55; p=0.036). Tukey's post hoc test analysis showed a significant interaction of the treatment in both regions (Fig. 5C).

In the case of PDE4D4 mRNA splicing form, acute fluoxetine treatment increased mRNA levels in frontal cortex and decreased them in frontoparietal cortex and medial septal nucleus (treatment×region interaction; $F_{2,18}$=4.69; p=0.023; Tukey's post hoc test $p<0.05$) (Fig. 3D). On the other hand, chronic treatment resulted in an increment of PDE4D4 mRNA hybridization signal in the frontal and frontoparietal cortex (Fig. 5D) and in a reduction in pontine, vestibular and cerebellar nuclei (treatment×region interaction; $F_{4,30}$=8.6; p=0.00009; Tukey's post hoc test $p<0.05$).

Hybridization signal intensity for PDE4D5 mRNA decreased in acute fluoxetine treated rats in the frontal and frontoparietal cortices, Ammon's horn, and area postrema (treatment effect; $F_{1,24}$=7.04; p=0.014; Tukey's post hoc test: $p<0.05$) (Fig. 3E). A reduction was also observed after chronic fluoxetine treatment in the dentate gyrus and medial habenular nucleus, whereas an increment was detected in the medial geniculate nucleus, pontine nucleus and area postrema (treatment effect; $F_{1,30}$=4.36; p=0.045; and treatment×region interaction; $F_{4,30}$=6.54; p=0.0006). Multiple comparison Tukey's post hoc test showed a significant difference between control and treated group in these regions; $p<0.05$ (Fig. 5E).

4. Discussion

The aim of the present work was to study the alterations on the transcription levels of the mRNAs coding for PDE4A, PDE4B, PDE4D and its five splice forms, in the rat brain upon acute and chronic treatment with a 5-HT transporter inhibitor, fluoxetine. When studying the correlation between the changes in the levels of both ^{3}H-rolipram binding sites and mRNA coding for each of the PDE4 isozymes analyzed, several considerations should be taken into account: (i) ^{3}H-rolipram labels only the high affinity site present in the PDE4 isozymes, but it does not label their catalytic site. (ii) In the brain, there is an anatomical component that makes it difficult to establish unequivocal correlations between mRNA and protein content, in a given area, due to its complex architecture. A coincidence in the changes observed for both parameters in a particular brain area would be consistent

with their somatodendritic localization, whereas a non-coincident alteration could be due to a location of the isozymes in neuronal terminals, far away from the cell bodies that contain the mRNA. (iii) The relationship between the transcription and the translation of the mRNA coding for the different PDE4 isozymes is not yet known in rat brain and it may be complex.

A role for the cAMP cascade in the long-term actions of antidepressant treatments has been suggested (Menkes et al., 1983; Nestler et al., 1989; Nibuya et al., 1996). The cortical and hippocampal cAMP systems are potential common targets for 5-HT and noradrenaline actions given the innervation of these territories by both transmitters and the convergence of intracellular signals resulting from the activation of adenylyl cyclase-coupled receptors (Duman et al., 1997). Indeed, rolipram and other family-selective PDE inhibitors (O'Donnell and Frith, 1999) display antidepressant properties.

Our data indicate that the different PDE4s are distinctly regulated by acute and chronic fluoxetine treatment. Acute fluoxetine treatment increased significantly [^{3}H]rolipram binding and the expression of the PDE4 family members, with the exception of PDE4D. This increase could result from an increase of cAMP levels (Duman et al., 1997) after the additional tone on 5-HT receptors positively coupled to adenylyl cyclase. Consistently, activation of the cAMP pathway increased the expression of PDE4 family members (Houslay, 1998). The unchanged expression of PDE4D appears to result from opposite changes in PDE4D2 (increase) and PDE4D5 (decrease) whereas the effects on PDE4D1 and PDE4D4 depend on the structure examined. In cultured cells, the activation of the cAMP system increased the expression of short PDE4D forms (PDE4D1 and PDE4D2) (Swinnen et al., 1989). Thus, elevated cAMP levels could explain the increase in PDE4D1 and PDE4D2 in the hippocampus.

Acute fluoxetine administration increases the expression of BDNF in cortex and the inferior olive while it decreases in other areas, including the hippocampus (CA fields and dentate gyrus). A trend to decreased BDNF mRNA levels (1.8 kb transcript by Northern blot analysis) in cortex and hippocampus was also observed after single treatment with sertraline (Nibuya et al., 1995).

Two-week fluoxetine treatment altered PDE4 mRNA expression in a manner different from acute treatment. Given the progressive and delayed onset of clinical antidepressant action, long-term changes may be related to the therapeutic effect of fluoxetine. Thus, changes in the mRNAs of PDE4 family members in several brain regions, could lead to an increase in cAMP accumulation with putative therapeutic benefits. Thus, a down-regulation of [^{3}H]rolipram binding sites is detected in corpus callosum, caudate-putamen, and several cortical areas. PDE4B mRNA decreases in the cerebellum (contrary to PDE4A), caudate-putamen, corpus callosum, pineal gland and inferior olive. PDE4A mRNA also decreases in the septum, anterior cingulate cortex and frontoparietal cortex. These results are in disagreement with the report by Takahashi et al. (1999) showing up-regulation of PDE4A and PDE4B mRNA in frontal cortex, and PDE4B mRNA in nucleus accumbens after chronic sertraline and fluoxetine (5 mg/kg day, 21 days). An increased PDE4B transcription in frontal cortex (but not hippocampus) was reported (Suda et al., 1998) after 21-day imipramine treatment. However, and in agreement with our observations, an increased hippocampal expression of PDE4A (no change in frontal cortex) has been reported after 2-week treatment with 5 mg/kg day fluoxetine (Ye et al., 2000).

Chronic treatment with fluoxetine resulted in a decrease of PDE4D expression in vestibular and cerebellar nuclei, and cerebellum, and in an increment in several other brain areas. Reductions in PDE4D1 mRNA levels are observed in six brain areas, including hippocampal CA fields and dentate gyrus (in common with PDE4D5). A marked increase in PDE4D mRNA (through the PDE4D5 isoform) occurs in the pontine nuclei. This area plays a key role in the regulation of sleep via cAMP pathway (Capece and Lydic, 1997). The increase in pontine PDE4D5 mRNA could be related to the long-term effects of antidepressants on sleep architecture, in particular the reduction of REM sleep (Sharpley and Cowen, 1995; Zajecka, 2000). Indeed, the pontine nuclei play a major role in the generation of REM sleep which is associated with increased (ACh) and decreased (5-HT and NE) neuronal activity (Jones, 1991).

Contrary to our expectations, the levels of BDNF mRNA decreased after chronic fluoxetine treatment in the hippocampus (DG and CA), increasing only in the pontine nuclei. Chronic antidepressant treatment was reported to augment CREB and BDNF expression in hippocampus (Nibuya et al., 1996). This led to the suggestion that the cAMP cascade may be a common cellular pathway for antidepressant drug action (Duman et al., 1997). However, 21-day treatments with two selective PDE4 inhibitors (rolipram and Ro 20-1724) or NE reuptake blockers (desipramine and Org, 4428) did not increase the expression of BDNF mRNA. This was only enhanced by combinations of the PDE4 inhibitor and NE reuptake inhibitors (Fujimaki et al., 2000). A closer inspection of the data by Nibuya et al. (1995) showed a moderate 20% increase of the 4.4. kb transcript (less abundant form in hippocampus (Hofer et al., 1990) after 21-day sertraline treatment.

The reasons for the discrepancy between our study and that of Nibuya et al. (1996) are unclear and perhaps involve methodological reasons. Indeed, in situ densitometric mRNA measurements done in brain areas are more accurate than those obtained after dissecting and homogenizing hippocampal tissue. Moreover, our stat-

istical analysis was done with a single averaged value per rat whereas Nibuya et al. (1996) used replicate measures per rat. In the present study fluoxetine was delivered by minipumps to prevent the injection-induced stress whereas Nibuya et al. (1996) administered the drugs i.p. Differences in the fluoxetine doses in both cases could also explain the discrepancy observed on BDNF changes. The dose used in the present study was intended to mimic the effects of the standard clinical dose since it produced a substantial increment (300% of controls) in cortical extracellular 5-HT after 14-day but not after single treatment (Hervas et al., 2001). The present data indicate that this dose elicits a substantial occupancy of the 5-HT transporter, as assessed by receptor autoradiography. Altogether, these data suggest that this fluoxetine dose was sufficient to increase the serotonergic function after repeated, but not single dosage, in parallel with its clinical effects.

The reduction of hippocampal BDNF expression is consistent with an increased tonic activation of hippocampal 5-HT_{1A} receptors produced by chronic antidepressant treatments (Haddjeri et al., 1998). 5-HT_{1A} receptors are very densely expressed in the hippocampus (Pompeiano et al., 1992) and are negatively coupled to adenylyl cyclase (De Vivo and Maayani, 1986). Thus, the antidepressant-induced activation of hippocampal postsynaptic 5-HT_{1A} receptors is more likely to reduce the hippocampal cAMP pathway (and possibly in BDNF expression) than the opposite. Also, since 5-HT_{1A} receptors are also coupled to a hyperpolarizing GIRK channel (Andrade et al., 1986) the additional activation of 5-HT_{1A} receptors during chronic fluoxetine argues against a CREB-mediated BDNF increase since CREB is activated by cellular depolarization (Sheng and Greenberg, 1990). Moreover, antidepressant drugs (including fluoxetine) have a direct inhibitory action on CRE-directed gene transcription by interfering with the above mechanism (Schwaninger et al., 1995). Furthermore, mechanisms other than the cAMP pathway can regulate BDNF mRNA expression since alternative usage of four promoters within the BDNF gene has been reported (Timmusk et al., 1993).

The results demonstrate that a clinically relevant dose of fluoxetine differentially modulates the expression of the cAMP-specific PDE4 isoenzymes, suggesting that these changes in expression may mediate the effects of antidepressant treatment.

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